55. A nucleic acid comprising a nucleotide sequence encoding a multivalent antibody comprising a sequence selected from the group consisting of SEQ ID NOs: 187, and 189; and a light chain sequence of SEQ ID NO: 185.

56. A nucleic acid comprising a nucleotide sequence encoding a multivalent antibody comprising at least 98% sequence identity to SEQ ID NOs: 187, and 189; and a light chain sequence of SEQ ID NO: 185.

57. A vector comprising the nucleic acid of claims 48-56.

58. A pharmaceutical composition comprising a multivalent antibody thereof having at least two receptor binding domains for two different binding sites of a target receptor selected from claims 1-47 and a pharmaceutically acceptable carrier.

59. A method of obtaining a multivalent antibody of claims 1, 17, and 35 comprising:(a) providing a first receptor binding domain which binds to a first binding site of an LRP6 target receptor; (b) providing a second receptor binding domain which binds to a second binding site of an LRP6 target receptor; and (c) linking the first receptor binding domain to the second receptor binding domain.

60. The method of claim 59, wherein the first or second receptor binding domain are selected from the group consisting of an IgG antibody, an scFv fragment, a single chain diabody, an antibody mimetic, and an antibody variable domain.

61. The method of claim 59, wherein the first and second receptor binding domains are linked together by a linker with a spatial distribution that permits the first and second receptor binding domains to bind to the first and second epitopes of LRP6, respectively.

62. The method of claim 61, wherein the linker is a Gly-Ser linker selected from the group consisting of $(Gly_4Ser)_4$, and $(Gly_4Ser)_3$.

63. The method of claim 59, further comprising introducing at least one amino acid mutation to the first or second receptor binding domain, such that the amino acid mutation improves stability of the first or second receptor binding domain compared with the unmutated first or second receptor binding domain, wherein the amino acid mutation is selected from Figures 29-32.

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64. A method of treating a cancer comprising selecting a subject having an LRP6 expressing cancer, administering to a subject in need thereof an effective amount of a composition comprising a multivalent antibody having at least two receptor binding domains for two different binding sites of a target receptor of claims 1-47.

65. The method of claim 64, wherein the subject is a human.

66. A method of treating a cancer comprising selecting a subject having an LRP6 expressing cancer, administering to a subject in need thereof an effective amount of a composition comprising a multivalent antibody having at least two receptor binding domains for two different binding sites of a target receptor of claims 1-47, wherein the cancer is selected from the group consisting of breast cancer, lung cancer, multiple myeloma, ovarian cancer, liver cancer, bladder cancer,gastric cancer, prostate cancer, acute myeloid leukemia, chronic myeloid leukemia, osteosarcoma, squamous cell carcinoma, and melanoma.

67. The method of claim 66, wherein the cancer is breast cancer.

68. A method of treating a disease mediated by a canonical Wnt signaling pathway using a multivalent antibody to LRP6.

69. A method of treating a cancer comprising selecting a subject having an LRP6 expressing cancer, administering to the subject an effective amount of a composition comprising multivalent antibody with heavy and light chain sequences selected from the group consisting of SEQ ID NOs: 166/170, 171/170, 173/170, 175/170, 201/199, 207/205, and 195/193 in combination with any standard of care cancer therapies.

70. Use of multivalent antibody of any one of the previous claims in the manufacture of a medicament for the treatment of a cancer selected from the group consiting of breast cancer, lung cancer, multiple myeloma, ovarian cancer, liver cancer, bladder cancer gastric cancer, prostate cancer, acute myeloid leukemia, chronic myeloid leukemia, osteosarcoma, squamous cell carcinoma, and melanoma.

71. A multivalent antibody having VH of SEQ ID NO: 14; VL of SEQ ID NO: 13; VH of SEQ ID NO: 82; VL of SEQ ID NO: 81 for use in treating a cancer mediated by a canonical Wnt signaling pathway.

72. A multivalent antibody having VH of SEQ ID NO: 14; VL of SEQ ID NO: 13; VH of SEQ ID NO: 82; VL of SEQ ID NO: 81 for use as a drug.

73. A multivalent antibody having SEQ ID NO: 166 and SEQ ID NO: 170 for use in treating a cancer mediated by a canonical Wnt signaling pathway.

74. A multivalent antibody having SEQ ID NO: 166 and SEQ ID NO: 170 for use as a drug.

75. A multivalent antibody of claims 1-47, for use as a medicament.

76. A multivalent antibody of claims 1-47, for use as a medicament for treatment of an LRP6 expressing cancer.

77. A multivalent antibody of claims 1-47, for use as a medicament for treatment of an LRP6 expressing cancer, wherein the cancer is selected from the group consisting of breast cancer, lung cancer, multiple myeloma, ovarian cancer, liver cancer, bladder cancer,gastric cancer, prostate cancer, acute myeloid leukemia, chronic myeloid leukemia, osteosarcoma, squamous cell carcinoma, and melanoma.

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Fig. 1A

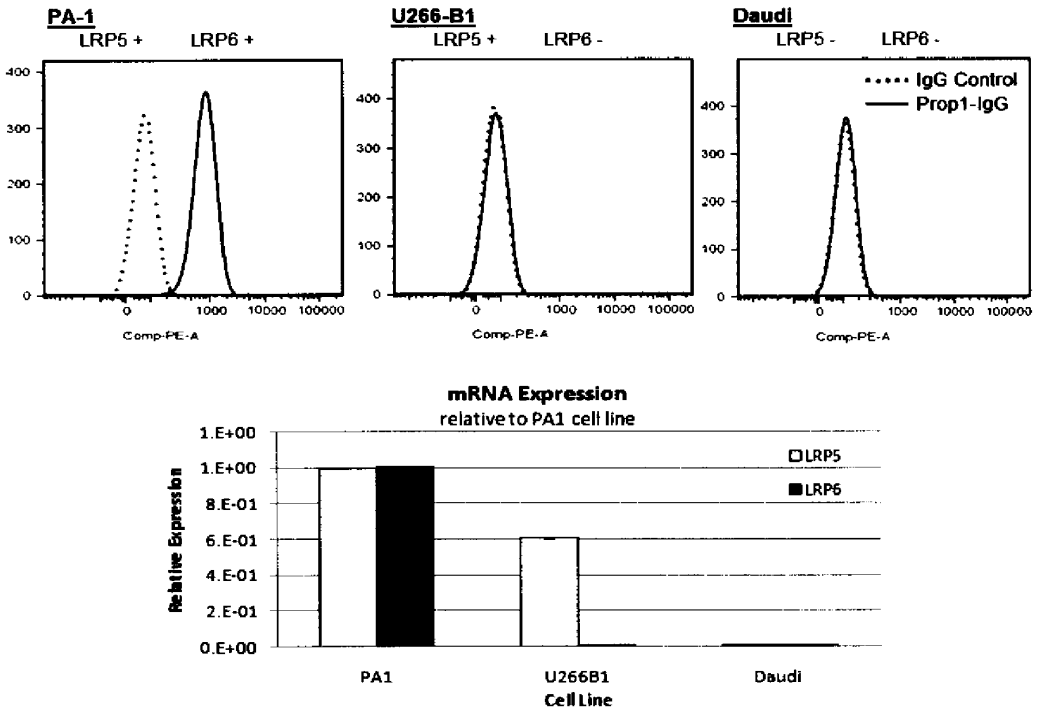

PA-1
LRP5 +
LRP6 +
U266-B1
LRP5 +
LRP6 -
Daudi
LRP5 -
LRP6 -
IgG Control
Prop1-IgG
Comp-PE-A
mRNA Expression
relative to PA1 cell line
LRP5
LRP6
Relative Expression
1.E+00
1.E+00
8.E-01
6.E-01
4.E-01
2.E-01
0.E+00
PA1
U266B1
Daudi
Cell Line

Fig. 1B

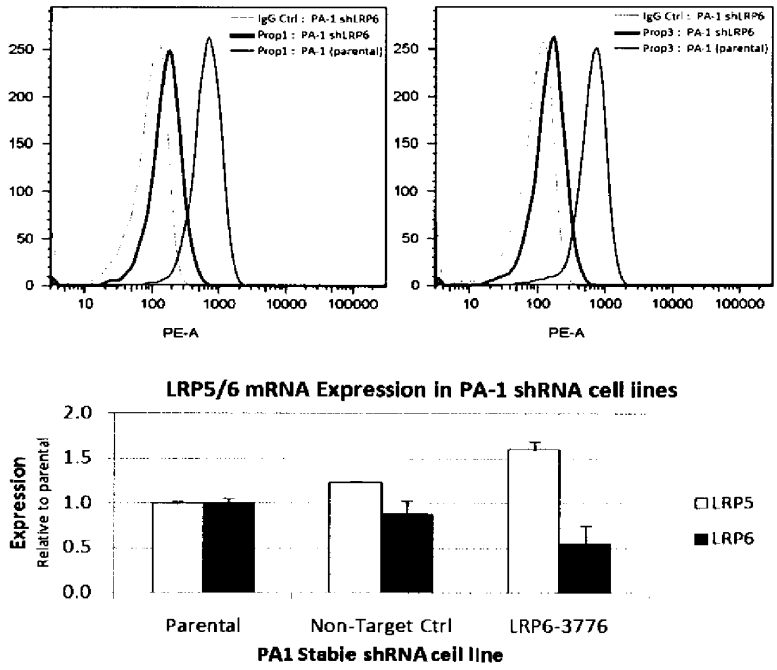

IgG Ctrl : PA-1 shLRP6
Prop1 : PA-1 shLRP6
Prop1 : PA-1 (parental)
IgG Ctrl : PA-1 shLRP6
Prop3 : PA-1 shLRP6
Prop3 : PA-1 (parental)
PE-A
PE-A
LRP5/6 mRNA Expression in PA-1 shRNA cell lines
Expression
Relative to parental
LRP5
LRP6
Parental
Non-Target Ctrl
LRP6-3776
PA1 Stable shRNA cell line

Fig. 2A

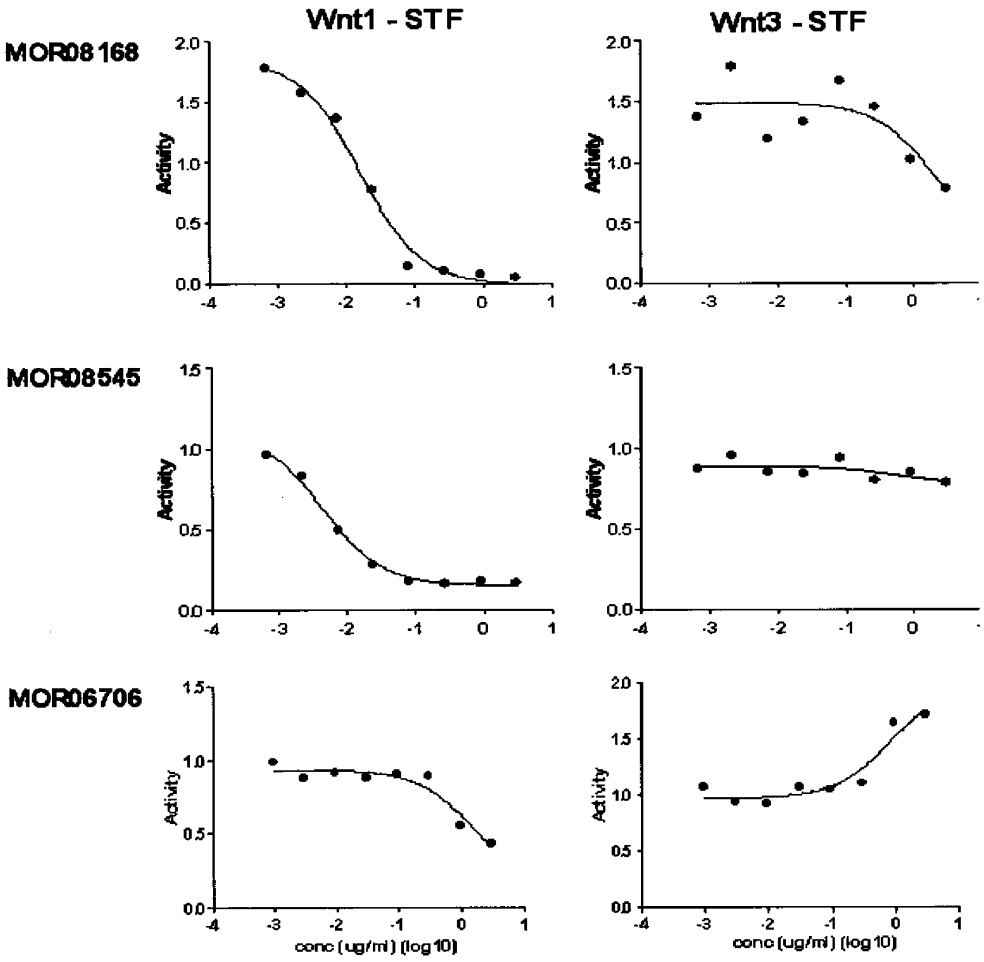

Wnt1 - STF
Wnt3 - STF
MOR08168
MOR08545
MOR06706
Activity
conc (ug/ml) (log10)

Fig. 2B

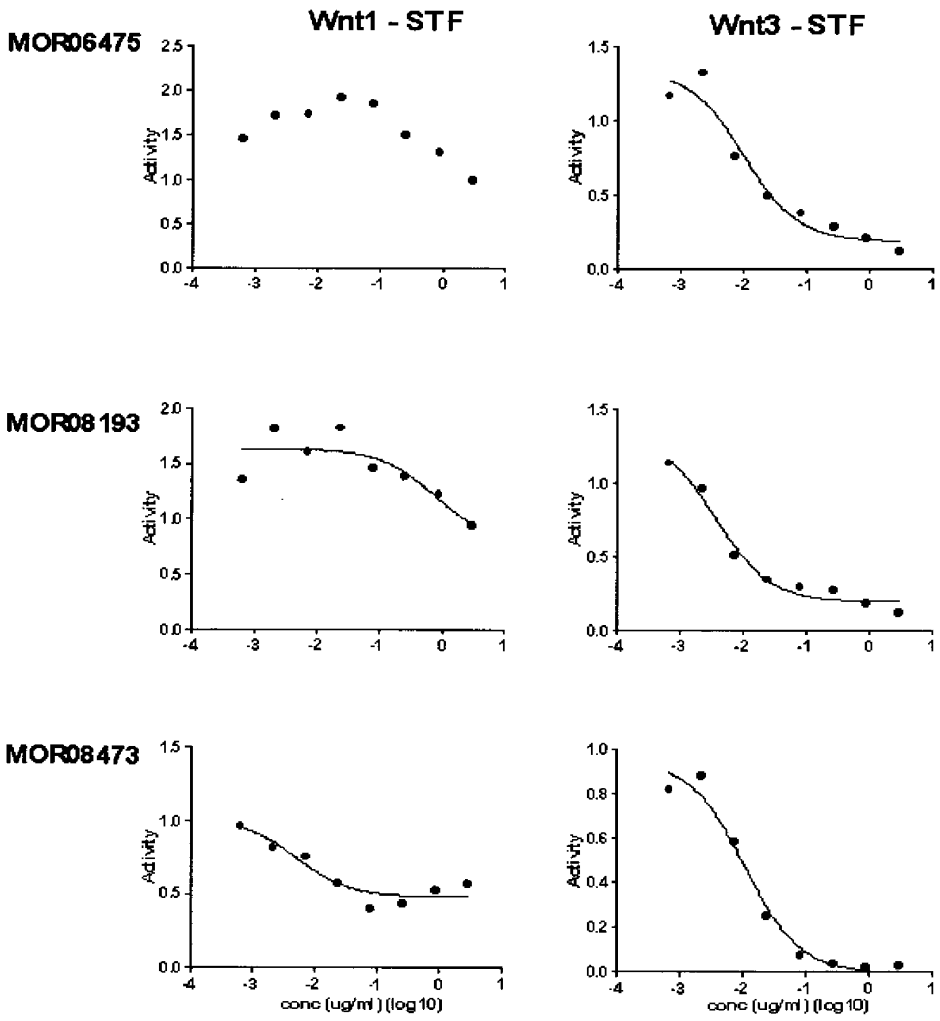

Wnt1 - STF
Wnt3 - STF
MOR06475
MOR08193
MOR08473
Activity
conc (ug/ml) (log10)

Fig. 3

	Prop 1			Prop3		
	MOR08168	MOR08545	MOR06706	MOR06475	MOR08193	MOR08473
human LRP6	0.28	0.09	0.14	0.31	0.36	0.74
mouse LRP6	0.12	0.06	n/a	0.96	0.08	0.46
cynomolgus LRP6	0.88	0.30	0.46	0.22	0.20	0.52

EC_{50} values expressed in nM.

Fig. 4

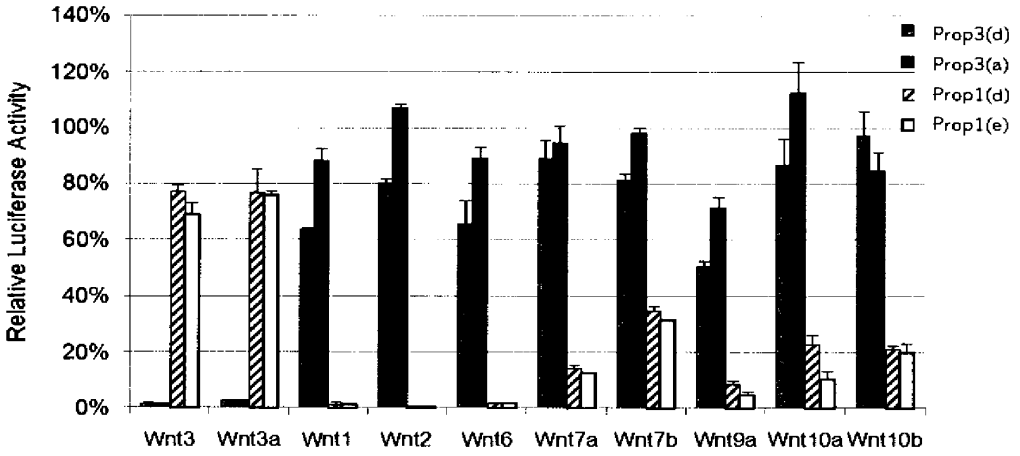

140%
120%
100%
80%
60%
40%
20%
0%
Relative Luciferase Activity
Prop3(d)
Prop3(a)
Prop1(d)
Prop1(e)
Wnt3
Wnt3a
Wnt1
Wnt2
Wnt6
Wnt7a
Wnt7b
Wnt9a
Wnt10a
Wnt10b

Fig. 5

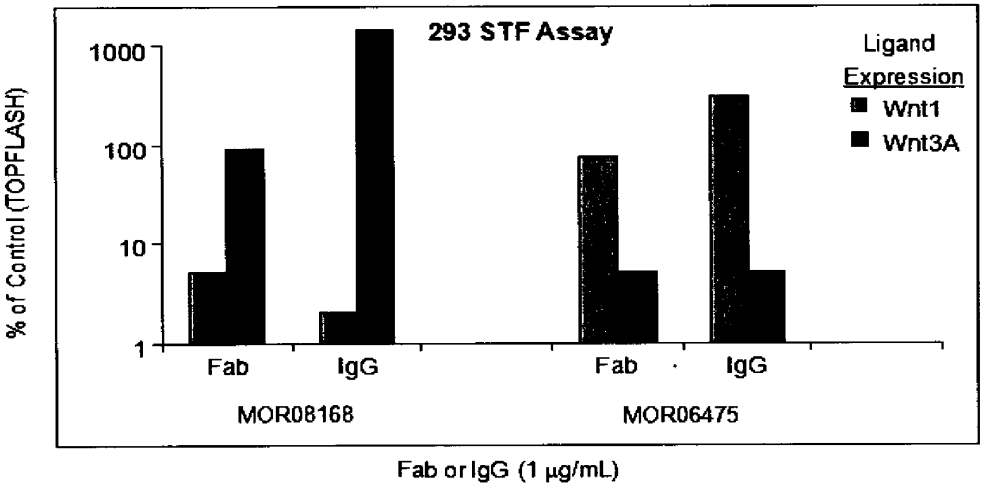

293 STF Assay
Ligand Expression
Wnt1
Wnt3A
% of Control (TOPFLASH)
1000
100
10
1
Fab
IgG
Fab
IgG
MOR08168
MOR06475
Fab or IgG (1 µg/mL)

Fig. 6

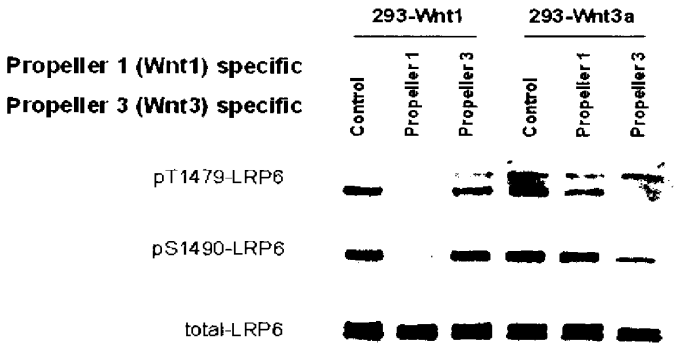
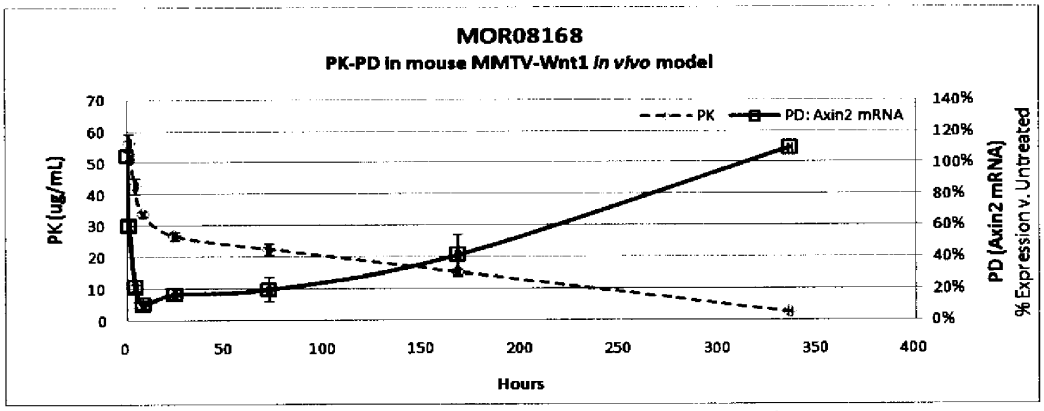

293-Wnt1
293-Wnt3a
Propeller 1 (Wnt1) specific
Propeller 3 (Wnt3) specific
Control
Propeller 1
Propeller 3
Control
Propeller 1
Propeller 3
pT1479-LRP6
pS1490-LRP6
total-LRP6

Fig. 7



Time points: 1, 4, 8 and 24 hours, 3, 7 and 14 days

Fig. 8A

Probe ID	Gene Symbol	Gene Title	Entrez Gene ID	log(2)fold change 0 h vs. 8 h	adj.P.Val 0 h vs. 8 h
1435154_at	AU018091	expressed sequence AU018091	245128	2.51	0.0081676
1418743_a_at	LOC100047138 /// Tesc	similar to Tescalcin /// tescalcin	100047138 /// 57816	2.42	0.0007069
1418744_s_at	LOC100047138 /// Tesc	similar to Tescalcin /// tescalcin	100047138 /// 57816	2.41	0.0011682
1447891_at	---	---	---	2.35	0.0012203
1417956_at	Cidea	cell death-inducing DNA fragmentation factor, alpha	12683	2.32	0.0061736
1436279_at	---	---	---	2.25	0.0007424
1451139_at	Slc39a4	solute carrier family 39 (zinc	72027	2.04	0.0003359
1417130_s_at	Angptl4	angiopoietin-like 4	57875	1.94	0.0007217
1434918_at	Sox6	SRY-box containing gene 6	20679	1.79	0.0021073
1451612_at	Mt1	metallothionein 1	17748	1.74	0.0046964
1441915_s_at	2310076L09Rik	RIKEN cDNA 2310076L09 gene	66968	1.71	0.0024997
1423860_at	Ptgds	prostaglandin D2 synthase (brain)	19215	1.66	0.0012372
1428283_at	Cyp2s1	cytochrome P450, family 2, subfamily s, polypeptide 1	74134	1.64	0.004778
1439036_a_at	Atp1b1	ATPase, Na+/K+ transporting, beta 1 polypeptide	11931	1.63	0.0070499
1451410_a_at	Crip3	cysteine-rich protein 3	114570	1.62	0.000223
1459898_at	Sbsn	suprabasin	282619	1.60	0.0022588
1433888_at	Atp2b2	ATPase, Ca++ transporting, plasma membrane 2	11941	1.58	0.0099753
1450435_at	L1cam	L1 cell adhesion molecule	16728	1.57	0.001168
1455506_at	Slc25a34	solute carrier family 25, member 34	384071	1.55	7.26E-05
1422820_at	Lipe	lipase, hormone sensitive	16890	1.52	0.0034308
1447655_x_at	Sox6	SRY-box containing gene 6	20679	1.51	0.003772
1451204_at	Scara5	scavenger receptor class A,	71145	1.51	0.0017923
1451828_a_at	Acsl4	acyl-CoA synthetase long-chain	50790	1.48	6.46E-05
1451331_at	Ppp1r1b	protein phosphatase 1, regulatory (inhibitor) subunit 1B	19049	1.44	0.0025364
1418453_a_at	Atp1b1	ATPase, Na+/K+ transporting, beta 1 polypeptide	11931	1.43	0.0049563
1419706_a_at	Akap12	A kinase (PRKA) anchor protein (gravin) 12	83397	1.42	0.0001178
1418911_s_at	Acsl4	acyl-CoA synthetase long-chain	50790	1.40	0.003297
1439630_x_at	Sbsn	suprabasin	282619	1.38	0.0007395
1438261_at	Cited4	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 4, mRNA (cDNA clone IMAGE:3670674)	56222	1.36	0.0067834

Fig. 8A CON

Probe ID	Gene Symbol	Gene Title	Entrez Gene ID	log(2)fold change 0 h vs. 8 h	adj.P.Val 0 h vs. 8 h
1429952_at	Mospd4	motile sperm domain containing 4	72076	1.36	0.0055785
1435595_at	1810011O10Rik	RIKEN cDNA 1810011O10 gene	69068	1.34	0.000592
1429352_at	Mocos	molybdenum cofactor sulfurase	68591	1.32	0.0090359
1459897_a_at	Sbsn	suprabasin	282619	1.32	0.0014474
1447845_s_at	Vnn1	vanin 1	22361	1.31	0.004465
1421841_at	Fgfr3	fibroblast growth factor receptor 3	14184	1.29	7.26E-05
1424937_at	2310076L09Rik	RIKEN cDNA 2310076L09 gene	66968	1.29	0.0001178
1449403_at	Pde9a	phosphodiesterase 9A	18585	1.27	0.0097294
1424218_a_at	Creb3l4	cAMP responsive element binding protein 3-like 4	78284	1.21	0.0020445
1424763_at	Rsph9	radial spoke head 9 homolog (Chlamydomonas)	75564	1.21	0.0067834
1420521_at	Papln	papilin, proteoglycan-like sulfated glycoprotein	170721	1.21	0.0045018
1434442_at	Stbd1	starch binding domain 1	52331	1.19	0.0047233
1438033_at	Tef	thyrotroph embryonic factor	21685	1.17	0.0013355
1442335_at	---	---	---	1.16	0.0061736
1448227_at	Grb7	growth factor receptor bound protein 7	14786	1.14	0.0012612
1416596_at	Slc44a4	solute carrier family 44, member 4	70129	1.14	0.0088984
1439620_at	Car13	carbonic anhydrase 13	71934	1.13	0.0032295
1418780_at	Cyp39a1	cytochrome P450, family 39, subfamily a, polypeptide 1	56050	1.12	0.0061736
1460409_at	Cpt1a	carnitine palmitoyltransferase 1a, liver	12894	1.10	0.003297
1449146_at	Notch4	Notch gene homolog 4 (Drosophila)	18132	1.10	0.0083512
1443841_x_at	Uap1l1	UDP-N-acteylglucosamine pyrophosphorylase 1-like 1	227620	1.08	0.0076401
1417273_at	Pdk4	pyruvate dehydrogenase kinase, isoenzyme 4	27273	1.08	0.0061736
1430278_a_at	Dqx1	DEAQ RNA-dependent ATPase	93838	1.07	0.0024997
1426065_a_at	Trib3	tribbles homolog 3 (Drosophila)	228775	1.04	0.0028588
1438038_at	4930402H24Rik	RIKEN cDNA 4930402H24 gene	228602	1.03	0.00599
1427537_at	Eppk1	epiplakin 1	223650	1.02	0.0011399
1416457_at	Ddah2	dimethylarginine dimethylaminohydrolase 2	51793	1.01	0.0057377

Fig. 8B

Probe ID	Gene Symbol	Gene Title	Entrez Gene	log(2)fold change 0 h vs. 8 h	adj.P.Val 0 h vs. 8 h
1449451_at	Serpinb11	serine (or cysteine) peptidase inhibitor, clade B (ovalbumin), member 11	66957	-3.95	7.37E-07
1451129_at	Calb2	calbindin 2	12308	-3.14	0.006783
1436845_at	Axin2	axin2	12006	-2.92	4.53E-07
1416468_at	Aldh1a1	aldehyde dehydrogenase family 1, subfamily A1	11668	-2.83	0.000506
1427508_at	Arsi	arylsulfatase i	545260	-2.55	0.000592
1418601_at	Aldh1a7	aldehyde dehydrogenase family 1, subfamily A7	26358	-2.55	0.005578
1432592_at	Pappa	pregnancy-associated plasma protein A	18491	-2.55	6.46E-05
1425985_s_at	Masp1	mannan-binding lectin serine peptidase 1	17174	-2.48	0.001787
1434802_s_at	Ntf3	neurotrophin 3	18205	-2.43	0.000914
1425978_at	Myocd	myocardin	214384	-2.39	0.00374
1436221_at	Ildr2	immunoglobulin-like domain containing receptor 2	100039795	-2.36	0.00296
1418678_at	Has2	hyaluronan synthase 2	15117	-2.31	0.000996
1436894_at	Ildr2	immunoglobulin-like domain containing receptor 2	100039795	-2.3	0.0025
1427633_a_at	Pappa	pregnancy-associated plasma protein A	18491	-2.21	0.002187
1436293_x_at	Ildr2	immunoglobulin-like domain containing receptor 2	100039795	-2.17	0.002799
1433959_at	Zmat4	zinc finger, matrin type 4	320158	-2.14	0.00638
1427600_at	---	---	---	-2.12	6.46E-05
1432591_at	Pappa	pregnancy-associated plasma protein A	18491	-2.07	0.001159
1448397_at	Gjb6	gap junction protein, beta 6	14623	-2.06	0.002796
1449926_at	Cd70	CD70 antigen	21948	-2.05	0.006174
1441807_s_at	---	---	---	-2.02	0.004956
1438602_s_at	Masp1	mannan-binding lectin serine peptidase 1	17174	-2.01	0.00255
1425212_a_at	Tnfrsf19	tumor necrosis factor receptor superfamily, member 19	29820	-1.94	6.46E-05
1428223_at	Mfsd2	major facilitator superfamily domain containing 2	76574	-1.93	0.006536
1420006_at	Bmp15	Bone morphogenetic protein 15 (Bmp15), mRNA	12155	-1.91	0.000707
1429506_at	Nkd1	naked cuticle 1 homolog (Drosophila)	93960	-1.91	0.001459
1433894_at	Jazf1	JAZF zinc finger 1	231986	-1.9	0.000592
1422699_at	Alox12	arachidonate 12-lipoxygenase	11684	-1.89	0.001168
1460449_at	Anks1b	ankyrin repeat and sterile alpha motif domain containing 1B	77531	-1.87	0.000592

Fig. 8B CON

Probe ID	Gene Symbol	Gene Title	Entrez Gene	log(2)fold change 0 h vs. 8 h	adj.P.Val 0 h vs. 8 h
1417275_at	Mal	myelin and lymphocyte protein, T-cell differentiation protein	17153	-1.87	0.002349
1449169_at	Has2	hyaluronan synthase 2	15117	-1.8	0.000707
1420005_s_at	Bmp15	bone morphogenetic protein 15	12155	-1.78	0.002107
1449033_at	Tnfrsf11b	tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	18383	-1.76	0.00402
1433990_at	Lhfpl3	lipoma HMGIC fusion partner-like 3	269629	-1.73	0.001503
1431229_at	Ipw	imprinted gene in the Prader-Willi syndrome region	16353	-1.71	0.0022
1429592_at	Lhfpl3	lipoma HMGIC fusion partner-like 3	269629	-1.7	0.004465
1457617_at	---	---	---	-1.64	0.008658
1434265_s_at	Ank2	ankyrin 2, brain	109676	-1.64	0.006936
1449634_a_at	Anks1b	ankyrin repeat and sterile alpha motif domain containing 1B	77531	-1.6	0.005666
1455436_at	Diras2	DIRAS family, GTP-binding RAS-like 2	68203	-1.56	0.00813
1457429_s_at	Gm106	gene model 106, (NCBI)	226866	-1.56	0.000118
1425425_a_at	Wif1	Wnt inhibitory factor 1	24117	-1.55	0.000336
1415921_a_at	Tnfrsf19	tumor necrosis factor receptor superfamily, member 19	29820	-1.53	8.15E-05
1457948_at	Gas7	Growth arrest-specific 7-cb protein (Gas7-cb)	14457	-1.53	0.006196
1439954_at	6430514M23Rik	RIKEN cDNA 6430514M23 gene	399595	-1.52	0.006504
1417709_at	Cyp46a1	cytochrome P450, family 46, subfamily a, polypeptide 1	13116	-1.52	0.004364
1445247_at	C530044C16Rik	RIKEN cDNA C530044C16 gene	319981	-1.52	0.000604
1428665_at	Pfn4	profilin family, member 4	382562	-1.51	0.006174
1448147_at	Tnfrsf19	tumor necrosis factor receptor superfamily, member 19	29820	-1.47	2.14E-08
1456335_at	Gm106	gene model 106, (NCBI)	226866	-1.47	0.001787
1440546_at	9630002D21Rik	RIKEN cDNA 9630002D21 gene	319560	-1.44	0.003664
1450506_a_at	Aen	apoptosis enhancing nuclease	68048	-1.43	0.005422
1453041_at	Ano9	anoctamin 9	71345	-1.41	0.000592
1453700_s_at	4933403O03Rik /// EG245263	RIKEN cDNA 4933403O03 gene /// predicted gene, EG245263	245263 /// 74	-1.41	0.004956
1449478_at	Mmp7	matrix metallopeptidase 7	17393	-1.39	0.009569
1452938_at	Anks1b	ankyrin repeat and sterile alpha motif domain containing 1B	77531	-1.37	0.006568
1444541_at	---	---	---	-1.37	0.000828
1450728_at	Fjx1	four jointed box 1 (Drosophila)	14221	-1.36	0.00323
1452728_at	Kirrel3	kin of IRRE like 3 (Drosophila)	67703	-1.36	0.005549

Fig. 8B CON

Probe ID	Gene Symbol	Gene Title	Entrez Gene	log(2)fold change 0 h vs. 8 h	adj.P.Val 0 h vs. 8 h
1430118_at	2700046A07Rik	RIKEN cDNA 2700046A07 gene	78449	-1.31	0.008169
1441272_at	Matr3	Matrin 3, mRNA (cDNA clone MGC:28206 IMAGE:3989914)	17184	-1.29	0.002269
1419539_at	Irx4	Iroquois related homeobox 4 (Drosophila)	50916	-1.26	0.005098
1442456_at	Spata5	spermatogenesis associated 5	57815	-1.26	0.006174
1456672_at	---	---	---	-1.24	0.006783
1439026_at	Trpm3	transient receptor potential cation channel, subfamily M, member 3	226025	-1.23	0.006174
1440446_at	---	---	---	-1.21	0.001125
1426139_a_at	Ccrl1	chemokine (C-C motif) receptor-like 1	252837	-1.2	0.009707
1435941_at	Rhbdl3	rhomboid, veinlet-like 3 (Drosophila)	246104	-1.18	0.004465
1453645_at	2700046A07Rik	RIKEN cDNA 2700046A07 gene	78449	-1.18	0.000692
1444905_at	D1Ertd705e	DNA segment, Chr 1, ERATO Doi 705, expressed	51940	-1.17	0.003297
1422733_at	Fjx1	four jointed box 1 (Drosophila)	14221	-1.14	0.007138
1423957_at	Aen	apoptosis enhancing nuclease	68048	-1.07	0.000391
1433084_at	4930402C16Rik	RIKEN cDNA 4930402C16 gene	73812	-1.07	0.006802
1421498_a_at	2010204K13Rik	RIKEN cDNA 2010204K13 gene	68355	-1.07	0.007469
1426981_at	Pcsk6	proprotein convertase subtilisin/kexin type 6	18553	-1.06	0.000506
1446846_at	---	---	---	-1.05	0.004364
1429861_at	Pcdh9	protocadherin 9	211712	-1.04	0.006174
1447958_at	---	---	---	-1.02	0.000592
1435608_at	LOC631806 /// Znrf3	similar to Goliath homolog precursor (Ring finger protein 130) (R-goliath) /// zinc and ring finger 3	407821 /// 63:	-1.02	0.000118
1456266_at	---	---	---	-1.02	0.002282
1421341_at	Axin2	axin2	12006	-1.02	0.001447
1418495_at	Zc3h8	zinc finger CCCH type containing 8	57432	-1.01	0.000233

Fig. 9A

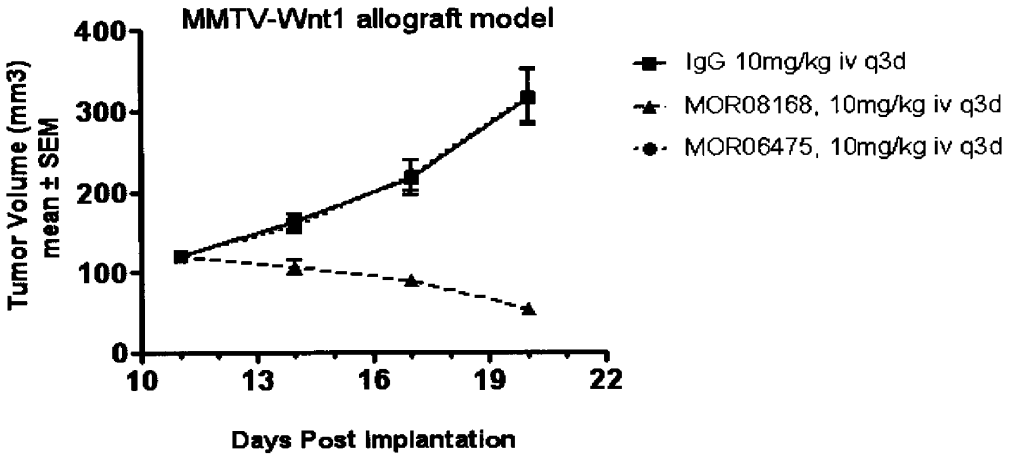

MMTV-Wnt1 allograft model
Tumor Volume (mm3) mean ± SEM
400
300
200
100
0
10
13
16
19
22
Days Post Implantation
IgG 10mg/kg iv q3d
MOR08168, 10mg/kg iv q3d
MOR06475, 10mg/kg iv q3d

Fig. 9B

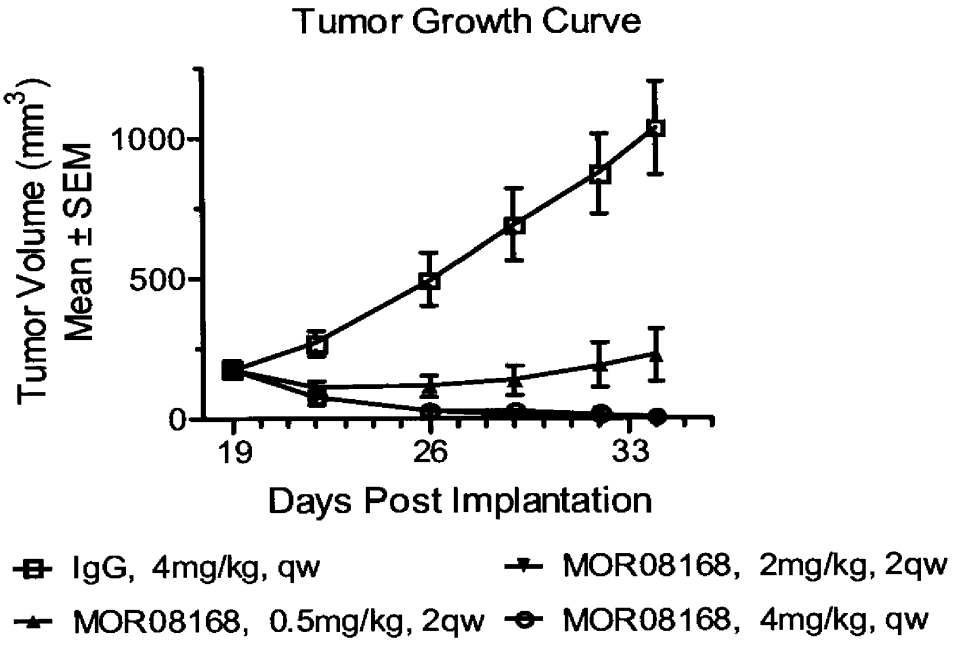

Tumor Growth Curve
Tumor Volume (mm3) Mean ± SEM
1000
500
0
19
26
33
Days Post Implantation
IgG, 4mg/kg, qw
MOR08168, 2mg/kg, 2qw
MOR08168, 0.5mg/kg, 2qw
MOR08168, 4mg/kg, qw

Fig 10

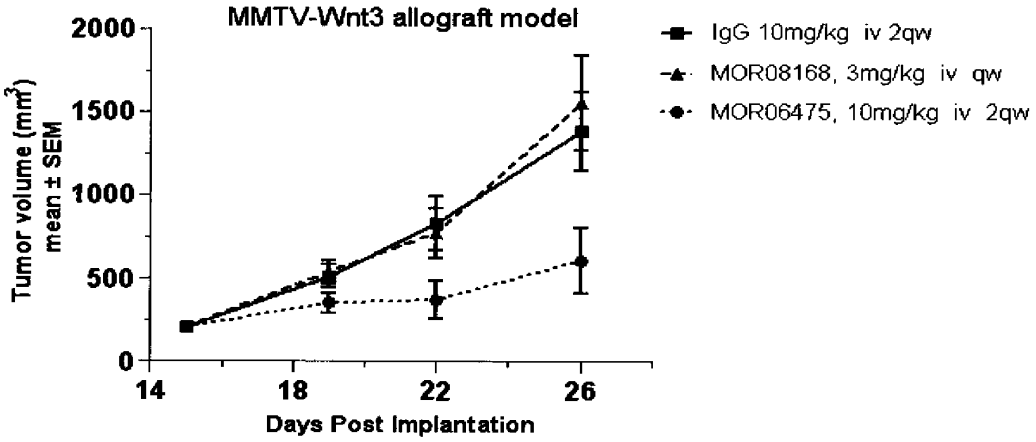

MMTV-Wnt3 allograft model
Tumor volume (mm3) mean ± SEM
2000
1500
1000
500
0
14
18
22
26
Days Post Implantation
IgG 10mg/kg iv 2qw
MOR08168, 3mg/kg iv qw
MOR06475, 10mg/kg iv 2qw

Fig 11

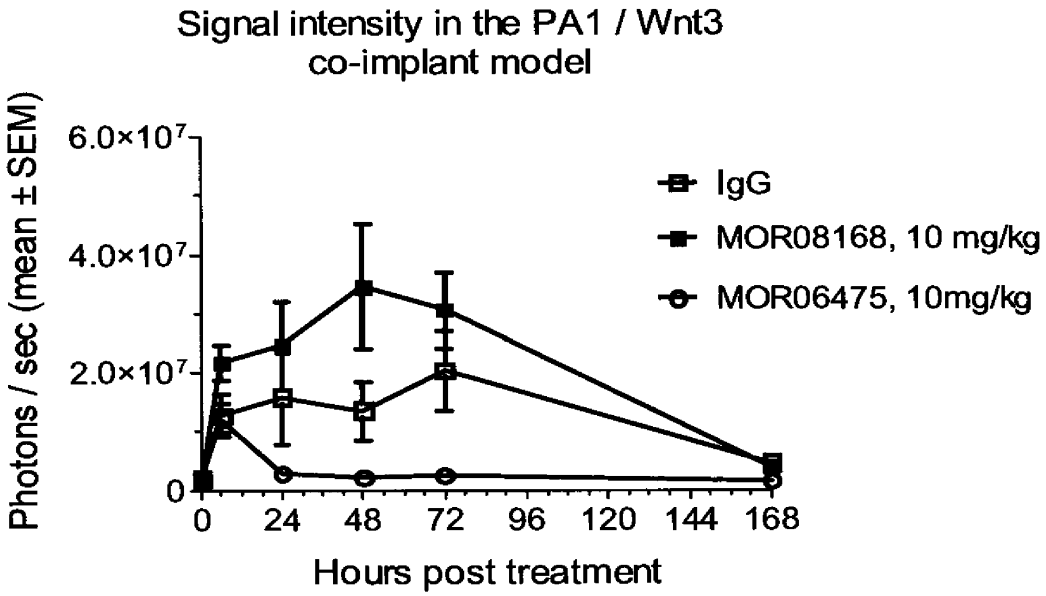

Signal intensity in the PA1 / Wnt3
co-implant model
Photons / sec (mean ± SEM)
6.0×10⁷
4.0×10⁷
2.0×10⁷
0
0
24
48
72
96
120
144
168
Hours post treatment
IgG
MOR08168, 10 mg/kg
MOR06475, 10mg/kg

Fig. 12A

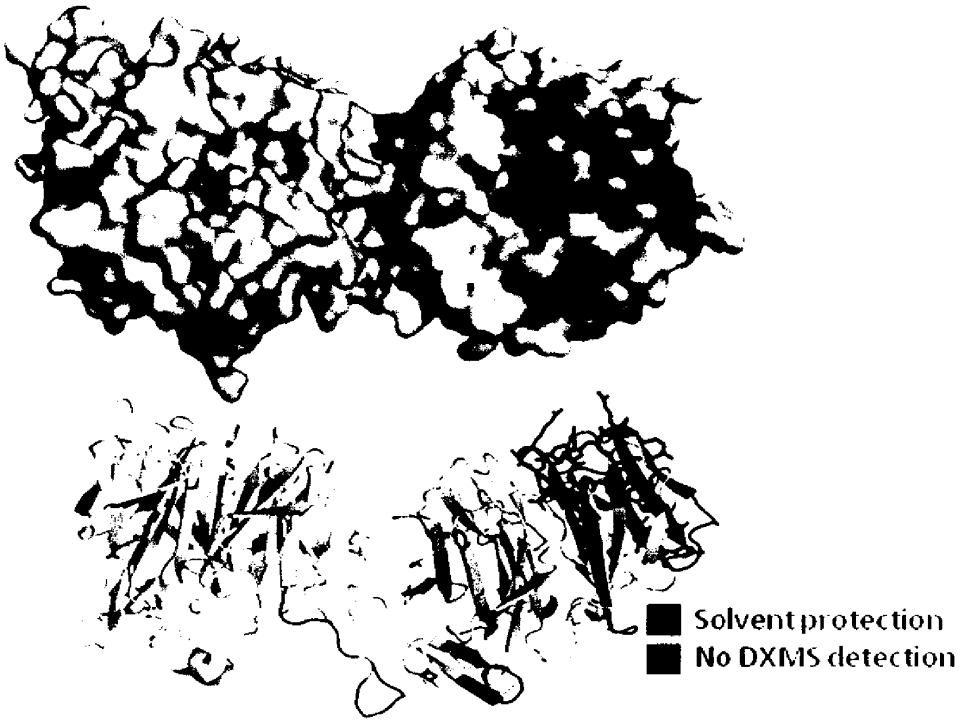


Fig. 12B

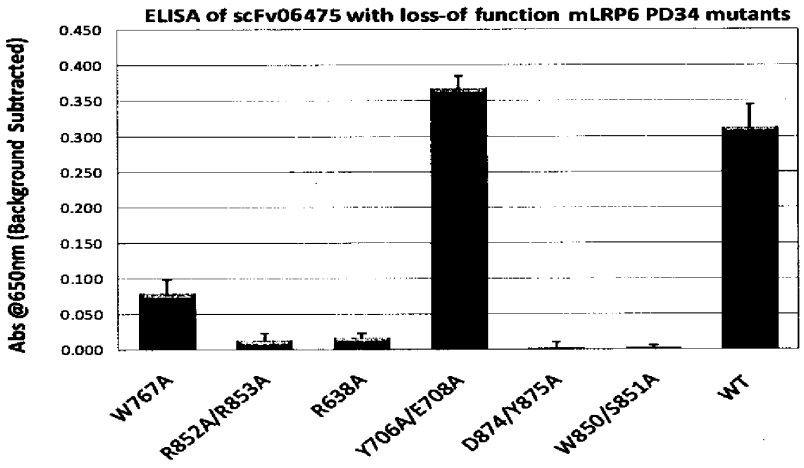

ELISA of scFv06475 with loss-of function mLRP6 PD34 mutants
Abs @650nm (Background Subtracted)
0.450
0.400
0.350
0.300
0.250
0.200
0.150
0.100
0.050
0.000
W767A
R852A/R853A
R638A
Y706A/E708A
D874/Y875A
W850/S851A
WT

Fig. 13

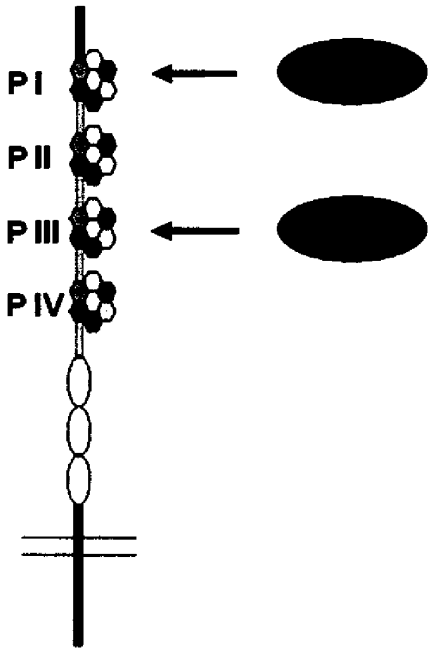


Fig. 14

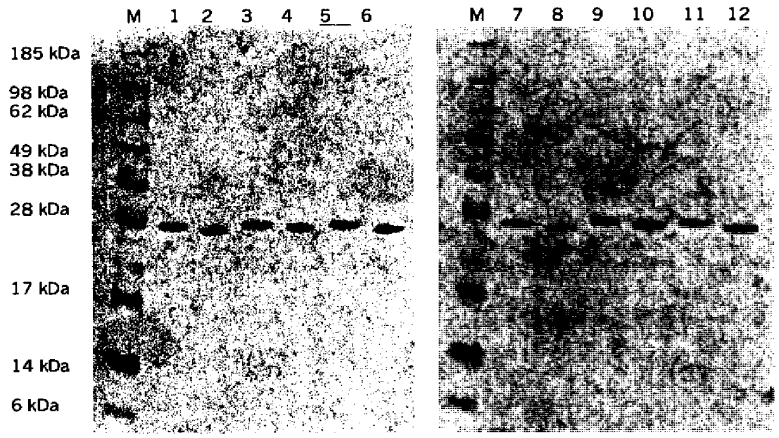

M 1 2 3 4 5 6
M 7 8 9 10 11 12
185 kDa
98 kDa
62 kDa
49 kDa
38 kDa
28 kDa
17 kDa
14 kDa
6 kDa

Fig. 15

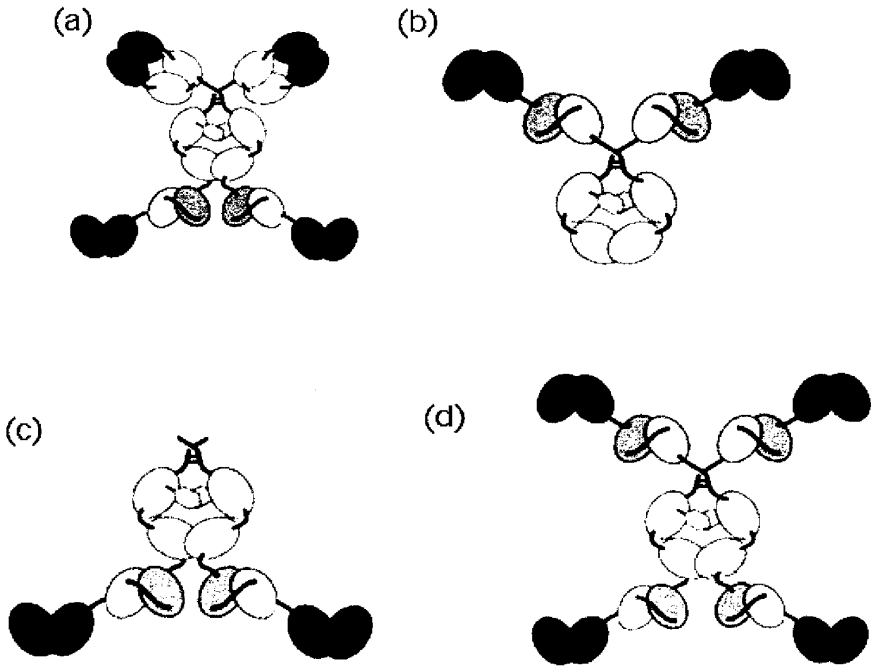


Fig. 16

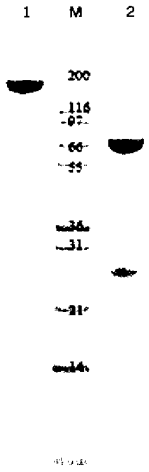


Fig. 17

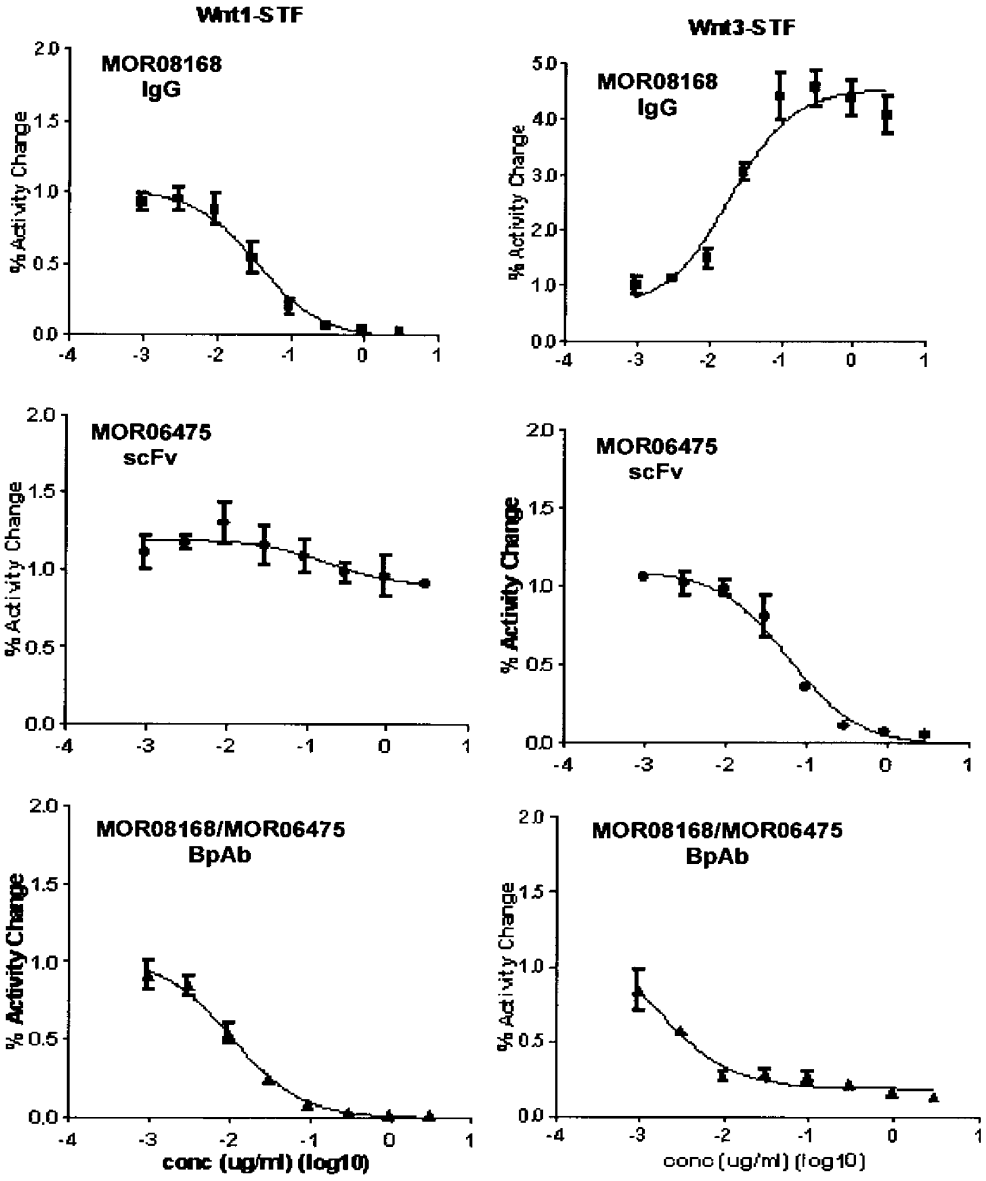

Wnt1-STF
MOR08168
IgG
% Activity Change
Wnt3-STF
MOR08168
IgG
% Activity Change
MOR06475
scFv
% Activity Change
MOR06475
scFv
% Activity Change
MOR08168/MOR06475
BpAb
% Activity Change
conc (ug/ml) (log10)
MOR08168/MOR06475
BpAb
% Activity Change
conc (ug/ml) (log10)

Fig. 18

	HEK STF IC50 (µg/ml) WNT1	WNT3a
MOR06475-sc-fv-Vl-Vh	n/a	0.07
MOR06475-sc-fv-Vh-Vl	n/a	0.06
MOR06475-sc-fv-Vl-Vh-(GGGGS)3	n/a	0.08
MOR06475-sc-fv-Vh-Vl-(GGGGS)3	n/a	0.08
MOR06475-Fab	n/a	0.11
MOR08168-sc-fv-Vl-Vh	0.05	n/a
MOR08168-sc-fv-Vh-Vl	0.06	n/a
MOR08168-sc-fv-Vl-Vh-(GGGGS)3	0.07	n/a
MOR08168-sc-fv-Vh-Vl-(GGGGS)3	0.1	n/a
MOR08168-Fab	0.15	n/a
MOR08545-sc-fv-Vl-Vh	0.53	n/a
MOR08545-sc-fv-Vh-Vl	0.06	n/a
MOR08545-sc-fv-Vl-Vh-(GGGGS)3	n/a	n/a
MOR08545-sc-fv-Vh-Vl-(GGGGS)3	0.05	n/a
MOR08545-Fab	0.05	n/a
anti-LRP6_MOR08168_hIgG1 LALA_6475scfv_at_VL	0.013	0.0071
anti-LRP6_MOR06475_hIgG1 LALA_8168scfvatCH3_(VH-3-VL)	0.013	0.0082
anti-LRP6_MOR06475_hIgG1 LALA_8168scfvatCH3_(VH-4-VL)	0.016	0.0094
anti-LRP6_MOR08168_hIgG1 LALA_6475scfvatCH3_opt_DPtoDA	0.02	0.0076
anti-LRP6_MOR08168_hIgG1 LALA_6475scfvatCH3_opt_DPtoTA	0.026	0.0098
anti-LRP6_MOR08168_hIgG1 LALA_6475scfvatCH3_opt_w/o-K	0.015	0.0064
MOR06475 IgG	n/a	0.0039
MOR08168 IgG	0.025	n/a
MOR08168hIgG1LALA 6475 scfv	0.016	0.011

Fig. 19

Antibody	K_D [µM], binding of Ab's to FcRn			
FcRn	huFcRn, pH6.0	huFcRn, pH7.4	cynoFcRn, pH6.0	cynoFcRn, pH7.4
[redacted]	0.023	LLB	0.28	LLB
[redacted]	0.021	LLB	0.12	LLB

LLB: low level binding

Fig. 20

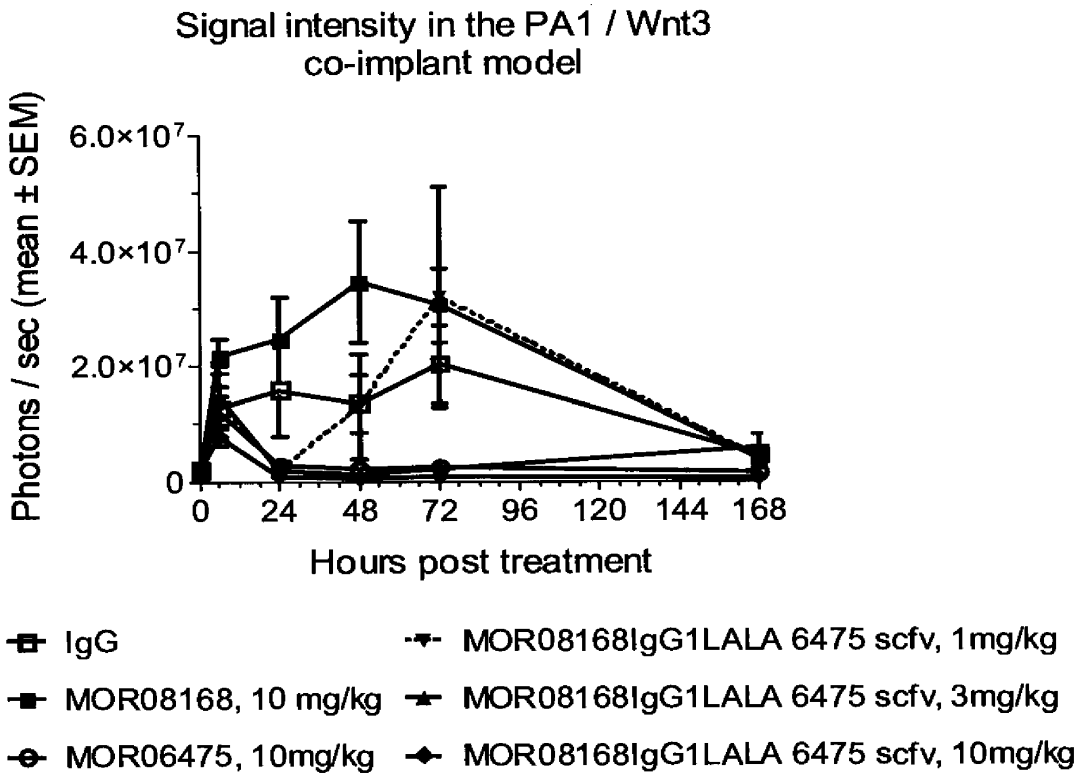

Signal intensity in the PA1 / Wnt3 co-implant model
Photons / sec (mean ± SEM)
6.0×10^7
4.0×10^7
2.0×10^7
0
0 24 48 72 96 120 144 168
Hours post treatment
IgG
MOR08168, 10 mg/kg
MOR06475, 10mg/kg
MOR08168IgG1LALA 6475 scfv, 1mg/kg
MOR08168IgG1LALA 6475 scfv, 3mg/kg
MOR08168IgG1LALA 6475 scfv, 10mg/kg

Fig. 21

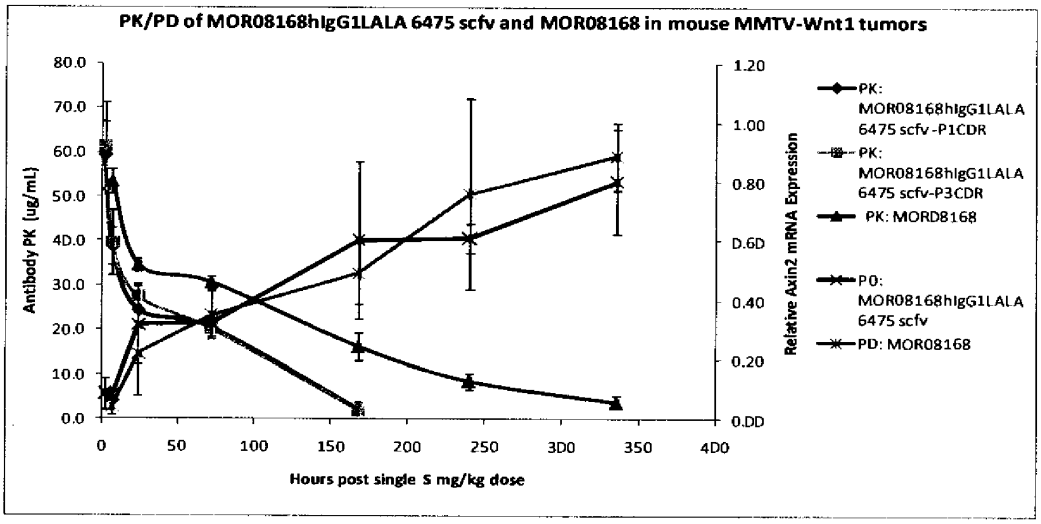

PK/PD of MOR08168hIgG1LALA 6475 scfv and MOR08168 in mouse MMTV-Wnt1 tumors
Antibody PK (ug/mL)
Relative Axin2 mRNA Expression
Hours post single 5 mg/kg dose
PK: MOR08168hIgG1LALA 6475 scfv-P1CDR
PK: MOR08168hIgG1LALA 6475 scfv-P3CDR
PK: MORD8168
PD: MOR08168hIgG1LALA 6475 scfv
PD: MOR08168

Fig. 22

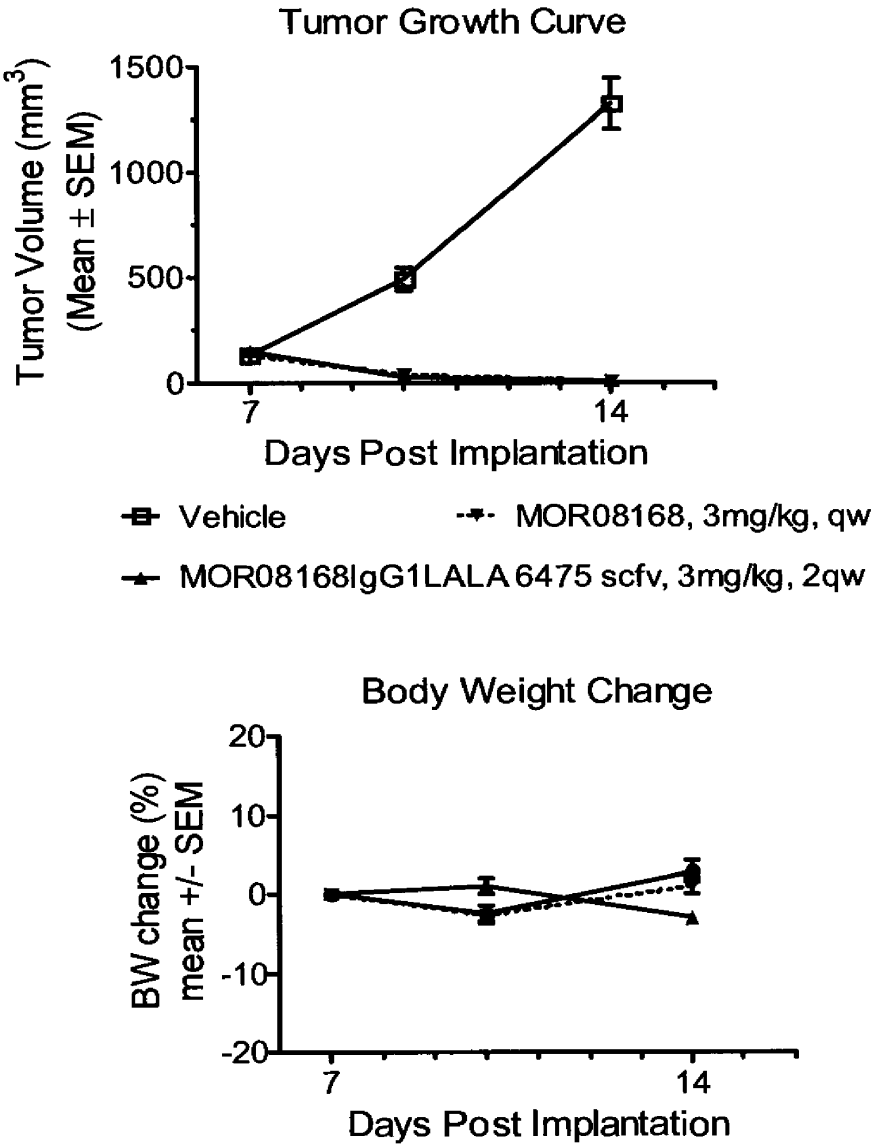

Tumor Growth Curve
Tumor Volume (mm3)
(Mean ± SEM)
1500
1000
500
0
7
14
Days Post Implantation
Vehicle
MOR08168, 3mg/kg, qw
MOR08168IgG1LALA 6475 scfv, 3mg/kg, 2qw
Body Weight Change
BW change (%)
mean +/- SEM
20
10
0
-10
-20
7
14
Days Post Implantation

Fig. 23

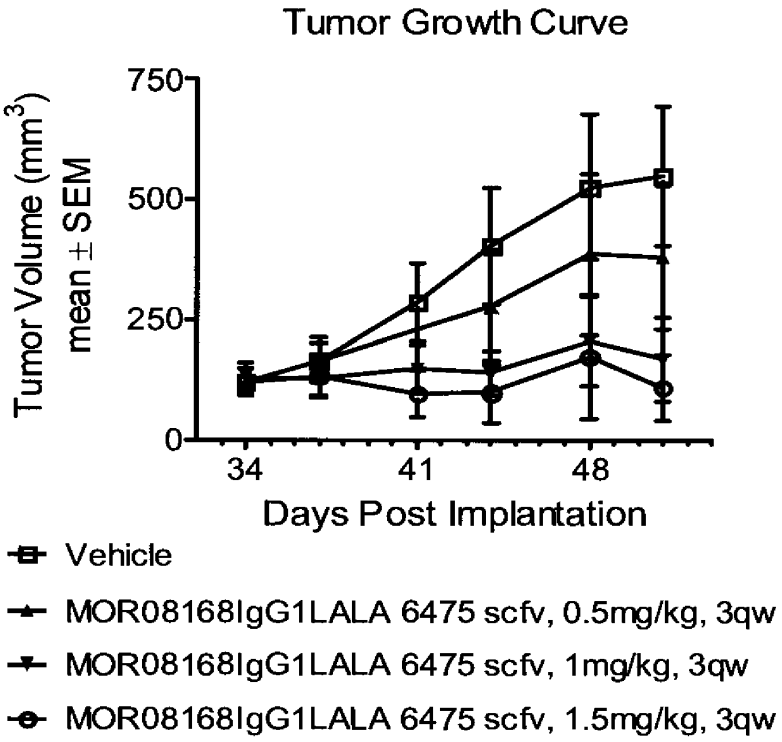

Tumor Growth Curve
Tumor Volume (mm3)
mean ± SEM
750
500
250
0
34
41
48
Days Post Implantation
Vehicle
MOR08168IgG1LALA 6475 scfv, 0.5mg/kg, 3qw
MOR08168IgG1LALA 6475 scfv, 1mg/kg, 3qw
MOR08168IgG1LALA 6475 scfv, 1.5mg/kg, 3qw

Fig. 24A

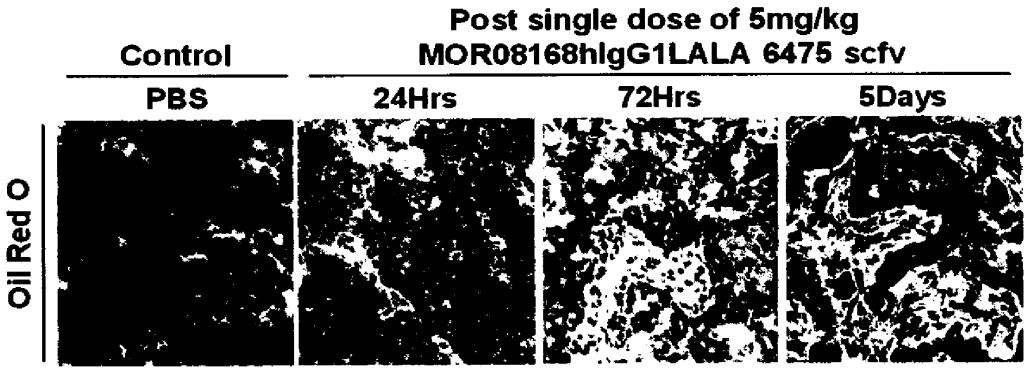

Post single dose of 5mg/kg
MOR08168hIgG1LALA 6475 scfv
Control
PBS
24Hrs
72Hrs
5Days
Oil Red O

Fig. 24B

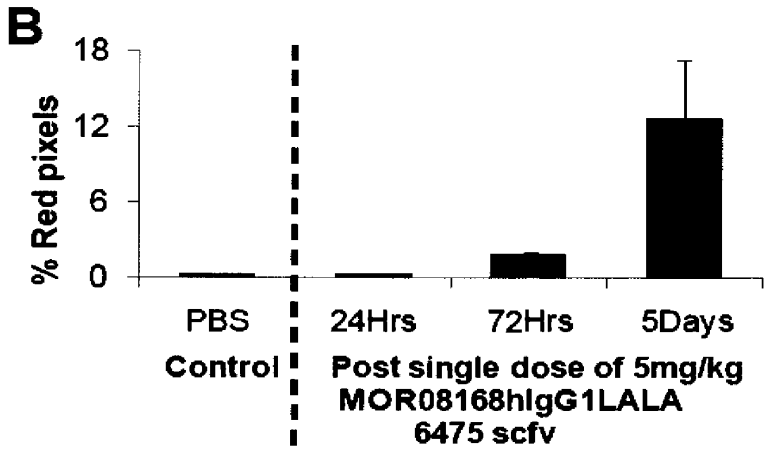

B
% Red pixels
18
12
6
0
PBS
24Hrs
72Hrs
5Days
Control
Post single dose of 5mg/kg
MOR08168hIgG1LALA
6475 scfv

Fig. 25

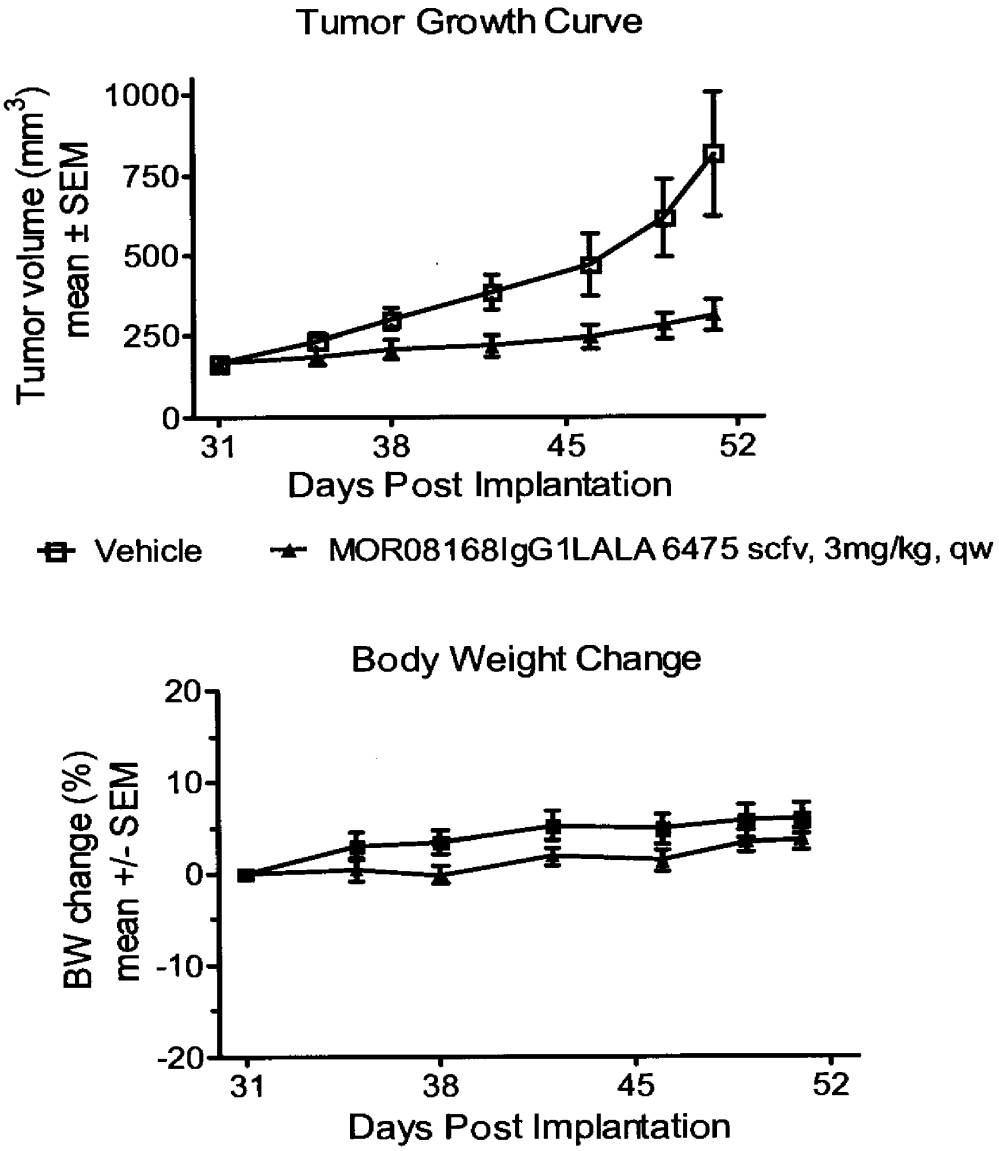

Tumor Growth Curve
Tumor volume (mm3) mean ± SEM
1000
750
500
250
0
31
38
45
52
Days Post Implantation
Vehicle
MOR08168IgG1LALA 6475 scfv, 3mg/kg, qw
Body Weight Change
BW change (%) mean +/- SEM
20
10
0
-10
-20
31
38
45
52
Days Post Implantation

Fig26A

Summary of KD measurement of MOR08168 IgG LALA 6475scFv, MOR08168 and MOR06475 binding to PD1/2 and PD3/4

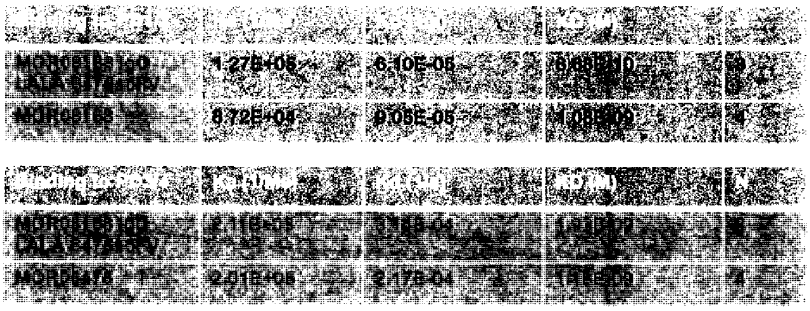


Fig26B


Binding to RD1/2

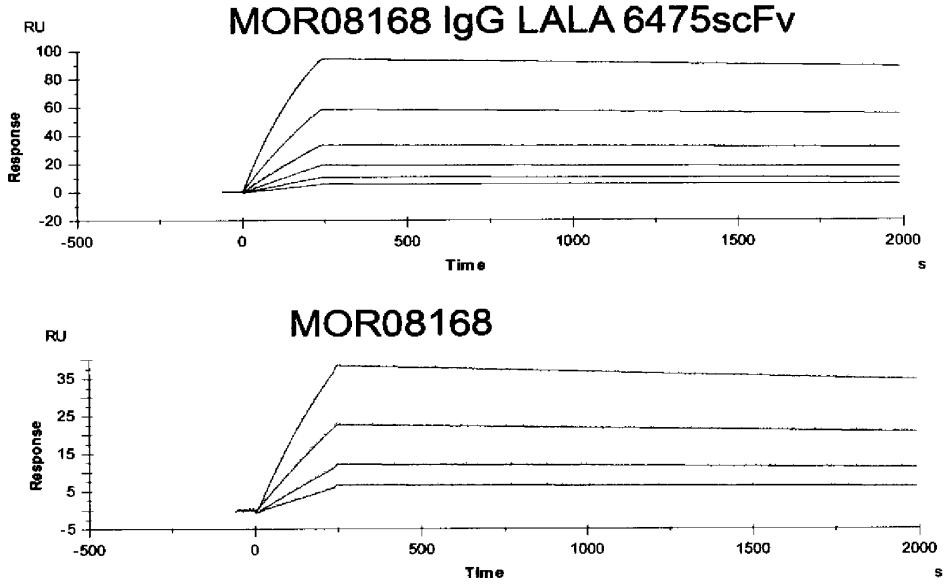
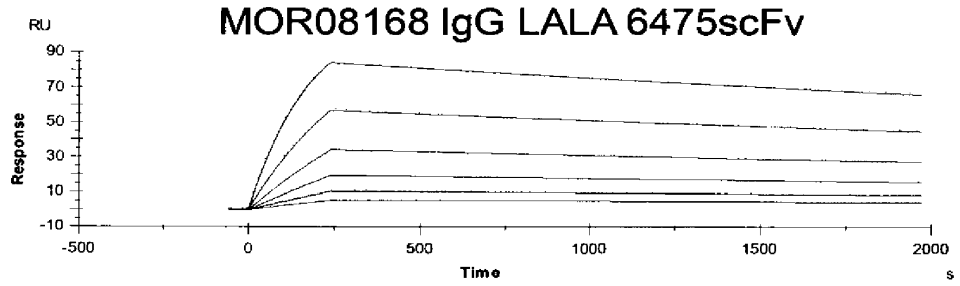

MOR08168 IgG LALA 6475scFv
RU
100
80
60
40
20
0
-20
Response
-500
0
500
1000
1500
2000
Time
s
MOR08168
RU
35
25
15
5
-5
Response
-500
0
500
1000
1500
2000
Time
s

Fig. 26B CON


Binding to PD3/4


MOR08168 IgG LALA 6475scFv
RU
Response
Time
s

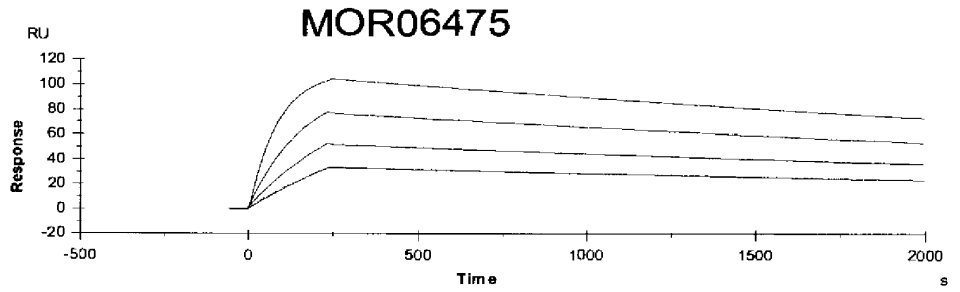

MOR06475
RU
Response
Time
s

Fig. 26C

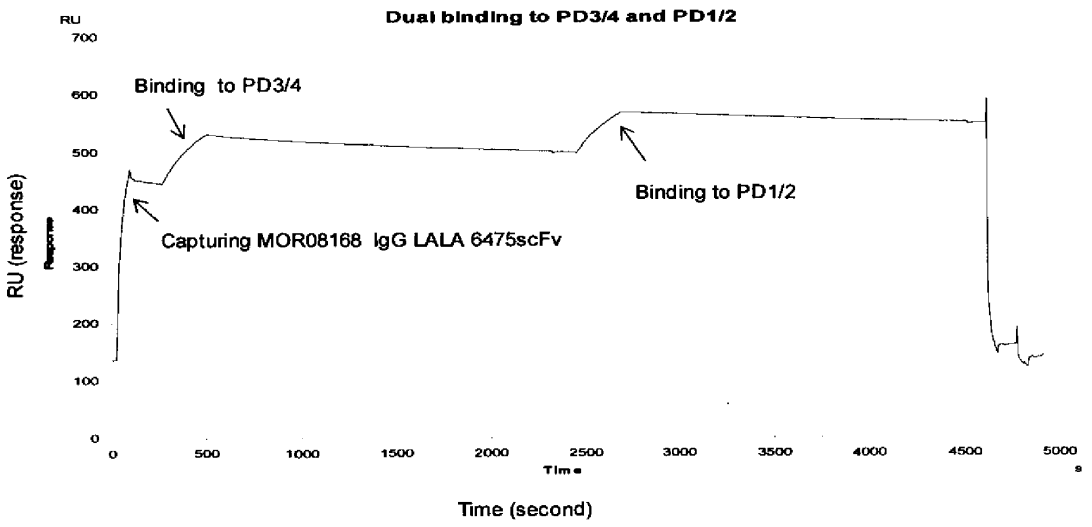
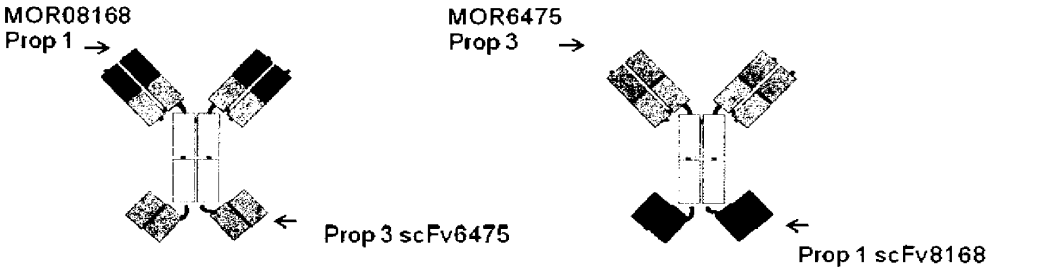

Dual binding to PD3/4 and PD1/2
RU
700
600
500
400
300
200
100
0
RU (response)
Binding to PD3/4
Capturing MOR08168 IgG LALA 6475scFv
Binding to PD1/2
0
500
1000
1500
2000
2500
3000
3500
4000
4500
5000
Time
Time (second)

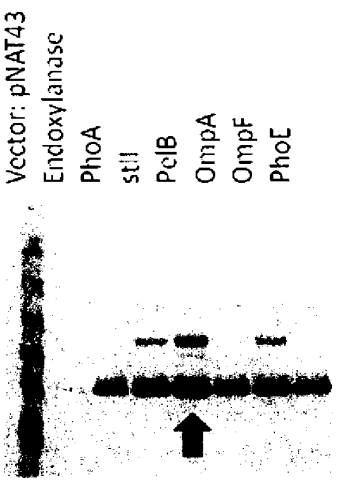
Fig. 27



901-MOR8168 IgG1LALA 6475scFv 902-MOR6475 IgG1 LALA 8168scFv

Fig. 28

a. Effect of leader sequences



b. Effect of Bacterial strains and constructs

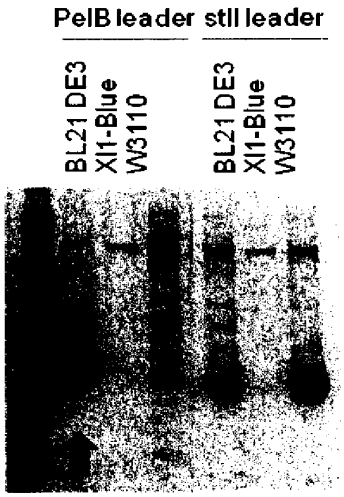


Fig. 29

Tm of single point mutations in scFv06475

Construct ID	mutation[a]	T_m on protein from E.coli	T_m on protein from mammalian
scFv06475 WT		59	61
6475-S2	VH:G34V	61	64
6475-S3	VH:I37F	61	64.5
6475-S6	VH:V85E	60	62.5
6475-S8	VH:M95F	61.5	64.5
6475-S9	VL:D93N	59.5	60.5

[a]Both kabat and Chothia numbering system have been used. The numbering is the same for all mutations but VH: G34V in scFv6475. This would be VH: G32V in Chothia numbering system. The numbering system in the text is Kabat system

Fig. 30

Tm of single point mutations in scFv08168

Construct ID	mutation	T_m on protein from E.coli [a]
WT	WT	48.50
B02	VH:V033N	50.50
B03-S1	VH:I034M	56.00
B04	VH:I034F	52.50
C05	VH:S049A	54.00
C07	VH:G050S	51.50
C08	VH:W052aG	55.50
C10	VH:H058Y	52.00
F11	VL:V047L	51.00
G02	VL:G064V	50.50
G07	VL:T078V	51.00

[a]Proteins were expressed from Acella strain .Samples were analyzed by DSF without removal of imidazole.

Fig. 31

Tm of single vs double mutations in scFv08168

Construct ID	Mutation	T_m on protein from E.coli[a]	T_m on protein from mammalian[b]	T_m on protein from mammalian
scFv08168 WT	WT	48.50	49	49
scFv08168 B02	VH:V33N	50.50		
scFv08168 B03-S1	VH:I34M	56.00	57	56.5
scFv08168 B04	VH:I34F	52.50		
scFv08168 C05	VH:S49A	54.00		
scFv08168 C07	VH:G50S	51.50		
scFv08168 C10	VH:H58Y	52.00		
scFv08168 F11	VL:V47L	51.00		
scFv08168 G02	VL:G64V	50.50		
scFv08168 G07	VL:T78V	51.00		
scFv08168 D1	VH S49A, I34M	61	61.5	62.5
scFv08168 D2	VH S49A, I34F	57.5	58	58.5
scFv08168 D4	**VH I34M, G50S**	**59.5**	**59.5**	**60**
scFv08168 D5	**VH I34M, H58Y**	**59**	**59**	**59**
scFv08168 D6	VH I34M, V48I	57	56	57.5
scFv08168 D7	VH I34M, VL S22T	57	57	58
scFv08168 D8	VH I34M, VL V47L	57.5	56	58.5
scFv08168 D9	VH I34M, VL G64V	57.5	58.5	57.5

[a]Proteins were expressed from Acella strain. Samples were analyzed by DSF without removal of imidazole
[b]Protein expressed from 293T suspension cells. Samples were analyzed by DSF without removal of imidazole

Fig. 32

Activity of scFv06475, scFv08168 and the variants in different assays

Construct ID	Mutations	EC50 by ELISA nM	Affinity by Proteon nM	IC50 by STF assay for Wnt1 inhibition (nM)	IC50 by STF assay for Wnt3a inhibition (nM)
scFv06475 WT		0.76	-	-	1.48
scFv06475-S2	VH:G34V	27.4	-	-	-
scFv06475-S3	VH:I37F	4.3	-	-	-
scFv06475-S6	VH:V85E	0.73	-	-	1.33
scFv06475-S8	**VH:M95F**	**1.0**	-	-	**0.96**
scFv06475-S9	VL:D93N	0.88	-	-	
scFv08168 WT		2.04	3.82	7.41	-
scFv08168 B03-S1	VH:I34M	0.98	-	5.19	-
scFv08168-D1	**VH S49A, I34M**	**1.61**	**2.55**	**2.44**	-
scFv08168-D2	VH S49A, I34F	1.68	-	2.59	-
scFv08168-D4	VH I34M, G50S	1.47	-	5.56	-
scFv08168-D5	VH I34M, H58Y	1.22	-	-	-
scFv08168-D6	VH I34M, V48I	1.24	-	0.74	-
scFv08168-D7	VH I34M, VL S22T	1.15	-	4.81	-
scFv08168-D8	VH I34M, VL V47L	0.91	-	-	-
scFv08168-D9	VH I34M, VL G64V	1.26	-	11.11	-

Fig. 33

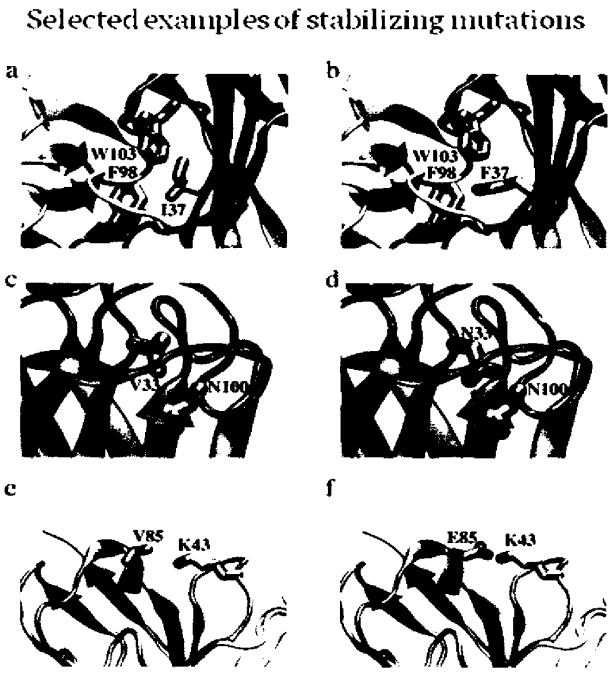

Selected examples of stabilizing mutations
a
W103
F98
E37
b
W103
F98
F37
c
V33
N100
d
N33
N100
e
V85
K43
f
E85
K43

Fig. 34

Thermostability measurement of HSA fusion and IgG based biparatopic molecules		
	T_m by DSF (ProteoSTAT)	T_m by DSC
MOR6475 IgG1-scFv8168 (902wt)	47, 72.5	ND
MOR6475 IgG1-scFv8168 (902 mutant)	62, 76	ND

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2011/057200

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K16/28 A61K39/395 A61P35/00 C07K16/46
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B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2009/056634 A2 (NOVARTIS AG [CH]; CONG FENG [US]; RANGWALA SHAMINA M [US]; ETTENBERG S) 7 May 2009 (2009-05-07) whole document, especially Examples 1, 3, 4, and paragraphs [00177-00178] -----	1-28, 32-48, 50, 52-68, 70-72, 75-77

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
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Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Luyten, Kattie

1

Form PCT/ISA/210 (second sheet) (April 2005)

MS&D Exhibit 1008, Page 3582 of 4223

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	S. A. ETTENBERG ET AL: "Inhibition of tumorigenesis driven by different Wnt proteins requires blockade of distinct ligand-binding regions by LRP6 antibodies", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, vol. 107, no. 35, 31 August 2010 (2010-08-31), pages 15473-15478, XP55002447, ISSN: 0027-8424, DOI: 10.1073/pnas.1007428107 the whole document -----	1-77

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2011/057200

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2009056634 A2	07-05-2009	AU 2008320823 A1	07-05-2009
		CA 2700433 A1	07-05-2009
		CN 101951954 A	19-01-2011
		EP 2209491 A2	28-07-2010
		JP 2011503025 A	27-01-2011
		KR 20100091170 A	18-08-2010
		US 2010254980 A1	07-10-2010

Form PCT/ISA/210 (patent family annex) (April 2005)

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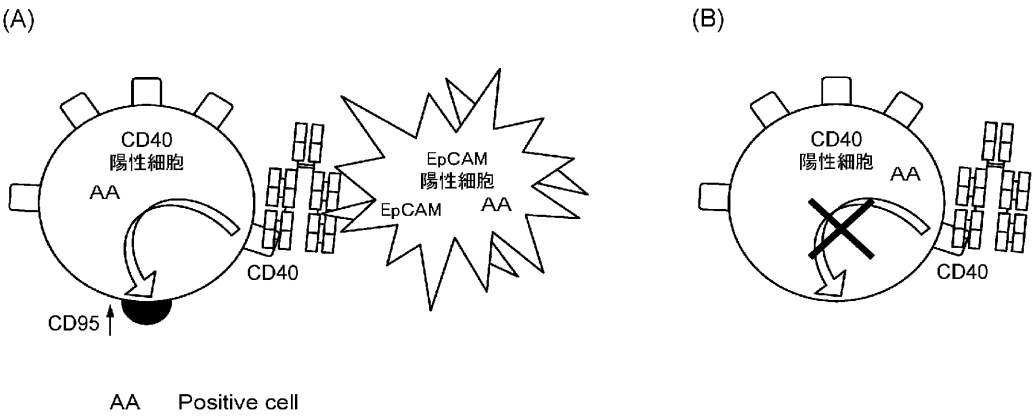
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(54) **Title:** BISPECIFIC ANTIBODY WHICH BINDS TO CD40 AND EpCAM

(54) 発明の名称： ＣＤ４０とＥｐＣＡＭに結合するバイスペシフィック抗体



(57) **Abstract:** The purpose of the present invention is to provide a bispecific antibody containing an antigen-binding domain which binds to CD40 and an antigen-binding domain which binds to the Epithelial Cell Adhesion Molecule (EpCAM). This invention pertains to a bispecific antibody containing an antigen-binding domain which binds to CD40 and an antigen-binding domain which binds to EpCAM.

(57) 要約：本発明は、ＣＤ４０に結合する抗原結合ドメインおよびＥｐｉｔｈｅｒｉａｌ Ｃｅｌｌ Ａｄｈｅｓｉｏｎ Ｍｏｌｅｃｕｌｅ（ＥｐＣＡＭ）に結合する抗原結合ドメインを含むバイスペシフィック抗体を提供することを課題とする。本発明は、ＣＤ４０に結合する抗原結合ドメインおよびＥｐＣＡＭに結合する抗原結合ドメインを含むバイスペシフィック抗体に関する。

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明　細　書

発明の名称 ：

ＣＤ４０とＥｐＣＡＭに結合するバイスペシフィック抗体

技術分野

[0001] 本発明は、ＣＤ４０に結合する抗原結合ドメインおよびＥｐｉｔｈｅｒｉａｌ　Ｃｅｌｌ　Ａｄｈｅｓｉｏｎ　Ｍｏｌｅｃｕｌｅ（ＥｐＣＡＭ）に結合する抗原結合ドメインを含むバイスペシフィック抗体、該バイスペシフィック抗体断片、該バイスペシフィック抗体または該バイスペシフィック抗体断片をコードするＤＮＡ、該ＤＮＡを含むベクター、該バイスペシフィック抗体または該バイスペシフィック抗体断片を生産するハイブリドーマおよび形質転換株、該バイスペシフィック抗体または該バイスペシフィック抗体断片の製造方法、該バイスペシフィック抗体または該バイスペシフィック抗体断片を含む治療および診断剤、該バイスペシフィック抗体または該バイスペシフィック抗体断片を用いる治療および診断方法、ならびに該バイスペシフィック抗体または該バイスペシフィック抗体断片を含む検出または測定試薬に関する。

背景技術

[0002] 抗体は、あらゆる哺乳動物の血清や組織体液中に存在する糖タンパク質であり、生体内において外来抗原を認識する。抗体は、補体系の活性化や、細胞表面に存在するレセプター（ＦｃＲ）への結合を介して、ＦｃＲ発現細胞の貪食能、抗体依存性細胞傷害能、メディエーターの遊離能、及び抗原提示能といったエフェクター機能を活性化し、生体防御に関与する。

[0003] １分子の抗体は、２本の相同な軽鎖（Ｌ鎖）と２本の相同な重鎖（Ｈ鎖）とから成り、２つの抗原結合部位を備える。抗体のクラス及びサブクラスはＨ鎖によって決定され、各クラス及びサブクラスは異なる固有の機能を有する。ヒトの抗体には、５つの異なるクラス、ＩｇＧ、ＩｇＡ、ＩｇＭ、ＩｇＤ及びＩｇＥが存在する。ＩｇＧはさらにＩｇＧ１、ＩｇＧ２、ＩｇＧ３及

びＩｇＧ４のサブクラスに、ＩｇＡは、ＩｇＡ１及びＩｇＡ２のサブクラスに各々分類される（Charles A. J. et. al., Immunobiology, 1997, Current Biology Ltd/Garland Publishing Inc.）。

[0004] 多価抗体は、１分子内に複数の抗原結合部位を備えた抗体である。多価抗体の例としては、まず、ハイブリッドハイブリドーマを用いて、二種類の異なる抗体由来のＨ鎖およびＬ鎖を１つの細胞で発現させることにより、異なる二種類の抗原にそれぞれ一価で結合する二価抗体を作製したことが報告された。（非特許文献１）。しかしながら、本法では、抗体のＨ鎖とＬ鎖との組み合わせが１０通り程度生じる。そのため、所望のＨ鎖とＬ鎖との組み合わせを有する多価抗体の生成量は低く、またそのような多価抗体を選択的に単離精製することも難しいため、所望の抗体の収量は減少する。

[0005] この問題点を克服するため、複数の抗原結合部位を連結して単一のポリペプチド鎖として発現することにより、サブユニット間の組み合わせのバリエーションを減らし、所望の組み合わせを有する抗体を生産する試みが報告されている。

[0006] 一例として、Ｈ鎖とＬ鎖の抗原結合部位を１つのポリペプチドで連結したｓｉｎｇｌｅ　ｃｈａｉｎ　Ｆｖ（ｓｃＦｖ）を含む抗体（非特許文献２）が知られている。さらに、ＩｇＧ１のＨ鎖定常領域のＣＨ１ドメインもしくは該ドメインの部分断片及びＬ鎖定常領域、又はフレキシブルリンカー（Ｇｌｙ－Ｇｌｙ－Ｇｌｙ－Ｇｌｙ－Ｓｅｒ）を用いて、二つの抗原結合部位を連結させた抗体などが報告されている（非特許文献４、特許文献１、特許文献２）。

[0007] これらの従来の多価抗体は、凝集し易く、安定性や生産性が低いという欠点を有していた。しかし一方で、単一のＨ鎖ポリペプチド中に複数の抗原結合部位を有し、抗体重鎖可変領域がイムノグロブリンドメインまたはその断片のアミノ酸配列を有するリンカーを介して結合している多価抗体は、安定性が高く、生産性も良いことが見出されている（特許文献３）。

[0008] ＣＤ４０は、ヒトＢ細胞表面に発現する抗原として同定され（非特許文献

４）、ＴＮＦ受容体ファミリーのメンバーの１つとして知られる。ＴＮＦ受容体ファミリー分子は、細胞外ドメインのシステインリッチリピートの存在により定義される。ＴＮＦ受容体ファミリーのメンバーとしては、ＣＤ４０の他に、例えば低親和性ＮＧＦレセプター、ＴＮＦレセプター、ＣＤ２７、ＯＸ４０およびＣＤ３０などが知られている。

[0009] ＴＮＦ受容体ファミリーを介したシグナルは、ホモ三量体のＴＮＦファミリー分子が３つのＴＮＦ受容体ファミリーに結合することにより誘導されるが、ＴＮＦ受容体ファミリー分子に特異的な抗体同士を架橋（クロスリンク）することによっても、細胞内にシグナルが伝達されることから、シグナル伝達にはＴＮＦ受容体ファミリー分子の会合が必要であると考えられている（非特許文献５、６）。

[0010] ＣＤ４０はタイプＩの膜型糖タンパク質であり、Ｂリンパ球、樹状細胞、単球上皮細胞および線維芽細胞などの多様な細胞タイプや、腫瘍性のヒトＢ細胞などのある種の腫瘍細胞にも発現していることが知られている。ＣＤ４０欠損マウスでは、胸腺依存の免疫グロブリンのクラススイッチや胚中心の形成が損なわれることが確認されており、細胞性と液性免疫反応でのＣＤ４０の重要な役割が実証されている（非特許文献７）。

[0011] ＣＤ４０のシグナルは免疫グロブリンのクラススイッチやＣＴＬの誘導に関与することから、腫瘍免疫の活性化やがんワクチンのアジュバントとしての医薬品への応用も期待されている（非特許文献８）。

[0012] 既存のＣＤ４０を標的とした抗体としては、Ｃｈｉ－Ｌｏｂ　７／４、ＨＣＤ－１２２、ＡＰＸ００５Ｍ、ＳＥＡ－ＣＤ４０およびＣＰ８７０，８９３（２１．４．１）等を挙げることができる（非特許文献９）。その中で、ＣＰ８７０，８９３は強力なＣＤ４０シグナル誘導能をもっており、固形腫瘍に対して全身性の免疫活性化を薬効メカニズムとする臨床試験が実施された。しかし、有効性を示すことができておらず、サイトカインシンドローム、血栓マーカーの上昇、肝臓パラメータの上昇など全身的な免疫活性化に由来する毒性の発現が報告されている（非特許文献１０）。

[0013] ＣＤ４０の生理学的リガンドはＣＤ４０　Ｌｉｇａｎｄ（ＣＤ１５４、ｇｐ３９）である。活性化したＴリンパ球にはＣＤ４０　Ｌｉｇａｎｄが発現しておりＢリンパ球の表面上のＣＤ４０との結合を介してＢリンパ球の分化や増殖、胚中心のＢリンパ球の自発的アポトーシスからの回避など重要な調節機構を担っている（非特許文献１１）。

[0014] ＣＤ４０のシグナル誘導能をもつ分子として、ＣＤ４０　Ｌｉｇａｎｄに抗体のＦｃ領域を融合させた分子が報告されている。さらに、腫瘍での効率的なＣＤ４０シグナルの誘導を目指してＣＤ４０　Ｌｉｇａｎｄとがん抗原に対する一本鎖抗体ｓｃＦｖの融合抗体が作製され、ＣＤ４０　Ｌｉｇａｎｄよりも２０倍程度低用量でＣＤ４０シグナルを誘導すると報告されている（非特許文献１２）。

[0015] ＣＤ４０を認識するバイスペシフィック抗体として、ヘテロ二量化した重鎖を有するＩｇＧ型抗ヒトＧＰＣ３／抗マウスＣＤ４０バイスペシフィック抗体が知られている（特許文献４）。また、ｎｅｃｔｉｎ－４、ＰＳＭＡまたはＥＧＦＲなどのがん抗原およびＣＤ４０に結合し、ＣＤ４０を活性化させるバイスペシフィックタンパク質が知られている（特許文献５）。さらに、ＣＤ４０およびがん細胞表面抗原に特異性を有するｄｉａｂｏｄｙなどのバイスペシフィック分子により、がん細胞近傍でＣＤ４０発現細胞を活性化させる方法が知られている（特許文献６）。

[0016] Ｅｐｉｔｈｅｌｉａｌ　ｃｅｌｌ　ａｄｈｅｓｉｏｎ　ｍｏｌｅｃｕｌｅ（ＥｐＣＡＭ、ＣＤ３２６、ＧＡ７３３－２、ＨＥＡ１２５、ＫＳ１／４、ＭＫ－１、ＭＨ９９、ＭＯＣ３１、３２３／Ａ３、１７－１Ａ、ＣＯ－１７Ａ、ＥＳＡ、ＥＧＰ－２、ＥＧＰ３４、ＥＧＰ４０、ＫＳＡ、ＫＳ１／４、ＴＲＯＰ－１、ＴＡＣＳＴ－１）は、細胞接着や細胞増殖、腫瘍の進行に関与する４０ｋＤａの膜貫通型膜糖タンパク質で、ホモタイプの細胞接着因子として機能する。

[0017] ＥｐＣＡＭは上皮由来の多くのがん細胞で高発現していることから、がん抗原として知られており、がん分子標的医薬品の標的分子や診断マーカー、

がんワクチン標的として期待されている（非特許文献１３、１４）。

[0018] ＥｐＣＡＭを標的とする医薬の例としては、ＥｐＣＡＭタンパク質や抗ＥｐＣＡＭ抗体の抗原認識部位に結合する抗イディオタイプ抗体を用いたワクチン療法、Ａｄｅｃａｔｕｍｕｍａｂ（ＭＴ２０１）、ＩＮＧ－１、３６２２Ｗ９４等の抗ＥｐＣＡＭ抗体が報告されている（非特許文献１５）。

[0019] さらに、抗ＥｐＣＡＭ抗体による細胞傷害活性を高める目的で、抗ＥｐＣＡＭ抗体と緑膿菌外毒素を融合させたＰｒｏｘｉｎｉｕｍｓ Ｖｉｖｅｎｄｉｕｍｓ（ＶＢ４－８４５）や、ＩＬ－２と融合させたＥＭＤ ２７３ ０６６（ｈｕＫＳ－ＩＬ２）が開発されている。抗ＣＤ３抗体を有することでＴ細胞とＥｐＣＡＭ陽性腫瘍細胞を架橋して腫瘍細胞を傷害するバイスペシフィック抗体Ｃａｔｕｍａｘｏｍａｂ（Ｒｅｍｏｖａｂ）は、欧州で承認されている（非特許文献１４、１６）。

先行技術文献

特許文献

[0020] 特許文献1：米国特許出願公開第２００７／００７１６７５号明細書

特許文献2：国際公開第２００１／０７７３４２号

特許文献3：国際公開第２００９／１３１２３９号

特許文献4：国際公開第２０１５／１５６２６８号

特許文献5：国際公開第２０１７／２０５７３８号

特許文献6：国際公開第９９／６１０５７号

非特許文献

[0021] 非特許文献1：Suresh et. al., Methods Enzymol. 121, 210-228, 1986

非特許文献2：Kranz et. al., J. Hematother. 5, 403-408, 1995

非特許文献3：Wu et. al., Nat. Biothech. 25, 1290-1297, 2007

非特許文献4：Clark et. al., PNAS USA 1986; 83:4494-4498

非特許文献5：Chuntharapai A et. al., J. Immunol. 166(8), 4891, 2001

非特許文献6：Ashkenazi A et. al., Nat Rev Cancer 2, 420, 2002

非特許文献7：Kawabe et. al., Immunity 1994; 1:167-168

非特許文献8：Diehl L et. al.、Nat Med. 1999 Jul;5(7):774-9.

非特許文献9：Beatty GL et. al., Expert Rev Anticancer Ther. 2017 Feb; 17(2):175-186.

非特許文献10：Vonderheide RH et. al., Oncoimmunology. 2013 Jan 1;2(1) :e23033.

非特許文献11：Bridges et. al., J Immumol 1987; 139:4242-4549

非特許文献12：1541376565889_0 et. al., Mol Cancer. 2014 Apr 17;13:85

非特許文献13：van der Gun BTet. al., Br J Cancer. 2011 Jul 12;105(2): 312-9.

非特許文献14：Chaudry MA et. al., Br J Cancer. 2007 Apr 10;96(7):1013 -9.

非特許文献15：Markus M et. al., Cancer Cell Int. 2010 Nov 2;10:44.

非特許文献16：Linke R et. al., MAbs. 2010, Vol.2(2), pp.129-136.

発明の概要

発明が解決しようとする課題

[0022] ＣＤ４０およびＥｐＣＡＭに対するバイスペシフィック抗体とその抗腫瘍効果は知られていない。したがって、本発明は、ＣＤ４０に結合する抗原結合ドメインおよびＥｐＣＡＭに結合する抗原結合ドメインを含むバイスペシフィック抗体、該バイスペシフィック抗体断片、該バイスペシフィック抗体または該バイスペシフィック抗体断片をコードするＤＮＡ、該ＤＮＡを含むベクター、該バイスペシフィック抗体または該バイスペシフィック抗体断片を生産するハイブリドーマおよび形質転換株、該バイスペシフィック抗体または該バイスペシフィック抗体断片の製造方法、該バイスペシフィック抗体または該バイスペシフィック抗体断片を含む治療および診断剤、該バイスペシフィック抗体または該バイスペシフィック抗体断片を用いる治療および診断方法、ならびに該バイスペシフィック抗体または該バイスペシフィック抗体断片を含む検出または測定試薬を提供することを目的とする。

課題を解決するための手段

[0023] 上記課題を解決するための手段として、本発明はＣＤ４０に結合する抗原結合ドメインおよびＥｐＣＡＭに結合する抗原結合ドメインを含むバイスペシフィック抗体または該バイスペシフィック抗体断片などを提供する。

[0024] すなわち本発明は、以下の（１）～（３５）に関する。

（１）ＣＤ４０に結合する抗原結合ドメインおよびＥｐｉｔｈｅｒｉａｌ ｃｅｌｌ ａｄｈｅｓｉｏｎ Ｍｏｌｅｃｕｌｅ（ＥｐＣＡＭ）に結合する抗原結合ドメインを含むバイスペシフィック抗体。

（２）ＣＤ４０およびＥｐＣＡＭにそれぞれ二価で結合する（１）記載のバイスペシフィック抗体。

（３）ＣＤ４０に結合する抗原結合ドメインがＣＤ４０に特異的に結合する抗体（抗ＣＤ４０抗体）の重鎖可変領域（ＶＨ）および軽鎖可変領域（ＶＬ）を含み、ＥｐＣＡＭに結合する抗原結合ドメインがＥｐＣＡＭに特異的に結合する抗体（抗ＥｐＣＡＭ抗体）のＶＨおよびＶＬを含む（１）または（２）記載のバイスペシフィック抗体。

（４）Ｎ末端から順にＶＨ１－Ｘ－ＶＨ２－Ｙの式

｛式中、

ＶＨ１は第一の抗体のＶＨを表し、

ＶＨ２は第二の抗体のＶＨを表し、

ＸおよびＹはそれぞれ抗体のＣＨ１を含むポリペプチド（ここでＸおよびＹの少なくとも一方はさらに抗体のヒンジ領域を含む）を表す｝

で表されるポリペプチドを含む同一の２本の重鎖、

および同一のＶＬを含む４本の軽鎖を有し、

該第一の抗体および該第二の抗体のいずれか一方が抗ＣＤ４０抗体であり、もう一方が抗ＥｐＣＡＭ抗体である（１）～（３）のいずれか１記載のバイスペシフィック抗体。

（５）軽鎖がκ鎖である（４）記載のバイスペシフィック抗体。

（６）前記式におけるＸがヒトＩｇＧのＣＨ１からなるポリペプチドであり、ＹがヒトＩｇＧのＣＨ１、ヒンジ領域、ＣＨ２およびＣＨ３からなるポリ

ペプチドである（４）または（５）記載のバイスペシフィック抗体または該バイスペシフィック抗体断片。

（７）前記式におけるＸが配列番号７５で表されるアミノ酸配列を含むポリペプチドであり、Ｙが配列番号７７で表されるアミノ酸配列を含むポリペプチドである（４）または（５）記載のバイスペシフィック抗体。

（８）前記式におけるＸがヒトＩｇＧのＣＨ１、ヒンジ領域、ＣＨ２およびＣＨ３を含むポリペプチドであり、Ｙがヒトｌｇ Ｇ のＣＨ１を含むポリペプチドである（４）または（５）記載のバイスペシフィック抗体。

（９）前記式におけるＸが配列番号７７で表されるアミノ酸配列を含むポリペプチドであり、Ｙが配列番号７５で表されるアミノ酸配列を含むポリペプチドである（４）または（５）記載のバイスペシフィック抗体。

（１０）ＶＬが、それぞれ配列番号２３～２５で表されるアミノ酸配列を含む相補性決定領域（ＣＤＲ）１～３を含むＶＬである（３）～（９）のいずれか１に記載のバイスペシフィック抗体。

（１１）ＶＬが、配列番号２２で表されるアミノ酸配列を含むＶＬである（３）～（１０）のいずれか１に記載のバイスペシフィック抗体。

（１２）ＣＤ４０アゴニスト活性を有する、（１）～（１１）のいずれか１に記載のバイスペシフィック抗体。

（１３）ＥｐＣＡＭを発現する細胞の非存在下ではＣＤ４０アゴニスト活性を示さず、ＥｐＣＡＭを発現する細胞の存在下でのみＣＤ４０アゴニスト活性を示す、（１）～（１２）のいずれか１に記載のバイスペシフィック抗体。

（１４）抗ＣＤ４０抗体がアゴニスト活性を有していない抗ＣＤ４０抗体である（３）～（１３）のいずれか１に記載のバイスペシフィック抗体。

（１５）第一の抗体が抗ＣＤ４０抗体であり、第二の抗体が抗ＥｐＣＡＭ抗体である（４）～（１４）のいずれか１に記載のバイスペシフィック抗体。

（１６）第一の抗体が抗ＥｐＣＡＭ抗体であり、第二の抗体が抗ＣＤ４０抗体である（４）～（１４）のいずれか１に記載のバイスペシフィック抗体。

（１７）抗ＣＤ４０抗体が、以下の（１ａ）～（１ｄ）からなる群から選ばれるいずれか１つの重鎖可変領域（ＶＨ）を含む抗ＣＤ４０抗体である、（３）～（１６）のいずれか１に記載のバイスペシフィック抗体。

（１ａ）それぞれ配列番号２８～３０で表されるアミノ酸配列を含むＣＤＲ１～３を含むＶＨ、

（１ｂ）それぞれ配列番号３３～３５で表されるアミノ酸配列を含むＣＤＲ１～３を含むＶＨ、

（１ｃ）それぞれ配列番号３８～４０で表されるアミノ酸配列を含むＣＤＲ１～３を含むＶＨ、および

（１ｄ）それぞれ配列番号４３～４５で表されるアミノ酸配列を含むＣＤＲ１～３を含むＶＨ。

（１８）抗ＣＤ４０抗体が、配列番号２７、３２、３７および４２のいずれか１つで表されるアミノ酸配列を含むＶＨを含む抗ＣＤ４０抗体である（３）～（１７）のいずれか１に記載のバイスペシフィック抗体。

（１９）抗ＥｐＣＡＭ抗体が、以下の（２ａ）～（２ｄ）からなる群から選ばれるいずれか１つのＶＨを含む抗ＥｐＣＡＭ抗体である、（３）～（１８）のいずれか１に記載のバイスペシフィック抗体。

（２ａ）それぞれ配列番号５２～５４で表されるアミノ酸配列を含むＣＤＲ１～３を含むＶＨ、

（２ｂ）それぞれ配列番号５７～５９で表されるアミノ酸配列を含むＣＤＲ１～３を含むＶＨ、

（２ｃ）それぞれ配列番号６２～６４で表されるアミノ酸配列を含むＣＤＲ１～３を含むＶＨ、および

（２ｄ）それぞれ配列番号６７～６９で表されるアミノ酸配列を含むＣＤＲ１～３を含むＶＨ。

（２０）抗ＥｐＣＡＭ抗体が、配列番号５１、５６、６１および６６のいずれか１つで表されるアミノ酸配列を含むＶＨを含む抗ＥｐＣＡＭ抗体である（３）～（１９）のいずれか１に記載のバイスペシフィック抗体。

（２１）（１）～（２０）のいずれか１に記載のバイスペシフィック抗体のバイスペシフィック抗体断片。

（２２）Ｆａｂ型バイスペシフィック抗体断片またはＦ（ａｂ’）２型バイスペシフィック抗体断片である（２１）に記載のバイスペシフィック抗体断片

（２３）（１）～（２０）のいずれか１に記載のバイスペシフィック抗体または（２１）または（２２）に記載のバイスペシフィック抗体断片をコードするＤＮＡ。

（２４）（２３）に記載のＤＮＡを含有する組換え体ベクター。

（２５）（２４）に記載の組換え体ベクターを宿主細胞に導入して得られる形質転換株。

（２６）（２５）に記載の形質転換株を培地に培養し、培養物中に（１）～（２０）のいずれか１に記載のバイスペシフィック抗体または（２１）もしくは（２２）に記載のバイスペシフィック抗体断片を生産蓄積させ、該培養物から該バイスペシフィック抗体または該バイスペシフィック抗体断片を採取することを特徴とする（１）～（２０）のいずれか１に記載のバイスペシフィック抗体または（２１）もしくは（２２）に記載のバイスペシフィック抗体断片の製造方法。

（２７）（１）～（２０）のいずれか１に記載のバイスペシフィック抗体または（２１）もしくは（２２）に記載のバイスペシフィック抗体断片を有効成分として含有する、ヒトＣＤ４０およびヒトＥｐＣＡＭの少なくとも一方が関係する疾患の治療および／または診断剤。

（２８）ヒトＣＤ４０およびヒトＥｐＣＡＭの少なくとも一方が関係する疾患ががんである（２７）に記載の剤。

（２９）（１）～（２０）のいずれか１に記載のバイスペシフィック抗体または（２１）もしくは（２２）に記載のバイスペシフィック抗体断片を用いる、ヒトＣＤ４０およびヒトＥｐＣＡＭの少なくともいずれか一方が関係する疾患の治療および／または診断方法。

（３０）ヒトＣＤ４０およびヒトＥｐＣＡＭの少なくとも一方が関係する疾患ががんである（２９）に記載の方法。

（３１）ヒトＣＤ４０およびヒトＥｐＣＡＭの少なくとも一方が関係する疾患の治療および／または診断に使用するための、（１）～（２０）のいずれか１に記載のバイスペシフィック抗体または（２１）もしくは（２２）に記載のバイスペシフィック抗体断片。

（３２）ヒトＣＤ４０およびヒトＥｐＣＡＭの少なくとも一方が関係する疾患ががんである（３１）に記載のバイスペシフィック抗体または（２１）もしくは（２２）に記載のバイスペシフィック抗体断片。

（３３）ヒトＣＤ４０およびヒトＥｐＣＡＭの少なくとも一方が関係する疾患の治療および／または診断剤の製造のための、（１）～（２０）のいずれか１に記載のバイスペシフィック抗体または（２１）もしくは（２２）に記載のバイスペシフィック抗体断片の使用。

（３４）ヒトＣＤ４０およびヒトＥｐＣＡＭの少なくとも一方が関係する疾患ががんである（３３）に記載の使用。

（３５）（１）～（２０）のいずれか１に記載のバイスペシフィック抗体または（２１）もしくは（２２）に記載のバイスペシフィック抗体断片を含む、ＥｐＣＡＭおよびＣＤ４０の少なくとも一方を検出または測定するための試薬。

発明の効果

[0025] 本発明により、ＣＤ４０に結合する抗原結合ドメインおよびＥｐＣＡＭに結合する抗原結合ドメインを含むバイスペシフィック抗体、該バイスペシフィック抗体断片、該バイスペシフィック抗体または該バイスペシフィック抗体断片をコードするＤＮＡ、該ＤＮＡを含むベクター、該バイスペシフィック抗体または該バイスペシフィック抗体断片を生産するハイブリドーマおよび形質転換株、該バイスペシフィック抗体または該バイスペシフィック抗体断片の製造方法、該バイスペシフィック抗体または該バイスペシフィック抗体断片を含む治療および診断剤、該バイスペシフィック抗体または該バイス

ペシフィック抗体断片を用いる治療および診断方法、ならびに該バイスペシフィック抗体または該バイスペシフィック抗体断片を含む検出または測定試薬を提供することができる。

図面の簡単な説明

[0026] [図1]図１は、本発明のバイスペシフィック抗体の構造の一例を示す。図１（Ａ）はＮ末端型バイスペシフィック抗体を表す。図１（Ｂ）はＣ末端型バイスペシフィック抗体を表す。

[図2]図２（Ａ）はＲａｍｏｓ細胞、図２（Ｂ）はＣｏｌｏ２０５細胞、図２（Ｃ）はＨＥＫ２９３細胞、図２（Ｄ）はヒトＥｐＣＡＭ／ＨＥＫ２９３細胞について、それぞれの細胞におけるＣＤ４０およびＥｐＣＡＭの発現をフローサイトメーターで評価した結果を示す。縦軸は細胞数を、横軸は蛍光強度を表す。破線は抗ＣＤ４０抗体２１．４．１の結合性、点線は抗ＥｐＣＡＭ抗体３６２２Ｗ９４の結合性、白抜きで示された線はコントロール抗体の抗ＤＮＰ抗体の結合性を表す。

[図3]図３（Ａ）および図３（Ｂ）は、抗ＣＤ４０抗体モノクローナル抗体のＣＤ４０シグナル誘導活性を、Ｒａｍｏｓ細胞上のＣＤ９５発現誘導を指標として評価した結果を示す。縦軸は、蛍光強度を示し、それぞれの抗体を１０、１または０．１μｇ／ｍＬ添加したときの、抗ＣＤ９５抗体のＲａｍｏｓ細胞に対する結合性を表す。比較対象にはネガティブコントロール抗体として抗ＤＮＰ抗体を、抗ＣＤ４０アゴニスト抗体として２１．４．１を用いた。

[図4]図４はＨＥＫ２９３細胞と共培養したＲａｍｏｓ細胞に対する、ＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体によるＣＤ４０シグナル誘導活性を示す。縦軸は蛍光強度を示し、それぞれの抗体を１０、１または０．１μｇ／ｍＬ添加したときの、抗ＣＤ９５抗体のＲａｍｏｓ細胞に対する結合性を表す。比較対象にはネガティブコントロール抗体として抗ＤＮＰ抗体、抗ＣＤ４０アゴニスト抗体として２１．４．１、抗ＥｐＣＡＭ抗体として３６２２Ｗ９４を用いた。

[図5]図５はヒトＥｐＣＡＭ／ＨＥＫ２９３細胞と共培養したＲａｍｏｓ細胞に対する、ＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体によるＣＤ４０シグナル誘導活性を示す。縦軸は蛍光強度を示し、それぞれの抗体を１０、１または０．１μｇ／ｍＬ添加したときの、抗ＣＤ９５抗体のＲａｍｏｓ細胞に対する結合性を表す。比較対象にはネガティブコントロール抗体として抗ＤＮＰ抗体、抗ＣＤ４０アゴニスト抗体として２１．４．１、抗ＥｐＣＡＭ抗体として３６２２Ｗ９４を用いた。

[図6]図６はＣｏｌｏ２０５細胞と共培養したＲａｍｏｓ細胞に対する、ＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体によるＣＤ４０シグナル誘導活性を示す。縦軸は蛍光強度を示し、それぞれの抗体を１０、１または０．１μｇ／ｍＬ添加したときの、抗ＣＤ９５抗体のＲａｍｏｓ細胞に対する結合性を表す。比較対象にはネガティブコントロール抗体として抗ＤＮＰ抗体、抗ＣＤ４０アゴニスト抗体として２１．４．１、抗ＥｐＣＡＭ抗体として３６２２Ｗ９４を用いた。

[図7]図７（Ａ）はＥｐＣＡＭ陽性細胞存在下での、ＣＤ４０陽性細胞に対する、ＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体の作用の模式図である。ＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体はＥｐＣＡＭ陽性細胞とＣＤ４０陽性細胞の両方に結合すると、ＣＤ４０陽性細胞にＣＤ４０シグナルを誘導する。その結果、ＣＤ４０陽性細胞上にＣＤ９５の発現が誘導される。図７（Ｂ）はＥｐＣＡＭ陽性細胞非存在下での、ＣＤ４０陽性細胞に対する、ＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体の作用の模式図である。ＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体はＣＤ４０陽性細胞にのみ結合したときにはＣＤ４０シグナルを誘導しない。

[図8]図８（Ａ）はＡｌｅｘａ６４７により蛍光標識したＨＥＲ２抗体によりＲａｍｏｓ細胞およびＣｏｌｏ２０５細胞を蛍光免疫染色した結果、図８（Ｂ）はＡｌｅｘａ４８８により蛍光標識したＣｔＲ１０９０Ｓ５５Ａ－Ｅｐ２０３によりＲａｍｏｓ細胞およびＣｏｌｏ２０５細胞を蛍光免疫染色した結果、図８（Ｃ）はこれらの重ね合わせを示す。矢印は強い蛍光が観察され

た箇所を示す。

[図9]図９（Ａ）～（Ｃ）はマウスを用いた毒性試験の結果を表す。図９（Ａ）の縦軸はマウスの体重（ｇ）を表す。図９（Ｂ）の縦軸はマウスの末梢血中のＡＳＴ（Ｕｎｉｔ／Ｌ）を表す。図９（Ｃ）の縦軸はマウスの末梢血中のＡＬＴ（Ｕｎｉｔ／Ｌ）を表す。それぞれの図中で灰色の棒グラフは投与前、黒色の棒グラフは投与２４時間後における測定値を示す。Ｒ１０９０（Ｎ）、Ｒ１０９０（Ｃ）およびＥｐｃ１１２（Ｃ）は、それぞれ、Ｒ１０９０Ｓ５５Ａ－Ｅｐ２０３、Ｃｔ　Ｒ１０９０Ｓ５５Ａ－Ｅｐ２０３およびＣｔ　Ｅｐｃ１１２－Ｒ１０６６を意味する。ネガティブコントロール抗体として抗ＤＮＰ抗体、抗ＣＤ４０アゴニスト抗体として２１．４．１を用いた。２１．４．１は１ｍｇ／ｋｇ、その他の抗体は１０ｍｇ／ｋｇを投与した。

[図10]図１０（Ａ）～（Ｄ）はマウスを用いた毒性試験の結果を示す。図１０（Ａ）は抗体投与２４時間後のマウスの白血球数を表す。図１０（Ｂ）は抗体投与２４時間後のマウスのリンパ球数を表す。図１０（Ｃ）は抗体投与２４時間後のマウスの単球数を表す。図１０（Ｄ）は抗体投与２４時間後のマウスの血小板数を表す。それぞれの図中においてグラフの縦軸はそれぞれの細胞の個数（1×10^3個）を表す。Ｒ１０９０（Ｎ）、Ｒ１０９０（Ｃ）およびＥｐｃ１１２（Ｃ）は、それぞれ、Ｒ１０９０Ｓ５５Ａ－Ｅｐ２０３、Ｃｔ　Ｒ１０９０Ｓ５５Ａ－Ｅｐ２０３およびＣｔ　Ｅｐｃ１１２－Ｒ１０６６を意味する。ネガティブコントロール抗体として抗ＤＮＰ抗体、抗ＣＤ４０アゴニスト抗体として２１．４．１を用いた。２１．４．１は１ｍｇ／ｋｇ、その他の抗体は１０ｍｇ／ｋｇを投与した。

[図11]図１１（Ａ）はｍＥｐＣＡＭ／Ｂ１６Ｆ１０を移植したマウスにおける、ＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体の抗腫瘍効果を評価した結果を示す。図１１（Ｂ）はＢ１６Ｆ１０を移植したマウスにおける、ＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体の抗腫瘍効果を評価した結果を示す。それぞれの図において縦軸は腫瘍体積（mm^3）、横軸は投与日を０日と

する日数を示す。ネガティブコントロール抗体として抗ＤＮＰ抗体、抗ＣＤ４０アゴニスト抗体として２１．４．１を用いた。２１．４．１は１ｍｇ／ｋｇ、その他の抗体は１０ｍｇ／ｋｇを投与した。

[図12]図１２（Ａ）はヘテロｌｇＧ型バイスペシフィック抗体の構造を表す。図１２（Ｂ）はＦａｂ型バイスペシフィック抗体断片の構造を表す。図１２（Ｃ）はＦ（ａｂ’）$_2$型バイスペシフィック抗体を表す。

[図13]図１３は、ヒトＥｐＣＡＭ／Ｅｘｐｉ２９３細胞と共培養したＲａｍｏｓ細胞に対する、ＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体によるＣＤ４０シグナル誘導活性を示す。横軸は蛍光強度を示し、それぞれの抗体を１μｇ／ｍＬ添加したときの、抗ＣＤ９５抗体のＲａｍｏｓ細胞に対する結合性を表す。比較対象にはネガティブコントロール抗体として抗ＤＮＰ抗体、抗ＣＤ４０アゴニスト抗体として２１．４．１を用いた。

[図14]図１４は、Ｅｘｐｉ２９３細胞と共培養したＲａｍｏｓ細胞に対する、ＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体によるＣＤ４０シグナル誘導活性を示す。横軸は蛍光強度を示し、それぞれの抗体を１μｇ／ｍＬ添加したときの、抗ＣＤ９５抗体のＲａｍｏｓ細胞に対する結合性を表す。比較対象にはネガティブコントロール抗体として抗ＤＮＰ抗体、抗ＣＤ４０アゴニスト抗体として２１．４．１を用いた。

[図15]図１５（Ａ）は、ヒトＥｐＣＡＭ／Ｅｘｐｉ２９３細胞と共培養したＲａｍｏｓ細胞に対する、ＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体断片によるＣＤ４０シグナル誘導活性を示す。図１５（Ｂ）は、Ｅｘｐｉ２９３細胞と共培養したＲａｍｏｓ細胞に対する、ＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体断片によるＣＤ４０シグナル誘導活性を示す。それぞれの図において、横軸は抗体濃度を示す。また縦軸は蛍光強度を示し、それぞれの抗体を添加したときの、抗ＣＤ９５抗体のＲａｍｏｓ細胞に対する結合性を表す。比較対象にはネガティブコントロール抗体として抗ＤＮＰ抗体、抗ＣＤ４０アゴニスト抗体として２１．４．１を用いた。

発明を実施するための形態

[0027] 本発明は、ＣＤ４０に結合する抗原結合ドメインおよびＥｐＣＡＭに結合する抗原結合ドメインを含むバイスペシフィック抗体または該バイスペシフィック抗体断片（以下、本発明のバイスペシフィック抗体または該バイスペシフィック抗体断片と記載する）に関する。

[0028] 本発明におけるＣＤ４０は、ＴＮＦ ｒｅｃｅｐｔｏｒ ｓｕｐｅｒｆａｍｉｌｙ ｍｅｍｂｅｒ ５（ＴＮＦＲＳＦ５）、Ｂｐ５０、ＣＤＷ４０、ＭＧＣ９０１３およびｐ５０と同義として使用される。ＣＤ４０としては例えば、ＮＣＢＩ（http://www.ncbi.nlm.nih.gov/）においてＧｅｎＢａｎｋ ａｃｃｅｓｓｉｏｎ Ｎｏ．ＮＰ＿００１２４１または配列番号６に示されるアミノ酸配列を含むヒトＣＤ４０、およびＧｅｎＢａｎｋ ａｃｃｅｓｓｉｏｎ Ｎｏ．ＸＰ＿００５５６９２７４または配列番号８に示されるアミノ酸配列を含むサルＣＤ４０などが挙げられる。また、例えば、配列番号６、ＧｅｎＢａｎｋ ａｃｃｅｓｓｉｏｎ Ｎｏ．ＮＰ＿００１２４１またはＧｅｎＢａｎｋ ａｃｃｅｓｓｉｏｎ Ｎｏ．ＸＰ＿００５５６９２７４に示されるアミノ酸配列において１つ以上のアミノ酸が欠失、置換または付加されたアミノ酸配列からなり、かつＣＤ４０の機能を有するポリペプチドが挙げられる。

[0029] 配列番号６、ＧｅｎＢａｎｋ ａｃｃｅｓｓｉｏｎ Ｎｏ．ＮＰ＿００１２４１ またはＧｅｎＢａｎｋ ａｃｃｅｓｓｉｏｎ Ｎｏ．ＸＰ＿００５５６９２７４に示されるアミノ酸配列と通常７０％以上、好ましくは８０％以上、さらに好ましくは９０％以上の相同性を有するアミノ酸配列を含むポリペプチド、最も好ましくは９５％、９６％、９７％、９８％および９９％以上の相同性を有するアミノ酸配列からなり、かつＣＤ４０の機能を有するポリペプチドも本発明のＣＤ４０に包含される。

[0030] 配列番号６、ＧｅｎＢａｎｋ ａｃｃｅｓｓｉｏｎ Ｎｏ．ＮＰ＿００１２４１またはＧｅｎＢａｎｋ ａｃｃｅｓｓｉｏｎ Ｎｏ．ＸＰ＿００５５６９２７４に示されるアミノ酸配列において１以上のアミノ酸残基が欠失、置換、または付加されたアミノ酸配列を有するポリペプチドは、部位特異的

変異導入法［Molecular Cloning,A Laboratory Manual,Second Edition, Cold Spring Harbor Laboratory Press (1989)、Current Protocols in Molecular Biology, John Wiley & Sons (1987-1997)、Nucleic Acids Research, 10, 6487 (1982)、Proc. Natl. Acad. Sci. USA, 79, 6409 (1982)、Gene, 34, 315 (1985)、Nucleic Acids Research, 13, 4431 (1985)、Proceeding of the National Academy of Sciences in USA, 82, 488 (1985)］などを用いて、例えば、配列番号６、ＧｅｎＢａｎｋ ａｃｃｅｓｓｉｏｎ Ｎｏ．ＮＰ＿００１２４１またはＧｅｎＢａｎｋ ａｃｃｅｓｓｉｏｎ Ｎｏ．ＸＰ＿００５５６９２７４に示されるアミノ酸配列をコードするＤＮＡに、部位特異的変異を導入することにより得ることができる。欠失、置換または付加されるアミノ酸の数は特に限定されないが、好ましくは１個～数十個、例えば、１～２０個、より好ましくは１個～数個、例えば、１～５個のアミノ酸である。

[0031] ＣＤ４０をコードする遺伝子としては、例えば、配列番号５またはＧｅｎＢａｎｋ ａｃｃｅｓｓｉｏｎ Ｎｏ．ＮＭ＿００１２５０に示されるヒトＣＤ４０の塩基配列、および配列番号７またはＧｅｎＢａｎｋ ａｃｃｅｓｓｉｏｎ Ｎｏ．ＸＭ＿０１１７６６９２２に示されるサルＣＤ４０の塩基配列などが挙げられる。また、例えば配列番号５またはＧｅｎＢａｎｋ ａｃｃｅｓｓｉｏｎ Ｎｏ．ＮＭ＿００１２５０に示される塩基配列において１以上の塩基が欠失、置換または付加された塩基配列からなり、かつＣＤ４０の機能を有するポリペプチドをコードするＤＮＡを含む遺伝子、配列番号５またはＧｅｎＢａｎｋ ａｃｃｅｓｓｉｏｎ Ｎｏ．ＮＭ＿００１２５０に示される塩基配列と好ましくは６０％以上の相同性を有する塩基配列、より好ましくは８０％以上の相同性を有する塩基配列、さらに好ましくは９５％以上の相同性を有する塩基配列からなり、かつＣＤ４０の機能を有するポリペプチドをコードするＤＮＡを含む遺伝子、並びに配列番号５またはＧｅｎＢａｎｋ ａｃｃｅｓｓｉｏｎ Ｎｏ．ＮＭ＿００１２５０に示される塩基配列からなるＤＮＡとストリンジェントな条件下でハイブリダイズするＤ

ＮＡからなり、かつＣＤ４０の機能を有するポリペプチドをコードするＤＮＡを含む遺伝子なども、本発明のＣＤ４０をコードする遺伝子に包含される。

[0032] ストリンジェントな条件下でハイブリダイズするＤＮＡとしては、例えば配列番号５またはＧｅｎＢａｎｋ ａｃｃｅｓｓｉｏｎ Ｎｏ．ＮＭ＿００１２５０に示される塩基配列を有するＤＮＡをプローブに用いた、コロニー・ハイブリダイゼーション法、プラーク・ハイブリダイゼーション法、サザンブロット・ハイブリダイゼーション法、またはＤＮＡマイクロアレイ法などにより得られるハイブリダイズ可能なＤＮＡを意味する。具体的には、ハイブリダイズしたコロニー若しくはプラーク由来のＤＮＡ、または該配列を有するＰＣＲ産物若しくはオリゴＤＮＡを固定化したフィルターまたはスライドガラスを用いて、０．７～１．０ｍｏｌ／Ｌの塩化ナトリウム存在下、６５℃にてハイブリダイゼーション［Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press (1989)、Current Protocols in Molecular Biology, John Wiley & Sons (1987-1997)、DNA Cloning 1: Core Techniques, A Practical Approach, Second Edition, Oxford University (1995)］を行った後、０．１～２倍濃度のＳＳＣ溶液（１倍濃度のＳＳＣ溶液の組成は、１５０ｍｍｏｌ／Ｌ塩化ナトリウム、１５ｍｍｏｌ／Ｌクエン酸ナトリウムよりなる）を用い、６５℃条件下でフィルターまたはスライドグラスを洗浄することにより同定できるＤＮＡを挙げることができる。ハイブリダイズ可能なＤＮＡとしては、例えば、配列番号５またはＧｅｎＢａｎｋ ａｃｃｅｓｓｉｏｎ Ｎｏ．ＮＭ＿００１２５０に示される塩基配列と好ましくは６０％以上の相同性を有するＤＮＡ、より好ましくは８０％以上の相同性を有するＤＮＡ、さらに好ましくは９５％以上の相同性を有するＤＮＡを挙げることができる。

[0033] 真核生物のタンパク質をコードする遺伝子の塩基配列には、しばしば遺伝子の多型が認められる。本発明において用いられる遺伝子内に、このような多型によって塩基配列に小規模な変異を生じた遺伝子も、本発明のＣＤ４０

をコードする遺伝子に包含される。

[0034] 本発明における相同性の数値は、特に明示した場合を除き、当業者に公知の相同性検索プログラムを用いて算出される数値であってよいが、塩基配列については、ＢＬＡＳＴ［J. Mol. Biol., 215, 403 (1990)］においてデフォルトのパラメータを用いて算出される数値など、アミノ酸配列については、ＢＬＡＳＴ２［Nucleic Acids Research,25, 3389 (1997)、Genome Research, 7, 649 (1997)、http://www.ncbi.nlm.nih.gov/Education/BLASTinfo/information3.html］においてデフォルトのパラメータを用いて算出される数値などが挙げられる。

[0035] デフォルトのパラメータとしては、Ｇ（Ｃｏｓｔ ｔｏ ｏｐｅｎ ｇａｐ）が塩基配列の場合は５、アミノ酸配列の場合は１１、－Ｅ（Ｃｏｓｔ ｔｏ ｅｘｔｅｎｄ ｇａｐ）が塩基配列の場合は２、アミノ酸配列の場合は１、－ｑ（Ｐｅｎａｌｔｙ ｆｏｒ ｎｕｃｌｅｏｔｉｄｅ ｍｉｓｍａｔｃｈ）が－３、－ｒ（ｒｅｗａｒｄ ｆｏｒ ｎｕｃｌｅｏｔｉｄｅ ｍａｔｃｈ）が１、－ｅ（ｅｘｐｅｃｔ ｖａｌｕｅ）が１０、－Ｗ（ｗｏｒｄｓｉｚｅ）が塩基配列の場合は１１残基、アミノ酸配列の場合は３残基、－ｙ［Ｄｒｏｐｏｆｆ（Ｘ）ｆｏｒ ｂｌａｓｔ ｅｘｔｅｎｓｉｏｎｓ ｉｎ ｂｉｔｓ］がｂｌａｓｔｎ の場合は２０、ｂｌａｓｔｎ以外のプログラムでは７、－Ｘ（Ｘ ｄｒｏｐｏｆｆ ｖａｌｕｅ ｆｏｒ ｇａｐｐｅｄ ａｌｉｇｎｍｅｎｔ ｉｎ ｂｉｔｓ）が１５および－Ｚ（ｆｉｎａｌ Ｘ ｄｒｏｐｏｆｆ ｖａｌｕｅ ｆｏｒ ｇａｐｐｅｄ ａｌｉｇｎｍｅｎｔ ｉｎ ｂｉｔｓ）がｂｌａｓｔｎの場合は５０、ｂｌａｓｔｎ以外のプログラムでは２５である（http://www.ncbi.nlm.nih.gov/blast/html/blastcgihelp.html）。

[0036] ＣＤ４０のアミノ酸配列の部分配列からなるポリペプチドは、当業者に公知の方法によって作製することができ、例えば、配列番号６、ＧｅｎＢａｎｋ ａｃｃｅｓｓｉｏｎ Ｎｏ．ＮＰ＿００１２４１またはＧｅｎＢａｎｋ ａｃｃｅｓｓｉｏｎ Ｎｏ．ＸＰ＿００５５６９２７４に示されるアミノ

酸配列をコードするＤＮＡの一部を欠失させ、これを含む発現ベクターを導入した形質転換体を培養することにより作製することができる。また、上記の方法で作製されるポリペプチドまたはＤＮＡに基づいて、上記と同様の方法により、例えば、配列番号６、ＧｅｎＢａｎｋ ａｃｃｅｓｓｉｏｎ Ｎｏ．ＮＰ＿００１２４１またはＧｅｎＢａｎｋ ａｃｃｅｓｓｉｏｎ Ｎｏ．ＸＰ＿００５５６９２７４に示されるアミノ酸配列の部分配列において１以上のアミノ酸が欠失、置換または付加されたアミノ酸配列を有するポリペプチドを得ることができる。さらに、ＣＤ４０のアミノ酸配列の部分配列からなるポリペプチド、またはＣＤ４０のアミノ酸配列の部分配列において１以上のアミノ酸が欠失、置換または付加されたアミノ酸配列を有するポリペプチドは、フルオレニルメチルオキシカルボニル（Ｆｍｏｃ）法、ｔ－ブチルオキシカルボニル（ｔＢｏｃ）法などの化学合成法によって製造することもできる。

[0037] 本発明におけるＣＤ４０の細胞外領域としては、例えば、ＧｅｎＢａｎｋ ａｃｃｅｓｓｉｏｎ Ｎｏ．ＮＰ＿００１２４１に示されるヒトＣＤ４０のアミノ酸配列を公知の膜貫通領域予測プログラムＳＯＳＵＩ（http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html）、ＴＭＨＭＭ ｖｅｒ．２（http://www.cbs.dtu.dk/services/TMHMM-2.0/）またはＥｘＰＡＳｙ Ｐｒｏｔｅｏｍｉｃｓ Ｓｅｒｖｅｒ（http://Ca.expasy.org/）などを用いて予測された領域などが挙げられる。具体的には、配列番号６またはＧｅｎＢａｎｋ ａｃｃｅｓｓｉｏｎ Ｎｏ．ＮＰ＿００１２４１の２１番目～１９４番目に示されるアミノ酸配列が挙げられる。

[0038] ＣＤ４０の機能としては、ＣＤ４０リガンドやアゴニストが結合したときにＣＤ４０シグナルを誘導し、種々の作用を引き起こすことが挙げられる。例えばがん細胞にＣＤ４０シグナルが誘導されると、該がん細胞の細胞死や増殖阻害などを引き起こす。Ｂリンパ球にＣＤ４０シグナルが誘導されると、例えば該Ｂリンパ球の活性化、ＣＤ９５の発現促進、クラススイッチ組換えおよびｓｏｍａｔｉｃ ｈｙｐｅｒｍｕｔａｔｉｏｎなどを惹起し、抗原

高親和性抗体産生などを誘導する。樹状細胞にＣＤ４０シグナルが誘導されると、例えば該樹状細胞の成熟化やＩＬ－１２産生を引き起こす。マクロファージにＣＤ４０シグナルが誘導されると、例えばＭ２マクロファージの表面マーカーの減少とＭ１マクロファージの表面マーカーの発現の誘導やｐｒｏ－ｉｎｆｌａｍｍａｔｏｒｙサイトカイン産生を引き起こす。

[0039] 本発明におけるＥｐＣＡＭは、ＣＤ３２６、ＧＡ７３３－２、ＨＥＡ１２５、ＫＳ１／４、ＭＫ－１、ＭＨ９９、ＭＯＣ３１、３２３／Ａ３、１７－１Ａ、ＣＯ－１７Ａ、ＥＳＡ、ＥＧＰ－２、ＥＧＰ３４、ＥＧＰ４０、ＫＳＡ、ＫＳ１／４、ＴＲＯＰ－１およびＴＡＣＳＴ－１と同義として用いられる。

[0040] ＥｐＣＡＭとしては、例えば、Ｇｅｎｂａｎｋ Ａｃｃｅｓｓｉｏｎ Ｎｏ．ＡＡＨ１４７８５若しくは配列番号１６に示されるアミノ酸配列を含むヒトＥｐＣＡＭ；Ｇｅｎｂａｎｋ Ａｃｃｅｓｓｉｏｎ Ｎｏ．ＮＰ＿００１０３５１１８若しくは配列番号１８に示されるアミノ酸配列を含むサルＥｐＣＡＭ；またはＧｅｎｂａｎｋ Ａｃｃｅｓｓｉｏｎ Ｎｏ．ＮＰ＿０３２５５８ 若しくは配列番号２０に示されるアミノ酸配列を含むマウスＥｐＣＡＭなどが挙げられる。また、例えば、配列番号１６、ＡＡＨ１４７８５、Ｇｅｎｂａｎｋ ａｃｃｅｓｓｉｏｎ Ｎｏ．ＮＰ＿００１０３５１１８またはＧｅｎｂａｎｋ ａｃｃｅｓｓｉｏｎ Ｎｏ．ＮＰ＿０３２５５８に示されるアミノ酸配列において１つ以上のアミノ酸が欠失、置換または付加されたアミノ酸配列からなり、かつＥｐＣＡＭの機能を有するポリペプチドが挙げられる。

[0041] 配列番号１６、ＡＡＨ１４７８５、Ｇｅｎｂａｎｋ ａｃｃｅｓｓｉｏｎ Ｎｏ．ＮＰ＿００１０３５１１８またはＧｅｎｂａｎｋ ａｃｃｅｓｓｉｏｎ Ｎｏ．ＮＰ＿０３２５５８に示されるアミノ酸配列と好ましくは７０％以上、より好ましくは８０％以上、さらに好ましくは９０％以上の相同性を有するアミノ酸配列を含むポリペプチド、最も好ましくは９５％、９６％、９７％、９８％および９９％以上の相同性を有するアミノ酸配列からなり

、かつＥｐＣＡＭの機能を有するポリペプチドも本発明のＥｐＣＡＭに包含される。

[0042] 配列番号１６、ＡＡＨ１４７８５、Ｇｅｎｂａｎｋ ａｃｃｅｓｓｉｏｎ Ｎｏ．ＮＰ＿００１０３５１１８またはＧｅｎｂａｎｋ ａｃｃｅｓｓｉｏｎ Ｎｏ．ＮＰ＿０３２５５８に示されるアミノ酸配列に示されるアミノ酸配列において１以上のアミノ酸残基が欠失、置換、または付加されたアミノ酸配列を有するポリペプチドは、前述の部位特異的変異導入法などを用いて、例えば配列番号１６、ＡＡＨ１４７８５、Ｇｅｎｂａｎｋ ａｃｃｅｓｓｉｏｎ Ｎｏ．ＮＰ＿００１０３５１１８またはＧｅｎｂａｎｋ ａｃｃｅｓｓｉｏｎ Ｎｏ．ＮＰ＿０３２５５８に示されるアミノ酸配列をコードするＤＮＡに、部位特異的変異を導入することにより得ることができる。欠失、置換または付加されるアミノ酸の数は特に限定されないが、好ましくは１個～数十個、例えば、１～２０個、より好ましくは１個～数個、例えば、１～５個のアミノ酸である。

[0043] 本発明におけるＥｐＣＡＭをコードする遺伝子としては、例えば、Ｇｅｎｂａｎｋ ａｃｃｅｓｓｉｏｎ Ｎｏ．ＮＭ＿００２３５４若しくは配列番号１５に示される塩基配列を含むヒトＥｐＣＡＭの遺伝子；Ｇｅｎｂａｎｋ ａｃｃｅｓｓｉｏｎ Ｎｏ．ＸＭ＿０１５４３３６８５若しくは配列番号１７に示される塩基配列を含むサルＥｐＣＡＭの遺伝子；またはＧｅｎｂａｎｋ ａｃｃｅｓｓｉｏｎ Ｎｏ．ＮＭ＿００８５３２若しくは配列番号１９に示される塩基配列を含むマウスＥｐＣＡＭの遺伝子が挙げられる。

[0044] また、例えば、配列番号１５、Ｇｅｎｂａｎｋ ａｃｃｅｓｓｉｏｎ Ｎｏ．ＮＭ＿００２３５４、Ｇｅｎｂａｎｋ ａｃｃｅｓｓｉｏｎ Ｎｏ．ＸＭ＿０１５４３３６８５またはＧｅｎｂａｎｋ ａｃｃｅｓｓｉｏｎ Ｎｏ．ＮＭ＿００８５３２に示される塩基配列において１以上の塩基が欠失、置換または付加された塩基配列からなり、かつＥｐＣＡＭの機能を有するポリペプチドをコードするＤＮＡを含む遺伝子、配列番号１５、Ｇｅｎｂａｎｋ ａｃｃｅｓｓｉｏｎ Ｎｏ．ＮＭ＿００２３５４、Ｇｅｎｂａｎｋ ａｃ

ｃｅｓｓｉｏｎ Ｎｏ．ＸＭ＿０１５４３３６８５またはＧｅｎｂａｎｋ ａｃｃｅｓｓｉｏｎ Ｎｏ．ＮＭ＿００８５３２に示される塩基配列に示される塩基配列と６０％以上の相同性を有する塩基配列、好ましくは８０％以上の相同性を有する塩基配列、さらに好ましくは９５％以上の相同性を有する塩基配列からなり、かつＥｐＣＡＭの機能を有するポリペプチドをコードするＤＮＡを含む遺伝子、及び配列番号１５、Ｇｅｎｂａｎｋ ａｃｃｅｓｓｉｏｎ Ｎｏ．ＮＭ＿００２３５４、Ｇｅｎｂａｎｋ ａｃｃｅｓｓｉｏｎ Ｎｏ．ＸＭ＿０１５４３３６８５またはＧｅｎ ａｃｃｅｓｓｉｏｎ Ｎｏ．ＮＭ＿００８５３２に示される塩基配列を含むＤＮＡとストリンジェントな条件下でハイブリダイズするＤＮＡからなり、かつＥｐＣＡＭの機能を有するポリペプチドをコードするＤＮＡを含む遺伝子なども、本発明のＥｐＣＡＭをコードする遺伝子に含まれる。

[0045] 本発明におけるＥｐＣＡＭの細胞外領域としては、例えば、ＧｅｎＢａｎｋ ａｃｃｅｓｓｉｏｎ Ｎｏ．ＡＡＨ１４７８５に示されるヒトＥｐＣＡＭのアミノ酸配列を公知の膜貫通領域予測プログラムＳＯＳＵＩ（http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html）、ＴＭＨＭＭ ｖｅｒ．２（http://www.cbs.dtu.dk/services/TMHMM-2.0/）またはＥｘＰＡＳｙ Ｐｒｏｔｅｏｍｉｃｓ Ｓｅｒｖｅｒ（http://Ca.expasy.org/）などを用いて予測された領域などが挙げられる。具体的には、配列番号１０またはＧｅｎＢａｎｋ ａｃｃｅｓｓｉｏｎ Ｎｏ．ＡＡＨ１４７８５の２２番目～２６５番目に示されるアミノ酸配列が挙げられる。

[0046] ＥｐＣＡＭの機能としては、例えばカルシウム非依存的な細胞と細胞の接着および、がん細胞におけるＣ－ＭｙｃやＣｙｃｌｉｎ Ａ／Ｅなどのアップレギュレーションを介した増殖促進などが挙げられる。

[0047] 抗体とは、イムノグロブリンを構成する重鎖の可変領域および重鎖の定常領域、並びに軽鎖の可変領域および軽鎖の定常領域の全部または一部をコードする遺伝子（「抗体遺伝子」と称する）に由来するタンパク質である。本発明の抗体は、いずれのイムノグロブリンクラスおよびサブクラスを有する

抗体または抗体断片をも包含する。

[0048] 重鎖（Ｈ鎖）とは、イムノグロブリン分子を構成する２種類のポリペプチドのうち、分子量が大きい方のポリペプチドを指す。重鎖は抗体のクラスとサブクラスを決定する。ＩｇＡ、ＩｇＤ、ＩｇＥ、ＩｇＧおよびＩｇＭは、それぞれα鎖、δ鎖、ε鎖、γ鎖およびμ鎖を重鎖として有し、重鎖の定常領域は異なるアミノ酸配列で特徴付けられる。軽鎖（Ｌ鎖）とは、イムノグロブリン分子を構成する２種類のポリペプチドのうち、分子量が小さい方のポリペプチドを指す。ヒトの抗体の場合、軽鎖にはκ鎖とλ鎖の２種類が存在する。

[0049] 可変領域（Ｖ領域）とは、通常は、イムノグロブリンのＮ末端側のアミノ酸配列内に存在する多様性に富んだ領域を指す。可変領域以外の部分は多様性の少ない構造をとることから、定常領域（Ｃ領域）と呼ばれる。重鎖と軽鎖の各可変領域は会合して抗原結合部位を形成し、抗原への抗体の結合特性を決定する。

[0050] ヒトの抗体の重鎖では、可変領域はＫａｂａｔらのＥＵインデックス（Kabat et. al., Sequences of proteins of immunological interest, 1991 Fifth edition）における１番目から１１７番目までのアミノ酸配列に該当し、定常領域は１１８番目以降のアミノ酸配列に該当する。ヒトの抗体の軽鎖ではＫａｂａｔらによる番号付け（Ｋａｂａｔ　ｎｕｍｂｅｒｉｎｇ）における１番目から１０７番目までのアミノ酸配列が可変領域に該当し、１０８番目以降のアミノ酸配列が定常領域に該当する。以下、重鎖可変領域または軽鎖可変領域を、ＶＨまたはＶＬと略記する。

[0051] 抗原結合部位は、抗体において抗原を認識し結合する部位であり、抗原決定基（エピトープ）と相補的な立体構造を形成する部位を指す。抗原結合部位は、抗原決定基との間に強い分子間相互作用を生じる。抗原結合部位は、少なくとも３つの相補性決定領域（ＣＤＲ）を含むＶＨおよびＶＬにより構成される。ヒトの抗体の場合、ＶＨおよびＶＬはそれぞれ３つのＣＤＲを有する。これらのＣＤＲを、それぞれＮ末端側から順番にＣＤＲ１、ＣＤＲ２

およびＣＤＲ３と称する。

[0052] 定常領域のうち、重鎖定常領域または軽鎖定常領域は、それぞれＣＨまたはＣＬと表記される。ＣＨは、重鎖のサブクラスであるα鎖、δ鎖、ε鎖、γ鎖およびμ鎖によって分類される。ＣＨは、Ｎ末端側より順に整列したＣＨ１ドメイン、ヒンジドメイン、ＣＨ２ドメイン、ＣＨ３ドメインから構成され、ＣＨ２ドメインとＣＨ３ドメインとを併せてＦｃ領域という。一方、ＣＬは、Ｃλ鎖およびＣκ鎖とよばれる２つのサブクラスに分類される。

[0053] 本発明において、抗ＣＤ４０抗体とは、ＣＤ４０の細胞外領域を特異的に認識し、かつ結合するモノクローナル抗体をいう。また、本発明において、抗ＥｐＣＡＭ抗体とは、ＥｐＣＡＭの細胞外領域を特異的に認識し、かつ結合するモノクローナル抗体をいう。また、本発明において抗体とは、ポリクローナル抗体およびオリゴクローナル抗体をも包含する。

[0054] 本発明において、抗体または該抗体断片がＣＤ４０およびＥｐＣＡＭの少なくとも一方に結合することは、例えば、公知の免疫学的検出法、好ましくは蛍光細胞染色法等を用いて、ＣＤ４０およびＥｐＣＡＭの少なくとも一方を発現した細胞と抗体との結合性を確認する方法により確認することができる。また、公知の免疫学的検出法［Monoclonal Antibodies - Principles and Practice, Third Edition, Academic Press (1996)、Antibodies - A Laboratory Manual, Cold Spring Harbor Laboratory (1988)、単クローン抗体実験マニュアル、講談社サイエンティフィック（1987）］などを組み合わせて用いることもできる。

[0055] モノクローナル抗体は、単一性（ｍｏｎｏｃｌｏｎａｌｉｔｙ）を保持した抗体産生細胞が分泌する抗体であり、単一のエピトープ（抗原決定基ともいう）を認識する。モノクローナル抗体分子同士は同一のアミノ酸配列（１次構造）を有し、単一の構造をとる。ポリクローナル抗体とは、異なるクローンの抗体産生細胞が分泌する抗体分子の集団をいう。オリゴクローナル抗体とは、複数の異なるモノクローナル抗体を混合した抗体分子の集団をいう。

[0056] エピトープは、抗体が認識し、結合する抗原の構造部位をいう。エピトープとしては、例えば、モノクローナル抗体が認識し、結合する単一のアミノ酸配列、アミノ酸配列からなる立体構造、糖鎖が結合したアミノ酸配列および糖鎖が結合したアミノ酸配列からなる立体構造などが挙げられる。

[0057] 本発明におけるモノクローナル抗体としては、ハイブリドーマにより産生される抗体、および抗体遺伝子を含む発現ベクターで形質転換した形質転換体により産生される遺伝子組換え抗体を挙げることができる。

[0058] ハイブリドーマは、例えば、抗原を調製し、該抗原を免疫した動物より抗原特異性を有する抗体産生細胞を取得し、さらに、該抗体産生細胞と骨髄腫細胞とを融合させることによって、調製することができる。該ハイブリドーマを培養するか、または該ハイブリドーマを動物に投与して該ハイブリドーマを腹水癌化させ、該培養液または腹水を分離、精製することにより、所望のモノクローナル抗体を取得することができる。抗原を免疫する動物としては、ハイブリドーマを作製することが可能であれば、いかなるものも用いることができるが、マウス、ラット、ハムスターおよびラビットなどが好適に用いられる。また、このような被免疫動物から抗体産生能を有する細胞を取得し、該細胞にｉｎ　ｖｉｔｒｏで免疫を施した後に、骨髄腫細胞と融合して、ハイブリドーマを作製することもできる。

[0059] 本発明における遺伝子組換え抗体としては、例えば、組換えマウス抗体、組換えラット抗体、組換えハムスター抗体、組換えラビット抗体、ヒト型キメラ抗体（キメラ抗体ともいう）、ヒト化抗体（ＣＤＲ移植抗体ともいう）およびヒト抗体などの、遺伝子組換え技術により製造される抗体が挙げられる。遺伝子組換え抗体においては、対象とする動物種や目的に応じて、どの動物種由来の重鎖および軽鎖の可変領域並びに定常領域を適用するかを決定することができる。例えば、対象とする動物種がヒトの場合には、可変領域をヒトまたはマウスなどの非ヒト動物由来とし、定常領域およびリンカーをヒト由来とすることができる。

[0060] キメラ抗体とは、ヒト以外の動物（非ヒト動物）の抗体のＶＨおよびＶＬ

と、ヒト抗体のＣＨおよびＣＬとからなる抗体を指す。非ヒト動物としては、マウス、ラット、ハムスターおよびラビットなど、ハイブリドーマを作製することが可能であれば、いかなるものも用いることができる。キメラ抗体は、モノクローナル抗体を生産する非ヒト動物由来のハイブリドーマより、ＶＨおよびＶＬをコードするｃＤＮＡを取得し、ヒト抗体のＣＨおよびＣＬをコードするＤＮＡを有する動物細胞用発現ベクターにそれぞれ挿入してキメラ抗体発現ベクターを構築し、動物細胞へ導入して発現させることによって、製造することができる。

[0061] ヒト化抗体とは、非ヒト動物抗体のＶＨおよびＶＬのＣＤＲをヒト抗体のＶＨおよびＶＬの対応するＣＤＲに移植した抗体を指す。ＶＨおよびＶＬのＣＤＲ以外の領域はフレームワーク領域（以下、ＦＲと表記する）と称される。ヒト化抗体は、非ヒト動物抗体のＶＨのＣＤＲのアミノ酸配列と任意のヒト抗体のＶＨのＦＲのアミノ酸配列からなるＶＨのアミノ酸配列をコードするｃＤＮＡと、非ヒト動物抗体のＶＬのＣＤＲのアミノ酸配列と任意のヒト抗体のＶＬのＦＲのアミノ酸配列からなるＶＬのアミノ酸配列をコードするｃＤＮＡを構築し、ヒト抗体のＣＨおよびＣＬをコードするＤＮＡを有する動物細胞用発現ベクターにそれぞれ挿入してヒト化抗体発現ベクターを構築し、動物細胞へ導入して発現させることによって、製造することができる。

[0062] ヒト抗体は、元来、ヒト体内に天然に存在する抗体をいうが、最近の遺伝子工学的、細胞工学的、発生工学的な技術の進歩により作製されたヒト抗体ファージライブラリーおよびヒト抗体産生トランスジェニック動物から得られる抗体なども含まれる。

[0063] ヒト体内に天然に存在する抗体は、例えば、ヒト末梢血リンパ球にＥＢウイルスなどを感染させて不死化し、クローニングすることにより、該抗体を産生するリンパ球を培養し、該培養上清より該抗体を精製することにより取得することができる。

[0064] ヒト抗体ファージライブラリーは、ヒトＢ細胞から調製した抗体遺伝子を

ファージ遺伝子に挿入することにより、Ｆａｂ、ｓｃＦｖなど抗体断片をファージ表面に発現させたライブラリーである。該ライブラリーより、抗原を固定化した基質に対する結合活性を指標として、所望の抗原結合活性を有する抗体断片を表面に発現しているファージを回収することができる。該抗体断片は、さらに、遺伝子工学的手法により２本の完全なＨ鎖および２本の完全なＬ鎖からなるヒト抗体分子へ変換することができる。

[0065] ヒト抗体産生トランスジェニック動物は、ヒト抗体遺伝子が細胞内に組み込まれた動物を意味する。具体的には、例えば、マウスＥＳ細胞へヒト抗体遺伝子を導入し、該ＥＳ細胞をマウスの初期胚へ移植後、個体を発生させることにより、ヒト抗体産生トランスジェニックマウスを作製することができる。ヒト抗体産生トランスジェニック動物由来のヒト抗体は、通常の非ヒト動物で行われているハイブリドーマ作製法を用いてハイブリドーマを取得し、培養することで、培養上清中に抗体を産生、蓄積させることにより調製できる。

[0066] 遺伝子組換え抗体のＣＨとしては、ヒトイムノグロブリンに属すればいかなるものでもよいが、ｈｕｍａｎ ｉｍｍｕｎｏｇｌｏｂｕｌｉｎ Ｇ（ｈＩｇＧ）クラスのものが好ましい。さらにｈＩｇＧクラスに属するｈＩｇＧ１、ｈＩｇＧ２、ｈＩｇＧ３およびｈＩｇＧ４といったサブクラスのいずれも用いることができる。また、遺伝子組換え抗体のＣＬとしては、ヒトイムノグロブリンに属すればいずれのものでもよく、κクラスまたはλクラスのものを用いることができる。

[0067] 本発明において、バイスペシフィック抗体とは、特異性が異なる２種類の抗原結合ドメインを有し、イムノグロブリンを構成する重鎖の可変領域および重鎖の定常領域、並びに軽鎖の可変領域および軽鎖の定常領域の全部または一部を含むタンパク質をいう。バイスペシフィック抗体のそれぞれの抗原結合ドメインは、単一の抗原の異なるエピトープに結合してもよいし、異なる抗原に結合してもよい。

[0068] 本発明において、ＣＤ４０またはＥｐＣＡＭに結合する抗原結合ドメイン

は、ＣＤ４０またはＥｐＣＡＭを特異的に認識し、結合するものであればいかなるものでもよい。例えば、抗体、リガンド、受容体、および天然に存在する相互作用分子など遺伝子組換え技術によって作製可能なポリペプチド、タンパク質分子およびその断片、並びに該タンパク質分子の低分子または天然物とのコンジュゲート体などいずれの形態であってもよい。

[0069] また、抗原結合ドメインとしては、抗体、リガンドおよび受容体など既知の結合分子の結合ドメインを利用して組換えた結合タンパク質でもよく、具体的には各抗原に結合する抗体のＣＤＲを含む組換えタンパク質、ＣＤＲを含む抗体可変領域、抗体可変領領域および各抗原に結合するリガンドの結合ドメインを含む組換えタンパク質などが挙げられる。なかでも、本発明においては、抗原結合ドメインは抗体の可変領域であることが好ましい。

[0070] 本発明のバイスペシフィック抗体または該バイスペシフィック抗体断片は、同一細胞上に発現しているＣＤ４０およびＥｐＣＡＭに結合してもよいし、異なる細胞上に発現しているＣＤ４０およびＥｐＣＡＭに結合してもよい。

[0071] ＣＤ４０を発現する細胞は例えば、Ｂ細胞、樹状細胞（ＤＣ）、マクロファージおよび単球などの抗原提示細胞や、Ｒａｍｏｓ細胞などのがん細胞などが挙げられる。

[0072] ＥｐＣＡＭを発現する細胞は例えば、頭頸部癌、肺癌、消化器癌、乳癌、泌尿器癌などのがん細胞が挙げられる。

[0073] 本発明のバイスペシフィック抗体または該バイスペシフィック抗体断片としては、例えばＣＤ４０アゴニスト活性を有するバイスペシフィック抗体または該バイスペシフィック抗体断片が挙げられる。本発明のバイスペシフィック抗体または該バイスペシフィック抗体断片としては、ＥｐＣＡＭ分子またはＥｐＣＡＭを発現する細胞の非存在下ではＣＤ４０アゴニスト活性を示さず、ＥｐＣＡＭ分子またはＥｐＣＡＭを発現する細胞の存在下でのみＣＤ４０アゴニスト活性を示すバイスペシフィック抗体または該バイスペシフィック抗体断片が好ましい。このようなバイスペシフィック抗体または該バイ

スペシフィック抗体断片は、ＥｐＣＡＭを発現する細胞が存在する、がんなどの病変部でのみＣＤ４０を活性化するため、全身的なＣＤ４０の活性化に伴う副作用を生じない点で好ましい。

[0074] 本発明のバイスペシフィック抗体または該バイスペシフィック抗体断片が有するＣＤ４０アゴニスト活性は、細胞上のＣＤ４０にバイスペシフィック抗体または該バイスペシフィック抗体断片が結合することにより、該ＣＤ４０を介したシグナルを惹起し、抗原提示細胞の活性化を誘導する活性、腫瘍細胞の細胞死を誘導する活性などをいう。

[0075] ＣＤ４０アゴニスト活性は、例えばＲａｍｏｓ細胞などのＣＤ４０を発現する細胞上のＣＤ９５発現量増加を評価することにより確認することができる。

[0076] すなわち、本発明のバイスペシフィック抗体または該バイスペシフィック抗体断片として、具体的には、ＥｐＣＡＭを発現する細胞の存在下で、ＥｐＣＡＭとＣＤ４０に結合したときに、ＣＤ４０を発現する抗原提示細胞の活性化および／または腫瘍細胞の細胞死を誘導するバイスペシフィック抗体または該バイスペシフィック抗体断片などが挙げられる。

本発明において、ＣＤ４０アンタゴニスト活性は、ＣＤ４０リガンドまたはＣＤ４０アゴニストによるＣＤ４０の活性化を阻害する活性などをいう。例えばＣＤ４０リガンドまたはＣＤ４０アゴニストのＣＤ４０への結合によるシグナル誘導を阻害する活性などをいう。

抗体のＣＤ４０アンタゴニスト活性は、例えばＣＤ４０リガンドによる、Ｒａｍｏｓ細胞などのＣＤ４０を発現する細胞へのＣＤ９５発現誘導が、抗体の添加により阻害されることにより確認することができる。

[0077] 一分子のバイスペシフィック抗体が有する、ある抗原に対する結合ドメインの数を、結合の価数と呼ぶ。例えば、本発明において、一分子のバイスペシフィック抗体が、ＣＤ４０に結合する抗原結合ドメインおよびＥｐＣＡＭに結合する抗原結合ドメインを二つずつ有する場合、かかるバイスペシフィック抗体は、ＣＤ４０およびＥｐＣＡＭに、それぞれ二価で結合する。

[0078] 本発明において、一分子あたりのバイスペシフィック抗体が、ＣＤ４０またはＥｐＣＡＭに何価で結合してもよいが、ＣＤ４０およびＥｐＣＡＭに、少なくともそれぞれ二価で結合することが好ましい。

[0079] また、イムノグロブリンドメインまたはその断片を含むリンカーなど、適切なリンカーを介して結合させた複数の抗原結合ドメインを含む抗体も本発明のバイスペシフィック抗体に含まれる。

[0080] 本発明のバイスペシフィック抗体において、ＣＤ４０に結合する抗原結合ドメインとＥｐＣＡＭに結合する抗原結合ドメインの位置は適宜選択することができる。

[0081] 本発明のバイスペシフィック抗体は、既存の作製技術（[Nature Protocols, 9, 2450-2463 (2014)]、国際公開第１９９８／０５０４３１号、国際公開第２００１／７７３４号、国際公開第２００２／００２７７３号および国際公開第２００９／１３１２３９号）などにより作製することができる。

[0082] 本発明のバイスペシフィック抗体において、ＣＤ４０に結合する抗原結合ドメインは、ＥｐＣＡＭに結合する抗原結合ドメインよりもＮ末端側に位置していてもよいし、Ｃ末端側に位置していてもよい。

[0083] 本発明のバイスペシフィック抗体としては、抗原結合ドメインとして抗体のＶ領域を用いることができ、例えば、一つの重鎖に複数のＶＨを含む重鎖を含む抗体、および一つのＶＨを含む重鎖を二つ含む抗体などが挙げられる。以下では一つの重鎖に複数のＶＨを含む重鎖を含む抗体のＮ末端から１番目のＶＨをＶＨ１、２番目のＶＨをＶＨ２、ｎ番目のＶＨをＶＨｎのように表すこともある。一つの重鎖に２つのＶＨを含む重鎖を含む抗体の場合、Ｎ末端側から１番目に位置するＶＨはＶＨ１、２番目に位置するＶＨはＶＨ２と表される。

[0084] 一つの重鎖に複数のＶＨを含む重鎖を含むバイスペシフィック抗体として、より具体的には、イムノグロブリンドメインまたはその断片を含むリンカーを用いて結合させた２つ以上のＶＨを含む重鎖を含む抗体が挙げられる。

[0085] ３つ以上のＶＨを結合させる場合には、リンカーとして、異なるイムノグ

ロブリンドメインまたはその断片を用いてもよいし、同じイムノグロブリンドメインまたはその断片を用いてもよい。また、２つ以上のＶＨを連結させる場合には、各ＶＨが特異的に抗原に結合できるようにイムノグロブリンドメインまたはその断片の長さや種類を変えることができる。

