IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Appln. No:	To Be Assigned
Applicants:	Michael Francis et al.
Filed:	Herewith
Title:	ALIGNED FIBER AND METHOD OF USE THEREOF
T.C./A.U.:	To Be Assigned
Examiner:	To Be Assigned
Confirmation No.:	To Be Assigned
Docket No.:	LNH-152US1

PRELIMINARY AMENDMENT

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Commissioner:

Please amend the above-identified application as follows:

☒ **Amendments to the Specification** begin on page **2** of this paper.

☒ **Amendments to the Claims** are reflected in the listing of claims which begins on page **3** of this paper.

☐ **Amendments to the Drawings** begin on page of this paper and include an attached replacement sheet(s).

☐ **Amendments to the Abstract** are on page of this paper. A clean version of the Abstract is on page of this paper.

☒ **Remarks/Arguments** begin on page **5** of this paper.

Amendments to the Specification:

Please add the following new heading and paragraph after the Title on page 1:

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a Divisional Application of U.S. Application No. 14/776,106, filed September 14, 2015, which is a U.S. National Phase Application of PCT International Application PCT/US2014/025636, filed March 13, 2014, which claims priority benefit of U.S. Application No. 61/785,031, filed March 14, 2013, the disclosures of which are incorporated herein in their entirety.

Amendments to the Claims:

This list of claims replaces all prior versions and lists of claims in the application:

1.-24. (Canceled)

25. (Currently Amended) A method of treating a tissue defect comprising applying a scaffold comprising one or more electrospun fibers, wherein a fast Fourier transform (FFT) analysis result of the fibers have adjacent major peaks with about 180° apart from each other, and wherein the scaffold is in a form of one or more elongated sheets, ~~the scaffold according to claim 23~~ to surround the tissue defect.

26. (Currently Amended) A method of treating a tissue defect or gap comprising applying a scaffold comprising one or more electrospun fibers, wherein a fast Fourier transform (FFT) analysis result of the fibers have adjacent major peaks with about 180° apart from each other, and wherein the scaffold is in the form of one or more elongated rolls, ~~the scaffold according to claim 24~~ between the tissue defect or gap.

27. (Currently Amended) A method of cell culture comprising culturing cells on a scaffold comprising one or more electrospun fibers, wherein a fast Fourier transform (FFT) analysis result of the fibers have adjacent major peaks with about 180° apart from each other ~~the scaffold according to claim 1~~ in conditions suitable for the cell culture.

28. (Original) The method of claim 27, wherein the cells are selected from the group consisting of stem cells, adipose derived stem cells, dental pulp stem cells, fibroblasts, and dorsal root ganglia.

29. (Original) The method of claim 27, wherein the cells are dorsal root ganglia.

30. (Original) The method of claim 27, wherein the cells are dorsal root ganglia, and the fibers comprise heart basement membrane extract, heart basement membrane extracellular matrix, placenta basement membrane extract, or placenta basement membrane extracellular matrix.

31. (Currently Amended) A method of promoting differentiation of stem cells into osteoblasts, chondrocytes, ligament or tendon, the method comprising culturing the cells on a

scaffold comprising one or more electrospun fibers, wherein a fast Fourier transform (FFT) analysis result of the fibers have adjacent major peaks with about 180° apart from each other ~~the scaffold according to claim 1~~ in conditions suitable for the cell differentiation.

32. (Original) The method of claim 31, wherein the stem cells are progenitor cells or adult stem cells.

33. (Original) The method of claim 32, wherein the progenitor cells or the adult stem cells are derived or isolated from placenta, bone marrow, adipose tissue, blood vessel, amniotic fluid, synovial fluid, synovial membrane, pericardium, periosteum, dura, peripheral blood, umbilical blood, menstrual blood, baby teeth, nucleus pulposus, brain, skin, hair follicle, intestinal crypt, neural tissue, or muscle.

34. (Original) The method of claim 31, wherein the stem cells are pluripotent stem cells.

35. (Original) The method of claim 34, wherein the stem cells are selected from the group consisting of adipose derived stem cells and dental pulp stem cells.

Remarks/Arguments:

Claims 1-35 were pending. Claims 1-24 are canceled as being directed to subject matter elected and prosecuted to allowance in parent application Serial No. 14/776,106. Claims 25-27 and 31 are amended to be rewritten in independent form, incorporating the subject matter of their intervening claims, which are now canceled. No new matter has been added. Early examination on the merits and allowance are earnestly solicited.

Respectfully submitted,

William J. Matney, Jr., Reg. No. 39,292
Glenn E. J. Murphy, Reg. No. 33,539
Attorneys for Applicants

WJM/GEJM/nm

Dated: July 25, 2018

2200 Renaissance Blvd
Suite 350
King of Prussia, PA 19406
(610) 407-0700

The Director is hereby authorized to charge or credit Deposit Account No. **18-0350** for any additional fees, or any underpayment or credit for overpayment in connection herewith.