[0086] 本発明のバイスペシフィック抗体は、具体的には、下記（ａ）～（ｅ）に示される少なくとも１の特徴を有する。

（ａ）１つの重鎖ポリペプチドに複数（例えば２～５個）の異なるＶＨを含み、ＶＨは互いに近接していないこと、

（ｂ）ＶＨが１０アミノ酸以上のポリペプチドリンカーを介してタンデム（縦列）に連結されていること、より具体的には、ＶＨが、例えばイムノグロブリンドメインの全部または一部のアミノ酸配列を含むリンカーを用いて連結されていること、

（ｃ）軽鎖と重鎖とが会合し、抗原結合部位を形成していること、

（ｄ）図１（Ａ）または図１（Ｂ）に示されるように２本の重鎖ポリペプチドと少なくとも４本の軽鎖ポリペプチドとからなる構造を有し、かつ２本の重鎖ポリペプチド間はヒンジ領域でジスルフィド結合により連結され、軽鎖ポリペプチドと重鎖ポリペプチドとの間もジスルフィド結合で連結されていること、および

（ｅ）重鎖の定常領域は、例えば天然型抗体重鎖の定常領域の全部または一部（例えば、ＣＨ１断片、ＣＨ１、ＣＨ２、ＣＨ３、ＣＨ１－ヒンジ、ＣＨ１－ヒンジ－ＣＨ２およびＣＨ１－ヒンジ－ＣＨ２－ＣＨ３など）からなること。

[0087] 本発明において、バイスペシフィック抗体に含まれる、抗ＣＤ４０抗体のＶＨ（抗ＣＤ４０抗体由来のＶＨを意味する）と抗ＥｐＣＡＭ抗体のＶＨ（抗ＥｐＣＡＭ抗体由来のＶＨを意味する）の位置は、適宜選択することができる。例えば、図１（Ａ）または図１（Ｂ）に示される構造のバイスペシフィック抗体については、抗ＣＤ４０抗体のＶＨは、抗ＥｐＣＡＭ抗体のＶＨよりもＮ末端側に位置していてもよいし、Ｃ末端側に位置していてもよいが

、Ｎ末端側に位置していることが好ましい。

[0088] 本発明において、バイスペシフィック抗体に含まれるＶＬは、同一のＶＬであっても、異なるＶＬであってもいずれでもよい。同一のＶＬを有するバイスペシフィック抗体であって、かつ二つの異なる抗原または同一抗原上の異なる二つのエピトープに結合することができるバイスペシフィック抗体のＶＨは、各可変領域がそれぞれの特異的な抗原またはエピトープに結合できるように、最適化または改変されたＶＨであればよく、例えば、アミノ酸改変によるスクリーニング、またはファージディプレイなどの方法を用いて適切なＶＨを選択することができる。

[0089] 本発明のバイスペシフィック抗体に含まれるＶＬは抗ＣＤ４０抗体または抗ＥｐＣＡＭ抗体のＶＬであればどのようなものでもよいが、それぞれ配列番号２３、２４および２５で示されるアミノ酸配列を含むＣＤＲ１、２および３を含むＶＬおよび配列番号２２で示されるアミノ酸配列を含むＶＬが好ましい。

[0090] 本発明において、リンカーとは、複数の抗原結合ドメインを結合させる化学構造を指し、好ましくはポリペプチドである。本発明のバイスペシフィック抗体に用いられるリンカーとしては、例えば、イムノグロブリンドメインの全部または一部のアミノ酸配列を含むリンカー、および複数のイムノグロブリンドメインからなるポリペプチドの全部または一部のアミノ酸配列を含むリンカーなどが挙げられる。

[0091] 本発明において、Ｃ末端側ポリペプチドとは複数の抗原結合ドメインのうち最もＣ末端側の抗原結合ドメインのＣ末端に結合するポリペプチド鎖を指す。本発明のバイスペシフィック抗体はＣ末端側ポリペプチドを有していても有していなくてもよいが、Ｃ末端側ポリペプチドを有しているバイスペシフィック抗体が好ましい。Ｃ末端側ポリペプチドとして、例えば、イムノグロブリンドメインの全部または一部のアミノ酸配列を含むポリペプチド、および複数のイムノグロブリンドメインからなるポリペプチドの全部または一部のアミノ酸配列を含むポリペプチドなどが挙げられる。

[0092] イムノグロブリンドメインの一部のアミノ酸配列を含むリンカーまたはＣ末端側ポリペプチドとして、イムノグロブリンから選ばれるアミノ酸配列は、断続的でも連続的でもよいが、連続するアミノ酸配列が好ましい。また、該アミノ酸配列は、ヒンジ領域を含んでいてもよい。

[0093] 本発明において、イムノグロブリンドメインとは、イムノグロブリンに類似したアミノ酸配列を持ち、少なくとも２個のシステイン残基が存在する約１００個のアミノ酸残基からなるペプチドを最小単位とする。本発明において、イムノグロブリンドメインは、上記の最小単位のイムノグロブリンドメインを複数含むポリペプチドをも包含する。イムノグロブリンドメインとしては、例えば、イムノグロブリン重鎖のＶＨ、ＣＨ１、ＣＨ２およびＣＨ３、並びにイムノグロブリン軽鎖のＶＬおよびＣＬなどが挙げられる。

[0094] イムノグロブリンの動物種は特に限定されないが、ヒトであることが好ましい。また、イムノグロブリン重鎖の定常領域のサブクラスは、ＩｇＤ、ＩｇＭ、ＩｇＧ１、ＩｇＧ２、ＩｇＧ３、ＩｇＧ４、ＩｇＡ１、ＩｇＡ２およびＩｇＥのいずれでもよく、好ましくは、ＩｇＧ由来およびＩｇＭ由来が挙げられる。また、イムノグロブリン軽鎖の定常領域のサブクラスは、κおよびλのいずれでもよい。

[0095] また、イムノグロブリンドメインはイムノグロブリン以外のタンパク質にも存在し、例えば主要組織適合抗原（ＭＨＣ）、ＣＤ１、Ｂ７およびＴ細胞受容体（ＴＣＲ）などのイムノグロブリンスーパーファミリーに属するタンパク質が含むイムノグロブリンドメインが挙げられる。本発明のバイスペシフィック抗体に用いるイムノグロブリンドメインとしては、いずれのイムノグロブリンドメインも適用することができる。

[0096] ヒトの抗体の場合、ＣＨ１は、ＥＵインデックスで示される１１８番目から２１５番目のアミノ酸配列を有する領域を指す。同様に、ＣＨ２はＫａｂａｔらのＥＵインデックスで示される２３１番目から３４０番目のアミノ酸配列を有する領域を、ＣＨ３はＫａｂａｔらのＥＵインデックスで示される３４１番目から４４６番目のアミノ酸配列を有する領域を各々指す。ＣＨ１

とＣＨ２の間には、ヒンジ（蝶番）領域（以下ヒンジと記載することもある）と呼ばれる柔軟性に富んだアミノ酸領域が存在する。ヒンジ領域は、Ｋａｂａｔ らのＥＵインデックスで示される２１６番目から２３０番目のアミノ酸配列を有する領域を指す。

[0097] ＣＬは、ヒトの抗体のκ鎖の場合には、Ｋａｂａｔ　ｎｕｍｂｅｒｉｎｇで示される１０８番目から２１４番目のアミノ酸配列を有する領域を、λ鎖の場合には、１０８番目から２１５番目のアミノ酸配列を有する領域を各々指す。

[0098] 本発明のバイスペシフィック抗体に用いられるリンカーおよびＣ末端側ポリペプチドとしては、Ｎ末端からＣ末端への方向に順に整列したＣＨ１－ヒンジ－ＣＨ２－ＣＨ３からなるイムノグロブリンドメイン、ＣＨ１－ヒンジ－ＣＨ２からなるイムノグロブリンドメイン、ＣＨ１－ヒンジからなるイムノグロブリンドメイン、ＣＨ１からなるイムノグロブリンドメイン、ＣＨ１のＮ末端側の断片、１４番目のアミノ酸残基がＣｙｓである１４アミノ酸残基からなるＣＨ１断片およびＣＨ１のＮ末端から１～１４番目のアミノ酸残基からなるＣＨ１断片、並びにこれらイムノグロブリンドメイン断片のアミノ酸配列において、１つ以上のアミノ酸残基が改変されたものを挙げることができるが、これらに限定されない。

[0099] また、本発明において、リンカーおよびＣ末端側ポリペプチドの一例として、抗体のＣＨ１、ヒンジ、ＣＨ２およびＣＨ３からなるアミノ酸配列の、全部または一部の断片を適宜組み合わせて用いることができる。また、それらのアミノ酸配列を部分的に欠損、または順番を入れ替えて使用することもできる。また、リンカーおよびＣ末端側ポリペプチドに使用する抗体のサブクラスは特に限定されないが、ＩｇＭまたはＩｇＧ４もしくはＩｇＧ４の重鎖定常領域の２２８番目のＳｅｒ残基をＰｒｏに、２３５番目のＬｅｕ残基をＡｓｎに、および４０９番目のＡｒｇ残基をＬｙｓにそれぞれ置換したＩｇＧ４変異体（以下、ＩｇＧ４ＰＥ　Ｒ４０９Ｋと記載する）であることが好ましく、ＩｇＧ４およびＩｇＧ４ＰＥ　Ｒ４０９Ｋがより好ましい。

[0100] 本発明において、リンカーおよびＣ末端側ポリペプチドの例としては、配列番号７５で表わされるＩｇＧ４のＣＨ１の、Ｎ末端の１～１４番目の１４アミノ酸残基からなるポリペプチド、配列番号７５で表わされるＩｇＧ４のＣＨ１からなるポリペプチドならびに配列番号７７で表されるＩｇＧ４ＰＥ　Ｒ４０９ＫのＣＨ（ＣＨ１、ヒンジ、ＣＨ２およびＣＨ３）からなるポリペプチドなどが挙げられ、配列番号７５で表わされるＩｇＧ４のＣＨ１からなるポリペプチドおよび配列番号７７で表されるＩｇＧ４ＰＥ　Ｒ４０９ＫのＣＨからなるポリペプチドがより好ましい。

[0101] 本発明のバイスペシフィック抗体に含まれるリンカーとＣ末端側ペプチドの組み合わせとしてはどのようなものでもよい。本発明のバイスペシフィック抗体として配列番号７５で表わされるアミノ酸配列を含むＩｇＧ４のＣＨ１からなるリンカーを含みかつ配列番号７７で表されるアミノ酸配列を含むＩｇＧ４ＰＥ　Ｒ４０９ＫのＣＨ（ＣＨ１、ヒンジ、ＣＨ２およびＣＨ３）からなるＣ末端側ペプチドを含むバイスペシフィック抗体、および配列番号７７で表されるアミノ酸配列を含むＩｇＧ４ＰＥ　Ｒ４０９ＫのＣＨからなるリンカーを含みかつ配列番号７５で表わされるアミノ酸配列を含むＩｇＧ４のＣＨ１からなるＣ末端側ペプチドを含むバイスペシフィック抗体が好ましい。

[0102] 本発明のバイスペシフィック抗体のうち、図１（Ａ）に示すように、Ｎ末端側から順に、ＶＨ１、ＣＨ１、ＶＨ２、ＣＨ１、ヒンジ、ＣＨ２およびＣＨ３のアミノ酸配列を含む２本の重鎖ならびに４本の軽鎖からなる抗体をＮ末端型バイスペシフィック抗体と称する。また、図１（Ｂ）に示すように、Ｎ末端側から順にＶＨ１、ＣＨ１、ヒンジ、ＣＨ２、ＣＨ３、ＶＨ２およびＣＨ１のアミノ酸配列を含む２本の重鎖ならびに４本の軽鎖からなる抗体をＣ末端型バイスペシフィック抗体と称する。

[0103] 本発明のバイスペシフィック抗体または該バイスペシフィック抗体断片を構成するアミノ酸配列において、１つ以上のアミノ酸残基が欠失、付加、置換または挿入され、かつ上述のバイスペシフィック抗体またはそのバイスペ

シフィック抗体断片と同様な活性を有するバイスペシフィック抗体またはそのバイスペシフィック抗体断片も、本発明のバイスペシフィック抗体またはそのバイスペシフィック抗体断片に包含される。

[0104] 欠失、置換、挿入および／または付加されるアミノ酸の数は１個以上でありその数は特に限定されないが、Molecular Cloning, The Second Edition, Cold Spring Harbor Laboratory Press (1989)、Current Protocols in Molecular Biology, John Willy & Sons (1987-1997)、Nucleic Acids Research, 10, 6487 (1982)、Proc. Natl. Acad. Sci., USA, 79, 6409 (1982)、Gene, 34, 315 (1985)、Nucleic Acids Research, 13, 4431 (1985)、Proc. Natl. Acad. Sci USA, 82, 488 (1985) などに記載の部位特異的変異導入法等の周知の技術により、欠失、置換、挿入若しくは付加できる程度の数である。例えば、通常１～数十個、好ましくは１～２０個、より好ましくは１～１０個、さらに好ましくは１～５個である。

[0105] 上記の本発明のバイスペシフィック抗体のアミノ酸配列において１つ以上のアミノ酸残基が欠失、置換、挿入または付加されたとは、次のことを示す。同一配列中の任意、かつ１若しくは複数のアミノ酸配列中において、１または複数のアミノ酸残基の欠失、置換、挿入または付加があることを意味する。また、欠失、置換、挿入または付加が同時に生じる場合もあり、置換、挿入または付加されるアミノ酸残基は天然型と非天然型いずれの場合もある。

[0106] 天然型アミノ酸残基としては、例えば、Ｌ－アラニン、Ｌ－アスパラギン、Ｌ－アスパラギン酸、Ｌ－グルタミン、Ｌ－グルタミン酸、グリシン、Ｌ－ヒスチジン、Ｌ－イソロイシン、Ｌ－ロイシン、Ｌ－リジン、Ｌ－アルギニン、Ｌ－メチオニン、Ｌ－フェニルアラニン、Ｌ－プロリン、Ｌ－セリン、Ｌ－スレオニン、Ｌ－トリプトファン、Ｌ－チロシン、Ｌ－バリンおよびＬ－システインなどが挙げられる。

[0107] 以下に、相互に置換可能なアミノ酸残基の好ましい例を示す。同一群に含まれるアミノ酸残基は相互に置換可能である。

[0108] Ａ群：ロイシン、イソロイシン、ノルロイシン、バリン、ノルバリン、アラニン、２－アミノブタン酸、メチオニン、Ｏ－メチルセリン、ｔ－ブチルグリシン、ｔ－ブチルアラニン、シクロヘキシルアラニン

Ｂ群：アスパラギン酸、グルタミン酸、イソアスパラギン酸、イソグルタミン酸、２－アミノアジピン酸、２－アミノスベリン酸

Ｃ群：アスパラギン、グルタミン

Ｄ群：リジン、アルギニン、オルニチン、２，４－ジアミノブタン酸、２，３－ジアミノプロピオン酸

Ｅ群：プロリン、３－ヒドロキシプロリン、４－ヒドロキシプロリン

Ｆ群：セリン、スレオニン、ホモセリン

Ｇ群：フェニルアラニン、チロシン

[0109] 本発明のバイスペシフィック抗体または該バイスペシフィック抗体断片は、翻訳後修飾されたいかなるアミノ酸残基を含む抗体をも包含する。翻訳後修飾としては、例えば、Ｈ鎖のＣ末端におけるリジン残基の欠失［リジン・クリッピング（ｌｙｓｉｎｅ　ｃｌｉｐｐｉｎｇ）］およびポリペプチドのＮ末端におけるグルタミン残基のピログルタミン（ｐｙｒｏＧｌｕ）への置換などが挙げられる［Beck et al, Analytical Chemistry, 85, 715-736(2013)］。

[0110] 本発明のバイスペシフィック抗体として具体的には、下記（１）～（３）からなる群より選ばれる、いずれか一つのバイスペシフィック抗体などが挙げられる。

（１）抗ＣＤ４０抗体のＶ領域（抗ＣＤ４０抗体由来のＶ領域を意味する）および抗ＥｐＣＡＭ抗体のＶ領域（抗ＥｐＣＡＭ抗体由来のＶ領域を意味する）を含むバイスペシフィック抗体、

（２）抗ＣＤ４０抗体のＶＨのＣＤＲ１～３およびＶＬのＣＤＲ１～３、並びに抗ＥｐＣＡＭ抗体のＶＨのＣＤＲ１～３およびＶＬのＣＤＲ１～３を含むバイスペシフィック抗体、並びに

（３）抗ＣＤ４０抗体のＶＨおよびＶＬ、並びに抗ＥｐＣＡＭ抗体のＶＨお

よびＶＬを含むバイスペシフィック抗体。

[0111] 上記（２）に記載のバイスペシフィック抗体は、抗ＣＤ４０抗体のＶＬのＣＤＲ１～３と抗ＥｐＣＡＭ抗体のＶＬのＣＤＲ１～３とがそれぞれ同一であってもよいし、異なっていてもよいが、同一であることが好ましい。

[0112] また、上記（３）に記載のバイスペシフィック抗体は、抗ＣＤ４０抗体のＶＬと抗ＥｐＣＡＭ抗体のＶＬとが同一であってもよいし、異なっていてもよいが、同一であることが好ましい。

[0113] 本発明の抗ＣＤ４０抗体は、ＣＤ４０アゴニスト活性を有していても有していなくてもよいが、ＣＤ４０アゴニスト活性を有していない抗ＣＤ４０抗体が好ましい。また、本発明のＣＤ４０抗体は、ＣＤ４０アンタゴニスト活性を有していても有していなくてもよいが、ＣＤ４０アンタゴニスト活性を有していない抗ＣＤ４０抗体が好ましい。

[0114] 本発明の抗ＣＤ４０抗体の一例として、それぞれ配列番号２３、２４および２５で示されるアミノ酸配列を含むＣＤＲ１、２および３を含むＶＬならびに以下の（１ａ）～（１ｄ）から選ばれるいずれか１つのＶＨを含む抗ＣＤ４０抗体が挙げられる。

（１ａ）それぞれ配列番号２８、２９および３０で示されるアミノ酸配列を含むＣＤＲ１、２および３を含むＶＨ、

（１ｂ）それぞれ配列番号３３、３４および３５で示されるアミノ酸配列を含むＣＤＲ１、２および３を含むＶＨ、

（１ｃ）それぞれ配列番号３８、３９および４０で示されるアミノ酸配列を含むＣＤＲ１、２および３を含むＶＨ、および

（１ｄ）それぞれ配列番号４３、４４および４５で示されるアミノ酸配列を含むＣＤＲ１、２および３を含むＶＨ。

[0115] 本発明の抗ＣＤ４０抗体は、上記（１ａ）～（１ｄ）のいずれか１つに示されるＶＨのＣＤＲ１～３のアミノ酸配列およびそれぞれ配列番号２３～２５で示されるＶＬのＣＤＲ１～３のアミノ酸配列と、それぞれ少なくとも３５％、少なくとも４０％、少なくとも４５％、少なくとも５０％、少なくと

も５５％、少なくとも６０％、少なくとも６５％、少なくとも７０％、少なくとも７５％、少なくとも８０％、少なくとも８５％、少なくとも９０％、少なくとも９５％、少なくとも９６％、少なくとも９７％、少なくとも９８％、少なくとも９９％相同である、ＶＨおよびＶＬのＣＤＲ１～３のアミノ酸配列を含む抗ＣＤ４０抗体をも包含する。

[0116] 本発明の抗ＣＤ４０抗体は、下記（ｉ）または（ｉｉ）に記載される抗体をも包含する。

（ｉ）それぞれ配列番号２３、２４および２５で示されるアミノ酸配列を含むＣＤＲ１、２および３を含むＶＬならびに上記（１ａ）～（１ｄ）のいずれか１つに示されるＶＨを含む抗ＣＤ４０抗体と、競合してＣＤ４０に結合する抗体。

（ｉｉ）それぞれ配列番号２３、２４および２５で示されるアミノ酸配列を含むＣＤＲ１、２および３を含むＶＬならびに上記（１ａ）～（１ｄ）のいずれか１つに示されるＶＨを含む抗ＣＤ４０抗体と同じエピトープに結合する抗体。

[0117] 本発明の抗ＣＤ４０抗体の他の一例として、配列番号２２で示されるアミノ酸配列を含むＶＬおよび配列番号２７、３２、３７または４２で示されるアミノ酸配列を含むＶＨを含む抗ＣＤ４０抗体が挙げられる。

[0118] 本発明の抗ＥｐＣＡＭ抗体の一例として、それぞれ配列番号２３、２４および２５で示されるアミノ酸配列を含むＣＤＲ１、２および３を含むＶＬならびに以下の（２ａ）～（２ｄ）から選ばれるいずれか１つのＶＨを含む抗ＥｐＣＡＭ抗体が挙げられる。

（２ａ）それぞれ配列番号５２、５３および５４で示されるアミノ酸配列を含むＣＤＲ１、２および３を含むＶＨ、

（２ｂ）それぞれ配列番号５７、５８および５９で示されるアミノ酸配列を含むＣＤＲ１、２および３を含むＶＨ、

（２ｃ）それぞれ配列番号６２、６３および６４で示されるアミノ酸配列を含むＣＤＲ１、２および３を含むＶＨ、

（２ｄ）それぞれ配列番号６７、６８および６９で示されるアミノ酸配列を含むＣＤＲ１、２および３を含むＶＨ。

[0119] 本発明の抗ＥｐＣＡＭ抗体は、上記（２ａ）～（２ｄ）のいずれか１つに示されるＶＨのＣＤＲ１～３のアミノ酸配列およびそれぞれ配列番号２３～２５で示されるＶＬのＣＤＲ１～３のアミノ酸配列と、それぞれ少なくとも３５％、少なくとも４０％、少なくとも４５％、少なくとも５０％、少なくとも５５％、少なくとも６０％、少なくとも６５％、少なくとも７０％、少なくとも７５％、少なくとも８０％、少なくとも８５％、少なくとも９０％、少なくとも９５％、少なくとも９６％、少なくとも９７％、少なくとも９８％、少なくとも９９％相同である、ＶＨおよびＶＬのＣＤＲ１～３のアミノ酸配列を含む抗ＥｐＣＡＭ抗体をも包含する。

[0120] 本発明の抗ＥｐＣＡＭ抗体は、下記（ｉ）または（ｉｉ）に記載される抗体をも包含する。

（ｉ）それぞれ配列番号２３、２４および２５で示されるアミノ酸配列を含むＣＤＲ１、２および３を含むＶＬならびに上記（２ａ）～（２ｄ）のいずれか１つに示されるＶＨを含む抗ＥｐＣＡＭ抗体と、競合してＥｐＣＡＭに結合する抗体。

（ｉｉ）それぞれ配列番号２３、２４および２５で示されるアミノ酸配列を含むＣＤＲ１、２および３を含むＶＬならびに上記（２ａ）～（２ｄ）のいずれか１つに示されるＶＨを含む抗ＥｐＣＡＭ抗体と同じエピトープに結合する抗体。

[0121] 本発明の抗ＥｐＣＡＭ抗体の他の例として、配列番号２２で示されるアミノ酸配列を含むＶＬおよび配列番号５１、５６、６１または６６で示されるアミノ酸配列を含むＶＨを含むバイスペシフィック抗体が挙げられる。

[0122] 本発明のバイスペシフィック抗体としてより具体的には、それぞれ配列番号２３、２４および２５で示されるアミノ酸配列を含むＣＤＲ１、２および３を含むＶＬならびに以下の（ｉ）または（ｉｉ）で表されるＶＨ１およびＶＨ２を含むバイスペシフィック抗体が挙げられる。

（ｉ）配列番号２８、２９および３０；配列番号３３、３４および３５；配列番号３８、３９および４０；または配列番号４３、４４および４５でそれぞれ示されるアミノ酸配列を含むＣＤＲ１、２および３を含む、ＣＤ４０に結合するＶＨ１、

ならびに配列番号５２、５３および５４；配列番号５７、５８および５９；配列番号６２、６３および６４；または配列番号６７、６８および６９でそれぞれ示されるアミノ酸配列を含むＣＤＲ１、２および３を含む、ＥｐＣＡＭに結合するＶＨ２。

（ｉｉ）配列番号５２、５３および５４；配列番号５７、５８および５９；配列番号６２、６３および６４；または配列番号６７、６８および６９でそれぞれ示されるアミノ酸配列を含むＣＤＲ１、２および３を含む、ＥｐＣＡＭに結合するＶＨ１、

ならびに配列番号２８、２９および３０；配列番号３３、３４および３５；配列番号３８、３９および４０；または配列番号４３、４４および４５でそれぞれ示されるアミノ酸配列を含むＣＤＲ１、２および３を含む、ＣＤ４０に結合するＶＨ２。

[0123] 本発明のバイスペシフィック抗体の別の具体的な例として、配列番号２２で示されるアミノ酸配列を含むＶＬおよび以下の（ｉ）または（ｉｉ）で表されるＶＨ１およびＶＨ２を含むバイスペシフィック抗体が挙げられる。

（ｉ）配列番号２７、３２、３７または４２で示されるアミノ酸配列を含む、ＣＤ４０に結合するＶＨ１、

および配列番号５１、５６、６１または６６で示されるアミノ酸配列を含む、ＥｐＣＡＭに結合するＶＨ２。

（ｉｉ）配列番号５１、５６、６１または６６で示されるアミノ酸配列を含む、ＥｐＣＡＭに結合するＶＨ１、

および配列番号２７、３２、３７または４２で示されるアミノ酸配列を含む、ＣＤ４０に結合するＶＨ２。

[0124] 本発明の一態様として、Ｎ末端から順にＶＨ１－Ｘ－ＶＨ２－Ｙの式｛式

中、ＶＨ１は第一の抗体のＶＨ（第一の抗体由来のＶＨを意味する）を表し、ＶＨ２は第二の抗体のＶＨ（第２の抗体由来のＶＨを意味する）を表し、ＸおよびＹはそれぞれポリペプチド（ここでＸおよびＹの少なくとも一方はさらに抗体のヒンジ領域を含む）を表す；以下式（Ｉ）と記載する｝で表されるポリペプチドからなる重鎖を含み、該第一の抗体および第二の抗体のいずれか一方は抗ＣＤ４０抗体であり、もう一方は抗ＥｐＣＡＭ抗体であるバイスペシフィック抗体が挙げられる。

[0125] 前記式（Ｉ）におけるＸおよびＹとして、例えば、それぞれ抗体のＣＨ１、ＣＨ２、ＣＨ３およびヒンジのいずれか１を含むポリペプチド（ここでＸおよびＹの少なくとも一方はさらに抗体のヒンジ領域を含む）が挙げられる。

[0126] 本発明の一態様として、前記式（Ｉ）におけるＸおよびＹのいずれか一方がＣＨ１であり、もう一方がＣＨ１、ＣＨ２、ＣＨ３およびヒンジを含むポリペプチドが挙げられる。具体的には、例えば、ＸはヒトＩｇＧ４またはヒトＩｇＧ１のＣＨ１、ヒンジ、ＣＨ２及びＣＨ３を含むポリペプチドであり、かつＹはヒトＩｇＧ４またはヒトＩｇＧ１のＣＨ１を含むポリペプチド；ＸはヒトＩｇＧ４の定常領域において２２８番目がＰｒｏ、２３５番目がＧｌｕおよび４０９番目がＬｙｓである定常領域を含むポリペプチドであり、かつＹはヒトＩｇＧ４またはヒトＩｇＧ１のＣＨ１を含むポリペプチド；ＸはヒトＩｇＧ４またはヒトＩｇＧ１のＣＨ１を含むポリペプチドであり、かつＹはヒトＩｇＧ４またはヒトＩｇＧ１のＣＨ１、ヒンジ、ＣＨ２及びＣＨ３を含むポリペプチド；ＸはヒトＩｇＧ４またはヒトＩｇＧ１のＣＨ１を含むポリペプチドであり、かつＹはヒトＩｇＧ４の定常領域において２２８番目がＰｒｏ、２３５番目がＧｌｕおよび４０９番目がＬｙｓである定常領域を含むポリペプチド；Ｘは配列番号７５で表されるアミノ酸配列を含むポリペプチドであり、Ｙは配列番号７７で表されるアミノ酸配列を含むポリペプチド；Ｘは配列番号７７で表されるアミノ酸配列を含むポリペプチドであり、Ｙは配列番号７５で表されるアミノ酸配列を含むポリペプチドが挙げられ

る。

[0127] 本発明の一態様として、式（Ｉ）で表されるポリペプチドからなる２本の同一の重鎖を含むバイスペシフィック抗体がより好ましく、式（Ｉ）で表されるポリペプチドからなる２本の同一の重鎖および同一の４本の軽鎖または２種類の軽鎖を２本ずつ含むバイスペシフィック抗体がさらに好ましく、式（Ｉ）で表されるポリペプチドからなる２本の同一の重鎖および同一の４本の軽鎖を含むバイスペシフィック抗体が一層好ましい。

[0128] 前記の好ましい態様において同一の２本の重鎖はヒンジ領域のシステイン残基を介してジスルフィド結合し、重合体を形成することが好ましい。また、１本の重鎖はＣＨ１のシステイン残基を介し、軽鎖の定常領域のシステイン残基とジスルフィド結合し、重合体を形成することが好ましい。したがって、本発明のバイスペシフィック抗体は２本の重鎖と４本の軽鎖が重合した、ヘテロヘキサマー構造を有することが好ましい。

[0129] 本発明の好ましい一態様として、式（Ｉ）｛Ｎ末端から順にＶＨ１－Ｘ－ＶＨ２－Ｙの式｛式中、ＶＨ１は第一の抗体のＶＨを表し、ＶＨ２は第二の抗体のＶＨを表し、Ｘは配列番号７５で表されるアミノ酸配列を含むポリペプチドを表し、Ｙは配列番号７７で表されるアミノ酸配列を含むポリペプチドを表す｝で表されるポリペプチドからなる２本の重鎖ならびにそれぞれ配列番号２３、２４および２５で示されるアミノ酸配列を含むＣＤＲ１、２および３を含むＶＬを含む４本の軽鎖を含むバイスペシフィック抗体であって、該ＶＨ１およびＶＨ２が下記の（ｉ）または（ｉｉ）に示されるＶＨであるバイスペシフィック抗体が挙げられる。

（ｉ）ＶＨ１が、配列番号２８、２９および３０；配列番号３３、３４および３５；配列番号３８、３９および４０；または配列番号４３、４４および４５でそれぞれ示されるアミノ酸配列を含むＣＤＲ１、２および３を含む、抗ヒトＣＤ４０抗体のＶＨであり、

ＶＨ２が、配列番号５２、５３および５４；配列番号５７、５８および５９；配列番号６２、６３および６４；または配列番号６７、６８および６９で

それぞれ示されるアミノ酸配列を含むＣＤＲ１、２および３を含む、抗ヒトＥｐＣＡＭ抗体のＶＨである。

（ｉｉ）ＶＨ１が、配列番号５２、５３および５４；配列番号５７、５８および５９；配列番号６２、６３および６４；または配列番号６７、６８および６９でそれぞれ示されるアミノ酸配列を含むＣＤＲ１、２および３を含む、抗ヒトＥｐＣＡＭ抗体のＶＨであり、

ＶＨ２が、配列番号２８、２９および３０；配列番号３３、３４および３５；配列番号３８、３９および４０；または配列番号４３、４４および４５でそれぞれ示されるアミノ酸配列を含むＣＤＲ１、２および３を含む、抗ヒトＣＤ４０抗体のＶＨである。

[0130] ここで、軽鎖はλ鎖であってもκ鎖であってもよいが、κ鎖であることが好ましい。

[0131] またここで、ＶＨ１がそれぞれ配列番号３３、３４および３５で示されるアミノ酸配列を含むＣＤＲ１、２および３を含む抗ヒトＣＤ４０抗体のＶＨであり、ＶＨ２がそれぞれ配列番号５７、５８および５９で示されるアミノ酸配列を含むＣＤＲ１、２および３を含む抗ヒトＥｐＣＡＭ抗体のＶＨである、バイスペシフィック抗体の一例として、Ｒ１０９０Ｓ５５Ａ－Ｅｐ２０３が挙げられる。

[0132] 本発明のさらに好ましい一態様として、式（Ｉ）｛Ｎ末端から順にＶＨ１－Ｘ－ＶＨ２－Ｙの式｛式中、ＶＨ１は第一の抗体のＶＨを表し、ＶＨ２は第二の抗体のＶＨを表し、Ｘは配列番号７５で表されるアミノ酸配列を含むポリペプチドを表し、Ｙは配列番号７７で表されるアミノ酸配列を含むポリペプチドを表す｝で表されるポリペプチドからなる２本の重鎖および配列番号２２で示されるアミノ酸配列を含むＶＬを含む４本の軽鎖を含むバイスペシフィック抗体であって、該ＶＨ１およびＶＨ２が下記の（ｉ）または（ｉｉ）に示されるＶＨであるバイスペシフィック抗体が挙げられる。

（ｉ）ＶＨ１が配列番号２７、３２、３７または４２で表されるアミノ酸配列を含む抗ヒトＣＤ４０抗体のＶＨであり、

ＶＨ２が配列番号５１、５６、６１または６６で表されるアミノ酸配列を含む抗ヒトＥｐＣＡＭ抗体のＶＨである。

（ｉｉ）ＶＨ１が配列番号５１、５６、６１または６６で表されるアミノ酸配列を含む抗ヒトＥｐＣＡＭ抗体のＶＨであり、

ＶＨ２が配列番号２７、３２、３７または４２で表されるアミノ酸配列を含む抗ヒトＣＤ４０抗体のＶＨである。

[0133] ここで、軽鎖はλ鎖であってもκ鎖であってもよいが、κ鎖であることが好ましい。

[0134] またここで、ＶＨ１が配列番号３２で示されるアミノ酸配列を含む抗ヒトＣＤ４０抗体のＶＨであり、ＶＨ２が配列番号５６で示されるアミノ酸配列を含む抗ヒトＥｐＣＡＭ抗体のＶＨである、バイスペシフィック抗体の一例として、Ｒ１０９０Ｓ５５Ａ－Ｅｐ２０３が挙げられる。

[0135] 本発明の好ましい一態様として、式（Ｉ）｛Ｎ末端から順にＶＨ１－Ｘ－ＶＨ２－Ｙの式｛式中、ＶＨ１は第一の抗体のＶＨを表し、ＶＨ２は第二の抗体のＶＨを表し、Ｘは配列番号７７で表されるアミノ酸配列を含むポリペプチドを表し、Ｙは配列番号７５で表されるアミノ酸配列を含むポリペプチドを表す｝で表されるポリペプチドからなる２本の重鎖ならびにそれぞれ配列番号２３、２４および２５で示されるアミノ酸配列を含むＣＤＲ１、２および３を含むＶＬを含む４本の軽鎖を含むバイスペシフィック抗体であって、該ＶＨ１およびＶＨ２が下記の（ｉ）または（ｉｉ）に示されるＶＨであるバイスペシフィック抗体が挙げられる。

（ｉ）ＶＨ１が、配列番号２８、２９および３０；配列番号３３、３４および３５；配列番号３８、３９および４０；または配列番号４３、４４および４５でそれぞれ示されるアミノ酸配列を含むＣＤＲ１、２および３を含む、抗ヒトＣＤ４０抗体のＶＨであり、

ＶＨ２が、配列番号５２、５３および５４；配列番号５７、５８および５９；配列番号６２、６３および６４；または配列番号６７、６８および６９でそれぞれ示されるアミノ酸配列を含むＣＤＲ１、２および３を含む、抗ヒト

ＥｐＣＡＭ抗体のＶＨである。

（ｉｉ）ＶＨ１が、配列番号５２、５３および５４；配列番号５７、５８および５９；配列番号６２、６３および６４；または配列番号６７、６８および６９でそれぞれ示されるアミノ酸配列を含むＣＤＲ１、２および３を含む、抗ヒトＥｐＣＡＭ抗体のＶＨであり、

ＶＨ２が、配列番号２８、２９および３０；配列番号３３、３４および３５；配列番号３８、３９および４０；または配列番号４３、４４および４５でそれぞれ示されるアミノ酸配列を含むＣＤＲ１、２および３を含む、抗ヒトＣＤ４０抗体のＶＨである。

[0136] ここで、軽鎖はλ鎖であってもκ鎖であってもよいが、κ鎖であることが好ましい。

[0137] またここで、ＶＨ１がそれぞれ配列番号３３、３４および３５で示されるアミノ酸配列を含むＣＤＲ１、２および３を含む抗ヒトＣＤ４０抗体のＶＨであり、ＶＨ２がそれぞれ配列番号５７、５８および５９で示されるアミノ酸配列を含むＣＤＲ１、２および３を含む抗ヒトＥｐＣＡＭ抗体のＶＨである、バイスペシフィック抗体の一例として、Ｃｔ　Ｒ１０９０Ｓ５５Ａ－Ｅｐ２０３が挙げられる。

[0138] また、ＶＨ１がそれぞれ配列番号６７、６８および６９で示されるアミノ酸配列を含むＣＤＲ１、２および３を含む抗ヒトＥｐＣＡＭ抗体のＶＨであり、ＶＨ２がそれぞれ配列番号２８、２９および３０で示されるアミノ酸配列を含むＣＤＲ１、２および３を含む抗ヒトＣＤ４０抗体のＶＨであるバイスペシフィック抗体の一例として、Ｃｔ　Ｅｐｃ１１２－Ｒ１０６６が挙げられる。

[0139] 本発明のさらに好ましい一態様として、式（Ｉ）｛Ｎ末端から順にＶＨ１－Ｘ－ＶＨ２－Ｙの式｛式中、ＶＨ１は第一の抗体のＶＨを表し、ＶＨ２は第二の抗体のＶＨを表し、Ｘは配列番号７７で表されるアミノ酸配列を含むポリペプチドを表し、Ｙは配列番号７５で表されるアミノ酸配列を含むポリペプチドを表す｝で表されるポリペプチドからなる２本の重鎖および配列番

号２２で示されるアミノ酸配列を含むＶＬを含む４本の軽鎖を含むバイスペシフィック抗体であって、該ＶＨ１およびＶＨ２が下記の（ｉ）または（ｉｉ）に示されるＶＨであるバイスペシフィック抗体が挙げられる。

（ｉ）ＶＨ１が配列番号２７、３２、３７または４２で表されるアミノ酸配列を含む抗ヒトＣＤ４０抗体のＶＨであり、

ＶＨ２が配列番号５１、５６、６１または６６で表されるアミノ酸配列を含む抗ヒトＥｐＣＡＭ抗体のＶＨである。

（ｉｉ）ＶＨ１が配列番号５１、５６、６１または６６で表されるアミノ酸配列を含む抗ヒトＥｐＣＡＭ抗体のＶＨであり、

ＶＨ２が配列番号２７、３２、３７または４２で表されるアミノ酸配列を含む抗ヒトＣＤ４０抗体のＶＨである。

[0140] ここで、軽鎖はλ鎖であってもκ鎖であってもよいが、κ鎖であることが好ましい。

[0141] またここで、ＶＨ１が配列番号３２で示されるアミノ酸配列を含む抗ヒトＣＤ４０抗体のＶＨであり、ＶＨ２が配列番号５６で示されるアミノ酸配列を含む抗ヒトＥｐＣＡＭ抗体のＶＨである、バイスペシフィック抗体の一例として、Ｃｔ　Ｒ１０９０Ｓ５５Ａ－Ｅｐ２０３が挙げられる。

[0142] またＶＨ１が配列番号６６で示されるアミノ酸配列を含む抗ヒトＥｐＣＡＭ抗体のＶＨであり、ＶＨ２が配列番号２７で示されるアミノ酸配列を含む抗ヒトＣＤ４０抗体のＶＨである、バイスペシフィック抗体の一例として、Ｃｔ　Ｅｐｃ１１２－Ｒ１０６６が挙げられる。

[0143] 本発明のバイスペシフィック抗体または該バイスペシフィック抗体断片には、エフェクター活性を有する抗体または該バイスペシフィック抗体断片も包含される。

[0144] エフェクター活性とは、抗体のＦｃ領域を介して引き起こされる抗体依存性の細胞傷害活性を指し、例えば、抗体依存性細胞傷害活性（Ａｎｔｉｂｏｄｙ－Ｄｅｐｅｎｄｅｎｔ　Ｃｅｌｌｕｌａｒ　Ｃｙｔｏｔｏｘｉｃｉｔｙ　ａｃｔｉｖｉｔｙ；ＡＤＣＣ活性）、補体依存性細胞傷害活性（Ｃｏｍｐ

ｌｅｍｅｎｔ－Ｄｅｐｅｎｄｅｎｔ Ｃｙｔｏｔｏｘｉｃｉｔｙ ａｃｔｉｖｉｔｙ； ＣＤＣ活性）、マクロファージや樹状細胞などの食細胞による抗体依存性貪食活性（Ａｎｔｉｂｏｄｙ－ｄｅｐｅｎｄｅｎｔ ｃｅｌｌｕｌａｒ ｐｈａｇｏｃｙｔｏｓｉｓ ａｃｔｉｖｉｔｙ；ＡＤＣＰ活性）およびオプソニン効果などが挙げられる。

[0145] 本発明においてＡＤＣＣ活性およびＣＤＣ活性は、公知の測定方法［Cancer Immunol. Immunother., 36, 373 (1993) ］を用いて測定することができる。

[0146] ＡＤＣＣ活性とは、標的細胞上の抗原に結合した抗体が、抗体のＦｃ領域を介して免疫細胞のＦｃ受容体と結合することで免疫細胞（ナチュラルキラー細胞など）を活性化し、標的細胞を傷害する活性をいう。

[0147] Ｆｃ受容体（ＦｃＲ）は、抗体のＦｃ領域に結合する受容体であり、抗体の結合によりさまざまなエフェクター活性を誘発する。各ＦｃＲは抗体のサブクラスに対応し、ＩｇＧ、ＩｇＥ、ＩｇＡ、ＩｇＭはそれぞれＦｃγＲ、ＦｃεＲ、Ｆｃαｒ、Ｆｃμｒに特異的に結合する。さらにＦｃγＲには、ＦｃγＲＩ（ＣＤ６４）、ＦｃγＲＩＩ（ＣＤ３２）およびＦｃγＲＩＩＩ（ＣＤ１６）のサブタイプが存在し、それぞれのサブタイプにはＦｃγＲＩＡ、ＦｃγＲＩＢ、ＦｃγＲＩＣ、ＦｃγＲＩＩＡ、ＦｃγＲＩＩＢ、ＦｃγＲＩＩＣ、ＦｃγＲＩＩＩＡおよびＦｃγＲＩＩＩＢのアイソフォームが存在する。これらの異なるＦｃγＲは異なる細胞上に存在する［Annu. Rev. Immunol. 9:457-492(1991)］。ヒトにおいては、ＦｃγＲＩＩＩＢは好中球に特異的に発現しており、ＦｃγＲＩＩＩＡは単球、ナチュラルキラー細胞（ＮＫ細胞）、マクロファージおよび一部のＴ細胞に発現する。ＦｃγＲＩＩＩＡへの抗体の結合を介して、ＮＫ細胞依存的なＡＤＣＣ活性が誘発される。

[0148] ＣＤＣ活性とは、標的細胞上の抗原に結合した抗体が血液中の補体関連タンパク質群からなる一連のカスケード（補体活性化経路）を活性化し、標的細胞を傷害する活性をいう。また、補体の活性化により生じるタンパク質断

片により、免疫細胞の遊走および活性化が誘導される。ＣＤＣ活性のカスケードは、まずＣ１ｑがＦｃ領域に結合し、次に２つのセリンプロテアーゼであるＣ１ｒおよびＣ１ｓと結合することで、Ｃ１複合体を形成し開始される。

[0149] 本発明のバイスペシフィック抗体または該バイスペシフィック抗体断片の抗原発現細胞に対するＣＤＣ活性、またはＡＤＣＣ活性は、公知の測定方法［Cancer Immunol. Immunother., 36, 373 (1993)］により評価することができる。

[0150] 本発明のバイスペシフィック抗体のエフェクター活性を制御する方法としては、抗体のＦｃ領域（ＣＨ２およびＣＨ３ドメインからなる定常領域）の２９７番目のアスパラギン（Ａｓｎ）に結合するＮ－結合複合型糖鎖の還元末端に存在するＮ－アセチルグルコサミン（ＧｌｃＮＡｃ）にα－１，６結合するフコース（コアフコースともいう）の量を制御する方法（国際公開第２００５／０３５５８６号、国際公開第２００２／３１１４０号および国際公開第００／６１７３９号）や、抗体のＦｃ領域のアミノ酸残基の改変により制御する方法（国際公開第００／４２０７２号）などが知られている。

[0151] バイスペシフィック抗体に付加するフコースの量を制御することで、抗体のＡＤＣＣ活性を増加または低下させることができる。例えば、抗体のＦｃに結合しているＮ－結合複合型糖鎖に結合するフコースの含量を低下させる方法として、α１，６－フコース転移酵素遺伝子が欠損した宿主細胞を用いてバイスペシフィック抗体を発現することで、高いＡＤＣＣを有するバイスペシフィック抗体を取得することができる。一方、バイスペシフィック抗体のＦｃに結合しているＮ－結合複合型糖鎖に結合するフコースの含量を増加させる方法として、α１，６－フコース転移酵素遺伝子を導入した宿主細胞を用いて抗体を発現させることで、低いＡＤＣＣ活性を有するバイスペシフィック抗体を取得することができる。

[0152] また、バイスペシフィック抗体のＦｃ領域のアミノ酸残基を改変することでＡＤＣＣ活性やＣＤＣ活性を増加または低下させることができる。例えば

、米国特許出願公開第２００７／０１４８１６５号明細書に記載のＦｃ領域のアミノ酸配列を用いることで、バイスペシフィック抗体のＣＤＣ活性を増加させることができる。また、米国特許第６，７３７，０５６号明細書、米国特許第７，２９７，７７５号明細書または米国特許第７，３１７，０９１号明細書などに記載のアミノ酸改変を行うことで、ＡＤＣＣ活性またはＣＤＣ活性を、増加させることも低下させることもできる。

[0153] さらに、上述の方法を組み合わせることにより、エフェクター活性が制御されたバイスペシフィック抗体を取得してもよい。

[0154] 本発明のバイスペシフィック抗体の安定性は、精製過程や一定条件下で保存されたサンプルにおいて形成される凝集体（オリゴマー）量を測定することによって評価することができる。すなわち、同一条件下で凝集体量が低減する場合を、抗体の安定性が向上したものと評価する。凝集体量は、ゲルろ過クロマトグラフィーを含む適当なクロマトグラフィーを用いて凝集した抗体と凝集していない抗体とを分離することによって測定することができる。

[0155] 本発明のバイスペシフィック抗体の生産性は、抗体産生細胞から培養液中に産生される抗体量を測定することによって評価することができる。より具体的には、培養液から産生細胞を除いた培養上清に含まれる抗体の量をＨＰＬＣ法やＥＬＩＳＡ法などの適当な方法で測定することによって評価することができる。

[0156] 本発明において、抗体断片とは、抗原結合ドメインを含み、該抗原に対する結合活性を有するタンパク質である。本発明において抗体断片としては、例えば、Ｆａｂ、Ｆａｂ’、Ｆ（ａｂ’）$_2$、ｓｃＦｖ、ｄｉａｂｏｄｙ、ｄｓＦｖまたはＣＤＲを含むペプチドなどが挙げられる。

[0157] Ｆａｂは、ＩｇＧ抗体をタンパク質分解酵素パパインで処理して得られる断片のうち（Ｈ鎖の２２４番目のアミノ酸残基で切断される）、Ｈ鎖のＮ末端側約半分とＬ鎖全体とがジスルフィド結合（Ｓ－Ｓ結合）で結合した、分子量約５万の抗原結合活性を有する抗体断片である。

[0158] Ｆ（ａｂ’）$_2$は、ＩｇＧをタンパク質分解酵素ペプシンで処理して得られ

る断片のうち（Ｈ鎖の２３４番目のアミノ酸残基で切断される）、Ｆａｂがヒンジ領域のＳ－Ｓ結合を介して結合されたものよりやや大きい、分子量約１０万の抗原結合活性を有する抗体断片である。

[0159] Ｆａｂ’は、上記Ｆ（ａｂ’）$_2$のヒンジ領域のＳ－Ｓ結合を切断した、分子量約５万の抗原結合活性を有する抗体断片である。

[0160] ｓｃＦｖは、１本のＶＨと１本のＶＬとを１２残基以上の適当なペプチドリンカー（Ｐ）を用いて連結した、ＶＨ－Ｐ－ＶＬまたはＶＬ－Ｐ－ＶＨポリペプチドであり、抗原結合活性を有する抗体断片である。

[0161] Ｄｉａｂｏｄｙは、抗原結合特異性の同じまたは異なるｓｃＦｖが２量体を形成した抗体断片であり、同じ抗原に対する二価の抗原結合活性、または異なる抗原に対し各々特異的な抗原結合活性を有する抗体断片である。

[0162] ｄｓＦｖは、ＶＨおよびＶＬ中の各１アミノ酸残基をシステイン残基に置換したポリペプチドを、該システイン残基間のＳ－Ｓ結合を介して結合させたものをいう。

[0163] ＣＤＲを含むペプチドは、ＶＨまたはＶＬのＣＤＲの少なくとも１領域を含んで構成される。複数のＣＤＲを含むペプチドは、ＣＤＲ同士を直接または適当なペプチドリンカーを介して結合させることで作製することができる。ＣＤＲを含むペプチドは、本発明のバイスペシフィック抗体のＶＨおよびＶＬのＣＤＲをコードするＤＮＡを構築し、該ＤＮＡを原核生物用発現ベクターまたは真核生物用発現ベクターに挿入し、該発現ベクターを原核生物または真核生物へ導入することにより発現させ、製造することができる。また、ＣＤＲを含むペプチドは、Ｆｍｏｃ法またはｔＢｏｃ法などの化学合成法によって製造することもできる。

[0164] 本発明のバイスペシフィック抗体断片は、本質的にバイスペシフィック抗体の構造の一部からなり、バイスペシフィック抗体の抗原結合部位特異性が異なる２種類の抗原結合ドメインを含み、該２種類の抗原のいずれに対しても結合活性を有するタンパク質である。

[0165] 本発明のバイスペシフィック抗体断片としては、例えば図１２（Ｂ）に示

されるＦａｂ型バイスペシフィック抗体断片、図１２（Ｃ）に示されるＦ（ａｂ’）２型バイスペシフィック抗体断片が挙げられる。

[0166] 抗体のエフェクター活性の増強または欠損、抗体の安定化、および血中半減期の制御を目的としたアミノ酸残基改変を含むＦｃ領域も、本発明のバイスペシフィック抗体に用いることができる。

[0167] 本発明のバイスペシフィック抗体またはバイスペシフィック抗体断片としては、本発明のバイスペシフィック抗体またはそのバイスペシフィック抗体断片に放射性同位元素、低分子の薬剤、高分子の薬剤、タンパク質または抗体医薬などを化学的または遺伝子工学的に結合させた抗体の誘導体を包含する。

[0168] 本発明における抗体の誘導体は、本発明のバイスペシフィック抗体またはそのバイスペシフィック抗体断片のＨ鎖またはＬ鎖のＮ末端側或いはＣ末端側、該バイスペシフィック抗体またはそのバイスペシフィック抗体断片中の適当な置換基或いは側鎖、さらには該バイスペシフィック抗体またはそのバイスペシフィック抗体断片中の糖鎖などに、放射性同位元素、低分子の薬剤、高分子の薬剤、免疫賦活剤、タンパク質または抗体医薬などを化学的手法［抗体工学入門、地人書館（１９９４）］により結合させることで製造することができる。

[0169] また、本発明における抗体の誘導体は、本発明のバイスペシフィック抗体またはバイスペシフィック抗体断片をコードするＤＮＡと、所望のタンパク質または抗体医薬をコードするＤＮＡとを連結させて、発現ベクターに挿入し、該発現ベクターを適当な宿主細胞へ導入し発現させる、遺伝子工学的手法により製造することができる。

[0170] 放射性同位元素としては、例えば、^{111}Ｉｎ、^{131}Ｉ、^{125}Ｉ、^{90}Ｙ、^{64}Ｃｕ、^{99}Ｔｃ、^{77}ＬｕまたはAt^{211}などが挙げられる。放射性同位元素は、クロラミンＴ法などによって抗体に直接結合させることができる。また、放射性同位元素をキレートする物質を抗体に結合させてもよい。キレート剤としては、例えば、１－イソチオシアネートベンジル－３－メチルジエチレン

トリアミンペンタ酢酸（ＭＸ－ＤＴＰＡ）などが挙げられる。

[0171] 低分子の薬剤としては、例えば、アルキル化剤、ニトロソウレア剤、代謝拮抗剤、抗生物質、植物アルカロイド、トポイソメラーゼ阻害剤、ホルモン療法剤、ホルモン拮抗剤、アロマターゼ阻害剤、Ｐ糖蛋白阻害剤、白金錯体誘導体、Ｍ期阻害剤若しくはキナーゼ阻害剤などの抗癌剤［臨床腫瘍学、癌と化学療法社（１９９６）］、ハイドロコーチゾン若しくはプレドニゾンなどのステロイド剤、アスピリン若しくはインドメタシンなどの非ステロイド剤、金チオマレート若しくはペニシラミンなどの免疫調節剤、サイクロフォスファミド若しくはアザチオプリンなどの免疫抑制剤またはマレイン酸クロルフェニラミン若しくはクレマシチンのような抗ヒスタミン剤などの抗炎症剤［炎症と抗炎症療法、医歯薬出版株式会社（１９８２）］などが挙げられる。

[0172] 抗癌剤としては、例えば、アミフォスチン（エチオール）、シスプラチン、ダカルバジン（ＤＴＩＣ）、ダクチノマイシン、メクロレタミン（ナイトロジェンマスタード）、ストレプトゾシン、シクロフォスファミド、イホスファミド、カルムスチン（ＢＣＮＵ）、ロムスチン（ＣＣＮＵ）、ドキソルビシン（アドリアマイシン）、エピルビシン、ゲムシタビン（ゲムザール）、ダウノルビシン、プロカルバジン、マイトマイシン、シタラビン、エトポシド、５－フルオロウラシル、フルオロウラシル、ビンブラスチン、ビンクリスチン、ブレオマイシン、ダウノマイシン、ペプロマイシン、エストラムスチン、パクリタキセル（タキソール）、ドセタキセル（タキソテア）、アルデスロイキン、アスパラギナーゼ、ブスルファン、カルボプラチン、オキサリプラチン、ネダプラチン、クラドリビン、カンプトテシン、１０－ヒドロキシ－７－エチル－カンプトテシン（ＳＮ３８）、フロクスウリジン、フルダラビン、ヒドロキシウレア、イダルビシン、メスナ、イリノテカン（ＣＰＴ－１１）、ノギテカン、ミトキサントロン、トポテカン、ロイプロリド、メゲストロール、メルファラン、メルカプトプリン、ヒドロキシカルバミド、プリカマイシン、ミトタン、ペガスパラガーゼ、ペントスタチン、ピポ

ブロマン、タモキシフェン、ゴセレリン、リュープロレニン、フルタミド、テニポシド、テストラクトン、チオグアニン、チオテパ、ウラシルマスタード、ビノレルビン、クロラムブシル、ハイドロコーチゾン、プレドニゾロン、メチルプレドニゾロン、ビンデシン、ニムスチン、セムスチン、カペシタビン、トムデックス、アザシチジン、ＵＦＴ、オキザロプラチン、ゲフィチニブ（イレッサ）、イマチニブ（ＳＴＩ５７１）、エルロチニブ、ＦＭＳ－ｌｉｋｅ ｔｙｒｏｓｉｎｅ ｋｉｎａｓｅ ３（ＦＩｔ３）阻害剤、ｖａｓｃｕｌａｒ ｅｎｄｏｔｈｅｌｉａｌ ｇｒｏｗｔｈ ｆａｃｏｔｒ ｒｅｃｅｐｔｏｒ（ＶＥＧＦＲ）阻害剤、ｆｉｂｒｏｂｌａｓｔ ｇｒｏｗｔｈ ｆａｃｔｏｒ ｒｅｃｅｐｔｏｒ（ＦＧＦＲ）阻害剤、タルセバなどのｅｐｉｄｅｒｍａｌ ｇｒｏｗｔｈ ｆａｃｔｏｒ ｒｅｃｅｐｔｏｒ（ＥＧＦＲ）阻害剤、ラディシコール、１７－アリルアミノ－１７－デメトキシゲルダナマイシン、ラパマイシン、アムサクリン、オール－トランスレチノイン酸、サリドマイド、レナリドマイド、アナストロゾール、ファドロゾール、レトロゾール、エキセメスタン、ブシラミン、ミゾリビン、シクロスポリン、ヒドロコルチゾン、ベキサロテン（ターグレチン）、デキサメタゾン、プロゲスチン類、エストロゲン類、アナストロゾール（アリミデックス）、ロイプリン、アスピリン、インドメタシン、セレコキシブ、アザチオプリン、ペニシラミン、金チオマレート、マレイン酸クロルフェニラミン、クロロフェニラミン、クレマシチン、トレチノイン、ベキサロテン、砒素、ボルテゾミブ、アロプリノール、カリケアマイシン、イブリツモマブチウキセタン、タルグレチン、オゾガミン、クラリスロマシン、ロイコボリン、ケトコナゾール、アミノグルテチミド、スラミン、メトトレキセート若しくはメイタンシノイドまたはその誘導体などが挙げられる。

[0173] 低分子の薬剤と本発明のバイスペシフィック抗体とを結合させる方法としては、例えば、グルタールアルデヒドを介して薬剤と該抗体のアミノ基間を結合させる方法、または水溶性カルボジイミドを介して薬剤のアミノ基と該抗体のカルボキシル基とを結合させる方法などが挙げられる。

[0174] 高分子の薬剤としては、ポリエチレングリコール（ＰＥＧ）、アルブミン、デキストラン、ポリオキシエチレン、スチレンマレイン酸コポリマー、ポリビニルピロリドン、ピランコポリマー、またはヒドロキシプロピルメタクリルアミドなどが挙げられる。これらの高分子化合物を本発明のバイスペシフィック抗体またはバイスペシフィック抗体断片に結合させることにより、（１）化学的、物理的若しくは生物的な種々の因子に対する安定性の向上、（２）血中半減期の顕著な延長、または（３）免疫原性の消失若しくは抗体産生の抑制、などの効果が期待される［バイオコンジュゲート医薬品、廣川書店（１９９３）］。

[0175] 例えば、ＰＥＧと本発明のバイスペシフィック抗体を結合させる方法としては、ＰＥＧ化修飾試薬と反応させる方法などが挙げられる［バイオコンジュゲート医薬品、廣川書店（１９９３）］。ＰＥＧ化修飾試薬としては、リジンのε－アミノ基への修飾剤（日本国特開昭６１－１７８９２６号公報）、アスパラギン酸およびグルタミン酸のカルボキシル基への修飾剤（日本国特開昭５６－２３５８７号公報）、またはアルギニンのグアニジノ基への修飾剤（日本国特開平２－１１７９２０号公報）などが挙げられる。

[0176] 免疫賦活剤としては、イムノアジュバントとして知られている天然物でもよく、具体例としては、免疫を亢進する薬剤が、β（１→３）グルカン（例えば、レンチナンまたはシゾフィラン）、またはαガラクトシルセラミド（ＫＲＮ７０００）などが挙げられる。

[0177] タンパク質としては、例えば、ＮＫ細胞、マクロファージまたは好中球などの免疫担当細胞を活性化するサイトカイン若しくは増殖因子または毒素タンパク質などが挙げられる。

[0178] サイトカインまたは増殖因子としては、例えば、インターフェロン（以下、ＩＦＮと記す）－α、ＩＦＮ－β、ＩＦＮ－γ、インターロイキン（以下、ＩＬと記す）－２、ＩＬ－１２、ＩＬ－１５、ＩＬ－１８、ＩＬ－２１、ＩＬ－２３、顆粒球コロニー刺激因子（Ｇ－ＣＳＦ）、顆粒球／マクロファージコロニー刺激因子（ＧＭ－ＣＳＦ）またはマクロファージコロニー刺激

因子（Ｍ－ＣＳＦ）などが挙げられる。

[0179] 毒素タンパク質としては、例えば、リシン、ジフテリアトキシンまたはＯＮＴＡＫなどが挙げられ、毒性を調節するためにタンパク質に変異を導入したタンパク毒素も含まれる。

[0180] タンパク質または抗体医薬との融合抗体は、本発明のバイスペシフィック抗体またはバイスペシフィック抗体断片をコードするｃＤＮＡにタンパク質をコードするｃＤＮＡを連結させ、融合抗体をコードするＤＮＡを構築し、該ＤＮＡを原核生物または真核生物用発現ベクターに挿入し、該発現ベクターを原核生物または真核生物へ導入することにより発現させ、製造することができる。

[0181] 上記抗体の誘導体を検出方法、定量方法、検出用試薬、定量用試薬または診断薬として使用する場合に、本発明のバイスペシフィック抗体またはそのバイスペシフィック抗体断片に結合する薬剤としては、通常の免疫学的検出または測定法で用いられる標識体が挙げられる。標識体としては、例えば、アルカリフォスファターゼ、ペルオキシダーゼ若しくはルシフェラーゼなどの酵素、アクリジニウムエステル若しくはロフィンなどの発光物質、またはフルオレセインイソチオシアネート（ＦＩＴＣ）若しくはテトラメチルローダミンイソチオシアネート（ＲＩＴＣ）、Ａｌｅｘａ（登録商標） Ｆｌｕｏｒ ４８８、Ｒ－ｐｈｙｃｏｅｒｙｔｈｒｉｎ（Ｒ－ＰＥ）などの蛍光物質などが挙げられる。

[0182] 本発明には、ＣＤＣ活性またはＡＤＣＣ活性などの細胞傷害活性を有するバイスペシフィック抗体およびそのバイスペシフィック抗体断片が包含される。本発明のバイスペシフィック抗体または該バイスペシフィック抗体断片の抗原発現細胞に対するＣＤＣ活性またはＡＤＣＣ活性は公知の測定方法［Cancer Immunol. Immunother., 36, 373 (1993)］により評価することができる。

[0183] また、本発明は、ＣＤ４０およびＥｐＣＡＭを特異的に認識し結合するバイスペシフィック抗体若しくはそのバイスペシフィック抗体断片を含む組成

物、または該バイスペシフィック抗体若しくは該バイスペシフィック抗体断片を有効成分として含有する、ＣＤ４０およびＥｐＣＡＭの少なくとも一方が関係する疾患、好ましくはＣＤ４０およびＥｐＣＡＭの発現細胞が関与する疾患の治療薬に関する。

[0184] ＣＤ４０およびＥｐＣＡＭの少なくとも一方が関係する疾患としては、例えば、ＣＤ４０およびＥｐＣＡＭの少なくとも一方が関係している疾患であればいかなるものでもよく、例えば、悪性腫瘍およびがんなどが挙げられる。

[0185] 本発明において、悪性腫瘍およびがんとしては、例えば、大腸がん、結腸直腸がん、肺がん、乳がん、グリオーマ、悪性黒色腫（メラノーマ）、甲状腺がん、腎細胞がん、白血病、リンパ腫、Ｔ細胞リンパ腫、胃がん、膵臓がん、子宮頚がん、子宮内膜がん、卵巣がん、胆管がん、食道がん、肝臓がん、頭頚部がん、皮膚がん、尿路がん、膀胱がん、前立腺がん、絨毛がん、咽頭がん、喉頭がん、中皮腫、胸膜腫、男性胚腫、子宮内膜過形成、子宮内膜症、胚芽腫、線維肉腫、カポジ肉腫、血管腫、海綿状血管腫、血管芽腫、網膜芽腫、星状細胞腫、神経線維腫、稀突起膠腫、髄芽腫、神経芽腫、神経膠腫、横紋筋肉腫、膠芽腫、骨原性肉腫、平滑筋肉腫およびウィルムス腫瘍などが挙げられる。

[0186] 本発明のバイスペシフィック抗体または該バイスペシフィック抗体断片、またはこれらの誘導体を含有する治療薬は、有効成分としての該抗体若しくは該バイスペシフィック抗体断片、またはこれらの誘導体のみを含むものであってもよいが、通常は薬理学的に許容される１以上の担体と一緒に混合し、製剤学の技術分野において公知の任意の方法により製造した医薬製剤として提供するのが好ましい。

[0187] 投与経路は、治療に際して最も効果的なものを使用するのが好ましく、例えば、経口投与、または口腔内、気道内、直腸内、皮下、筋肉内または静脈内などの非経口投与が挙げられる。中でも、静脈内投与が好ましい。

[0188] 投与形態としては、例えば、噴霧剤、カプセル剤、錠剤、散剤、顆粒剤、

シロップ剤、乳剤、座剤、注射剤、軟膏またはテープ剤などが挙げられる。

[0189] 投与量または投与回数は、目的とする治療効果、投与方法、治療期間、年齢および体重などにより異なるが、通常成人１日当たり１０μｇ／ｋｇ～１０ｍｇ／ｋｇである。

[0190] さらに、本発明は、本発明のバイスペシフィック抗体またはそのバイスペシフィック抗体断片を含有する、ＣＤ４０およびＥｐＣＡＭの少なくとも一方の免疫学的検出用若しくは測定用試薬、またはＣＤ４０およびＥｐＣＡＭの少なくとも一方が関係する疾患、好ましくはＣＤ４０およびＥｐＣＡＭの発現細胞が関与する疾患の診断薬に関する。また、本発明は、本発明のバイスペシフィック抗体またはそのバイスペシフィック抗体断片を用いた、ＣＤ４０およびＥｐＣＡＭの少なくとも一方の免疫学的検出用若しくは測定用方法、ＣＤ４０およびＥｐＣＡＭの少なくとも一方が関係する疾患、好ましくはＣＤ４０およびＥｐＣＡＭの発現細胞が関与する疾患の治療方法、またはＣＤ４０およびＥｐＣＡＭの少なくとも一方が関係する疾患、好ましくはＣＤ４０およびＥｐＣＡＭの発現細胞が関与する疾患の診断方法に関する。

[0191] 本発明においてＣＤ４０およびＥｐＣＡＭの少なくとも一方の量を検出または測定する方法としては、任意の公知の方法が挙げられる。例えば、免疫学的検出または測定方法などが挙げられる。

[0192] 免疫学的検出または測定方法とは、標識を施した抗原または抗体を用いて、抗体量または抗原量を検出または測定する方法である。免疫学的検出または測定方法としては、例えば、放射免疫測定法（ＲＩＡ）、酵素免疫測定法（ＥＩＡまたはＥＬＩＳＡ）、蛍光免疫測定法（ＦＩＡ）、発光免疫測定法（ｌｕｍｉｎｅｓｃｅｎｔ ｉｍｍｕｎｏａｓｓａｙ）、ウエスタンブロット法または物理化学的手法などが挙げられる。

[0193] 本発明のバイスペシフィック抗体または該バイスペシフィック抗体断片を用いてＣＤ４０およびＥｐＣＡＭの少なくとも一方が発現した細胞を検出または測定することにより、ＣＤ４０およびＥｐＣＡＭの少なくとも一方が関係する疾患、好ましくはＣＤ４０およびＥｐＣＡＭの発現細胞が関与する疾

患を診断することができる。

[0194] ＣＤ４０およびＥｐＣＡＭの少なくとも一方が発現している細胞の検出には、公知の免疫学的検出法を用いることができるが、例えば、免疫沈降法、免疫細胞染色法、免疫組織染色法または蛍光抗体染色法などが挙げられる。また、例えば、ＦＭＡＴ８１００ＨＴＳシステム（アプライドバイオシステム社製）などの蛍光抗体染色法なども挙げられる。

[0195] 本発明においてＣＤ４０およびＥｐＣＡＭの少なくとも一方を検出または測定する対象となる生体試料としては、例えば、組織細胞、血液、血漿、血清、膵液、尿、糞便、組織液または培養液など、ＣＤ４０およびＥｐＣＡＭの少なくとも一方が発現した細胞を含む可能性のあるものであれば特に限定されない。

[0196] 本発明のバイスペシフィック抗体若しくは該バイスペシフィック抗体断片、またはこれらの誘導体を含有する診断薬は、目的とする診断法に応じて、抗原抗体反応を行なうための試薬、該反応の検出用試薬を含んでもよい。抗原抗体反応を行なうための試薬としては、例えば、緩衝剤、塩などが挙げられる。

[0197] 検出用試薬としては、例えば、前記バイスペシフィック抗体若しくは該バイスペシフィック抗体断片、またはこれらの誘導体に結合する標識された二次抗体、または標識に対応した基質などの通常の免疫学的検出または測定法に用いられる試薬が挙げられる。

[0198] 以下、本発明のバイスペシフィック抗体の作製方法、該バイスペシフィック抗体または該バイスペシフィック抗体断片の活性評価方法、並びに該バイスペシフィック抗体または該バイスペシフィック抗体断片を用いた疾患の治療方法および診断方法について具体的に記載する。

[0199] １．モノクローナル抗体の作製方法

本発明におけるモノクローナル抗体の製造方法は、下記の作業工程を包含する。すなわち、（１）免疫原として使用する抗原の精製および細胞表面に抗原が過剰発現した細胞の作製の少なくとも一方、（２）抗原を動物に免疫

した後、血液を採取しその抗体価を検定して脾臓などを摘出する時期を決定し、抗体産生細胞を調製する工程、（３）骨髄腫細胞（ミエローマ）の調製、（４）抗体産生細胞とミエローマとの細胞融合、（５）目的とする抗体を産生するハイブリドーマ群の選別、（６）ハイブリドーマ群からの単クローン（ｍｏｎｏｃｌｏｎａｌ）細胞の分離（クローニング）、（７）場合によっては、モノクローナル抗体を大量に製造するためのハイブリドーマの培養、またはハイブリドーマを移植した動物の飼育、（８）このようにして製造されたモノクローナル抗体の生理活性およびその抗原結合特異性の検討、または標識試薬としての特性の検定、などである。

[0200] 以下、本発明におけるＣＤ４０およびＥｐＣＡＭに結合するバイスペシフィック抗体を作製するために使用する、ＣＤ４０に結合するモノクローナル抗体およびＥｐＣＡＭに結合するモノクローナル抗体の作製方法を上記の工程に沿って詳述する。該抗体の作製方法はこれに制限されず、例えば脾臓細胞以外の抗体産生細胞およびミエローマを使用することもできる。

[0201] （１）抗原の精製

ＣＤ４０およびＥｐＣＡＭの少なくとも一方を発現させた細胞は、ＣＤ４０およびＥｐＣＡＭの少なくとも一方の全長またはその部分長をコードするｃＤＮＡを含む発現ベクターを、大腸菌、酵母、昆虫細胞または動物細胞などに導入することにより、得ることができる。また、ＣＤ４０およびＥｐＣＡＭの少なくとも一方を多量に発現している各種ヒト腫瘍培養細胞またはヒト組織などからＣＤ４０およびＥｐＣＡＭの少なくとも一方を精製し、抗原として使用することができる。また、該腫瘍培養細胞または該組織などをそのまま抗原として用いることもできる。さらに、Ｆｍｏｃ法またはｔＢｏｃ法などの化学合成法によりＣＤ４０およびＥｐＣＡＭの少なくとも一方の部分配列を有する合成ペプチドを調製し、抗原に用いることもできる。

[0202] 本発明で用いられるＣＤ４０およびＥｐＣＡＭの少なくとも一方は、Molecular Cloning,A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press (1989)やCurrent Protocols In Molecular Biology, John

Wiley & Sons(1987-1997)などに記載された方法などを用い、例えば以下の方法により、該ＣＤ４０およびＥｐＣＡＭの少なくとも一方をコードするＤＮＡを宿主細胞中で発現させることで製造することができる。

[0203] ＣＤ４０およびＥｐＣＡＭの少なくとも一方をコードする部分を含む完全長ｃＤＮＡを適当な発現ベクターのプロモーターの下流に挿入することにより、組換えベクターを作製する。上記完全長ｃＤＮＡの代わりに、完全長ｃＤＮＡをもとにして調製された、ポリペプチドをコードする部分を含む適当な長さのＤＮＡ断片を用いてもよい。次に、得られた該組換えベクターを、該発現ベクターに適合した宿主細胞に導入することにより、ＣＤ４０およびＥｐＣＡＭの少なくとも一方を生産する形質転換体を得ることができる。

[0204] 発現ベクターとしては、使用する宿主細胞における自律複製または染色体中への組込みが可能で、かつＣＤ４０およびＥｐＣＡＭの少なくとも一方をコードするＤＮＡを転写できる位置に適当なプロモーターを含有しているものであれば、いずれも用いることができる。

[0205] 宿主細胞としては、例えば、大腸菌などのエシェリヒア属などに属する微生物、酵母、昆虫細胞または動物細胞など、目的とする遺伝子を発現できるものであればいずれも用いることができる。

[0206] 大腸菌などの原核生物を宿主細胞として用いる場合、組換えベクターは、原核生物中で自律複製が可能であるとともに、プロモーター、リボソーム結合配列、ＣＤ４０およびＥｐＣＡＭの少なくとも一方をコードする部分を含むＤＮＡ、並びに転写終結配列を含むベクターであることが好ましい。また、該組換えベクターには、転写終結配列は必ずしも必要ではないが、構造遺伝子の直下に転写終結配列を配置することが好ましい。さらに、該組換えベクターは、プロモーターを制御する遺伝子を含んでもよい。

[0207] 該組換えベクターとしては、リボソーム結合配列であるシャイン・ダルガルノ配列と開始コドンとの間を適当な距離（例えば、６～１８塩基）に調節したプラスミドを用いることが好ましい。

[0208] また、該ＣＤ４０およびＥｐＣＡＭの少なくとも一方をコードするＤＮＡ

の塩基配列としては、宿主内での発現に最適なコドンとなるように塩基を置換することができ、これにより目的とするＣＤ４０およびＥｐＣＡＭの少なくとも一方の生産率を向上させることができる。

[0209] 発現ベクターとしては、使用する宿主細胞中で機能を発揮できるものであればいずれも用いることができ、例えば、ｐＢＴｒｐ２、ｐＢＴａｃ１、ｐＢＴａｃ２（以上、ロシュ・ダイアグノスティックス社製）、ｐＫＫ２３３－２（ファルマシア社製）、ｐＳＥ２８０（インビトロジェン社製）、ｐＧＥＭＥＸ－１（プロメガ社製）、ｐＱＥ－８（キアゲン社製）、ｐＫＹＰ１０（日本国特開昭５８－１１０６００号公報）、ｐＫＹＰ２００［Agricultural Biological Chemistry, 48, 669 (1984)］、ｐＬＳＡ１［Agric. Biol. Chem., 53, 277 (1989)］、ｐＧＥＬ１［Proc. Natl. Acad. Sci. USA, 82, 4306 (1985)］、ｐＢｌｕｅｓｃｒｉｐｔ ＩＩ ＳＫ（－）（ストラタジーン社製）、ｐＴｒｓ３０［大腸菌ＪＭ１０９／ｐＴｒＳ３０（ＦＥＲＭ ＢＰ－５４０７）より調製］、ｐＴｒｓ３２［大腸菌ＪＭ１０９／ｐＴｒＳ３２（ＦＥＲＭ ＢＰ－５４０８）より調製］、ｐＧＨＡ２［大腸菌ＩＧＨＡ２（ＦＥＲＭ ＢＰ－４００）より調製、日本国特開昭６０－２２１０９１号公報］、ｐＧＫＡ２［大腸菌ＩＧＫＡ２（ＦＥＲＭ ＢＰ－６７９８）より調製、日本国特開昭６０－２２１０９１号公報］、ｐＴｅｒｍ２（米国特許第４，６８６，１９１号明細書、米国特許第４，９３９，０９４号明細書、米国特許第５，１６０，７３５号明細書）、ｐＳｕｐｅｘ、ｐＵＢ１１０、ｐＴＰ５、ｐＣ１９４、ｐＥＧ４００［J. Bacteriol., 172, 2392 (1990)］、ｐＧＥＸ（ファルマシア社製）、ｐＥＴシステム（ノバジェン社製）またはｐＭＥ１８ＳＦＬ３（東洋紡社製）などが挙げられる。

[0210] プロモーターとしては、使用する宿主細胞中で機能するものであればいかなるものでもよい。例えば、ｔｒｐプロモーター（Ｐｔｒｐ）、ｌａｃプロモーター、ＰＬプロモーター、ＰＲプロモーターまたはＴ７プロモーターなどの、大腸菌またはファージなどに由来するプロモーターが挙げられる。また、例えば、Ｐｔｒｐを２つ直列させたタンデムプロモーター、ｔａｃプロ

モーター、ｌａｃＴ７プロモーター、またはｌｅｔ　Ｉプロモーターなどの人為的に設計改変されたプロモーターなども挙げられる。

[0211] 宿主細胞としては、例えば、大腸菌ＸＬ１－Ｂｌｕｅ、大腸菌ＸＬ２－Ｂｌｕｅ、大腸菌ＤＨ１、大腸菌ＭＣ１０００、大腸菌ＫＹ３２７６、大腸菌Ｗ１４８５、大腸菌ＪＭ１０９、大腸菌ＨＢ１０１、大腸菌Ｎｏ．４９、大腸菌Ｗ３１１０、大腸菌ＮＹ４９、または大腸菌ＤＨ５αなどが挙げられる。

[0212] 宿主細胞への組換えベクターの導入方法としては、使用する宿主細胞へＤＮＡを導入する方法であればいずれも用いることができ、例えば、カルシウムイオンを用いる方法［Proc. Natl. Acad. Sci. USA, 69, 2110 (1972)、Gene, 17, 107 (1982)、Molecular & General Genetics, 168, 111 (1979)］が挙げられる。

[0213] 動物細胞を宿主として用いる場合、発現ベクターとしては、動物細胞中で機能するものであればいずれも用いることができ、例えば、ｐｃＤＮＡＩ（インビトロジェン社製）、ｐｃＤＭ８（フナコシ社製）、ｐＡＧＥ１０７［日本国特開平３－２２９７９号公報；Cytotechnology, 3, 133 (1990)］、ｐＡＳ３－３（日本国特開平２－２２７０７５号公報）、ｐＣＤＭ８［Nature, 329, 840 (1987)］、ｐｃＤＮＡＩ／Ａｍｐ（インビトロジェン社製）、ｐｃＤＮＡ３．１（インビトロジェン社製）、ｐＲＥＰ４（インビトロジェン社製）、ｐＡＧＥ１０３［J. Biochemistry, 101, 1307 (1987)］、ｐＡＧＥ２１０、ｐＭＥ１８ＳＦＬ３またはｐＫＡＮＴＥＸ９３（国際公開第９７／１０３５４号）などが挙げられる。

[0214] プロモーターとしては、動物細胞中で機能を発揮できるものであればいずれも用いることができ、例えば、サイトメガロウイルス（ＣＭＶ）のｉｍｍｅｄｉａｔｅ　ｅａｒｌｙ（ＩＥ）遺伝子のプロモーター、ＳＶ４０の初期プロモーター、レトロウイルスのプロモーター、メタロチオネインプロモーター、ヒートショックプロモーター、ＳＲαプロモーターまたはモロニーマウス白血病ウイルスのプロモーター若しくはエンハンサーが挙げられる。ま

た、ヒトＣＭＶのＩＥ遺伝子のエンハンサーをプロモーターと共に用いてもよい。

[0215] 宿主細胞としては、例えば、ヒトバーキットリンパ腫細胞Ｎａｍａｌｗａ、アフリカミドリザル腎臓由来細胞ＣＯＳ、チャイニーズハムスター卵巣由来細胞ＣＨＯ、またはヒト白血病細胞ＨＢＴ５６３７（日本国特開昭６３－０００２９９号公報）などが挙げられる。

[0216] 宿主細胞への組換えベクターの導入方法としては、動物細胞にＤＮＡを導入する方法であればいずれも用いることができ、例えば、エレクトロポレーション法［Cytotechnology, 3, 133（1990）］、リン酸カルシウム法（日本国特開平２－２２７０７５号公報）、またはリポフェクション法［Proc. Natl. Acad. Sci. USA, 84, 7413（1987）］などが挙げられる。

[0217] 以上のようにして得られるＣＤ４０およびＥｐＣＡＭの少なくとも一方をコードするＤＮＡを組み込んだ組換えベクターを保有する微生物、または動物細胞などに由来する形質転換体を培地中で培養し、培養物中に該ＣＤ４０およびＥｐＣＡＭの少なくとも一方を生成蓄積させ、該培養物から採取することにより、ＣＤ４０およびＥｐＣＡＭの少なくとも一方を製造することができる。該形質転換体を培地中で培養する方法は、宿主の培養に用いられる通常の方法に従って行うことができる。

[0218] 真核生物由来の細胞で発現させた場合には、糖または糖鎖が付加されたＣＤ４０およびＥｐＣＡＭの少なくとも一方を得ることができる。

[0219] 誘導性のプロモーターを用いた組換えベクターで形質転換した微生物を培養する際には、必要に応じてインデューサーを培地に添加してもよい。例えば、ｌａｃプロモーターを用いた組換えベクターで形質転換した微生物を培養する場合にはイソプロピル－β－Ｄ－チオガラクトピラノシドなどを、ｔｒｐプロモーターを用いた組換えベクターで形質転換した微生物を培養する場合にはインドールアクリル酸などを、各々培地に添加してもよい。

[0220] 動物細胞を宿主として得られた形質転換体を培養する培地としては、例えば、一般に使用されているＲＰＭＩ１６４０培地［The Journal of the Amer

ican Medical Association, 199, 519 (1967)]、ＥａｇｌｅのＭＥＭ培地 [Science, 122, 501 (1952)]、ダルベッコ改変ＭＥＭ培地[Virology, 8, 396 (1959)]、199培地 [Proc. Soc. Exp. Biol. Med., 73, 1 (1950)]、Ｉｓｃｏｖｅ’ｓ Ｍｏｄｉｆｉｅｄ Ｄｕｌｂｅｃｃｏ’ｓ Ｍｅｄｉｕｍ（ＩＭＤＭ）培地、またはこれら培地に牛胎児血清（ＦＢＳ）などを添加した培地などが挙げられる。培養は、通常ｐＨ６～８、３０～４０℃、５％CO_2存在下などの条件下で１～７日間行う。また、培養中に、必要に応じて、カナマイシン、ペニシリンなどの抗生物質を培地に添加してもよい。

[0221] ＣＤ４０およびＥｐＣＡＭの少なくとも一方をコードする遺伝子の発現方法としては、直接発現以外に、分泌生産または融合タンパク質発現などの方法 [Molecular Cloning, A Laboratory Manual,Second Edition,Cold Spring Harbor Laboratory Press (1989)] を用いることができる。ＣＤ４０およびＥｐＣＡＭの少なくとも一方の生産方法としては、例えば、宿主細胞内に生産させる方法、宿主細胞外に分泌させる方法、または宿主細胞外膜上に生産させる方法が挙げられ、使用する宿主細胞や、生産させるＣＤ４０およびＥｐＣＡＭの少なくとも一方の構造を変えることにより、適切な方法を選択することができる。

[0222] 例えば、細胞外領域のアミノ酸配列をコードするＤＮＡに、抗体のＦｃ領域をコードするＤＮＡ、グルタチオンＳ－トランスフェラーゼ（ＧＳＴ）をコードするＤＮＡ、またはＦＬＡＧタグをコードするＤＮＡまたはＨｉｓｔｉｄｉｎｅタグをコードするＤＮＡなどを連結したＤＮＡを作製して、発現し精製することで抗原融合タンパク質を作製することができる。具体的には、例えば、ＣＤ４０およびＥｐＣＡＭの少なくとも一方の細胞外領域をヒトＩｇＧのＦｃ領域に結合させたＦｃ融合タンパク質、ＣＤ４０およびＥｐＣＡＭの少なくとも一方の細胞外領域とグルタチオンＳ－トランスフェラーゼ（ＧＳＴ）との融合タンパク質が挙げられる。

[0223] ＣＤ４０およびＥｐＣＡＭの少なくとも一方が宿主細胞内または宿主細胞外膜上に生産される場合、ポールソンらの方法 [J. Biol. Chem., 264, 1761

9（1989）］、ロウらの方法［Proc. Natl. Acad. Sci., USA, 86, 8227（1989）、Genes Develop., 4, 1288（1990）］、日本国特開平０５－３３６９６３号公報、または国際公開第９４／２３０２１号などに記載の方法を用いることにより、ＣＤ４０およびＥｐＣＡＭの少なくとも一方を宿主細胞外に積極的に分泌させることができる。また、ジヒドロ葉酸還元酵素遺伝子などを用いた遺伝子増幅系（日本国特開平２－２２７０７５号公報）を利用してＣＤ４０およびＥｐＣＡＭの少なくとも一方の生産量を上昇させることもできる。

[0224] 生産したＣＤ４０およびＥｐＣＡＭの少なくとも一方は、例えば、以下のようにして単離、精製することができる。

[0225] ＣＤ４０およびＥｐＣＡＭの少なくとも一方が細胞内に溶解状態で発現した場合には、培養終了後に細胞を遠心分離により回収し、水系緩衝液に懸濁後、超音波破砕機、フレンチプレス、マントンガウリンホモゲナイザー、またはダイノミルなどを用いて細胞を破砕し、無細胞抽出液を得る。該無細胞抽出液を遠心分離して得られる上清から、通常のタンパク質の単離精製法、すなわち溶媒抽出法、硫安などによる塩析法、脱塩法、有機溶媒による沈殿法、ジエチルアミノエチル（ＤＥＡＥ）－セファロース、ＤＩＡＩＯＮ　ＨＰＡ－７５（三菱化学社製）などのレジンを用いた陰イオン交換クロマトグラフィー法、Ｓ－Ｓｅｐｈａｒｏｓｅ　ＦＦ（ファルマシア社製）などのレジンを用いた陽イオン交換クロマトグラフィー法、ブチルセファロース、フェニルセファロースなどのレジンを用いた疎水性クロマトグラフィー法、分子篩を用いたゲルろ過法、アフィニティークロマトグラフィー法、クロマトフォーカシング法、または等電点電気泳動などの電気泳動法などの手法を、単独でまたは組み合わせて用いることで、精製タンパク質を得ることができる。

[0226] ＣＤ４０およびＥｐＣＡＭの少なくとも一方が細胞内に不溶体を形成して発現した場合は、上記と同様に細胞を回収後破砕し、遠心分離を行うことにより、沈殿画分として該ＣＤ４０およびＥｐＣＡＭの少なくとも一方の不溶

体を回収する。回収した該ＣＤ４０およびＥｐＣＡＭの少なくとも一方の不溶体をタンパク質変性剤で可溶化する。該可溶化液を希釈または透析することにより、該ＣＤ４０およびＥｐＣＡＭの少なくとも一方を正常な立体構造に戻した後、上記と同様の単離精製法によりポリペプチドの精製タンパク質を得ることができる。

[0227] ＣＤ４０およびＥｐＣＡＭの少なくとも一方またはその糖修飾体などの誘導体が細胞外に分泌された場合には、培養上清において該ＣＤ４０およびＥｐＣＡＭの少なくとも一方、またはその糖修飾体などの誘導体を回収することができる。該培養上清を上記と同様に遠心分離などの手法を用いて処理することにより、可溶性画分を取得し、該可溶性画分から上記と同様の単離精製法を用いることにより、精製タンパク質を得ることができる。

[0228] また、本発明において用いられるＣＤ４０およびＥｐＣＡＭの少なくとも一方は、Ｆｍｏｃ法またはｔＢｏｃ法などの化学合成法によっても製造することができる。具体的には、例えば、アドバンストケムテック社製、パーキン・エルマー社製、ファルマシア社製、プロテインテクノロジインストルメント社製、シンセセル－ベガ社製、パーセプチブ社製、または島津製作所社製などのペプチド合成機を利用して化学合成することができる。

[0229] （２）抗体産生細胞の調製工程

３～２０週令のマウス、ラットまたはハムスターなどの動物に、（１）で得られる抗原を免疫して、その動物の脾臓、リンパ節または末梢血中の抗体産生細胞を採取する。また、動物としては、例えば、富塚らの文献［Tomizuka. et al., Proc Natl Acad Sci USA., 97、722,（2000）］に記載されているヒト由来抗体を産生するトランスジェニックマウス、免疫原性を高めるためにＣＤ４０またはＥｐＣＡＭのコンディショナルノックアウトマウスなどが被免疫動物として挙げられる。

[0230] 免疫は、フロイントの完全アジュバント、または水酸化アルミニウムゲルと百日咳菌ワクチンなどの適当なアジュバントとともに抗原を投与することにより行う。マウス免疫の際の免疫原投与法は、皮下注射、腹腔内注射、静

脈内注射、皮内注射、筋肉内注射または足蹠注射などいずれでもよいが、腹腔内注射、足蹠注射または静脈内注射が好ましい。抗原が部分ペプチドである場合には、ＢＳＡ（ウシ血清アルブミン）、またはＫＬＨ（Ｋｅｙｈｏｌｅ Ｌｉｍｐｅｔ ｈｅｍｏｃｙａｎｉｎ）などのキャリアタンパク質とのコンジュゲートを作製し、これを免疫原として用いる。

[0231] 抗原の投与は、１回目の投与の後、１～２週間おきに５～１０回行う。各投与後３～７日目に眼底静脈叢より採血し、その血清の抗体価を酵素免疫測定法［Antibodies - A Laboratory Manual, Cold Spring Harbor Laboratory（1988）］などを用いて測定する。免疫に用いた抗原に対し上記の血清が十分な抗体価を示した動物を、融合用抗体の産生細胞の供給源として用いれば、以後の操作の効果を高めることができる。

[0232] 抗原の最終投与後３～７日目に、免疫した動物より脾臓などの抗体産生細胞を含む組織を摘出し、抗体産生細胞を採取する。抗体産生細胞は、形質細胞およびその前駆細胞であるリンパ球であり、これは個体のいずれの部位から得てもよく、一般には脾臓、リンパ節、骨髄、扁桃、末梢血、またはこれらを適宜組み合わせたもの等から得ることができるが、脾臓細胞が最も一般的に用いられる。脾臓細胞を用いる場合には、脾臓を細断してほぐした後、遠心分離し、さらに赤血球を除去することにより、融合用抗体産生細胞を取得する。

[0233] （３）ミエローマの調製工程

ミエローマとしては、マウス、ラット、モルモット、ハムスター、ウサギまたはヒト等の哺乳動物に由来する自己抗体産生能のない細胞を用いることが出来るが、一般的にはマウスから得られた株化細胞、例えば、８－アザグアニン耐性マウス（ＢＡＬＢ／ｃ由来）ミエローマ細胞株Ｐ３－Ｘ６３Ａｇ８－Ｕ１（Ｐ３－Ｕ１）［Current Topics in Microbiology and Immunology, 18, 1（1978）］、Ｐ３－ＮＳ１／１－Ａｇ４１（ＮＳ－１）［European J. Immunology, 6, 511（1976）］、ＳＰ２／０－Ａｇ１４（ＳＰ－２）［Nature, 276, 269（1978）］、Ｐ３－Ｘ６３－Ａｇ８６５３（６５３）［J. Im

munology, 123, 1548 (1979)]、またはＰ３－Ｘ６３－Ａｇ８（Ｘ６３）[Nature, 256, 495 (1975)]などが用いられる。該細胞株は、適当な培地、例えば８－アザグアニン培地［グルタミン、２－メルカプトエタノール、ゲンタマイシン、ＦＣＳおよび８－アザグアニンを加えたＲＰＭＩ－１６４０培地］、イスコフ改変ダルベッコ培地（Ｉｓｃｏｖｅ’ｓ　Ｍｏｄｉｆｉｅｄ　Ｄｕｌｂｅｃｃｏ’ｓ　Ｍｅｄｉｕｍ；以下「ＩＭＤＭ」という）、またはダルベッコ改変イーグル培地（Ｄｕｌｂｅｃｃｏ’ｓ　Ｍｏｄｉｆｉｅｄ　Ｅａｇｌｅ　Ｍｅｄｉｕｍ；以下「ＤＭＥＭ」という）などの培地で継代培養する。細胞融合の３～４日前に上記の細胞株を正常培地（例えば、１０％　ＦＣＳを含むＤＭＥＭ培地）で継代培養し、融合を行う当日に２×１０^{7}個以上の細胞数を確保する。

[0234] （４）細胞融合

（２）で得られる融合用抗体産生細胞と（３）で得られるミエローマ細胞を、Ｍｉｎｉｍｕｍ　Ｅｓｓｅｎｔｉａｌ　Ｍｅｄｉｕｍ（ＭＥＭ）培地またはＰＢＳ（リン酸二ナトリウム１．８３ｇ、リン酸一カリウム０．２１ｇ、食塩７．６５ｇ、蒸留水１リットル、ｐＨ７．２）でよく洗浄し、融合用抗体産生細胞：ミエローマ細胞＝５：１～１０：１になるように混合し、遠心分離した後、上清を除く。沈澱した細胞集塊をよくほぐした後、ポリエチレングリコール－１０００（ＰＥＧ－１０００）、ＭＥＭ培地およびジメチルスルホキシドの混合液を、３７℃にて攪拌しながら加える。さらに１～２分間毎にＭＥＭ培地１～２ｍＬを数回加えた後、ＭＥＭ培地を加えて全量が５０ｍＬになるようにする。遠心分離後、上清を除き、沈澱した細胞集塊を緩やかにほぐした後、ＨＡＴ培地［ヒポキサンチン、チミジンおよびアミノプテリンを加えた正常培地］中に緩やかに細胞を懸濁する。この懸濁液を５％CO_2インキュベーター中、３７℃にて７～１４日間培養する。

[0235] また、以下の方法でも細胞融合を行うことができる。脾臓細胞とミエローマ細胞とを無血清培地（例えばＤＭＥＭ）、またはリン酸緩衝生理食塩液（以下「リン酸緩衝液」という）でよく洗浄し、脾臓細胞とミエローマ細胞の

細胞数の比が５：１～１０：１程度になるように混合し、遠心分離する。上清を除去し、沈澱した細胞集塊をよくほぐした後、撹拌しながら１ｍＬの５０％（ｗ／ｖ）ポリエチレングリコール（分子量１０００～４０００）を含む無血清培地を滴下する。その後、１０ｍＬの無血清培地をゆっくりと加えた後、遠心分離する。再び上清を捨て、沈澱した細胞を適量のヒポキサンチン・アミノプテリン・チミジン（ＨＡＴ）液およびヒトインターロイキン－２（ＩＬ－２）を含む正常培地（以下、ＨＡＴ培地という）中に懸濁して培養用プレート（以下、プレートという）の各ウェルに分注し、５％炭酸ガス存在下、３７℃にて２週間程度培養する。途中適宜ＨＡＴ培地を補う。

[0236] （５）ハイブリドーマ群の選択

融合に用いたミエローマ細胞が８－アザグアニン耐性株である場合、すなわちヒポキサンチン・グアニン・ホスホリボシルトランスフェラーゼ（ＨＧＰＲＴ）欠損株である場合は、融合しなかったミエローマ細胞およびミエローマ細胞同士の融合細胞は、ＨＡＴ培地中では生存することができない。一方、抗体産生細胞同士の融合細胞および抗体産生細胞とミエローマ細胞とのハイブリドーマは、ＨＡＴ培地中で生存することができるが、抗体産生細胞同士の融合細胞はやがて寿命に達する。したがって、ＨＡＴ培地中での培養を続けることによって、抗体産生細胞とミエローマ細胞とのハイブリドーマのみが生き残り、結果的にハイブリドーマを取得することができる。

[0237] コロニー状に生育してきたハイブリドーマについて、ＨＡＴ培地からアミノプテリンを除いた培地（以下、ＨＴ培地という）への培地交換を行う。その後、培養上清の一部を採取し、後述する抗体価測定法を用いて、抗体を産生するハイブリドーマを選択することができる。抗体価の測定方法としては、例えば、放射性同位元素免疫定量法（ＲＩＡ法）、固相酵素免疫定量法（ＥＬＩＳＡ法）、蛍光抗体法および受身血球凝集反応法など種々の公知技術が挙げられるが、検出感度、迅速性、正確性および操作の自動化の可能性などの観点から、ＲＩＡ法またはＥＬＩＳＡ法が好ましい。

[0238] 抗体価を測定することにより、所望の抗体を産生することが判明したハイ

ブリドーマを、別のプレートに移しクローニングを行う。このクローニング法としては、例えば、プレートの１ウェルに１個の細胞が含まれるように希釈して培養する限界希釈法、軟寒天培地中で培養しコロニーを回収する軟寒天法、マイクロマニュピレーターによって１個の細胞を単離する方法、セルソーターによって１個の細胞を単離する方法などが挙げられる。

[0239] 抗体価の認められたウェルについて、例えば限界希釈法によるクローニングを２～４回繰り返し、安定して抗体価の認められたものを、ヒトＣＤ４０またはＥｐＣＡＭに対するモノクローナル抗体を産生するハイブリドーマ株として選択する。

[0240] （６）モノクローナル抗体の調製

プリスタン処理［２，６，１０，１４－テトラメチルペンタデカン（Ｐｒｉｓｔａｎｅ）０．５ｍＬを腹腔内投与し、２週間飼育する］した８～１０週令のマウスまたはヌードマウスに、（５）で得られるモノクローナル抗体産生ハイブリドーマを腹腔内に注射する。１０～２１日でハイブリドーマは腹水癌化する。このマウスから腹水を採取し、遠心分離して固形分を除去後、４０～５０％硫酸アンモニウムで塩析し、カプリル酸沈殿法、ＤＥＡＥ－セファロースカラム、プロテインＡカラムまたはゲル濾過カラムによる精製を行い、ＩｇＧまたはＩｇＭ画分を集め、精製モノクローナル抗体とする。また、同系統のマウス（例えば、ＢＡＬＢ／ｃ）若しくはＮｕ／Ｎｕマウス、ラット、モルモット、ハムスターまたはウサギ等の腹腔内で該ハイブリドーマを増殖させることにより、ＣＤ４０またはＥｐＣＡＭに結合するモノクローナル抗体を大量に含む腹水を得ることができる。

[0241] （５）で得られたモノクローナル抗体産生ハイブリドーマを、１０％ＦＢＳ添加を添加したＲＰＭＩ１６４０培地などで培養した後、遠心分離により上清を除き、ＧＩＴ培地、または５％ダイゴＧＦ２１を添加したＨｙｂｒｉｄｏｍａ　ＳＦＭ培地等に懸濁し、フラスコ培養、スピナー培養またはバック培養などにより３～７日間培養する。得られた細胞懸濁液を遠心分離し、得られた上清よりプロテインＡカラムまたはプロテインＧカラムによる精製

を行ない、ＩｇＧ画分を集め、精製モノクローナル抗体を得ることもできる。精製の簡便な方法としては、市販のモノクローナル抗体精製キット（例えば、ＭＡｂＴｒａｐ　ＧＩＩキット；アマシャムファルマシアバイオテク社製）等を利用することもできる。

[0242] 抗体のサブクラスの決定は、サブクラスタイピングキットを用いて酵素免疫測定法により行う。蛋白量の定量は、ローリー法および２８０ｎｍにおける吸光度［１．４（OD_{280}）＝イムノグロブリン１ｍｇ／ｍＬ］より算出する方法により行うことができる。

[0243] （７）ＣＤ４０またはＥｐＣＡＭに対するモノクローナル抗体の結合アッセイ

ＣＤ４０またはＥｐＣＡＭに対するモノクローナル抗体の結合活性は、オクテルロニー（Ｏｕｃｈｔｅｒｌｏｎｙ）法、ＥＬＩＳＡ法、ＲＩＡ法、フローサイトメトリー法（ＦＣＭ）または表面プラズモン共鳴法（ＳＰＲ）などのバインディングアッセイ系により測定することができる。

[0244] オクテルロニー法は簡便ではあるが、抗体の濃度が低い場合には濃縮操作が必要である。一方、ＥＬＩＳＡ法またはＲＩＡ法を用いた場合は、培養上清をそのまま抗原吸着固相と反応させ、さらに二次抗体として各種イムノグロブリンアイソタイプ、サブクラスに対応する抗体を用いることにより、抗体のアイソタイプ、サブクラスを同定すると共に、抗体の結合活性を測定することが可能である。

[0245] 手順の具体例としては、精製または部分精製した組換えＣＤ４０およびＥｐＣＡＭの少なくとも一方をＥＬＩＳＡ用９６穴プレート等の固相表面に吸着させ、さらに抗原が吸着していない固相表面を抗原と無関係なタンパク質、例えばウシ血清アルブミン（ＢＳＡ）によりブロッキングを行う。ＥＬＩＳＡプレートをｐｈｏｓｐｈａｔｅ　ｂｕｆｆｅｒ　ｓａｌｉｎｅ（ＰＢＳ）および０．０５％　Ｔｗｅｅｎ２０を含むＰＢＳ（Ｔｗｅｅｎ－ＰＢＳ）などで洗浄後、段階希釈した第１抗体（例えばマウス血清、培養上清など）を反応させ、プレートに固定化された抗原へ抗体を結合させる。次に、第２

抗体としてビオチン、酵素（ｈｏｒｓｅ　ｒａｄｉｓｈ　ｐｅｒｏｘｉｄａｓｅ；ＨＲＰ、ａｌｋａｌｉｎｅ　ｐｈｏｓｐｈａｔａｓｅ；ＡＬＰなど）、化学発光物質または放射線化合物などで標識した抗イムノグロブリン抗体を分注して、プレートに結合した第１抗体に第２抗体を反応させる。Ｔｗｅｅｎ－ＰＢＳでよく洗浄した後、第２抗体の標識物質に応じた反応を行い、標的抗原に対し特異的に反応するモノクローナル抗体を選択する。

[0246] ＦＣＭ法では、抗体の抗原発現細胞に対する結合活性を測定することができる［Cancer Immunol. Immunother., 36, 373(1993)］。抗体が細胞膜上に発現している膜タンパク質抗原に結合することは、当該抗体が天然に存在する抗原の立体構造を認識し、結合することを意味する。

[0247] ＳＰＲ法としては、Ｂｉａｃｏｒｅによるｋｉｎｅｔｉｃｓ解析が挙げられる。例えば、Ｂｉａｃｏｒｅ　Ｔ１００を用い、抗原と被験物との間の結合におけるｋｉｎｅｔｉｃｓを測定し、その結果を機器付属の解析ソフトウエアで解析をする。手順の具体例としては、抗マウスＩｇＧ抗体をセンサーチップＣＭ５にアミンカップリング法により固定した後、ハイブリドーマ培養上清または精製モノクローナル抗体などの被験物質を流して適当量を結合させ、さらに濃度既知の複数濃度の抗原を流して、結合および解離を測定する。次に、得られたデータについて、機器付属のソフトウエアを用いて１：１バインディングモデルによるｋｉｎｅｔｉｃｓ解析を行い、各種パラメータを取得する。または、ＣＤ４０およびＥｐＣＡＭの少なくとも一方をセンサーチップ上に、例えばアミンカップリング法により固定した後、濃度既知の複数濃度の精製モノクローナル抗体を流して、結合および解離を測定する。得られたデータについて、機器付属のソフトウエアを用いてバイバレントバインディングモデルによるｋｉｎｅｔｉｃｓ解析を行い、各種パラメータを取得する。

[0248] また、本発明において、ＣＤ４０またはＥｐＣＡＭに対する抗体と競合してＣＤ４０またはＥｐＣＡＭに結合する抗体は、上述のバインティングアッセイ系に被検抗体を共存させて反応させることにより、選択することができ

る。すなわち、被検抗体を加えた時に抗原との結合が阻害される抗体をスクリーニングすることにより、ＣＤ４０またはＥｐＣＡＭへの結合について、前記で取得した抗体と競合する抗体を得ることができる。

[0249] （８）ＣＤ４０またはＥｐＣＡＭに対するモノクローナル抗体のエピトープの同定

本発明において、抗体が認識し結合するエピトープの同定は以下のようにして行うことができる。

[0250] 例えば、抗原の部分欠損体、種間で異なるアミノ酸残基を改変した変異体、または特定のドメインを改変した変異体を作製し、該欠損体または変異体に対する抗体の反応性が低下すれば、欠損部位またはアミノ酸改変部位が該抗体のエピトープであることが明らかになる。このような抗原の部分欠損体および変異体は、適当な宿主細胞、例えば大腸菌、酵母、植物細胞または哺乳動物細胞などを用いて、分泌タンパク質として取得してもよいし、宿主細胞の細胞膜上に発現させて抗原発現細胞として調製してもよい。膜型抗原の場合は、抗原の立体構造を保持したまま発現させるために、宿主細胞の膜上に発現させることが好ましい。また、抗原の１次構造または立体構造を模倣した合成ペプチドを作製し、抗体の反応性を確認することもできる。合成ペプチドは、公知のペプチド合成技術を用いて、その分子の様々な部分ペプチドを作製する方法等が挙げられる。

[0251] 例えば、ヒトおよびマウスのＣＤ４０またはＥｐＣＡＭの細胞外領域について、各領域を構成するドメインを適宜組み合わせたキメラタンパク質を作製し、該タンパク質に対する抗体の反応性を確認することで、抗体のエピトープを同定することができる。その後、さらに細かく、その対応部分のオリゴペプチド、または該ペプチドの変異体等を、当業者に周知のオリゴペプチド合成技術を用いて種々合成し、該ペプチドに対する抗体の反応性を確認することでエピトープを特定することができる。多種類のオリゴペプチドを得るための簡便な方法として、市販のキット［例えば、ＳＰＯＴｓキット（ジェノシス・バイオテクノロジーズ社製）、マルチピン合成法を用いた一連の

マルチピン・ペプチド合成キット（カイロン社製）等］を利用することもできる。

[0252] ＣＤ４０またはＥｐＣＡＭに結合する抗体が結合するエピトープと同じエピトープに結合する抗体は、上述のバインティングアッセイ系で得た抗体のエピトープを同定し、該エピトープの部分的な合成ペプチド、該エピトープの立体構造を模した合成ペプチド、または該エピトープの組換え体等を作製し、免疫することで取得することができる。

[0253] 例えば、エピトープが膜タンパク質であれば、全細胞外領域または一部の細胞外ドメインを、適当なタグ、例えば、ＦＬＡＧタグ、Ｈｉｓｔｉｄｉｎｅタグ、ＧＳＴタンパク質または抗体Ｆｃ領域などに連結した組換え融合タンパク質を作製し、該組換えタンパク質を免疫することで、より効率的に該エピトープ特異的な抗体を作製することができる。

[0254] ２．遺伝子組換え抗体の作製

遺伝子組換え抗体の作製例として、P. J. Delves., ANTIBODY PRODUCTION ESSENTIAL TECHNIQUES., 1997 WILEY、P. Shepherd and C. Dean. Monoclonal Antibodies., 2000 OXFORD UNIVERSITY PRESSおよびJ. W. Goding., Monoclonal Antibodies:principles and practice., 1993 ACADEMIC PRESSなどに概説されているが、以下にキメラ抗体、ヒト化抗体およびヒト抗体の作製方法を示す。また、遺伝子組換えマウス、ラット、ハムスターおよびラビット抗体についても、同様の方法で作製することができる。

[0255] （１）ハイブリドーマからのモノクローナル抗体のＶ領域をコードするｃＤＮＡの取得

モノクローナル抗体のＶＨおよびＶＬをコードするｃＤＮＡの取得は、例えば以下のようにして行うことができる。

[0256] まず、モノクローナル抗体を産生するハイブリドーマよりｍＲＮＡを抽出し、ｃＤＮＡを合成する。次に、合成したｃＤＮＡをファージまたはプラスミドなどのベクターにクローニングしてｃＤＮＡライブラリーを作製する。該ライブラリーより、抗体のＣ領域部分またはＶ領域部分をコードするＤＮ

Ａをプローブとして用いて、ＶＨまたはＶＬをコードするｃＤＮＡを有する組換えファージまたは組換えプラスミドをそれぞれ単離する。単離した組換えファージまたは組換えプラスミド内のＶＨまたはＶＬの全塩基配列を決定し、当該塩基配列よりＶＨまたはＶＬの全アミノ酸配列を推定する。

[0257] ハイブリドーマの作製に用いる非ヒト動物としては、マウス、ラット、ハムスターまたはウサギなどを用いるが、ハイブリドーマを作製することが可能であれば、いかなる動物も用いることができる。

[0258] ハイブリドーマからの全ＲＮＡの調製には、チオシアン酸グアニジン－トリフルオロ酢酸セシウム法［Methods in Enzymol., 154, 3 （1987）］、またはＲＮＡ ｅａｓｙ ｋｉｔ（キアゲン社製）などのキットなどを用いる。

[0259] 全ＲＮＡからのｍＲＮＡの調製には、オリゴ（ｄＴ）固定化セルロースカラム法［Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press (1989) ］、またはＯｌｉｇｏ－ｄＴ３０＜Ｓｕｐｅｒ＞ｍＲＮＡ Ｐｕｒｉｆｉｃａｔｉｏｎ Ｋｉｔ（タカラバイオ社製）などのキットなどを用いる。また、Ｆａｓｔ Ｔｒａｃｋ ｍＲＮＡ Ｉｓｏｌａｔｉｏｎ Ｋｉｔ（インビトロジェン社製）またはＱｕｉｃｋＰｒｅｐ ｍＲＮＡ Ｐｕｒｉｆｉｃａｔｉｏｎ Ｋｉｔ（ファルマシア社製）などのキットを用いて、ｍＲＮＡを調製することもできる。

[0260] ｃＤＮＡの合成およびｃＤＮＡライブラリーの作製には、公知の方法［Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press (1989) 、Current Protocols in Molecular Biology, Supplement 1, John Wiley & Sons (1987-1997) ］またはＳｕｐｅｒＳｃｒｉｐｔ Ｐｌａｓｍｉｄ Ｓｙｓｔｅｍ ｆｏｒ ｃＤＮＡ Ｓｙｎｔｈｅｓｉｓ ａｎｄ Ｐｌａｓｍｉｄ Ｃｌｏｎｉｎｇ（インビトロジェン社製）若しくはＺＡＰ－ｃＤＮＡ Ｓｙｎｔｈｅｓｉｓ Ｋｉｔ（ストラタジーン社製）などのキットなどを用いる。

[0261] ｃＤＮＡライブラリーの作製の際に、ハイブリドーマから抽出したｍＲＮ

Ａを鋳型として合成したｃＤＮＡを組み込むベクターとしては、該ｃＤＮＡを組み込めるベクターであればいかなるものでも用いることができる。

[0262] 例えば、ＺＡＰ Ｅｘｐｒｅｓｓ［Strategies, 5, 58 (1992)］、ｐＢＩｕｅｓｃｒｉｐｔ ＩＩＳＫ（＋）［Nucleic Acids Research, 17, 9494 (1989)］、λＺＡＰＩＩ（Ｓｔｒａｔａｇｅｎｅ社製）、λｇｔ１０、λｇｔ１１［DNA Cloning: A Practical Approach, I, 49 (1985)］、Ｌａｍｂｄａ ＢｌｕｅＭｉｄ（クローンテック社製）、λＥｘＣｅｌｌ、ｐＴ７Ｔ３－１８Ｕ（ファルマシア社製）、ｐｃＤ２［Mol. Cell. Biol., 3, 280 (1983)］またはｐＵＣ１８［Gene, 33, 103 (1985)］などを用いる。

[0263] ファージまたはプラスミドベクターにより構築されるｃＤＮＡライブラリーを導入する大腸菌には、該ｃＤＮＡライブラリーを導入、発現および維持できるものであればいかなるものでも用いることができる。例えば、ＸＬ１－Ｂｌｕｅ ＭＲＦ’［Strategies, 5, 81 (1992)］、Ｃ６００［Genetics, 39, 440 (1954)］、Ｙ１０８８、Ｙ１０９０［Science, 222, 778 (1983)］、ＮＭ５２２［J. Mol. Biol., 166, 1 (1983)］、Ｋ８０２［J. Mol. Biol., 16, 118 (1966)］、またはＪＭ１０５［Gene, 38, 275 (1985)］などを用いる。

[0264] ｃＤＮＡライブラリーからの非ヒト抗体のＶＨまたはＶＬをコードするｃＤＮＡクローンの選択には、アイソトープ若しくは蛍光標識したプローブを用いたコロニー・ハイブリダイゼーション法、またはプラーク・ハイブリダイゼーション法［Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press (1989)］などを用いる。

[0265] また、プライマーを調製し、ｍＲＮＡから合成したｃＤＮＡまたはｃＤＮＡライブラリーを鋳型として、Ｐｏｌｙｍｅｒａｓｅ Ｃｈａｉｎ Ｒｅａｃｔｉｏｎ法［以下、ＰＣＲ法と表記する、Molecular Cloning, A Laboratory Manual, Second Edition , Cold Spring Harbor Laboratory Press (1989)、Current Protocols in Molecular Biology, Supplement 1, John Wiley & Sons (1987-1997)］を行うことにより、ＶＨまたはＶＬをコードするｃＤ

ＮＡを調製することもできる。

[0266] 選択されたｃＤＮＡを、適当な制限酵素などで切断した後、ｐＢｌｕｅｓｃｒｉｐｔ ＳＫ（－）（ストラタジーン社製）などのプラスミドにクローニングし、通常用いられる塩基配列解析方法などにより該ｃＤＮＡの塩基配列を決定する。例えば、ジデオキシ法［Proc. Natl. Acad. Sci. USA, 74, 5463（1977）］などの反応を行った後、Ａ．Ｌ．Ｆ．ＤＮＡシークエンサー（ファルマシア社製）などの塩基配列自動分析装置などを用いて解析する。

[0267] 決定した全塩基配列からＶＨおよびＶＬの全アミノ酸配列をそれぞれ推定し、既知の抗体のＶＨおよびＶＬの全アミノ酸配列［Sequences of Proteins of Immunological Interest, US Dept. Health and Human Services (1991)］と比較することにより、取得したｃＤＮＡが、分泌シグナル配列を含めて抗体のＶＨおよびＶＬ各々の完全なアミノ酸配列をコードしているか否かを確認する。

[0268] 分泌シグナル配列を含む抗体のＶＨおよびＶＬ各々の完全なアミノ酸配列に関しては、既知の抗体のＶＨおよびＶＬの全アミノ酸配列［Sequences of Proteins of Immunological Interest, US Dept. Health and Human Services (1991)］と比較することにより、分泌シグナル配列の長さおよびＮ末端アミノ酸配列を推定することができ、さらにそれらが属するサブグループを同定することができる。

[0269] また、ＶＨおよびＶＬの各ＣＤＲのアミノ酸配列は、既知の抗体のＶＨおよびＶＬのアミノ酸配列［Sequences of Proteins of Immunological Interest, US Dept. Health and Human Services (1991)］と比較することによって推定することができる。

[0270] また、得られたＶＨおよびＶＬの完全なアミノ酸配列について、例えば、ＳＷＩＳＳ－ＰＲＯＴまたはＰＩＲ－Ｐｒｏｔｅｉｎなどの任意のデータベースを用いてＢＬＡＳＴ法［J. Mol. Biol., 215, 403 (1990)］などによる相同性検索を行うことで、当該ＶＨおよびＶＬの完全なアミノ酸配列が新規なものであるか否かを確認することができる。

[0271] （２）遺伝子組換え抗体発現用ベクターの構築

遺伝子組換え抗体発現用ベクターは、動物細胞用発現ベクターにヒト抗体のＣＨおよびＣＬの少なくとも一方をコードするＤＮＡをクローニングすることにより構築することができる。

[0272] ヒト抗体のＣ領域としては、任意のヒト抗体のＣＨおよびＣＬを用いることができ、例えば、ヒト抗体のγ１サブクラスのＣＨおよびκクラスのＣＬなどを用いることができる。ヒト抗体のＣＨおよびＣＬをコードするＤＮＡとしてはｃＤＮＡを用いるが、エキソンとイントロンからなる染色体ＤＮＡを用いることもできる。

[0273] 動物細胞用発現ベクターとしては、ヒト抗体のＣ領域をコードする遺伝子を組み込んで発現できるものであれば、いかなるものでも用いることができ、例えば、ｐＡＧＥ１０７［Cytotechnol., 3, 133 (1990)］、ｐＡＧＥ１０３［J. Biochem., 101, 1307 (1987)］、ｐＨＳＧ２７４［Gene, 27, 223 (1984)］、ｐＫＣＲ［Proc. Natl. Acad. Sci. USA, 78, 1527 (1981)］、pSG1bd2-4［Cytotechnol., 4, 173 (1990)］、またはｐＳＥ１ＵＫ１Ｓｅｄ１－３［Cytotechnol., 13, 79 (1993)］、ＩＮＰＥＰ４（Ｂｉｏｇｅｎ－ＩＤＥＣ社製）、Ｎ５ＫＧ１ｖａｌ（米国特許第６，００１，３５８号明細書）、Ｎ５ＫＧ４ＰＥ　Ｒ４０９Ｋ（国際公開第２００６／０３３３８６号に記載）、Ｎ５ＫＧ２ベクター（国際公開第２００３／０３３５３８号に記載）、トランスポゾンベクター（国際公開第２０１０／１４３６９８号）などを用いることができる。

[0274] 動物細胞用発現ベクターのプロモーターとエンハンサーとしては、ＳＶ４０の初期プロモーター［J. Biochem., 101, 1307 (1987)］、モロニーマウス白血病ウイルスＬＴＲ［Biochem. Biophys. Res. Commun., 149, 960 (1987)］、ＣＭＶプロモーター（米国特許第５，１６８，０６２号明細書）または免疫グロブリンH鎖のプロモーター［Cell, 41, 479 (1985)］とエンハンサー［Cell, 33, 717 (1983)］などを用いることができる。

[0275] 遺伝子組換え抗体の発現には、ベクターの構築の容易さ、動物細胞への導

入の容易さ、細胞内における抗体Ｈ鎖およびＬ鎖の発現量の均衡性などの観点から、抗体Ｈ鎖およびＬ鎖の両遺伝子を搭載したベクター（タンデム型ベクター）［J. Immunol. Methods, 167, 271 （1994）］を用いるが、抗体Ｈ鎖とＬ鎖の各遺伝子を別々に搭載した複数のベクター（セパレート型ベクター）を組み合わせて用いることもできる。

[0276] タンデム型の遺伝子組換え抗体発現用ベクターとしては、ｐＫＡＮＴＥＸ９３（国際公開第９７／１０３５４号）、ｐＥＥ１８［Hybridoma, 17, 559 （1998）］、Ｎ５ＫＧ１ｖａｌ（米国特許第６，００１，３５８号明細書）、Ｎ５ＫＧ４ＰＥ　Ｒ４０９Ｋ（国際公開第２００６／０３３３８６号に記載）、Ｎ５ＫＧ２ベクター（国際公開第２００３／０３３５３８号に記載）、Ｔｏｌ２トランスポゾンベクター（国際公開第２０１０／１４３６９８号）などを用いる。

[0277] （３）キメラ抗体発現ベクターの構築

（２）で得られる遺伝子組換え抗体発現用ベクター内のヒト抗体のＣＨまたはＣＬをコードする遺伝子の各々の上流に、（１）で得られる非ヒト抗体のＶＨまたはＶＬをコードするｃＤＮＡを各々クローニングすることで、キメラ抗体発現ベクターを構築することができる。

[0278] まず、非ヒト抗体のＶＨまたはＶＬをコードするｃＤＮＡの３’末端側と、ヒト抗体のＣＨまたはＣＬの５’末端側とを連結するために、連結部分の塩基配列が適切なアミノ酸をコードし、かつ適当な制限酵素認識配列になるように設計したＶＨおよびＶＬのｃＤＮＡを作製する。次に、作製したＶＨおよびＶＬのｃＤＮＡを、（２）で得られる遺伝子組換え抗体発現用ベクター内のヒト抗体のＣＨまたはＣＬをコードする遺伝子の各々の上流に、それらが適切な形で発現する様にそれぞれクローニングし、キメラ抗体発現ベクターを構築する。

[0279] また、非ヒト抗体のＶＨまたはＶＬをコードするｃＤＮＡを、適当な制限酵素の認識配列を両端に有する合成ＤＮＡを用いてＰＣＲ法によりそれぞれ増幅し、（２）で得られる遺伝子組換え抗体発現用ベクターにクローニング

することで、キメラ抗体発現ベクターを構築することもできる。

[0280] （４）ヒト化抗体のＶ領域をコードするｃＤＮＡの作製

ヒト化抗体のＶＨまたはＶＬをコードするｃＤＮＡは、以下のようにして作製することができる。まず、（１）で得られる非ヒト抗体のＶＨまたはＶＬのＣＤＲのアミノ酸配列を移植するヒト抗体のＶＨまたはＶＬのフレームワーク領域（以下、ＦＲと表記する）のアミノ酸配列をそれぞれ選択する。

[0281] 選択するＦＲのアミノ酸配列には、ヒト抗体由来のものであればいずれのものでも用いることができる。例えば、Ｐｒｏｔｅｉｎ Ｄａｔａ Ｂａｎｋなどのデータベースに登録されているヒト抗体のＦＲのアミノ酸配列、またはヒト抗体のＦＲの各サブグループの共通アミノ酸配列［Sequences of Proteins of Immunological Interest, US Dept. Health and Human Services（1991）］などを用いる。抗体の結合活性の低下を抑えるために、元の非ヒト抗体のＶＨまたはＶＬのＦＲのアミノ酸配列とできるだけ高い相同性（６０％以上）を有するヒトＦＲのアミノ酸配列を選択する。

[0282] 次に、選択したヒト抗体のＶＨまたはＶＬのＦＲのアミノ酸配列に、元の非ヒト抗体のＣＤＲのアミノ酸配列をそれぞれ移植し、ヒト化抗体のＶＨまたはＶＬのアミノ酸配列をそれぞれ設計する。設計したアミノ酸配列を、抗体遺伝子の塩基配列に見られるコドンの使用頻度［Sequences of Proteins of Immunological Interest, US Dept. Health and Human Services (1991)］を考慮してＤＮＡ配列に変換することで、ヒト化抗体のＶＨまたはＶＬのｃＤＮＡ配列をそれぞれ設計する。

[0283] 設計したｃＤＮＡ配列に基づき、１００～１５０塩基前後の長さからなる数本の合成ＤＮＡを合成し、それらを用いてＰＣＲ反応を行う。この場合、ＰＣＲ反応における反応効率および合成可能なＤＮＡ長の観点から、好ましくはＨ鎖およびＬ鎖に対し各々４～６本の合成ＤＮＡを設計する。また、可変領域全長の合成ＤＮＡを合成して用いることもできる。

[0284] さらに、両端に位置する合成ＤＮＡの５’末端に適当な制限酵素の認識配列を導入することで、（２）で得られる遺伝子組換え抗体発現用ベクターに

、容易にヒト化抗体のＶＨまたはＶＬをコードするｃＤＮＡをクローニングすることができる。ＰＣＲ反応後、増幅産物をｐＢｌｕｅｓｃｒｉｐｔ　ＳＫ（－）（ストラタジーン社製）などのプラスミドにそれぞれクローニングし、（１）に記載の方法と同様の方法により塩基配列を決定し、所望のヒト化抗体のＶＨまたはＶＬのアミノ酸配列をコードするＤＮＡ配列を有するプラスミドを取得する。

[0285] （５）ヒト化抗体のＶ領域のアミノ酸配列の改変

ヒト化抗体は、非ヒト抗体のＶＨおよびＶＬのＣＤＲのみをヒト抗体のＶＨおよびＶＬのＦＲに移植しただけでは、その抗原結合活性は元の非ヒト抗体に比べて低下する［BIO/TECHNOLOGY, 9, 266（1991）］。そのため、ヒト抗体のＶＨおよびＶＬのＦＲのアミノ酸配列のうち、直接抗原との結合に関与しているアミノ酸残基、ＣＤＲのアミノ酸残基と相互作用するアミノ酸残基、および抗体の立体構造を維持し間接的に抗原との結合に関与しているアミノ酸残基を同定し、それらのアミノ酸残基を元の非ヒト抗体のアミノ酸残基に置換することにより、低下したヒト化抗体の抗原結合活性を上昇させることができる。

[0286] 抗原結合活性に関わるＦＲのアミノ酸残基を同定するために、Ｘ線結晶解析［J. Mol. Biol., 112, 535（1977）］またはコンピューターモデリング［Protein Engineering, 7, 1501（1994）］などを用いることにより、抗体の立体構造の構築および解析を行うことができる。また、それぞれの抗体について数種の改変体を作製し、それぞれの抗原結合活性との相関を検討することを繰り返し、試行錯誤することで、必要な抗原結合活性を有する改変ヒト化抗体を取得できる。

[0287] ヒト抗体のＶＨおよびＶＬのＦＲのアミノ酸残基は、改変用合成ＤＮＡを用いて（４）に記載のＰＣＲ反応を行うことにより、改変することができる。ＰＣＲ反応後の増幅産物について、（１）に記載の方法により、塩基配列を決定し、目的とする改変が施されたことを確認する。

[0288] （６）ヒト化抗体発現ベクターの構築

（２）で得られる遺伝子組換え抗体発現用ベクターのヒト抗体のＣＨまたはＣＬをコードするそれぞれの遺伝子の上流に、構築したヒト化抗体のＶＨまたはＶＬをコードするｃＤＮＡをそれぞれクローニングし、ヒト化抗体発現ベクターを構築することができる。

[0289] 例えば、（４）および（５）で得られるヒト化抗体のＶＨまたはＶＬを構築する際に用いる合成ＤＮＡのうち、両端に位置する合成ＤＮＡの５’末端に適当な制限酵素の認識配列を導入することで、（２）で得られる遺伝子組換え抗体発現用ベクター内のヒト抗体のＣＨまたはＣＬをコードする各遺伝子の上流に、それらが適切な形で発現するようにそれぞれクローニングする。

[0290] （７）ヒト抗体発現ベクターの構築

ヒト抗体を産生する動物を被免疫動物として用いて、モノクローナル抗体を産生するハイブリドーマを樹立した場合には、（１）において、ヒト抗体のＶＨおよびＶＬのアミノ酸配列およびｃＤＮＡ配列を得ることができる。そこで、（２）で得られる遺伝子組換え抗体発現用ベクターのヒト抗体のＣＨまたはＣＬをコードするそれぞれの遺伝子の上流に、（１）で得たヒト抗体のＶＨまたはＶＬをコードする遺伝子をそれぞれクローニングすることで、ヒト抗体発現ベクターを構築することができる。

[0291] （８）遺伝子組換え抗体の一過性発現

（３）、（６）および（７）で得られる遺伝子組換え抗体発現ベクター、またはそれらを改変した発現ベクターを用いて遺伝子組換え抗体を一過性に発現させ、得られた多種類の遺伝子組換え抗体の抗原結合活性を効率的に評価することができる。

[0292] 発現ベクターを導入する宿主細胞には、遺伝子組換え抗体を発現できる宿主細胞であれば、いかなる細胞でも用いることができるが、例えばＣＯＳ－７細胞［American Type Culture Collection（ATCC）番号：CRL1651］を用いる。ＣＯＳ－７細胞への発現ベクターの導入には、ＤＥＡＥ－デキストラン法［Methods in Nucleic Acids Res., CRC press（1991）］、またはリポフ

エクション法［Proc. Natl. Acad. Sci. USA, 84, 7413 (1987)］などを用いる。

[0293] 発現ベクターの導入後、培養上清中の遺伝子組換え抗体の発現量および抗原結合活性を、酵素免疫抗体法［Monoclonal Antibodies-Principles and practice, Third edition, Academic Press (1996)、Antibodies - A Laboratory Manual, Cold Spring Harbor Laboratory (1988)、単クローン抗体実験マニュアル、講談社サイエンティフィック（１９８７）］などを用いて測定する。

[0294] （９）遺伝子組換え抗体の安定的発現株の取得と遺伝子組換え抗体の調製

（３）、（６）および（７）で得られた遺伝子組換え抗体発現ベクターを適当な宿主細胞に導入することにより遺伝子組換え抗体を安定的に発現する形質転換株を得ることができる。

[0295] 宿主細胞への発現ベクターの導入としては、例えば、エレクトロポレーション法［日本国特開平２－２５７８９１号公報、Cytotechnology, 3, 133 (1990)］、カルシウムイオン方法、エレクトロポレーション法、スフェロプラスト法、酢酸リチウム法、リン酸カルシウム法、リポフェクション法などが挙げられる。また、後述の動物に遺伝子を導入する方法としては、例えば、マイクロインジェクション法、ＥＳ細胞にエレクトロポレーションやリポフェクション法を用いて遺伝子を導入する方法、および核移植法などが挙げられる。

[0296] 遺伝子組換え抗体発現ベクターを導入する宿主細胞としては、遺伝子組換え抗体を発現させることができる宿主細胞であれば、いかなる細胞でも用いることができる。例えば、マウスＳＰ２／０－Ａｇ１４細胞（ＡＴＣＣ ＣＲＬ１５８１）、マウスＰ３Ｘ６３－Ａｇ８．６５３細胞（ＡＴＣＣ ＣＲＬ１５８０）、チャイニーズハムスターＣＨＯ－Ｋ１細胞（ＡＴＣＣ ＣＣＬ－６１）、ＤＵＫＸＢ１１（ＡＴＣＣ ＣＣＬ－９０９６）、Ｐｒｏ－５細胞（ＡＴＣＣ ＣＣＬ－１７８１）、ＣＨＯ－Ｓ細胞（Ｌｉｆｅ Ｔｅｃｈｎｏｌｏｇｉｅｓ、Ｃａｔ Ｎｏ．１１６１９）、ジヒドロ葉酸還元酵素

遺伝子（ｄｈｆｒ）が欠損したＣＨＯ細胞（ＣＨＯ／ＤＧ４４細胞）［Proc. Natl. Acad. Sci. USA, 77, 4216 (1980)］、レクチン耐性を獲得したＬｅｃ１３細胞［Somatic Cell and Molecular genetics, 12, 55 (1986)］、α１，６－フコース転移酵素遺伝子が欠損したＣＨＯ細胞（国際公開第２００５／０３５５８６号、国際公開第０２／３１１４０号）、ラットＹＢ２／３ＨＬ．Ｐ２．Ｇ１１．１６Ａｇ．２０細胞（ＡＴＣＣ番号：ＣＲＬ１６６２）などを用いる。

[0297] また、細胞内糖ヌクレオチドＧＤＰ－フコースの合成に関与する酵素などのタンパク質、Ｎ－グリコシド結合複合型糖鎖の還元末端のＮ－アセチルグルコサミンの６位にフコースの１位がα結合する糖鎖修飾に関与する酵素などのタンパク質、または細胞内糖ヌクレオチドＧＤＰ－フコースのゴルジ体への輸送に関与するタンパク質などの活性が低下または欠失した宿主細胞、例えばα１，６－フコース転移酵素遺伝子が欠損したＣＨＯ細胞（国際公開第２００５／０３５５８６号、国際公開第０２／３１１４０号）などを用いることもできる。

[0298] 発現ベクターの導入後、遺伝子組換え抗体を安定的に発現する形質転換株を、Ｇ４１８硫酸塩（以下、Ｇ４１８と表記する）などの薬剤を含む動物細胞培養用培地で培養することにより選択する（日本国特開平２－２５７８９１号公報）。

[0299] 動物細胞培養用培地には、ＲＰＭＩ１６４０培地（インビトロジェン社製）、ＧＩＴ培地（日本製薬社製）、ＥＸ－ＣＥＬＬ３０１培地（ジェイアールエイチ社製）、ＥＸ－ＣＥＬＬ３０２培地（ジェイアールエイチ社製）、ＥＸ－ＣＥＬＬ３２５培地（ジェイアールエイチ社製）、ＩＭＤＭ培地（インビトロジェン社製）若しくはＨｙｂｒｉｄｏｍａ－ＳＦＭ培地（インビトロジェン社製）、またはこれらの培地にＦＢＳなどの各種添加物を添加した培地などを用いる。得られた形質転換株を培地中で培養することで、培養上清中に遺伝子組換え抗体を発現、蓄積させる。培養上清中の遺伝子組換え抗体の発現量および抗原結合活性はＥＬＩＳＡ法などにより測定することがで

きる。また、ＤＨＦＲ増幅系（日本国特開平２－２５７８９１号公報）などを利用して、形質転換株の産生する遺伝子組換え抗体の発現量を上昇させることができる。

[0300] 遺伝子組換え抗体は、形質転換株の培養上清よりプロテインＡカラムを用いて精製することができる［Monoclonal Antibodies - Principles and practice, Third edition, Academic Press (1996)、Antibodies - A Laboratory Manual, Cold Spring Harbor Laboratory (1988)］。また、ゲル濾過、イオン交換クロマトグラフィーおよび限外濾過など、タンパク質の精製で用いられる方法を組み合わせて精製することもできる。

[0301] 精製した遺伝子組換え抗体のＨ鎖、Ｌ鎖または抗体分子全体の分子量は、ポリアクリルアミドゲル電気泳動法［Nature, 227, 680 (1970)］、またはウェスタンブロッティング法［Monoclonal Antibodies - Principles and practice, Third edition, Academic Press (1996)、Antibodies - A Laboratory Manual, Cold Spring Harbor Laboratory (1988)］など用いて測定することができる。

[0302] ３．バイスペシフィック抗体または該バイスペシフィック抗体断片の作製

本発明のバイスペシフィック抗体は、例えば、まず、異なるエピトープに結合する複数のモノクローナル抗体を上記１．に記載の方法を用いて取得し、次に、各抗体のＶＨおよびＶＬのｃＤＮＡ配列を上記２．に記載の方法を用いて決定し、各抗体の抗原結合部位を連結したバイスペシフィック抗体または該バイスペシフィック抗体断片を設計することによって作製することができる。

[0303] 具体的には、各抗体の抗原結合部位とリンカーとを適宜組み合わせたＤＮＡを合成し、上記２．（２）に記載の遺伝子組換え抗体発現ベクターに組み込み、当該バイスペシフィック抗体または該バイスペシフィック抗体断片を発現させることで、作製することができる。より具体的には、リンカーを介して各抗体のＶＨを連結したポリペプチドをコードするＤＮＡを合成すると共に、各抗体のＶＬをコードするＤＮＡを合成し、これらのＤＮＡを上記２

．（２）に記載の遺伝子組換え抗体発現ベクターに組み込み、当該バイスペシフィック抗体または該バイスペシフィック抗体断片を発現させることで、作製することができる。また、２．（２）のＣＨまたはＣＬをコードする遺伝子の代わりに、任意のポリペプチド鎖をコードする遺伝子を導入した動物細胞用発現ベクターを用いることにより、最もＣ末端に近い抗原結合部位のＣ末端側に任意のポリペプチド鎖（Ｃ末端側ポリペプチド）を有するバイスペシフィック抗体または該バイスペシフィック抗体断片を作製することができる。任意のポリペプチド鎖をコードする遺伝子はリンカーを介して各抗体のＶＨを連結したポリペプチドをコードするＤＮＡと結合した上で動物細胞用発現ベクターに組み込んでも良いし、リンカーを介して各抗体のＶＨを連結したポリペプチドをコードするＤＮＡとは別々に動物細胞用発現ベクターに組み込んでもよい。

[0304] 抗原結合部位は、上記１．に記載のハイブリドーマを用いた方法の他、Ｐｈａｇｅ　Ｄｉｓｐｌａｙ法、Ｙｅａｓｔ　ｄｉｓｐｌａｙ法などの技術により単離し、取得することが出来る［Emmanuelle Laffy et. al., Human Antibodies 14, 33-55, （2005）］。

[0305] 上記のリンカーおよびＣ末端側ポリペプチドとして例えばポリペプチド鎖が挙げられる。具体的には例えば複数の抗原結合ドメインを結合させたポリペプチドを指す。例えばＮ末端からＣ末端への方向に順に整列したＣＨ１－ヒンジ－ＣＨ２－ＣＨ３からなるイムノグロブリンドメイン、ＣＨ１－ヒンジ－ＣＨ２からなるイムノグロブリンドメイン、ＣＨ１－ヒンジからなるイムノグロブリンドメイン、ＣＨ１からなるイムノグロブリンドメイン、ＣＨ１のＮ末端側の断片、１４番目のアミノ酸残基がＣｙｓである１４アミノ酸残基からなるＣＨ１断片およびＣＨ１のＮ末端から１～１４番目のアミノ酸残基からなるＣＨ１断片、並びにこれらイムノグロブリンドメイン断片のアミノ酸配列において、１つ以上のアミノ酸残基が改変されたものを挙げることができる。

[0306] 上記のリンカーおよびＣ末端側ポリペプチドとしてさらに具体的には、例

えば、配列番号７５で表わされるＩｇＧ４のＣＨ１のＮ末端の１～１４番目の１４アミノ酸残基からなるポリペプチド、配列番号７５で表わされるＩｇＧ４のＣＨ１からなるポリペプチドならびに配列番号７７で表されるＩｇＧ４ＰＥ　Ｒ４０９ＫのＣＨ（ＣＨ１、ヒンジ、ＣＨ２およびＣＨ３）からなるポリペプチドなどが挙げられる。

[0307] また、複数のＶＨと単一のＶＬから成るバイスペシフィック抗体または該バイスペシフィック抗体断片を作製する場合には、バイスペシフィック抗体に含まれる各抗原結合部位が各々の特異的抗原に反応するように、ファージディスプレイ法等を用いたスクリーニングを行い、単一のＶＬに最も適した各々のＶＨを選択する。

[0308] 具体的には、まず、上記１．に記載の方法を用いて、第１の抗原で動物を免疫してその脾臓からハイブリドーマを作製し、第１の抗原結合部位をコードするＤＮＡ配列をクローニングする。次に、第２の抗原で動物を免疫して、その脾臓からｃＤＮＡライブラリーを調製し、該ライブラリーからＰＣＲによりＶＨのアミノ酸配列をコードするＤＮＡを取得する。

[0309] 続いて、第２の抗原の免疫で得られたＶＨと第１の抗原結合部位のＶＬとを連結したｓｃＦｖを発現するファージライブラリーを作製し、該ファージライブラリーを用いたパニングにより、第２の抗原に特異的に結合するｓｃＦｖを提示したファージを選択する。選択したファージから、第２の抗原結合部位のＶＨのアミノ酸配列をコードするＤＮＡ配列をクローニングする。

[0310] さらに、第１の抗原結合部位のＶＨと第２の抗原結合部位のＶＨとを上述のリンカーを介して連結したポリペプチドのアミノ酸配列をコードするＤＮＡ配列を設計し、該ＤＮＡ配列と単一のＶＬのアミノ酸配列をコードするＤＮＡ配列とを、例えば上記２．（２）に記載の遺伝子組換え抗体発現ベクターに挿入することにより、本発明のバイスペシフィック抗体または該バイスペシフィック抗体断片の発現ベクターを構築することができる。また２．（２）のＣＨをコードする遺伝子の代わりに、任意のポリペプチド鎖をコードする遺伝子を導入した動物細胞用発現ベクターを用いることにより、第二の

抗原結合部位のＶＨのＣ末端側に任意のポリペプチド鎖（Ｃ末端側ポリペプチド）を有するバイスペシフィック抗体または該バイスペシフィック抗体断片を作製することができる。。

[0311] ４．本発明のバイスペシフィック抗体または該バイスペシフィック抗体断片の活性評価

精製したバイスペシフィック抗体または該バイスペシフィック抗体断片の活性評価は、以下のように行うことができる。

[0312] ＣＤ４０およびＥｐＣＡＭの少なくとも一方を発現した細胞株に対する本発明のバイスペシフィック抗体または該バイスペシフィック抗体断片の結合活性は、前述の１．（７）記載のバインディングアッセイ系を用いて測定することができる。

[0313] ＣＤ４０およびＥｐＣＡＭの少なくとも一方を発現した細胞に対するＣＤＣ活性、またはＡＤＣＣ活性は公知の測定方法［Cancer Immunol. Immunother., 36, 373 (1993)］により測定することができる。

[0314] 本発明のバイスペシフィック抗体または該バイスペシフィック抗体断片の細胞死誘導活性は次の方法で測定することができる。例えば、細胞を９６穴プレートに播種し、抗体を添加して一定期間培養した後に、ＷＳＴ－８試薬（Ｄｏｊｉｎｄｏ社製）を反応させ、プレートリーダーにより４５０ｎｍの吸光度を測定することにより、細胞の生存率を測定する。

[0315] ５．本発明のバイスペシフィック抗体または該バイスペシフィック抗体断片を用いた疾患の治療方法

本発明のバイスペシフィック抗体または該バイスペシフィック抗体断片は、ＣＤ４０およびＥｐＣＡＭの少なくとも一方が関係する疾患、好ましくはＣＤ４０およびＥｐＣＡＭの発現細胞が関与する疾患の治療に用いることができる。ＣＤ４０およびＥｐＣＡＭの少なくとも一方が関係する疾患としては、例えば、悪性腫瘍およびがんなどが挙げられる。

[0316] 悪性腫瘍およびがんとしては、例えば、大腸がん、結腸直腸がん、肺がん、乳がん、グリオーマ、悪性黒色腫（メラノーマ）、甲状腺がん、腎細胞が

ん、白血病、リンパ腫、Ｔ細胞リンパ腫、胃がん、膵臓がん、子宮頚がん、子宮内膜がん、卵巣がん、胆管がん、食道がん、肝臓がん、頭頚部扁平上皮がん、皮膚がん、尿路がん、膀胱がん、前立腺がん、絨毛がん、咽頭がん、喉頭がん、胸膜腫、男性胚腫、子宮内膜過形成、子宮内膜症、胚芽腫、線維肉腫、カポジ肉腫、血管腫、海綿状血管腫、血管芽腫、網膜芽腫、星状細胞腫、神経線維腫、稀突起膠腫、髄芽腫、神経芽腫、神経膠腫、横紋筋肉腫、膠芽腫、骨原性肉腫、平滑筋肉腫およびウィルムス腫瘍などが挙げられる。

[0317] 本発明のバイスペシフィック抗体または該バイスペシフィック抗体断片、またはこれらの誘導体を含有する治療薬は、有効成分としての該抗体若しくは該バイスペシフィック抗体断片、またはこれらの誘導体のみを含むものであってもよいが、通常は薬理学的に許容される１以上の担体と一緒に混合し、製剤学の技術分野において公知の方法により製造した医薬製剤として提供される。

[0318] 投与経路としては、例えば、経口投与、または口腔内、気道内、直腸内、皮下、筋肉内若しくは静脈内などの非経口投与が挙げられる。投与形態としては、例えば、噴霧剤、カプセル剤、錠剤、散剤、顆粒剤、シロップ剤、乳剤、座剤、注射剤、軟膏またはテープ剤などが挙げられる。各種製剤は、通常用いられている賦形剤、増量剤、結合剤、浸潤剤、崩壊剤、表面活性剤、滑沢剤、分散剤、緩衝剤、保存剤、溶解補助剤、防腐剤、着色料、香味剤、および安定化剤などを用いて常法により製造することができる。

[0319] 賦形剤としては、例えば、乳糖、果糖、ブドウ糖、コーンスターチ、ソルビット、結晶セルロース、滅菌水、エタノール、グリセロール、生理食塩水および緩衝液などが挙げられる。崩壊剤としては、例えば、澱粉、アルギン酸ナトリウム、ゼラチン、炭酸カルシウム、クエン酸カルシウム、デキストリン、炭酸マグネシウムおよび合成ケイ酸マグネシウムなどが挙げられる。

[0320] 結合剤としては、例えば、メチルセルロースまたはその塩、エチルセルロース、アラビアゴム、ゼラチン、ヒドロキシプロピルセルロースおよびポリビニルピロリドンなどが挙げられる。滑沢剤としては、例えば、タルク、ス

テアリン酸マグネシウム、ポリエチレングリコールおよび硬化植物油などが挙げられる。

[0321] 安定化剤としては、例えば、アルギニン、ヒスチジン、リジン、メチオニンなどのアミノ酸、ヒト血清アルブミン、ゼラチン、デキストラン４０、メチルセルロース、亜硫酸ナトリウム、メタ亜硫酸ナトリウムなどが挙げられる。

[0322] その他の添加剤としては、例えば、シロップ、ワセリン、グリセリン、エタノール、プロピレングリコール、クエン酸、塩化ナトリウム、亜硝酸ソーダおよびリン酸ナトリウムなどが挙げられる。

[0323] 経口投与に適当な製剤としては、例えば、乳剤、シロップ剤、カプセル剤、錠剤、散剤または顆粒剤などが挙げられる。

[0324] 乳剤またはシロップ剤のような液体調製物は、水、ショ糖、ソルビトール若しくは果糖などの糖類、ポリエチレングリコール若しくはプロピレングリコールなどのグリコール類、ごま油、オリーブ油若しくは大豆油などの油類、ｐ－ヒドロキシ安息香酸エステル類などの防腐剤、またはストロベリーフレーバー若しくはペパーミントなどのフレーバー類などを添加剤として用いて製造される。

[0325] カプセル剤、錠剤、散剤または顆粒剤などは、乳糖、ブドウ糖、ショ糖若しくはマンニトールなどの賦形剤、デンプン若しくはアルギン酸ナトリウムなどの崩壊剤、ステアリン酸マグネシウム若しくはタルクなどの滑沢剤、ポリビニルアルコール、ヒドロキシプロピルセルロース若しくはゼラチンなどの結合剤、脂肪酸エステルなどの界面活性剤、またはグリセリンなどの可塑剤などを添加剤として用いて製造される。

[0326] 非経口投与に適当な製剤としては、例えば、注射剤、座剤または噴霧剤などが挙げられる。注射剤は、塩溶液、ブドウ糖溶液、またはその両者の混合物からなる担体などを用いて製造される。

[0327] 座剤はカカオ脂、水素化脂肪またはカルボン酸などの担体を用いて製造される。噴霧剤は、受容者の口腔および気道粘膜を刺激せず、かつ本発明のバ

イスペシフィック抗体または該バイスペシフィック抗体断片を微細な粒子として分散させ、吸収を容易にさせる担体などを用いて製造される。担体としては、例えば、乳糖またはグリセリンなどが挙げられる。また、エアロゾルまたはドライパウダーとして製造することもできる。さらに、上記非経口剤においても、経口投与に適当な製剤で添加剤として例示した成分を添加することもできる。

[0328] 本発明のバイスペシフィック抗体の有効量と適切な希釈剤および薬理学的に使用し得るキャリアとの組合せとして投与される有効量は、１回につき体重１ｋｇあたり０．０００１ｍｇ～１００ｍｇであり、２日から８週間間隔で投与される。

[0329] ６．本発明のバイスペシフィック抗体または該バイスペシフィック抗体断片を用いた疾患の診断方法

本発明のバイスペシフィック抗体または該バイスペシフィック抗体断片を用いて、ＣＤ４０およびＥｐＣＡＭの少なくとも一方が発現した細胞を検出または測定することにより、ＣＤ４０およびＥｐＣＡＭの少なくとも一方が関係する疾患、好ましくはＣＤ４０およびＥｐＣＡＭの発現細胞が関与する疾患を診断することができる。

[0330] ＣＤ４０およびＥｐＣＡＭの少なくとも一方が関係する疾患である悪性腫瘍またはがんの診断は、例えば、以下のようにＣＤ４０およびＥｐＣＡＭの少なくとも一方を検出または測定して行うことができる。

[0331] まず、複数の健常者の生体から採取した生体試料について、本発明のバイスペシフィック抗体若しくは該バイスペシフィック抗体断片、またはこれらの誘導体を用い、下記の免疫学的手法を用いて、ＣＤ４０およびＥｐＣＡＭの少なくとも一方の検出または測定を行い、健常者の生体試料中のＣＤ４０およびＥｐＣＡＭの少なくとも一方の存在量を調べる。

[0332] 次に、被験者の生体試料中についても同様にＣＤ４０およびＥｐＣＡＭの少なくとも一方の存在量を調べ、その存在量を健常者の存在量と比較する。被験者のＣＤ４０およびＥｐＣＡＭの少なくとも一方の存在量が健常者と比

較して増加している場合には、該被験者はがんであると診断される。その他のＣＤ４０およびＥｐＣＡＭの少なくとも一方が関係する疾患の診断についても、同様の方法で診断できる。

[0333] 免疫学的手法とは、標識を施した抗原または抗体を用いて、抗体量または抗原量を検出または測定する方法である。例えば、放射性物質標識免疫抗体法、酵素免疫測定法、蛍光免疫測定法、発光免疫測定法、ウェスタンブロット法または物理化学的手法などが挙げられる。

[0334] 放射性物質標識免疫抗体法としては、例えば、抗原または抗原を発現した細胞などに、本発明のバイスペシフィック抗体または該バイスペシフィック抗体断片を反応させ、さらに放射線標識を施した抗イムノグロブリン抗体または結合断片を反応させた後、シンチレーションカウンターなどで測定する方法が挙げられる。

[0335] 酵素免疫測定法としては、例えば、抗原または抗原を発現した細胞などに、本発明のバイスペシフィック抗体または該バイスペシフィック抗体断片を反応させ、さらに標識を施した抗イムノグロブリン抗体または結合断片を反応させた後、発色色素を吸光光度計で測定する方法が挙げられる。例えば、サンドイッチＥＬＩＳＡ法などが挙げられる。

[0336] 酵素免疫測定法で用いる標識体としては、公知の酵素標識［酵素免疫測定法、医学書院（１９８７）］を用いることができる。例えば、アルカリフォスファターゼ標識、ペルオキシダーゼ標識、ルシフェラーゼ標識、またはビオチン標識などを用いる。

[0337] サンドイッチＥＬＩＳＡ法は、固相に抗体を結合させた後、検出または測定対象である抗原をトラップさせ、トラップされた抗原に第２の抗体を反応させる方法である。該ＥＬＩＳＡ法では、検出または測定したい抗原に結合する抗体または抗体断片であって、抗原結合部位の異なる２種類の抗体を準備し、そのうち、第１の抗体または抗体断片をあらかじめプレート（例えば、９６穴プレート）に吸着させ、次に第２の抗体または抗体断片をＦＩＴＣなどの蛍光物質、ペルオキシダーゼなどの酵素、またはビオチンなどで標識

しておく。上記の抗体が吸着したプレートに、生体内から分離された細胞若しくはその破砕液、組織若しくはその破砕液、細胞培養上清、血清、胸水、腹水、または眼液などを反応させた後、標識した抗体または抗体断片を反応させ、標識物質に応じた検出反応を行う。濃度既知の抗原を段階的に希釈して作製した検量線より、被験サンプル中の抗原濃度を算出する。

[0338] サンドイッチＥＬＩＳＡ法に用いる抗体としては、ポリクローナル抗体またはモノクローナル抗体のいずれを用いてもよく、Ｆａｂ、Ｆａｂ’、またはＦ（ａｂ）$_2$などの抗体断片を用いてもよい。サンドイッチＥＬＩＳＡ法で用いる２種類の抗体の組み合わせとしては、異なるエピトープに結合するモノクローナル抗体または該抗体断片の組み合わせでもよいし、ポリクローナル抗体とモノクローナル抗体またはその抗体断片との組み合わせでもよい。

[0339] 蛍光免疫測定法としては、例えば、文献［Monoclonal Antibodies-Principles and practice, Third edition,Academic Press (1996)、単クローン抗体実験マニュアル、講談社サイエンティフィック（１９８７）］などに記載された方法で測定する。蛍光免疫測定法で用いる標識体としては、公知の蛍光標識［蛍光抗体法、ソフトサイエンス社（１９８３）］を用いることができる。例えば、ＦＩＴＣまたはＲＩＴＣなどを用いる。

[0340] 発光免疫測定法としては、例えば、文献［生物発光と化学発光　臨床検査４２、廣川書店（１９９８）］などに記載された方法で測定する。発光免疫測定法で用いる標識体としては、公知の発光体標識が挙げられ、例えば、アクリジニウムエステルまたはロフィンなどを用いる。

[0341] ウェスタンブロット法としては、抗原または抗原を発現した細胞などをＳＤＳ（ドデシル硫酸ナトリウム）－ＰＡＧＥ［Antibodies - A Laboratory Manual Cold Spring Harbor Laboratory (1988)］で分画した後、該ゲルをポリフッ化ビニリデン（ＰＶＤＦ）膜またはニトロセルロース膜にブロッティングし、該膜に抗原に結合する抗体または抗体断片を反応させ、さらにＦＩＴＣなどの蛍光物質、ペルオキシダーゼなどの酵素標識、またはビオチン標識などを施した抗ＩｇＧ抗体またはその抗体断片を反応させた後、該標識を

可視化することによって測定する。一例を以下に示す。

[0342] まず、所望のアミノ酸配列を有するポリペプチドを発現している細胞や組織を溶解し、還元条件下でレーンあたりのタンパク量として０．１～３０μｇをＳＤＳ－ＰＡＧＥ法により泳動する。次に、泳動されたタンパク質をＰＶＤＦ膜にトランスファーし１～１０％ＢＳＡを含むＰＢＳ（以下、ＢＳＡ－ＰＢＳと表記する）に室温で３０分間反応させブロッキング操作を行う。ここで本発明のバイスペシフィック抗体を反応させ、０．０５～０．１％のＴｗｅｅｎ－２０を含むＰＢＳ（Ｔｗｅｅｎ－ＰＢＳ）で洗浄し、ペルオキシダーゼ標識したヤギ抗マウスＩｇＧを室温で２時間反応させる。Ｔｗｅｅｎ－ＰＢＳで洗浄し、ＥＣＬ Ｗｅｓｔｅｒｎ Ｂｌｏｔｔｉｎｇ Ｄｅｔｅｃｔｉｏｎ Ｒｅａｇｅｎｔｓ（アマシャム社製）などを用いて該抗体が結合したバンドを検出することにより、抗原を検出する。ウェスタンブロッティングでの検出に用いられる抗体としては、天然型の立体構造を保持していないポリペプチドに結合できる抗体が用いられる。

[0343] 物理化学的手法としては、例えば、抗原であるＣＤ４０およびＥｐＣＡＭの少なくとも一方と本発明のバイスペシフィック抗体またはそのバイスペシフィック抗体断片とを結合させることにより、凝集体を形成させて、該凝集体を検出する。この他に物理化学的手法として、毛細管法、一次元免疫拡散法、免疫比濁法またはラテックス免疫比濁法［臨床検査法提要、金原出版（１９９８）］などを用いることもできる。

[0344] ラテックス免疫比濁法では、抗体または抗原を感作させた粒径０．１～１μｍ程度のポリスチレンラテックスなどの担体を用い、対応する抗原または抗体により抗原抗体反応を起こさせると、反応液中の散乱光は増加し、透過光は減少する。この変化を吸光度または積分球濁度として検出することにより、被験サンプル中の抗原濃度などを測定する。

[0345] 一方、ＣＤ４０およびＥｐＣＡＭの少なくとも一方が発現している細胞の検出または測定には、公知の免疫学的検出法を用いることができるが、好ましくは免疫沈降法、免疫細胞染色法、免疫組織染色法または蛍光抗体染色法

などを用いる。

[0346] 免疫沈降法としては、ＣＤ４０およびＥｐＣＡＭの少なくとも一方を発現した細胞などを本発明のバイスペシフィック抗体またはそのバイスペシフィック抗体断片と反応させた後、プロテインＧセファロースなどのイムノグロブリンに特異的な結合能を有する担体を加えて、抗原抗体複合体を沈降させる。

[0347] または、以下のような方法によっても行なうことができる。まず、ＥＬＩＳＡ用９６穴プレートに本発明のバイスペシフィック抗体またはそのバイスペシフィック抗体断片を固相化した後、ＢＳＡ－ＰＢＳによりブロッキングする。次に、ＢＳＡ－ＰＢＳを捨てＰＢＳでよく洗浄した後、ＣＤ４０およびＥｐＣＡＭの少なくとも一方を発現している細胞や組織の溶解液を反応させる。よく洗浄した後のプレートより、免疫沈降物をＳＤＳ－ＰＡＧＥ用サンプルバッファーで抽出し、上記のウェスタンブロッティングにより検出する。

[0348] 免疫細胞染色法または免疫組織染色法とは、抗原を発現した細胞または組織などを、場合によっては抗体の透過性を良くするために界面活性剤やメタノールなどで処理した後、本発明のバイスペシフィック抗体と反応させ、さらにＦＩＴＣなどの蛍光標識、ペルオキシダーゼなどの酵素標識またはビオチン標識などを施した抗イムノグロブリン抗体またはその結合断片と反応させた後、該標識を可視化し、顕微鏡にて顕鏡する方法である。また、蛍光標識の抗体と細胞を反応させ、フローサイトメーターにて解析する蛍光抗体染色法［Monoclonal Antibodies - Principles and practice, Third edition, Academic Press (1996)、単クローン抗体実験マニュアル、講談社サイエンティフィック（１９８７）］により検出を行うことができる。特に、本発明のバイスペシフィック抗体またはそのバイスペシフィック抗体断片は、蛍光抗体染色法により、細胞膜上に発現しているＣＤ４０およびＥｐＣＡＭの少なくとも一方を検出することができる。

[0349] また、蛍光抗体染色法のうち、ＦＭＡＴ８１００ＨＴＳシステム（アプラ

イドバイオシステム社製）などを用いた場合には、形成された抗体－抗原複合体と、抗体－抗原複合体の形成に関与していない遊離の抗体または抗原とを分離することなく、抗原量または抗体量を測定することができる。

実施例

[0350] 以下、本発明を実施例により具体的に説明するが、本発明は下記実施例に限定されるものではない。

[0351] ［実施例１］可溶性ヒトおよびサルＣＤ４０抗原ならびに可溶性ヒト、サルおよびマウスＥｐＣＡＭ抗原の調製

１．ヒトＣＤ４０およびサルＣＤ４０の可溶性抗原の調製

Ｃ末端端にＦＬＡＧ－Ｆｃが付加されたヒトおよびサルＣＤ４０の細胞外ドメインタンパク質をそれぞれ以下に記載する方法で作製した。ヒトＣＤ４０細胞外ドメインをコードする塩基配列を配列番号１に、当該塩基配列から推定されるアミノ酸配列を配列番号２に、サルＣＤ４０細胞外ドメインをコードする塩基配列を配列番号３に、当該塩基配列から推定されるアミノ酸配列を配列番号４に示す。

[0352] （１）ヒトおよびサルＣＤ４０－ＦＬＡＧ－Ｆｃベクターの作製

ヒトＣＤ４０遺伝子配列（Ｇｅｎｂａｎｋ Ａｃｃｅｓｓｉｏｎ Ｎｕｍｂｅｒ：ＮＭ_００１２５０、配列番号５；該遺伝子がコードするアミノ酸配列を配列番号６に示す）を基に配列番号１で示される塩基配列からなるヒトＣＤ４０の細胞外ドメインの遺伝子断片を作製した。

[0353] ＦＬＡＧ－ｔａｇおよびヒトＩｇＧのＦｃ領域を含むＩＮＰＥＰ４ベクター（Ｂｉｏｇｅｎ－ＩＤＥＣ社製）を制限酵素ＫｐｎＩおよびＸｂａＩで消化し、配列番号１で示される塩基配列の１～６０番目の塩基配列からなるヒトＣＤ４０シグナル配列コード領域を加えた該細胞外ドメインの遺伝子断片を適切な部位に挿入して、ヒトＣＤ４０－ＦＬＡＧ－Ｆｃ発現ベクターを作製した。

[0354] 同様の方法で、サル末梢血単核球（ＰＢＭＣ）からクローニングしたサルＣＤ４０遺伝子配列（配列番号７；該遺伝子がコードするアミノ酸配列を配

列番号８に示す）を基に、配列番号３で示される塩基配列からなるサルＣＤ４０の細胞外ドメインの遺伝子断片を含むサルＣＤ４０－ＦＬＡＧ－Ｆｃ発現ベクターを作製した。

[0355] （２）ヒトおよびサルＣＤ４０－ＦＬＡＧ－Ｆｃタンパク質の作製

ＦｒｅｅＳｔｙｌｅ（商標） ２９３ Ｅｘｐｒｅｓｓｉｏｎ Ｓｙｓｔｅｍ （Ｔｈｅｒｍｏ Ｆｉｓｈｅｒ社製）を用いて、１－（１）で作製したヒトＣＤ４０－ＦＬＡＧ－Ｆｃ発現ベクターをＨＥＫ２９３細胞に導入して培養し、一過性発現系でタンパク質を発現させた。ベクター導入５日後に培養上清を回収し、孔径０．２２μｍのメンブランフィルター（ＭＩＬＬＩＰＯＲＥ社製）で濾過した。

[0356] この培養上清をＰｒｏｔｅｉｎ Ａ樹脂（ＭａｂＳｅｌｅｃｔ、ＧＥヘルスケア社製）を用いてアフィニティー精製を行った。プロテインＡに吸着させた抗体を、ダルベッコリン酸緩衝生理食塩水［Ｄ－ＰＢＳ（－） ｗｉｔｈｏｕｔ Ｃａ ａｎｄ Ｍｇ，ｌｉｑｕｉｄ；以下Ｄ－ＰＢＳ（－）と記載する。ナカライテスク社製］にて洗浄し、２０ｍＭクエン酸ナトリウム、５０ｍＭ ＮａＣｌ緩衝液（ｐＨ３．４）により溶出して、１Ｍリン酸ナトリウム緩衝液（ｐＨ７．０）を含むチューブに回収した。

[0357] 次に、ＶＩＶＡＳＰＩＮ（Ｓａｒｔｒｉｕｓ ｓｔｅａｌｉｎ社製）を用いた限外ろ過により、Ｄ－ＰＢＳ（－）に置換した後、孔径０．２２μｍのメンブレンフィルターＭｉｌｌｅｘ－Ｇｖ（Ｍｉｌｌｉｐｏｒｅ社製）でろ過滅菌し、ヒトＣＤ４０－ＦＬＡＧ－Ｆｃタンパク質を作製した。同様の方法で、１－（１）で作製したサルＣＤ４０－ＦＬＡＧ－Ｆｃ発現ベクターを用い、サルＣＤ４０－ＦＬＡＧ－Ｆｃタンパク質を作製した。取得したタンパク質の濃度は、波長２８０ｎｍの吸光度を測定して、各タンパク質のアミノ酸配列から推定される吸光係数を用いて算出した。

[0358] （３）ヒトおよびサルＣＤ４０－ＧＳＴベクターの作製

ＧＳＴ領域を含むＮ５ベクター（Ｂｉｏｇｅｎ－ＩＤＥＣ社製）を制限酵素ＢｇｌＩＩおよびＫｐｎＩで消化して、１－（１）記載の配列番号１で示

される塩基配列からなるヒトＣＤ４０の細胞外ドメインの遺伝子断片を適切な部位に挿入し、ヒトＣＤ４０－ＧＳＴ発現ベクターを作製した。同様の方法で、配列番号３で示される塩基配列からなる細胞外ドメインの遺伝子断片を含むサルＣＤ４０－ＧＳＴ発現ベクターを作製した。

[0359] （４）ヒトおよびサルＣＤ４０－ＧＳＴタンパク質の作製

１－（３）で作製したヒトＣＤ４０－ＧＳＴベクターを、１－（２）と同様の方法でＨＥＫ２９３細胞に導入し、該細胞を培養後、培養上清をメンブランフィルターでろ過した。この培養上清をＧｌｕｔａｔｈｉｏｎｅ　Ｓｅｐｈａｒｏｓｅ　４Ｂ（ＧＥヘルスケア社製）に反応させ、Ｄ－ＰＢＳ（－）で洗浄して、溶出緩衝液として１０ｍＭ　Ｇｌｕｔａｔｈｉｏｎｅ　ｉｎ　５０ｍＭ　Ｔｒｉｓ－ＨＣｌ（ｐＨ８．０）を用いてアフィニティー精製した。

[0360] 溶出された融合タンパク溶液に対し１－（２）と同様の方法で限外ろ過およびメンブレンフィルターによるろ過滅菌を行い、ヒトＣＤ４０－ＧＳＴタンパク質を得た。また、サルＣＤ４０－ＧＳＴベクターを用い、同様の方法でサルＣＤ４０－ＧＳＴタンパク質を得た。取得したタンパク質の濃度は、波長２８０ｎｍの吸光度を測定して、各タンパク質のアミノ酸配列から推定される吸光係数を用いて算出した。

[0361] ２．ヒト、サルおよびマウスＥｐＣＡＭの可溶性抗原の調製

Ｃ末端にＦＬＡＧ－ＦｃまたはＧＳＴが付加されたヒト、サルまたはマウスＥｐＣＡＭの細胞外ドメインタンパク質をそれぞれ以下に記載する方法で作製した。ヒトＥｐＣＡＭ細胞外ドメインをコードする塩基配列を配列番号９に、当該塩基配列から推定されるアミノ酸配列を配列番号１０に、サルＥｐＣＡＭ細胞外ドメインをコードする塩基配列を配列番号１１に、当該塩基配列から推定されるアミノ酸配列を配列番号１２に、マウスＥｐＣＡＭ細胞外ドメインをコードする塩基配列を配列番号１３に、当該塩基配列から推定されるアミノ酸配列を配列番号１４に示す。

[0362] 配列番号１５に示すヒトＥｐＣＡＭ遺伝子（Ｇｅｎｂａｎｋ　Ａｃｃｅｓ

ｓｉｏｎ　Ｎｕｍｂｅｒ：ＮＭ＿００２３５４、該遺伝子がコードするアミノ酸配列を配列番号１６に示す）、配列番号１７に示すサルＥｐＣＡＭ遺伝子（Ｇｅｎｂａｎｋ　Ａｃｃｅｓｓｉｏｎ　Ｎｕｍｂｅｒ：ＸＭ＿０１５４３３６８５、該遺伝子がコードするアミノ酸配列を配列番号１８に示す）および配列番号１９に示すマウスＥｐＣＡＭ遺伝子（Ｇｅｎｂａｎｋ　Ａｃｃｅｓｓｉｏｎ　Ｎｕｍｂｅｒ：ＮＭ＿００８５３２、該遺伝子がコードするアミノ酸配列を配列番号２０に示す）を基に、１－（１）および（２）に記載の方法と同様にして、ヒト、サルおよびマウスＥｐＣＡＭ－ＦＬＡＧ－Ｆｃタンパク質をそれぞれ得た。

[0363] なお、ＥｐＣＡＭ－ＦＬＡＧ－Ｆｃ発現ベクターには、シグナル配列コード領域として、配列番号１５に示す塩基配列の１～６３番目からなる塩基配列を組み込んだ。また、１－（３）および（４）に記載の方法と同様にして、ヒト、サルおよびマウスＥｐＣＡＭ－ＧＳＴタンパク質をそれぞれ得た。取得したタンパク質の濃度は、波長２８０ｎｍの吸光度を測定して、各タンパク質のアミノ酸配列から推定される吸光係数を用い算出した。

[0364] ［実施例２］膜発現ヒトＥｐＣＡＭ発現ＨＥＫ２９３細胞の作製

ｐＥＦ６／Ｖ５－Ｈｉｓ　ＴＯＰＯ　ＴＡ　Ｅｘｐｒｅｓｓｉｏｎ　Ｋｉｔ（Ｉｎｖｉｔｒｏｇｅｎ社製）を用いて配列番号１５に示すヒトＥｐＣＡＭの遺伝子をＴＯＰＯクローニングし、順方向にヒトＥｐＣＡＭ遺伝子が挿入されているクローンを選択することにより、膜発現用ヒトＥｐＣＡＭ発現ベクターｐＥＦ６－ヒトＥｐＣＡＭ　ｆｕｌｌを得た。

[0365] 取得した発現ベクターｐＥＦ６－ヒトＥｐＣＡＭ　ｆｕｌｌを、ＦｒｅｅＳｔｙｌｅ（商標）　２９３　Ｅｘｐｒｅｓｓｉｏｎ　Ｓｙｓｔｅｍ（Ｔｈｅｒｍｏ　Ｆｉｓｈｅｒ社製）を用いてＨＥＫ２９３細胞に導入して培養し、一過性発現系でタンパク質を発現させた。遺伝子導入後、２４時間振とう培養し、遠心分離によりヒトＥｐＣＡＭ／ＨＥＫ２９３細胞を得た。

[0366] ［実施例３］抗ＣＤ４０抗体の取得

１．ＣＤ４０免疫ヒト抗体Ｍ１３ファージライブラリーの作製

ヒト抗体産生マウス［Ishida & Lonberg, IBC’s 11th Antibody Engineering, Abstract 2000; Ishida, I. et al., Cloning & Stem Cells 4, 85-96 (2002)および石田　功（２００２）実験医学２０，６，８４６－８５１］に、免疫原として、実施例１で作製したヒトＣＤ４０－ＦＬＡＧ－Ｆｃを計４回腹腔内投与した。初回免疫時のみ、アジュバントとしてＡｌｕｍゲル（２ｍｇ／匹）および百日咳ワクチン（１×１０^{9}個／匹）を添加した。

[0367] 初回免疫から２週間後に２回目免疫、その１週間後に３回目免疫、３回目免疫から１０日後に最終免疫を行い、最終免疫から４日後に解剖して脾臓を外科的に摘出した。摘出した脾臓をセルストレイナー（ファルコン社製）上に乗せてシリコン棒で軽く潰しながら細胞をチューブへ移し、遠心分離して細胞を沈殿させた後、赤血球除去試薬（シグマアルドリッチ社製）と氷中で３分間反応させ、更に遠心分離を行った。

[0368] 得られた脾臓細胞からＲＮｅａｓｙ　Ｍｉｎｉ　ｋｉｔ（ＱＩＡＧＥＮ社製）を用いてＲＮＡを抽出し、ＳＭＡＲＴｅｒ　ＲＡＣＥ　ｃＤＮＡ増幅キット（Ｃｌｏｎｔｅｃｈ社製）にてｃＤＮＡを増幅し、さらにＰＣＲにてＶＨ遺伝子断片を増幅させた。該ＶＨ遺伝子断片およびヒトの抗体ｇｅｒｍ－ｌｉｎｅ配列であり、配列番号２１で示される塩基配列からなるＬ６配列を含むＶＬ遺伝子断片をファージミドｐＣＡＮＴＡＢ　５Ｅ（Ａｍｅｒｓｈａｍ　Ｐｈａｒｍａｃｉａ社製）に挿入し、大腸菌ＴＧ１（Ｌｕｃｉｇｅｎ社製）を形質転換してプラスミドを得た。

[0369] なお、Ｌ６配列は配列番号２２で示されるアミノ酸配列からなる、ヒト抗体の軽鎖可変領域（ＶＬ）をコードしており、該ＶＬのＣＤＲ１、２および３（それぞれＬＣＤＲ１、２および３とも表す）のアミノ酸配列はそれぞれ配列番号２３、２４および２５で示される。

[0370] 得られたプラスミドをＶＣＳＭ１３　Ｉｎｔｅｒｆｅｒｅｎｃｅ　Ｒｅｓｉｓｔａｎｔ　Ｈｅｌｐｅｒ　Ｐｈａｇｅ（Ａｇｉｌｅｎｔ　Ｔｅｃｈｎｏｌｏｇｉｅｓ社製）に感染させることで、Ｌ６配列からなるＶＬ遺伝子を有し、ＶＨ遺伝子がライブラリー化されたＣＤ４０免疫ヒト抗体Ｍ１３ファー

ジライブラリーを得た。

[0371] ２．抗ＣＤ４０モノクローナル抗体の取得

このＣＤ４０免疫ヒト抗体Ｍ１３ファージライブラリーを用いて、下記のファージディスプレイ法により、Ｌ６にコードされるＶＬを有する抗ＣＤ４０モノクローナル抗体を取得した。実施例１で取得したヒトＣＤ４０－ＧＳＴを固相化し、ＳｕｐｅｒＢｌｏｃｋ　Ｂｌｏｃｋｉｇ　Ｂｕｆｆｅｒ（Ｔｈｅｒｍｏ　Ｆｉｓｈｅｒ社製）を用いてヒトＣＤ４０－ＧＳＴが結合していない部位をブロックしたＭＡＸＩＳＯＲＰ　ＳＴＡＲＴＵＢＥ（ＮＵＮＣ社製）と、ヒト抗体Ｍ１３ファージライブラリーを室温下で１～２時間反応させ、Ｄ－ＰＢＳ（－）および０．１％　Ｔｗｅｅｎ２０含有ＰＢＳ（以下ＰＢＳ－Ｔと記載する。和光純薬工業社製）でそれぞれ３回洗浄後に０．１ＭのＧｌｙ－ＨＣｌ（ｐＨ２．２）でファージを溶出した。

[0372] 溶出したファージは、ＴＧ１コンピテントセルに感染させ、ファージを増幅し、再度ＭＡＸＩＳＯＲＰ　ＳＴＡＲＴＵＢＥに固相化したヒトＣＤ４０－ＧＳＴと反応させて、Ｄ－ＰＢＳ（－）およびＰＢＳ－Ｔでそれぞれ５回洗浄後に０．１ＭのＧｌｙ－ＨＣｌ（ｐＨ２．２）によるファージの溶出を行った。

[0373] この操作を２回または３回繰り返し、ヒトＣＤ４０に特異的に結合するｓｃＦｖを提示したファージを濃縮した。濃縮されたファージをＴＧ１に感染させ、ＳＯＢＡＧプレート（２．０％　トリプトン、０．５％　Ｙｅａｓｔ　ｅｘｔｒａｃｔ、０．０５％　ＮａＣｌ、２．０％　グルコース、１０ｍＭ　$MgCl_2$、１００μｇ／ｍＬ　アンピシリン、１．５％　アガー）に播種してコロニーを形成させた。

[0374] コロニーを植菌して培養後、ＶＣＳＭ１３　Ｉｎｔｅｒｆｅｒｅｎｃｅ　Ｒｅｓｉｓｔａｎｔ　Ｈｅｌｐｅｒ　Ｐｈａｇｅを感染させて、再度培養することで単クローンのファージを得た。得られた単クローンのファージを用いて、ＥＬＩＳＡにてヒトおよびサルＣＤ４０－ＧＳＴのいずれにも結合するクローンを選択した。

[0375] ＥＬＩＳＡには、実施例１のヒトまたはサルＣＤ４０－ＧＳＴを各ウェルに固相化し、ＳｕｐｅｒＢｌｏｃｋ Ｂｌｏｃｋｉｇ Ｂｕｆｆｅｒ（Ｔｈｅｒｍｏ Ｆｉｓｈｅｒ社製）を用いてヒトまたはサルＣＤ４０－ＧＳＴが結合していない部位をブロックしたＭＡＸＩＳＯＲＰ（ＮＵＮＣ社製）を用いた。各ウェルに各々のファージクローンを加え、室温下で３０分間反応させた後、各ウェルをＰＢＳ－Ｔで３回洗浄した。

[0376] 次いで、西洋ワサビペルオキシダーゼで標識された抗Ｍ１３抗体（ＧＥヘルスケア社製）を１０％ブロックエース（大日本製薬株式会社製）含有ＰＢＳ－Ｔで５０００倍に希釈し、各ウェルに５０μＬずつ加え、室温下３０分間インキュベートした。マイクロプレートを、ＰＢＳ－Ｔで４回洗浄後、ＴＭＢ発色基質液（ＤＡＫＯ社製）を各ウェルに５０μＬずつ加え、室温下で１０分間インキュベートした。各ウェルに２Ｎ ＨＣｌ溶液（５０μＬ／ｗｅｌｌ）を加えて発色反応を止め、波長４５０ｎｍ（参照波長５７０ｎｍ）での吸光度をプレートリーダー（Ｅｍａｘ；Ｍｏｌｅｃｕｌａｒ Ｄｅｖｉｃｅｓ社）で測定した。

[0377] ヒトおよびサルＣＤ４０のいずれにも結合したクローンについて、配列解析を行い、Ｌ６にコードされるＶＬを有する抗ＣＤ４０抗体Ｒ１０６６、Ｒ１０９０Ｓ５５Ａ、Ｒ２０８９およびＲ２１７８を取得した。表１に、取得したそれぞれのＣＤ４０抗体のＶＨをコードする全塩基配列および当該塩基配列から推定されるアミノ酸配列、ならびにＶＨのＣＤＲ１～３（以下ではＨＣＤＲ１～３と記載することもある）のアミノ酸配列を示す。

[0378] [表1]

抗ヒトＣＤ４０抗体ＶＨの配列情報

クローン名	R1066	R1090S55A	R2089	R2178
VHをコードする塩基配列	配列番号26	配列番号31	配列番号36	配列番号41
VHのアミノ酸配列	配列番号27	配列番号32	配列番号37	配列番号42
HCDR1のアミノ酸配列	配列番号28	配列番号33	配列番号38	配列番号43
HCDR2のアミノ酸配列	配列番号29	配列番号34	配列番号39	配列番号44
HCDR3のアミノ酸配列	配列番号30	配列番号35	配列番号40	配列番号45

[0379] 取得した抗ＣＤ４０抗体Ｒ１０６６、Ｒ１０９０Ｓ５５Ａ、Ｒ２０８９およびＲ２１７８の遺伝子が組み込まれた可溶性ＩｇＧの発現ベクターをそれぞれ作製した。まず、Ｒ１０６６、Ｒ１０９０Ｓ５５Ａ、Ｒ２０８９およびＲ２１７８の共通のＶＬをコードするＬ６遺伝子をＮ５ＫＧ４ＰＥ　Ｒ４０９Ｋ（国際公開第２００６／０３３３８６号に記載）のＢｇＩＩＩ－ＢｓｉＷＩサイトにサブクローニングした。

[0380] その後、Ｒ１０６６、Ｒ１０９０Ｓ５５Ａ、Ｒ２０８９およびＲ２１７８のそれぞれのＶＨ遺伝子をＮ５ＫＧ４ＰＥ　Ｒ４０９ＫのＳａＩＩ－ＮｈｅＩサイトへサブクローニングして、ヒトＩｇＧ４ＰＥ　Ｒ４０９Ｋの定常領域をもつ抗ＣＤ４０モノクローナル抗体Ｒ１０６６、Ｒ１０９０Ｓ５５Ａ、Ｒ２０８９およびＲ２１７８の発現ベクターであるＮ５ＫＧ４ＰＥ　Ｒ４０９Ｋ＿Ｒ１０６６、Ｎ５ＫＧ４ＰＥ　Ｒ４０９Ｋ＿Ｒ１０９０Ｓ５５Ａ、Ｎ５ＫＧ４ＰＥ　Ｒ４０９Ｋ＿Ｒ２０８９およびＮ５ＫＧ４ＰＥ　Ｒ４０９Ｋ＿Ｒ２１７８をそれぞれ得た。

[0381] また、国際公開第２００３／０４０１７０号に記載された抗ＣＤ４０モノクローナル抗体２１．４．１を抗ＣＤ４０抗体の陽性コントロール抗体として作製するため、発現ベクターを作製した。２１．４．１のＶＨの塩基配列を配列番号４６に、該配列から推定されたＶＨのアミノ酸配列を配列番号４７に、それぞれ示す。また、２１．４．１のＶＬの塩基配列を配列番号４８、該配列から推定されたＶＬのアミノ酸配列を配列番号４９にそれぞれ示す。

[0382] ２１．４．１のＶＨおよびＶＬをコードする遺伝子を合成し、Ｎ５ＫＧ２ベクター（国際公開第２００３／０３３５３８号に記載）のＳａＩＩ－ＮｈｅＩおよびＢｇＩＩＩ－ＢｓｉＷＩサイトにそれぞれサブクローニングして、ヒトＩｇＧ２の定常領域をもつ抗ＣＤ４０モノクローナル抗体２１．４．１の発現ベクターＮ５ＫＧ２＿２１．４．１を得た。

[0383] ［実施例４］抗ＥｐＣＡＭ抗体の取得

１．ＥｐＣＡＭ免疫ヒト抗体Ｍ１３ファージライブラリーの作製

実施例１のヒトＥｐＣＡＭ－Ｆｃを用い、実施例３の１と同様の方法により、Ｌ６配列からなるＶＬ遺伝子を有しＶＨ遺伝子がライブラリー化されたＥｐＣＡＭ免疫ヒト抗体Ｍ１３ファージライブラリーを得た。

[0384] ２．抗ＥｐＣＡＭ抗体の取得

実施例１で取得したマウスＥｐＣＡＭ－ＧＳＴを固相化し、ＳｕｐｅｒＢｌｏｃｋ　Ｂｌｏｃｋｉｇ　Ｂｕｆｆｅｒ（Ｔｈｅｒｍｏ　Ｆｉｓｈｅｒ社製）を用いてマウスＥｐＣＡＭ－ＧＳＴが結合していない部位をブロックしたＭＡＸＩＳＯＲＰ　ＳＴＡＲＴＵＢＥ（ＮＵＮＣ社製）と、ＥｐＣＡＭ免疫ヒト抗体Ｍ１３ファージライブラリーを用い、実施例３の２と同様の方法によりマウスＥｐＣＡＭに特異的に結合するｓｃＦｖを提示したファージを単クローン化した。

[0385] なお、一部実験ロットでは、２回目以降のマウスＥｐＣＡＭ－ＧＳＴを用いたバイオパニング操作の代わりに、溶出したファージを、実施例２のヒトＥｐＣＡＭ／ＨＥＫ２９３をＣＦＳＥ染色した細胞とＥｐＣＡＭ陰性のＨＥＫ２９３細胞に添加し、氷中で１時間反応後に洗浄し、ＣＦＳＥ陽性細胞をＦＡＣＳ　Ａｒｉａ　ＩＩＩ（ＢＤ社製）にてｓｏｒｔｉｎｇし、０．１ＭのＧｌｙ－ＨＣｌ（ｐＨ２．２）でファージを溶出する操作によりヒトＥｐＣＡＭに特異的に結合するファージの濃縮を行った。単クローン化したファージから、ＥＬＩＳＡにてヒト、サル、およびマウスＥｐＣＡＭ－ＧＳＴへの結合性を有するクローンを選択した。

[0386] ＥＬＩＳＡは、実施例１のヒト、サル、またはマウスＥｐＣＡＭ－ＧＳＴを固相化し、ＳｕｐｅｒＢｌｏｃｋ　Ｂｌｏｃｋｉｇ　Ｂｕｆｆｅｒ（Ｔｈｅｒｍｏ　Ｆｉｓｈｅｒ社製）を用いてヒト、サル、またはマウスＥｐＣＡＭ－ＧＳＴが結合していない部位をブロックしたＭＡＸＩＳＯＲＰ（ＮＵＮＣ社製）を用い、実施例３の２と同様の方法により行った。

[0387] ヒト、サル、およびマウスＥｐＣＡＭのいずれにも結合したクローンについて、配列解析を行い、Ｌ６にコードされるＶＬを有する抗ＥｐＣＡＭ抗体Ｅｐ５９、Ｅｐ２０３、Ｅｐｃ０５１およびＥｐｃ１１２を取得した。表１

と同様の表示方法で、各ＥｐＣＡＭ抗体のＶＨの配列情報を表２に示す。

[0388]

[表2]

抗ＥｐＣＡＭ抗体ＶＨの配列情報

クローン名	Ep59	Ep203	Epc051	Epc112
VHをコードする塩基配列	配列番号50	配列番号55	配列番号60	配列番号65
VHのアミノ酸配列	配列番号51	配列番号56	配列番号61	配列番号66
HCDR1のアミノ酸配列	配列番号52	配列番号57	配列番号62	配列番号67
HCDR2のアミノ酸配列	配列番号53	配列番号58	配列番号63	配列番号68
HCDR3のアミノ酸配列	配列番号54	配列番号59	配列番号64	配列番号69

[0389] また、抗ＥｐＣＡＭ抗体の陽性コントロール抗体として、国際公開第２００３／０４０７２５号に記載された抗ＥｐＣＡＭモノクローナル抗体３６２２Ｗ９４の可変領域をもつ抗体を作製した。３６２２Ｗ９４のＶＨの塩基配列を配列番号７０に、該配列から推定されたＶＨのアミノ酸配列を配列番号７１に、それぞれ示す。また、３６２２Ｗ９４のＶＬの塩基配列を配列番号７２、該配列から推定されたＶＬのアミノ酸配列を配列番号７３にそれぞれ示す。

[0390] ３６２２Ｗ９４のＶＨおよびＶＬをコードする遺伝子を合成し、国際公開第２０１０／１４３６９８号に記載されているＴｏｌ２トランスポゾン発現ベクターｐＫＴＡＢＥＸ－ＴＣ２６の制限酵素サイトＢｇｌＩＩ－ＢｓｉＷＩにＶＬを、制限酵素サイトＳａｌＩ－ＮｈｅＩにＶＨをそれぞれサブクローニングし、ヒトＩｇＧ１の定常領域をもつ抗ＥｐＣＡＭモノクローナル抗体３６２２Ｗ９４の発現ベクターｐＫＴＡＢＥＸ－ＴＣ２６＿３６２２Ｗ９４を得た。

[0391] ［実施例５］ＣＤ４０およびＥｐＣＡＭに結合するバイスペシフィック抗体の発現ベクターの構築

図１（Ａ）および図１（Ｂ）に記載する構造を有する、ヒトおよびサルＣＤ４０の少なくとも一方ならびにヒト、サルおよびマウスＥｐＣＡＭの少なくともいずれか１つに結合するバイスペシフィック抗体を以下の方法で作製

した。バイスペシフィック抗体の形状には国際公開第２００９／１３１２３９号に記載の形状を採用した。

[0392] 当該バイスペシフィック抗体のうち、Ｎ末端側から順に、ＶＨ１、ＣＨ１、ＶＨ２、ＣＨ１、ヒンジ、ＣＨ２およびＣＨ３のアミノ酸配列を含む２本の重鎖ならびに４本の軽鎖からなる抗体をＮ末端型バイスペシフィック抗体と称する。また、Ｎ末端側から順にＶＨ１、ＣＨ１、ヒンジ、ＣＨ２、ＣＨ３、ＶＨ２およびＣＨ１のアミノ酸配列を含む２本の重鎖ならびに４本の軽鎖からなる抗体をＣ末端型バイスペシフィック抗体と称する。ＶＨ１およびＶＨ２は抗ＣＤ４０抗体のＶＨまたは抗ＥｐＣＡＭ抗体のＶＨのいずれかであり、片方が抗ＣＤ４０抗体のＶＨ、もう一方が抗ＥｐＣＡＭ抗体のＶＨである。

[0393] 以下の記載では、ＶＨ１とＶＨ２の間をつなぐポリペプチドをリンカーと称し、リンカーのアミノ酸配列をコードする遺伝子をリンカー遺伝子と称する。また、ＶＨ２のＣ末端側に結合するポリペプチドをＣ末端側ポリペプチドと称し、Ｃ末端側ポリペプチドをコードする遺伝子をＣ末端側配列遺伝子と称する。

[0394] 以下の工程で作製されるＮ末端型バイスペシフィック抗体はリンカーとして、ＩｇＧ４のＣＨ１（塩基配列を配列番号７４、アミノ酸配列を配列番号７５で示す）を有する。またＶＨ２のＣ末端側ポリペプチドとして国際公開第２００６／０３３３８６号記載のＩｇＧ４ＰＥ　Ｒ４０９ＫのＣＨ（ＣＨ１、ヒンジ、ＣＨ２およびＣＨ３）からなるポリペプチド（塩基配列を配列番号７６、アミノ酸配列を配列番号７７で示す）を有する。

[0395] Ｃ末端型バイスペシフィック抗体はリンカーとして、ＩｇＧ４ＰＥ　Ｒ４０９ＫのＣＨからなるポリペプチド（塩基配列を配列番号７６、アミノ酸配列を配列番号７７で示す）を有する。また、Ｃ末端側ポリペプチドとしてＩｇＧ４のＣＨ１（塩基配列を配列番号７４、アミノ酸配列を配列番号７５で示す）を有する。また、以下の工程で作成されるバイスペシフィック抗体は、Ｌ６にコードされるＶＬを含む軽鎖を有する。

[0396] 当該バイスペシフィック抗体の名称、当該バイスペシフィック抗体の構造、当該バイスペシフィック抗体作製に使用した抗ＣＤ４０抗体のクローンおよび抗ＥｐＣＡＭ抗体のクローンを表３に示す。なお以下では、Ｎ末端型バイスペシフィック抗体の名称にＮｔと付記することもある。

[0397]

[表3]

バイスペシフィック抗体の名称	バイスペシフィック抗体の構造	VH1抗体クローン	VH2抗体クローン
R1066-Ep59	N末端型	R1066	Ep59
R1066-Ep203	N末端型	R1066	Ep203
R1066-Epc051	N末端型	R1066	Epc051
R1066-Epc112	N末端型	R1066	Epc112
R1090S55A-Ep59	N末端型	R1090S55A	Ep59
R1090S55A-Ep203	N末端型	R1090S55A	Ep203
R1090S55A-Epc051	N末端型	R1090S55A	Epc051
R1090S55A-Epc112	N末端型	R1090S55A	Epc112
R2089-Ep59	N末端型	R2089	Ep59
R2089-Ep203	N末端型	R2089	Ep203
R2089-Epc051	N末端型	R2089	Epc051
R2089-Epc112	N末端型	R2089	Epc112
R2178-Ep59	N末端型	R2178	Ep59
R2178-Ep203	N末端型	R2178	Ep203
R2178-Epc051	N末端型	R2178	Epc051
R2178-Epc112	N末端型	R2178	Epc112
Ep59-R1066	N末端型	Ep59	R1066
Ep59-R1090S55A	N末端型	Ep59	R1090S55A
Ep59-R2089	N末端型	Ep59	R2089
Ep59-R2178	N末端型	Ep59	R2178
Ep203-R1066	N末端型	Ep203	R1066
Ep203-R1090S55A	N末端型	Ep203	R1090S55A
Ep203-R2089	N末端型	Ep203	R2089
Ep203-R2178	N末端型	Ep203	R2178
Ct R1066-Ep59	C末端型	R1066	Ep59
Ct R1066-Ep203	C末端型	R1066	Ep203
Ct R1090S55A-Ep59	C末端型	R1090S55A	Ep59
Ct R1090S55A-Ep203	C末端型	R1090S55A	Ep203
Ct Ep59-R1066	C末端型	Ep59	R1066
Ct Ep59-R1090S55A	C末端型	Ep59	R1090S55A
Ct Ep203-R1066	C末端型	Ep203	R1066
Ct Ep203-R1090S55A	C末端型	Ep203	R1090S55A
Ct R1066-Epc051	C末端型	R1066	Epc051
Ct R1066-Epc112	C末端型	R1066	Epc112
Ct Epc112-R1066	C末端型	Epc112	R1066

[0398] １．Ｎ末端型バイスペシフィック抗体の発現ベクターの作製

表３に記載されたバイスペシフィック抗体のうち、Ｎ末端型バイスペシフィック抗体について、発現ベクターを以下に記載する方法で作製した。

[0399] 実施例３で取得したＲ１０６６、Ｒ１０９０Ｓ５５Ａ、Ｒ２０８９およびＲ２１７８ならびに実施例４で取得したＥｐ５９、Ｅｐ２０３、Ｅｐｃ０５１およびＥｐｃ１１２の共通のＶＬをコードするＬ６遺伝子（配列番号２１）をＮ５ＫＧ４ＰＥ　Ｒ４０９Ｋ（国際公開第２００６／０３３３８６号に記載）のＢｇｌｌ－ＢｓｉＷＩサイトにサブクローニングした。

[0400] その後、リンカーであるヒトＩｇＧ４のＣＨ１と同じアミノ酸配列をコードし、使用コドンを変更したリンカー遺伝子（配列番号７４）を合成した。このリンカー遺伝子および実施例３で取得した抗ＣＤ４０抗体または実施例４で取得した抗ＥｐＣＡＭ抗体のＶＨ遺伝子を鋳型とし、ＫＯＤ　ＦＸ　Ｎｅｏ　ＤＮＡ　Ｐｏｌｙｍｅｒａｓｅ（東洋紡社製）を用いて、ＰＣＲによりリンカー遺伝子およびＶＨ２部分の遺伝子断片を増幅させた。増幅させた遺伝子断片を、ＮｈｅＩで開裂させたＮ５ＫＧ４ＰＥ　Ｒ４０９Ｋに連結することにより、Ｎ末端型バイスペシフィック抗体発現プラスミドベクターを得た。

[0401] ２．Ｃ末端型バイスペシフィック抗体の発現ベクターの作製

表３に記載されたバイスペシフィック抗体のうち、Ｃ末端型バイスペシフィック抗体について、発現ベクターを以下に記載する方法で作製した。

[0402] 実施例３で取得した抗ＣＤ４０抗体Ｒ１０６６、Ｒ１０９０Ｓ５５Ａ、Ｒ２０８９およびＲ２１７８ならびに実施例４で取得した抗ＥｐＣＡＭ抗体Ｅｐ５９、Ｅｐ２０３、Ｅｐｃ０５１およびＥｐｃ１１２の共通のＶＬをコードするＬ６遺伝子（配列番号２１）をＮ５ＫＧ４ＰＥ　Ｒ４０９Ｋ（国際公開第２００６／０３３３８６号に記載）のＢｇｌｌ－ＢｓｉＷＩサイトにサブクローニングした。

[0403] その後、Ｃ末端側ポリペプチドであるヒトＩｇＧ４のＣＨ１と同じアミノ

酸配列をコードし、使用コドンを変更した、停止コドンを含むＣ末端側配列遺伝子（配列番号７８）を合成した。実施例３で取得した抗ＣＤ４０抗体、または実施例４で取得した抗ＥｐＣＡＭ抗体のＶＨ遺伝子および合成したＣ末端側配列遺伝子を鋳型とし、ＫＯＤ　ＦＸ　Ｎｅｏ（東洋紡社製）を用いて、ＰＣＲによりＶＨ２およびＣ末端側配列遺伝子断片を増幅させた。

[0404] さらに、Ｎ５ＫＧ４ＰＥ　Ｒ４０９Ｋを鋳型として、ＫＯＤ　ＦＸ　Ｎｅｏ（東洋紡社製）を用いて、ＰＣＲによりリンカー遺伝子断片を増幅させた。増幅させた遺伝子断片を、ＮｈｅＩおよびＢａｍＨＩで開裂させたＮ５ＫＧ４ＰＥ　Ｒ４０９Ｋに連結することにより、表３に記載するＣ末端型バイスペシフィック抗体発現プラスミドベクターを得た。

[0405] ［実施例６］ＣＤ４０に結合するモノクローナル抗体、ＥｐＣＡＭに結合するモノクローナル抗体およびＣＤ４０とＥｐＣＡＭに結合するバイスペシフィック抗体の調製

実施例３から実施例５で抗体発現プラスミドベクターＮ５ＫＧ４ＰＥ　Ｒ４０９ＫおよびＮ５ＫＧ２のいずれかにサブクローニングした抗ＣＤ４０モノクローナル抗体、抗ＥｐＣＡＭモノクローナル抗体、ならびにＣＤ４０およびＥｐＣＡＭの各結合部位を有するバイスペシフィック抗体（ＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体）をそれぞれ発現させ、精製した。

[0406] １．抗ＣＤ４０モノクローナル抗体およびＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体の作製

抗ＣＤ４０モノクローナル抗体の発現ベクターをＥｘｐｉ２９３（商標）Ｅｘｐｒｅｓｓｉｏｎ　Ｓｙｓｔｅｍ（Ｔｈｅｒｍｏ　Ｆｉｓｈｅｒ社製）によりＥｘｐｉ２９３細胞に共遺伝子導入し、１６時間後にＴｒａｎｓｆｅｃｔｉｏｎ　Ｅｎｈａｎｃｅｒを添加して、一過性発現系で抗体を発現させた。

[0407] ベクター導入４日後に培養上清を回収し、孔径０．２２μｍのメンブランフィルター（ＭＩＬＬＩＰＯＲＥ製）で濾過した後、Ｐｒｏｔｅｉｎ　Ａ樹脂（ＭａｂＳｅｌｅｃｔ、ＧＥヘルスケア社製）を用いて抗体をアフィニテ

ィー精製した。洗浄液としてＤ－ＰＢＳ（－）を用いた。Ｐｒｏｔｅｉｎ Ａに吸着させた抗体を、２０ｍＭクエン酸ナトリウム、５０ｍＭ ＮａＣｌ緩衝液（ｐＨ３．０）により溶出し、２００ｍＭリン酸ナトリウム緩衝液（ｐＨ７．０）を含むチューブに回収した。

[0408] 次に、ＶＩＶＡＳＰＩＮ（Ｓａｒｔｒｉｕｓ ｓｔｅａｌｉｎ社製）を用いて濃縮し、Ｎａｐ Ｃｏｌｕｍｎ（ＧＥヘルスケア社製）を用いてＤ－ＰＢＳ（－）へ緩衝液を置換した。さらに、ＡＫＴＡ ＦＰＬＣ（ＧＥヘルスケア社製）およびＳｕｐｅｒｄｅｘ Ｈｉｇｈ－ｐｅｒｆｏｒｍａｎｃｅ Ｃｏｌｕｍｎｓ（ＧＥヘルスケア社製）を用いて、該抗体溶液より単量体画分を分取した。ＡＫＴＡシステム孔径０．２２μｍのメンブランフィルター（Ｍｉｌｌｅｘ－ＧＶ、ＭＩＬＬＩＰＯＲＥ社製）でろ過滅菌することにより精製抗体を得た。

[0409] 抗体溶液の波長２８０ｎｍの吸光度を測定し、各抗体のアミノ酸配列から推定される吸光係数を用いて、精製抗体の濃度を算出した。ＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体も同様の方法で作製した。

[0410] ２．抗ＥｐＣＡＭモノクローナル抗体の作製

実施例４で作製した抗ＥｐＣＡＭモノクローナル抗体３６２２Ｗ９４発現ベクターｐＫＴＡＢＥＸ－ＴＣ２６＿３６２２Ｗ９４をＴｏｌ２トランスポゼース発現ベクターと共に、ＣＨＯ－Ｋ１細胞にエレクロポレーション法によりｔｒａｎｓｆｅｃｔｉｏｎした。３μｇ／ｍｌ シクロヘキシミド（シグマアルドリッチ社製）を添加した培地で耐性株を選択した。１４－２１日後に可溶性ＩｇＧ ＦｃサンドウィッチＥＬＩＳＡにより、抗体産生ｗｅｌｌを選択した。

[0411] 抗体産生量の多いｗｅｌｌは、拡大培養を行い、さらに抗体量を再度測定して、発現量の多い株を３６２２Ｗ９４発現株として樹立した。樹立した３６２２Ｗ９４発現株は、ＥＸＣＥＬ３０２培地（シグマアルドリッチ社製）で培養して、上清を回収し、孔径０．２２μｍのメンブランフィルター（ＭＩＬＬＩＰＯＲＥ社製）で濾過した後、Ｐｒｏｔｅｉｎ Ａ樹脂（ＭａｂＳ

ｅｌｅｃｔ、ＧＥヘルスケア社製）を用いて抗体をアフィニティー精製した。洗浄液としてＤ－ＰＢＳ（－）を用いた。

[0412] Ｐｒｏｔｅｉｎ Ａに吸着させた抗体を、２０ｍＭクエン酸ナトリウム、５０ｍＭ ＮａＣｌ緩衝液（ｐＨ３．０）により溶出し、２００ｍＭリン酸ナトリウム緩衝液（ｐＨ７．０）を含むチューブに回収した。次に、ＶＩＶＡＳＰＩＮ（Ｓａｒｔｒｉｕｓ ｓｔｅａｌｉｎ社製）を用いて濃縮し、Ｎａｐ Ｃｏｌｕｍｎ（ＧＥヘルスケア社製）を用いてＤ－ＰＢＳ（－）へ緩衝液を置換した。

[0413] さらに、ＡＫＴＡ ＦＰＬＣ（ＧＥヘルスケア社製）およびＳｕｐｅｒｄｅｘ Ｈｉｇｈ－ｐｅｒｆｏｒｍａｎｃｅ Ｃｏｌｕｍｎｓ（ＧＥヘルスケア社製）を用いて、該抗体溶液より単量体画分を分取した。孔径０．２２μｍのメンブランフィルター（Ｍｉｌｌｅｘ－ＧＶ、ＭＩＬＬＩＰＯＲＥ社製）でろ過滅菌を行うことにより精製抗体を得た。抗体溶液の波長２８０ｎｍの吸光度を測定し、各抗体のアミノ酸配列から推定される吸光係数を用いて、精製抗体の濃度を算出した。

[0414] ［実施例７］フローサイトメーターによる細胞株のＣＤ４０およびＥｐＣＡＭ発現評価

バーキットリンパ腫細胞Ｒａｍｏｓ細胞（ＡＴＣＣ Ｎｏ．ＣＣＬ-１５９６）、ヒト大腸癌細胞Ｃｏｌｏ２０５細胞（ＡＴＣＣ Ｎｏ．ＣＣＬ-２２２）、ヒト胚性腎細胞ＨＥＫ２９３細胞（ＡＴＣＣＮｏ．ＣＲＬ-１５７３）および実施例２で得たヒトＥｐＣＡＭ／ＨＥＫ２９３細胞の細胞表面上のＣＤ４０およびＥｐＣＡＭの発現を、以下の手順に従いｆｌｕｏｒｅｓｃｅｎｃｅ ａｃｔｉｖａｔｅｄ ｃｅｌｌ ｓｏｒｔｉｎｇ（ＦＡＣＳ）法により評価した。評価には実施例６で得た抗ＣＤ４０抗体２１．４．１および抗ＥｐＣＡＭ抗体３６２２Ｗ９４を用いた。

[0415] Ｒａｍｏｓ細胞を、１×10^{6}ｃｅｌｌｓ／ｍＬの濃度で０．１％ NaN_{3}、１％ ＦＢＳ含有Ｄ－ＰＢＳ（－）のＳｔａｉｎｉｎｇ Ｂｕｆｆｅｒ（ＳＢ）に懸濁し、１００μＬ／ｗｅｌｌで９６穴丸底プレート（ファルコン

社製）に分注した。遠心分離（２０００ｒｐｍ、４℃、２分間）の後、上清を除去し、ペレットへ実施例６で得た１０μｇ／ｍＬの抗ＣＤ４０抗体２１．４．１及び抗ＥｐＣＡＭ抗体３６２２Ｗ９４を５０μＬ／ｗｅｌｌ加えて懸濁した後、氷温下で３０分間静置した。

[0416] さらに遠心分離（２０００ｒｐｍ、４℃、２分間）して上清を除去し、ペレットを２００μＬ／ｗｅｌｌのＳＢで３回洗浄した後に、１μｇ／ｍＬのＧｏａｔ Ｆ（ａｂ’）$_2$ Ａｎｔｉ－Ｈｕｍａｎ ＩｇＧ（γ ｃｈａｉｎ ｓｐｅｃｉｆｉｃ） Ｒ－ｐｈｙｃｏｅｒｙｔｈｒｉｎ（Ｒ－ＰＥ） Ｃｏｎｊｕｇａｔｅ（Ｓｏｕｔｈｅｒｎ Ｂｉｏｔｅｃｈ社製）を５０μＬ／ｗｅｌｌで加え、氷温下３０分間インキュベートした。ＳＢで２回洗浄した後、２００μＬ／ｗｅｌｌのＳＢに懸濁し、フローサイトメーターＦＡＣＳＣＡＮＴＯ ＩＩ、（ベクトンディッキンソン社製）で各細胞の蛍光強度を測定した。

[0417] 同様にして、Ｃｏｌｏ２０５細胞、ＨＥＫ２９３細胞およびヒトＥｐＣＡＭ／ＨＥＫ２９３細胞をそれぞれ評価した。陰性コントロールにはMol Immunol. 1996 Jun;33(9):759-68.に記載の２，４－ｄｉｎｉｔｒｏｐｈｅｎｏｌ（ＤＮＰ）抗体のＶＬおよびＶＨ（ＧｅｎＢａｎｋアクセッションＮｏ．：ＶＬ Ｕ１６６８８、ＶＨ Ｕ１１６６８７）をコードするベクターを用い、国際公開第２００６／０３３３８６号の実施例５記載の方法に準じて作製したＩｇＧ４抗体Ｓ２２８Ｐ、Ｌ２３５Ｅ、Ｒ４０９Ｋ変異体（以下抗ＤＮＰ抗体と記載する）を使用した。

[0418] 図２（Ａ）にはＲａｍｏｓ細胞、図２（Ｂ）にはＣｏｌｏ２０５細胞、図２（Ｃ）にはＨＥＫ２９３細胞、図２（Ｄ）にはヒトＥｐＣＡＭ／ＨＥＫ２９３細胞に対して測定した結果を示した。

[0419] 図２（Ａ）に示すように、Ｒａｍｏｓ細胞に対しては、抗ＣＤ４０抗体である２１．４．１が結合活性を示し、抗ＥｐＣＡＭ抗体である３６２２Ｗ９４は結合活性を示さなかった。一方で、図２（Ｂ）および図２（Ｄ）に示すように、Ｃｏｌｏ２０５細胞およびヒトＥｐＣＡＭ／ＨＥＫ２９３細胞に対

しては、２１．４．１は結合活性を示さず、３６２２Ｗ９４は結合活性を示した。また、図２（Ｃ）に示すように、ＨＥＫ２９３細胞に対しては、２１．４．１および３６２２Ｗ９４はいずれも結合活性を示さなかった。

[0420] これにより、Ｒａｍｏｓ細胞はＣＤ４０陽性でＥｐＣＡＭ陰性、Ｃｏｌｏ２０５細胞およびヒトＥｐＣＡＭ／ＨＥＫ２９３細胞はＣＤ４０陰性でＥｐＣＡＭ陽性、ＨＥＫ２９３細胞はＣＤ４０およびＥｐＣＡＭのいずれも陰性であることが確認できた。

[0421] ［実施例８］フローサイトメトリーによるＣＤ９５発現量解析によるＣＤ４０モノクローナル抗体のＣＤ４０シグナル誘導活性の評価

Ｒａｍｏｓ細胞上のＣＤ９５の発現量増加を指標として、実施例６で得られたＣＤ４０モノクローナル抗体のＣＤ４０シグナル誘導能を以下のようにＦＣＭ法で評価した。

[0422] ２×１０^{6}ｃｅｌｌｓ／ｍＬのＲａｍｏｓ細胞を５０μＬ／ｗｅｌｌでＵ底９６ｗｅｌｌプレート（ファルコン社製）に播種し、１０％ＦＢＳを含むＲＰＭＩ１６４０培地（シグマアルドリッチ社製）で希釈した０．２、２または２０μｇ／ｍｌ（終濃度０．１、１または１０μｇ／ｍｌ）の被験抗体を５０μｇ／ｍＬ添加し、３７℃、５．０％炭酸ガス下で１６時間培養した。

[0423] 遠心分離（２０００ｒｐｍ、４℃、２分間）の後、上清を除去し、ペレットを２００μＬ／ｗｅｌｌのＳＢで３回洗浄した。遠心分離（２０００ｒｐｍ、４℃、２分間）の後、上清を除去し、ペレットへＰＥ　ｍｏｕｓｅ　ａｎｔｉ－ｈｕｍａｎ　ＣＤ９５（ベクトンディッキンソン社製）抗体を加えて懸濁した後、氷温下で３０分間静置した。

[0424] さらに遠心分離（２０００ｒｐｍ、４℃、２分間）して上清を除去し、ペレットを２００μＬ／ｗｅｌｌのＳＢで３回洗浄した後、１００倍希釈した７－アミノアクチノマイシン（７ＡＡＤ）（ベクトンディッキンソン社製）を２００μＬ／ｗｅｌｌのＳＢに懸濁し、フローサイトメーターＦＡＣＳＣＡＮＴＯ　ＩＩ（ベクトンディッキンソン社製）でＲａｍｏｓ細胞上のＣＤ９５の蛍光強度を測定した。陰性コントロールには抗ＤＮＰ抗体を使用した

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[0425] 図３（Ａ）には抗ＣＤ４０抗体２１．４．１、Ｒ１０６６、Ｒ２０８９およびＲ２１７８、図３（Ｂ）には抗ＣＤ４０抗体２１．４．１、Ｒ１０６６およびＲ１０９０Ｓ５５Ａならびに抗ＥｐＣＡＭ抗体３６２２Ｗ９４の評価結果を示す。

[0426] これらの評価結果より、被験抗体２１．４．１、Ｒ１０６６、Ｒ２０８９、Ｒ２１７８、Ｒ１０９０Ｓ５５Ａおよび３６２２Ｗ９４の内、抗ＣＤ４０アゴニスト抗体２１．４．１を添加した時のみ、Ｒａｍｏｓ細胞上のＣＤ９５の発現が上昇することがわかった。

[0427] すなわち、２１．４．１のみがＣＤ４０のシグナルを誘導するアゴニスト抗体であり、実施例３において取得された抗ＣＤ４０モノクローナル抗体Ｒ１０６６、Ｒ２０８９、Ｒ２１７８およびＲ１０９０Ｓ５５Ａは、ＣＤ４０へのシグナルを誘導しない非アゴニスト抗体であることが示された。また、ＣＤ４０リガンドの存在下で同様の試験を行ったところ、抗ＣＤ４０モノクローナル抗体Ｒ１０６６、Ｒ２０８９、Ｒ２１７８およびＲ１０９０Ｓ５５ＡはいずれもＣＤ４０リガンドによるＲａｍｏｓ細胞上のＣＤ９５の発現を阻害しなかった（データ記載なし）。すなわち、抗ＣＤ４０モノクローナル抗体Ｒ１０６６、Ｒ２０８９、Ｒ２１７８およびＲ１０９０Ｓ５５Ａはいずれも、ＣＤ４０リガンドによるＣＤ４０へのシグナル誘導を阻害しない、非アンタゴニスト抗体であることが示された。

[0428] ［実施例９］ビアコアによるＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体のＥｐＣＡＭに対する結合性の評価

実施例６で得られたＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体のヒト、サルおよびマウスＥｐＣＡＭそれぞれに対する結合活性と種交差性を確認することを目的として、実施例１で作製したヒト、サルおよびマウスＥｐＣＡＭ－ＧＳＴを用いて、表面プラズモン共鳴法（ＳＰＲ法）による結合性試験を実施した。測定機器として、Ｂｉａｃｏｒｅ Ｔ１００（ＧＥヘルスケア社製）を使用した。

[0429] Ａｎｔｉ－ｈｕｍａｎ ＩｇＧ ａｎｔｉｂｏｄｙを、Ｈｕｍａｎ Ａｎｔｉｂｏｄｙ Ｃａｐｔｕｒｅ Ｋｉｔ（ＧＥヘルスケア社製）を用いて、添付文書に従いＣＭ５センサーチップ（ＧＥヘルスケア社製）に固定化した。フローセルに、１～３μｇ／ｍＬに調製した被験抗体を１０μＬ／ｍｉｎの流速で１０秒間添加した。

[0430] 次いで、アナライトとして１０または１００ｎＭより５段階に２倍希釈したヒト、サルおよびマウスＥｐＣＡＭ－ＧＳＴタンパク質溶液（０．１％ＢＳＡを含むＨＢＳ－ＥＰ＋（ＧＥヘルスケア社製）で希釈）をそれぞれ３０μＬ／ｍｉｎの流速で添加し、各抗体とアナライトとの結合反応を２分間、解離反応を１０分間測定した。

[0431] 測定はシングルサイクルカイネティクス法で行った。得られたセンサーグラムを、Ｂｉａ Ｅｖａｌｕａｔｉｏｎ Ｓｏｆｔｗａｒｅ（ＧＥヘルスケア社製）を用いて解析し、各抗体の速度論定数を算出した。

[0432] 算出された各バイスペシフィック抗体のヒト、サルおよびマウスＥｐＣＡＭそれぞれに対する解離定数［ｋｄ／ｋａ＝K_D］ならびにヒトＥｐＣＡＭに対する解離定数をサルＥｐＣＡＭまたはマウスＥｐＣＭに対する解離定数で割った値を表４に示す。

[0433]

[表4]

K_D値	ヒトEpCAM [nM]	サルEpCAM [nM]	ヒトEpCAM [nM]/ サルEpCAM [nM]	マウスEpCAM [nM]	ヒトEpCAM [nM]/ マウスEpCAM [nM]
R1066-Ep59	9.60	73.73	0.13	15.26	0.63
R1066-Ep203	12.82	22.22	0.58	15.82	0.81
R1066-Epc051	11.27	75.35	0.15	21.87	0.52
R1066-Epc112	8.31	14.76	0.56	16.46	0.51
R1090S55A-Ep59	11.40	14.13	0.81	28.68	0.40
R1090S55A-Ep203	9.51	16.47	0.58	16.72	0.57
R1090S55A-Epc051	7.43	50.05	0.15	20.00	0.37
R1090S55A-Epc112	7.90	13.09	0.60	19.64	0.40
R2087-Ep59	8.88	10.39	0.85	17.80	0.50
R2087-Ep203	9.62	9.02	1.07	17.67	0.54
R2089-Epc051	7.60	5.48	1.39	24.21	0.31
R2089-Epc112	7.83	4.13	1.90	21.13	0.37
R2178-Ep59	9.99	4.29	2.33	203.70	0.05
R2178-Ep203	8.67	2.12	4.08	241.80	0.04
R2178-Epc051	7.66	1.78	4.31	16.56	0.46
R2178-Epc112	7.15	9.11	0.78	14.84	0.48
Ep59-R1066	5.00	5.09	0.98	6.18	0.81
Ep59-R1090S55A	3.11	3.37	0.92	3.86	0.80
Ep59-R2089	4.69	4.48	1.05	5.47	0.86
Ep59-R2178	5.23	6.54	0.80	6.55	0.80
Ep203-R1066	6.49	6.54	0.99	13.25	0.49
Ep203-R1090S55A	4.98	6.90	0.72	11.77	0.42
Ep203-R2089	5.80	5.87	0.99	11.52	0.50
Ep203-R2178	4.15	5.11	0.81	11.68	0.36
Ct R1066-Ep59	10.19	8.96	1.14	51.98	0.20
Ct R1066-Ep203	25.74	14.01	1.84	24.30	1.06
Ct R1090S55A-Ep59	11.94	10.84	1.10	18.10	0.66
Ct R1090S55A-Ep203	14.54	13.31	1.09	24.23	0.60
Ct Ep59-R1066	6.63	5.93	1.12	13.36	0.50
Ct Ep59-R1090S55A	5.78	5.25	1.10	13.78	0.42
Ct Ep203-R1066	6.11	5.98	1.02	18.69	0.33
Ct Ep203-R1090S55A	4.47	4.29	1.04	17.28	0.26

[0434] 表４に示す通り、ＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体のヒト、サルおよびマウスＥｐＣＡＭに対するK_D値は、マウスＥｐＣＡＭに対するＲ２１７８－Ｅｐ５９およびＲ２１７８－Ｅｐ２０３の結合を除き、10^{-8}Ｍオーダー以下の強い結合を示した。

[0435] ＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体は、Ｒ１０６６－Ｅｐ５９、Ｒ１０６６－Ｅｐｃ０５１およびＲ１０９０Ｓ５５Ａ－Ｅｐｃ０５１以外はサルＥｐＣＡＭに対するK_D値がヒトＥｐＣＡＭに対するK_D値の１／５～５倍の間にあり、高いヒト－サルＥｐＣＡＭの種交差性があることが示され

た。

[0436] また、ＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体は、Ｒ２１７８－Ｅｐ５９およびＲ２１７８－Ｅｐ２０３以外はマウスＥｐＣＡＭに対するK_D値がヒトＥｐＣＡＭに対するK_D値の１／５～５倍の間にあり、高いヒト－マウスＥｐＣＡＭの種交差性があることが示された。

[0437] ［実施例１０］ビアコアによるＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体のＣＤ４０に対する結合性の評価

実施例６で得られたＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体のヒトおよびサルＣＤ４０それぞれに対する結合活性ならびに種交差性を確認することを目的として、実施例１で作製したヒトおよびサルＣＤ４０－ＧＳＴを用いて、表面プラズモン共鳴法（ＳＰＲ法）による結合性試験を実施した。測定機器として、ＢｉａｃｏｒｅＴ１００（ＧＥヘルスケア社製）を使用した。

[0438] Ａｎｔｉ－ｈｕｍａｎ ＩｇＧ ａｎｔｉｂｏｄｙを、Ｈｕｍａｎ Ａｎｔｉｂｏｄｙ Ｃａｐｔｕｒｅ Ｋｉｔ（ＧＥヘルスケア社製）を用いて、添付文書に従いＣＭ５センサーチップ（ＧＥヘルスケア社製）に固定化した。フローセルに、１～３μｇ／ｍＬに調製した被験抗体を１０μＬ／ｍｉｎの流速で１０秒間添加した。

[0439] 次いで、アナライトとして１．２５ｎＭより５段階に２倍希釈したヒトおよびサルＣＤ４０－ＧＳＴタンパク質溶液（０．１％ＢＳＡを含むＨＢＳ－ＥＰ＋で希釈）をそれぞれ３０μＬ／ｍｉｎ分の流速で添加し、各抗体とアナライトとの結合反応を２分間、解離反応を１０分間測定した。測定はシングルサイクルカイネティクス法で行った。

[0440] 得られたセンサーグラムを、Ｂｉａ Ｅｖａｌｕａｔｉｏｎ Ｓｏｆｔｗａｒｅ（ＧＥヘルスケア社製）を用いて解析し、各抗体の速度論定数を算出した。算出された各バイスペシフィック抗体のヒトおよびサルＣＤ４０それぞれに対する解離定数［ｋｄ／ｋａ＝K_D］ならびにヒトＣＤ４０に対する解離定数をサルＣＤ４０に対する解離定数で割った値を表５に示す。

[0441]

[表5]

K_D値	ヒトCD40 [nM]	サルCD40 [nM]	ヒトCD40 [nM]/ サルCD40 [nM]
R1066-Ep59	2.23	1.19	1.87
R1066-Ep203	2.97	1.04	2.87
R1066-Epc051	4.03	0.29	13.92
R1066-Epc112	2.57	1.18	2.18
R1090S55A-Ep59	1.31	0.77	1.70
R1090S55A-Ep203	1.30	0.80	1.62
R1090S55A-Epc051	1.21	0.71	1.70
R1090S55A-Epc112	1.30	0.81	1.60
R2089-Ep59	1.48	1.71	0.86
R2089-Ep203	1.60	2.21	0.72
R2089-Epc051	1.05	1.71	0.61
R2089-Epc112	0.52	2.12	0.25
R2178-Ep59	21.70	1.46	14.85
R2178-Ep203	0.16	1.46	0.11
R2178-Epc051	2.30	1.78	1.29
R2178-Epc112	2.59	2.71	0.95
Ep59-R1066	NC	NC	-
Ep59-R1090S55A	NC	NC	-
Ep59-R2089	1.29	1.65	0.78
Ep59-R2178	0.34	0.61	0.56
Ep203-R1066	NC	NC	-
Ep203-R1090S55A	NC	NC	-
Ep203-R2089	0.29	3.35	0.09
Ep203-R2178	NC	NC	-
Ct R1066-Ep59	0.40	1.10	0.36
Ct R1066-Ep203	7.43	2.71	2.74
Ct R1090S55A-Ep59	0.98	1.54	0.64
Ct R1090S55A-Ep203	1.17	1.67	0.70
Ct Ep59-R1066	NC	NC	-
Ct Ep59-R1090S55A	NC	NC	-
Ct Ep203-R1066	NC	NC	-
Ct Ep203-R1090S55A	NC	NC	-

NC：Not Calculated

[0442] 表５に示す通り、ＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体Ｒ１０６６－Ｅｐ５９、Ｒ１０６６－Ｅｐ２０３、Ｒ１０６６－Ｅｐｃ０５１、Ｒ１０６６－Ｅｐｃ１１２、Ｒ１０９０Ｓ５５Ａ－Ｅｐ５９、Ｒ１０９０Ｓ５５Ａ－Ｅｐ２０３、Ｒ１０９０Ｓ５５Ａ－Ｅｐｃ０５１、Ｒ１０９０Ｓ５５Ａ－Ｅｐｃ１１２、Ｒ２０８９－Ｅｐ５９、Ｒ２０８９－Ｅｐ２０３、Ｒ２０８９－Ｅｐｃ０５１、Ｒ２０８９－Ｅｐｃ１１２、Ｒ２１７８－Ｅｐ２０３、Ｒ２１７８－Ｅｐｃ０５１、Ｒ２１７８－Ｅｐｃ１１２、Ｅｐ５９－Ｒ２０８９、Ｅｐ５９－Ｒ２１７８、Ｅｐ２０３－Ｒ２０８９、Ｃｔ Ｒ１０６６－Ｅｐ５９、Ｃｔ Ｒ１０６６－Ｅｐ２０３、Ｃｔ Ｒ１０９０Ｓ５５Ａ－Ｅｐ５９およびＣｔ Ｒ１０９０Ｓ５５Ａ－Ｅｐ２０３それぞれの、ヒトＣＤ４０およびサルＣＤ４０に対するK_D値はいずれも、10^{-9}Ｍオーダー以下の強い結合を示した。

[0443] また、ＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体Ｒ１０６６－Ｅｐ５９、Ｒ１０６６－Ｅｐ２０３、Ｒ１０６６－Ｅｐｃ１１２、Ｒ１０９０Ｓ５５Ａ－Ｅｐ５９、Ｒ１０９０Ｓ５５Ａ－Ｅｐ２０３、Ｒ１０９０Ｓ５５Ａ－Ｅｐｃ０５１、Ｒ１０９０Ｓ５５Ａ－Ｅｐｃ１１２、Ｒ２０８９－Ｅｐ５９、Ｒ２０８９－Ｅｐ２０３、Ｒ２０８９－Ｅｐｃ０５１、Ｒ２０８９－Ｅｐｃ１１２、Ｒ２１７８－Ｅｐｃ０５１、Ｒ２１７８－Ｅｐｃ１１２、Ｅｐ５９－Ｒ２０８９、Ｅｐ５９－Ｒ２１７８、Ｃｔ Ｒ１０６６－Ｅｐ５９、Ｃｔ Ｒ１０６６－Ｅｐ２０３、Ｃｔ Ｒ１０９０Ｓ５５Ａ－Ｅｐ５９およびＣｔ Ｒ１０９０Ｓ５５Ａ－Ｅｐ２０３それぞれのサルＣＤ４０に対するK_D値は、ヒトＣＤ４０に対するK_D値の１／５～５倍の間にあり、高いヒト－サルＣＤ４０の種交差性があることが示された。

[0444] ［実施例１１］フローサイトメトリーによるＣＤ９５発現量解析によるＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体のＣＤ４０シグナル誘導活性の評価

Ｒａｍｏｓ細胞上のＣＤ９５の発現量増加を指標として、実施例６で得られたＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体のＥｐＣＡＭ陽性または

陰性細胞共存下におけるＲａｍｏｓ細胞へのＣＤ４０シグナル誘導活性を以下のようにＦＣＭ法で評価した。

[0445] ４×１０^{6}ｃｅｌｌｓ／ｍＬのＲａｍｏｓ細胞を２５μＬ／ｗｅｌｌでＵ底９６ｗｅｌｌプレート（ファルコン社製）に播種し、１０％ＦＢＳを含むＲＰＭＩ１６４０培地（シグマアルドリッチ社製）で希釈した０．２、２または２０μｇ／ｍＬ（終濃度０．１、１または１０μｇ／ｍＬ）の被験抗体を５０μｇ／ｍＬ添加し、さらにＥｘｐｉ２９３細胞、ヒトＥｐＣＡＭ発現ＨＥＫ２９３細胞またはＣｏｌｏ２０５細胞４×１０^{6}ｃｅｌｌｓ／ｍＬを２５μＬ／ｗｅｌｌで加え、３７℃、５．０％炭酸ガス下で１６時間培養した。

[0446] 遠心分離（２０００ｒｐｍ、４℃、２分間）の後、上清を除去し、ペレットを２００μＬ／ｗｅｌｌのＳＢで３回洗浄した。遠心分離（２０００ｒｐｍ、４℃、２分間）の後、上清を除去し、ペレットへＰＥ　ｍｏｕｓｅ　ａｎｔｉ－ｈｕｍａｎ　ＣＤ９５抗体（ベクトンディッキンソン社製）およびＦＩＴＣ　ｍｏｕｓｅ　ａｎｔｉ－ｈｕｍａｎ　ＣＤ２０を加えて懸濁した後、氷温下で３０分間静置した。

[0447] さらに遠心分離（２０００ｒｐｍ、４℃、２分間）して上清を除去し、ペレットを２００μＬ／ｗｅｌｌのＳＢで３回洗浄した後、１００倍希釈した７ＡＡＤ（ベクトンディッキンソン社製）を２００μＬ／ｗｅｌｌのＳＢに懸濁し、フローサイトメーターＦＡＣＳＣＡＮＴＯ　ＩＩ（ベクトンディッキンソン社製）でＣＤ２０陽性画分に存在するＲａｍｏｓ細胞上のＣＤ９５の蛍光強度を測定した。陰性コントロールには抗ＤＮＰ抗体を使用した。

[0448] ＨＥＫ２９３細胞と共培養したＲａｍｏｓ細胞の結果を図４に、ヒトＥｐＣＡＭ／ＨＥＫ２９３細胞と共培養したＲａｍｏｓ細胞の結果を図５に、Ｃｏｌｏ２０５細胞と共培養したＲａｍｏｓ細胞の結果を図６に示す。

[0449] 図４に示すように、実施例３で作製した抗ＣＤ４０抗体２１．４．１存在下で、ＨＥＫ２９３細胞、ヒトＥｐＣＡＭ／ＨＥＫ２９３細胞およびＣｏｌｏ２０５細胞のいずれかの１つの細胞とＲａｍｏｓ細胞を共培養した場合、Ｒａｍｏｓ細胞上のＣＤ９５の発現量が増加し、ＣＤ４０のシグナルが誘導

されていることがわかった。

[0450] また、図４～６に示すように、実施例４で作製した抗ＥｐＣＡＭ抗体３６２２Ｗ９４存在下で、ヒトＥｐＣＡＭ／ＨＥＫ２９３細胞またはＣｏｌｏ２０５細胞とＲａｍｏｓ細胞を共培養した場合、Ｒａｍｏｓ細胞上のＣＤ９５の発現量はネガティブコントロールと同等であり、ＣＤ４０シグナルが誘導されていないことが示された。

[0451] 一方で、図４に示すようにＥｐＣＡＭ陰性のＨＥＫ２９３細胞とＲａｍｏｓ細胞を共培養下では、いずれのＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体クローンもＣＤ４０のシグナルを誘導しなかったが、図５および６に示すようにＥｐＣＡＭ陽性のヒトＥｐＣＡＭ／ＨＥＫ２９３細胞またはＣｏｌｏ２０５細胞とＲａｍｏｓ細胞を共培養した場合には、ＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体がＲａｍｏｓ細胞上のＣＤ９５の発現量を増加させ、ＣＤ４０のシグナルを誘導していることが示された。

[0452] これらの結果は、図７（Ｂ）に示すようにＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体がＲａｍｏｓ細胞上のＣＤ４０に単独で結合したときにはＣＤ４０シグナルを誘導せず、図７（Ａ）に示すようにＥｐＣＡＭ陽性細胞が共存する時にのみ、ＣＤ４０シグナルを誘導することを示唆している。

[0453] 親抗体である抗ＣＤ４０抗体および抗ＥｐＣＡＭ抗体はいずれもＥｐＣＡＭ陽性細胞の共存下でＲａｍｏｓ細胞にＣＤ４０シグナルを誘導しないことが確認されている［抗ＣＤ４０抗体については図３（Ａ）および（Ｂ）に記載。抗ＥｐＣＡＭ抗体についてのデータは省略］が、ＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体は、親抗体が有していないＣＤ４０シグナル誘導活性を有することが分かった。

[0454] これにより、本発明のＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体は、腫瘍など、ＥｐＣＡＭが発現する細胞が存在する病変部位特異的に、免疫細胞や腫瘍細胞などのＣＤ４０陽性細胞にシグナルを誘導することが示唆された。

[0455] また、図５に示す通り、ｈＥｐＣＡＭ／ＨＥＫ細胞とＲａｍｏｓ細胞を共

培養した際のＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体のＣＤ４０シグナル誘導活性はＶＨ１が抗ＣＤ４０抗体のＶＨからなり、ＶＨ２が抗ＥｐＣＡＭ抗体のＶＨからなるＣ末端型バイスペシフィック抗体、ＶＨ１が抗ＥｐＣＡＭ抗体のＶＨからなり、ＶＨ２が抗ＣＤ４０抗体のＶＨからなるＣ末端型バイスペシフィック抗体、ＶＨ１が抗ＣＤ４０抗体のＶＨからなり、ＶＨ２が抗ＥｐＣＡＭ抗体のＶＨからなるＮ末端型バイスペシフィック抗体の順に強かった。

[0456] 図６に示す通り、Ｃｏｌｏ２０５細胞とＲａｍｏｓ細胞を共培養した場合もほぼ同様の傾向が確認できた。一方、ＶＨ１が抗ＥｐＣＡＭ抗体のＶＨからなり、ＶＨ２が抗ＣＤ４０抗体のＶＨからなるＮ末端型バイスペシフィック抗体は、ＣＤ４０シグナル誘導活性を有しないことが示された。

[0457] すなわち、ＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体のもつ、ＥｐＣＡＭ陽性細胞の存在に依存したＣＤ４０アゴニスト活性の強さは、ＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体の形状および抗ＣＤ４０抗体のＶＨおよび抗ＥｐＣＡＭ抗体のＶＨの配置に依存しており、Ｃ末端型バイスペシフィック抗体の活性はＮ末端型バイスペシフィック抗体よりも強かった。すなわち、ＶＨ１が抗ＣＤ４０抗体のＶＨからなり、ＶＨ２が抗ＥｐＣＡＭ抗体のＶＨからなるバイスペシフィック抗体の活性はＶＨ１が抗ＥｐＣＡＭ抗体のＶＨからなり、ＶＨ２が抗ＣＤ４０抗体のＶＨからなるバイスペシフィック抗体よりも強いことが示された。

[0458] ［実施例１２］蛍光標識抗体の作製

ＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体の細胞表面上での局在を蛍光免疫染色法により解析するために、ＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体および細胞表面マーカー抗体の蛍光標識を行った。

Ａｌｅｘａ Ｆｌｕｏｒ ４８８ Ｍｉｃｒｏｓｃａｌｅ Ｐｒｏｔｅｉｎ Ｌａｂｅｌｉｎｇ Ｋｉｔ（Ｍｏｌｅｃｕｌａｒ Ｐｒｏｂｅｓ社製）を用い、添付文書に記載の方法に従って、ＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体Ｃｔ Ｒ１０９０Ｓ５５Ａ－Ｅｐ２０３にＡｌｅｘａ４８８を

標識した蛍光標識抗体を作製した。

[0459] また、Ｒａｍｏｓ細胞に発現しておらずＣｏｌｏ２０５細胞に発現している表面マーカーとしてＨＥＲ２を選択し、Ａｌｅｘａ　Ｆｌｕｏｒ　６４７　Ｍｉｃｒｏｓｃａｌｅ　Ｐｒｏｔｅｉｎ　Ｌａｂｅｌｉｎｇ　Ｋｉｔ（Ｍｏｌｅｃｕｌａｒ　Ｐｒｏｂｅｓ社製）を用い、添付文書に記載の方法に従って、公知の抗ＨＥＲ２抗体（国際公開第１９９２／０２２６５３号に記載）にＡｌｅｘａ６４７を標識した抗体を作製した。

[0460] ［実施例１３］ＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体によるＥｐＣＡＭ陽性細胞及びＣＤ４０陽性細胞の架橋部位への集積の確認

ＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体がＲａｍｏｓ細胞とＥｐＣＡＭ陽性細胞に結合する様子を確認するため、Ｃｔ　Ｒ１０９０Ｓ５５Ａ－Ｅｐ２０３－Ａｌｅｘａ　４８８を用い、蛍光免疫染色法による評価を行った。

[0461] １×１０^{6}ｃｅｌｌｓ／ｍＬのＲａｍｏｓ細胞を５０μＬ／ｗｅｌｌで平底９６ｗｅｌｌプレート（グライナー社製）に播種し、実施例１３で調製したＣｔ　Ｒ１０９０Ｓ５５Ａ－Ｅｐ２０３－Ａｌｅｘａ　４８８およびＨＥＲ２　Ａｂ－Ａｌｅｘａ　６４７をフェノールレッド不含のＲＰＭＩ－１６４０培地（Ｇｉｂｃｏ社製）で希釈し、それぞれ２μｇ／ｍｌ（終濃度１μｇ／ｍｌ）および１００μｇ／ｍｌ添加した。

[0462] さらにＣｏｌｏ２０５細胞１×１０^{6}ｃｅｌｌｓ／ｍＬを５０μＬ／ｗｅｌｌで加えて、３７℃、５．０％炭酸ガス下で１４時間培養し、ＩｎＣｅｌｌ　Ａｎａｌｙｚｅｒ６０００（ＧＥヘルスケア社製）にて観察した。

[0463] 図８（Ａ）にＨＥＲ２抗体に標識したＡｌｅｘａ６４７によりＣｏｌｏ２０５細胞を検出した結果を、図８（Ｂ）にＣｔ　Ｒ１０９０Ｓ５５Ａ－Ｅｐ２０３に標識したＡｌｅｘａ４８８を検出した結果を示し、それらの重ね合わせ図８（Ｃ）を示す。

[0464] ＨＥＲ２はＲａｍｏｓ細胞には発現しておらずＣｏｌｏ２０５細胞には発現しているため、図８（Ａ）ではＣｏｌｏ２０５細胞のみから蛍光が観察さ

れている。したがって、図８（Ａ）で観察された細胞がＣｏｌｏ２０５細胞であり、図８（Ｂ）で観察されて図８（Ａ）で観察されていない細胞がＲａｍｏｓ細胞である。

[0465] 図８（Ｂ）においてＣｔ　Ｒ１０９０Ｓ５５Ａ－Ｅｐ２０３－Ａｌｅｘａ４８８は、Ｒａｍｏｓ細胞およびＣｏｌｏ２０５細胞の両細胞から蛍光が観察されている。これにより、Ｃｔ　Ｒ１０９０Ｓ５５Ａ－Ｅｐ２０３が細胞表面上でもＣＤ４０およびＥｐＣＡＭに結合することが確認できた。

[0466] 図８（Ｂ）および図８（Ｃ）に示す通り、Ｃｔ　Ｒ１０９０Ｓ５５Ａ－Ｅｐ２０３－Ａｌｅｘａ　４８８は、Ｃｏｌｏ２０５細胞とＲａｍｏｓ細胞の接触面に集積し、強く蛍光を発している様子が観察された（矢印部分）。この結果から、ＣＤ４０陽性細胞とＥｐＣＡＭ陽性細胞が架橋され、その接触面にＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体が集積していることが示された。

[0467] ＣＤ４０を含むＴＮＦＲＳＦは３量体以上の会合体を形成するとシグナルを誘導することが知られており、細胞の接触面においてＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体が集積することにより、ＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体が結合したＣＤ４０も集積したため、ＣＤ４０のシグナルが誘導されたのだと考えられる。

[0468] また、細胞接触面に集積したＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体は細胞内へ内在化していることも観察されており、これはＣＤ４０シグナルが誘導されたことにより、ＣＤ４０の内在化が促進されていることを示唆している。

[0469] ［実施例１４］抗体のマウスにおける毒性試験

ＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体Ｃｔ　Ｒ１０９０Ｓ５５Ａ－Ｅｐ２０３、Ｃｔ　Ｅｐｃ１１２－Ｒ１０６６、Ｒ１０９０Ｓ５５Ａ－Ｅｐ２０３および抗ＣＤ４０アゴニスト抗体２１．４．１はいずれもマウスＣＤ４０に対する交差反応性を有しないため、マウスモデルを用いた検討では、Ｃ１７ＢＬ／６Ｊ　ＪｃｌマウスにヒトＣＤ４０を含むＢＡＣベクターを

導入した、ヒトＣＤ４０　ＢＡＣ　Ｔｇマウス（以下、ｈＣＤ４０Ｔｇマウスと称する）を用いた。

[0470] ｈＣＤ４０ＴｇマウスはＢＡＣ　ｃｌｏｎｅ（ＣＴＤ－２５３２Ｉ１９）（Ｉｎｖｉｔｒｏｇｅｎ社）を精製後に受精卵に導入して作製した。作製したｈＣＤ４０ＴｇマウスはＣ１７ＢＬ／６Ｊ　ＪｃｌマウスとＦ１交配し、ヒトＣＤ４０遺伝子を有する事をＰＣＲ法で確認した後に試験に供した。

[0471] ＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体Ｃｔ　Ｒ１０９０Ｓ５５Ａ－Ｅｐ２０３、Ｃｔ　Ｅｐｃ１１２－Ｒ１０６６、Ｒ１０９０Ｓ５５Ａ－Ｅｐ２０３、抗ＣＤ４０アゴニスト抗体２１．４．１およびコントロール抗体である抗ＤＮＰ抗体をｈＣＤ４０Ｔｇマウス各群５匹にそれぞれ尾静脈から投与し、投与前と投与２４時間後の体重、投与２４時間後の末梢血液細胞数、血漿中アスパラギンアミノトランスフェラーゼ（ＡＳＴ）、アラニンアミノトランスフェラーゼ（ＡＬＴ）濃度を測定した。抗体投与量は、２１．４．１以外は１０ｍｇ／ｋｇ、２１．４．１については毒性により１０ｍｇ／ｋｇでの投与は困難なため、１ｍｇ／ｋｇとした。

[0472] その結果、図９（Ａ）～（Ｃ）および図１０（Ａ）～（Ｄ）に示すように、２１．４．１を投与した群では、投与後に体重の減少、肝逸脱酵素であるＡＳＴおよびＡＬＴの上昇ならびに末梢血中白血球、リンパ球、単球および血小板の減少が認められたが、Ｃｔ　Ｒ１０９０Ｓ５５Ａ－Ｅｐ２０３、Ｃｔ　Ｅｐｃ１１２－Ｒ１０６６およびＲ１０９０Ｓ５５Ａ－Ｅｐ２０３を投与した群は、陰性コントロールであるＤＮＰ抗体を投与した群と同様、変化は認められなかった。

[0473] 以上より、本発明のＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体が先行抗体に比べ、全身性の毒性を顕著に低減していることが示された。

[0474] ［実施例１５］抗体のマウスシンジェニックモデルにおける抗腫瘍効果

ＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体Ｃｔ　Ｒ１０９０Ｓ５５Ａ－Ｅｐ２０３、Ｃｔ　Ｅｐｃ１１２－Ｒ１０６６、Ｒ１０９０Ｓ５５Ａ－Ｅｐ２０３および抗ＣＤ４０アゴニスト抗体２１．４．１のマウスにおける抗

腫瘍効果を検討するため、マウスシンジェニックモデルを用い、検討を行った。

[0475] １．腫瘍細胞ｍＥｐＣＡＭ／Ｂ１６Ｆ１０の作製

移植する腫瘍細胞として、以下の方法によりマウスＥｐＣＡＭを発現していないマウスメラノーマ細胞株Ｂ１６－Ｆ１０（ＡＴＣＣ　ＣＲＬ－６４７５）とＢ１６－Ｆ１０にマウスＥｐＣＡＭを発現させたｍＥｐＣＡＭ／Ｂ１６Ｆ１０を作製した。

[0476] 配列番号１９に示すマウスＥｐＣＡＭ遺伝子を用いて、実施例２と同様の方法により作製した、膜発現用マウスＥｐＣＡＭ発現ベクターｐＥＦ６－マウスＥｐＣＡＭ　ＦｕｌｌからｍＥｐＣＡＭ（９４５ｂｐ）をＢａｍＨＩ／ＮｏｔＩで切り出し、同じくＢａｍＨＩ／ＮｏｔＩで処理したｐｃＤＮＡ３．１（＋）にｌｉｇａｔｉｏｎしｐｃＤＮＡ３－マウスＥｐＣＡＭ　ｆｕｌｌを得た。

[0477] 取得した発現ベクターｐｃＤＮＡ３．１－マウスＥｐＣＡＭ　ｆｕｌｌを、ｌｉｐｏｆｅｃｔａｍｉｎｅ　３０００（Ｔｈｅｒｍｏ　Ｆｉｓｈｅｒ　Ｓｃｉｅｎｔｉｆｉｃ社製）を用いてＢ１６Ｆ１０細胞に導入して培養し、翌日より３ｍｇ／ｍｌ　Ｇ４１８でセレクションを行った。セルソーターにより、高発現画分のソーティングを３回繰り返し、マウスＥｐＣＡＭを高発現するＢ１６Ｆ１０細胞を得た。

[0478] ２．マウスシンジェニックモデルを用いた抗腫瘍試験

Ｂ１６－Ｆ１０またはｍＥｐＣＡＭ／Ｂ１６Ｆ１０をｈＣＤ４０Ｔｇマウス皮下に1×10^{6}細胞移植（Ｄａｙ－８）した。Ｄａｙ０、３および７に各抗体を各群５匹に尾静脈から投与した。抗体投与量は、２１．４．１以外は１０ｍｇ／ｋｇ、２１．４．１については毒性により１０ｍｇ／ｋｇでの投与は困難なため、１ｍｇ／ｋｇとした。ノギスを用いて腫瘍の長径、短径を測定し、短径×短径×長径／２の式により腫瘍体積を算出した。結果を図１１（Ａ）および図１１（Ｂ）に示す。

[0479] 図１１（Ａ）に示すように、ＣＤ４０－ＥｐＣＡＭバイスペシフィック抗

体Ｃｔ　Ｒ１０９０Ｓ５５Ａ－Ｅｐ２０３、Ｃｔ　Ｅｐｃ１１２－Ｒ１０６６およびＲ１０９０Ｓ５５Ａ－Ｅｐ２０３はいずれも、ｍＥｐＣＡＭ／Ｂ１６Ｆ１０に対しては、顕著な薬効を示した。一方、図１１（Ｂ）に示すように、ＥｐＣＡＭを発現しないＢ１６－Ｆ１０に対しては薬効を示さなかった。以上より、作製したＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体はＥｐＣＡＭ依存的な薬効を有する事が示された。

[0480] ［実施例１６］ＣＤ４０およびＥｐＣＡＭに結合するバイスペシフィック抗体または抗体断片の発現ベクターの構築

１．バイスペシフィック抗体または抗体断片の設計

（１）ヘテロＩｇＧ型バイスペシフィック抗体の設計

ＣＤ４０およびＥｐＣＡＭにそれぞれ１価で結合するバイスペシフィック抗体として、図１２（Ａ）に記載する構造を有するバイスペシフィック抗体（以下、ヘテロＩｇＧ型バイスペシフィック抗体と記載する）を設計した。ヘテロＩｇＧ型バイスペシフィック抗体のＦｃ領域には国際公開第２０１６／０７１００４号に記載のＦｃ領域を採用した。

[0481] ヘテロＩｇＧ型バイスペシフィック抗体は、異なる２つの重鎖定常領域（ＣＨ）を有しており、それぞれを以下ではＣＨａおよびＣＨｂと記載する。ＣＨａの塩基配列およびアミノ酸配列を配列番号７９および８０で示す。またＣＨｂの塩基配列およびアミノ酸配列を配列番号８１および８２で示す。ＣＨａおよびＣＨｂのＮ末端側にはそれぞれ抗体の重鎖可変領域が結合し、重鎖を形成する。以下ではＣＨａおよびＣＨｂに結合する重鎖可変領域をそれぞれＶＨａおよびＶＨｂと記載する。ヘテロＩｇＧ型バイスペシフィック抗体はＮ末端側よりＶＨａおよびＣＨａが連結してなる１本の重鎖、Ｎ末端側よりＶＨｂおよびＣＨｂが連結してなる１本の重鎖ならびに同一の２本の軽鎖からなる。

[0482] （２）Ｆａｂ型バイスペシフィック抗体断片の設計

ＣＤ４０およびＥｐＣＡＭにそれぞれ１価で結合するバイスペシフィック抗体断片として、図１２（Ｂ）に記載する構造を有する抗体断片（以下では

Ｆａｂ型バイスペシフィック抗体断片と記載する）を設計した。Ｆａｂ型バイスペシフック抗体断片の構造には、国際公開第２００９／１３１２３９号に記載の抗体のＮ末端からヒンジの途中までの構造を採用し、重鎖のＣ末端側にＦＬＡＧ－Ｔａｇを連結させた。すなわち、Ｆａｂ型バイスペシフィック抗体断片は、Ｎ末端側から順に、ＶＨ１、ＣＨ１、ＶＨ２、ＣＨ１、ヒンジの一部およびＦＬＡＧ－Ｔａｇが連結してなる１本の重鎖ならびに２本の軽鎖からなる。Ｆａｂ型バイスペシフィック抗体断片のＶＨ２のＣ末端側に結合するＣＨ１からＦＬＡＧ－Ｔａｇまでの遺伝子配列を配列番号８３に、アミノ酸配列を配列番号８４に記載する。

[0483] （３）Ｆ（ａｂ’）$_2$型バイスペシフィック抗体断片の設計

ＣＤ４０およびＥｐＣＡＭにそれぞれ二価で結合するバイスペシフィック抗体断片として、図１２（Ｃ）に記載する構造を有する抗体断片（以下ではＦ（ａｂ’）$_2$型バイスペシフィック抗体断片と記載する）を設計した。Ｆ（ａｂ’）$_2$型バイスペシフィック抗体断片の構造には、国際公開第２００９／１３１２３９号に記載の抗体のＮ末端からヒンジまでの構造を採用し、重鎖のＣ末端側にＦＬＡＧ－Ｔａｇを連結させた。すなわち、Ｆ（ａｂ’）$_2$型バイスペシフィック抗体断片は、Ｎ末端側から順に、ＶＨ１、ＣＨ１、ＶＨ２、ＣＨ１、ヒンジおよびＦＬＡＧ－Ｔａｇが連結してなる２本の重鎖ならびに４本の軽鎖からなる。Ｆ（ａｂ’）$_2$型バイスペシフィック抗体断片のＶＨ２のＣ末端側に結合するＣＨ１からＦＬＡＧ－Ｔａｇまでの遺伝子配列を配列番号８５に、アミノ酸配列を配列番号８６に記載する。

[0484] 以下の工程で作製されるヘテロＩｇＧ型バイスペシフィック抗体のＶＨａは抗ＣＤ４０抗体のＶＨであり、ＶＨｂは抗ＥｐＣＡＭ抗体のＶＨである。またＦａｂ型およびＦ（ａｂ’）$_2$型バイスペシフィック抗体断片のＶＨ１は抗ＣＤ４０抗体のＶＨであり、ＶＨ２は抗ＥｐＣＡＭ抗体のＶＨである。

[0485] 以下の工程で作製されるＦａｂ型およびＦ（ａｂ’）$_2$型バイスペシフィック抗体はＶＨ１とＶＨ２の間のリンカーとして、ＩｇＧ４のＣＨ１（塩基配列を配列番号７４、アミノ酸配列を配列番号７５で示す）を有する。

[0486] また、以下の工程で作製されるヘテロＩｇＧ型バイスペシフィック抗体、Ｆａｂ型バイスペシフィック抗体断片およびＦ（ａｂ’）$_2$型バイスペシフィック抗体断片は、Ｌ６にコードされるＶＬを含む軽鎖を有する。

[0487] 当該バイスペシフィック抗体／抗体断片の名称、バイスペシフィック抗体／抗体断片の構造、当該抗体／抗体断片作製に使用した抗ＣＤ４０抗体のクローンおよび抗ＥｐＣＡＭ抗体のクローンを表４に示す。

[0488] [表6]

抗体／抗体断片の名称	抗体／抗体断片の構造	VHa／VH1	VHb／VH2
heteroIgG R1090S55A-Ep203	ヘテロIgG型	R1090S55A	Ep203
heteroIgG R1090S55A-Ep59	ヘテロIgG型	R1090S55A	Ep59
heteroIgG R1066-Ep203	ヘテロIgG型	R1066	Ep203
heteroIgG R1066-Epc112	ヘテロIgG型	R1066	Epc112
heteroIgG R1066-Ep59	ヘテロIgG型	R1066	Ep59
heteroIgG R2089-Epc112	ヘテロIgG型	R2089	Epc112
R1090S55A-Ep203 Fab	Fab型	R1090S55A	Ep203
R1066-Ep203 Fab	Fab型	R1066	Ep203
R1090S55A-Ep203 F(ab')2	F(ab')2型	R1090S55A	Ep203
R1066-Ep203 F(ab')2	F(ab')2型	R1066	Ep203

[0489] ２．バイスペシフィック抗体または抗体断片の発現ベクターの作製

（１）ヘテロＩｇＧ型バイスペシフィック抗体の発現ベクターの作製

表４に記載されたヘテロＩｇＧ型バイスペシフィック抗体について、発現ベクターを以下に記載する方法で作製した。

[0490] 実施例３で取得したＲ１０６６、Ｒ１０９０Ｓ５５ＡまたはＲ２０８９のＶＨをコードする塩基配列に、配列番号７９で表される、ＣＨａをコードする塩基配列を連結した遺伝子を合成し、ｐＣＩ－Ｈｙｇｒｏ２．０１（Ｐｒｏｍｅｇａ社製ｐＣＩベクターを基に合成）のＢｇＩＩＩ－ＢａｍＨＩサイトに連結した。

[0491] また、実施例４で取得したＥｐ５９、Ｅｐ２０３およびＥｐｃ１１２のそれぞれのＶＨ遺伝子を実施例３で取得したＮ５ＫＧ４ＰＥ　Ｒ４０９Ｋ＿Ｒ１０６６のＳａｌＩ－ＮｈｅＩサイトへサブクローニングした。その後、ＶＬをコードするＬ６遺伝子（配列番号２１）からＶＨの領域までの遺伝子断片をＰｒｉｍｅＳＴＡＲ Ｍａｘ ＤＮＡ Ｐｏｌｙｍｅｒａｓｅ（タカラバイ

オ社製）を用いたＰＣＲ反応により増幅し、配列番号８１で表されるＣＨｂをコードする合成塩基配列を、ｐＣＩ－Ｈｙｇｒｏ２．０１のＮｈｅＩ－ＢａｍＨＩサイトにサブクローニングしたプラスミドのＸｂａＩ－ＮｈｅＩサイトに連結することにより、表４に記載するヘテロＩｇＧ型バイスペシフィック抗体発現プラスミドベクターを得た。

[0492] （２）Ｆａｂ型バイスペシフィック抗体断片の発現ベクターの作製

表４に記載された、Ｆａｂ型バイスペシフィック抗体断片について、発現ベクターを以下に記載する方法で作製した。

[0493] 実施例５－１で作製したＮ末端型バイスペシフィック抗体Ｒ１０６６－Ｅｐ２０３およびＲ１０９０Ｓ５５Ａ－Ｅｐ２０３発現ベクターを鋳型とし、ＶＬからヒンジ領域のＮ末端側の４残基までの配列にＦＬＡＧ－Ｔａｇを付加した遺伝子断片をＰｒｉｍｅＳＴＡＲ Ｍａｘ ＤＮＡ Ｐｏｌｙｍｅｒａｓｅ（タカラバイオ社製）を用いたＰＣＲ反応により増幅し、ｐＣＩ－Ｈｙｇｒｏ２．０１のＸｂａＩ－ＢａｍＨＩサイトに連結することにより、表４に記載するＦａｂ型バイスペシフィック抗体断片発現プラスミドベクターを得た。Ｆａｂ型バイスペシフィック抗体断片のＶＨ２のＣ末端側に結合するＣＨ１からＦＬＡＧ－Ｔａｇまでの遺伝子配列を配列番号８３に、アミノ酸配列を配列番号８４に記載する。

[0494] （３）Ｆ（ａｂ’）$_2$型バイスペシフィック抗体断片の発現ベクターの作製

表４に記載されたＦ（ａｂ’）$_2$型バイスペシフィック抗体断片について、発現ベクターを以下に記載する方法で作製した。

[0495] 実施例５－１で作製したＮ末端型バイスペシフィック抗体Ｒ１０６６－Ｅｐ２０３およびＲ１０９０Ｓ５５Ａ－Ｅｐ２０３発現ベクターを鋳型とし、ＶＬからヒンジの配列にＦＬＡＧ－Ｔａｇを付加した遺伝子断片をＰｒｉｍｅＳＴＡＲ Ｍａｘ ＤＮＡ Ｐｏｌｙｍｅｒａｓｅ（タカラバイオ社製）を用いたＰＣＲ反応により増幅し、ｐＣＩ－Ｈｙｇｒｏ２．０１のＸｂａＩ－ＢａｍＨＩサイトに連結することにより、表４に記載するＦ（ａｂ’）$_2$型バイスペシフィック抗体断片発現プラスミドベクターを得た。Ｆ（ａｂ’）$_2$型バ

イスペシフィック抗体断片のＶＨ２のＮ末端側に結合するＣＨ１からＦＬＡＧ－Ｔａｇまでの遺伝子配列を配列番号８５に、アミノ酸配列を配列番号８６に記載する。

[0496] ［実施例１７］ＣＤ４０とＥｐＣＡＭに結合するバイスペシフィック抗体の調製

実施例１６で作製した抗体発現プラスミドベクターｐＣＩ－Ｈｙｇｒｏ２．０１にサブクローニングしたＣＤ４０およびＥｐＣＡＭの各結合部位を有するバイスペシフィック抗体または抗体断片を、以下の方法によりそれぞれ発現させ、精製した。

[0497] １．ヘテロＩｇＧ型バイスペシフィック抗体の作製

実施例１６－２（１）で作製したＶＨａおよびＣＨａからなる重鎖をコードする発現ベクターならびにＶＨｂおよびＣＨｂからなる重鎖をコードする発現ベクターをＥｘｐｉ２９３（商標） Ｅｘｐｒｅｓｓｉｏｎ Ｓｙｓｔｅｍ（Ｔｈｅｒｍｏ Ｆｉｓｈｅｒ社製）によりＥｘｐｉ２９３細胞に共遺伝子導入し、１６時間後にＴｒａｎｓｆｅｃｔｉｏｎ Ｅｎｈａｎｃｅｒを添加して、一過性発現系で抗体を発現させた。

[0498] ベクター導入４日後に培養上清を回収し、孔径０．２２μｍのメンブランフィルター（ＭＩＬＬＩＰＯＲＥ製）で濾過した後、Ｐｒｏｔｅｉｎ Ａ樹脂（ＭａｂＳｅｌｅｃｔ、ＧＥヘルスケア社製）を用いて抗体をアフィニティー精製した。洗浄液としてＤ－ＰＢＳ（－）を用いた。Ｐｒｏｔｅｉｎ Ａに吸着させた抗体を、１００ｍＭクエン酸ナトリウム、５０ｍＭ ＮａＣｌ緩衝液（ｐＨ３．５）により溶出し、１Ｍ Ｔｒｉｓ－ＨＣｌ緩衝液（ｐＨ９．０）を含むチューブに回収した。

[0499] 次に、ＶＩＶＡＳＰＩＮ（Ｓａｒｔｒｉｕｓ ｓｔｅａｌｉｎ社製）を用いて濃縮し、Ｎａｐ Ｃｏｌｕｍｎ（ＧＥヘルスケア社製）を用いてＤ－ＰＢＳ（－）へ緩衝液を置換した。さらに、ＡＫＴＡ ＦＰＬＣ（ＧＥヘルスケア社製）およびＳｕｐｅｒｄｅｘ Ｈｉｇｈ－ｐｅｒｆｏｒｍａｎｃｅ Ｃｏｌｕｍｎｓ（ＧＥヘルスケア社製）を用いて、該抗体溶液より単量体画

分を分取した。ＡＫＴＡシステム孔径０．２２μｍのメンブランフィルター（Ｍｉｌｌｅｘ－ＧＶ、ＭＩＬＬＩＰＯＲＥ社製）でろ過滅菌することにより精製抗体を得た。

[0500] 抗体溶液の波長２８０ｎｍの吸光度を測定し、各抗体のアミノ酸配列から推定される吸光係数を用いて、精製抗体の濃度を算出した。また、調製したヘテロＩｇＧ型バイスペシフィック抗体の糖鎖を酵素消化した上で、Ｓｙｎａｐｔ Ｇ２（Ｗａｔｅｒｓ社製）による質量分析を行い、ＶＨａおよびＣＨａからなる重鎖ならびにＶＨｂおよびＣＨｂからなる重鎖をそれぞれ１本ずつ有するヘテロＩｇＧ型バイスペシフィック抗体になっていることを確認した。

[0501] ２．Ｆａｂ型バイスペシフィック抗体断片およびＦ（ａｂ’）$_2$型バイスペシフィック抗体断片の作製

実施例１６－２（２）で作製したＦａｂ型バイスペシフィック抗体断片発現ベクターをＥｘｐｉ２９３（商標） Ｅｘｐｒｅｓｓｉｏｎ Ｓｙｓｔｅｍ（Ｔｈｅｒｍｏ Ｆｉｓｈｅｒ社製）によりＥｘｐｉ２９３細胞に遺伝子導入し、１６時間後にＴｒａｎｓｆｅｃｔｉｏｎ Ｅｎｈａｎｃｅｒを添加して、一過性発現系で抗体断片を発現させた。

[0502] ベクター導入４日後に培養上清を回収し、孔径０．２２μｍのメンブランフィルター（ＭＩＬＬＩＰＯＲＥ製）で濾過した後、Ｐｒｏｔｅｉｎ Ｌ樹脂（プロテイン・エクスプレス社製）を用いて抗体断片をアフィニティー精製した。洗浄液としてＤ－ＰＢＳ（－）を用いた。Ｐｒｏｔｅｉｎ Ｌに吸着させた抗体断片を、１００ｍＭグリシン緩衝液（ｐＨ２．５）により溶出し、１Ｍ ＴｒｉｓＨＣｌ緩衝液（ｐＨ８．０）を含むチューブに回収した。

[0503] 次に、ＶＩＶＡＳＰＩＮ（Ｓａｒｔｒｉｕｓ ｓｔｅａｌｉｎ社製）を用いて濃縮し、Ｎａｐ Ｃｏｌｕｍｎ（ＧＥヘルスケア社製）を用いて、緩衝液をＤ－ＰＢＳ（－）に置換した。さらに、ＡＫＴＡ ＦＰＬＣ（ＧＥヘルスケア社製）およびＳｕｐｅｒｄｅｘ Ｈｉｇｈ－ｐｅｒｆｏｒｍａｎｃｅ

Ｃｏｌｕｍｎｓ（ＧＥヘルスケア社製）を用いて、該抗体断片溶液よりそれぞれＦａｂ型バイスペシフィック抗体断片の分子量に溶出する画分を分取した。ＡＫＴＡシステム孔径０．２２μｍのメンブランフィルター（Ｍｉｌｅｘ－ＧＶ、ＭＩＬＬＩＰＯＲＥ社製）でろ過滅菌することにより精製抗体断片を得た。

[0504] また、実施例１６－２（３）で作製したＦ（ａｂ’）$_2$型バイスペシフィック抗体断片発現ベクターを用い、上記と同様の方法でＦ（ａｂ’）$_2$型バイスペシフィック抗体断片を得た。精製されたＦ（ａｂ’）$_2$型バイスペシフィック抗体断片をＳＤＳ－ＰＡＧＥにより評価し、重鎖の間のジスルフィド結合が還元されたＦａｂ’体の含量が低いことを確認した。

[0505] 抗体断片溶液の波長２８０ｎｍの吸光度を測定し、各抗体断片のアミノ酸配列から推定される吸光係数を用いて、精製抗体断片の濃度を算出した。

[0506] ［実施例１８］ヒトＥｐＣＡＭ発現Ｅｘｐｉ２９３細胞の作製

実施例２で得られた膜発現用ヒトＥｐＣＡＭ発現ベクターｐＥＦ６－ヒトＥｐＣＡＭ　ｆｕｌｌを、Ｅｘｐｉ２９３（商標）　Ｅｘｐｒｅｓｓｉｏｎ　Ｓｙｓｔｅｍ（Ｔｈｅｒｍｏ　Ｆｉｓｈｅｒ社製）によりＥｘｐｉ２９３細胞に遺伝子導入して培養し、一過性発現系でタンパク質を発現させた。遺伝子導入後、２４時間振とう培養し、遠心分離により、細胞膜上にヒトＥｐＣＡＭが発現したＥｘｐｉ２９３細胞（以下ヒトＥｐＣＡＭ／Ｅｘｐｉ２９３細胞と記載する）を得た。

[0507] ［実施例１９］フローサイトメトリーによるＣＤ９５発現量解析によるヘテロバイスペシフィック抗体のＣＤ４０シグナル誘導活性の評価

Ｒａｍｏｓ細胞上のＣＤ９５の発現量増加を指標として、実施例１７－１で得られたヘテロＩｇＧ型バイスペシフィック抗体の、ＥｐＣＡＭ陽性または陰性細胞共存下におけるＲａｍｏｓ細胞へのＣＤ４０シグナル誘導活性を以下のようにＦＣＭ法で評価した。

[0508] 添加する被験抗体濃度を２μｇ／ｍＬ（終濃度１μｇ／ｍＬ）にし、さらにＲａｍｏｓ細胞と共培養する細胞株をＥｘｐｉ２９３細胞または実施例１

８で得られたヒトＥｐＣＡＭ／Ｅｘｐｉ２９３細胞に変更した以外は実施例１１と同様の方法で評価を行った。

[0509] ヒトＥｐＣＡＭ／Ｅｘｐｉ２９３細胞と共培養したＲａｍｏｓ細胞の結果を図１３に、Ｅｘｐｉ２９３細胞と共培養したＲａｍｏｓ細胞の結果を図１４に示す。

[0510] 図１３および図１４に示すように、実施例３で作製した抗ＣＤ４０抗体２１．４．１存在下で、Ｅｘｐｉ２９３細胞およびヒトＥｐＣＡＭ／Ｅｘｐｉ２９３細胞のいずれか１つの細胞とＲａｍｏｓ細胞を共培養した場合、Ｒａｍｏｓ細胞上のＣＤ９５の発現量が増加しており、ＣＤ４０のシグナルが誘導されていることが確認された。

[0511] 図１４に示すようにＥｐＣＡＭ陰性のＥｘｐｉ２９３細胞とＲａｍｏｓ細胞を共培養した場合、ヘテロＩｇＧ型、Ｎ末端型およびＣ末端型ＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体のいずれのクローンもＣＤ４０のシグナルを誘導しなかった。

[0512] 一方で、図１３に示すようにＥｐＣＡＭ陽性のヒトＥｐＣＡＭ／Ｅｘｐｉ２９３細胞とＲａｍｏｓ細胞を共培養した場合には、ほとんどのＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体によりＲａｍｏｓ細胞上のＣＤ９５の発現量が増加しており、ＣＤ４０のシグナルを誘導していることが示された。そのシグナル誘導活性の強さは抗ＣＤ４０抗体のＶＨおよび抗ＥｐＣＡＭ抗体のＶＨの組み合わせにより異なるものの、同じ抗ＣＤ４０抗体のＶＨおよび抗ＥｐＣＡＭ抗体のＶＨの組み合わせのバイスペシフィック抗体の中ではＣ末端型ＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体、Ｎ末端型ＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体、ヘテロＩｇＧ型ＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体の順であった。

[0513] これにより、ＣＤ４０およびＥｐＣＡＭにそれぞれ二価で結合するＣ末端型およびＮ末端型ＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体は、ＣＤ４０およびＥｐＣＡＭにそれぞれ１価で結合するヘテロＩｇＧ型バイスペシフィック抗体よりも強いＥｐＣＡＭ依存的なＣＤ４０シグナル誘導活性を有す

ることが示された。

[0514] ［実施例２０］フローサイトメトリーによるＣＤ９５発現量解析によるＦａｂ型およびＦ（ａｂ’）$_2$型バイスペシフィック抗体断片のＣＤ４０シグナル誘導活性の比較

Ｒａｍｏｓ細胞上のＣＤ９５の発現量増加を指標として、実施例１７－２で得られたＦａｂ型およびＦ（ａｂ’）$_2$型バイスペシフィック抗体断片のＥｐＣＡＭ陽性または陰性細胞共存下におけるＲａｍｏｓ細胞へのＣＤ４０シグナル誘導活性を以下のようにＦＣＭ法で評価した。

[0515] 添加する被抗体を１３３．３ｎＭ（終濃度６６．７ｎＭ）から３倍希釈系列で６点の濃度でデータを取得した以外は、実施例１９と同様の方法で評価を実施した。ヒトＥｐＣＡＭ／Ｅｘｐｉ２９３細胞と共培養したＲａｍｏｓ細胞の結果を図１５（Ａ）に、Ｅｘｐｉ２９３細胞と共培養したＲａｍｏｓ細胞の結果を図１５（Ｂ）に示す。

[0516] 図１５（Ａ）および図１５（Ｂ）に示すように、実施例３で作製した抗ＣＤ４０抗体２１．４．１存在下で、Ｅｘｐｉ２９３細胞およびヒトＥｐＣＡＭ／Ｅｘｐｉ２９３細胞のいずれかの１つの細胞とＲａｍｏｓ細胞を共培養した場合、Ｒａｍｏｓ細胞上のＣＤ９５の発現量が増加し、ＣＤ４０のシグナルが誘導されていることが確認された。

[0517] 図１５（Ｂ）に示すようにＦａｂ型およびＦ（ａｂ’）$_2$型ＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体断片の存在下、ＥｐＣＡＭ陰性のＥｘｐｉ２９３細胞とＲａｍｏｓ細胞を共培養した場合、いずれのクローンもＣＤ４０のシグナルを誘導しなかった。一方で、図１５（Ａ）に示すようにＥｐＣＡＭ陽性のヒトＥｐＣＡＭ／Ｅｘｐｉ２９３細胞とＲａｍｏｓ細胞を共培養した場合には、いずれのＦａｂ型およびＦ（ａｂ’）$_2$型ＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体断片もＲａｍｏｓ細胞上のＣＤ９５の発現量を増加させ、ＣＤ４０のシグナルを誘導していることが示された。そのシグナル誘導活性は、抗ＣＤ４０抗体のＶＨおよび抗ＥｐＣＡＭ抗体のＶＨの組み合わせにより異なるものの、Ｆ（ａｂ’）$_2$型ＣＤ４０－ＥｐＣＡＭバイスペシフ

ィック抗体断片の方がＦａｂ型ＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体断片よりも強かった。

[0518] Ｆ（ａｂ’）$_2$型バイスペシフィック抗体断片は、ヒンジ領域に含まれるシステインによるジスルフィド結合によりＦａｂ型バイスペシフィック抗体断片が２量体化した構造に類似した形状をしている。ＶＨ１とＶＨ２の組み合わせが同じ場合、Ｆａｂ型およびＦ（ａｂ’）$_2$型バイスペシフィック抗体断片のアミノ酸配列は、ヒンジを除き、同一である。Ｆ（ａｂ’）$_2$型バイスペシフィック抗体の方が長いヒンジを有しているが、ヒンジ領域は２量体化以外に直接的な抗原への結合などの機能は有していない。そのため、ＶＨ１とＶＨ２の組み合わせが同じ場合、Ｆａｂ型およびＦ（ａｂ’）$_2$型バイスペシフィック抗体のＥｐＣＡＭ依存的ＣＤ４０シグナル誘導活性の違いは、結合価数の違いによるものであると考えられる。

[0519] Ｆａｂ型およびＦ（ａｂ’）$_2$型ＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体のＥｐＣＡＭ依存的ＣＤ４０シグナル誘導活性を比較すると、Ｆ（ａｂ’）$_2$型ＣＤ４０－ＥｐＣＡＭバイスペシフィック抗体の方が強かったことから、ＥｐＣＡＭ依存的ＣＤ４０シグナル誘導活性はＣＤ４０およびＥｐＣＡＭにそれぞれ１価で結合する抗体断片よりもＣＤ４０およびＥｐＣＡＭにそれぞれ二価で結合する抗体断片の方が強力な活性をもつことが示唆される。

[0520] 本発明を特定の態様を用いて詳細に説明したが、本発明の意図と範囲を離れることなく様々な変更および変形が可能であることは、当業者にとって明らかである。なお、本出願は、２０１７年１１月８日付けで出願された日本特許出願（特願２０１７－２１５８３４号）に基づいており、その全体が引用により援用される。

配列表フリーテキスト

[0521] 配列番号２３－人工配列の説明：Ｌ６　ＬＣＤＲ１のアミノ酸配列

配列番号２４－人工配列の説明：Ｌ６　ＬＣＤＲ２のアミノ酸配列

配列番号２５－人工配列の説明：Ｌ６　ＬＣＤＲ３のアミノ酸配列

配列番号２８－人工配列の説明：Ｒ１０６６　ＨＣＤＲ１のアミノ酸配列

配列番号２９－人工配列の説明：Ｒ１０６６　ＨＣＤＲ２のアミノ酸配列

配列番号３０－人工配列の説明：Ｒ１０６６　ＨＣＤＲ３のアミノ酸配列

配列番号３３－人工配列の説明：Ｒ１０９０Ｓ５５Ａ　ＨＣＤＲ１のアミノ酸配列

配列番号３４－人工配列の説明：Ｒ１０９０Ｓ５５Ａ　ＨＣＤＲ２のアミノ酸配列

配列番号３５－人工配列の説明：Ｒ１０９０Ｓ５５Ａ　ＨＣＤＲ３のアミノ酸配列

配列番号３８－人工配列の説明：Ｒ２０８９　ＨＣＤＲ１のアミノ酸配列

配列番号３９－人工配列の説明：Ｒ２０８９　ＨＣＤＲ２のアミノ酸配列

配列番号４０－人工配列の説明：Ｒ２０８９　ＨＣＤＲ３のアミノ酸配列

配列番号４３－人工配列の説明：Ｒ２１７８　ＨＣＤＲ１のアミノ酸配列

配列番号４４－人工配列の説明：Ｒ２１７８　ＨＣＤＲ２のアミノ酸配列

配列番号４５－人工配列の説明：Ｒ２１７８　ＨＣＤＲ３のアミノ酸配列

配列番号５２－人工配列の説明：Ｅｐ５９　ＨＣＤＲ１のアミノ酸配列

配列番号５３－人工配列の説明：Ｅｐ５９　ＨＣＤＲ２のアミノ酸配列

配列番号５４－人工配列の説明：Ｅｐ５９　ＨＣＤＲ３のアミノ酸配列

配列番号７０－人工配列の説明：３６２２Ｗ９４　ＶＨの塩基配列

配列番号７１－人工配列の説明：合成コンストラクトのアミノ酸配列

配列番号７２－人工配列の説明：３６２２Ｗ９４　ＶＬの塩基配列

配列番号７３－人工配列の説明：合成コンストラクトのアミノ酸配列

配列番号７４－人工配列の説明：ＩｇＧ４　ＣＨ１の塩基配列

配列番号７５－人工配列の説明：合成コンストラクトのアミノ酸配列

配列番号７６－人工配列の説明：ＩｇＧ４ＰＥ　Ｒ４０９Ｋ　ＣＨの塩基配列

配列番号７７－人工配列の説明：合成コンストラクトのアミノ酸配列

配列番号７８－人工配列の説明：停止コドンを含むＩｇＧ４　ＣＨ１の塩基配列

配列番号７９－人工配列の説明：ヘテロＩｇＧ型バイスペシフィック抗体ＣＨａの塩基配列

配列番号８０－人工配列の説明：合成コンストラクトのアミノ酸配列

配列番号８１－人工配列の説明：ヘテロＩｇＧ型バイスペシフィック抗体ＣＨｂの塩基配列

配列番号８２－人工配列の説明：合成コンストラクトのアミノ酸配列

配列番号８３－人工配列の説明：Ｆａｂ型バイスペシフィック抗体のＣＨ１－ｔａｇの塩基配列

配列番号８４－人工配列の説明：合成コンストラクトのアミノ酸配列

配列番号８５－人工配列の説明：Ｆ（ａｂ’）$_2$型バイスペシフィック抗体のＣＨ１－ｔａｇの塩基配列

配列番号８６－人工配列の説明：合成コンストラクトのアミノ酸配列

請求の範囲

[請求項1] ＣＤ４０に結合する抗原結合ドメインおよびＥｐｉｔｈｅｒｉａｌ Ｃｅｌｌ Ａｄｈｅｓｉｏｎ Ｍｏｌｅｃｕｌｅ（ＥｐＣＡＭ）に結合する抗原結合ドメインを含むバイスペシフィック抗体。

[請求項2] ＣＤ４０およびＥｐＣＡＭにそれぞれ二価で結合する請求項１記載のバイスペシフィック抗体。

[請求項3] ＣＤ４０に結合する抗原結合ドメインがＣＤ４０に特異的に結合する抗体（抗ＣＤ４０抗体）の重鎖可変領域（ＶＨ）および軽鎖可変領域（ＶＬ）を含み、ＥｐＣＡＭに結合する抗原結合ドメインがＥｐＣＡＭに特異的に結合する抗体（抗ＥｐＣＡＭ抗体）のＶＨおよびＶＬを含む請求項１または２記載のバイスペシフィック抗体。

[請求項4] Ｎ末端から順にＶＨ１－Ｘ－ＶＨ２－Ｙの式
｛式中、
ＶＨ１は第一の抗体のＶＨを表し、
ＶＨ２は第二の抗体のＶＨを表し、
ＸおよびＹはそれぞれ抗体のＣＨ１を含むポリペプチド（ここでＸおよびＹの少なくとも一方はさらに抗体のヒンジ領域を含む）を表す｝
で表されるポリペプチドを含む同一の２本の重鎖、
および同一のＶＬをを含む４本の軽鎖を有し、
該第一の抗体および該第二の抗体のいずれか一方が抗ＣＤ４０抗体であり、もう一方が抗ＥｐＣＡＭ抗体である請求項１～３のいずれか１項記載のバイスペシフィック抗体。

[請求項5] 軽鎖がκ鎖である請求項４記載のバイスペシフィック抗体。

[請求項6] 前記式におけるＸがヒトＩｇＧのＣＨ１からなるポリペプチドであり、ＹがヒトＩｇＧのＣＨ１、ヒンジ領域、ＣＨ２およびＣＨ３からなるポリペプチドである請求項４または５記載のバイスペシフィック抗体または該バイスペシフィック抗体断片。

[請求項7] 前記式におけるＸが配列番号７５で表されるアミノ酸配列を含むポ

リペプチドであり、Ｙが配列番号７７で表されるアミノ酸配列を含むポリペプチドである請求項４または５記載のバイスペシフィック抗体。

[請求項8] 前記式におけるＸがヒトＩｇＧのＣＨ１、ヒンジ領域、ＣＨ２およびＣＨ３を含むリペプチドであり、ＹがヒトＩｇＧのＣＨ１を含むポリペプチドである請求項４または５記載のバイスペシフィック抗体。

[請求項9] 前記式におけるＸが配列番号７７で表されるアミノ酸配列を含むポリペプチドであり、Ｙが配列番号７５で表されるアミノ酸配列を含むポリペプチドである請求項４または５記載のバイスペシフィック抗体。

[請求項10] ＶＬが、それぞれ配列番号２３～２５で表されるアミノ酸配列を含む相補性決定領域（ＣＤＲ）１～３を含むＶＬである請求項３～９のいずれか１項に記載のバイスペシフィック抗体。

[請求項11] ＶＬが、配列番号２２で表されるアミノ酸配列を含むＶＬである請求項３～１０のいずれか１項に記載のバイスペシフィック抗体。

[請求項12] ＣＤ４０アゴニスト活性を有する、請求項１～１１のいずれか１項に記載のバイスペシフィック抗体。

[請求項13] ＥｐＣＡＭを発現する細胞の非存在下ではＣＤ４０アゴニスト活性を示さず、ＥｐＣＡＭを発現する細胞の存在下でのみＣＤ４０アゴニスト活性を示す、請求項１～１２のいずれか１項に記載のバイスペシフィック抗体。

[請求項14] 抗ＣＤ４０抗体がアゴニスト活性を有していない抗ＣＤ４０抗体である請求項３～１３のいずれか１項に記載のバイスペシフィック抗体。

[請求項15] 第一の抗体が抗ＣＤ４０抗体であり、第二の抗体が抗ＥｐＣＡＭ抗体である請求項４～１４のいずれか１項に記載のバイスペシフィック抗体。

[請求項16] 第一の抗体が抗ＥｐＣＡＭ抗体であり、第二の抗体が抗ＣＤ４０抗

体である請求項４～１４のいずれか１項に記載のバイスペシフィック抗体。

[請求項17] 抗ＣＤ４０抗体が、以下の（１ａ）～（１ｄ）からなる群から選ばれるいずれか１つの重鎖可変領域（ＶＨ）を含む抗ＣＤ４０抗体である、請求項３～１６のいずれか１項に記載のバイスペシフィック抗体。

（１ａ）それぞれ配列番号２８～３０で表されるアミノ酸配列を含むＣＤＲ１～３を含むＶＨ、

（１ｂ）それぞれ配列番号３３～３５で表されるアミノ酸配列を含むＣＤＲ１～３を含むＶＨ、

（１ｃ）それぞれ配列番号３８～４０で表されるアミノ酸配列を含むＣＤＲ１～３を含むＶＨ、および

（１ｄ）それぞれ配列番号４３～４５で表されるアミノ酸配列を含むＣＤＲ１～３を含むＶＨ。

[請求項18] 抗ＣＤ４０抗体が、配列番号２７、３２、３７および４２のいずれか１つで表されるアミノ酸配列を含むＶＨを含む抗ＣＤ４０抗体である請求項３～１７のいずれか１項に記載のバイスペシフィック抗体。

[請求項19] 抗ＥｐＣＡＭ抗体が、以下の（２ａ）～（２ｄ）からなる群から選ばれるいずれか１つのＶＨを含む抗ＥｐＣＡＭ抗体である、請求項３～１８のいずれか１項に記載のバイスペシフィック抗体。

（２ａ）それぞれ配列番号５２～５４で表されるアミノ酸配列を含むＣＤＲ１～３を含むＶＨ、

（２ｂ）それぞれ配列番号５７～５９で表されるアミノ酸配列を含むＣＤＲ１～３を含むＶＨ、

（２ｃ）それぞれ配列番号６２～６４で表されるアミノ酸配列を含むＣＤＲ１～３を含むＶＨ、および

（２ｄ）それぞれ配列番号６７～６９で表されるアミノ酸配列を含むＣＤＲ１～３を含むＶＨ。

[請求項20] 抗ＥｐＣＡＭ抗体が、配列番号５１、５６、６１および６６のいずれか１つで表されるアミノ酸配列を含むＶＨを含む抗ＥｐＣＡＭ抗体である請求項３～１９のいずれか１項に記載のバイスペシフィック抗体。

[請求項21] 請求項１～２０のいずれか１項に記載のバイスペシフィック抗体のバイスペシフィック抗体断片。

[請求項22] Ｆａｂ型バイスペシフィック抗体断片またはＦ（ａｂ’）２型バイスペシフィック抗体断片である請求項２１に記載のバイスペシフィック抗体断片。

[請求項23] 請求項１～２０のいずれか１項に記載のバイスペシフィック抗体または請求項２１もしくは２２に記載のバイスペシフィック抗体断片をコードするＤＮＡ。

[請求項24] 請求項２３に記載のＤＮＡを含有する組換え体ベクター。

[請求項25] 請求項２４に記載の組換え体ベクターを宿主細胞に導入して得られる形質転換株。

[請求項26] 請求項２５に記載の形質転換株を培地に培養し、培養物中に請求項１～２０のいずれか１項に記載のバイスペシフィック抗体または請求項２１もしくは２２に記載のバイスペシフィック抗体断片を生産蓄積させ、該培養物から該バイスペシフィック抗体または該バイスペシフィック抗体断片を採取することを特徴とする請求項１～２０のいずれか１項に記載のバイスペシフィック抗体または請求項２１もしくは２２に記載のバイスペシフィック抗体断片の製造方法。

[請求項27] 請求項１～２０のいずれか１項に記載のバイスペシフィック抗体または請求項２１もしくは２２に記載のバイスペシフィック抗体断片を有効成分として含有する、ヒトＣＤ４０およびヒトＥｐＣＡＭの少なくとも一方が関係する疾患の治療および／または診断剤。

[請求項28] ヒトＣＤ４０およびヒトＥｐＣＡＭの少なくとも一方が関係する疾患ががんである請求項２７に記載の剤。

[請求項29] 請求項１～２０のいずれか１項に記載のバイスペシフィック抗体または請求項２１もしくは２２に記載のバイスペシフィック抗体断片を用いる、ヒトＣＤ４０およびヒトＥｐＣＡＭの少なくともいずれか一方が関係する疾患の治療および／または診断方法。

[請求項30] ヒトＣＤ４０およびヒトＥｐＣＡＭの少なくとも一方が関係する疾患ががんである請求項２９に記載の方法。

[請求項31] ヒトＣＤ４０およびヒトＥｐＣＡＭの少なくとも一方が関係する疾患の治療および／または診断に使用するための、請求項１～２０のいずれか１項に記載のバイスペシフィック抗体または請求項２１もしくは２２に記載のバイスペシフィック抗体断片。

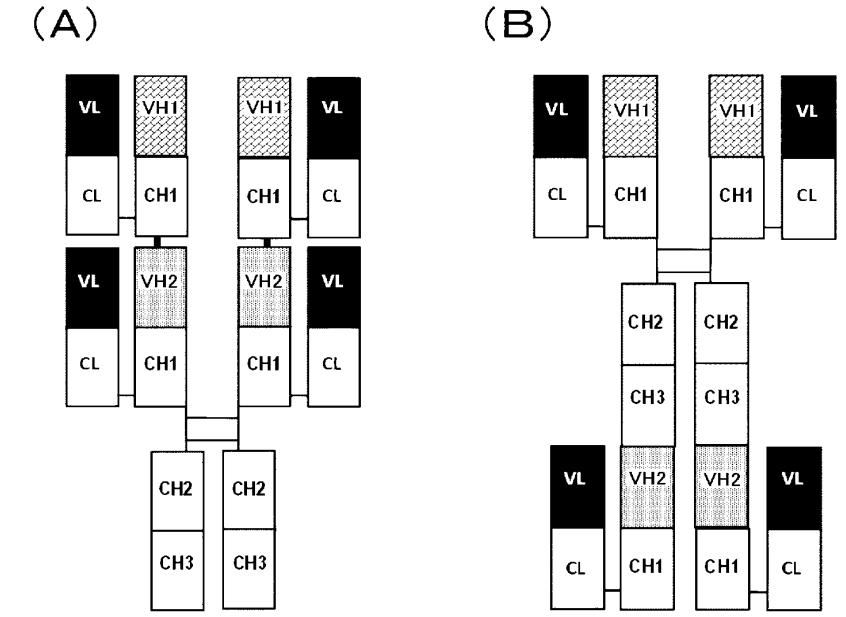
[請求項32] ヒトＣＤ４０およびヒトＥｐＣＡＭの少なくとも一方が関係する疾患ががんである請求項３１に記載のバイスペシフィック抗体または請求項２１もしくは２２に記載のバイスペシフィック抗体断片。

[請求項33] ヒトＣＤ４０およびヒトＥｐＣＡＭの少なくとも一方が関係する疾患の治療および／または診断剤の製造のための、請求項１～２０のいずれか１項に記載のバイスペシフィック抗体または請求項２１もしくは２２に記載のバイスペシフィック抗体断片の使用。

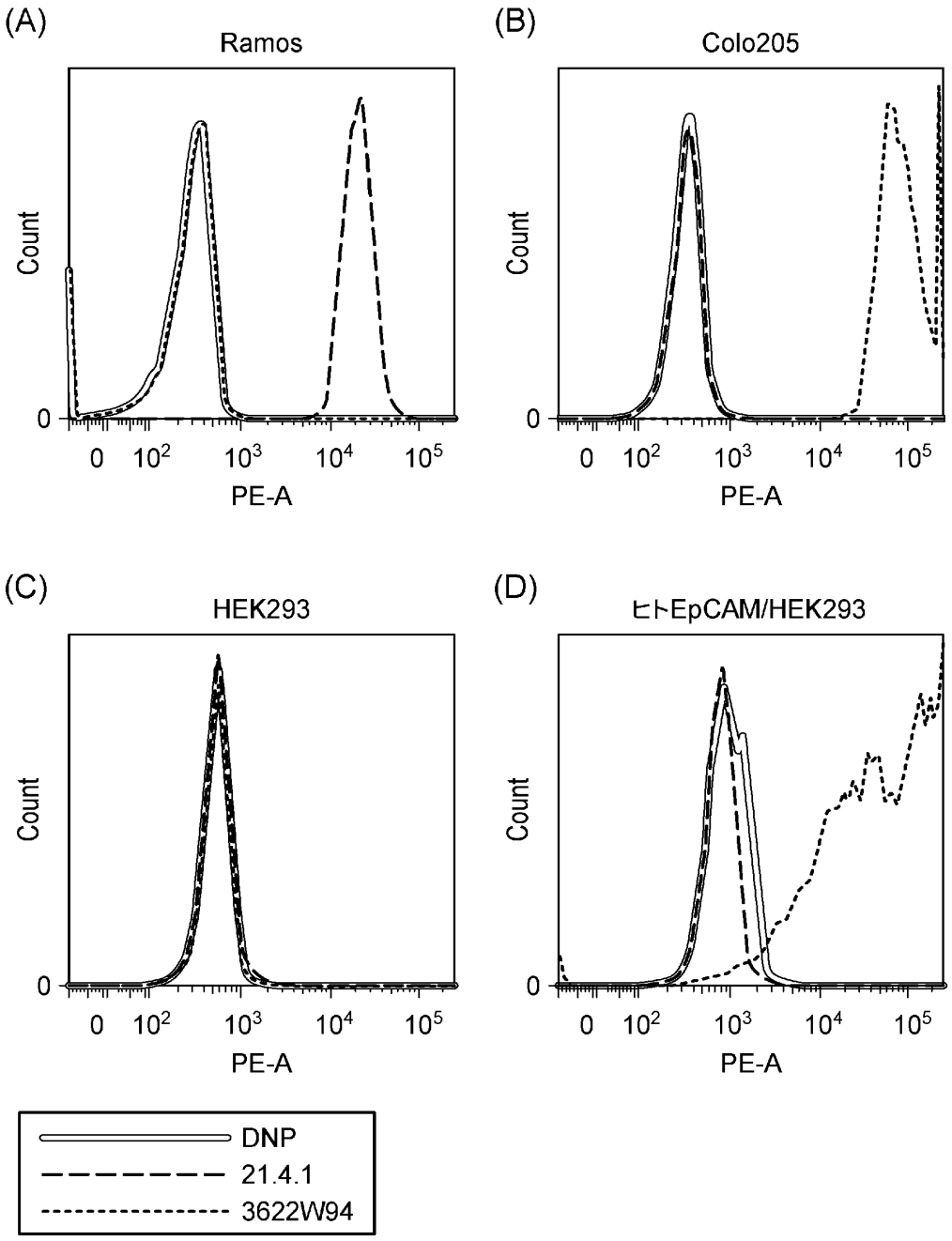
[請求項34] ヒトＣＤ４０およびヒトＥｐＣＡＭの少なくとも一方が関係する疾患ががんである請求項３３に記載の使用。

[請求項35] 請求項１～２０のいずれか１項に記載のバイスペシフィック抗体または請求項２１もしくは２２に記載のバイスペシフィック抗体断片を含む、ＥｐＣＡＭおよびＣＤ４０の少なくとも一方を検出または測定するための試薬。

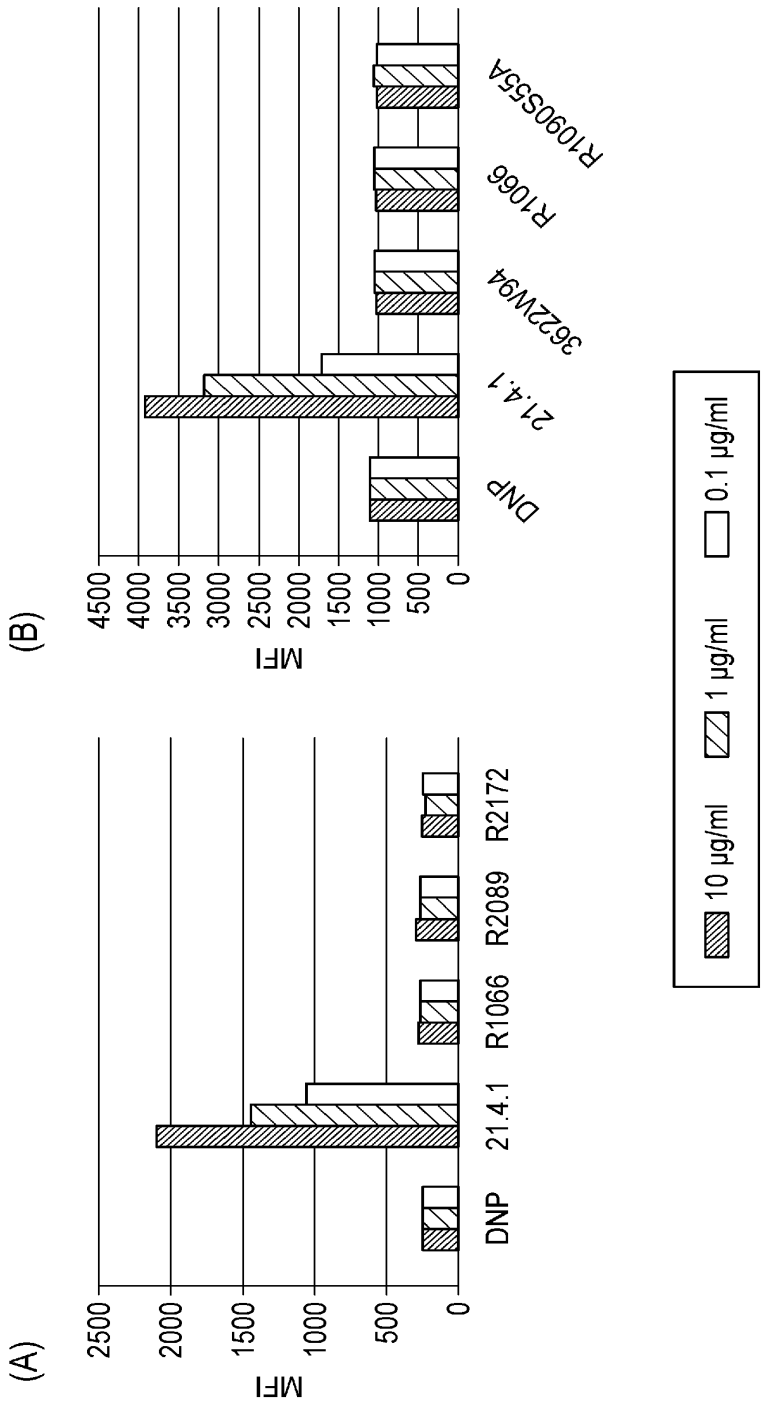
[図1]


(A)
VL
VH1
VH1
VL
CL
CH1
CH1
CL
VL
VH2
VH2
VL
CL
CH1
CH1
CL
CH2
CH2
CH3
CH3
(B)
VL
VH1
VH1
VL
CL
CH1
CH1
CL
CH2
CH2
CH3
CH3
VL
VH2
VH2
VL
CL
CH1
CH1
CL

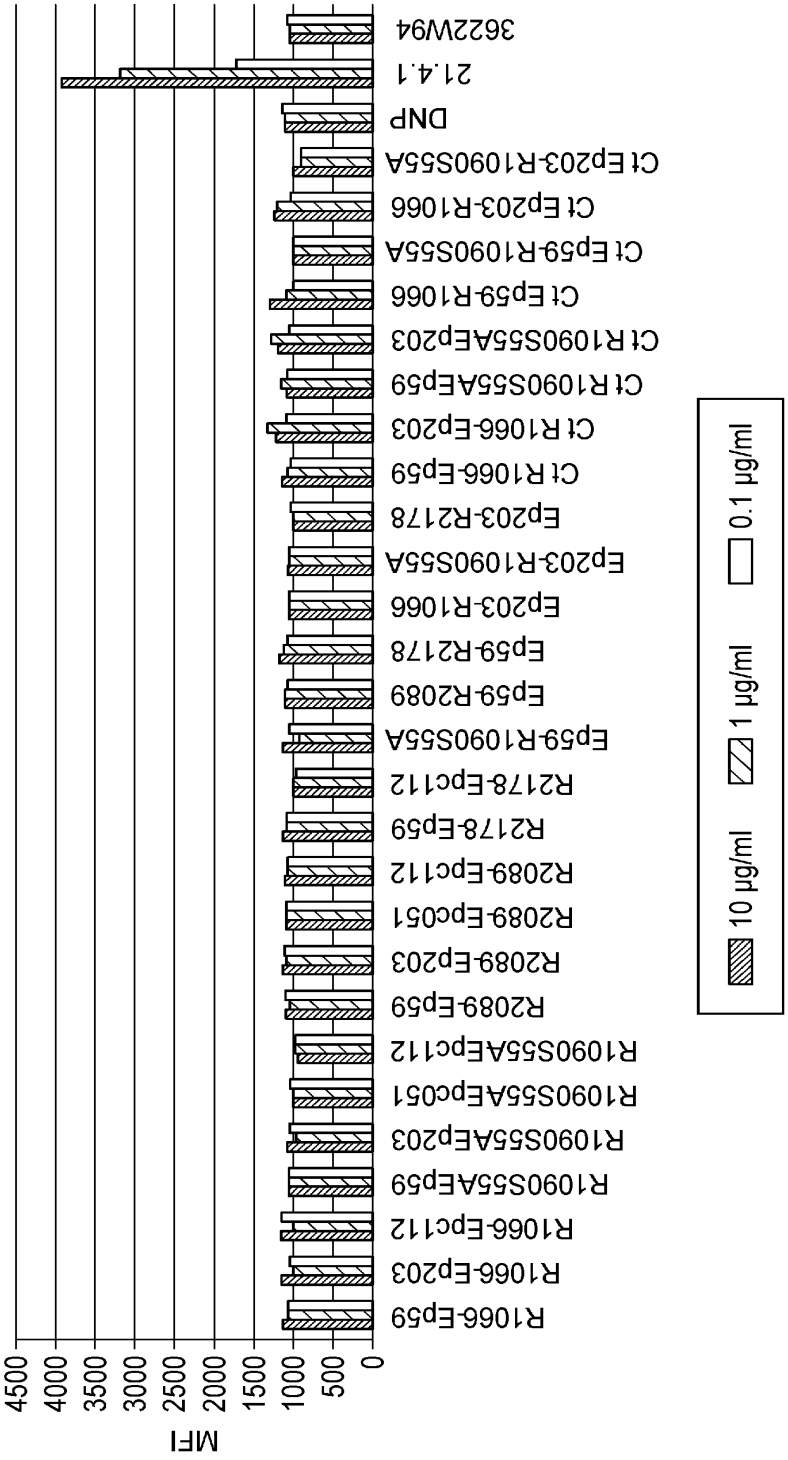
[図2]


(A)
Ramos
Count
0
0 10^2 10^3 10^4 10^5
PE-A
(B)
Colo205
Count
0
0 10^2 10^3 10^4 10^5
PE-A
(C)
HEK293
Count
0
0 10^2 10^3 10^4 10^5
PE-A
(D)
ヒトEpCAM/HEK293
Count
0
0 10^2 10^3 10^4 10^5
PE-A
DNP
21.4.1
3622W94

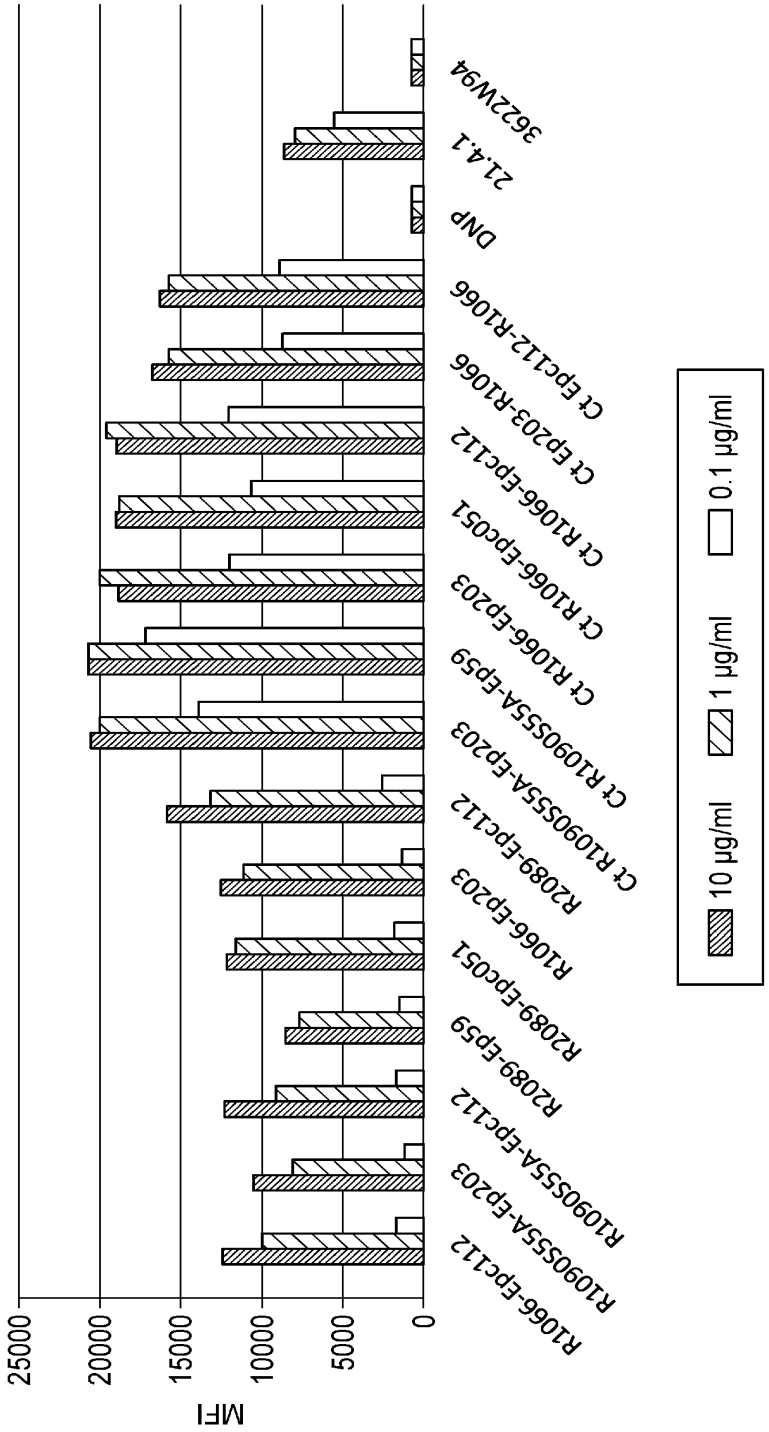
[図3]


(A)
MFI
2500
2000
1500
1000
500
0
DNP
21.4.1
R1066
R2089
R2172
(B)
MFI
4500
4000
3500
3000
2500
2000
1500
1000
500
0
DNP
21.4.1
3622W94
R1066
R1090S55A
10 µg/ml
1 µg/ml
0.1 µg/ml

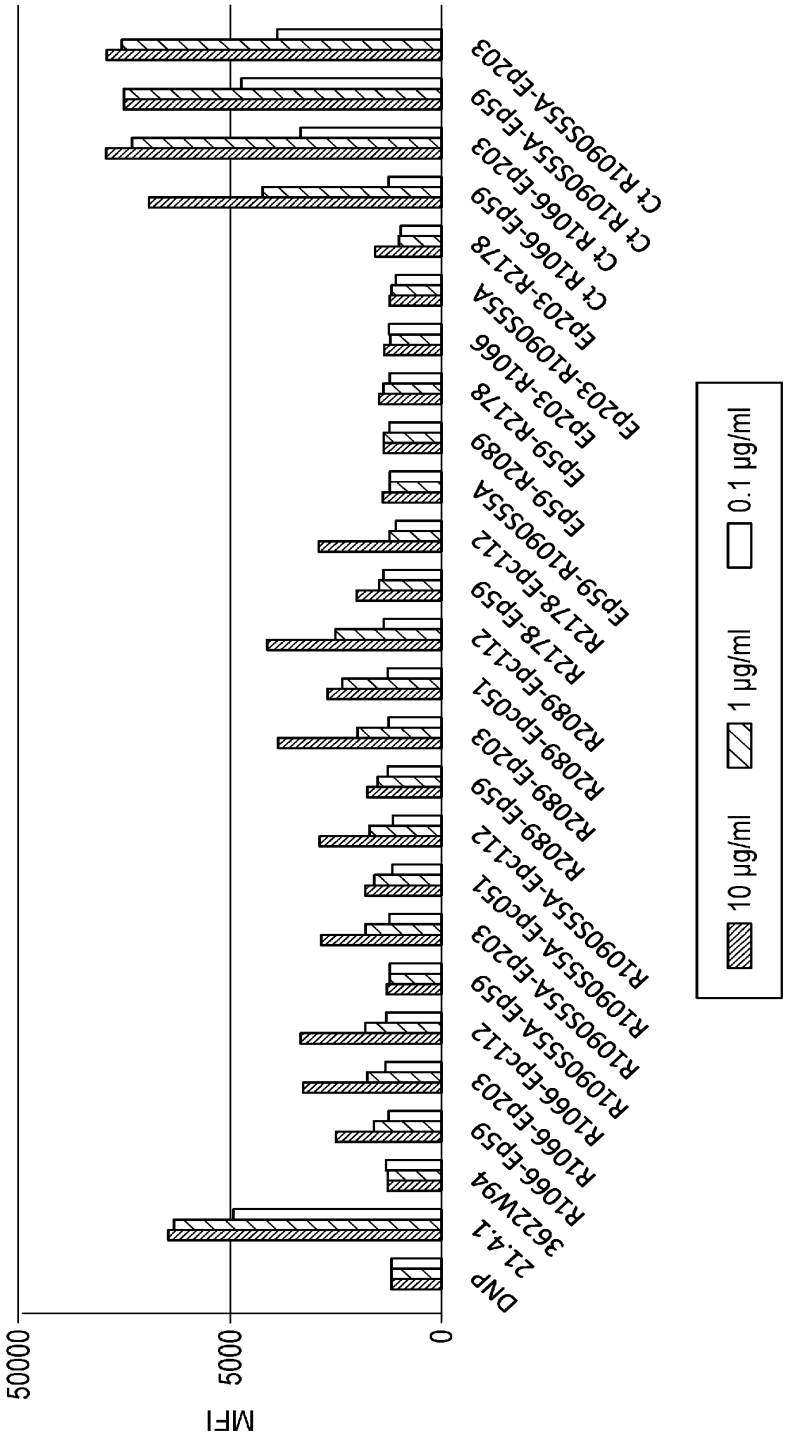
[図4]


MFI
4500
4000
3500
3000
2500
2000
1500
1000
500
0
10 μg/ml
1 μg/ml
0.1 μg/ml
R1066-Ep59
R1066-Ep203
R1066-Epc112
R1090S55AEp59
R1090S55AEp203
R1090S55AEpc051
R1090S55AEpc112
R2089-Ep59
R2089-Ep203
R2089-Epc051
R2089-Epc112
R2178-Ep59
R2178-Epc112
Ep59-R1090S55A
Ep59-R2089
Ep59-R2178
Ep203-R1066
Ep203-R1090S55A
Ep203-R2178
Ct R1066-Ep59
Ct R1066-Ep203
Ct R1090S55AEp59
Ct R1090S55AEp203
Ct Ep59-R1066
Ct Ep59-R1090S55A
Ct Ep203-R1066
Ct Ep203-R1090S55A
DNP
21.4.1
3622W94

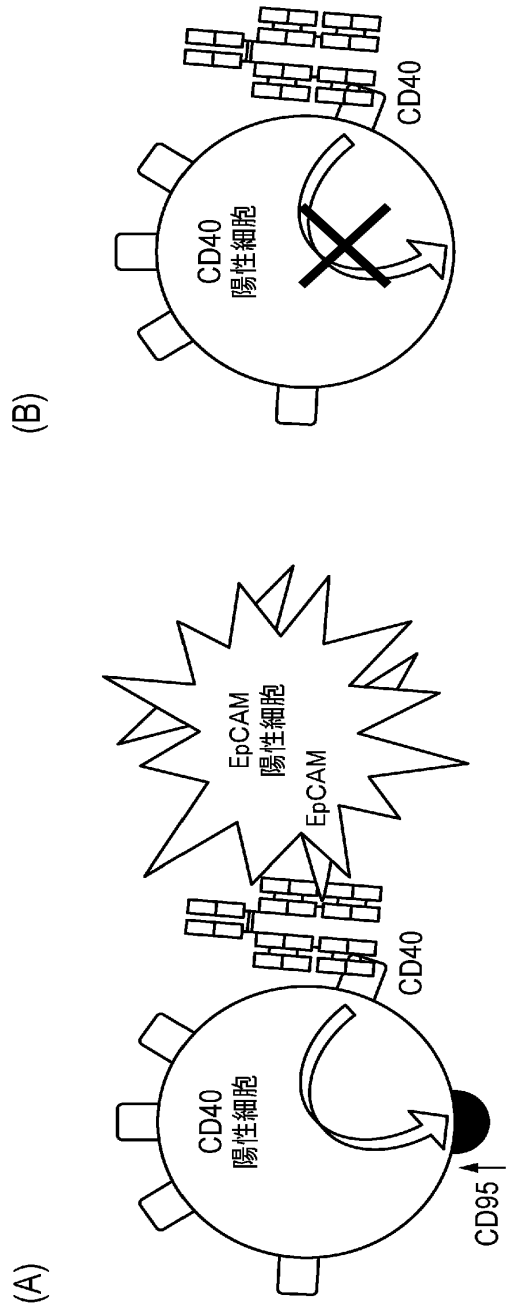
[図5]


MFI
25000
20000
15000
10000
5000
0
R1066-Epc112
R1090S55A-Ep203
R1090S55A-Epc112
R2089-Ep59
R2089-Epc051
R1066-Ep203
R2089-Epc112
Ct R1090S55A-Ep203
Ct R1090S55A-Ep59
Ct R1066-Ep203
Ct R1066-Epc051
Ct R1066-Epc112
Ct Ep203-R1066
Ct Epc112-R1066
DNP
21.4.1
3622W94
10 µg/ml
1 µg/ml
0.1 µg/ml

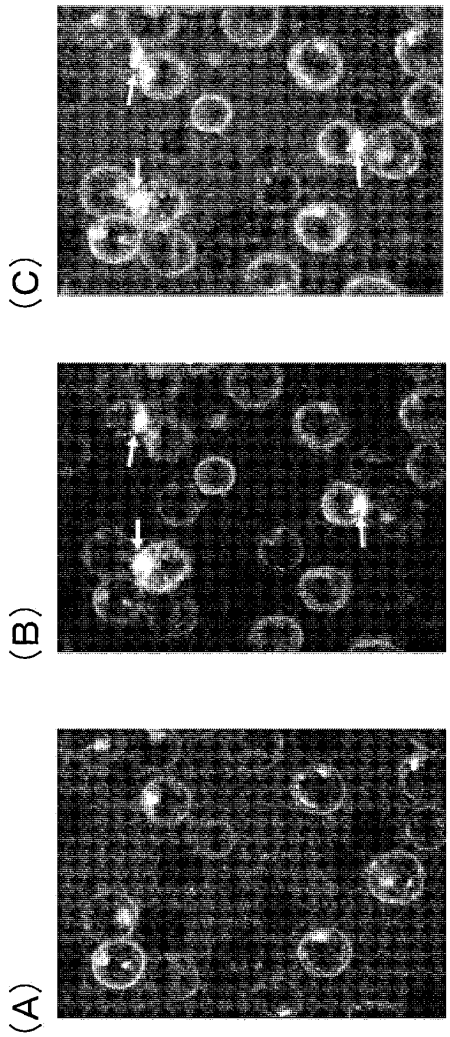
[図6]


MFI
50000
5000
0
DNP
21.4.1
3622W94
R1066-Ep59
R1066-Ep203
R1066-Epc112
R1090S55A-Ep59
R1090S55A-Ep203
R1090S55A-Epc051
R2089-Epc112
R2089-Ep59
R2089-Ep203
R2089-Epc051
R2178-Epc112
R2178-Ep59
Ep59-R1090S55A
Ep59-R2089
Ep59-R2178
Ep203-R1066
Ep203-R1090S55A
Ct R2178
Ct R1066-Ep59
Ct R1066-Ep203
Ct R1090S55A-Ep59
Ct R1090S55A-Ep203
10 μg/ml
1 μg/ml
0.1 μg/ml

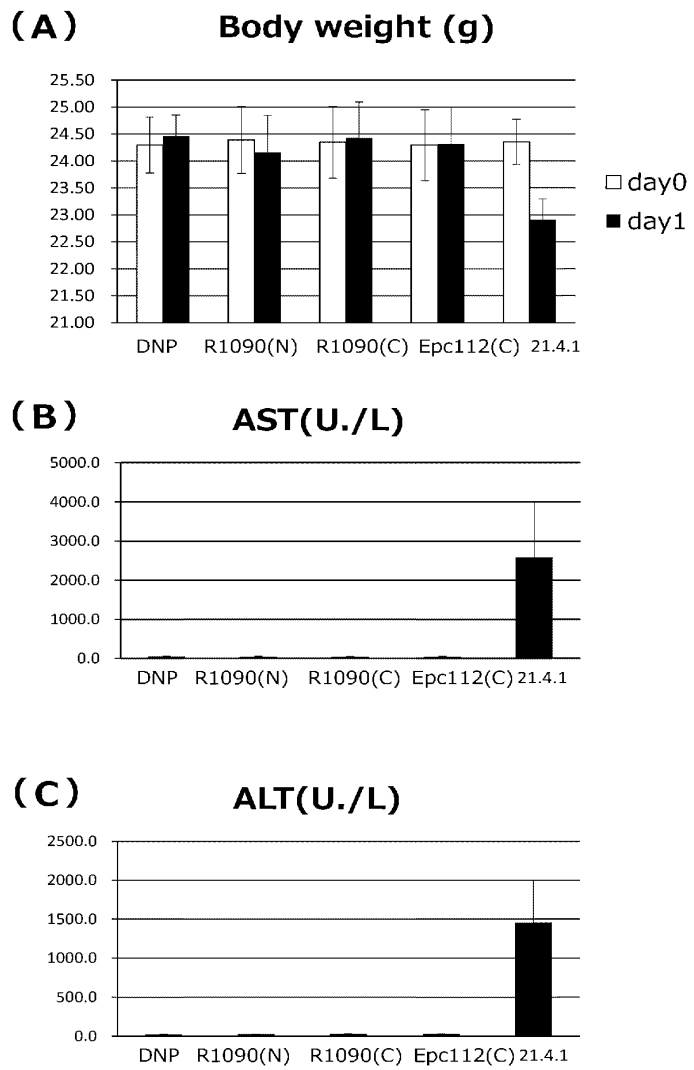
［図7］



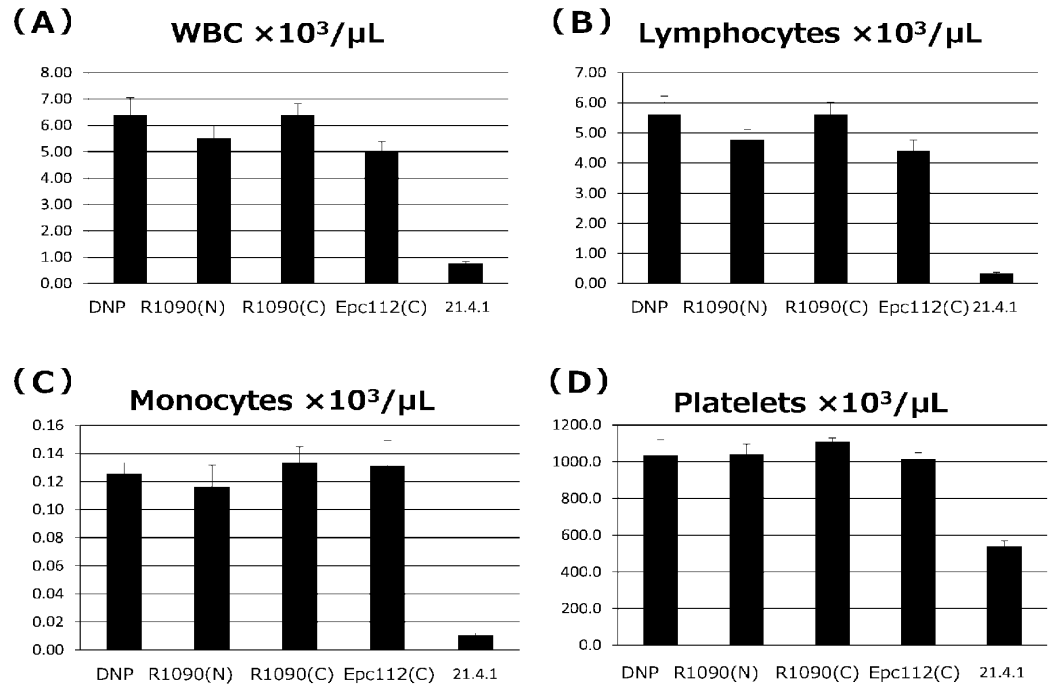
[図8]



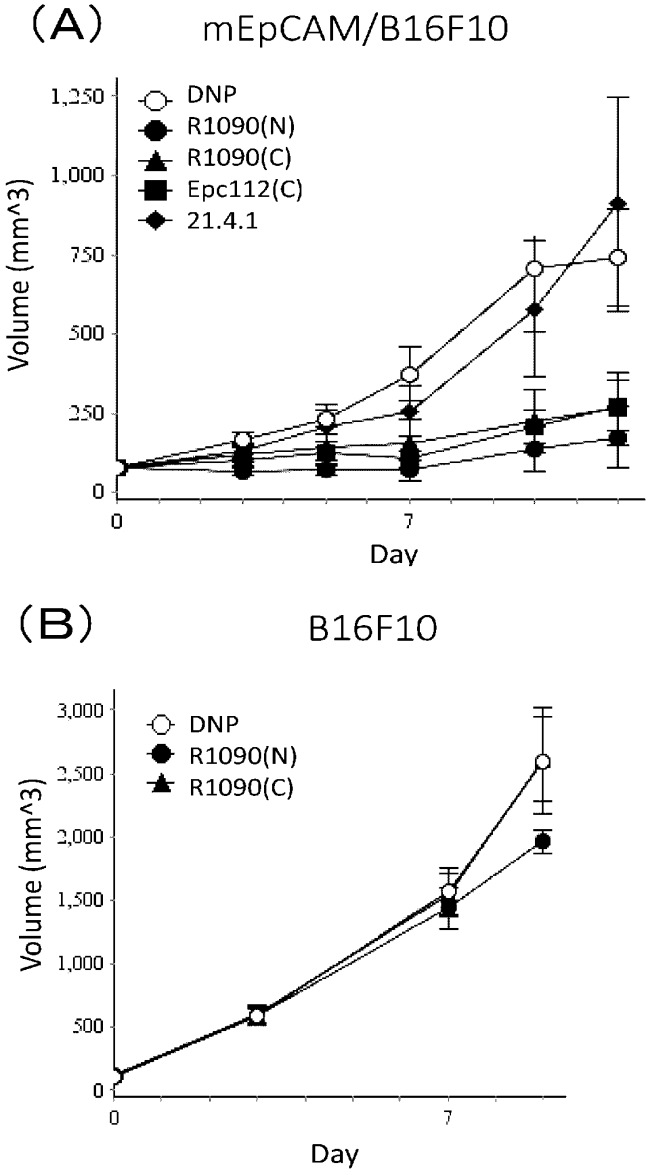
[図9]


(A)
Body weight (g)
25.50
25.00
24.50
24.00
23.50
23.00
22.50
22.00
21.50
21.00
DNP
R1090(N)
R1090(C)
Epc112(C)
21.4.1
day0
day1
(B)
AST(U./L)
5000.0
4000.0
3000.0
2000.0
1000.0
0.0
DNP
R1090(N)
R1090(C)
Epc112(C)
21.4.1
(C)
ALT(U./L)
2500.0
2000.0
1500.0
1000.0
500.0
0.0
DNP
R1090(N)
R1090(C)
Epc112(C)
21.4.1

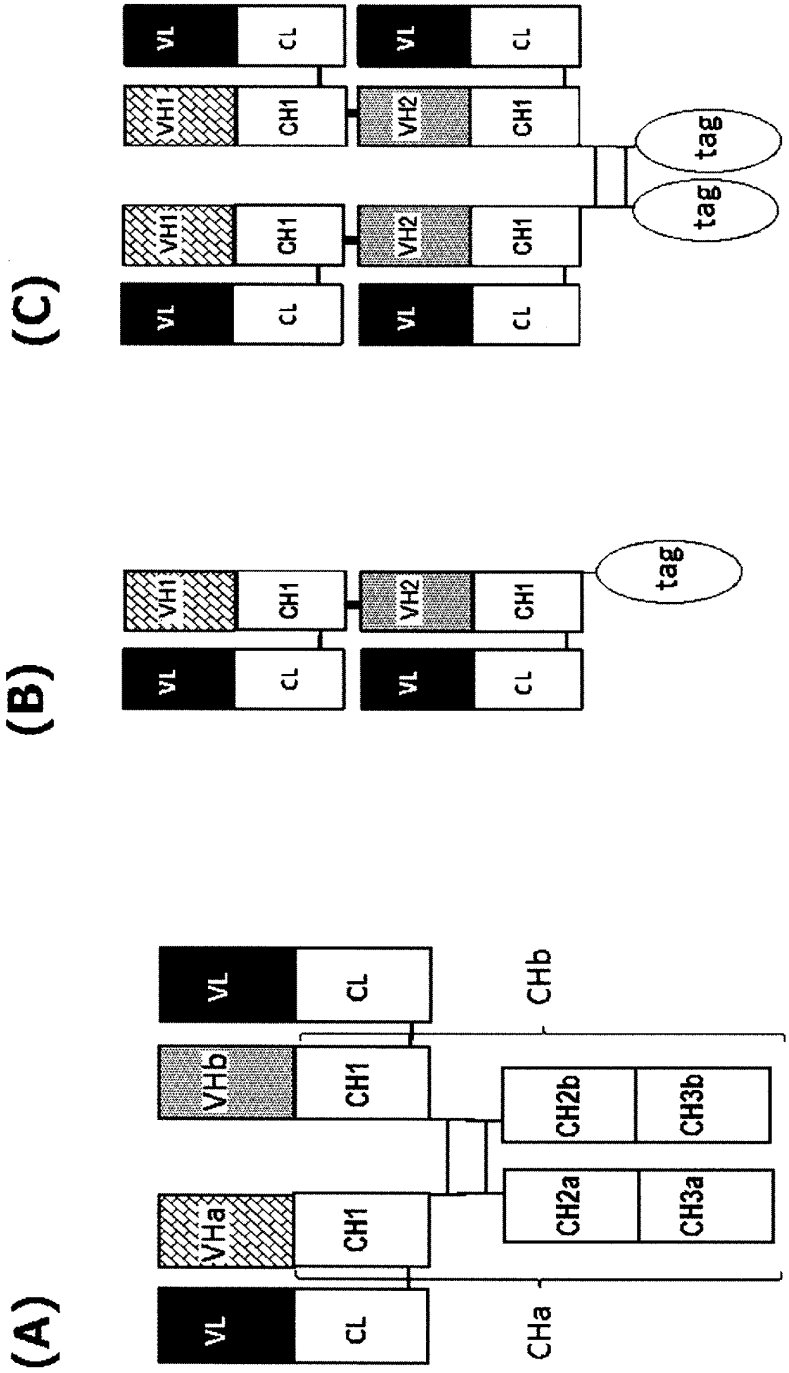
[図10]


(A)
WBC ×10³/μL
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
DNP R1090(N) R1090(C) Epc112(C) 21.4.1
(B)
Lymphocytes ×10³/μL
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
DNP R1090(N) R1090(C) Epc112(C) 21.4.1
(C)
Monocytes ×10³/μL
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
DNP R1090(N) R1090(C) Epc112(C) 21.4.1
(D)
Platelets ×10³/μL
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1000.0
800.0
600.0
400.0
200.0
0.0
DNP R1090(N) R1090(C) Epc112(C) 21.4.1

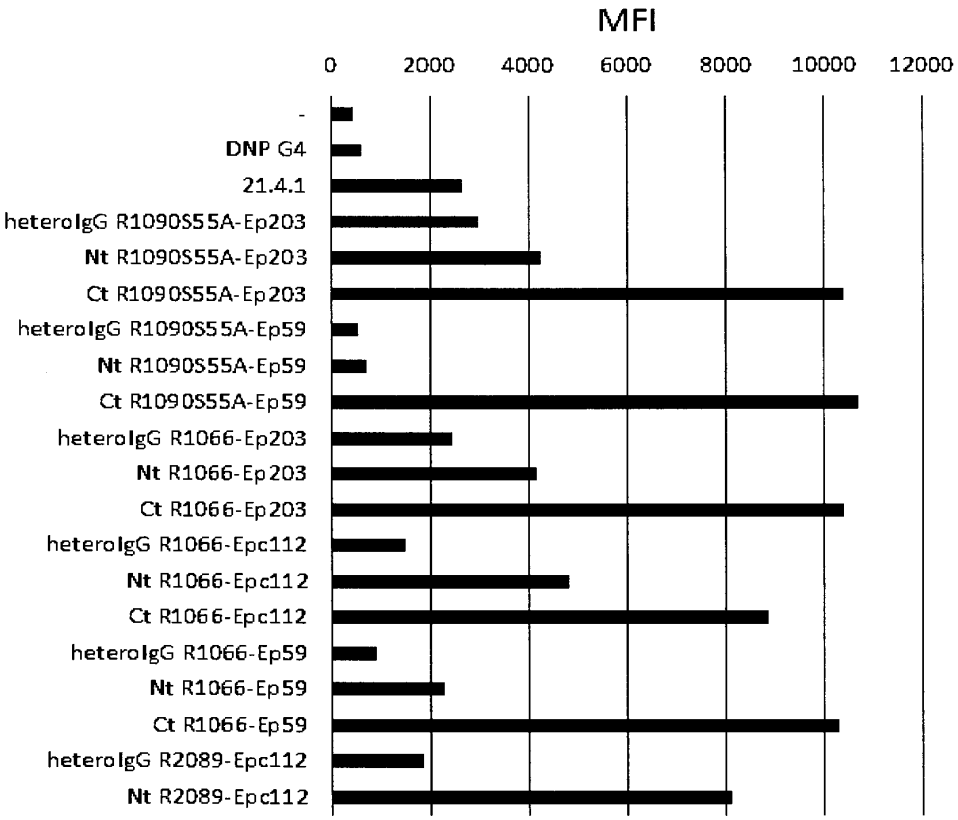
[図11]


(A)
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R1090(N)
R1090(C)
Epc112(C)
21.4.1
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1,000
750
500
250
0
0
7
Day
(B)
B16F10
DNP
R1090(N)
R1090(C)
Volume (mm^3)
3,000
2,500
2,000
1,500
1,000
500
0
0
7
Day

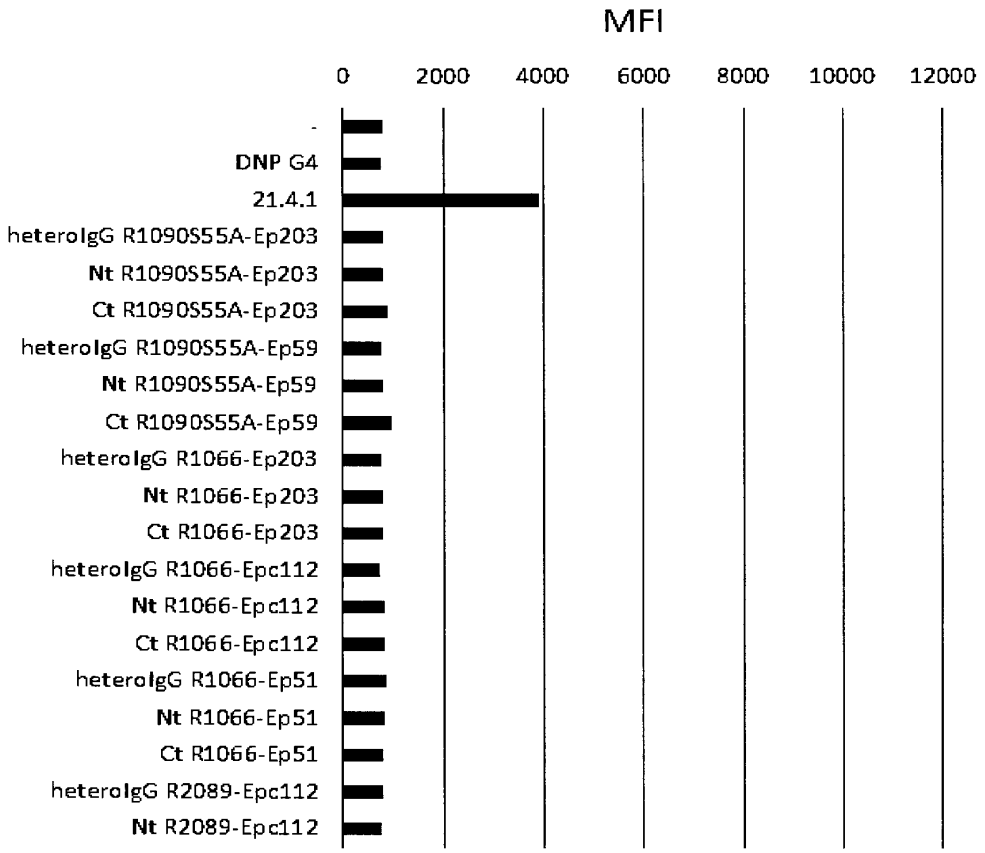
[図12]



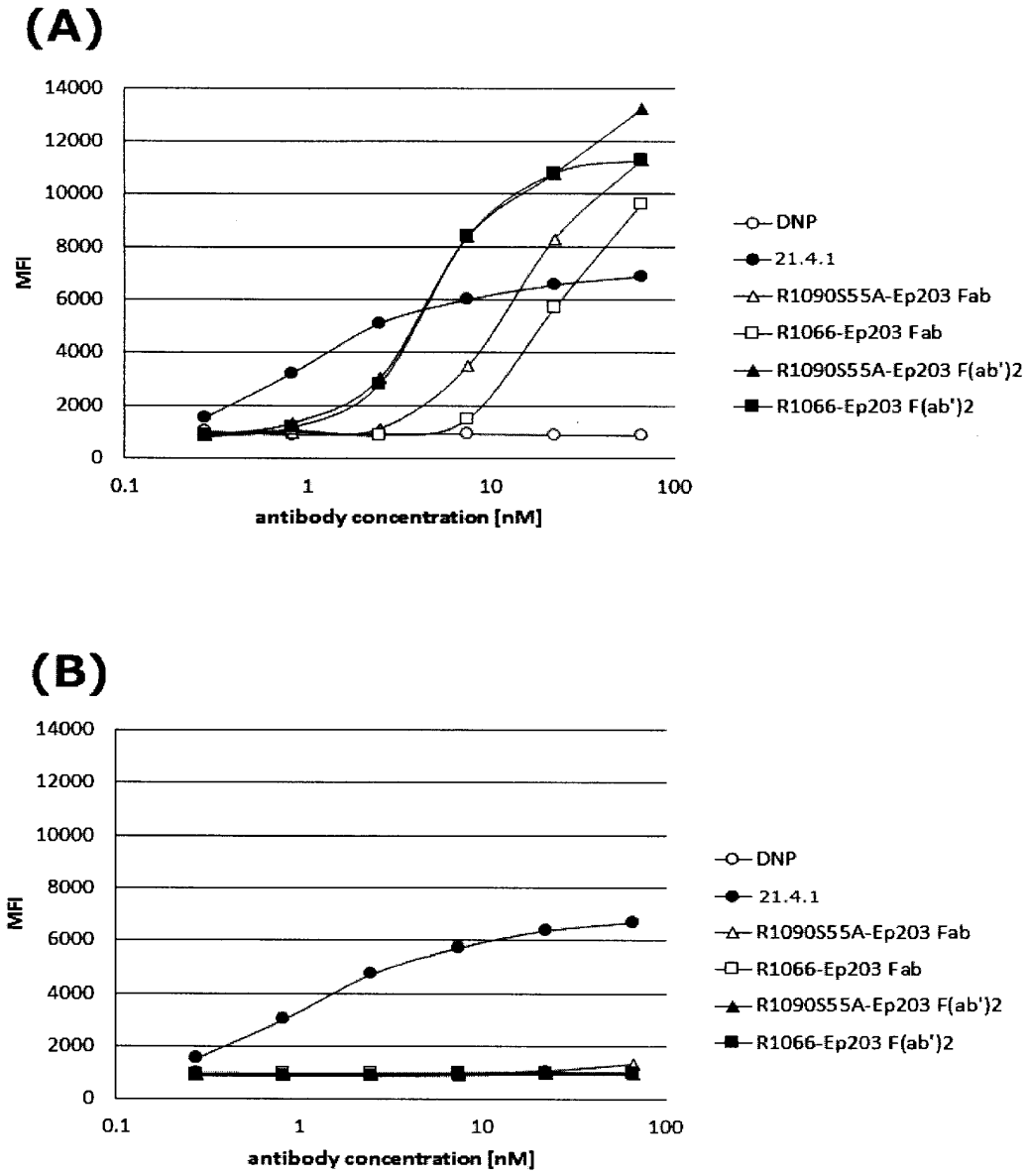
[図13]


MFI
0
2000
4000
6000
8000
10000
12000
-
DNP G4
21.4.1
heterolgG R1090S55A-Ep203
Nt R1090S55A-Ep203
Ct R1090S55A-Ep203
heterolgG R1090S55A-Ep59
Nt R1090S55A-Ep59
Ct R1090S55A-Ep59
heterolgG R1066-Ep203
Nt R1066-Ep203
Ct R1066-Ep203
heterolgG R1066-Epc112
Nt R1066-Epc112
Ct R1066-Epc112
heterolgG R1066-Ep59
Nt R1066-Ep59
Ct R1066-Ep59
heterolgG R2089-Epc112
Nt R2089-Epc112

[図14]


MFI
0
2000
4000
6000
8000
10000
12000
-
DNP G4
21.4.1
heterolgG R1090S55A-Ep203
Nt R1090S55A-Ep203
Ct R1090S55A-Ep203
heterolgG R1090S55A-Ep59
Nt R1090S55A-Ep59
Ct R1090S55A-Ep59
heterolgG R1066-Ep203
Nt R1066-Ep203
Ct R1066-Ep203
heterolgG R1066-Epc112
Nt R1066-Epc112
Ct R1066-Epc112
heterolgG R1066-Ep51
Nt R1066-Ep51
Ct R1066-Ep51
heterolgG R2089-Epc112
Nt R2089-Epc112

[図15]


(A)
14000
12000
10000
8000
6000
4000
2000
0
MFI
0.1
1
10
100
antibody concentration [nM]
DNP
21.4.1
R1090S55A-Ep203 Fab
R1066-Ep203 Fab
R1090S55A-Ep203 F(ab')2
R1066-Ep203 F(ab')2
(B)
14000
12000
10000
8000
6000
4000
2000
0
MFI
0.1
1
10
100
antibody concentration [nM]
DNP
21.4.1
R1090S55A-Ep203 Fab
R1066-Ep203 Fab
R1090S55A-Ep203 F(ab')2
R1066-Ep203 F(ab')2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2018/041250

A. CLASSIFICATION OF SUBJECT MATTER
Int.Cl. See extra sheet

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
Int.Cl. C07K16/46, A61K39/395, A61K49/16, A61P35/00, C07K16/30, C12N1/15, C12N1/19, C12N1/21, C12N5/10, C12N15/13, C12N15/61, C12P21/08, G01N33/53

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Published examined utility model applications of Japan	1922-1996
Published unexamined utility model applications of Japan	1971-2019
Registered utility model specifications of Japan	1996-2019
Published registered utility model applications of Japan	1994-2019

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
JSTPlus/JMEDPlus/JST7580 (JDreamIII), CAplus/MEDLINE/EMBASE/BIOSIS (STN), UniProt/GeneSeq

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	BRUNEKREEFT, Kim L. et al., "Targeted deli very of CD40L promotes restricted activation of antigen-presenting cells and induction of cancer cell death", Molecular Cancer, 2014, vol. 13:85, pp. 1-13	1, 3, 10-12, 15-35 1-35
Y	WO 2009/131239 A1 (KYOWA HAKKO KIRIN COMPANY, LIMITED) 29 October 2009, paragraphs [0025], [0242], examples & US 2011/0076722 A1, paragraphs [0025], [0313], examples & EP 2281845 A1	2-35

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search 30 January 2019 (30.01.2019)	Date of mailing of the international search report 12 February 2019 (12.02.2019)
Name and mailing address of the ISA/ Japan Patent Office 3-4-3, Kasumigaseki, Chiyoda-ku, Tokyo 100-8915, Japan	Authorized officer Telephone No.

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2018/041250

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2006/033386 A1 (KIRIN BREWERY CO., LTD.) 30 March 2006, examples, sequence 48 & US 2008/0063635 A1, examples, sequence 48 & EP 1810979 A1	7, 9-35
Y	US 2007/0238665 A1 (LAZAR, Gregory Alan et al.) 11 October 2007, sequence 4 & JP 2008-537941 A & WO 2006/085967 A2 & EP 1871808 A1 & KR 10-2005-0116400 A & CN 1867583 A	7, 9-35
Y	YE, Shiming et al., "Enhancement of tumor specific immunity by activation of CD40 through a biospecific molecule targeting CD40 and a tumor surface antigen", Journal for Immuno Therapy of Cancer, 07 November 2017, 5(Supple 2):87, p, 182, P370	1-35
A	WO 99/61057 A2 (TANOX INC.) 02 December 1999, abstract, claims & AU 4009899 A	1-35

INTERNATIONAL SEARCH REPORT

International application No.

CLASSIFICATION OF SUBJECT MATTER

C07K16/46(2006.01)i, A61K39/395(2006.01)i, A61K49/16(2006.01)i,
A61P35/00(2006.01)i, C07K16/30(2006.01)i, C12N1/15(2006.01)i,
C12N1/19(2006.01)i, C12N1/21(2006.01)i, C12N5/10(2006.01)i,
C12N15/13(2006.01)i, C12N15/61(2006.01)i, C12P21/08(2006.01)i,
G01N33/53(2006.01)i

国際調査報告

国際出願番号　ＰＣＴ／ＪＰ２０１８／０４１２５０

Ａ．　発明の属する分野の分類（国際特許分類（ＩＰＣ））

Int.Cl.　特別ページ参照

Ｂ．　調査を行った分野

調査を行った最小限資料（国際特許分類（ＩＰＣ））

Int.Cl.　C07K16/46, A61K39/395, A61K49/16, A61P35/00, C07K16/30, C12N1/15, C12N1/19, C12N1/21, C12N5/10, C12N15/13, C12N15/61, C12P21/08, G01N33/53

最小限資料以外の資料で調査を行った分野に含まれるもの

日本国実用新案公報	１９２２－１９９６年
日本国公開実用新案公報	１９７１－２０１９年
日本国実用新案登録公報	１９９６－２０１９年
日本国登録実用新案公報	１９９４－２０１９年

国際調査で使用した電子データベース（データベースの名称、調査に使用した用語）

JSTPlus/JMEDPlus/JST7580 (JDreamIII), CAplus/MEDLINE/EMBASE/BIOSIS (STN), UniProt/GeneSeq

Ｃ．　関連すると認められる文献

引用文献のカテゴリー＊	引用文献名　及び一部の箇所が関連するときは、その関連する箇所の表示	関連する請求項の番号
X	BRUNEKREEFT, Kim L et al., Targeted delivery of CD40L promotes restricted activation of antigen-presenting cells and induction	1,3,10-12,15-35
Y	of cancer cell death, Molecular Cancer, 2014, vol.13:85, p.1-13	1-35
Y	WO 2009/131239 A1（協和発酵キリン株式会社）2009.10.29, 段落００２５、００２４２、実施例 & US 2011/0076722 A1, [0025], [0313], examples & EP 2281845 A1	2-35

☑　Ｃ欄の続きにも文献が列挙されている。　　☐　パテントファミリーに関する別紙を参照。

＊　引用文献のカテゴリー
「Ａ」特に関連のある文献ではなく、一般的技術水準を示すもの
「Ｅ」国際出願日前の出願または特許であるが、国際出願日以後に公表されたもの
「Ｌ」優先権主張に疑義を提起する文献又は他の文献の発行日若しくは他の特別な理由を確立するために引用する文献（理由を付す）
「Ｏ」口頭による開示、使用、展示等に言及する文献
「Ｐ」国際出願日前で、かつ優先権の主張の基礎となる出願の日の後に公表された文献
「Ｔ」国際出願日又は優先日後に公表された文献であって出願と矛盾するものではなく、発明の原理又は理論の理解のために引用するもの
「Ｘ」特に関連のある文献であって、当該文献のみで発明の新規性又は進歩性がないと考えられるもの
「Ｙ」特に関連のある文献であって、当該文献と他の１以上の文献との、当業者にとって自明である組合せによって進歩性がないと考えられるもの
「＆」同一パテントファミリー文献

国際調査を完了した日	国際調査報告の発送日
３０．０１．２０１９	１２．０２．２０１９

国際調査機関の名称及びあて先	特許庁審査官（権限のある職員）	４Ｂ	９７３９
日本国特許庁（ＩＳＡ／ＪＰ） 郵便番号１００－８９１５ 東京都千代田区霞が関三丁目４番３号	田中　晴絵 電話番号　０３－３５８１－１１０１　内線　３４４８		

様式ＰＣＴ／ＩＳＡ／２１０（第２ページ）（２０１５年１月）

Ｃ（続き）．　関連すると認められる文献

引用文献のカテゴリー＊	引用文献名　及び一部の箇所が関連するときは、その関連する箇所の表示	関連する請求項の番号
Y	WO 2006/033386 A1（麒麟麦酒株式会社）2006.03.30, 実施例、配列番号４８ & US 2008/0063635 A1,examples,sequence48 & EP 1810979 A1	7,9-35
Y	US 2007/0238665 A1 (LAZAR,Gregory Alan et al.) 2007.10.11, sequence4 & JP 2008-537941 A & WO 2006/085967 A2 & EP 1871808 A1 & KR 10-2005-0116400 A & CN 1867583 A	7,9-35
Y	YE,Shiming et al., Enhancement of tumor specific immunity by activation of CD40 through a biospecific molecule targeting CD40 and a tumor surface antigen, Journal for Immuno Therapy of Cancer, 2017.11.07, 5(Supple 2):87, p,182,P370	1-35
A	WO 99/61057 A2 (TANOX INC.) 1999.12.02, 要約、特許請求の範囲 & AU 4009899 A	1-35

MS&D Exhibit 1008, Page 3751 of 4223

発明の属する分野の分類
C07K16/46(2006.01)i, A61K39/395(2006.01)i, A61K49/16(2006.01)i, A61P35/00(2006.01)i, C07K16/30(2006.01)i, C12N1/15(2006.01)i, C12N1/19(2006.01)i, C12N1/21(2006.01)i, C12N5/10(2006.01)i, C12N15/13(2006.01)i, C12N15/61(2006.01)i, C12P21/08(2006.01)i, G01N33/53(2006.01)i

MS&D Exhibit 1008, Page 3752 of 4223

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
22 February 2024 (22.02.2024)

WIPO | PCT

(10) International Publication Number
WO 2024/040118 A2

(51) International Patent Classification:
C07K 16/28 (2006.01) *A61K 39/395* (2006.01)

(21) International Application Number:
PCT/US2023/072317

(22) International Filing Date:
16 August 2023 (16.08.2023)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
63/398,502 16 August 2022 (16.08.2022) US
63/472,938 14 June 2023 (14.06.2023) US

(71) Applicant: **SURROZEN OPERATING, INC.** [US/US]; 171 Oyster Point Blvd., Suite 400, South San Francisco, California 94080 (US).

(72) Inventor: **LI, Yang**; 1142 Cuesta Drive, Mountain View, California 94040 (US).

(74) Agent: **LAHERTY, Carol D.** et al.; COOLEY LLP, 1299 Pennsylvania Ave. NW, Suite 700, Washington, District of Columbia 20004-2400 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CV, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IQ, IR, IS, IT, JM, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, MG, MK, MN, MU, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, CV, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SC, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, ME, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

(54) Title: CELL TARGETING MULTICOMPONENT POLYPEPTIDE

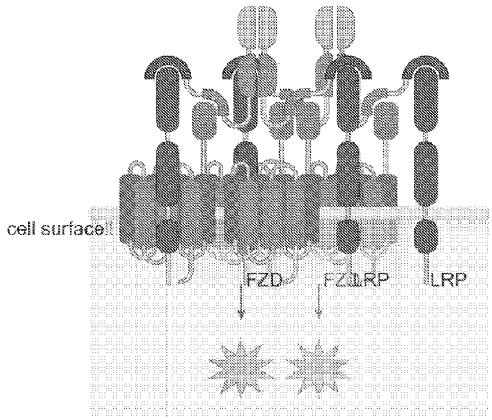


Fig. 1A

(57) Abstract: The present invention provides a cell targeting multicomponent polypeptides complexes, to modulate signaling pathways having tissue specificity, by binding to homo- or hetero-oligomeric receptor complexes.

[Continued on next page]

WO 2024/040118 A2

Declarations under Rule 4.17:

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

— *with sequence listing part of description (Rule 5.2(a))*

CELL TARGETING MULTICOMPONENT POLYPEPTIDE

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Patent Application Serial No. 63/398,502, filed August 16, 2022, and U.S. Provisional Patent Application Serial No. 63/472,938, filed June 14, 2023, which are incorporated by reference in their entireties.

SEQUENCE LISTINGS

[0002] The Sequence Listing XML associated with this application is provided in XML file format and is hereby incorporated by reference into the specification. The name of the XML file containing the Sequence Listing XML is SRZN_026_01WO_ST26.xml. The XML file is 39,279 bytes, and created on August 8, 2023, and is being submitted electronically via USPTO Patent Center.

FIELD OF THE INVENTION

[0003] The present disclosure provides cell targeting multicomponent polypeptides that modulate signaling pathways having cell and tissue specificity by binding to hetero-oligomeric receptor complexes.

BACKGROUND OF THE INVENTION

[0004] Receptor-mediated cellular signaling is fundamental to cell-cell communication, cellular growth and differentiation, maintenance of tissue function and homeostasis, and regulation of injury repair. When a cell-specific response is necessary, nature achieves that selectivity through various mechanisms such as cell-specific receptor, ligand gradient, short-range ligand, direct cell-cell contacts. However, most ligand-receptor systems have pleiotropic effects due to broad expressions of receptors on multiple cell types. While this may be important when a coordinated response in numerous cell types or tissues is needed, modulating cell-specific signaling has been a major challenge for research and drug development to avoid systemic toxicity or off-target tissue effects. In addition, it is often difficult to identify differences between normal and diseased cells for a particular signaling pathway of interest, making it challenging, if not impossible, to selectively modulate that signaling pathway either in diseased or normal cells. Therefore, effective cell targeting is needed for both research and therapeutic development.

[0005] Cell-targeted antagonists are more accessible to achieve than cell-targeted agonists, as antagonists have less stringent requirements on affinity, epitope, and geometry of the molecules than agonists (Dickopf et al., 2020). Previous efforts to engineer cell selective growth factors and cytokines employed variations of the "chimeric activators" concept (Cironi et al., 2008), which invariably involved attaching a cell targeting arm ("targeting element") to either the wild-type ligand or a mutated ligand with reduced affinity toward signaling receptor ("activity element"). Such an approach has been applied to several signaling pathways, such as the erythropoietin (Taylor et al., 2010, Burrill et al., 2016), interferon (Cironi et al., 2008, Garcin et al., 2014), and interleukin-2 (Ghasemi et al., 2016, Lazear et al., 2017) pathways.

[0006] While little selectivity could be achieved by attaching a targeting arm to a wild-type ligand, selectivity of up to 1000-fold has been reported when a mutated ligand was used (Garcin et al., 2014). The "chimeric activators" approach is based on the cooperativity concept. In this approach, mutations that weaken the affinity of a natural ligand to its receptor are selected as the "activity element". Due to the weakened affinity, the mutant "activity element" alone should display a significantly right-shifted dose-response curve (or lower potency) in an activity assay on both target and non-target cells that express the signaling receptors.

[0007] When a "targeting element" is tethered to the mutant "activity element," the "targeting element" helps to increase the local concentration of the mutant "activity element" on the desired target cell surface, driving engagement of signaling receptor and subsequent intracellular signaling activation, effectively left shift the activity dose-response curve (increase potency) on the target cell and create the selectivity between target vs. non-target cells.

[0008] While conceptually elegant, identifying the appropriate mutations that achieve the just-right reduction of affinity that could be rescued by the "targeting element" is not trivial. In some cases, while the potency could be rescued, the maximal signaling strength remains compromised, resulting in a partial agonist. In addition, the selectivity is not exquisite; a modest 10-fold separation between targeting vs. non-targeting cells is reported in most literature examples.

[0009] WNT ligands and their signals play key roles in the control of development, homeostasis and regeneration of many essential organs and tissues, including bone, liver, skin, stomach, intestine, kidney, central nervous system, mammary gland, taste bud, ovary, cochlea and many other tissues (reviewed, e.g., by Clevers, Loh, and Nusse (2014) *Science*; 346:54).

Modulation of WNT signaling pathways has potential for treatment of degenerative diseases and tissue injuries.

[0010] The WNT pathway is highly conserved across species and crucial for embryonic development, and adult tissue homeostasis and regeneration (Nusse and Clevers, 2017). WNT-induced signaling through β-catenin stabilization has been widely studied and is achieved by ligand binding to frizzled (FZD) and low-density lipoprotein receptor-related protein (LRP) family of receptors. There are nineteen mammalian WNTs, 10 FZDs (FZD_{1-10}), and 2 LRPs (LRP5 and LRP6).

[0011] WNTs are highly hydrophobic due to lipidation that is required for function and are promiscuous, capable of binding and activating multiple FZD and LRP pairs (Janda et al., 2012, Kadowaki et al., 1996, Dijksterhuis et al., 2015). Elucidating the functions of individual FZDs in tissues has been hampered by difficulties in producing the ligands and lack of receptor and tissue selectivity. Recent breakthroughs in the development of WNT-mimetic molecules have largely resolved the production and receptor specificity challenges (Janda et al., 2017, Chen et al., 2020, Tao et al., 2019, Miao et al., 2020).

[0012] While tissue selectivity could be partly achieved by tissue injury as damaged tissues seem more sensitive to WNTs (Xie et al., 2022), it would still represent a significant technical advancement to be able to target WNTs to specific cells and tissues.

[0013] Antibodies are a well-established and rapidly growing drug class with at least 45 antibody-based products currently marketed for imaging or therapy in the United States and/or Europe with ~$100 billion in total worldwide sales. This major clinical and commercial success with antibody therapeutics has fueled much interest in developing the next generation antibody drugs including bispecific antibodies. As their name implies, bispecific antibodies or multispecific antibodies (collectively "MsAbs") bind to at least two different antigens, or at least two different epitopes on the same antigen, as first demonstrated more than 50 years ago. Engineering monospecific antibodies for multispecificity opens up many new potential therapeutic applications as evidenced by >30 BsAb in clinical development.

[0014] Bispecific or multispecific antibodies are a class of engineered antibody and antibody-like proteins that, in contrast to 'regular' monospecific antibodies, combine two or more different specific antigen binding elements in a single construct. Since bispecific antibodies do not typically occur in nature, they are constructed either chemically or biologically, using techniques such as cell fusion or recombinant DNA technologies. The

ability to bind two or more different epitopes with a single molecule offers a number of potential advantages. One approach is to use the specificity of one arm as a targeting site for individual molecules, cellular markers or organisms, such as bacteria and viruses. While the other arm functions as an effector site for the recruitment of effector cells or delivery of molecular payloads to the target, such as drugs, cytokines or toxins. Alternatively, bispecifics can be used to dual target, allowing detection or binding of a target cell type with much higher specificity than monospecific antibodies.

[0015] The modular architecture of immunoglobulins has been exploited to create a growing number (>60) of alternative MsAb formats (see, e.g., Spiess et al (2015) *Mol. Immunol.* 67:95-106). MsAb are classified here into five distinct structural groups: (i) bispecific IgG (BsIgG) (ii) IgG appended with an additional antigen-binding moiety (iii) MsAb fragments (iv) Multispecific fusion proteins and (v) MsAb conjugates. Each of these different MsAb formats brings different properties in binding valency for each antigen, geometry of antigen-binding sites, pharmacokinetic half-life, and in some cases effector functions.

[0016] For antagonistic MsAbs antibodies, which represent the vast majority of the MsAb molecules in development, the geometry of the antigen binding modules is less critical. However, for agonistic MsAbs, these molecules need to faithfully mimic the activity of the natural ligand, the binding geometry could be crucial (see, e.g., Shi, et al. (2018) *J. Biol. Chem.* 293:5909-5919). Such is true of WNT multicomponent polypeptide molecules, which are required to bind and activate two spatially separated WNT receptors, FZD and LRP.

[0017] WNT multicomponent polypeptide molecules which can bind to the hetero-oligomeric WNT/LRP receptor complex have been described previously (see, e.g., WO2019/126398 and WO2020/010308) as have WNT enhancers using RSPO (see, e.g., WO2018/140821, WO2018/132572, and WO2020/014271).

[0018] Previous attempts to create multicomponent receptor molecules have been successful for antagonist molecules because lower affinities and tissue selectivity were not as critical. Creating agonist multicomponent receptor molecules has been challenging because of this reduced affinity and lack of selectivity. The present invention addresses this need by the use of a binding composition specific for a bridging molecule, which resides on the cell surface, tethered to binding domains specific for signaling receptor components. This use of a bridging molecule binding domain has provided acceptable affinity for agonist signaling, as well as the ability to confer specificity by target tissue and cell specific cell surface antigens.

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BRIEF DESCRIPTION OF THE DRAWINGS

[0019] Fig. 1A shows an optimized design for a WNT mimetic that is a tetravalent bispecific antibody-based molecule.

[0020] Fig. 1B shows a cell targeted WNT mimetic based on the "chimeric activator" concept where an "targeting element" is tethered to the WNT mimetic.

[0021] Fig. 1C illustrates two separate polypeptides: one FZD binder with additional binding domain that binds "bridging molecule" and one LRP binder that binds "bridging molecule", where the two molecules bind different epitopes of the "bridging molecule"

[0022] Fig. 1D illustrates a WNT mimetic with two FZD binding domains tethered to a bridging molecule by two binding domains specific for two epitopes on the bridging molecule and two LRP binding domains tethered to the bridging molecule by two binding domains specific for two epitopes on the bridging molecule, wherein the two bridging binding epitopes are linked together and each separate molecule could be tethered to "bridging element" by binding to a different epitope on the bridging receptor, creating the final receptor complex.

[0023] Fig. 1E shows a WNT mimetic with two FZD binding domain tethered to a bridging molecule by a binding domain specific for a first epitope on the bridging molecule and two LRP binding domains tethered to the bridging molecule by a binding domain specific for a second epitope on the bridging molecule, wherein the two bridging binding epitopes are identical and each separate molecule could be tethered to "bridging element" binding to a epitope on the multimeric bridging receptor, creating the final receptor complex.

[0024] Fig. 1F shows a FZD binding domain linked to one LRP binding domain which is tethered to the bridging molecule by a binding domain specific for a first epitope on the bridging molecule and an FZD binding domain linked to one LRP binding domain which is tethered to the bridging molecule by a binding domain specific for a second epitope on the bridging molecule, and each separate molecule could be tethered to a "bridging element" binding to a different epitope on the bridging receptor, creating the final receptor complex.

[0025] Fig. 1G shows a FZD binding domain linked to one LRP binding domain which is tethered to the bridging molecule by two binding domains specific for two epitopes on the bridging molecule wherein the two bridging binding epitopes are linked together, and each separate molecule could be tethered to a "bridging element" binding to a different epitope on the bridging receptor, creating the final receptor complex.

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[0026] Fig. 1H shows FZD binding domain linked to one LRP binding domain which is tethered to the bridging molecule by a binding domain specific for a epitope on the bridging molecule, wherein each separate molecule tethered to a "bridging element" binding to a multimeric bridging receptor, creating the final receptor complex.

[0027] Figs. 2A-2C show selected βKlotho and endocrine fibroblast growth factor 21 (FGF21) ligand system as the bridging receptor system, with the FZD and LRP binding domains. The graphic representations of the binders and the various combinations F12578 (binds FZD1,2,5,7,8) and L (binds LRP5/6). Fig. 2A shows elements that are used in the split molecules. LRP6 binder element is in scFv format; FZD binder is in the IgG1 format; both αGFP IgG1 and αGFP scFv are used for assembly of the negative control molecules. Two types of βKlotho binders are used: Binder No.1, 39F7 IgG1, a βKlotho monoclonal antibody; Binder No. 2, FGF21FL, and different deletion variants. Fig. 2B illustrates the diagram of the assembled molecules. Fig. 2C shows the diagram of the assembled negative control molecules.

[0028] Figs. 2D-2G show binding of various F-FGF21 fusion proteins to FZD7 and βKlotho. Bindings of FZD7 and β-Klotho to F-FGF21FL (Fig. 2D), F-FGF21ΔN (Fig. 2E), F-FGF21ΔC (Fig. 2F), or F-FGF21ΔNΔC (Fig. 2G) were determined by Octet.

[0029] Figs. 2H-2J illustrate binding of LRP6 and βKlotho to L-39F7 (Fig. 2H), αGFP-39F7 (Fig. 2I), or L-αGFP (Fig. 2J) were determined by Octet. Mean KD values were calculated from all 7 binding curves with global fits (red dotted lines) using 1:1 Langmuir binding model.

[0030] Figs. 2K-2L show step bindings, Fig. 2K is step binding of FZD7 and βKlotho to various F-FGF21 fusion proteins. Sequential binding of F-FGF21FL (blue sensorgram), F-FGF21ΔN (red sensorgram), F-FGF21ΔC (light green sensorgram), or F-FGF21ΔNΔC (green sensorgram), followed by FZD7 CRD, then followed by addition of βKlotho on Octet shows simultaneous engagement of both FZD7 and βKlotho to the indicated F-FGF21 proteins. Sensorgrams for FZD7 and βKlotho area (dashed box on left) are enlarged at the right. Fig. 2L shows step binding of LRP6 and βKlotho to L-39F7 and its control proteins. Sequential binding L-39F7 (blue sensorgram), L-αGFP (light blue sensorgram), αGFP-39F7 (red sensorgram), or 39F7 IgG (green sensorgram), followed by LRP6E3E4, then followed by addition of βKlotho on Octet shows simultaneous engagement of both LRP6 and βKlotho to the indicated L-39F7 and its control proteins. Sensorgrams for LRP6 and βKlotho area (dashed box on left) are enlarged on the right.

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[0031] Figs. 3A-3F illustrate dose dependent STF assay of SWIFT molecules in HEK293 and Huh7 cells the combination of F12578-FGF21FL or FGF12578-FGF21ΔN with L-39F7 resulted in WNT/β-catenin signaling in a liver cell line, Huh7 cells, which express the bridging receptor βKlotho, but not in 293 cells where βKlotho is not expressed. This signaling depends on the presence of both FZD and LRP binding arms and the ability to bind the bridging receptor, as the removal of LRP binding arm L from L-39F7 or inactivation of βKlotho binding (use of FGF21ΔNΔC) resulted in no activity in either cell.

[0032] Fig. 3G illustrates expression of bridging receptor βKlotho (KLB) in HEK293 and Huh7 cells.

[0033] Figs. 4A and 4B show the FZD binder (F12578), LRP binder (L), and the two non-competing bridging receptor (βKlotho) binders (FGF21FL and 39F7) were combined in the molecule, F12578-FGF21FL-39F7-L (Fig. 4A), and their activity WNT/β-catenin signaling in Huh7 cells that expresses the bridging receptor βKlotho but not in 293 cells where βKlotho is not expressed (Fig. 4B).

[0034] Figs. 5A-5D illustrate activity of targeted molecules in primary human cells. Fig. 5A is presentative images of primary human hepatocytes cultures in 2D or human small intestinal organoids. Scale bars 200 μm. Fig. 5B shows expression of bridging receptor βKlotho (KLB) in hepatocytes or small intestinal cells. Fig. 5C illustrates WNT target gene AXIN2 expression normalized to control treatment after 24-hour treatment with 10 nM of indicated molecules in human hepatocytes. Fig. 5D shows WNT target gene AXIN2 expression normalized to control treatment after 24-hour treatment with 10 nM of indicated molecules in human small intestinal organoids.

SUMMARY OF THE INVENTION

[0035] In various embodiments, the present invention provides a cell targeting multicomponent polypeptide and related uses thereof.

[0036] The present invention provides a cell targeting multicomponent polypeptide molecule comprising at least one first antigen binding domain that binds to a first signaling receptor component, at least one-second antigen binding domain that binds to a second signaling receptor component receptor, and at least one different antigen binding domain that binds to a bridging molecule, and the first receptor and second receptor can be different or identical.

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[0037] In some embodiments, the binding domain that binds to the bridging molecule comprises at least two binding domains that bind to different epitopes on the bridging molecule or bind to the same epitope on the bridging molecule.

[0038] In another embodiment, the first antigen binding domain that binds to a first signaling receptor component is tethered to at least one binding domain that binds to the bridging receptor, and the second antigen binding domain that binds to a second signaling receptor component is tethered to at least one binding domain that binds to the bridging molecule.

[0039] In a further embodiment, the antigen binding domain is joined directly or by a linker to the binding domain that binds to the bridging molecule.

[0040] In another embodiment, the bridging molecule is monomeric or multimeric.

[0041] In a further embodiment, the binding domains are each independently selected from the group consisting of: an scFv, a VHH/sdAb, a Fab, and a Fab´2.

[0042] In some embodiment, the binding domains are joined through a linker by a peptide linker comprising from 1-100 amino acids.

[0043] In yet another embodiment, the binding domains are attached to the N-terminus of an antibody Fc domain. In another embodiment, a nucleic acid encodes the multicomponent polypeptide.

[0044] In a further embodiment, the nucleic acid is an expression vector comprises a promoter sequence operatively linked to the nucleic acid sequence; optionally wherein the vector is a virus comprising a promoter operatively linked to the nucleic acid.

[0045] In another embodiment, a host cell comprises the vector.

[0046] In related embodiment provides a process for producing the multicomponent polypeptide comprising culturing the host cell under conditions wherein the multicomponent polypeptide is expressed by the expression vector.

[0047] The present invention further provides a multicomponent WNT molecule comprises at least one FZD binding domain, at least one LRP binding domain, and at least one bridging molecule; the multicomponent WNT multicomponent polypeptide molecule modulates WNT signaling.

[0048] In yet another embodiment,the multicomponent WNT molecule comprises: one or two FZD binding domains tethered to the bridging molecule by a binding domain specific for a first epitope on the bridging molecule; one or two LRP binding domains tethered to the bridging molecule by a binding domain specific for a second epitope on the bridging molecule, and the molecule can activate WNT signaling.

[0049] In some embodiments, the multicomponent WNT molecule comprises the binding domain specific for the first epitope of the bridging molecule and the FZD binding domain(s) are joined directly or joined by a linker; and the binding domain specific for the second epitope of the bridging molecule and the LRP binding domain(s) are joined directly or joined by a linker; the binding domain specific for the first epitope and the binding domain specific for the second epitope of the bridging molecule are identical or different.

[0050] In another embodiment, the linker is a peptide or non-peptide linker.

[0051] In yet another embodiment, the FZD binding domain(s) and the LRP binding domain(s) are each independently selected from the group consisting of: a scFv, a VHH/sdAb, a Fab, and a Fab´2.

[0052] In a further embodiment, the FZD binding domain(s) and the LRP binding domains(s) are joined by a peptide linker comprising from 1-100 amino acids.

[0053] In yet another embodiment, the FZD binding domain(s) and the LRP binding domain(s) are attached to the N-terminus of an antibody Fc domain.

[0054] In some embodiment, a nucleic acid encoding the multicomponent WNT molecule.

[0055] In another embodiment, the vector is an expression vector comprises a promoter sequence operatively linked to the nucleic acid sequence; optionally wherein the vector is a virus comprising a promoter operatively linked to the nucleic acid.

[0056] In a further embodiment, a host cell comprises the vector.

[0057] In some embodiments, a process for producing the multicomponent WNT molecule culturing the host cell under conditions wherein the multicomponent polypeptide is expressed by the expression vector.

[0058] In some embodiment, a pharmaceutical composition to modulate the WNT/β-catenin signaling pathway comprises an effective amount of the multicomponent WNT molecule, and a pharmaceutically acceptable diluent, adjuvant, or carrier.

[0059] In yet another embodiment, provides a method of treating or preventing a disease or disorder in a subject in need thereof, the method comprises by providing to the subject an effective amount of the multicomponent WNT molecule.

[0060] The present invention also provides a multicomponent WNT molecule comprises at least one FZD binding domain linked to one LRP binding domain which is tethered to the bridging molecule by a binding domain specific for a first epitope on the bridging molecule; at least one FZD binding domain linked to one LRP binding domain which is tethered to the bridging molecule by a binding domain specific for a second epitope on the bridging molecule; and the tethering of the FZD and LRP binding domains to the bridging receptor forms a WNT mimetic capable of activating WNT signaling.

[0061] In a further embodiment, the binding domain specific for a first epitope of the bridging molecule and the FZD/LRP binding domains are joined directly or by a linker; and the binding domain specific for a second epitope of the bridging molecule and the FZD/LRP binding domains are joined directly, or by a linker the binding domain of the first epitope and second epitope of the bridging molecule are identical or different; and the binding domain of the first epitope and the second epitope of the bridging molecule could be linked together to FZD/LRP.

[0062] In some embodiments, the bridging molecule is monomeric or multimeric.

[0063] In another embodiment, at least one of the FZD or LRP binding domains is selected from the group consisting of: a scFv, a VHH/sdAb, a Fab, and a Fab´2.

[0064] In yet another embodiment, the binding domains are joined through a linker by a peptide link comprising from 1-100 amino acids.

[0065] In related embodiment, the binding domains are attached to the N-terminus of an antibody Fc domain.

[0066] In some embodiment, a nucleic acid encoding the multicomponent WNT molecule.

[0067] In another embodiment, the vector is an expression vector comprises a promoter sequence operatively linked to the nucleic acid sequence; optionally wherein the vector is a virus comprising a promoter operatively linked to the nucleic acid.

[0068] In a further embodiment, a host cell comprises the vector.

[0069] In some embodiments, a process for producing the multicomponent WNT molecule culturing the host cell under conditions wherein the multicomponent polypeptide is expressed by the expression vector.

[0070] In some embodiment, a pharmaceutical composition to modulate the WNT/β-catenin signaling pathway comprises an effective amount of the multicomponent WNT molecule, and a pharmaceutically acceptable diluent, adjuvant, or carrier.

[0071] In yet another embodiment, provides a method of treating or preventing a disease or disorder in a subject in need thereof, the method comprises by providing to the subject an effective amount of the multicomponent WNT molecule.

DETAILED DESCRIPTION

[0072] As used herein, including the appended claims, the singular forms of words such as "a," "an," and "the," include their corresponding plural references unless the context clearly dictates otherwise.

[0073] Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

[0074] Each embodiment in this specification is to be applied *mutatis mutandis* to every other embodiment unless expressly stated otherwise.

[0075] All references cited herein are incorporated by reference to the same extent as if each individual publication, patent application, or patent, was specifically and individually indicated to be incorporated by reference.

I. Definitions.

[0076] "Activity" of a molecule may describe or refer to the binding of the molecule to a ligand or to a receptor, to catalytic activity, to the ability to stimulate gene expression, to antigenic activity, to the modulation of activities of other molecules, and the like. "Activity" of a molecule may also refer to activity in modulating or maintaining cell-to-cell interactions, e.g., adhesion, or activity in maintaining a structure of a cell, e.g., cell membranes or cytoskeleton. "Activity" may also mean specific activity, e.g., [catalytic activity]/[mg protein], or [immunological activity]/[mg protein], or the like.

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[0077] The terms “tethered” or “bound” or “joined” or “linked”, as used herein, refer to different binding domains of the multicomponent molecule being fastened together. It is understood that different binding domains of the multicomponent molecule are fastened together and could be used interchangeably.

[0078] The terms “targeting element”, “bridging element”, “bridging molecule”, or “bridging receptor” as used herein, refer to cell surface receptors also known as transmembrane receptors, such as, e.g., ion channel-linked receptors, G-protein coupled receptors, and enzyme-linked receptors. A bridging receptor can be a cell surface glycan.

[0079] The terms “chimeric activator”, as used herein, refers to the cooperativity concept of different ligands by enhancing agonist activity to the targeted cell.

[0080] The terms “multicomponent polypeptide”, “multicomponent polypeptide molecule”, and “multicomponent molecule”, as used herein, are interchangeable.

[0081] The terms “multicomponent WNT molecule”, “WNT multicomponent polypeptide molecule”, and “WNT mimetic” as used herein, are interchangeable.

[0082] The terms "administering" or "introducing" or “providing”, as used herein, refer to delivery of a composition to a cell, to cells, tissues and/or organs of a subject, or to a subject. Such administering or introducing may take place in vivo, in vitro or ex vivo.

[0083] As is well known in the art, an antibody is an immunoglobulin molecule capable of specific binding to a target, such as a carbohydrate, polynucleotide, lipid, polypeptide, etc., through at least one epitope recognition site, located in the variable region of the immunoglobulin molecule. As used herein, the term encompasses not only intact polyclonal or monoclonal antibodies, but also fragments thereof (such as dAb, Fab, Fab', F(ab')2, Fv), single chain (scFv), V_{HH}, synthetic variants thereof, naturally occurring variants, fusion proteins comprising an antibody or an antigen-binding fragment thereof, humanized antibodies, chimeric antibodies, and any other modified configuration of the immunoglobulin molecule that comprises an antigen-binding site or fragment (epitope recognition site) of the required specificity. "Diabodies", multivalent or multispecific fragments constructed by gene fusion (WO94/13804; P. Holliger et al (1993)., *Proc. Natl. Acad. Sci. USA* 90 6444-6448) are also a particular form of antibody contemplated herein. Minibodies comprising a scFv joined to a CH3 domain are also included herein (See e.g., S. Hu et al. (1996), *Cancer Res.*, 56:3055-3061; Ward, E. S. et al. (1989) *Nature* 341:544-546; Bird et al.(1988) *Science* 242:423-426; Huston et al. (1988) *Proc. Natl. Acad. Sci. USA* 85:5879-5883; PCT/US92/09965; WO94/13804; P.

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Holliger et al.(1993) *Proc. Natl. Acad. Sci. USA* 90:6444-6448; and Y. Reiter et al. (1996) *Nature Biotech.* 14:1239-1245).

[0084] The term "antigen-binding fragment" as used herein refers to a polypeptide fragment that contains at least one CDR of an immunoglobulin heavy and/or light chain, or of a V_{HH}, that binds to the antigen of interest, in particular to one or more FZD receptor or LRP5 or LRP6 receptor. In this regard, an antigen-binding fragment of the herein described antibodies may comprise 1, 2, 3, 4, 5, or all 6 CDRs of a VH and VL sequence set forth herein from antibodies that bind one or more FZD receptor or LRP5 and/or LRP6. In particular embodiments, an antigen-binding fragment may comprise all three VH CDRs or all three VL CDRs. Similarly, an antigen binding fragment thereof may comprise all three CDRs of a V_{HH} binding fragment. An antigen-binding fragment of a FZD-specific antibody is capable of binding to a FZD receptor. An antigen- binding fragment of a LRP5/6-specific antibody is capable of binding to a LRP5 and/or LRP6 receptor. As used herein, the term encompasses not only isolated fragments but also polypeptides comprising an antigen-binding fragment of an antibody disclosed herein, such as, for example, fusion proteins comprising an antigen-binding fragment of an antibody disclosed herein, such as, e.g., a fusion protein comprising a V_{HH} that binds one or more FZD receptors and a V_{HH} that binds LRP5 and/or LRP6.

[0085] The term "antigen" refers to a molecule or a portion of a molecule capable of being bound by a selective binding agent, such as an antibody, and additionally capable of being used in an animal to produce antibodies capable of binding to an epitope of that antigen. In certain embodiments, a binding agent (e.g., a multicomponent WNT molecule or binding region thereof) is said to specifically bind an antigen when it preferentially recognizes its target antigen in a complex mixture of proteins and/or macromolecules. In certain embodiments, a multicomponent WNT molecule or binding region thereof (e.g., an antibody or antigen-binding fragment thereof) is said to specifically bind an antigen when the equilibrium dissociation constant is $\leq 10^{-7}$ or $\leq 10^{-8}$ M. In some embodiments, the equilibrium dissociation constant may be $\leq 10^{-9}$ M or $\leq 10^{-10}$ M.

[0086] As used herein, the term "CDR" refers to at least one of the three hypervariable regions of a heavy or light chain variable (V) region. Proceeding from the N-terminus of a heavy or light chain, these regions are denoted as "CDR1," "CDR2," and "CDR3" respectively. An antigen-binding site, therefore, includes six CDRs, comprising the CDR set from each of a heavy and a light chain V region. A polypeptide comprising a single CDR, (e.g., a CDR1, CDR2 or CDR3) is referred to herein as a "molecular recognition unit." Crystallographic

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analysis of a number of antigen-antibody complexes has demonstrated that the amino acid residues of CDRs form extensive contact with bound antigen, wherein the most extensive antigen contact is with the heavy chain CDR3. Thus, the molecular recognition units are primarily responsible for the specificity of an antigen-binding site. In certain embodiments, antibodies and antigen-binding fragments thereof as described herein include a heavy chain and a light chain CDRs, respectively interposed between a heavy chain and a light chain framework regions (FRs)which provide support to the CDRs and define the spatial relationship of the CDRs relative to each other.

[0087] As used herein, the term "FRs" refer to the four flanking amino acid sequences which frame the CDRs of a heavy or light chain V region. Some FR residues may contact bound antigen; however, FRs are primarily responsible for folding the V region into the antigen-binding site, particularly the FR residues directly adjacent to the CDRs. Within FRs, certain amino residues and certain structural features are very highly conserved. In this regard, all V region sequences contain an internal disulfide loop of around 90 amino acid residues. When the V regions fold into a binding-site, the CDRs are displayed as projecting loop motifs which form an antigen-binding surface. It is generally recognized that there are conserved structural regions of FRs which influence the folded shape of the CDR loops into certain "canonical" structures—regardless of the precise CDR amino acid sequence. Further, certain FR residues are known to participate in non-covalent interdomain contacts which stabilize the interaction of the antibody heavy and light chains. The structures and locations of immunoglobulin CDRs and variable domains may be determined by reference to Kabat, E. A. et al., Sequences of Proteins of Immunological Interest. 4th Edition. US Department of Health and Human Services. 1987, and updates thereof, now available on the Internet (immuno.bme.nwu.edu).

[0088] A "monoclonal antibody" refers to a homogeneous antibody population wherein the monoclonal antibody is comprised of amino acids (naturally occurring and non-naturally occurring) that are involved in the selective binding of an epitope. Monoclonal antibodies are highly specific, being directed against a single epitope. The term "monoclonal antibody" encompasses not only intact monoclonal antibodies and full-length monoclonal antibodies, but also fragments thereof (such as Fab, Fab', F(ab')2, Fv), single chain (scFv), V_{HH}, variants thereof, fusion proteins comprising an antigen-binding fragment of a monoclonal antibody, humanized monoclonal antibodies, chimeric monoclonal antibodies, and any other modified configuration of the immunoglobulin molecule that comprises an antigen- binding fragment

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(epitope recognition site) of the required specificity and the ability to bind to an epitope, including multicomponent WNT molecules disclosed herein. It is not intended to be limited as regards the source of the antibody or the manner in which it is made (e.g., by hybridoma, phage selection, recombinant expression, transgenic animals, etc.). The term includes whole immunoglobulins as well as the fragments etc. described above under the definition of "antibody"

[0089] The term "co-receptor" refers to a first cell surface receptor that binds signaling molecule or ligand in conjunction with another receptor to facilitate ligand recognition and initiate a biological process, such as WNT pathway signaling.

[0090] The term "agonist activity" refers to the ability of an agonist to mimic the effect or activity of a naturally occurring protein.

[0091] As used herein "peptide linker" or "linker moiety" refers to a sequence of sometimes repeating amino acid residues, usually glycine and serine, that are used to join the various antigen binding domains described below. The length of the linker sequence determines the flexibility of the antigen binding domains in MsAbs, in particular, in the binding of epitopes on co-receptors such as FZD receptors, LRP5 and/or LRP6, and/or ZNRF3/RNF43.

[0092] As used herein, the term "enhances" refers to a measurable increase in the level of receptor signaling modulated by a ligand or ligand agonist compared with the level in the absence of the agonist, e.g., a multicomponent WNT molecule. In particular embodiments, the increase in the level of receptor signaling is at least 10%, at least 20%, at least 50%, at least two-fold, at least five-fold, at least 10-fold, at least 20-fold, at least 50- fold, or at least 100-fold as compared to the level of receptor signaling in the absence of the agonist, e.g., in the same cell type.

[0093] An antigen or epitope that "specifically binds" or "preferentially binds" (used interchangeably herein) to an antibody or antigen-binding fragment thereof is a term well understood in the art, and methods to determine such specific or preferential binding are also well known in the art. A molecule, e.g., a multicomponent WNT molecule, is said to exhibit "specific binding" or "preferential binding" if it reacts or associates more frequently, more rapidly, with greater duration and/or with greater affinity with a particular cell or substance than it does with alternative cells or substances. A molecule or binding region thereof, e.g., a multicomponent WNT molecule or binding region thereof, "specifically binds" or "preferentially binds" to a target antigen, e.g., a FZD receptor, if it binds with greater affinity,

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avidity, more readily, and/or with greater duration than it binds to other substances. It is also understood by reading this definition that, for example, a multicomponent WNT molecule or binding region thereof that specifically or preferentially binds to a first target may or may not specifically or preferentially bind to a second target. As such, "specific binding" or "preferential binding" does not necessarily require (although it can include) exclusive binding. Generally, but not necessarily, reference to binding means preferential binding.

[0094] The term "operably linked" means that the components to which the term is applied are in a relationship that allows them to carry out their inherent functions under suitable conditions. For example, a transcription control sequence "operably linked" to a protein coding sequence is ligated thereto so that expression of the protein coding sequence is achieved under conditions compatible with the transcriptional activity of the control sequences.

[0095] The term "control sequence" as used herein refers to polynucleotide sequences that can affect expression, processing or intracellular localization of coding sequences to which they are ligated or operably linked. The nature of such control sequences may depend upon the host organism. In particular embodiments, transcription control sequences for prokaryotes may include a promoter, ribosomal binding site, and transcription termination sequence. In other particular embodiments, transcription control sequences for eukaryotes may include promoters comprising one or a plurality of recognition sites for transcription factors, transcription enhancer sequences, transcription termination sequences and polyadenylation sequences. In certain embodiments, "control sequences" can include leader sequences and/or fusion partner sequences.

[0096] The term "polynucleotide" as referred to herein means single- stranded or double-stranded nucleic acid polymers. In certain embodiments, the nucleotides comprising the polynucleotide can be ribonucleotides or deoxyribonucleotides or a modified form of either type of nucleotide. Said modifications include base modifications such as bromouridine, ribose modifications such as arabinoside and 2',3'-dideoxyribose and internucleotide linkage modifications such as phosphorothioate, phosphorodithioate, phosphoroselenoate, phosphorodiselenoate, phosphoroanilothioate, phoshoraniladate and phosphoroamidate. The term "polynucleotide" specifically includes single and double stranded forms of DNA.

[0097] The term "naturally occurring nucleotides" includes deoxyribonucleotides and ribonucleotides. The term "modified nucleotides" includes nucleotides with modified or substituted sugar groups and the like. The term "oligonucleotide linkages" includes

oligonucleotide linkages such as phosphorothioate, phosphorodithioate, phosphoroselenoate, phosphorodiselenoate, phosphoroanilothioate, phoshoraniladate, phosphoroamidate, and the like. See, e.g., LaPlanche et al. (1986) *Nucl. Acids Res.* 14:9081; Stec et al. (1984) *J. Am. Chem. Soc.* 106:6077; Stein et al. (1988) *Nucl. Acids Res.* 16:3209; Zon et al. (1991) *Anti-Cancer Drug Design*, 6:539; Zon et al. (1991) Oligonucleotides and Analogues: A Practical Approach, pp. 87-108 (F. Eckstein, Ed.), Oxford University Press, Oxford England; Stec et al., U.S. Pat. No. 5,151,510; Uhlmann and Peyman (1990) *Chem. Rev.* 90:543, the disclosures of which are hereby incorporated by reference for any purpose. An oligonucleotide can include a detectable label to enable detection of the oligonucleotide or hybridization thereof.

[0098] The term "vector" is used to refer to any molecule (e.g., nucleic acid, plasmid, or virus) used to transfer coding information to a host cell. The term "expression vector" refers to a vector that is suitable for transformation of a host cell and contains nucleic acid sequences that direct and/or control expression of inserted heterologous nucleic acid sequences. Expression includes, but is not limited to, processes such as transcription, translation, and RNA splicing, if introns are present.

[0099] The term "host cell" is used to refer to a cell into which has been introduced, or which is capable of having introduced into it, a nucleic acid sequence encoding one or more of the herein described polypeptides, and which further expresses or is capable of expressing a selected gene of interest, such as a gene encoding any herein described polypeptide. The term includes the progeny of the parent cell, whether or not the progeny are identical in morphology or in genetic make-up to the original parent, so long as the selected gene is present. Accordingly, there is also contemplated a method comprising introducing such nucleic acid into a host cell. The introduction may employ any available technique. For eukaryotic cells, suitable techniques may include calcium phosphate transfection, DEAE-Dextran, electroporation, liposome- mediated transfection and transduction using retrovirus or other virus, *e.g.*, vaccinia or, for insect cells, baculovirus. For bacterial cells, suitable techniques may include calcium chloride transformation, electroporation and transfection using bacteriophage. The introduction may be followed by causing or allowing expression from the nucleic acid, *e.g.*, by culturing host cells under conditions for expression of the gene. In one embodiment, the nucleic acid is integrated into the genome (*e.g.*, chromosome) of the host cell. Integration may be promoted by inclusion of sequences which promote recombination with the genome, in accordance-with standard techniques.

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[0100] The term "transfection" is used to refer to the uptake of foreign or exogenous DNA by a cell, and a cell has been "transfected" when the exogenous DNA has been introduced inside the cell membrane. A number of transfection techniques are well known in the art and are disclosed herein. See, e.g., Graham et al., 1973, Virology 52:456; Sambrook et al., 2001, MOLECULAR CLONING, A LABORATORY MANUAL, Cold Spring Harbor Laboratories; Davis et al., 1986, BASIC METHODS IN MOLECULAR BIOLOGY, Elsevier; and Chu et al., 1981, Gene 13:197. Such techniques can be used to introduce one or more exogenous DNA moieties into suitable host cells.

[0101] "Transduction" refers to the acquisition and transfer of eukaryotic cellular sequences by viruses, e.g., retroviruses.

[0102] The term "transformation" as used herein refers to a change in a cell's genetic characteristics, and a cell has been transformed when it has been modified to contain a new DNA. For example, a cell is transformed where it is genetically modified from its native state. Following transfection or transduction, the transforming DNA may recombine with that of the cell by physically integrating into a chromosome of the cell, or may be maintained transiently as an episomal element without being replicated, or may replicate independently as a plasmid. A cell is considered to have been stably transformed when the DNA is replicated with the division of the cell.

[0103] The term "naturally occurring" or "native" when used in connection with biological materials such as nucleic acid molecules, polypeptides, host cells, and the like, refers to materials which are found in nature and are not manipulated by a human. Similarly, "non-naturally occurring" or "non-native" as used herein refers to a material that is not found in nature or that has been structurally modified or synthesized by a human.

[0104] The terms "polypeptide" "protein" and "peptide" and "glycoprotein" are used interchangeably and mean a polymer of amino acids not limited to any particular length. The term does not exclude modifications such as myristylation, sulfation, glycosylation, phosphorylation and addition or deletion of signal sequences. The terms "polypeptide" or "protein" means one or more chains of amino acids, wherein each chain comprises amino acids covalently linked by peptide bonds, and wherein said polypeptide or protein can comprise a plurality of chains non-covalently and/or covalently linked together by peptide bonds, having the sequence of native proteins, that is, proteins produced by naturally-occurring and specifically non-recombinant cells, or genetically-engineered or recombinant cells, and

comprise molecules having the amino acid sequence of the native protein, or molecules having deletions from, additions to, and/or substitutions of one or more amino acids of the native sequence. The terms "polypeptide" and "protein" specifically encompass multicomponent WNT molecules, FZD binding regions thereof, LRP5/6 binding regions thereof, antibodies and antigen-binding fragments thereof that bind to a FZD receptor or a LRP5 or LRP6 receptor disclosed herein, or sequences that have deletions from, additions to, and/or substitutions of one or more amino acid of any of these polypeptides. Thus, a "polypeptide" or a "protein" can comprise one (termed "a monomer") or a plurality (termed "a multimer") of amino acid chains. Polypeptides and proteins include glycoproteins.

[0105] The term "isolated protein," "or "isolated antibody" referred to herein means that a subject protein, multicomponent molecule, or antibody: (1) is free of at least some other proteins with which it would typically be found in nature; (2) is essentially free of other proteins from the same source, e.g., from the same species, (3) is expressed by a cell from a different species; (4) has been separated from at least about 50 percent of polynucleotides, lipids, carbohydrates, or other materials with which it is associated in nature; (5) is not associated (by covalent or noncovalent interaction) with portions of a protein with which the "isolated protein" is associated in nature; (6) is operably associated (by covalent or noncovalent interaction) with a polypeptide with which it is not associated in nature; or (7) does not occur in nature. Such an isolated protein can be encoded by genomic DNA, cDNA, mRNA or other RNA, or may be of synthetic origin, or any combination thereof. In certain embodiments, an isolated protein may comprise naturally-occurring and/or artificial polypeptide sequences. In certain embodiments, the isolated protein is substantially free from proteins or polypeptides or other contaminants that are found in its natural environment that would interfere with its use (therapeutic, diagnostic, prophylactic, research or otherwise).

[0106] A "WNT super agonist" is a molecule having enhanced WNT agonist activity. As used herein, the WNT super agonists have both WNT signaling and WNT signal enhancing activity. In some embodiments, the WNT super agonist molecule will bind both at least one FZD receptor and at least one LRP receptor, as well as binding and activating at least one E3 ubiquitin ligase receptor, thereby stabilizing the FZD and/or LRP receptors.

II. General

[0107] The present invention provides combinations of antigen binding molecules that act as multicomponent polypeptide and enhancing molecules by binding to and modulating co-

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receptor signaling, for example, antigen binding molecules that bind to one or more FZD receptor and one or more LRP5 or LRP6 receptor, and a bridging receptor, which in turn modulates a downstream WNT signaling pathway. In particular embodiments, the multicomponent complex activates or increases a signaling pathway associated with one or both co-receptors by homo- or hetero-dimerization after interaction of a bridging element with the bridging receptor. In particular embodiments, the multicomponent polypeptide molecules disclosed herein comprise: (i) one or more antibodies or antigen-binding fragments thereof that specifically bind to one or more first co-receptor, including antibodies or antigen-binding fragments thereof having particular co-receptor specificity and/or functional properties; and (ii) one or more antibodies or antigen-binding fragments thereof that specifically bind to one or more second co-receptors, and one or more bridging molecule that bind to one or more bridging receptor. Certain embodiments encompass specific structural formats or arrangements of the first and second co-receptor binding region(s) of the multicomponent molecules advantageous in increasing downstream signaling and related biological effects.

[0108] The practice of the present invention will employ, unless indicated specifically to the contrary, conventional methods of virology, immunology, microbiology, molecular biology and recombinant DNA techniques within the skill of the art, many of which are described below for the purpose of illustration. Such techniques are explained fully in the literature. See, e.g., Current Protocols in Molecular Biology or Current Protocols in Immunology, John Wiley & Sons, New York, N.Y.(2009); Ausubel et al., Short Protocols in Molecular Biology, 3rd ed., Wiley & Sons, 1995; Sambrook and Russell, Molecular Cloning: A Laboratory Manual (3rd Edition, 2001); Maniatis et al. Molecular Cloning: A Laboratory Manual (1982); DNA Cloning: A Practical Approach, vol. I & II (D. Glover, ed.); Oligonucleotide Synthesis (N. Gait, ed., 1984); Nucleic Acid Hybridization (B. Hames & S. Higgins, eds., 1985); Transcription and Translation (B. Hames & S. Higgins, eds., 1984); Animal Cell Culture (R. Freshney, ed., 1986); Perbal, A Practical Guide to Molecular Cloning (1984) and other like references.

[0109] Sequences of illustrative antibodies, or antigen-binding fragments, or complementarity determining regions (CDRs) thereof, that bind to one or more FZD receptors, are set forth in WO2019126399. Sequences of illustrative LRP5 and/or LRP6 antibodies, or antigen-binding fragments, or complementarity determining regions (CDRs) thereof, are set forth in WO2019126401. Sequences of antigen binding molecules that bind one or more FZD receptor and LRP5 and/or LRP6 are set forth in U.S. Provisional application nos. 62/607,875,

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62/641,217, and 62/680,522, titled WNT Signaling Pathway Agonists, filed December 19, 2017, March 9, 2018, and June 4, 2018, respectively.

[0110] Antibodies and antibody fragments thereof may be prepared by methods well known in the art. For example, the proteolytic enzyme papain preferentially cleaves IgG molecules to yield several fragments, two of which (the F(ab) fragments) each comprise a covalent heterodimer that includes an intact antigen-binding site. The enzyme pepsin is able to cleave IgG molecules to provide several fragments, including the F(ab')2 fragment which comprises both antigen-binding sites. An Fv fragment for use according to certain embodiments of the present invention can be produced by preferential proteolytic cleavage of an IgM, and on rare occasions of an IgG or IgA immunoglobulin molecule. Fv fragments are, however, more commonly derived using recombinant techniques known in the art. The Fv fragment includes a non-covalent VH:VL heterodimer including an antigen-binding site which retains much of the antigen recognition and binding capabilities of the native antibody molecule. (See, e.g., Inbar *et al.* (1972) *Proc. Nat. Acad. Sci. USA 69*:2659-2662; Hochman *et al.* (1976) *Biochem 15*:2706- 2710; and Ehrlich *et al.* (1980) *Biochem 19*:4091-4096).

[0111] In certain embodiments, single chain Fv or scFV antibodies are contemplated. For example, Kappa bodies (Ill *et al.* (1997), *Prot. Eng.* 10: 949-57; minibodies (Martin *et al.*(1994) *EMBO J* 13: 5305-9; diabodies (Holliger *et al.* (1993) *PNAS* 90: 6444-8; or janusins (Traunecker *et al.*(1991) *EMBO J* 10: 3655-59; and Traunecker *et al.* (1992) *Int. J. Cancer Suppl.* 7: 51-52.), may be prepared using standard molecular biology techniques following the teachings of the present application with regard to selecting antibodies having the desired specificity. In still other embodiments, bispecific or chimeric antibodies may be made that encompass the ligands of the present disclosure. For example, a chimeric antibody may comprise CDRs and framework regions from different antibodies, while bispecific antibodies may be generated that bind specifically to one or more FZD receptor through one binding domain and to a second molecule through a second binding domain and bridging molecule with identical or different bridging epitopes for the first or second binding domains. These antibodies may be produced through recombinant molecular biological techniques or may be physically conjugated together.

[0112] A single chain Fv (scFv) polypeptide is a covalently linked VH::VL heterodimer which is expressed from a gene fusion including VH- and VL- encoding genes linked by an encoded peptide linker. Huston *et al.* (1988) *Proc. Nat. Acad. Sci. USA 85*(16):5879-5883. A number of methods have been described to discern chemical structures for converting the

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naturally aggregated—but chemically separated—light and heavy polypeptide chains from an antibody V region into an scFv molecule which will fold into a three dimensional structure substantially similar to the structure of an antigen-binding site. See, *e.g.*, U.S. Pat. Nos. 5,091,513 and 5,132,405, to Huston *et al.*; and U.S. Pat. No. 4,946,778, to Ladner *et al.*

[0113] In certain embodiments, an antibody as described herein is in the form of a diabody. Diabodies are multimers of polypeptides, each polypeptide comprising a first domain comprising a binding region of an immunoglobulin light chain and a second domain comprising a binding region of an immunoglobulin heavy chain, the two domains being linked (*e.g.* by a peptide linker) but unable to associate with each other to form an antigen binding site: antigen binding sites are formed by the association of the first domain of one polypeptide within the multimer with the second domain of another polypeptide within the multimer (WO94/13804). A dAb fragment of an antibody consists of a VH domain (Ward, E. S. *et al.* (1989) Nature 341:544-546).

[0114] Where bispecific antibodies are to be used, these may be conventional bispecific antibodies, which can be manufactured in a variety of ways (Holliger, P. and Winter G. (1993) *Curr. Op. Biotechnol.* 4:446-449), *e.g.,* prepared chemically or from hybrid hybridomas, or may be any of the bispecific antibody fragments mentioned above. Diabodies and scFv can be constructed without an Fc region, using only variable domains, potentially reducing the effects of anti-idiotypic reaction.

[0115] Bispecific diabodies, as opposed to bispecific whole antibodies, may also be particularly useful because they can be readily constructed and expressed in *E. coli*. Diabodies (and many other polypeptides such as antibody fragments) of appropriate binding specificities can be readily selected using phage display (WO94/13804) from libraries. If one arm of the diabody is to be kept constant, for instance, with a specificity directed against antigen X, then a library can be made where the other arm is varied and an antibody of appropriate specificity selected. Bispecific whole antibodies may be made by knobs-into-holes engineering (J. B. B. Ridgeway et al. (1996) *Protein Eng.*, 9:616- 621).

[0116] In certain embodiments, the antibodies described herein may be provided in the form of a UniBody®. A UniBody® is an IgG4 antibody with the hinge region removed (see GenMab Utrecht, The Netherlands; see also, *e.g.*, US20090226421). This proprietary antibody technology creates a stable, smaller antibody format with an anticipated longer therapeutic window than current small antibody formats. IgG4 antibodies are considered inert and thus do

not interact with the immune system. Fully human IgG4 antibodies may be modified by eliminating the hinge region of the antibody to obtain half-molecule fragments having distinct stability properties relative to the corresponding intact IgG4 (GenMab, Utrecht). Halving the IgG4 molecule leaves only one area on the UniBody® that can bind to cognate antigens (*e.g.*, disease targets) and the UniBody® therefore binds univalently to only one site on target cells.

[0117] In certain embodiments, the antibodies of the present disclosure may take the form of a single variable domain fragment known as a V_{HH}. The V_{HH} technology was originally developed following the discovery and identification that *camelidae* (e.g., camels and llamas) possess fully functional antibodies that consist of heavy chains only and therefore lack light chains. These heavy-chain only antibodies contain a single V_{HH} domain and two constant domains (CH2, CH3). The cloned and isolated V_{HH} domains have full antigen binding capacity and are very stable. These V_{HH} domains are encoded by single genes and are efficiently produced in almost all prokaryotic and eukaryotic hosts *e.g., E. coli* (see *e.g.* U.S. Pat. No. 6,765,087), molds (for example *Aspergillus* or *Trichoderma*) and yeast (for example *Saccharomyces, Kluyvermyces, Hansenula* or *Pichia* (see *e.g.* U.S. Pat. No. 6,838,254). The production process is scalable and multi-kilogram quantities of VHHs have been produced. VHHs may be formulated as a ready-to-use solution having a long shelf life. The Nanoclone® method (see, e.g., WO 06/079372) is a proprietary method for generating VHHs against a desired target, based on automated high-throughput selection of B-cells. VHH antibodies typically have a small size of around 15 kDa.

[0118] In certain embodiments, the antibodies or antigen-binding fragments thereof as disclosed herein are humanized. This refers to a chimeric molecule, generally prepared using recombinant techniques, having an antigen- binding site derived from an immunoglobulin from a non-human species and the remaining immunoglobulin structure of the molecule based upon the structure and/or sequence of a human immunoglobulin. The antigen-binding site may comprise either complete variable domains fused onto constant domains or only the CDRs grafted onto appropriate framework regions in the variable domains. Epitope binding sites may be wild type or modified by one or more amino acid substitutions. This eliminates the constant region as an immunogen in human individuals, but the possibility of an immune response to the foreign variable region remains (LoBuglio, A. F. et al., (1989*) Proc Natl Acad Sci USA* 86:4220-4224; Queen et al. (1988) *Proc Natl Acad Sci USA* 86:10029-10033; and Riechmann et al. (1988) *Nature* 332:323-327). Illustrative methods for humanization of the anti- FZD antibodies disclosed herein include the methods described in U.S. patent no. 7,462,697.

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[0119] Another approach focuses not only on providing human-derived constant regions, but modifying the variable regions as well so as to reshape them as closely as possible to human form. It is known that the variable regions of both heavy and light chains contain three complementarity- determining regions (CDRs) which vary in response to the epitopes in question and determine binding capability, flanked by four framework regions (FRs) which are relatively conserved in a given species and which putatively provide a scaffolding for the CDRs. When nonhuman antibodies are prepared with respect to a particular epitope, the variable regions can be "reshaped" or "humanized" by grafting CDRs derived from nonhuman antibody on the FRs present in the human antibody to be modified. Application of this approach to various antibodies has been reported by Sato, K., et al., (1993) *Cancer Res* 53:851-856. Riechmann, L., et al., (1988) *supra*; Verhoeyen, M., et al., (1988) *Science* 239:1534-1536; Kettleborough, C. A., et al., (1991) *Protein Engg* 4:773-3783; Maeda, H., et al., (1991) *Human Antibodies Hybridoma* 2:124-134; Gorman, S. D., et al., (1991) *Proc Natl Acad Sci USA* 88:4181-4185; Tempest, P. R., et al., (1991) *Bio/Technol.* 9:266-271; Co, M. S., et al., (1991) *Proc Natl Acad Sci USA* 88:2869-2873; Carter, P., et al., (1992) *Proc Natl Acad Sci USA* 89:4285-4289; and Co, M. S. et al., (1992) *J Immunol* 148:1149-1154. In some embodiments, humanized antibodies preserve all CDR sequences (for example, a humanized mouse antibody which contains all six CDRs from the mouse antibodies). In other embodiments, humanized antibodies have one or more CDRs (one, two, three, four, five, six) which are altered with respect to the original antibody, which are also termed one or more CDRs "derived from" one or more CDRs from the original antibody.

[0120] In certain embodiments, the antibodies of the present disclosure may be chimeric antibodies. In this regard, a chimeric antibody is comprised of an antigen-binding fragment of an antibody operably linked or otherwise fused to a heterologous Fc portion of a different antibody. In certain embodiments, the heterologous Fc domain is of human origin. In other embodiments, the heterologous Fc domain may be from a different Ig class from the parent antibody, including IgA (including subclasses IgA1 and IgA2), IgD, IgE, IgG (including subclasses IgG1, IgG2, IgG3, and IgG4), and IgM. In further embodiments, the heterologous Fc domain may be comprised of CH2 and CH3 domains from one or more of the different Ig classes. As noted above with regard to humanized antibodies, the antigen-binding fragment of a chimeric antibody may comprise only one or more of the CDRs of the antibodies described herein (e.g., 1, 2, 3, 4, 5, or 6 CDRs of the antibodies described herein), or may comprise an entire variable domain (VL, VH or both).

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III. Structures of Multicomponent Receptor Complexes

[0121] The disclosure provides, in certain aspects, a cell targeting multicomponent polypeptide having a one or more first antigen binding domain that binds to a first receptor, a one or more second antigen binding domain that binds to a second receptor, and one or more different antigen binding domain that binds to one or more bridging molecule that activates the signaling pathways.

[0122] In certain illustrative embodiments of the tissue-specific bridging molecules disclosed herein: the tissue is bone tissue, and the cell surface receptor is parathyroid hormone receptor 1 (PTH1R); or the tissue is liver tissue, and the cell surface receptor is asialoglycoprotein receptor 1 (ASGR1), asialoglycoprotein receptor 2 (ASGR2), transferrin receptor 2 (TFR2) or solute carrier family 10 member 1 (SLC10A1), or the tissue is oral mucous tissue, and the cell surface receptor is LY6/PLAUR Domain Containing 3 (LYPD3) or Desmoglein 3 (DSG3).

[0123] In certain illustrative embodiments of the tissue-specific bridging molecules disclosed herein: the cell surface molecule is a PTH1, and at least one different antigen binding domain specifically binds PTH1R; the cell surface molecule is ASGR1, and at least one different antigen binding domain specifically binds ASGR1; the cell surface molecule is ASGR2, and third binding at least one different antigen binding domain binds ASGR2; the cell surface molecule is SLC10A1, and at least one different antigen binding domain specifically binds SLC10A1; or the cell surface molecule is TFR2, and at least one different antigen binding domain specifically binds TFR2, the cell surface molecule is LYPD3, and at least one different antigen binding domain specifically binds LYPD3; or the cell surface molecule is DSG3, at least one different antigen binding domain sequence specifically binds DSG3, at least one different antigen binding domain is an antibody or fragment thereof, a small molecule, or a ligand, or fragment or variant thereof, of the cell surface molecule.

[0124] In certain embodiment, the tethering of the targeting element or the bridging molecule to the activity element or the ensemble of the first antigen binding domain that binds to a first receptor, a one or more second antigen binding domain that binds to a second receptor, increase the local concentration of the activity element on the desired target cell surface, by engagement of signaling receptor and subsequent intracellular signaling activation. Thus, the tethering of the first and second binding domains to the bridging receptor forms a chimeric activator capable of activating the targeted cell signaling.

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[0125] In certain embodiments, a multicomponent molecule is capable of modulating signaling events associated with at least one of the co-receptors that it binds, in a cell contacted with the multicomponent polypeptide molecule. In certain embodiments, the multicomponent polypeptide molecule increases receptor signaling. As an example, a WNT multicomponent polypeptide molecule specifically modulates the biological activity of a human WNT/β-catenin signaling pathway.

[0126] Multicomponent polypeptide molecules of the present invention are biologically active in binding to one or more of a first receptor and to one or more of a second receptor, and as an example, in the activation of WNT signaling, the WNT multicomponent polypeptide molecule is a WNT agonist. The term "agonist activity" refers to the ability of an agonist to mimic the effect or activity of a naturally occurring protein binding to a first and second receptor. The ability of the multicomponent polypeptide molecules and other receptor agonists disclosed herein to mimic the activity of the natural ligand can be confirmed by a number of assays. As an example, WNT multicomponent polypeptide molecules, some of which are disclosed herein activate, enhance or increase the canonical WNT/β-catenin signaling pathway.

[0127] In particular embodiments, the structures of the multicomponent polypeptide molecules disclosed herein are bispecific, i.e., they specifically bind to two or more different epitopes, e.g., one or more epitopes of a first receptor, and one or more epitopes of a second receptor.

[0128] In particular embodiments, multicomponent molecules disclosed herein are multivalent, e.g., they comprise two or more regions that each specifically bind to the same epitope, e.g., two or more regions that bind to an epitope within one or more first co-receptor and/or two or more regions that bind to an epitope within a second co-receptor. In particular embodiments, they comprise two or more regions that bind to an epitope within a first co-receptor and two or more regions that bind to an epitope within a second co-receptor. In certain embodiments, multicomponent polypeptide molecules comprise a ratio of the number of regions that bind one or more first co-receptor to the number of regions that a second co-receptor of or about: 1:1, 2:1, 3:1, 4:1, 5:1, 6:1, 2:3, 2:5, 2:7, 7:2, 5:2, 3:2, 3:4, 3:5, 3:7, 3:8, 8:3, 7:3, 5:3, 4:3, 4:5, 4:7, 4:9, 9:4, 7:4, 5:4, 6:7, 7:6, 1:2, 1:3, 1:4, 1:5, or 1:6. In certain embodiments, the multicomponent polypeptide molecules are bispecific and multivalent.

[0129] In particular embodiment, the multicomponent WNT molecule described herein, are bispecific and can simultaneously bind to FZDs and LRPs binding domains. The optimal

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stoichiometry (at least for the antibody based molecules) are tetravalent bispecific (2:2 format), requiring two FZD binders and two LRP binders in the same molecule to achieve efficient signaling (Tao et al., 2019, Chen et al., 2020). Taking advantage of the fact that two FZD binders alone, two LRP binders alone, or a molecule with one FZD binder and one LRP binder (1:1 format) do not signal. Cell specificity is achieved by attaching an "targeting element" (capable of binding to another cell surface receptor, called a bridging receptor here) to these three inactive molecules, and signaling competent receptor complexes consisting of two FZDs and two LRPs could then be assemble via the bridging receptor on the target cell surface. This approach reduces or eliminates the need to mutate and reduce the affinity of the "active elements" toward signaling receptors, and creates a highly cell specific activation of the signaling pathway as the individual components are inactive. In particular embodiments, the antigen binding domain(s) binds to a signaling receptor or a signaling receptor component.

[0130] The structures of the multicomponent polypeptide molecules disclosed herein may have any of a variety of different structural formats or configurations. The multicomponent polypeptide molecules may comprise polypeptides and/or non-polypeptide binding moieties, e.g., small molecules. In particular embodiments, the multicomponent polypeptide molecules comprise both a polypeptide region and a non-polypeptide binding moiety. In certain embodiments, the multicomponent polypeptide molecules may comprise a single polypeptide, or they may comprise two or more, three or more, or four or more polypeptides.

[0131] In certain embodiments, one or more polypeptides of a multicomponent polypeptide molecule are antibodies or antigen-binding fragments thereof. In certain embodiments, surrogates comprise two antibodies or antigen binding fragments thereof, one that binds one or more first co-receptor and one that binds on or more second co-receptor. In certain embodiments, the molecules, e.g., surrogates, comprise one, two, three, or four polypeptides, e.g., linked or bound to each other or fused to each other.

[0132] When the multicomponent polypeptide molecules comprise a single polypeptide, they may be a fusion protein comprising one or more first co-receptor binding domain and one or more second co-receptor binding domain and one or more bridging binding domains. The binding domains may be directly fused or they may be connected via a linker, e.g., a polypeptide linker, including but not limited to any of those disclosed herein.

[0133] When the multicomponent polypeptide molecules comprise two or more polypeptides, the polypeptides may be linked via covalent bonds, such as, e.g., disulfide bonds,

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and/or noncovalent interactions. For example, heavy chains of human immunoglobulin IgG interact at the level of their CH3 domains directly, whereas, at the level of their CH2 domains, they interact via the carbohydrate attached to the asparagine (Asn) N84.4 in the DE turn. In particular embodiments, the multicomponent polypeptide molecules comprise one or more regions derived from an antibody or antigen-binding fragment thereof, e.g., antibody heavy chains or antibody light chains or fragments thereof. In certain embodiments, a surrogate polypeptide comprises two antibody heavy chain regions (e.g., hinge regions) bound together via one or more disulfide bond. In certain embodiments, a surrogate polypeptide comprises an antibody light chain region (e.g., a CL region) and an antibody heavy chain region (e.g., a CH1 region) bound together via one or more disulfide bond.

[0134] The multicomponent molecules, e.g., surrogate polypeptides, may be engineered to facilitate binding between two polypeptides. For example, Knob-into-holes amino acid modifications may be introduced into two different polypeptides to facilitate their binding. Knobs-into-holes amino acid (AA) changes is a rational design strategy developed in antibody engineering, used for heterodimerization of the heavy chains, in the production of bispecific IgG antibodies. AA changes are engineered in order to create a knob on the CH3 of the heavy chains from a first antibody and a hole on the CH3 of the heavy chains of a second antibody. The knob may be represented by a tyrosine (Y) that belongs to the 'very large' IMGT volume class of AA, whereas the hole may be represented by a threonine (T) that belongs to the 'small' IMGT volume class. Other means of introducing modifications into polypeptides to facilitate their binding are known and available in the art. For example, specific amino acids may be introduced and used for cross-linking, such as Cysteine to form an intermolecular disulfide bond.

[0135] Multicomponent molecules may have a variety of different structural formats, including but not limited to those as described in WO2019126398 and WO2020010308.

[0136] In one embodiment, a multicomponent molecule comprises an scFv or antigen-binding fragment thereof fused to a VHH or antigen-binding fragment thereof. In certain embodiments, the scFv specifically binds one or more first receptor, and the VHH specifically binds to one or more second receptor. In certain embodiments, the scFv specifically binds LRP5 and/or LRP6, and the VHH specifically binds one or more FZD receptor. In particular embodiments, the scFv or antigen-binding fragment thereof is fused directly to the VHH or antigen-binding fragment thereof, whereas in other embodiments, the two binding regions are fused via a linker moiety. In particular embodiments, the VHH is fused or linked to the N-

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terminus of the scFV, while in other embodiments, the VHH is fused to the C-terminus of the scFv.

[0137] In various embodiments, including but not limited to those depicted in WO2019126398, WO2020010308, Table 2, Table 3, and Figures 1-3, a multicomponent polypeptide molecule comprises one or more Fab or antigen-binding fragment thereof and one or more VHH or antigen- binding fragment thereof (or alternatively, one or more scFv or antigen-binding fragment thereof). In certain embodiments, the Fab specifically binds one or more FZD receptor, and the VHH (or scFv) specifically binds LRP5 and/or LRP6. In certain embodiments, the Fab specifically binds LRP5 and/or LRP6, and the VHH (or scFv) specifically binds one or more FZD receptor. In certain embodiments, the VHH (or scFv) is fused to the N- terminus of the Fab, while in some embodiments, the VHH (or scFv) is fused to the C-terminus of the Fab. In particular embodiments, the Fab is present in a full IgG format, and the VHH (or scFv) is fused to the N-terminus and/or C-terminus of the IgG light chain. In particular embodiments, the Fab is present in a full IgG format, and the VHH (or scFv) is fused to the N-terminus and/or C-terminus of the IgG heavy chain.

[0138] In particular embodiments, two or more VHHs (or scFvs) are fused to the IgG at any combination of these locations.

[0139] Fabs may be converted into a full IgG format that includes both the Fab and Fc fragments, for example, using genetic engineering to generate a fusion polypeptide comprising the Fab fused to an Fc region, i.e., the Fab is present in a full IgG format. The Fc region for the full IgG format may be derived from any of a variety of different Fcs, including but not limited to, a wild-type or modified IgG1, IgG2, IgG3, IgG4 or other isotype, e.g., wild-type or modified human IgG1, human IgG2, human IgG3, human IgG4, human IgG4Pro (comprising a mutation in core hinge region that prevents the formation of IgG4 half molecules), human IgA, human IgE, human IgM, or the modified IgG1 referred to as IgG1 LALAPG. The L235A, P329G (LALA-PG) variant has been shown to eliminate complement binding and fixation as well as Fc-γ dependent antibody-dependent cell-mediated cytotoxity (ADCC) in both murine IgG2a and human IgG1. These LALA-PG substitutions allow a more accurate translation of results generated with an "effectorless" antibody framework scaffold between mice and primates. In particular embodiments of any of the IgG disclosed herein, the IgG comprises one or more of the following amino acid substitutions: N297G, N297A, N297E, L234A, L235A, or P236G.

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[0140] Non-limiting examples of bivalent and bispecific multicomponent polypeptide molecules of co-receptors that are bivalent towards both the one or more first receptor and one or more second receptor (e.g., FZD and LRP) are provided as the top four structures depicted in WO2019126398 and WO2020010308, where the VHH or scFv is depicted in white or grey, and the Fab or IgG is depicted in black. As shown, the VHH (or scFvs) may be fused to the N-termini of both light chains, to the N-termini of both heavy chains, to the C- termini of both light chains, or to the C-termini of both heavy chains. It is further contemplated, e.g., that VHH (or scFvs) could be fused to both the N-termini and C-termini of the heavy and/or light chains, to the N-termini of the light chains and the heavy chains, to the C-termini of the heavy and light chains, to the N-termini of the heavy chains and C-termini of the light chains, or to the C-termini of the heavy chains and the N-termini of the light chains. In other related embodiments, two or more VHH (or scFvs) may be fused together, optionally via a linker moiety, and fused to the Fab or IgG at one or more of these locations. In a related embodiment, the multicomponent polypeptide molecule has a Hetero-IgG format, whereas the Fab is present as a half antibody, and one or more VHH (or scFv) is fused to one or more of the N-terminus of the Fc, the N-terminus of the Fab, the C-terminus of the Fc, or the C-terminus of the Fab. A bispecific but monovalent to each receptor version of this format is depicted at Figure 6. In certain embodiments, the Fab or antigen-binding fragment (or IgG) thereof is fused directly to the VHH (or scFv) or antigen-binding fragment thereof, whereas in other embodiments, the binding regions are fused via a linker moiety. In particular embodiments, the Fab is described herein or comprises any of the CDR sets described herein.

[0141] In various embodiments, including but not limited to those depicted in WO2019126398, WO2020010308, Table 2, Table 3, and Figures 1-3, an antigen binding molecule comprises one or more Fab or antigen-binding fragment thereof that binds one or more first receptor (e.g., FZD receptors) and one or more Fab or antigen-binding fragment thereof that binds to at least one or more second receptor (e.g., LRP5 and/or LRP6). In a particular embodiment, it comprises two Fab or antigen-binding fragments thereof that bind one or more first co-receptor and/or two Fab or antigen-binding fragments thereof that bind to one or more second co-receptor. In further embodiments, one or more of the Fab is present in a full IgG format, and in certain embodiments, both Fab are present in a full IgG format. In certain embodiments, the Fab in full IgG format specifically binds one or more first receptor (e.g., one or more FZD receptor), and the other Fab specifically binds at least one second receptor (e.g., LRP5 and/or LRP6). For example, the Fab specifically binds one or more FZD

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receptor, and the Fab in full IgG format specifically binds LRP5 and/or LRP6. In certain embodiments, the Fab specifically binds LRP5 and/or LRP6, and the Fab in full IgG format specifically binds one or more FZD receptor. In certain embodiments, the Fab is fused to the N-terminus of the IgG, e.g., to the heavy chain or light chain N-terminus, optionally via a linker. In certain embodiments, the Fab is fused to the N-terminus of the heavy chain of the IgG and not fused to the light chain. In particular embodiments, the two heavy chains can be fused together directly or via a linker. An example of such a bispecific and bivalent with respect to both receptors is shown in Figure 1A. In other related embodiments, two or more VHHs may be fused together, optionally via a linker moiety, and fused to the Fab or IgG at one or more of these locations. In a related embodiment, the WNT multicomponent polypeptide molecule has a Hetero-IgG format, whereas one of the Fab is present as a half antibody, and the other Fab is fused to one or more of the N-terminus of the Fc, the N-terminus of the Fab, or the C- terminus of the Fc. A bispecific but monovalent to each receptor version of this format is depicted at Figure 6. In certain embodiments, the Fab or antigen-binding fragment thereof is fused directly to the other Fab or IgG or antigen-binding fragment thereof, whereas in other embodiments, the binding regions are fused via a linker moiety. In particular embodiments, the one or both of the two Fabs are described herein or comprise any of the CDR sets described herein.

[0142] In certain embodiments, the antigen binding molecules have a format as described in PCT Application Publication No. WO2017/136820, e.g., a Fabs- in-tandem IgG (FIT-IG) format. Shiyong Gong, Fang Ren, Danqing Wu, Xuan Wu & Chengbin Wu (2017). FIT-IG also include the formats disclosed in, e.g., Gong, et al (2017) *mAbs* 9:118-1128. In certain embodiments, FIT-IGs combine the functions of two antibodies into one molecule by re-arranging the DNA sequences of two parental monoclonal antibodies into two or three constructs and co-expressing them in mammalian cells. Examples of FIT-IG formats and constructs are provided in FIGS. 1A and 1B and FIGS. 2A and 2B of PCT Application Publication No. WO2017/136820. In certain embodiments, FIT-IGs require no Fc mutation; no scFv elements; and no linker or peptide connector. The Fab-domains in each arm work "in tandem" forming a tetravalent bi-specific antibody with four active and independent antigen binding sites that retain the biological function of their parental antibodies In particular embodiments, WNT surrogates comprises a Fab and an IgG. In certain embodiments, the Fab binder LC is fused to the HC of the IgG, e.g., by a linker of various length in between. In various embodiment, the Fab binder HC can be fused or unfused to the LC of the IgG. A variation of this format has been called Fabs-in-tandem IgG (or FIT-Ig).

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[0143] In certain embodiments, the WNT multicomponent polypeptide molecules have a format described in PCT Application Publication No. WO2009/080251 (Klein et al.), e.g., a CrossMab format. CrossMabs formats are also described in Schaefer et al. (2011) *Proc. Natl. Acad. Sci USA* 108:11187-11192. The CrossMab format allows correct assembly of two heavy chains and two light chains derived from existing antibodies to form a bispecific, bivalent IgG antibodies. The technology is based on the cross over the antibody domain within one Fab-arm of a bispecific IgG antibody in order to enable correct chain association. Various portions of the Fab can be exchanged, e.g., the entire Fab, the variable heavy and light chains, or the CH1-CL chains can be exchanged.

[0144] In further embodiments of the present invention, the FiT-Ig and CrossMab technologies are combined to create a multispecific, multivalent antigen binding molecule, Cross-FiT, as depicted in Figure 1A and Table 2. Also contemplated is a linker between the crossed CL domain of the Fab and the Ig domains rather than between the CH1 and Ig domains. Additional antigen binding fragments, e.g., Fabs, VHH/sdAbs, and/or scFvs, can be appended to the Cross-FiT structure at various sites, e.g., the heavy or light chains and/or the C-terminus of the Fc domain to create multispecific antibodies.

[0145] In particular embodiments, multicomponent polypeptide molecules comprise two or more VHHs/sdAbs (or scFvs), including at least one that binds one or more first receptor and at least one that binds at least one second receptor. In certain embodiments, one of the binding regions is a VHH/sdAbs and the other is an scFv. Multicomponent polypeptide molecules comprising two or more VHH/sdAbs (or scFvs) may be formatted in a variety of configurations, including but not limited to those depicted in WO2019126398 and WO2020010308 . In certain bispecific, bivalent formats, two or more VHH/sdAbs (or scFvs) are fused in tandem or fused to two different ends of an Fc, optionally via one or more linkers. Where linkers are present, the linker and its length may be the same or different between the VHH/sdAb (or scFv) and the other VHH/sdAb (or scFv), or between the VHH and Fc. For example, in certain embodiments, the VHH/sdAb is fused to the N-terminus, at either the heavy or light chain, and/or C-terminus of the IgG heavy chain. In particular embodiments, two or more VHH/sdAbs are fused to the IgG at any combination of these locations. In various embodiments, both VHH/sdAbs may be fused to the N-termini of the Fc, to the C-termini of the Fc, or one or more VHH/sdAb may be fused to either or both of an N-terminus or C-terminus of the Fc. In a related embodiment, the multicomponent polypeptide molecule has a Hetero-IgG format, whereas one VHH/sdAb is present as a half antibody, and the other is fused

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to the N-terminus of the Fc or the C-terminus of the Fc.. In certain embodiments, the VHH/sdAb is fused directly to the other VHH/sdAb whereas in other embodiments, the binding regions are fused via a linker moiety. In particular embodiments, the VHH/sdAb are described herein or comprises any of the CDR sets described herein. In various embodiments, any of these formats may comprise one or more scFvs in place of one or more VHH/sdAbs.

[0146] In certain embodiments, a multicomponent polypeptide molecule is formatted as a diabody. The binders against the two co-receptors can also be linked together in a diabody (or DART) configuration. The diabody can also be in a single chain configuration. If the diabody is fused to an Fc, this will create a bivalent bispecific format. Without fusion to Fc, this would be a monovalent bispecific format. In certain embodiments, a diabody is a noncovalent dimer scFv fragment that consists of the heavy-chain variable (VH) and light-chain variable (VL) regions connected by a small peptide linker. Another form of diabody is a single-chain (Fv)2 in which two scFv fragments are covalently linked to each other.

[0147] As discussed, the multicomponent polypeptide molecules, in various embodiments, comprise one or more antibodies or antigen-binding fragments thereof disclosed herein. Thus, in particular embodiments, the surrogate comprises two polypeptides, wherein each polypeptide comprises an Nab or scFv that binds at least one first co-receptor and an Nab or scFv that binds at least one second co-receptor, optionally wherein one of the binding domains is an scFv and the other is an Nab. In certain embodiments, a surrogate comprises three polypeptides, wherein the first polypeptide comprises an antibody heavy chain and the second polypeptide comprises an antibody light chain, wherein the antibody heavy chain and light chain bind either receptor, and wherein the third polypeptide comprises a VHH/sdAb fused to a heavy chain Fc region or the light chain of the antibody, wherein the VHH/sdAb binds to either co-receptor. In other embodiments, the surrogates comprise four polypeptides, including two heavy chain polypeptides and two light chain polypeptides, wherein the two heavy chains and two light chains bind one or more first receptor, and further comprise one or more VHH/sdAb or scFv fused to one or more of the heavy chains and/or light chains, wherein the VHH/sdAb or scFv binds to one or more second co-receptor. In an illustrative embodiment, a WNT surrogate comprises at least four polypeptides, including two heavy chain polypeptides and two light chain polypeptides that bind either LRP5/6 or one or more FZDs, wherein the WNT surrogate further comprises a Fab that binds either LRP5/6 or one or more FZDs. For example, the Fab may comprise two polypeptides, each fused to one of the two heavy chain polypeptides, and two polypeptides, each fused to one of the two light chain polypeptides, or

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it may comprise two polypeptides each fused to one of the two heavy chain polypeptides and two additional polypeptides, each bound to one of the two polypeptides fused to the heavy chain polypeptides, thus making a second Fab. Other configurations disclosed herein may be used to produce different multicomponent polypeptide molecules.

[0148] Also contemplated are Ig molecules where the VL and VH domains of one Ig are appended with the VL and VH domains of a second antibody. This format is call Fv-Ig or 2Fv-Ig for a homodimer. The VL and VH domains from the second Ig are appended to the N-terminus of the VL and VH domains of the first Ig via short peptide linkers. This format preserves the natural antibody's avidity to cell surface receptors or to more than one receptor or co-receptor complexes (see, e.g., Wu, et al (2007) *Nature Biotechnol.* 25:1290-1297).

[0149] In certain embodiments, the antigen binding formats are multicomponent polypeptide molecules that comprise one or more polypeptides comprising two or more binding regions. For illustrative purposes, the two or more binding regions may be a first receptor binding regions or a second receptor binding regions, or they may comprise one or more first receptor binding region and one or more second receptor binding region. The binding regions may be directly joined or contiguous, or may be separated by a linker, e.g. a polypeptide linker, or a non-peptidic linker, etc. The length of the linker, and therefore the spacing between the binding domains can be used to modulate the signal strength, and can be selected depending on the desired use of the multicomponent polypeptide molecule. The enforced distance between binding domains can vary, but in certain embodiments may be less than about 100 angstroms, less than about 90 angstroms, less than about 80 angstroms, less than about 70 angstroms, less than about 60 angstroms, or less than about 50 angstroms. In some embodiments the linker is a rigid linker, in other embodiments the linker is a flexible linker. In certain embodiments where the linker is a peptide linker, it may be from about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 or more amino acids in length, and is of sufficient length and amino acid composition to enforce the distance between binding domains. In some embodiments, the linker comprises or consists of one or more glycine and/or serine residues.

[0150] The multicomponent polypeptide molecule can be multimerized, e.g., through an Fc domain, by concatenation, coiled coils, polypeptide zippers, biotin/avidin or streptavidin multimerization, and the like. The multicomponent polypeptide molecules can also be joined to a moiety such as PEG, Fc, etc., as known in the art to enhance stability in vivo.

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[0151] In certain embodiments, a multicomponent polypeptide molecule enhances or increases the co-receptors pathway signaling, e.g., in the case of WNT - β-catenin signaling, by at least 30%, 35%, 40%, 45%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 100%,110%, 150%, 200%, 250%, 300%, 400% or 500%, as compared to the β- catenin signaling induced by a neutral substance or negative control as measured in an assay described above, for example as measured in the TOPFlash assay (see, e.g., Molinaar (1996) *Cell* 86:391-399). A negative control may be included in these assays. By way of example, WNT multicomponent polypeptide molecules may enhance β-catenin signaling by a factor of 2x, 5x, 10x, 100x, 1000x, 10000x or more as compared to the activity in the absence of the WNT multicomponent polypeptide molecule when measured, for example when measured in the TOPFlash assay.

[0152] In certain embodiments, functional properties of the multicomponent polypeptide molecules may be assessed using a variety of methods known to the skilled person, including e.g., affinity/binding assays (for example, surface plasmon resonance, competitive inhibition assays), cytotoxicity assays, cell viability assays, cell proliferation or differentiation assays in response to the native molecule/ligand, cancer cell and/or tumor growth inhibition using in vitro or in vivo models, including but not limited to any described herein. The multicomponent polypeptide molecules may also be tested for effects on one or both co-receptor internalization, in vitro and in vivo efficacy, etc. Such assays may be performed using well-established protocols known to the skilled person (see e.g., Current Protocols in Molecular Biology (Greene Publ. Assoc. Inc. & John Wiley & Sons, Inc., NY, NY); Current Protocols in Immunology (Edited by: John E. Coligan, Ada M. Kruisbeek, David H. Margulies, Ethan M. Shevach, Warren Strober 2001 John Wiley & Sons, NY, NY); or commercially available kits.

[0153] In certain embodiments, a binding region of a multicomponent polypeptide molecule (e.g., an antigen-binding fragment of an anti-FZD antibody) comprises one or more of the CDRs of the anti-co-receptor antibodies. In this regard, it has been shown in some cases that the transfer of only the VHCDR3 of an antibody can be performed while still retaining desired specific binding (Barbas et al., PNAS (1995) 92: 2529-2533). See also, McLane et al., PNAS (1995) 92:5214- 5218, Barbas et al., J. Am. Chem. Soc. (1994) 116:2161-2162).

[0154] Also disclosed herein is a method for obtaining an antibody or antigen binding domain specific for a co-receptor, the method comprising providing by way of addition, deletion, substitution or insertion of one or more amino acids in the amino acid sequence of a VH domain set out herein or a VH domain which is an amino acid sequence variant of the VH domain, optionally combining the VH domain thus provided with one or more VL domains,

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and testing the VH domain or VH/VL combination or combinations to identify a specific binding member or an antibody antigen binding domain specific for one or more co-receptors and optionally with one or more desired properties. The VL domains may have an amino acid sequence which is substantially as set out herein. An analogous method may be employed in which one or more sequence variants of a VL domain disclosed herein are combined with one or more VH domains.

[0155] Immunological binding generally refers to the non-covalent interactions of the type which occur between an immunoglobulin molecule and an antigen for which the immunoglobulin is specific, for example by way of illustration and not limitation, as a result of electrostatic, ionic, hydrophilic and/or hydrophobic attractions or repulsion, steric forces, hydrogen bonding, van der Waals forces, and other interactions. The strength, or affinity of immunological binding interactions can be expressed in terms of the dissociation constant (Kd) of the interaction, wherein a smaller Kd represents a greater affinity. Immunological binding properties of selected polypeptides can be quantified using methods well known in the art. One such method entails measuring the rates of antigen-binding site/antigen complex formation and dissociation, wherein those rates depend on the concentrations of the complex partners, the affinity of the interaction, and on geometric parameters that equally influence the rate in both directions. Thus, both the "on rate constant" (Kon) and the "off rate constant" (Koff) can be determined by calculation of the concentrations and the actual rates of association and dissociation. The ratio of Koff /Kon enables cancellation of all parameters not related to affinity, and is thus equal to the dissociation constant Kd. See, generally, Davies et al. (1990) Annual Rev. Biochem. 59:439-473.

[0156] In certain embodiments, the multicomponent polypeptide molecules or binding regions thereof described herein have an affinity of less than about 10,000, less than about 1000, less than about 100, less than about 10, less than about 1 or less than about 0.1 nM, and in some embodiments, the antibodies may have even higher affinity for one or more co-receptors.

[0157] The constant regions of immunoglobulins show less sequence diversity than the variable regions, and are responsible for binding a number of natural proteins to elicit important biochemical events. In humans, there are five different classes of antibodies including IgA (which includes subclasses IgA1 and IgA2), IgD, IgE, IgG (which includes subclasses IgG1, IgG2, IgG3, and IgG4), and IgM. The distinguishing features between these antibody classes are their constant regions, although subtler differences may exist in the V region.

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[0158] The Fc region of an antibody interacts with a number of Fc receptors and ligands, imparting an array of important functional capabilities referred to as effector functions. For IgG, the Fc region comprises Ig domains CH2 and CH3 and the N-terminal hinge leading into CH2. An important family of Fc receptors for the IgG class are the Fc gamma receptors (FcγRs). These receptors mediate communication between antibodies and the cellular arm of the immune system (Raghavan et al., 1996, Annu Rev Cell Dev Biol 12:181- 220; Ravetch et al., 2001, Annu Rev Immunol 19:275-290). In humans this protein family includes FcγRI (CD64), including isoforms FcγRIa, FcγRIb, and FcγRIc; FcγRII (CD32), including isoforms FcγRIIa (including allotypes H131 and R131), FcγRIIb (including FcγRIIb-1 and FcγRIIb-2), and FcγRIIc; and FcγRIII (CD16), including isoforms FcγRIIIa (including allotypes V158 and F158) and FcγRIIIb (including allotypes FcγRIIIb-NA1 and FcγRIIIb-NA2) (Jefferis et al., 2002, Immunol Lett 82:57-65). These receptors typically have an extracellular domain that mediates binding to Fc, a membrane spanning region, and an intracellular domain that may mediate some signaling event within the cell. These receptors are expressed in a variety of immune cells including monocytes, macrophages, neutrophils, dendritic cells, eosinophils, mast cells, platelets, B cells, large granular lymphocytes, Langerhans' cells, natural killer (NK) cells, and T cells. Formation of the Fc/FcγR complex recruits these effector cells to sites of bound antigen, typically resulting in signaling events within the cells and important subsequent immune responses such as release of inflammation mediators, B cell activation, endocytosis, phagocytosis, and cytotoxic attack.

[0159] The ability to mediate cytotoxic and phagocytic effector functions is a potential mechanism by which antibodies destroy targeted cells. The cell- mediated reaction wherein nonspecific cytotoxic cells that express FcγRs recognize bound antibody on a target cell and subsequently cause lysis of the target cell is referred to as antibody dependent cell-mediated cytotoxicity (ADCC) (Raghavan et al., 1996, Annu Rev Cell Dev Biol 12:181-220; Ghetie et al., 2000, Annu Rev Immunol 18:739-766; Ravetch et al., 2001, Annu Rev Immunol 19:275-290). The cell-mediated reaction wherein nonspecific cytotoxic cells that express FcγRs recognize bound antibody on a target cell and subsequently cause phagocytosis of the target cell is referred to as antibody dependent cell-mediated phagocytosis (ADCP). All FcγRs bind the same region on Fc, at the N-terminal end of the Cg2 (CH2) domain and the preceding hinge. This interaction is well characterized structurally (Sondermann et al., 2001, J Mol Biol 309:737-749), and several structures of the human Fc bound to the extracellular domain of human FcγRIIIb have been solved (pdb accession code 1E4K) (Sondermann et al., 2000,

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Nature 406:267- 273.) (pdb accession codes 1IIS and 1IIX) (Radaev et al., 2001, J Biol Chem 276:16469-16477.)

[0160] The different IgG subclasses have different affinities for the FcγRs, with IgG1 and IgG3 typically binding substantially better to the receptors than IgG2 and IgG4 (Jefferis et al., 2002, Immunol Lett 82:57-65). All FcγRs bind the same region on IgG Fc, yet with different affinities: the high affinity binder FcγRI has a Kd for IgG1 of 10^{-8} M^{-1}, whereas the low affinity receptors FcγRII and FcγRIII generally bind at 10^{-6} and 10^{-5} respectively. The extracellular domains of FcγRIIIa and FcγRIIIb are 96% identical; however, FcγRIIIb does not have an intracellular signaling domain. Furthermore, whereas FcγRI, FcγRIIa/c, and FcγRIIIa are positive regulators of immune complex-triggered activation, characterized by having an intracellular domain that has an immunoreceptor tyrosine-based activation motif (ITAM), FcγRIIb has an immunoreceptor tyrosine-based inhibition motif (ITIM) and is therefore inhibitory. Thus the former are referred to as activation receptors, and FcγRIIb is referred to as an inhibitory receptor. The receptors also differ in expression pattern and levels on different immune cells. Yet another level of complexity is the existence of a number of FcγR polymorphisms in the human proteome. A particularly relevant polymorphism with clinical significance is V158/F158 FcγRIIIa. Human IgG1 binds with greater affinity to the V158 allotype than to the F158 allotype. This difference in affinity, and presumably its effect on ADCC and/or ADCP, has been shown to be a significant determinant of the efficacy of the anti-CD20 antibody rituximab (Rituxan®, a registered trademark of IDEC Pharmaceuticals Corporation). Subjects with the V158 allotype respond favorably to rituximab treatment; however, subjects with the lower affinity F158 allotype respond poorly (Cartron et al., 2002, Blood 99:754-758). Approximately 10-20% of humans are V158/V158 homozygous, 45% are V158/F158 heterozygous, and 35-45% of humans are F158/F158 homozygous (Lehrnbecher et al., 1999, Blood 94:4220-4232; Cartron et al., 2002, Blood 99:754-758). Thus 80-90% of humans are poor responders,, they have at least one allele of the F158 FcγRIIIa.

[0161] The Fc region is also involved in activation of the complement cascade. In the classical complement pathway, C1 binds with its C1q subunits to Fc fragments of IgG or IgM, which has formed a complex with antigen(s). In certain embodiments of the invention, modifications to the Fc region comprise modifications that alter (either enhance or decrease) the ability of a FZD-specific antibody as described herein to activate the complement system (see e.g., U.S. Patent 7,740,847). To assess complement activation, a complement-dependent

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cytotoxicity (CDC) assay may be performed (See, e.g., Gazzano- Santoro et al., J. Immunol. Methods, 202:163 (1996).

[0162] Thus in certain embodiments, the present invention provides the multicomponent polypeptide molecules having a modified Fc region with altered functional properties, such as reduced or enhanced CDC, ADCC, or ADCP activity, or enhanced binding affinity for a specific FcγR or increased serum half-life. Other modified Fc regions contemplated herein are described, for example, in issued U.S. Patents 7,317,091; 7,657,380; 7,662,925; 6,538,124; 6,528,624; 7,297,775; 7,364,731; Published U.S. Applications US2009092599; US20080131435; US20080138344; and published International Applications WO2006/105338; WO2004/063351; WO2006/088494; WO2007/024249.

[0163] Structurally, the Fc region can be important for proper assembly of the msAb. In particular, modifications to the CH3 domain such as knobs-in-hole (see, e.g., WO1996/027011; and WO1998/050431) or Azymetric mutations (see, e.g., WO2012/58768) can prevent heavy chain mispairing. The present invention utilizes these mutations in certain Fc embodiments.

[0164] The multicomponent molecules disclosed herein may also be modified to include an epitope tag or label, e.g., for use in purification or diagnostic applications. There are many linking groups known in the art for making antibody conjugates, including, for example, those disclosed in U.S. Pat. No. 5,208,020 or EP Patent 0 425 235 B1, and Chari et al., Cancer Research 52: 127-131 (1992). The linking groups include disulfide groups, thioether groups, acid labile groups, photolabile groups, peptidase labile groups, or esterase labile groups, as disclosed in the above-identified patents, disulfide and thioether groups being preferred.

[0165] In certain embodiments, and antigen- binding fragments thereof against one co-receptor and/or antibodies and antigen-binding fragments thereof against the other co-receptor present within a multicomponent polypeptide molecule are monoclonal. In certain embodiments, they are humanized.

[0166] The present invention further provides in certain embodiments an isolated nucleic acid encoding a polypeptide present in a multicomponent polypeptide molecule. Nucleic acids include DNA and RNA. These and related embodiments may include polynucleotides encoding antibody fragments that bind one or more co-receptors. The term "isolated polynucleotide" as used herein shall mean a polynucleotide of genomic, cDNA, or synthetic origin, or some combination thereof, which by virtue of its origin, the isolated polynucleotide: (1) is not associated with all or a portion of a polynucleotide in which the isolated polynucleotide is found

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in nature; (2) is linked to a polynucleotide to which it is not linked in nature, or (3) does not occur in nature as part of a larger sequence. An isolated polynucleotide may include naturally occurring and/or artificial sequences.

[0167] As will be understood by those skilled in the art, polynucleotides may include genomic sequences, extra-genomic and plasmid-encoded sequences and smaller engineered gene segments that express, or may be adapted to express, proteins, polypeptides, peptides and the like. Such segments may be naturally isolated, or modified synthetically by the skilled person.

[0168] As will be also recognized by the skilled artisan, polynucleotides may be single-stranded (coding or antisense) or double-stranded, and may be DNA (genomic, cDNA or synthetic) or RNA molecules. RNA molecules may include HnRNA molecules, which contain introns and correspond to a DNA molecule in a one-to-one manner, and mRNA molecules, which do not contain introns. Additional coding or non-coding sequences may, but need not, be present within a polynucleotide according to the present disclosure, and a polynucleotide may, but need not, be linked to other molecules and/or support materials. Polynucleotides may comprise a native sequence or may comprise a sequence that encodes a variant or derivative of such a sequence.

[0169] It will be appreciated by those of ordinary skill in the art that, as a result of the degeneracy of the genetic code, there are many nucleotide sequences that encodes an antibody as described herein. Some of these polynucleotides bear minimal sequence identity to the nucleotide sequence of the native or original polynucleotide sequence encoding a polypeptide within a multicomponent polypeptide molecule. Nonetheless, polynucleotides that vary due to differences in codon usage are expressly contemplated by the present disclosure. In certain embodiments, sequences that have been codon- optimized for mammalian expression are specifically contemplated.

[0170] Therefore, in another embodiment of the invention, a mutagenesis approach, such as site-specific mutagenesis, may be employed for the preparation of variants and/or derivatives of the polypeptides described herein. By this approach, specific modifications in a polypeptide sequence can be made through mutagenesis of the underlying polynucleotides that encode them. These techniques provide a straightforward approach to prepare and test sequence variants, for example, incorporating one or more of the foregoing considerations, by introducing one or more nucleotide sequence changes into the polynucleotide.

[0171] Site-specific mutagenesis allows the production of mutants through the use of specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Mutations may be employed in a selected polynucleotide sequence to improve, alter, decrease, modify, or otherwise change the properties of the polynucleotide itself, and/or alter the properties, activity, composition, stability, or primary sequence of the encoded polypeptide.

[0172] In certain embodiments, the inventors contemplate the mutagenesis of the polynucleotide sequences that encode a polypeptide present in a multicomponent polypeptide molecule, to alter one or more properties of the encoded polypeptide, such as the binding affinity, or the function of a particular Fc region, or the affinity of the Fc region for a particular FcγR. The techniques of site-specific mutagenesis are well-known in the art, and are widely used to create variants of both polypeptides and polynucleotides. For example, site- specific mutagenesis is often used to alter a specific portion of a DNA molecule. In such embodiments, a primer comprising typically about 14 to about 25 nucleotides or so in length is employed, with about 5 to about 10 residues on both sides of the junction of the sequence being altered.

[0173] As will be appreciated by those of skill in the art, site-specific mutagenesis techniques have often employed a phage vector that exists in both a single stranded and double stranded form. Typical vectors useful in site- directed mutagenesis include vectors such as the M13 phage. These phages are readily commercially-available and their use is generally well-known to those skilled in the art. Double-stranded plasmids are also routinely employed in site directed mutagenesis that eliminates the step of transferring the gene of interest from a plasmid to a phage.

[0174] The preparation of sequence variants of the selected peptide- encoding DNA segments using site-directed mutagenesis provides a means of producing potentially useful species and is not meant to be limiting as there are other ways in which sequence variants of peptides and the DNA sequences encoding them may be obtained. For example, recombinant vectors encoding the desired peptide sequence may be treated with mutagenic agents, such as hydroxylamine, to obtain sequence variants. Specific details regarding these methods and protocols are found in the teachings of Maloy et al., 1994; Segal, 1976; Prokop and Bajpai, 1991; Kuby, 1994; and Maniatis et al., 1982, each incorporated herein by reference, for that purpose.

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[0175] In many embodiments, one or more nucleic acids encoding a polypeptide of multicomponent WNT molecule are introduced directly into a host cell, and the cell incubated under conditions sufficient to induce expression of the encoded polypeptides. The surrogate polypeptides of this disclosure may be prepared using standard techniques well known to those of skill in the art in combination with the polypeptide and nucleic acid sequences provided herein. The polypeptide sequences may be used to determine appropriate nucleic acid sequences encoding the particular polypeptide disclosed thereby. The nucleic acid sequence may be optimized to reflect particular codon "preferences" for various expression systems according to standard methods well known to those of skill in the art.

[0176] According to certain related embodiments there is provided a recombinant host cell which comprises one or more constructs as described herein, e.g., a vector comprising a nucleic acid encoding a multicomponent polypeptide molecule or polypeptide thereof; and a method of production of the encoded product, which method comprises expression from encoding nucleic acid therefor. Expression may conveniently be achieved by culturing under appropriate conditions recombinant host cells containing the nucleic acid. Following production by expression, an antibody or antigen-binding fragment thereof, may be isolated and/or purified using any suitable technique, and then used as desired.

[0177] Polypeptides, and encoding nucleic acid molecules and vectors, may be isolated and/or purified, e.g. from their natural environment, in substantially pure or homogeneous form, or, in the case of nucleic acid, free or substantially free of nucleic acid or genes of origin other than the sequence encoding a polypeptide with the desired function. Nucleic acid may comprise DNA or RNA and may be wholly or partially synthetic. Reference to a nucleotide sequence as set out herein encompasses a DNA molecule with the specified sequence, and encompasses a RNA molecule with the specified sequence in which U is substituted for T, unless context requires otherwise.

[0178] Systems for cloning and expression of a polypeptide in a variety of different host cells are well known. Suitable host cells include bacteria, mammalian cells, yeast and baculovirus systems. Mammalian cell lines available in the art for expression of a heterologous polypeptide include Chinese hamster ovary cells, HeLa cells, baby hamster kidney cells, NSO mouse melanoma cells and many others. A common, preferred bacterial host is E. coli.

[0179] The expression of polypeptides, e.g., antibodies and antigen- binding fragments thereof, in prokaryotic cells such as E. coli is well established in the art. For a review, see for

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example Pluckthun, A. Bio/Technology 9: 545-551 (1991). Expression in eukaryotic cells in culture is also available to those skilled in the art as an option for production of antibodies or antigen-binding fragments thereof, see recent reviews, for example Ref, M. E. (1993) Curr. Opinion Biotech. 4: 573-576; Trill J. J. et al. (1995) Curr. Opinion Biotech 6: 553-560.

[0180] Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator sequences, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. Vectors may be plasmids, viral e.g. phage, or phagemid, as appropriate. For further details see, for example, Molecular Cloning: a Laboratory Manual: 2nd edition, Sambrook et al., 1989, Cold Spring Harbor Laboratory Press. Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in Current Protocols in Molecular Biology, Second Edition, Ausubel et al. eds., John Wiley & Sons, 1992, or subsequent updates thereto.

[0181] The present invention also provides, in certain embodiments, a method which comprises using a construct as stated above in an expression system in order to express a particular polypeptide such as a WNT chimeric activator molecule. The term "transduction" is used to refer to the transfer of genes from one bacterium to another, usually by a phage.

[0182] Amino acid sequence modification(s) of any of the polypeptides described herein are contemplated. For example, it may be desirable to improve the binding affinity and/or other biological properties of the multicomponent polypeptide molecule. For example, amino acid sequence variants of a multicomponent polypeptide molecule may be prepared by introducing appropriate nucleotide changes into a polynucleotide that encodes the antibody, or a chain thereof, or by peptide synthesis. Such modifications include, for example, deletions from, and/or insertions into and/or substitutions of, residues within the amino acid sequences of the antibody. Any combination of deletion, insertion, and substitution may be made to arrive at the final multicomponent polypeptide molecule, provided that the final construct possesses the desired characteristics (e.g., high affinity binding to one or more co-receptors). The amino acid changes also may alter post-translational processes of the antibody, such as changing the number or position of glycosylation sites. Any of the variations and modifications described above for polypeptides of the present invention may be included in antibodies of the present invention.

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[0183] The present disclosure provides variants of any of the polypeptides (e.g., multicomponent molecule, multicomponent polypeptide molecules, or antibodies or antigen-binding fragments thereof) disclosed herein. In certain embodiments, a variant has at least 90%, at least 95%, at least 98%, or at least 99% identity to a polypeptide disclosed herein. In certain embodiments, such variant polypeptides bind to one or more first co-receptors, and/or to one or more second co-receptors, at least about 50%, at least about 70%, and in certain embodiments, at least about 90% as well as a t multicomponent polypeptide molecule specifically set forth herein. In further embodiments, such variant multicomponent polypeptide molecules bind to one or more first co-receptor, and/or to one or more second co-receptor, with greater affinity than the multicomponent polypeptide molecules set forth herein, for example, that bind quantitatively at least about 105%, 106%, 107%, 108%, 109%, or 110% as well as an antibody sequence specifically set forth herein.

[0184] In particular embodiments, the multicomponent polypeptide molecule or a binding region thereof, e.g., a Fab, scFv, or VHH may comprise: a) a heavy chain variable region comprising: i. a CDR1 region that is identical in amino acid sequence to the heavy chain CDR1 region of a selected antibody described herein; ii. a CDR2 region that is identical in amino acid sequence to the heavy chain CDR2 region of the selected antibody; and iii. a CDR3 region that is identical in amino acid sequence to the heavy chain CDR3 region of the selected antibody; and/or b) a light chain variable domain comprising: i. a CDR1 region that is identical in amino acid sequence to the light chain CDR1 region of the selected antibody; ii. a CDR2 region that is identical in amino acid sequence to the light chain CDR2 region of the selected antibody; and iii. a CDR3 region that is identical in amino acid sequence to the light chain CDR3 region of the selected antibody; wherein the antibody specifically binds a selected target. In a further embodiment, the antibody, or antigen-binding fragment thereof, is a variant antibody or antigen-binding fragment thereof wherein the variant comprises a heavy and light chain identical to the selected antibody except for up to 8, 9, 10, 11, 12, 13, 14, 15, or more total amino acid substitutions in the CDR regions of the VH and VL regions. In this regard, there may be 1, 2, 3, 4, 5, 6, 7, 8, or in certain embodiments, 9, 10, 11, 12, 13, 14, 15 more total amino acid substitutions in the (collective) CDR regions of the selected antibody. Substitutions may be in CDRs either in the VH and/or the VL regions. (See e.g., Muller, 1998, Structure 6:1153- 1167).

[0185] In particular embodiments, the multicomponent polypeptide molecule or a binding region thereof, e.g., a Fab, scFv, or VHH/sdAb, may have: a) a heavy chain variable region

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having an amino acid sequence that is at least 80% identical, at least 95% identical, at least 90%, at least 95% or at least 98% or 99% identical, to the heavy chain variable region of an antibody or antigen- binding fragments thereof described herein; and/or b) a light chain variable region having an amino acid sequence that is at least 80% identical, at least 85%, at least 90%, at least 95% or at least 98% or 99% identical, to the light chain variable region of an antibody or antigen-binding fragments thereof described herein.

[0186] A polypeptide has a certain percent "sequence identity" to another polypeptide, meaning that, when aligned, that percentage of amino acids are the same when comparing the two sequences. Sequence similarity can be determined in a number of different manners. To determine sequence identity, sequences can be aligned using the methods and computer programs, including BLAST, available over the world wide web at ncbi.nlm.nih.gov/BLAST/. Another alignment algorithm is FASTA, available in the Genetics Computing Group (GCG) package, from Madison, Wis., USA, a wholly owned subsidiary of Oxford Molecular Group, Inc. Other techniques for alignment are described in Methods in Enzymology, vol. 266: Computer Methods for Macromolecular Sequence Analysis (1996), ed. Doolittle, Academic Press, Inc., a division of Harcourt Brace & Co., San Diego, Calif., USA. Of particular interest are alignment programs that permit gaps in the sequence. The Smith-Waterman is one type of algorithm that permits gaps in sequence alignments. See Meth. Mol. Biol. 70: 173-187 (1997). Also, the GAP program using the Needleman and Wunsch alignment method can be utilized to align sequences. See J. Mol. Biol. 48: 443-453 (1970).

[0187] Of interest is the BestFit program using the local homology algorithm of Smith and Waterman (Advances in Applied Mathematics 2: 482- 489 (1981) to determine sequence identity. The gap generation penalty will generally range from 1 to 5, usually 2 to 4 and in many embodiments will be 3. The gap extension penalty will generally range from about 0.01 to 0.20 and in many instances will be 0.10. The program has default parameters determined by the sequences inputted to be compared. Preferably, the sequence identity is determined using the default parameters determined by the program. This program is available also from Genetics Computing Group (GCG) package, from Madison, Wis., USA.

[0188] Another program of interest is the FastDB algorithm. FastDB is described in Current Methods in Sequence Comparison and Analysis, Macromolecule Sequencing and Synthesis, Selected Methods and Applications, pp. 127-149, 1988, Alan R. Liss, Inc. Percent sequence identity is calculated by FastDB based upon the following parameters: Mismatch Penalty: 1.00; Gap Penalty: 1.00; Gap Size Penalty: 0.33; and Joining Penalty: 30.0.

[0189] In particular embodiments, the multicomponent polypeptide molecule or a binding region thereof, e.g., a Fab, scFv, or VHH may comprise: a) a heavy chain variable region comprising: i. a CDR1 region that is identical in amino acid sequence to the heavy chain CDR1 region of a selected antibody described herein; ii. a CDR2 region that is identical in amino acid sequence to the heavy chain CDR2 region of the selected antibody; and iii. a CDR3 region that is identical in amino acid sequence to the heavy chain CDR3 region of the selected antibody; and b) a light chain variable domain comprising: i. a CDR1 region that is identical in amino acid sequence to the light chain CDR1 region of the selected antibody; ii. a CDR2 region that is identical in amino acid sequence to the light chain CDR2 region of the selected antibody; and iii. a CDR3 region that is identical in amino acid sequence to the light chain CDR3 region of the selected antibody; wherein the antibody specifically binds a selected target (e.g., a FZD receptor, such as FZD1). In a further embodiment, the antibody, or antigen-binding fragment thereof, is a variant antibody wherein the variant comprises a heavy and light chain identical to the selected antibody except for up to 8, 9, 10, 11, 12, 13, 14, 15, or more amino acid substitutions in the CDR regions of the VH and VL regions. In this regard, there may be 1, 2, 3, 4, 5, 6, 7, 8, or in certain embodiments, 9, 10, 11, 12, 13, 14, 15 more amino acid substitutions in the CDR regions of the selected antibody. Substitutions may be in CDRs either in the VH and/or the VL regions. (See e.g., Muller, 1998, Structure 6:1153-1167).

[0190] Determination of the three-dimensional structures of representative polypeptides (e.g., variant FZD binding regions or LRP5/6 binding regions and bridging regions of WNT multicomponent polypeptide molecules as provided herein) may be made through routine methodologies such that substitution, addition, deletion or insertion of one or more amino acids with selected natural or non-natural amino acids can be virtually modeled for purposes of determining whether a so derived structural variant retains the space-filling properties of presently disclosed species. See, for instance, Donate et al., 1994 Prot. Sci. 3:2378; Bradley et al., Science 309: 1868-1871 (2005); Schueler-Furman et al., Science 310:638 (2005); Dietz et al., Proc. Nat. Acad. Sci. USA 103:1244 (2006); Dodson et al., Nature 450:176 (2007); Qian et al., Nature 450:259 (2007); Raman et al. Science 327:1014-1018 (2010). Some additional non-limiting examples of computer algorithms that may be used for these and related embodiments, such as for rational design of binding regions include VMD which is a molecular visualization program for displaying, animating, and analyzing large biomolecular systems using 3-D graphics and built-in scripting (see the website for the Theoretical and Computational Biophysics Group, University of Illinois at Urbana-Champagne, at

ks.uiuc.edu/Research/vmd/. Many other computer programs are known in the art and available to the skilled person and which allow for determining atomic dimensions from space-filling models (van der Waals radii) of energy-minimized conformations; GRID, which seeks to determine regions of high affinity for different chemical groups, thereby enhancing binding, Monte Carlo searches, which calculate mathematical alignment, and CHARMM (Brooks et al. (1983) J. Comput. Chem. 4:187-217) and AMBER (Weiner et al (1981) J. Comput. Chem. 106: 765), which assess force field calculations, and analysis (see also, Eisenfield et al. (1991) Am. J. Physiol. 261:C376-386; Lybrand (1991) J. Pharm. Belg. 46:49-54; Froimowitz (1990) Biotechniques 8:640-644; Burbam et al. (1990) Proteins 7:99-111; Pedersen (1985) Environ. Health Perspect. 61:185-190; and Kini et al. (1991) J. Biomol. Struct. Dyn. 9:475-488). A variety of appropriate computational computer programs are also commercially available, such as from Schrödinger (Munich, Germany).

IV. WNT Signal Enhancing Molecules (WNT Enhancers)

[0191] In an embodiment, a multicomponent WNT molecule, as described herein, possesses at least one FZD binding domain, at least one LRP binding domain, and at least one bridging molecule; the WNT multicomponent polypeptide molecule modulates WNT signaling. In particular embodiments, the binding domains bind to their target when the target is present on the cell surface, e.g., they may bind to an epitope within the extracellular domain of their target.

[0192] In certain embodiments, the multicomponent WNT molecule, described herein, possesses two FZD binding domains tethered to the bridging molecule by a binding domain specific for a first epitope on the bridging molecule; two LRP binding domains tethered to the bridging molecule by a binding domain specific for a second epitope at least one different antigen binding domain molecule; and, the tethering of the FZD and LRP binding domains to the bridging molecule forms a WNT mimetic capable of activating WNT signaling.

[0193] In certain embodiments, the multicomponent WNT molecule, described herein, possesses the binding domain specific for a first epitope of the bridging molecule and the FZD binding domain are joined directly or by a linker, the binding domain specific for a second epitope of the bridging molecule and the LRP binding domain are joined directly, or by a linker, and the binding domain of the first epitope and second epitope of the bridging molecule are identical or different.

[0194] In another embodiment, the multicomponent WNT molecule , reported herein, possess at least one FZD binding domain linked to one LRP binding domain which is tethered to at least one different antigen binding domain by a binding domain specific for a first epitope on the bridging molecule, at least one FZD binding domain linked to one LRP binding domain which is tethered to at least one different antigen binding domain by a binding domain specific for a second epitope on the bridging molecule, and the tethering of the FZD and LRP binding domains to the bridging receptor forms a WNT mimetic capable of activating WNT signaling.

[0195] In certain embodiments, the multicomponent WNT molecule, described herein, possesses the binding domain specific for a first epitope of the bridging molecule and the FZD binding domain are joined directly or by a linker, and the binding domain specific for a second epitope of the bridging molecule and the LRP binding domain are joined directly, or by a linker, wherein the binding domain of the first epitope and second epitope of the bridging molecule are identical or different, and could monomeric or multimeric.

V. Targeting Molecules

[0196] Specific cell types and cells within specific tissue may comprise one or more cell- or tissue-specific surface molecule, such as a cell surface receptor. As used herein, the molecule is said to be cell- or tissue-specific if a greater amount of the molecule is present on the specific cell or tissue type as compared to one or more other cell or tissue types, or any other cell or tissue type. In certain embodiments, the greater amount is at least two-fold, at least five-fold, at least 10-fold, at least 20-fold, at least 50-fold, or at least 100-fold as compared to the amount in the one or more other cell or tissue types, or any other cell or tissue type. In particular embodiments, the cell-specific surface molecule has increased or enhanced expression on a target organ, tissue or cell type, e.g., an organ, tissue or cell type in which it is desirous to enhance WNT signaling, e.g., to treat or prevent a disease or disorder, e.g., as compared to one or more other non-targeted organs, tissues or cell types. In certain embodiments, the cell-specific surface molecule is preferentially expressed on the surface of the target organ, tissue or cell type as compared to one or more other organ, tissue or cell types, respectively. For example, in particular embodiments, a cell surface receptor is considered to be a tissue-specific or cell-specific cell surface molecule if it is expressed at levels at least two-fold, at least five-fold, at least 10-fold, at least 20-fold, at least 30-fold, at least 40-fold, at least 50-fold, at least 100-fold, at least 500-fold, or at least 1000-fold higher in the target organ, tissue or cell than it is expressed in one or more, five or more, all other organs, tissues or cells, or an average of all

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other organs, tissue or cells, respectively. In certain embodiments, the tissue-specific or cell-specific cell surface molecule is a cell surface receptor, e.g., a polypeptide receptor comprising a region located within the cell surface membrane and an extracellular region to which the targeting module can bind. In various embodiments, the methods described herein may be practiced by specifically targeting cell surface molecules that are only expressed on the target tissue or a subset of tissues including the target tissue, or by specifically targeting cell surface molecules that have higher levels of expression on the target tissue as compared to all, most, or a substantial number of other tissues, e.g., higher expression on the target tissue than on at least two, at least five, at least ten, or at least twenty other tissues.

[0197] The targeted tissue may be bound by a targeting module, e.g., a binding domain that specifically binds to the tissue specific receptor. The targeted tissue may be any tissue, e.g., any mammalian tissue or cell type. In certain embodiments, the targeted tissue may be present in any organ. In certain embodiments, the target tissue is bone tissue, liver tissue, skin tissue, stomach tissue, intestine tissue, oral mucosa tissue, kidney tissue, central nervous system tissue, mammary gland tissue, taste bud tissue, ovary tissue, inner ear tissue (including cochlear and vestibular tissues), hair follicles, pancreas tissue, retina tissue, cornea tissue, heart tissue or lung tissue, and the targeting module binds to a tissue-specific cell surface molecule (e.g., a cell surface receptor) preferentially expressed on bone tissue, liver tissue, skin tissue, stomach tissue, intestine tissue, oral mucosa tissue, kidney tissue, central nervous system tissue, mammary gland tissue, taste bud tissue, ovary tissue, inner ear tissue (including cochlear and vestibular tissues), hair follicles, pancreas tissue, retina tissue, cornea tissue, heart tissue or lung tissue, respectively.

[0198] The targeting module may bind to any cell type, e.g., any cell within any tissue, organ or animal, including but not limited to mammals, such as humans. In certain embodiments, the tissue-specific WNT surrogate-signal enhancing combination molecule binds to specific cell types, e.g., specific cell types associated with a target tissue. For example, in liver tissue, the targeting module may bind to hepatocytes, precursors and stem cells of hepatocytes, biliary tract cells, and/or endothelial or other vascular cells. Examples of liver specific targeting molecules are provided, e.g., in WO 2018/140821 and WO 2020/014271, both of which are incorporated by reference herein. For example, in bone tissue, the targeting module may bind osteoblasts, precursors of osteoblasts, mesenchymal stem cells, stem cells and precursor cells that give rise to bone, cartilage and/or other cells present in bone tissue. Cell types present in various tissues, including but not limited to the tissues described herein,

are known in the art, and in various embodiments, the tissue specific WNT signal enhancing molecules described herein may bind any of them.

VI. Linkers

[0199] In certain embodiments, the multicomponent targeting molecule, for example FZD molecule, LRP molecule, and the bridging molecule, are bound or fused directly to each other, whereas in other embodiments, they are separated by a linker, e.g., a polypeptide linker, or a non-peptidyl linker, *etc*. In particular embodiments, a linker is an Fc linker, e.g., a region of an antibody Fc domain capable of dimerizing with another Fc linker, e.g., via one or more disulfide bonds. In another particular embodiment, a linker is albumin, e.g., human serum albumin, where the targeting and action modules are on the N- and C- termini of albumin.

[0200] In certain embodiments, particularly when joining two polypeptides, the linker is made up of amino acids linked together by peptide bonds. In particular embodiments, the linker comprises, in length, from 1 up to about 40 amino acid residues, from 1 up to about 20 amino acid residues, or from 1 to about 10 amino acid residues. In certain embodiments, the amino acid residues in the linker are from among the twenty canonical amino acids, and in certain embodiments, selected from cysteine, glycine, alanine, proline, asparagine, glutamine, and/or serine. In certain embodiments, a linker comprises one or more non-natural amino acids. In some embodiments, a peptidyl linker is made up of a majority of amino acids that are sterically unhindered, such as glycine, serine, and alanine linked by a peptide bond. Certain linkers include polyglycines, polyserines, and polyalanines, or combinations of any of these. Some exemplary peptidyl linkers are poly(Gly)1-8, particularly (Gly)3, (Gly)4 (SEQ ID NO: 14), (Gly)5 (SEQ ID NO: 15), (Gly)6 (SEQ ID NO: 16), (Gly)7 (SEQ ID NO: 17), and (Gly)8 (SEQ ID NO: 18) as well as, poly(Gly)4 Ser (SEQ ID NO: 19), poly(Gly-Ala)2 (SEQ ID NO: 20), poly(Gly-Ala)3 (SEQ ID NO: 21), poly(Gly-Ala)4 (SEQ ID NO: 22) and poly(Ala)1-8 (SEQ ID NO: 23-27). Other specific examples of peptidyl linkers include (Gly)5Lys (SEQ ID NO: 28), and (Gly)5LysArg (SEQ ID NO: 29). To explain the above nomenclature, for example, (Gly)3Lys(Gly)4 means Gly-Gly-Gly-Lys-Gly-Gly-Gly-Gly (SEQ ID NO: 30). Other combinations of Gly and Ala are also useful. Additionally, a peptidyl linker can also comprise a non-peptidyl segment such as a 6 carbon aliphatic molecule of the formula --CH2--CH2--CH2--CH2--CH2--CH2--. The peptidyl linkers can be altered to form derivatives as described herein.

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[0201] Illustrative non-peptidyl linkers include, for example, alkyl linkers such as --NH--(CH2) s--C(O)--, wherein s=2-20. These alkyl linkers may further be substituted by any non-sterically hindering group such as lower alkyl (e.g., C1-C6) lower acyl, halogen (e.g., Cl, Br), CN, NH2, phenyl, etc. Non-peptide portions of the inventive composition of matter, such as non-peptidyl linkers or non-peptide half-life extending moieties can be synthesized by conventional organic chemistry reactions. Chemical groups that find use in linking binding domains include carbamate; amide (amine plus carboxylic acid); ester (alcohol plus carboxylic acid), thioether (haloalkane plus sulfhydryl; maleimide plus sulfhydryl), Schiff's base (amine plus aldehyde), urea (amine plus isocyanate), thiourea (amine plus isothiocyanate), sulfonamide (amine plus sulfonyl chloride), disulfide; hydrazone, lipids, and the like, as known in the art.

[0202] The linkage between domains may comprise spacers, e.g. alkyl spacers, which may be linear or branched, usually linear, and may include one or more unsaturated bonds; usually having from one to about 300 carbon atoms; more usually from about one to 25 carbon atoms; and may be from about three to 12 carbon atoms. Spacers of this type may also comprise heteroatoms or functional groups, including amines, ethers, phosphodiesters, and the like. Specific structures of interest include: $(CH_2CH_2O)n$ where n is from 1 to about 12; $(CH_2CH_2NH)n$, where n is from 1 to about 12; $[(CH_2)n(C=O)NH(CH_2)_m]_z$, where n and m are from 1 to about 6, and z is from 1 to about 10; $[(CH_2)nOPO_3(CH_2)_m]_z$ where n and m are from 1 to about 6, and z is from 1 to about 10. Such linkers may include polyethylene glycol, which may be linear or branched.

[0203] In certain embodiments, the domains may be joined through a homo- or heterobifunctional linker. Illustrative entities include: azidobenzoyl hydrazide, N-[4-(p-azidosalicylamino)butyl]-3'-[2'-pyridyldithio]propionamide), bis-sulfosuccinimidyl suberate, dimethyladipimidate, disuccinimidyltartrate, N-γ-maleimidobutyryloxysuccinimide ester, N-hydroxy sulfosuccinimidyl-4-azidobenzoate, N-succinimidyl [4-azidophenyl]-1,3'-dithiopropionate, N-succinimidyl [4-iodoacetyl]aminobenzoate, glutaraldehyde, NHS-PEG-MAL; succinimidyl 4-[N-maleimidomethyl]cyclohexane-1-carboxylate; 3-(2-pyridyldithio)propionic acid N-hydroxysuccinimide ester (SPDP); N, N'-(1,3-phenylene) bismaleimide; N, N'-ethylene-bis-(iodoacetamide); or 4-(N-maleimidomethyl)-cyclohexane-1-carboxylic acid N-hydroxysuccinimide ester (SMCC); m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS), and succinimide 4-(p-maleimidophenyl)butyrate (SMPB), an extended chain analog of MBS. In certain embodiments, the succinimidyl group of these

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cross-linkers reacts with a primary amine, and the thiol-reactive maleimide forms a covalent bond with the thiol of a cysteine residue.

[0204] Other reagents useful include: homobifunctional cross-linking reagents including bismaleimidohexane ("BMH"); p,p'-difluoro-m,m'-dinitrodiphenylsulfone (which forms irreversible cross-linkages with amino and phenolic groups); dimethyl adipimidate (which is specific for amino groups); phenol-1,4-disulfonylchloride (which reacts principally with amino groups); hexamethylenediisocyanate or diisothiocyanate, or azophenyl-p-diisocyanate (which reacts principally with amino groups); disdiazobenzidine (which reacts primarily with tyrosine and histidine); O-benzotriazolyloxy tetramethuluronium hexafluorophosphate (HATU), dicyclohexyl carbodiimde, bromo-tris (pyrrolidino) phosphonium bromide (PyBroP); N,N-dimethylamino pyridine (DMAP); 4-pyrrolidino pyridine; N-hydroxy benzotriazole; and the like.

VII. Cell Targeting activators via bridging receptor(s)

[0205] The present invention encompasses a novel concept to achieve cell targeting for ligand/receptor systems that involves multicomponent receptor complexes, where a productive signaling competent receptor complex formation is mediated through a "bridging element", using "chimeric activator" approach based on the cooperativity concept. When an "targeting element" is tethered to the mutant "activity element", the "targeting element" helps to increase the local concentration of the mutant "activity element" on the desired target cell surface, driving engagement of signaling receptor and subsequent intracellular signaling activation, effectively left shift the activity dose response curve (increase potency) on target cell and create the selectivity between target vs non-target cells.

[0206] The concept of cell targeting for ligand/receptor systems that involves multicomponent receptor complexes, where a productive signaling competent receptor complex formation is mediated through a "bridging element" is tested using the WNT/β-catenin signaling pathway as a model system.

[0207] WNT pathway is highly conserved across species and crucial for embryonic development, and adult tissue homeostasis and regeneration (Nusse and Clevers, 2017). WNT induced signaling through β-catenin stabilization has been widely studied and is achieved by ligand binding to frizzled (FZD) and low-density lipoprotein receptor-related protein (LRP) family of receptors. There are nineteen mammalian WNTs, 10 FZDs (FZD1-10), and 2 LRPs (LRP5 and LRP6). WNTs are highly hydrophobic due to lipidation that is required for function

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and are promiscuous, capable of binding and activating multiple FZD and LRP pairs (Janda et al., 2012, Kadowaki et al., 1996, Dijksterhuis et al., 2015). Elucidating the functions of individual FZDs in tissues has been hampered by difficulties in producing the ligands and lack of receptor and tissue selectivity. Recent breakthroughs in the development of WNT-mimetic molecules have largely resolved the production and receptor specificity challenges (Janda et al., 2017, Chen et al., 2020, Tao et al., 2019, Miao et al., 2020). While tissue selectivity could be partly achieved by tissue injury as damaged tissues seem more sensitive to WNTs (Xie et al., 2022), it would still represent a significant technical advancement to be able to target WNTs to specific cells and tissues.

[0208] The WNT mimetics reported so far are all bispecific and can simultaneously bind to FZDs and LRPs, and the optimal stoichiometry (at least for the antibody based molecules) are tetravalent bispecific (2:2 format), requiring two FZD binders and two LRP binders in the same molecule to achieve efficient signaling (Tao et al., 2019, Chen et al., 2020). We took advantage of the fact that two FZD binders alone, two LRP binders alone, or a molecule with one FZD binder and one LRP binder (1:1 format) do not signal. Cell specificity could be achieved by attaching an "targeting element" (capable of binding to another cell surface receptor, called a bridging receptor or bridging molecule here) to these three inactive molecules, and signaling competent receptor complexes consisting of two FZDs and two LRPs could then be assemble via the bridging receptor on the target cell surface. This approach reduces or eliminates the need to mutate and reduce the affinity of the "active elements" toward signaling receptors, and creates a highly cell specific activation of the signaling pathway as the individual components are inactive.

[0209] Fig. 1A. shows one optimized design for a WNT mimetic that is a tetravalent bispecific antibody-based molecule. It is important to highlight that efficient WNT/β-catenin signaling requires two FZD binding domains and two LRP binding domains in one molecule (Chen et al., 2020). Fig. 1B shows a cell targeted WNT mimetic based on the "chimeric activator" concept where an "targeting element" is tethered to the WNT mimetic. These molecules are representative illustrations, orientations and attachment locations of the different elements can be varied. The adjustments of the affinities of the different binding components can influence the separation between targeting vs non-targeting cells.

[0210] Figures 1C-1E show the cell targeting approach using the bridging receptor concept. The first step of this concept is to split the active molecule into inactive components. Using the tetravalent bispecific WNT mimetic as an example, among many different ways of splitting

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this molecule, we explored two ways for illustration purpose. The first method is to split the tetravalent WNT mimetic into one molecule having two FZD binding arms (2:0) and a second molecule having two LRP binding arms (0:2) (Fig. 1C-1E). These two inactive components can be assembled by a cell specific bridging receptor in several ways. If the bridging receptor exists as a monomer, as depicted in Fig. 1C, each separate molecule could be tethered to a "bridging element" binding to a different epitope on the bridging receptor, creating a final receptor complex on the cell surface consisting of two FZDs and two LRPs that would be competent to trigger signaling. The two different "bridging elements" could also be tethered to the two separate FZD and LRP binders as depicted in Fig. 1D for the assembly of the active receptor complex on the cell surface. If the bridging receptor exists as a multimeric complex of its own, a single "bridging element" could be tethered to the two different FZD and LRP molecules as depicted in Fig. 1E.

[0211] Figures 1F-1H show the method to split the tetravalent WNT mimetic is to split into two identical 1:1 molecules that consists of one FZD binding arm and one LRP binding arm. Similar to the scenario in Fig. 1C, if the bridging receptor exists as a monomer, two "bridging elements" binding to two different epitopes on the bridging receptor could be tethered to two separate 1:1 "activity element", which can then assemble via bridging receptor to create a competent productive signaling complex (Fig. 1F). The two different "bridging elements" could also be tethered together to the one 1:1 "activity element" (Fig. 1G). Unlike the scenarios shown in Fig. 1C-1F which employs two distinct "activity elements" for signaling, in the scenario in Fig. 1G, only a single molecule is needed to assemble a productive receptor signaling complex with cell targeting capability. If the bridging receptor exist as a multimeric complex of its own, a single "bridging element" could be tethered to a single "activity element" to create one molecule that achieve cell targeting (Fig. 1H). Additional variations of how the elements can be combined could be envisioned under the bridging receptor concept.

[0212] Tandem scFv multicomponent polypeptide are generated and assembled by linking or directly fusing a first scFv to either the C- or N-terminus of a second scFv molecule. In one format, the first scFv can bind to one or more FZD receptors and the second scFv can bind to one or more LRP receptors. In an alternative format, the first scFv can bind to one or more LRP receptors, and the second scFv can bind to one or more FZD receptors. One of the scFv molecules can also be linked or directly fused at its C-terminus to the N-terminus of an Fc molecule. In certain embodiments the WNT enhancer is linked or fused to the N-terminus of

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a first scFv, which in turn is linked or fused to the N-terminus of the second scFv, which is linked or fused to the N-terminus of the Fc molecule. In alternative embodiments, the WNT enhancer is linked or fused to the C-terminus of the Fc molecule, which in turn is linked or fused to the C-terminus of one scFv molecule, which is linked or fused at its N-terminus to the C-terminus of a second scFv molecule.

[0213] Fab-IgG molecules, where the FZD and LRP binders are Fabs can be assembled in various approaches, such as charge pairing, knobs-in-holes, crossover of heavy and light chains of the Fabs, etc. In charge pairing the heavy chain (VH-CH1) domain of an anti-LRP6 Fab or an anti-FZD Fab, through direct fusion or a linker of 5, 10, or 15-mer amino acids, are fused in tandem with the N-terminus of the heavy chain (VH-CH1-CH2-CH3) of an anti-FZD or anti-LRP binder. In certain embodiments, also known as Fabs-in tandem (FiT), both VH-CH1 domains of anti-LRP6 and anti-FZD contain three amino acid mutations (Q39D, Q105D, S183K in the anti-LRP6 Fab; Q39K, Q105K, S183E in anti-FZD Fab) each for proper paring with their own partner light chains, which also contain three complementary amino acid mutations (Q38K, A/S43K, S176E in anti-LRP6 light chain; Q38D, A/S43D, S176K in the anti-FZD light chain). The order of the anti-LRP6 and anti-FZD Fabs could be reversed, where the anti-FZD binder is a Fab and is fused to anti-LRP binder which is in IgG format. In certain embodiments, the WNT enhancer can be attached to the Fab to the N-terminus of either the Vh or Vl the Fab furthest from the IgG domain. In other embodiments, the WNT enhancer is attached to C-terminus of the IgG domain.

[0214] HC-LC cross over approach for Fab-on-IgG format: The light chain (VL-CL) domains of anti-LRP6 binder is, through direct fusion or a linker of 5, 10, or 15-mer amino acids, fused in tandem with the N-terminus of the heavy chain (VH-CH1-CH2-CH3) of an anti-FZD binder. The second construct was VH-CH1 of the anti-LRP6 binder and the third construct was VL-CL of the anti-FZD binder. Similar to the example above, the order of the anti-LRP6 and the anti-FZD binders could be reversed, where anti-FZD binder Fab is fused to the N-terminus of the anti-LRP binder which is in IgG format. Also as above the WNT enhancer can be attached to N-terminus of the VH or VL of the crossover Fab furthest from the IgG domain, or attached to the C-terminus of the IgG domain.

[0215] In certain embodiments, the molecules comprise one or more polypeptide(s) comprising or consisting of a sequence selected from any of SEQ ID NOs:1-13, or a functional fragment or variant thereof. In particular embodiments, a variant comprises at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% identity to any one of SEQ

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ID NOs:1-13. In certain embodiments, a fragment comprises at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 98% of a contiguous sequence of any of SEQ ID NOs:1-13 or a variant of any of SEQ ID NOs:1-13. In certain embodiments, a functional fragment comprises an FGF21 sequence, an F12578 sequence, and/or a L sequence present in any of SEQ ID NOs:1-13, or a variant thereof.

VIII. Compositions

[0216] Pharmaceutical compositions comprising a multicomponent polypeptide molecule described herein and one or more pharmaceutically acceptable diluent, carrier, or excipient are also disclosed.

[0217] In further embodiments, pharmaceutical compositions comprising a polynucleotide comprising a nucleic acid sequence encoding a multicomponent polypeptide molecule described herein and one or more pharmaceutically acceptable diluent, carrier, or excipient are also disclosed. In particular embodiments, the pharmaceutical composition further comprises one or more polynucleotides comprising a nucleic acid sequence encoding a naturally occurring co-receptor ligand polypeptide. In certain embodiments, the polynucleotides are DNA or mRNA, e.g., a modified mRNA. In particular embodiments, the polynucleotides are modified mRNAs further comprising a 5' cap sequence and/or a 3' tailing sequence, e.g., a polyA tail. In other embodiments, the polynucleotides are expression cassettes comprising a promoter operatively linked to the coding sequences. In certain embodiments, the nucleic acid sequence encoding the multicomponent polypeptide molecule and the nucleic acid sequence encoding naturally occurring co-receptor ligand polypeptide are present in the same polynucleotide.

[0218] In further embodiments, pharmaceutical compositions comprising an expression vector, e.g., a viral vector, comprising a polynucleotide comprising a nucleic acid sequence encoding a multicomponent polypeptide molecule described herein and one or more pharmaceutically acceptable diluent, carrier, or excipient are also disclosed. In particular embodiments, the pharmaceutical composition further comprises an expression vector, e.g., a viral vector, comprising a polynucleotide comprising a nucleic acid sequence encoding a naturally occurring co-receptor ligand polypeptide. In certain embodiments, the nucleic acid sequence encoding the multicomponent polypeptide molecule and the nucleic acid sequence encoding the naturally occurring co-receptor ligand polypeptide are present in the same polynucleotide, e.g., expression cassette.

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[0219] The present invention further contemplates a pharmaceutical composition comprising a cell comprising an expression vector comprising a polynucleotide comprising a promoter operatively linked to a nucleic acid encoding a multicomponent polypeptide molecule and one or more pharmaceutically acceptable diluent, carrier, or excipient. In particular embodiments, the pharmaceutical composition further comprises a cell comprising an expression vector comprising a polynucleotide comprising a promoter operatively linked to a nucleic acid sequence encoding a polypeptide corresponding to the natural ligand of the receptors. In particular embodiments, the cell is a heterologous cell or an autologous cell obtained from the subject to be treated. In particular embodiments, the cell is a stem cell, e.g., an adipose-derived stem cell or a hematopoietic stem cell.

[0220] The subject molecules, alone or in combination, can be combined with pharmaceutically-acceptable carriers, diluents, excipients and reagents useful in preparing a formulation that is generally safe, non-toxic, and desirable, and includes excipients that are acceptable for mammalian, e.g., human or primate, use. Such excipients can be solid, liquid, semisolid, or, in the case of an aerosol composition, gaseous. Examples of such carriers, diluents and excipients include, but are not limited to, water, saline, Ringer's solutions, dextrose solution, and 5% human serum albumin. Supplementary active compounds can also be incorporated into the formulations. Solutions or suspensions used for the formulations can include a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial compounds such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating compounds such as ethylenediaminetetraacetic acid (EDTA); buffers such as acetates, citrates or phosphates; detergents such as Tween 20 to prevent aggregation; and compounds for the adjustment of tonicity such as sodium chloride or dextrose. The pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. In particular embodiments, the pharmaceutical compositions are sterile.

[0221] Pharmaceutical compositions may further include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, or phosphate buffered saline (PBS). In some cases, the composition is sterile and should be fluid such that it can be drawn into a syringe or delivered to a subject from a syringe. In certain embodiments, it is stable under the conditions of manufacture and storage and is preserved against the contaminating action of microorganisms such as bacteria

and fungi. The carrier can be, e.g., a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the internal compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

[0222] Sterile solutions can be prepared by incorporating the multicomponent polypeptide molecule (or encoding polynucleotide or cell comprising the same) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, methods of preparation are vacuum drying and freeze-drying that yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

[0223] In one embodiment, the pharmaceutical compositions are prepared with carriers that will protect the antibody or antigen-binding fragment thereof against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially. Liposomal suspensions can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art.

[0224] It may be advantageous to formulate the pharmaceutical compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active antibody or antigen-binding fragment

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thereof calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms are dictated by and directly dependent on the unique characteristics of the antibody or antigen-binding fragment thereof and the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active antibody or antigen-binding fragment thereof for the treatment of individuals.

[0225] The pharmaceutical compositions can be included in a container, pack, or dispenser, e.g. syringe, e.g. a prefilled syringe, together with instructions for administration.

[0226] The pharmaceutical compositions of the invention encompass any pharmaceutically acceptable salts, esters, or salts of such esters, or any other compound which, upon administration to an animal comprising a human, is capable of providing (directly or indirectly) the biologically active antibody or antigen-binding fragment thereof.

[0227] The present invention includes pharmaceutically acceptable salts of a WNT multicomponent polypeptide molecule described herein. The term "pharmaceutically acceptable salt" refers to physiologically and pharmaceutically acceptable salts of the compounds of the invention: i.e., salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto. A variety of pharmaceutically acceptable salts are known in the art and described, e.g., in "Remington's Pharmaceutical Sciences", 17th edition, Alfonso R. Gennaro (Ed.), Mark Publishing Company, Easton, PA, USA, 1985 (and more recent editions thereof), in the "Encyclopaedia of Pharmaceutical Technology", 3rd edition, James Swarbrick (Ed.), Informa Healthcare USA (Inc.), NY, USA, 2007, and in J. Pharm. Sci. 66: 2 (1977). Also, for a review on suitable salts, see "Handbook of Pharmaceutical Salts: Properties, Selection, and Use" by Stahl and Wermuth (Wiley-VCH, 2002).

[0228] Pharmaceutically acceptable base addition salts are formed with metals or amines, such as alkali and alkaline earth metals or organic amines. Metals used as cations comprise sodium, potassium, magnesium, calcium, and the like. Amines comprise N-N'-dibenzylethylenediamine, chloroprocaine, choline, diethanolamine, dicyclohexylamine, ethylenediamine, N-methylglucamine, and procaine (see, for example, Berge et al., "Pharmaceutical Salts," J. Pharma Sci., 1977, 66, 119). The base addition salts of said acidic compounds are prepared by contacting the free acid form with a sufficient amount of the desired base to produce the salt in the conventional manner. The free acid form may be

regenerated by contacting the salt form with an acid and isolating the free acid in the conventional manner. The free acid forms differ from their respective salt forms somewhat in certain physical properties such as solubility in polar solvents, but otherwise the salts are equivalent to their respective free acid for purposes of the present invention.

[0229] In some embodiments, the pharmaceutical composition provided herein comprise a therapeutically effective amount of a WNT multicomponent polypeptide molecule or pharmaceutically acceptable salt thereof in admixture with a pharmaceutically acceptable carrier, diluent and/or excipient, for example saline, phosphate buffered saline, phosphate and amino acids, polymers, polyols, sugar, buffers, preservatives and other proteins. Exemplary amino acids, polymers and sugars and the like are octylphenoxy polyethoxy ethanol compounds, polyethylene glycol monostearate compounds, polyoxyethylene sorbitan fatty acid esters, sucrose, fructose, dextrose, maltose, glucose, mannitol, dextran, sorbitol, inositol, galactitol, xylitol, lactose, trehalose, bovine or human serum albumin, citrate, acetate, Ringer's and Hank's solutions, cysteine, arginine, carnitine, alanine, glycine, lysine, valine, leucine, polyvinylpyrrolidone, polyethylene and glycol. Preferably, this formulation is stable for at least six months at 4° C.

[0230] In some embodiments, the pharmaceutical composition provided herein comprises a buffer, such as phosphate buffered saline (PBS) or sodium phosphate/sodium sulfate, tris buffer, glycine buffer, sterile water and other buffers known to the ordinarily skilled artisan such as those described by Good et al. (1966) Biochemistry 5:467. The pH of the buffer may be in the range of 6.5 to 7.75, preferably 7 to 7.5, and most preferably 7.2 to 7.4.

IX. Methods of Use

[0231] For illustrative purposes only, the cell targeting multicomponent WNT molecule can be used as to treat various diseases or disorders where tissue regeneration is necessary. Such diseases include, but are not limited to: increase bone growth or regeneration, bone grafting, healing of bone fractures, treatment of osteoporosis and osteoporotic fractures, vertebral compression fractures, spinal fusion, osseointegration of orthopedic devices, tendon-bone integration, tooth growth and regeneration, dental implantation, periodontal diseases, maxillofacial reconstruction, and osteonecrosis of the jaw. Also contemplated are: treatment of alopecia; enhancing regeneration of sensory organs, e.g. treatment of hearing loss, including internal and external auditory hair cells, treatment of vestibular hypofunction, treatment of macular degeneration, treatment of vitreoretinopathy, diabetic retinopathy, other diseases of

retinal degeneration, Fuchs' dystrophy, other cornea disease, etc.; treatment of stroke, traumatic brain injury, Alzheimer's disease, multiple sclerosis and other conditions affecting the blood brain barrier; treatment of spinal cord injuries, other spinal cord diseases. The compositions of this invention may also be used in treatment of oral mucositis, treatment of short bowel syndrome, inflammatory bowel diseases (IBD), other gastrointestinal disorders; treatment of metabolic syndrome, dyslipidemia, treatment of diabetes, treatment of pancreatitis, conditions where exocrine or endocrine pancreas tissues are damaged; conditions where enhanced epidermal regeneration is desired, e.g., epidermal wound healing, treatment of diabetic foot ulcers, syndromes involving tooth, nail, or dermal hypoplasia, etc., conditions where angiogenesis is beneficial; treatment of myocardial infarction, coronary artery disease, heart failure; enhanced growth of hematopoietic cells, e.g. enhancement of hematopoietic stem cell transplants from bone marrow, mobilized peripheral blood, treatment of immunodeficiencies, graft versus host diseases, etc.; treatment of acute kidney injuries, chronic kidney diseases; treatment of lung diseases, chronic obstructive pulmonary diseases (COPD), idiopathic pulmonary fibrosis (IPF) enhanced regeneration of lung tissues. The compositions of the present invention may also be used in enhanced regeneration of liver cells, e.g. liver regeneration, treatment of cirrhosis, enhancement of liver transplantations, treatment of acute liver failure, treatment of chronic liver diseases with hepatitis C or B virus infection or post-antiviral drug therapies, alcoholic liver diseases, alcoholic hepatitis, non-alcoholic liver diseases with steatosis or steatohepatitis, and the like. The compositions of this invention may treat diseases and disorders including, without limitation, conditions in which regenerative cell growth is desired.

[0232] In particular embodiments, a pharmaceutical composition is administered parenterally, e.g., intravenously, orally, rectally, or by injection. In some embodiments, it is administered locally, e.g., topically or intramuscularly. In some embodiments, a composition is administered to target tissues, e.g., to bone, joints, ear tissue, eye tissue, gastrointestinal tract, skin, a wound site or spinal cord. Methods of the invention may be practiced in vivo or ex vivo. In some embodiments, the contacting of a target cell or tissue with a multicomponent polypeptide molecule is performed ex vivo, with subsequent implantation of the cells or tissues, e.g., activated stem or progenitor cells, into the subject. The skilled artisan can determine an appropriate site of and route of administration based on the disease or disorder being treated.

[0233] The dose and dosage regimen may depend upon a variety of factors readily determined by a physician, such as the nature of the disease or disorder, the characteristics of

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the subject, and the subject's history. In particular embodiments, the amount of a multicomponent polypeptide molecule administered or provided to the subject is in the range of about 0.01 mg/kg to about 50 mg/kg, 0.1 mg/kg to about 500 mg/kg, or about 0.1 mg/kg to about 50 mg/kg of the subject's body weight.

[0234] All of the above U.S. patents, U.S. patent application publications, U.S. patent applications, foreign patents, foreign patent applications and non-patent publications referred to in this specification and/or listed in the Application Data Sheet, are incorporated herein by reference, in their entirety.

[0235] From the foregoing it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

EXAMPLES

[0236] The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the present invention, and are not intended to limit the scope of what the inventors regard as their invention nor are they intended to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees Centigrade, and pressure is at or near atmospheric.

[0237] General methods in molecular biology, cell biology and biochemistry can be found in such standard textbooks as "Molecular Cloning: A Laboratory Manual, 3rd Ed." (Sambrook et al., Harbor Laboratory Press 2001); "Short Protocols in Molecular Biology, 4th Ed." (Ausubel et al. eds., John Wiley & Sons 1999); "Protein Methods" (Bollag et al., John Wiley & Sons 1996); "Nonviral Vectors for Gene Therapy" (Wagner et al. eds., Academic Press 1999); "Viral Vectors" (Kaplift & Loewy eds., Academic Press 1995); "Immunology Methods Manual" (I. Lefkovits ed., Academic Press 1997); and "Cell and Tissue Culture: Laboratory Procedures in Biotechnology" (Doyle & Griffiths, John Wiley & Sons 1998), the disclosures of which are incorporated herein by reference. Reagents, cloning vectors, and kits for genetic manipulation referred to in this disclosure are available from commercial vendors such as BioRad, Stratagene, Invitrogen, Sigma-Aldrich, and ClonTech.

[0238] Recombinant molecules were generated that combine agonists for the WNT receptors, FZD and/or LRP co-receptors, together with bridging receptor, to create WNT signaling "cell targeting multicomponent polypeptide"

[0239] Materials and methods employed in the following Examples include the following.

[0240] **Protein production:** All recombinant proteins were produced in Expi293F cells (Thermo Fisher Scientific) by transient transfection unless otherwise specified. All IgG-based and Fc-containing constructs were first purified with Protein-A resin and eluted with 0.1 M glycine pH 3.5. All proteins were then polished by a size exclusion column in HBS buffer (10 mM HEPES pH 7.2, 150 mM NaCl). Proteins were supplemented with glycerol to 10% for long term storage at -80°C.

[0241] **SuperTop Flash (STF) assay:** WNT signaling activity is measured using cell lines containing a luciferase gene controlled by a WNT-responsive promoter (Super Top Flash reporter assay, STF) as reported (Janda et al., 2017; Nature 545:234). In brief, cells are seeded at a density of 10,000 per well in 96-well plates 24 hr prior to treatment, then treated with RSPO or mimetic proteins overnight either alone or together with 30% WNT3a-conditioned media. WNT3a conditioned media are prepared from ATCC-CRL-2647 WNT3a secreting L cells following vendor recommended conditions. Cells were lysed with Luciferase Cell Culture Lysis Reagent (Promega) and activity are measured with Luciferase Assay System (Promega) using vendor suggested procedures. Data are plotted as average -/+ standard deviation of triplicates and fitted by non-linear regression using Prism (GraphPad Software).

Affinity Measurement and Step-Binding Assay

[0242] Binding kinetics of F-FGF21 series (F-FGF21FL, F-FGF21ΔN, F-FGF21ΔC, and F-FGF21ΔNΔC) to human FZD7 CRD and βKlotho (Fisher Scientific) or L-39F7 series (L-39F7, αGFP-39F7, and L-αGFP) to human LRP6E3E4 and βKlotho, respectively, were determined by bio-layer interferometry (BLI) using an Octet Red 96 (PALL ForteBio) instrument at 30°C, 1000 rpm with AHC biosensors (Sartorius). Various F-FGF21 or L-39F7 proteins were diluted to 50 nM in the running buffer and captured to the AHC biosensor, followed by dipping into wells containing the FZD7 CRD, LRP6E3E4 and βKlotho at different concentrations in a running buffer or into a well with only the running buffer as reference channel. The dissociation of the interaction was followed with the running buffer. The monovalent KD for each binder was calculated by Octet System software, based on fitting to a 1:1 binding model.

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[0243] Step-binding assay was performed with the BLI using the Octet Red 96 instrument at 30°C, 1000 rpm with AHC biosensors. Various F-FGF21 or L-39F7 proteins were diluted to 50 nM in the running buffer and captured to the AHC biosensor, followed by dipping into wells containing the 100 nM FZD7 CRD or 100 nM LRP6E3E4, respectively. The sensor chips next moved into 150 nM βKlothos containing 100 nM FZD7 CRD or containing 100 nM LRP6E3E4 to check the additional bindings of β-Klotho. Sensorgram slopes were compared for βKlotho bindings.

Primary human cells

[0244] Human hepatocytes were purchased from BioIVT (10-donor pooled cryoplateable X008001-P) and cultured in LONZA hepatocyte maintenance medium (CC-3198). In short, plastic culture plates were coated with 20% Matrigel Matrix (CB40230C) and cells were plated in plating medium (BioIVT Z990003). After four hours the medium was changed to maintenance medium and refreshed every day for three days prior to the 24h experiment.

[0245] Human small intestinal organoids were a gift from the Calvin Kuo Lab at Stanford. Organoids were maintained and expanded as previously described32. In short, adapted expansion medium contained Advanced DMEM, 10 mM HEPES, 1x GlutaMAX, 1X Penicillin-Streptomycin, 1x B27, 1x N2, 1.25 mM N-acetylcysteine, 10 mM Nicotinamide, 50 ng/mL recombinant human EGF, 50 ng/mL recombinant human Noggin, 20 nM R-Spondin 2, 0.1 nM L-F Wnt mimetic, 10 nM recombinant Gastrin, 500 nM A83-01 and 10 μM SB202190.

[0246] Treatment of molecules was done in the presence of 20 nM R-spondin 2 for 24h at a concentration of 10 nM. After 24 hours the cells were harvested and RNA collected for qPCR. Each experiment with both primary human hepatocytes and human small intestinal organoids was repeated three times.

Quantitative polymerase chain reaction analysis of gene expression

[0247] RNAs from HEK293, Huh7 cells, or primary human cells were extracted using the Qiagen RNeasy Micro Kit (Qiagen). cDNA was produced using the SuperScript IV VILO cDNA Synthesis Kit (Thermo Fisher). βKlotho (KLB) RNA was quantified using Maxima SYBR Green qPCR master mix on a Bio-Rad CFX96 real time PCR machine. Cycle threshold (Ct) values were normalized to the expression of constitutive ACTINB RNA using the following oligo's:

[0248] ACTB_F1: CTGGAACGGTGAAGGTGACA (SEQ ID NO: 31);

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[0249] ACTB_R1: AAGGGACTTCCTGTAACAATGCA (SEQ ID NO: 32);

[0250] KLB_F1: ATCTAGTGGCTTGGCATGGG (SEQ ID NO: 33); KLB_R1:CCAAACTTTCGAGTGAGCCTTG (SEQ ID NO: 34); KLB_F2:CACTGAATCTGTTCTTAAGCCCG (SEQ ID NO: 35);

[0251] KLB_R2: GGCGTTCCACACGTACAGA (SEQ ID NO: 36);

[0252] KLB_F3: GGAGGTGCTGAAAGCATACCT (SEQ ID NO: 37); and

[0253] KLB_R3: TCTCTTCAGCCAGTTTGAATGC (SEQ ID NO: 38).

Example 1

Multicomponent WNT Molecule for Concept of the 2:0/0:2 Split Design

[0254] To evaluate the structures shown in Fig. 1C, the βKlotho and endocrine fibroblast growth factor 21 (FGF21) ligand system were tested as the bridging receptors. FGF21 is an endocrine hormone produced by the liver that regulates metabolic homeostasis (BonDurant and Potthoff, 2018). FGF21 signals through FGFR1c, FGFR2c, and FGFR3c in the presence of co-receptor βKlotho (Zhang and Li, 2015). The binding of FGF21 to the receptor complex is primarily driven by its affinity toward βKlotho via its C-terminal domain (Lee et al., 2018, Shi et al., 2018), while its N-terminal domain is important for FGFR interaction and signaling (Yie et al., 2012, Micanovic et al., 2009). βKlotho binding antibodies have also been identified that could induce βKlotho/FGFR signaling, and one particular agonistic βKlotho antibody binds to a different epitope on βKlotho from FGF21 and does not compete with FGF21 binding (Min et al., 2018). Therefore, the following bridging receptor (βKlotho) binding elements were selected to test the cell targeting concept:

[0255] FGF21FL (full length FGF21) that can bind to βKlotho and is competent to induce FGFR signaling;

[0256] FGF21ΔC (FGF21 without the C-terminal βKlotho interaction domain) that does not bind βKlotho and is not capable of inducing FGFR signaling;

[0257] FGF21ΔN (FGF21 without the N-terminal FGFR interaction domain) that binds βKlotho but does not signal;

[0258] FGF21ΔNΔC that cannot bind βKlotho and cannot signal; and

[0259] 39F7 IgG that binds βKlotho and can induce FGFR signaling.

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[0260] The FZD and LRP binding domains selected were F12578 (binds FZD1,2,5,7,8) and L (binds LRP6), previously named F1 and L2, respectively (Chen et al., 2020).

[0261] The graphic representations of the binders and the various combinations are shown in Fig. 2A-2C.

[0262] To test the structures described in Fig. 1C, the FZD binder (F12578) was combined with one of two versions of the bridging receptor (βKlotho) binder, F12578-FGF21FL or F12578-FGF21ΔN (Fig. 2B), and the LRP binder (L) was combined with the other bridging receptor (βKlotho) binder, 39F7, as L-39F7 (Fig. 2B). As shown in Fig. 2D and 2E, the combination of F12578-FGF21FL or FGF12578-FGF21ΔN with L-39F7 resulted in WNT/β-catenin signaling in a liver cell line, Huh7 cells, which express the bridging receptor βKlotho, but not in 293 cells, where βKlotho is not expressed. This signaling depended on the presence of both the FZD and LRP binding arms and the ability to bind the bridging receptor, as the removal of LRP binding arm L from L-39F7 or inactivation of βKlotho binding (use of FGF21ΔNΔC) resulted in no activity in either cell type (Fig. 2D and 2E). This provided experimental evidence supporting the concept presented in Fig. 1C.

[0263] Table 1 describes the different components/formats tested.

Table 1: Formats and sequences of multicomponent WNT polypeptide with 2:0 / 0:2 split design:

VH = Italic;
Human CH1CH2CH3 with LALAPG mutations (LALAPG mutations are only marked in the first HC_IgG1_LALAPG) = Italic bold;
VL = Italic underlined:
Constant of lambda LC = Italic underlined dark gray; and
Constant of Kappa LC = Italic underlined bold;
Linker = light grey;
hFGF21ΔN = bold;
hFGF21FL= bold underline;
hFGF21ΔNΔC = light gray bold italic;
hFGF21ΔC = light gray italic; and
LALAPG mutations = bold dark gray underlined.

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Structure	SEQ ID NO.	Sequences
L-39F7 LC	1	*EVQLVESGGGLVQPGGSLRLSCAASGFTFTSYYISWVRQAPGKGLEWVAEISP YSGSTYYADSVKGRFTISADTSK NTAYLQMNSLRAEDTAVYYCALRARPPIRLHPRGSVMDYWGQGTLVTVSSGS AASGSSGGSSSQA*<u>*DIQMTQS PSSLSASVGDRVTITCRASQDVSTAVAWYQQKPGKAPKLLIYSASFLYSGVPSRF SGSGSGTDFTLTISSLQPEDFA TYYCQQSYTTPPTFGQGTKVEIK*</u>*GSGSG*<u>*EIVLTQSPGTLSLSPGERATLSCRAS QSVSSTYLAWYQQKPGQAPRLL IYGASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQSGSSPLTFGGGT EVEIK***RTVAAPSVFIFPPSDEQL KSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYS LSSTLTLSKADYEKHKVYACEVTHQ GLSSPVTKSFNRGEC***</u>*
39F7 HC	2	*QVQLVESGGGVVQPGRSLRLSCAASGFTFSNYGIHWVRQAPGKGLEWVAVIW YDGSIKYYADSVKGRFTISR DNSKNTLYLQMNSLRAEDTAVYYCARDRAAAGLHYYYGMDVWGQGTTVTVSS* ***ASTKGPSVFPLAPSSKSTSG GTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVT VPSSSLGTQTYICNVNHKPSNTKV DKKVEPKSCDKTHTCPPCPAPE[14]GGPSVFLFPPKPKDTLMISRTPEVTC VVVDVSHEDPEVKFNWYVDGVEV HNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKAL[G]APIE KTISKAKGQPREPQVYTLPPSREEM TKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYS KLTVDKSRWQQGNVFSCSVMHEA LHNHYTQKSLSLSPGK***</u>*
F12758-FGF21FL LC	3	*DIELTQPPSVSVAPGQTARISCSGDNIGSFYVHWYQQKPGQAPVLVIYDKSNRP SGIPERFSGSNSGNTATLTISG TQAEDEADYYCQSYANTLSLVFGGGTKLTVLGQPKAAPSVTLFPPSSEELQA NKATLVCLISDFYPGAVTVAWKA DSSPVKAGVETTTPSKQSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEG STVEKTVAPTECS[G][G][G][G]S*<u>**HPIPDSSPL LQFGGQVRQRYLYTDDAQQTEAHLEIREDGTVGGAADQSPESLLQL KALKPGVIQILGVKTSRFLCQRPDGALYGSLHFDPEACSFRERLLED GYNVYQSEAHGLPLHLPGNKSPHRDPAPRGPARFLPLPGLPPALPEP PGILAPQPPDVGSSDPLSMVGGSQGRSPSYES**</u>
F12758 HC	4	*EVQLVESGGGLVQPGGSLRLSCAASGFTFSHYTLSWVRQAPGKGLEWVSVISG DGSYTYYADSVKGRFTISSDN SKNTLYLQMNSLRAEDTAVYYCARNFIKYVFANWGQGTLVTVSS****ASTKGPSVF PLAPSSKSTSGGTAALGCLVKD YFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYI CNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPK DTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY NSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREP QVYTLPPSREEMTKNQVSLTCLVGFYPSDIAVEWESNGQPENNYKTTPPV LDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG K***</u>

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F12758-FGF21ΔN LC	5	*DIELTQPPSVSVAPGQTARISCSGDNIGSFYVHWYQQKPGQAPVLVIYDKSNRP SGIPERFSGSNSGNTATLTISGT QAEDEADYYCQSYANTLSLVFGGGTKLTVL*GQPKAAPSVTLFPPSSEELQAN KATLVCLISDFYPGAVTVAWKADS SPVKAGVETTTPSKQSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEGST VEKTVAPTECS**QRYLYTDDAQ QTEAHLEIREDGTVGGAADQSPESLLQLKALKPGVIQILGVKTSRFL CQRPDGALYGSLHFDPEACSFRERLLEDGYNVYQSEAHGLPLHLPGN KSPHRDPAPRGPARFLPLPGLPPALPEPPGILAPQPPDVGSSDPLSMV GGSQGRSPSYES**
L-αGFP LC	6	*EVQLVESGGGLVQPGGSLRLSCAASGFTFTSYYISWVRQAPGKGLEWVAEISP YSGSTYYADSVKGRFTISADT SKNTAYLQMNSLRAEDTAVYYCALRARPPIRLHPRGSVMDYWGQGTLVTVSS*GG GGSGGGGSGGGGS*DIQMT QSPSSLSASVGDRVTITCRASQDVSTAVAWYQQKPGKAPKLLIYSASFLYSGVPS RFSGSGSGTDFTLTISSLQPE DFATYYCQQSYTTPPTFGQGTKVEIK*GSGSGGSGSGSGGSGG*DIELTQPPSVSV APGQTARISCSGDNLGKKYVY WYQQKPGQAPVLVIYGDDERPSGIPERFSGSNSGNTATLTISGTQAEDEADYY CASYDSSHILIVFGGGTKLTVL* GQPKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVK AGVETTTPSKQSNNKYAASSYLSL TPEQWKSHRSYSCQVTHEGSTVEKTVAPTECS
αGFP HC	7	*QVQLVESGGGLVQPGGSLRLSCAASGFTFSRYGMHWVRQAPGKGLEWVSGIS SIGSNTYYADSVKGRFTISRD NSKNTLYLQMNSLRAEDTAVYYCARWYKTYIDVWGQGTLVTVSS**ASTKGPSV FPLAPSSKSTSGGTAALGCLVK DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTY ICNVNHKPSNTKVDKKVEPKSCDK THTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPE VKFNWYVDGVEVHNAKTKPREEQ YNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPRE PQVYTLPPSREEMTKNQVSLTCLVK GFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQ GNVFSCSVMHEALHNHYTQKSLSLSPGK***
F12758-FGF21ΔC LC	8	*DIELTQPPSVSVAPGQTARISCSGDNIGSFYVHWYQQKPGQAPVLVIYDKSNRP SGIPERFSGSNSGNTATLTISGT QAEDEADYYCQSYANTLSLVFGGGTKLTVL*GQPKAAPSVTLFPPSSEELQAN KATLVCLISDFYPGAVTVAWKADS SPVKAGVETTTPSKQSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEGST VEKTVAPTECS*GGSGGS*HPIPDSSPLLQ FGGQVRQRYLYTDDAQQTEAHLEIREDGTVGGAADQSPESLLQLKALKP GVIQILGVKTSRFLCQRPDGALYGSLH FDPEACSFRERLLEDGYNVYQSEAHGLPLHLPGNKSPHRDPAPRGPARFL PLPGLPPALPEPPGILAPQPPDVG
F12758-FGF21ΔNΔC LC	9	*DIELTQPPSVSVAPGQTARISCSGDNIGSFYVHWYQQKPGQAPVLVIYDKSNRP SGIPERFSGSNSGNTATLTISG TQAEDEADYYCQSYANTLSLVFGGGTKLTVL*GQPKAAPSVTLFPPSSEELQA NKATLVCLISDFYPGAVTVAWKA DSSPVKAGVETTTPSKQSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEG STVEKTVAPTECS*GGSGGSGGSGGS*

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Example 2

Multicomponent WNT Molecule for Concept of the 1:1 Split Design

[0264] To test the concept depicted in Fig. 1G, the FZD binder (F12578), LRP binder (L), and the two non-competing bridging receptor (βKlotho) binders (FGF21FL and 39F7) were combined in the molecule, F12578-FGF21FL-39F7-L, depicted in Fig. 3A. As shown in Fig. 3B, F12578-FGF21FL-39F7-L activated WNT/β-catenin signaling in Huh7 cells that express the bridging receptor βKlotho but not in 293 cells where βKlotho is not expressed (Fig. 3B). No activity was observed in 293 cells. This provided validation for the concept presented in Fig. 1G. Table 2 describes the different components/formats tested.

[0265] Table 2: Formats and sequences of multicomponent WNT polypeptide with 1:0 / 01: split design:

VH= Italic;

Human CH1CH2CH3 with LALAPG mutations (LALAPG mutations are only marked in the first HC_IgG1_LALAPG)=Italic bold;

VL = Italic underlined;

Constant of lambda LC = dark gray;

Constant of Kappa LC = Italic underlined bold;

G4S linker = light grey;

hFGF21ΔN =bold;

hFGF21FL=bold underline;

hFGF21ΔNΔC = light gray bold italic;

hFGF21ΔC = light gray italic;

Amino acids mutations in SEQ 11 and SEQ 12 = bold dark gray underlined.

Structure	SEQ ID NO.	Sequences
39F7 LC	10	*EIVLTQSPGTLSLSPGERATLSCRASQSVSSTYLAWYQQKPGQAPRLLIYGASSRATGIP DRFSGSGSGTD*FTLTISRLEPEDFAVYYCQQSGSSP*LTFGGGTEVEIK**RTVAAPSVF IFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKD STYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC***
F12758_ Hole _Flag HC	11	*EVQLVESGGGLVQPGGSLRLSCAASGFTFSHYTLSWVRQAPGKGLEWVSVISGDGSYT YYADSVKGRFTISSDNSKNTLYLQMNSLRAEDTAVYYCARNFIKYVFANWGQGTLVTVS* ***SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFP AVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCP PCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDG VEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKT***

		ISKAKGQPREPQVYTLPPSREEMTKNQVSLSCAVKGFYPSDIAVEWESNGQPEN NYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSL SPGK[illegible] T366S L368A Y407V are mutations for the "Hole" of Knob and Hole
39F7_ Knob _HC	12	*QVQLVESGGGVVQPGRSLRLSCAASGFTFSNYGIHWVRQAPGKGLEWVAVIWYDGSI KYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCARDRAAAGLHYYYGMDVW GQGTTVTVSS**ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGAL TSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKS CDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEV KFNWYVDGV EVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTI SAKGQPREPQVYTLPPSREEMTKNQVSLSCLVKGFYPSDIAVEWESNGQPENNY KTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVM HEALHNHYTQKSLSLSPGK*** T366W is the "Knob" of Knob and Hole
α GFP-39F7 LC	13	*QVQLVESGGGLVQPGGSLRLSCAASGFTFSRYGMHWVRQAPGKGLEWVSGISSIGSN TYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCARWYKTYIDVWGQGTLVTV SS[illegible]DIELTQPPSVSVAPGQTARISCSGDNLGKKYVYWYQQKP GQAPVLVIYGDDERPSGIPERFSGSNSGNTATLTISGTQAEDEADYYCASYDSSHILIVF GGGTKLTVL[illegible]EIVLTQSPGTLSLSPGERATLSCRASQSVSSTYLAWYQQKPGQA PRLLIYGASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQSGSSPLTFGGGTE VEIK**RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGN SQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGE C***</u>
F12758-FGF21FL LC	3	*DIELTQPPSVSVAPGQTARISCSGDNIGSFYVHWYQQKPGQAPVLVIYDKSNRPSGIPE RFSGSNSGNTATLTISGTQAEDEADYYCQSYANTLSLVFGGGTKLTVL*[illegible]**HPIPDSSPL LQFGGQVRQRYLYTDDAQQTEAHLEIREDGTVGGAADQSPESLLQLKALK PGVIQILGVKTSRFLCQRPDGALYGSLHFDPEACSFRERLLEDGYNVYQSEA HGLPLHLPGNKSPHRDPAPRGPARFLPLPGLPPALPEPPGILAPQPPDVGSSD PLSMVGGSQGRSPSYES**

[0266] The various embodiments described above can be combined to provide further embodiments. All of the U.S. patents, U.S. patent application publications, U.S. patent applications, foreign patents, foreign patent applications, and non-patent publications referred to in this specification and/or listed in the Application Data Sheet are incorporated herein by reference, in their entirety. Aspects of the embodiments can be modified, if necessary to employ concepts of the various patents, applications, and publications to provide yet further embodiments. These and other changes can be made to the embodiments considering the above-detailed description. In general, in the following claims, the terms used should not be construed to limit the claims to the specific embodiments disclosed in the specification and the claims but should be construed to include all possible embodiments along with the full scope of equivalents to which such claims are entitled. Accordingly, the claims are not limited by the disclosure.

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What is Claimed is:

1. A cell targeting multicomponent polypeptide molecule comprising:

a) at least one first antigen binding domain that binds to a first signaling receptor component,

b) at least one second antigen binding domain that binds to a second signaling receptor component, and

c) at least one different antigen binding domain that binds to a bridging molecule,

wherein the first signaling receptor and second signaling receptor can be different or identical.

2. The cell targeting multicomponent polypeptide molecule of claim 1, wherein the binding domain that binds to the bridging molecule comprises at least two binding domains that:

a) bind to different epitopes on the bridging molecule; or

b) bind to the same epitope on the bridging molecule.

3. The cell targeting multicomponent polypeptide molecule of claim 1 or claim 2, wherein

a) the first antigen binding domain that binds to a first signaling receptor component is tethered to at least one binding domain that binds to the bridging molecule; and

b) the second antigen binding domain that binds to a second signaling receptor component is tethered to at least one binding domain that binds to the bridging molecule.

4. The multicomponent polypeptide molecule of claim 3, wherein the antigen binding domain is joined directly or by a linker to the binding domain that binds to the bridging molecule.

5. The multicomponent polypeptide molecule of claim 3, wherein the bridging molecule is monomeric or multimeric.

6. The multicomponent polypeptide molecule of claim 3, wherein the binding domains are each independently selected from the group consisting of: an scFv, a VHH/sdAb, a Fab, and a Fab′2.

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7. The multicomponent polypeptide molecule of claim 3, wherein the binding domains are joined through a linker by a peptide linker comprising from 1-100 amino acids.

8. The multicomponent polypeptide molecule of claim 3, wherein the binding domains are attached to the N-terminus of an antibody Fc domain.

9. A nucleic acid encoding the multicomponent polypeptide molecule of any one of claims 1-8 or a polypeptide component thereof.

10. A vector comprising the nucleic acid of claim 9, wherein the vector is an expression vector comprising a promoter sequence operatively linked to the nucleic acid sequence; optionally wherein the vector is a virus comprising a promoter operatively linked to the nucleic acid.

11. A host cell comprising the vector of claim 10.

12. A process for producing the multicomponent polypeptide molecule of any one of claims 1-8 or a polypeptide component thereof, comprising culturing the host cell of claim 11 under conditions wherein the multicomponent polypeptide molecule or polypeptide component thereof is expressed by the expression vector.

13. A multicomponent WNT molecule comprising:

a) at least one FZD binding domain;

b) at least one LRP binding domain; and

c) at least one bridging molecule,

wherein the multicomponent WNT multicomponent polypeptide molecule modulates WNT signaling.

14. The multicomponent WNT molecule of claim 13, comprising:

a) one or two FZD binding domains tethered to the bridging molecule by a binding domain specific for a first epitope on the bridging molecule; and

b) one or two LRP binding domains tethered to the bridging molecule by a binding domain specific for a second epitope on the bridging molecule,

wherein the multicomponent WNT molecule is capable of activating WNT signaling.

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15. The multicomponent WNT molecule of claim 13 or claim 14, wherein:

a) the binding domain specific for the first epitope of the bridging molecule and the FZD binding domain(s) are joined directly or joined by a linker;

b) the binding domain specific for the second epitope of the bridging molecule and the LRP binding domain(s) are joined directly, or joined by a linker; and

c) the binding domain specific for the first epitope and the binding domain specific for the second epitope of the bridging molecule are identical or different.

16. The multicomponent WNT molecule of claim 15, wherein the linker is a peptide or non-peptide linker.

17. The multicomponent WNT molecule of claim 13, wherein the FZD binding domain(s) and the LRP binding domain(s) are each independently selected from the group consisting of: a scFv, a VHH/sdAb, a Fab, and a Fab´2.

18. The multicomponent WNT molecule of claim 13, wherein the FZD binding domain(s) and the LRP binding domains(s) are joined by a peptide linker comprising from 1-100 amino acids.

19. The multicomponent WNT molecule of claim 13, wherein the FZD binding domain(s) and the LRP binding domain(s) are attached to the N-terminus of an antibody Fc domain.

20. A nucleic acid encoding the multicomponent WNT molecule of any one of claims 13-19 or a polypeptide component thereof.

21. A vector comprising the nucleic acid of claim 20, wherein the vector is an expression vector comprising a promoter sequence operatively linked to the nucleic acid sequence; optionally wherein the vector is a virus comprising a promoter operatively linked to the nucleic acid.

22. A host cell comprising the vector of claim 21.

23. A process for producing the multicomponent WNT molecule or polypeptide component thereof of claim 13, comprising culturing the host cell of claim 22 under conditions

wherein the multicomponent WNT protein or polypeptide component thereof is expressed by the expression vector.

24. A pharmaceutical composition to modulate the WNT/β-catenin signaling pathway comprising:

a) an effective amount of the multicomponent WNT molecule of any one of claims 13-19; and

b) a pharmaceutically acceptable diluent, adjuvant, or carrier.

25. A method of treating or preventing a disease or disorder in a subject in need thereof, the method comprising providing to the subject an effective amount of the multicomponent WNT molecule of any one of claims 13-19 or the pharmaceutical composition of claim 24.

26. A multicomponent WNT molecule comprising:

a) at least one FZD binding domain linked to one LRP binding domain which is tethered to the bridging molecule by a binding domain specific for a first epitope on the bridging molecule; and

b) at least one FZD binding domain linked to one LRP binding domain which is tethered to the bridging molecule by a binding domain specific for a second epitope on the bridging molecule;

wherein the tethering of the FZD and LRP binding domains to the bridging receptor forms a WNT mimetic capable of activating WNT signaling.

27. The multicomponent WNT molecule of claim 26, wherein:

a) the binding domain specific for a first epitope of the bridging molecule and the FZD/LRP binding domains are joined directly or by a linker;

b) the binding domain specific for a second epitope of the bridging molecule and the FZD/LRP binding domains are joined directly, or by a linker;

c) the binding domain of the first epitope and second epitope of the bridging molecule are identical or different; and/or

d) the binding domain of the first epitope and the second epitope of the bridging molecule could be linked together to FZD/LRP.

28. The multicomponent WNT molecule of claim 26 or claim 27, wherein the bridging molecule is monomeric or multimeric.

29. The multicomponent WNT molecule of any one of claims 26-28, wherein at least one of the FZD or LRP binding domains is selected from the group consisting of: a scFv, a VHH/sdAb, a Fab, and a Fab´2.

30. The multicomponent WNT molecule of any one of claims 26-29, wherein the binding domains are joined through a linker by a peptide link comprising from 1-100 amino acids.

31. The multicomponent WNT molecule of any one of claims 26-30, wherein the binding domains are attached to the N-terminus of an antibody Fc domain.

32. A nucleic acid encoding the multicomponent WNT molecule of any one of claims 26-31 or a polypeptide component thereof.

33. A vector comprising the nucleic acid of claim 32, wherein the vector is an expression vector comprising a promoter sequence operatively linked to the nucleic acid sequence; optionally wherein the vector is a virus comprising a promoter operatively linked to the nucleic acid.

34. A host cell comprising the vector of claim 33.

35. A process for producing the multicomponent WNT molecule or component thereof of any one of claims 26-31, comprising culturing the host cell of claim 36 under conditions wherein multicomponent WNT or polypeptide component thereof is expressed by the expression vector.

36. A pharmaceutical composition to modulate the WNT/β-catenin signaling pathway comprising:

a) an effective amount of the multicomponent WNT of any one of claims 26-31; and

b) a pharmaceutically acceptable diluent, adjuvant, or carrier.

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37. A method of treating or preventing a disease or disorder in a subject in need thereof, the method comprising providing to the subject an effective amount of the multicomponent WNT molecule of any one of claims 26-31 or the pharmaceutical composition of claim 36.

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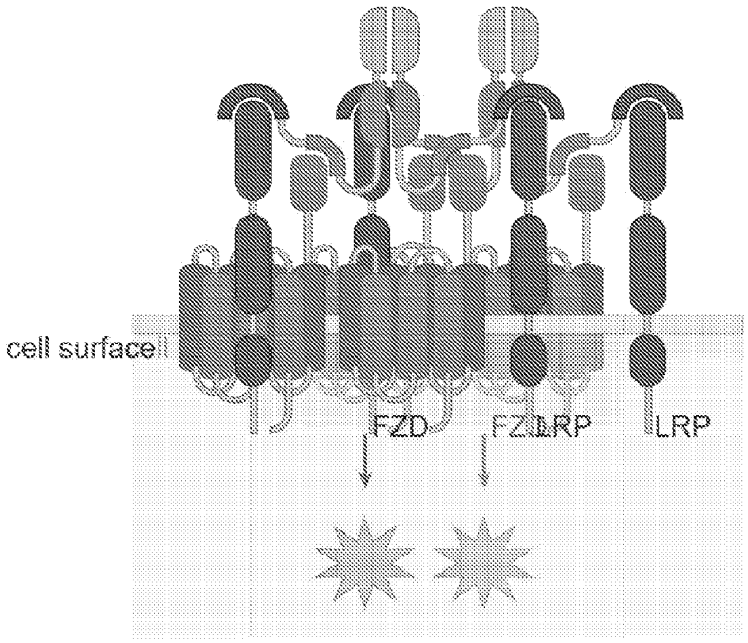


Fig. 1A

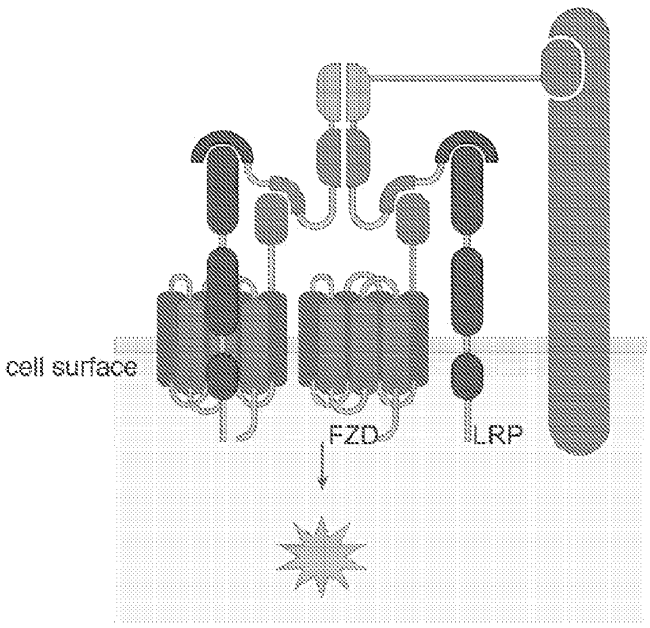


Fig. 1B

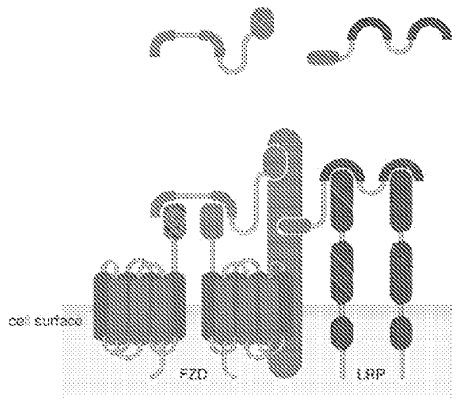


Fig. 1C

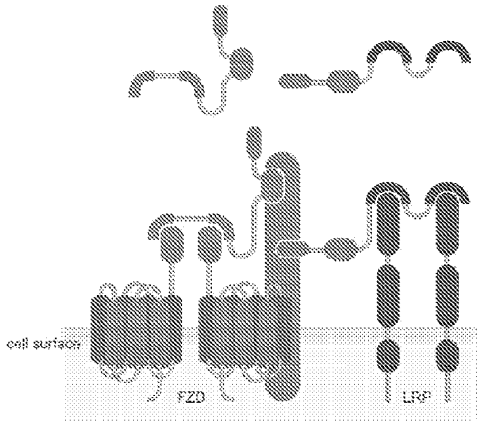


Fig. 1D

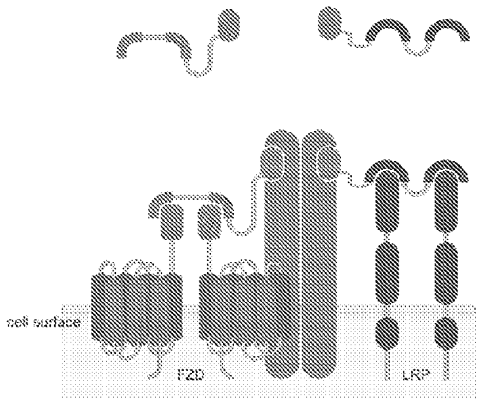


Fig. 1E

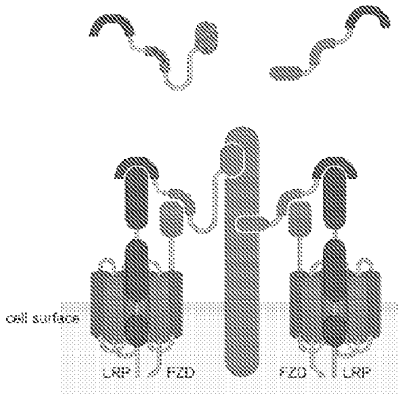


Fig. 1F

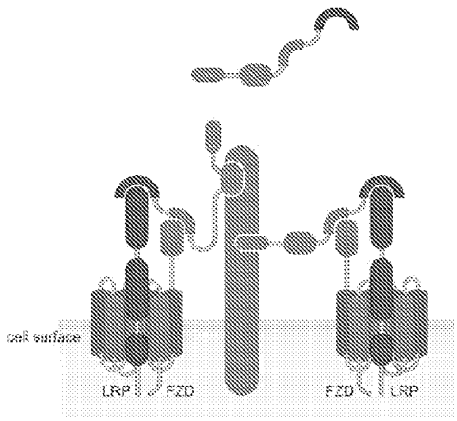


Fig. 1G

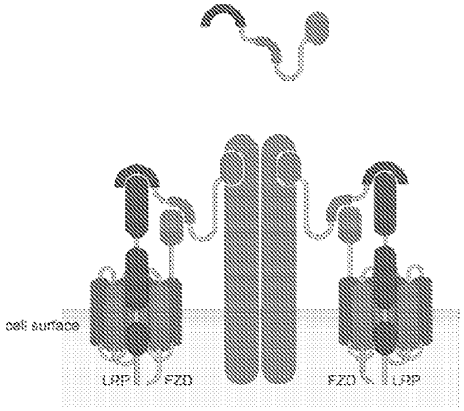


Fig. 1H

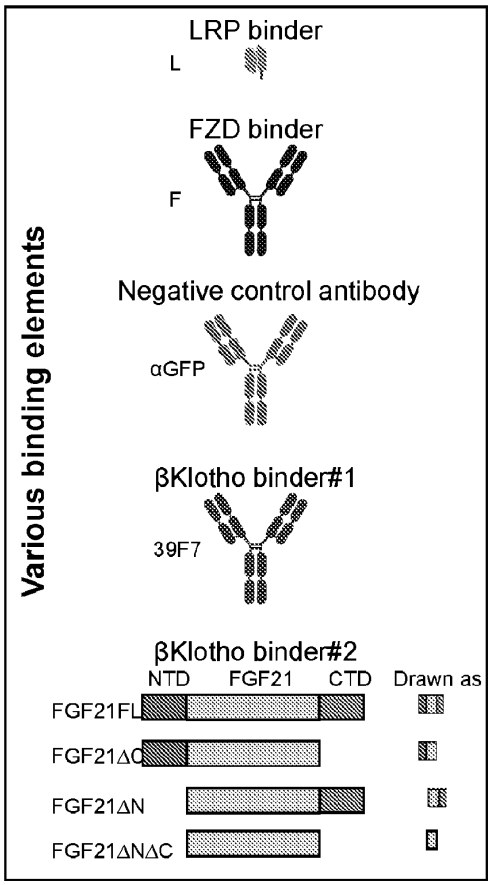

Various binding elements
LRP binder
L
FZD binder
F
Negative control antibody
αGFP
βKlotho binder#1
39F7
βKlotho binder#2
NTD
FGF21
CTD
Drawn as
FGF21FL
FGF21ΔC
FGF21ΔN
FGF21ΔNΔC

Fig. 2A

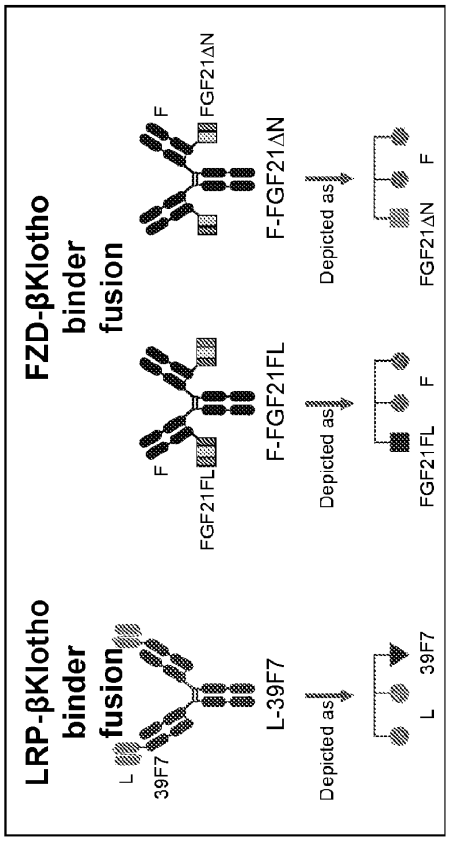

LRP-βKlotho binder fusion
L
39F7
L-39F7
Depicted as
L 39F7
FZD-βKlotho binder fusion
F
FGF21FL
F-FGF21FL
Depicted as
FGF21FL F
F
FGF21ΔN
F-FGF21ΔN
Depicted as
FGF21ΔN F

Fig. 2B

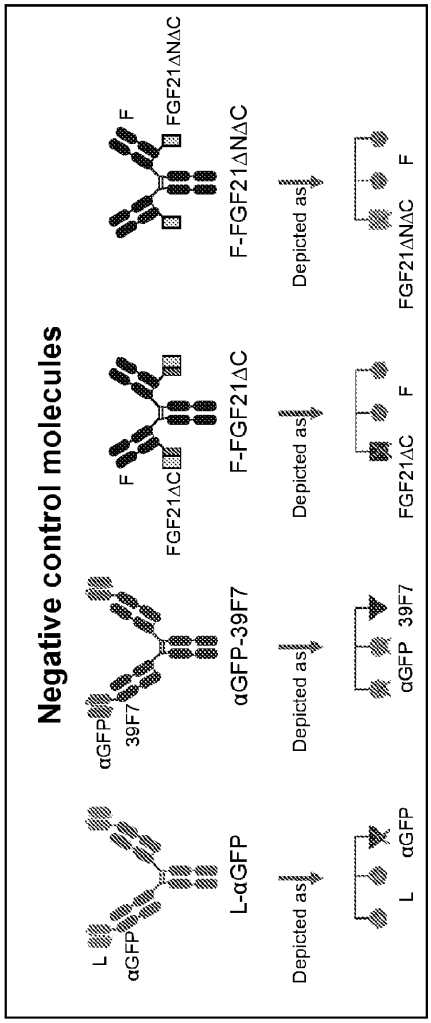

Negative control molecules
L
αGFP
L-αGFP
αGFP
39F7
αGFP-39F7
F
FGF21ΔC
F-FGF21ΔC
F
FGF21ΔNΔC
F-FGF21ΔNΔC
Depicted as
Depicted as
Depicted as
Depicted as
L
αGFP
αGFP
39F7
FGF21ΔC
F
FGF21ΔNΔC
F

Fig. 2C

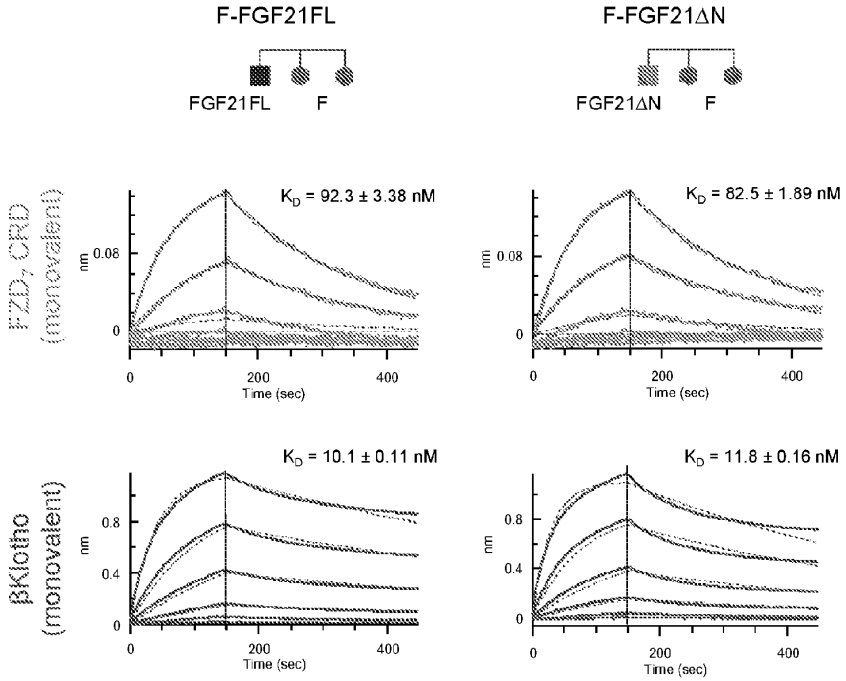

F-FGF21FL
FGF21FL
F
F-FGF21ΔN
FGF21ΔN
F
FZD7 CRD (monovalent)
βKlotho (monovalent)
nm
Time (sec)
K_D = 92.3 ± 3.38 nM
K_D = 82.5 ± 1.89 nM
K_D = 10.1 ± 0.11 nM
K_D = 11.8 ± 0.16 nM

Fig. 2D Fig. 2E

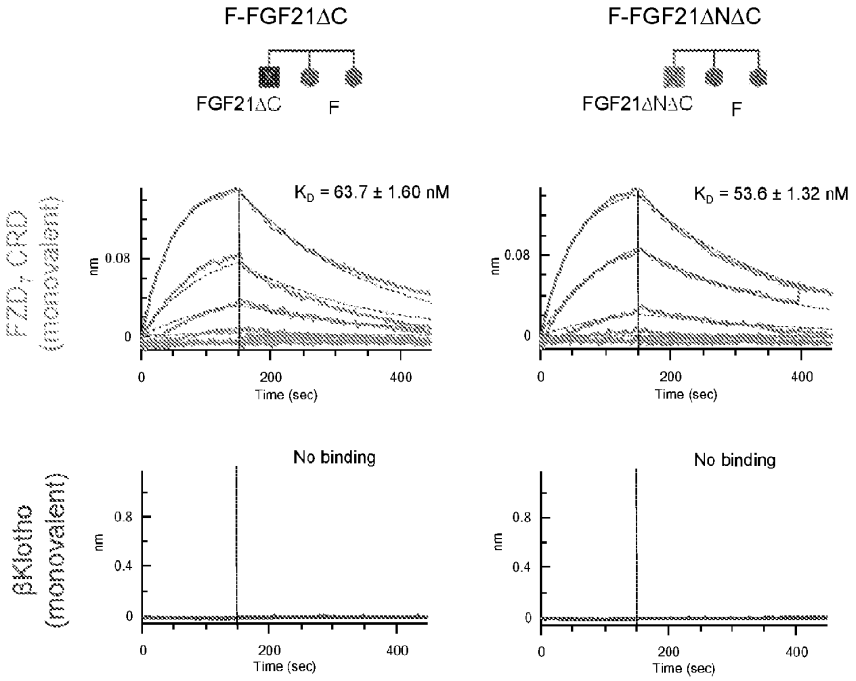

F-FGF21ΔC
FGF21ΔC
F
F-FGF21ΔNΔC
FGF21ΔNΔC
F
FZD7 CRD (monovalent)
K_D = 63.7 ± 1.60 nM
K_D = 53.6 ± 1.32 nM
nm
0.08
0
200
400
Time (sec)
βKlotho (monovalent)
No binding
0.8
0.4

Fig. 2F

Fig. 2G

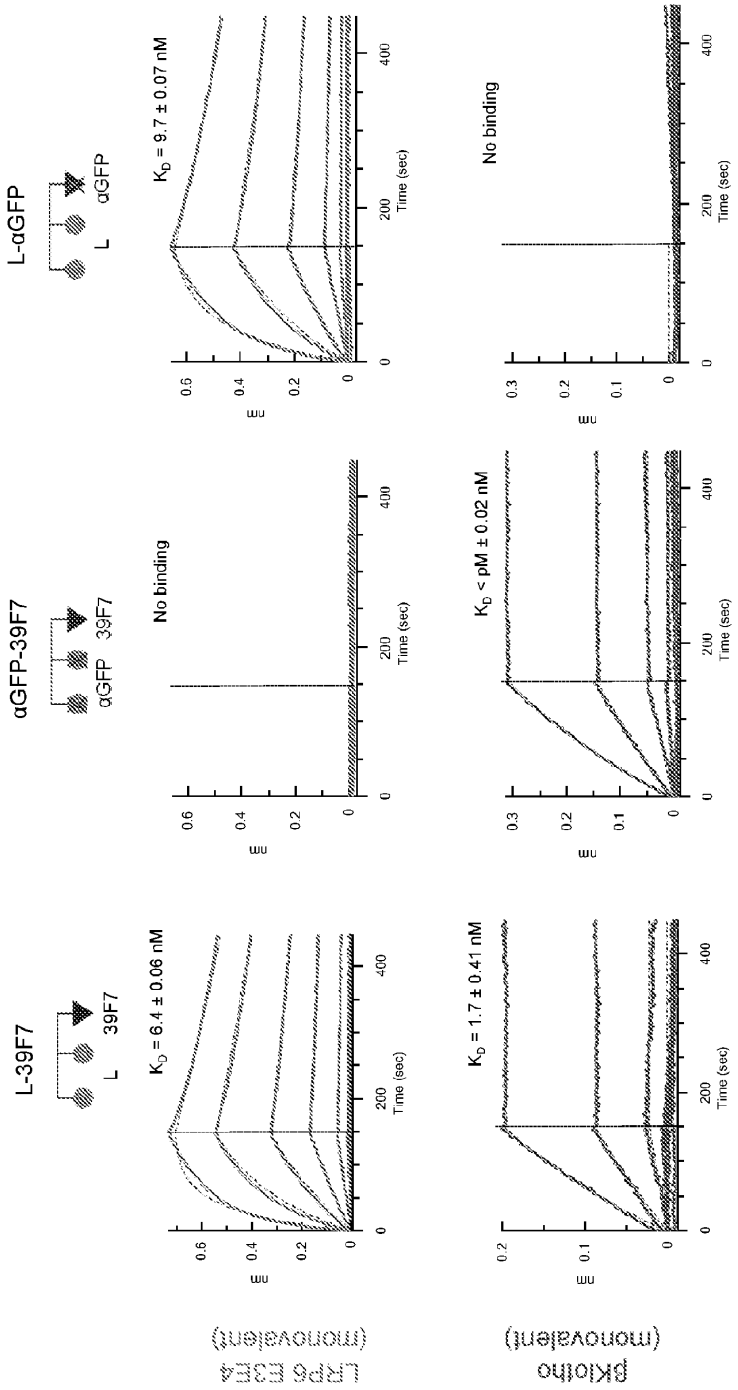


Fig. 2H Fig. 2I Fig. 2J

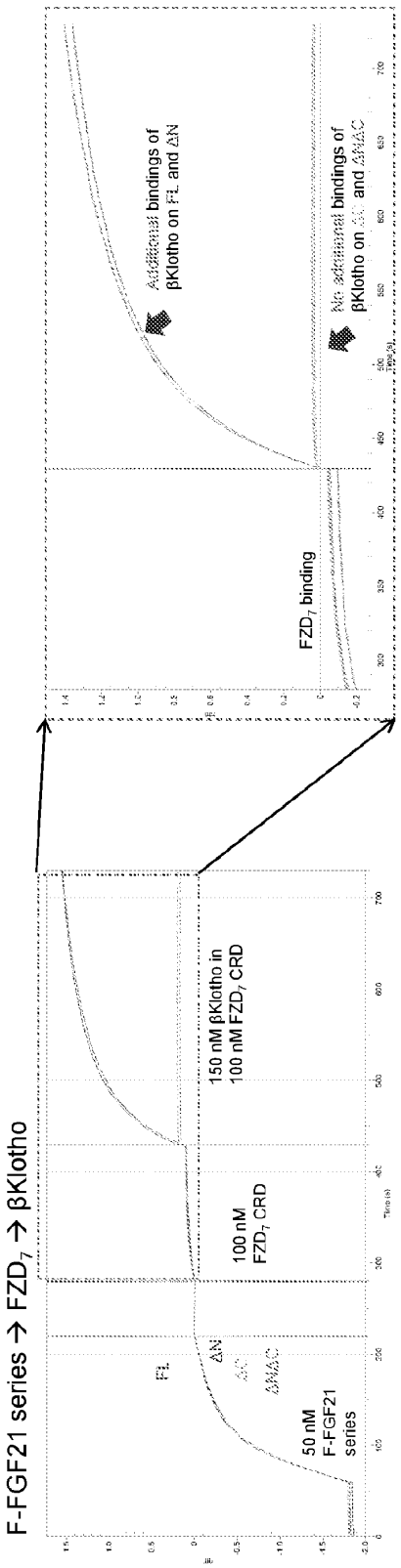

F-FGF21 series → FZD7 → βKlotho
50 nM F-FGF21 series
100 nM FZD7 CRD
150 nM βKlotho in 100 nM FZD7 CRD
FZD7 binding

Fig. 2K

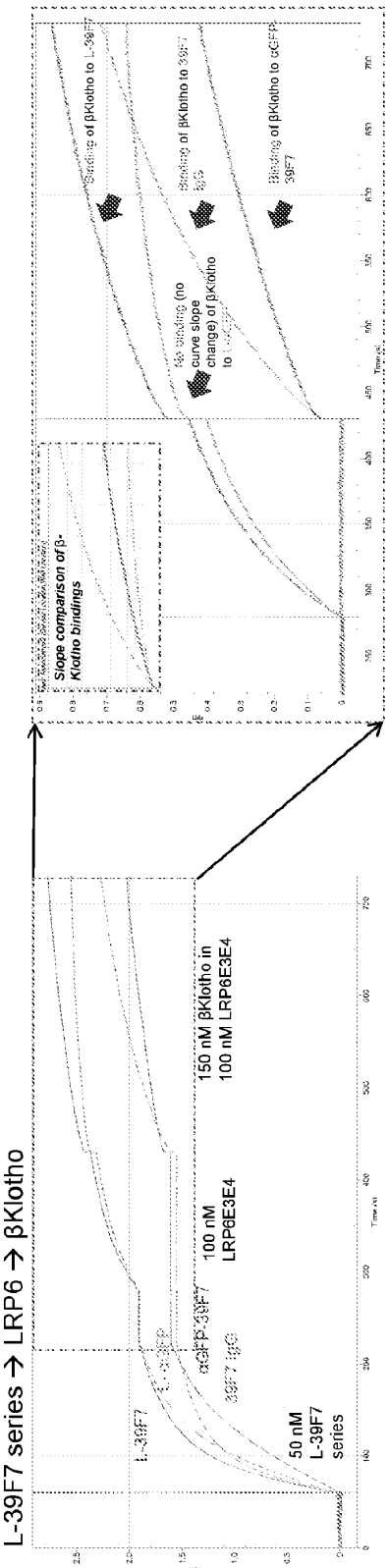

L-39F7 series → LRP6 → βKlotho
50 nM L-39F7 series
100 nM LRP6E3E4
150 nM βKlotho in 100 nM LRP6E3E4
Slope comparison of β-Klotho bindings

Fig. 2L

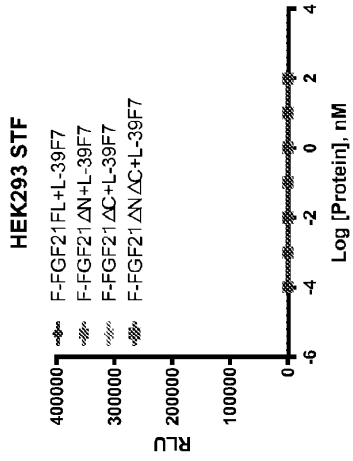


Fig. 3A

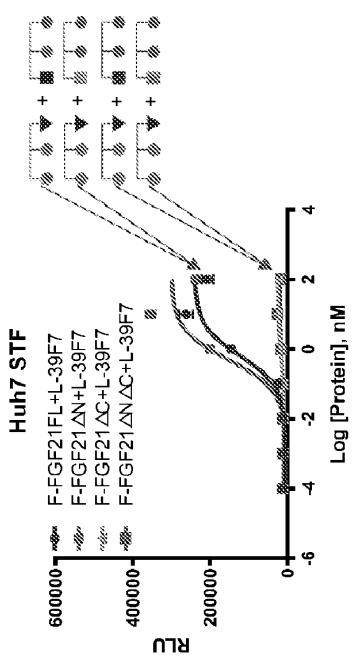


Fig. 3B

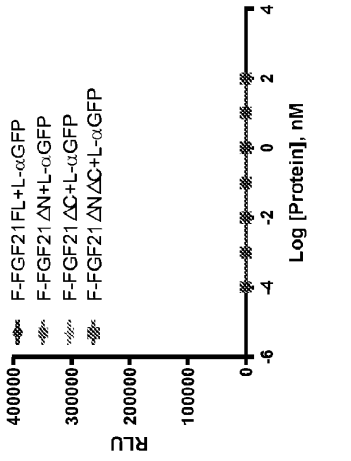


Fig. 3C

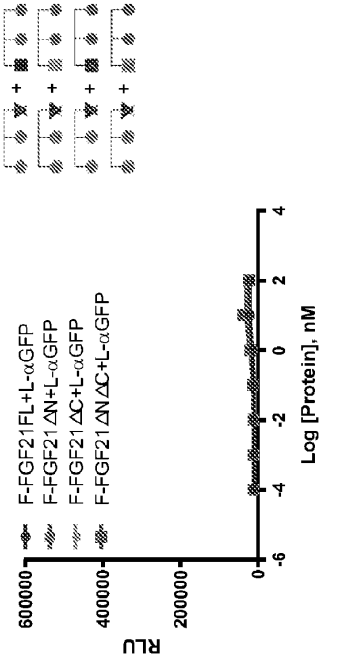


Fig. 3D

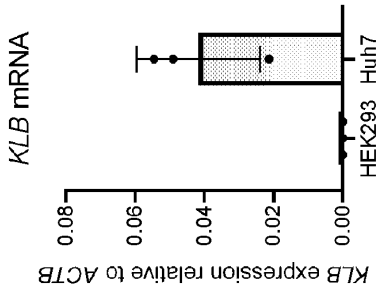


Fig. 3G

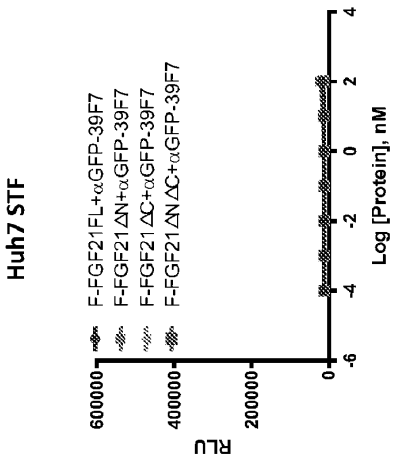
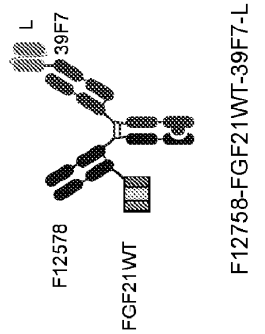


Fig. 3F

Fig. 3E



F12758-FGF21WT-39F7-L

Fig. 4A

Fig. 4B

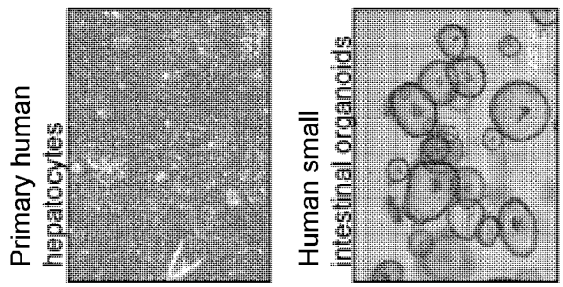


Fig. 5A

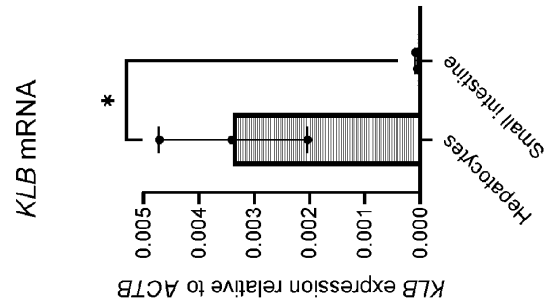


Fig. 5B

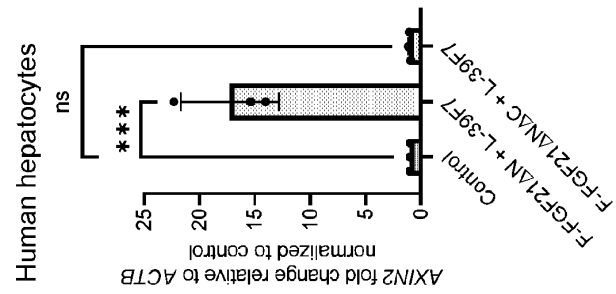


Fig. 5C

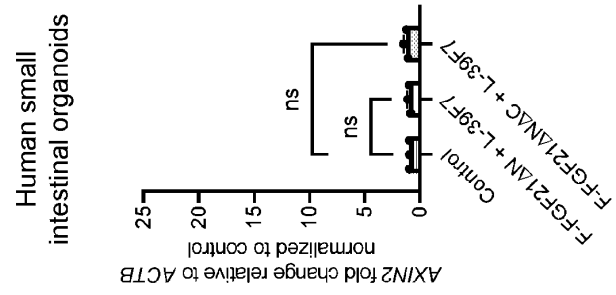


Fig. 5D

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(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, CV, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SC, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, ME, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

(54) Title: CELL TARGETING MULTICOMPONENT POLYPEPTIDE

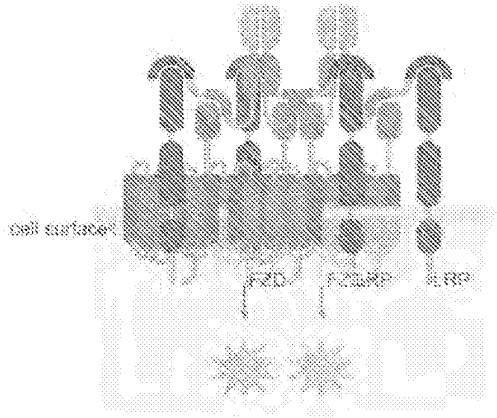


Fig. 1A

(57) Abstract: The present invention provides a cell targeting multicomponent polypeptides complexes, to modulate signaling pathways having tissue specificity, by binding to homo- or hetero-oligomeric receptor complexes.

[Continued on next page]

WO 2024/040118 A2

Declarations under Rule 4.17:

- *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*
- *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

Published:

- *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*
- *with sequence listing part of description (Rule 5.2(a))*

CELL TARGETING MULTICOMPONENT POLYPEPTIDE

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Patent Application Serial No. 63/398,502, filed August 16, 2022, and U.S. Provisional Patent Application Serial No. 63/472,938, filed June 14, 2023, which are incorporated by reference in their entireties.

SEQUENCE LISTINGS

[0002] The Sequence Listing XML associated with this application is provided in XML file format and is hereby incorporated by reference into the specification. The name of the XML file containing the Sequence Listing XML is SRZN_026_01WO_ST26.xml. The XML file is 39,279 bytes, and created on August 8, 2023, and is being submitted electronically via USPTO Patent Center.

FIELD OF THE INVENTION

[0003] The present disclosure provides cell targeting multicomponent polypeptides that modulate signaling pathways having cell and tissue specificity by binding to hetero-oligomeric receptor complexes.

BACKGROUND OF THE INVENTION

[0004] Receptor-mediated cellular signaling is fundamental to cell-cell communication, cellular growth and differentiation, maintenance of tissue function and homeostasis, and regulation of injury repair. When a cell-specific response is necessary, nature achieves that selectivity through various mechanisms such as cell-specific receptor, ligand gradient, short-range ligand, direct cell-cell contacts. However, most ligand-receptor systems have pleiotropic effects due to broad expressions of receptors on multiple cell types. While this may be important when a coordinated response in numerous cell types or tissues is needed, modulating cell-specific signaling has been a major challenge for research and drug development to avoid systemic toxicity or off-target tissue effects. In addition, it is often difficult to identify differences between normal and diseased cells for a particular signaling pathway of interest, making it challenging, if not impossible, to selectively modulate that signaling pathway either in diseased or normal cells. Therefore, effective cell targeting is needed for both research and therapeutic development.

[0005] Cell-targeted antagonists are more accessible to achieve than cell-targeted agonists, as antagonists have less stringent requirements on affinity, epitope, and geometry of the molecules than agonists (Dickopf et al., 2020). Previous efforts to engineer cell selective growth factors and cytokines employed variations of the "chimeric activators" concept (Cironi et al., 2008), which invariably involved attaching a cell targeting arm ("targeting element") to either the wild-type ligand or a mutated ligand with reduced affinity toward signaling receptor ("activity element"). Such an approach has been applied to several signaling pathways, such as the erythropoietin (Taylor et al., 2010, Burrill et al., 2016), interferon (Cironi et al., 2008, Garcin et al., 2014), and interleukin-2 (Ghasemi et al., 2016, Lazear et al., 2017) pathways.

[0006] While little selectivity could be achieved by attaching a targeting arm to a wild-type ligand, selectivity of up to 1000-fold has been reported when a mutated ligand was used (Garcin et al., 2014). The "chimeric activators" approach is based on the cooperativity concept. In this approach, mutations that weaken the affinity of a natural ligand to its receptor are selected as the "activity element". Due to the weakened affinity, the mutant "activity element" alone should display a significantly right-shifted dose-response curve (or lower potency) in an activity assay on both target and non-target cells that express the signaling receptors.

[0007] When a "targeting element" is tethered to the mutant "activity element," the "targeting element" helps to increase the local concentration of the mutant "activity element" on the desired target cell surface, driving engagement of signaling receptor and subsequent intracellular signaling activation, effectively left shift the activity dose-response curve (increase potency) on the target cell and create the selectivity between target vs. non-target cells.

[0008] While conceptually elegant, identifying the appropriate mutations that achieve the just-right reduction of affinity that could be rescued by the "targeting element" is not trivial. In some cases, while the potency could be rescued, the maximal signaling strength remains compromised, resulting in a partial agonist. In addition, the selectivity is not exquisite; a modest 10-fold separation between targeting vs. non-targeting cells is reported in most literature examples.

[0009] WNT ligands and their signals play key roles in the control of development, homeostasis and regeneration of many essential organs and tissues, including bone, liver, skin, stomach, intestine, kidney, central nervous system, mammary gland, taste bud, ovary, cochlea and many other tissues (reviewed, e.g., by Clevers, Loh, and Nusse (2014) *Science*, 346:54).

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Modulation of WNT signaling pathways has potential for treatment of degenerative diseases and tissue injuries.

[0010] The WNT pathway is highly conserved across species and crucial for embryonic development, and adult tissue homeostasis and regeneration (Nusse and Clevers, 2017). WNT-induced signaling through β-catenin stabilization has been widely studied and is achieved by ligand binding to frizzled (FZD) and low-density lipoprotein receptor-related protein (LRP) family of receptors. There are nineteen mammalian WNTs, 10 FZDs (FZD_{1-10}), and 2 LRPs (LRP5 and LRP6).

[0011] WNTs are highly hydrophobic due to lipidation that is required for function and are promiscuous, capable of binding and activating multiple FZD and LRP pairs (Janda et al., 2012, Kadowaki et al., 1996, Dijksterhuis et al., 2015). Elucidating the functions of individual FZDs in tissues has been hampered by difficulties in producing the ligands and lack of receptor and tissue selectivity. Recent breakthroughs in the development of WNT-mimetic molecules have largely resolved the production and receptor specificity challenges (Janda et al., 2017, Chen et al., 2020, Tao et al., 2019, Miao et al., 2020).

[0012] While tissue selectivity could be partly achieved by tissue injury as damaged tissues seem more sensitive to WNTs (Xie et al., 2022), it would still represent a significant technical advancement to be able to target WNTs to specific cells and tissues.

[0013] Antibodies are a well-established and rapidly growing drug class with at least 45 antibody-based products currently marketed for imaging or therapy in the United States and/or Europe with ~$100 billion in total worldwide sales. This major clinical and commercial success with antibody therapeutics has fueled much interest in developing the next generation antibody drugs including bispecific antibodies. As their name implies, bispecific antibodies or multispecific antibodies (collectively "MsAbs") bind to at least two different antigens, or at least two different epitopes on the same antigen, as first demonstrated more than 50 years ago. Engineering monospecific antibodies for multispecificity opens up many new potential therapeutic applications as evidenced by >30 BsAb in clinical development.

[0014] Bispecific or multispecific antibodies are a class of engineered antibody and antibody-like proteins that, in contrast to 'regular' monospecific antibodies, combine two or more different specific antigen binding elements in a single construct. Since bispecific antibodies do not typically occur in nature, they are constructed either chemically or biologically, using techniques such as cell fusion or recombinant DNA technologies. The

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ability to bind two or more different epitopes with a single molecule offers a number of potential advantages. One approach is to use the specificity of one arm as a targeting site for individual molecules, cellular markers or organisms, such as bacteria and viruses. While the other arm functions as an effector site for the recruitment of effector cells or delivery of molecular payloads to the target, such as drugs, cytokines or toxins. Alternatively, bispecifics can be used to dual target, allowing detection or binding of a target cell type with much higher specificity than monospecific antibodies.

[0015] The modular architecture of immunoglobulins has been exploited to create a growing number (>60) of alternative MsAb formats (see, e.g., Spiess et al (2015) *Mol. Immunol.* 67:95-106). MsAb are classified here into five distinct structural groups: (i) bispecific IgG (BsIgG) (ii) IgG appended with an additional antigen-binding moiety (iii) MsAb fragments (iv) Multispecific fusion proteins and (v) MsAb conjugates. Each of these different MsAb formats brings different properties in binding valency for each antigen, geometry of antigen-binding sites, pharmacokinetic half-life, and in some cases effector functions.

[0016] For antagonistic MsAbs antibodies, which represent the vast majority of the MsAb molecules in development, the geometry of the antigen binding modules is less critical. However, for agonistic MsAbs, these molecules need to faithfully mimic the activity of the natural ligand, the binding geometry could be crucial (see, e.g., Shi, et al. (2018) *J. Biol. Chem.* 293:5909-5919). Such is true of WNT multicomponent polypeptide molecules, which are required to bind and activate two spatially separated WNT receptors, FZD and LRP.

[0017] WNT multicomponent polypeptide molecules which can bind to the hetero-oligomeric WNT/LRP receptor complex have been described previously (see, e.g., WO2019/126398 and WO2020/010308) as have WNT enhancers using RSPO (see, e.g., WO2018/140821, WO2018/132572, and WO2020/014271).

[0018] Previous attempts to create multicomponent receptor molecules have been successful for antagonist molecules because lower affinities and tissue selectivity were not as critical. Creating agonist multicomponent receptor molecules has been challenging because of this reduced affinity and lack of selectivity. The present invention addresses this need by the use of a binding composition specific for a bridging molecule, which resides on the cell surface, tethered to binding domains specific for signaling receptor components. This use of a bridging molecule binding domain has provided acceptable affinity for agonist signaling, as well as the ability to confer specificity by target tissue and cell specific cell surface antigens.

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BRIEF DESCRIPTION OF THE DRAWINGS

[0019] Fig. 1A shows an optimized design for a WNT mimetic that is a tetravalent bispecific antibody-based molecule.

[0020] Fig. 1B shows a cell targeted WNT mimetic based on the "chimeric activator" concept where an "targeting element" is tethered to the WNT mimetic.

[0021] Fig. 1C illustrates two separate polypeptides: one FZD binder with additional binding domain that binds "bridging molecule" and one LRP binder that binds "bridging molecule", where the two molecules bind different epitopes of the "bridging molecule"

[0022] Fig. 1D illustrates a WNT mimetic with two FZD binding domains tethered to a bridging molecule by two binding domains specific for two epitopes on the bridging molecule and two LRP binding domains tethered to the bridging molecule by two binding domains specific for two epitopes on the bridging molecule, wherein the two bridging binding epitopes are linked together and each separate molecule could be tethered to "bridging element" by binding to a different epitope on the bridging receptor, creating the final receptor complex.

[0023] Fig. 1E shows a WNT mimetic with two FZD binding domain tethered to a bridging molecule by a binding domain specific for a first epitope on the bridging molecule and two LRP binding domains tethered to the bridging molecule by a binding domain specific for a second epitope on the bridging molecule, wherein the two bridging binding epitopes are identical and each separate molecule could be tethered to "bridging element" binding to a epitope on the multimeric bridging receptor, creating the final receptor complex.

[0024] Fig. 1F shows a FZD binding domain linked to one LRP binding domain which is tethered to the bridging molecule by a binding domain specific for a first epitope on the bridging molecule and an FZD binding domain linked to one LRP binding domain which is tethered to the bridging molecule by a binding domain specific for a second epitope on the bridging molecule, and each separate molecule could be tethered to a "bridging element" binding to a different epitope on the bridging receptor, creating the final receptor complex.

[0025] Fig. 1G shows a FZD binding domain linked to one LRP binding domain which is tethered to the bridging molecule by two binding domains specific for two epitopes on the bridging molecule wherein the two bridging binding epitopes are linked together, and each separate molecule could be tethered to a "bridging element" binding to a different epitope on the bridging receptor, creating the final receptor complex.

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[0026] Fig. 1H shows FZD binding domain linked to one LRP binding domain which is tethered to the bridging molecule by a binding domain specific for a epitope on the bridging molecule, wherein each separate molecule tethered to a "bridging element" binding to a multimeric bridging receptor, creating the final receptor complex.

[0027] Figs. 2A-2C show selected βKlotho and endocrine fibroblast growth factor 21 (FGF21) ligand system as the bridging receptor system, with the FZD and LRP binding domains. The graphic representations of the binders and the various combinations F12578 (binds FZD1,2,5,7,8) and L (binds LRP5/6). Fig. 2A shows elements that are used in the split molecules. LRP6 binder element is in scFv format; FZD binder is in the IgG1 format; both αGFP IgG1 and αGFP scFv are used for assembly of the negative control molecules. Two types of βKlotho binders are used: Binder No.1, 39F7 IgG1, a βKlotho monoclonal antibody; Binder No. 2, FGF21FL, and different deletion variants. Fig. 2B illustrates the diagram of the assembled molecules. Fig. 2C shows the diagram of the assembled negative control molecules.

[0028] Figs. 2D-2G show binding of various F-FGF21 fusion proteins to FZD7 and βKlotho. Bindings of FZD7 and β-Klotho to F-FGF21FL (Fig. 2D), F-FGF21ΔN (Fig. 2E), F-FGF21ΔC (Fig. 2F), or F-FGF21ΔNΔC (Fig. 2G) were determined by Octet.

[0029] Figs. 2H-2J illustrate binding of LRP6 and βKlotho to L-39F7 (Fig. 2H), αGFP-39F7 (Fig. 2I), or L-αGFP (Fig. 2J) were determined by Octet. Mean KD values were calculated from all 7 binding curves with global fits (red dotted lines) using 1:1 Langmuir binding model.

[0030] Figs. 2K-2L show step bindings. Fig. 2K is step binding of FZD7 and βKlotho to various F-FGF21 fusion proteins. Sequential binding of F-FGF21FL (blue sensorgram), F-FGF21ΔN (red sensorgram), F-FGF21ΔC (light green sensorgram), or F-FGF21ΔNΔC (green sensorgram), followed by FZD7 CRD, then followed by addition of βKlotho on Octet shows simultaneous engagement of both FZD7 and βKlotho to the indicated F-FGF21 proteins. Sensorgrams for FZD7 and βKlotho area (dashed box on left) are enlarged at the right. Fig. 2L shows step binding of LRP6 and βKlotho to L-39F7 and its control proteins. Sequential binding L-39F7 (blue sensorgram), L-αGFP (light blue sensorgram), αGFP-39F7 (red sensorgram), or 39F7 IgG (green sensorgram), followed by LRP6E3E4, then followed by addition of βKlotho on Octet shows simultaneous engagement of both LRP6 and βKlotho to the indicated L-39F7 and its control proteins. Sensorgrams for LRP6 and βKlotho area (dashed box on left) are enlarged on the right.

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[0031] Figs. 3A-3F illustrate dose dependent STF assay of SWIFT molecules in HEK293 and Huh7 cells the combination of F12578-FGF21FL or FGF12578-FGF21ΔN with L-39F7 resulted in WNT/β-catenin signaling in a liver cell line, Huh7 cells, which express the bridging receptor βKlotho, but not in 293 cells where βKlotho is not expressed. This signaling depends on the presence of both FZD and LRP binding arms and the ability to bind the bridging receptor, as the removal of LRP binding arm L from L-39F7 or inactivation of βKlotho binding (use of FGF21ΔNΔC) resulted in no activity in either cell

[0032] Fig. 3G illustrates expression of bridging receptor βKlotho (KLB) in HEK293 and Huh7 cells.

[0033] Figs. 4A and 4B show the FZD binder (F12578), LRP binder (L), and the two non-competing bridging receptor (βKlotho) binders (FGF21FL and 39F7) were combined in the molecule, F12578-FGF21FL-39F7-L (Fig. 4A), and their activity WNT/β-catenin signaling in Huh7 cells that expresses the bridging receptor βKlotho but not in 293 cells where βKlotho is not expressed (Fig. 4B).

[0034] Figs. 5A-5D illustrate activity of targeted molecules in primary human cells. Fig. 5A is presentative images of primary human hepatocytes cultures in 2D or human small intestinal organoids. Scale bars 200 μm. Fig. 5B shows expression of bridging receptor βKlotho (KLB) in hepatocytes or small intestinal cells. Fig. 5C illustrates WNT target gene AXIN2 expression normalized to control treatment after 24-hour treatment with 10 nM of indicated molecules in human hepatocytes. Fig. 5D shows WNT target gene AXIN2 expression normalized to control treatment after 24-hour treatment with 10 nM of indicated molecules in human small intestinal organoids.

SUMMARY OF THE INVENTION

[0035] In various embodiments, the present invention provides a cell targeting multicomponent polypeptide and related uses thereof.

[0036] The present invention provides a cell targeting multicomponent polypeptide molecule comprising at least one first antigen binding domain that binds to a first signaling receptor component, at least one-second antigen binding domain that binds to a second signaling receptor component receptor, and at least one different antigen binding domain that binds to a bridging molecule, and the first receptor and second receptor can be different or identical

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[0037] In some embodiments, the binding domain that binds to the bridging molecule comprises at least two binding domains that bind to different epitopes on the bridging molecule or bind to the same epitope on the bridging molecule.

[0038] In another embodiment, the first antigen binding domain that binds to a first signaling receptor component is tethered to at least one binding domain that binds to the bridging receptor, and the second antigen binding domain that binds to a second signaling receptor component is tethered to at least one binding domain that binds to the bridging molecule.

[0039] In a further embodiment, the antigen binding domain is joined directly or by a linker to the binding domain that binds to the bridging molecule.

[0040] In another embodiment, the bridging molecule is monomeric or multimeric.

[0041] In a further embodiment, the binding domains are each independently selected from the group consisting of: an scFv, a VHH/sdAb, a Fab, and a Fab`2.

[0042] In some embodiment, the binding domains are joined through a linker by a peptide linker comprising from 1-100 amino acids.

[0043] In yet another embodiment, the binding domains are attached to the N-terminus of an antibody Fc domain. In another embodiment, a nucleic acid encodes the multicomponent polypeptide.

[0044] In a further embodiment, the nucleic acid is an expression vector comprises a promoter sequence operatively linked to the nucleic acid sequence; optionally wherein the vector is a virus comprising a promoter operatively linked to the nucleic acid.

[0045] In another embodiment, a host cell comprises the vector.

[0046] In related embodiment provides a process for producing the multicomponent polypeptide comprising culturing the host cell under conditions wherein the multicomponent polypeptide is expressed by the expression vector.

[0047] The present invention further provides a multicomponent WNT molecule comprises at least one FZD binding domain, at least one LRP binding domain, and at least one bridging molecule; the multicomponent WNT multicomponent polypeptide molecule modulates WNT signaling.

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[0048] In yet another embodiment,the multicomponent WNT molecule comprises: one or two FZD binding domains tethered to the bridging molecule by a binding domain specific for a first epitope on the bridging molecule; one or two LRP binding domains tethered to the bridging molecule by a binding domain specific for a second epitope on the bridging molecule, and the molecule can activate WNT signaling.

[0049] In some embodiments, the multicomponent WNT molecule comprises the binding domain specific for the first epitope of the bridging molecule and the FZD binding domain(s) are joined directly or joined by a linker; and the binding domain specific for the second epitope of the bridging molecule and the LRP binding domain(s) are joined directly or joined by a linker; the binding domain specific for the first epitope and the binding domain specific for the second epitope of the bridging molecule are identical or different.

[0050] In another embodiment, the linker is a peptide or non-peptide linker.

[0051] In yet another embodiment, the FZD binding domain(s) and the LRP binding domain(s) are each independently selected from the group consisting of: a scFv, a VHH/sdAb, a Fab, and a Fab'2.

[0052] In a further embodiment, the FZD binding domain(s) and the LRP binding domains(s) are joined by a peptide linker comprising from 1-100 amino acids.

[0053] In yet another embodiment, the FZD binding domain(s) and the LRP binding domain(s) are attached to the N-terminus of an antibody Fc domain.

[0054] In some embodiment, a nucleic acid encoding the multicomponent WNT molecule.

[0055] In another embodiment, the vector is an expression vector comprises a promoter sequence operatively linked to the nucleic acid sequence; optionally wherein the vector is a virus comprising a promoter operatively linked to the nucleic acid.

[0056] In a further embodiment, a host cell comprises the vector.

[0057] In some embodiments, a process for producing the multicomponent WNT molecule culturing the host cell under conditions wherein the multicomponent polypeptide is expressed by the expression vector.

[0058] In some embodiment, a pharmaceutical composition to modulate the WNT/β-catenin signaling pathway comprises an effective amount of the multicomponent WNT molecule, and a pharmaceutically acceptable diluent, adjuvant, or carrier.

[0059] In yet another embodiment, provides a method of treating or preventing a disease or disorder in a subject in need thereof, the method comprises by providing to the subject an effective amount of the multicomponent WNT molecule.

[0060] The present invention also provides a multicomponent WNT molecule comprises at least one FZD binding domain linked to one LRP binding domain which is tethered to the bridging molecule by a binding domain specific for a first epitope on the bridging molecule, at least one FZD binding domain linked to one LRP binding domain which is tethered to the bridging molecule by a binding domain specific for a second epitope on the bridging molecule; and the tethering of the FZD and LRP binding domains to the bridging receptor forms a WNT mimetic capable of activating WNT signaling.

[0061] In a further embodiment, the binding domain specific for a first epitope of the bridging molecule and the FZD/LRP binding domains are joined directly or by a linker; and the binding domain specific for a second epitope of the bridging molecule and the FZD/LRP binding domains are joined directly, or by a linker the binding domain of the first epitope and second epitope of the bridging molecule are identical or different, and the binding domain of the first epitope and the second epitope of the bridging molecule could be linked together to FZD/LRP.

[0062] In some embodiments, the bridging molecule is monomeric or multimeric.

[0063] In another embodiment, at least one of the FZD or LRP binding domains is selected from the group consisting of: a scFv, a VHH/sdAb, a Fab, and a Fab'2.

[0064] In yet another embodiment, the binding domains are joined through a linker by a peptide link comprising from 1-100 amino acids.

[0065] In related embodiment, the binding domains are attached to the N-terminus of an antibody Fc domain.

[0066] In some embodiment, a nucleic acid encoding the multicomponent WNT molecule.

[0067] In another embodiment, the vector is an expression vector comprises a promoter sequence operatively linked to the nucleic acid sequence; optionally wherein the vector is a virus comprising a promoter operatively linked to the nucleic acid.

[0068] In a further embodiment, a host cell comprises the vector.

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[0069] In some embodiments, a process for producing the multicomponent WNT molecule culturing the host cell under conditions wherein the multicomponent polypeptide is expressed by the expression vector.

[0070] In some embodiment, a pharmaceutical composition to modulate the WNT/β-catenin signaling pathway comprises an effective amount of the multicomponent WNT molecule, and a pharmaceutically acceptable diluent, adjuvant, or carrier.

[0071] In yet another embodiment, provides a method of treating or preventing a disease or disorder in a subject in need thereof, the method comprises by providing to the subject an effective amount of the multicomponent WNT molecule.

DETAILED DESCRIPTION

[0072] As used herein, including the appended claims, the singular forms of words such as "a," "an," and "the," include their corresponding plural references unless the context clearly dictates otherwise.

[0073] Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

[0074] Each embodiment in this specification is to be applied *mutatis mutandis* to every other embodiment unless expressly stated otherwise.

[0075] All references cited herein are incorporated by reference to the same extent as if each individual publication, patent application, or patent, was specifically and individually indicated to be incorporated by reference.

I. Definitions.

[0076] "Activity" of a molecule may describe or refer to the binding of the molecule to a ligand or to a receptor, to catalytic activity, to the ability to stimulate gene expression, to antigenic activity, to the modulation of activities of other molecules, and the like. "Activity" of a molecule may also refer to activity in modulating or maintaining cell-to-cell interactions, e.g., adhesion, or activity in maintaining a structure of a cell, e.g., cell membranes or cytoskeleton. "Activity" may also mean specific activity, e.g., [catalytic activity]/[mg protein], or [immunological activity]/[mg protein], or the like.

[0077] The terms "tethered" or "bound" or "joined" or "linked", as used herein, refer to different binding domains of the multicomponent molecule being fastened together. It is understood that different binding domains of the multicomponent molecule are fastened together and could be used interchangeably.

[0078] The terms "targeting element", "bridging element", "bridging molecule", or "bridging receptor" as used herein, refer to cell surface receptors also known as transmembrane receptors, such as, e.g., ion channel-linked receptors, G-protein coupled receptors, and enzyme-linked receptors. A bridging receptor can be a cell surface glycan.

[0079] The terms "chimeric activator", as used herein, refers to the cooperativity concept of different ligands by enhancing agonist activity to the targeted cell.

[0080] The terms "multicomponent polypeptide", "multicomponent polypeptide molecule", and "multicomponent molecule", as used herein, are interchangeable.

[0081] The terms "multicomponent WNT molecule", "WNT multicomponent polypeptide molecule", and "WNT mimetic" as used herein, are interchangeable.

[0082] The terms "administering" or "introducing" or "providing", as used herein, refer to delivery of a composition to a cell, to cells, tissues and/or organs of a subject, or to a subject. Such administering or introducing may take place in vivo, in vitro or ex vivo.

[0083] As is well known in the art, an antibody is an immunoglobulin molecule capable of specific binding to a target, such as a carbohydrate, polynucleotide, lipid, polypeptide, etc., through at least one epitope recognition site, located in the variable region of the immunoglobulin molecule. As used herein, the term encompasses not only intact polyclonal or monoclonal antibodies, but also fragments thereof (such as dAb, Fab, Fab', F(ab')2, Fv), single chain (scFv), V_{HH}, synthetic variants thereof, naturally occurring variants, fusion proteins comprising an antibody or an antigen-binding fragment thereof, humanized antibodies, chimeric antibodies, and any other modified configuration of the immunoglobulin molecule that comprises an antigen-binding site or fragment (epitope recognition site) of the required specificity. "Diabodies", multivalent or multispecific fragments constructed by gene fusion (WO94/13804; P. Holliger et al (1993), *Proc. Natl. Acad. Sci. USA* 90 6444-6448) are also a particular form of antibody contemplated herein. Minibodies comprising a scFv joined to a CH3 domain are also included herein (See e.g., S. Hu et al. (1996), *Cancer Res.*, 56:3055-3061; Ward, E. S. et al. (1989) *Nature* 341:544-546; Bird et al.(1988) *Science* 242:423-426; Huston et al. (1988) *Proc. Natl. Acad. Sci. USA* 85:5879-5883; PCT/US92/09965; WO94/13804; P.

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Holliger et al.(1993) *Proc. Natl. Acad. Sci. USA* 90:6444-6448; and Y. Reiter et al. (1996) *Nature Biotech.* 14:1239-1245).

[0084] The term "antigen-binding fragment" as used herein refers to a polypeptide fragment that contains at least one CDR of an immunoglobulin heavy and/or light chain, or of a V_{HH}, that binds to the antigen of interest, in particular to one or more FZD receptor or LRP5 or LRP6 receptor. In this regard, an antigen-binding fragment of the herein described antibodies may comprise 1, 2, 3, 4, 5, or all 6 CDRs of a VH and VL sequence set forth herein from antibodies that bind one or more FZD receptor or LRP5 and/or LRP6. In particular embodiments, an antigen-binding fragment may comprise all three VH CDRs or all three VL CDRs. Similarly, an antigen binding fragment thereof may comprise all three CDRs of a V_{HH} binding fragment. An antigen-binding fragment of a FZD-specific antibody is capable of binding to a FZD receptor. An antigen- binding fragment of a LRP5/6-specific antibody is capable of binding to a LRP5 and/or LRP6 receptor. As used herein, the term encompasses not only isolated fragments but also polypeptides comprising an antigen-binding fragment of an antibody disclosed herein, such as, for example, fusion proteins comprising an antigen-binding fragment of an antibody disclosed herein, such as, e.g., a fusion protein comprising a V_{HH} that binds one or more FZD receptors and a V_{HH} that binds LRP5 and/or LRP6.

[0085] The term "antigen" refers to a molecule or a portion of a molecule capable of being bound by a selective binding agent, such as an antibody, and additionally capable of being used in an animal to produce antibodies capable of binding to an epitope of that antigen. In certain embodiments, a binding agent (e.g., a multicomponent WNT molecule or binding region thereof) is said to specifically bind an antigen when it preferentially recognizes its target antigen in a complex mixture of proteins and/or macromolecules. In certain embodiments, a multicomponent WNT molecule or binding region thereof (e.g., an antibody or antigen-binding fragment thereof) is said to specifically bind an antigen when the equilibrium dissociation constant is $\leq 10^{-7}$ or $\leq 10^{-8}$ M. In some embodiments, the equilibrium dissociation constant may be $\leq 10^{-9}$ M or $\leq 10^{-10}$ M.

[0086] As used herein, the term "CDR" refers to at least one of the three hypervariable regions of a heavy or light chain variable (V) region. Proceeding from the N-terminus of a heavy or light chain, these regions are denoted as "CDR1," "CDR2," and "CDR3" respectively. An antigen-binding site, therefore, includes six CDRs, comprising the CDR set from each of a heavy and a light chain V region. A polypeptide comprising a single CDR, (e.g., a CDR1, CDR2 or CDR3) is referred to herein as a "molecular recognition unit." Crystallographic

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analysis of a number of antigen-antibody complexes has demonstrated that the amino acid residues of CDRs form extensive contact with bound antigen, wherein the most extensive antigen contact is with the heavy chain CDR3. Thus, the molecular recognition units are primarily responsible for the specificity of an antigen-binding site. In certain embodiments, antibodies and antigen-binding fragments thereof as described herein include a heavy chain and a light chain CDRs, respectively interposed between a heavy chain and a light chain framework regions (FRs)which provide support to the CDRs and define the spatial relationship of the CDRs relative to each other.

[0087] As used herein, the term "FRs" refer to the four flanking amino acid sequences which frame the CDRs of a heavy or light chain V region. Some FR residues may contact bound antigen; however, FRs are primarily responsible for folding the V region into the antigen-binding site, particularly the FR residues directly adjacent to the CDRs. Within FRs, certain amino residues and certain structural features are very highly conserved. In this regard, all V region sequences contain an internal disulfide loop of around 90 amino acid residues. When the V regions fold into a binding-site, the CDRs are displayed as projecting loop motifs which form an antigen-binding surface. It is generally recognized that there are conserved structural regions of FRs which influence the folded shape of the CDR loops into certain "canonical" structures—regardless of the precise CDR amino acid sequence. Further, certain FR residues are known to participate in non-covalent interdomain contacts which stabilize the interaction of the antibody heavy and light chains. The structures and locations of immunoglobulin CDRs and variable domains may be determined by reference to Kabat, E. A. et al., Sequences of Proteins of Immunological Interest. 4th Edition. US Department of Health and Human Services. 1987, and updates thereof, now available on the Internet (immuno bme.nwu.edu).

[0088] A "monoclonal antibody" refers to a homogeneous antibody population wherein the monoclonal antibody is comprised of amino acids (naturally occurring and non-naturally occurring) that are involved in the selective binding of an epitope. Monoclonal antibodies are highly specific, being directed against a single epitope. The term "monoclonal antibody" encompasses not only intact monoclonal antibodies and full-length monoclonal antibodies, but also fragments thereof (such as Fab, Fab', F(ab')2, Fv), single chain (scFv), V_{HH}, variants thereof, fusion proteins comprising an antigen-binding fragment of a monoclonal antibody, humanized monoclonal antibodies, chimeric monoclonal antibodies, and any other modified configuration of the immunoglobulin molecule that comprises an antigen- binding fragment

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(epitope recognition site) of the required specificity and the ability to bind to an epitope, including multicomponent WNT molecules disclosed herein. It is not intended to be limited as regards the source of the antibody or the manner in which it is made (e.g., by hybridoma, phage selection, recombinant expression, transgenic animals, etc.). The term includes whole immunoglobulins as well as the fragments etc. described above under the definition of "antibody"

[0089] The term "co-receptor" refers to a first cell surface receptor that binds signaling molecule or ligand in conjunction with another receptor to facilitate ligand recognition and initiate a biological process, such as WNT pathway signaling.

[0090] The term "agonist activity" refers to the ability of an agonist to mimic the effect or activity of a naturally occurring protein.

[0091] As used herein "peptide linker" or "linker moiety" refers to a sequence of sometimes repeating amino acid residues, usually glycine and serine, that are used to join the various antigen binding domains described below. The length of the linker sequence determines the flexibility of the antigen binding domains in MsAbs, in particular, in the binding of epitopes on co-receptors such as FZD receptors, LRP5 and/or LRP6, and/or ZNRF3/RNF43.

[0092] As used herein, the term "enhances" refers to a measurable increase in the level of receptor signaling modulated by a ligand or ligand agonist compared with the level in the absence of the agonist, e.g., a multicomponent WNT molecule. In particular embodiments, the increase in the level of receptor signaling is at least 10%, at least 20%, at least 50%, at least two-fold, at least five-fold, at least 10-fold, at least 20-fold, at least 50- fold, or at least 100-fold as compared to the level of receptor signaling in the absence of the agonist, e.g., in the same cell type.

[0093] An antigen or epitope that "specifically binds" or "preferentially binds" (used interchangeably herein) to an antibody or antigen-binding fragment thereof is a term well understood in the art, and methods to determine such specific or preferential binding are also well known in the art. A molecule, e.g., a multicomponent WNT molecule, is said to exhibit "specific binding" or "preferential binding" if it reacts or associates more frequently, more rapidly, with greater duration and/or with greater affinity with a particular cell or substance than it does with alternative cells or substances. A molecule or binding region thereof, e.g., a multicomponent WNT molecule or binding region thereof, "specifically binds" or "preferentially binds" to a target antigen, e.g., a FZD receptor, if it binds with greater affinity,

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avidity, more readily, and/or with greater duration than it binds to other substances. It is also understood by reading this definition that, for example, a multicomponent WNT molecule or binding region thereof that specifically or preferentially binds to a first target may or may not specifically or preferentially bind to a second target. As such, "specific binding" or "preferential binding" does not necessarily require (although it can include) exclusive binding. Generally, but not necessarily, reference to binding means preferential binding.

[0094] The term "operably linked" means that the components to which the term is applied are in a relationship that allows them to carry out their inherent functions under suitable conditions. For example, a transcription control sequence "operably linked" to a protein coding sequence is ligated thereto so that expression of the protein coding sequence is achieved under conditions compatible with the transcriptional activity of the control sequences.

[0095] The term "control sequence" as used herein refers to polynucleotide sequences that can affect expression, processing or intracellular localization of coding sequences to which they are ligated or operably linked. The nature of such control sequences may depend upon the host organism. In particular embodiments, transcription control sequences for prokaryotes may include a promoter, ribosomal binding site, and transcription termination sequence. In other particular embodiments, transcription control sequences for eukaryotes may include promoters comprising one or a plurality of recognition sites for transcription factors, transcription enhancer sequences, transcription termination sequences and polyadenylation sequences. In certain embodiments, "control sequences" can include leader sequences and/or fusion partner sequences.

[0096] The term "polynucleotide" as referred to herein means single- stranded or double-stranded nucleic acid polymers. In certain embodiments, the nucleotides comprising the polynucleotide can be ribonucleotides or deoxyribonucleotides or a modified form of either type of nucleotide. Said modifications include base modifications such as bromouridine, ribose modifications such as arabinoside and 2',3'-dideoxyribose and internucleotide linkage modifications such as phosphorothioate, phosphorodithioate, phosphoroselenoate, phosphorodiselenoate, phosphoroanilothioate, phoshoraniladate and phosphoroamidate. The term "polynucleotide" specifically includes single and double stranded forms of DNA.

[0097] The term "naturally occurring nucleotides" includes deoxyribonucleotides and ribonucleotides. The term "modified nucleotides" includes nucleotides with modified or substituted sugar groups and the like. The term "oligonucleotide linkages" includes

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oligonucleotide linkages such as phosphorothioate, phosphorodithioate, phosphoroselenoate, phosphorodiselenoate, phosphoroanilothioate, phoshoraniladate, phosphoroamidate, and the like. See, e.g., LaPlanche et al. (1986) *Nucl. Acids Res.* 14:9081; Stec et al. (1984) *J. Am. Chem. Soc.* 106:6077; Stein et al. (1988) *Nucl. Acids Res.* 16:3209; Zon et al. (1991) *Anti-Cancer Drug Design*, 6:539; Zon et al. (1991) Oligonucleotides and Analogues: A Practical Approach, pp. 87-108 (F. Eckstein, Ed.), Oxford University Press, Oxford England; Stec et al., U.S. Pat. No. 5,151,510; Uhlmann and Peyman (1990) *Chem. Rev.* 90:543, the disclosures of which are hereby incorporated by reference for any purpose. An oligonucleotide can include a detectable label to enable detection of the oligonucleotide or hybridization thereof.

[0098] The term "vector" is used to refer to any molecule (e.g., nucleic acid, plasmid, or virus) used to transfer coding information to a host cell. The term "expression vector" refers to a vector that is suitable for transformation of a host cell and contains nucleic acid sequences that direct and/or control expression of inserted heterologous nucleic acid sequences. Expression includes, but is not limited to, processes such as transcription, translation, and RNA splicing, if introns are present.

[0099] The term "host cell" is used to refer to a cell into which has been introduced, or which is capable of having introduced into it, a nucleic acid sequence encoding one or more of the herein described polypeptides, and which further expresses or is capable of expressing a selected gene of interest, such as a gene encoding any herein described polypeptide. The term includes the progeny of the parent cell, whether or not the progeny are identical in morphology or in genetic make-up to the original parent, so long as the selected gene is present. Accordingly, there is also contemplated a method comprising introducing such nucleic acid into a host cell. The introduction may employ any available technique. For eukaryotic cells, suitable techniques may include calcium phosphate transfection, DEAE-Dextran, electroporation, liposome- mediated transfection and transduction using retrovirus or other virus, *e.g.*, vaccinia or, for insect cells, baculovirus. For bacterial cells, suitable techniques may include calcium chloride transformation, electroporation and transfection using bacteriophage. The introduction may be followed by causing or allowing expression from the nucleic acid, *e.g.*, by culturing host cells under conditions for expression of the gene. In one embodiment, the nucleic acid is integrated into the genome (*e.g.*, chromosome) of the host cell. Integration may be promoted by inclusion of sequences which promote recombination with the genome, in accordance with standard techniques.

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[0100] The term "transfection" is used to refer to the uptake of foreign or exogenous DNA by a cell, and a cell has been "transfected" when the exogenous DNA has been introduced inside the cell membrane. A number of transfection techniques are well known in the art and are disclosed herein. See, e.g., Graham et al., 1973, Virology 52:456; Sambrook et al., 2001, MOLECULAR CLONING, A LABORATORY MANUAL, Cold Spring Harbor Laboratories; Davis et al., 1986, BASIC METHODS IN MOLECULAR BIOLOGY, Elsevier; and Chu et al., 1981, Gene 13:197. Such techniques can be used to introduce one or more exogenous DNA moieties into suitable host cells.

[0101] "Transduction" refers to the acquisition and transfer of eukaryotic cellular sequences by viruses, e.g., retroviruses.

[0102] The term "transformation" as used herein refers to a change in a cell's genetic characteristics, and a cell has been transformed when it has been modified to contain a new DNA. For example, a cell is transformed where it is genetically modified from its native state. Following transfection or transduction, the transforming DNA may recombine with that of the cell by physically integrating into a chromosome of the cell, or may be maintained transiently as an episomal element without being replicated, or may replicate independently as a plasmid. A cell is considered to have been stably transformed when the DNA is replicated with the division of the cell.

[0103] The term "naturally occurring" or "native" when used in connection with biological materials such as nucleic acid molecules, polypeptides, host cells, and the like, refers to materials which are found in nature and are not manipulated by a human. Similarly, "non-naturally occurring" or "non-native" as used herein refers to a material that is not found in nature or that has been structurally modified or synthesized by a human.

[0104] The terms "polypeptide" "protein" and "peptide" and "glycoprotein" are used interchangeably and mean a polymer of amino acids not limited to any particular length. The term does not exclude modifications such as myristylation, sulfation, glycosylation, phosphorylation and addition or deletion of signal sequences. The terms "polypeptide" or "protein" means one or more chains of amino acids, wherein each chain comprises amino acids covalently linked by peptide bonds, and wherein said polypeptide or protein can comprise a plurality of chains non-covalently and/or covalently linked together by peptide bonds, having the sequence of native proteins, that is, proteins produced by naturally-occurring and specifically non-recombinant cells, or genetically-engineered or recombinant cells, and

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comprise molecules having the amino acid sequence of the native protein, or molecules having deletions from, additions to, and/or substitutions of one or more amino acids of the native sequence. The terms "polypeptide" and "protein" specifically encompass multicomponent WNT molecules, FZD binding regions thereof, LRP5/6 binding regions thereof, antibodies and antigen-binding fragments thereof that bind to a FZD receptor or a LRP5 or LRP6 receptor disclosed herein, or sequences that have deletions from, additions to, and/or substitutions of one or more amino acid of any of these polypeptides. Thus, a "polypeptide" or a "protein" can comprise one (termed "a monomer") or a plurality (termed "a multimer") of amino acid chains. Polypeptides and proteins include glycoproteins.

[0105] The term "isolated protein," "or "isolated antibody" referred to herein means that a subject protein, multicomponent molecule, or antibody: (1) is free of at least some other proteins with which it would typically be found in nature; (2) is essentially free of other proteins from the same source, e.g., from the same species; (3) is expressed by a cell from a different species; (4) has been separated from at least about 50 percent of polynucleotides, lipids, carbohydrates, or other materials with which it is associated in nature; (5) is not associated (by covalent or noncovalent interaction) with portions of a protein with which the "isolated protein" is associated in nature; (6) is operably associated (by covalent or noncovalent interaction) with a polypeptide with which it is not associated in nature; or (7) does not occur in nature. Such an isolated protein can be encoded by genomic DNA, cDNA, mRNA or other RNA, or may be of synthetic origin, or any combination thereof. In certain embodiments, an isolated protein may comprise naturally-occurring and/or artificial polypeptide sequences. In certain embodiments, the isolated protein is substantially free from proteins or polypeptides or other contaminants that are found in its natural environment that would interfere with its use (therapeutic, diagnostic, prophylactic, research or otherwise).

[0106] A "WNT super agonist" is a molecule having enhanced WNT agonist activity. As used herein, the WNT super agonists have both WNT signaling and WNT signal enhancing activity. In some embodiments, the WNT super agonist molecule will bind both at least one FZD receptor and at least one LRP receptor, as well as binding and activating at least one E3 ubiquitin ligase receptor, thereby stabilizing the FZD and/or LRP receptors.

II. General

[0107] The present invention provides combinations of antigen binding molecules that act as multicomponent polypeptide and enhancing molecules by binding to and modulating co-

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receptor signaling, for example, antigen binding molecules that bind to one or more FZD receptor and one or more LRP5 or LRP6 receptor, and a bridging receptor, which in turn modulates a downstream WNT signaling pathway. In particular embodiments, the multicomponent complex activates or increases a signaling pathway associated with one or both co-receptors by homo- or hetero-dimerization after interaction of a bridging element with the bridging receptor. In particular embodiments, the multicomponent polypeptide molecules disclosed herein comprise: (i) one or more antibodies or antigen-binding fragments thereof that specifically bind to one or more first co-receptor, including antibodies or antigen-binding fragments thereof having particular co-receptor specificity and/or functional properties; and (ii) one or more antibodies or antigen-binding fragments thereof that specifically bind to one or more second co-receptors, and one or more bridging molecule that bind to one or more bridging receptor. Certain embodiments encompass specific structural formats or arrangements of the first and second co-receptor binding region(s) of the multicomponent molecules advantageous in increasing downstream signaling and related biological effects.

[0108] The practice of the present invention will employ, unless indicated specifically to the contrary, conventional methods of virology, immunology, microbiology, molecular biology and recombinant DNA techniques within the skill of the art, many of which are described below for the purpose of illustration. Such techniques are explained fully in the literature. See, e.g., Current Protocols in Molecular Biology or Current Protocols in Immunology, John Wiley & Sons, New York, N.Y.(2009); Ausubel et al., Short Protocols in Molecular Biology, 3rd ed., Wiley & Sons, 1995; Sambrook and Russell, Molecular Cloning: A Laboratory Manual (3rd Edition, 2001); Maniatis et al. Molecular Cloning: A Laboratory Manual (1982); DNA Cloning: A Practical Approach, vol. I & II (D. Glover, ed.); Oligonucleotide Synthesis (N. Gait, ed., 1984); Nucleic Acid Hybridization (B. Hames & S. Higgins, eds., 1985); Transcription and Translation (B. Hames & S. Higgins, eds., 1984); Animal Cell Culture (R. Freshney, ed., 1986); Perbal, A Practical Guide to Molecular Cloning (1984) and other like references.

[0109] Sequences of illustrative antibodies, or antigen-binding fragments, or complementarity determining regions (CDRs) thereof, that bind to one or more FZD receptors, are set forth in WO2019126399. Sequences of illustrative LRP5 and/or LRP6 antibodies, or antigen-binding fragments, or complementarity determining regions (CDRs) thereof, are set forth in WO2019126401. Sequences of antigen binding molecules that bind one or more FZD receptor and LRP5 and/or LRP6 are set forth in U.S. Provisional application nos. 62/607,875,

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62/641,217, and 62/680,522, titled WNT Signaling Pathway Agonists, filed December 19, 2017, March 9, 2018, and June 4, 2018, respectively.

[0110] Antibodies and antibody fragments thereof may be prepared by methods well known in the art. For example, the proteolytic enzyme papain preferentially cleaves IgG molecules to yield several fragments, two of which (the F(ab) fragments) each comprise a covalent heterodimer that includes an intact antigen-binding site. The enzyme pepsin is able to cleave IgG molecules to provide several fragments, including the F(ab')2 fragment which comprises both antigen-binding sites. An Fv fragment for use according to certain embodiments of the present invention can be produced by preferential proteolytic cleavage of an IgM, and on rare occasions of an IgG or IgA immunoglobulin molecule. Fv fragments are, however, more commonly derived using recombinant techniques known in the art. The Fv fragment includes a non-covalent VH::VL heterodimer including an antigen-binding site which retains much of the antigen recognition and binding capabilities of the native antibody molecule. (See, e g., Inbar *et al.* (1972) *Proc. Nat. Acad. Sci. USA 69*:2659-2662; Hochman *et al.* (1976) *Biochem 15*:2706- 2710; and Ehrlich *et al.* (1980) *Biochem 19*:4091-4096).

[0111] In certain embodiments, single chain Fv or scFV antibodies are contemplated. For example, Kappa bodies (Ill *et al.* (1997), *Prot. Eng.* 10: 949-57; minibodies (Martin *et al.*(1994) *EMBO J.* 13: 5305-9; diabodies (Holliger *et al.* (1993) *PNAS* 90: 6444-8; or janusins (Traunecker *et al.*(1991) *EMBO J.* 10: 3655-59; and Traunecker *et al.* (1992) *Int. J. Cancer Suppl.* 7: 51-52), may be prepared using standard molecular biology techniques following the teachings of the present application with regard to selecting antibodies having the desired specificity. In still other embodiments, bispecific or chimeric antibodies may be made that encompass the ligands of the present disclosure. For example, a chimeric antibody may comprise CDRs and framework regions from different antibodies, while bispecific antibodies may be generated that bind specifically to one or more FZD receptor through one binding domain and to a second molecule through a second binding domain and bridging molecule with identical or different bridging epitopes for the first or second binding domains. These antibodies may be produced through recombinant molecular biological techniques or may be physically conjugated together.

[0112] A single chain Fv (scFv) polypeptide is a covalently linked VH::VL heterodimer which is expressed from a gene fusion including VH- and VL- encoding genes linked by an encoded peptide linker. Huston *et al.* (1988) *Proc. Nat. Acad. Sci. USA 85*(16):5879-5883. A number of methods have been described to discern chemical structures for converting the

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naturally aggregated—but chemically separated—light and heavy polypeptide chains from an antibody V region into an scFv molecule which will fold into a three dimensional structure substantially similar to the structure of an antigen-binding site. See, *e.g.*, U.S. Pat. Nos. 5,091,513 and 5,132,405, to Huston *et al.*; and U.S. Pat. No. 4,946,778, to Ladner *et al.*

[0113] In certain embodiments, an antibody as described herein is in the form of a diabody. Diabodies are multimers of polypeptides, each polypeptide comprising a first domain comprising a binding region of an immunoglobulin light chain and a second domain comprising a binding region of an immunoglobulin heavy chain, the two domains being linked (*e.g.* by a peptide linker) but unable to associate with each other to form an antigen binding site: antigen binding sites are formed by the association of the first domain of one polypeptide within the multimer with the second domain of another polypeptide within the multimer (WO94/13804). A dAb fragment of an antibody consists of a VH domain (Ward, E. S. *et al.* (1989) Nature 341.544-546).

[0114] Where bispecific antibodies are to be used, these may be conventional bispecific antibodies, which can be manufactured in a variety of ways (Holliger, P. and Winter G. (1993) *Curr. Op. Biotechnol.* 4:446-449), *e.g.*, prepared chemically or from hybrid hybridomas, or may be any of the bispecific antibody fragments mentioned above. Diabodies and scFv can be constructed without an Fc region, using only variable domains, potentially reducing the effects of anti-idiotypic reaction.

[0115] Bispecific diabodies, as opposed to bispecific whole antibodies, may also be particularly useful because they can be readily constructed and expressed in *E. coli*. Diabodies (and many other polypeptides such as antibody fragments) of appropriate binding specificities can be readily selected using phage display (WO94/13804) from libraries. If one arm of the diabody is to be kept constant, for instance, with a specificity directed against antigen X, then a library can be made where the other arm is varied and an antibody of appropriate specificity selected. Bispecific whole antibodies may be made by knobs-into-holes engineering (J. B. B. Ridgeway et al. (1996) *Protein Eng.*, 9:616- 621).

[0116] In certain embodiments, the antibodies described herein may be provided in the form of a UniBody®. A UniBody® is an IgG4 antibody with the hinge region removed (see GenMab Utrecht, The Netherlands; see also, *e.g.*, US20090226421). This proprietary antibody technology creates a stable, smaller antibody format with an anticipated longer therapeutic window than current small antibody formats. IgG4 antibodies are considered inert and thus do

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not interact with the immune system. Fully human IgG4 antibodies may be modified by eliminating the hinge region of the antibody to obtain half-molecule fragments having distinct stability properties relative to the corresponding intact IgG4 (GenMab, Utrecht). Halving the IgG4 molecule leaves only one area on the UniBody® that can bind to cognate antigens (*e.g.*, disease targets) and the UniBody® therefore binds univalently to only one site on target cells.

[0117] In certain embodiments, the antibodies of the present disclosure may take the form of a single variable domain fragment known as a V_{HH}. The V_{HH} technology was originally developed following the discovery and identification that *camelidae* (e.g., camels and llamas) possess fully functional antibodies that consist of heavy chains only and therefore lack light chains. These heavy-chain only antibodies contain a single V_{HH} domain and two constant domains (CH2, CH3). The cloned and isolated V_{HH} domains have full antigen binding capacity and are very stable. These V_{HH} domains are encoded by single genes and are efficiently produced in almost all prokaryotic and eukaryotic hosts *e.g.*, *E. coli* (see *e.g.* U.S. Pat. No. 6,765,087), molds (for example *Aspergillus* or *Trichoderma*) and yeast (for example *Saccharomyces, Kluyvermyces, Hansenula* or *Pichia* (see *e.g.* U.S. Pat. No. 6,838,254). The production process is scalable and multi-kilogram quantities of VHHs have been produced. VHHs may be formulated as a ready-to-use solution having a long shelf life. The Nanoclone® method (see, e.g., WO 06/079372) is a proprietary method for generating VHHs against a desired target, based on automated high-throughput selection of B-cells. VHH antibodies typically have a small size of around 15 kDa.

[0118] In certain embodiments, the antibodies or antigen-binding fragments thereof as disclosed herein are humanized. This refers to a chimeric molecule, generally prepared using recombinant techniques, having an antigen- binding site derived from an immunoglobulin from a non-human species and the remaining immunoglobulin structure of the molecule based upon the structure and/or sequence of a human immunoglobulin. The antigen-binding site may comprise either complete variable domains fused onto constant domains or only the CDRs grafted onto appropriate framework regions in the variable domains. Epitope binding sites may be wild type or modified by one or more amino acid substitutions. This eliminates the constant region as an immunogen in human individuals, but the possibility of an immune response to the foreign variable region remains (LoBuglio, A. F. et al., (1989*) Proc Natl Acad Sci USA* 86:4220-4224; Queen et al. (1988) *Proc Natl Acad Sci USA* 86:10029-10033; and Riechmann et al. (1988) *Nature* 332:323-327). Illustrative methods for humanization of the anti- FZD antibodies disclosed herein include the methods described in U.S. patent no. 7,462,697.

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[0119] Another approach focuses not only on providing human-derived constant regions, but modifying the variable regions as well so as to reshape them as closely as possible to human form. It is known that the variable regions of both heavy and light chains contain three complementarity- determining regions (CDRs) which vary in response to the epitopes in question and determine binding capability, flanked by four framework regions (FRs) which are relatively conserved in a given species and which putatively provide a scaffolding for the CDRs. When nonhuman antibodies are prepared with respect to a particular epitope, the variable regions can be "reshaped" or "humanized" by grafting CDRs derived from nonhuman antibody on the FRs present in the human antibody to be modified. Application of this approach to various antibodies has been reported by Sato, K., et al., (1993) *Cancer Res* 53:851-856. Riechmann, L., et al., (1988) *supra*; Verhoeyen, M., et al., (1988) *Science* 239:1534-1536; Kettleborough, C. A., et al., (1991) *Protein Engg* 4:773-3783; Maeda, H., et al., (1991) *Human Antibodies Hybridoma* 2:124-134; Gorman, S. D., et al., (1991) *Proc Natl Acad Sci USA* 88:4181-4185; Tempest, P. R., et al., (1991) *Bio/Technol.* 9:266-271; Co, M. S., et al., (1991) *Proc Natl Acad Sci USA* 88:2869-2873; Carter, P., et al., (1992) *Proc Natl Acad Sci USA* 89:4285-4289; and Co, M. S. et al., (1992) *J Immunol* 148:1149-1154. In some embodiments, humanized antibodies preserve all CDR sequences (for example, a humanized mouse antibody which contains all six CDRs from the mouse antibodies). In other embodiments, humanized antibodies have one or more CDRs (one, two, three, four, five, six) which are altered with respect to the original antibody, which are also termed one or more CDRs "derived from" one or more CDRs from the original antibody.

[0120] In certain embodiments, the antibodies of the present disclosure may be chimeric antibodies. In this regard, a chimeric antibody is comprised of an antigen-binding fragment of an antibody operably linked or otherwise fused to a heterologous Fc portion of a different antibody. In certain embodiments, the heterologous Fc domain is of human origin. In other embodiments, the heterologous Fc domain may be from a different Ig class from the parent antibody, including IgA (including subclasses IgA1 and IgA2), IgD, IgE, IgG (including subclasses IgG1, IgG2, IgG3, and IgG4), and IgM. In further embodiments, the heterologous Fc domain may be comprised of CH2 and CH3 domains from one or more of the different Ig classes. As noted above with regard to humanized antibodies, the antigen-binding fragment of a chimeric antibody may comprise only one or more of the CDRs of the antibodies described herein (e.g., 1, 2, 3, 4, 5, or 6 CDRs of the antibodies described herein), or may comprise an entire variable domain (VL, VH or both).

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III. Structures of Multicomponent Receptor Complexes

[0121] The disclosure provides, in certain aspects, a cell targeting multicomponent polypeptide having a one or more first antigen binding domain that binds to a first receptor, a one or more second antigen binding domain that binds to a second receptor, and one or more different antigen binding domain that binds to one or more bridging molecule that activates the signaling pathways.

[0122] In certain illustrative embodiments of the tissue-specific bridging molecules disclosed herein: the tissue is bone tissue, and the cell surface receptor is parathyroid hormone receptor 1 (PTH1R), or the tissue is liver tissue, and the cell surface receptor is asialoglycoprotein receptor 1 (ASGR1), asialoglycoprotein receptor 2 (ASGR2), transferrin receptor 2 (TFR2) or solute carrier family 10 member 1 (SLC10A1), or the tissue is oral mucous tissue, and the cell surface receptor is LY6/PLAUR Domain Containing 3 (LYPD3) or Desmoglein 3 (DSG3).

[0123] In certain illustrative embodiments of the tissue-specific bridging molecules disclosed herein: the cell surface molecule is a PTH1, and at least one different antigen binding domain specifically binds PTH1R; the cell surface molecule is ASGR1, and at least one different antigen binding domain specifically binds ASGR1; the cell surface molecule is ASGR2, and third binding at least one different antigen binding domain binds ASGR2; the cell surface molecule is SLC10A1, and at least one different antigen binding domain specifically binds SLC10A1, or the cell surface molecule is TFR2, and at least one different antigen binding domain specifically binds TFR2, the cell surface molecule is LYPD3, and at least one different antigen binding domain specifically binds LYPD3; or the cell surface molecule is DSG3, at least one different antigen binding domain sequence specifically binds DSG3, at least one different antigen binding domain is an antibody or fragment thereof, a small molecule, or a ligand, or fragment or variant thereof, of the cell surface molecule.

[0124] In certain embodiment, the tethering of the targeting element or the bridging molecule to the activity element or the ensemble of the first antigen binding domain that binds to a first receptor, a one or more second antigen binding domain that binds to a second receptor, increase the local concentration of the activity element on the desired target cell surface, by engagement of signaling receptor and subsequent intracellular signaling activation. Thus, the tethering of the first and second binding domains to the bridging receptor forms a chimeric activator capable of activating the targeted cell signaling.

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[0125] In certain embodiments, a multicomponent molecule is capable of modulating signaling events associated with at least one of the co-receptors that it binds, in a cell contacted with the multicomponent polypeptide molecule. In certain embodiments, the multicomponent polypeptide molecule increases receptor signaling. As an example, a WNT multicomponent polypeptide molecule specifically modulates the biological activity of a human WNT/β-catenin signaling pathway.

[0126] Multicomponent polypeptide molecules of the present invention are biologically active in binding to one or more of a first receptor and to one or more of a second receptor, and as an example, in the activation of WNT signaling, the WNT multicomponent polypeptide molecule is a WNT agonist. The term "agonist activity" refers to the ability of an agonist to mimic the effect or activity of a naturally occurring protein binding to a first and second receptor. The ability of the multicomponent polypeptide molecules and other receptor agonists disclosed herein to mimic the activity of the natural ligand can be confirmed by a number of assays. As an example, WNT multicomponent polypeptide molecules, some of which are disclosed herein activate, enhance or increase the canonical WNT/β-catenin signaling pathway.

[0127] In particular embodiments, the structures of the multicomponent polypeptide molecules disclosed herein are bispecific, i.e., they specifically bind to two or more different epitopes, e.g., one or more epitopes of a first receptor, and one or more epitopes of a second receptor.

[0128] In particular embodiments, multicomponent molecules disclosed herein are multivalent, e.g., they comprise two or more regions that each specifically bind to the same epitope, e.g., two or more regions that bind to an epitope within one or more first co-receptor and/or two or more regions that bind to an epitope within a second co-receptor. In particular embodiments, they comprise two or more regions that bind to an epitope within a first co-receptor and two or more regions that bind to an epitope within a second co-receptor. In certain embodiments, multicomponent polypeptide molecules comprise a ratio of the number of regions that bind one or more first co-receptor to the number of regions that a second co-receptor of or about: 1:1, 2:1, 3:1, 4:1, 5:1, 6:1, 2:3, 2:5, 2:7, 7:2, 5:2, 3:2, 3:4, 3:5, 3:7, 3:8, 8:3, 7:3, 5:3, 4:3, 4:5, 4:7, 4:9, 9:4, 7:4, 5:4, 6:7, 7:6, 1:2, 1:3, 1:4, 1:5, or 1:6. In certain embodiments, the multicomponent polypeptide molecules are bispecific and multivalent.

[0129] In particular embodiment, the multicomponent WNT molecule described herein, are bispecific and can simultaneously bind to FZDs and LRPs binding domains. The optimal

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stoichiometry (at least for the antibody based molecules) are tetravalent bispecific (2:2 format), requiring two FZD binders and two LRP binders in the same molecule to achieve efficient signaling (Tao et al., 2019, Chen et al., 2020). Taking advantage of the fact that two FZD binders alone, two LRP binders alone, or a molecule with one FZD binder and one LRP binder (1:1 format) do not signal. Cell specificity is achieved by attaching an "targeting element" (capable of binding to another cell surface receptor, called a bridging receptor here) to these three inactive molecules, and signaling competent receptor complexes consisting of two FZDs and two LRPs could then be assemble via the bridging receptor on the target cell surface. This approach reduces or eliminates the need to mutate and reduce the affinity of the "active elements" toward signaling receptors, and creates a highly cell specific activation of the signaling pathway as the individual components are inactive. In particular embodiments, the antigen binding domain(s) binds to a signaling receptor or a signaling receptor component.

[0130] The structures of the multicomponent polypeptide molecules disclosed herein may have any of a variety of different structural formats or configurations. The multicomponent polypeptide molecules may comprise polypeptides and/or non-polypeptide binding moieties, e.g., small molecules. In particular embodiments, the multicomponent polypeptide molecules comprise both a polypeptide region and a non-polypeptide binding moiety. In certain embodiments, the multicomponent polypeptide molecules may comprise a single polypeptide, or they may comprise two or more, three or more, or four or more polypeptides.

[0131] In certain embodiments, one or more polypeptides of a multicomponent polypeptide molecule are antibodies or antigen-binding fragments thereof. In certain embodiments, surrogates comprise two antibodies or antigen binding fragments thereof, one that binds one or more first co-receptor and one that binds on or more second co-receptor. In certain embodimenis, the molecules, e.g., surrogates, comprise one, two, three, or four polypeptides, e.g., linked or bound to each other or fused to each other.

[0132] When the multicomponent polypeptide molecules comprise a single polypeptide, they may be a fusion protein comprising one or more first co-receptor binding domain and one or more second co-receptor binding domain and one or more bridging binding domains. The binding domains may be directly fused or they may be connected via a linker, e.g., a polypeptide linker, including but not limited to any of those disclosed herein.

[0133] When the multicomponent polypeptide molecules comprise two or more polypeptides, the polypeptides may be linked via covalent bonds, such as, e.g., disulfide bonds,

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and/or noncovalent interactions. For example, heavy chains of human immunoglobulin IgG interact at the level of their CH3 domains directly, whereas, at the level of their CH2 domains, they interact via the carbohydrate attached to the asparagine (Asn) N84.4 in the DE turn. In particular embodiments, the multicomponent polypeptide molecules comprise one or more regions derived from an antibody or antigen-binding fragment thereof, e.g., antibody heavy chains or antibody light chains or fragments thereof. In certain embodiments, a surrogate polypeptide comprises two antibody heavy chain regions (e.g., hinge regions) bound together via one or more disulfide bond. In certain embodiments, a surrogate polypeptide comprises an antibody light chain region (e.g., a CL region) and an antibody heavy chain region (e.g., a CH1 region) bound together via one or more disulfide bond

[0134] The multicomponent molecules, e.g., surrogate polypeptides, may be engineered to facilitate binding between two polypeptides. For example, Knob-into-holes amino acid modifications may be introduced into two different polypeptides to facilitate their binding. Knobs-into-holes amino acid (AA) changes is a rational design strategy developed in antibody engineering, used for heterodimerization of the heavy chains, in the production of bispecific IgG antibodies. AA changes are engineered in order to create a knob on the CH3 of the heavy chains from a first antibody and a hole on the CH3 of the heavy chains of a second antibody. The knob may be represented by a tyrosine (Y) that belongs to the 'very large' IMGT volume class of AA, whereas the hole may be represented by a threonine (T) that belongs to the 'small' IMGT volume class. Other means of introducing modifications into polypeptides to facilitate their binding are known and available in the art. For example, specific amino acids may be introduced and used for cross-linking, such as Cysteine to form an intermolecular disulfide bond.

[0135] Multicomponent molecules may have a variety of different structural formats, including but not limited to those as described in WO2019126398 and WO2020010308.

[0136] In one embodiment, a multicomponent molecule comprises an scFv or antigen-binding fragment thereof fused to a VHH or antigen-binding fragment thereof. In certain embodiments, the scFv specifically binds one or more first receptor, and the VHH specifically binds to one or more second receptor. In certain embodiments, the scFv specifically binds LRP5 and/or LRP6, and the VHH specifically binds one or more FZD receptor. In particular embodimenis, the scFv or antigen-binding fragment thereof is fused directly to the VHH or antigen-binding fragment thereof, whereas in other embodiments, the two binding regions are fused via a linker moiety. In particular embodiments, the VHH is fused or linked to the N-

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terminus of the scFV, while in other embodiments, the VHH is fused to the C-terminus of the scFv.

[0137] In various embodiments, including but not limited to those depicted in WO2019126398, WO2020010308, Table 2, Table 3, and Figures 1-3, a multicomponent polypeptide molecule comprises one or more Fab or antigen-binding fragment thereof and one or more VHH or antigen- binding fragment thereof (or alternatively, one or more scFv or antigen-binding fragment thereof). In certain embodiments, the Fab specifically binds one or more FZD receptor, and the VHH (or scFv) specifically binds LRP5 and/or LRP6. In certain embodiments, the Fab specifically binds LRP5 and/or LRP6, and the VHH (or scFv) specifically binds one or more FZD receptor. In certain embodiments, the VHH (or scFv) is fused to the N- terminus of the Fab, while in some embodiments, the VHH (or scFv) is fused to the C-terminus of the Fab. In particular embodiments, the Fab is present in a full IgG format, and the VHH (or scFv) is fused to the N-terminus and/or C-terminus of the IgG light chain. In particular embodiments, the Fab is present in a full IgG format, and the VHH (or scFv) is fused to the N-terminus and/or C-terminus of the IgG heavy chain.

[0138] In particular embodiments, two or more VHHs (or scFvs) are fused to the IgG at any combination of these locations.

[0139] Fabs may be converted into a full IgG format that includes both the Fab and Fc fragments, for example, using genetic engineering to generate a fusion polypeptide comprising the Fab fused to an Fc region, i.e., the Fab is present in a full IgG format. The Fc region for the full IgG format may be derived from any of a variety of different Fcs, including but not limited to, a wild-type or modified IgG1, IgG2, IgG3, IgG4 or other isotype, e.g., wild-type or modified human IgG1, human IgG2, human IgG3, human IgG4, human IgG4Pro (comprising a mutation in core hinge region that prevents the formation of IgG4 half molecules), human IgA, human IgE, human IgM, or the modified IgG1 referred to as IgG1 LALAPG. The L235A, P329G (LALA-PG) variant has been shown to eliminate complement binding and fixation as well as Fc-γ dependent antibody-dependent cell-mediated cytotoxity (ADCC) in both murine IgG2a and human IgG1. These LALA-PG substitutions allow a more accurate translation of results generated with an "effectorless" antibody framework scaffold between mice and primates. In particular embodiments of any of the IgG disclosed herein, the IgG comprises one or more of the following amino acid substitutions: N297G, N297A, N297E, L234A, L235A, or P236G.

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[0140] Non-limiting examples of bivalent and bispecific multicomponent polypeptide molecules of co-receptors that are bivalent towards both the one or more first receptor and one or more second receptor (e.g., FZD and LRP) are provided as the top four structures depicted in WO2019126398 and WO2020010308, where the VHH or scFv is depicted in white or grey, and the Fab or IgG is depicted in black. As shown, the VHH (or scFvs) may be fused to the N-termini of both light chains, to the N-termini of both heavy chains, to the C- termini of both light chains, or to the C-termini of both heavy chains. It is further contemplated, e.g., that VHH (or scFvs) could be fused to both the N-termini and C-termini of the heavy and/or light chains, to the N-termini of the light chains and the heavy chains, to the C-termini of the heavy and light chains, to the N-termini of the heavy chains and C-termini of the light chains, or to the C-termini of the heavy chains and the N-termini of the light chains. In other related embodiments, two or more VHH (or scFvs) may be fused together, optionally via a linker moiety, and fused to the Fab or IgG at one or more of these locations. In a related embodiment, the multicomponent polypeptide molecule has a Hetero-IgG format, whereas the Fab is present as a half antibody, and one or more VHH (or scFv) is fused to one or more of the N-terminus of the Fc, the N-terminus of the Fab, the C-terminus of the Fc, or the C-terminus of the Fab. A bispecific but monovalent to each receptor version of this format is depicted at Figure 6. In certain embodiments, the Fab or antigen-binding fragment (or IgG) thereof is fused directly to the VHH (or scFv) or antigen-binding fragment thereof, whereas in other embodiments, the binding regions are fused via a linker moiety. In particular embodiments, the Fab is described herein or comprises any of the CDR sets described herein.

[0141] In various embodiments, including but not limited to those depicted in WO2019126398, WO2020010308, Table 2, Table 3, and Figures 1-3, an antigen binding molecule comprises one or more Fab or antigen-binding fragment thereof that binds one or more first receptor (e.g., FZD receptors) and one or more Fab or antigen-binding fragment thereof that binds to at least one or more second receptor (e.g., LRP5 and/or LRP6). In a particular embodiment, it comprises two Fab or antigen-binding fragments thereof that bind one or more first co-receptor and/or two Fab or antigen-binding fragments thereof that bind to one or more second co-receptor. In further embodiments, one or more of the Fab is present in a full IgG format, and in certain embodiments, both Fab are present in a full IgG format. In certain embodiments, the Fab in full IgG format specifically binds one or more first receptor (e.g., one or more FZD receptor), and the other Fab specifically binds at least one second receptor (e.g., LRP5 and/or LRP6). For example, the Fab specifically binds one or more FZD

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receptor, and the Fab in full IgG format specifically binds LRP5 and/or LRP6. In certain embodiments, the Fab specifically binds LRP5 and/or LRP6, and the Fab in full IgG format specifically binds one or more FZD receptor. In certain embodiments, the Fab is fused to the N-terminus of the IgG, e.g., to the heavy chain or light chain N-terminus, optionally via a linker. In certain embodiments, the Fab is fused to the N-terminus of the heavy chain of the IgG and not fused to the light chain. In particular embodiments, the two heavy chains can be fused together directly or via a linker. An example of such a bispecific and bivalent with respect to both receptors is shown in Figure 1A. In other related embodiments, two or more VHHs may be fused together, optionally via a linker moiety, and fused to the Fab or IgG at one or more of these locations. In a related embodiment, the WNT multicomponent polypeptide molecule has a Hetero-IgG format, whereas one of the Fab is present as a half antibody, and the other Fab is fused to one or more of the N-terminus of the Fc, the N-terminus of the Fab, or the C- terminus of the Fc. A bispecific but monovalent to each receptor version of this format is depicted at Figure 6. In certain embodiments, the Fab or antigen-binding fragment thereof is fused directly to the other Fab or IgG or antigen-binding fragment thereof, whereas in other embodiments, the binding regions are fused via a linker moiety. In particular embodiments, the one or both of the two Fabs are described herein or comprise any of the CDR sets described herein.

[0142] In certain embodiments, the antigen binding molecules have a format as described in PCT Application Publication No. WO2017/136820, e.g., a Fabs- in-tandem IgG (FIT-IG) format. Shiyong Gong, Fang Ren, Danqing Wu, Xuan Wu & Chengbin Wu (2017). FIT-IG also include the formats disclosed in, e.g., Gong, et al (2017) *mAbs* 9:1118-1128. In certain embodiments, FIT-IGs combine the functions of two antibodies into one molecule by re-arranging the DNA sequences of two parental monoclonal antibodies into two or three constructs and co-expressing them in mammalian cells. Examples of FIT-IG formats and constructs are provided in FIGS. 1A and 1B and FIGS. 2A and 2B of PCT Application Publication No. WO2017/136820. In certain embodiments, FIT-IGs require no Fc mutation, no scFv elements, and no linker or peptide connector. The Fab-domains in each arm work "in tandem" forming a tetravalent bi-specific antibody with four active and independent antigen binding sites that retain the biological function of their parental antibodies. In particular embodiments, WNT surrogates comprises a Fab and an IgG. In certain embodiments, the Fab binder LC is fused to the HC of the IgG, e.g., by a linker of various length in between. In various embodiment, the Fab binder HC can be fused or unfused to the LC of the IgG. A variation of this format has been called Fabs-in-tandem IgG (or FIT-Ig).

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[0143] In certain embodiments, the WNT multicomponent polypeptide molecules have a format described in PCT Application Publication No. WO2009/080251 (Klein et al.), e.g., a CrossMab format. CrossMabs formats are also described in Schaefer et al. (2011) *Proc. Natl. Acad. Sci USA* 108:11187-11192. The CrossMab format allows correct assembly of two heavy chains and two light chains derived from existing antibodies to form a bispecific, bivalent IgG antibodies. The technology is based on the cross over the antibody domain within one Fab-arm of a bispecific IgG antibody in order to enable correct chain association. Various portions of the Fab can be exchanged, e.g., the entire Fab, the variable heavy and light chains, or the CH1-CL chains can be exchanged.

[0144] In further embodiments of the present invention, the FIT-Ig and CrossMab technologies are combined to create a multispecific, multivalent antigen binding molecule, Cross-FIT, as depicted in Figure 1A and Table 2. Also contemplated is a linker between the crossed CL domain of the Fab and the Ig domains rather than between the CH1 and Ig domains. Additional antigen binding fragments, e.g., Fabs, VHH/sdAbs, and/or scFvs, can be appended to the Cross-FIT structure at various sites, e.g., the heavy or light chains and/or the C-terminus of the Fc domain to create multispecific antibodies.

[0145] In particular embodiments, multicomponent polypeptide molecules comprise two or more VHHs/sdAbs (or scFvs), including at least one that binds one or more first receptor and at least one that binds at least one second receptor. In certain embodiments, one of the binding regions is a VHH/sdAbs and the other is an scFv. Multicomponent polypeptide molecules comprising two or more VHH/sdAbs (or scFvs) may be formatted in a variety of configurations, including but not limited to those depicted in WO2019126398 and WO2020010308 . In certain bispecific, bivalent formats, two or more VHH/sdAbs (or scFvs) are fused in tandem or fused to two different ends of an Fc, optionally via one or more linkers. Where linkers are present, the linker and its length may be the same or different between the VHH/sdAb (or scFv) and the other VHH/sdAb (or scFv), or between the VHH and Fc. For example, in certain embodiments, the VHH/sdAb is fused to the N-terminus, at either the heavy or light chain, and/or C-terminus of the IgG heavy chain. In particular embodiments, two or more VHH/sdAbs are fused to the IgG at any combination of these locations. In various embodiments, both VHH/sdAbs may be fused to the N-termini of the Fc, to the C-termini of the Fc, or one or more VHH/sdAb may be fused to either or both of an N-terminus or C-terminus of the Fc. In a related embodiment, the multicomponent polypeptide molecule has a Hetero-IgG format, whereas one VHH/sdAb is present as a half antibody, and the other is fused

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to the N-terminus of the Fc or the C-terminus of the Fc. In certain embodiments, the VHH/sdAb is fused directly to the other VHH/sdAb whereas in other embodiments, the binding regions are fused via a linker moiety. In particular embodiments, the VHH/sdAb are described herein or comprises any of the CDR sets described herein. In various embodiments, any of these formats may comprise one or more scFvs in place of one or more VHH/sdAbs.

[0146] In certain embodiments, a multicomponent polypeptide molecule is formatted as a diabody. The binders against the two co-receptors can also be linked together in a diabody (or DART) configuration. The diabody can also be in a single chain configuration. If the diabody is fused to an Fc, this will create a bivalent bispecific format. Without fusion to Fc, this would be a monovalent bispecific format. In certain embodiments, a diabody is a noncovalent dimer scFv fragment that consists of the heavy-chain variable (VH) and light-chain variable (VL) regions connected by a small peptide linker. Another form of diabody is a single-chain (Fv)2 in which two scFv fragments are covalently linked to each other.

[0147] As discussed, the multicomponent polypeptide molecules, in various embodiments, comprise one or more antibodies or antigen-binding fragments thereof disclosed herein. Thus, in particular embodiments, the surrogate comprises two polypeptides, wherein each polypeptide comprises an Nab or scFv that binds at least one first co-receptor and an Nab or scFv that binds at least one second co-receptor, optionally wherein one of the binding domains is an scFv and the other is an Nab. In certain embodiments, a surrogate comprises three polypeptides, wherein the first polypeptide comprises an antibody heavy chain and the second polypeptide comprises an antibody light chain, wherein the antibody heavy chain and light chain bind either receptor, and wherein the third polypeptide comprises a VHH/sdAb fused to a heavy chain Fc region or the light chain of the antibody, wherein the VHH/sdAb binds to either co-receptor. In other embodiments, the surrogates comprise four polypeptides, including two heavy chain polypeptides and two light chain polypeptides, wherein the two heavy chains and two light chains bind one or more first receptor, and further comprise one or more VHH/sdAb or scFv fused to one or more of the heavy chains and/or light chains, wherein the VHH/sdAb or scFv binds to one or more second co-receptor. In an illustrative embodiment, a WNT surrogate comprises at least four polypeptides, including two heavy chain polypeptides and two light chain polypeptides that bind either LRP5/6 or one or more FZDs, wherein the WNT surrogate further comprises a Fab that binds either LRP5/6 or one or more FZDs. For example, the Fab may comprise two polypeptides, each fused to one of the two heavy chain polypeptides, and two polypeptides, each fused to one of the two light chain polypeptides, or

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it may comprise two polypeptides each fused to one of the two heavy chain polypeptides and two additional polypeptides, each bound to one of the two polypeptides fused to the heavy chain polypeptides, thus making a second Fab. Other configurations disclosed herein may be used to produce different multicomponent polypeptide molecules.

[0148] Also contemplated are Ig molecules where the VL and VH domains of one Ig are appended with the VL and VH domains of a second antibody. This format is call Fv-Ig or 2Fv-Ig for a homodimer. The VL and VH domains from the second Ig are appended to the N-terminus of the VL and VH domains of the first Ig via short peptide linkers. This format preserves the natural antibody's avidity to cell surface receptors or to more than one receptor or co-receptor complexes (see, e.g., Wu, et al (2007) *Nature Biotechnol.* 25:1290-1297).

[0149] In certain embodiments, the antigen binding formats are multicomponent polypeptide molecules that comprise one or more polypeptides comprising two or more binding regions. For illustrative purposes, the two or more binding regions may be a first receptor binding regions or a second receptor binding regions, or they may comprise one or more first receptor binding region and one or more second receptor binding region. The binding regions may be directly joined or contiguous, or may be separated by a linker, e.g. a polypeptide linker, or a non-peptidic linker, etc. The length of the linker, and therefore the spacing between the binding domains can be used to modulate the signal strength, and can be selected depending on the desired use of the multicomponent polypeptide molecule. The enforced distance between binding domains can vary, but in certain embodiments may be less than about 100 angstroms, less than about 90 angstroms, less than about 80 angstroms, less than about 70 angstroms, less than about 60 angstroms, or less than about 50 angstroms. In some embodiments the linker is a rigid linker, in other embodiments the linker is a flexible linker. In certain embodiments where the linker is a peptide linker, it may be from about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 or more amino acids in length, and is of sufficient length and amino acid composition to enforce the distance between binding domains. In some embodiments, the linker comprises or consists of one or more glycine and/or serine residues.

[0150] The multicomponent polypeptide molecule can be multimerized, e.g., through an Fc domain, by concatenation, coiled coils, polypeptide zippers, biotin/avidin or streptavidin multimerization, and the like. The multicomponent polypeptide molecules can also be joined to a moiety such as PEG, Fc, etc., as known in the art to enhance stability in vivo.

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[0151] In certain embodiments, a multicomponent polypeptide molecule enhances or increases the co-receptors pathway signaling, e.g., in the case of WNT - β-catenin signaling, by at least 30%, 35%, 40%, 45%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 100%,110%, 150%, 200%, 250%, 300%, 400% or 500%, as compared to the β- catenin signaling induced by a neutral substance or negative control as measured in an assay described above, for example as measured in the TOPFlash assay (see, e.g., Molinaar (1996) *Cell* 86:391-399). A negative control may be included in these assays. By way of example, WNT multicomponent polypeptide molecules may enhance β-catenin signaling by a factor of 2x, 5x, 10x, 100x, 1000x, 10000x or more as compared to the activity in the absence of the WNT multicomponent polypeptide molecule when measured, for example when measured in the TOPFlash assay.

[0152] In certain embodiments, functional properties of the multicomponent polypeptide molecules may be assessed using a variety of methods known to the skilled person, including e.g., affinity/binding assays (for example, surface plasmon resonance, competitive inhibition assays), cytotoxicity assays, cell viability assays, cell proliferation or differentiation assays in response to the native molecule/ligand, cancer cell and/or tumor growth inhibition using in vitro or in vivo models, including but not limited to any described herein. The multicomponent polypeptide molecules may also be tested for effects on one or both co-receptor internalization, in vitro and in vivo efficacy, etc. Such assays may be performed using well-established protocols known to the skilled person (see e.g., Current Protocols in Molecular Biology (Greene Publ. Assoc. Inc. & John Wiley & Sons, Inc., NY, NY); Current Protocols in Immunology (Edited by: John E. Coligan, Ada M. Kruisbeek, David H. Margulies, Ethan M. Shevach, Warren Strober 2001 John Wiley & Sons, NY, NY), or commercially available kits.

[0153] In certain embodiments, a binding region of a multicomponent polypeptide molecule (e.g., an antigen-binding fragment of an anti-FZD antibody) comprises one or more of the CDRs of the anti-co-receptor antibodies. In this regard, it has been shown in some cases that the transfer of only the VHCDR3 of an antibody can be performed while still retaining desired specific binding (Barbas et al., PNAS (1995) 92: 2529-2533). See also, McLane et al., PNAS (1995) 92:5214- 5218, Barbas et al., J. Am. Chem. Soc. (1994) 116:2161-2162).

[0154] Also disclosed herein is a method for obtaining an antibody or antigen binding domain specific for a co-receptor, the method comprising providing by way of addition, deletion, substitution or insertion of one or more amino acids in the amino acid sequence of a VH domain set out herein or a VH domain which is an amino acid sequence variant of the VH domain, optionally combining the VH domain thus provided with one or more VL domains,

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and testing the VH domain or VH/VL combination or combinations to identify a specific binding member or an antibody antigen binding domain specific for one or more co-receptors and optionally with one or more desired properties. The VL domains may have an amino acid sequence which is substantially as set out herein. An analogous method may be employed in which one or more sequence variants of a VL domain disclosed herein are combined with one or more VH domains.

[0155] Immunological binding generally refers to the non-covalent interactions of the type which occur between an immunoglobulin molecule and an antigen for which the immunoglobulin is specific, for example by way of illustration and not limitation, as a result of electrostatic, ionic, hydrophilic and/or hydrophobic attractions or repulsion, steric forces, hydrogen bonding, van der Waals forces, and other interactions. The strength, or affinity of immunological binding interactions can be expressed in terms of the dissociation constant (Kd) of the interaction, wherein a smaller Kd represents a greater affinity. Immunological binding properties of selected polypeptides can be quantified using methods well known in the art. One such method entails measuring the rates of antigen-binding site/antigen complex formation and dissociation, wherein those rates depend on the concentrations of the complex partners, the affinity of the interaction, and on geometric parameters that equally influence the rate in both directions. Thus, both the "on rate constant" (Kon) and the "off rate constant" (Koff) can be determined by calculation of the concentrations and the actual rates of association and dissociation. The ratio of Koff /Kon enables cancellation of all parameters not related to affinity, and is thus equal to the dissociation constant Kd. See, generally, Davies et al. (1990) Annual Rev. Biochem. 59:439-473.

[0156] In certain embodiments, the multicomponent polypeptide molecules or binding regions thereof described herein have an affinity of less than about 10,000, less than about 1000, less than about 100, less than about 10, less than about 1 or less than about 0.1 nM, and in some embodiments, the antibodies may have even higher affinity for one or more co-receptors.

[0157] The constant regions of immunoglobulins show less sequence diversity than the variable regions, and are responsible for binding a number of natural proteins to elicit important biochemical events. In humans, there are five different classes of antibodies including IgA (which includes subclasses IgA1 and IgA2), IgD, IgE, IgG (which includes subclasses IgG1, IgG2, IgG3, and IgG4), and IgM. The distinguishing features between these antibody classes are their constant regions, although subtler differences may exist in the V region.

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[0158] The Fc region of an antibody interacts with a number of Fc receptors and ligands, imparting an array of important functional capabilities referred to as effector functions. For IgG, the Fc region comprises Ig domains CH2 and CH3 and the N-terminal hinge leading into CH2. An important family of Fc receptors for the IgG class are the Fc gamma receptors (FcγRs). These receptors mediate communication between antibodies and the cellular arm of the immune system (Raghavan et al., 1996, Annu Rev Cell Dev Biol 12:181- 220; Ravetch et al., 2001, Annu Rev Immunol 19:275-290). In humans this protein family includes FcγRI (CD64), including isoforms FcγRIa, FcγRIb, and FcγRIc; FcγRII (CD32), including isoforms FcγRIIa (including allotypes H131 and R131), FcγRIIb (including FcγRIIb-1 and FcγRIIb-2), and FcγRIIc; and FcγRIII (CD16), including isoforms FcγRIIIa (including allotypes V158 and F158) and FcγRIIIb (including allotypes FcγRIIIb-NA1 and FcγRIIIb-NA2) (Jefferis et al., 2002, Immunol Lett 82:57-65). These receptors typically have an extracellular domain that mediates binding to Fc, a membrane spanning region, and an intracellular domain that may mediate some signaling event within the cell. These receptors are expressed in a variety of immune cells including monocytes, macrophages, neutrophils, dendritic cells, eosinophils, mast cells, platelets, B cells, large granular lymphocytes, Langerhans' cells, natural killer (NK) cells, and T cells. Formation of the Fc/FcγR complex recruits these effector cells to sites of bound antigen, typically resulting in signaling events within the cells and important subsequent immune responses such as release of inflammation mediators, B cell activation, endocytosis, phagocytosis, and cytotoxic attack.

[0159] The ability to mediate cytotoxic and phagocytic effector functions is a potential mechanism by which antibodies destroy targeted cells. The cell- mediated reaction wherein nonspecific cytotoxic cells that express FcγRs recognize bound antibody on a target cell and subsequently cause lysis of the target cell is referred to as antibody dependent cell-mediated cytotoxicity (ADCC) (Raghavan et al., 1996, Annu Rev Cell Dev Biol 12:181-220; Ghetie et al., 2000, Annu Rev Immunol 18:739-766; Ravetch et al., 2001, Annu Rev Immunol 19:275-290). The cell-mediated reaction wherein nonspecific cytotoxic cells that express FcγRs recognize bound antibody on a target cell and subsequently cause phagocytosis of the target cell is referred to as antibody dependent cell-mediated phagocytosis (ADCP). All FcγRs bind the same region on Fc, at the N-terminal end of the Cg2 (CH2) domain and the preceding hinge. This interaction is well characterized structurally (Sondermann et al., 2001, J Mol Biol 309:737-749), and several structures of the human Fc bound to the extracellular domain of human FcγRIIIb have been solved (pdb accession code 1E4K) (Sondermann et al., 2000,

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Nature 406:267- 273.) (pdb accession codes 1IIS and 1IIX) (Radaev et al., 2001, J Biol Chem 276:16469-16477.)

[0160] The different IgG subclasses have different affinities for the FcγRs, with IgG1 and IgG3 typically binding substantially better to the receptors than IgG2 and IgG4 (Jefferis et al., 2002, Immunol Lett 82:57-65). All FcγRs bind the same region on IgG Fc, yet with different affinities: the high affinity binder FcγRI has a Kd for IgG1 of 10^{-8} M^{-1}, whereas the low affinity receptors FcγRII and FcγRIII generally bind at 10^{-6} and 10^{-5} respectively. The extracellular domains of FcγRIIIa and FcγRIIIb are 96% identical; however, FcγRIIIb does not have an intracellular signaling domain. Furthermore, whereas FcγRI, FcγRIIa/c, and FcγRIIIa are positive regulators of immune complex-triggered activation, characterized by having an intracellular domain that has an immunoreceptor tyrosine-based activation motif (ITAM), FcγRIIb has an immunoreceptor tyrosine-based inhibition motif (ITIM) and is therefore inhibitory. Thus the former are referred to as activation receptors, and FcγRIIb is referred to as an inhibitory receptor. The receptors also differ in expression pattern and levels on different immune cells. Yet another level of complexity is the existence of a number of FcγR polymorphisms in the human proteome. A particularly relevant polymorphism with clinical significance is V158/F158 FcγRIIIa. Human IgG1 binds with greater affinity to the V158 allotype than to the F158 allotype. This difference in affinity, and presumably its effect on ADCC and/or ADCP, has been shown to be a significant determinant of the efficacy of the anti-CD20 antibody rituximab (Rituxan®, a registered trademark of IDEC Pharmaceuticals Corporation). Subjects with the V158 allotype respond favorably to rituximab treatment; however, subjects with the lower affinity F158 allotype respond poorly (Cartron et al., 2002, Blood 99:754-758). Approximately 10-20% of humans are V158/V158 homozygous, 45% are V158/F158 heterozygous, and 35-45% of humans are F158/F158 homozygous (Lehrnbecher et al., 1999, Blood 94:4220-4232; Cartron et al., 2002, Blood 99:754-758). Thus 80-90% of humans are poor responders,, they have at least one allele of the F158 FcγRIIIa.

[0161] The Fc region is also involved in activation of the complement cascade. In the classical complement pathway, C1 binds with its C1q subunits to Fc fragments of IgG or IgM, which has formed a complex with antigen(s). In certain embodiments of the invention, modifications to the Fc region comprise modifications that alter (either enhance or decrease) the ability of a FZD-specific antibody as described herein to activate the complement system (see e.g., U.S. Patent 7,740,847). To assess complement activation, a complement-dependent

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cytotoxicity (CDC) assay may be performed (See, e.g., Gazzano- Santoro et al., J. Immunol. Methods, 202:163 (1996).

[0162] Thus in certain embodiments, the present invention provides the multicomponent polypeptide molecules having a modified Fc region with altered functional properties, such as reduced or enhanced CDC, ADCC, or ADCP activity, or enhanced binding affinity for a specific FcγR or increased serum half-life. Other modified Fc regions contemplated herein are described, for example, in issued U.S. Patents 7,317,091; 7,657,380; 7,662,925; 6,538,124; 6,528,624; 7,297,775; 7,364,731; Published U.S. Applications US2009092599; US20080131435; US20080138344; and published International Applications WO2006/105338; WO2004/063351; WO2006/088494; WO2007/024249.

[0163] Structurally, the Fc region can be important for proper assembly of the msAb. In particular, modifications to the CH3 domain such as knobs-in-hole (see, e.g., WO1996/027011; and WO1998/050431) or Azymetric mutations (see, e.g., WO2012/58768) can prevent heavy chain mispairing. The present invention utilizes these mutations in certain Fc embodiments.

[0164] The multicomponent molecules disclosed herein may also be modified to include an epitope tag or label, e.g., for use in purification or diagnostic applications. There are many linking groups known in the art for making antibody conjugates, including, for example, those disclosed in U.S. Pat. No. 5,208,020 or EP Patent 0 425 235 B1, and Chari et al., Cancer Research 52: 127-131 (1992). The linking groups include disulfide groups, thioether groups, acid labile groups, photolabile groups, peptidase labile groups, or esterase labile groups, as disclosed in the above-identified patents, disulfide and thioether groups being preferred.

[0165] In certain embodiments, and antigen- binding fragments thereof against one co-receptor and/or antibodies and antigen-binding fragments thereof against the other co-receptor present within a multicomponent polypeptide molecule are monoclonal. In certain embodiments, they are humanized.

[0166] The present invention further provides in certain embodiments an isolated nucleic acid encoding a polypeptide present in a multicomponent polypeptide molecule. Nucleic acids include DNA and RNA. These and related embodiments may include polynucleotides encoding antibody fragments that bind one or more co-receptors. The term "isolated polynucleotide" as used herein shall mean a polynucleotide of genomic, cDNA, or synthetic origin, or some combination thereof, which by virtue of its origin, the isolated polynucleotide: (1) is not associated with all or a portion of a polynucleotide in which the isolated polynucleotide is found

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in nature, (2) is linked to a polynucleotide to which it is not linked in nature, or (3) does not occur in nature as part of a larger sequence. An isolated polynucleotide may include naturally occurring and/or artificial sequences.

[0167] As will be understood by those skilled in the art, polynucleotides may include genomic sequences, extra-genomic and plasmid-encoded sequences and smaller engineered gene segments that express, or may be adapted to express, proteins, polypeptides, peptides and the like. Such segments may be naturally isolated, or modified synthetically by the skilled person.

[0168] As will be also recognized by the skilled artisan, polynucleotides may be single-stranded (coding or antisense) or double-stranded, and may be DNA (genomic, cDNA or synthetic) or RNA molecules. RNA molecules may include HnRNA molecules, which contain introns and correspond to a DNA molecule in a one-to-one manner, and mRNA molecules, which do not contain introns. Additional coding or non-coding sequences may, but need not, be present within a polynucleotide according to the present disclosure, and a polynucleotide may, but need not, be linked to other molecules and/or support materials. Polynucleotides may comprise a native sequence or may comprise a sequence that encodes a variant or derivative of such a sequence.

[0169] It will be appreciated by those of ordinary skill in the art that, as a result of the degeneracy of the genetic code, there are many nucleotide sequences that encodes an antibody as described herein. Some of these polynucleotides bear minimal sequence identity to the nucleotide sequence of the native or original polynucleotide sequence encoding a polypeptide within a multicomponent polypeptide molecule. Nonetheless, polynucleotides that vary due to differences in codon usage are expressly contemplated by the present disclosure. In certain embodiments, sequences that have been codon- optimized for mammalian expression are specifically contemplated.

[0170] Therefore, in another embodiment of the invention, a mutagenesis approach, such as site-specific mutagenesis, may be employed for the preparation of variants and/or derivatives of the polypeptides described herein. By this approach, specific modifications in a polypeptide sequence can be made through mutagenesis of the underlying polynucleotides that encode them. These techniques provide a straightforward approach to prepare and test sequence variants, for example, incorporating one or more of the foregoing considerations, by introducing one or more nucleotide sequence changes into the polynucleotide.

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[0171] Site-specific mutagenesis allows the production of mutants through the use of specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Mutations may be employed in a selected polynucleotide sequence to improve, alter, decrease, modify, or otherwise change the properties of the polynucleotide itself, and/or alter the properties, activity, composition, stability, or primary sequence of the encoded polypeptide.

[0172] In certain embodiments, the inventors contemplate the mutagenesis of the polynucleotide sequences that encode a polypeptide present in a multicomponent polypeptide molecule, to alter one or more properties of the encoded polypeptide, such as the binding affinity, or the function of a particular Fc region, or the affinity of the Fc region for a particular FcγR. The techniques of site-specific mutagenesis are well-known in the art, and are widely used to create variants of both polypeptides and polynucleotides. For example, site- specific mutagenesis is often used to alter a specific portion of a DNA molecule. In such embodiments, a primer comprising typically about 14 to about 25 nucleotides or so in length is employed, with about 5 to about 10 residues on both sides of the junction of the sequence being altered.

[0173] As will be appreciated by those of skill in the art, site-specific mutagenesis techniques have often employed a phage vector that exists in both a single stranded and double stranded form. Typical vectors useful in site- directed mutagenesis include vectors such as the M13 phage. These phages are readily commercially-available and their use is generally well-known to those skilled in the art. Double-stranded plasmids are also routinely employed in site directed mutagenesis that eliminates the step of transferring the gene of interest from a plasmid to a phage.

[0174] The preparation of sequence variants of the selected peptide- encoding DNA segments using site-directed mutagenesis provides a means of producing potentially useful species and is not meant to be limiting as there are other ways in which sequence variants of peptides and the DNA sequences encoding them may be obtained. For example, recombinant vectors encoding the desired peptide sequence may be treated with mutagenic agents, such as hydroxylamine, to obtain sequence variants. Specific details regarding these methods and protocols are found in the teachings of Maloy et al., 1994; Segal, 1976; Prokop and Bajpai, 1991; Kuby, 1994, and Maniatis et al., 1982, each incorporated herein by reference, for that purpose.

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[0175] In many embodiments, one or more nucleic acids encoding a polypeptide of multicomponent WNT molecule are introduced directly into a host cell, and the cell incubated under conditions sufficient to induce expression of the encoded polypeptides. The surrogate polypeptides of this disclosure may be prepared using standard techniques well known to those of skill in the art in combination with the polypeptide and nucleic acid sequences provided herein. The polypeptide sequences may be used to determine appropriate nucleic acid sequences encoding the particular polypeptide disclosed thereby. The nucleic acid sequence may be optimized to reflect particular codon "preferences" for various expression systems according to standard methods well known to those of skill in the art.

[0176] According to certain related embodiments there is provided a recombinant host cell which comprises one or more constructs as described herein, e.g., a vector comprising a nucleic acid encoding a multicomponent polypeptide molecule or polypeptide thereof, and a method of production of the encoded product, which method comprises expression from encoding nucleic acid therefor. Expression may conveniently be achieved by culturing under appropriate conditions recombinant host cells containing the nucleic acid. Following production by expression, an antibody or antigen-binding fragment thereof, may be isolated and/or purified using any suitable technique, and then used as desired.

[0177] Polypeptides, and encoding nucleic acid molecules and vectors, may be isolated and/or purified, e.g. from their natural environment, in substantially pure or homogeneous form, or, in the case of nucleic acid, free or substantially free of nucleic acid or genes of origin other than the sequence encoding a polypeptide with the desired function. Nucleic acid may comprise DNA or RNA and may be wholly or partially synthetic. Reference to a nucleotide sequence as set out herein encompasses a DNA molecule with the specified sequence, and encompasses a RNA molecule with the specified sequence in which U is substituted for T, unless context requires otherwise.

[0178] Systems for cloning and expression of a polypeptide in a variety of different host cells are well known. Suitable host cells include bacteria, mammalian cells, yeast and baculovirus systems. Mammalian cell lines available in the art for expression of a heterologous polypeptide include Chinese hamster ovary cells, HeLa cells, baby hamster kidney cells, NSO mouse melanoma cells and many others. A common, preferred bacterial host is E. coli.

[0179] The expression of polypeptides, e.g., antibodies and antigen- binding fragments thereof, in prokaryotic cells such as E. coli is well established in the art. For a review, see for

example Pluckthun, A. Bio/Technology 9: 545-551 (1991). Expression in eukaryotic cells in culture is also available to those skilled in the art as an option for production of antibodies or antigen-binding fragments thereof, see recent reviews, for example Ref, M. E. (1993) Curr. Opinion Biotech. 4: 573-576; Trill J. J. et al. (1995) Curr. Opinion Biotech 6: 553-560.

[0180] Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator sequences, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. Vectors may be plasmids, viral e.g. phage, or phagemid, as appropriate. For further details see, for example, Molecular Cloning: a Laboratory Manual: 2nd edition, Sambrook et al., 1989, Cold Spring Harbor Laboratory Press. Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in Current Protocols in Molecular Biology, Second Edition, Ausubel et al. eds., John Wiley & Sons, 1992, or subsequent updates thereto.

[0181] The present invention also provides, in certain embodiments, a method which comprises using a construct as stated above in an expression system in order to express a particular polypeptide such as a WNT chimeric activator molecule. The term "transduction" is used to refer to the transfer of genes from one bacterium to another, usually by a phage.

[0182] Amino acid sequence modification(s) of any of the polypeptides described herein are contemplated. For example, it may be desirable to improve the binding affinity and/or other biological properties of the multicomponent polypeptide molecule. For example, amino acid sequence variants of a multicomponent polypeptide molecule may be prepared by introducing appropriate nucleotide changes into a polynucleotide that encodes the antibody, or a chain thereof, or by peptide synthesis. Such modifications include, for example, deletions from, and/or insertions into and/or substitutions of, residues within the amino acid sequences of the antibody. Any combination of deletion, insertion, and substitution may be made to arrive at the final multicomponent polypeptide molecule, provided that the final construct possesses the desired characteristics (e.g., high affinity binding to one or more co-receptors). The amino acid changes also may alter post-translational processes of the antibody, such as changing the number or position of glycosylation sites. Any of the variations and modifications described above for polypeptides of the present invention may be included in antibodies of the present invention.

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[0183] The present disclosure provides variants of any of the polypeptides (e.g., multicomponent molecule, multicomponent polypeptide molecules, or antibodies or antigen-binding fragments thereof) disclosed herein. In certain embodiments, a variant has at least 90%, at least 95%, at least 98%, or at least 99% identity to a polypeptide disclosed herein. In certain embodiments, such variant polypeptides bind to one or more first co-receptors, and/or to one or more second co-receptors, at least about 50%, at least about 70%, and in certain embodiments, at least about 90% as well as a t multicomponent polypeptide molecule specifically set forth herein. In further embodiments, such variant multicomponent polypeptide molecules bind to one or more first co-receptor, and/or to one or more second co-receptor, with greater affinity than the multicomponent polypeptide molecules set forth herein, for example, that bind quantitatively at least about 105%, 106%, 107%, 108%, 109%, or 110% as well as an antibody sequence specifically set forth herein.

[0184] In particular embodiments, the multicomponent polypeptide molecule or a binding region thereof, e.g., a Fab, scFv, or VHH may comprise: a) a heavy chain variable region comprising: i. a CDR1 region that is identical in amino acid sequence to the heavy chain CDR1 region of a selected antibody described herein; ii. a CDR2 region that is identical in amino acid sequence to the heavy chain CDR2 region of the selected antibody; and iii. a CDR3 region that is identical in amino acid sequence to the heavy chain CDR3 region of the selected antibody; and/or b) a light chain variable domain comprising: i. a CDR1 region that is identical in amino acid sequence to the light chain CDR1 region of the selected antibody; ii. a CDR2 region that is identical in amino acid sequence to the light chain CDR2 region of the selected antibody; and iii. a CDR3 region that is identical in amino acid sequence to the light chain CDR3 region of the selected antibody; wherein the antibody specifically binds a selected target. In a further embodiment, the antibody, or antigen-binding fragment thereof, is a variant antibody or antigen-binding fragment thereof wherein the variant comprises a heavy and light chain identical to the selected antibody except for up to 8, 9, 10, 11, 12, 13, 14, 15, or more total amino acid substitutions in the CDR regions of the VH and VL regions. In this regard, there may be 1, 2, 3, 4, 5, 6, 7, 8, or in certain embodiments, 9, 10, 11, 12, 13, 14, 15 more total amino acid substitutions in the (collective) CDR regions of the selected antibody. Substitutions may be in CDRs either in the VH and/or the VL regions. (See e.g., Mulier, 1998, Structure 6:1153-1167).

[0185] In particular embodiments, the multicomponent polypeptide molecule or a binding region thereof, e.g., a Fab, scFv, or VHH/sdAb, may have: a) a heavy chain variable region

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having an amino acid sequence that is at least 80% identical, at least 95% identical, at least 90%, at least 95% or at least 98% or 99% identical, to the heavy chain variable region of an antibody or antigen- binding fragments thereof described herein; and/or b) a light chain variable region having an amino acid sequence that is at least 80% identical, at least 85%, at least 90%, at least 95% or at least 98% or 99% identical, to the light chain variable region of an antibody or antigen-binding fragments thereof described herein.

[0186] A polypeptide has a certain percent "sequence identity" to another polypeptide, meaning that, when aligned, that percentage of amino acids are the same when comparing the two sequences. Sequence similarity can be determined in a number of different manners. To determine sequence identity, sequences can be aligned using the methods and computer programs, including BLAST, available over the world wide web at ncbi nlm nih gov/BLAST/ Another alignment algorithm is FASTA, available in the Genetics Computing Group (GCG) package, from Madison, Wis., USA, a wholly owned subsidiary of Oxford Molecular Group, Inc. Other techniques for alignment are described in Methods in Enzymology, vol. 266: Computer Methods for Macromolecular Sequence Analysis (1996), ed. Doolittle, Academic Press, Inc., a division of Harcourt Brace & Co., San Diego, Calif., USA. Of particular interest are alignment programs that permit gaps in the sequence. The Smith-Waterman is one type of algorithm that permits gaps in sequence alignments. See Meth. Mol. Biol. 70: 173-187 (1997). Also, the GAP program using the Needleman and Wunsch alignment method can be utilized to align sequences. See J. Mol. Biol. 48: 443-453 (1970).

[0187] Of interest is the BestFit program using the local homology algorithm of Smith and Waterman (Advances in Applied Mathematics 2: 482- 489 (1981) to determine sequence identity. The gap generation penalty will generally range from 1 to 5, usually 2 to 4 and in many embodiments will be 3. The gap extension penalty will generally range from about 0.01 to 0.20 and in many instances will be 0.10. The program has default parameters determined by the sequences inputted to be compared. Preferably, the sequence identity is determined using the default parameters determined by the program. This program is available also from Genetics Computing Group (GCG) package, from Madison, Wis., USA.

[0188] Another program of interest is the FastDB algorithm. FastDB is described in Current Methods in Sequence Comparison and Analysis, Macromolecule Sequencing and Synthesis, Selected Methods and Applications, pp. 127-149, 1988, Alan R. Liss, Inc. Percent sequence identity is calculated by FastDB based upon the following parameters: Mismatch Penalty: 1.00; Gap Penalty: 1.00; Gap Size Penalty: 0.33; and Joining Penalty: 30.0.

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[0189] In particular embodiments, the multicomponent polypeptide molecule or a binding region thereof, e.g., a Fab, scFv, or VHH may comprise: a) a heavy chain variable region comprising: i. a CDR1 region that is identical in amino acid sequence to the heavy chain CDR1 region of a selected antibody described herein; ii. a CDR2 region that is identical in amino acid sequence to the heavy chain CDR2 region of the selected antibody; and iii. a CDR3 region that is identical in amino acid sequence to the heavy chain CDR3 region of the selected antibody; and b) a light chain variable domain comprising: i. a CDR1 region that is identical in amino acid sequence to the light chain CDR1 region of the selected antibody; ii. a CDR2 region that is identical in amino acid sequence to the light chain CDR2 region of the selected antibody; and iii. a CDR3 region that is identical in amino acid sequence to the light chain CDR3 region of the selected antibody; wherein the antibody specifically binds a selected target (e.g., a FZD receptor, such as FZD1). In a further embodiment, the antibody, or antigen-binding fragment thereof, is a variant antibody wherein the variant comprises a heavy and light chain identical to the selected antibody except for up to 8, 9, 10, 11, 12, 13, 14, 15, or more amino acid substitutions in the CDR regions of the VH and VL regions. In this regard, there may be 1, 2, 3, 4, 5, 6, 7, 8, or in certain embodiments, 9, 10, 11, 12, 13, 14, 15 more amino acid substitutions in the CDR regions of the selected antibody. Substitutions may be in CDRs either in the VH and/or the VL regions. (See e.g., Muller, 1998, Structure 6:1153-1167).

[0190] Determination of the three-dimensional structures of representative polypeptides (e.g., variant FZD binding regions or LRP5/6 binding regions and bridging regions of WNT multicomponent polypeptide molecules as provided herein) may be made through routine methodologies such that substitution, addition, deletion or insertion of one or more amino acids with selected natural or non-natural amino acids can be virtually modeled for purposes of determining whether a so derived structural variant retains the space-filling properties of presently disclosed species. See, for instance, Donate et al., 1994 Prot. Sci. 3:2378; Bradley et al., Science 309: 1868-1871 (2005); Schueler-Furman et al., Science 310:638 (2005); Dietz et al., Proc. Nat. Acad. Sci. USA 103:1244 (2006); Dodson et al., Nature 450:176 (2007); Qian et al., Nature 450:259 (2007); Raman et al. Science 327:1014-1018 (2010). Some additional non-limiting examples of computer algorithms that may be used for these and related embodiments, such as for rational design of binding regions include VMD which is a molecular visualization program for displaying, animating, and analyzing large biomolecular systems using 3-D graphics and built-in scripting (see the website for the Theoretical and Computational Biophysics Group, University of Illinois at Urbana-Champagne, at

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ks.uiuc.edu/Research/vmd/. Many other computer programs are known in the art and available to the skilled person and which allow for determining atomic dimensions from space-filling models (van der Waals radii) of energy-minimized conformations; GRID, which seeks to determine regions of high affinity for different chemical groups, thereby enhancing binding; Monte Carlo searches, which calculate mathematical alignment, and CHARMM (Brooks et al. (1983) J. Comput. Chem. 4:187-217) and AMBER (Weiner et al (1981) J. Comput. Chem. 106: 765), which assess force field calculations, and analysis (see also, Eisenfield et al. (1991) Am. J. Physiol. 261:C376-386; Lybrand (1991) J. Pharm. Belg. 46:49-54; Froimowitz (1990) Biotechniques 8:640-644; Burbam et al. (1990) Proteins 7:99-111; Pedersen (1985) Environ. Health Perspect. 61:185-190; and Kini et al. (1991) J. Biomol. Struct. Dyn. 9:475-488). A variety of appropriate computational computer programs are also commercially available, such as from Schrödinger (Munich, Germany).

IV. WNT Signal Enhancing Molecules (WNT Enhancers)

[0191] In an embodiment, a multicomponent WNT molecule, as described herein, possesses at least one FZD binding domain, at least one LRP binding domain, and at least one bridging molecule; the WNT multicomponent polypeptide molecule modulates WNT signaling. In particular embodiments, the binding domains bind to their target when the target is present on the cell surface, e.g., they may bind to an epitope within the extracellular domain of their target.

[0192] In certain embodiments, the multicomponent WNT molecule, described herein, possesses two FZD binding domains tethered to the bridging molecule by a binding domain specific for a first epitope on the bridging molecule; two LRP binding domains tethered to the bridging molecule by a binding domain specific for a second epitope at least one different antigen binding domain molecule; and, the tethering of the FZD and LRP binding domains to the bridging molecule forms a WNT mimetic capable of activating WNT signaling.

[0193] In certain embodiments, the multicomponent WNT molecule, described herein, possesses the binding domain specific for a first epitope of the bridging molecule and the FZD binding domain are joined directly or by a linker, the binding domain specific for a second epitope of the bridging molecule and the LRP binding domain are joined directly, or by a linker, and the binding domain of the first epitope and second epitope of the bridging molecule are identical or different.

[0194] In another embodiment, the multicomponent WNT molecule , reported herein, possess at least one FZD binding domain linked to one LRP binding domain which is tethered to at least one different antigen binding domain by a binding domain specific for a first epitope on the bridging molecule, at least one FZD binding domain linked to one LRP binding domain which is tethered to at least one different antigen binding domain by a binding domain specific for a second epitope on the bridging molecule, and the tethering of the FZD and LRP binding domains to the bridging receptor forms a WNT mimetic capable of activating WNT signaling.

[0195] In certain embodiments, the multicomponent WNT molecule, described herein, possesses the binding domain specific for a first epitope of the bridging molecule and the FZD binding domain are joined directly or by a linker, and the binding domain specific for a second epitope of the bridging molecule and the LRP binding domain are joined directly, or by a linker, wherein the binding domain of the first epitope and second epitope of the bridging molecule are identical or different, and could monomeric or multimeric.

V. Targeting Molecules

[0196] Specific cell types and cells within specific tissue may comprise one or more cell- or tissue-specific surface molecule, such as a cell surface receptor. As used herein, the molecule is said to be cell- or tissue-specific if a greater amount of the molecule is present on the specific cell or tissue type as compared to one or more other cell or tissue types, or any other cell or tissue type. In certain embodiments, the greater amount is at least two-fold, at least five-fold, at least 10-fold, at least 20-fold, at least 50-fold, or at least 100-fold as compared to the amount in the one or more other cell or tissue types, or any other cell or tissue type. In particular embodiments, the cell-specific surface molecule has increased or enhanced expression on a target organ, tissue or cell type, e.g., an organ, tissue or cell type in which it is desirous to enhance WNT signaling, e.g., to treat or prevent a disease or disorder, e.g., as compared to one or more other non-targeted organs, tissues or cell types. In certain embodiments, the cell-specific surface molecule is preferentially expressed on the surface of the target organ, tissue or cell type as compared to one or more other organ, tissue or cell types, respectively. For example, in particular embodiments, a cell surface receptor is considered to be a tissue-specific or cell-specific cell surface molecule if it is expressed at levels at least two-fold, at least five-fold, at least 10-fold, at least 20-fold, at least 30-fold, at least 40-fold, at least 50-fold, at least 100-fold, at least 500-fold, or at least 1000-fold higher in the target organ, tissue or cell than it is expressed in one or more, five or more, all other organs, tissues or cells, or an average of all

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other organs, tissue or cells, respectively. In certain embodiments, the tissue-specific or cell-specific cell surface molecule is a cell surface receptor, e.g., a polypeptide receptor comprising a region located within the cell surface membrane and an extracellular region to which the targeting module can bind. In various embodiments, the methods described herein may be practiced by specifically targeting cell surface molecules that are only expressed on the target tissue or a subset of tissues including the target tissue, or by specifically targeting cell surface molecules that have higher levels of expression on the target tissue as compared to all, most, or a substantial number of other tissues, e.g., higher expression on the target tissue than on at least two, at least five, at least ten, or at least twenty other tissues.

[0197] The targeted tissue may be bound by a targeting module, e.g., a binding domain that specifically binds to the tissue specific receptor. The targeted tissue may be any tissue, e.g., any mammalian tissue or cell type. In certain embodiments, the targeted tissue may be present in any organ. In certain embodiments, the target tissue is bone tissue, liver tissue, skin tissue, stomach tissue, intestine tissue, oral mucosa tissue, kidney tissue, central nervous system tissue, mammary gland tissue, taste bud tissue, ovary tissue, inner ear tissue (including cochlear and vestibular tissues), hair follicles, pancreas tissue, retina tissue, cornea tissue, heart tissue or lung tissue, and the targeting module binds to a tissue-specific cell surface molecule (e.g., a cell surface receptor) preferentially expressed on bone tissue, liver tissue, skin tissue, stomach tissue, intestine tissue, oral mucosa tissue, kidney tissue, central nervous system tissue, mammary gland tissue, taste bud tissue, ovary tissue, inner ear tissue (including cochlear and vestibular tissues), hair follicles, pancreas tissue, retina tissue, cornea tissue, heart tissue or lung tissue, respectively.

[0198] The targeting module may bind to any cell type, e.g., any cell within any tissue, organ or animal, including but not limited to mammals, such as humans. In certain embodiments, the tissue-specific WNT surrogate-signal enhancing combination molecule binds to specific cell types, e.g., specific cell types associated with a target tissue. For example, in liver tissue, the targeting module may bind to hepatocytes, precursors and stem cells of hepatocytes, biliary tract cells, and/or endothelial or other vascular cells. Examples of liver specific targeting molecules are provided, e.g., in WO 2018/140821 and WO 2020/014271, both of which are incorporated by reference herein. For example, in bone tissue, the targeting module may bind osteoblasts, precursors of osteoblasts, mesenchymal stem cells, stem cells and precursor cells that give rise to bone, cartilage and/or other cells present in bone tissue. Cell types present in various tissues, including but not limited to the tissues described herein,

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are known in the art, and in various embodiments, the tissue specific WNT signal enhancing molecules described herein may bind any of them.

VI. Linkers

[0199] In certain embodiments, the multicomponent targeting molecule, for example FZD molecule, LRP molecule, and the bridging molecule, are bound or fused directly to each other, whereas in other embodiments, they are separated by a linker, e.g., a polypeptide linker, or a non-peptidyl linker, *etc.* In particular embodiments, a linker is an Fc linker, e.g., a region of an antibody Fc domain capable of dimerizing with another Fc linker, e.g., via one or more disulfide bonds. In another particular embodiment, a linker is albumin, e.g., human serum albumin, where the targeting and action modules are on the N- and C- termini of albumin.

[0200] In certain embodiments, particularly when joining two polypeptides, the linker is made up of amino acids linked together by peptide bonds. In particular embodiments, the linker comprises, in length, from 1 up to about 40 amino acid residues, from 1 up to about 20 amino acid residues, or from 1 to about 10 amino acid residues. In certain embodiments, the amino acid residues in the linker are from among the twenty canonical amino acids, and in certain embodiments, selected from cysteine, glycine, alanine, proline, asparagine, glutamine, and/or serine. In certain embodiments, a linker comprises one or more non-natural amino acids. In some embodiments, a peptidyl linker is made up of a majority of amino acids that are sterically unhindered, such as glycine, serine, and alanine linked by a peptide bond. Certain linkers include polyglycines, polyserines, and polyalanines, or combinations of any of these. Some exemplary peptidyl linkers are poly(Gly)1-8, particularly (Gly)3, (Gly)4 (SEQ ID NO: 14), (Gly)5 (SEQ ID NO: 15), (Gly)6 (SEQ ID NO: 16), (Gly)7 (SEQ ID NO: 17), and (Gly)8 (SEQ ID NO: 18) as well as, poly(Gly)4 Ser (SEQ ID NO: 19), poly(Gly-Ala)2 (SEQ ID NO: 20), poly(Gly-Ala)3 (SEQ ID NO: 21), poly(Gly-Ala)4 (SEQ ID NO: 22) and poly(Ala)1-8 (SEQ ID NO: 23-27). Other specific examples of peptidyl linkers include (Gly)5Lys (SEQ ID NO: 28), and (Gly)5LysArg (SEQ ID NO: 29). To explain the above nomenclature, for example, (Gly)3Lys(Gly)4 means Gly-Gly-Gly-Lys-Gly-Gly-Gly-Gly (SEQ ID NO: 30). Other combinations of Gly and Ala are also useful. Additionally, a peptidyl linker can also comprise a non-peptidyl segment such as a 6 carbon aliphatic molecule of the formula --CH2--CH2--CH2--CH2--CH2--CH2--. The peptidyl linkers can be altered to form derivatives as described herein.

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[0201] Illustrative non-peptidyl linkers include, for example, alkyl linkers such as --NH--(CH2) s--C(O)--, wherein s=2-20. These alkyl linkers may further be substituted by any non-sterically hindering group such as lower alkyl (e g., C1-C6) lower acyl, halogen (e.g , Cl, Br), CN, NH2, phenyl, etc. Non-peptide portions of the inventive composition of matter, such as non-peptidyl linkers or non-peptide half-life extending moieties can be synthesized by conventional organic chemistry reactions. Chemical groups that find use in linking binding domains include carbamate; amide (amine plus carboxylic acid); ester (alcohol plus carboxylic acid), thioether (haloalkane plus sulfhydryl; maleimide plus sulfhydryl), Schiff's base (amine plus aldehyde), urea (amine plus isocyanate), thiourea (amine plus isothiocyanate), sulfonamide (amine plus sulfonyl chloride), disulfide; hydrazone, lipids, and the like, as known in the art.

[0202] The linkage between domains may comprise spacers, e g. alkyl spacers, which may be linear or branched, usually linear, and may include one or more unsaturated bonds; usually having from one to about 300 carbon atoms; more usually from about one to 25 carbon atoms; and may be from about three to 12 carbon atoms. Spacers of this type may also comprise heteroatoms or functional groups, including amines, ethers, phosphodiesters, and the like. Specific structures of interest include: $(CH_2CH_2O)n$ where n is from 1 to about 12; $(CH_2CH_2NH)n$, where n is from 1 to about 12; $[(CH_2)n(C=O)NH(CH_2)_m]_z$, where n and m are from 1 to about 6, and z is from 1 to about 10; $[(CH_2)nOPO_3(CH_2)_m]_z$ where n and m are from 1 to about 6, and z is from 1 to about 10. Such linkers may include polyethylene glycol, which may be linear or branched.

[0203] In certain embodiments, the domains may be joined through a homo- or heterobifunctional linker. Illustrative entities include: azidobenzoyl hydrazide, N-[4-(p-azidosalicylamino)butyl]-3'-[2'-pyridyldithio]propionamide), bis-sulfosuccinimidyl suberate, dimethyladipimidate, disuccinimidyltartrate, N-γ-maleimidobutyryloxysuccinimide ester, N-hydroxy sulfosuccinimidyl-4-azidobenzoate, N-succinimidyl [4-azidophenyl]-1,3'-dithiopropionate, N-succinimidyl [4-iodoacetyl]aminobenzoate, glutaraldehyde, NHS-PEG-MAL; succinimidyl 4-[N-maleimidomethyl]cyclohexane-1-carboxylate; 3-(2-pyridyldithio)propionic acid N-hydroxysuccinimide ester (SPDP); N, N'-(1,3-phenylene) bismaleimide; N, N'-ethylene-bis-(iodoacetamide); or 4-(N-maleimidomethyl)-cyclohexane-1-carboxylic acid N-hydroxysuccinimide ester (SMCC); m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS), and succinimide 4-(p-maleimidophenyl)butyrate (SMPB), an extended chain analog of MBS. In certain embodiments, the succinimidyl group of these

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cross-linkers reacts with a primary amine, and the thiol-reactive maleimide forms a covalent bond with the thiol of a cysteine residue.

[0204] Other reagents useful include: homobifunctional cross-linking reagents including bismaleimidohexane ("BMH"); p,p'-difluoro-m,m'-dinitrodiphenylsulfone (which forms irreversible cross-linkages with amino and phenolic groups); dimethyl adipimidate (which is specific for amino groups); phenol-1,4-disulfonylchloride (which reacts principally with amino groups); hexamethylenediisocyanate or diisothiocyanate, or azophenyl-p-diisocyanate (which reacts principally with amino groups); disdiazobenzidine (which reacts primarily with tyrosine and histidine); O-benzotriazolyloxy tetramethuluronium hexafluorophosphate (HATU); dicyclohexyl carbodiimde; bromo-tris (pyrrolidino) phosphonium bromide (PyBroP); N,N-dimethylamino pyridine (DMAP); 4-pyrrolidino pyridine; N-hydroxy benzotriazole; and the like.

VII. Cell Targeting activators via bridging receptor(s)

[0205] The present invention encompasses a novel concept to achieve cell targeting for ligand/receptor systems that involves multicomponent receptor complexes, where a productive signaling competent receptor complex formation is mediated through a "bridging element", using "chimeric activator" approach based on the cooperativity concept. When an "targeting element" is tethered to the mutant "activity element", the "targeting element" helps to increase the local concentration of the mutant "activity element" on the desired target cell surface, driving engagement of signaling receptor and subsequent intracellular signaling activation, effectively left shift the activity dose response curve (increase potency) on target cell and create the selectivity between target vs non-target cells.

[0206] The concept of cell targeting for ligand/receptor systems that involves multicomponent receptor complexes, where a productive signaling competent receptor complex formation is mediated through a "bridging element" is tested using the WNT/β-catenin signaling pathway as a model system.

[0207] WNT pathway is highly conserved across species and crucial for embryonic development, and adult tissue homeostasis and regeneration (Nusse and Clevers, 2017). WNT induced signaling through β-catenin stabilization has been widely studied and is achieved by ligand binding to frizzled (FZD) and low-density lipoprotein receptor-related protein (LRP) family of receptors. There are nineteen mammalian WNTs, 10 FZDs (FZD1-10), and 2 LRPs (LRP5 and LRP6). WNTs are highly hydrophobic due to lipidation that is required for function

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and are promiscuous, capable of binding and activating multiple FZD and LRP pairs (Janda et al., 2012, Kadowaki et al., 1996, Dijksterhuis et al., 2015). Elucidating the functions of individual FZDs in tissues has been hampered by difficulties in producing the ligands and lack of receptor and tissue selectivity. Recent breakthroughs in the development of WNT-mimetic molecules have largely resolved the production and receptor specificity challenges (Janda et al., 2017, Chen et al., 2020, Tao et al., 2019, Miao et al., 2020). While tissue selectivity could be partly achieved by tissue injury as damaged tissues seem more sensitive to WNTs (Xie et al., 2022), it would still represent a significant technical advancement to be able to target WNTs to specific cells and tissues.

[0208] The WNT mimetics reported so far are all bispecific and can simultaneously bind to FZDs and LRPs, and the optimal stoichiometry (at least for the antibody based molecules) are tetravalent bispecific (2:2 format), requiring two FZD binders and two LRP binders in the same molecule to achieve efficient signaling (Tao et al., 2019, Chen et al., 2020). We took advantage of the fact that two FZD binders alone, two LRP binders alone, or a molecule with one FZD binder and one LRP binder (1:1 format) do not signal. Cell specificity could be achieved by attaching an "targeting element" (capable of binding to another cell surface receptor, called a bridging receptor or bridging molecule here) to these three inactive molecules, and signaling competent receptor complexes consisting of two FZDs and two LRPs could then be assemble via the bridging receptor on the target cell surface. This approach reduces or eliminates the need to mutate and reduce the affinity of the "active elements" toward signaling receptors, and creates a highly cell specific activation of the signaling pathway as the individual components are inactive.

[0209] Fig. 1A shows one optimized design for a WNT mimetic that is a tetravalent bispecific antibody-based molecule. It is important to highlight that efficient WNT/β-catenin signaling requires two FZD binding domains and two LRP binding domains in one molecule (Chen et al., 2020). Fig. 1B shows a cell targeted WNT mimetic based on the "chimeric activator" concept where an "targeting element" is tethered to the WNT mimetic. These molecules are representative illustrations, orientations and attachment locations of the different elements can be varied. The adjustments of the affinities of the different binding components can influence the separation between targeting vs non-targeting cells.

[0210] Figures 1C-1E show the cell targeting approach using the bridging receptor concept. The first step of this concept is to split the active molecule into inactive components. Using the tetravalent bispecific WNT mimetic as an example, among many different ways of splitting

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this molecule, we explored two ways for illustration purpose. The first method is to split the tetravalent WNT mimetic into one molecule having two FZD binding arms (2:0) and a second molecule having two LRP binding arms (0:2) (Fig. 1C-1E). These two inactive components can be assembled by a cell specific bridging receptor in several ways. If the bridging receptor exists as a monomer, as depicted in Fig. 1C, each separate molecule could be tethered to a "bridging element" binding to a different epitope on the bridging receptor, creating a final receptor complex on the cell surface consisting of two FZDs and two LRPs that would be competent to trigger signaling. The two different "bridging elements" could also be tethered to the two separate FZD and LRP binders as depicted in Fig. 1D for the assembly of the active receptor complex on the cell surface. If the bridging receptor exists as a multimeric complex of its own, a single "bridging element" could be tethered to the two different FZD and LRP molecules as depicted in Fig. 1E.

[0211] Figures 1F-1H show the method to split the tetravalent WNT mimetic is to split into two identical 1:1 molecules that consists of one FZD binding arm and one LRP binding arm. Similar to the scenario in Fig. 1C, if the bridging receptor exists as a monomer, two "bridging elements" binding to two different epitopes on the bridging receptor could be tethered to two separate 1:1 "activity element", which can then assemble via bridging receptor to create a competent productive signaling complex (Fig. 1F). The two different "bridging elements" could also be tethered together to the one 1:1 "activity element" (Fig. 1G). Unlike the scenarios shown in Fig. 1C-1F which employs two distinct "activity elements" for signaling, in the scenario in Fig. 1G, only a single molecule is needed to assemble a productive receptor signaling complex with cell targeting capability. If the bridging receptor exist as a multimeric complex of its own, a single "bridging element" could be tethered to a single "activity element" to create one molecule that achieve cell targeting (Fig. 1H). Additional variations of how the elements can be combined could be envisioned under the bridging receptor concept.

[0212] Tandem scFv multicomponent polypeptide are generated and assembled by linking or directly fusing a first scFv to either the C- or N-terminus of a second scFv molecule. In one format, the first scFv can bind to one or more FZD receptors and the second scFv can bind to one or more LRP receptors. In an alternative format, the first scFv can bind to one or more LRP receptors, and the second scFv can bind to one or more FZD receptors. One of the scFv molecules can also be linked or directly fused at its C-terminus to the N-terminus of an Fc molecule. In certain embodiments the WNT enhancer is linked or fused to the N-terminus of

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a first scFv, which in turn is linked or fused to the N-terminus of the second scFv, which is linked or fused to the N-terminus of the Fc molecule. In alternative embodiments, the WNT enhancer is linked or fused to the C-terminus of the Fc molecule, which in turn is linked or fused to the C-terminus of one scFv molecule, which is linked or fused at its N-terminus to the C-terminus of a second scFv molecule.

[0213] Fab-IgG molecules, where the FZD and LRP binders are Fabs can be assembled in various approaches, such as charge pairing, knobs-in-holes, crossover of heavy and light chains of the Fabs, etc. In charge pairing the heavy chain (VH-CH1) domain of an anti-LRP6 Fab or an anti-FZD Fab, through direct fusion or a linker of 5, 10, or 15-mer amino acids, are fused in tandem with the N-terminus of the heavy chain (VH-CH1-CH2-CH3) of an anti-FZD or anti-LRP binder. In certain embodiments, also known as Fabs-in tandem (FiT), both VH-CH1 domains of anti-LRP6 and anti-FZD contain three amino acid mutations (Q39D, Q105D, S183K in the anti-LRP6 Fab; Q39K, Q105K, S183E in anti-FZD Fab) each for proper paring with their own partner light chains, which also contain three complementary amino acid mutations (Q38K, A/S43K, S176E in anti-LRP6 light chain; Q38D, A/S43D, S176K in the anti-FZD light chain). The order of the anti-LRP6 and anti-FZD Fabs could be reversed, where the anti-FZD binder is a Fab and is fused to anti-LRP binder which is in IgG format. In certain embodiments, the WNT enhancer can be attached to the Fab to the N-terminus of either the Vh or Vl the Fab furthest from the IgG domain. In other embodiments, the WNT enhancer is attached to C-terminus of the IgG domain.

[0214] HC-LC cross over approach for Fab-on-IgG format. The light chain (VL-CL) domains of anti-LRP6 binder is, through direct fusion or a linker of 5, 10, or 15-mer amino acids, fused in tandem with the N-terminus of the heavy chain (VH-CH1-CH2-CH3) of an anti-FZD binder. The second construct was VH-CH1 of the anti-LRP6 binder and the third construct was VL-CL of the anti-FZD binder. Similar to the example above, the order of the anti-LRP6 and the anti-FZD binders could be reversed, where anti-FZD binder Fab is fused to the N-terminus of the anti-LRP binder which is in IgG format. Also as above the WNT enhancer can be attached to N-terminus of the VH or VL of the crossover Fab furthest from the IgG domain, or attached to the C-terminus of the IgG domain.

[0215] In certain embodiments, the molecules comprise one or more polypeptide(s) comprising or consisting of a sequence selected from any of SEQ ID NOs: 1-13, or a functional fragment or variant thereof. In particular embodiments, a variant comprises at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% identity to any one of SEQ

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ID NOs:1-13. In certain embodiments, a fragment comprises at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 98% of a contiguous sequence of any of SEQ ID NOs:1-13 or a variant of any of SEQ ID NOs:1-13. In certain embodiments, a functional fragment comprises an FGF21 sequence, an F12578 sequence, and/or a L sequence present in any of SEQ ID NOs:1-13, or a variant thereof.

VIII. Compositions

[0216] Pharmaceutical compositions comprising a multicomponent polypeptide molecule described herein and one or more pharmaceutically acceptable diluent, carrier, or excipient are also disclosed.

[0217] In further embodiments, pharmaceutical compositions comprising a polynucleotide comprising a nucleic acid sequence encoding a multicomponent polypeptide molecule described herein and one or more pharmaceutically acceptable diluent, carrier, or excipient are also disclosed. In particular embodiments, the pharmaceutical composition further comprises one or more polynucleotides comprising a nucleic acid sequence encoding a naturally occurring co-receptor ligand polypeptide. In certain embodiments, the polynucleotides are DNA or mRNA, e.g., a modified mRNA. In particular embodiments, the polynucleotides are modified mRNAs further comprising a 5' cap sequence and/or a 3' tailing sequence, e.g., a polyA tail. In other embodiments, the polynucleotides are expression cassettes comprising a promoter operatively linked to the coding sequences. In certain embodiments, the nucleic acid sequence encoding the multicomponent polypeptide molecule and the nucleic acid sequence encoding naturally occurring co-receptor ligand polypeptide are present in the same polynucleotide.

[0218] In further embodiments, pharmaceutical compositions comprising an expression vector, e.g., a viral vector, comprising a polynucleotide comprising a nucleic acid sequence encoding a multicomponent polypeptide molecule described herein and one or more pharmaceutically acceptable diluent, carrier, or excipient are also disclosed. In particular embodiments, the pharmaceutical composition further comprises an expression vector, e.g., a viral vector, comprising a polynucleotide comprising a nucleic acid sequence encoding a naturally occurring co-receptor ligand polypeptide. In certain embodiments, the nucleic acid sequence encoding the multicomponent polypeptide molecule and the nucleic acid sequence encoding the naturally occurring co-receptor ligand polypeptide are present in the same polynucleotide, e.g., expression cassette.

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[0219] The present invention further contemplates a pharmaceutical composition comprising a cell comprising an expression vector comprising a polynucleotide comprising a promoter operatively linked to a nucleic acid encoding a multicomponent polypeptide molecule and one or more pharmaceutically acceptable diluent, carrier, or excipient. In particular embodiments, the pharmaceutical composition further comprises a cell comprising an expression vector comprising a polynucleotide comprising a promoter operatively linked to a nucleic acid sequence encoding a polypeptide corresponding to the natural ligand of the receptors. In particular embodiments, the cell is a heterologous cell or an autologous cell obtained from the subject to be treated. In particular embodiments, the cell is a stem cell, e.g., an adipose-derived stem cell or a hematopoietic stem cell.

[0220] The subject molecules, alone or in combination, can be combined with pharmaceutically-acceptable carriers, diluents, excipients and reagents useful in preparing a formulation that is generally safe, non-toxic, and desirable, and includes excipients that are acceptable for mammalian, e.g., human or primate, use. Such excipients can be solid, liquid, semisolid, or, in the case of an aerosol composition, gaseous. Examples of such carriers, diluents and excipients include, but are not limited to, water, saline, Ringer's solutions, dextrose solution, and 5% human serum albumin. Supplementary active compounds can also be incorporated into the formulations. Solutions or suspensions used for the formulations can include a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial compounds such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating compounds such as ethylenediaminetetraacetic acid (EDTA); buffers such as acetates, citrates or phosphates; detergents such as Tween 20 to prevent aggregation; and compounds for the adjustment of tonicity such as sodium chloride or dextrose. The pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. In particular embodiments, the pharmaceutical compositions are sterile.

[0221] Pharmaceutical compositions may further include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, or phosphate buffered saline (PBS). In some cases, the composition is sterile and should be fluid such that it can be drawn into a syringe or delivered to a subject from a syringe. In certain embodiments, it is stable under the conditions of manufacture and storage and is preserved against the contaminating action of microorganisms such as bacteria

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and fungi. The carrier can be, e.g., a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the internal compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

[0222] Sterile solutions can be prepared by incorporating the multicomponent polypeptide molecule (or encoding polynucleotide or cell comprising the same) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, methods of preparation are vacuum drying and freeze-drying that yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

[0223] In one embodiment, the pharmaceutical compositions are prepared with carriers that will protect the antibody or antigen-binding fragment thereof against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially. Liposomal suspensions can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art.

[0224] It may be advantageous to formulate the pharmaceutical compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active antibody or antigen-binding fragment

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thereof calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms are dictated by and directly dependent on the unique characteristics of the antibody or antigen-binding fragment thereof and the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active antibody or antigen-binding fragment thereof for the treatment of individuals.

[0225] The pharmaceutical compositions can be included in a container, pack, or dispenser, e.g. syringe, e.g. a prefilled syringe, together with instructions for administration.

[0226] The pharmaceutical compositions of the invention encompass any pharmaceutically acceptable salts, esters, or salts of such esters, or any other compound which, upon administration to an animal comprising a human, is capable of providing (directly or indirectly) the biologically active antibody or antigen-binding fragment thereof.

[0227] The present invention includes pharmaceutically acceptable salts of a WNT multicomponent polypeptide molecule described herein. The term "pharmaceutically acceptable salt" refers to physiologically and pharmaceutically acceptable salts of the compounds of the invention: i.e., salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto. A variety of pharmaceutically acceptable salts are known in the art and described, e.g., in "Remington's Pharmaceutical Sciences", 17th edition, Alfonso R. Gennaro (Ed.), Mark Publishing Company, Easton, PA, USA, 1985 (and more recent editions thereof), in the "Encyclopaedia of Pharmaceutical Technology", 3rd edition, James Swarbrick (Ed.), Informa Healthcare USA (Inc.), NY, USA, 2007, and in J. Pharm. Sci. 66: 2 (1977). Also, for a review on suitable salts, see "Handbook of Pharmaceutical Salts: Properties, Selection, and Use" by Stahl and Wermuth (Wiley-VCH, 2002).

[0228] Pharmaceutically acceptable base addition salts are formed with metals or amines, such as alkali and alkaline earth metals or organic amines. Metals used as cations comprise sodium, potassium, magnesium, calcium, and the like. Amines comprise N-N'-dibenzylethylenediamine, chloroprocaine, choline, diethanolamine, dicyclohexylamine, ethylenediamine, N-methylglucamine, and procaine (see, for example, Berge et al., "Pharmaceutical Salts," J. Pharma Sci., 1977, 66, 119). The base addition salts of said acidic compounds are prepared by contacting the free acid form with a sufficient amount of the desired base to produce the salt in the conventional manner. The free acid form may be

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regenerated by contacting the salt form with an acid and isolating the free acid in the conventional manner. The free acid forms differ from their respective salt forms somewhat in certain physical properties such as solubility in polar solvents, but otherwise the salts are equivalent to their respective free acid for purposes of the present invention.

[0229] In some embodiments, the pharmaceutical composition provided herein comprise a therapeutically effective amount of a WNT multicomponent polypeptide molecule or pharmaceutically acceptable salt thereof in admixture with a pharmaceutically acceptable carrier, diluent and/or excipient, for example saline, phosphate buffered saline, phosphate and amino acids, polymers, polyols, sugar, buffers, preservatives and other proteins. Exemplary amino acids, polymers and sugars and the like are octylphenoxy polyethoxy ethanol compounds, polyethylene glycol monostearate compounds, polyoxyethylene sorbitan fatty acid esters, sucrose, fructose, dextrose, maltose, glucose, mannitol, dextran, sorbitol, inositol, galactitol, xylitol, lactose, trehalose, bovine or human serum albumin, citrate, acetate, Ringer's and Hank's solutions, cysteine, arginine, carnitine, alanine, glycine, lysine, valine, leucine, polyvinylpyrrolidone, polyethylene and glycol. Preferably, this formulation is stable for at least six months at 4° C.

[0230] In some embodiments, the pharmaceutical composition provided herein comprises a buffer, such as phosphate buffered saline (PBS) or sodium phosphate/sodium sulfate, tris buffer, glycine buffer, sterile water and other buffers known to the ordinarily skilled artisan such as those described by Good et al. (1966) Biochemistry 5:467. The pH of the buffer may be in the range of 6.5 to 7.75, preferably 7 to 7.5, and most preferably 7.2 to 7.4.

IX. Methods of Use

[0231] For illustrative purposes only, the cell targeting multicomponent WNT molecule can be used as to treat various diseases or disorders where tissue regeneration is necessary. Such diseases include, but are not limited to. increase bone growth or regeneration, bone grafting, healing of bone fractures, treatment of osteoporosis and osteoporotic fractures, vertebral compression fractures, spinal fusion, osseointegration of orthopedic devices, tendon-bone integration, tooth growth and regeneration, dental implantation, periodontal diseases, maxillofacial reconstruction, and osteonecrosis of the jaw. Also contemplated are: treatment of alopecia, enhancing regeneration of sensory organs, e g. treatment of hearing loss, including internal and external auditory hair cells, treatment of vestibular hypofunction, treatment of macular degeneration, treatment of vitreoretinopathy, diabetic retinopathy, other diseases of

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retinal degeneration, Fuchs' dystrophy, other cornea disease, etc.; treatment of stroke, traumatic brain injury, Alzheimer's disease, multiple sclerosis and other conditions affecting the blood brain barrier; treatment of spinal cord injuries, other spinal cord diseases. The compositions of this invention may also be used in treatment of oral mucositis, treatment of short bowel syndrome, inflammatory bowel diseases (IBD), other gastrointestinal disorders, treatment of metabolic syndrome, dyslipidemia, treatment of diabetes, treatment of pancreatitis, conditions where exocrine or endocrine pancreas tissues are damaged; conditions where enhanced epidermal regeneration is desired, e.g., epidermal wound healing, treatment of diabetic foot ulcers, syndromes involving tooth, nail, or dermal hypoplasia, etc., conditions where angiogenesis is beneficial; treatment of myocardial infarction, coronary artery disease, heart failure; enhanced growth of hematopoietic cells, e g. enhancement of hematopoietic stem cell transplants from bone marrow, mobilized peripheral blood, treatment of immunodeficiencies, graft versus host diseases, etc.; treatment of acute kidney injuries, chronic kidney diseases; treatment of lung diseases, chronic obstructive pulmonary diseases (COPD), idiopathic pulmonary fibrosis (IPF) enhanced regeneration of lung tissues. The compositions of the present invention may also be used in enhanced regeneration of liver cells, e.g. liver regeneration, treatment of cirrhosis, enhancement of liver transplantations, treatment of acute liver failure, treatment of chronic liver diseases with hepatitis C or B virus infection or post-antiviral drug therapies, alcoholic liver diseases, alcoholic hepatitis, non-alcoholic liver diseases with steatosis or steatohepatitis, and the like. The compositions of this invention may treat diseases and disorders including, without limitation, conditions in which regenerative cell growth is desired.

[0232] In particular embodiments, a pharmaceutical composition is administered parenterally, e.g., intravenously, orally, rectally, or by injection. In some embodiments, it is administered locally, e.g., topically or intramuscularly. In some embodiments, a composition is administered to target tissues, e.g., to bone, joints, ear tissue, eye tissue, gastrointestinal tract, skin, a wound site or spinal cord. Methods of the invention may be practiced in vivo or ex vivo. In some embodiments, the contacting of a target cell or tissue with a multicomponent polypeptide molecule is performed ex vivo, with subsequent implantation of the cells or tissues, e.g., activated stem or progenitor cells, into the subject. The skilled artisan can determine an appropriate site of and route of administration based on the disease or disorder being treated.

[0233] The dose and dosage regimen may depend upon a variety of factors readily determined by a physician, such as the nature of the disease or disorder, the characteristics of

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the subject, and the subject's history. In particular embodiments, the amount of a multicomponent polypeptide molecule administered or provided to the subject is in the range of about 0.01 mg/kg to about 50 mg/kg, 0.1 mg/kg to about 500 mg/kg, or about 0.1 mg/kg to about 50 mg/kg of the subject's body weight.

[0234] All of the above U.S. patents, U.S. patent application publications, U.S. patent applications, foreign patents, foreign patent applications and non-patent publications referred to in this specification and/or listed in the Application Data Sheet, are incorporated herein by reference, in their entirety.

[0235] From the foregoing it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

EXAMPLES

[0236] The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the present invention, and are not intended to limit the scope of what the inventors regard as their invention nor are they intended to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees Centigrade, and pressure is at or near atmospheric.

[0237] General methods in molecular biology, cell biology and biochemistry can be found in such standard textbooks as "Molecular Cloning: A Laboratory Manual, 3rd Ed." (Sambrook et al., Harbor Laboratory Press 2001); "Short Protocols in Molecular Biology, 4th Ed." (Ausubel et al. eds., John Wiley & Sons 1999); "Protein Methods" (Bollag et al., John Wiley & Sons 1996); "Nonviral Vectors for Gene Therapy" (Wagner et al. eds., Academic Press 1999); "Viral Vectors" (Kaplift & Loewy eds., Academic Press 1995); "Immunology Methods Manual" (I. Lefkovits ed., Academic Press 1997); and "Cell and Tissue Culture: Laboratory Procedures in Biotechnology" (Doyle & Griffiths, John Wiley & Sons 1998), the disclosures of which are incorporated herein by reference. Reagents, cloning vectors, and kits for genetic manipulation referred to in this disclosure are available from commercial vendors such as BioRad, Stratagene, Invitrogen, Sigma-Aldrich, and ClonTech.

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[0238] Recombinant molecules were generated that combine agonists for the WNT receptors, FZD and/or LRP co-receptors, together with bridging receptor, to create WNT signaling "cell targeting multicomponent polypeptide"

[0239] Materials and methods employed in the following Examples include the following.

[0240] **Protein production:** All recombinant proteins were produced in Expi293F cells (Thermo Fisher Scientific) by transient transfection unless otherwise specified. All IgG-based and Fc-containing constructs were first purified with Protein-A resin and eluted with 0.1 M glycine pH 3.5. All proteins were then polished by a size exclusion column in HBS buffer (10 mM HEPES pH 7.2, 150 mM NaCl). Proteins were supplemented with glycerol to 10% for long term storage at -80°C.

[0241] **SuperTop Flash (STF) assay:** WNT signaling activity is measured using cell lines containing a luciferase gene controlled by a WNT-responsive promoter (Super Top Flash reporter assay, STF) as reported (Janda et al., 2017; Nature 545:234). In brief, cells are seeded at a density of 10,000 per well in 96-well plates 24 hr prior to treatment, then treated with RSPO or mimetic proteins overnight either alone or together with 30% WNT3a-conditioned media. WNT3a conditioned media are prepared from ATCC-CRL-2647 WNT3a secreting L cells following vendor recommended conditions. Cells were lysed with Luciferase Cell Culture Lysis Reagent (Promega) and activity are measured with Luciferase Assay System (Promega) using vendor suggested procedures. Data are plotted as average -/+ standard deviation of triplicates and fitted by non-linear regression using Prism (GraphPad Software).

Affinity Measurement and Step-Binding Assay

[0242] Binding kinetics of F-FGF21 series (F-FGF21FL, F-FGF21ΔN, F-FGF21ΔC, and F-FGF21ΔNΔC) to human FZD7 CRD and βKlotho (Fisher Scientific) or L-39F7 series (L-39F7, αGFP-39F7, and L-αGFP) to human LRP6E3E4 and βKlotho, respectively, were determined by bio-layer interferometry (BLI) using an Octet Red 96 (PALL ForteBio) instrument at 30°C, 1000 rpm with AHC biosensors (Sartorius). Various F-FGF21 or L-39F7 proteins were diluted to 50 nM in the running buffer and captured to the AHC biosensor, followed by dipping into wells containing the FZD7 CRD, LRP6E3E4 and βKlotho at different concentrations in a running buffer or into a well with only the running buffer as reference channel. The dissociation of the interaction was followed with the running buffer. The monovalent KD for each binder was calculated by Octet System software, based on fitting to a 1:1 binding model.

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[0243] Step-binding assay was performed with the BLI using the Octet Red 96 instrument at 30°C, 1000 rpm with AHC biosensors. Various F-FGF21 or L-39F7 proteins were diluted to 50 nM in the running buffer and captured to the AHC biosensor, followed by dipping into wells containing the 100 nM FZD7 CRD or 100 nM LRP6E3E4, respectively. The sensor chips next moved into 150 nM βKlothos containing 100 nM FZD7 CRD or containing 100 nM LRP6E3E4 to check the additional bindings of β-Klotho. Sensorgram slopes were compared for βKlotho bindings.

Primary human cells

[0244] Human hepatocytes were purchased from BioIVT (10-donor pooled cryoplateable X008001-P) and cultured in LONZA hepatocyte maintenance medium (CC-3198). In short, plastic culture plates were coated with 20% Matrigel Matrix (CB40230C) and cells were plated in plating medium (BioIVT Z990003). After four hours the medium was changed to maintenance medium and refreshed every day for three days prior to the 24h experiment.

[0245] Human small intestinal organoids were a gift from the Calvin Kuo Lab at Stanford. Organoids were maintained and expanded as previously described32. In short, adapted expansion medium contained Advanced DMEM, 10 mM HEPES, 1x GlutaMAX, 1X Penicillin-Streptomycin, 1x B27, 1x N2, 1.25 mM N-acetylcysteine, 10 mM Nicotinamide, 50 ng/mL recombinant human EGF, 50 ng/mL recombinant human Noggin, 20 nM R-Spondin 2, 0.1 nM L-F Wnt mimetic, 10 nM recombinant Gastrin, 500 nM A83-01 and 10 μM SB202190.

[0246] Treatment of molecules was done in the presence of 20 nM R-spondin 2 for 24h at a concentration of 10 nM. After 24 hours the cells were harvested and RNA collected for qPCR. Each experiment with both primary human hepatocytes and human small intestinal organoids was repeated three times.

Quantitative polymerase chain reaction analysis of gene expression

[0247] RNAs from HEK293, Huh7 cells, or primary human cells were extracted using the Qiagen RNeasy Micro Kit (Qiagen). cDNA was produced using the SuperScript IV VILO cDNA Synthesis Kit (Thermo Fisher). βKlotho (KLB) RNA was quantified using Maxima SYBR Green qPCR master mix on a Bio-Rad CFX96 real time PCR machine. Cycle threshold (Ct) values were normalized to the expression of constitutive ACTINB RNA using the following oligo's:

[0248] ACTB_F1: CTGGAACGGTGAAGGTGACA (SEQ ID NO: 31);

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[0249] ACTB_R1: AAGGGACTTCCTGTAACAATGCA (SEQ ID NO: 32);

[0250] KLB_F1: ATCTAGTGGCTTGGCATGGG (SEQ ID NO: 33); KLB_R1:CCAAACTTTCGAGTGAGCCTTG (SEQ ID NO: 34); KLB_F2:CACTGAATCTGTTCTTAAGCCCG (SEQ ID NO: 35);

[0251] KLB_R2: GGCGTTCCACACGTACAGA (SEQ ID NO: 36);

[0252] KLB_F3: GGAGGTGCTGAAAGCATACCT (SEQ ID NO: 37); and

[0253] KLB_R3: TCTCTTCAGCCAGTTTGAATGC (SEQ ID NO: 38).

Example 1

Multicomponent WNT Molecule for Concept of the 2:0/0:2 Split Design

[0254] To evaluate the structures shown in Fig. 1C, the βKlotho and endocrine fibroblast growth factor 21 (FGF21) ligand system were tested as the bridging receptors. FGF21 is an endocrine hormone produced by the liver that regulates metabolic homeostasis (BonDurant and Potthoff, 2018). FGF21 signals through FGFR1c, FGFR2c, and FGFR3c in the presence of co-receptor βKlotho (Zhang and Li, 2015). The binding of FGF21 to the receptor complex is primarily driven by its affinity toward βKlotho via its C-terminal domain (Lee et al., 2018, Shi et al., 2018), while its N-terminal domain is imporiant for FGFR interaction and signaling (Yie et al., 2012, Micanovic et al., 2009). βKlotho binding antibodies have also been identified that could induce βKlotho/FGFR signaling, and one particular agonistic βKlotho antibody binds to a different epitope on βKlotho from FGF21 and does not compete with FGF21 binding (Min et al., 2018). Therefore, the following bridging receptor (βKlotho) binding elements were selected to test the cell targeting concept:

[0255] FGF21FL (full length FGF21) that can bind to βKlotho and is competent to induce FGFR signaling;

[0256] FGF21ΔC (FGF21 without the C-terminal βKlotho interaction domain) that does not bind βKlotho and is not capable of inducing FGFR signaling;

[0257] FGF21ΔN (FGF21 without the N-terminal FGFR interaction domain) that binds βKlotho but does not signal;

[0258] FGF21ΔNΔC that cannot bind βKlotho and cannot signal; and

[0259] 39F7 IgG that binds βKlotho and can induce FGFR signaling.

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[0260] The FZD and LRP binding domains selected were F12578 (binds FZD1,2,5,7,8) and L (binds LRP6), previously named F1 and L2, respectively (Chen et al., 2020).

[0261] The graphic representations of the binders and the various combinations are shown in Fig. 2A-2C.

[0262] To test the structures described in Fig. 1C, the FZD binder (F12578) was combined with one of two versions of the bridging receptor (βKlotho) binder, F12578-FGF21FL or F12578-FGF21ΔN (Fig. 2B), and the LRP binder (L) was combined with the other bridging receptor (βKlotho) binder, 39F7, as L-39F7 (Fig. 2B). As shown in Fig. 2D and 2E, the combination of F12578-FGF21FL or FGF12578-FGF21ΔN with L-39F7 resulted in WNT/β-catenin signaling in a liver cell line, Huh7 cells, which express the bridging receptor βKlotho, but not in 293 cells, where βKlotho is not expressed. This signaling depended on the presence of both the FZD and LRP binding arms and the ability to bind the bridging receptor, as the removal of LRP binding arm L from L-39F7 or inactivation of βKlotho binding (use of FGF21ΔNΔC) resulted in no activity in either cell type (Fig. 2D and 2E). This provided experimental evidence supporting the concept presented in Fig. 1C.

[0263] Table 1 describes the different components/formats tested.

Table 1: Formats and sequences of multicomponent WNT polypeptide with 2:0 / 0:2 split design:

VH = Italic;
Human CH1CH2CH3 with LALAPG mutations (LALAPG mutations are only marked in the first HC_IgG1_LALAPG) = Italic bold;
VL = Italic underlined;
Constant of lambda LC = Italic underlined dark gray; and
Constant of Kappa LC = Italic underlined bold;
Linker = light grey;
hFGF21ΔN = bold;
hFGF21FL= bold underline;
hFGF21ΔNΔC = light gray bold italic;
hFGF21ΔC = light gray italic; and
LALAPG mutations = bold dark gray underlined.

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Structure	SEQ ID NO.	Sequences
L-39F7 LC	1	*EVQLVESGGGLVQPGGSLRLSCAASGFTFSTYTISWVRQAPGKGLEWVAEISP YSGNTYYADSVKGRFTISADTSK NTAYLQMNSLRAEDTAVYYCALRARPPIRLHPRGSYADYWGQGTLVTVSS[illegible] [illegible]* ***DIQMTQS PSSLSASVGDRVTITCRASQDVSTAVAWYQQKPGKAPKLLIYSASFLYSGVPSRF SGSGSGTDFTLTISSLQPEDFA TYYCQQSYTTPPTFGQGTKVEIK*** *RTVAAPSVFIFPPSDEQL KSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYS LSSTLTLSKADYEKHKVYACEVTHQ GLSSPVTKSFNRGEC*
39F7 HC	2	*QVQLVESGGGVVQPGRSLRLSCAASGFTFSNYGIHWVRQAPGKGLEWVALIW YDGSKKYYADSVKGRFTISR DNSKNTLYLQMNSLRAEDTAVYYCARDRAAAGLHYYYGMDVWGQGTTVTVSS* ***ASTKGPSVFPLAPSSKSTSG GTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVT VPSSSLGTQTYICNVNHKPSNTKV DKKVEPKSCDKTHTCPPCPAPE[illegible]GGPSVFLFPPKPKDTLMISRTPEVTC VVVDVSHEDPEVKFNWYVDGVEV HNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIE KTISKAKGQPREPQVYTLPPSREEM TKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYS KLTVDKSRWQQGNVFSCSVMHEA LHNHYTQKSLSLSPGK***
F12758-FGF21FL LC	3	*DIELTQPPSVSVAPGQTARISCSGDNIGSFYVHWYQQKPGQAPVLVIYDKSNRP SGIPERFSGSNSGNTATLTIS GTQAEDEADYYCQSYANTLSLVFGGGTKLTVLGQPKAAPSVTLFPPSSEELQA NKATLVCLISDFYPGAVTVAWKA DSSPVKAGVETTTPSKQSNNKYAASSYLSLTPEQWESHRSYSCQVTHEG STVEKTVAPTECS[illegible]* ***HPIPDSSPL LQFGGQVRQRYLYTDDAQQTEAHLEIREDGTVGGAADQSPESLLQL KALKPGVIQILGVKTSRFLCQRPDGALYGSLHFDPEACSFRERLLED GYNVYQSEAHGLPLHLPGNKSPHRDPAPRGPARFLPLPGLPPALPEP PGILAPQPPDVGSSDPLSMVGPSQGRSPSYES***
F12758 HC	4	*EVQLVESGGGLVQPGGSLRLSCAASGFTFSHYTLSWVRQAPGKGLEWVSTISG DGSTTYYADSVKGRFTISSDN SKNTLYLQMNSLRAEDTAVYYCARMPIKTVFANWGQGTLVTVSS****ASTKGPSVF PLAPSSKSTSGGTAALGCLVKD YFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYI CNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPK DTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY NSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREP QVYTLPPSREEMTKNQVSLTCLVGFYPSDIAVEWESNGQPENNYKTTPPV LDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG K***

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F12758-FGF21ΔN LC	5	*DIELTQPPSLSVAPGQTARISCSGDNIGSFYVHWYQQKPGQAPVLVIYDKSNRP SGIPERFSGSNSGNTATLTISGT QAEDEADYYCQSYANTLSLVFGGGTKLTVL*GQPKAAPSVTLFPPSSEELQAN KATLVCLISDFYPGAVTVAWKADS SPVKAGVETTTPSKQSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEGST VEKTVAPTECS [illegible] **QRYLYTDDAQ QTEAHLEIREDGTVGGAADQSPESLLQLKALKPGVIQILGVKTSRFL CQRPDGALYGSLHFDPEACSFRERLLEDGYNVYQSEAHGLPLHLPGN KSPHRDPAPRGPARFLPLPGLPPALPEPPGILAPQPPDVGSSDPLSMV GGSQGRSPSYES**
L-αGFP LC	6	*EVQLVESGGGLVQPGGSLRLSCAASGFTFSTYTISWVRQAPGKGLEWVAEISP YSGNTYYADSVKGRFTISADT SKNTAYLQMNSLRAEDTAVYYCALRARPPIRLHPRGSYMDYWGQGTLVTVSS [illegible] *DIQMT QSPSSLSASVGDRVTITCRASQDISLYLAWYQQKPGKAPKLLIYSASFLYSGVPS RFSGSGSGTDFTLTISSLQPE DFATYYCQQSYTTPPTFGQGTKVEIK* [illegible] *DIELTQPPSVSV APGQTARISCSGDNLGKKYV HWYQQKPGQAPVLVIYGDDERPSGIPERFSGSNSGNTATLTISGTQAEDEADYY CASYDSSTHLIFGGGTKLTVL* GQPKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVK AGVETTTPSKQSNNKYAASSYLS [illegible] TPEQWKSHRSYSCQVTHEGSTVEKTVAPTECS
αGFP HC	7	*QVQLVESGGGLVQPGGSLRLSCAASGFTFSRYGMHWVRQAPGKGLEWVSGIS SIGSNTYYADSVKGRFTISRD NSKNTLYLQMNSLRAEDTAVYYCARWYKTYIDYWGQGTLVTVSS**ASTKGPSV FPLAPSSKSTSGGTAALGCLVK DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTY ICNVNHKPSNTKVDKKVEPKSCDK THTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPE VKFNWYVDGVEVHNAKTKPREEQ YNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPRE PQVYTLPPSREEMTKNQVSLTCLVK GFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQ GNVFSCSVMHEALHNHYTQKSLSLSPGK***
F12758-FGF21ΔC LC	8	*DIELTQPPSLSVAPGQTARISCSGDNIGSFYVHWYQQKPGQAPVLVIYDKSNRP SGIPERFSGSNSGNTATLTISGT QAEDEADYYCQSYANTLSLVFGGGTKLTVL*GQPKAAPSVTLFPPSSEELQAN KATLVCLISDFYPGAVTVAWKADS SPVKAGVETTTPSKQSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEGST VEKTVAPTECS [illegible] HPIPDSSPLLQ FGGQVRQRYLYTDDAQQTEAHLEIREDGTVGGAADQSPESLLQLKALKP GVIQILGVKTSRFLCQRPDGALYGSLH FDPEACSFRERLLEDGYNVYQSEAHGLPLHLPGNKSPHRDPAPRGPARFL PLPGLPPALPEPPGILAPQPPDVG*
F12758-FGF21ΔNΔC LC	9	*DIELTQPPSLSVAPGQTARISCSGDNIGSFYVHWYQQKPGQAPVLVIYDKSNRP SGIPERFSGSNSGNTATLTISG TQAEDEADYYCQSYANTLSLVFGGGTKLTVL*GQPKAAPSVTLFPPSSEELQA NKATLVCLISDFYPGAVTVAWKA DSSPVKAGVETTTPSKQSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEG STVEKTVAPTECS [illegible]

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Example 2

Multicomponent WNT Molecule for Concept of the 1:1 Split Design

[0264] To test the concept depicted in Fig. 1G, the FZD binder (F12578), LRP binder (L), and the two non-competing bridging receptor (βKlotho) binders (FGF21FL and 39F7) were combined in the molecule, F12578-FGF21FL-39F7-L, depicted in Fig. 3A. As shown in Fig. 3B, F12578-FGF21FL-39F7-L activated WNT/β-catenin signaling in Huh7 cells that express the bridging receptor βKlotho but not in 293 cells where βKlotho is not expressed (Fig. 3B). No activity was observed in 293 cells. This provided validation for the concept presented in Fig. 1G. Table 2 describes the different components/formats tested.

[0265] Table 2: Formats and sequences of multicomponent WNT polypeptide with 1:0 / 01: split design:

VH= Italic;

Human CH1CH2CH3 with LALAPG mutations (LALAPG mutations are only marked in the first HC_IgG1_LALAPG)=Italic bold;

VL = Italic underlined;

Constant of lambda LC = dark gray;

Constant of Kappa LC = Italic underlined bold;

G4S linker = light grey;

hFGF21ΔN =bold;

hFGF21FL=bold underline;

hFGF21ΔNΔC = light gray bold italic;

hFGF21ΔC = light gray italic;

Amino acids mutations in SEQ 11 and SEQ 12 = bold dark gray underlined

Structure	SEQ ID NO.	Sequences
39F7 LC	10	*EIVLTQSPGTLSLSPGERATLSCRASQSVSSTYLAWYQQKPGQAPRLLIYGASSRATGIP DRFSGSGSGTDFTLTISRLEPEDFAVYYCQQSGSSPLTFGGGTEVEIKRTVAAPSVF IFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKD STYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC*
F12578_ Hole _Flag HC	11	*EVQLVESGGGLVQPGGSLRLSCAASGFTFSHYTLSWVRQAPGKGLEWVSTISGDGSTT YYADSVKGRFTISSDNSKNTLYLQMNSLRAEDTAVYYCARNFIKYVFANWGQGTLVTVS SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFP AVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCP PCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDG VEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKT*

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		ISKAKGQPREPQVYTLPPSREEMTKNQVSLSCAVKGFYPSDIAVEWESNGQPEN NYKTTPPVLDSDGSFFLVSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSL SPGK[illegible] T366S L368A Y407V are mutations for the "Hole" of Knob and Hole
39F7_Knob _HC	12	QVQLVESGGGVVQPGRSLRLSCAASGFTFSNYGIHWVRQAPGKGLEWVAVIWYDGSI KYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCARDRAAGLIDYYGMDVW GQGTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGAL TSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKS CDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEV KFNWYVDGV EVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTI SAKGQPREPQVYTLPPSREEMTKNQVSLWCLVKGFYPSDIAVEWESNGQPENNY KTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVM HEALHNHYTQKSLSLSPGK T366W is the "Knob" of Knob and Hole
α GFP-39F7 LC	13	QVQLVESGGGLVQPGGSLRLSCAASGFTFSRYGMHWVRQAPGKGLEWVSGISSIGSN TYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCARVYKTYIDVWGQGTLVTV SS[illegible]DIELTQPPSVSVAPGQTARISCSGDNLGKKYVYWYQQKP GQAPVLVIYGDDERPSGIPERFSGSNSGNTATLTISGTQAEDEADYYCASYDSSHILVF GGGTKLTVL[illegible]EIVLTQSPGTLSLSPGERATLSCRASQSVSSTYLAWYQQKPGQA PRLLIYGASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQSGSSPLTFGGGTE VEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGN SQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGE C
F12758-FGF21FL LC	3	DIELTQPPSVSVAPGQTARISCSGDNIGSFYVHWYQQKPGQAPVLVIYDKSNRPSGIPE RFSGSNSGNTATLTISGTQAEDEADYYCQSYANTLSLVFGGGTKLTVLGQPKAAPSVT LFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNNK YAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTECS[illegible]HPIPDSSPL LQFGGQVRQRYLYTDDAQQTEAHLEIREDGTVGGAADQSPESLLQLKALK PGVIQILGVKTSRFLCQRPDGALYGSLHFDPEACSFRERLLEDGYNVYQSEA HGLPLHLPGNKSPHRDPAPRGPARFLPLPGLPPALPEPPGILAPQPPDVGSSD PLSMVGGSQGRSPSYES

[0266] The various embodiments described above can be combined to provide further embodiments. All of the U.S. patents, U.S. patent application publications, U.S. patent applications, foreign patents, foreign patent applications, and non-patent publications referred to in this specification and/or listed in the Application Data Sheet are incorporated herein by reference, in their entirety. Aspects of the embodiments can be modified, if necessary to employ concepts of the various patents, applications, and publications to provide yet further embodiments. These and other changes can be made to the embodiments considering the above-detailed description. In general, in the following claims, the terms used should not be construed to limit the claims to the specific embodiments disclosed in the specification and the claims but should be construed to include all possible embodiments along with the full scope of equivalents to which such claims are entitled. Accordingly, the claims are not limited by the disclosure.

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What is Claimed is:

1. A cell targeting multicomponent polypeptide molecule comprising:

a) at least one first antigen binding domain that binds to a first signaling receptor component,

b) at least one second antigen binding domain that binds to a second signaling receptor component, and

c) at least one different antigen binding domain that binds to a bridging molecule,

wherein the first signaling receptor and second signaling receptor can be different or identical.

2. The cell targeting multicomponent polypeptide molecule of claim 1, wherein the binding domain that binds to the bridging molecule comprises at least two binding domains that:

a) bind to different epitopes on the bridging molecule; or

b) bind to the same epitope on the bridging molecule.

3. The cell targeting multicomponent polypeptide molecule of claim 1 or claim 2, wherein

a) the first antigen binding domain that binds to a first signaling receptor component is tethered to at least one binding domain that binds to the bridging molecule, and

b) the second antigen binding domain that binds to a second signaling receptor component is tethered to at least one binding domain that binds to the bridging molecule.

4. The multicomponent polypeptide molecule of claim 3, wherein the antigen binding domain is joined directly or by a linker to the binding domain that binds to the bridging molecule.

5. The multicomponent polypeptide molecule of claim 3, wherein the bridging molecule is monomeric or multimeric.

6. The multicomponent polypeptide molecule of claim 3, wherein the binding domains are each independently selected from the group consisting of: an scFv, a VHH/sdAb, a Fab, and a Fab'2.

7. The multicomponent polypeptide molecule of claim 3, wherein the binding domains are joined through a linker by a peptide linker comprising from 1-100 amino acids.

8. The multicomponent polypeptide molecule of claim 3, wherein the binding domains are attached to the N-terminus of an antibody Fc domain.

9. A nucleic acid encoding the multicomponent polypeptide molecule of any one of claims 1-8 or a polypeptide component thereof.

10. A vector comprising the nucleic acid of claim 9, wherein the vector is an expression vector comprising a promoter sequence operatively linked to the nucleic acid sequence; optionally wherein the vector is a virus comprising a promoter operatively linked to the nucleic acid.

11. A host cell comprising the vector of claim 10.

12. A process for producing the multicomponent polypeptide molecule of any one of claims 1-8 or a polypeptide component thereof, comprising culturing the host cell of claim 11 under conditions wherein the multicomponent polypeptide molecule or polypeptide component thereof is expressed by the expression vector.

13. A multicomponent WNT molecule comprising:

a) at least one FZD binding domain;

b) at least one LRP binding domain; and

c) at least one bridging molecule,

wherein the multicomponent WNT multicomponent polypeptide molecule modulates WNT signaling.

14. The multicomponent WNT molecule of claim 13, comprising:

a) one or two FZD binding domains tethered to the bridging molecule by a binding domain specific for a first epitope on the bridging molecule; and

b) one or two LRP binding domains tethered to the bridging molecule by a binding domain specific for a second epitope on the bridging molecule,

wherein the multicomponent WNT molecule is capable of activating WNT signaling.

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15. The multicomponent WNT molecule of claim 13 or claim 14, wherein:

a) the binding domain specific for the first epitope of the bridging molecule and the FZD binding domain(s) are joined directly or joined by a linker;

b) the binding domain specific for the second epitope of the bridging molecule and the LRP binding domain(s) are joined directly, or joined by a linker; and

c) the binding domain specific for the first epitope and the binding domain specific for the second epitope of the bridging molecule are identical or different.

16. The multicomponent WNT molecule of claim 15, wherein the linker is a peptide or non-peptide linker.

17. The multicomponent WNT molecule of claim 13, wherein the FZD binding domain(s) and the LRP binding domain(s) are each independently selected from the group consisting of: a scFv, a VHH/sdAb, a Fab, and a Fab'2.

18. The multicomponent WNT molecule of claim 13, wherein the FZD binding domain(s) and the LRP binding domains(s) are joined by a peptide linker comprising from 1-100 amino acids.

19. The multicomponent WNT molecule of claim 13, wherein the FZD binding domain(s) and the LRP binding domain(s) are attached to the N-terminus of an antibody Fc domain.

20. A nucleic acid encoding the multicomponent WNT molecule of any one of claims 13-19 or a polypeptide component thereof.

21. A vector comprising the nucleic acid of claim 20, wherein the vector is an expression vector comprising a promoter sequence operatively linked to the nucleic acid sequence; optionally wherein the vector is a virus comprising a promoter operatively linked to the nucleic acid.

22. A host cell comprising the vector of claim 21.

23. A process for producing the multicomponent WNT molecule or polypeptide component thereof of claim 13, comprising culturing the host cell of claim 22 under conditions

MS&D Exhibit 1008, Page 3927 of 4223

wherein the multicomponent WNT protein or polypeptide component thereof is expressed by the expression vector.

24. A pharmaceutical composition to modulate the WNT/β-catenin signaling pathway comprising:

a) an effective amount of the multicomponent WNT molecule of any one of claims 13-19; and

b) a pharmaceutically acceptable diluent, adjuvant, or carrier.

25. A method of treating or preventing a disease or disorder in a subject in need thereof, the method comprising providing to the subject an effective amount of the multicomponent WNT molecule of any one of claims 13-19 or the pharmaceutical composition of claim 24.

26. A multicomponent WNT molecule comprising:

a) at least one FZD binding domain linked to one LRP binding domain which is tethered to the bridging molecule by a binding domain specific for a first epitope on the bridging molecule; and

b) at least one FZD binding domain linked to one LRP binding domain which is tethered to the bridging molecule by a binding domain specific for a second epitope on the bridging molecule;

wherein the tethering of the FZD and LRP binding domains to the bridging receptor forms a WNT mimetic capable of activating WNT signaling.

27. The multicomponent WNT molecule of claim 26, wherein:

a) the binding domain specific for a first epitope of the bridging molecule and the FZD/LRP binding domains are joined directly or by a linker;

b) the binding domain specific for a second epitope of the bridging molecule and the FZD/LRP binding domains are joined directly, or by a linker;

c) the binding domain of the first epitope and second epitope of the bridging molecule are identical or different; and/or

d) the binding domain of the first epitope and the second epitope of the bridging molecule could be linked together to FZD/LRP.

28. The multicomponent WNT molecule of claim 26 or claim 27, wherein the bridging molecule is monomeric or multimeric.

29. The multicomponent WNT molecule of any one of claims 26-28, wherein at least one of the FZD or LRP binding domains is selected from the group consisting of: a scFv, a VHH/sdAb, a Fab, and a Fab'2.

30. The multicomponent WNT molecule of any one of claims 26-29, wherein the binding domains are joined through a linker by a peptide link comprising from 1-100 amino acids.

31. The multicomponent WNT molecule of any one of claims 26-30, wherein the binding domains are attached to the N-terminus of an antibody Fc domain.

32. A nucleic acid encoding the multicomponent WNT molecule of any one of claims 26-31 or a polypeptide component thereof.

33. A vector comprising the nucleic acid of claim 32, wherein the vector is an expression vector comprising a promoter sequence operatively linked to the nucleic acid sequence; optionally wherein the vector is a virus comprising a promoter operatively linked to the nucleic acid.

34. A host cell comprising the vector of claim 33.

35. A process for producing the multicomponent WNT molecule or component thereof of any one of claims 26-31, comprising culturing the host cell of claim 36 under conditions wherein multicomponent WNT or polypeptide component thereof is expressed by the expression vector.

36. A pharmaceutical composition to modulate the WNT/β-catenin signaling pathway comprising:

a) an effective amount of the multicomponent WNT of any one of claims 26-31; and

b) a pharmaceutically acceptable diluent, adjuvant, or carrier.

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37. A method of treating or preventing a disease or disorder in a subject in need thereof, the method comprising providing to the subject an effective amount of the multicomponent WNT molecule of any one of claims 26-31 or the pharmaceutical composition of claim 36.

MS&D Exhibit 1008, Page 3930 of 4223

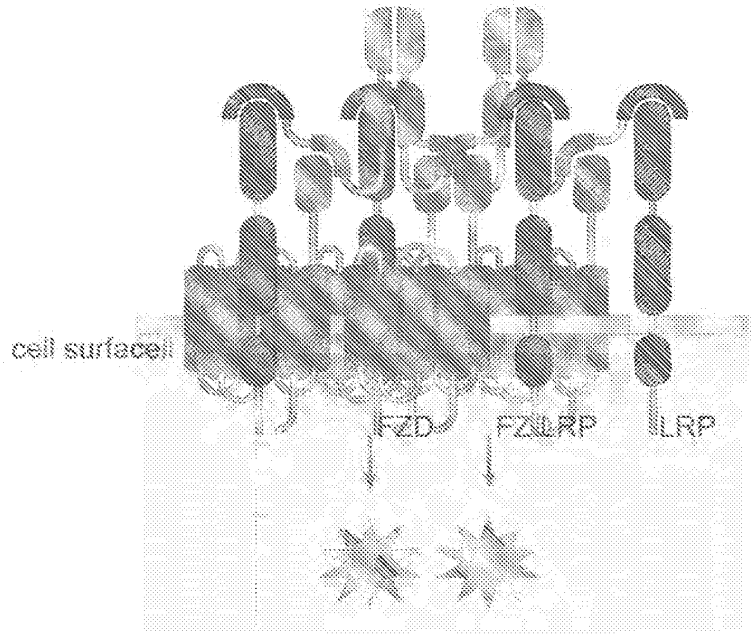


Fig. 1A

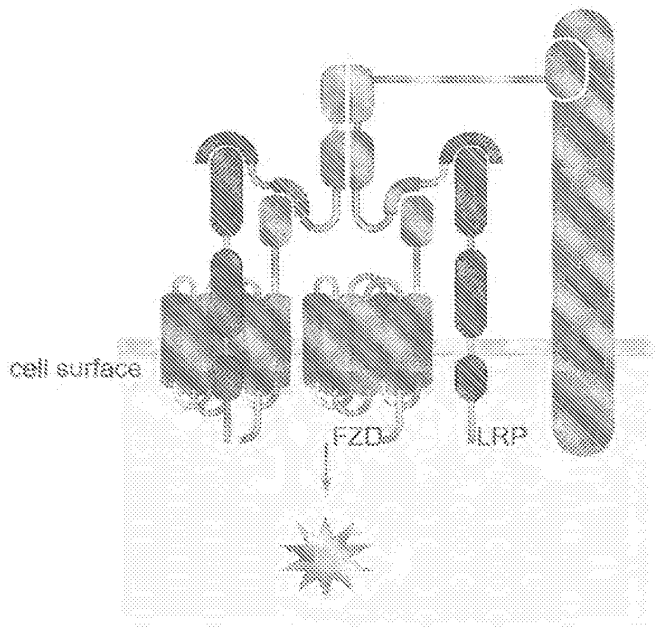


Fig. 1B

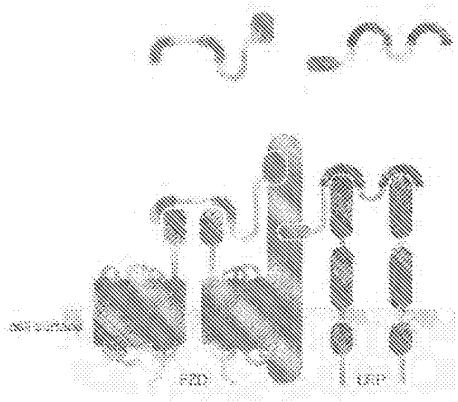


Fig. 1C

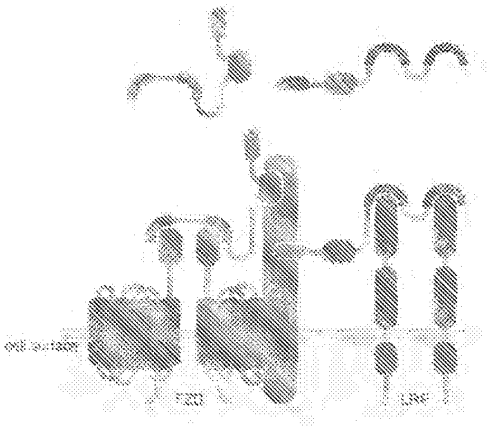


Fig. 1D

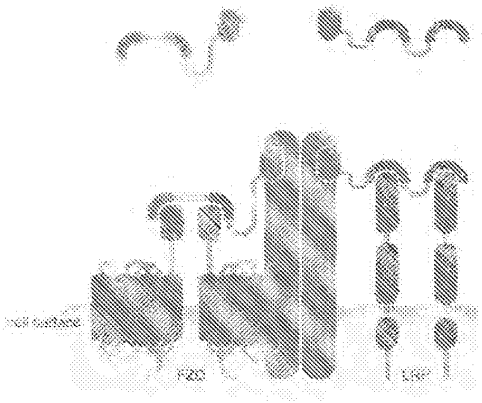


Fig. 1E

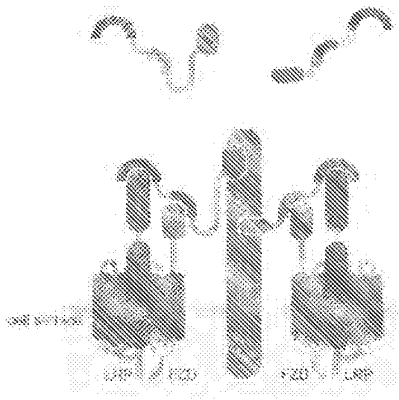


Fig. 1F

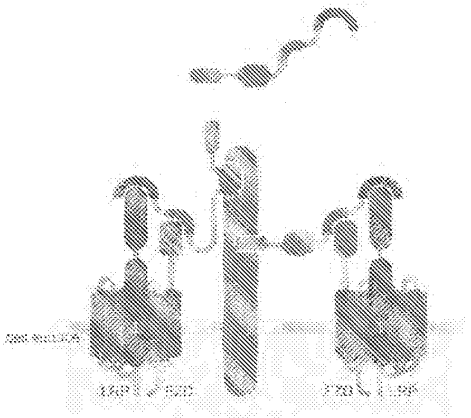


Fig. 1G

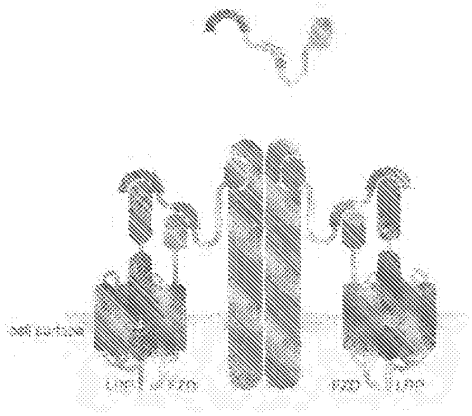


Fig. 1H

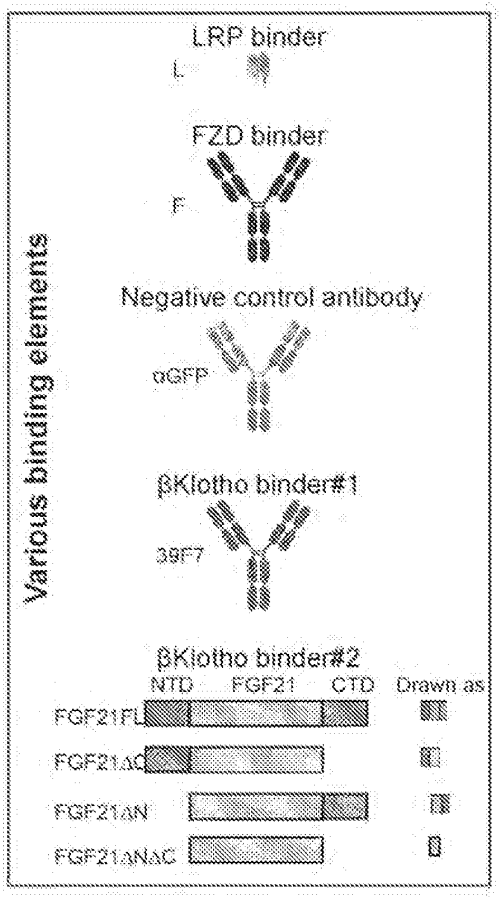

Various binding elements
LRP binder
L
FZD binder
F
Negative control antibody
αGFP
βKlotho binder#1
39F7
βKlotho binder#2
NTD
FGF21
CTD
Drawn as
FGF21FL
FGF21ΔC
FGF21ΔN
FGF21ΔNΔC

Fig. 2A

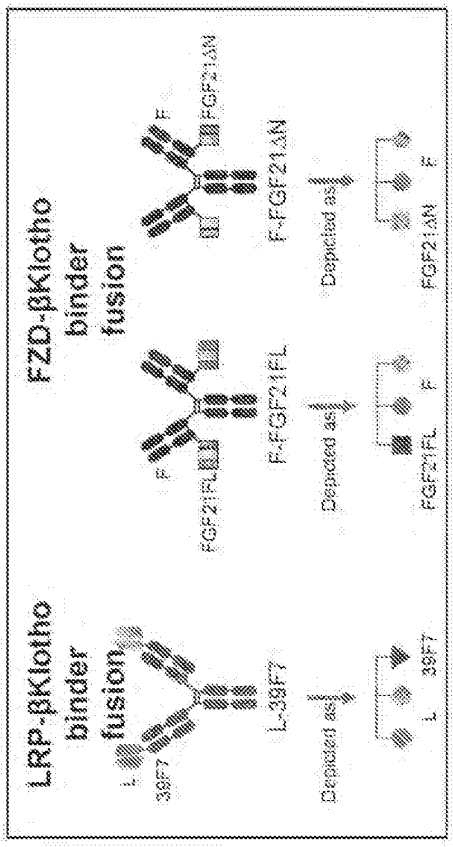

LRP-βKlotho binder fusion
L
39F7
L-39F7
Depicted as
L
39F7
FZD-βKlotho binder fusion
F
FGF21FL
F-FGF21FL
Depicted as
FGF21FL
F
F
FGF21ΔN
F-FGF21ΔN
Depicted as
FGF21ΔN
F

Fig. 2B

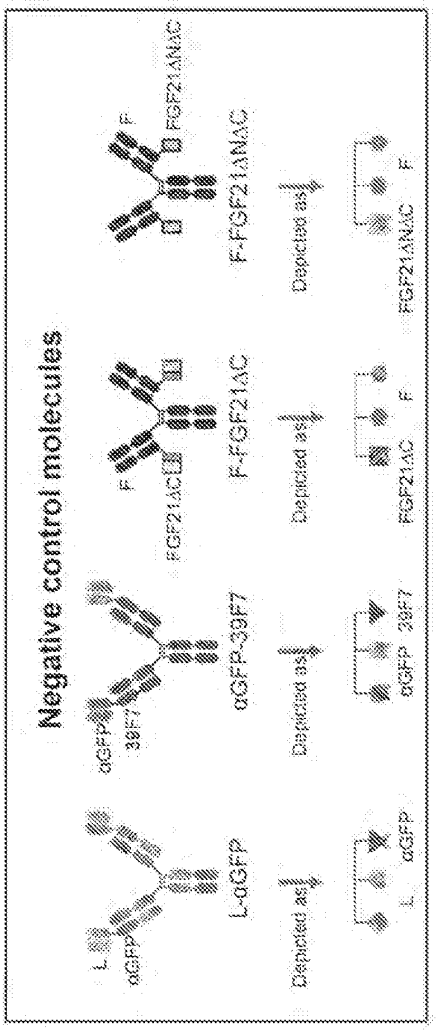

Negative control molecules
L
αGFP
L-αGFP
αGFP
39F7
αGFP-39F7
F
FGF21ΔC
F-FGF21ΔC
F
FGF21ΔNΔC
F-FGF21ΔNΔC
Depicted as
Depicted as
Depicted as
L
αGFP
αGFP
39F7
FGF21ΔC
F
FGF21ΔNΔC
F

Fig. 2C

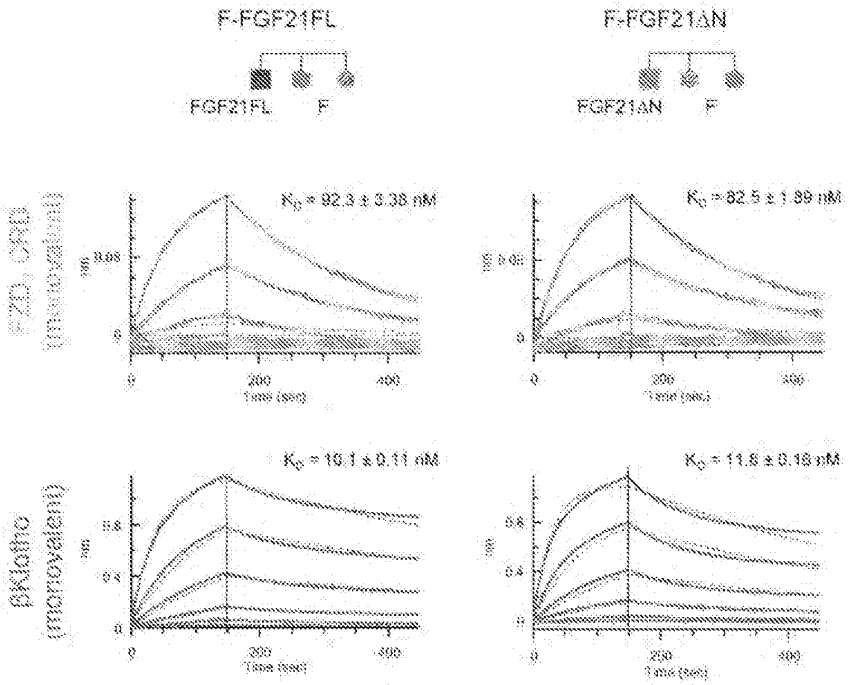

F-FGF21FL
FGF21FL
F
F-FGF21ΔN
FGF21ΔN
F
FZD, CNS (microvalent)
βKlotho (microvalent)
K_D = 92.3 ± 3.38 nM
K_D = 82.5 ± 1.89 nM
K_D = 19.1 ± 0.11 nM
K_D = 11.6 ± 0.16 nM

Fig. 2D Fig. 2E

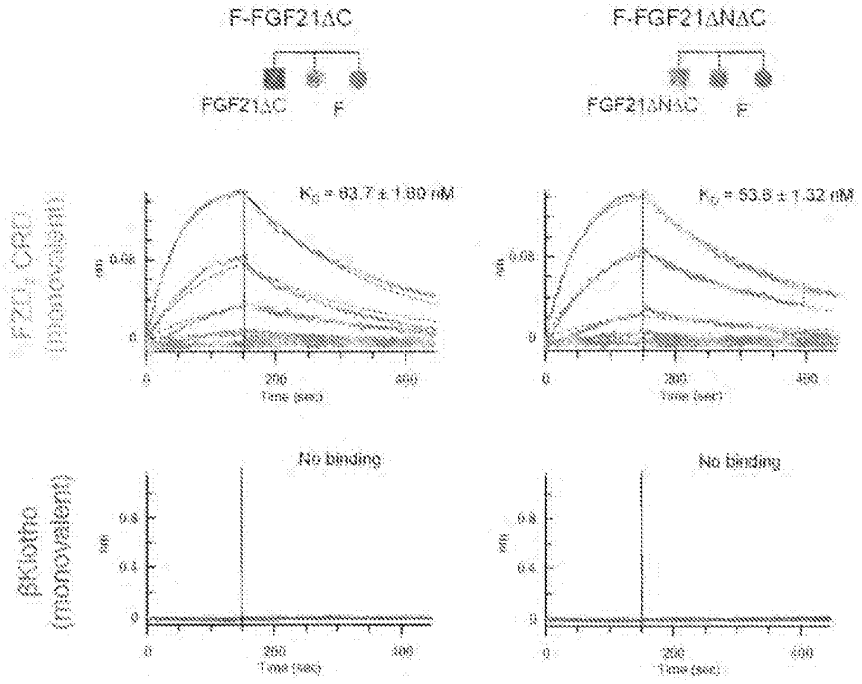

F-FGF21ΔC
FGF21ΔC
F
F-FGF21ΔNΔC
FGF21ΔNΔC
F
FZD1 CRD (monovalent)
βKlotho (monovalent)
No binding
No binding
Time (sec)

Fig. 2F Fig. 2G

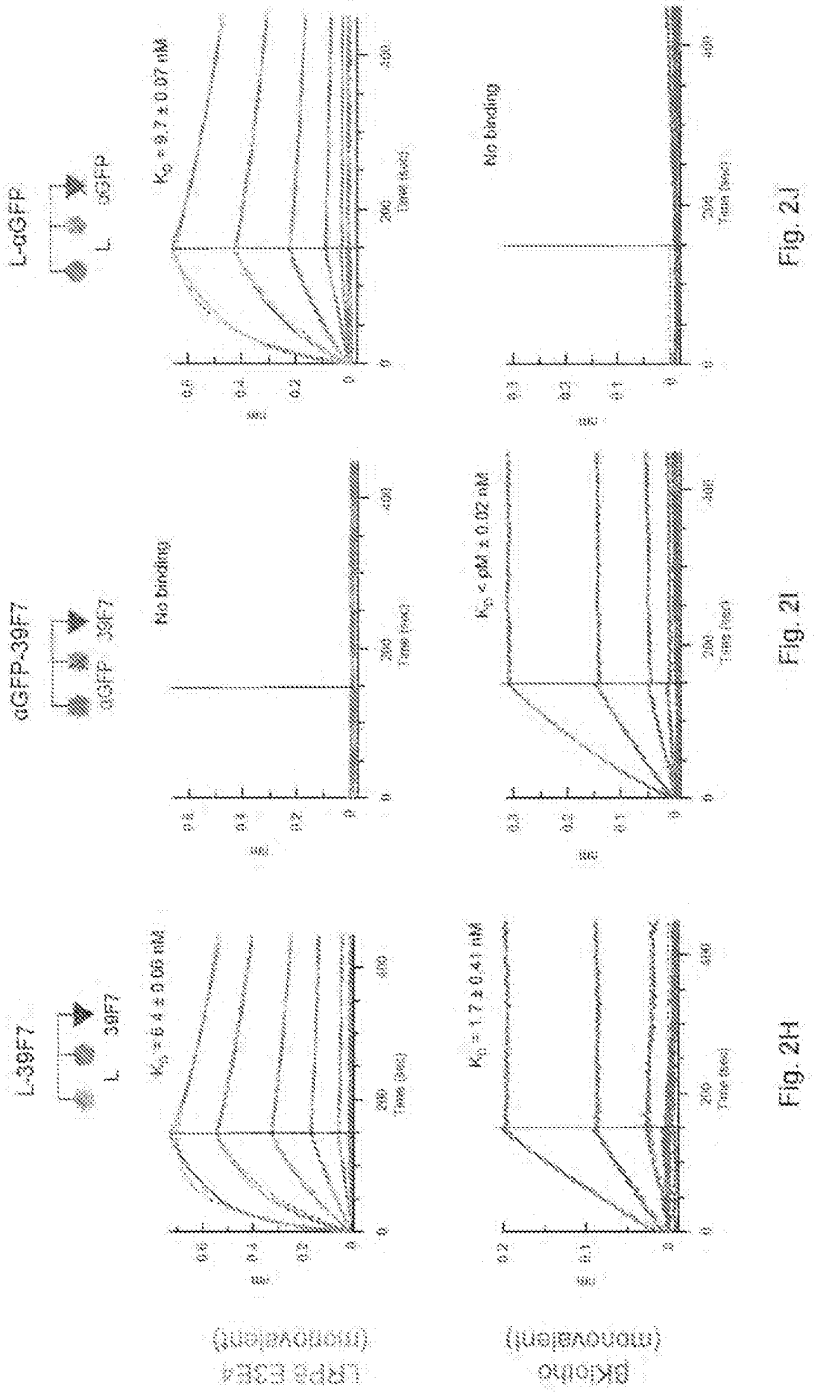


Fig. 2H Fig. 2I Fig. 2J

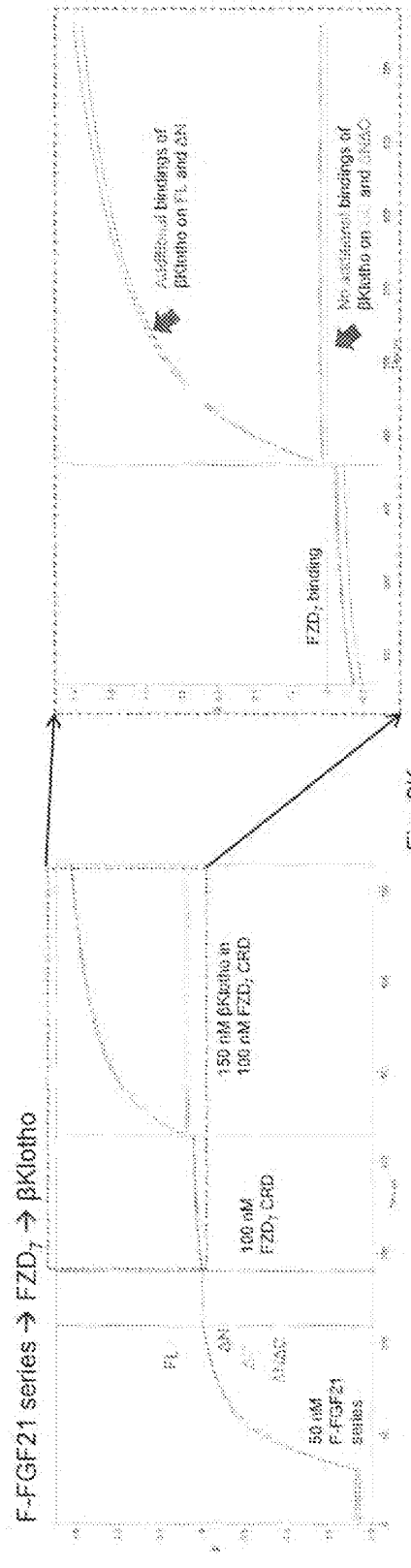

F-FGF21 series → FZD7 → βKlotho
FL
ΔN
ΔNΔC
50 nM F-FGF21 series
100 nM FZD7 CRD
150 nM βKlotho in 100 nM FZD7 CRD
FZD7 binding
Additional bindings of βKlotho on FL and ΔN
No additional bindings of βKlotho on ΔC and ΔNΔC

Fig. 2K

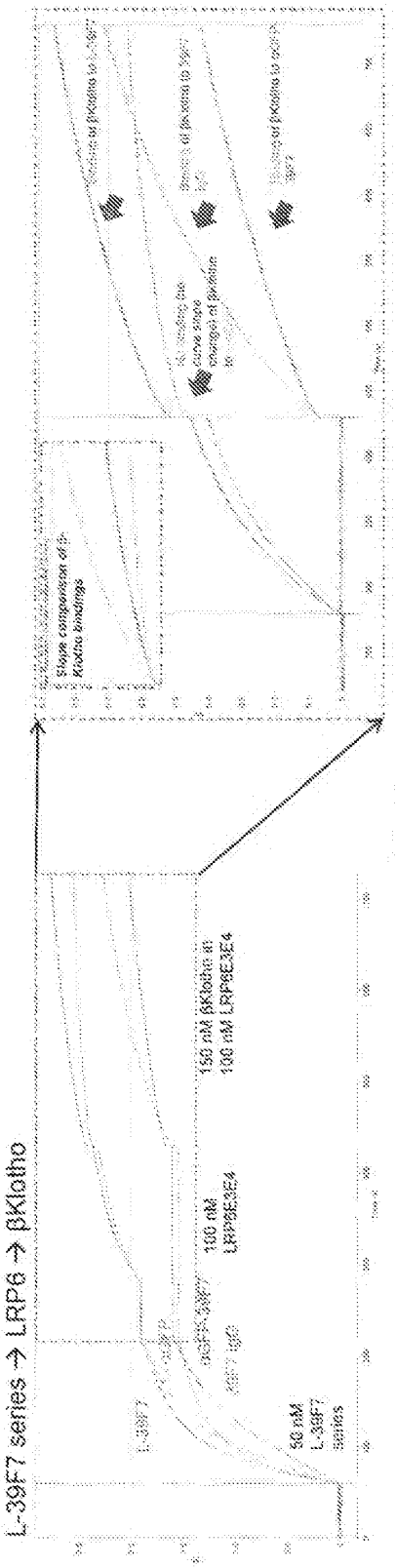

L-39F7 series → LRP6 → βKlotho
L-39F7
100 nM LRP6E3E4
150 nM βKlotho in 100 nM LRP6E3E4
50 nM L-39F7 series

Fig. 2L

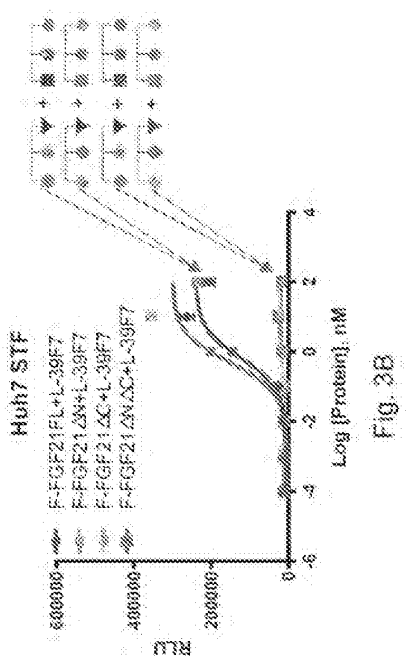


Fig. 3B

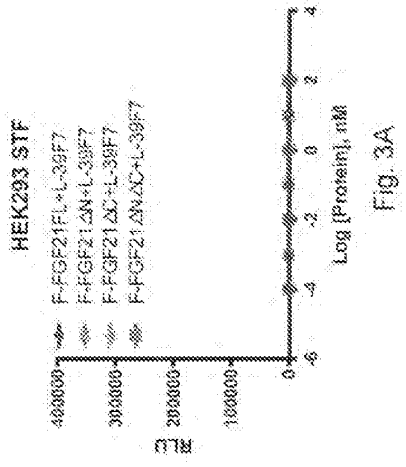


Fig. 3A

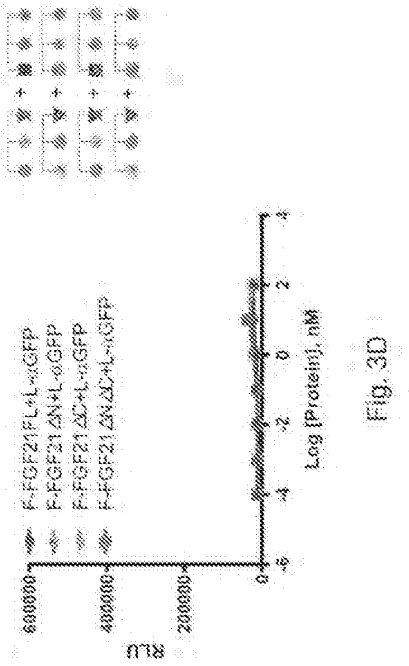


Fig. 3D

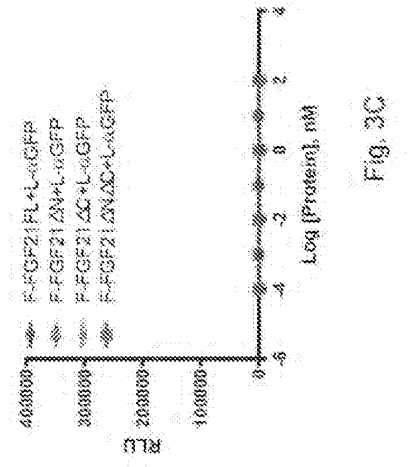


Fig. 3C

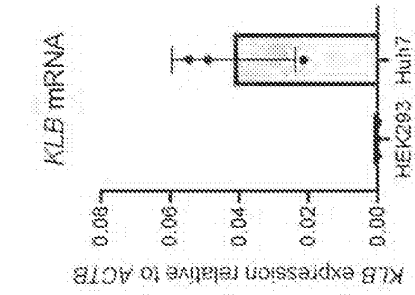


Fig. 3G

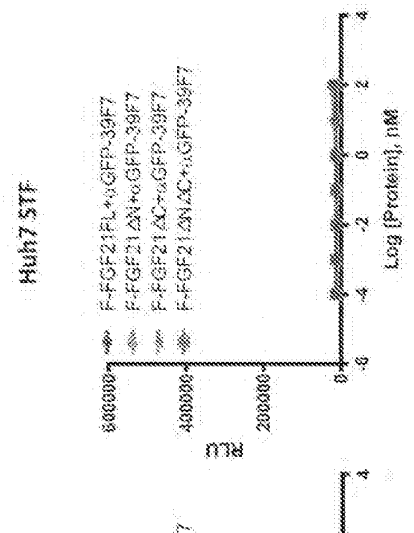


Fig. 3F

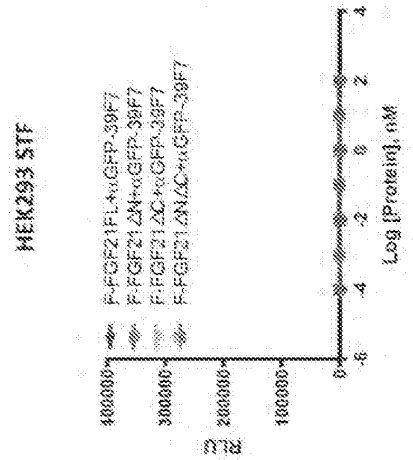
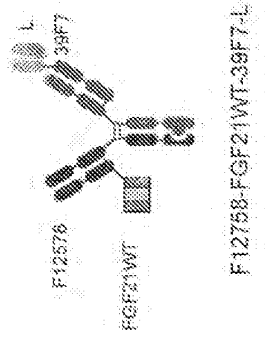


Fig. 3E



F12758-FGF21WT-39F7-L

Fig. 4A

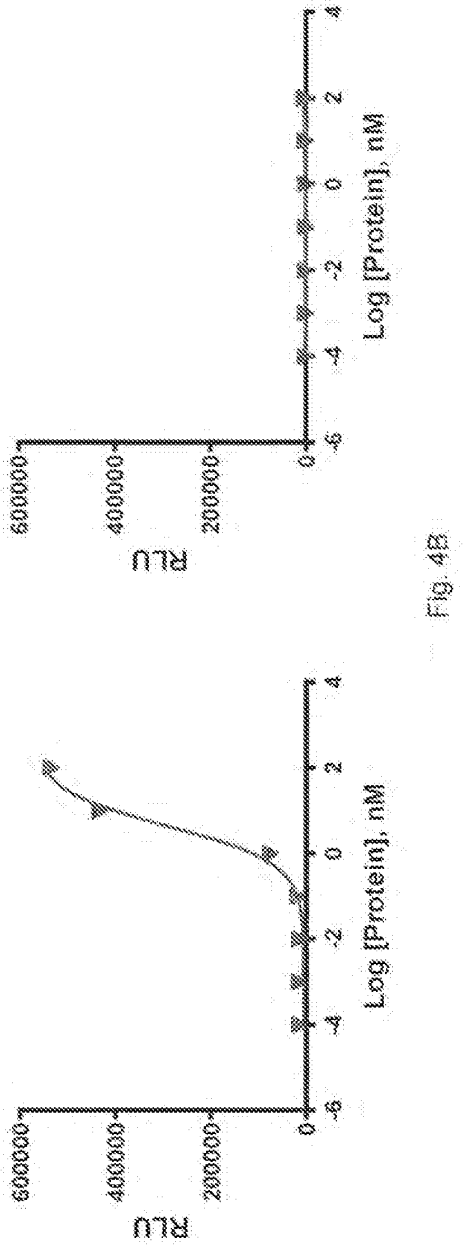


Fig. 4B

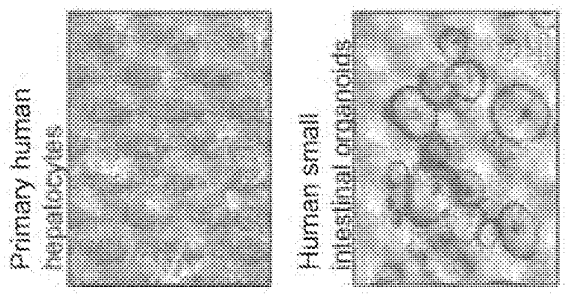


Fig. 5A

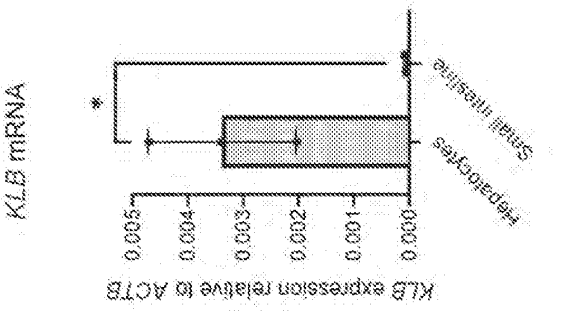


Fig. 5B

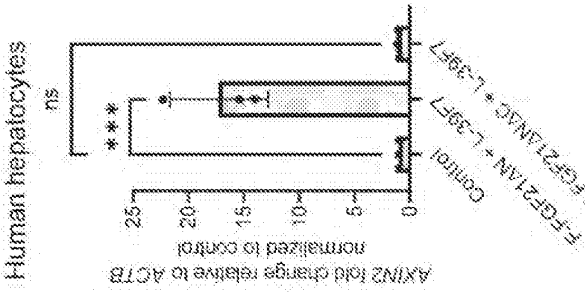


Fig. 5C

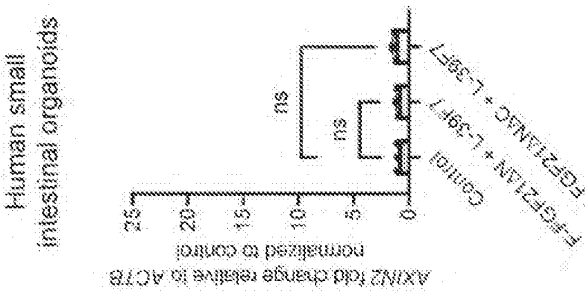


Fig. 5D

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Alexandria, VA 22313 - 1450
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ELECTRONIC ACKNOWLEDGEMENT RECEIPT

APPLICATION #	RECEIPT DATE / TIME	ATTORNEY DOCKET #
18/361,782	**10/11/2024 06:06:41 PM Z ET**	**SRZN-006/04US 328202-2203**

Title of Invention

WNT SURROGATE MOLECULES AND USES THEREOF

Application Information

APPLICATION TYPE	Utility - Nonprovisional Application under 35 USC 111(a)	PATENT #	-
CONFIRMATION #	9001	FILED BY	Susan Clingerman
PATENT CENTER #	67542042	FILING DATE	07/28/2023
CUSTOMER #	157070	FIRST NAMED INVENTOR	Yang LI
CORRESPONDENCE ADDRESS	-	AUTHORIZED BY	Carol Laherty

Documents

TOTAL DOCUMENTS: 3

DOCUMENT		PAGES	DESCRIPTION	SIZE (KB)
SRZN006_04US_RESPONSE.pdf		14	-	181 KB
SRZN006_04US_RESPONSE-A....pdf	(1-1)	1	Amendment/Request for Reconsideration-After Non-Final Rejection	129 KB
SRZN006_04US_RESPONSE-CLM.pdf	(2-3)	2	Claims	132 KB
SRZN006_04US_RESPONSE-REM.pdf	(4-14)	11	Applicant Arguments/Remarks Made in an Amendment	171 KB

Digest

DOCUMENT	MESSAGE DIGEST(SHA-512)
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This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

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PATENT AND TRADEMARK OFFICE

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ELECTRONIC PAYMENT RECEIPT

APPLICATION #	RECEIPT DATE / TIME	ATTORNEY DOCKET #
18/361,782	**10/11/2024 06:06:41 PM Z ET**	**SRZN-006/04US 328202-2203**

Title of Invention

WNT SURROGATE MOLECULES AND USES THEREOF

Application Information

APPLICATION TYPE	Utility - Nonprovisional Application under 35 USC 111(a)	PATENT #	-
CONFIRMATION #	9001	FILED BY	Susan Clingerman
PATENT CENTER #	67542042	AUTHORIZED BY	Carol Laherty
CUSTOMER #	157070	FILING DATE	07/28/2023
CORRESPONDENCE ADDRESS	-	FIRST NAMED INVENTOR	Yang LI

Payment Information

PAYMENT METHOD	PAYMENT TRANSACTION ID	PAYMENT AUTHORIZED BY
DA / 501283	**E20240AI07347256**	**Susan Clingerman**

PRE-AUTHORIZED ACCOUNT	PRE-AUTHORIZED CATEGORY
501283	**37 CFR 1.16 (National application filing, search, and examination fees); 37 CFR 1.17 (Patent application and reexamination processing fees); 37 CFR 1.19 (Document supply fees); 37 CFR 1.20 (Post Issuance fees); 37 CFR 1.21 (Miscellaneous fees and charges)**

FEE CODE	DESCRIPTION	ITEM PRICE($)	QUANTITY	ITEM TOTAL($)
1251	EXTENSION FOR RESPONSE WITHIN FIRST MONTH	220.00	1	220.00
			TOTAL AMOUNT:	**$220.00**

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for filing date (see 37 CFR 1.53(b)-(d)

and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

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Docket No.: SRZN-006/04US 328202-2203
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Inventor:	Yang LI	Confirmation No.:	9001
Application No.:	18/361,782	Group Art Unit:	1645
Filed:	July 28, 2023	Examiner:	C. Cheong

For: WNT SURROGATE MOLECULES AND USES THEREOF

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

AMENDMENT/RESPONSE TO OFFICE ACTION

In response to the non-final Office Action dated June 13, 2024, to which the deadline for responding is October 14, 2024, with a one-month extension of time, Applicant submits the following Amendments and/or Remarks, and respectfully requests reconsideration of the application in view thereof.

Any extensions of time necessary to prevent abandonment of this application are hereby petitioned for under 37 C.F.R. §1.136(a), and any additional fees required (including fees for net addition of claims) are hereby authorized to be charged to our Deposit Account No. 50-1283.

Amendments to the Claims are reflected in the listing of the claims which begins on page 2 of this paper.

Remarks begin on page 4 of this paper.

IN THE CLAIMS:

Set forth below in ascending order, with status identifiers, is a complete listing of all claims currently under examination. Changes to any amended claims are indicated by [[double brackets]], ~~strikethrough~~ and/or underlining. This listing also reflects any cancellation and/or addition of claims.

1. (Currently Amended) A soluble, tetravalent, multispecific Wnt surrogate molecule, wherein the Wnt surrogate molecule comprises:
(i) two Fzd binding regions that specifically bind to two Frizzled (Fzd) receptors, wherein each of the two Fzd binding regions comprises an antibody or antigen-binding fragment of the antibody[[,]]; and
(ii) two LRP5/6 binding regions that specifically bind to a Low-density lipoprotein (LDL) receptor-related protein 5 (LRP5) and/or a Low-density lipoprotein (LDL) receptor-related protein 6 (LRP6), wherein each of the two LRP5/6 binding regions comprises an antibody or antigen-binding fragment of the antibody, and
wherein each of the two LRP5/6 binding regions is fused to one of the two Fzd binding regions via a linker moiety.

2.-13. (Canceled)

14. (Currently Amended) The Wnt surrogate molecule of claim 1, wherein the two antibodies or antigen-binding fragments of the two Fzd binding regions each comprise: a CDRH1 of SEQ ID NO: 248, a CDRH2 of SEQ ID NO:600, a CDRH3 of SEQ ID NO:757, a CDRL1 of SEQ ID NO: 1098, a CDRL2 of SEQ ID NO: 1175, and a CDRL3 of SEQ ID NO: 1372; and
wherein the two antibodies or antigen-binding fragments of the two LRP5/6 binding regions each comprises: a CDRH1 of SEQ ID NO: 1617, a CDRH2 of SEQ ID NO: 1716, and a CDRH3 of SEQ ID NO: 2065.

15. (Previously Presented) The Wnt surrogate molecule of claim 14, wherein the Fzd binding regions comprise a full immunoglobulin G (IgG), comprising two light chains and two heavy chains, and the LRP5/6 binding regions comprise a VHH.

16. (Canceled)

17. (Previously Presented) The Wnt surrogate molecule of claim 1, wherein the LRP5/6 binding region is fused to the N-terminus or the C-terminus of the heavy chain or is fused to the N-terminus or the C-terminus of the light chain.

18.-54. (Canceled)

55. (Previously Presented) A pharmaceutical composition comprising a physiologically acceptable excipient, diluent, or carrier, and the Wnt surrogate molecule according to claim 1.

56.-70. (Canceled)

71. (Previously Presented) The Wnt Surrogate molecule of claim 14, wherein each of the two LRP5/6 binding regions is fused to one of the light chains via a linker moiety.

72. (Previously Presented) The Wnt surrogate molecule of claim 14, wherein the Wnt surrogate molecule comprises two polypeptides having at least 90% identity to the sequence set forth in SEQ ID NO: 144 and two polypeptides having at least 90% identity to the sequence set forth in SEQ ID NO: 155.

73. (Previously Presented) A pharmaceutical composition comprising the physiologically acceptable excipient, diluent, or carrier, and the Wnt surrogate molecule of claim 72.

REMARKS

Upon entry of these amendments, claims 1, 14-15, 17, 55, and 71-73 are pending in the present application and under consideration. Claims 1-13, 16, 18-54, and 56-70 were previously cancelled, without prejudice or disclaimer. By this response, claims 1 and 14 are amended.

The above-noted amendments are supported by the application as originally filed and do not introduce any new matter. The amendments are made without prejudice to the prosecution of any canceled or amended subject matter in one or more continuing application(s). Amendment and cancellation of certain claims is not to be construed as a dedication to the public of any of the subject matter of the claims as previously presented.

Claim Objections

Claim 14 is objected to for reciting "Wnt surrogate of claim 1" instead of "Wnt surrogate molecule of claim 1." Claim 1 has been amended to recite "Wnt surrogate molecule." Applicant respectfully requests the rejection to be withheld).

Claim Rejections – 35 USC §103

Claims 1, 17 and 55 stand rejected under 35 U.S.C. 103 as allegedly being unpatentable over Garcia et al (US2017/0306029; 8/7/2023 IDS) in view of Wu et al (Nature Biotechnology, vol 25, 1290-1297 (2007); PTO-892).

Applicant respectfully disagrees.

The rationale to support a conclusion that the claim would have been obvious is that all the claimed elements were known in the prior art, and one skilled in the art could have combined the elements as claimed by known methods with no change in their respective functions, and the combination yielded nothing more than predictable results to one of ordinary skill in the art. *KSR, 550 U.S. at 416, 82 USPQ2d at 1395; B/E Aerospace, Inc. v. C&D Zodiac, Inc., 962 F.3d 1373, 1379, 2020 USPQ2d 10706 (Fed. Cir. 2020); Sakraida v. AG Pro, Inc., 425 U.S. 273, 282,*

189 USPQ 449, 453 (1976); Anderson's-Black Rock, Inc. v. Pavement Salvage Co., 396 U.S. 57, 62-63, 163 USPQ 673, 675 (1969); Great Atl. & P. Tea Co. v. Supermarket Equip. Corp., 340 U.S. 147, 152, 87 USPQ 303, 306 (1950). "[I]t can be important to identify a reason that would have prompted a person of ordinary skill in the relevant field to combine the elements in the way the claimed new invention does." *KSR, 550 U.S. at 418, 82 USPQ2d at 1396.* If any of these findings cannot be made, then this rationale cannot be used to support a conclusion that the claim would have been obvious to one of ordinary skill in the art.

The Examiner asserts that "[r]egarding claim 1, Garcia teaches that the molecules of the invention are water-soluble; bind with high affinity to both (i) frizzled (Fzd) proteins and (ii) Lrp5/6; and directly activate canonical Wnt pathway signaling." However, the Examiner states that "[h]owever, Garcia does not teach dual variable domain (DVD) format of bispecific antibody."

The Examiner asserts that Wu teaches a generally applicable model of a dual-specific, tetravalent immunoglobulin G (lgG)-like molecule - termed dual-variable-domain immunoglobulin (DVD-Ig) - that can be engineered from any two monoclonal antibodies. The Examiner alleges that it would have been obvious to one of ordinary skill in the art before the filing date of the claimed invention to have prepared a bispecific antibody of Garcia in the format of DVD-lg taught by Wu, because Wu teaches that DVD-lg format bispecific antibody is highly stable and exhibits excellent, IgG-like physicochemical and pharmacokinetic properties, and because making a bispecific antibody in a different format is well known in the art and routine experimentation. The Examiner concludes that the invention as a whole would have been *prima facie* obvious to one of ordinary skill in the art before the effective filing date of the claimed invention.

Applicant recognizes that Garcia teaches agonists of Wnt pathway signaling that are bivalent and bispecific. However, the agonists exemplified in Garcia comprise an LRP binding arm that is a decoy protein based on DKK1 (see, e.g., Examples 1 and 2 and Figures 4 and 5, which describe B12-DKK1c and scFv-DKK1c constructs).

Garcia, alone or in combination with Wu, fails to motivate the skilled artisan to make variants or other structural changes in the Wnt agonists disclosed therein, in order to change the LRP binding arm from the decoy protein based on DKK1 to an LRP binding domain of the present invention. Garcia, alone or in combination with Wu, also fails to motivate the skilled artisan to prepare molecules that are tetravalent and bispecific.

In formulating the instant rejection, the Examiner has, in essence, selected Garcia's disclosure to serve as the lead compound, and has proposed the modification of this lead compound by making a dual variable domain (DVD) format. As clarified by the courts in various post-KSR chemical obviousness cases, the prior art must have led a person of ordinary skill in the art to identify Garcia's as the lead, and motivate the person of ordinary skill to make the specific modifications the inventors have made. *Takeda Chemical Industries, Ltd. v. Alphapharm Pty., Ltd., 492 F.3d 1350 (Fed. Cir. 2007), cert. denied, 128 S.Ct. 1739 (March 31, 2008)*.

As pointed out above, Garcia describes Wnt agonists well suited for their intended purpose, which contain an LRP binding arm comprising a decoy protein based on DKK1, and which are bivalent and bispecific. Thus, the Examiner has failed to "identify some reason that would have prompted a researcher to modify the prior art compounds [Wnt agonist of Garcia] to arrive at the claimed compounds." *Genetics Institute, LLC v. Novartis Vaccines and Diagnostics, Inc., 655 F.3d 1291 (Fed. Cir. 2011)*. Accordingly, Garcia cannot be interpreted as teaching or suggesting modifying the bispecific Wnt agonists disclosed therein to include non-DKK1 LRP binding regions.

Wu fails to remedy the essential insufficiency of the rejection. Wu neither discloses nor suggests a tetravalent, bispecific Wnt agonist comprising both FZD and LRP binding domains. As discussed above, Garcia does not suggest a tetravalent, bispecific Wnt agonist with a non-DKK1 LRP binding region, nor is this essential disclosure is found in Wu's reference.

Accordingly, the asserted combination of references does not provide one of ordinary skill in the art with the required motivation or any expectation of success in achieving the claimed invention. Moreover, as noted above, the asserted combination needs to disclose or

suggest all elements of the present claims. In the absence of a combination of references disclosing all of the elements of the claims, one of skill would not have a reasonable expectation of success in making the claimed invention. Moreover, the prior art would not have supplied one of ordinary skill in the art with a reason or motivation to modify Garcia to make the claimed invention with a reasonable expectation of success. Thus, the Examiner has formulated the instant rejection of claims by relying on impermissible hindsight reconstruction. *Otsuka Pharmaceutical Co., Ltd. v. Sandoz, Inc., 678 F.3d 1280 (Fed. Cir. 2012), cert denied, 133 S.Ct. 940 (Jan. 14, 2013)*.

Each of the arguments above is equally applicable to the dependent claims.

In the absence of some suggestion in the references supporting the proposed combination, the *prima facie case* of obviousness fails for lack of motivation to combine the references.

In view of the above facts and arguments, Applicant submits that a proper *prima facie* case of obviousness for claim 1, and dependent claims 17 and 55, has not been set forth, and respectfully requests that all rejections under 35 U.S.C. § 103(a) be withdrawn.

Double Patenting

Claims 1, 17 and 55 stand rejected on the ground of nonstatutory double patenting as allegedly being unpatentable over claims 1-10 of U.S. Patent No. 11,773,171 (hereinafter the '171 patent; 1/18/2024 IDS).

Without acquiescence to this rejection, Applicant submits a terminal disclaimer with respect to the '171 patent, and respectfully requests that this rejection be withdrawn.

Claims 1 and 55 stand provisionally rejected on the ground of nonstatutory double patenting as allegedly being unpatentable over claims 1 and 15-16 of copending Application No. 18/148,448 (hereinafter the '448 application; US2023/0295347; PTO-892). Although the claims at issue are not identical, they are not patentably distinct from each other.

Applicant respectfully disagrees with the Examiner. Claim 1 of the '448 application is directed to a very specific compound with specified CDR sequences (see below). The antibody described in claim 1 of the '448 application is a humanized polished antibody, whereas claim 1 of the instant application is not specifically directed to a single humanized antibody.

1. (Original) A multispecific antibody or antibody fragment comprising at least:

(A) a first antigen-binding region which specifically binds to frizzled class receptor 4 (Fzd4) and comprises a first heavy chain variable domain (VHI) and a first light chain variable domain (VLI),

(B) a second antigen-binding region which specifically binds to low density lipoprotein receptor-related protein 5 and/or 6 (LRP5 and/or LRP6) and comprises at least a second heavy chain variable domain (VH2)

wherein:

(A) the VHI comprises:

(i) a heavy chain complementarity-determining region I (CDRHI) which has an amino acid sequence which:

(i-1) has at least 80% identity to the amino acid sequence of SEQ ID NO: 121 or to the CDRHI amino acid sequence contained in SEQ ID NO: 181, optionally defined according to Kabat; or

(i-2) comprises or consists of the amino acid sequence of SEQ ID NO: 121, or comprises or consists of the CDRHI amino acid sequence contained in SEQ ID NO: 181, optionally defined according to Kabat;

(ii) a heavy chain complementarity-determining region 2 (CDRH2) which has an amino acid sequence which:

(ii-I) has at least 90% identity to the amino acid sequence of SEQ ID NO: 141 oto the CDRH2 amino acid sequence contained in SEQ ID NO: 181, optionally defined according to Kabat; or

(ii-2) comprises or consists of: the amino acid sequence of SEQ ID NO: 141; or comprises or consists of the CDRH2 amino acid sequence contained in SEQ ID NO: 181, optionally defined by Kabat; and

(iii) a heavy chain complementarity-determining region 3 (CDRH3) which has an amino acid sequence which:

(iii-I) has at least 90% identity to the amino acid sequence of SEQ ID NO: 161 or to the CDRH3 amino acid sequence contained in SEQ ID NO: 181, optionally defined by Kabat; or (iii-2) comprises or consists of the amino acid sequence of SEQ ID NO: 161, or comprises or consists of the CDRH3 amino acid sequence contained in SEQ ID NO: 181, optionally defined by Kabat; and

the VLI comprises:

(iv) a light chain complementarity-determining region I (CDRLI) which has an amino acid sequence which:

(iv-I) has at least 90% identity to the amino acid sequence of SEQ ID NO: 221 or to the CDRLI amino acid sequence contained in SEQ ID NO: 281, optionally defined by Kabat; or

(iv-2) comprises or consists of the amino acid sequence of SEQ ID NO: 221, or comprises or consists of the CDRLI amino acid sequence contained in SEQ ID NO: 281, optionally defined according to Kabat; (v) a light chain complementarity-determining region 2 (CDRL2) which has an amino acid sequence which comprises or consists of:

(v-1) the amino acid sequence of SEQ ID NO: 241; or

(v-2) the CDRL2 amino acid sequence contained in SEQ ID NO: 281, optionally defined according to Kabat; and

(vi) a light chain complementarity-determining region 3 (CDRL3) which has an amino acid sequence which comprises or consists of:

(vi-I) the amino acid sequence of SEQ ID NO: 261; or

(vi-2) the CDRL3 amino acid sequence contained in SEQ ID NO: 281, optionally defined according to Kabat; and (B) the VH2 comprises:

(i) a CDRHI which has an amino acid sequence which comprises or consists of:

(i-1) the amino acid sequence of SEQ ID NO: 321; or

(i-2) the CDRHI amino acid sequence contained in SEQ ID NO: 381, optionally defined according to IMGT or Kabat;

(ii) a CDRH2 which has an amino acid sequence which:

(ii-I) has at least 90% identity to the amino acid sequence of SEQ ID NO: 341 or to the CDRH3 amino acid sequence contained in SEQ ID NO: 381, optionally defined according to IMGT or Kabat;

(ii-2) comprises or consists of the amino acid sequence of SEQ ID NO: 341; or (ii-3) comprises or consists of the CDRH2 amino acid sequence contained in SEQ ID NO: 381, optionally defined according to IMGT or Kabat; and

(iii) a CDRH3 which has an amino acid sequence which:

(iii-I) has at least 90% identity to the amino acid sequence of SEQ ID NO: 361 or to the CDRH3 amino acid sequence contained in SEQ ID NO: 381, optionally defined according to IMGT or Kabat; or

(iii-2) comprises or consists of the amino acid sequence of any one of SEQ ID NOs: 361 and 363-366, or comprises or consists of the CDRH3 amino acid sequence contained in any one of SEQ ID NOS: 381 and 393-396, optionally defined according to IMGT or Kabat.

Applicant respectfully submits that the instant application and cited application are directed to different claimed subject matter, such that the instant claims would not be obvious over the pending claims of the cited application, and respectfully requests that the Examiner reconsider and withdraw the provisional obviousness type double patenting rejection.

Claims 1, 17 and 55 stand provisionally rejected on the ground of nonstatutory double patenting as allegedly being unpatentable over claims 1, 10-12 and 26 of copending Application No. 18/652,070 (hereinafter the '070 application).

Without acquiescence to this rejection, Applicant submits a terminal disclaimer with respect to the '070 application, and respectfully requests that this rejection be withdrawn.

Claims 1, 14-15, 17, 55 and 71-73 stand provisionally rejected on the ground of nonstatutory double patenting as allegedly being unpatentable over claims 1, 27, 34, 39 and 46 of copending Application No. 17/257,817 (hereinafter the '817 application; US2021/0292422; PTO-892).

Without acquiescence to this rejection, Applicant submits a terminal disclaimer with respect to the '817 application, and respectfully requests that this rejection be withdrawn.

Claims 1, 17 and 55 stand rejected on the ground of nonstatutory double patenting as allegedly being unpatentable over claims 1-10 of U.S. Patent No. 11,746,150 (hereinafter the '150 patent; IDS).

Without acquiescence to this rejection, Applicant submits a terminal disclaimer with respect to the '150 patent, and respectfully requests that this rejection be withdrawn.

Claims 1, 17 and 55 stand provisionally rejected on the ground of nonstatutory double patenting as allegedly being unpatentable over claims 1, 6-7 and 23 of copending Application No. 17/623,006 (hereinafter the '006 application; US2022/0275095; 8/7/2023 IDS).

Without acquiescence to this rejection, Applicant submits a terminal disclaimer with respect to the '006 application, and respectfully requests that this rejection be withdrawn.

Claims 1, 17 and 55 stand provisionally rejected on the ground of nonstatutory double patenting as allegedly being unpatentable over claims 1, 3-4, 9-10, 20 and 43 of copending Application No. 17/801,624 (hereinafter the '624 application; US2023/0138045; 8/7/2023 IDS).

Without acquiescence to this rejection, Applicant submits a terminal disclaimer with respect to the '624 application, and respectfully requests that this rejection be withdrawn.

Claims 1, 17 and 55 stand provisionally rejected on the ground of nonstatutory double patenting as allegedly being unpatentable over claims 1-3 and 6 of copending Application No. 18/281,253 (hereinafter the '253 application; US2024/0150473; PTO-892.

Applicant respectfully disagrees with the Examiner. Claim 1 of the '253 application is directed to a very specific compound with the polypeptide sequences recited (see below). The antibody described in the claim 1 of '253 application is a humanized polished antibody. Claim 1 of the instant application is not specifically directed to a humanized antibody.

1. (Currently Pending) An engineered WNT agonist comprising:
(a) one or more binding domains that bind to one or more FZD; and
(b) one or more binding domains that bind to LRP5, LRP6, or both LRP5 and LRP6,
wherein the engineered WNT agonist comprises a polypeptide comprising a sequence having at least 90%, at least 95%, at least 98%, or at least 99% sequence identity to SEQ ID NO:9 and a polypeptide comprising a sequence having at least 90%, at least 95%, at least 98%, or at least 99% sequence identity to SEQ ID NO:10, and
wherein the one or more binding domains that bind to one or more FZD bind to:

FZD 5 and FZD 8.

Applicant respectfully submits that the instant application and cited application are directed to different claimed subject matter, such that the instant claims would not be obvious over the pending claims of the cited application, and respectfully requests that the Examiner reconsider and withdraw the provisional obviousness type double patenting rejection.

CONCLUSION

In view of the foregoing, Applicant respectfully submits that no further impediments exist to the allowance of this application and, therefore, requests an indication of allowability. However, the Examiner is requested to call the undersigned if any questions or comments arise.

The Director is hereby authorized to charge any appropriate fees under 37 C.F.R. §§1.16, 1.17, and 1.21 that may be required by this paper, and to credit any overpayment, to Deposit Account No. 50-1283 referencing Docket No. SRZN-006/04US 328202-2203. However, the Director is not authorized to charge the cost of the issue fee to the Deposit Account.

Applicant believes no fee is due with this response. However, if a fee is due, please charge our Deposit Account No. 50-1283, under Order No. SRZN-006/04US 328202-2203 from which the undersigned is authorized to draw.

Dated: October 11, 2024

Respectfully submitted,
COOLEY LLP

Electronic signature: /Carol D. Laherty/
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USPTO CUSTOMER NO. 157070

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Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	18/361,782	LI et al.
	Examiner	Art Unit
	CHEOM-GIL CHEONG	1645

CPC - Searched*		
Symbol	Date	Examiner

CPC Combination Sets - Searched*		
Symbol	Date	Examiner

US Classification - Searched*			
Class	Subclass	Date	Examiner

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

Search Notes		
Search Notes	Date	Examiner
Reviewed prosecution history of parent case 16/954,484	06/05/2024	cc
Inventor name search	06/05/2024	cc
Reviewed pending claims of 18/311,082; 18/148,448; 16/954,482; 17/257,817; 18/652,070; 17/623,006; 17/801,624; 17/414,265; 17/429,584; 18/281,253; 17/438,303; 17/438,806;	06/05/2024	cc
Reviewed issued claims of US11746150, US11773171	06/05/2024	cc
Sequence search - see SCV	06/05/2024	cc
PE2E search – see attached	06/05/2024	cc
Google Scholar search – see attached	06/06/2024	cc
Inventor name search	12/19/2024	cc
PE2E search – see attached	12/19/2024	cc

/CHEOM-GIL CHEONG/ Examiner, Art Unit 1645	/Meera Natarajan/ Primary Examiner, Art Unit 1643

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	18/361,782	LI et al.
	Examiner	Art Unit
	CHEOM-GIL CHEONG	1645

Interference Search			
US Class/CPC Symbol	US Subclass/CPC Group	Date	Examiner
	Sequence search - see SCV	06/05/2024	cc

/CHEOM-GIL CHEONG/ Examiner, Art Unit 1645	/Meera Natarajan/ Primary Examiner, Art Unit 1643

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 1 of 6

Complete if Known	
Application Number	18/361,782
Filing Date	07-28-2023
First Named Inventor	LI; Yang
Art Unit	1645
Examiner Name	CHEONG, CHEOM-GIL
Attorney Docket Number	SRZN-006/04US 328202-2203

U. S. PATENT DOCUMENTS

Examiner Initials*		Cite No.[1]	Document Number Number-Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
		001	US-12006368	06-11-2024	LI; Yang et al.	
		002	US-20210032352	02-04-2021	ANGERS; Stephane et al.	
		003	US-20210309704	10-07-2021	VANHOLLEBEKE; Benoit	
		004	US-20230027249	01-26-2023	ADHIKARATH BALAN; Arun et al.	
		005	US-20240150458	05-09-2024	LI; Yang et al.	
		006	US-20240262918	08-08-2024	LI; Yang	

FOREIGN PATENT DOCUMENTS

Examiner Initials*		Cite No.[1]	Foreign Patent Document Country Code[3]Number[4]Kind Code[5] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages Or Relevant Figures Appear	T[6]
		007	WO-2011138391-A1	11-10-2011	NOVARTIS AG., et al.		☐
		008	WO-2017093478-A1	06-08-2017	BOEHRINGER INGELHEIM INT.		☐
		009	WO-2019093342-A1	05-16-2019	KYOWA HAKKO KIRIN CO LTD.		☒
		010	WO-2022130341-A1	06-23-2022	ANTLERA THERAPEUTICS INC.		☐
		011	WO-2024040118-A2	02-22-2024	SURROZEN OPERATING INC.		☐

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. [1] Applicant's unique citation designation number (optional). [2] See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. [3] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [6] Applicant is to place a check mark here if English language Translation is attached.

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Sheet 2 of 6

Complete if Known	
Application Number	18/361,782
Filing Date	07-28-2023
First Named Inventor	LI; Yang
Art Unit	1645
Examiner Name	CHEONG, CHEOM-GIL
Attorney Docket Number	SRZN-006/04US 328202-2203

NON-PATENT LITERATURE DOCUMENTS				
Examiner Initials*		Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
		012	AHMAD et al. "scFv Antibody: Principles and Clinical Application" Clinical and Developmental Immunology, (2012); 2012(Article)980250:1-16.	☐
		013	ALFTHAN et al. "Properties of a single-chain antibody containing different linker peptides" Protein Engineering, Design and Selection (1995); 8(7):725-731.	☐
		014	AUNG et al. "A new tool for technical standardization of the Ki67 immunohistochemical assay" Modern Pathology (2021); 34(7):1261-1270.	☐
		015	BANNAS et al. "Nanobodies and nanobody-based human heavy chain antibodies as antitumor therapeutics" Frontiers in Immunology (2017); 8:(1603):1-13.	☐
		016	BANNIER-HÉLAOUËT et al. "Exploring the human lacrimal gland using organoids and single-cell sequencing" Cell Stem Cell (2021); 28(7):1221-1232.	☐
		017	BASOVA et al. "Origin and lineage plasticity of endogenous lacrimal gland epithelial stem/progenitor cells" Iscience (2020); 23(6): 40 pages.	☐
		018	BEVER et al. "VHH antibodies: emerging reagents for the analysis of environmental chemicals" Analytical and Bioanalytical Chemistry (2016); 408(22):5985-6002.	☐
		019	CHEN, H., et al.; "BRAIDing receptors for cell-specific targeting," Elife, 9 January 2024; 12:RP90221, 14 pages.	☐
		020	CHEN, H., et al.; "BRAIDing receptors for cell-specific targeting," November 1, 2023, pre-print bioRxiv 2023.07.18.549566 [retrieved online February 2, 2024] URL:https://www.biorxiv.org/content/10.1101/2023.07.18.549566v2; 27 pages.	☐
		021	CHEN, Q. "Canonical Wnt signaling in diabetic retinopathy" Vision Research (2017); 139:47-58.	☐

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. [1] Applicant's unique citation designation number (optional). [2] Applicant is to place a check mark here if English language Translation is attached.

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /C.C./

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Sheet 3 of 6

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NON-PATENT LITERATURE DOCUMENTS

Examiner Initials*		Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
		022	CHEN, Weizao, et al. "Improving the CH1-CK heterodimerization and pharmacokinetics of 4Dm2m, a novel potent CD4-antibody fusion protein against HIV-1" MAbs. (2016); 8(4):761-774.	☐
		023	COLMAN, P.M. (1994) "Effects of amino acid sequence changes on antibody-antigen interactions" Research in Immunology, 145(1):33-36.	☐
		024	DAILEY et al. Norrin Treatment Improves Ganglion Cell Survival in an Oxygen-induced Retinopathy Model of Retinal Ischemia. Exp Eye Res. 2017. 164:129-138. (Year: 2017).	☐
		025	D'ANGELO et al. "Many routes to an antibody heavy-chain CDR3: necessary, yet insufficient, for specific binding" Frontiers in Immunology (2018); 9(895):336672, 1-13.	☐
		026	DARTT "Signal transduction and control of lacrimal gland protein secretion: a review" Current Eye Research (1989); 8(6):619-636.	☐
		027	DEAN et al. "Canonical Wnt signaling negatively regulates branching morphogenesis of the lung and lacrimal gland" Developmental Biology (2005); 286(1):270-286.	☐
		028	DVORIANTCHIKOVA et al. "Molecular profiling of the developing lacrimal gland reveals putative role of notch signaling in branching morphogenesis" Investigative Ophthalmology & Visual Science (2017); 58(2):1098-1109.	☐
		029	FARMER et al. "Defining epithelial cell dynamics and lineage relationships in the developing lacrimal gland" Development (2017); 144(13):2517-2528.	☐
		030	GARG et al. "Lacrimal gland development: From signaling interactions to regenerative medicine" Developmental Dynamics (2017); 246(12):970-980.	☐
		031	GOEL et al. "Plasticity within the Antigen-Combining Site May Manifest as Molecular Mimicry in the Hu moral Immune Response" The Journal of Immunology (2004); 173(12):7358-7367.	☐

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. [1] Applicant's unique citation designation number (optional). [2] Applicant is to place a check mark here if English language Translation is attached.

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Substitute for form 1449/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 4 of 6

Complete if Known	
Application Number	18/361,782
Filing Date	07-28-2023
First Named Inventor	LI; Yang
Art Unit	1645
Examiner Name	CHEONG, CHEOM-GIL
Attorney Docket Number	SRZN-006/04US 328202-2203

NON-PATENT LITERATURE DOCUMENTS

Examiner Initials*		Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
		032	HASSANI et al. "Construction of a chimeric antigen receptor bearing a nanobody against prostate a specific membrane antigen in prostate cancer" J Cell Biochem (2019); 120(6):10787–10795.	☐
		033	HASSANZADEH-GHASSABEH, G. et al. (2013) "Nanobodies and their potential applications" Nanomedicine, 8(6):1013-1026.	☐
		034	HODGES et al. "Regulatory pathways in lacrimal gland epithelium" International Review of Cytology (2003); 231:129-196.	☐
		035	HUSSACK et al. "Characterization of single-domain antibodies with an engineered disulfide bond" Single Domain Antibodies: Methods and Protocols (2012); 911:417-429.	☐
		036	JAKOB et al. "Structure reveals function of the dual variable domain immunoglobulin (DVD-Ig™) molecule" mAbs (2013); 5(3):358–363.	☐
		037	JENSEN et al. "Salivary gland dysfunction and xerostomia in Sjögren's syndrome" Oral and Maxillofacial Surgery Clinics (2014); 26(1):35-53.	☐
		038	KAWAKITA "Regeneration of lacrimal gland function to maintain the health of the ocular surface" Investigative Ophthalmology & Visual Science (2018); 59(14):DES169-DES173.	☐
		039	KHAN et al. "Adjustable Locks and Flexible Keys: Plasticity of Epitope-Paratope Interactions in Germline Antibodies" J. Immunol. (2014); 192:5398-5405.	☐
		040	KIM TY et al. "Prolonged halflife of small-sized therapeutic protein using serum albumin-specific protein binder" J Control Release (2019); 315:31–39.	☐
		041	KLEIN et al. "Design and characterization of structured protein linkers with differing flexibilities" Protein Engineering, Design & Selection (2014); 27(10):325-330.	☐

Examiner Signature		Date Considered	

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 5 of 6

Complete if Known	
Application Number	18/361,782
Filing Date	07-28-2023
First Named Inventor	LI; Yang
Art Unit	1645
Examiner Name	CHEONG, CHEOM-GIL
Attorney Docket Number	SRZN-006/04US 328202-2203

NON-PATENT LITERATURE DOCUMENTS

Examiner Initials*		Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
		042	LE GALL et al. "Effect of linker sequences between the antibody variable domains on the formation, stability and biological activity of a bispecific tandem diabody" Protein Engineering Design and Selection (2004); 17(4):357-366.	☐
		043	LIU, Y., et al. "A ligation of the lacrimal excretory duct in mouse induces lacrimal gland inflammation with proliferative cells" Stem Cells International (2017); 2017(1):4923426, 9 pages.	☐
		044	MA, X., et al. "Dry eye management in a Sjögren's syndrome mouse model by inhibition of p38-MAPK pathway" Diagnostic Pathology (2014); 9(5):1-6.	☐
		045	MACMILLAN, JOHN A. "Diseases of the lacrimal gland and ocular complications" Journal of the American Medical Association (1948); 138(11):801-805.	☐
		046	PARFITT et al. "Renewal of the holocrine meibomian glands by label-retaining, unipotent epithelial progenitors" Stem Cell Reports (2016); 7(3):399-410.	☐
		047	PICHE-NICHOLAS et al. "Changes in complementarity-determining regions significantly alter IgG binding to the neonatal Fc receptor (FcRn) and pharmacokinetics" mAbs (2018); 10(1):81-94.	☐
		048	POOSARLA et al. "Computational De Novo Design of Antibodies Binding to a Peptide With High Affinity" Biotech. Bioeng. (2017); 114(6):1331-1342.	☐
		049	RUDIKOFF, S., et al., "Single amino acid substitution altering antigen-binding specificity," Proc Natl Acad Sci USA. 1982 Mar; 79(6):1979-83.	☐
		050	TIWARI, S. et al. "Human lacrimal gland regeneration: Perspectives and review of literature." Saudi Journal of Ophthalmology (2014); 28(1):12-18.	☐
		051	US Patent Application No. 18/652,070, filed May 1, 2024, by Yang LI, et al.	☐

Examiner Signature		Date Considered	

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 6 of 6

Complete if Known	
Application Number	18/361,782
Filing Date	07-28-2023
First Named Inventor	LI; Yang
Art Unit	1645
Examiner Name	CHEONG, CHEOM-GIL
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NON-PATENT LITERATURE DOCUMENTS

Examiner Initials*		Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
		052	US Patent Application No. 18/691,419 filed March 12, 2024, by Russell FLETCHER, et al.	☐
		053	US Patent Application No. 18/719,798 filed June 13, 2024, by Yang LI, et al.	☐
		054	US Patent Application No. 18/888,027, filed September 17, 2024, by Kenan Christopher GARCIA, et al.	☐
		055	US Patent Application No. 18/893,740, filed September 23, 2024, by Kenan Christopher GARCIA, et al.	☐
		056	VAN AUDENHOVE et al. "Nanobodies as Versatile Tools to Understand, Diagnose, Visualize and Treat Cancer" EBioMedicine (2016); 8:40–48.	☐
		057	VITALI, C., et al. "European Study Group on Classification Criteria for Sjögren's Syndrome. Classification criteria for Sjögren's syndrome: a revised version of the European criteria proposed by the American-European Consensus Group" Annals of the Rheumatic Diseases (2002); 61:554.	☐
		058	WHITLOW et al. "An improved linker for single-chain Fv with reduced aggregation and enhanced proteolytic stability" Protein Engineering, Design and Selection (1993); 6(8):989-995.	☐
		059	YAO, Y. et al. "The lacrimal gland: development, wound repair and regeneration" Biotechnology Letters (2017); 39:939-949.	☐
		060	ZOUKHRI et al. "A single injection of interleukin-1 induces reversible aqueous-tear deficiency, lacrimal gland inflammation, and acinar and ductal cell proliferation" Experimental Eye Research (2007); 84(5):894-904.	☐

Examiner Signature	/CHEOM-GIL CHEONG/	Date Considered	12/19/2024

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. [1] Applicant's unique citation designation number (optional). [2] Applicant is to place a check mark here if English language Translation is attached.

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PE2E SEARCH - Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	British Equivalents	Time Stamp
L17	102271	(C07K16/2863 OR C07K16/468 OR A61K2039/505 OR C07K2317/30 OR C07K2317/55 OR C07K2317/569 OR C07K2317/622 OR C07K2317/92).cpc.	(US-PGPUB; USPAT; USOCR; FIT (WO))	ADJ	ON	ON	2024/12/19 01:58 PM
L18	4	(C07K16/2863 AND C07K16/468 AND A61K2039/505 AND C07K2317/30 AND C07K2317/55 AND C07K2317/569 AND C07K2317/622 AND C07K2317/92).cpc.	(US-PGPUB; USPAT; USOCR; FIT (WO))	ADJ	ON	ON	2024/12/19 01:58 PM
L19	35	(Artificial Intelligence) More like doc: WO-2019126398-A1 Text: (CA-2705923-A1 OR BR-112012022044-A2 OR JP-2014527818-A OR BR-112014017518-A2 OR WO-2015109212-A1 OR JP-2022520084-A OR WO-2020206005-A1 OR KR-20240126877-A OR CA-3085782-A1 OR AU-2018393073-A1 OR JP-2021506297-A OR EP-3732201-A1 OR WO-2019126398-A1 OR CN-111727203-A OR JP-2023113950-A OR US-20200308287-A1 OR CN-118271444-A OR US-11773171-B2 OR JP-2024057106-A OR CN-111727203-B).did.	(US-PGPUB; USPAT; USOCR; FIT (AU, AP, AT, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, OA, RU, SU, WO, MC, MD, MY, NL, NO, NZ, PH, PL, PT, RO, RS, SE, SG, SI, SK, TH, TN, TR, TW, UA, VN); FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2024/12/19 03:29 PM
L20	17	((("SURROZEN") near3 ("OPERATING") near3 ("INC"))).AS,AANM.	(US-PGPUB; USPAT)	ADJ	ON	ON	2024/12/19 03:29 PM
L21	4559	((("LI") near3 ("Yang")) OR (("YUAN") near3 ("Tom")) OR (("SATO") near3 ("Aaron")) OR (("YEH") near3 ("Wen-Chen")) OR (("JANDA")	(US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT)	ADJ	ON	ON	2024/12/19 03:34 PM

		near3 ("Claudia")) OR (("FOWLER") near3 ("Tristan")) OR (("BARIBAULT") near3 ("Helene")) OR (("LAI") near3 ("Kuo-Pao")) OR (("XIE") near3 ("Liqin")) OR (("BREZSKI") near3 ("Randall")) OR (("LU") near3 ("Chenggang"))).INV.					
L22	61	((("LI") near3 ("Yang")) OR (("YUAN") near3 ("Tom")) OR (("SATO") near3 ("Aaron")) OR (("YEH") near3 ("Wen-Chen")) OR (("JANDA") near3 ("Claudia")) OR (("FOWLER") near3 ("Tristan")) OR (("BARIBAULT") near3 ("Helene")) OR (("LAI") near3 ("Kuo-Pao")) OR (("XIE") near3 ("Liqin")) OR (("BREZSKI") near3 ("Randall")) OR (("LU") near3 ("Chenggang"))).INV. AND (Fzd OR Frizzled) AND (LRP5 OR LRP6)	(US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT)	ADJ	ON	ON	2024/12/19 03:34 PM
L23	27	((("LI") near3 ("Yang")) OR (("YUAN") near3 ("Tom")) OR (("SATO") near3 ("Aaron")) OR (("YEH") near3 ("Wen-Chen")) OR (("JANDA") near3 ("Claudia")) OR (("FOWLER") near3 ("Tristan")) OR (("BARIBAULT") near3 ("Helene")) OR (("LAI") near3 ("Kuo-Pao")) OR (("XIE") near3 ("Liqin")) OR (("BREZSKI") near3 ("Randall")) OR (("LU") near3 ("Chenggang"))).INV. AND (Fzd OR Frizzled).clm. AND (LRP5 OR LRP6).clm.	(US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT)	ADJ	ON	ON	2024/12/19 03:40 PM
L24	19	(Artificial Intelligence) Similar to: 18/361,782 with 0 CPC Selections and 0 Text Selections Text: (WO-2019126398-A1	(US-PGPUB; USPAT; USOCR; FIT (WO))	ADJ	ON	ON	2024/12/19 03:41 PM

		OR AU-2018393073-A1 OR US-20200308287-A1 OR CA-3085782-A1 OR JP-2021506297-A OR CN-111727203-A OR US-20240141049-A1 OR CN-111727203-B OR US-11773171-B2 OR CN-112566940-A OR US-20210292422-A1 OR WO-2020010308-A1 OR JP-7418332-B2 OR JP-2024500839-A OR CA-3104526-A1 OR CN-111655729-B OR JP-2021529796-A OR WO-2022130341-A1 OR JP-2024001287-A OR JP-2023554500-A OR JP-2021508247-A OR CN-116964102-A OR KR-20230122077-A OR WO-2022130342-A1 OR CA-3140580-A1 OR CN-111655729-A OR CN-114423784-A OR US-20230118983-A1 OR CN-116917332-A OR JP-2022539212-A OR WO-2020250156-A1 OR US-20210087280-A1 OR US-20220315659-A1 OR JP-7377288-B2 OR AU-2020291208-A1 OR CA-3144499-A1 OR AU-2018393074-A1 OR CA-3085596-A1 OR WO-2019126399-A1 OR KR-20220024460-A OR CN-117946267-A OR US-20220275095-A1 OR WO-2023250402-A2 OR JP-2023515505-A OR AU-2020300618-A1 OR WO-2021003416-A1 OR US-11746150-B2 OR US-20230138045-A1 OR US-20160235844-A1 OR EP-2911691-B1).did.					
L25	367	Frizzled near11 receptor near55 LRP5 near55 LRP6	(US-PGPUB; USPAT; USOCR; FIT (WO))	ADJ	ON	ON	2024/12/19 03:44 PM
L26	232	Frizzled near11	(US-PGPUB; USPAT;	ADJ	ON	ON	2024/12/19

		receptor near55 LRP5 near55 LRP6 AND @ad<"20171219"	USOCR; FIT (WO))				03:44 PM
L27	11	Frizzled near11 receptor near55 LRP5 near55 LRP6 AND tetravalent AND @ad<"20171219"	(US-PGPUB; USPAT; USOCR; FIT (WO))	ADJ	ON	ON	2024/12/19 03:46 PM
L28	10	Frizzled near11 receptor near55 LRP5/6 AND tetravalent AND @ad<"20171219"	(US-PGPUB; USPAT; USOCR; FIT (WO))	ADJ	ON	ON	2024/12/19 03:54 PM
L29	8	Frizzled near11 receptor near55 LRP5/6 AND VHH AND tetravalent AND @ad<"20171219"	(US-PGPUB; USPAT; USOCR; FIT (WO))	ADJ	ON	ON	2024/12/19 03:54 PM
L30	0	Frizzled near11 receptor near55 LRP5/6 near55 bispecific AND VHH AND tetravalent AND @ad<"20171219"	(US-PGPUB; USPAT; USOCR; FIT (WO))	ADJ	ON	ON	2024/12/19 04:19 PM
L31	8	Frizzled near11 receptor near55 LRP5/6 AND bispecific AND VHH AND tetravalent AND @ad<"20171219"	(US-PGPUB; USPAT; USOCR; FIT (WO))	ADJ	ON	ON	2024/12/19 04:19 PM
L32	12	(US-20230027249-$ | US-12006368-$ | US-20210032352-$ | US-20210309704-$ | US-20240150458-$ | US-20240262918-$).DID.	(US-PGPUB; USPAT; USOCR; FIT (WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2024/12/19 05:37 PM
L33	14	(AI-2011138391-$ | AI-2017093478-$ | AI-2019093342-$ | AI-2022130341-$ | WO-2011138391-$ | WO-2017093478-$ | WO-2019093342-$ | WO-2022130341-$ | WO-2024040118-$).DID.	(US-PGPUB; USPAT; USOCR; FIT (WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2024/12/19 05:38 PM
L34	26	L32 OR L33	(US-PGPUB; USPAT; USOCR; FIT (WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2024/12/19 05:38 PM
L35	8	L34 AND (Fzd SAME LRP$3)	(US-PGPUB; USPAT; USOCR; FIT (WO))	ADJ	ON	ON	2024/12/19 05:39 PM
L36	4	L34 AND (Fzd SAME LRP$3).clm.	(US-PGPUB; USPAT; USOCR; FIT (WO))	ADJ	ON	ON	2024/12/19 05:39 PM

PE2E SEARCH - Search History (Interference)

There are no Interference searches to show.

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
18/361,782	07/28/2023	Yang LI	SRZN-006/04US 328202-2203	9001

157070 7590 12/27/2024

Cooley LLP / Surrozen Operating, Inc.
Attn: IP Docketing Department
1299 Pennsylvania Avenue, NW, Suite 700
Washington, DC 20004

EXAMINER	
CHEONG, CHEOM-GIL	

ART UNIT	PAPER NUMBER
1645	

NOTIFICATION DATE	DELIVERY MODE
12/27/2024	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

zIPPatentDocketingMailboxUS@cooley.com

Office Action Summary	**Application No.** 18/361,782	**Applicant(s)** LI et al.	
	Examiner CHEOM-GIL CHEONG	**Art Unit** 1645	**AIA (FITF) Status** Yes

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☑ Responsive to communication(s) filed on 10/11/2024.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☑ This action is **FINAL.** 2b) ☐ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5) ☑ Claim(s) 1,14-15,17,55 and 71-73 is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6) ☐ Claim(s) _____ is/are allowed.

7) ☑ Claim(s) 1,17 and 55 is/are rejected.

8) ☑ Claim(s) 14-15 and 71-73 is/are objected to.

9) ☐ Claim(s) _____ are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☑ The drawing(s) filed on 1/18/2024 is/are: a)☑ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)

2) ☑ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.

DETAILED ACTION

Notice of Pre-AIA or AIA Status

The present application, filed on or after March 16, 2013, is being examined under the first inventor to file provisions of the AIA.

Claim Status

Claim(s) 2-13, 16, 18-54, and 56-70 were/was canceled.

Claims 1, 14-15, 17, 55 and 71-73 are pending and under consideration.

Terminal Disclaimer

The terminal disclaimer filed on 10/11/2024 disclaiming the terminal portion of any patent granted on this application which would extend beyond the expiration date of the full statutory term of prior patent numbers **US11773171** and **US11746150**; and any patent granted on pending Application Numbers **18/652070, 17/801624, 17/257817, 17/623006** has been reviewed and is accepted. The terminal disclaimer has been recorded.

Withdrawn Rejections

Objection of claim 14 is withdrawn. Applicant amended the claim 14, thereby obviating this objection.

Rejection of Claim 1, 17 and 55 on the ground of nonstatutory double patenting as being unpatentable over claim 1-10 of U.S. Patent No. 11773171 is withdrawn. Applicant filed Terminal Disclaimer to overcome this rejection.

Provisional Rejection of Claim 1, 17 and 55 on the ground of nonstatutory double patenting as being unpatentable over claim 1, 10-12 and 26 of copending Application No. 18/652,070 is withdrawn. Applicant filed Terminal Disclaimer to overcome this rejection.

Provisional rejection of Claim 1, 14-15, 17, 55 and 71-73 on the ground of nonstatutory double patenting as being unpatentable over claim 1, 27, 34, 39 and 46 of copending Application No. 17/257,817 is withdrawn. Applicant filed Terminal Disclaimer to overcome this rejection.

Rejection of Claim 1, 17 and 55 on the ground of nonstatutory double patenting as being unpatentable over claim 1-10 of U.S. Patent No. 11746150 is withdrawn. Applicant filed Terminal Disclaimer to overcome this rejection.

Provisional rejection of Claim 1, 17 and 55 on the ground of nonstatutory double patenting as being unpatentable over claim 1, 6-7 and 23 of copending Application No. 17/623,006 is withdrawn. Applicant filed Terminal Disclaimer to overcome this rejection.

Provisional rejection of Claim 1, 17 and 55 on the ground of nonstatutory double patenting as being unpatentable over claim 1, 3-4, 9-10, 20 and 43 of copending Application No. 17/801,624 is withdrawn. Applicant filed Terminal Disclaimer to overcome this rejection.

Maintained - Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103 which forms the basis for all obviousness rejections set forth in this Office action:

> A patent for a claimed invention may not be obtained, notwithstanding that the claimed invention is not identically disclosed as set forth in section 102, if the differences between the claimed invention and the prior art are such that the claimed invention as a whole would have been obvious before the effective filing date of the claimed invention to a person having ordinary skill in the art to which the claimed invention pertains. Patentability shall not be negated by the manner in which the invention was made.

The factual inquiries for establishing a background for determining obviousness under 35 U.S.C. 103 are summarized as follows:

1. Determining the scope and contents of the prior art.

2. Ascertaining the differences between the prior art and the claims at issue.

3. Resolving the level of ordinary skill in the pertinent art.

4. Considering objective evidence present in the application indicating obviousness or nonobviousness.

Claims 1, 17 and 55 are rejected under 35 U.S.C. 103 as being unpatentable over Garcia et al (US2017/0306029; 8/7/2023 IDS) in view of Wu et al (Nature Biotechnology, vol 25, 1290-1297 (2007)).

Regarding claim 1, Garcia teaches that the molecules of the invention are water soluble; bind with high affinity to both (i) frizzled (Fzd) proteins and (ii) Lrp5/6; and directly activate canonical wnt pathway signaling (paragraph 011).

Regarding claim 55, Garcia teaches a pharmaceutical composition as claimed (claim 34).

However, Garcia does not teach dual variable domain (DVD) format of bispecific antibody.

Regarding claim 1 and 17, Wu teaches "Here, we describe a generally applicable model of a dual-specific, tetravalent immunoglobulin G (IgG)-like molecule—termed **dual-variable-domain immunoglobulin (DVD-Ig)**—that can be engineered from any two monoclonal antibodies while preserving activities of the parental antibodies. This molecule can be efficiently produced from mammalian cells and exhibits **good physicochemical and pharmacokinetic properties**. Preclinical studies of a DVD-Ig protein in an animal disease model demonstrate its potential for therapeutic application in human diseases" (abstract). Wu further teaches "The DVD-Ig protein is an IgG-like molecule that can be efficiently produced by conventional mammalian expression systems as a single species **for easy manufacturing and purification**, while maintaining the affinities and potencies of the two parental mAbs. In addition, it is **highly stable and exhibits excellent, IgG-like physicochemical and pharmacokinetic properties**" (page 1290, right column, first paragraph).

It would have been obvious to one of ordinary skill in the art before the filing date of the claimed invention to have prepared bispecific antibody of Garcia in the format of DVD-Ig taught by Wu because Wu teaches that DVD-Ig format bispecific antibody is highly stable and exhibits excellent, IgG-like physicochemical and pharmacokinetic properties. One of ordinary skill in the art would be motivated to use DVD-Ig format to prepare bispecific antibody because DVD-Ig format provides advantageous properties for therapeutics for human disease. Therefore, the invention as a whole would have been obvious to one of ordinary skill in the art.

From the teachings of the references, it is apparent that one of ordinary skill in the art would have had a reasonable expectation of success because Wu teaches that DVD-Ig format bispecific antibody is highly stable and exhibits excellent, IgG-like physicochemical and pharmacokinetic properties and because making bispecific antibody in a different format is well known in the art and routine experimentation. Therefore, the invention as a whole would have been *prima facie* obvious to one of ordinary skill in the art before the effective filing date of the claimed invention, as evidenced by the references, especially in the absence of evidence to the contrary.

Response to Arguments

In the response filed on 10/11/2024, Applicant argued at page 5-6,

> Applicant recognizes that Garcia teaches agonists of Wnt pathway signaling that are bivalent and bispecific. However, the agonists exemplified in Garcia comprise an LRP binding arm that is a decoy protein based on DKK1 (see, e.g., Examples 1 and 2 and Figures 4 and 5, which describe B12-DKK1c and scFv-DKK1c constructs).

> Garcia, alone or in combination with Wu, fails to motivate the skilled artisan to make variants or other structural changes in the Wnt agonists disclosed therein, in order to change the LRP binding arm from the decoy protein based on DKK1 to an LRP binding domain of the present invention. Garcia, alone or in combination with Wu, also fails to motivate the skilled artisan to prepare molecules that are tetravalent and bispecific.

Applicant's arguments have been fully considered but they are not persuasive. Instant claim 1 **does not define specific LRP5/6 binding region** like instant claim 14. Therefore, DKK1 taught by Garcia is still encompassed by generically defined "LRP5/6 binding region" of instant claim 1. Thus, one of ordinary skill in the art would not need to replace DKK1 with LRP5/6 binding region of instant invention to arrive at instant claim 1 and dependent claims 17 and 55.

Furthermore, Garcia teaches "the Lrp5/6 binding domain or element may be selected from **any domain that binds Lrp5/6** at high affinity" [0015] and "Lrp5/6 binding proteins or polypeptides, including without limitation, Norrin, DKK1, DKK2, DKK3, DKK4, sclerostin; and the like. In **certain** embodiments the Lrp5/6 binding domain is a c-terminal portion of **DKK1**" [0015]. Therefore, LRP5/6 binding domain taught by Garcia is NOT limited only to DKK1.

As discussed above, Garcia teaches that the molecules of the invention are water soluble; bind with high affinity to both (i) frizzled (Fzd) proteins and (ii) Lrp5/6; and directly activate canonical wnt pathway signaling (paragraph 011). Wu teaches that DVD-Ig format bispecific antibody is highly stable and exhibits excellent, IgG-like physicochemical and pharmacokinetic properties. One of ordinary skill in the art would be motivated to make bispecific antibody in DVD-Ig format and test its therapeutic property because therapeutics may show better characteristics in a different format.

One of ordinary skill in the art would also have a reasonable expectation of success because Wu teaches that DVD-Ig format provides advantageous properties for therapeutics for human disease.

Maintained - Double Patenting

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory double patenting rejection is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on nonstatutory double patenting provided the reference application or patent either is shown to be commonly owned with the examined application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement. See MPEP § 717.02 for applications subject to examination under the first inventor to file

provisions of the AIA as explained in MPEP § 2159. See MPEP § 2146 *et seq.* for applications not subject to examination under the first inventor to file provisions of the AIA. A terminal disclaimer must be signed in compliance with 37 CFR 1.321(b).

The filing of a terminal disclaimer by itself is not a complete reply to a nonstatutory double patenting (NSDP) rejection. A complete reply requires that the terminal disclaimer be accompanied by a reply requesting reconsideration of the prior Office action. Even where the NSDP rejection is provisional the reply must be complete. See MPEP § 804, subsection I.B.1. For a reply to a non-final Office action, see 37 CFR 1.111(a). For a reply to final Office action, see 37 CFR 1.113(c). A request for reconsideration while not provided for in 37 CFR 1.113(c) may be filed after final for consideration. See MPEP §§ 706.07(e) and 714.13.

The USPTO Internet website contains terminal disclaimer forms which may be used. Please visit www.uspto.gov/patent/patents-forms. The actual filing date of the application in which the form is filed determines what form (e.g., PTO/SB/25, PTO/SB/26, PTO/AIA/25, or PTO/AIA/26) should be used. A web-based eTerminal Disclaimer may be filled out completely online using web-screens. An eTerminal Disclaimer that meets all requirements is auto-processed and approved immediately upon submission. For more information about eTerminal Disclaimers, refer to www.uspto.gov/patents/apply/applying-online/eterminal-disclaimer.

Claim 1 and 55 are provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claim 1 and 15-16 of copending Application No. 18/148,448 (hereinafter application'448; US2023/0295347).

Although the claims at issue are not identical, they are not patentably distinct from each other because of the following reasons.

Regarding claim 1, claim 1, 15 and 16(III)(e) of application'448 claims as follows.

1. (Original) A multispecific antibody or antibody fragment comprising at least:

(A) a first antigen-binding region which specifically binds to frizzled class receptor 4 (Fzd4) and comprises a first heavy chain variable domain (VH1) and a first light chain variable domain (VL1),

(B) a second antigen-binding region which specifically binds to low density lipoprotein receptor-related protein 5 and/or 6 (LRP5 and/or LRP6) and comprises at least a second heavy chain variable domain (VH2).

15. (Currently Amended) The multispecific antibody or antibody fragment of ~~any one of claims 1-13~~claim 1, which comprises:

(A) two or more Fzd4-binding regions, wherein at least one of said Fzd4-binding regions is, comprises, or is comprised in said first antigen-binding region; and/or

(B) two or more LRP5 and/or LRP6-binding regions, wherein at least one of said LRP5 and/or LRP6-binding regions is, comprises, or is comprised in said second antigen-binding region.

16. (Original) The multispecific antibody or antibody fragment of claim 15, wherein:

(III) the multispecific antibody or antibody fragment comprises:

(e) two identical Fzd4-binding regions, each of which is, comprises, or is comprised in said first antigen-binding region; and two identical LRP5 and/or LRP6-binding region, each of which is, comprises, or is comprised in said second antigen-binding region;

Regarding claim 55, claim 23 of application'448 claims pharmaceutical composition as claimed.

This is a provisional nonstatutory double patenting rejection because the patentably indistinct claims have not in fact been patented.

Claim 1, 17 and 55 are provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claim 1-3 and 6 of copending Application No. 18/281,253 (hereinafter application'253; US2024/0150473). Although the claims at issue are not identical, they are not patentably distinct from each other because of the following reasons.

Regarding claim 1 and 17, claim 1-3 of application'253 claims

1. (Currently Amended) An engineered WNT agonist comprising:
(a) one or more binding domains that bind to one or more FZD, and
(b) one or more binding domains that bind to LRP5, LRP6, or both LRP5 and LRP6,
wherein the engineered WNT agonist comprises a polypeptide comprising a sequence having at least 90%, at least 95%, at least 98%, or at least 99% sequence identity to ~~any of~~ SEQ ID NOs ~~4-259~~ and a polypeptide comprising a sequence having at least 90%, at least 95%, at least 98%, or at least 99% sequence identity to SEQ ID NO:10, ~~or a polypeptide sequence~~

2. (Currently Amended) The engineered WNT agonist of claim 1, comprising:

(a) ~~a~~ two polypeptides comprising a sequence having at least 90%, at least 95%, at least 98%, or at least 99% sequence identity to ~~any one of~~ SEQ ID NOs: ~~9-25 or a sequence disclosed in Table 3~~; or

(b) ~~a~~ two polypeptides comprising a sequence having at least 90%, at least 95%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 10~~comprising two or three of the CDR~~

As evidenced by page 92 of specification of application'253, SEQ ID NO: 9 and 10 claimed by claims 1-3 of application'253 have same structure recited by instant claim 1 and 17.

Regarding claim 55, claim 6 of application'253 claims

6. (Currently Amended) A pharmaceutical composition comprising the engineered WNT agonist of ~~any one of~~ claims ~~1-5~~ and a pharmaceutically acceptable carrier, diluent, or excipient.

This is a provisional nonstatutory double patenting rejection because the patentably indistinct claims have not in fact been patented.

Response to Arguments

In the response filed on /2024, Applicant argued at page 11,

Applicant respectfully submits that the instant application and cited application are directed to different claimed subject matter, such that the instant claims would not be obvious over the pending claims of the cited application, and respectfully requests that the Examiner reconsider and withdraw the provisional obviousness type double patenting rejection.

Applicant's arguments have been fully considered but they are not persuasive. Although instant claim 14 claims specific antibody for Fzd binding region and LRP5/6 binding region, instant claim 1 claims **genus of binding regions which only requires binding to Fzd receptor and LRP5/6**. Therefore, the antibody of reference

application'448 is SPECIES antibody encompassed by GENUS claimed by instant claim 1. Since GENUS is anticipated by SPECIES, Applicant's argument is not persuasive.

Claim Objection

Claims 14-15 and 71-73 are objected to as being dependent on rejected base claim.

Conclusion

No claim is allowed.

THIS ACTION IS MADE FINAL. Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to CHEOM-GIL CHEONG whose telephone number is

(571)272-6251. The examiner can normally be reached Monday - Friday 9:00 am - 5:00 pm.

Examiner interviews are available via telephone, in-person, and video conferencing using a USPTO supplied web-based collaboration tool. To schedule an interview, applicant is encouraged to use the USPTO Automated Interview Request (AIR) at http://www.uspto.gov/interviewpractice.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Gary Nickol can be reached on 571-272-0835. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of published or unpublished applications may be obtained from Patent Center. Unpublished application information in Patent Center is available to registered users. To file and manage patent submissions in Patent Center, visit: https://patentcenter.uspto.gov. Visit https://www.uspto.gov/patents/apply/patent-center for more information about Patent Center and https://www.uspto.gov/patents/docx for information about filing in DOCX format. For additional questions, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/CHEOM-GIL CHEONG/
Examiner, Art Unit 1645

/ANAND U DESAI/
Primary Examiner, Art Unit 1656
December 20, 2024

PTO/SB/06 (09-11)
Approved for use through 1/31/2014. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 18/361,782	Filing Date 07/28/2023	☐ To be Mailed

ENTITY: ☑ LARGE ☐ SMALL ☐ MICRO

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA		RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A		N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A		N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A		N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*		x $100 =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*		x $480 =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).				
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))					
* If the difference in column 1 is less than zero, enter "0" in column 2.				TOTAL	

APPLICATION AS AMENDED - PART II

		(Column 1)		(Column 2)	(Column 3)			
AMENDMENT	02/06/2025	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 5	Minus	** 20	= 0		x $200 =	0
	Independent (37 CFR 1.16(h))	* 1	Minus	*** 3	= 0		x $600 =	0
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	0

		(Column 1)		(Column 2)	(Column 3)			
AMENDMENT		CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=		x $0 =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=		x $0 =	
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.	LIE
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".	/MARSHA R RICHARDS/
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".	
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.	

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Docket No.: SRZN-006/04US 328202-2203
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Inventor:	Yang LI	Confirmation No.:	9001
Application No.:	18/361,782	Group Art Unit:	1645
Filed:	July 28, 2023	Examiner:	C. Cheong

For: WNT SURROGATE MOLECULES AND USES THEREOF

Mail Stop RCE
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

AMENDMENT/RESPONSE TO FINAL OFFICE ACTION

In response to the final Office Action dated December 27, 2024 (the "Office Action"), to which the deadline for responding is March 27, 2025, Applicant submits the following Amendments and/or Remarks, and respectfully requests reconsideration of the application in view thereof.

Any extensions of time necessary to prevent abandonment of this application are hereby petitioned for under 37 C.F.R. §1.136(a), and any additional fees required (including fees for net addition of claims) are hereby authorized to be charged to our Deposit Account No. 50-1283.

Amendments to the Claims are reflected in the listing of the claims which begins on page 2 of this paper.

Remarks begin on page 4 of this paper.

UNITED STATES
PATENT AND TRADEMARK OFFICE

P.O. Box 1450
Alexandria, VA 22313 - 1450
www.uspto.gov

ELECTRONIC ACKNOWLEDGEMENT RECEIPT

APPLICATION #	RECEIPT DATE / TIME	ATTORNEY DOCKET #
18/361,782	**02/06/2025 01:39:04 PM Z ET**	**SRZN-006/04US 328202-2203**

Title of Invention

WNT SURROGATE MOLECULES AND USES THEREOF

Application Information

APPLICATION TYPE	Utility - Nonprovisional Application under 35 USC 111(a)	PATENT #	-
CONFIRMATION #	9001	FILED BY	Susan Clingerman
PATENT CENTER #	69076317	FILING DATE	07/28/2023
CUSTOMER #	157070	FIRST NAMED INVENTOR	Yang LI
CORRESPONDENCE ADDRESS	-	AUTHORIZED BY	Carol Laherty

Documents

TOTAL DOCUMENTS: 4

DOCUMENT		PAGES	DESCRIPTION	SIZE (KB)
SRZN006_04US_RCE.pdf		3	Request for Continued Examination (RCE)	98 KB
Warning: This is not a USPTO supplied RCE fillable form. Data in the form cannot be automatically loaded to other USPTO systems.				
SRZN006_04US_RESPONSE_1.114.pdf		12	-	201 KB
SRZN006_04US_RESPONSE_1.114-AMSB.pdf	(1-1)	1	Amendment Submitted/Entered with Filing of Continued Prosecution Application (CPA)/Request for Continued Examination(RCE)	110 KB
SRZN006_04US_RESPONSE_1.114-CLM.pdf	(2-3)	2	Claims	122 KB

SRZN006_04US_RESPONSE_1.114-REM.pdf	(4-12)	9	Applicant Arguments/Remarks Made in an Amendment	192 KB

Digest

DOCUMENT	MESSAGE DIGEST(SHA-512)
SRZN006_04US_RCE.pdf	FC8479B086EF84ABDB09521475F6D080E691858160DEE9558 424A594F89BD04D20877DD792A1CBF487A50377324AD524B8 6153161E9F34EF536F3FE23CD60D8D
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SRZN006_04US_RESPONSE_1.114-REM.pdf	1802753166CF61B1E9526AA4D65457E69EA0CC45CF2FC4A45 5794AF99270F3113C2E3B50D028E700FFD64F494BF2AC2F07 1302A45AB5170B2BBB1937009C3C90

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PTO/SB/30EFS (01-22)
Approved for use through 05/31/2024 OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

REQUEST FOR CONTINUED EXAMINATION(RCE)TRANSMITTAL
(Submitted Only via EFS-Web)

Application Number	18361782	Filing Date	2023-07-28	Docket Number (if applicable)	SRZN-006/04US 328202-220	Art Unit	1645
First Named Inventor	Yang LI			Examiner Name	C. Cheong		

This is a Request for Continued Examination (RCE) under 37 CFR 1.114 of the above-identified application.
Request for Continued Examination (RCE) practice under 37 CFR 1.114 does not apply to any utility or plant application filed prior to June 8, 1995, or to any design application. The Instruction Sheet for this form is located at WWW.USPTO.GOV

SUBMISSION REQUIRED UNDER 37 CFR 1.114

Note: If the RCE is proper, any previously filed unentered amendments and amendments enclosed with the RCE will be entered in the order in which they were filed unless applicant instructs otherwise. If applicant does not wish to have any previously filed unentered amendment(s) entered, applicant must request non-entry of such amendment(s).

☐ Previously submitted. If a final Office action is outstanding, any amendments filed after the final Office action may be considered as a submission even if this box is not checked.

- ☐ Consider the arguments in the Appeal Brief or Reply Brief previously filed on _____
- ☐ Other _____

☒ Enclosed

- ☒ Amendment/Reply
- ☐ Information Disclosure Statement (IDS)
- ☐ Affidavit(s)/ Declaration(s)
- ☐ Other _____

MISCELLANEOUS

☐ Suspension of action on the above-identified application is requested under 37 CFR 1.103(c) for a period of months _____
(Period of suspension shall not exceed 3 months; Fee under 37 CFR 1.17(i) required)

☐ Other _____

FEES

☒ **The RCE fee under 37 CFR 1.17(e) is required by 37 CFR 1.114 when the RCE is filed.**
The Director is hereby authorized to charge any underpayment of fees, or credit any overpayments, to Deposit Account No 50-1283

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT REQUIRED

☒ Patent Practitioner Signature

☐ Applicant Signature

PTO/SB/30EFS (01-22)
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Signature of Registered U.S. Patent Practitioner			
Signature	/Carol D. Laherty/	Date (YYYY-MM-DD)	2025-02-06
Name	Carol D. Laherty	Registration Number	51909

This collection of information is required by 37 CFR 1.114. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450.

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Docket No.: SRZN-006/04US 328202-2203
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Inventor:	Yang LI	Confirmation No.:	9001
Application No.:	18/361,782	Group Art Unit:	1645
Filed:	July 28, 2023	Examiner:	C. Cheong

For: WNT SURROGATE MOLECULES AND USES THEREOF

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AMENDMENT/RESPONSE TO FINAL OFFICE ACTION

In response to the final Office Action dated December 27, 2024 (the "Office Action"), to which the deadline for responding is March 27, 2025, Applicant submits the following Amendments and/or Remarks, and respectfully requests reconsideration of the application in view thereof.

Any extensions of time necessary to prevent abandonment of this application are hereby petitioned for under 37 C.F.R. §1.136(a), and any additional fees required (including fees for net addition of claims) are hereby authorized to be charged to our Deposit Account No. 50-1283.

Amendments to the Claims are reflected in the listing of the claims which begins on page 2 of this paper.

Remarks begin on page 4 of this paper.

IN THE CLAIMS:

Set forth below in ascending order, with status identifiers, is a complete listing of all claims currently under examination. Changes to any amended claims are indicated by [[double brackets]], ~~strikethrough~~ and/or underlining. This listing also reflects any cancellation and/or addition of claims.

1. (Previously Presented) A soluble, tetravalent, multispecific Wnt surrogate molecule, wherein the Wnt surrogate molecule comprises:
(i) two Fzd binding regions that specifically bind to two Frizzled (Fzd) receptors, wherein each of the two Fzd binding regions comprises an antibody or antigen-binding fragment of the antibody; and
(ii) two LRP5/6 binding regions that specifically bind to a Low-density lipoprotein (LDL) receptor-related protein 5 (LRP5) and/or a Low-density lipoprotein (LDL) receptor-related protein 6 (LRP6), wherein each of the two LRP5/6 binding regions comprises an antibody or antigen-binding fragment of the antibody, and
wherein each of the two LRP5/6 binding regions is fused to one of the two Fzd binding regions via a linker moiety.

2.-14. (Canceled)

15. (Currently Amended) The Wnt surrogate molecule of claim ~~14~~1, wherein the Fzd binding regions comprise a full immunoglobulin G (IgG), comprising two light chains and two heavy chains, and the LRP5/6 binding regions comprise a VHH.

16. (Canceled)

17. (Previously Presented) The Wnt surrogate molecule of claim 1, wherein the LRP5/6 binding region is fused to the N-terminus or the C-terminus of the heavy chain or is fused to the N-terminus or the C-terminus of the light chain.

18.-54. (Canceled)

55. (Previously Presented) A pharmaceutical composition comprising a physiologically acceptable excipient, diluent, or carrier, and the Wnt surrogate molecule according to claim 1.

56.-70. (Canceled)

71. (Currently Amended) The Wnt Surrogate molecule of claim ~~1~~41, wherein each of the two LRP5/6 binding regions is fused to one of the light chains via a linker moiety.

72.-73. (Canceled)

REMARKS

By this amendment, claims 15 and 71 are amended, and claims 14, 72 and 73 are cancelled, without prejudice or disclaimer. Support for the claim amendments can be found throughout the application as originally filed. No new matter is added.

The above-noted amendments are supported by the application as originally filed and do not introduce any new matter. The amendments are made without prejudice to the prosecution of any canceled or amended subject matter in this or one or more continuing application(s). Amendment and cancellation of certain claims is not to be construed as a dedication to the public of any of the subject matter of the claims as previously presented.

Claim Objection

Claims 14-15 and 71-73 are objected to as being dependent on a rejected base claim.

Applicants respectfully request that this objection is withdrawn with the rejection of the base claims.

Claim Rejections – 35 USC §103

Claims 1, 17 and 55 stand rejected under 35 U.S.C. 103 as allegedly being unpatentable over Garcia et al. ("Garcia"; US 2017/0306029; 8/7/2023 IDS) in view of Wu et al. (("Wu"; Nature Biotechnology, Vol 25, 1290-1297 (2007)).

The Office Action asserts that Garcia teaches water soluble molecules that bind with high affinity to both: (i) Fzd proteins; and (ii) Lrp5/6, and directly activate canonical Wnt pathway signaling. The Examiner acknowledges that Garcia does not teach a dual variable domain (DVD) format of bispecific antibody, but asserts that Wu teaches a DVD format of bispecific antibody, and that it would have been obvious to prepare a bispecific antibody of Garcia in the DVD-Ig format taught be Wu, since Wu teaches a "DVD-Ig format bispecific antibody that is highly stable and exhibits excellent, IgG-like physicochemical and pharmacokinetic properties" (Office

Action at page 6). Moreover, the Office Action asserts that the skilled artisan would be motivated to use the DVD-Ig format, since it provides advantageous properties for therapeutics for human disease, and would have a reasonable expectation of success in view of Wu's teachings related to the stability and IgG-like properties of the DVD-Ig format, and "because making bispecific antibody in a different format is well known in the art and routine experimentation" (Office Action at page 6).

Applicant traverses this rejection.

In order to establish obviousness, the Office must first set forth a *prima facie* case, supported by evidence, showing why the claims at issue would have been obvious in light of the prior art (M.P.E.P. 2142). To establish a *prima facie* case of obviousness, the Examiner is required to analyze the Graham factors; that is, the Examiner must: (a) determine the scope and content of the prior art; (b) ascertain the differences between the claimed invention and the prior art; and (c) resolve the level of ordinary skill in the art (MPEP 2141 (II); citing *KSR International Co., v. Teleflex Inc.*, 550 U.S. 398, 399 (2007). Once the Examiner has performed this factual inquiry, the Examiner is required to provide "some articulated reasoning with some rational underpinning to support the legal conclusion of obviousness." MPEP §2142 (citing *KSR* at 418).

At a minimum, to establish a *prima facie* case of obviousness, the cited reference(s) must teach or suggest all the features of the claim. Even assuming, *arguendo,* that the reference(s) teach each feature, the Office must provide some articulated reasoning with some rational underpinning regarding why a person having ordinary skill in the art would combine these features in the manner claimed by the Applicants with a reasonable expectation of successfully achieving the claimed subject matter. See *KSR International Co. v. Teleflex, Inc.,* 550 U.S. 398, 401 (2007) ("[A] patent composed of several elements is not proved obvious merely by demonstrating that each of its elements was, independently, known in the prior art.").

Thus, for a *prima facie* case of obviousness in the present case, the Examiner must establish that the cited references provide motivation to modify the bispecific molecule disclosed

by Garcia to a DVD-IgG format disclosed by Wu, and that the skilled artisan would reasonably have expected this molecule to have Wnt surrogate activity.

Applicant submits that the Office Action fails to provide the requisite reasoning as to why a person having ordinary skill in the art would have been motivated to combine features of the distinct molecules disclosed in Garcia and Wu with a reasonable expectation of successfully achieving the claimed subject matter. Thus, the Office Action fails to establish a *prima facie* case of obviousness.

Applicant submits that claim 1 is directed to a tetravalent, multispecific Wnt surrogate molecule comprising two Fzd binding regions and two Lrp5/6 binding regions, thus having a 2:2 stoichiometry of 2 Fzd binders:2 LRP5/6 binders (2:2 Fzd binders/Lrp5/6 binders). In contrast, the Wnt surrogates disclosed in Garcia include one Fzd binding region and one LRP binding region, and are bivalent, bispecific molecules comprised of a single polypeptide and having a stoichiometry of 1:1 Fzd binder:LRP5/6 binder. The specific molecules disclosed in Garcia include two different Fzd binding regions: 1) a single-chain variable fragment (scFv) of an antagonistic antibody, vantictumab (18R5), with FZD1,2,5,7,8 specificity; and 2) a de novo designed four-helix bundle protein, B12, which binds FZD5,8. The LRP5/6 binding region of both molecules is a C-terminal domain of the human WNT antagonist, DKK1 (DKK1c). These two WNT surrogates, 18R5scFv-DKK1c and B12-DKK1c both have the stoichiometry of 1:1 Fzd binder:LRP5/6 binder. Garcia does not disclose or contemplate any other stoichiometry between Fzd binding regions and LRP5/6 binding regions. Moreover, Garcia does not recognize or highlight any requirement for higher valency (meaning having more than one FZD binding domain or having more than one LRP binding domain) as a requirement for Wnt signaling activity.

The Office Action rejects Applicant's previous argument that the skilled artisan would not be motivated to change the LRP5/6 binding region from DKK1c to an Lrp5/6 binding region as defined in claim 1, on the alleged basis that "DKK1 taught by Garcia is still encompassed by generically defined 'LRP5/6 binding region' of instant claim 1" (Office Action at page 7), stating that "thus, one of ordinary skill in the art would not need to replace DKK1 with LRP5/6 binding

region of instant invention to arrive at instant claim 1 and dependent claims 17 and 55" (Office Action at page 7).

Applicants submit that the premise of the Examiner's analysis is unfounded, since claim 1 specifically recites that "each of the two LRP5/6 binding regions comprises an antibody or antigen-binding fragment of the antibody." DKK1c is not an antibody or antigen-binding fragment thereof. Accordingly, Applicant reiterates that Garcia, alone or in combination with Wu, fails to motivate the skilled artisan to modify the Lrp5/6 binding region from DKK1c to an antibody or antigen-binding fragment thereof, as claimed.

Moreover, Applicant submits that the skilled artisan would appreciate that the teachings of Wu relate to bivalent molecules that allow the simultaneous blockade of multiple targets (e.g., IL-12 and IL-18), which is very different than the claimed molecules, which simultaneously engage two cell surface coeffector molecules (Fzds and Lrp5/6) required to agonize the canonical Wnt signaling cascade. In particular, Wu describes DVD-Ig format bispecific antibodies that block multiple disease-associated targets, e.g., disease mediators that are playing redundant roles in inducing the same signaling cascade, such as interleukin-1α. Wu describes that DVD-Ig molecules "can be engineered from any two monoclonal antibodies while preserving activities of the parental antibodies" (Wu, Abstract). Wu fails to disclose DVD-IgG molecules or any other molecules with a 2:2 Fzd binders:Lrp5/6 binders stoichiometry that simultaneously bind to coreceptors to induce a signaling pathway, let alone DVD-IgG molecules that bind to Fzd(s) and Lrp5/6 to initiate Wnt signaling. The skilled artisan would appreciate that the ability of a molecule to bind two receptors to block their signaling would not necessarily require that both receptors are bound simultaneously, whereas the claimed molecules have the ability to simultaneously bind both Fzd and Lrp5/6 to initiate Wnt signaling. This represents an important biological distinction between the molecules of Wu and those presently claimed.

Indeed, it is only the present inventors who invented a tetravalent, mutispecific Wnt agonist molecule capable of agonizing a Wnt signaling pathway in cells. Prior to this invention, the need of a tetravalent, multispecific format for Wnt surrogate molecules was neither taught nor contemplated, and the skilled artisan would not have been motivated to alter the teachings of

Garcia to produce a tetravalent, multispecific DVD-Ig format bispecific antibody as disclosed by Wu, since the teachings of Wu are limited to blocking multiple targets and not binding coreceptors to agonize a single signaling pathway. There is no objective reason provided by the teachings of Garcia and Wu that would lead the skilled artisan to combine these references, nor is there any evidence that the resultant combination of these references would lead the skilled artisan to arrive at the claimed invention with predictable results. Instead, the understanding in the art actually teaches away from the claimed Wnt surrogate molecules, as described in the accompanying Declaration of Dr. Yang Li. In this Declaration, Dr. Li explains that the belief in the art at the time of the claimed invention was the inaccurate notion that bivalent molecules with 1:1 Fzd/Lrp5/6 binding stoichiometry were capable of agonizing a Wnt signaling pathway.

Further, even if the Office had established a *prima facie* case of obviousness (which it has not), evidence of surprising or unexpected advantageous properties, such as superiority over the prior art, can rebut *prima facie* obviousness. *See* M.P.E.P. 716.02(a).

Applicant submits that the claimed tetravalent, multispecific Wnt surrogate molecules represent a surprising and unexpected advantage over the prior art. The Declaration of Dr. Li points out that Garcia's teaching fails to render the claimed invention obvious. The need for tetravalent/multivalent design of the WNT surrogate/mimetics molecules was neither taught nor contemplated. Instead, the claimed tetravalent, multispecific Wnt surrogate represent surprising and unexpected superior properties over those described in Garcia, particularly in view of the limited teachings of Wu.

As described in the accompanying Declaration of Dr. Li and Post et al. (submitted herewith), in contrast to WNT mimetics in which one FZD binder was linked to one LRP binder (a bivalent bispecific format denoted1:1design herein), WNT mimetics using antibody fragments for both FZD and LRP binding in the 1:1 format resulted in little to no activity. In one such study of bivalent bispecific antibody-based molecules, WNT mimetic activity was present in crudely purified protein preparations but was lost after the final size exclusion chromatography. Analysis of all size exclusion chromatography fractions surprisingly and unexpectedly indicated recovery of activity in fractions containing species with molecular weights of two or more times the

expected molecular weights of the monomers, e.g., multivalent molecules with a tetravalent bispecific format, or 2:2 Fzd binder:Lrp5/6 binder stoichiometry.

In view of the above, Applicant submits that the claimed tetravalent, mutispecific Wnt surrogate molecules are associated with surprising and unexpected advantages over the prior art, including increased activity, which further establishes their nonobviousness.

The rationale to support a conclusion that the claim would have been obvious is that all the claimed elements were known in the prior art and one skilled in the art could have combined the elements as claimed by known methods with no change in their respective functions, and the combination yielded nothing more than predictable results to one of ordinary skill in the art. None of these cited references teach or suggest 2FZD:2LRP tetravalent, multispecific Wnt signaling modulator, and indeed, certain prior art teaches away from such a format, as discusses in the Declaration of Dr. Li. As such, it is clear that Garcia and Wu, either alone or in combination, fail to teach or suggest the claimed invention, and there is no rational to support the conclusion that any of the pending claims have been obvious over Garcia in view of Wu.

For at least the foregoing reasons, Applicant submits that a case of obviousness has not been established, and respectfully requests withdrawal of the rejection under 35 U.S.C. § 103.

Double Patenting

Claims 1 and 55 stand provisionally rejected on the ground of nonstatutory double patenting as allegedly being unpatentable over claims 1 and 15-16 of copending Application No. 18/148,448 (hereinafter application '448; US2023/0295347).

Claims 1, 17 and 55 stand provisionally rejected on the ground of nonstatutory double patenting as allegedly being unpatentable over claims 1-3 and 6 of copending Application No. 18/281,253 (hereinafter application '253; US2024/0150473).

Applicant respectfully submits that each of these cited references has an effective filing date after the instant application. Accordingly, if these are the only remaining rejections, they

must be withdrawn pursuant to MPEP 804.I.B.1.(b)(i), which indicates that "if a provisional nonstatutory double patenting rejection is the only rejection remaining in an application having the earlier patent term filing date, the examiner should withdraw the rejection in the application having the earlier patent term filing date and permit that application to issue as a patent."

In addition, Applicant respectfully submits that the instant application and the two cited applications are directed to different claimed subject matter, such that the instant claims would not be obvious over the pending claims of the cited applications.

The instant application is directed to a soluble tetravalent, mutispecific Wnt surrogate molecule comprising two Fzd binding regions and two Lrp5/6 binding regions.

In contrast, co-pending application '448 claims a multispecific antibody, as shown below.

1. A multispecific antibody or antibody fragment comprising at least:(A) a first antigen-binding region that specifically binds to frizzled class receptor 4 (Fzd4) and comprises a first heavy chain variable domain (VHl) and a first light chain variable domain (VLl), (B) a second antigen-binding region which specifically binds to low-density lipoprotein receptor-related protein 5 and/or 6 (LRP5 and/or LRP6) and comprises at least a second heavy chain variable domain (VH2),

wherein:

(A) the VHl comprises:

(i) a heavy chain complementarity-determining region 1 (CDRHl) which has an amino acid sequence which: (i-1) has at least 80% identity to the amino acid sequence of SEQ ID NO: 121 or to the CDRHl amino acid sequence contained in SEQ ID NO: 181, optionally defined according to Kabat; or (i-2) comprises or consists of the amino acid sequence of SEQ ID NO: 121, or comprises or consists of the CDRHl amino acid sequence contained in SEQ ID NO: 181, optionally defined according to Kabat;

(ii) a heavy chain complementarity-determining region 2 (CDRH2) which has an amino acid sequence which:

(ii-1) has at least 90% identity to the amino acid …..

Co-pending application '253 claims an engineered WNT agonist as shown below.

1. An engineered WNT agonist comprising:

(a) one or more binding domains that bind to one or more FZD; and

(b) one or more binding domains that bind to LRP5, LRP6, or both LRP5 and LRP6,

wherein the engineered WNT agonist comprises a polypeptide comprising a sequence having at least 90%, at least 95%, at least 98%, or at least 99% sequence identity to SEQ ID NO:9 and a polypeptide comprising a sequence having at least 90%, at least 95%, at least 98%, or at least 99% sequence identity to SEQ ID NO:10, and wherein the one or more binding domains that bind to one or more FZD bind to FZD 5 and FZD 8.

Since the instant claims in the co-pending applications are directed to Wnt surrogates with specific sequences, Applicant submits that they are not obvious over the claims of the cited co-pending application and respectfully requests that the Examiner reconsider and withdraw the provisional obviousness-type double patenting rejections.

CONCLUSION

In view of the foregoing, Applicant respectfully submits that no further impediments exist to the allowance of this application and, therefore, requests an indication of allowability. However, the Examiner is requested to call the undersigned if any questions or comments arise.

The Director is hereby authorized to charge any appropriate fees under 37 C.F.R. §§1.16, 1.17, and 1.21 that may be required by this paper, and to credit any overpayment, to Deposit Account No. 50-1283 referencing Docket No. SRZN-006/04US 328202-2203. However, the Director is not authorized to charge the cost of the issue fee to the Deposit Account.

Dated: February 6, 2025

Respectfully submitted,
COOLEY LLP

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ELECTRONIC PAYMENT RECEIPT

APPLICATION #	RECEIPT DATE / TIME	ATTORNEY DOCKET #
18/361,782	**02/06/2025 01:39:04 PM Z ET**	**SRZN-006/04US 328202-2203**

Title of Invention

WNT SURROGATE MOLECULES AND USES THEREOF

Application Information

APPLICATION TYPE	Utility - Nonprovisional Application under 35 USC 111(a)	PATENT #	-
CONFIRMATION #	9001	FILED BY	Susan Clingerman
PATENT CENTER #	69076317	AUTHORIZED BY	Carol Laherty
CUSTOMER #	157070	FILING DATE	07/28/2023
CORRESPONDENCE ADDRESS	-	FIRST NAMED INVENTOR	Yang LI

Payment Information

PAYMENT METHOD	PAYMENT TRANSACTION ID	PAYMENT AUTHORIZED BY
DA / 501283	**E202526D39576929**	**Susan Clingerman**

FEE CODE	DESCRIPTION	ITEM PRICE($)	QUANTITY	ITEM TOTAL($)
1801	REQUEST FOR CONTINUED EXAMINATION (RCE) - 1ST REQUEST (SEE 37 CFR 1.114)	1500.00	1	1500.00
			TOTAL AMOUNT:	**$1,500.00**

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C.

371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Docket No.: SRZN-006/04US 328202-2203
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Inventor:	Yang LI	Confirmation No.:	9001
Application No.:	18/361,782	Group Art Unit:	1645
Filed:	July 28, 2023	Examiner:	C. Cheong

For: WNT SURROGATE MOLECULES AND USES THEREOF

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INFORMATION DISCLOSURE STATEMENT

In accordance with the duty of disclosure set forth in 37 CFR § 1.56, Applicant hereby submits this Information Disclosure Statement ("IDS") along with the attached Form used in lieu of PTO/SB/08 ("SB/08") and Written Assertion used in lieu of PTO/SB/08C in compliance with 37 CFR §§ 1.97 and 1.98. It is respectfully requested that the information be expressly considered during the prosecution of this Application, and the references be made of record therein and appear among the "References Cited" on any patent to issue therefrom.

In accordance with 37 CFR § 1.97(b), no additional fee for submission of this Information Disclosure Statement is required, as it is filed within any one of the time periods:

[ ] within three months from the filing date of this national Application other than a CPA under 37 CFR § 1.53(d);

[ ] within three months from the date of entry of the national stage as set forth in 37 CFR § 1.491 in this international Application;

[ ] before the mailing date of a first Office action on the merits; or

[X] before the mailing of a first Office action after the filing of a request for continued examination under 37 CFR § 1.114.

In agreement with 37 CFR § 1.97(g), this Information Disclosure Statement shall not be construed as a representation that a search has been made.

In agreement with 37 CFR § 1.97(h), the filing of this Information Disclosure Statement shall not be construed to be an admission that the information cited in the statement is, or is considered to be, relevant, analogous, or material to patentability as defined by 37 CFR § 1.56(b).

As required by 37 CFR § 1.98(a)(2), Applicant submits a copy of any foreign patent and non-patent literature document cited on the SB/08.

As authorized by 37 CFR § 1.98(a)(2)(ii), Applicant does not submit a copy of any U.S. publication or patent information cited on the SB/08.

IDS SIZE FEE - WRITTEN ASSERTION UNDER 37 CFR § 1.98

As required by 37 CFR § 1.98(a)(4), Applicant asserts that with the submission of this Information Disclosure Statement, the cumulative number of items of information that have been provided to the Office during the pendency of the application is 540. As the filing of this Information Disclosure Statement does not cause the cumulative number of items of information to exceed any IDS size threshold specified in 37 CFR §§ 1.17(v)(1), (v)(2), or (v)(3), no IDS size fee under 37 CFR § 1.17(v) is required at this time.

The Director is hereby authorized to charge the fee set forth in 37 CFR § 1.17(p) as indicated above for this Information Disclosure Statement submitted herewith, and to credit any overpayment, to Deposit Account No. 50-1283, referencing Docket No. SRZN-006/04US 328202-2203.

It is respectfully requested that the Examiner consider the above-noted information and return an initialed copy of the SB/08 to the undersigned. The Examiner is encouraged to contact the undersigned by telephone if it is felt that a telephone interview would advance the prosecution of the present Application.

Dated: February 6, 2025

Respectfully submitted,
COOLEY LLP

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(54) Title: WNT SURROGATE AGENTS AND METHODS FOR LACRIMAL GLAND REGENERATION

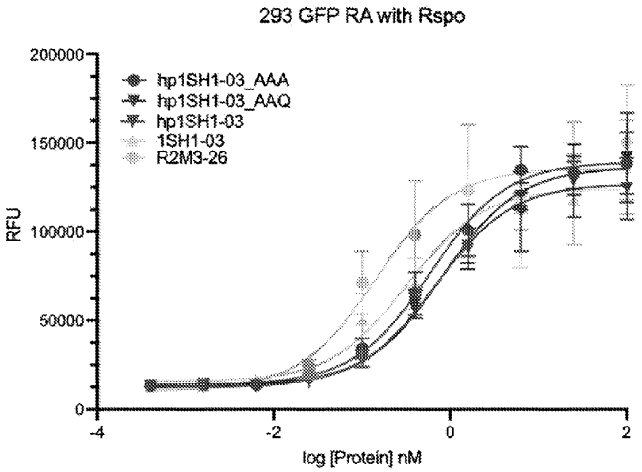


FIG. 7

(57) Abstract: Methods and compositions for regenerating lacrimal gland cells for treatment of dry eye disorders are provided. In particular, the present disclosure provides methods and compositions for modulating WNT signaling by administering engineered WNT signaling agonist in a subject to modulate regeneration of tear-producing acinar cells to treat aqueous-deficient type dry eye diseases.

[Continued on next page]

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Declarations under Rule 4.17:

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

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WNT SURROGATE AGENTS AND METHODS FOR LACRIMAL GLAND REGENERATION

CROSS REFERENCE TO RELATED APPLICATION

[0001] This application claims priority to U.S. Provisional Patent Application Serial No. 63/469,496, filed May 29, 2023, which is incorporated herein by reference in its entirety.

SEQUENCE LISTING

[0002] The Sequence Listing XML associated with this application is provided in XML file format and is hereby incorporated by reference into the specification. The name of the XML file containing the Sequence Listing XML is SRZN_029_01WO_ST26.xml. The XML file is 75,154 bytes, and created on May 24, 2024, and is being submitted electronically via USPTO Patent Center.

FIELD OF THE INVENTION

[0003] The disclosure provides methods and compositions for regenerating lacrimal gland cells for treatment of dry eye disorders are provided. In particular, the disclosure provides methods and compositions for modulating WNT signaling by administering engineered WNT signaling agonist in a subject to modulate regeneration of tear-producing acinar cells to treat aqueous-deficient type dry eye diseases.

BACKGROUND OF THE INVENTION

[0004] The lacrimal gland is an exocrine gland positioned under the outer lateral portion of the upper eyelid in most vertebrates. The lacrimal gland produces a tear film, which is secreted by lacrimal ducts, and is associated with ocular health (*See* Yao & Zhang at 939). The film keeps the cornea surface and inner eyelids moistened and protects the corneal and conjunctival epithelia from physical damage, as well as from immune reactions (*See id.*).

[0005] Lacrimal gland disorders and damage can cause significant ocular disease pathologies, especially dry eye disease (Dartt, OCULAR DISEASE at 105). Dry eye disease is characterized by increased osmolarity of the tear film, and inflammation of the ocular surface (*See id.*). Inadequate production of tears can lead to irritation, pain, and potentially damage to the ocular surface. In addition, dry eye can lead to ocular irritation and discomfort, as well as severe ocular surface diseases (e.g., ocular infections, corneal ulcerations, and ocular surface

keratinization). These severe diseases can be induced by an atrophied or injured lacrimal gland (*See* Tetsuya Kawakita).

[0006] There are two major classes of dry eye diseases: aqueous-deficient and evaporative. In turn, aqueous deficiency is subclassed into Sjögren's syndrome (autoimmune) dry eye and non-Sjögren's syndrome (non-autoimmune) dry eye.

[0007] In cases of aqueous-deficient dry eye, the lacrimal gland is frequently inflamed, leading to atrophy and death of the tear fluid-producing acinar cells. Following loss of acinar cells, tear production problems worsen, leading to a reinforcing vicious inflammation-atrophy cycle. Accordingly, the lacrimal gland is a target in aqueous-deficient dry eye diseases.

[0008] Presently, both aqueous-deficient and evaporative dry eye diseases are treated by topical administration on the ocular surface of artificial tears and/or anti-inflammatory agents. (*See id.* at 106.) Only a few treatments target the lacrimal gland itself, by systemic administration of anti-inflammatory agents, so-called secretagogue agents, immunosuppressive agents, and sex hormones (*See id.*). None of these treatment approaches deal directly with atrophy and/or damage to the lacrimal gland, including the tear-producing acinar cells. Consequently, there is a great unmet need for agents and methods for regeneration of acinar cells, restoring endogenous tear production, and breaking the cycle of inflammation and atrophy.

BRIEF SUMMARY

[0009] The present description provides an engineered multivalent bispecific WNT signaling modulator, wherein the WNT signaling modulator comprises a) at least one first antigen binding domain that binds to at least one of Lrp6 and/or Lrp5; and b) at least one-second antigen binding domains bind to at least one FZD receptor.

[0010] In some embodiments, the engineered WNT signaling modulator is a tetravalent bispecific molecule. In particular embodiments, the engineered WNT signaling modulator of comprises: a) two light chains, each comprising a polypeptide sequence having at least 95% identity to SEQ ID NO:3; and b) two heavy chains, each comprising a polypeptide sequence having at least 95% identity to SEQ ID NO: 4, or 5, or 6.

[0011] In some embodiments of the engineered WNT signaling modulator, each of the two light chains comprises a VHH domain comprising one or more or more of the following amino acid residue substitutions: N29Q and/or VHH3-H4 DDD substituted with DED.

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[0012] In some embodiments of the engineered WNT signaling modulator, each heavy chain comprises amino acid residues: a CDRH1 of SEQ ID NO: 20, a CDRH2 of SEQ ID NO: 21, and a CDRH3 of SEQ ID NO: 22.

[0013] In another embodiments, the engineered WNT signaling modulator comprises amino acid modifications comprising: L234A, L235A, and P329G amino acid substitutions, as numbered according to the EU index. In some embodiments, the heavy chain of SEQ ID NO: 5 comprises Fc region mutation with at least one of the three amino acid modifications selected from the group consisting of: I253A, H310A, and H435A, as numbered according to the EU index. In another embodiments, the heavy chain of SEQ ID NO: 6 comprises Fc region mutation with at least one of the three amino acid modifications selected from the group consisting of: I253A, H310A and H435Q, as numbered according to the EU index.

[0014] In some embodiments, the engineered WNT signaling modulator is a trivalent bispecific molecule. In particular embodiments, the engineered WNT signaling modulator comprises: a) a first antigen binding domain that bind to one of Lrp6 and/or Lrp5; and b) two second antigen binding domains that each bind to one or more or two or more FZD receptors. In certain embodiments, the engineered Wnt signaling molecule binds to two or more FZD receptors.

[0015] In some embodiments of the engineered WNT signaling modulator, the anti-LRP 5/6 is an N-terminal 'Hole' appended, comprising a polypeptides sequence having 95% identity to the combination of SEQ ID NOs: 8, 7, and 9.

[0016] In another embodiments of the engineered WNT signaling modulator, the anti-LRP 5/6 is a C-terminal 'Hole' appended, comprising a polypeptides sequence having 95% identity to the combination of SEQ ID NOs: 13, 7, and 9.

[0017] In particular embodiments of the engineered WNT signaling modulator, the anti-LRP 5/6 is an N-terminal 'Knob' appended, comprising a polypeptides sequence having 95% identity to the combination of SEQ ID NOs: 11, 7, and 12.

[0018] In some embodiments of the engineered WNT signaling modulator, the anti-LRP 5/6 is a C-terminal 'Knob' appended, comprising a polypeptides sequence having 95% identity to the combination of SEQ ID NOs: 10, 7, and 12.

[0019] In some embodiments, the description provides a nucleic acid comprising a sequence encoding the engineered multivalent bispecific WNT signaling modulator, or a polypeptide chain thereof, wherein the nucleic acid(s) is/are DNA, cDNA, RNA, mRNA, modified mRNA, or a DNA/RNA hybrid. In some embodiments, the description provides a vector comprising the nucleic acid wherein the vector is an expression vector comprising a

promoter operatively linked to the nucleic acid. In another embodiments, the description provides a pharmaceutical composition comprising a pharmaceutically acceptable diluent, excipient or carrier, and: the engineered WNT signaling modulator, or the nucleic acid and /or the vector.

[0020] In some embodiments, the disclosure provides a method of regenerating lacrimal gland cells in a subject, comprising administering a WNT signaling modulator to the subject, wherein the subject is a mammal animal or human patient. In another embodiments, the lacrimal gland cells are acinar cells, progenitor cells, ductal cells, myoepithelial cells, or immune cells. In particular embodiments the engineered WNT signaling modulator is a WNT agonist. In some embodiments, the engineered WNT signaling agonist is administered to i) one or more of the subject's eyes, optionally intra-lacrimal gland or via ocular drops; ii) locally administering, optionally to the eye, ear, nose (optionally intranasally), skin (optionally trans dermally or epicutaneously), mucosa, skin, or vagina, or by inhalation; or iii) parenterally administering, optionally by injection (optionally intravenous, intramuscular, subcutaneous, intradermal, intrathecal, intra-arterial, intraarticular, intraosseous, or intraperitoneal administration) or by inhalation; or, buccally, or rectally.

BRIEF DESCRIPTION OF THE DRAWINGS

[0021] FIG. 1 provides the amino acid sequences of a tetravalent bispecific WNT signaling agonist, 1SH1-03 (SEQ ID NOs: 1 and 2), which comprises two heavy chains and two light chains of an anti-FZD antibody, 1SH1, wherein an anti-LRP5/6 VHH3 domain is fused to the N-terminus of each anti-FZD antibody light chain via the linker sequence, GGSGS (SEQ ID NO:26), and its associated IgG-like antibody structure, wherein the two heavy chains are joined via disulfide bonds, and each light chain is joined to a heavy chain via a disulfide bond. The VL sequence of the anti-FZD antibody is:
DVVMTQSPLSLPVTPGEPASISCRSS***QSLLHSNGYNY***LDWYLQKPGQSPQLLIY***LGS***K
RASGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYC***MQALQIPPT***FGQGTKVEIK (SEQ ID NO: 27), and the CDRs are indicated in bold italic underlined. The VH sequence of the anti-FZD antibody is:
EVQLVESGPGLVKPSQTLSLTCTVS***GGSISSGGYY***WSWIRQHPGKGLEWIGY***IYYSGS***
TYYNPSLKSRVTISVDTSKNQFSLKLNSVTAADTAVYYC***ARHAGFYGLADYFDY***WG
QGTLVTVSS (SEQ ID NO: 28), and the CDRs are indicated in bold italic underlined. The VHH domain sequence is:
DVQLVESGGGLVQPGGSLRLSCTSS***ANINSIET***LGWYRQAPGKQRELIAN***MRGGGY***

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MKYAGSLKGRFTMSTESAKNTMYLQMNSLKPEDTAVYYC***YVKLRDDDYVY***RGQGT QVTVSS (SEQ ID NO: 29), and the CDRs are indicated in bold italic underlined. The linker sequence is: GGSGS (SEQ ID NO: 26). The FC sequence is: DKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNW YVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPI EKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPEN NYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP GK (SEQ ID NO: 30).

[0022] FIG. 2 shows various amino acids substitution made to 1SH1-03 molecule to eliminate liable amino acids, including the substitution of N29 with Q (N29Q) in CDR1 of the VHH3 and the substitution of D102 with E (D102E; DDD to DED) in CDR3 of the VHH3, and the substitution of V124 with I (V124I) in the 1SH1 light chain, and the substitutions of V5 with Q (V5Q) and N85 with S (N85S) in the 1SH1 heavy chain. Light chain numbering is provided in the context of the fusion of the VHH3 and 1SH1 light chain for V124I, and in the context of only the 1SH1 light chain domain for N33G and N36T. Thus, V124I corresponds to V2I in the context of the 1SH1 light chain domain.

[0023] FIG. 3 provides the amino acid sequences of the humanized tetravalent bispecific WNT signaling agonist, 1SH1-03 (SEQ ID NOs: 3 and 4), comprising the modifications noted above to the parental 1SH1-03 molecule. The construct has the same general structure as the parental construct. The VHH domain sequence is: EVQLVESGGGLVQPGGSLRLSCASS***ANIQSIET***LGWYRQAPGKQRELIAN***MRGGGY M***KYADSLKGRFTMSTDNSKNTMYLQMNSLRAEDTAVYYC***YVKLRDEDYVY***RGQG TQVTVSS (SEQ ID NO: 31). The VL sequence is: DIVMTQSPLSLPVTPGEPASISCRSS***QSLLHSGGYTY***LDWYLQKPGQSPQLLIY***LGS***KR ASGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYC***MQALQIPPT***FGQGTKVEIKR (SEQ ID NO: 32). The VH sequence is: EVQL**Q**ESGPGLVKPSQTLSLTCTVS***GGSISSGGYY***WSWIRQHPGKGLEWIGY***IYYSGS T***YYNPSLKSRVTISVDTSKNQFSLKL**S**SVTAADTAVYYC***ARHAGFYGLADYFDY***WG QGTLVTVSS (SEQ ID NO: 33). The linker sequence is: GGGGS (SEQ ID NO: 34). The FC sequence is: *DKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVD GVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAK GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPVLDSD GSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK* (SEQ ID NO: 35).

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{0024} FIG. 4 shows the amino acid sequences of humanized tetravalent bispecific WNT signaling agonist, 1SH1-03 (SEQ ID NOs: 3 and 5), with additional mutations in the Fc domain to decrease the serum half-life, including I253A, H310A, and H435A amino acid substitutions. The VHH domain sequence is: EVQLVESGGGLVQPGGSLRLSCASS***ANIQSIET***LGWYRQAPGKQRELIAN***MRGGGY M***KYADSLKGRFTMSTDNSKNTMYLQMNSLRAEDTAVYYC***YVKLRDEDYVY***RGQG TQVTVSS (SEQ ID NO: 31). The VL domain sequence is: DIVMTQSPLSLPVTPGEPASISCRSS***QSLLHSGGYTY***LDWYLQKPGQSPQLLIY***LGS***KR ASGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYC***MQALQIPPT***FGQGTKVEIKR (SEQ ID NO: 32). The VH sequence is: EVQL**Q**ESGPGLVKPSQTLSLTCTVS***GGSISSGGYY***WSWIRQHPGKGLEWIGY***IYYSGS T***YYNPSLKSRVTISVDTSKNQFSLKL**S**SVADTAVYYC***ARHAGFYGLADYFDY***WGQG TLVTVSS (SEQ ID NO: 36). The FC sequence is: *APEAAGGPSVFLFPPKPKDTLMASRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKT KPREEQYNSTYRVVSVLTVLAQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVYT LPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPVLDSDGSFFLYSKLTV DKSRWQQGNVFSCSVMHEALHNAYTQKSLSLSPGK* (SEQ ID NO: 37). The linker sequence is: GGGGS (SEQ ID NO: 34).

{0025} FIG. 5 provides the amino acid sequences of humanized tetravalent bispecific WNT signaling agonist, 1SH1-03 (SEQ ID NOs.: 3 and 6), with additional mutations in the Fc domain to decrease the serum half-life, I253A, H310A, and H435Q. The VHH sequence is: EVQLVESGGGLVQPGGSLRLSCASS***ANIQSIET***LGWYRQAPGKQRELIAN***MRGGGY M***KYADSLKGRFTMSTDNSKNTMYLQMNSLRAEDTAVYYC***YVKLRDEDYVY***RGQG TQVTVSS (SEQ ID NO: 31). The VL sequence is: DIVMTQSPLSLPVTPGEPASISCRSS***QSLLHSGGYTY***LDWYLQKPGQSPQLLIY***LGS***KR ASGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYC***MQALQIPPT***FGQGTKVEIKR (SEQ ID NO: 32). The VH sequence is: EVQL**Q**ESGPGLVKPSQTLSLTCTVS***GGSISSGGYY***WSWIRQHPGKGLEWIGY***IYYSGS T***YYNPSLKSRVTISVDTSKNQFSLKL**S**SVTAADTAVYYC***ARHAGFYGLADYFDY***WG QGTLVTVSS (SEQ ID NO: 38). The FC sequence is: *DKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMASRTPEVTCVVVDVSHEDPEVKFNWYV DGVEVHNAKTKPREEQYNSTYRVVSVLTVLAQDWLNGKEYKCKVSNKALGAPIEKTISKAK GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPVLDSD*

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GSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNQYTQKSLSLSPGK (SEQ ID NO: 39). The linker sequence is: GGGGS (SEQ ID NO: 34).

[0026] FIGs. 6A-6D illustrate constructs of four trivalent bispecific WNT signaling agonist variants of hp1SH1-03, each comprising two heavy chains and two light chains of an anti-FZD antibody, 1SH1, and an anti-LRP5/6 VHH3 domain appended to the N-terminus of either the knob – heavy chain (FIG. 6C) or hole-heavy chain (FIG. 6A) or to the C-terminus of either the knob – heavy chain (FIG. 6D) or hole-heavy chain (FIG. 6B).

[0027] FIG. 7 depicts the response of the WNT reporter cell line HEK293-GFP-RA (TCF-GFP reporter assay) to positive control (R2M3-26), parental 1SH1-03, and humanized polished (hp) 1SH1-03 with and without FcRn mutations AAA and AAQ in the presence of 20 nM RSPO.

[0028] FIG. 8 shows response of the WNT reporter cell line HEK293-GFP-RA (TCF-GFP reporter assay) to positive control (R2M3-26), parental 1SH1-03, humanized polished (hp) 1SH1-03, and trivalent bispecific N-term and C-term hole appended molecules.

[0029] FIG. 9 shows WNT target gene, Axin2, expression 24h after treatment of primary mouse lacrimal gland cells ex vivo (explant assay). Treatments included negative control (R2M3-null), parental 1SH1-03, and the different versions of humanized polished (hp) 1SH1-03 at the concentrations indicated on the X-axis in nM.

[0030] FIG. 10 shows WNT target gene Axin2 expression 24h after local injection of the indicated WNT signaling agonist constructs in the IL-1a model. All treatment groups were treated with a single dose of 10 ug injection. Data was normalized to the anti-GFP control group.

[0031] FIG. 11 depicts WNT target gene Axin2 expression 24h after local treatment in the duct ligation damage model. All treatment groups were treated with a single dose of 10 ug injection. Data was normalized to the R2M3-null negative control group.

[0032] FIG. 12 shows tear volume measurements using phenol red thread in the IL-1a model. Treatments included three different doses of hp1SH1-03 (10 ug, 2 ug and 0.4 ug) as compared to parental 1SH1-03 and the negative control, R2M3-null. At day 3, the lines from top to bottom correspond to: hp1SH1-03 2 ug, 1SH1-03 10 ug, hp1SH1-03 10 ug, R2M3null 10 ug, and hp1SH1-03 0.4 ug.

[0033] FIG. 13 shows tear volume measurements using phenol red thread in the IL-1a model. Data represents an average of twelve animals. Treatments included two different doses of hp1SH1-03 and hp1SH1-03-AAA (10 ug and 2 ug) as compared to parental 1SH1-03 and the negative control, R2M3-null. At day 3, the lines from top to bottom correspond to:

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hp1SH1-03_AAA 2 ug, hp1SH1-03_AAA 10ug, 1SH1-03 10 ug, hp1SH1-03 2 ug, hp1SH1-03 10 ug, and R2M3null 10 ug.

[0034] FIG. 14 describes tear volume measurements using phenol red thread in the mouse duct ligation model of dry eye. Treatments included two different doses of hp1SH1-03 and hp1SH1-03-AAA (10 and 2 ug) and compared to parental 1SH1-03 and negative control R2M3-null. The lines from top to bottom at day 14 correspond to: hp1SH1-03_AAA 10 ug X2, hp1SH1-03_AAA 2 ug, hp1SH1-03 2 ug, 1SH1-03 10 ug, hp1SH1-03_AAA 10 ug, hp1SH1-03 10 ug, R2M3null 10 ug X2, and R2M3null 10 ug.

[0035] FIG. 15 shows proliferation of cornea endothelial cells from human skirt material after 14 days of treatment.

[0036] FIGs. 16A-16D illustrate WNT target gene expression (AXIN2 (FIG. 16A), LEF1 (FIG. 16B), RNF43 (FIG. 16C)) and proliferation marker (CCND1 (FIG. 16D)) after 24h treatment in human endothelial cell .

[0037] FIG. 17 shows the proliferation of cornea endothelial cells from human skirt material after 14 days of treatment with humanized polished (hp) 1SH1-03 (hp1SH1-03_AAA) alone or in combination with engineered FGF TTHX1114.

[0038] FIG. 18 describes cornea WNT target gene Axin2 expression 24h after treatment of non-injured mouse eyes through intracameral injection with the indicated amounts of hp1SH1-03_AAA.

[0039] FIG. 19 shows cornea WNT target gene Axin2 expression 24h after treatment of cryoinjured mouse eyes through intracameral injection with the indicated amounts of hp1SH1-03_AAA.

[0040] FIG. 20 illustrates the measurement of central corneal thickness (CCT) following corneal freeze (cryoinjury) and treatment over the course of 8 days.

[0041] FIG. 21 shows the measurement of central corneal thickness (CCT) following corneal freeze (cryoinjury) and treatment over the course of 7 days.

[0042] FIG. 22 describes pharmacokinetic measurement of serum drug concentration following IV injection (3mpk) of hp1SH1-03 with or without FcRn mutation AAA.

DETAILED DESCRIPTION

I. Definitions

[0043] As used herein, including the appended claims, the singular forms of words such as "a," "an," and "the," include their corresponding plural references unless the context clearly dictates otherwise.

[0044] All references cited herein are incorporated by reference to the same extent as if each individual publication, patent application, or patent, was specifically and individually indicated to be incorporated by reference.

[0045] The terms "Engineered WNT signaling modulator", "Engineered WNT signaling agonist", "WNT surrogate molecule", and "WNT mimetic" are used interchangeably.

[0046] "Activity" of a molecule may describe or refer to the binding of the molecule to a ligand or to a receptor, to catalytic activity, to the ability to stimulate gene expression, to antigenic activity, to the modulation of activities of other molecules, and the like. "Activity" of a molecule may also refer to activity in modulating or maintaining cell-to-cell interactions, e.g., adhesion, or activity in maintaining a structure of a cell, e.g., cell membranes or cytoskeleton. "Activity" may also mean specific activity, e.g., [catalytic activity]/[mg protein], or [immunological activity]/[mg protein], or the like.

[0047] The terms "administering" or "introducing" or "providing", as used herein, refer to delivery of a composition to a cell, to cells, tissues and/or organs of a subject, or to a subject. Such administering or introducing may take place *in vivo*, *in vitro* or *ex vivo*.

[0048] As used herein, the term "antibody" means an isolated or recombinant binding agent that comprises the necessary variable region sequences to specifically bind an antigenic epitope. Therefore, an antibody is any form of antibody or fragment thereof that exhibits the desired biological activity, e.g., binding the specific target antigen. Thus, it is used in the broadest sense and specifically covers monoclonal antibodies (including full-length monoclonal antibodies), polyclonal antibodies, human antibodies, humanized antibodies, chimeric antibodies, nanobodies, diabodies, multispecific antibodies (e.g., bispecific antibodies), and antibody fragments including, but not limited to, scFv, Fab, Fab2, Fab'2, diabody, VHH, single domain antibody, so long as they exhibit the desired biological activity.

[0049] "Antibody fragments" comprise a portion of an intact antibody, for example, the antigen-binding or variable region of the intact antibody. Examples of antibody fragments include Fab, Fab', F(ab')2, and Fv fragments; diabodies; linear antibodies (e.g., Zapata et al.,

Protein Eng. 8(10): 1057-1062 (1995)); single-chain antibody molecules (e.g., scFv); and multispecific antibodies formed from antibody fragments. Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, a designation reflecting the ability to crystallize readily. Pepsin treatment yields an F(ab')2 fragment that has two antigen combining sites and is still capable of cross-linking antigen.

[0050] The term "antigen" refers to a molecule or a portion of a molecule capable of being bound by a selective binding agent, such as an antibody, and 30 additionally capable of being used in an animal to produce antibodies capable of binding to an epitope of that antigen. In certain embodiments, a binding agent (e.g., a WNT surrogate molecule or binding region thereof, or a WNT antagonist) is said to specifically bind an antigen when it preferentially recognizes its target antigen in a complex mixture of proteins and/or macromolecules.

[0051] The term "antigen-binding fragment" as used herein refers to a polypeptide fragment that contains at least one complementarity-determining region (CDR) of an immunoglobulin heavy and/or light chain, or of a VHH/sdAb (single domain antibody) or Nanobody® (Nab), that binds to the antigen of interest, in particular to one or more Fzd receptors, or to LRP5 and/or LRP6. In this regard, an antigen-binding fragment of the herein described antibodies may comprise 1, 2, 3, 4, 5, or all 6 CDRs of a VH and VL from antibodies that bind one or more Fzd receptors or LRP5 and/or LRP6.

[0052] As used herein, the terms "biological activity" and "biologically active" refer to the activity attributed to a particular biological element in a cell. For example, the "biological activity" of a WNT agonist, or fragment or variant thereof refers to the ability to mimic or enhance WNT signals. As another example, the biological activity of a polypeptide or functional fragment or variant thereof refers to the ability of the polypeptide or functional fragment or variant thereof to carry out its native functions of, e.g., binding, enzymatic activity, etc. In some embodiments, a functional fragment or variant retains at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, or at least 100% of an activity of the corresponding native protein or nucleic acid. As a third example, the biological activity of a gene regulatory element, e.g., promoter, enhancer, Kozak sequence, and the like, refers to the ability of the regulatory element or functional fragment or variant thereof to regulate, i.e., promote, enhance, or activate the translation of, respectively, the expression of the gene to which it is operably linked.

[0053] The term "bifunctional antibody," as used herein, refers to an antibody that comprises a first arm having a specificity for one antigenic site and a second arm having a specificity for a different antigenic site, i.e., the bifunctional antibodies have a dual specificity.

[0054] "Bispecific antibody" refers to a full-length antibody that is generated by but not limited to quadroma technology (*see* Milstein et al., *Nature*, 305(5934): 537-540 (1983)), by chemical conjugation of two different monoclonal antibodies (*see* Staerz et al., *Nature*, 314(6012): 628-631 (1985)), or by knob-into-hole or similar approaches, which introduce mutations in the Fc region (*see* Holliger et al., *Proc. Natl. Acad. Sci. USA*, 90(14): 6444–6448 (1993)), resulting in multiple different immunoglobulin species of which only one is the functional bispecific antibody. A bispecific antibody binds one antigen (or epitope) on one of its two binding arms (one pair of HC/LC or VHH), and binds a different antigen (or epitope) on its second arm (a different pair of HC/LC or VHH). By this definition, a bispecific antibody has two distinct antigen-binding arms (in both specificity and CDR sequences) and is monovalent for each antigen to which it binds.

[0055] By "comprising," it is meant that the recited elements are required in, for example, the composition, method, kit, etc., but other elements may be included to form the, for example, composition, method, kit etc. within the scope of the claim. For example, an expression cassette "comprising" a gene encoding a therapeutic polypeptide operably linked to a promoter is an expression cassette that may include other elements in addition to the gene and promoter, e.g., poly-adenylation sequence, enhancer elements, other genes, linker domains, etc.

[0056] By "consisting essentially of," it is meant a limitation of the scope of the, for example, composition, method, kit, etc., described to the specified materials or steps that do not materially affect the basic and novel characteristic(s) of the, for example, composition, method, kit, etc. For example, an expression cassette "consisting essentially of" a gene encoding a therapeutic polypeptide operably linked to a promoter and a polyadenylation sequence may include additional sequences, e.g., linker sequences, so long as they do not materially affect the transcription or translation of the gene. As another example, a variant, or mutant, polypeptide fragment "consisting essentially of" a recited sequence has the amino acid sequence of the recited sequence plus or minus about 10 amino acid residues at the boundaries of the sequence based upon the full length naïve polypeptide from which it was derived, e.g., 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 residue less than the recited bounding amino acid

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residue, or 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 residues more than the recited bounding amino acid residue.

[0057] By "consisting of," it is meant the exclusion from the composition, method, or kit of any element, step, or ingredient not specified in the claim. For example, a polypeptide or polypeptide domain "consisting of" a recited sequence contains only the recited sequence.

[0058] A "control element" or "control sequence" is a nucleotide sequence involved in an interaction of molecules that contributes to the functional regulation of a polynucleotide, including replication, duplication, transcription, splicing, translation, or degradation of the polynucleotide. The regulation may affect the frequency, speed, or specificity of the process, and may be enhancing or inhibitory in nature. Control elements known in the art include, for example, transcriptional regulatory sequences such as promoters and enhancers. A promoter is a DNA region capable under certain conditions of binding RNA polymerase and initiating transcription of a coding region usually located downstream (in the 3' direction) from the promoter.

[0059] An "expression vector" is a vector, e.g., plasmid, minicircle, viral vector, liposome, and the like as discussed herein or as known in the art, comprising a region which encodes a gene product of interest, and is used for effecting the expression of the gene product in an intended target cell. An expression vector also comprises control elements, e.g., promoters, enhancers, untranslated regions (UTRs), miRNA targeting sequences, etc., operatively linked to the encoding region to facilitate expression of the gene product in the target. The combination of control elements and a gene or genes to which they are operably linked for expression is sometimes referred to as an "expression cassette," a large number of which are known and available in the art or can be readily constructed from components that are available in the art.

[0060] As used herein, the term "FR set" refers to the four flanking amino acid sequences which frame the CDRs of a CDR set of a heavy or light chain V region. Some FR residues may contact bound antigen; however, FRs are primarily responsible for folding the V region into the antigen-binding site, particularly the FR residues directly adjacent to the CDRs. Within FRs, certain amino residues and certain structural features are very highly conserved. In this regard, all V region sequences contain an internal disulfide loop of around 90 amino acid residues. When the V regions fold into a binding-site, the CDRs are displayed as projecting loop motifs which form an antigen-binding surface. It is generally recognized that there are conserved structural regions of FRs which influence the folded shape of the CDR loops into certain "canonical" structures—regardless of the precise CDR amino acid

sequence. Further, certain FR residues are known to participate in non-covalent interdomain contacts which stabilize the interaction of the antibody heavy and light chains.

[0061] As used herein, the term "HC" refers to the heavy chain and the term "LC" refers to the light chain.

[0062] The terms "individual," "host," "subject," and "patient" are used interchangeably herein, and refer to a mammal, including, but not limited to, human and non-human primates, including simians and humans; mammalian sport animals (e.g., horses); mammalian farm animals (e.g., sheep, goats, etc.); mammalian pets (dogs, cats, etc.); and rodents (e.g., mice, rats, etc.).

[0063] "Humanized" antibodies or fragments thereof refers to antibodies or fragments thereof from non-human species whose protein sequences have been modified to increase their similarity to antibody variants produced naturally in humans. The process of "humanization" is usually applied to monoclonal antibodies developed for administration to humans.

[0064] A "monoclonal antibody" refers to a homogeneous antibody population wherein the monoclonal antibody is comprised of amino acids (naturally occurring and non-naturally occurring) that are involved in the selective binding of an epitope. Monoclonal antibodies are highly specific, being directed against a single epitope. The term "monoclonal antibody" encompasses not only intact monoclonal antibodies and full-length monoclonal antibodies, but also fragments thereof (such as Fab, Fab', F(ab')2, Fv), single chain (scFv), single domain antibodies (sdAbs, also known as Nanobodies), variants thereof, fusion proteins comprising an antigen-binding fragment of a monoclonal antibody, humanized monoclonal antibodies, chimeric monoclonal antibodies, and any other modified configuration of the immunoglobulin molecule that comprises an antigen- binding fragment (epitope recognition site) of the required specificity and the ability to bind to an epitope, including WNT surrogate molecules disclosed herein. It is not intended to be limited as regards the source of the antibody or the manner in which it is made (e.g., by hybridoma, phage selection, recombinant expression, transgenic animals, etc.). The term includes whole immunoglobulins as well as the fragments etc. described above under the definition of "antibody".

[0065] The term "native" or "wild-type" as used herein refers to a nucleotide sequence, e.g., gene, or gene product, e.g., RNA or protein, that is present in a wild-type cell, tissue, organ, or organism. The term "variant" as used herein refers to a mutant of a reference polynucleotide or polypeptide sequence, for example, a native polynucleotide or polypeptide sequence, i.e., having less than 100% sequence identity with the reference polynucleotide or

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polypeptide sequence. Put another way, a variant comprises at least one amino acid difference (e.g., amino acid substitution, amino acid insertion, amino acid deletion) relative to a reference polynucleotide sequence, e.g., a native polynucleotide or polypeptide sequence. For example, a variant may be a polynucleotide having a sequence identity of 50% or more, 60% or more, or 70% or more with a full-length native polynucleotide sequence, e.g., an identity of 75% or 80% or more, such as 85%, 90%, or 95% or more, for example, 98% or 99% identity with the full-length native polynucleotide sequence. As another example, a variant may be a polypeptide having a sequence identity of 70% or more with a full-length native polypeptide sequence, e.g., an identity of 75% or 80% or more, such as 85%, 90%, or 95% or more, for example, 98% or 99% identity with the full-length native polypeptide sequence. Variants may also include variant fragments of a reference, e.g., native, sequence sharing a sequence identity of 70% or more with a fragment of the reference, e.g., native, sequence, e.g., an identity of 75% or 80% or more, such as 85%, 90%, or 95% or more, for example, 98% or 99% identity with the native sequence.

[0066] "Operatively linked" or "operably linked" refers to a juxtaposition of genetic elements, wherein the elements are in a relationship permitting them to operate in the expected manner. For instance, a promoter is operatively linked to a coding region if the promoter helps initiate transcription of the coding sequence. There may be intervening residues between the promoter and coding region so long as this functional relationship is maintained.

[0067] As used herein, the terms "polypeptide," "peptide," and "protein" refer to polymers of amino acids of any length. The terms also encompass an amino acid polymer that has been modified; for example, to include disulfide bond formation, glycosylation, lipidation, phosphorylation, or conjugation with a labeling component.

[0068] The term "polynucleotide" refers to a polymeric form of nucleotides of any length, including deoxyribonucleotides or ribonucleotides, or analogs thereof. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs, and may be interrupted by non-nucleotide components. If present, modifications to the nucleotide structure may be imparted before or after assembly of the polymer. The term polynucleotide, as used herein, refers interchangeably to double-stranded and single-stranded molecules. Unless otherwise specified or required, any embodiment of the invention described herein that is a polynucleotide encompasses both the double-stranded form and each of two complementary single-stranded forms known or predicted to make up the double-stranded form.

[0069] A polynucleotide or polypeptide has a certain percent "sequence identity" to another polynucleotide or polypeptide, meaning that, when aligned, that percentage of bases or amino acids are the same when comparing the two sequences. The terms "identical" or "identity" when used in the context of two or more nucleic acids or polypeptide sequences, refer to the number or percentage of residues that are the same in a sequence of interest and a reference sequence. The percentage can be calculated by optimally aligning the sequence of interest to the reference sequence; comparing the two sequences over the entire length of the reference sequence; determining the number of positions at which the identical amino acid residue or nucleic acid base occurs in both sequences to yield the number of matched positions; dividing the number of matched positions by the total number of positions in the reference sequence adjusted by adding the number of gap positions introduced into the reference sequence in generating the alignment; and multiplying the result by 100 to yield the percentage of sequence identity. When comparing DNA and RNA, thymine (T) and uracil (U) can be considered equivalent Sequence "identity" may be determined by using the stand-alone executable BLAST engine program for blasting two sequences (bl2seq), which can be retrieved from the National Center for Biotechnology Information (NCBI) ftp site or over the worldwide web at ncbi.nlm.nih.gov/BLAST/, using the default parameters (Tatusova and Madden, FEMS Microbiol Lett., 1999, 174, 247-250; which is incorporated herein by reference in its entirety).

[0070] A "promoter" as used herein encompasses a DNA sequence that directs the binding of RNA polymerase and thereby promotes RNA synthesis, i.e., a minimal sequence sufficient to direct transcription. Promoters and corresponding protein or polypeptide expression may be ubiquitous, meaning strongly active in a wide range of cells, tissues and species or cell-type specific, tissue-specific, or species specific. Promoters may be "constitutive," meaning continually active, or "inducible," meaning the promoter can be activated or deactivated by the presence or absence of biotic or abiotic factors. Also included in the nucleic acid constructs or vectors of the invention are enhancer sequences that may or may not be contiguous with the promoter sequence. Enhancer sequences influence promoter-dependent gene expression and may be located in the 5' or 3' regions of the native gene.

[0071] "Recombinant," as applied to a polynucleotide means that the polynucleotide is the product of various combinations of cloning, restriction or ligation steps, and other procedures that result in a construct that is distinct from a polynucleotide found in nature.

[0072] As used herein "Sjögren's syndrome" or "Sjogren's syndrome" is a chronic autoimmune disease affecting the lacrimal and salivary glands, defined according to

customary international diagnostic criteria such as described in Vitali et al (2002) *Ann.Rheum. Dis.* 61:554. Sjögren's syndrome is one of two major subclasses of aqueous-deficient dry eye diseases, distinguished from non-Sjögren's syndrome type.

[0073] As used herein, "SWAP™" (Surrozen WNT-signal activating proteins) refers to WNT mimetic compounds comprising engineered bi-specific full-length immunoglobulin-G (IgG) antibodies that, like WNT proteins, directly activate the canonical WNT-signaling pathway in a target tissue, e.g., lacrimal gland tissue.

[0074] As used herein, "SWEETS™" (Surrozen WNT-signal enhancer engineered for tissue specificity) refers to antibody-based R-spondin mimetic compounds, e.g., as described in US20200048324.

[0075] The terms "treatment", "treating" and the like are used herein to generally mean obtaining a desired pharmacologic and/or physiologic effect. The effect may be prophylactic in terms of completely or partially preventing a disease or symptom thereof, e.g., reducing the likelihood that the disease or symptom thereof occurs in the subject, and/or may be therapeutic in terms of a partial or complete cure for a disease and/or adverse effect attributable to the disease. "Treatment" as used herein covers any treatment of a disease in a mammal and includes: (a) inhibiting the disease, i.e., arresting or slowing its development; or (b) relieving the disease, i.e., causing regression or reduction of the disease or reducing the severity of the disease. The therapeutic agent may be administered before, during or after the onset of disease or injury. The treatment of ongoing disease, where the treatment stabilizes or reduces the undesirable clinical symptoms of the patient, is of particular interest. Such treatment is desirably performed prior to complete loss of function in the affected tissues. The subject therapy will desirably be administered during the symptomatic stage of the disease, and in some cases after the symptomatic stage of the disease.

[0076] The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, molecular biology techniques), microbiology, biochemistry and immunology, which are within the scope of those of skill in the art. Such techniques are explained fully in the literature, such as, "Molecular Cloning: A Laboratory Manual", second edition (Sambrook et al., 1989); "Oligonucleotide Synthesis" (M. J. Gait, ed., 1984); "Animal Cell Culture" (R. I. Freshney, ed., 1987); "Methods in Enzymology" (Academic Press, Inc.); "Handbook of Experimental Immunology" (D. M. Weir & C. C. Blackwell, eds.); "Gene Transfer Vectors for Mammalian Cells" (J. M. Miller & M. P. Calos, eds., 1987); "Current Protocols in Molecular Biology" (F. M. Ausubel et al., eds., 1987); "PCR: The Polymerase Chain Reaction", (Mullis et al., eds., 1994); and "Current Protocols in

Immunology" (J. E. Coligan et al., eds., 1991), each of which is expressly incorporated by reference herein.

[0077] Several aspects of the invention are described below with reference to example applications for illustration. It should be understood that numerous specific details, relationships, and methods are set forth to provide a full understanding of the invention. One having ordinary skill in the relevant art, however, will readily recognize that the invention can be practiced without one or more of the specific details or with other methods. The present invention is not limited by the illustrated ordering of acts or events, as some acts may occur in different orders and/or concurrently with other acts or events. Furthermore, not all illustrated acts or events are required to implement a methodology in accordance with the present invention.

[0078] The terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting of the invention. As used herein, the singular forms "a", "an" and "the" are intended to include the plural forms as well, unless the context clearly indicates otherwise. Furthermore, to the extent that the terms "including", "includes", "having", "has", "with", or variants thereof are used in either the detailed description and/or the claims, such terms are intended to be inclusive in a manner similar to the term "comprising".

[0079] The term "about" or "approximately" means within an acceptable error range for the particular value as determined by one of ordinary skill in the art, which will depend in part on how the value is measured or determined, i.e., the limitations of the measurement system. For example, "about" can mean within 1 or more than 1 standard deviation, per the practice in the art. Alternatively, "about" can mean a range of up to 20%, preferably up to 10%, more preferably up to 5%, and more preferably still up to 1% of a given value. Alternatively, particularly with respect to biological systems or processes, the term can mean within an order of magnitude, preferably within 5-fold, and more preferably within 2-fold, of a value. Where particular values are described in the application and claims, unless otherwise stated the term "about" meaning within an acceptable error range for the particular value should be assumed.

[0080] All publications mentioned herein are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited. It is understood that the present disclosure supersedes any disclosure of an incorporated publication to the extent there is a contradiction.

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[0081] It is further noted that the claims may be drafted to exclude any optional element. As such, this statement is intended to serve as antecedent basis for use of such exclusive terminology as "solely", "only" and the like in connection with the recitation of claim elements, or the use of a "negative" limitation.

[0082] Unless otherwise indicated, all terms used herein have the same meaning as they would to one skilled in the art and the practice of the present invention will employ, conventional techniques of microbiology and recombinant DNA technology, which are within the knowledge of those of skill of the art.

II. General

[0083] The present invention provides methods of modulating WNT signals and WNT signaling pathways to treat lacrimal gland disorders due to, including but not limited to, Sjögren's syndrome disorder, chronic graft versus host disease (cGHVD), rheumatoid arthritis (RA), Stephen's Johnson syndrome, Ocular Rosacea, chemotherapy, radiation oncology treatments, diabetes, roasacea, or lupus, etc.

[0084] WNT ("Wingless-related integration site" or "Wingless and Int-1" or "Wingless-Int") ligands and their signals play key roles in the control of development, homeostasis and regeneration of many essential organs and tissues, including bone, liver, skin, stomach, intestine, kidney, central nervous system, mammary gland, taste bud, ovary, cochlea, lung, and many other tissues (reviewed, e.g., by Clevers, Loh, and Nusse, 2014; 346:1248012). Modulation of WNT signaling pathways has potential for treatment of degenerative diseases and tissue injuries.

[0085] One of the challenges for modulating WNT signaling as a therapeutic is the existence of multiple WNT ligands and WNT receptors, Frizzled 1-10 (Fzd1-10), with many tissues expressing multiple and overlapping Fzds. Canonical WNT signals also involve Low-density lipoprotein (LDL) receptor-related protein 5 (LRP5) or Low-density lipoprotein (LDL) receptor-related protein 6 (LRP6) as co-receptors, which are broadly expressed in various tissues, in addition to Fzds. R-spondins 1–4 are a family of ligands that amplify WNT signals. Each of the R-spondins work through a receptor complex that contains Zinc and Ring Finger 3 (ZNRF3) or Ring Finger Protein 43 (RNF43) on one end and a Leucine-rich repeat-containing G-protein coupled receptor 4-6 (LGR4-6) on the other (reviewed, e.g., by Knight & Hankenson 2014, *Matrix Biol.*; 37: 157-161). R-spondins might also work through additional mechanisms of action. ZNRF3 and RNF43 are two membrane-bound E3 ligases specifically targeting WNT receptors (Fzd1-10 and LRP5 or LRP6) for degradation. Binding

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of an R-spondin to ZNRF3/RNF43 and LGR4-6 causes clearance or sequestration of the ternary complex, which removes E3 ligases from WNT receptors and stabilizes WNT receptors, resulting in enhanced WNT signals. Each R-spondin contains two Furin domains (1 and 2), with Furin domain 1 binding to ZNRF3/RNF43, and Furin domain 2 binding to LGR4-6. Fragments of R-spondins containing Furin domains 1 and 2 are sufficient for amplifying WNT signaling. While R-spondin effects depend on WNT signals, since both LGR4-6 and ZNRF3/RNF43 are widely expressed in various tissues, the effects of R-spondins are not tissue-specific.

[0086] Activating WNT signaling by a WNT agonist may be used for the treatment of a variety of lacrimal gland diseases and disorders, including dry eye diseases. Similarly, amplifying WNT signaling by RSPO or an RSPO mimetic may be used for the treatment of a variety of lacrimal gland diseases and disorders, including various dry eye and salivary gland diseases. A WNT agonist molecule may also be used for the treatment of dry eye and salivary gland disorders. In particular, active WNT signaling can provide a major stem cell maintenance signal and plays a key role in regulating regeneration of the acinar cells, e.g., in salivary glands.

III. Engineered WNT Agonists

[0087] The present disclosure provides engineered WNT agonists and contemplates the use of engineered WNT agonists to stimulate, agonize, or promote WNT signaling, e.g., through the canonical WNT/β-catenin signaling pathway. Such engineered WNT agonists may also be referred to as WNT/ β -catenin signaling agonists or WNT mimetics.

[0088] The disclosure provides engineered WNT mimetics with drug-like properties, particularly in the form of recombinant, multivalent bi-specific antibodies that bring together Fzd and Lrp to stimulate signaling, mimicking endogenous WNT ligands. The WNT mimetics of the disclosure may freely diffuse, access damaged tissues and guide tissue repair where WNT signals are needed. The disclosure also provides WNT mimetics that are capable of repairing damaged lacrimal or salivary gland tissue without being combined with RSPO.

[0089] In some embodiments, the WNT/β-catenin signaling antagonist or agonist can include binding agents or epitope binding domains that bind one or more Fzd receptors and inhibit or enhance WNT signaling. In certain embodiments, the agent or antibody specifically binds to the cysteine-rich domain (CRD) within the human frizzled receptor(s) to which it binds.

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[0090] Additionally, antagonistic binding agents containing epitope binding domains against LRP can also be used. In some embodiments, the WNT/β-catenin antagonist possesses binding agents or epitope binding domains that bind E3 ligases ZNRF3/RNF43 and one or more FZD receptors or one or more LRP co-receptors to promote the degradation of FZD or LRP receptors, and this molecule can also contain a binding domain that binds a cell type specific epitope for targeting. The E3 ligase agonist antibodies or fragments thereof can be single molecules or combined with other WNT antagonists, e.g., Fzd receptor antagonists, LRP receptor antagonists, etc.

[0091] As is well known in the art, an antibody is an immunoglobulin molecule capable of specific binding to a target such as a carbohydrate, polynucleotide, lipid, polypeptide, etc., through at least on epitope binding domain, located on the variable region of the immunoglobulin molecule. As used herein, the term encompasses not only intact polyclonal or monoclonal antibodies, but also fragments thereof containing epitope binding domains (e.g., dAb, Fab, Fab', $(F(ab')_2$, Fv, single chain (scFv), VHH (i.e., Nanobodies®) or single domain antibodies (sdAb), DVD-Igs (also known as Fv-Igs), synthetic variants thereof, naturally occurring variants, fusion proteins comprising and epitope binding domain, humanized antibodies, chimeric antibodies, and any other modified configuration of the immunoglobulin molecule that comprises an antigen-binding site or fragment (epitope recognition site) of the required specificity. "Diabodies," multivalent or multispecific fragments constructed by gene fusion (WO94/13804; P. Holliger et al., *Proc. Natl. Acad. Sci. USA* 90 6444-6448, 1993) are also a particular form of antibody contemplated herein. Minibodies comprising a scFv joined to a CH3 domain are also included herein (S. Hu et al., *Cancer Res.*, 56, 3055-3061, 1996). *See e.g.*, Ward, E. S. et al., *Nature* 341, 544-546 (1989); Bird et al., *Science*, 242, 423-426, 1988; Huston et al., *Proc. Natl. Acad. Sci. USA*, 85, 5879-5883, 1988); PCT/US92/09965; WO94/13804; P. Holliger et al., *Proc. Natl. Acad. Sci. USA* 90 6444–6448, 1993; Y. Reiter et al., *Nature Biotech*, 14, 1239-1245, 1996; S. Hu et al., *Cancer Res.*, 56, 3055-3061, 1996; C. Bever et al., *Anal Bioanal Chem.* 2016 Sept; 408(22); 5985–6002.

[0092] The proteolytic enzyme papain preferentially cleaves IgG molecules to yield several fragments, two of which (the F(ab) fragments) each comprise a covalent heterodimer that includes an intact antigen-binding site. The enzyme pepsin is able to cleave IgG molecules to provide several fragments, including the F(ab')2 fragment which comprises both antigen-binding sites. An Fv fragment for use according to certain embodiments of the

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present disclosure can be produced by preferential proteolytic cleavage of an IgM, and on rare occasions of an IgG or IgA immunoglobulin molecule. Fv fragments are, however, more commonly derived using recombinant techniques known in the art. The Fv fragment includes a non-covalent VH::VL heterodimer including an antigen-binding site which retains much of the antigen recognition and binding capabilities of the native antibody molecule. Inbar et al. (1972) *Proc. Nat. Acad. Sci. USA* 69:2659-2662; Hochman et al. (1976) *Biochem* 15:2706-2710; and Ehrlich et al. (1980) *Biochem* 19:4091-4096.

[0093] In certain embodiments, single chain Fv or scFV antibodies are contemplated. For example, Kappa bodies (Ill et al., *Prot. Eng.* 10: 949-57 (1997)); minibodies (Martin et al., *EMBO J* 13: 5305-9 (1994)); diabodies (Holliger et al., *Proc. Nat. Acad. Sci.* 90: 6444-8 (1993)); or Janusins (Traunecker et al., *EMBO J* 10: 3655-59 (1991) and Traunecker et al., *Int. J. Cancer Suppl.* 7: 51-52 (1992)), may be prepared using standard molecular biology techniques following the teachings of the present application with regard to selecting antibodies having the desired specificity. In still other embodiments, bispecific or chimeric antibodies may be made that encompass the ligands of the present disclosure. For example, a chimeric antibody may comprise CDRs and framework regions from different antibodies, while bispecific antibodies may be generated that bind specifically to one or more Fzd receptors through one binding domain and to a second molecule through a second binding domain. These antibodies may be produced through recombinant molecular biological techniques or may be physically conjugated together.

[0094] A single chain Fv (scFv) polypeptide is a covalently linked VH:VL heterodimer which is expressed from a gene fusion including VH- and VL-encoding genes linked by a peptide-encoding linker. Huston et al. (1988) *Proc. Nat. Acad. Sci. USA* 85(16):5879-5883. A number of methods have been described to discern chemical structures for converting the naturally aggregated—but chemically separated—light and heavy polypeptide chains from an antibody V region into an scFv molecule which will fold into a three-dimensional structure substantially similar to the structure of an antigen-binding site. *See, e.g.*, U.S. Pat. Nos. 5,091,513 and 5,132,405, to Huston et al.; and U.S. Pat. No. 4,946,778, to Ladner et al.

[0095] In certain embodiments, an antibody as described herein is in the form of a diabody. Diabodies are multimers of polypeptides, each polypeptide comprising a first domain comprising a binding region of an immunoglobulin light chain and a second domain comprising a binding region of an immunoglobulin heavy chain, the two domains being linked (e.g., by a peptide linker) but unable to associate with each other to form an antigen binding site: antigen binding sites are formed by the association of the first domain of one

polypeptide within the multimer with the second domain of another polypeptide within the multimer (WO94/13804).

[0096] A dAb fragment of an antibody consists of a VH domain (Ward, E. S. et al., (1989) *Nature* 341:544-546).

[0097] Where bispecific antibodies are to be used, these may be conventional bispecific antibodies, which can be manufactured in a variety of ways (Holliger, P. & Winter G., *Curr. Opin. Biotech.* 4, 446-449 (1993)), e.g., prepared chemically or from hybrid hybridomas, or may be any of the bispecific antibody fragments mentioned above. Diabodies and scFv can be constructed without an Fc region, using only variable domains, potentially reducing the effects of anti-idiotypic reaction.

[0098] Bispecific diabodies, as opposed to bispecific whole antibodies, may also be particularly useful because they can be readily constructed and expressed in E. coli. Diabodies (and many other polypeptides such as antibody fragments) of appropriate binding specificities can be readily selected using phage display (WO94/13804) from libraries. If one arm of the diabody is to be kept constant, for instance, with a specificity directed against antigen X, then a library can be made where the other arm is varied and an antibody of appropriate specificity selected. Bispecific whole antibodies may be made by knobs-into-holes engineering (J. B. B. Ridgeway et al., *Protein Eng.*, 9, 616-621 (1996)).

[0099] In certain embodiments, the antibodies described herein may be provided in the form of a UniBody®. A UniBody® is an IgG4 antibody with the hinge region removed (see GenMab Utrecht, The Netherlands; see also, e.g., US2009/0226421). This proprietary antibody technology creates a stable, smaller antibody format with an anticipated longer therapeutic window than current small antibody formats. IgG4 antibodies are considered inert and thus do not interact with the immune system. Fully human IgG4 antibodies may be modified by eliminating the hinge region of the antibody to obtain half-molecule fragments having distinct stability properties relative to the corresponding intact IgG4 (GenMab, Utrecht). Halving the IgG4 molecule leaves only one area on the UniBody® that can bind to cognate antigens (e.g., disease targets) and the UniBody® therefore binds univalently to only one site on target cells.

[0100] In certain embodiments, antibodies and antigen-binding fragments thereof as described herein include a heavy chain and a light chain CDR set, respectively interposed between a heavy chain and a light chain framework region (FR) set which provide support to the CDRs and define the spatial relationship of the CDRs relative to each other. As used herein, the term "CDR set" refers to the three hypervariable regions of a heavy or light chain

V region. Proceeding from the N-terminus of a heavy or light chain, these regions aredenoted as "CDR1," "CDR2," and "CDR3" respectively. An antigen-binding site, therefore, includes six CDRs, comprising the CDR set from each of a heavy and a light chain V region. A polypeptide comprising a single CDR, (e.g., a CDR1, CDR2 or CDR3) is referred to herein as a "molecular recognition unit." Crystallographic analysis of a number of antigen-antibody complexes has demonstrated that the amino acid residues of CDRs form extensive contact with bound antigen, wherein the most extensive antigen contact is with the heavy chain CDR3. Thus, the molecular recognition units are primarily responsible for the specificity of an antigen-binding site.

[0101] As used herein, the term "FR set" refers to the four flanking amino acid sequences which frame the CDRs of a CDR set of a heavy or light chain V region. Some FR residues may contact bound antigen; however, FRs are primarily responsible for folding the V region into the antigen-binding site, particularly the FR residues directly adjacent to the CDRs. Within FRs, certain amino residues and certain structural features are very highly conserved. In this regard, all V region sequences contain an internal disulfide loop of around 90 amino acid residues. When the V regions fold into a binding-site, the CDRs are displayed as projecting loop motifs which form an antigen-binding surface. It is generally recognized that there are conserved structural regions of FRs which influence the folded shape of the CDR loops into certain "canonical" structures—regardless of the precise CDR amino acid sequence. Further, certain FR residues are known to participate in non-covalent interdomain contacts which stabilize the interaction of the antibody heavy and light chains.

[0102] A "monoclonal antibody" refers to a homogeneous antibody population wherein the monoclonal antibody is comprised of amino acids (naturally occurring and non-naturally occurring) that are involved in the selective binding of an epitope. Monoclonal antibodies are highly specific, being directed against a single epitope. The term "monoclonal antibody" encompasses not only intact monoclonal antibodies and full-length monoclonal antibodies, but also fragments thereof (such as Fab, Fab', F(ab')2, Fv), single chain (scFv), Nanobodies®, variants thereof, fusion proteins comprising an antigen-binding fragment of a monoclonal antibody, humanized monoclonal antibodies, chimeric monoclonal antibodies, and any other modified configuration of the immunoglobulin molecule that comprises an antigen- binding fragment (epitope recognition site) of the required specificity and the ability to bind to an epitope, including WNT surrogate molecules disclosed herein. It is not intended to be limited as regards the source of the antibody or the manner in which it is made (e.g., by hybridoma, phage selection, recombinant expression, transgenic animals, etc.). The term includes whole

immunoglobulins as well as the fragments etc. described above under the definition of "antibody".

[0103] In certain embodiments, the antibodies of the present disclosure may take the form of a single domain antibody (sdAb). SdAb technology was originally developed following the discovery and identification that camelidae (e.g., camels, alpacas, and llamas) possess fully functional antibodies that consist of heavy chains only and therefore lack light chains. These heavy-chain only antibodies contain a single variable domain (VHH) and two constant domains (CH2, CH3). The cloned and isolated single variable domains have full antigen binding capacity and are very stable. These single variable domains, with their unique structural and functional properties, form the basis of sdAbs. SdAb are encoded by single genes and are efficiently produced in almost all prokaryotic and eukaryotic hosts, e.g., E. coli (see, e.g., U.S. Pat. No. 6,765,087), molds (for example Aspergillus or Trichoderma) and yeast (for example Saccharomyces, Kluyvermyces, Hansenula, or Pichia (see, e.g., U.S. Pat. No. 6,838,254). The production process is scalable and multi-kilogram quantities of sdAbs have been produced. SdAbs may be formulated as a ready-to-use solution having a long shelf life. The Nanoclone® method (see, e.g., WO 06/079372) is a proprietary method for generating sdAbs against a desired target, based on automated high-throughput selection of B-cells. SdAb are single-domain antigen-binding fragments of camelid-specific heavy-chain only antibodies. sdAb, also referred to as VHH antibodies, typically have a small size of around 15 kDa. *See* C. Bever et al., *Anal Bioanal Chem.* 2016 Sept; 408(22); 5985–6002.

[0104] Another antibody fragment contemplated is a dual-variable domain-immunoglobulin (DVD-Ig or Fv-Ig) is an engineered protein that combines the function and specificity of two monoclonal antibodies in one molecular entity. An Fv-Ig is designed as an IgG-like molecule, except that each light chain and heavy chain contains two variable domains in tandem through a short peptide linkage, instead of one variable domain in IgG. The fusion orientation of the two variable domains and the choice of linker sequence are critical to functional activity and efficient expression of the molecule. An Fv-Ig can be produced by conventional mammalian expression systems as a single species for manufacturing and purification. An Fv-Ig has the specificity of the parental antibodies, is stable in vivo, and exhibits IgG-like physicochemical and pharmacokinetic properties. Fv-Igs and methods for making them are described in Wu, C., et al., *Nat Biotech*, 25:1290–1297 (2007)).

[0105] In certain embodiments, the antibodies or antigen-binding fragments thereof as disclosed herein are humanized. This refers to a chimeric molecule, generally prepared using

recombinant techniques, having an antigen- binding site derived from an immunoglobulin from a non-human species and the remaining immunoglobulin structure of the molecule based upon the structure and/or sequence of a human immunoglobulin. The antigen-binding site may comprise either complete variable domains fused onto constant domains or only the CDRs grafted onto appropriate framework regions in the variable domains. Epitope binding sites may be wild type or modified by one or more amino acid substitutions. This eliminates the constant region as an immunogen in human individuals, but the possibility of an immune response to the foreign variable region remains (LoBuglio, A. F. et al., (1989) *Proc Natl Acad Sci USA* 86:4220–4224; Queen et al., *PNAS* (1988) 86:10029–10033; Riechmann et al., *Nature* (1988) 332:323–327). Illustrative methods for humanization of the anti-Fzd or LRP antibodies disclosed herein include the methods described in U.S. Pat. No. 7,462,697.

[0106] Another approach focuses not only on providing human-derived constant regions but modifying the variable regions as well so as to reshape them as closely as possible to human form. It is known that the variable regions of both heavy and light chains contain three complementarity-determining regions (CDRs) which vary in response to the epitopes in question and determine binding capability, flanked by four framework regions (FRs) which are relatively conserved in a given species and which putatively provide a scaffolding for the CDRs. When nonhuman antibodies are prepared with respect to a particular epitope, the variable regions can be "reshaped" or "humanized" by grafting CDRs derived from nonhuman antibody on the FRs present in the human antibody to be modified. Application of this approach to various antibodies has been reported by Sato, K., et al., (1993) *Cancer Res* 53:851-856; Riechmann, L., et al., (1988) *Nature* 332:323-327; Verhoeyen, M., et al., (1988) *Science* 239:1534-1536; Kettleborough, C. A., et al., (1991) *Protein Engineering* 4:773-3783; Maeda, H., et al., (1991) Human Antibodies Hybridoma 2:124-134; Gorman, S. D., et al., (1991) *Proc Natl Acad Sci USA* 88:4181-4185; Tempest, P. R., et al., (1991) *Bio/Technology* 9:266-271; Co, M. S., et al., (1991) *Proc Natl Acad Sci USA* 88:2869-2873; Carter, P., et al., (1992) *Proc Natl Acad Sci USA* 89:4285-4289; and Co, M. S. et al., (1992) *J Immunol* 148:1149-1154. In some embodiments, humanized antibodies preserve all CDR sequences (for example, a humanized mouse antibody which contains all six CDRs from the mouse antibodies). In other embodiments, humanized antibodies have one or more CDRs (one, two, three, four, five, six) which are altered with respect to the original antibody, which are also termed one or more CDRs "derived from" one or more CDRs from the original antibody.

[0107] In certain embodiments, the antibodies of the present disclosure may be chimeric antibodies. In this regard, a chimeric antibody is comprised of an antigen-binding fragment of

an antibody operably linked or otherwise fused to a heterologous Fc portion of a different antibody. In certain embodiments, the heterologous Fc domain is of human origin. In other embodiments, the heterologous Fc domain may be from a different Ig class from the parent antibody, including IgA (including subclasses IgA1 and IgA2), IgD, IgE, IgG (including subclasses IgG1, IgG2, IgG3, and IgG4), and IgM. In further embodiments, the heterologous Fc domain may be comprised of CH2 and CH3 domains from one or more of the different Ig classes. As noted above with regard to humanized antibodies, the antigen-binding fragment of a chimeric antibody may comprise only one or more of the CDRs of the antibodies described herein (e.g., 1, 2, 3, 4, 5, or 6 CDRs of the antibodies described herein), or may comprise an entire variable domain (VL, VH or both).

[0108] The structures and locations of immunoglobulin CDRs and variable domains may be determined by reference to Kabat, E. A. et al., SEQUENCES OF PROTEINS OF IMMUNOLOGICAL INTEREST, 4th Edition, US Department of Health and Human Services. 1987, and updates thereof, now available on the Internet (immuno.bme.nwu.edu).

[0109] In certain embodiments, the antagonist or agonist binding agent binds with a dissociation constant (K_D) of about 1 μM or less, about 100 nM or less, about 40 nM or less, about 20 nM or less, or about 10 nM or less. For example, in certain embodiments, a FZD binding agent or antibody described herein that binds to more than one FZD, binds to those FZDs with a K_D of about 100nM or less, about 20 nM or less, or about 10 nM or less. In certain embodiments, the binding agent binds to one or more its target antigen with an EC50 of about 1 μM or less, about 100 nM or less, about 40 nM or less, about 20 nM or less, about 10 nM or less, or about 1 nM 20 or less.

[0110] The antibodies or other agents of the present invention can be assayed for specific binding by any method known in the art. Immunoassays that can be used include, but are not limited to, competitive and non-competitive assay systems using techniques such as biolayer interferometry (BLI) analysis, FACS analysis, immunofluorescence, immunocytochemistry, Western blots, radioimmunoassays, ELISA, "sandwich" immunoassays, immunoprecipitation assays, precipitation reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays, and protein A immunoassays. Such assays are routine and well known in the art (*see, e.g.*, Ausubel et al, eds, 1994, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, Vol. 1, John Wiley & Sons, Inc., New York, which is incorporated by reference herein in its entirety).

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[0111] For example, the specific binding of an antibody to a target antigen may be determined using ELISA. An ELISA assay comprises preparing antigen, coating wells of a 96 well microtiter plate with antigen, adding the antibody or other binding agent conjugated to a detectable compound such as an enzymatic substrate (e.g., horse-radish peroxidase or alkaline phosphatase) to the well, incubating for a period of time and detecting the presence of the antigen. In some embodiments, the antibody or agent is not conjugated to a detectable compound, but instead a second conjugated antibody that recognizes the first antibody or agent is added to the well. In some embodiments, instead of coating the well with the antigen, the antibody or agent can be coated to the well and a second antibody conjugated to a detectable compound can be added following the addition of the antigen to the coated well. One of skill in the art would be knowledgeable as to the parameters that can be modified to increase the signal detected as well as other variations of ELISAs known in the art (*see, e.g.*, Ausubel et al, eds, 1994, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, Vol. 1, John Wiley & Sons, Inc., New York at 11.2.1).

[0112] The binding affinity of an antibody or other agent to a target antigen and the off-rate of the antibody-antigen interaction can be determined by competitive binding assays. One example of a competitive binding assay is a radioimmunoassay comprising the incubation of labeled antigen (e.g., Fzd, LRP), or fragment or variant thereof, with the antibody of interest in the presence of increasing amounts of unlabeled antigen followed by the detection of the antibody bound to the labeled antigen. The affinity of the antibody and the binding off-rates can be determined from the data by scattered plot analysis. In some embodiments, BLI analysis is used to determine the binding on and off rates of antibodies or agents. BLI kinetic analysis comprises analyzing the binding and dissociation of antibodies from chips with immobilized antigens on their surface.

IV. Compositions and Methods of Use of WNT Agonists

[0113] The present description provides methods of treating lacrimal gland diseases and disorders, including but not limited to dry eye disorders, e.g., by activation and/or regeneration of lacrimal gland acinar cells, and compositions therefore.

[0114] It will be readily understood to persons skilled in the art that lacrimal gland is homologous to Meibomian gland and accessory lacrimal glands, and the methods and compositions described herein may be applied for treatment of the Meibomian glands and/or accessory lacrimal glands, e.g., in aqueous-deficient or evaporative dry eye. Meibomian glands are oil glands which line the margin of eyelids. These glands cause the eyelids to

secrete oils that blend with natural tears, preventing excessive evaporation of the natural tears. Patients suffering from ocular rosacea often have Meibomian gland dysfunction (MGD) where the eyelids secrete fewer oils resulting dry eyes. MGD is thought to be the leading cause of dry eye disease. A WNT agonist molecule may also be used for the treatment of Meibomian gland disorders. In particular, active WNT signaling can potentially provide a maintenance signal to basal progenitor cells and plays a key role in regulating regeneration of the meibocytes (see, e.g., Parfitt et al (2016) *Stem Cell Rep.*7:399-410). In one aspect, the present invention comprises a method of regenerating lacrimal gland acinar cells in a subject, comprising administering a WNT signaling modulator to the subject. In an embodiment, the WNT signaling modulator may be an engineered WNT signaling modulator such as a SWAP™ compound. In another embodiment, the WNT signaling modulator is an engineered WNT agonist or an engineered WNT antagonist. In yet a further embodiment, the WNT signaling modulator is a tissue-specific WNT signal enhancing molecule, such as a SWEETS™ molecule. A WNT signaling modulator may also be a combination of a WNT agonist and a tissue-specific WNT signal enhancer.

[0115] In particular embodiments of any of the methods disclosed herein, the WNT agonist is selected from those disclosed in any of the following: PCT Application Publication No. WO 2016/040895; US Application Publication No. US 2017-0306029; US Application Publication No. US 2017-0349659; PCT Application Publication No. WO 2019/126398; PCT Application Publication No. WO 2020/01030, PCT Application Publication No. WO 2021/173726, or U.S. Application No. 17/806,624, all of which are herein incorporated by reference in their entireties. In particular embodiments of any of the methods disclosed herein, the tissue-specific WNT signal enhancing molecule is selected from those disclosed in any of the following: PCT Application Publication No. WO 2018/140821; US Application Publication No. US 2020-0048324; or PCT Application Publication No. WO 2020/14271, all of which are herein incorporated by reference in their entireties. In particular embodiments of any of methods disclosed herein, WNT surrogate agents and methods for lacrimal gland generation is selected from those disclosed in the PCT Application No. PCT/US2022/081884, which is incorporated by reference in its entirety.

[0116] In an embodiment of the method of regenerating lacrimal gland acinar cells, the WNT signaling modulator may comprise at least one engineered bi-specific full-length IgG antibody that directly activates a canonical WNT signaling pathway. In any embodiment, the engineered WNT agonist may be selected from: (*i*) WNT3a; (*ii*) a WNT mimetic; or (*iii*) an R-spondin mimetic. The WNT mimetic may be a SWAP™ compound. The R-spondin

mimetic may be a SWEETS™ compound. The WNT mimetics may comprise one or more polypeptides comprising or having a polypeptide sequence set forth in any of SEQ ID Nos: 1-13 isoforms and homologs thereof, and suitable expression vectors therefor. The WNT mimetics may comprise one or more polypeptides having 80% to 100% homology (or at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% identity) with any of the polypeptide sequences set forth in SEQ ID Nos 1-13. In certain embodiments, the WNT mimetic comprises two polypeptide sequences, each having 80% to 100% homology (or at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% identity) with any of the polypeptide sequences set forth in SEQ ID Nos 1-13.

[0117] In certain embodiments, the WNT mimetic comprises a light chain variable region sequence and/or a heavy chain variable region sequence, each independently comprising at least one, at least two, or all three CDR sequences disclosed in any of SEQ ID NOs: 1-13. In certain embodiments, the light chain variable region and/or heavy chain variable region each independently comprise zero, less than two, less than three, less than four, less than five, less than six, less than seven, or less than eight amino acid substitutions within the CDRs. In certain embodiments, the Wnt agonist comprises zero, less than two, less than three, less than four, less than five, less than six, less than seven, less than eight, less than nine, less than ten, less than eleven, or less than twelve amino acid substitutions within the CDRs.

[0118] In certain embodiments, the engineered WNT signaling modulator is a tetravalent bispecific molecule comprising two heavy chain and two light chain polypeptide sequences, each having 80% to 100% homology (or at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% identity) with any of the polypeptide sequences set forth in SEQ ID NOs 1-6. In particular embodiments, the heavy chain and light chain polypeptides present in the WNT mimetic have at least 80%, at least 90%, at least 95%, at least 98%, or 100% identity to any of following combinations: SEQ ID NOs: 1 and 2; SEQ ID NOs: 3 and 4; SEQ ID NOs: 3 and 5; or SEQ ID NOs: 3 and 6. In particular embodiments fo the engineered WNT signaling modulator, each of the two heavy chain and two light chain polypeptides, each having 80% to 100% homology (or at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% identity) with any of the polypeptide sequences set forth in SEQ ID NOs: 1-6, comprise VHH, light chain and heavy chain CDR sequences set forth in any of SEQ ID NOs: 1-6. In particular embodiments, the light chain polypeptides comprise the VHH CDRs set forth in SEQ ID NOs: 14-16 or SEQ ID NOs: 23, 15, and 24. In particular embodiments, the light chain polypeptides comprise the light chain variable region CDRs set forth in SEQ ID NOs: 17-19 or SEQ ID NOs: 25, 18, and 19. In

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particular embodiments, the heavy chain polypeptides comprise the heavy chain CDRs set forth in SEQ ID NOs: 20-22. In particular embodiments, the WNT mimetic has an IgG antibody structure comprising two heavy and two light chains, wherein the two heavy chains are bound to each other, and each of the light chains is bound to a different one of the two heavy chains. In certain embodiments, the WNT signaling modulator comprises a VHH sequence comprising at least one, at least two, or all three VHH CDR sequences disclosed in any of SEQ ID NOs: 1-6. In certain embodiments, the VHH region comprise zero, less than two, less than three, less than four, less than five, less than six, less than seven, or less than eight amino acid substitutions within the VHH CDRs. In certain embodiments, the WNT signaling modulator comprises a light chain variable region sequence and/or a heavy chain variable region sequence, each independently comprising at least one, at least two, or all three CDR sequences disclosed in any of SEQ ID NOs: 1-6. In certain embodiments, the light chain variable region and/or heavy chain variable region each independently comprise zero, less than two, less than three, less than four, less than five, less than six, less than seven, or less than eight amino acid substitutions within the CDRs. In certain embodiments, the Wnt agonist comprises zero, less than two, less than three, less than four, less than five, less than six, less than seven, less than eight, less than nine, less than ten, less than eleven, or less than twelve amino acid substitutions within the CDRs. In particular embodiments, Wnt signaling modulators and variants thereof, and heavy chains and light chains thereof (e.g., those having at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% identity to any one of SEQ ID NOs: 1-6) comprise one or more the amino acid modifications introduced into hp1SH1-03, hp1SH1-03_AAA, or hp1SH1-03_AAQ as compared to 1SH1-03, e.g., one or more (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or all) of the amino acid modifications shown in any of FIGs. 2-5. In particular embodiments, the WNT mimetic comprises amino acid modifications corresponding to one or more of N29Q or D102E in the VHH, N33, N36T, or V124I in the 1SH1 light chain, or V5Q or N85S in the 1SH1 heavy chain. In particular embodiments, the Wnt mimetic comprises a light chain polypeptide comprising the CDRs set forth in SEQ ID NO: 1 or SEQ ID NO:3 and one or more of the amino acid substitutions corresponding to N29Q or D102E in the VHH, N33, N36T, or V124I in the 1SH1 light chain, or V5Q or N85S in the 1SH1 heavy chain.

[0119] In particular embodiments, the engineered WNT signaling modulator is a trivalent bispecific molecule, wherein the anti-LRP 5 and/or LRP 6 binder is a VHH appended either from N- or C- terminal of either hole or knob of the heavy chain polypeptide sequence each having 80% to 100% homology (or at least 80%, at least 85%, at least 90%, at least 95%, at

least 98%, at least 99%, or 100% identity) with any of the polypeptide sequences set forth in SEQ ID NOs: 7-13. In particular embodiments, the heavy chain and light chain polypeptides present in the WNT mimetic have at least 80%, at least 90%, at least 95%, at least 98%, or 100% identity to any of following combinations: SEQ ID NOs: 7, 8, and 9; SEQ ID NOs: 7, 9 13; SEQ ID NOs: 7, 11, 12; SEQ ID NOs: 7,10 and 12. In certain embodiments, the WNT signaling modulator comprises a light chain variable region sequence and/or a heavy chain variable region sequence, each independently comprising at least one, at least two, or all three CDR sequences disclosed in any of SEQ ID NOs: 7-13. In certain embodiments, the light chain variable region and/or heavy chain variable region each independently comprise zero, less than two, less than three, less than four, less than five, less than six, less than seven, or less than eight amino acid substitutions within the CDRs. In certain embodiments, the Wnt agonist comprises zero, less than two, less than three, less than four, less than five, less than six, less than seven, less than eight, less than nine, less than ten, less than eleven, or less than twelve amino acid substitutions within the CDRs. . In particular embodiments, Wnt signaling modulators and variants thereof, and heavy chains and light chains thereof (e.g., those having at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% identity to any one of SEQ ID NOs: 7-13) comprise one or more the amino acid modifications introduced into 1SH1-03, e.g., one or more (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or all) of the amino acid modifications shown in any of FIGs. 2-5.

[0120] In certain embodiments, the disclosure provides WNT signaling agonists, including but not limited to hp1SH1-03, and also fragments and variants thereof. In particular embodiments, the WNT signaling agonist has a structure diagrammed in Fig. 3, which is a humanized structure of the antibody showed in Fig1. Fig. 3 shows hp1SH1-03 comprises one or both of the sequences disclosed in Fig. 3, or any functional fragment or variant thereof, e.g., an antigen-binding fragment thereof, such as a VHH domain, VL domain, or VH domain, or an Fc domain, or a variant of any of these functional domains, e.g., a variant having at least 90%, at least 95%, at least 98%, or at least 99% sequence identity to any functional fragment of antibody sequence disclosed herein. In some embodiments, a WNT signaling agonist comprises a variant sequence having at least 90% identity (e.g., 95%, 98% or 100% identity) to a sequence disclosed in Fig. 3 or set forth in SEQ ID Nos: 3 and /or 4, or any or any functional fragment thereof, e.g., an antigen-binding fragment thereof. In some embodiments, a WNT signaling agonist comprises a wild type or variant sequence having at least 90% identity (e.g., 95%, 98% or 100% identity) to a light chain sequence disclosed in Fig. 3 or set forth in SEQ ID NO: 3, and a wild type or variant

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sequence having at least 90% identity (e.g., 95%, 98% or 100% identity) to a heavy chain sequence disclosed in Fig. 3 or set forth in SEQ ID NO:4. In particular embodiments, any variant sequence comprises one or more of the amino acid residues introduced into the parental molecule, 1SH1-03, to generate hp1SH1-03, including but not limited to, e.g., those in VHH3-H4 in Fig. 2, VHH3-H4 N29Q, VHH3-H4 DDD substituted with DED in Fig. 2, 1SH1 HC CDR1 N31S, and CDR2 D62E in Fig 3, and/or those in the humanized 1SH1 framework, as shown in Fig. 2. In certain embodiments, CDRH3 D108 and D111 are maintained. In certain embodiments, VHH3 M51 and M57 in VHH CDR3 are maintained. In further embodiments, mutations may be present in 1SH1 IgG framework on the light chain V2I, and/or on the heavy chain, e.g., V5Q and N92S, which mutations were made to de-risk immunogenicity to closest germline sequence of hp-1SH1; the sequences of hp1SH1-03 is shown in Fig. 3. In certain embodiments, the WNT signaling modulator comprises a light chain variable region sequence and/or a heavy chain variable region sequence, each independently comprising at least one, at least two, or all three CDR sequences disclosed in Fig. 3, or variants thereof, wherein the sequence comprises zero, less than two, less than three, less than four, less than five, less than six, less than seven, or less than eight amino acid modifications, e.g., substitutions, within the CDRs. In certain embodiments, the light chain variable region and/or heavy chain variable region each independently comprise zero, less than two, less than three, less than four, less than five, less than six, less than seven, or less than eight amino acid substitutions within the CDRs. In certain embodiments, the Wnt agonist comprises zero, less than two, less than three, less than four, less than five, less than six, less than seven, less than eight, less than nine, less than ten, less than eleven, or less than twelve amino acid substitutions within the CDRs.

{0121} In some embodiments, the hp1SH1-03 comprises one or more (e.g., one, two, or three) of the following amino acid modifications: L234A, L235A, and P329G amino acid substitutions, and optionally all three. In particular embodiments of any of the IgG disclosed herein, the Fc of the IgG comprises one or more of the following amino acid substitutions: N297G, N297A, N297E, L234A, L235A, P236G. In further embodiments, in an effort to generate variants with shorter in vivo circulation time, the Fc region of hp1SH1-03 was modified to further have the following amino acid substitutions: I253A, H310A, and H435A (also referred to as IAHAHA or AAA herein); or I253A, H310A, and H435Q (also referred to as IAHAHQ or AAQherein). The hp1SH1-03 variant that additionally comprises IAHAHA was named hp1SH1-03 AAA, and the hp1SH1-03 variant that additionally comprises IAHAHQ was named hp1SH1-03 AAQ. No changes were made to the light chain of

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hp1SH1-03. Amino acid sequences of the heavy and light chains of hp1SH1-03AAA (SEQ ID NOs: 3 and 5), and hp1SH1-03 AAQ (SEQ ID NOs: 3 and 6) are shown in FIGS. 4 and 5, respectively. Without wishing to be bound by theory, reduced in vivo circulation time (half-life, T½) may provide reduced toxicity.

[0122] In an embodiment of the method of regenerating lacrimal gland acinar cells, the WNT signaling modulator may affect expression of any one or more of: Fzd1, Fzd2, Fzd5, Fzd7, Fzd8, and Lrp6, and/or Lrp5. For example, the WNT signaling modulator may target any one or more of the group consisting of: Fzd1, Fzd2, and Fzd7; or any one or more of the group consisting of: Fzd5 and Fzd8, while also targeting Lrp6 and/or Lrp5. For another example, the WNT signaling modulator may affect expression of any one or more of: Fzd1, Fzd2, and Fzd7. The WNT signaling modulator may cause an increase or decrease in any of these Fzds.

[0123] In one embodiment of the method of regenerating lacrimal gland acinar cells, the method further comprises the step of administering at least one of the group consisting of: RSPO, RSPO fragment, and engineered RSPO mimetic.

[0124] In some embodiment the WNT signaling modulator may be a superagonist of WNT platform. This superagonist activity was observed with LRP, FZD, and RSPO fused WNT molecule acting as a robust activator of WNT signaling pathway. the WNT superagonist is selected from those disclosed in the following: PCT Application Publication No. WO 2021/173726.

[0125] In some embodiments, the WNT signaling modulator may be present in a pharmaceutical composition in a concentration ≥1μM. For example, the WNT signaling modulator may be in a concentration of 1μM, 1.1μM, 1.5μM, 2.0μM, 2.5μM, 3.0μM, 3.5μM, 4.0μM, 4.5μM, 5.0μM. In certain embodiments, the concentration of WNT signaling modulator may be ≥5μM. In any embodiment, the WNT signaling modulator may be in a concentration ≥1nM. For example, the WNT signaling modulator may be in a concentration of 1nM, 1.1nM, 1.5nM, 2.0nM, 2.5nM, 3.0nM, 3.5nM, 4.0nM, 4.5nM, 5.0nM. In certain embodiments, the concentration of WNT signaling modulator may be ≥5nM.

[0126] In any embodiment, the WNT signaling modulator may be in a concentration ≥100 pM. For example, the WNT signaling modulator may be in a concentration of 100 pM, 200 pM, 400 pM, 800p M, 1.0 nM, 10,0 nM, 100.0nM, 1 μM, 2 uM, 3μM, 5.0μM. In certain embodiments, the concentration of WNT signaling modulator may be ≥5nM.

[0127] In any embodiment, the WNT signaling modulator may be administered in a therapeutically effective amount.

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[0128] In any embodiment, the subject may be a live mammal. For example, the subject may be mouse, rat, dog, cat, horse, or cow. In any embodiment, the subject may be a mammal, e.g., a human patient.

[0129] In any embodiment, the WNT signaling modulator may be administered systemically or locally. For example, the WNT signaling modulator may be administered locally via aqueous eyedrop solution or local intra-lacrimal gland injection.

[0130] In another aspect, the present invention comprises a method of treating a lacrimal gland disorder in a subject, comprising administering a WNT signaling modulator to the subject. In an embodiment, the WNT signaling modulator may be an engineered WNT signaling modulator. In another embodiment, the WNT signaling modulator is an engineered WNT agonist In an embodiment of the method of treating a lacrimal gland disorder, the WNT signaling modulator may affect expression of any one or more of: Fzd1, Fzd2, Fzd5, Fzd7, Fzd8, and Lrp6 and/or Lrp5. For example, the WNT signaling modulator may target any one or more of the group consisting of: Fzd1, Fzd2, and Fzd7; or any one or more of the group consisting of: Fzd5 and Fzd8, while also targeting Lrp6 and/or Lrp5. For another example, the WNT signaling modulator may affect expression of any one or more of: Fzd1, Fzd2, and Fzd7.

[0131] In one embodiment of the method of treating a lacrimal gland disorder, the method further comprises the step of administering at least one of the group consisting of: RSPO2, RSPO2 fragment, and engineered RSPO2 mimetic.

[0132] In any embodiment, the subject may be a live mammal. For example, the subject may be mouse, rat, dog, cat, horse, or cow. In any embodiment, the subject may be a human patient.

[0133] In any embodiment, the WNT signaling modulator may be administered systemically or locally. For example, i) to one or more of the subject's eyes, optionally intravitreally or via ocular drops; ii)locally administering, optionally to the eye, ear, nose (optionally intranasally), skin (optionally transdermally or epicutaneously), mucosa, skin, or vagina, or by inhalation; or iii) parenterally administering, optionally by injection (optionally intravenous, intramuscular, subcutaneous, intradermal, intrathecal, intra-arterial, intraarticular, intraosseous, or intraperitoneal administration) or by inhalation; or iv) enterally administering, optionally orally, sublingually, buccally, or rectally.

[0134] In still another aspect, the present invention comprises a composition for the treatment of a dry eye disorder in a subject, the composition comprising a WNT signaling

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modulator. In any embodiment of the composition, the dry eye disorder may be a Sjögren's syndrome disorder.

[0135] In an embodiment of the composition, the WNT signaling modulator may comprise at least one engineered bi-specific full-length IgG antibody that directly activates a canonical WNT signaling pathway. The at least one engineered bi-specific full-length IgG antibody may be specific for any one or more of Fzd1, Fzd,2, Fzd5, Fzd7, Fzd8, and Lrp6 or Lrp5.

[0136] In an embodiment, the composition may further comprise an at least one additional agent, including an anti-inflammatory agent, an artificial tear agent, or an lacrimal gland secretagogue. In particular, the anti-inflammatory agent may be antibiotics or steroids including cyclosporine A (e.g., Restasis®) and lifitegrast ophthalmic solution (e.g., Xiidra®). Artificial tears agent may include non-prescription eye drops that simulate tears, e.g., hydroxypropyl cellulose (Lacrisert®) inserts. Lacrimal gland secretagogue agents may include varenicline nasal spray, which selectively agonizes nicotinic acetylcholine receptors. Also, contemplated are autologous blood serum eye drops.

[0137] In any embodiment of the composition, the composition may comprise therapeutically effective amounts of each of its components.

[0138] In any embodiment of the composition, the subject may be a live mammal. For example, the subject may be mouse, rat, dog, cat, horse, or cow. In any embodiment of the composition, the subject may be a human patient.

[0139] In further embodiments, pharmaceutical compositions comprising an expression vector, e.g., a viral vector, comprising a polynucleotide comprising a nucleic acid sequence encoding a WNT antagonist/agonist molecule described herein and one or more pharmaceutically acceptable diluent, carrier, or excipient are also disclosed. In certain embodiments, the nucleic acid sequence encoding the WNT antagonist molecule and the nucleic acid sequence encoding the WNT agonist are in the same polynucleotide, e.g., expression cassette.

[0140] The present disclosure further contemplates a pharmaceutical composition comprising a cell comprising an expression vector comprising a polynucleotide comprising a promoter operatively linked to a nucleic acid encoding a WNT antagonist/agonist molecule and one or more pharmaceutically acceptable diluent, carrier, or excipient. In particular embodiments, the pharmaceutical composition further comprises a cell comprising an expression vector comprising a polynucleotide comprising a promoter operatively linked to a nucleic acid sequence encoding a WNT antagonist and a WNT agonist. In certain

embodiments, the nucleic acid sequence encoding the WNT antagonist molecule and the nucleic acid sequence encoding the WNT agonist molecule are present in the same polynucleotide, e.g., expression cassette and/or in the same cell. In particular embodiments, the cell is a heterologous cell or an autologous cell obtained from the subject to be treated.

[0141] In particular embodiments, the cell is a stem cell, e.g., an adipose-derived stem cell or a hematopoietic stem cell. The present disclosure contemplates pharmaceutical compositions comprising a first molecule for delivery of a WNT antagonist molecule as a first active agent, and a WNT agonist as a second molecule. The first and second molecule may be the same type of molecule or different types of molecules. For example, in certain embodiments, the first and second molecule may each be independently selected from the following types of molecules: polypeptides, small organic molecules, nucleic acids encoding the first or second active agent (optionally DNA or mRNA, optionally modified RNA), vectors comprising a nucleic acid sequence encoding the first or second active agent (optionally expression vectors or viral vectors), and cells comprising a nucleic acid sequence encoding the first or second active agent (optionally an expression cassette).

[0142] The subject molecules, alone or in combination, can be combined with pharmaceutically acceptable carriers, diluents, excipients and reagents useful in preparing a formulation that is generally safe, non-toxic, and desirable, and includes excipients that are acceptable for mammalian, e.g., human or primate, use. Such excipients can be solid, liquid, semisolid, or, in the case of an aerosol composition, gaseous. Examples of such carriers, diluents and excipients include, but are not limited to, water, saline, Ringer's solutions, dextrose solution, and 5% human serum albumin. Supplementary active compounds can also be incorporated into the formulations. Solutions or suspensions used for the formulations can include a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial compounds such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating compounds such as ethylenediaminetetraacetic acid (EDTA); buffers such as acetates, citrates or phosphates; detergents such as Tween 20 to prevent aggregation; and compounds for the adjustment of tonicity such as sodium chloride or dextrose. The pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. In particular embodiments, the pharmaceutical compositions are sterile.

[0143] Pharmaceutical compositions may further include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological

saline, bacteriostatic water, or phosphate buffered saline (PBS). In some cases, the composition is sterile and should be fluid such that it can be drawn into a syringe or delivered to a subject from a syringe. In certain embodiments, it is stable under the conditions of manufacture and storage and is preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be, e.g., a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the internal compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

[0144] Sterile solutions can be prepared by incorporating the WNT antagonist/agonist antibody or antigen-binding fragment thereof (or encoding polynucleotide or cell comprising the same) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, methods of preparation are vacuum drying and freeze-drying that yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile- filtered solution thereof.

[0145] In one embodiment, the pharmaceutical compositions are prepared with carriers that will protect the antibody or antigen-binding fragment thereof against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially. Liposomal suspensions can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art.

[0146] It may be advantageous to formulate the pharmaceutical compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active antibody or antigen-binding fragment thereof calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms is dictated by and directly dependent on the unique characteristics of the antibody or antigen-binding fragment thereof and the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active antibody or antigen-binding fragment thereof for the treatment of individuals.

[0147] The pharmaceutical compositions can be included in a container, pack, or dispenser, e.g., eye dropper, e.g., a prefilled eye dropper, together with instructions for administration.

[0148] The pharmaceutical compositions of the present disclosure may be delivered to a subject in the form of a pill, capsule, cream, salve, syrup, dermal patch, suppository, intravenous drip, local injection aqueous solution, non-aqueous solution, eye wash solution, or any combination of thereof.

[0149] The pharmaceutical compositions of the present disclosure may be delivered to a subject by direct ophthalmic application, muscular injection, intra-lacrimal gland injection, subconjunctival injection, meibomian gland injection, intravenous injection, peritoneal injection, nasally, orally, rectally, or any combination thereof.

[0150] The pharmaceutical compositions of the present disclosure encompass any pharmaceutically acceptable salts, esters, or salts of such esters, or any other compound which, upon administration to an animal comprising a human, is capable of providing (directly or indirectly) the biologically active antibody or antigen-binding fragment thereof.

[0151] The present disclosure includes pharmaceutically acceptable salts of a WNT antagonist/agonist molecule described herein. The term "pharmaceutically acceptable salt" refers to physiologically and pharmaceutically acceptable salts of the compounds of the present disclosure: i.e., salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto. A variety of pharmaceutically acceptable salts are known in the art and described, e.g., in "Remington's Pharmaceutical Sciences", 17th edition, Alfonso R. Gennaro (Ed.), Mark Publishing Company, Easton, PA, USA, 1985 (and more recent editions thereof), in the "Encyclopedia of Pharmaceutical Technology", 3rd edition, James Swarbrick (Ed.), Informa Healthcare USA (Inc.), NY, USA,

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2007, and in *J. Pharm. Sci.* 66:2 (1977). Also, for a review on suitable salts, see "Handbook of Pharmaceutical Salts: Properties, Selection, and Use" by Stahl and Wermuth (Wiley-VCH, 2002). Pharmaceutically acceptable base addition salts are formed with metals or amines, such as alkali and alkaline earth metals or organic amines.

[0152] Metals used as cations comprise sodium, potassium, magnesium, calcium, and the like. Amines comprise N-N'-dibenzylethylenediamine, chloroprocaine, choline, diethanolamine, dicyclohexylamine, ethylenediamine, N- methylglucamine, and procaine (see, for example, Berge et al., "Pharmaceutical Salts," *J. Pharma Sci.*, 1977, 66, 119). The base addition salts of said acidic compounds are prepared by contacting the free acid form with a sufficient amount of the desired base to produce the salt in the conventional manner. The free acid form may be regenerated by contacting the salt form with an acid and isolating the free acid in the conventional manner. The free acid forms differ from their respective salt forms somewhat in certain physical properties such as solubility in polar solvents, but otherwise the salts are equivalent to their respective free acid for purposes of the present disclosure.

[0153] In some embodiments, the pharmaceutical composition provided herein comprise a therapeutically effective amount of a WNT antagonist/agonist molecule or pharmaceutically acceptable salt thereof in admixture with a pharmaceutically acceptable carrier, diluent and/or excipient, for example saline, phosphate buffered saline, phosphate and amino acids, polymers, polyols, sugar, buffers, preservatives and other proteins. Exemplary amino acids, polymers and sugars and the like are octylphenoxy polyethoxy ethanol compounds, polyethylene glycol monostearate compounds, polyoxyethylene sorbitan fatty acid esters, sucrose, fructose, dextrose, maltose, glucose, mannitol, dextran, sorbitol, inositol, galactitol, xylitol, lactose, trehalose, bovine or human serum albumin, citrate, acetate, Ringer's and Hank's solutions, cysteine, arginine, carnitine, alanine, glycine, lysine, valine, leucine, polyvinylpyrrolidone, polyethylene and glycol. Preferably, this formulation is stable for at least six months at 4°C.

[0154] In some embodiments, the pharmaceutical composition provided herein comprises a buffer, such as phosphate buffered saline (PBS) or sodium phosphate/sodium sulfate, tris buffer, glycine buffer, sterile water and other buffers known to the ordinarily skilled artisan such as those described by Good et al. (1966) *Biochemistry* 5:467. The pH of the buffer may be in the range of 6.5 to 7.75, preferably 7 to 7.5, and most preferably 7.2 to 7.4.

[0155] From the foregoing it will be appreciated that, although specific embodiments of the present disclosure have been described herein for purposes of illustration, various

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modifications may be made without deviating from the spirit and scope of the present disclosure. The broad scope of this invention is best understood with reference to the following examples, which are not intended to limit the inventions to the specific embodiments.

EXAMPLES

Methods

[0156] Standard methods in molecular biology were used, including methods described, e.g., in Maniatis et al. (1982) *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.; Sambrook and Russell (2001) *Molecular Cloning*, 3rd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.; Wu (1993) *Recombinant DNA*, Vol. 217, Academic Press, San Diego, Calif. Standard methods also appear in Ausbel et al. (2001) *Current Protocols in Molecular Biology, Vols.* 1-4, John Wiley and Sons, Inc. New York, N.Y., which describes cloning in bacterial cells and DNA mutagenesis (Vol. 1), cloning in mammalian cells and yeast (Vol. 2), glycoconjugates and protein expression (Vol. 3), and bioinformatics (Vol. 4).

[0157] Methods for protein purification including immunoprecipitation, chromatography, electrophoresis, centrifugation, and crystallization are described, e.g., in Coligan et al. (2000) *Current Protocols in Protein Science, Vol.* 1, John Wiley and Sons, Inc., New York. Chemical analysis, chemical modification, post-translational modification, production of fusion proteins, glycosylation of proteins are described in, e.g., Coligan et al. (2000) *Current Protocols in Protein Science, Vol.* 2, John Wiley and Sons, Inc., New York; Ausubel et al. (2001) *Current Protocols in Molecular Biology, Vol.* 3, John Wiley and Sons, Inc., NY, N.Y., pp. 16.0.5-16.22.17; Sigma-Aldrich, Co. (2001) *Products for Life Science Research*, St. Louis, Mo.; pp. 45-89; Amersham Pharmacia Biotech (2001) *BioDirectory*, Piscataway, N.J., pp. 384-391. Production, purification, and fragmentation of polyclonal and monoclonal antibodies are described, e.g., in Coligan et al. (2001) *Current Protocols in Immunology, Vol.* 1, John Wiley and Sons, Inc., New York; Harlow and Lane (1999) *Using Antibodies*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.; Harlow and Lane, supra. Standard techniques for characterizing ligand/receptor interactions are available in, e.g., Coligan et al. (2001) *Current Protocols in Immunology, Vol.* 4, John Wiley, Inc., New York.

[0158] Methods for flow cytometry, including fluorescence activated cell sorting detection systems (FACS®), are available in, e.g., Owens et al. (1994) *Flow Cytometry*

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Principles for Clinical Laboratory Practice, John Wiley and Sons, Hoboken, N.J.; Givan (2001) *Flow Cytometry*, 2nd ed.; Wiley-Liss, Hoboken, N.J.; Shapiro (2003) *Practical Flow Cytometry*, John Wiley and Sons, Hoboken, N.J. Fluorescent reagents suitable for modifying nucleic acids, including nucleic acid primers and probes, polypeptides, and antibodies, for use, e.g., as diagnostic reagents, are available In, e.g., Molecular Probes (2003) *Catalogue*, Molecular Probes, Inc., Eugene, Oreg.; Sigma-Aldrich (2003) *Catalogue*, St. Louis, Mo.

[0159] Standard methods of histology of the immune system are described in, e.g., Muller-Harmelink (ed.) (1986) *Human Thymus: Histopathology and Pathology*, Springer Verlag, New York, N.Y.; Hiatt, et al. (2000) *Color Atlas of Histology*, Lippincott, Williams, and Wilkins, Phila, Pa.; Louis, et al. (2002) *Basic Histology: Text and Atlas*, McGraw-Hill, New York, N.Y.

[0160] Software packages and databases for determining, e.g., antigenic fragments, leader sequences, protein folding, functional domains, glycosylation sites, and sequence alignments, are available, in, e.g., GenBank, Vector NTI® Suite (Informax, Inc, Bethesda, Md.); GCG Wisconsin Package (Accelrys, Inc., San Diego, Calif.); DeCypher® (TimeLogic Corp., Crystal Bay, Nev.); Menne et al. (2000) *Bioinformatics* 16: 741-742; Menne et al. (2000) *Bioinformatics Applications Note* 16:741-742; Wren et al. (2002) *Comput. Methods Programs Biomed.* 68:177-181; von Heijne (1983) *Eur. J. Biochem.* 133:17-21; von Heijne (1986) *Nucleic Acids Res.* 14:4683-4690.

[0161] Female C57BL/6 mice (8–10-week-old) were purchased from Jackson Laboratory (Bar Harbor, ME). All animal procedures were performed in accordance with IACUC committee's regulations. Mice were anesthetized and the left extraorbital lacrimal glands were left untreated while the right lacrimal glands were injected with IL-1α or a cocktail of IL-1α and SWAP™, i.e., WNT mimetic, as shown.

Example 1

Activity of WNT Signaling Agonists

[0162] GFP reporter WNT signaling activity was measured using Hek293T cells containing a GFP-reporter gene controlled by a WNT-responsive promoter (GFP reporter assay). In brief, cells were seeded at a density of 20,000 per well in 96-well plates 24 hours prior to treatment in the presence of 3 μM IWP2 to inhibit the production of endogenous WNTs. The recombinant WNT mimetics were then added to the cells overnight. After removing the culture media, GFP fluorescence was measured using a microplate reader with

a positive control (R2M3-26), parental 1SH1-03 and the humanized polished hp1SH1-03 with and without FcRn mutations AAA and AAQ shown in Fig 7; molecules are active with slight difference in EC_{50}.

[0163] Fig. 8 describes GFP reporter for engineered trivalent bispecific using WNT signaling modulator reader with a positive control (R2M3-26), parental 1SH1-03 and the humanized polished hp1SH1-03.

[0164] Mouse *Axin2* induction in Surrogate WNT screen was performed in mouse lacrimal gland explant cultures, derived from fresh mouse lacrimal gland tissue, in complete expansion medium. Cells were plated in Matrigel as described above and exposed to different surrogate WNTs (R2M3-null, as negaticve control,1SH1-03, hp1SH1-03, hp1SH1-03, hp1SH1-03AAA, hp1SH1-03AAQ) at different concentrations. After 24-hour exposure, the RNA was extracted using the QIAGEN miniprep kit according to manufacturer's protocol. Expression of *Axin2* was determined by qPCR using SYBR Green (Thermo Fisher) according to manufacturer's protocol (primers sequences provided below), and the results are shown in Fig. 9.

[0165] Fig.10 shows WNT target gene Axin2 expression 24h after local injection in IL-1a model. All treatment groups were given a single dose of 10 ug injection. Data was normalized to anti-GFP control group. Significant elevation was observed in all 1SH1-03 groups but not in negative control R2M3-null.

[0166] Fig. 11 shows WNT target gene Axin2 expression 24h after local treatment in duct ligation damage mouse model of dry eye. All treatment groups at a single dose of 10 ug injection. Data normalized to R2M3-null negative control group. Significant elevation all 1SH1-03 groups except hp1SH1-03-AAQ.

Example 2

Intra-Lacrimal Gland Injection

[0167] Intra-lacrimal gland injection was performed as previously described with minor alteration in Zoukhri et al. A Single Injection of Interleukin-1 Induces a Reversible Aqueous-tear Deficiency, Lacrimal Gland Inflammation, and Acinar and Ductal Cell Proliferation, 84 EXP. EYE RES. 894-904 (2007). Briefly, a small incision anterior to the ear of isoflurane-anesthetized animal was made to expose the extraorbital lacrimal gland. A volume of 1 μL was injected 3 times (total volume of 3 μL) to the exposed lacrimal gland.

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[0168] The following procedure was established for lacrimal gland duct ligation, adapted from Liu et al. 2017. Animals were anesthetized with isoflurane. After the mouse was no longer responsive to pinches on two extremities in two different body quadrants, it was moved to the stage of a dissecting scope and fashioned with a nosecone to continue anesthesia by isoflurane. A small incision anterior to the ear was made to expose the extraorbital lacrimal gland which is lifted to expose the main excretory duct. A size 4-0 silk thread was then used to ligate the duct. After duct ligation, the skin incision was closed, sutured, and antibiotic ointment is then applied to the skin. The contralateral lacrimal gland was not operated on and served as a control. After three days, the skin suture was reopened to expose the lacrimal gland. The duct ligation was then released by cutting the silk suture with micro scissors. In the same surgery, treatment groups were injected with 2 mL of test article into the lacrimal gland ipsilateral to the ligation. After injection, the skin incision was closed, sutured, and antibiotic ointment was then applied. The animals were then sacrificed, and ipsilateral and contralateral lacrimal glands collected 24h, 3 days or 14 days after releasing the ligation.

[0169] For induction of dry eye, 3 μL of recombinant human IL-1α (Peprotech) at 2 μg/μL was injected to the lacrimal gland. For local SWAP™ treatment, SWAP™ (3.5 μg/μL) was mixed with IL-1α (2 μg/μL) in a total volume of 3 μL then injected to the lacrimal gland. For systemic SWAP™ treatment, SWAP™ was intraperitoneally injected at 10 mg/kg twice a week for 2 weeks.

[0170] Aqueous tear secretion was measured using phenol-impregnated cotton threads (Zone-Quick, Menicon) on restrained, unanesthetized mice. The threads were held with forceps and applied to the lateral canthus of both eyes for 30 seconds. Wetting of the thread which turned from yellow to red in contact with tears was measured in millimeters. Tear volume measurements using phenol red thread in the IL-1a model. Data represents an average of twelve animals. Treatments included three different doses of hp1SH1-03 (10, 2 and 0.4 ug) and compared to parental 1SH1-03 and negative control R2M3-null. Significant tear volume elevation in several treatment groups on day 2 and 3 compared to R2M3-null negative control except the lowest dose, and the results are shown in Fig. 12.

[0171] FIG. 13 describes tear volume measurements using phenol red thread in the IL-1a model. Data represents an average of twelve animals. Treatments included two different doses of hp1SH1-03 and hp1SH1-03-AAA (10 and 2 ug) and compared to parental 1SH1-03 and negative control R2M3-null. Significant tear volume elevation in several treatment groups on day 3 compared to R2M3-null negative control.

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[0172] Fig. 14 describes tear volume measurements using phenol red thread in the mouse duct ligation model of dry eye. Data represents an average of twelve animals per group per timepoint. Treatments included 1SH1-03, hp1SH1-03, hp1SH1-03-AAA at 2 or 10 ug compared to 10 ug negative control R2M3-null. Two groups were dosed again for a second time on day 7 (indicated with x2). Significant tear volume increase starts at day 7 for some groups and all treatments reach statistically significant increase at day 14. The group with 2x dosing of hp1SH1-03-AAA trends the highest.

Example 3

WNT Agonist Treatment in Primary Human Corneal Endothelium Cell Culture

[0173] Human corneal endothelial cells with attached Descemet membrane were received from Eversight (Ann Arbor, MI). Multiple donor samples were pooled and digested in 2 mg/mL collagenase A in MEM-alpha + 15% FBS + 20 μg/mL gentamicin sulfate for 3 hours at 37°C with occasional trituration. Samples were then washed with MEM-alpha + 15% FBS + 20 μg/mL gentamicin before resuspension and plating in Human Endothelial SFM + 5% FBS + 2 ng/mL EGF + 20 μg/mL gentamicin sulfate.

[0174] Endothelial cells were treated with test proteins (5 nM hp1SH1-03 and anti GFP antibody as a negative control) or left untreated in Human Endothelial SFM + 5% FBS + 2ng/mL EGF + 20 μg/mL gentamicin sulfate. After 14 days, cells were fixed and immunofluorescence performed for Ki67 (Abcam #ab16667, 1:100) and Na+K+ ATPase (Sigma #05-369, 1:100) prior to nuclear staining with DAPI. Ki67 is a well-known marker for cell proliferation. See discussion in, e.g., Aung TN et al., Mod Pathol (2021), https://doi.org/10.1038/s41379-021-00745-6. Samples were imaged on a Leica Dmi8 microscope. The percentage of Ki67+ cells (# of Ki67+ cells / total # of cells) in the resulting images were quantified using a custom Python script. Treatment with humanized polished (hp) 1SH1-03 significantly increased the number of proliferating cells compared to negative control anti-GFP antibody. Data normalized to negative control matched per experiment. Pooled material from seven endothelial skirts are used for one experiment result depicted in Fig. 15.

[0175] Treatment with hp1SH1-03-AAA (5nM) increased WNT target gene expression (AXIN2, LEF1, RNF43) and proliferation marker (CCND1) compared to the negative control anti-GFP antibody (5nM) after 24h. Data are normalized to the negative control treatment.

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Cells were expanded for two weeks from primary cell material prior to the 24h treatment; results are depicted in Fig. 16 A to Fig. 16 D.

[0176] Proliferation of cornea endothelial cells from human skirt material after 14 days of treatment. Treatment with humanized polished hp1SH1-03 in combination with engineered FGF TTHX1114 significantly increased the number of proliferating cells compared to negative control anti-GFP antibody. All treatments at 5nM final concentrations. Data was normalized to negative control matched per experiment. Pooled material from seven endothelial skirts are used for one experiment. This demonstrates the potential utilization of combining WNT and FGF pathway activation in cornea endothelial cell disease (Fig. 17).

Example 4

WNT Agonist Treatments in Naïve Mice by Intracameral and Intravitreal Injection

[0177] 6 to 12-week-old female Balb/C mice were anesthetized by placing the animal in an induction chamber connected to an oxygen source and isoflurane vaporizer and adjusting oxygen flow to 0.9 liters/min and the isoflurane vaporizer to 3-4%. As soon as the mouse became unresponsive and had shallow breathing, it was transferred to the anesthesia platform with the nose cone. The head was positioned so that the eye to be injected is facing up towards the microscope objective. Only one eye per mouse was injected, the contralateral used as control. 1 drop of Proparacaine was placed on the cornea for 30 seconds.

[0178] The cornea was punctured closely anterior to the iridocorneal angle. The fluid that emanates from the anterior chamber was wiped off with a weck-cell before 1 microliter of wnt agonist hp1SH1-03 (0.1 µg and 0.001 µg), hp1SH1-03-AAA (0.1 µg and 0.001 µg), and vehicle was injected into the anterior chamber (intracamerally) or posterior chamber (intravitreally) using a 33 gauge needle attached to Hamilton syringe. The mice were allowed to recover from the anesthesia. The mice were taken down the next day, the eyes harvested, and the corneas were dissected, fixed in 10% formalin, and embedded in paraffin.

[0179] For half of the eye samples, sections of paraffin-embedded tissue were made and Axin2 levels measured using an RNAscope® kit including RNAscope Multiplex Fluorescent Reagent Kit v2 (ACDBio) according to the manufacturer's instructions. For the remaining eye samples, RT- qPCR for Wnt target gene Axin2 was carried out using TaqMan™ Gene Expression Assay reagents (ThermoFisher) on a CFX96™ thermocycler (BioRad). β-actin was used for normalization. Cornea WNT target gene Axin2 expression 24h after treatment

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of non-injured mouse eyes through intracameral injection. Only cornea cell material was isolated for gene expression analysis. Treatment includes negative control vehicle injection and several different doses of hp1SH1-03-AAA. All treatment groups increase target gene expression with a significant increase in the lowest two doses. Results from such studies are described in Fig. 18.

Example 5

Cryoinjury Model

[0180] Mice were randomly divided into two groups, and 2 cycles of cryoinjury were applied to the right eye of each mouse (Han et al., 2013). Under Isoflurane anesthesia, transcorneal freezing was initiated by gently placing a cryoprobe made of stainless steel (2.5 mm indiameter; flat tip; ERBE Elektromedizin GmbH, Tübingen, Germany), precooled in liquid nitrogen for a minute on the central cornea. A cryoprobe with a diameter of 2.5 mm was be chosen as it is similar to the corneal diameter (2.6 mm) of C57BL/6 mice. be chosen as it is similar to the corneal diameter (2.6 mm) of C57BL/6 mice. No pressure was applied to avoid damage to adjacent tissue including the lens and the trabecular meshwork. The cryoprobe was kept on the corneal surface until an ice ball was formed on the cornea and covered the entire corneal surface, which corresponds to the duration of 3 seconds, as the defect on the endothelium was shown to be the same size as the ice ball. This injury was repeated after a 1min interval. Immediately after freezing, the cryoprobe was freed from the corneal surface with irrigation with a balanced salt solution, and the cornea was allowed to thaw spontaneously. Only the right eye was treated with the cryoprobe and the left eye served as an untreated control. We followed the recovery of the cornea over 4-5 days typically by following corneal thickness via OCT and corneal clarity via bright field imaging (both Phoenix micron IV).

[0181] Fig. 19 describes cornea WNT target gene Axin2 expression 24h after treatment of cryoinjured mouse eyes through intracameral injection. Only cornea cell material was isolated for gene expression analysis. Treatment included negative control vehicle injection and several different doses of hp1SH1-03-AAA. All treatment groups increased target gene expression with a significant increase in the lowest three doses.

[0182] Central corneal thickness (CCT) following corneal freeze (cryoinjury) and treatment over the course of 8 days was measured. Treatment groups included negative control vehicle and two different doses of hp1SH1-03-AAA. Corneal freeze was performed

on day 0 which led to an increase in CCT to around 300 μm. Treatment with hp1SH1-03-AAA lowered the corneal thickness compared to vehicle control. The difference was significantly lower on day 7 for the lowest dose (Fig. 20 and Fig. 21).

[0183] Fig. 22 describes pharmacokinetic measurement of serum drug concentration following IV injection (3mg/kg) of hp1SH1-03 with or without the FcRn mutation AAA. The addition of AAA mutation (hp1SH1-03-AAA) led to lower exposure, faster clearance and shorter residence time. The experiment was performed in humanized FcRn mice (JAX#014565).

Example 6

ISH1-03 Humanization (hp-1SH1)

VHH 03 Humanization

[0184] In order to humanize the camelid antibody (VHH3) sequence, the sequence database of human germline IgG heavy chain (HC) was searched. 3-66*01 was found as the closest match. Four different mutants (H1-H4) were generated and purified. The "VHH3-H4," which showed the best yield of production, was picked as a humanized VHH3.

Polishing VHH3

[0185] To de-risk potential liabilities of deamidation, isomerization, and oxidation in VHH3 CDR regions, VHH3 mutants were generated.

N29Q mutation at CDR1

[0186] Three different N29 mutants (N29Q, N29A, N29S) of VHH3 were generated and purified, but N29Q VHH3 only showed good yield for high purity expression. The mutation in SWAP, combined with the 1RC07 (1RC07-3_H4_N29Q) showed no changes in STF response. The asparagine in CDR1 is replaced to glutamine to mitigate the potential liability of deamidation.

D102E mutation at CDR3

[0187] Five different mutants (DED, SDE, EDE ESD, SSD) in VHH3 CDR3 were generated and purified to de-risk "DD" motif isomerization in VHH3. All mutants in SWAP, combined with the 1RC07 (1RC07-3 formats) showed no changes in STF response. The DED

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mutant, which shows good expression and may have minimal structural change, was picked. The aspartic acid in CDR3 was replaced to glutamic acid to mitigate the potential liability of isomerization.

No changes of M51 and M57 in VHH3 CDR2.

[0188] Three different mutants at each methionine residue in VHH3 CDR2 (M51K, M51L, M51I. M57K, M57L, M57I) were generated and purified to de-risk potential oxidation liability. All mutants showed a big decrease of expression, and it is decided to keep both methionine residues, and to check their oxidation in the further Mass-spec analyses.

Polishing and germlining 1SH1 IgG

V124I mutation in 1SH1 LC

[0189] To de-risk immunogenicity, the position was mutated to the closest germline sequence.

N155G mutation in 1SH1 LC

[0190] To de-risk potential deamidation, all possible point mutants were made and assessed for impact on binding to target Fzd, with those showing the least impact to STF activity when paired with other polishing positions being identified.

N158T mutation in 1SH1 LC

[0191] To de-risk potential deamidation, all possible point mutants were made and assessed for impact on binding to target Fzd. Final candidate selected based on least impact to STF activity when paired with other polishing positions

V5Q mutation in 1SH1 HC

[0192] To de-risk immunogenicity, position was mutated to closest germline sequence.

N85S mutation in 1SH1 HC

[0193] To de-risk immunogenicity, position was mutated to closest germline sequence.

D108 position in 1SH1 HC

[0194] Theoretical isomerization position could not be mutated to any residue that maintained binding.

D111 position in 1SH1 HC

[0195] Theoretical isomerization position could not be mutated to any residue that maintained binding.

Reagents and Materials

[0196] WNT mimetics were constructed as described in WO 2020/010308 A1, which is incorporated herein in its entirety. All recombinant proteins were produced in Expi293F™ cells (Thermo Fisher Scientific) by transient transfection unless otherwise specified. All IgG-based and Fc-containing constructs were first purified with Protein-A resin and eluted with 0.1 M glycine pH 3.5. All proteins were then polished by a size exclusion column in HBS buffer (10 mM HEPES pH 7.2, 150 mM NaCl). Proteins were supplemented with glycerol to 10% for long term storage at -80°C.

[0197] Reagents used in lacrimal gland regeneration experiments included (but were not limited to):

Reagent List	
Component	*Catalog number*
Advanced DMEM/F-12	Thermo Scientific 12634-010
HEPES	Thermo Scientific 15630080
GlutaMAX	Thermo Scientific 35050061
Penicillin-Streptomycin	Thermo Scientific 15140122
B27	Thermo Scientific 17504044
N2	Thermo Scientific 17502048
Human Growth Factors	Peprotech
Recombinant Human R-Spondin 1	R&D 4645RS
Surrogate WNT	In-house production
Matrigel™ GFR Membrane Matrix	Corning CB40230C
Y-27632	MilliporeSigma 5092280001

[0198] Primers used in the experiments included:

Primer name	*Primer sequence*	*SEQ ID No.*
Mouse forward *Axin2*	CAGCCCAAGAACCGGGAAAT	40
Mouse reverse *Axin2*	GAGCCTCCTCTCTTTTACAGCA	41
Mouse forward *Actb*	ACCTTCTACAATGAGCTGCGT	42

Mouse reverse *Actb*	AGGTCTCAAACATGATCTGGGT	43
Mouse forward Rnf43	CGGAGCCGTACTGGGTTAAG	44
Mouse reverse Rnf43	GGCACGGGGATGAAACAAAT	45
Mouse forward Mist1	CCAAGATCGAGACCCTCACG	46
Mouse reverse Mist1	GCGGCTGCTGGACATAGTAA	47
Mouse forward Krt7	GCGGAGATGAACCGCTCTAT	48
Mouse reverse Krt7	TCTAACTTGGCACGCTGGTT	49
Human forward *AXIN2*	TCATTTCCCGAGAACCCACC	50
Human reverse *AXIN2*	AGCTTCAGCTTTTCCAGCCT	51
Human forward *ACTB*	CTGGAACGGTGAAGGTGACA	52
Human reverse *ACTB*	AAGGGACTTCCTGTAACAATGCA	53
Mouse forward Fzd1	CATCGAGGCCAACTCACAGT	54
Mouse reverse Fzd1	TGAGCCCCACAAAACACACT	55
Mouse forward Fzd2	AGCGATTTCGCTACCCAGAG	56
Mouse reverse Fzd2	AGAACGAAGCCCGCAATGTA	57
Mouse forward Fzd3	GCCCTTTGTGAGACCAGGTT	58
Mouse reverse Fzd3	ATATGCCTGCAGGTCTCAGC	59
Mouse forward Fzd4	CCTGAGCGCCCCATCATATT	60
Mouse reverse Fzd4	CTTTCCCGGCCTACAGTCAG	61
Mouse forward Fzd5	CCGCCACAGGTACCTAGCTT	62
Mouse reverse Fzd5	AAGGACAGAACTCTCGGAGGA	63
Mouse forward Fzd6	CCCGAGGTGAGGCGTCC	64
Mouse reverse Fzd6	TGAATTCTGGGGCAACTGCT	65
Mouse forward Fzd7	ATGCTCTATGTACGCACCCG	66
Mouse reverse Fzd7	CGAACTTGTTCATGAGCGCC	67
Mouse forward Fzd8	TGTTGGAAGTGACCTCGCTC	68
Mouse reverse Fzd8	AACCGATGCCTTTGCACAAC	69
Mouse forward Fzd9	TAAGGACTTCGCGCTGGTTT	70
Mouse reverse Fzd9	AAGATAATCGGGCGCTCTGG	71
Mouse forward Fzd10	CCTTCCTCATCGACCCATCG	72
Mouse reverse Fzd10	TAGCCCACCGAATAAACGCA	73
Mouse forward Lrp5	CCATTGTGTTGCACCCTGTG	74

Mouse reverse Lrp5	CAGGACATGCCGATCTCTCC	75
Mouse forward Lrp6	TGCAAACAGACGGGACTTGA	76
Mouse reverse Lrp6	CCAAACACAAAGTCCACCGC	77

[0199] WNT mimetic constructs used in the experiments comprised the following amino acid sequences (Bold = LRP binding variable domain; underline = linker; italics = FZD binding variable domain; Plain (non bold, non italic, no underline=constant domain).

1SH1-03	Light Chain SEQ ID NO: 1 **DVQLVESGGGLVQPGGSLRLSCTSSANINSIETLGWYRQAPGKQRELI** **ANMRGGGYMKYAGSLKGRFTMSTESAKNTMYLQMNSLKPEDTAVY** **YCYVKLRDDDYVYRGQGTQVTVSS**GGSGS*DVVMTQSPLSLPVTPGEPASI* *SCRSSQSLLHSNGYNYLDWYLQKPGQSPQLLIYLGSKRASGVPDRFSGSGSGTD* *FTLKISRVEAEDVGVYYCMQALQIPPTFGQGTKVEIKR*TVAAPSVFIFPPSDEQ LKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTY SLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
1SH1-03	Heavy Chain SEQ ID NO: 2 *EVQLVESGPGLVKPSQTLSLTCTVSGGSISSGGYYWSWIRQHPGKGLEWIGYIYY* *SGSTYYNPSLKSRVTISVDTSKNQFSLKLNSVTAADTAVYYCARHAGFYGLADYF* *DYWGQGTLVTVSS*ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVT VSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKP SNTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTP EVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSV LTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVYTLPPSR EEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFF LYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
hp1SH1-03	Light Chain SEQ ID NO: 3 **EVQLVESGGGLVQPGGSLRLSCASSANIQSIETLGWYRQAPGKQRELI** **ANMRGGGYMKYADSLKGRFTMSTDNSKNTMYLQMNSLRAEDTAVY** **YCYVKLRDEDYVYRGQGTQVTVSS**GGGGS*DIVMTQSPLSLPVTPGEPASI* *SCRSSQSLLHSGGYTYLDWYLQKPGQSPQLLIYLGSKRASGVPDRFSGSGSGTD* *FTLKISRVEAEDVGVYYCMQALQIPPTFGQGTKVEIKRTV*AAPSVFIFPPSDEQ
hp1SH1-03 AAA	
hp1SH1-03 AAQ	

	LKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTY SLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
hp1SH1-03	heavy Chain SEQ ID NO: 4 *EVQLQESGPGLVKPSQTLSLTCTVSGGSISSGGYYWSWIRQHPGKGLEWIGYIY* *YSGSTYYNPSLKSRVTISVDTSKNQFSLKLSSVTA* *ADTAVYYCARHAGFYGLADYFDYWGQGTLVTVSS*ASTKGPSVFPLAPSSKSTS GGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVL QSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHT CPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVD VSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDW LNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVYTL PPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPVLDSDG SFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSL SPGK
hp1SH1-03 AAA	heavy Chain SEQ ID NO: 5 *EVQLQESGPGLVKPSQTLSLTCTVSGGSISSGGYYWSWIRQHPGKGLEWIGYIY* *YSGSTYYNPSLKSRVTISVDTSKNQFSLKLSSV* *ADTAVYYCARHAGFYGLADYFDYWGQGTLVTVSS*ASTKGPSVFPLAPSSKSTS GGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVL QSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHT CPPCPAPEAAGGPSVFLFPPKPKDTLMASRTPEVTCVVVD VSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLAQDW LNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVYTL PPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPVLDSDG SFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNAYTQKSLSL SPGK
hp1SH1-03 AAQ	heavy Chain SEQ ID NO: 6

	*EVQLQESGPGLVKPSQTLSLTCTVSGGSISSGGYYWSWIRQHPGKGLEWIGYIY YSGSTYYNPSLKSRVTISVDTSKNQFSLKLSSVTA ADTAVYYCARHAGFYGLADYFDYWGQGTLVTVSS*ASTKGPSVFPLAPSSKSTS GGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVL QSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHT CPPCPAPEAAGGPSVFLFPPKPKDTLMASRTPEVTCVVVD VSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLAQDW LNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVYTL PPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPVLDSDG SFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNQYTQKSLSL SPGK
hp1SH1	Light chain SEQ ID NO:7 *DIVMTQSPLSLPVTPGEPASISCRSSQSLLHSGGYTYLDWYLQKPGQSPQLLIYL GSKRASGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCMQALQIPPTFGQGTK VEIKR*TVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNAL QSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSP VTKSFNRGEC
hpVhh03_hp1SH1_HC_hole_T366S_L368A_Y407V	Heavy Chain SEQ ID NO:8 **EVQLVESGGGLVQPGGSLRLSCASSANIQSIETLGWYRQAPGKQRELI ANMRGGGYMKYADSLKGRFTMSTDNSKNTMYLQMNSLRAEDTAVY YCYVKLRDEDYVYRGQGTQVTVSS**GGGGS*EVQLQESGPGLVKPSQTLSL TCTVSGGSISSGGYYWSWIRQHPGKGLEWIGYIYYSGSTYYNPSLKSRVTISVDTS KNQFSLKLSSVTAADTAVYYCARHAGFYGLADYFDYWGQGTLVTVSS*ASTKGP SVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVL QSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHT CPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFN WYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVS NKALGAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLSCAVKGFYPS

	DIAVEWESNGQPENNYKTTPPVLDSDGSFFLVSKLTVDKSRWQQGNVFSC SVMHEALHNHYTQKSLSLSPGK
hp1SH1_knob	Heavy Chain SEQ ID NO:9 *EVQLQESGPGLVKPSQTLSLTCTVSGGSISSGGYYWSWIRQHPGKGLEWIGYIY YSGSTYYNPSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYYCARHAGFYGLADY FDYWGQGTLVTVSS*ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVT VSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKP SNTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTP EVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSV LTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVYTLPPSR EEMTKNQVSLWCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFF LYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
hp1SH1_HC_knob_T366W_hpVHH03	Heavy chain SEQ ID NO: 10 *EVQLQESGPGLVKPSQTLSLTCTVSGGSISSGGYYWSWIRQHPGKGLEWIGYIY YSGSTYYNPSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYYCARHAGFYGLADY FDYWGQGTLVTVSS*ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVT VSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKP SNTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTP EVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSV LTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVYTLPPSR EEMTKNQVSLWCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFF LYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP<u>GGGGGS</u>**EVQ LVESGGGLVQPGGSLRLSCASSANIQSIETLGWYRQAPGKQRELIANM RGGGYMKYADSLKGRFTMSTDNSKNTMYLQMNSLRAEDTAVYYCY VKLRDEDYVYRGQGTQVTVSS**
hpVHH03_1SH1_HC_	Heavy Chain SEQ ID NO: 11

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knob_T366W	**EVQLVESGGGLVQPGGSLRLSCASSANIQSIETLGWYRQAPGKQRELI ANMRGGGYMKYADSLKGRFTMSTDNSKNTMYLQMNSLRAEDTAVY YCYVKLRDEDYVYRGQGTQVTVSS**GGGGS*EVQLQESGPGLVKPSQTLSL TCTVSGGSISSGGYYWSWIRQHPGKGLEWIGYIYYSGSTYYNPSLKSRVTISVDTS KNQFSLKLSSVTAADTAVYYCARHAGFYGLADYFDYWGQGTLVTVSS*ASTKGP SVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVL QSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHT CPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFN WYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVS NKALGAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLWCLVKGFYP SDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFS CSVMHEALHNHYTQKSLSLSPGK
hp1SH1_HC_hole_T366S_L368A_Y407V	Heavy Chain SEQ ID NO: 12 *EVQLQESGPGLVKPSQTLSLTCTVSGGSISSGGYYWSWIRQHPGKGLEWIGYIY YSGSTYYNPSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYYCARHAGFYGLADY FDYWGQGTLVTV*SSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPV TVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHK PSNTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRT PEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVS VLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVYTLPPS REEMTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSF FLVSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

hp1SH1_HC_hole_T366S_L368A_Y407V_hpVHH03	Heavy chains SEQ ID NO: 13 *EVQLQESGPGLVKPSQTLSLTCTVSGGSISSGGYYWSWIRQHPGKGLEWIGYIY YSGSTYYNPSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYYCARHAGFYGLADY FDYWGQGTLVTVSS*ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVT VSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKP SNTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTP EVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSV LTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVYTLPPSR EEMTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFF LVSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGGGGGS**EVQ LVESGGGLVQPGGSLRLSCASSANIQSIETLGWYRQAPGKQRELIANM RGGGYMKYADSLKGRFTMSTDNSKNTMYLQMNSLRAEDTAVYYCY VKLRDEDYVYRGQGTQVTVSS**
VHH03 CDR1	SEQ ID NO: 14 ANINSIET
VHH03 CDR2	SEQ ID NO: 15 MRGGGYM
VHH03 CDR3	SEQ ID NO: 16 YVKLRDDDYVY
1SH1-CDRL1	SEQ ID NO: 17 QSLLHSNGYNY

1SH1 - CDRL2	LGS (SEQ ID NO: 18)
1SH1- CDRL3	SEQ ID NO: 19 MQALQIPPT
1SH1- CDRH1	SEQ ID NO: 20 GGSISSGGYY
1SH1- CDRH2	SEQ ID NO: 21 IYYSGST
1SH1- CDRH3	SEQ ID NO: 22 ARHAGFYGLADYFDY
hp-VHH03 CDR1	SEQ ID NO: 23 ANIQSIET
hp-VHH03 CDR 2	SEQ ID NO: 15 MRGGGYM
hp-VHH03 CDR 3	SEQ ID NO: 24 YVKLRDEDYVY

hp-1SH1-CDRL1	SEQ ID NO: 25 QSLLHSGGYTY
hp-1SH1-CDRL2	LGS (SEQ ID NO: 18)
hp-1SH1-CDRL3	SEQ ID NO: 19 MQALQIPPT
hp-1SH1-CDRH1	SEQ ID NO: 20 GGSISSGGYY
hp-1SH1-CDRH2	SEQ ID NO: 21 IYYSGST
hp-1SH1-CDRH2	SEQ ID NO: 22 ARHAGFYGLADYFDY
Linker	SEQ IDNO: 26 GGSGS

{0200} It will be readily understood to those skilled in the art that the above constructs may be altered and expressed as various homologs and isoforms, may be edited at non-binding-domain sequences, and may be expressed using various synonymous nucleotide sequences using various suitable expression vector systems.

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CLAIMS

WHAT IS CLAIMED IS:

1. An engineered multivalent bispecific WNT signaling modulator, wherein the WNT signaling modulator comprises:

a. at least one first antigen binding domain that binds to at least one of Lrp6 and/or Lrp5; and

b. at least one-second antigen binding domains bind to at least one FZD receptors.

2. The engineered WNT signaling modulator of claim 1, wherein the modulator is a tetravalent bispecific molecule.

3. The engineered WNT signaling modulator of claim 2, wherein the WNT signaling modulator comprises:

a. two light chains, each comprising a polypeptide sequence having at least 95% identity to SEQ ID NO:3; and

b. two heavy chains, each comprising a polypeptide sequence having at least 95% identity to SEQ ID NO: 4, 5, or 6.

4. The engineered WNT signaling modulator of claim 3, wherein each of the two light chains comprises a VHH domain comprising one or more or more of the following amino acid residues: N29Q and/or VHH3-H4 DDD substituted with DED.

5. The engineered WNT signaling modulator of claim 3, wherein each heavy chain comprises amino acid residues: a CDRH1 SEQ ID NO: 20, a CDRH2 SEQ ID NO: 21, and CDRH3 SEQ ID NO: 22.

6. The engineered WNT signaling modulator of any one of claims 2-5, wherein the WNT signaling modulator molecule further has amino acid modifications comprising: L234A, L235A, and P329G amino acid substitutions, as numbered according to the EU index.

7. The engineered WNT signaling modulator of claims 2-6 wherein the heavy chain of SEQ ID NO: 5 comprises Fc region mutation with at least three amino acid modifications selected from the group consisting of: I253A, H310A, and H435A, as numbered according to the EU index.

8. The engineered WNT signaling modulator of claims 2-6, wherein the heavy chain of SEQ ID NO: 6 comprises Fc region mutation with at least three amino acid modifications selected from the group consisting of: I253A, H310A and H435Q, as numbered according to the EU index.

9. The engineered WNT signaling modulator of claim 1, wherein the modulator is a trivalent bispecific molecule.

10. The engineered WNT signaling modulator of claim 9, wherein the WNT signaling modulator comprises:

a. a first antigen binding domain that bind to one of Lrp6 and/or Lrp5; and

b. two second antigen binding domains that bind to at least two FZD receptors.

11. The engineered WNT signaling modulator of claim 10, wherein the anti-LRP 5/6 is an N-terminal 'Hole' appended comprising a polypeptides sequence having 95% identity to the combination of SEQ ID NOs: 8, 7, and 9.

12. The engineered WNT signaling modulator of claim 10, wherein the anti-LRP 5/6 is a C-terminal 'Hole' appended comprising a polypeptides sequence having 95% identity to the combination of SEQ ID NOs: 13, 7, and 9.

13. The engineered WNT signaling modulator of claim10, wherein the anti-LRP 5/6 is an N-terminal 'Knob' appended comprising a polypeptides sequence having 95% identity to the combination of SEQ ID NOs: 11, 7, and 12.

14. The engineered WNT signaling modulator of claim 10, wherein the anti-LRP 5/6 is a C-terminal 'Knob' appended comprising a polypeptides sequence having 95% identity to the combination of SEQ ID NOs: 10, 7, and 12.

15. A nucleic acid comprising a sequence encoding the engineered multivalent bispecific WNT signaling modulator of any one of claims 1-14 optionally wherein the nucleic acid(s) is/are DNA, cDNA, RNA, mRNA, modified mRNA, or a DNA/RNA hybrid.

16. A vector comprising the nucleic acid of claim 15.

17. The vector of claim 16, wherein the vector is an expression vector comprising a promoter operatively linked to the nucleic acid of claim 15.

18. A pharmaceutical composition comprising a pharmaceutically acceptable diluent, excipient or carrier, and:

a. the engineered WNT signaling modulator of any one of claims 1-14;

b. the nucleic acid of any one of claim 15; and/or

c. the vector of any one of claims 16-17.

19. A method of regenerating lacrimal gland cells in a subject, comprising administering a WNT signaling modulator to the subject.

20. The method of claim 19, wherein the subject is a mammal animal or human patient.

21. The method of claim 19, wherein the lacrimal gland cells are acinar cells, progenitor cells, ductal cells, myoepithelial cells, or immune cells.

22. The method of claim 19, wherein the engineered WNT signaling modulator is a WNT agonist.

23. The method of claim 19-22, wherein the engineered WNT signaling agonist is administered:

i. to one or more of the subject's eyes, optionally intra-lacrimal gland or via ocular drops;

ii. locally administering, optionally to the eye, ear, nose (optionally intranasally), skin (optionally transdermally or epicutaneously), mucosa, skin, or vagina, or by inhalation; or

iii. parenterally administering, optionally by injection (optionally intravenous, intramuscular, subcutaneous, intradermal, intrathecal, intra-arterial, intraarticular, intraosseous, or intraperitoneal administration) or by inhalation; or

iv. enterally administering, optionally orally, sublingually, buccally, or rectally.

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1SH1-03

Light Chain (LC)

DVQLVESGGGLVQPGGSLRLSCTSS***ANINSIET***LGWYRQAPGKQRELIAN***MRGGGYM***KYAGSLKGRFTMSTESAKNTMYLQMN
SLKPEDTAVYYC***YVKLRDDDYVY***RGQGTQVTVSSGGSGGSDVVMTQSPLSLPVTPGEPASISCRSS***QSLLHSNGYNY***LDWYLQK
PGQSPQLLIY***LGS***KRASGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYC***MQALQIPPT***FGQGTKVEIKRTVAAPSVFIFPPSDEQL
KSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSF
NRGEC (SEQ ID NO:1)

Heavy Chain (HC)

EVQLVESGPGLVKPSQTLSLTCTVS***GGSISSGGYY***WSWIRQHPGKGLEWIGY***IYYSGST***YYNPSLKSRVTISVDTSKNQFSLKLNS
VTAADTAVYYC***ARHAGFYGLADYFDY***WGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALT
SGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKD
TLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIE
KTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQ
QGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO:2)

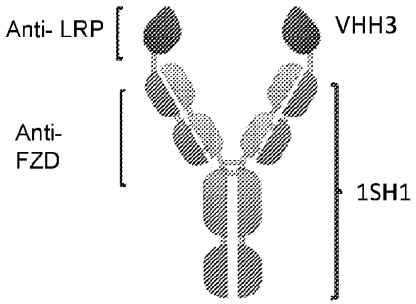


FIG. 1

VHH domain
VL
VH
FC
Linker Sequence
CDR: Black bold font with underline

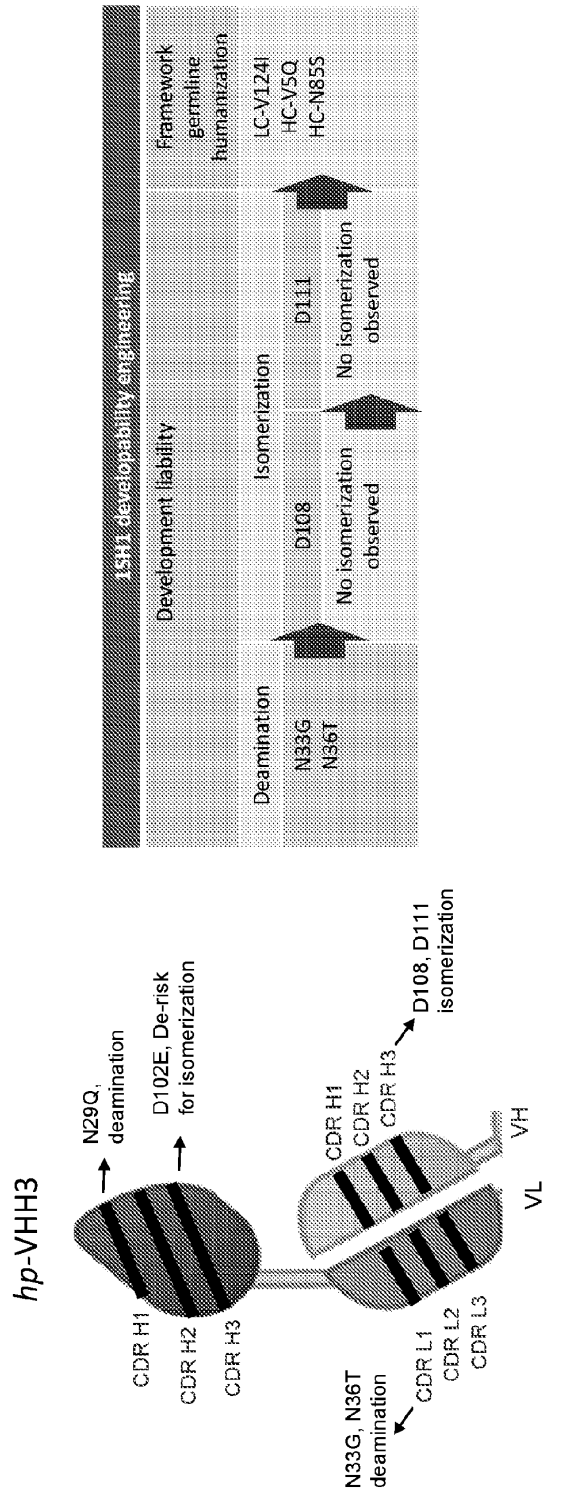

De-liability on variable fragment of 1SH1-03
hp-VHH3
N29Q, deamination
D102E, De-risk for isomerization
CDR H1
CDR H2
CDR H3
N33G, N36T deamination
CDR L1
CDR L2
CDR L3
VL
CDR H1
CDR H2
CDR H3
D108, D111 isomerization
VH
1SH1 developability engineering
Development liability
Framework germline humanization
LC-V124I
HC-V5Q
HC-N85S
Isomerization
Deamination
N33G
N36T
D108
No isomerization observed
D111
No isomerization observed

FIG. 2

***hp*-1SH1-03**

LC

EVQLVESGGGLVQPGGSLRLSCAASS***ANI*AD*SIET***LGWYRQAPGKQRELIAN***MRGGGYM***KYADSLKGRFTMSTDNSKNTMYLQMN
SLRAEDTAVYYC***YVKLRD*ED*DYVY***RGQGTQVTVSS***GGGGS***DVMTQSPLSLPVTPGEPASISCRSS***QSLLHSGGYTYL***DWYLQKP
GQSPQLLIY***LGS***KRASGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYC***MQALQIPPT***FGQGTKVEIKRTVAAPSVFIFPPSDEQLK
SGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFN
RGEC(SEQ ID NO:3)

HC

EVQLQESGPGLVKPSQTLSLTCTVS***GGSISSGGYY***WSWIRQHPGKGLEWIGY***IYYSGSTY***YNPSLKSRVTISVDTSKNQFSLKLS
SVTAADTAVYYC***ARHAGFYGLADYFDY***WGQGTLVTVSS***ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGA***
LTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKP
KDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGA
PIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPVLDSDGSFFLYSKLTVDKSR
WQQGNVFSCSVMHEALHNHYTQKSLSLSPGK(SEQ ID NO:4)

VHH domain
VL
VH
CDR: Black bold font with underline

Fc
Linker Sequence

Changes from parental (1SH1-03) sequence
Red: Humanization in VHH3 (D1E, T23A, G61D, ESA(72-74)DNS, KP(86-87)RA)
Brown bold italics: Liability de-risking in VHH3 CDRs (N29Q, D102E)
Dark blue bold: Germline humanization in framework

FIG. 3

***hp*-1SH1-03_AAA**

LC

EVQLVESGGGLVQPGGSLRLSCASS***ANI***K***SIET***LGWYRQAPGKQRELIAN***MRGGGYM***KYADSLKGRFTMSTDNSKNTMYLQMN
SLRAEDTAVYYC***YVKLRD***K***DYVY***RGQGTQVTVSS***GGGGS***DIVMTQSPLSLPVTPGEPASISCRSS***QSLLHSGGYTY***LDWYLQKP
GQSPQLLIY***LGS***KRASGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYC***MQALQIPPT***FGQGTKVEIKRTVAAPSVFIFPPSDEQLK
SGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFN
RGEC(SEQ ID NO:3)

HC

EVQLQESGPGLVKPSQTLSLTCTVS***GGSISSGGYY***WSWIRQHPGKGLEWIGY***IYYSGST***YYNPSLKSRVTISVDTSKNQFSLKL
SSVADTAVYYC***ARHAGFYGLADYFDY***WGQGTLVTVSS*ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGA*
LTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPK
PKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKA
LGAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPVLDSDGSFFLYSKLTV
DKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK(SEQ ID NO:5)

VHH domain
VL
VH
CDR: Black bold font with underline
Mutation in FC: I253A, H310A,H435A

FC
Linker Sequence

Changes from parental (1SH1-03) sequence

Red: Humanization in VHH3
Brown: Liability de-risking in VHH3 CDRs
Dark blue: Germline humanization in framework

FIG. 4

***hp*-1SH1-03_AAQ**

LC

EVQLVESGGGLVQPGGSLRLSCASS***AMQSIET***LGWYRQAPGKQRELI***ANMRGGGYMK***YADSLKGRFTMSTDNSKNTMYLQMN
SLRAEDTAVYYC***YYKLRDEDYV***YRGQGTQVTVSS**[VHH domain]**DIVMTQSPLSLPVTPGEPASISCRSS***QSLLHSGGYTY***LDWYLQKP
GQSPQLLIY***LGSK***RASGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYC***MQALQIPPTT***FGQGTKVEIKRTVAAPSVFIFPPSDEQLK
SGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFN
RGEC(SEQ ID NO:3)

HC

EVQLQESGPGLVKPSQTLSLTCTVS***GGSISSGGYY***WSWIRQHPGKGLEWIGY***IYYSGST***YYNPSLKSRVTISVDTSKNQFSLKLSS
VTAADTAVYYC***ARHAGFYGLADYFDY***WGQGTLVTVSSA*STKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALT*
SGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC[FC]*DKTHTCPPCPAPEAAGGPSVFLFPPKPKDT*
*LM*ISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVL*QDWLNGKEYKCKVSNKALGAPIEK*
TISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQG
NVFSCSVMHEALHN[Linker Sequence]*YTQKSLSLSPGK*(SEQ ID NO:6)

VHH domain FC
VL Linker Sequence
VH
CDR: Black bold font with underline
Mutation in FC: I253A, H310A,H435Q

Changes from parental (1SH1-03) sequence

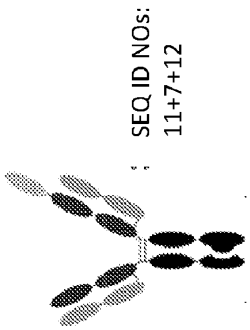
Red: Humanization in VHH3
Brown: Liability de-risking in VHH3 CDRs
Dark blue: Germline humanization in framework

FIG. 5

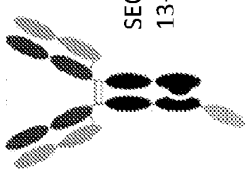
Wnt Surrogate Molecule With One LRP and Two FZD Binders



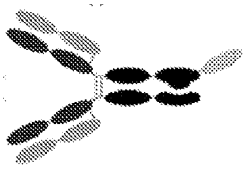
N-terminal ''Hole"A
FIG. 6A



N-terminal ''Knob"A
FIG. 6C



C-terminal ''Hole"A
FIG. 6B



C-terminal ''Knob"A
FIG. 6D

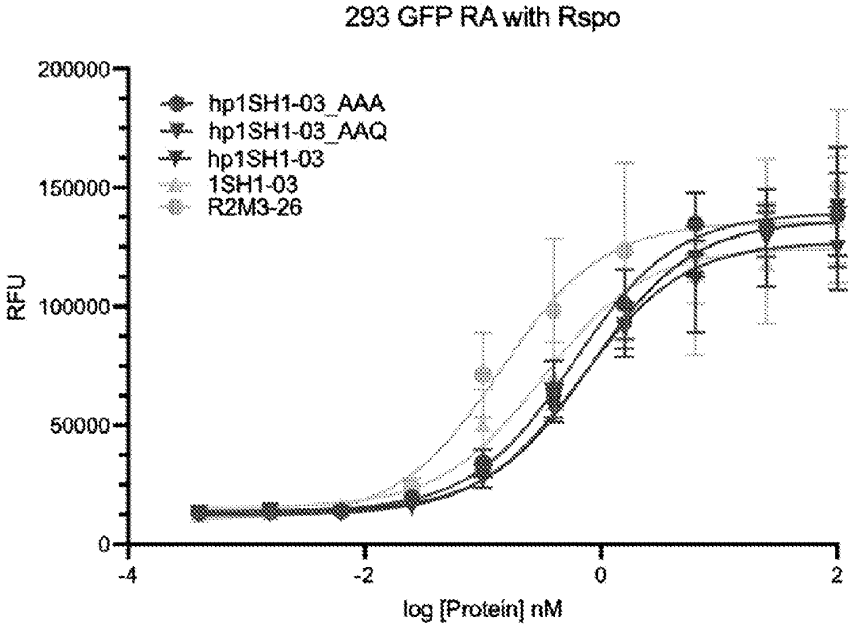

293 GFP RA with Rspo
200000
150000
100000
50000
0
RFU
hp1SH1-03_AAA
hp1SH1-03_AAQ
hp1SH1-03
1SH1-03
R2M3-26
-4
-2
0
2
log [Protein] nM

FIG. 7

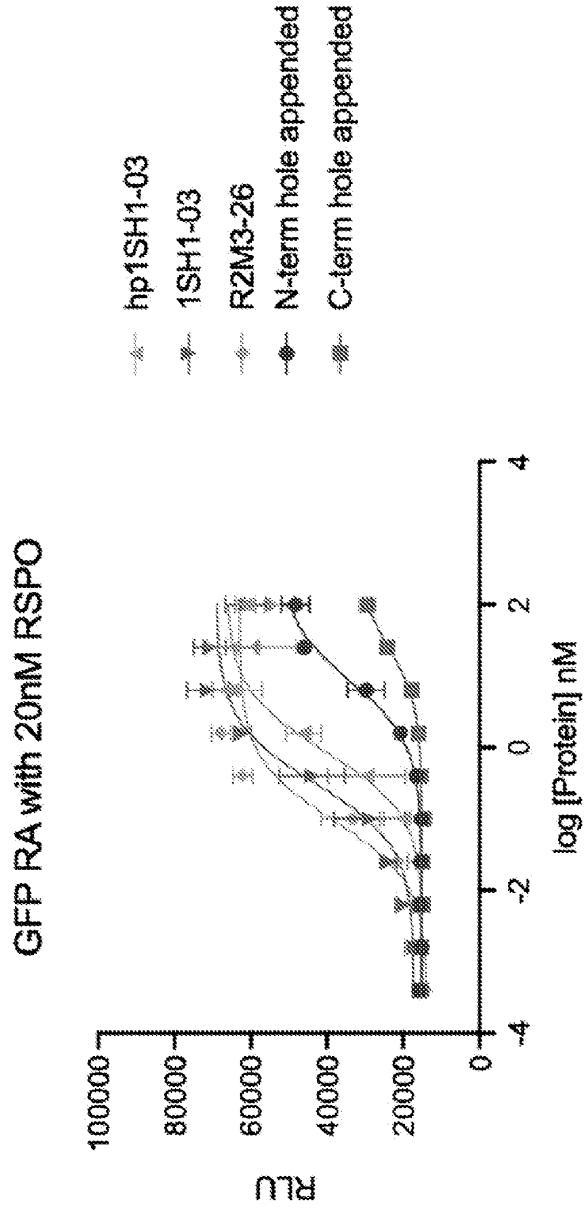

GFP RA with 20nM RSPO
hp1SH1-03
1SH1-03
R2M3-26
N-term hole appended
C-term hole appended
RLU
100000
80000
60000
40000
20000
0
-4
-2
0
2
4
log [Protein] nM

FIG. 8

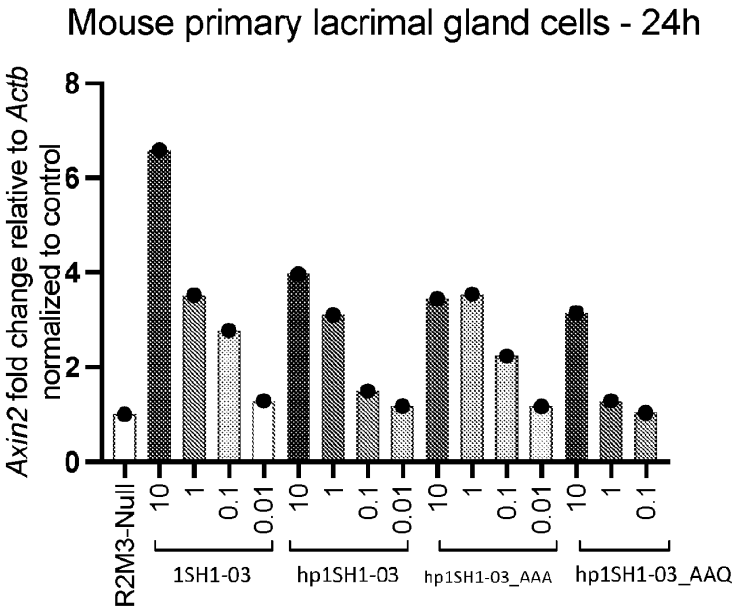

Mouse primary lacrimal gland cells - 24h
Axin2 fold change relative to Actb
normalized to control
8
6
4
2
0
R2M3-Null
10
1
0.1
0.01
1SH1-03
hp1SH1-03
hp1SH1-03_AAA
hp1SH1-03_AAQ

FIG. 9

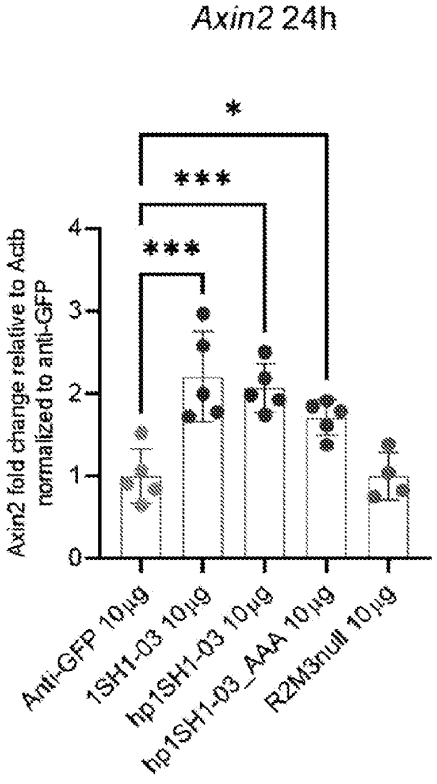

Axin2 24h
*
***
***
Axin2 fold change relative to Actb normalized to anti-GFP
0
1
2
3
4
Anti-GFP 10µg
1SH1-03 10µg
hp1SH1-03 10µg
hp1SH1-03_AAA 10µg
R2M3null 10µg

FIG. 10

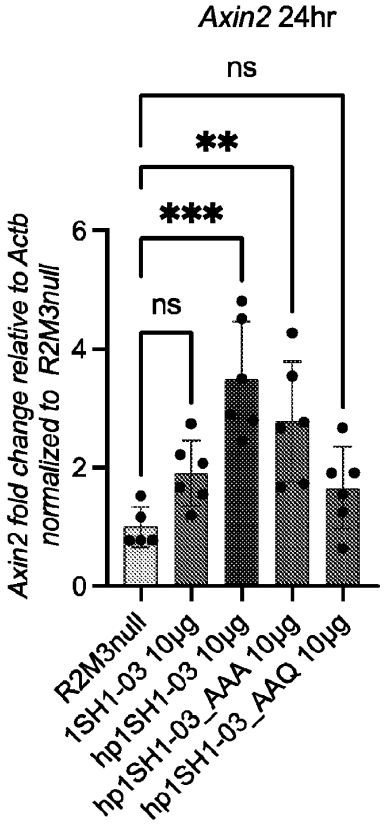

Axin2 24hr
ns
**
***
ns
Axin2 fold change relative to Actb
normalized to R2M3null
6
4
2
0
R2M3null
1SH1-03 10µg
hp1SH1-03 10µg
hp1SH1-03_AAA 10µg
hp1SH1-03_AAQ 10µg

FIG. 11

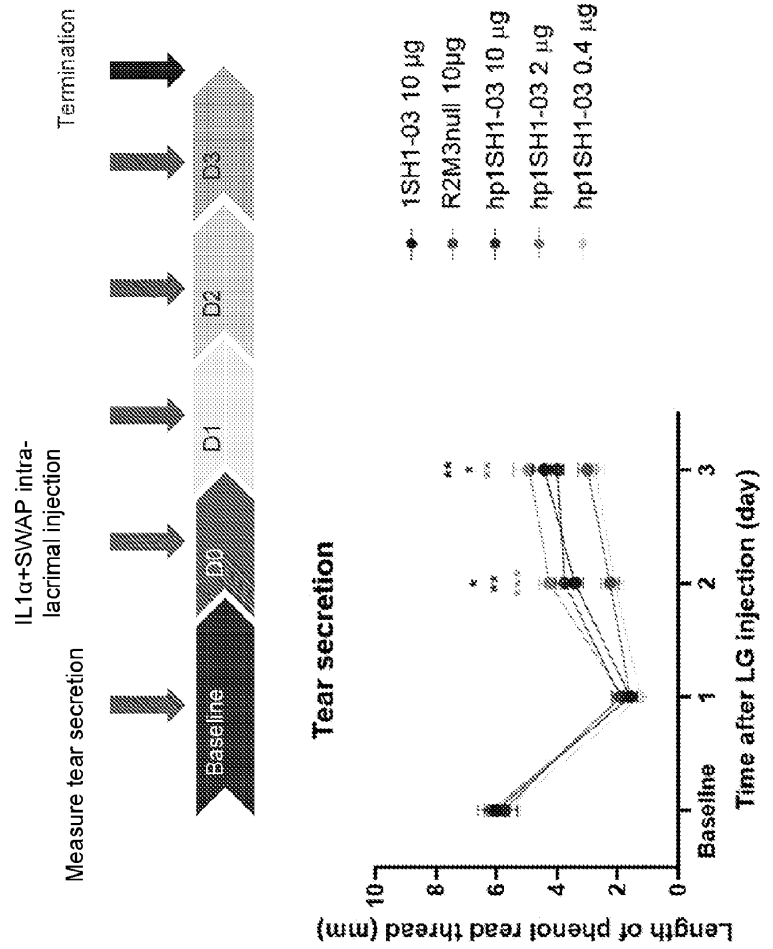

IL1α+SWAP intra-lacrimal injection
Measure tear secretion
Termination
Baseline
D0
D1
D2
D3
Tear secretion
1SH1-03 10 µg
R2M3null 10µg
hp1SH1-03 10 µg
hp1SH1-03 2 µg
hp1SH1-03 0.4 µg
Length of phenol read thread (mm)
Time after LG injection (day)
Baseline
1
2
3
0
2
4
6
8
10

FIG. 12

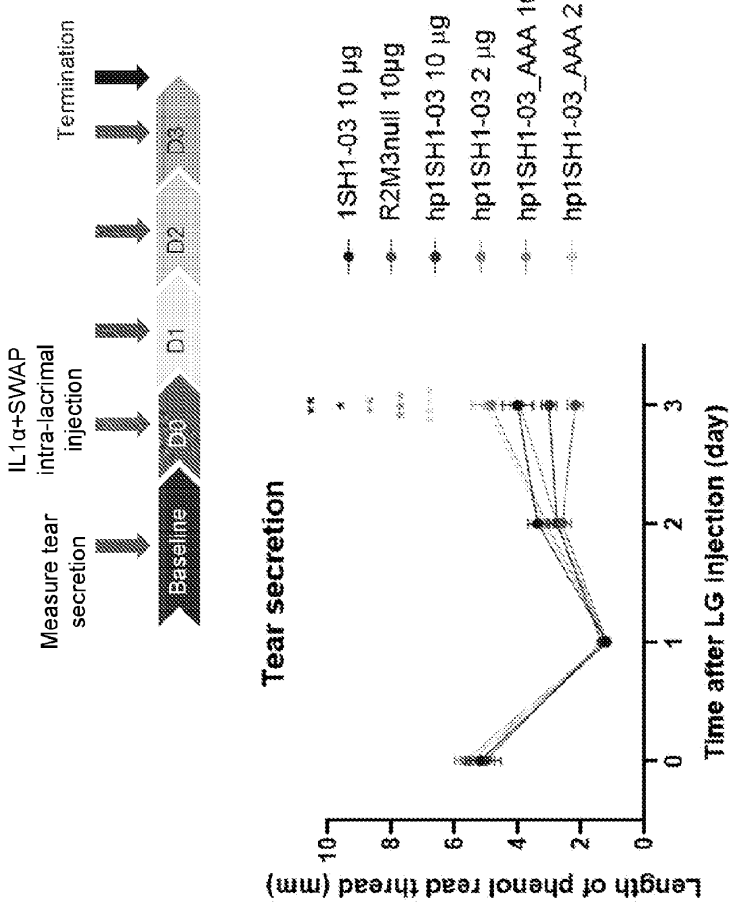

Measure tear secretion
IL1α+SWAP intra-lacrimal injection
Termination
Baseline
D1
D2
D3
Tear secretion
1SH1-03 10 µg
R2M3null 10µg
hp1SH1-03 10 µg
hp1SH1-03 2 µg
hp1SH1-03_AAA 10 µg
hp1SH1-03_AAA 2 µg
Length of phenol read thread (mm)
Time after LG injection (day)

FIG. 13

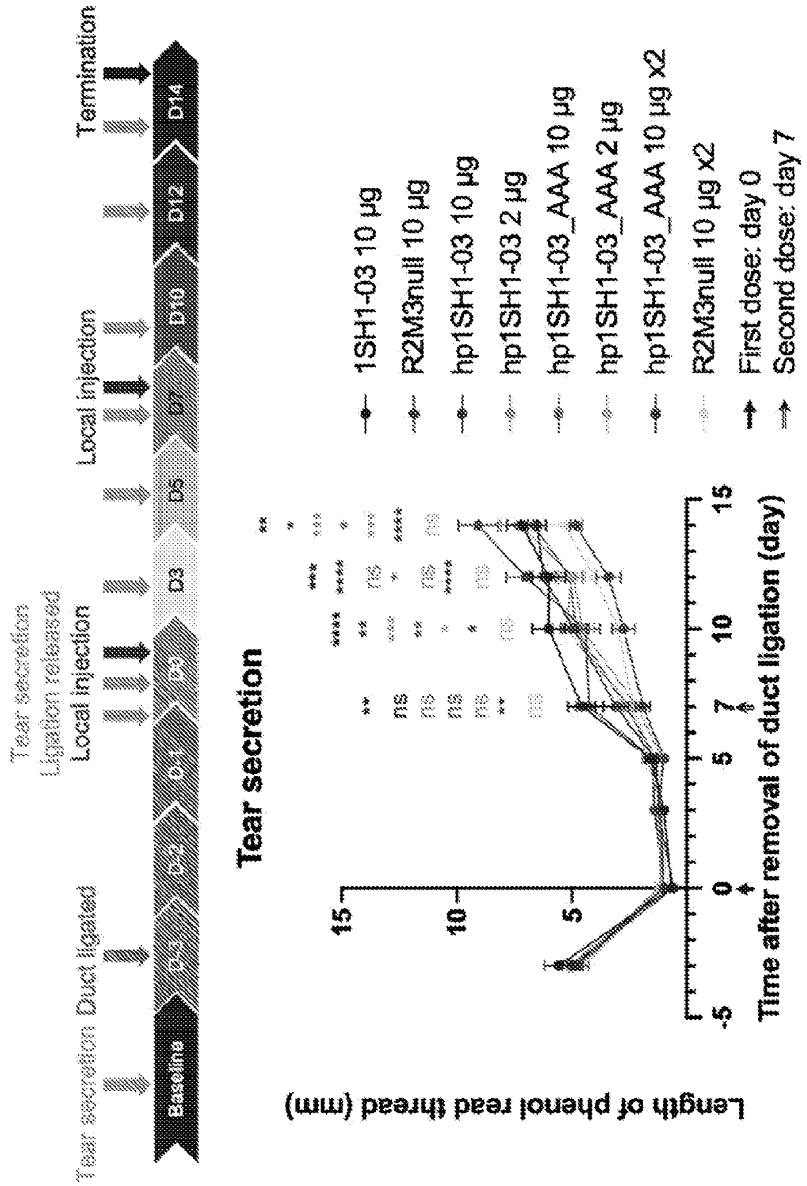

Tear secretion
Ligation released
Local injection
Tear secretion Duct ligated
Local injection
Termination
Baseline
D1
D2
D3
D5
D7
D10
D12
D14
Tear secretion
Length of phenol read thread (mm)
Time after removal of duct ligation (day)
1SH1-03 10 µg
R2M3null 10 µg
hp1SH1-03 10 µg
hp1SH1-03 2 µg
hp1SH1-03_AAA 10 µg
hp1SH1-03_AAA 2 µg
hp1SH1-03_AAA 10 µg x2
R2M3null 10 µg x2
First dose: day 0
Second dose: day 7

FIG. 14

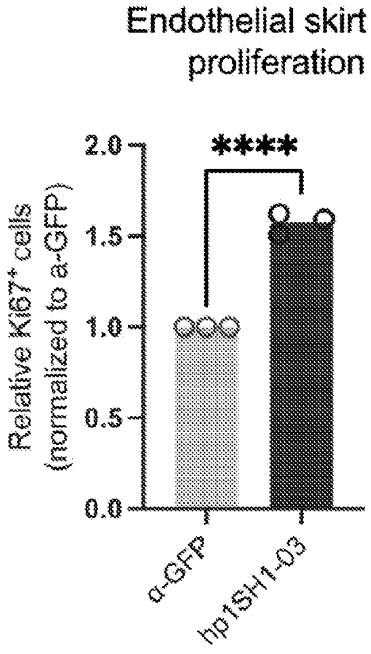

Endothelial skirt cell proliferation
Relative Ki67+ cells (normalized to a-GFP)
2.0
1.5
1.0
0.5
0.0
****
a-GFP
hp1SH1-03

FIG. 15

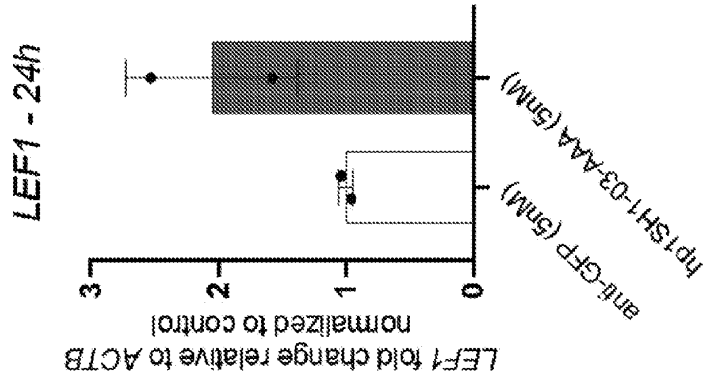


FIG. 16B

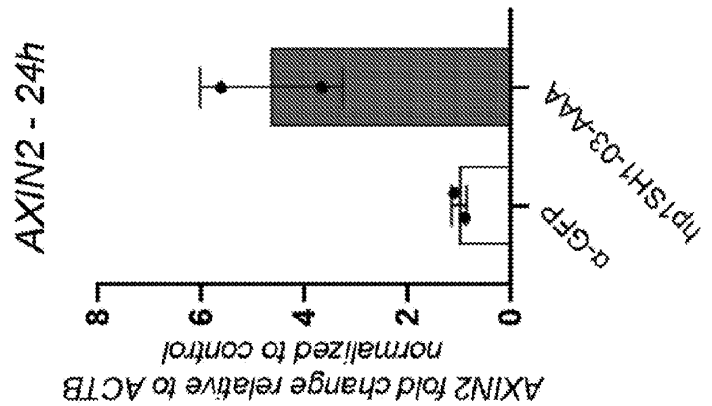


FIG. 16A

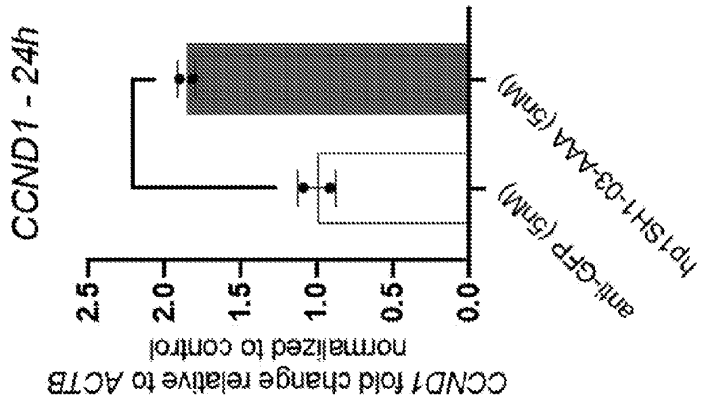


FIG. 16D

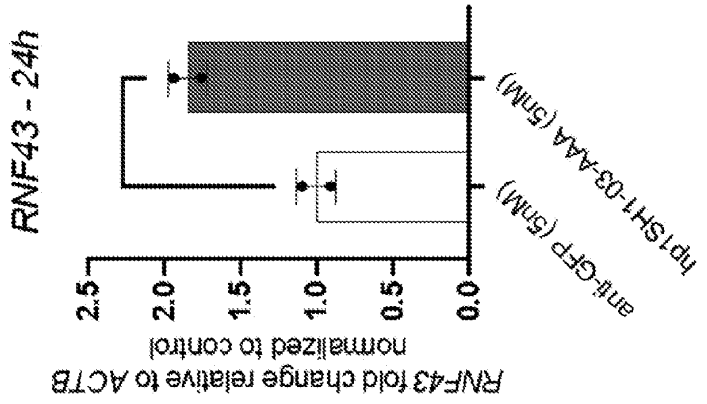


FIG. 16C

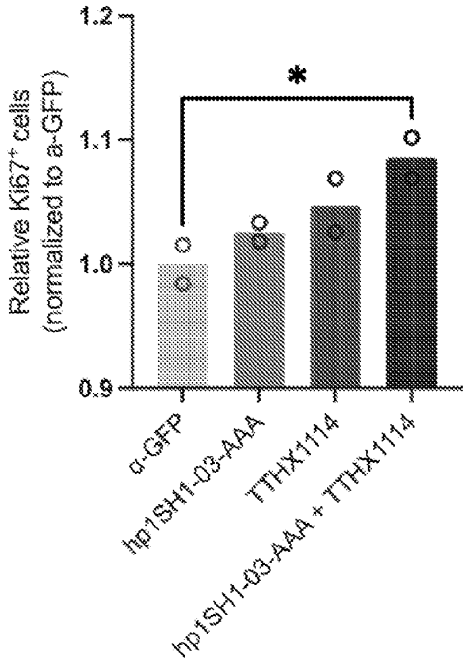

Relative Ki67+ cells
(normalized to α-GFP)
1.2
1.1
1.0
0.9
*
α-GFP
hp1SH1-03-AAA
TTHX1114
hp1SH1-03-AAA + TTHX1114

FIG. 17

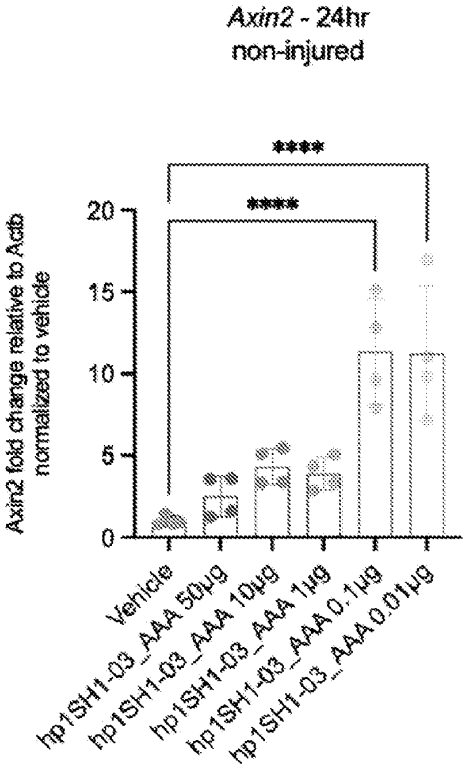

Axin2 - 24hr
non-injured
****
****
Axin2 fold change relative to Actb
normalized to vehicle
20
15
10
5
0
Vehicle
hp1SH1-03_AAA 50µg
hp1SH1-03_AAA 10µg
hp1SH1-03_AAA 1µg
hp1SH1-03_AAA 0.1µg
hp1SH1-03_AAA 0.01µg

FIG. 18

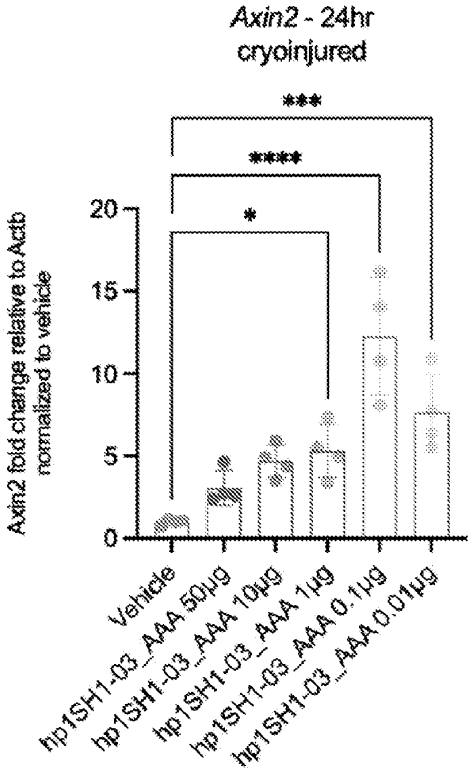

Axin2 - 24hr
cryoinjured
***
****
*
Axin2 fold change relative to Actb
normalized to vehicle
20
15
10
5
0
Vehicle
hp1SH1-03_AAA 50µg
hp1SH1-03_AAA 10µg
hp1SH1-03_AAA 1µg
hp1SH1-03_AAA 0.1µg
hp1SH1-03_AAA 0.01µg

FIG. 19

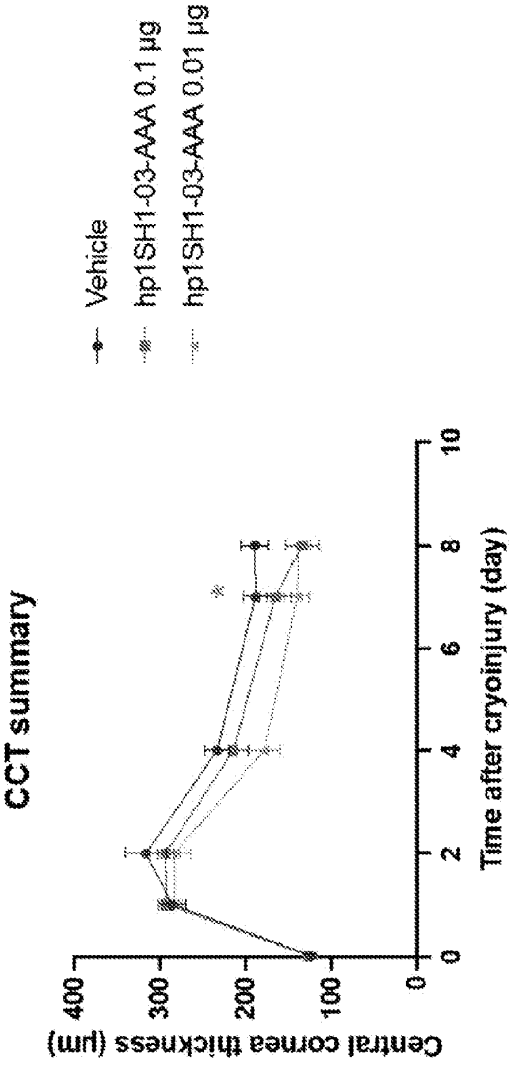

CCT summary
Vehicle
hp1SH1-03-AAA 0.1 µg
hp1SH1-03-AAA 0.01 µg
Central cornea thickness (µm)
400
300
200
100
0
0
2
4
6
8
10
Time after cryoinjury (day)

FIG. 20

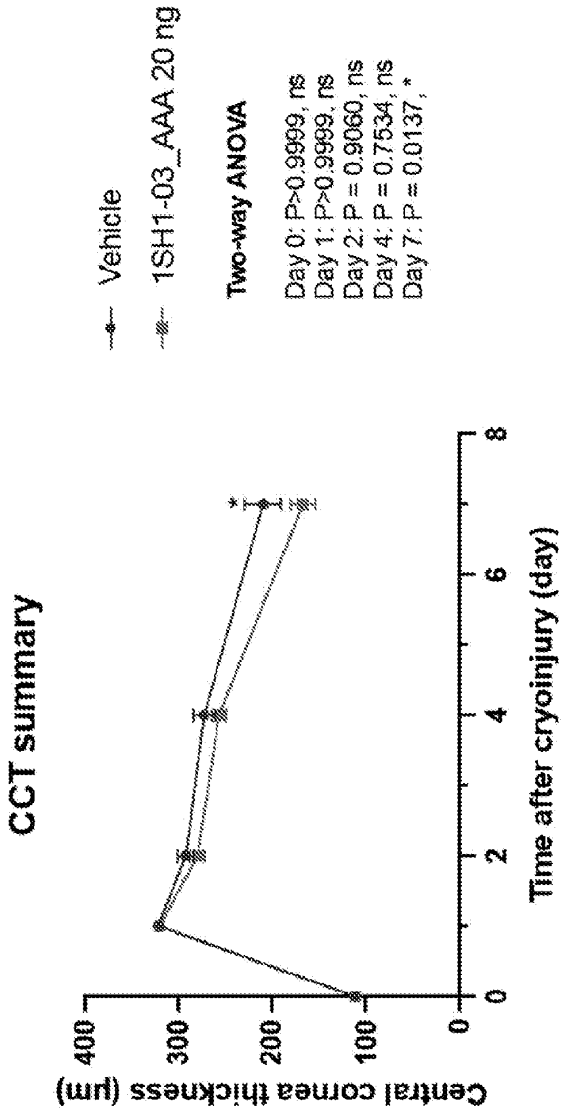

CCT summary
Vehicle
1SH1-03_AAA 20 ng
Two-way ANOVA
Day 0: P>0.9999, ns
Day 1: P>0.9999, ns
Day 2: P = 0.9060, ns
Day 4: P = 0.7534, ns
Day 7: P = 0.0137, *
Central cornea thickness (μm)
400
300
200
100
0
0
2
4
6
8
Time after cryoinjury (day)

FIG. 21

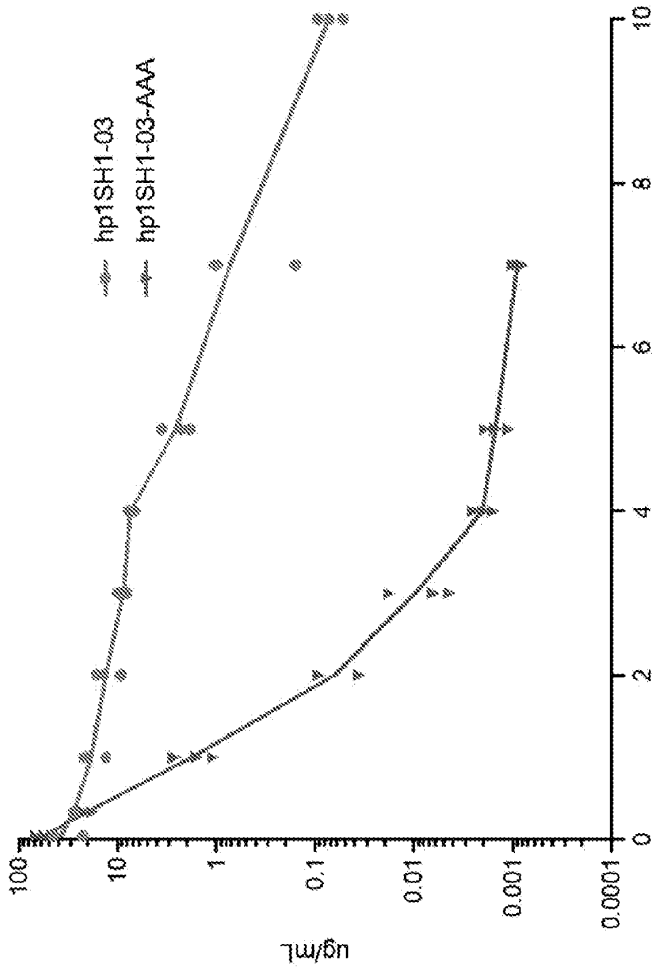

hp1SH1-03
hp1SH1-03-AAA
ug/mL
100
10
1
0.1
0.01
0.001
0.0001
0
2
4
6
8
10
Days

FIG. 22

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ELECTRONIC ACKNOWLEDGEMENT RECEIPT

APPLICATION #	RECEIPT DATE / TIME	ATTORNEY DOCKET #
18/361,782	**02/06/2025 05:06:06 PM Z ET**	**SRZN-006/04US 328202-2203**

Title of Invention

WNT SURROGATE MOLECULES AND USES THEREOF

Application Information

APPLICATION TYPE	Utility - Nonprovisional Application under 35 USC 111(a)	PATENT #	-
CONFIRMATION #	9001	FILED BY	MaxVal Group
PATENT CENTER #	69077612	FILING DATE	07/28/2023
CUSTOMER #	157070	FIRST NAMED INVENTOR	Yang LI
CORRESPONDENCE ADDRESS	-	AUTHORIZED BY	Ashley Sherrid

Documents

TOTAL DOCUMENTS: 21

DOCUMENT	PAGES	DESCRIPTION	SIZE (KB)
SRZN-006_04US_328202-2203_IDS_Transmittal_2025-02-06.pdf	3	Transmittal Letter	111 KB
SRZN-006_04US_328202-2203_IDS_SB08_20250206111043.pdf	3	Information Disclosure Statement (IDS) Form (SB08)	116 KB
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FOR_WO2024249444A2_EFS.pdf	90	Foreign Reference	4195 KB
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Interrelationship-3-13-2018.pdf			
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NPL_Chen_H-Protocol-to-Generate_2020.pdf	20	Non Patent Literature	16525 KB
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NPL_WODARZ-Mechanisms-59-88-1998.pdf	30	Non Patent Literature	17931 KB
NPL_ZHANG-A_Small-Molecule-2020-23pages.pdf	23	Non Patent Literature	16179 KB
NPL_ZHANG-Wnt_BETA-catenin-3383-3393-2015.pdf	11	Non Patent Literature	11758 KB

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Application Number	18/361,782
Filing Date	07-28-2023
First Named Inventor	LI; Yang
Art Unit	1645
Examiner Name	CHEONG, CHEOM-GIL
Attorney Docket Number	SRZN-006/04US 328202-2203

U. S. PATENT DOCUMENTS

Examiner Initials*		Cite No.[1]	Document Number Number-Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
		001	US-20240368287	11-07-2024	LI; Yang et al.	
		002	US-20240391991	11-28-2024	FLETCHER; Russell et al.	

FOREIGN PATENT DOCUMENTS

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		003	WO-2020018445-A1	01-23-2020	UNIV LELAND STANFORD JUNIOR., et al.		☐
		004	WO-2024249444-A2	12-05-2024	SURROZEN OPERATING INC.		☐

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Application Number	18/361,782
Filing Date	07-28-2023
First Named Inventor	LI; Yang
Art Unit	1645
Examiner Name	CHEONG, CHEOM-GIL
Attorney Docket Number	SRZN-006/04US 328202-2203

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		005	ACKERS, I., et al.; "Interrelationship of canonical and non-canonical Wnt signalling pathways in chronic metabolic diseases," Diab Vasc Dis Res.; 15(1):3-13 (2018).	☐
		006	CARMON et al. "R-spondins function as ligands of the orphan receptors LGR4 and LGR5 to regulate Wnt/ß-catenin signaling" Proceedings of the National Academy of Sciences (2011); 108(28):11452-11457.	☐
		007	CHAWLA, S., et al. "Establishment of in vitro model of corneal scar pathophysiology" J Cell Physiol. (2017); 1-14.	☐
		008	CHEN, H. et al. "Protocol to Generate and Characterize Potent and Selective WNT Mimetic Molecules" STAR Protocols (2020); 100043, 20 pages.	☐
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		010	FLANAGAN et al. "Frizzled7 functions as a Wnt receptor in intestinal epithelial Lgr5+ stem cells" Stem Cell Reports (2015); 4(5):759-767.	☐
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		013	MIAO et al. "Next-generation surrogate Wnts support organoid growth and deconvolute frizzled pleiotropy in vivo" Cell Stem Cell (2020); 27(5):840-851.	☐
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Complete if Known	
Application Number	18/361,782
Filing Date	07-28-2023
First Named Inventor	LI; Yang
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		015	NUSSE et al. "Wnt/ß-catenin signaling, disease, and emerging therapeutic modalities" Cell (2017); 169(6):985-999.	☐
		016	POST, Y., et al.; "Design principles and therapeutic applications of novel synthetic WNT signaling agonists," iScience; 27(6):109938; pp. 1-18 (2024).	☐
		017	SATO et al. "Paneth cells constitute the niche for Lgr5 stem cells in intestinal crypts" Nature (2011); 469(7330):415-418.	☐
		018	VAN ES et al. "Wnt signalling induces maturation of Paneth cells in intestinal crypts" Nature Cell Biology (2005); 7(4):381-386.	☐
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		020	ZHANG, C., et al.; "A Small-Molecule Wnt Mimic Improves Human Limbal Stem Cell Ex Vivo Expansion," iScience; 23(5):101075; pp. 1 -23 (2020).	☐
		021	ZHANG, Y., et al.; "Wnt/ß-catenin signaling modulates corneal epithelium stratification via inhibition of Bmp4 during mouse development," Development; 142(19):3383-3393 (2015).	☐

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(71) Applicants: **THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY** [US/US]; Office of the General Counsel, Building 170, 3rd Floor, Main Quad P.O., Box 20386, Stanford, California 94305-2038 (US). **UNIVERSITY OF WASHINGTON** [US/US]; 4311 Eleventh Avenue, NE, Suite 500, Seattle, Washington 98105-4608 (US).

(72) Inventors; and
(71) Applicants: **GARCIA, Kenan Christopher**; 580 Kenwood Drive, Menlo Park, California 94025 (US). **MIAO, Yi**; 736 Grandview Ter., San Jose, California 95133 (US). **BAKER, David**; 1628 22nd Ave. NE, Seattle, Washington 98112 (US). **PARK, Keunwan** . **DANG, Luke**; 11427 W. Lake Joy Dr. NE, Carnation, Washington 98014 (US).

(74) Agent: **SHERWOOD, Pamela J.**; 201 Redwood Shores Parkway, Suite 200, Redwood City, California 94065 (US).

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(54) Title: FRIZZLED SPECIFIC WNT AGONISTS AND ANTAGONISTS

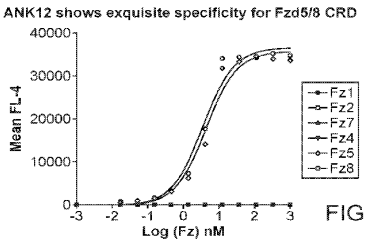


FIG. 1A

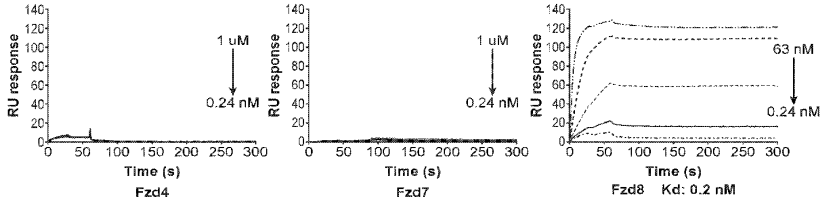


FIG. 1B

(57) Abstract: Wnt signaling antagonist and agonist compositions and methods for their use are provided.

[Continued on next page]

WO 2020/018445 A1

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FRIZZLED SPECIFIC WNT AGONISTS AND ANTAGONISTS

CROSS REFERENCE

[0001] This application claims the benefit of United States Provisional Application No. 62/698,576, filed July 16, 2018, which is incorporated herein by reference in its entirety.

BACKGROUND

[0002] Wnts (*Wingless* and Int-1) are central mediators of vertebrate and invertebrate development, due to their influences on cell proliferation, differentiation, and migration. Wnts act through activation of cell surface receptors on responder cells which activate at least three different signaling pathways including the "canonical" β-catenin pathway, and the "non-canonical" planar cell polarity (PCP) and Ca^{2+} pathways.

[0003] Wnt/β-catenin signal transduction results in the cytoplasmic protein β-catenin entering the nucleus to modulate transcription. When the pathway is not activated, β-catenin is subject to a cycle of continual synthesis and destruction by the β-catenin destruction complex, comprised of the scaffold proteins Axin and APC and the kinases GSK3 and casein kinase 1 (CK1). Wnt signaling removes APC from the complex and relocalizes the other components to the plasma membrane via the adaptor Dsh, thus stabilizing β-catenin which enters the nucleus to mediate transcription.

[0004] The seven-pass transmembrane receptor Frizzled (Fz) is critical for nearly all Wnt signaling, and the N-terminal Fz cysteine rich domain (CRD) serves as the Wnt binding domain. In addition to Fz, the Wnt/β-catenin pathway requires the Low-density lipoprotein receptor related proteins 5 and 6 (Lrp5/6) to serve as co-receptors. LRP5 and LRP6 are functionally redundant single-pass transmembrane receptors. Biochemical studies of LRP6 indicate that different Wnts may bind to different extracellular domains of the LRP5/6 protein. The LRP6 extracellular domain contains four repeating sequences of β-propeller and epidermal growth factor-like (βP--E) domains. The crystal structures of the extracellular LRP6 regions indicate that the βP--E repeats represent two discrete, compact, rigid structures, each containing two βP--E pairs. Wnt9b binds the first two βP--E repeats on the extracellular domain of LRP6, whereas Wnt3a binds the last two βP--E domains. Binding of Wnt ligands to Fz and LRP5/6 results in the production of phosphatidylinositol (4,5)-bisphosphate (PIP2). Increased PIP2 induces oligomerization and clustering of LRP5/6. Increased PIP2 induces recruitment of Axin to LRP5/6. This recruitment may be due, in part, to the action of Amer1/WTX (APC membrane recruitment 1 or Wilms tumor gene on the X chromosome), a tumor suppressor mutated in Wilms' tumor that binds to Axin,

CK1γ, and GSK3. Amer1/WTX is recruited to the plasma membrane in a PIP2-dependent manner.

[0005] The development of pharmaceutically active Wnt agonist and antagonists that are water soluble is therefore of great interest.

SUMMARY

[0006] Wnt is a lipidated protein that is difficult to express in a recombinant form. However, a number of *in vitro* cell culture systems, particularly those comprising stem cells, such as organoid cultures, may require the presence of Wnt, or molecules that act as Wnt signaling agonists. Provided herein are engineered proteins that act as Wnt signaling agonists or antagonists, which are readily expressed in conventional recombinant systems, and can be used in culture media to provide Wnt signaling agonist or antagonist activity. The engineered proteins are water soluble and bind with high affinity to frizzled (Fzd) proteins. A benefit of the proteins disclosed herein is their selectivity for binding to frizzled sub-types.

[0007] In one embodiment, engineered Wnt signaling antagonist DFBs proteins are provided. These proteins are exemplified by the sequences set forth in SEQ ID NO:1-4. The proteins were engineered from an ankyrin-repeat (ANK) based protein scaffold by amino acid substitutions and selections to provide for high affinity binding to selected Fzd proteins. The polypeptide binds one or more human Fzd proteins at high affinity, e.g. a Kd of less than about 1×10^{-7} M, less than about 1×10^{-8} M, less than about 1×10^{-9} M, or less than about 1×10^{-10} M. Fzd binding domain can also be affinity selected to enhance binding to a desired Fzd protein or plurality of Fzd proteins, e.g. to provide tissue selectivity. In some embodiments the Fzd binding domain binds to one, two, three, four, five or more different frizzled proteins, e.g. one or more of human frizzled proteins Fz1, Fz2, Fz3, Fz4, Fz5, Fz6, Fz7, Fz8, Fz9, Fz10. The frizzled binding domain can have a specificity for the one or more desired frizzled protein of at least 10-fold, 25-fold, 50-fold, 100-fold, 200-fold or more relative to other frizzled proteins.

[0008] In another embodiment, engineered Wnt signaling agonist proteins are provided, which are comprised of a Wnt signaling antagonist, e.g. comprising a sequence of any of SEQ ID NO:1-4, joined to an Lrp-binding domain. These proteins are exemplified by the sequences set forth in SEQ ID NO:5-8. The Wnt signaling agonist molecule is therefore comprised of separate or contiguous binding domains or elements for Fzd, and for Lrp5/6. The Fzd binding domain/element and the Lrp5/6 binding domain/element may be directly fused, or may be separated by a linker, e.g. a polypeptide linker, or a non-peptidic linker, *etc.* Where the linker is a peptide linker, it may be from about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20

21, 22, 23, 24, 25, 26, 27, 28, 29, 30 or more amino acids in length, and is of sufficient length and amino acid composition to enforce the distance between binding domains. In some embodiments the linker comprises or consists of one or more glycine and/or serine residues. The Lrp5/6 binding domain or element binds Lrp5/6 at high affinity, e.g. a K_D of less than about 1 x 10^{-7} M, less than about 1 x 10^{-8} M, less than about 1 x 10^{-9} M, less than about 1 x 10^{-10} M. Suitable Lrp5/6 binding domains include DKK1, DKK2, DKK3, DKK4; and the like. In certain embodiments the Lrp5/6 binding domain is the c-terminal portion of DKK1.

[0009] A Wnt signaling agonist or antagonist described herein can be multimerized, e.g. through an Fc domain, by concatenation, coiled coils, polypeptide zippers, biotin/avidin or streptavidin multimerization, and the like. The Wnt signaling agonist can also be joined to a moiety such as PEG, Fc, etc. as known in the art to enhance stability in vivo.

[0010] Compositions of interest include, without limitation, an effective dose of an agonist or antagonist described herein in culture media, or a pharmaceutically acceptable excipient. Such compositions may comprise additional agents, *e.g.* adjuvants, additional growth factors, and the like. Wnt signaling agonists or antagonists described herein may be produced synthetically; by various suitable recombinant methods, and the like, as known in the art. In addition, a benefit of the water soluble polypeptides provided herein is the lack of a requirement for formulation additives, e.g. lipids, detergents, *etc.* that might limit their therapeutic utility.

[0011] In some aspects of the invention, a method is provided for altering, activating, increasing or enhancing Wnt signaling in a cell, including without limitation a cell grown in medium *in vitro*, e.g. an organoid culture. In such methods, a cell expressing a frizzled receptor is contacted with a concentration of a Wnt signaling agonist that is effective to increase signaling, *e.g.* to increase signaling by 25%, 50%, 75%, 90%, 95%, or more, relative to the signaling in the absence of the Wnt signaling agonist. Such signaling activation may induce proliferation of the targeted cell, which cells include without limitation organoids, stem cells, *etc.*

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is best understood from the following detailed description when read in conjunction with the accompanying drawings. It is emphasized that, according to common practice, the various features of the drawings are not to-scale. On the contrary, the dimensions of the various features are arbitrarily expanded or reduced for clarity. Included in the drawings are the following figures.

[0012] **FIG. 1A-1B**. (**FIG. 1A**) ANK12 yeast titration with individual Fzd CRD. (**FIG. 1B**) SPR results of ANK12 to Fzd4, 7 and 8.

[0013] **FIG. 2**. Affinity measurement of 1AF34, 1AF34F7 and 4AF30 towards different Fzds.

[0014] **FIG. 3**. Affinity measurement of 1AF34F7.

[0015] **FIG. 4**. Affinity measurement of 4AF30.

[0016] **FIG. 5A-5D**. (**FIG. 5A**) Activity of DFBs agonists on HEK293T cell line with Fzd1/2/4/5/7/8 knockout. (**FIG. 5B**) Activity of agonists against HEK293T cell line with Fzd1/2/4/5/7/8 knockout with transfected Fzd4 receptor. (**FIG. 5C**) Activity of agonists against HEK293T cell line with Fzd1/2/4/5/7/8 knockout with transfected Fzd7 receptor. (**FIG. 5D**) Activity of agonists against HEK293T cell line with Fzd1/2/4/5/7/8 knockout with transfected Fzd8 receptor. Going from left to right, starting concentrations are 100 nM with 2-fold dilution to 0.39 nM.

[0017] **FIG. 6**. Activity of Wnt agonist in supporting human colon organoid growth.

[0018] **FIG. 7**. Activity of Wnt agonist in supporting human rectal organoid.

[0019] **FIG. 8**. A schematic and sequence of an exemplary expression construct, in which the Wnt signaling agonist is expressed with a cystatin signal sequence, a linker, and an Fc tag joined by a cleavable linker. The complete construct is shown as SEQ ID NO:14. The coding sequence is provided as SEQ ID NO:15. The mature protein comprises the sequence of SEQ ID NO:5.

DETAILED DESCRIPTION OF THE INVENTION

[0020] Before the present methods and compositions are described, it is to be understood that this invention is not limited to particular method or composition described, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims.

[0021] Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limits of that range is also specifically disclosed. Each smaller range between any stated value or intervening value in a stated range and any other stated or intervening value in that stated range is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included or excluded in the range, and each range where either, neither or both limits are included in the smaller ranges is also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the invention.

[0022] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention

belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, some potential and preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited. It is understood that the present disclosure supersedes any disclosure of an incorporated publication to the extent there is a contradiction.

[0023] It must be noted that as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a cell" includes a plurality of such cells and reference to "the peptide" includes reference to one or more peptides and equivalents thereof, e.g. polypeptides, known to those skilled in the art, and so forth.

[0024] The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention. Further, the dates of publication provided may be different from the actual publication dates which may need to be independently confirmed.

[0025] By "comprising" it is meant that the recited elements are required in the composition/method/kit, but other elements may be included to form the composition/method/kit etc. within the scope of the claim. For example, a composition comprising a Wnt agonist or antagonist described herein is a composition that may comprise other elements in addition to Wnt agonist or antagonist described herein, e.g. functional moieties such as polypeptides, small molecules, or nucleic acids bound, e.g. covalently bound, to the Wnt agonist or antagonist described herein; agents that promote the stability, agents that promote solubility, adjuvants, etc. as will be readily understood in the art, with the exception of elements that are encompassed by any negative provisos.

[0026] By "consisting essentially of", it is meant a limitation of the scope of composition or method described to the specified materials or steps that do not materially affect the basic and novel characteristic(s) of the subject invention. For example, a Wnt agonist or antagonist "consisting essentially of" a disclosed sequence has the amino acid sequence of the disclosed sequence plus or minus about 5 amino acid residues at the boundaries of the sequence based upon the sequence from which it was derived, e.g. about 5 residues, 4 residues, 3 residues, 2 residues or about 1 residue less than the recited bounding amino acid residue, or about 1 residue, 2 residues, 3 residues, 4 residues, or 5 residues more than the recited bounding amino acid residue.

[0027] By "consisting of", it is meant the exclusion from the composition, method, or kit of any element, step, or ingredient not specified in the claim. For example, a Wnt agonist or antagonist "consisting of" a disclosed sequence consists only of the disclosed amino acid sequence.

[0028] General methods in molecular and cellular biochemistry can be found in such standard textbooks as Molecular Cloning: A Laboratory Manual, 3rd Ed. (Sambrook et al., CSH Laboratory Press 2001); Short Protocols in Molecular Biology, 4th Ed. (Ausubel et al. eds., John Wiley & Sons 1999); Protein Methods (Bollag et al., John Wiley & Sons 1996); Nonviral Vectors for Gene Therapy (Wagner et al. eds., Academic Press 1999); Viral Vectors (Kaplift & Loewy eds., Academic Press 1995); Immunology Methods Manual (I. Lefkovits ed., Academic Press 1997); and Cell and Tissue Culture: Laboratory Procedures in Biotechnology (Doyle & Griffiths, John Wiley & Sons 1998), the disclosures of which are incorporated herein by reference. Reagents, cloning vectors, and kits for genetic manipulation referred to in this disclosure are available from commercial vendors such as BioRad, Stratagene, Invitrogen, Sigma-Aldrich, and ClonTech.

Compositions

[0029] Wnt signaling agonist or antagonist proteins and methods for their use are provided. These and other objects, advantages, and features of the invention will become apparent to those persons skilled in the art upon reading the details of the compositions and methods as more fully described below.

[0030] Wnt signaling agonist or antagonist proteins of the present invention are biologically active in binding to a cognate Frizzled receptor, resulting in activation or inhibition of Wnt signaling. The term "Wnt signaling agonist activity" refers to the ability of an agonist to mimic the effect or activity of a Wnt protein binding to a frizzled protein and Lrp, thereby activating the Wnt signaling pathway. The term "Wnt signaling antagonist activity" refers to the ability of an antagonist to reduce or otherwise block Wnt protein binding to a frizzled protein, thereby reducing activity of the Wnt signaling pathway.

[0031] The ability of the engineered proteins to mimic or inhibit the activity of Wnt can be confirmed by a number of assays. The engineered proteins typically initiate a reaction or activity that is similar to or the same as that initiated by the receptor's natural ligand. In particular, agonists of the invention enhance the canonical Wnt/β-catenin signaling pathway while antagonists inhibit the pathway. As used herein, the term "enhances" refers to a measurable increase in the level of Wnt/β-catenin signaling compared with the level in the absence of an agonist of the invention.

[0032] Various methods are known in the art for measuring the level of canonical Wnt/β-catenin signaling. These include, but are not limited to assays that measure: Wnt/β-catenin target gene expression; TCF reporter gene expression; beta-catenin stabilization; LRP phosphorylation; Axin translocation from cytoplasm to cell membrane and binding to LRP. The canonical Wnt/β-catenin signaling pathway ultimately leads to changes in gene expression through the transcription factors TCF7, TCF7L1, TCF7L2 and LEF. The transcriptional response to Wnt activation has been characterized in a number of cells and tissues. As such, global transcriptional profiling by methods well known in the art can be used to assess Wnt/β-catenin signaling activation.

[0033] Changes in Wnt-responsive gene expression are generally mediated by TCF and LEF transcription factors. A TCF reporter assay assesses changes in the transcription of TCF/LEF controlled genes to determine the level of Wnt/.beta.-catenin signaling. A TCF reporter assay was first described by Korinek, V. et al., 1997. Also known as TOP/FOP this method involves the use of three copies of the optimal TCF motif CCTTTGATC, or three copies of the mutant motif CCTTTGGCC, upstream of a minimal c-Fos promoter driving luciferase expression (pTOPFLASH and pFOPFLASH, respectively) to determine the transactivational activity of endogenous β-catenin/TCF4. A higher ratio of these two reporter activities (TOP/FOP) indicates higher β-catenin/TCF4 activity.

[0034] Various other reporter transgenes that respond to Wnt signals exist intact in animals and therefore, effectively reflect endogenous Wnt signaling. These reporters are based on a multimerized TCF binding site, which drives expression of LacZ or GFP, which are readily detectable by methods known in the art. These reporter genes include: TOP-GAL, BAT-GAL, ins-TOPEGFP, ins-TOPGAL, LEF-EGFP, Axin2-LacZ, Axin2-d2EGFP, Lgr5tm1(cre/ERT2), TOPdGFP.

[0035] The recruitment of dephosphorylated β–catenin to the membrane, stabilisation and phosphorylation status of β-catenin and translocation of β-catenin to the nucleus (Klapholz-Brown Z et al., PLoS One. 2(9) e945, 2007) in some cases mediated by complex formation with TCF transcription factors and TNIK are key steps in the Wnt signaling pathway. Stabilisation is mediated by Disheveled family proteins that inhibit the "destruction" complex so that degradation of intracellular β-catenin is reduced, and translocation of β-catenin to the nucleus follows thereafter. Therefore, measuring the level and location of β-catenin in a cell is a good reflection of the level of Wnt/β-catenin signaling. A non-limiting example of such an assay is the "BioImage β-Catenin Redistribution Assay" (Thermo Scientific) which provides recombinant U2OS cells that stably express human β-catenin fused to the C-terminus of enhanced green fluorescent protein

(EGFP). Imaging and analysis is performed with a fluorescence microscope or HCS platform allowing the levels and distribution of EGFP-β-catenin to be visualized.

[0036] Another way, in which the destruction complex is inhibited, is by removal of Axin by recruitment of Axin to the cytoplasmic tail of the Wnt co-receptor LRP. Axin has been shown to bind preferentially to a phosphorylated form of the LRP tail. Visualisation of Axin translocation, for example with a GFP-Axin fusion protein, is therefore another method for assessing levels of Wnt/β-catenin signaling.

[0037] In certain embodiments, the agonist or antagonist of the invention alter β-catenin signaling by at least 30%, 35%, 40%, 45%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 100%, 110%, 150%, 200%, 250%, 300%, 400% or 500% compared to the β-catenin signaling induced by a neutral substance or negative control as measured in an assay described above, for example as measured in the TOPFlash assay. A negative control may be included in these assays.

[0038] "Wnt gene product" or "Wnt polypeptide" when used herein encompass native sequence Wnt polypeptides, Wnt polypeptide variants, Wnt polypeptide fragments and chimeric Wnt polypeptides. In particular embodiments, a Wnt polypeptide is a native human full length mature Wnt protein. For example, human native sequence Wnt proteins of interest in the present application include the following: Wnt-1 (GenBank Accession No. NM_005430); Wnt-2 (GenBank Accession No. NM_003391); Wnt-2B (Wnt-13) (GenBank Accession No. NM_004185 (isoform 1), NM_024494.2 (isoform 2)), Wnt-3 (RefSeq.: NM_030753), Wnt3a (GenBank Accession No. NM_033131), Wnt-4 (GenBank Accession No. NM_030761), Wnt-5A (GenBank Accession No. NM_003392), Wnt-5B (GenBank Accession No. NM_032642), Wnt-6 (GenBank Accession No. NM_006522), Wnt-7A (GenBank Accession No. NM_004625), Wnt-7B (GenBank Accession No. NM_058238), Wnt-8A (GenBank Accession No. NM_058244), Wnt-8B (GenBank Accession No. NM_003393), Wnt-9A (Wnt-14) (GenBank Accession No. NM_003395), Wnt-9B (Wnt-15) (GenBank Accession No. NM_003396), Wnt-10A (GenBank Accession No. NM_025216), Wnt-10B (GenBank Accession No. NM_003394), Wnt-11 (GenBank Accession No. NM_004626), Wnt-16 (GenBank Accession No. NM_016087)). Although each member has varying degrees of sequence identity with the family, all encode small (i.e., 39-46 kD), acylated, palmitoylated, secreted glycoproteins that contain 23-24 conserved cysteine residues whose spacing is highly conserved (McMahon, A P et al., Trends Genet. 1992; 8: 236-242; Miller, J R. Genome Biol. 2002; 3(1): 3001.1-3001.15).

[0039] "Wnt protein signaling" or "Wnt signaling" is used herein to refer to the mechanism by which a biologically active Wnt exerts its effects upon a cell to modulate a cell's activity. Wnt proteins modulate cell activity by binding to Wnt receptors, including proteins from the Frizzled (Fz) family of proteins, proteins from the ROR family of proteins, the proteins LRP5, LRP6 from the LRP family of proteins, the protein FRL1/crypto, and the protein Derailed/Ryk. Once activated by Wnt binding, the Wnt receptor(s) will activate one or more intracellular signaling cascades. These include the canonical Wnt signaling pathway; the Wnt/planar cell polarity (Wnt/PCP) pathway; the Wnt-calcium (Wnt/Ca^{2+}) pathway (Giles, RH et al. (2003) Biochim Biophys Acta 1653, 1-24; Peifer, M. et al. (1994) Development 120: 369-380; Papkoff, J. et al (1996) Mol. Cell Biol. 16: 2128-2134; Veeman, M. T. et al. (2003) Dev. Cell 5: 367-377); and other Wnt signaling pathways as is well known in the art. Activation of the canonical Wnt signaling pathway results in the inhibition of phosphorylation of the intracellular protein ß-catenin, leading to an accumulation of ß-catenin in the cytosol and its subsequent translocation to the nucleus where it interacts with transcription factors, e.g. TCF/LEF, to activate target genes. A "biologically active Wnt signaling agonist" is a Wnt signaling agonist composition that is able to specifically bind to a Fzd receptor and activate Wnt signaling when provided to a cell *in vitro* or *in vivo*, that is, when present in cell culture media, *etc.*

[0040] In certain embodiments, a Wnt signaling agonist or antagonist alters signaling of the canonical Wnt pathway by at least about 50%, about 75%, about 100%, about 150%, about 200%, about 300%, about 400%, about 5-fold, about 10-fold, and may increase or decrease signaling by 50-fold, 100-fold, 500-fold, or more, relative to the level of Wnt signaling in the absence of the protein.

[0041] The term "specific binding" refers to that binding which occurs between such paired species as enzyme/substrate, receptor/ligand, *etc.* which may be mediated by covalent or non-covalent interactions or a combination of covalent and non-covalent interactions. Accordingly, "specific binding" occurs between a paired species where there is interaction between the two which produces a bound complex. Methods known in the art may be used to determine the affinity of the interaction.

[0042] By "water soluble" it is meant a composition that is soluble in aqueous buffers in the absence of detergent, usually soluble at a concentration that provides a biologically effective dose of the polypeptide. Compositions that are water soluble form a substantially homogenous composition that has a specific activity that is at least about 5% that of the starting material from

which it was purified, usually at least about 10%, 20%, or 30% that of the starting material, more usually about 40%, 50%, or 60% that of the starting material, and may be about 50%, about 90% or greater. Engineered proteins compositions of the present invention typically form a substantially homogeneous aqueous solution at concentrations of at least 25 μM and higher, e.g. at least 25 μM, 40 μM, or 50 μM, usually at least 60 μM, 70 μM, 80 μM, or 90 μM, sometimes as much as 100 μM, 120 μM, or 150μM. In other words, proteins of the present invention typically form a substantially homogeneous aqueous solution at concentrations of about 0.1 mg/ml, about 0.5 mg/ml, of about 1 mg/ml or more.

[0043] *Fzd binding domain.* The Fzd binding domain is an engineered protein as described herein and exemplified by the polypeptides of any of SEQ ID NO:1-4. The polypeptides bind to at least one Fzd protein at high affinity, e.g. a K_D of less than about 1×10^{-7} M, less than about 1×10^{-8} M, less than about 1×10^{-9} M, less than about 1×10^{-10} M. Suitable Fzd binding domains are engineered Fzd binding proteins based on a DFBs scaffold; and the like. The Fzd binding domains have been affinity selected to enhance binding to a desired Fzd protein or plurality of Fzd proteins, e.g. to provide tissue selectivity. Methods of affinity selection for this purpose utilize one or more rounds of selection by introducing targeted amino acid changes and generating a library of candidate coding sequences, transforming a population of cells with the candidate coding sequence, e.g. into yeast cells, selecting (for example using paramagnetic microbeads) for the desired specificity. Typically multiple rounds of selection are performed, and the resulting vectors sequenced and used as the basis for protein engineering. The Fzd binding domain may bind to one, two, three, four, five or more different frizzled proteins, e.g. one or more of human frizzled proteins Fz1, Fz2, Fz3, Fz4, Fz5, Fz6, Fz7, Fz8, Fz9, Fz10.

[0044] *Lrp5/6 binding domain.* An Lrp5/6 may be selected from any domain that binds Lrp5 or Lrp6 at high affinity, e.g. with a K_D of less than about 1×10^{-7} M, less than about 1×10^{-8} M, less than about 1×10^{-9} M, less than about 1×10^{-10} M.

[0045] "LRP", "LRP proteins" and "LRP receptors" is used herein to refer to proteins of the low density lipoprotein receptor-related protein family. These receptors are single-pass transmembrane proteins that bind and internalize ligands in the process of receptor- mediated endocytosis. LRP proteins LRP5 (GenBank Accession No. NM 002335.2) and LRP6 (GenBank Accession No. NM 002336.2) are included in the Wnt receptor complex.

[0046] Suitable Lrp5/6 binding domains include, without limitation, de novo designed Lrp5/6 binding proteins, antibody derived binding proteins, *e.g.* scFv, Fab, *etc.* and other portions of

antibodies that specifically bind to one or more Fzd proteins; nanobody derived binding domains; knottin-based engineered scaffolds; naturally occurring Lrp5/6, including particularly limitation, DKK1, DKK2, DKK3, DKK4, sclerostin; Wise; fusions proteins comprising any of the above; derivatives of any of the above; variants of any of the above; and biologically active fragments of any of the above, and the like.

[0047] Members of the Dickkopf (Dkk) gene family (see Krupnik et al. (1999) Gene 238(2):301-13) include Dkk-1, Dkk-2, Dkk-3, and Dkk-4, and the Dkk-3 related protein Soggy (Sgy). hDkks 1-4 contain two distinct cysteine-rich domains in which the positions of 10 cysteine residues are highly conserved between family members. Exemplary sequences of human Dkk genes and proteins are publicly available, e.g. Genbank accession number NM_014419 (soggy-1); NM_014420 (DKK4); AF177394 (DKK-1); AF177395 (DKK-2); NM_015881 (DKK3); and NM_014421 (DKK2). In some embodiments of the invention, the Lrp6 binding moiety is a DKK1 peptide, including without limitation the C-terminal domain of human DKK1. The C-terminal domain may comprise the sequence (SEQ ID NO:12) KMYHTKGQEGSVCLRSSDCASGLCCARHFWSKICKPVLKEGQVCTKHRRKGSHGLEIFQRCY CGEGLSCRIQKDHHQASNSSRLHTCQRH (see Genbank accession number NP_036374) or a biologically active fragment thereof.

[0048] Binding of DKK proteins to LRP5/6 are discussed, for example in Brott and Sokol Mol. Cell. Biol. 22 (17), 6100-6110 (2002); and Li et al. J. Biol. Chem. 277 (8), 5977-5981 (2002), each herein specifically incorporated by reference. The corresponding region of human DKK2 (Genbank reference NP_055236) may comprise the sequence (SEQ ID NO:13) KMSHIKGHEGDPCLRSSDCIEGFCCARHFWTKICKPVLHQGEVCTKQRKKGSHGLEIFQRCDC AKGLSCKVWKDATYSSKARLHVCQK or a biologically active fragment thereof.

[0049] *Variants.* The polypeptides provided herein may be modified to generate derivatives, variants, and biologically active fragments. A "variant" polypeptide means a biologically active polypeptide as defined below having less than 100% sequence identity with a provided sequence. Such variants include polypeptides comprising one or more amino acid modifications, e.g., insertions, deletions or substitutions, as compared to the provided sequence, e.g., wherein one or more amino acid residues are added at the N- or C-terminus of, or within, the native sequence; from about one to forty amino acid residues are deleted, and optionally substituted by one or more amino acid residues; and derivatives of the above polypeptides, wherein an amino acid residue has been covalently modified so that the resulting product has a non-naturally occurring amino acid. Ordinarily, a biologically active variant will have an amino acid sequence having at least

about 90% amino acid sequence identity with a native sequence polypeptide, preferably at least about 95%, more preferably at least about 99%.

[0050] A "functional derivative" of a sequence is a compound having a qualitative biological property in common with an initial sequence. "Functional derivatives" include, but are not limited to, fragments of a sequence and derivatives of a sequence, provided that they have a biological activity in common. The term "derivative" encompasses both amino acid sequence variants of polypeptide and covalent modifications thereof.

[0051] Wnt signaling agonists or antagonists for use in the subject compositions and methods may be modified using ordinary molecular biological techniques and synthetic chemistry so as to improve their resistance to proteolytic degradation or to optimize solubility properties or to render them more suitable as a therapeutic agent. Analogs of such polypeptides include those containing residues other than naturally occurring L-amino acids, e.g. D-amino acids or non-naturally occurring synthetic amino acids. D-amino acids may be substituted for some or all of the amino acid residues.

[0052] The Wnt signaling agonist or antagonists may be prepared by *in vitro* synthesis, using conventional methods as known in the art. Various commercial synthetic apparatuses are available, for example, automated synthesizers by Applied Biosystems, Inc., Beckman, etc. By using synthesizers, naturally occurring amino acids may be substituted with unnatural amino acids. The particular sequence and the manner of preparation will be determined by convenience, economics, purity required, and the like. If desired, various groups may be introduced into the peptide during synthesis or during expression, which allow for linking to other molecules or to a surface. Thus cysteines can be used to make thioethers, histidines for linking to a metal ion complex, carboxyl groups for forming amides or esters, amino groups for forming amides, and the like.

[0053] A Wnt signaling agonist or antagonist may be fused or bonded to an additional polypeptide sequence. Examples include signal sequences for secretion of the polypeptides, immunoadhesins, which combine a Wnt signaling agonist or antagonist with an immunoglobulin sequence, particularly an Fc sequence, and epitope tagged polypeptides, which comprise a native inhibitors polypeptide or portion thereof fused to a "tag polypeptide". The tag polypeptide has enough residues to provide an epitope against which an antibody can be made, yet is short enough such that it does not interfere with biological activity of the original polypeptide. Suitable tag polypeptides generally have at least six amino acid residues and usually between about 6-60 amino acid residues. The Wnt agonist or antagonist may also be fused or combined in a

formulation, or co-administered with an agent that enhances Wnt activity, *e.g.* R-spondin 1, R-spondin 2, anti-sclerosin antibody, *etc.*

[0054] *Linker.* The Fzd binding sequence and the Lrp5/6 binding sequence may be separated by a linker, e.g. a polypeptide linker, or a non-peptidic linker, *etc.* In some embodiments the linker is a rigid linker, in other embodiments the linker is a flexible linker. In some embodiments, the linker moiety is a peptide linker. In some embodiments, the peptide linker comprises 2 to 100 amino acids. In some embodiments, the peptide linker comprises 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 but no greater than 100 amino acids. In some embodiments, the peptide linker is between 5 to 75, 5 to 50, 5 to 25, 5 to 20, 5 to 15, 5 to 10 or 5 to 9 amino acids in length. Exemplary linkers include linear peptides having at least two amino acid residues such as Gly-Gly, Gly-Ala-Gly, Gly-Pro-Ala, Gly-Gly-Gly-Gly-Ser. Suitable linear peptides include poly glycine, polyserine, polyproline, polyalanine and oligopeptides consisting of alanyl and/or serinyl and/or prolinyl and/or glycyl amino acid residues. In some embodiments, the peptide linker comprises the amino acid sequence selected from the group consisting of Gly_9, Glu_9, Ser_9, Gly_5-Cys-Pro_2-Cys, $(Gly_4\text{-}Ser)_3$, Ser-Cys-Val-Pro-Leu-Met-Arg-Cys-Gly-Gly-Cys-Cys-Asn, Pro-Ser-Cys-Val-Pro-Leu-Met-Arg-Cys-Gly-Gly-Cys-Cys-Asn, Gly-Asp-Leu-Ile-Tyr-Arg-Asn-Gln-Lys, and Gly_9-Pro-Ser-Cys-Val-Pro-Leu-Met-Arg-Cys-Gly-Gly-Cys-Cys-Asn. In one embodiment a linker comprises the amino acid sequence GSTSGSGKSSEGKG, or (GGGGS)n, where n is 1, 2, 3, 4, 5, etc.; however many such linkers are known and used in the art and may serve this purpose.

[0055] The term "*cell culture*" or "*culture*" means the maintenance of cells in an artificial, in vitro environment. It is to be understood, however, that the term "cell culture" is a generic term and may be used to encompass the cultivation not only of individual cells, but also of tissues or organs.

[0056] The term "*culture system*" is used herein to refer to the culture conditions in which the subject explants are grown that promote prolonged tissue expansion with proliferation, multilineage differentiation and recapitulation of cellular and tissue ultrastructure.

[0057] By "*container*" is meant a glass, plastic, or metal vessel that can provide an aseptic environment for culturing cells.

[0058] *Organoid Culture.* Organoids are in-vitro derived 3-dimensional cell aggregates derived from primary tissue or stem cells that are capable of self-renewal, self-organization and exhibit organ functionality. Organoids provide a 3-dimensional model system that recapitulates body processes and functions from the molecular to whole organism level. 3D cell culture models are a more accurate representation of the natural environment experienced by cells in the living organism as opposed to growing cells on 2D flat surfaces.

[0059] Generally organoids harbor small population of self-renewing stem cells that can differentiate into cells of all major cell lineages, with similar frequency as in physiological condition. For example, organoids may be generated either from primary tissues or pluripotent stem cells (induced pluripotent stem cells (iPSC) or embryonic stem cells (ESCs)) by providing appropriate physical and biochemical cue. As such, organoids are biologically relevant to any model system and are amenable to manipulate niche components and gene sequence. However, the presence of the stem cells requires that suitable growth factors also be present, which may include Wnt proteins. The difficulty in producing Wnt protein for this purpose is addressed by the use of the engineered molecules, particularly Wnt agonist polypeptides, described herein.

[0060] Organoid cultures are useful as in vitro model systems, to study processes including tumorigenesis, infection, gene correction, drug toxicity and efficacy testing, etc. Organoids may also provide cells for regenerative medicine. Organoids are also useful in personalized medicine applications, for example organoids derived from adult stem cell of individual patients allows ex-vivo testing of drug response and treatment options.

[0061] See, for example, Yin et al. (2016) Cell Stem Cell 18, 25–38; Lancaster and Knoblich (2014) Science 345, 1247125; Clevers (2016) Cell 165, 1586–1597; Eiraku and Sasai (2012) Curr. Opin. Neurobiol. 22, 768–777; Nakano et al. (2012) Cell Stem Cell 10, 771–785; McCracken et al. (2014) Nature 516, 400–404; Wong et al. (2012) Nat. Biotechnol. 30, 876–882; Huang et al. (2014) Nat. Biotechnol. 32, 84–91; Takebe et al. (2013) Nature 499, 481–484; Takasato et al. (2015) Nature 526, 564–568; Sato and Clevers (2013) Science 340, 1190–1194; Huch et al. (2015) Cell 160, 299–312; Linnemann et al. (2015) Dev. Camb. Engl. 142, 3239–3251; Boj et al. (2015) Cell 160, 324–338; Ciancanelliet al. (2015) Science 348, 448–453; Qian, et al. (2016) Cell 165, 1238–1254; Spence et al. (2011) Nature 470, 105–109.

[0062] The terms "stem cell" as used herein, refer to a cell that has the property of self-renewal and has the developmental potential to differentiate into multiple cell types. A stem cell is capable of proliferation and giving rise to more such stem cells while maintaining its developmental potential. Stem cells can divide asymmetrically, with one daughter cell retaining the

developmental potential of the parent stem cell and the other daughter cell expressing some distinct other specific function, phenotype and/or developmental potential from the parent cell. The daughter cells themselves can be induced to proliferate and produce progeny that subsequently differentiate into one or more mature cell types, while also retaining one or more cells with parental developmental potential. A differentiated cell may derive from a multipotent cell, which itself is derived from a multipotent cell, and so on. While each of these multipotent cells may be considered stem cells, the range of cell types each such stem cell can give rise to, i.e., their developmental potential, can vary considerably. Alternatively, some of the stem cells in a population can divide symmetrically into two stem cells, known as stochastic differentiation, thus maintaining some stem cells in the population as a whole, while other cells in the population give rise to differentiated progeny only. Accordingly, the term "stem cell" refers to any subset of cells that have the developmental potential, under particular circumstances, to differentiate to a more specialized or differentiated phenotype, and which retain the capacity, under certain circumstances, to proliferate without substantially differentiating.

[0063] The term "somatic stem cell" is used herein to refer to any pluripotent or multipotent stem cell derived from non-embryonic tissue, including fetal, juvenile, and adult tissue. Natural somatic stem cells have been isolated from a wide variety of adult tissues including blood, bone marrow, brain, olfactory epithelium, skin, pancreas, skeletal muscle, and cardiac muscle. The term "progenitor cell" is used herein to refer to cells that are at an earlier stage along a developmental pathway or progression, relative to a cell which it can give rise to by differentiation. Often, progenitor cells have significant or very high proliferative potential. Progenitor cells can give rise to multiple distinct differentiated cell types, or to a single differentiated cell type, depending on the developmental pathway and on the environment in which the cells develop and differentiate.

[0064] *Expression construct*: In the present methods, a Wnt signaling agonist or antagonist, if a polypeptide, may be produced by recombinant methods. The nucleic acid encoding the Wnt agonist or antagonist can be inserted into a replicable vector for expression. Many such vectors are available. The vector components generally include, but are not limited to, one or more of the following: an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence. Exemplary coding sequences for a Wnt signaling agonist are provided as SEQ ID NO:9 and SEQ ID NO:10. Codon usage may be optimized for the desired host cell.

[0065] Expression vectors will contain a promoter that is recognized by the host organism and is operably linked to the Wnt agonist or antagonist coding sequence. Promoters are untranslated

sequences located upstream (5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of particular nucleic acid sequence to which they are operably linked. Such promoters typically fall into two classes, inducible and constitutive. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, e.g., the presence or absence of a nutrient or a change in temperature.

[0066] Promoters suitable for use with prokaryotic hosts include the β-lactamase and lactose promoter systems, alkaline phosphatase, a tryptophan (trp) promoter system, and hybrid promoters such as the tac promoter. However, other known bacterial promoters are also suitable. Such nucleotide sequences have been published, thereby enabling a skilled worker operably to ligate them to a DNA coding sequence. Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the coding sequence.

[0067] Promoter sequences are known for eukaryotes. Examples of suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglyceratekinase or other glycolytic enzymes, such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in EP 73,657. Yeast enhancers also are advantageously used with yeast promoters.

[0068] Transcription from vectors in mammalian host cells may be controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus, adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and most preferably Simian Virus 40 (SV40), from heterologous mammalian promoters, e.g., the actin promoter, PGK (phosphoglycerate kinase), or an immunoglobulin promoter, from heat-shock promoters, provided such promoters are compatible with the host cell systems. The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that also contains the SV40 viral origin of replication. The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment.

[0069] Transcription by higher eukaryotes is often increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp, which act on a promoter to increase its transcription. Enhancers are relatively orientation and position independent, having been found 5' and 3' to the transcription unit, within an intron, as well as within the coding sequence itself. Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, α-fetoprotein, and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin, the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. The enhancer may be spliced into the expression vector at a position 5' or 3' to the coding sequence, but is preferably located at a site 5' from the promoter.

[0070] Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) may also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3', untranslated regions of eukaryotic or viral DNAs or cDNAs.

[0071] Construction of suitable vectors containing one or more of the above-listed components employs standard techniques. Isolated plasmids or DNA fragments can be cleaved, tailored, and re-ligated in the form desired to generate the plasmids required. For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures are used to transform host cells, and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction endonuclease digestion, and/or sequenced.

[0072] Suitable host cells for cloning or expressing the DNA in the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes for this purpose include eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteriaceae such as Escherichia, e.g., E. coli, Enterobacter, Erwinia, Klebsiella, Proteus, Salmonella, e.g., Salmonella typhimurium, Serratia, e.g., Serratia marcescans, and Shigella, as well as Bacilli such as B. subtilis and B. licheniformis, Pseudomonas such as P. aeruginosa, and Streptomyces. These examples are illustrative rather than limiting.

[0073] In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable expression hosts. Saccharomyces cerevisiae, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as

Schizosaccharomyces pombe; Kluyveromyces hosts such as K. lactis, K. fragilis, etc.; Pichia pastoris; Candida; Neurospora crassa; Schwanniomyces such as Schwanniomyces occidentalis; and filamentous fungi such as Penicillium, Tolypocladium, and Aspergillus hosts such as A. nidulan, and A. niger.

[0074] Examples of useful mammalian host cell lines are mouse L cells (L-M[TK-], ATCC#CRL-2648), monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture; baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO); mouse sertoli cells (TM4); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1 587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells; MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2).

[0075] Host cells are transfected with the above-described expression vectors for Wnt signaling agonist or antagonist production, and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences. Mammalian host cells may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), Sigma), RPMI 1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM), Sigma) are suitable for culturing the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics, trace elements, and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

Methods of Use

[0076] In certain methods of the present invention, an effective amount of a composition comprising a Wnt agonist or antagonist is provided to cells, *e.g.* by contacting the cell with an effective amount of that composition to achieve a desired effect, e.g. to enhance Wnt signaling, proliferation, *etc.* In particular embodiments, the contacting occurs in vitro, ex vivo or in vivo. In

particular embodiments, the cells are derived from or present within a subject in need or increased Wnt signaling.

[0077] In some methods of the invention, an effective amount of the subject composition is provided to enhance Wnt signaling in a cell. Biochemically speaking, an effective amount or effective dose of a Wnt agonist or antagonist is an amount to alter Wnt signaling in a cell by at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or by 100% relative to the signaling in the absence of the Wnt agonist or antagonist. The amount of modulation of a cell's activity can be determined by a number of ways known to one of ordinary skill in the art of Wnt biology.

[0078] In a clinical sense, an effective dose of a Wnt agonist or antagonist composition is the dose that, when administered to a subject for a suitable period of time, e.g., at least about one week, and maybe about two weeks, or more, up to a period of about 4 weeks, 8 weeks, or longer, will evidence an alteration in the symptoms associated with lack of Wnt signaling. In some embodiments, an effective dose may not only slow or halt the progression of the disease condition but may also induce the reversal of the condition. It will be understood by those of skill in the art that an initial dose may be administered for such periods of time, followed by maintenance doses, which, in some cases, will be at a reduced dosage.

[0079] Cells *in vitro* may be contacted with a composition comprising a Wnt agonist or antagonist by any of a number of well-known methods in the art. For example, the composition may be provided to the cells in the media in which the subject cells are being cultured. Nucleic acids encoding the Wnt agonist or antagonist may be provided to the subject cells or to cells co-cultured with the subject cells on vectors under conditions that are well known in the art for promoting their uptake, for example electroporation, calcium chloride transfection, and lipofection. Alternatively, nucleic acids encoding the Wnt agonist or antagonist may be provided to the subject cells or to cells cocultured with the subject cells via a virus, i.e. the cells are contacted with viral particles comprising nucleic acids encoding the Wnt peptide agonist or antagonist polypeptide. Retroviruses, for example, lentiviruses, are particularly suitable to the method of the invention, as they can be used to transfect non-dividing cells (see, for example, Uchida *et al.* (1998) P.N.A.S. 95(20):11939-44). Commonly used retroviral vectors are "defective", *i.e.* unable to produce viral proteins required for productive infection. Rather, replication of the vector requires growth in a packaging cell line.

[0080] Likewise, cells *in vivo* may be contacted with the subject Wnt agonist or antagonist compositions by any of a number of well-known methods in the art for the administration of peptides, small molecules, or nucleic acids to a subject. The Wnt agonist or antagonist

composition can be incorporated into a variety of formulations or pharmaceutical compositions, which in some embodiments will be formulated in the absence of detergents, liposomes, *etc.*, as have been described for the formulation of full-length Wnt proteins.

[0081] Wnt signaling is a key component of stem cell culture. For example, the stem cell culture media as described in WO2010/090513, WO2012/014076, Sato et al., 2011 (GASTROENTEROLOGY 2011; 141:1762-1772) and Sato et al., 2009 (Nature 459, 262-5). The Wnt signaling agonists of the invention are suitable alternatives to Rspondin for use in these stem cell culture media, or may be combined with Rspondin.

[0082] Accordingly, in one embodiment, the invention provides a method for enhancing the proliferation of stem cells comprising supplying stem cells with agonists of the invention. In one embodiment, the invention provides a cell culture medium comprising one or more agonists of the invention. In some embodiments, the cell culture medium may be any cell culture medium already known in the art that normally comprises Wnt or Rspondin, but wherein the Wnt or Rspondin is replaced (wholly or partially) or supplemented by agonists of the invention. For example, the culture medium may be as described in as described in WO2010/090513, WO2012/014076, Sato et al., 2011 (GASTROENTEROLOGY 2011; 141:1762-1772) and Sato et al., 2009 (Nature 459, 262-5), which are hereby incorporated by reference in their entirety.

[0083] Stem cell culture media often comprise additional growth factors. This method may thus additionally comprise supplying the stem cells with a growth factor. Growth factors commonly used in cell culture medium include epidermal growth factor (EGF, (Peprotech), Transforming Growth Factor-alpha (TGF-alpha, Peprotech), basic Fibroblast Growth Factor (bFGF, Peprotech), brain-derived neurotrophic factor (BDNF, R&D Systems), Human Growth Factor (HGF) and Keratinocyte Growth Factor (KGF, Peprotech, also known as FGF7). EGF is a potent mitogenic factor for a variety of cultured ectodermal and mesodermal cells and has a profound effect on the differentiation of specific cells in vivo and in vitro and of some fibroblasts in cell culture. The EGF precursor exists as a membrane-bound molecule which is proteolytically cleaved to generate the 53-amino acid peptide hormone that stimulates cells. EGF or other mitogenic growth factors may thus be supplied to the stem cells. During culturing of stem cells, the mitogenic growth factor may be added to the culture medium every second day, while the culture medium is refreshed preferably every fourth day. In general, a mitogenic factor is selected from the groups consisting of: i) EGF, TGF-.alpha. and KGF, ii) EGF, TGF-.alpha. and FGF7; iii) EGF, TGF-.alpha. and FGF; iv) EGF and KGF; v) EGF and FGF7; vi) EGF and a FGF; vii) TGF-α and KGF; viii) TGF-.alpha. and FGF7; ix) or from TGF α and a FGF.

[0084] These methods of enhancing proliferation of stem cells can be used to grow new organoids and tissues from stem cells, as for example described in WO2010/090513 WO2012/014076, Sato et al., 2011 (GASTROENTEROLOGY 2011; 141:1762-1772) and Sato et al., 2009 (Nature 459, 262-5).

Compositions

[0085] For use in cell culture the Wnt signaling agonist or antagonist may be provided in culture medium, or may be provided in a form useful to diluting into culture medium. For therapeutic applications, the Wnt signaling agonist or antagonist may be administered to a mammal, preferably a human, in a physiologically acceptable dosage form, including those that may be administered to a human intravenously as a bolus or by continuous infusion over a period of time. Alternative routes of administration include topical, intramuscular, intraperitoneal, intra-cerobrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. The Wnt agonist or antagonists also are suitably administered by intratumoral, peritumoral, intralesional, or perilesional routes or to the lymph, to exert local as well as systemic therapeutic effects.

[0086] Pharmaceutical compositions may also comprise combinations of the molecules of the invention with cells, including stem cells, progenitor cells, and the like. In some embodiments, the compositions comprise the molecules of the invention in combination with regenerative somatic stem cells, e.g. epithelial stem cells, neural stem cells, liver stem cells, hematopoietic stem cells, osteoblasts, muscle stem cells, mesenchymal stem cells, pancreatic stem cells, etc. In such combinations, cells can be pre-treated with a molecule of the invention prior to use, e.g. ex vivo treatment of cells with the Wnt agonist or antagonist; cells can be administered concomitantly with a molecule of the invention in a separate or combined formulation; cells can be provided to an individual prior to treatment with a molecule of the invention, and the like.

[0087] Pharmaceutical compositions can include, depending on the formulation desired, pharmaceutically-acceptable, non-toxic carriers of diluents, which are defined as vehicles commonly used to formulate pharmaceutical compositions for animal or human administration. The diluent is selected so as not to affect the biological activity of the combination. Examples of such diluents are distilled water, buffered water, physiological saline, PBS, Ringer's solution, dextrose solution, and Hank's solution. In addition, the pharmaceutical composition or formulation can include other carriers, adjuvants, or non-toxic, nontherapeutic, nonimmunogenic stabilizers, excipients and the like. The compositions can also include additional substances to approximate physiological conditions, such as pH adjusting and buffering agents, toxicity adjusting agents,

wetting agents and detergents. Further guidance regarding formulations that are suitable for various types of administration can be found in Remington's Pharmaceutical Sciences, Mace Publishing Company, Philadelphia, Pa., 17th ed. (1985). For a brief review of methods for drug delivery, see, Langer, Science 249:1527-1533 (1990).

[0088] The components used to formulate the pharmaceutical compositions are preferably of high purity and are substantially free of potentially harmful contaminants (e.g., at least National Food (NF) grade, generally at least analytical grade, and more typically at least pharmaceutical grade). Moreover, compositions intended for in vivo use are usually sterile. To the extent that a given compound must be synthesized prior to use, the resulting product is typically substantially free of any potentially toxic agents, particularly any endotoxins, which may be present during the synthesis or purification process. Compositions for parental administration are also sterile, substantially isotonic and made under GMP conditions.

[0089] In another embodiment of the invention, an article of manufacture containing materials useful for the treatment of the conditions described herein is provided. The article of manufacture comprises a container and a label. Suitable containers include, for example, bottles, vials, syringes, and test tubes. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition that is effective for treating the condition and may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). The active agent in the composition is the Wnt agonist or antagonist. The label on, or associated with, the container indicates that the composition is used for treating the condition of choice. Further container(s) may be provided with the article of manufacture which may hold, for example, a pharmaceutically-acceptable buffer, such as phosphate-buffered saline, Ringer's solution or dextrose solution. The article of manufacture may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, syringes, and package inserts with instructions for use.

[0090] The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the present invention, and are not intended to limit the scope of what the inventors regard as their invention nor are they intended to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (e.g. amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated

otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees Centigrade, and pressure is at or near atmospheric.

Example 1.

[0091] DFBss are genetically engineered antibody mimetic proteins typically exhibiting highly specific and high-affinity target protein binding. They are derived from natural ankyrin proteins, one of the most common classes of binding proteins in nature, which are responsible for diverse functions such as cell signaling, regulation and structural integrity of the cell. DFBs consist of at least three, repeat motifs proteins, and usually consist of four or five, of which the first (N-capping repeat) and last (C-capping repeat) serve to provide a hydrophilic surface. Their molecular mass is about 14 or 18 kDa for four- or five-repeat DFBs, respectively.

[0092] DFBs are derived from naturally occurring ankyrin proteins, a protein class that mediates high-affinity protein-protein interactions in nature. DFBs can be expressed in the cytoplasm of Escherichia coli at high levels in soluble form. The proteins exhibit high thermal and thermodynamic stability increasing with increasing repeat number.

[0093] A protein scaffold was based on the Rosetta-built idealized Ankyrin repeat protein (PDB: 4GPM, see Fallas et al. (2017) Nat. Chem. 9:353-360). Starting from this scaffold, we combined computational design and in vitro evolution with yeast surface display to evolve high affinity Wnt antagonists. Rosetta was used to build the idealized Ankyrin repeat protein and we further allowed randomization at positions permitted by Rosetta calculation to retain original packing.

[0094] SEQ ID NO:1-4 provide the amino acid sequences of the antagonist proteins thus developed, which have the following sequences and frizzled specificity:

[0095] 1AF34 comprises the amino acid sequence set forth in SEQ ID NO:1. It binds with high affinity to human frizzled proteins Fzd1, Fzd2, Fzd5, Fzd7, Fzd8. The sequence of the mature protein is as follows, although it may be noted that the last two residues (LE) provide a flexible loop but may be deleted without affecting activity.

SEQ ID NO:1

SELGTRLIRAALDGNKDRVKDLIENGADVNASLMSGATPLHAAAMNGHKEVVKLLISKGADVNA
QSVAGSTPLDAAAFSGHKEVVKLLISKGADVNAVNAAGLTPLHAAADNGHKEVVKLLISKGADV
NAKADHGMTPLHFAAQRGHKEVVKLLISKGADLNTSAKDGATPLDMARESGNEEVVKLLEKQL
E

[0096] ANK12 comprises the amino acid sequence set forth in SEQ ID NO:2. It binds with high affinity to human frizzled proteins Fzd5, Fzd8. The sequence of the mature protein is as follows:

SEQ ID NO:2

SELGKRLIMAALDGNKDRVKDLIENGADVNASLVSGATPLHAAAMNGHKEVVKLLISKGADVNA
QSAAGSTPLAAAAINGHKEVVKLLISKGADVNAVTAAGMTPLHAAAANGHKEVVKLLISKGADV
NAKADRGMTPLHFAAWRGHKEVVKLLISKGADLNTSAKDGATPLDMARESGNEEVVKLLEKQL
E

[0097] 1AF34F7 comprises the amino acid sequence set forth in SEQ ID NO:3. It binds with high affinity to human frizzled proteins Fzd1, Fzd2, Fzd7. The sequence of the mature protein is as follows:

SEQ ID NO:3

SELGTRLIRAALDGNKDRVKDLIENGADVNASLMSGATPLHAAAMNGHKEVVKLLISKGADVNA
QSVAGSTPLDAAAFSGHKEVVKLLISKGADVNAVNAAGLTPLHDAADDGHNEVVKLLISKGADV
NAKADHGMTPLHFAAQRGHKEVVKLLISKGADLNTSAKDGATPLDMARESGNEEVVKLLEKQL
E

[0098] 4AF30 comprises the amino acid sequence set forth in SEQ ID NO:4. It binds with high affinity to human frizzled protein Fzd4. The sequence of the mature protein is as follows:

SEQ ID NO:4

SELGKRLIRAALDGNKDRVKDLIENGADVNASLMSGTTPLYAAAMNGHKEVVKLLISKGADVNA
QSVAGSTPLVAAANFGHNEVVKLLISKGADVNAVTAFGVTPLHAAAADGHKEVVKLLISKGADV
NAKAGRGMTPLHIAAFRGHKEVVKLLISKGADLNTSAKDGATPLDMARESGNEEVVKLLEKQLE

[0099] To understand the affinity of individual DFBs towards different Fzd CRDs, two different methods were used to measure the specificity and affinity. DFBs were expressed on yeast surface and the yeast stained with biotinylated Fzd CRDs. The cells were washed and Streptavidin, Alexa Fluor™ 647 conjugates were used to stain the yeasts. The fluorescence signals were read on flow cytometry.

[00100] Second, individual DFB proteins were purified and surface Plasmon Resonance (SPR) used to measure the affinity between Fzd CRD with the DFB. The biotinylated Fzd CRD was captured on SA sensor chip at low density. A gradient concentration of DFB analyte was flown over the chip. The data was analyzed by BIACore accompanying software.

[00101] The results of these experiments are shown in Figures 1A and 1B. Figure 1A shows the results of ANK12 yeast titration with individual Fzd CRD. ANK12 showed EC_{50} of 4.4 nM and 3.6 nM for Fz5 and Fz8 while not interacting with Fz1, 2, 4 and 7. Figure 1B shows the SPR results of

ANK12 to Fzd4, 7 and 8. ANK12 showed no binding to Fzd4 or Fzd7 while showing a Kd of 200 pM for Fzd8. Collectively, ANK12 showed high specificity and affinity to Fzd5 and Fzd8.

[00102] Similar affinity measurement of 1AF34, 1AF34F7 and 4AF30 towards different Fzds using both methods were performed, as shown in Figure 2. 1AF34 showed EC_{50} of 19.0, 31.6 and 5.6 nM to Fz1, 2 and 7. 1AF34 showed weaker staining to Fz5/8 with EC_{50} not available. By SPR, 1AF34 binds to Fzd7 at 1 nM affinity and Fzd8 at 23 nM affinity. Shown in Figure 3, 1AF34F7 showed EC_{50} of 7.5 nM, 9.9 nM and 1.8 nM to Fz1, 2 and 7, respectively. 1AF34F7 showed no cross-reactivity to Fz5/8 up to 1 μM concentration. Using SPR, 1AF34F7 shows 1.6 nM affinity to Fzd7 CRD. Shown in Figure 4, 4AF30 only binds to Fz4 with EC_{50} of 1.6 nM. Using SPR, 4AF30 shows 1.4 nM affinity to Fzd4 and 400 nM for Fzd8.

[00103] To generate Fzd subtype specific Wnt agonists, the DFBs were fused with DKK1c to generate bi-specific molecules that bridge specific Fzd receptors with co-receptors Lrp5/6 to activate canonical Wnt signaling. The amino acid sequence of all four DFBs agonists are set forth in SEQ ID NO:5-8.

[00104] SEQ ID NO:5-8 provide the amino acid sequences of the agonist proteins thus developed, which have the following sequences and frizzled specificity:

[00105] 1AF34-DKK1c comprises the amino acid sequence set forth in SEQ ID NO:5. It binds with high affinity to human frizzled proteins Fzd1, Fzd2, Fzd5, Fzd7, Fzd8. The sequence of the mature protein is as follows. Amino acid residues 1-192 correspond to SEQ ID NO:1, residues 193-202 are a 10mer GS linker, residues 203-292 correspond to human DKK1c.

SEQ ID NO:5

SELGTRLIRAALDGNKDRVKDLIENGADVNASLMSGATPLHAAAMNGHKEVVKLLISKGADVNA
QSVAGSTPLDAAAFSGHKEVVKLLISKGADVNAVNAAGLTPLHAAADNGHKEVVKLLISKGADV
NAKADHGMTPLHFAAQRGHKEVVKLLISKGADLNTSAKDGATPLDMARESGNEEVVKLLEKQL
EGSGSGGSGSGKMYHTKGQEGSVCLRSSDCASGLCCARHFWSKICKPVLKEGQVCTKHRRK
GSHGLEIFQRCYCGEGLSCRIQKDHHQASNSSRLHTCQRH

[00106] ANK12-DKK1c comprises the amino acid sequence set forth in SEQ ID NO:6. It binds with high affinity to human frizzled proteins Fzd5, Fzd8. The sequence of the mature protein is as follows. Amino acid residues 1-192 correspond to SEQ ID NO:2, residues 193-202 are a 10mer GS linker, residues 203-292 correspond to human DKK1c.

SEQ ID NO:6

SELGKRLIMAALDGNKDRVKDLIENGADVNASLVSGATPLHAAAMNGHKEVVKLLISKGADVNA
QSAAGSTPLAAAAINGHKEVVKLLISKGADVNAVTAAGMTPLHAAAANGHKEVVKLLISKGADV

NAKADRGMTPLHFAAWRGHKEVVKLLISKGADLNTSAKDGATPLDMARESGNEEVVKLLEKQL
EGSGSGGSGSGKMYHTKGQEGSVCLRSSDCASGLCCARHFWSKICKPVLKEGQVCTKHRRK
GSHGLEIFQRCYCGEGLSCRIQKDHHQASNSSRLHTCQRH

[00107] 1AF34F7-DKK1c comprises the amino acid sequence set forth in SEQ ID NO:7. It binds with high affinity to human frizzled proteins Fzd1, Fzd2, Fzd7. The sequence of the mature protein is as follows. Amino acid residues 1-192 correspond to SEQ ID NO:3, residues 193-202 are a 10mer GS linker, residues 203-292 correspond to human DKK1c.

SEQ ID NO:7

SELGTRLIRAALDGNKDRVKDLIENGADVNASLMSGATPLHAAAMNGHKEVVKLLISKGADVNA
QSVAGSTPLDAAAFSGHKEVVKLLISKGADVNAVNAAGLTPLHDAADDGHNEVVKLLISKGADV
NAKADHGMTPLHFAAQRGHKEVVKLLISKGADLNTSAKDGATPLDMARESGNEEVVKLLEKQL
EGSGSGGSGSGKMYHTKGQEGSVCLRSSDCASGLCCARHFWSKICKPVLKEGQVCTKHRRK
GSHGLEIFQRCYCGEGLSCRIQKDHHQASNSSRLHTCQRH

[00108] 4AF30-DKK1c comprises the amino acid sequence set forth in SEQ ID NO:8. It binds with high affinity to human frizzled protein Fzd4. The sequence of the mature protein is as follows. Amino acid residues 1-90 correspond to human DKK1c, residues 91-100 are a 10mer GS linker, residues 101-292 correspond to SEQ ID NO:4.

SEQ ID NO:8

KMYHTKGQEGSVCLRSSDCASGLCCARHFWSKICKPVLKEGQVCTKHRRKGSHGLEIFQRCY
CGEGLSCRIQKDHHQASNSSRLHTCQRHGSGSGGSGSGSELGKRLIRAALDGNKDRVKDLIE
NGADVNASLMSGTTPLYAAAMNGHKEVVKLLISKGADVNAQSVAGSTPLVAAANFGHNEVVKL
LISKGADVNAVTAFGVTPLHAAAADGHKEVVKLLISKGADVNAKAGRGMTPLHIAAFRGHKEVV
KLLISKGADLNTSAKDGATPLDMARESGNEEVVKLLEKQLE

[00109] To confirm the Fz specificity of each DFBs agonist of SEQ ID NO:5-8, the HEK293T cell line with Fzd1/2/4/5/7/8 knockout was used. Shown in Figure 5A, the Wnt agonists show no activity in this knockout cell line. Mouse Fzd4 receptor was transfected into the cell line and stimulated with all four types of Wnt agonists. Only Fzd4 specific agonist (4AF30-DKK1c, shown in Figure 5B) showed potent activation of Wnt signaling, shown by SuperTop Flash Wnt reporter assays.

[00110] Shown in Figure 5C and D, mouse Fzd7 and Fzd8 receptor was transfected into the cell line and stimulated with all four types of agonist or antagonist agonists. In the cells with transfected Fz7 receptor, two of the agonists (1AF34-DKK1c and 1AF34F7-DKK1c) showed potent activation of Wnt signaling, shown by SuperTop Flash assays. In the cells transfected with Fz8 receptors, two of the agonists (1AF34-DKK1c and ANK12-DKK1c) showed potent activation

of Wnt signaling. Fzd4 specific agonist (4AF30-DKK1c) showed activation of Wnt signaling, but the activity is diminished significantly at low concentration (< 3 nM). The Fzd agonist specificity is consistent with their specificity to Fzd CRD, as shown by SPR.

[00111] Having confirmed the specificity of our DFBs based Wnt agonists, it was determined if the agonist was active in supporting organoid growth. The Wnt agonist 1AF34-DKK1c (SEQ ID NO:5), which has broad spectrum of activity for most Fz receptors (Fz1/2/5/7/8) was tested, shown in Figure 6. Wnt3a conditioned media (CM) was used as control to our Wnt agonist (1AF34-DKK1c at 0.25 nM) in supporting human colon organoid growth. The agonist or antagonist Wnt agonist not only support human colon organoid growth but also shows better growth compared to Wnt3a CM. Shown in Figure 7 are similar experiments on human rectal organoid. The agonist or antagonist Wnt agonist also supports rectal organoid and shows better response compared to Wnt3a CM.

[00112] The coding sequence for SEQ ID NO:5 was designed, in one iteration, as SEQ ID NO:9:

GGATCCTCTGAACTGGGTACGCGTCTGATCAGGGCAGCATTAGACGGTAACAAAGACCGT
GTTAAAGACCTCATTGAAAATGGTGCTGACGTTAACGCGTCCCTTATGTCTGGGGCGACTC
CGTTACACGCCGCCGCCATGAACGGCCACAAAGAGGTTGTGAAGTTACTGATCTCCAAGG
GCGCAGATGTGAATGCTCAGTCCGTTGCGGGTTCTACACCTCTGGATGCGGCGGCGTTTA
GTGGACATAAAGAAGTGGTAAAACTGCTGATAAGTAAAGGAGCAGACGTCAATGCTGTTAA
TGCGGCTGGATTGACCCCCCTACATGCTGCTGCTGATAATGGGCACAAGGAAGTAGTGAA
GTTGCTTATTTCTAAGGGGGCCGACGTAAATGCGAAAGCTGACCATGGCATGACTCCACTC
CATTTCGCAGCACAGCGCGGTCATAAGGAAGTCGTTAAACTATTAATCAGCAAAGGTGCGG
ATTTGAACACCTCTGCCAAAGACGGTGCAACCCCGCTTGACATGGCGCGTGAATCTGGCA
ATGAGGAGGTTGTCAAGCTCTTGGAAAAGCAACTCGAGGGAAGCGGTTCCGGAGGTTCTG
GCTCCGGAAAAATGTATCACACTAAGGGACAGGAAGGCAGCGTGTGCCTGAGGTCCTCTG
ACTGTGCATCTGGCCTGTGCTGCGCCAGACACTTTTGGTCCAAAATCTGCAAGCCGGTTCT
GAAAGAGGGCCAGGTGTGCACAAAGCATCGACGAAAGGGCAGCCATGGACTCGAGATCTT
TCAGAGGTGCTACTGCGGGGAGGGTCTGTCTTGCAGAATCCAGAAAGATCATCATCAGGC
CTCCAACTCCTCCCGCCTGCACACCTGCCAGCGACATGCGGCCGC

[00113] In another version, the coding sequence for SEQ ID NO:5 was optimized for expression in human cells, e.g. in HEK cells, show as SEQ ID NO:10:

GGATCCAGCGAACTCGGCACCCGACTTATCAGAGCTGCCCTTGATGGAAATAAGGACCGG
GTCAAAGACCTGATAGAGAATGGGGCGGACGTTAATGCAAGTCTTATGTCCGGTGCTACC
CCGTTGCATGCCGCTGCAATGAATGGCCACAAGGAGGTTGTAAAATTGCTCATAAGTAAAG
GCGCAGACGTAAACGCACAATCCGTGGCGGGCAGTACCCCCTTGGATGCCGCTGCGTTCT

CAGGCCATAAAGAGGTCGTAAAACTGCTGATCTCCAAAGGAGCGGACGTCAATGCAGTAA
ATGCTGCAGGACTTACCCCCTTGCACGCCGCTGCCGACAATGGACACAAGGAGGTTGTAA
AACTTCTCATATCTAAAGGCGCGGACGTCAACGCTAAAGCGGACCACGGAATGACGCCGC
TCCACTTTGCGGCACAGAGGGGCCATAAAGAGGTAGTGAAACTTCTGATCTCAAAAGGAG
CCGACTTGAATACGAGCGCTAAGGATGGTGCAACGCCATTGGATATGGCCAGGGAGTCCG
GAAATGAAGAGGTGGTGAAGCTGCTTGAGAAGCAACTCGAAGGTAGCGGGAGCGGCGGG
AGTGGCTCTGGCAAGATGTACCATACCAAGGGCCAAGAAGGGAGTGTGTGCCTGCGGTCA
AGCGACTGCGCATCAGGCTTGTGCTGTGCTAGGCATTTCTGGTCCAAGATTTGTAAGCCAG
TATTGAAGGAAGGCCAGGTGTGTACTAAACATAGGCGCAAGGGAAGTCATGGTCTCGAGA
TATTCCAACGCTGTTACTGTGGCGAGGGATTGTCTTGCCGCATACAAAAAGACCACCACCA
GGCGTCTAACTCTAGTAGGCTTCACACATGCCAACGGCACGCGGCCGC

[00114] For some purposes, for example protein stability and expression, the Wnt agonist proteins described herein are joined to an Fc sequences, including for example human IgG1, shown in SEQ ID NO:11

GCTAGCACCAAGGGCCCATCGGTCTTCCCCCTGGCACCCTCCTCCAAGAGCACCTCTGGG
GGCACAGCGGCCCTGGGCTGCCTGGTCAAGGACTACTTCCCCGAACCGGTGACGGTGTC
GTGGAACTCAGGCGCCCTGACCAGCGGCGTGCACACCTTCCCGGCTGTCCTACAGTCCTC
AGGACTCTACTCCCTCAGCAGCGTGGTGACCGTGCCCTCCAGCAGCTTGGGCACCCAGAC
CTACATCTGCAACGTGAATCACAAGCCCAGCAACACCAAGGTGGACAAGAAAGTTGAGCC
CAAATCTTGTGACAAAACTCACACATGCCCACCGTGCCCAGCACCTGAACTCCTGGGGGG
ACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGACACCCTCATGATCTCCCGGACCCCT
GAGGTCACATGCGTGGTGGTGGACGTGAGCCACGAAGACCCTGAGGTCAAGTTCAACTG
GTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTACA
ACAGCACGTACCGTGTGGTCAGCGTCCTCACCGTCCTGCACCAGGACTGGCTGAATGGCA
AGGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCATCGAGAAAACCATCT
CCAAAGCCAAAGGGCAGCCCCGAGAACCACAGGTGTACACCCTGCCCCCATCCCGGGAG
GAGATGACCAAGAACCAGGTCAGCCTGACCTGCCTGGTCAAAGGCTTCTATCCCAGCGAC
ATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACTACAAGACCACGCCTCC
CGTGCTGGACTCCGACGGCTCCTTCTTCCTCTACAGCAAGCTCACCGTGGACAAGAGCAG
GTGGCAGCAGGGGAACGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTA
CACGCAGAAGAGCCTCTCCCTGTCTCCGGGTAAATGA

The coding sequence of the construct for expression may have the sequence of SEQ ID NO:15:

ATGGCCCGGCCTCTGTGTACCCTGCTACTCCTGATGGCTACCCTGGCTGGGGCTCTGGCC
GGATCCAGCGAACTCGGCACCCGACTTATCAGAGCTGCCCTTGATGGAAATAAGGACCGG
GTCAAAGACCTGATAGAGAATGGGGCGGACGTTAATGCAAGTCTTATGTCCGGTGCTACC
CCGTTGCATGCCGCTGCAATGAATGGCCACAAGGAGGTTGTAAAATTGCTCATAAGTAAAG
GCGCAGACGTAAACGCACAATCCGTGGCGGGCAGTACCCCCTTGGATGCCGCTGCGTTCT
CAGGCCATAAAGAGGTCGTAAAACTGCTGATCTCCAAAGGAGCGGACGTCAATGCAGTAA
ATGCTGCAGGACTTACCCCCTTGCACGCCGCTGCCGACAATGGACACAAGGAGGTTGTAA
AACTTCTCATATCTAAAGGCGCGGACGTCAACGCTAAAGCGGACCACGGAATGACGCCGC
TCCACTTTGCGGCACAGAGGGGCCATAAAGAGGTAGTGAAACTTCTGATCTCAAAAGGAG
CCGACTTGAATACGAGCGCTAAGGATGGTGCAACGCCATTGGATATGGCCAGGGAGTCCG
GAAATGAAGAGGTGGTGAAGCTGCTTGAGAAGCAACTCGAAGGTAGCGGGAGCGGCGGG
AGTGGCTCTGGCAAGATGTACCATACCAAGGGCCAAGAAGGGAGTGTGTGCCTGCGGTCA
AGCGACTGCGCATCAGGCTTGTGCTGTGCTAGGCATTTCTGGTCCAAGATTTGTAAGCCAG
TATTGAAGGAAGGCCAGGTGTGTACTAAACATAGGCGCAAGGGAAGTCATGGTCTCGAGA
TATTCCAACGCTGTTACTGTGGCGAGGGATTGTCTTGCCGCATACAAAAAGACCACCACCA
GGCGTCTAACTCTAGTAGGCTTCACACATGCCAACGGCACGCGGCCGCAGAGAACCTGTA
CTTCCAGGGATCTTCCGAGCCCAAATCTTGTGACAAAACTCACACATGCCCACCGTGCCCA
GCACCTGAACTCCTGGGGGGACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGACACC
CTCATGATCTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAGCCACGAAGAC
CCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAG
CCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCCTCACCGTCCTGCA
CCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCCAGC
CCCCATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAACCACAGGTGTACAC
CCTGCCCCCATCCCGGGATGAGCTGACCAAGAACCAGGTCAGCCTGACCTGCCTGGTCAA
AGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACA
ACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGCTCCTTCTTCCTCTACAGCAAGC
TCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCTCATGCTCCGTGATGCATG
AGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCGGGTAAAGGGGCCG
CATAG

[00115] The preceding merely illustrates the principles of the invention. It will be appreciated that those skilled in the art will be able to devise various arrangements which, although not explicitly described or shown herein, embody the principles of the invention and are included within its spirit and scope. Furthermore, all examples and conditional language recited herein are principally

intended to aid the reader in understanding the principles of the invention and the concepts contributed by the inventors to furthering the art, and are to be construed as being without limitation to such specifically recited examples and conditions. Moreover, all statements herein reciting principles, aspects, and embodiments of the invention as well as specific examples thereof, are intended to encompass both structural and functional equivalents thereof. Additionally, it is intended that such equivalents include both currently known equivalents and equivalents developed in the future, i.e., any elements developed that perform the same function, regardless of structure. The scope of the present invention, therefore, is not intended to be limited to the exemplary embodiments shown and described herein. Rather, the scope and spirit of the present invention is embodied by the appended claims.

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WHAT IS CLAIMED IS:

1. A Wnt signaling antagonist polypeptide comprising an amino acid sequence set forth in any of SEQ ID NO:1-4, or a variant thereof.

2. A Wnt signaling agonist comprising a polypeptide of claim 1, linked to a binding domain having high affinity to one or both of Lrp5 and Lrp6 protein.

3. The Wnt signaling agonist of claim 2, wherein the binding domains is joined to the polypeptide of claim 1 through a linker.

4. The Wnt signaling agonist of claim 2 or claim 3, wherein the Fzd binding domain binds one or more Fzd proteins with a K_D of at least about 1 x 10^{-7} M.

5. The Wnt signaling agonist of any of claims 2-4, wherein the Lrp5/6 binding domain binds one or both of Lrp5 and Lrp6 proteins with a K_D of at least about 1 x 10^{-7} M.

6. The Wnt signaling agonist of claim 5, wherein the Lrp5/6 binding domain is a polypeptide.

7. The Wnt signaling agonist of Claim 6, wherein the Lrp5/6 binding domain comprises a binding portion of a DKK protein.

8. The Wnt signaling agonist of Claim 7, wherein the Lrp5/6 binding domain comprises the C-terminal domain of human DKK1.

9. A Wnt signaling agonist comprising the amino acid sequence set forth in any of SEQ ID NO:5, 6, 7, 8 or 14.

10. A polynucleotide encoding the Wnt signaling agonist of claim 9.

11. The polynucleotide of claim 10, comprising the coding sequence set forth in any of SEQ ID NO:9, 10 or 15.

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12. A cell culture medium, comprising an effective dose of a Wnt signaling agonist according to any of claims 2-9.

13. A method of activating or enhancing Wnt signaling, the method comprising contacting a cell expressing a frizzled receptor with an effective dose of a Wnt signaling agonist according to any one of Claims 2-9.

14. The method of claim 13, wherein the cell is present as a population in an in vitro culture medium.

15. The method of claim 14, wherein the cell population comprises stem cells.

16. The method of claim 14, wherein the cell population comprises organoids.

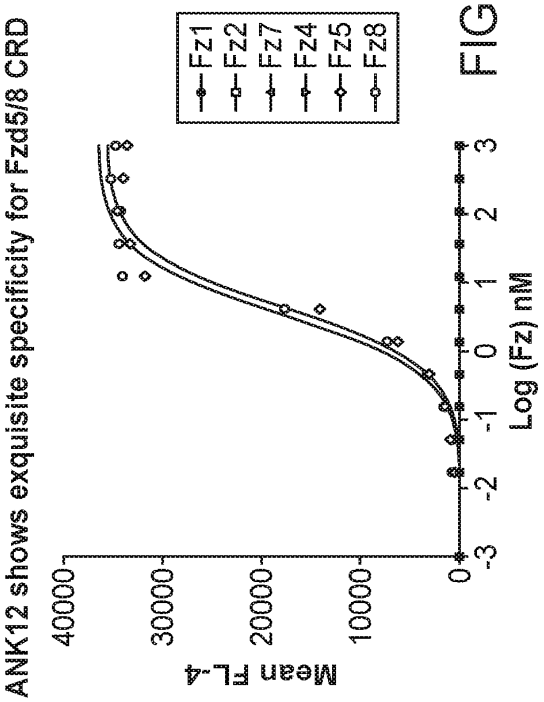

ANK12 shows exquisite specificity for Fzd5/8 CRD
Mean FL-4
40000
30000
20000
10000
0
-3
-2
-1
0
1
2
3
Log (Fz) nM
Fz1
Fz2
Fz7
Fz4
Fz5
Fz8

FIG. 1A

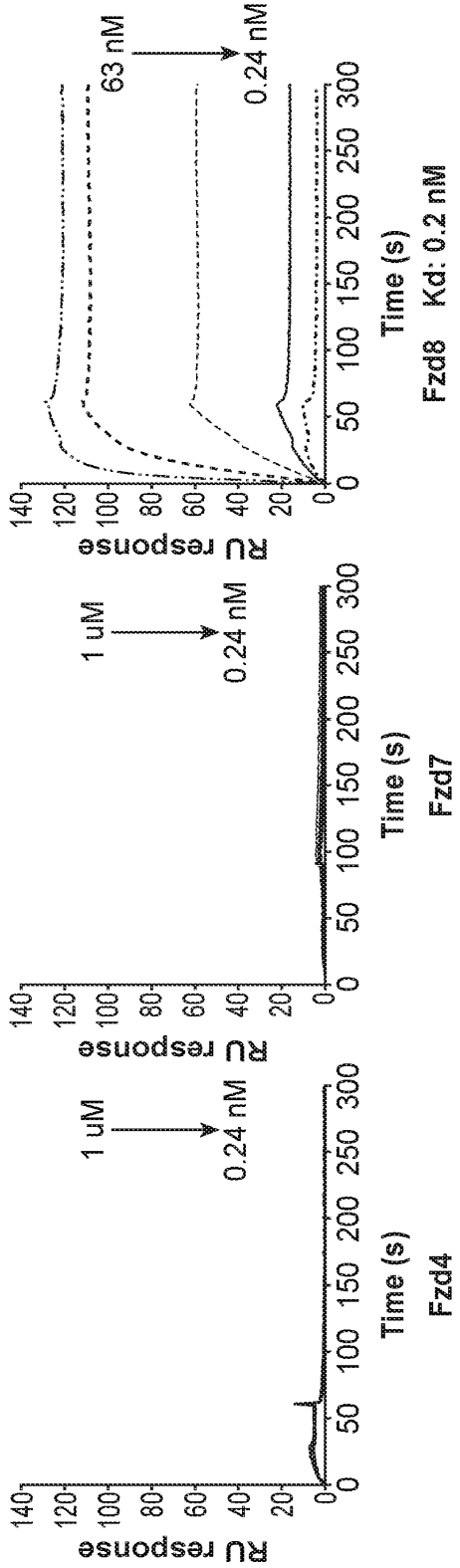

RU response
Time (s)
Fzd4
1 uM
0.24 nM
Fzd7
Fzd8 Kd: 0.2 nM
63 nM
0.24 nM

FIG. 1B

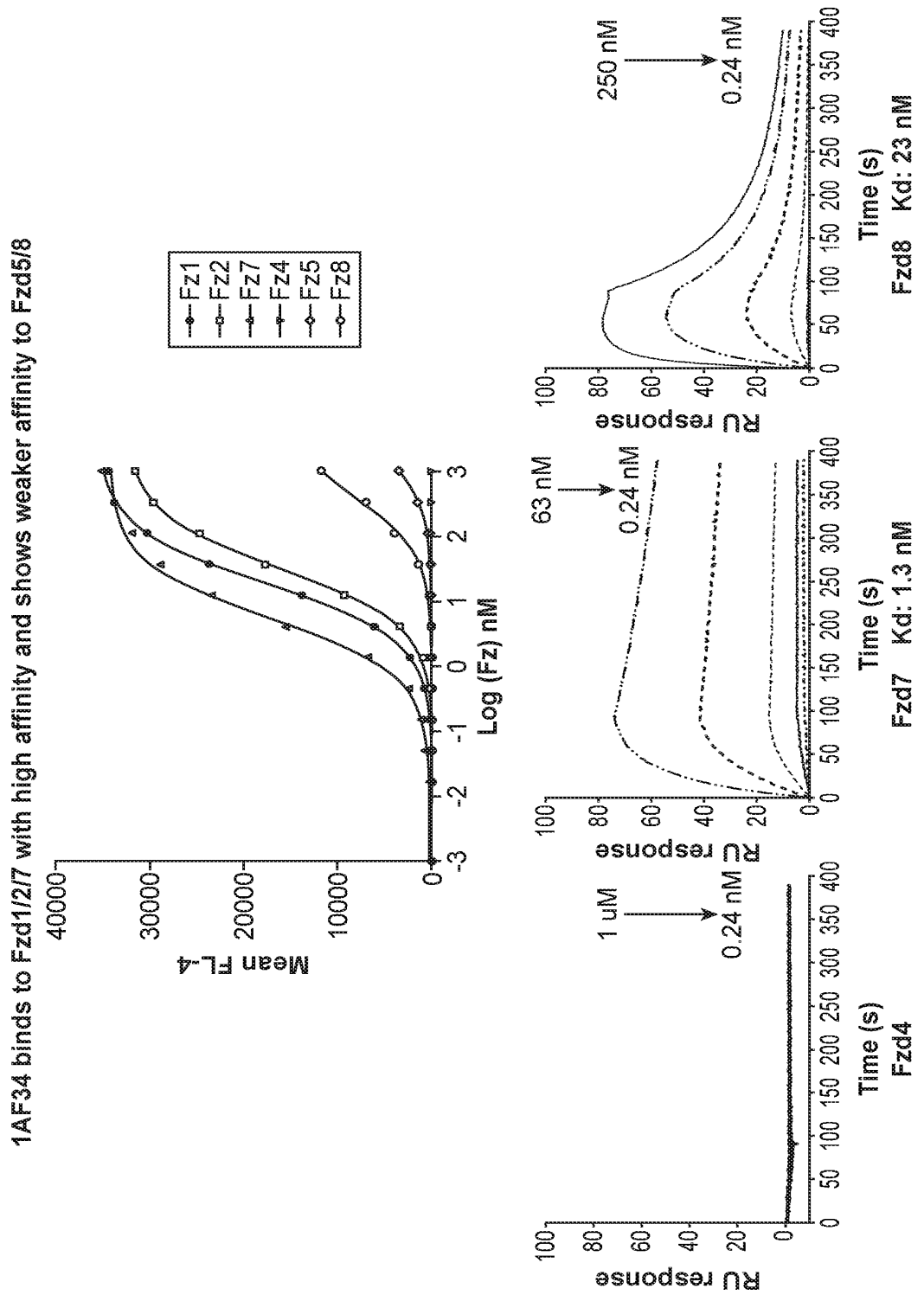

1AF34 binds to Fzd1/2/7 with high affinity and shows weaker affinity to Fzd5/8
Mean FL-4
Log (Fz) nM
Fz1
Fz2
Fz7
Fz4
Fz5
Fz8
RU response
Time (s)
1 uM
0.24 nM
Fzd4
63 nM
0.24 nM
Fzd7 Kd: 1.3 nM
250 nM
0.24 nM
Fzd8 Kd: 23 nM

FIG. 2

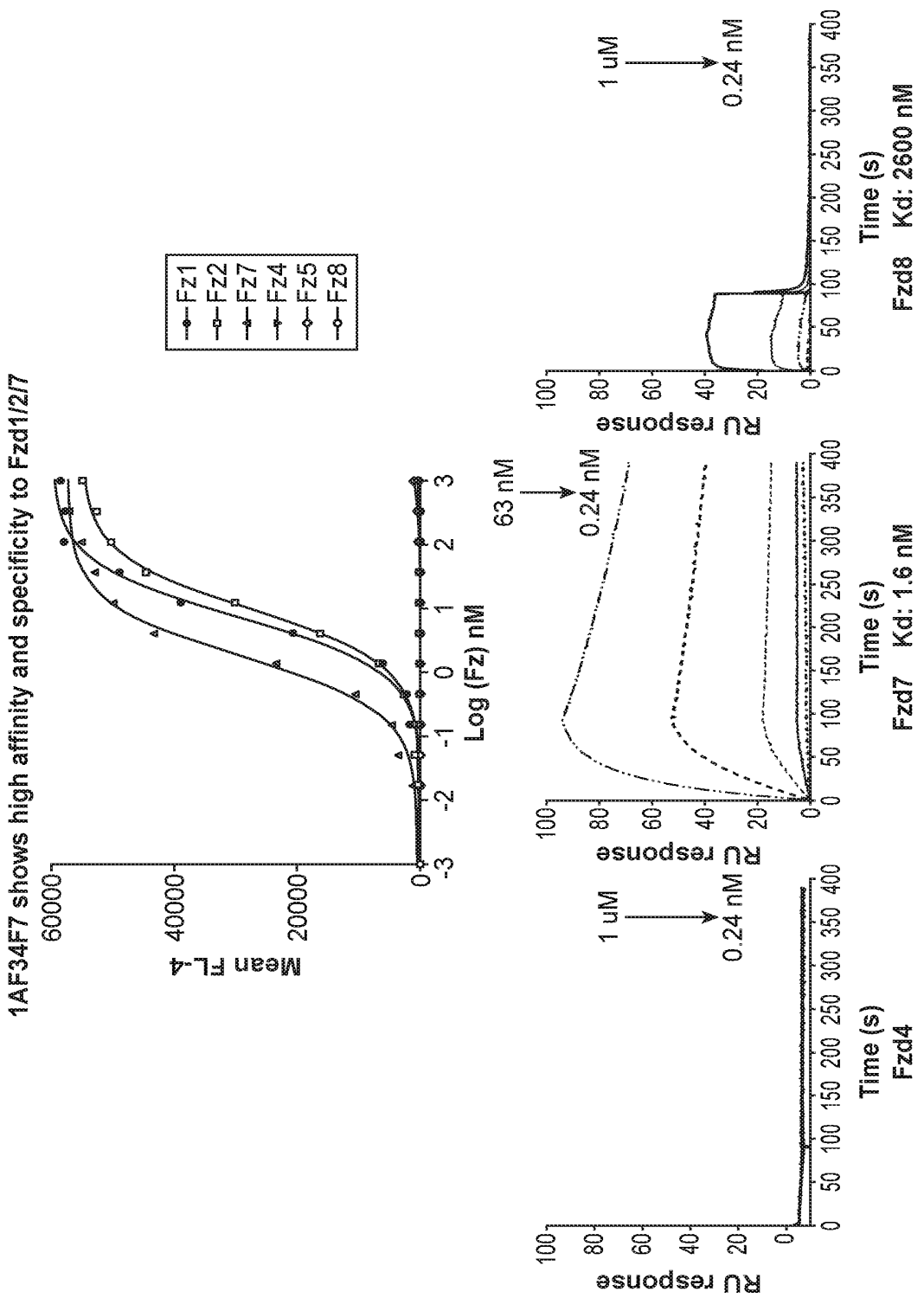

1AF34F7 shows high affinity and specificity to Fzd1/2/7
Mean FL-4
60000
40000
20000
0
-3
-2
-1
0
1
2
3
Log (Fz) nM
Fz1
Fz2
Fz7
Fz4
Fz5
Fz8
RU response
100
80
60
40
20
0
0 50 100 150 200 250 300 350 400
Time (s)
1 uM
0.24 nM
Fzd4
63 nM
0.24 nM
Fzd7 Kd: 1.6 nM
1 uM
0.24 nM
Fzd8 Kd: 2600 nM

FIG. 3

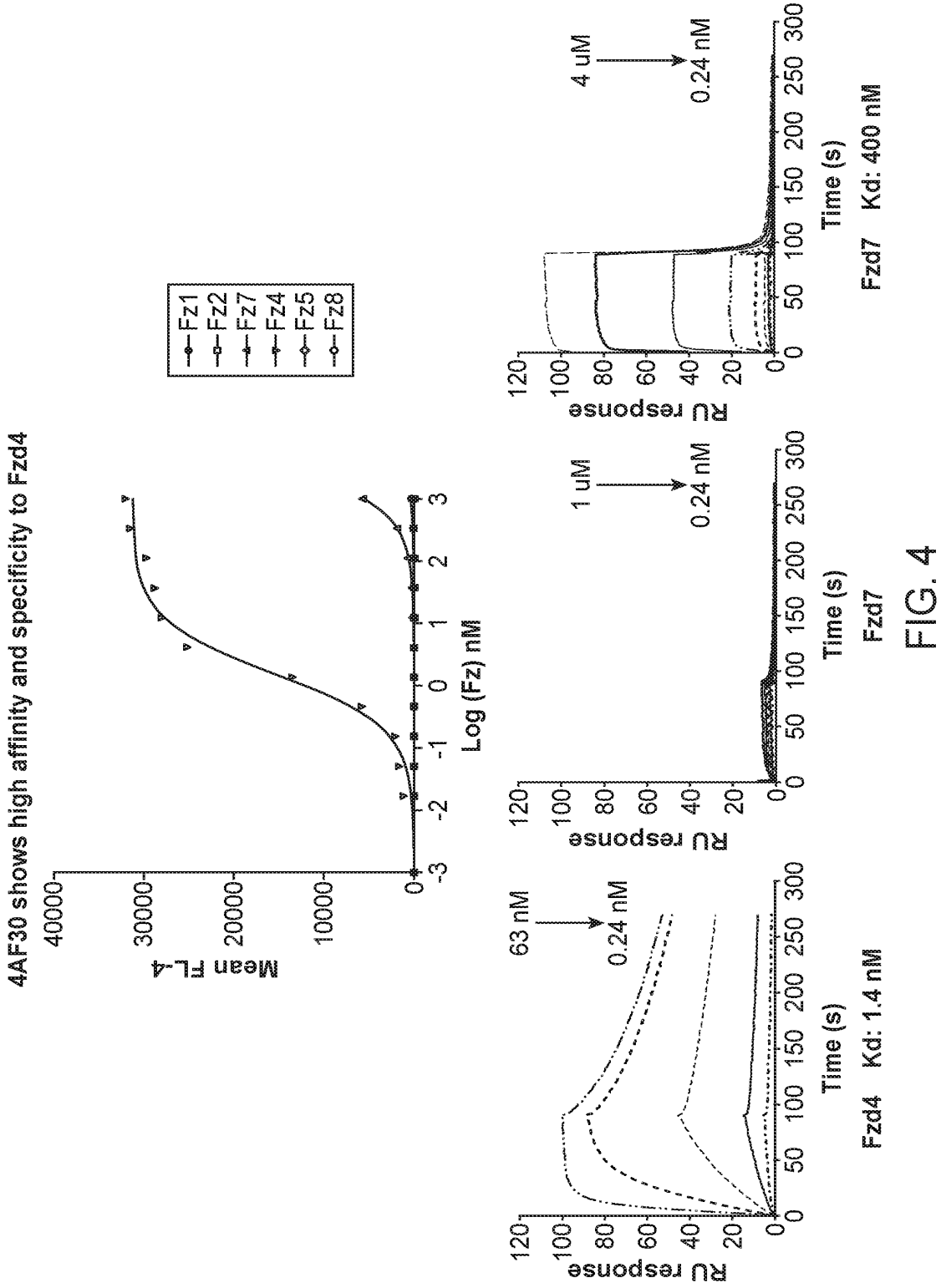

4AF30 shows high affinity and specificity to Fzd4
Mean FL-4
Log (Fz) nM
Fz1
Fz2
Fz7
Fz4
Fz5
Fz8
RU response
Time (s)
63 nM
0.24 nM
Fzd4 Kd: 1.4 nM
1 uM
0.24 nM
Fzd7
4 uM
0.24 nM
Fzd7 Kd: 400 nM

FIG. 4

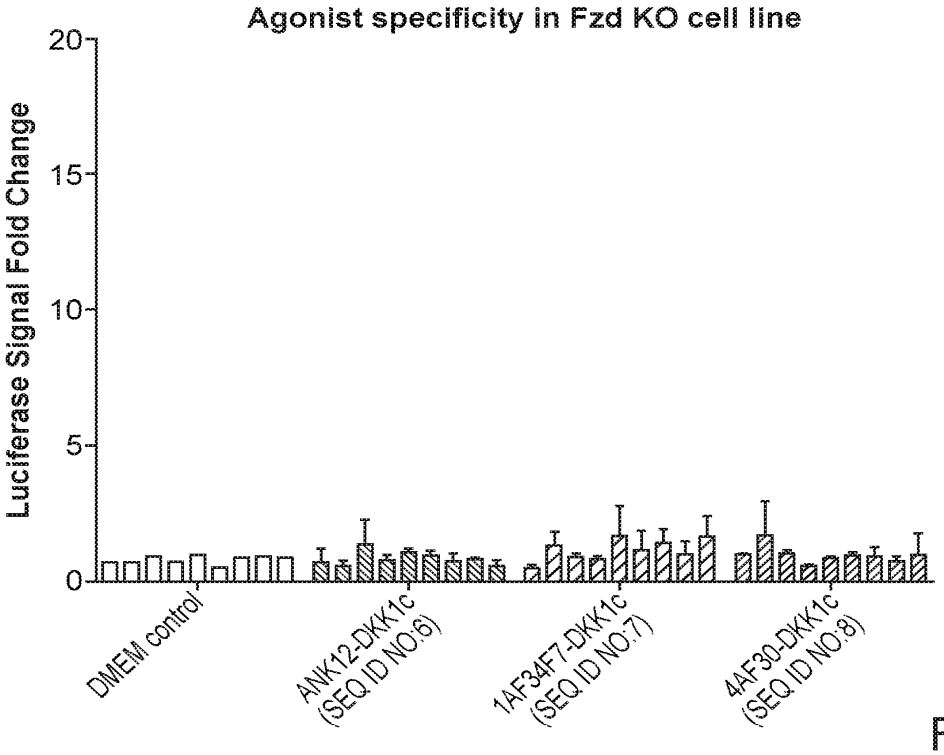

Agonist specificity in Fzd KO cell line
Luciferase Signal Fold Change
20
15
10
5
0
DMEM control
ANK12-DKK1c (SEQ ID NO:6)
1AF34F7-DKK1c (SEQ ID NO:7)
4AF30-DKK1c (SEQ ID NO:8)

FIG. 5A

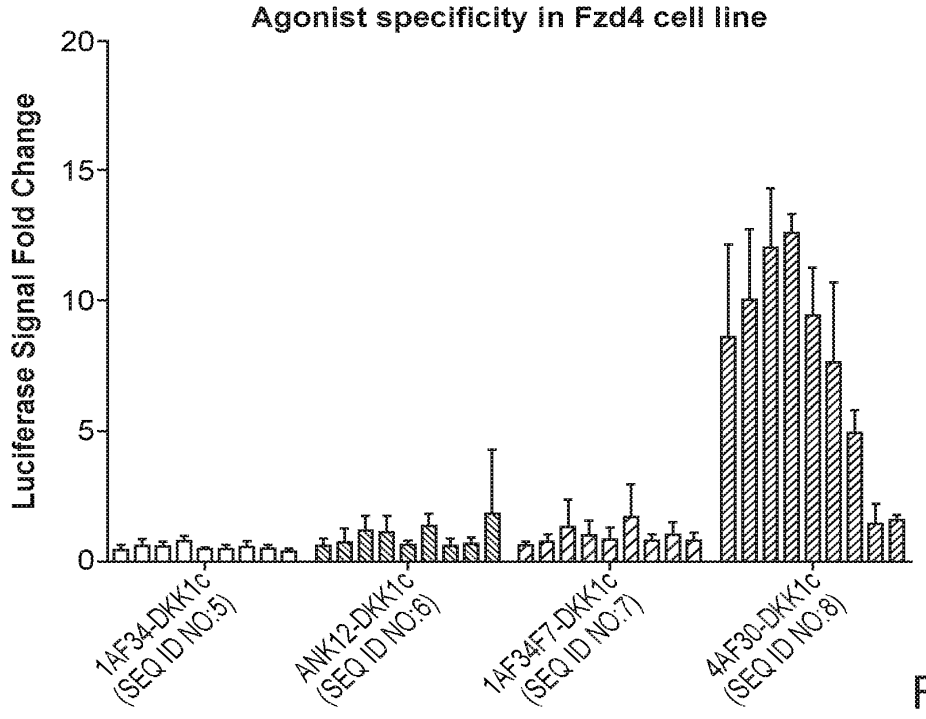

Agonist specificity in Fzd4 cell line
Luciferase Signal Fold Change
20
15
10
5
0
1AF34-DKK1c (SEQ ID NO:5)
ANK12-DKK1c (SEQ ID NO:6)
1AF34F7-DKK1c (SEQ ID NO:7)
4AF30-DKK1c (SEQ ID NO:8)

FIG. 5B

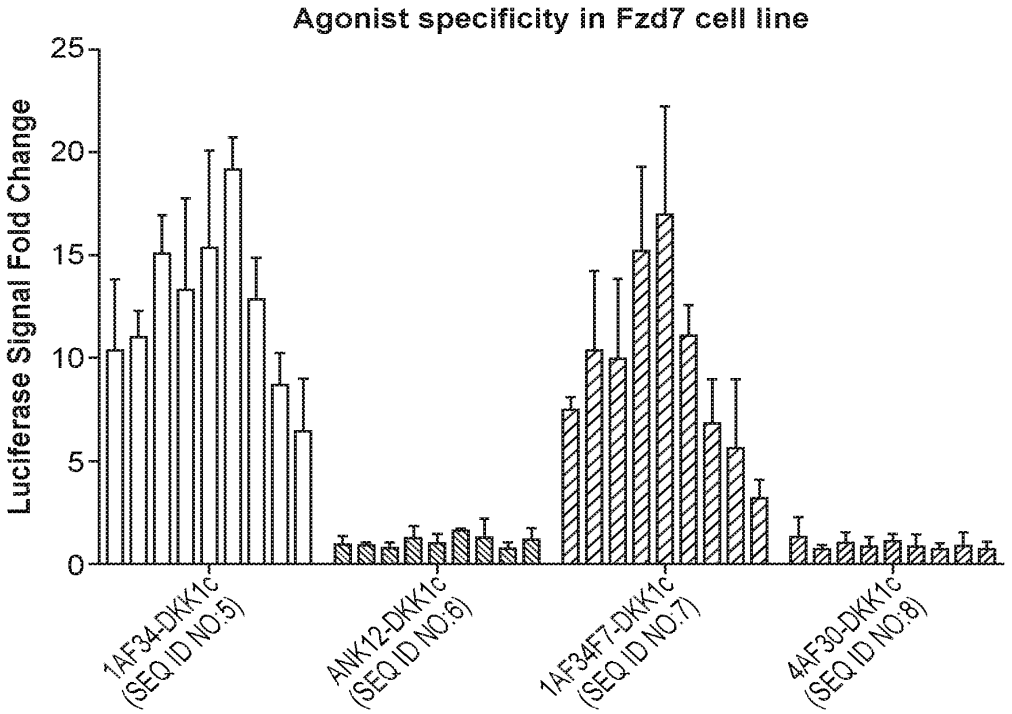

Agonist specificity in Fzd7 cell line
Luciferase Signal Fold Change
25
20
15
10
5
0
1AF34-DKK1c (SEQ ID NO:5)
ANK12-DKK1c (SEQ ID NO:6)
1AF34F7-DKK1c (SEQ ID NO:7)
4AF30-DKK1c (SEQ ID NO:8)

FIG. 5C

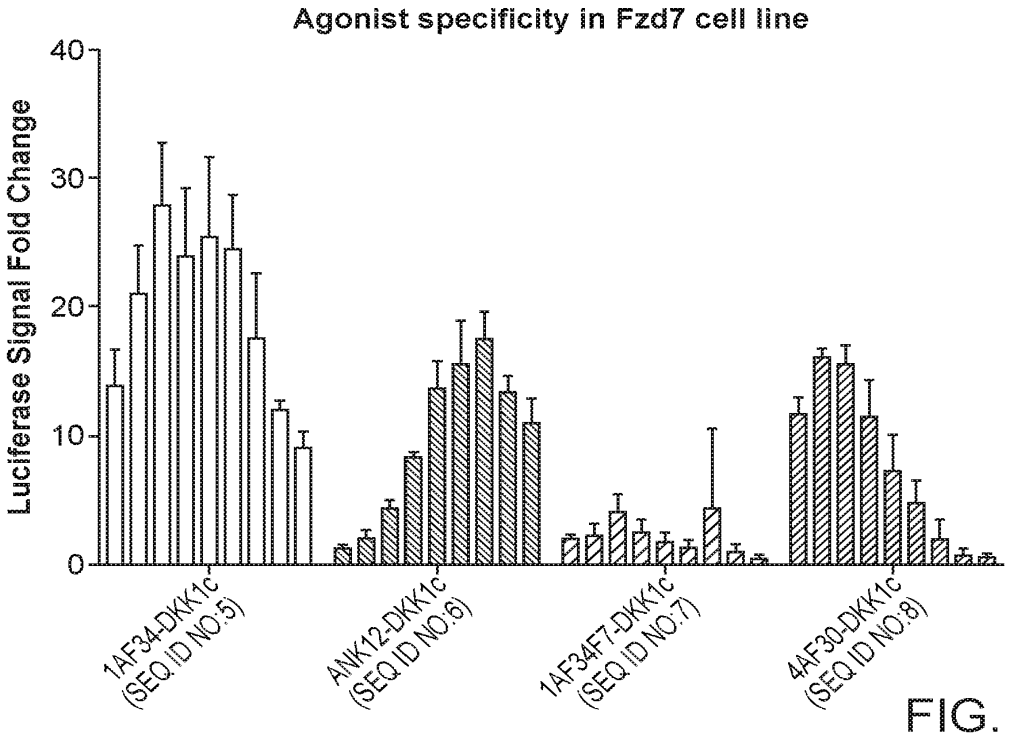

Agonist specificity in Fzd7 cell line
Luciferase Signal Fold Change
40
30
20
10
0
1AF34-DKK1c (SEQ ID NO:5)
ANK12-DKK1c (SEQ ID NO:6)
1AF34F7-DKK1c (SEQ ID NO:7)
4AF30-DKK1c (SEQ ID NO:8)

FIG. 5D

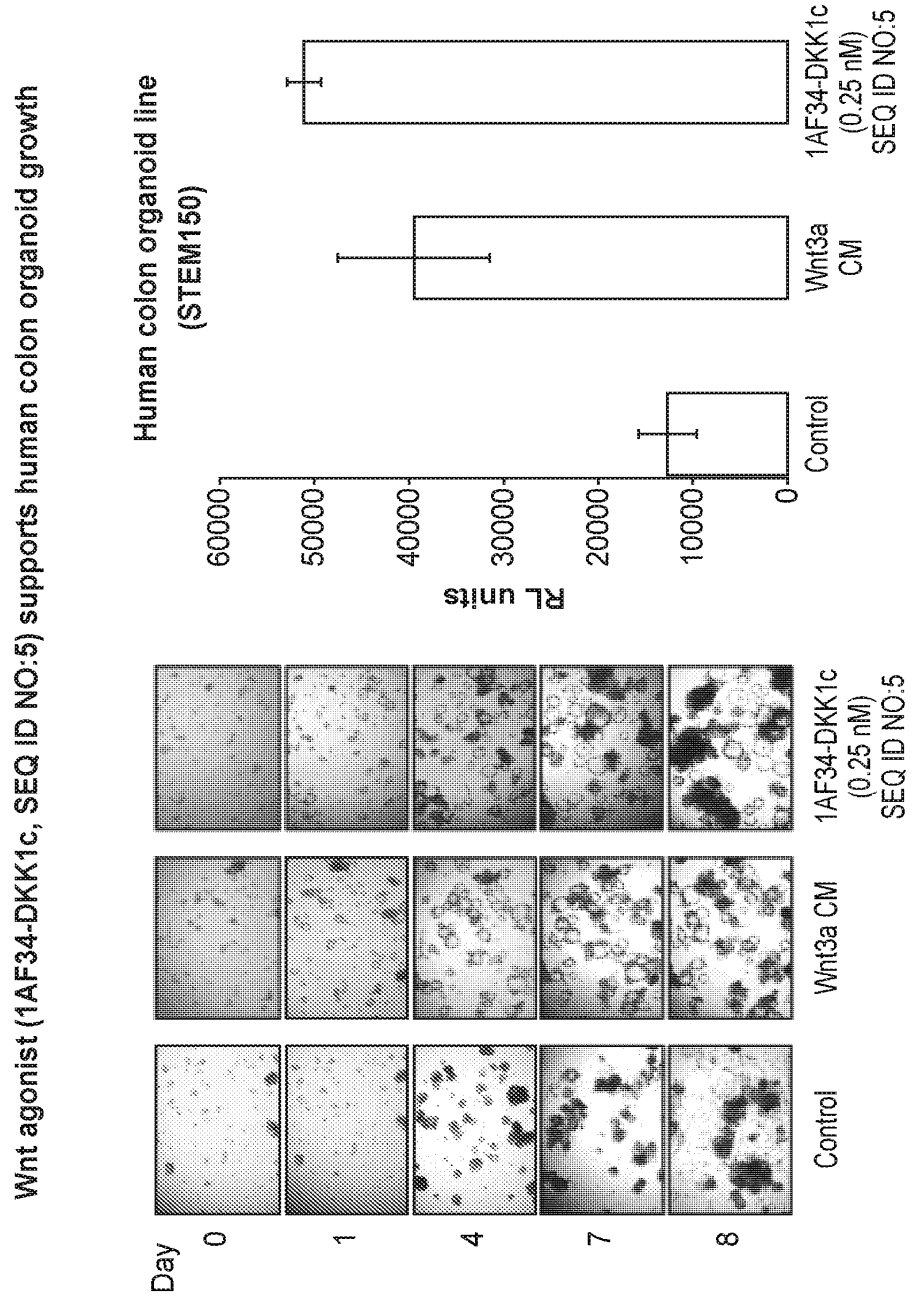

Wnt agonist (1AF34-DKK1c, SEQ ID NO:5) supports human colon organoid growth
Day
0
1
4
7
8
Control
Wnt3a CM
1AF34-DKK1c (0.25 nM) SEQ ID NO:5
Human colon organoid line (STEM150)
RL units
60000
50000
40000
30000
20000
10000
0
Control
Wnt3a CM
1AF34-DKK1c (0.25 nM) SEQ ID NO:5

FIG. 6

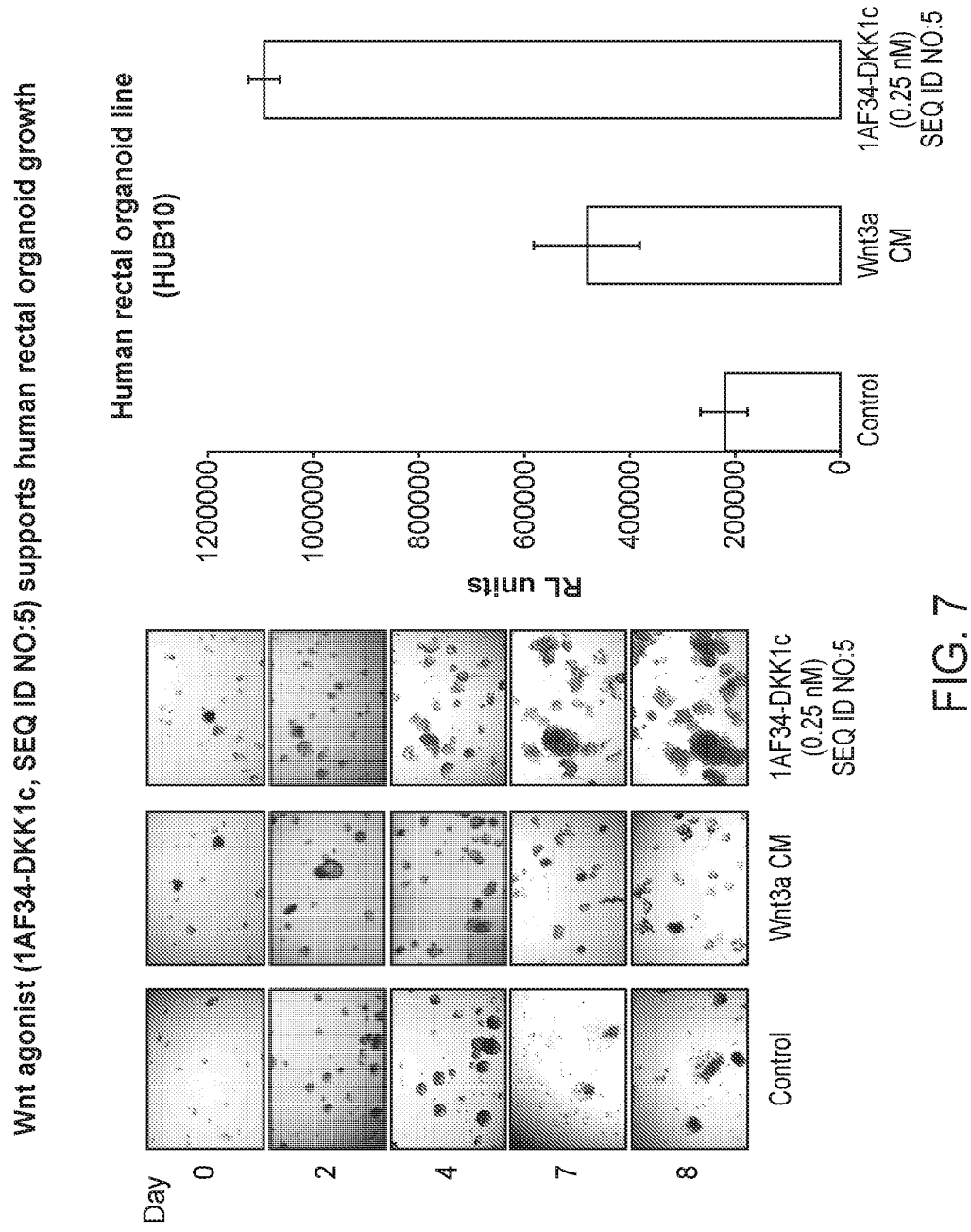

Wnt agonist (1AF34-DKK1c, SEQ ID NO:5) supports human rectal organoid growth
Day
0
2
4
7
8
Control
Wnt3a CM
1AF34-DKK1c (0.25 nM) SEQ ID NO:5
Human rectal organoid line (HUB10)
RL units
1200000
1000000
800000
600000
400000
200000
0
Control
Wnt3a CM
1AF34-DKK1c (0.25 nM) SEQ ID NO:5

FIG. 7

1-20 Cystatin signal sequence
MARPLCTLLLLMATLAGALA

21-22 GlySer linker (BamHI-site)
GS

23-314 Wnt agonist sequence
SELGTRLIRAALDGNKDRVKDLIENGADVNASLMSGATPLHAAAMNGHKEVVKLLISKGADVNAQSVAG
STPLDAAAFSGHKEVVKLLISKGADVNAVNAAGLTPLHAAAADNGHKEVVKLLISKGADVNAKADHGMTP
LHTAAQRGHKEVVKLLISKGADLNTSAKDGATPLDMARESGNEEVVKLLEKQLEGSGSGGSGSGKMYHT
KGQEGSVCLRSSDCASGLCCARHFWSKICKPVLKEGQVCTKHRRKGSHGLEIFQRCYCGEGLSCRIQKDHH
QASNSSRLHTCQRH

315-317 AlaAlaALa linker (NotI site)
AAA

318-325 Te cleavage site
ENLYFQGS

326-561 hFc tag
SERKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVH
NAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRD
ELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSV
MHEALHNHYTQKSLSLSPGKGAA

FIG. 8

MS&D Exhibit 1008, Page 4156 of 4223

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US19/41852

A. CLASSIFICATION OF SUBJECT MATTER

IPC - A61K 38/17; C07K 14/435, 14/47, 16/28 (2019.01)

CPC - A61K 38/17; C07K 14/435, 14/47, 14/4703, 16/28, 16/2863

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2014/0200179 A1 (GARCIA, KC et al.) 17 July 2014; paragraphs [0041], [0058], [0091]	1-3, 4/2-3, 9-11
A	US 2018/0137234 A1 (UNIVERSITY OF WASHINGTON) 17 May 2018; paragraph [0043]	1-3, 4/2-3, 9-11
A	US 2017/0349659 A1 (THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY) 7 December 2017; paragraphs [0043], [0070]	1-3, 4/2-3, 9-11
P, X	(DANG, LT et al.) Receptor subtype discrimination using extensive shape complementary designed interfaces. Nature Structural & Molecular Biology. June 2019, Epub 13 May 2019, Vol 26, No. 6; pages 407-414; DOI: 10.1038/s41594-019-0224-z	1

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"D" document cited by the applicant in the international application
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 December 2019 (02.12.2019)	19 DEC 2019
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Shane Thomas Telephone No. PCT Helpdesk: 571-272-4300

Form PCT/ISA/210 (second sheet) (July 2019)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US19/41852

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 5-8 and 12-16
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

-***-Please See Supplemental Page-***-

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Groups I+, Claims 1-4, 9-11; SEQ ID NO: 1 (Wnt signaling antagonist polypeptide sequence); SEQ ID NO: 5 (Wnt signaling agonist amino acid sequence); SEQ ID NO: 9 (Wnt signaling agonist polynucleotide sequence)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

-***-Continued from Box No. III: Observations where unity of invention is lacking-***-

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I+, Claims 1-4, 9-11; SEQ ID NO: 1 (Wnt signaling antagonist polypeptide sequence); SEQ ID NO: 5 (Wnt signaling agonist amino acid sequence); and SEQ ID NO: 9 (Wnt signaling agonist polynucleotide sequence) are directed toward Wnt signaling antagonists and agonists.

The antagonists and agonists will be searched to the extent that they encompass SEQ ID NO: 1 (first exemplary Wnt signaling antagonist polypeptide sequence); SEQ ID NO: 5 (first exemplary Wnt signaling agonist amino acid sequence); and SEQ ID NO: 9 (first exemplary Wnt signaling agonist polynucleotide sequence). Applicant is invited to elect additional antagonist(s) and/or agonist(s), with specified SEQ ID NO: for each, or with specified substitution(s) at specified site(s) of a SEQ ID NO:, such that the sequence of each elected species is fully specified (i.e. no optional or variable residues or substituents), and available as an option within at least one searchable claim, to be searched. Additional antagonist(s) and/or agonist(s) will be searched upon the payment of additional fees. It is believed that claims 1-4 (each in-part), and 9-11 (each in-part) encompass this first named invention and thus these claims will be searched without fee to the extent that they encompass SEQ ID NO: 1 (Wnt signaling antagonist polypeptide sequence); SEQ ID NO: 5 (Wnt signaling agonist amino acid sequence); and SEQ ID NO: 9 (Wnt signaling agonist polynucleotide sequence). Applicants must specify the searchable claims that encompass any additionally elected antagonist(s) and/or agonist(s). Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined. An exemplary election would be SEQ ID NO: 2 (Wnt signaling antagonist polypeptide); SEQ ID NO: 6 (Wnt signaling agonist amino acid sequence); SEQ ID NO: 10 (Wnt signaling agonist polynucleotide sequence).

No technical features are shared between the antagonists and/or agonists. of Groups I+ and, accordingly, these groups lack unity a priori.

Additionally, even if Groups I+ were considered to share the technical features including: a Wnt signaling antagonist polypeptide comprising an amino acid sequence; a Wnt signaling agonist comprising said polypeptide, linked to a binding domain having high affinity to one or both of Lrp5 and Lrp6 protein; a Wnt signaling agonist comprising an amino acid sequence; a polynuceotide encoding the Wnt signaling agonist; these shared technical features are previously disclosed by WO 2016/040895 A1 (THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY, et al.) (hereinafter 'Stanford') in view of US 2014/0200179 A1 to Garcia et al. (hereinafter 'Garcia').

California discloses a Wnt signaling agonist comprising a polypeptide (a Wnt signaling agonist comprising a polypeptide; abstract), linked to a binding domain having high affinity to one or both of Lrp5 and Lrp6 protein (linked to a binding domain having high affinity to one or both of Lrp5 and Lrp6 protein; abstract); a Wnt signaling agonist comprising an amino acid sequence (a Wnt signaling agonist comprising an amino acid sequence; abstract; paragraph [00104]); a polynuceotide encoding the Wnt signaling agonist (a polynuceotide encoding the Wnt signaling agonist; paragraph [00105]).

California does not disclose a Wnt signaling antagonist polypeptide comprising an amino acid sequence.

Garcia discloses a Wnt signaling antagonist polypeptide comprising an amino acid sequence (polypeptides that inhibit Wnt signaling; abstract; paragraphs [0041], [0091], [0108])

It would have been obvious to one of ordinary skill in the art at the time of the invention to modify the disclosure of California, to include a Wnt signaling antagonist polypeptide comprising an amino acid sequence, as disclosed by Garcia, in order to provide superior compositions for the treatment of conditions involving Wnt signaling.

Since none of the special technical features of the Groups I+ inventions is found in more than one of the inventions, and since all of the shared technical features are previously disclosed by a combination of the Stanford and Garcia references, unity of invention is lacking.

UNITED STATES
PATENT AND TRADEMARK OFFICE

P.O. Box 1450
Alexandria, VA 22313 - 1450
www.uspto.gov

ELECTRONIC ACKNOWLEDGEMENT RECEIPT

APPLICATION #	RECEIPT DATE / TIME	ATTORNEY DOCKET #
18/361,782	**02/07/2025 05:37:25 PM Z ET**	**SRZN-006/04US 328202-2203**

Title of Invention

WNT SURROGATE MOLECULES AND USES THEREOF

Application Information

APPLICATION TYPE	Utility - Nonprovisional Application under 35 USC 111(a)	PATENT #	-
CONFIRMATION #	9001	FILED BY	Elizabeth Hearn
PATENT CENTER #	69101834	FILING DATE	07/28/2023
CUSTOMER #	157070	FIRST NAMED INVENTOR	Yang LI
CORRESPONDENCE ADDRESS	-	AUTHORIZED BY	Ashley Sherrid

Documents

TOTAL DOCUMENTS: 5

DOCUMENT		PAGES	DESCRIPTION	SIZE (KB)
SRZN-006_04US_328202-2203_IDS_Transmittal_2025-02-07.pdf		3	-	106 KB
SRZN-006_04US_328202-2203_IDS_Transmittal_2025-02-07-TRAN.LET.pdf	(1-2)	2	Transmittal Letter	101 KB
SRZN-006_04US_328202-2203_IDS_Transmittal_2025-02-07-IDS.SIZEFEE.pdf	(3-3)	1	Assertion regarding Information Disclosure Statement (IDS) Size Fee (SB/08C)	97 KB
SRZN-006_04US_328202-2203_IDS_SB08_20250207142321.pdf		2	Information Disclosure Statement (IDS) Form (SB08)	104 KB

Warning: This is not a USPTO supplied IDS fillable form. Data in the form cannot be

automatically loaded to other USPTO systems.

WU-Amelioration-2011.pdf	11	Non Patent Literature	12984 KB
KOLUMAM_2015.pdf	14	Non Patent Literature	15682 KB

Digest

DOCUMENT	MESSAGE DIGEST(SHA-512)
SRZN-006_04US_328202-2203_IDS_Transmittal_2025-02-07.pdf	491C4490FD7456E6CD8EA4450D50F6B1F1987C79D6ADB81A1499EC26DF04E293655A20A72811B34A511776D9B391F0743EC1FCAC92BB74F7A5044E6154B69A81
SRZN-006_04US_328202-2203_IDS_Transmittal_2025-02-07-TRAN.LET.pdf	9551DC508D20D46789921352597F5C67B08D424413E051B066A7B37E85D33049767BDACD05C33B0F9A8991D7B725CF533AA4A68EB85ED867F29481D78D1746E4
SRZN-006_04US_328202-2203_IDS_Transmittal_2025-02-07-IDS.SIZEFEE.pdf	549D1097233B952D1AA30FEE4E7FBCC2D808953BB765EB8441E55B19DD09A5B97CA8FE53AF6F689EFA3CCE285FE13FD5E582F065123D36CDACDB63C3BF47B8E1
SRZN-006_04US_328202-2203_IDS_SB08_20250207142321.pdf	4958D45C0E35C90F9F535389196065D517673E5BA07927259E263D4DC7A0D596DD2F07DBE6FA14B548F7D9278E60C082E43FAFDEB5293E9B5EA76372AF32712C
WU-Amelioration-2011.pdf	A3EB08A768123C3C50D6AB23DFCF9312BEFD3103F0A066FE85D6BE70164C938CCB089CAED882B57841E516BFCC0C74DFD9D5700DCEEF773B91D9C84FFCE17239
KOLUMAM_2015.pdf	7E9335F95985A6F47F8DFE06F3E5F691E3F1CDD904C66718EAAF24F12F95E0E1C1663E8E39A20131B60D08AAB03A4545B4E47A6329A6D0EE4C0E881EFFA99096

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Form used in lieu of PTO/SB/08a/b (07-09)
Approved for use through 05/31/2024. OMB 0651-0031
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Substitute for form 1449/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 1 of 2

Complete if Known	
Application Number	18/361,782
Filing Date	07-28-2023
First Named Inventor	LI; Yang
Art Unit	1645
Examiner Name	CHEONG, CHEOM-GIL
Attorney Docket Number	SRZN-006/04US 328202-2203

U. S. PATENT DOCUMENTS

Examiner Initials*		Cite No.[1]	Document Number / Number-Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
			None			

FOREIGN PATENT DOCUMENTS

Examiner Initials*		Cite No.[1]	Foreign Patent Document / Country Code[3] Number[4] Kind Code[5] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages Or Relevant Figures Appear	T[6]
			None				

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. [1] Applicant's unique citation designation number (optional). [2] See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. [3] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [6] Applicant is to place a check mark here if English language Translation is attached.

Form used in lieu of PTO/SB/08a/b (07-09)
Approved for use through 05/31/2024. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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Substitute for form 1449/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 2 of 2

Complete if Known	
Application Number	18/361,782
Filing Date	07-28-2023
First Named Inventor	LI; Yang
Art Unit	1645
Examiner Name	CHEONG, CHEOM-GIL
Attorney Docket Number	SRZN-006/04US 328202-2203

NON-PATENT LITERATURE DOCUMENTS				
Examiner Initials*		Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
		001	KOLUMAM et al. "Sustained brown fat stimulation and insulin sensitization by a humanized bispecific antibody agonist for fibroblast growth factor receptor 1/ßKlotho complex" EBioMedicine (2015); 2(7):730-743.	☐
		002	WU, Ai-Luen, et al. "Amelioration of type 2 diabetes by antibody-mediated activation of fibroblast growth factor receptor 1" Science Translational Medicine (2011); 3(113):113ra126, 11 pages.	☐

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. [1] Applicant's unique citation designation number (optional). [2] Applicant is to place a check mark here if English language Translation is attached.

Docket No.: SRZN-006/04US 328202-2203
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Inventor:	Yang LI	Confirmation No.:	9001
Application No.:	18/361,782	Group Art Unit:	1645
Filed:	July 28, 2023	Examiner:	C. Cheong

For: WNT SURROGATE MOLECULES AND USES THEREOF

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

INFORMATION DISCLOSURE STATEMENT

In accordance with the duty of disclosure set forth in 37 CFR § 1.56, Applicant hereby submits this Information Disclosure Statement ("IDS") along with the attached Form used in lieu of PTO/SB/08 ("SB/08") and Written Assertion used in lieu of PTO/SB/08C in compliance with 37 CFR §§ 1.97 and 1.98. It is respectfully requested that the information be expressly considered during the prosecution of the above-identified Application, and the references be made of record therein and appear among the "References Cited" on any patent to issue therefrom.

In accordance with 37 CFR § 1.97(b), no additional fee for submission of this Information Disclosure Statement is required, as it is filed within any one of the time periods:

[ ] within three months from the filing date of this national Application other than a CPA under 37 CFR § 1.53(d);

[ ] within three months from the date of entry of the national stage as set forth in 37 CFR § 1.491 in this international Application;

[ ] before the mailing date of a first Office action on the merits; or

[X] before the mailing of a first Office action after the filing of a request for continued examination under 37 CFR § 1.114.

In agreement with 37 CFR § 1.97(g), this Information Disclosure Statement shall not be construed as a representation that a search has been made.

In agreement with 37 CFR § 1.97(h), the filing of this Information Disclosure Statement shall not be construed to be an admission that the information cited in the statement is, or is considered to be, relevant, analogous, or material to patentability as defined by 37 CFR § 1.56(b).

As required by 37 CFR § 1.98(a)(2), Applicant submits a copy of any foreign patent and non-patent literature document cited on the SB/08.

IDS SIZE FEE - WRITTEN ASSERTION UNDER 37 CFR § 1.98

As required by 37 CFR § 1.98(a)(4), Applicant asserts that with the submission of this Information Disclosure Statement, the cumulative number of items of information that have been provided to the Office during the pendency of the present Application is 542. As the filing of this Information Disclosure Statement does not cause the cumulative number of items of information to exceed any IDS size threshold specified in 37 CFR §§ 1.17(v)(1), (v)(2), or (v)(3), no IDS size fee under 37 CFR § 1.17(v) is required at this time.

The Director is hereby authorized to charge the fee set forth in 37 CFR § 1.17(p) as indicated above for this Information Disclosure Statement submitted herewith, and to credit any overpayment, to Deposit Account No. 50-1283, referencing Docket No. SRZN-006/04US 328202-2203.

It is respectfully requested that the Examiner consider the above-noted information and return an initialed copy of the SB/08 to the undersigned. The Examiner is encouraged to contact the undersigned by telephone if it is felt that a telephone interview would advance the prosecution of the present Application.

Dated: February 7, 2025

Respectfully submitted,
COOLEY LLP

USPTO CUSTOMER No. 157070

COOLEY LLP
ATTN: IP Docketing Department
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Electronic signature: /Ashley M. Sherrid/
Ashley M. Sherrid
Reg. No. 81,446
Carol D. Laherty
Reg. No. 51,909

Docket No.: SRZN-006/04US 328202-2203
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Inventor:	Yang LI	Confirmation No.:	9001
Application No.:	18/361,782	Group Art Unit:	1645
Filed:	July 28, 2023	Examiner:	C. Cheong

For: WNT SURROGATE MOLECULES AND USES THEREOF

Mail Stop AF
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF DR. YANG LI UNDER 37 CFR § 1.132

I, Yang LI, do hereby declare and state that:

1. I am the Executive Vice President of Research at Surrozen, Inc. I hold a Ph.D. degree in Cell Biology from Stanford University and dual B.S. degrees in Chemistry and Molecular and Cell Biology from Pennsylvania State University. I have over 26 years of experience in the biopharmaceutical industry, and I have authored more than 90 scientific papers in the field of biomedical research and am a named inventor on over 40 patents or patent applications. My *curriculum vitae* is attached hereto as **Exhibit 1**. I am also a named inventor of the subject matter described and claimed in the above-identified application.

2. I am thoroughly familiar with the subject matter described and claimed in the above-identified application, and I have reviewed the Final Office Action mailed December 27, 2024 and the documents cited in the rejections therein. I understand that claims 1, 17, and 55 stand rejected under 35 U.S.C. 103 as allegedly being obvious over Garcia

et al. (US2017/0306029), in view of Wu et al. (Nature Biotechnology, Vol. 5, 1290-1297 (2007)).

3. I submit this declaration to establish that the claimed invention is not obvious over the cited references, and to provide further evidence of surprising and unexpected advantages associated with the claimed molecules.

4. I submit that Wnt surrogate molecules having a 2:2 Fzd binders:Lrp5/6 binders stoichiometry were only first described in the instant application. Previously, it was believed in the art that the appropriate stoichiometry for Wnt surrogate molecules was 1:1 Fzd binder:Lrp5/6 binder. Garcia et al. only described Wnt surrogate molecules having 1:1 Fzd binder:Lrp5/6 binder stoichiometry, and did not suggest or provide any motivation for a person skilled in the art to design a tetravalent molecule as claimed. This is further evidenced by the fact that Garcia's research group not only continued to teach the original 1:1 Fzd binder:Lrp5/6 binder stoichiometry, but also presented arguments against the superiority of the tetravalent format (Miao et al., 2020, WO2020/018445). Thus, the understanding in the art at the time of the present invention actually teaches away from the claimed tetravalent Wnt surrogate molecules with 2:2 stoichiometry.

5. I further submit that a skilled artisan, when developing a Wnt surrogate molecule, which must bind both Fzd receptor(s) and Lrp5/6 receptors simultaneously, in order to agonize the Wnt signaling pathway, would not be motivated to modify the Wnt surrogate molecules disclosed in Garcia et al. by adopting the DVD-Ig format disclosed by Wu et al. in the context of signaling pathway inhibitors. Wu et al. is not related to Wnt surrogate agonists. Instead, Wu et al. relates to bivalent molecules that allow the simultaneous blockade of multiple targets (e.g., IL-12 and IL-18), which is very different than the claimed molecules, which simultaneously engage two cell surface coeffector molecules (Fzd and Lrp5/6) required to agonize the canonical Wnt signaling cascade. In particular, Wu et al. describes DVD-Ig format bispecific

antibodies that block multiple disease-associated targets, e.g., disease mediators that are playing redundant roles in inducing the same signaling cascade, such as interleukin-1α. Wu et al. describes that DVD-Ig molecules "can be engineered from any two monoclonal antibodies while preserving activities of the parental antibodies" (Wu et al., Abstract). Wu et al. fails to disclose DVD-IgG molecules or any other molecules with a 2:2 stoichiometry that simultaneously bind to coreceptors to induce a signaling pathway, let alone DVD-IgG molecules that bind to Fzd(s) and Lrp5/6 to initiate Wnt signaling. The skilled artisan would appreciate that the ability of a molecule to bind two receptors to block their signaling would not necessarily require that both receptors are bound simultaneously, whereas, in contrast, the claimed molecules have the ability to simultaneously bind both Fzd and Lrp5/6 to initiate Wnt signaling. This represents an important biological distinction between the molecules of Wu et al. and those presently claimed, such that one of skill in the art would have no motivation to prepare DVD-IgG Wnt surrogate molecules, and certainly no real expectation that they would be capable of agonizing the Wnt signaling pathway in view of the teachings of Garcia et al. and Wu et al. In addition, one of skill in the art would be dissuaded from preparing a tetravalent Wnt surrogate molecule, since they would worry that any resulting signaling would not represent the same signaling as the natural Wnt ligand. For example, Wu et al. 2011 showed that an antibody containing two FGFR1c binding regions did not need to include any β-Klotho binding regions to induce the FGF-21 signaling pathway, which normally requires FGF-21 binding to both FGF-21 coreceptors, FGFR1c and β-Klotho. Therefore, it was expected that a tetravalent molecule that included two FGFR1c binding regions and two β-Klotho binding regions, would activate FGFR1c signaling, but the signaling would not be co-receptor β-Klotho dependent. Such a tetravalent molecule would not be a true FGF-21 mimetic. Moreover, given that FGFR1 is broadly expressed, but β-Klotho expression is more limited, it was believed that such a tetravalent molecule could result in undesirable side effects. Because of this, this group elected to design a FGF-21 mimetic antibody in a bivalent bispecific format where there is only one FGFR1c binding region and only one β-Klotho binding region (Kolumam et al.

2015), a 1:1 design similar to what Garcia et al. used for Wnt surrogates. Based on this, one of skill in the art would have similar concerns that a DVD-IgG Wnt surrogate, even if it was able to activate Wnt signaling, may lead to undesirable activation of Wnt signaling that was not dependent on both FZD and LRP5/6 receptors.

6. I also submit that the claimed tetravalent, multispecific Wnt surrogate molecules surprisingly and unexpectedly possess greatly superior activity as compared to those having 1:1 Fzd binder:Lrp5/6 binder stoichiometry, as disclosed by Garcia et al. This serendipitous discovery was surprising and unexpected in view of the prior art and understanding in the field. The nonobvious and unexpected finding of the superiority of multivalency resulted from our serendipitous analysis of numerous fractions obtained from various purification processes, including size exclusion chromatography (SEC), following synthesis of the tandem scFv format in the 1:1 configuration (with a single scFv Fzd binding linked to a single scFv Lrp5/6 binding domain) described by Garcia et al. The eluted proteins from these various fractions were tested in a WNT-responsive cell-based assay. These fractions included the monomer tandem scFv species and other fractions collected before or after the monomer peak, which may contain contaminants, such as higher molecular weight aggregated species or lower molecular weight degradants or misfolded proteins. This careful analysis of all SEC fractions revealed the unexpected finding that the peak of WNT activity across the SEC fractions did not correlate with the monomer protein product peak, but rather the activity was found in higher molecular weight species-containing fractions and correlated in particular with proteins having molecular weights of two or more times the expected molecular weights of the monomers (WO2019/126398, Chen et al., 2020b, Chen et al., 2020a). Therefore, the activity previously observed with pooled material around the monomer protein peak was "bleed through" or spill over/contamination of higher molecular weight products into the monomer fractions, and the monomer peak containing proteins having only one Fzd binding region and one Lrp5/6 binding region were largely inactive. The higher

molecular weight species associated with Wnt activity were formed from the aggregation of two or three monomeric tandem scFv molecules, resulting in the presence of two or more binding domains for FZD and two or more binding domains for LRP in one molecule. This unexpected and serendipitous finding suggested to us that efficient WNT surrogate signaling might require the clustering of receptor complexes of more than one FZD and one LRP. To further prove this finding, we generated and tested tetravalent formats, such as the 2:2 design antibody-based molecules containing two FZD binding domains and two LRP binding domains in one molecule. The FZD binding domains we tested included those having broad, subfamily, or monospecific FZD specificity (as described in WO2019/126398). These studies provided definitive evidence that efficient WNT signaling induction required more than one FZD binder and one LRP binder in the same molecule. The tetravalent (or higher order) requirement for WNT surrogate molecules is now supported by subsequent work, conducted after the filing of the instant application, on a very diverse set of molecules having many different formats and various antibody fragments, such as scFvs-Fc, VHHs-Fc, Fv-IgG (DVD), VHH-IgG, Fab-IgG, diabody-Fc-Diabody, diabody-Fc-Fab, etc. formats (Post et al., 2024). Only the tetravalent molecules were active when all of these formats were tested. The 1:1 fusions of antibody fragments, such as tandem scFv, Fv-Fab, Fab-Fab, VHH-Fab, etc. (which all have only one FZD binding domain and one LRP binding domain), were very weakly active or completely inactive (Post et al., 2024). These experiments explicitly showed that more than one FZD and one LRP must be present in the signaling complex to induce efficient signaling, as later reported by us in Chen *et al.*, 2020b, such as Figures 5B&C. This was a surprising and unexpected result, particularly in view of the teachings of Garcia et al.

7. As further evidence of the surprising and unexpected nature of our finding that the claimed tetravalent, multispecific Wnt surrogates have superior activity, I submit that three separate studies from three independent groups (including our own) who were developing Wnt modulators all based their initial work on the teaching of Garcia et

al., and thus all three groups started with molecules having a 1:1 stoichiometry of FZD binder:LRP binder. This demonstrates a shared belief in the field that this was the appropriate format for Wnt signaling activity. These groups included: (1) our own, which took an initial approach based on 1:1 stoichiometry (as discussed above and in WO2019/126398, Chen et al., 2020b, and Chen et al., 2020a); (2) a University of Toronto group, who's initial approach was based on 1:1 stoichiometry (see, e.g., WO2019/159084, Tao et al., 2019); and (3) a UCLA group (see, e.g., Zhang et al., 2020), who's initial work was also based on 1:1 stoichiometry. Different groups developed molecules having different binding domains, such as different antibody fragments, different regions of DKK, or small molecules, but there was no leap in conceptual changes in the format from 1:1 to 2:2. That was only first disclosed in the instant application.

8. In view of the above, I submit that the claimed tetravalent, multispecific Wnt surrogate molecules are clearly not obvious. Not only would they not be obvious in view of disparate and unrelated teachings of Garcia et al. and Wu et al., but this claimed format demonstrates surprising and unexpectedly superior activity as compared to the prior art Wnt surrogate molecules.

9. I, the undersigned, declare that all statements made herein of our knowledge are accurate and that all statements made on information and belief are believed to be accurate; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: February 7, 2025



Yang LI

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Exhibit 1

Yang Li, Ph.D.

EDUCATION:

Ph.D. in Cell Biology 9/1990-3/1996
Stanford University, Stanford, CA. Advisor: Dr. Roger D. Kornberg (Nobel Laureate)

Dual B.S. degrees in Chemistry and Molecular & Cell Biology with honor 9/1986-6/1990
Pennsylvania State University, PA.

PROFESSIONAL EXPERIENCE:

Executive Vice President, Research, Surrozen, Inc. 8/2023-present
Senior Vice President, Biology, Surrozen, Inc. 1/2021-7/2023
Vice President, Biology, Surrozen, Inc. 10/2017–12/2020
Senior Director, Head of Discovery Biology and Head of Protein Sciences, Surrozen, Inc. 1/2017–9/2017

- Built and oversaw both Discovery Biology and Protein Sciences Departments till July 2023, and now with expanded responsibility to oversee the entire research organization
- One of the main inventors of Surrozen science and platform technologies, sole inventor on four technology applications
- Key decision maker in research strategy, disease focus, and technology development
- Shepherd two programs into the clinic, two in IND enabling phase, and additional programs toward candidate nomination.
- Member of the executive management team, involved in setting corporate strategies, R&D strategies, BD/investor related activities
- Joint Steering Committee chair of CMC partnership with Lonza
- Joint Steering Committee co-chair of retinopathy partnership with Boehringer-Ingelheim

Scientific Director, Cardiometabolic Department, Amgen, Inc. 1/2010 – 1/2017

- Reported to the Cardiometabolic Department Vice President and served as a member of the senior leaders' team advising the VP on department research strategies, resource allocations, portal progressions, etc.
- Supervise/manage a group of 14 scientists at all levels on research activities in diabetes, obesity, kidney, and cardiovascular diseases.
- Served as Global Discovery Research Lead on a clinical program and managed and wrote research study reports in aid of regulatory filing, and provided research support for clinical development.
- Effectively built and managed several academic collaborations with Stanford University, UT South Western, Texas A&M, and Shanghai Tech University.
- Worked with BD to evaluate various in-licensing opportunities.
- Program leader for a cross-site, cross-function FGF21 program. Pioneered agonistic antibody and several other industry leading approaches.
- Managed and led a cross-function, cross-site discovery research effort by establishing a high-throughput in vivo genomic screening platform. Led the team to the identification of over a dozen novel secreted hormones and receptors. The most advanced target from this effort is currently in late preclinical development.

Principal Scientist, Metabolic Disorders, Amgen, Inc. 3/2006 – 12/2009

- Core team member on two clinical programs for obesity and diabetes, contributed to biology research, research study reports writing in aid of regulatory filing.

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- Established and successfully led the FGF21 mimetic program. Several noted achievements including the discovery of receptors for FGF21 and related family members, pathways and target tissues identifications, and setting strategies to explore novel modalities for therapeutic development.
- Successfully launched and led several small molecule GPCR programs, large molecule programs, and a large cross-site, cross-function discovery efforts to address pipeline needs.

Senior Scientist, Dept. of Biology, Amgen, Inc. 4/2004 – 2/2006
- Led 6 GPCR projects for metabolic diseases.
- Led an effort to develop novel PPARγ ligands for treating diabetes.

Scientist, Dept. of Biology, Tularik, Inc. 6/1998 – 4/2004
- Team member on a clinical stage small molecule ONR program. Led the research team to support regulatory filing and clinical development.
- Led a team on the development of novel small molecular drugs against HCV.

Postdoctoral Research Fellow 4/1996 – 5/1998
Dr. Roger D. Kornberg advisor, Dept. of Structural Biology, Stanford University.

Consultant, BioPore Corporation, Palo Alto, CA. 2/1995 - 6/1995

Research Assistant, Supelco, Inc., PA. 6/1990 - 9/1990
Development of HPLC columns for chiral separations.

PUBLICATIONS:

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ACCEPTED INVITED MEETING PRESENTATIONS:

1. TIDES, Oligonucleotide & Peptide Therapeutics, Boston, MA, 2022.
2. PEGS Protein Engineering & Cell Therapy Summit, Boston, MA, 2020.
3. Gordon Research Conference on Fibroblast Growth Factors in Development & Disease, Hong Kong, China, 2016.
4. Gordon Research Conference on Fibroblast Growth Factors in Development & Disease, Ventura, CA, 2014.
5. Chinese-American Bio/Pharmaceutical Society (CABS) Science Workshop, San Francisco, CA, 2013.
6. Gordon Research Conference on Fibroblast Growth Factors in Development & Disease, Les Diablerets, Switzerland, 2012.
7. Keystone Symposia, Genetic and Molecular Basis of Obesity and Body Weight Regulation, Santa Fe, NM, 2012.
8. GTCbio, 4th Diabetes Drug Discovery & Development, Philadelphia, PA, 2011.

9. Cambridge Healthtech Institute, 8th Discovery On Target, Boston, MA, 2010.
10. Gordon Research Conference on Fibroblast Growth Factors In Development & Disease, Ventura, CA, 2010.
11. GTCbio, 4th Annual Metabolic Diseases World Summit, Boston, MA, 2008.
12. BIT, 6th Annual International Drug Discovery Science and Technology, Beijing, China, 2008.
13. The Knowledge Foundation, Orphan & Nuclear Receptors – New Therapeutic Developments, San Diego, CA, 2003.

PATENTS AND PATENT APPLICATIONS:

1. Li, Y., Nguyen, H.T., Post, Y., Yeh, W.-C., Lopez, T., and Brezski, R.J. (2024) WO2024/249444 WNT SURROGATE AGENTS AND METHODS FOR LACRIMAL GLAND REGENERATION
2. Li, Y., Go, H., Chen, H., Bell, S.M., Lopez, T. and Sampathkumar, P. (2024) WO2024/227103 MOLECULES MODULATING WNT SIGNALING PATHWAYS AND USES THEREOF.
3. Zhang, Z., Li, Y., Yeh, W.C., Yuan, T.Z., Baribault, H., Lai, K.P. (2024) US11,958,891 Tissue-specific Wnt signal enhancing molecules and uses thereof.
4. Li, Y. (2024) WO2024/040118A2 CELL TARGETING MULTICOMPONENT POLYPEPTIDE
5. Handa, P., Lee, S.J., Li, Y., Sanman, L., Vanhove, G., Yeh, W.C. (2023) WO2023/250291A2 Modulation of Wnt signalling in corneal disorders.
6. Li, Y., Yuan, T.Z., Sato, A.K., Yeh, W.C., Janda, C.Y., Fowler, T.W., Baribault, H., Lai, K.P., Xie, L., Brezski, R., Lu, C. (2023) US11,773,171 Wnt surrogate molecules and uses thereof.
7. Li, Y., Yuan, T.Z., Sato, A.K., Yeh, W.C., Sampathkumar, P. (2023) US11,746,150 Anti-Lrp5/6 antibodies and methods of use.
8. Chen, H., Lee, S., Li, Y., and Yeh, W.C. (2023) WO2023/130055A2 WNT RECEPTOR-SPECIFIC COMPOUND AND METHOD RELATING HERETO
9. Li, Y., Hguyen, H., Yorick, P., Yeh, W.C. (2023) WO2023/115048A1, WNT-SURROGATE AGENTS AND METHODS FOR LACRIMAL GLAND REGENERATION.
10. Fletcher, R., Lai, K.P., Li, Y., Yeh, W.C. (2023) WO2023/044348A1, Modulation of Wnt signaling in pulmonary disorders.
11. Fletcher, R., Lee, S.J., Li, Y., Chenggang, L., Pathkumar, P., Geertrui, V., Yeh, W.C., Xie, L., Presta, L. (2022) WO2022/192445A1, Modulation of Wnt signaling in gastrointestinal disorders.
12. Li, Y., Zhang, Z., Brezski, R.J., Presta, L., Lopez, T., Chen, H., Baribault, H., Yeh, W.C., Tu, S. (2022) WO2022/104280A1, Liver-specific wnt signal enhancing molecules and uses thereof.
13. Li, Y., Stevens, J., King, C., Foltz, I., Kannan, G., Yie, J., and Hu, S. (2022) US11,248,052B2, Human antigen binding proteins that bind to a complex comprising β-Klotho and an FGF receptor.
14. Li, Y. (2021) WO2021/173726A1, WNT SUPER AGONISTS.
15. Li, Y., Lopez, T.S., Wang, I.C., Sampathkumar, P. (2021) WO2021/003416A1, Monospecific anti-frizzled antibodies and methods of use.
16. Ollmann, M., Li, Y., Zhang, J., Homann, O., Miranda, L.P., Murray, J.K., Wu, B., Yoon, O.K., Allen, J.G., Netirojjanakul, C. (2020) US10,870,856B2, RNAi constructs for inhibiting ASGR1 expression and methods of use thereof.
17. Xiong, Y., Li, Y., Yeh, W., Shan, B., and Sheng, J. (2020) US10,752,664B2, Method of treating or ameliorating metabolic disorders using growth differentiation factor 15 (GDF-15).
18. Lee, S., Li, Y., Yeh, W.C. (2020) WO2020/206005A1 Modulation of Wnt signaling in auditory disorders.
19. Li, Y., Lu, C., Baribault, H., Yeh, W.C., Xie, L. (2020) WO2020/185960A1 Modulation of Wnt signaling in gastrointestinal disorders.
20. Li, Y., Tu, S., Lee, S., Yeh, W.C. (2020) WO2020/167848A1 Modulation of Wnt signaling in ocular disorders.
21. Li, Y. and Sampathkumar, P. (2020) WO2020/132356A1 Antigen binding formats for receptor complexes.

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22. Hu, S., Foltz, I., King, C., Li, Y., and Arora, T. (2020) US10,570,205B2, Human Antigen Binding Proteins That Bind Beta-Klotho, FGF Receptors And Complexes Thereof.
23. Li, Y., (2020) WO2020/010308A1 Multi-specific Wnt surrogate molecules and uses thereof.
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39. Jean, J., Piper, D., Powers, J., Walker, N., and Li, Y. (2001) WO/2001/077091A2, NS5B HCV Polymerase Inhibitors.

AWARDS AND HONORS:

1996 Recipient of **Medical Research Fellowship**, Giannini Cancer Foundation, CA.
1996 Recipient of **Dean's Fellowship**, Stanford Medical School, Stanford, CA.
1988-89 **Teas Scholarship**, Dept. of Chem., Penn State University, PA.
1987-88 **Weidner Scholarship**, Dept. of Chem., Penn State University, PA.
1986 **Outstanding chemistry student**, awarded by Chemcut Co., State College, PA.
1986 **Member** of the Central Pennsylvania Team in the American Regional Mathematics Competition.

UNITED STATES
PATENT AND TRADEMARK OFFICE

P.O. Box 1450
Alexandria, VA 22313 - 1450
www.uspto.gov

ELECTRONIC ACKNOWLEDGEMENT RECEIPT

APPLICATION #	RECEIPT DATE / TIME	ATTORNEY DOCKET #
18/361,782	**02/07/2025 06:14:35 PM Z ET**	**SRZN-006/04US 328202-2203**

Title of Invention

WNT SURROGATE MOLECULES AND USES THEREOF

Application Information

APPLICATION TYPE	Utility - Nonprovisional Application under 35 USC 111(a)	PATENT #	-
CONFIRMATION #	9001	FILED BY	Susan Clingerman
PATENT CENTER #	69102554	FILING DATE	07/28/2023
CUSTOMER #	157070	FIRST NAMED INVENTOR	Yang LI
CORRESPONDENCE ADDRESS	-	AUTHORIZED BY	Carol Laherty

Documents

TOTAL DOCUMENTS: 1

DOCUMENT	PAGES	DESCRIPTION	SIZE (KB)
SRZN006_04US_1132_DEC_LI.pdf	20	Affidavit-traversing rejections or objections rule 132	251 KB

Digest

DOCUMENT	MESSAGE DIGEST(SHA-512)
SRZN006_04US_1132_DEC_LI.pdf	7CF90511F164460F6E7F51C74DDCCB9CBE4A90EEF07DA080591AEBBB3063F89C5B9DC55BB24868F79BCA6AA61FF5B7A99415868861FC123E282F79304C2CB63D

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

United States Patent and Trademark Office

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Document Code:WFEE

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Sale Accounting Date:02/18/2025

Sale Item Reference Number	Effective Date
18361782	02/07/2025

Document Number	Fee Code	Fee Code Description	Amount Paid	Payment Method
I20252HD16466405	1806	SUBMISSION- INFORMATION DISCLOSURE STMT	$280.00	Deposit Account

Examiner-Initiated Interview Summary	**Application No.** 18/361,782	**Applicant(s)** LI et al.		
	Examiner CHEOM-GIL CHEONG	**Art Unit** 1645	**AIA (First Inventor to File) Status** Yes	**Page** 1 of 1

All Participants (applicant, applicants representative, PTO personnel)	**Title**	**Type**
CHEOM-GIL CHEONG	Examiner	Telephonic
Anand Desai	Primary Examiner	
Carol Laherty	Attorney	

Date of Interview: 11 March 2025

Issues Discussed:

35 U.S.C. 112

Examiner proposed Examiner's Amendment to amend claim 17 and claim 71 to depend from claim 15 instead of claim 1 because claim 1 does not recite heavy chain and light chain and therefore it raise 112(b) issue for antecedent basis. Applicant agreed.

/CHEOM-GIL CHEONG/ Examiner, Art Unit 1645	/ANAND U DESAI/ Primary Examiner, Art Unit 1656

Applicant is reminded that a complete written statement as to the substance of the interview must be made of record in the application file. It is the applicants responsibility to provide the written statement, unless the interview was initiated by the Examiner and the Examiner has indicated that a written summary will be provided. See MPEP 713.04
Please further see:
MPEP 713.04
Title 37 Code of Federal Regulations (CFR) § 1.133 Interviews, paragraph (b)
37 CFR § 1.2 Business to be transacted in writing

Applicant recordation instructions: It is not necessary for applicant to provide a separate record of the substance of interview.

Examiner recordation instructions: Examiners must summarize the substance of any interview of record. A complete and proper recordation of the substance of an interview should include the items listed in MPEP 713.04 for complete and proper recordation including the identification of the general thrust of each argument or issue discussed, a general indication of any other pertinent matters discussed regarding patentability and the general results or outcome of the interview, to include an indication as to whether or not agreement was reached on the issues raised.

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	18/361,782	LI et al.
	Examiner	Art Unit
	CHEOM-GIL CHEONG	1645

CPC									
Symbol								Type	Version
C07K	/	16	/	2863				F	2013-01-01
C07K	/	16	/	468				I	2013-01-01
A61K	/	2039	/	505				A	2013-01-01
C07K	/	2317	/	30				A	2013-01-01
C07K	/	2317	/	55				A	2013-01-01
C07K	/	2317	/	569				A	2013-01-01
C07K	/	2317	/	622				A	2013-01-01
C07K	/	2317	/	92				A	2013-01-01

CPC Combination Sets									
Symbol								Type	Set	Ranking	Version
	/		/								

/CHEOM-GIL CHEONG/ Examiner, Art Unit 1645	12 March 2025	Total Claims Allowed:	
(Assistant Examiner)	(Date)	5	
/ANAND U DESAI/ Primary Examiner, Art Unit 1656	14 March 2025	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	none

U.S. Patent and Trademark Office — Part of Paper No.: 20250312

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	18/361,782	LI et al.
	Examiner	Art Unit
	CHEOM-GIL CHEONG	1645

INTERNATIONAL CLASSIFICATION					
CLAIMED					
C07K16/28	/	16	/	28	
A61K39/00	/	39	/	00	

NON-CLAIMED				
	/		/	

US ORIGINAL CLASSIFICATION	
CLASS	SUBCLASS

CROSS REFERENCES(S)						
CLASS	SUBCLASS (ONE SUBCLASS PER BLOCK)					

/CHEOM-GIL CHEONG/ Examiner, Art Unit 1645	12 March 2025	Total Claims Allowed:	
(Assistant Examiner)	(Date)	5	
/ANAND U DESAI/ Primary Examiner, Art Unit 1656	14 March 2025	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	none

U.S. Patent and Trademark Office — Part of Paper No.: 20250312

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	18/361,782	LI et al.
	Examiner	**Art Unit**
	CHEOM-GIL CHEONG	1645

☑ Claims renumbered in the same order as presented by applicant ☐ CPA ☑ T.D. ☐ R.1.47

CLAIMS

Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original

/CHEOM-GIL CHEONG/ Examiner, Art Unit 1645	12 March 2025	**Total Claims Allowed:**	
(Assistant Examiner)	(Date)	5	
/ANAND U DESAI/ Primary Examiner, Art Unit 1656	14 March 2025	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	none

U.S. Patent and Trademark Office Part of Paper No.: 20250312

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	18/361,782	LI et al.
	Examiner	Art Unit
	CHEOM-GIL CHEONG	1645

CPC - Searched*		
Symbol	Date	Examiner

CPC Combination Sets - Searched*		
Symbol	Date	Examiner

US Classification - Searched*			
Class	Subclass	Date	Examiner

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

/CHEOM-GIL CHEONG/ Examiner, Art Unit 1645	

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	18/361,782	LI et al.
	Examiner	Art Unit
	CHEOM-GIL CHEONG	1645

Search Notes		
Search Notes	**Date**	**Examiner**
Reviewed prosecution history of parent case 16/954,484	06/05/2024	cc
Inventor name search	06/05/2024	cc
Reviewed pending claims of 18/311,082; 18/148,448; 16/954,482; 17/257,817; 18/652,070; 17/623,006; 17/801,624; 17/414,265; 17/429,584; 18/281,253; 17/438,303; 17/438,806;	06/05/2024	cc
Reviewed issued claims of US11746150, US11773171	06/05/2024	cc
Sequence search - see SCV	06/05/2024	cc
PE2E search – see attached	06/05/2024	cc
Google Scholar search – see attached	06/06/2024	cc
Inventor name search	12/19/2024	cc
PE2E search – see attached	12/19/2024	cc
Inventor name search	03/12/2025	cc
PE2E search – see attached	03/12/2025	cc

Interference Search			
US Class/CPC Symbol	**US Subclass/CPC Group**	**Date**	**Examiner**
	Sequence search - see SCV	06/05/2024	cc
	PE2E search – see attached	03/12/2025	cc

/CHEOM-GIL CHEONG/ Examiner, Art Unit 1645	

PE2E SEARCH - Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	British Equivalents	Time Stamp
L37	104279	(C07K16/2863 OR C07K16/468 OR A61K2039/505 OR C07K2317/30 OR C07K2317/55 OR C07K2317/569 OR C07K2317/622 OR C07K2317/92).cpc.	(US-PGPUB; USPAT; USOCR; FIT (WO))	ADJ	ON	ON	2025/03/12 07:39 PM
L38	4	(C07K16/2863 AND C07K16/468 AND A61K2039/505 AND C07K2317/30 AND C07K2317/55 AND C07K2317/569 AND C07K2317/622 AND C07K2317/92).cpc.	(US-PGPUB; USPAT; USOCR; FIT (WO))	ADJ	ON	ON	2025/03/12 07:39 PM
L39	40	(Artificial Intelligence) More like doc: WO-2019126398-A1 Text: (EP-3211011-A1 OR JP-2014527818-A OR NZ-626520-A OR WO-2015109212-A1 OR JP-6796059-B2 OR AU-2018393076-A1 OR CN-111655729-B OR WO-2020185960-A1 OR WO-2020206005-A1 OR US-20240150473-A1 OR KR-20240126877-A OR JP-2021506297-A OR AU-2018393073-A1 OR US-20200308287-A1 OR CA-3085782-A1 OR EP-3732201-A1 OR CN-111727203-A OR US-20240141049-A1 OR WO-2019126398-A1 OR JP-7604560-B2).did.	(US-PGPUB; USPAT; USOCR; FIT (AU, AP, AT, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, OA, RU, SU, WO, MC, MD, MY, NL, NO, NZ, PH, PL, PT, RO, RS, SE, SG, SI, SK, TH, TN, TR, TW, UA, VN); FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2025/03/12 07:43 PM
L40	19	((("SURROZEN") near3 ("OPERATING") near3 ("INC"))).AS,AANM.	(US-PGPUB; USPAT)	ADJ	ON	ON	2025/03/12 07:43 PM
L41	4661	((("LI") near3 ("Yang")) OR (("YUAN") near3 ("Tom")) OR (("SATO") near3 ("Aaron")) OR (("YEH") near3 ("Wen-Chen")) OR (("JANDA")	(US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT)	ADJ	ON	ON	2025/03/12 07:43 PM

		near3 ("Claudia")) OR (("FOWLER") near3 ("Tristan")) OR (("BARIBAULT") near3 ("Helene")) OR (("LAI") near3 ("Kuo-Pao")) OR (("XIE") near3 ("Liqin")) OR (("BREZSKI") near3 ("Randall")) OR (("LU") near3 ("Chenggang"))).INV.					
L42	64	((("LI") near3 ("Yang")) OR (("YUAN") near3 ("Tom")) OR (("SATO") near3 ("Aaron")) OR (("YEH") near3 ("Wen-Chen")) OR (("JANDA") near3 ("Claudia")) OR (("FOWLER") near3 ("Tristan")) OR (("BARIBAULT") near3 ("Helene")) OR (("LAI") near3 ("Kuo-Pao")) OR (("XIE") near3 ("Liqin")) OR (("BREZSKI") near3 ("Randall")) OR (("LU") near3 ("Chenggang"))).INV. AND (Fzd OR Frizzled) AND (LRP5 OR LRP6)	(US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT)	ADJ	ON	ON	2025/03/12 07:43 PM
L43	29	((("LI") near3 ("Yang")) OR (("YUAN") near3 ("Tom")) OR (("SATO") near3 ("Aaron")) OR (("YEH") near3 ("Wen-Chen")) OR (("JANDA") near3 ("Claudia")) OR (("FOWLER") near3 ("Tristan")) OR (("BARIBAULT") near3 ("Helene")) OR (("LAI") near3 ("Kuo-Pao")) OR (("XIE") near3 ("Liqin")) OR (("BREZSKI") near3 ("Randall")) OR (("LU") near3 ("Chenggang"))).INV. AND (Fzd OR Frizzled).clm. AND (LRP5 OR LRP6).clm.	(US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT)	ADJ	ON	ON	2025/03/12 07:45 PM
L44	19	(Artificial Intelligence) Similar to: 18/361,782 with 0 CPC Selections and 0 Text Selections Text: (WO-2019126398-A1	(US-PGPUB; USPAT; USOCR; FIT (WO))	ADJ	ON	ON	2025/03/12 07:45 PM

		OR AU-2018393073-A1 OR US-20200308287-A1 OR CA-3085782-A1 OR JP-2021506297-A OR CN-111727203-A OR US-20240141049-A1 OR CN-111727203-B OR US-11773171-B2 OR CN-112566940-A OR US-20210292422-A1 OR WO-2020010308-A1 OR JP-7418332-B2 OR JP-2024500839-A OR CA-3104526-A1 OR CN-111655729-B OR JP-2021529796-A OR WO-2022130341-A1 OR JP-2024001287-A OR JP-2023554500-A OR JP-2021508247-A OR CN-116964102-A OR KR-20230122077-A OR WO-2022130342-A1 OR CA-3140580-A1 OR CN-111655729-A OR CN-114423784-A OR US-20230118983-A1 OR CN-116917332-A OR JP-2022539212-A OR WO-2020250156-A1 OR US-20210087280-A1 OR US-20220315659-A1 OR JP-7377288-B2 OR AU-2020291208-A1 OR CA-3144499-A1 OR AU-2018393074-A1 OR CA-3085596-A1 OR WO-2019126399-A1 OR KR-20220024460-A OR CN-117946267-A OR US-20220275095-A1 OR WO-2023250402-A2 OR JP-2023515505-A OR AU-2020300618-A1 OR WO-2021003416-A1 OR US-11746150-B2 OR US-20230138045-A1 OR US-20160235844-A1 OR EP-2911691-B1).did.					
L45	372	Frizzled near11 receptor near55 LRP5 near55 LRP6	(US-PGPUB; USPAT; USOCR; FIT (WO))	ADJ	ON	ON	2025/03/12 07:45 PM
L46	232	Frizzled near11	(US-PGPUB; USPAT;	ADJ	ON	ON	2025/03/12

		receptor near55 LRP5 near55 LRP6 AND @ad<"20171219"	USOCR; FIT (WO))				07:45 PM
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L48	10	Frizzled near11 receptor near55 LRP5/6 AND tetravalent AND @ad<"20171219"	(US-PGPUB; USPAT; USOCR; FIT (WO))	ADJ	ON	ON	2025/03/12 07:51 PM
L49	8	Frizzled near11 receptor near55 LRP5/6 AND VHH AND tetravalent AND @ad<"20171219"	(US-PGPUB; USPAT; USOCR; FIT (WO))	ADJ	ON	ON	2025/03/12 07:51 PM
L50	0	Frizzled near11 receptor near55 LRP5/6 near55 bispecific AND VHH AND tetravalent AND @ad<"20171219"	(US-PGPUB; USPAT; USOCR; FIT (WO))	ADJ	ON	ON	2025/03/12 07:51 PM
L51	8	Frizzled near11 receptor near55 LRP5/6 AND bispecific AND VHH AND tetravalent AND @ad<"20171219"	(US-PGPUB; USPAT; USOCR; FIT (WO))	ADJ	ON	ON	2025/03/12 07:52 PM

PE2E SEARCH - Search History (Interference)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	British Equivalents	Time Stamp
N1	5	(Frizzled near11 receptor near55 LRP5 near55 LRP6).clm.	(US-PGPUB; USPAT)	ADJ	ON	ON	2025/03/12 08:01 PM
N2	1	(Frizzled near11 receptor near55 LRP5 near55 LRP6 AND tetravalent).clm.	(US-PGPUB; USPAT)	ADJ	ON	ON	2025/03/12 08:02 PM
N3	1	(Frizzled near11 receptor near55 LRP5/6 AND tetravalent).clm.	(US-PGPUB; USPAT)	ADJ	ON	ON	2025/03/12 08:04 PM
N4	1	(Frizzled near11 receptor near55 LRP5/6 AND VHH AND tetravalent).clm.	(US-PGPUB; USPAT)	ADJ	ON	ON	2025/03/12 08:06 PM
N5	0	(Frizzled near11 receptor near55 LRP5/6 near55 bispecific AND VHH AND tetravalent).clm.	(US-PGPUB; USPAT)	ADJ	ON	ON	2025/03/12 08:07 PM

Form used in lieu of PTO/SB/08a/b (07-09)
Approved for use through 05/31/2024. OMB 0651-0031
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 1 of 3

Complete if Known	
Application Number	18/361,782
Filing Date	07-28-2023
First Named Inventor	LI; Yang
Art Unit	1645
Examiner Name	CHEONG, CHEOM-GIL
Attorney Docket Number	SRZN-006/04US 328202-2203

U. S. PATENT DOCUMENTS

Examiner Initials*		Cite No.[1]	Document Number Number-Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
		001	US-20240368287	11-07-2024	LI; Yang et al.	
		002	US-20240391991	11-28-2024	FLETCHER; Russell et al.	

FOREIGN PATENT DOCUMENTS

Examiner Initials*		Cite No.[1]	Foreign Patent Document Country Code[3] Number[4] Kind Code[5] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages Or Relevant Figures Appear	T[6]
		003	WO-2020018445-A1	01-23-2020	UNIV LELAND STANFORD JUNIOR., et al.		☐
		004	WO-2024249444-A2	12-05-2024	SURROZEN OPERATING INC.		☐

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. [1] Applicant's unique citation designation number (optional). [2] See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. [3] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [6] Applicant is to place a check mark here if English language Translation is attached.

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /C.C./

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Approved for use through 05/31/2024. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 2 of 3

Complete if Known	
Application Number	18/361,782
Filing Date	07-28-2023
First Named Inventor	LI; Yang
Art Unit	1645
Examiner Name	CHEONG, CHEOM-GIL
Attorney Docket Number	SRZN-006/04US 328202-2203

NON-PATENT LITERATURE DOCUMENTS

Examiner Initials*		Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
		005	ACKERS, I., et al.; "Interrelationship of canonical and non-canonical Wnt signalling pathways in chronic metabolic diseases," Diab Vasc Dis Res.; 15(1):3-13 (2018).	☐
		006	CARMON et al. "R-spondins function as ligands of the orphan receptors LGR4 and LGR5 to regulate Wnt/ß-catenin signaling" Proceedings of the National Academy of Sciences (2011); 108(28):11452-11457.	☐
		007	CHAWLA, S., et al. "Establishment of in vitro model of corneal scar pathophysiology" J Cell Physiol. (2017); 1-14.	☐
		008	CHEN, H. et al. "Protocol to Generate and Characterize Potent and Selective WNT Mimetic Molecules" STAR Protocols (2020); 100043, 20 pages.	☐
		009	DANG et al. "Receptor subtype discrimination using extensive shape complementary designed interfaces" Nature Structural & Molecular Biology (2019); 26(6):407-414.	☐
		010	FLANAGAN et al. "Frizzled7 functions as a Wnt receptor in intestinal epithelial Lgr5+ stem cells" Stem Cell Reports (2015); 4(5):759-767.	☐
		011	GLINKA et al. "LGR4 and LGR5 are R-spondin receptors mediating Wnt/ß-catenin and Wnt/PCP signalling" EMBO Reports (2011); 12(10):1055-1061.	☐
		012	MAURIZI, E., et al.; "A fine-tuned ß-catenin regulation during proliferation of corneal endothelial cells revealed using proteomics analysis," Sci Rep.; 10(1):13841; pp. 1-16 (2020).	☐
		013	MIAO et al. "Next-generation surrogate Wnts support organoid growth and deconvolute frizzled pleiotropy in vivo" Cell Stem Cell (2020); 27(5):840-851.	☐
		014	NAKATSU, M.N., et al.; "Wnt/ß-catenin signaling regulates proliferation of human cornea epithelial stem/progenitor cells," Invest Ophthalmol Vis Sci.; 52(7):4734-4741 (2011).	☐

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. [1] Applicant's unique citation designation number (optional). [2] Applicant is to place a check mark here if English language Translation is attached.

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /C.C./

Form used in lieu of PTO/SB/08a/b (07-09)
Approved for use through 05/31/2024. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Substitute for form 1449/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 3 of 3

Complete if Known	
Application Number	18/361,782
Filing Date	07-28-2023
First Named Inventor	LI; Yang
Art Unit	1645
Examiner Name	CHEONG, CHEOM-GIL
Attorney Docket Number	SRZN-006/04US 328202-2203

NON-PATENT LITERATURE DOCUMENTS

Examiner Initials*		Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
		015	NUSSE et al. "Wnt/ß-catenin signaling, disease, and emerging therapeutic modalities" Cell (2017); 169(6):985-999.	☐
		016	POST, Y., et al.; "Design principles and therapeutic applications of novel synthetic WNT signaling agonists," iScience; 27(6):109938; pp. 1-18 (2024).	☐
		017	SATO et al. "Paneth cells constitute the niche for Lgr5 stem cells in intestinal crypts" Nature (2011); 469(7330):415-418.	☐
		018	VAN ES et al. "Wnt signalling induces maturation of Paneth cells in intestinal crypts" Nature Cell Biology (2005); 7(4):381-386.	☐
		019	WODARZ, A., et al.; "Mechanisms of Wnt signaling in development," Annu Rev Cell Dev Biol.; 14:59-88 (1998).	☐
		020	ZHANG, C., et al.; "A Small-Molecule Wnt Mimic Improves Human Limbal Stem Cell Ex Vivo Expansion," iScience; 23(5):101075; pp. 1 -23 (2020).	☐
		021	ZHANG, Y., et al.; "Wnt/ß-catenin signaling modulates corneal epithelium stratification via inhibition of Bmp4 during mouse development," Development; 142(19):3383-3393 (2015).	☐

Examiner Signature	/CHEOM-GIL CHEONG/	Date Considered	03/12/2025

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. [1] Applicant's unique citation designation number (optional). [2] Applicant is to place a check mark here if English language Translation is attached.

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /C.C./

Form used in lieu of PTO/SB/08a/b (07-09)
Approved for use through 05/31/2024. OMB 0651-0031
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Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Substitute for form 1449/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 1 of 2

Complete if Known	
Application Number	18/361,782
Filing Date	07-28-2023
First Named Inventor	LI; Yang
Art Unit	1645
Examiner Name	CHEONG, CHEOM-GIL
Attorney Docket Number	SRZN-006/04US 328202-2203

U. S. PATENT DOCUMENTS

Examiner Initials*		Cite No.[1]	Document Number / Number-Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
			None			

FOREIGN PATENT DOCUMENTS

Examiner Initials*		Cite No.[1]	Foreign Patent Document / Country Code[3] Number[4] Kind Code[5] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages Or Relevant Figures Appear	T[6]
			None				

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. [1] Applicant's unique citation designation number (optional). [2] See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. [3] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [6] Applicant is to place a check mark here if English language Translation is attached.

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /C.C./

Form used in lieu of PTO/SB/08a/b (07-09)
Approved for use through 05/31/2024. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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Substitute for form 1449/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 2 of 2

Complete if Known	
Application Number	18/361,782
Filing Date	07-28-2023
First Named Inventor	LI; Yang
Art Unit	1645
Examiner Name	CHEONG, CHEOM-GIL
Attorney Docket Number	SRZN-006/04US 328202-2203

NON-PATENT LITERATURE DOCUMENTS

Examiner Initials*		Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
		001	KOLUMAM et al. "Sustained brown fat stimulation and insulin sensitization by a humanized bispecific antibody agonist for fibroblast growth factor receptor 1/ßKlotho complex" EBioMedicine (2015); 2(7):730-743.	☐
		002	WU, Ai-Luen, et al. "Amelioration of type 2 diabetes by antibody-mediated activation of fibroblast growth factor receptor 1" Science Translational Medicine (2011); 3(113):113ra126, 11 pages.	☐

Examiner Signature	/CHEOM-GIL CHEONG/	Date Considered	03/12/2025

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. [1] Applicant's unique citation designation number (optional). [2] Applicant is to place a check mark here if English language Translation is attached.

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /C.C./

Bibliographic Data

Application No: 18/361,782

Foreign Priority claimed: ○ Yes ◉ No

35 USC 119 (a-d) conditions met: ☐ Yes ☑ No ☐ Met After Allowance

Verified and Acknowledged: /CHEOM-GIL CHEONG/ Examiner's Signature | Initials

Title: WNT SURROGATE MOLECULES AND USES THEREOF

FILING or 371(c) DATE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
07/28/2023	424	1645	SRZN-006/04US 328202-2203
RULE			

APPLICANTS

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CONTINUING DATA

This application is a CON of 16954484 06/16/2020 PAT 11773171

16954484 is a 371 of PCT/US2018/066616 12/19/2018

PCT/US2018/066616 has PRO of 62680522 06/04/2018

PCT/US2018/066616 has PRO of 62641217 03/09/2018

PCT/US2018/066616 has PRO of 62607875 12/19/2017

FOREIGN APPLICATIONS

IF REQUIRED, FOREIGN LICENSE GRANTED**

09/12/2023

STATE OR COUNTRY

UNITED STATES

ADDRESS

Cooley LLP / Surrozen Operating, Inc.
Attn: IP Docketing Department
1299 Pennsylvania Avenue, NW, Suite 700
Washington, DC 20004
UNITED STATES

FILING FEE RECEIVED

$5,140

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

NOTICE OF ALLOWANCE AND FEE(S) DUE

157070 7590 03/25/2025

Cooley LLP / Surrozen Operating, Inc.
Attn: IP Docketing Department
1299 Pennsylvania Avenue, NW, Suite 700
Washington, DC 20004

EXAMINER	
CHEONG, CHEOM-GIL	
ART UNIT	PAPER NUMBER
1645	

DATE MAILED: 03/25/2025

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
18/361,782	07/28/2023	Yang LI	SRZN-006/04US 328202-2203	9001

TITLE OF INVENTION: WNT SURROGATE MOLECULES AND USES THEREOF

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$1290	$0.00	$0.00	$1290	06/25/2025

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.

HOW TO REPLY TO THIS NOTICE:

I. Review the ENTITY STATUS shown above. If the ENTITY STATUS is shown as SMALL or MICRO, verify whether entitlement to that entity status still applies.

If the ENTITY STATUS is the same as shown above, pay the TOTAL FEE(S) DUE shown above.

If the ENTITY STATUS is changed from that shown above, on PART B - FEE(S) TRANSMITTAL, complete section number 5 titled "Change in Entity Status (from status indicated above)".

For purposes of this notice, small entity fees are 40% the amount of undiscounted fees, and micro entity fees are 20% the amount of undiscounted fees.

II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

IMPORTANT REMINDER: Maintenance fees are due in utility patents issuing on applications filed on or after Dec. 12, 1980. It is patentee's responsibility to ensure timely payment of maintenance fees when due. More information is available at www.uspto.gov/PatentMaintenanceFees.

PTOL-85 (Rev. 11/23)

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), by mail or fax, or via the USPTO patent electronic filing system.

By mail, send to: Mail Stop ISSUE FEE
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

By fax, send to: (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications. **Because electronic patent issuance may occur shortly after issue fee payment, any desired continuing application should preferably be filed prior to payment of this issue fee in order not to jeopardize copendency.**

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

157070 7590 03/25/2025
Cooley LLP / Surrozen Operating, Inc.
Attn: IP Docketing Department
1299 Pennsylvania Avenue, NW, Suite 700
Washington, DC 20004

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being transmitted to the USPTO via the USPTO patent electronic filing system or by facsimile to (571) 273-2885, on the date below.

(Typed or printed name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
18/361,782	07/28/2023	Yang LI	SRZN-006/04US 328202-2203	9001

TITLE OF INVENTION: WNT SURROGATE MOLECULES AND USES THEREOF

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$1290	$0.00	$0.00	$1290	06/25/2025

EXAMINER	ART UNIT	CLASS-SUBCLASS
CHEONG, CHEOM-GIL	1645	424-133100

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

❑ Change of correspondence address (or Change of Correspondence Address form PTO/AIA/122 or PTO/SB/122) attached.

❑ "Fee Address" indication (or "Fee Address" Indication form PTO/AIA/47 or PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list
(1) The names of up to 3 registered patent attorneys or agents OR, alternatively,
(2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1 _____
2 _____
3 _____

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document must have been previously recorded, or filed for recordation, as set forth in 37 CFR 3.11 and 37 CFR 3.81(a). Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE (B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent) : ❑ Individual ❑ Corporation or other private group entity ❑ Government

4a. Fees submitted: ❑ Issue Fee ❑ Publication Fee (if required)

4b. Method of Payment: *(Please first reapply any previously paid fee shown above)*

❑ Electronic Payment via the USPTO patent electronic filing system ❑ Enclosed check ❑ Non-electronic payment by credit card (Attach form PTO-2038)

❑ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment to Deposit Account No. _____

5. **Change in Entity Status** (from status indicated above)

❑ Applicant certifying micro entity status. See 37 CFR 1.29

NOTE: Absent a valid certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.

❑ Applicant asserting small entity status. See 37 CFR 1.27

NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.

❑ Applicant changing to regular undiscounted fee status.

NOTE: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature _____ Date _____

Typed or printed name _____ Registration No. _____

MS&D Exhibit 1008, Page 4208 of 4223

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
18/361,782	07/28/2023	Yang LI	SRZN-006/04US ~~328202-2203~~	9001

157070 7590 03/25/2025

Cooley LLP / Surrozen Operating, Inc.
Attn: IP Docketing Department
1299 Pennsylvania Avenue, NW, Suite 700
Washington, DC 20004

EXAMINER	
CHEONG, CHEOM-GIL	
ART UNIT	**PAPER NUMBER**
1645	

DATE MAILED: 03/25/2025

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(Applications filed on or after May 29, 2000)

The Office has discontinued providing a Patent Term Adjustment (PTA) calculation with the Notice of Allowance.

Section 1(h)(2) of the AIA Technical Corrections Act amended 35 U.S.C. 154(b)(3)(B)(i) to eliminate the requirement that the Office provide a patent term adjustment determination with the notice of allowance. See Revisions to Patent Term Adjustment, 78 Fed. Reg. 19416, 19417 (Apr. 1, 2013). Therefore, the Office is no longer providing an initial patent term adjustment determination with the notice of allowance. The Office will continue to provide a patent term adjustment determination with the Issue Notification Letter that is mailed to applicant approximately three weeks prior to the issue date of the patent, and will include the patent term adjustment on the patent. Any request for reconsideration of the patent term adjustment determination (or reinstatement of patent term adjustment) should follow the process outlined in 37 CFR 1.705.

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200.

OMB Clearance and PRA Burden Statement for PTOL-85 Part B

The Paperwork Reduction Act (PRA) of 1995 requires Federal agencies to obtain Office of Management and Budget approval before requesting most types of information from the public. When OMB approves an agency request to collect information from the public, OMB (i) provides a valid OMB Control Number and expiration date for the agency to display on the instrument that will be used to collect the information and (ii) requires the agency to inform the public about the OMB Control Number's legal significance in accordance with 5 CFR 1320.5(b).

The information collected by PTOL-85 Part B is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 30 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450. Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. The United States Patent and Trademark Office (USPTO) collects the information in this record under authority of 35 U.S.C. 2. The USPTO's system of records is used to manage all applicant and owner information including name, citizenship, residence, post office address, and other information with respect to inventors and their legal representatives pertaining to the applicant's/ owner's activities in connection with the invention for which a patent is sought or has been granted. The applicable Privacy Act System of Records Notice for the information collected in this form is COMMERCE/PAT-TM-7 Patent Application Files, available in the Federal Register at 78 FR 19243 (March 29, 2013).
https://www.govinfo.gov/content/pkg/FR-2013-03-29/pdf/2013-07341.pdf

Routine uses of the information in this record may include disclosure to:

1) law enforcement, in the event that the system of records indicates a violation or potential violation of law;
2) a federal, state, local, or international agency, in response to its request;
3) a contractor of the USPTO having need for the information in order to perform a contract;
4) the Department of Justice for determination of whether the Freedom of Information Act (FOIA) requires disclosure of the record;
5) a Member of Congress submitting a request involving an individual to whom the record pertains, when the individual has requested the Member's assistance with respect to the subject matter of the record;
6) a court, magistrate, or administrative tribunal, in the course of presenting evidence, including disclosures to opposing counsel in the course of settlement negotiations;
7) the Administrator, General Services Administration (GSA), or their designee, during an inspection of records conducted by GSA under authority of 44 U.S.C. 2904 and 2906, in accordance with the GSA regulations and any other relevant (i.e., GSA or Commerce) directive, where such disclosure shall not be used to make determinations about individuals;
8) another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c));
9) the Office of Personnel Management (OPM) for personnel research purposes; and
10) the Office of Management and Budget (OMB) for legislative coordination and clearance.

If you do not furnish the information requested on this form, the USPTO may not be able to process and/or examine your submission, which may result in termination of proceedings, abandonment of the application, and/or expiration of the patent.

Notice of Allowability	Application No. 18/361,782	Applicant(s) LI et al.	
	Examiner CHEOM-GIL CHEONG	Art Unit 1645	AIA (FITF) Status Yes

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☑ This communication is responsive to 2/6/2025.
   ☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2. ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

3. ☑ The allowed claim(s) is/are 1,15,17,55 and 71 . As a result of the allowed claim(s), you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information , please see **http://www.uspto.gov/patents/init_events/pph/index.jsp** or send an inquiry to **PPHfeedback@uspto.gov.**

4. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

   Certified copies:

   a) ☐ All b) ☐ Some* c) ☐ None of the:

   1. ☐ Certified copies of the priority documents have been received.
   2. ☐ Certified copies of the priority documents have been received in Application No. _____ .
   3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

   * Certified copies not received: _____ .

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
   ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____ .

   Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).

6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☐ Notice of References Cited (PTO-892)
2. ☑ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date _____.
3. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material _____.
4. ☑ Interview Summary (PTO-413), Paper No./Mail Date. _____.
5. ☑ Examiner's Amendment/Comment
6. ☐ Examiner's Statement of Reasons for Allowance
7. ☐ Other _____.

/CHEOM-GIL CHEONG/ Examiner, Art Unit 1645	/ANAND U DESAI/ Primary Examiner, Art Unit 1656

DETAILED ACTION

Notice of Pre-AIA or AIA Status

The present application, filed on or after March 16, 2013, is being examined under the first inventor to file provisions of the AIA.

Continued Examination Under 37 CFR 1.114

A request for continued examination under 37 CFR 1.114, including the fee set forth in 37 CFR 1.17(e), was filed in this application after final rejection. Since this application is eligible for continued examination under 37 CFR 1.114, and the fee set forth in 37 CFR 1.17(e) has been timely paid, the finality of the previous Office action has been withdrawn pursuant to 37 CFR 1.114. Applicant's submission filed on 2/6/2025 has been entered.

EXAMINER'S AMENDMENT

An examiner's amendment to the record appears below. Should the changes and/or additions be unacceptable to applicant, an amendment may be filed as provided by 37 CFR 1.312. To ensure consideration of such an amendment, it MUST be submitted no later than the payment of the issue fee.

Authorization for this examiner's amendment was given in an interview with Carol Laherty on 3/11/2025.

The claim has been amended as follows:

Claim 17. The Wnt surrogate molecule of ~~claim 1~~ claim 15, wherein the LRP5/6 binding region is fused to the N-terminus or the C-terminus of the heavy chain or is fused to the N-terminus or the C-terminus of the light chain.

Claim 71. The Wnt Surrogate molecule of ~~claim 1~~ claim 15, wherein each of the two LRP5/6 binding regions is fused to one of the light chains via a linker moiety.

Conclusion

Claims 1, 15, 17, 55 and 71 are allowed.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to CHEOM-GIL CHEONG whose telephone number is (571)272-6251. The examiner can normally be reached Monday - Friday 9:00 am - 5:00 pm.

Examiner interviews are available via telephone, in-person, and video conferencing using a USPTO supplied web-based collaboration tool. To schedule an interview, applicant is encouraged to use the USPTO Automated Interview Request (AIR) at http://www.uspto.gov/interviewpractice.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Gary Nickol can be reached on 571-272-0835. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of published or unpublished applications may be obtained from Patent Center. Unpublished application information in Patent Center is available to registered users. To file and manage patent submissions in Patent Center, visit: https://patentcenter.uspto.gov. Visit https://www.uspto.gov/patents/apply/patent-center for more information about Patent Center and https://www.uspto.gov/patents/docx for information about filing in DOCX format. For additional questions, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/CHEOM-GIL CHEONG/
Examiner, Art Unit 1645

/ANAND U DESAI/
Primary Examiner, Art Unit 1656
March 14, 2025

UNITED STATES
PATENT AND TRADEMARK OFFICE

P.O. Box 1450
Alexandria, VA 22313 - 1450
www.uspto.gov

ELECTRONIC ACKNOWLEDGEMENT RECEIPT

APPLICATION #	RECEIPT DATE / TIME	ATTORNEY DOCKET #
18/361,782	**03/27/2025 04:39:32 PM Z ET**	**SRZN-006/04US 328202-2203**

Title of Invention

WNT SURROGATE MOLECULES AND USES THEREOF

Application Information

APPLICATION TYPE	Utility - Nonprovisional Application under 35 USC 111(a)	PATENT #	-
CONFIRMATION #	9001	FILED BY	Susan Clingerman
PATENT CENTER #	69764803	FILING DATE	07/28/2023
CUSTOMER #	157070	FIRST NAMED INVENTOR	Yang LI
CORRESPONDENCE ADDRESS	-	AUTHORIZED BY	Carol Laherty

Documents

TOTAL DOCUMENTS: 1

DOCUMENT	PAGES	DESCRIPTION	SIZE (KB)
SRZN006_04US_ISSUE_FEE_TRANSMITTAL.pdf	1	Issue Fee Payment (PTO-85B)	287 KB

Digest

DOCUMENT	MESSAGE DIGEST(SHA-512)
SRZN006_04US_ISSUE_FEE_TRANSMITTAL.pdf	6436942FE0A6D7F8BC10F99BBC9C289BF037F0B1B212ECC961BC722AAF1A3711F470C465EE3A777752E0985273F4844B343A0458A7DC3C5537906BCDE5AEE9F4

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), by mail or fax, or via the USPTO patent electronic filing system.

By mail, send to: Mail Stop ISSUE FEE
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

By fax, send to: (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications. **Because electronic patent issuance may occur shortly after issue fee payment, any desired continuing application should preferably be filed prior to payment of this issue fee in order not to jeopardize copendency.**

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

157070 7590 03/25/2025
Cooley LLP / Surrozen Operating, Inc.
Attn: IP Docketing Department
1299 Pennsylvania Avenue, NW, Suite 700
Washington, DC 20004

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being transmitted to the USPTO via the USPTO patent electronic filing system or by facsimile to (571) 273-2885, on the date below.

(Typed or printed name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
18/361,782	07/28/2023	Yang LI	SRZN-006/04US 328202-2203	9001

TITLE OF INVENTION: WNT SURROGATE MOLECULES AND USES THEREOF

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$1290	$0.00	$0.00	$1290	06/25/2025

EXAMINER	ART UNIT	CLASS-SUBCLASS
CHEONG, CHEOM-GIL	1645	424-133100

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

❑ Change of correspondence address (or Change of Correspondence Address form PTO/AIA/122 or PTO/SB/122) attached.

❑ "Fee Address" indication (or "Fee Address" Indication form PTO/AIA/47 or PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list
(1) The names of up to 3 registered patent attorneys or agents OR, alternatively,
(2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1 Cooley LLP
2
3

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document must have been previously recorded, or filed for recordation, as set forth in 37 CFR 3.11 and 37 CFR 3.81(a). Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE: Surrozen Operating, Inc.

(B) RESIDENCE: (CITY and STATE OR COUNTRY): South San Francisco, California

Please check the appropriate assignee category or categories (will not be printed on the patent) : ❑ Individual ☒ Corporation or other private group entity ❑ Government

4a. Fees submitted: ☒ Issue Fee ❑ Publication Fee (if required)

4b. Method of Payment: *(Please first reapply any previously paid fee shown above)*

☒ Electronic Payment via the USPTO patent electronic filing system ❑ Enclosed check ❑ Non-electronic payment by credit card (Attach form PTO-2038)

☒ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment to Deposit Account No. 50-1283

5. **Change in Entity Status** (from status indicated above)

❑ Applicant certifying micro entity status. See 37 CFR 1.29

NOTE: Absent a valid certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.

❑ Applicant asserting small entity status. See 37 CFR 1.27

NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.

❑ Applicant changing to regular undiscounted fee status.

NOTE: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature /Carol D. Laherty/ Date March 27, 2025

Typed or printed name Carol D. Laherty Registration No. 51,909

PTOL-85 Part B (11/23) Approved for use through 03/31/2026 OMB 0651-0033 U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

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ELECTRONIC PAYMENT RECEIPT

APPLICATION #	RECEIPT DATE / TIME	ATTORNEY DOCKET #
18/361,782	**03/27/2025 04:39:32 PM Z ET**	**SRZN-006/04US 328202-2203**

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CUSTOMER #	157070	FILING DATE	07/28/2023
CORRESPONDENCE ADDRESS	-	FIRST NAMED INVENTOR	Yang LI

Payment Information

PAYMENT METHOD	PAYMENT TRANSACTION ID	PAYMENT AUTHORIZED BY
DA / 501283	**E20253QG40347619**	**Susan Clingerman**

FEE CODE	DESCRIPTION	ITEM PRICE($)	QUANTITY	ITEM TOTAL($)
1501	UTILITY ISSUE FEE	1290.00	1	1290.00
			TOTAL AMOUNT:	**$1,290.00**

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

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Alexandria, Virginia 22313-1450
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APPLICATION NO.	ISSUE DATE	PATENT NO.	ATTORNEY DOCKET NO.	CONFIRMATION NO.
18/361,782	05/13/2025	12297278	SRZN-006/04US 328202-2203	9001

157070 7590 04/30/2025

Cooley LLP / Surrozen Operating, Inc.
Attn: IP Docketing Department
1299 Pennsylvania Avenue, NW, Suite 700
Washington, DC 20004

ISSUE NOTIFICATION

The projected patent number and issue date are specified above. The patent will issue electronically. The electronically issued patent is the official patent grant pursuant to 35 U.S.C. § 153. The patent may be accessed on or after the issue date through Patent Center at https://patentcenter.uspto.gov/. The patent will be available in both the public and the private sides of Patent Center. Further assistance in electronically accessing the patent, or about Patent Center, is available by calling the Patent Electronic Business Center at 1-888-217-9197.

The USPTO is implementing electronic patent issuance with a transition period, during which period the USPTO will mail a ceremonial paper copy of the electronic patent grant to the correspondence address of record. Additional copies of the patent (i.e., certified and presentation copies) may be ordered for a fee from the USPTO's Certified Copy Center at https://certifiedcopycenter.uspto.gov/index.html. The Certified Copy Center may be reached at (800)972-6382.

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(application filed on or after May 29, 2000)

The Patent Term Adjustment is 0 day(s). Any patent to issue from the above-identified application will include an indication of the adjustment on the front page.

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Center (https://patentcenter.uspto.gov).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Application Assistance Unit (AAU) of the Office of Patents Stakeholder Experience (OPSE), Stakeholder Support Division (SSD) at (571)-272-4200.

INVENTOR(s) (Please see PATENT CENTER site https://patentcenter.uspto.gov for additional inventors):

Yang LI, Mountain View, CA;
Tom Zhiye YUAN, Union City, CA;
Aaron Ken SATO, Burlingame, CA;
Wen-Chen YEH, Belmont, CA;
Claudia Yvonne JANDA, Palo Alto, CA;

APPLICANT(s) (Please see PATENT CENTER site https://patentcenter.uspto.gov for additional applicants):

Surrozen Operating, Inc., South San Francisco, CA

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
18/361,782	07/28/2023	Yang LI	SRZN-006/04US 328202-2203	9001

157070 7590 05/13/2025

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Washington, DC 20004

EXAMINER	
CHEONG, CHEOM-GIL	

ART UNIT	PAPER NUMBER
1645	

NOTIFICATION DATE	DELIVERY MODE
05/13/2025	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

zIPPatentDocketingMailboxUS@cooley.com

APPLICATION NO.	ISSUE DATE	PATENT NO.
18/361,782	13-May-2025	12297278

Cooley LLP / Surrozen Operating, Inc.
Attn: IP Docketing Department
Washington, DC 20004

EGRANT NOTIFICATION

Your electronic patent grant (eGrant) is now available, which can be accessed via Patent Center at https://patentcenter.uspto.gov

The electronic patent grant is the official patent grant under 35 U.S.C. 153. For more information, please visit https://www.uspto.gov/electronicgrants