4071102

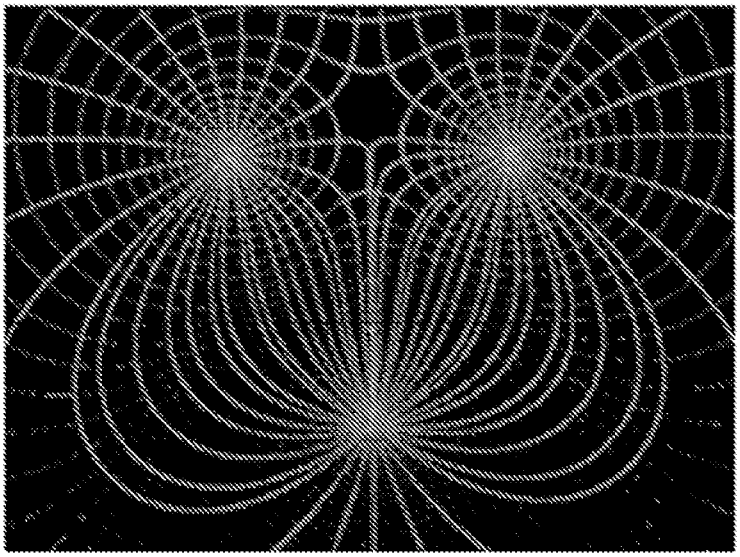


FIG. 1A

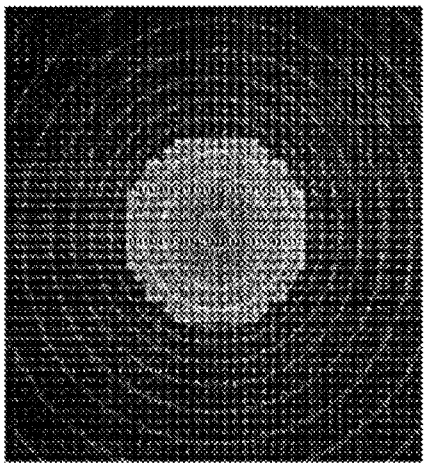


FIG. 1B

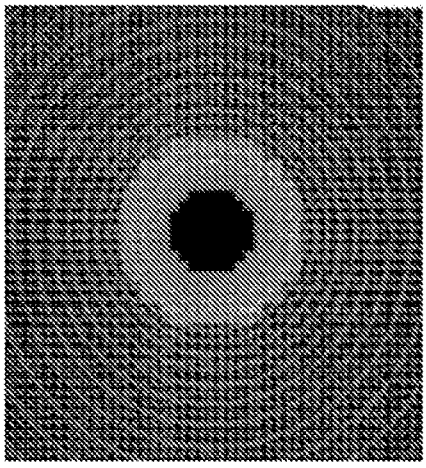


FIG. 1C

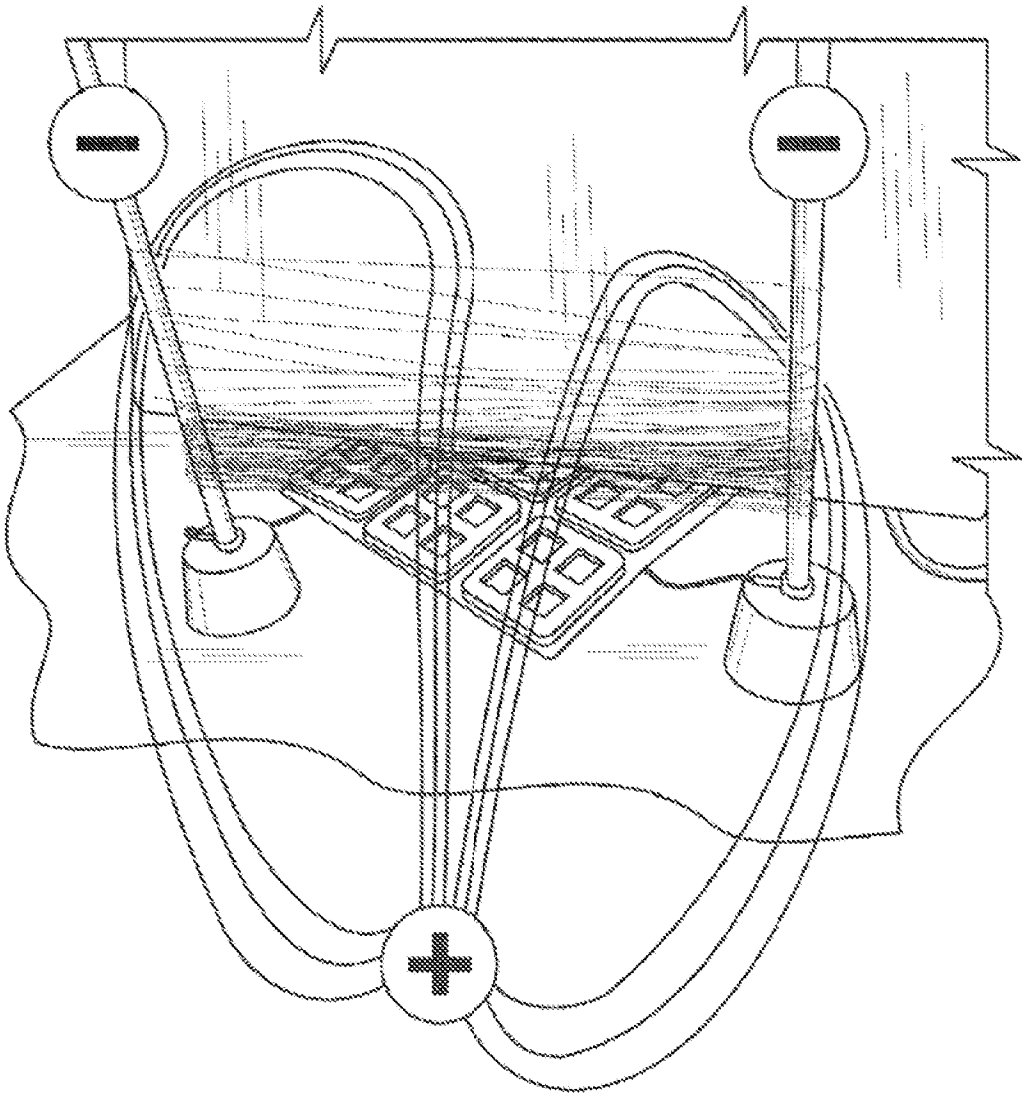
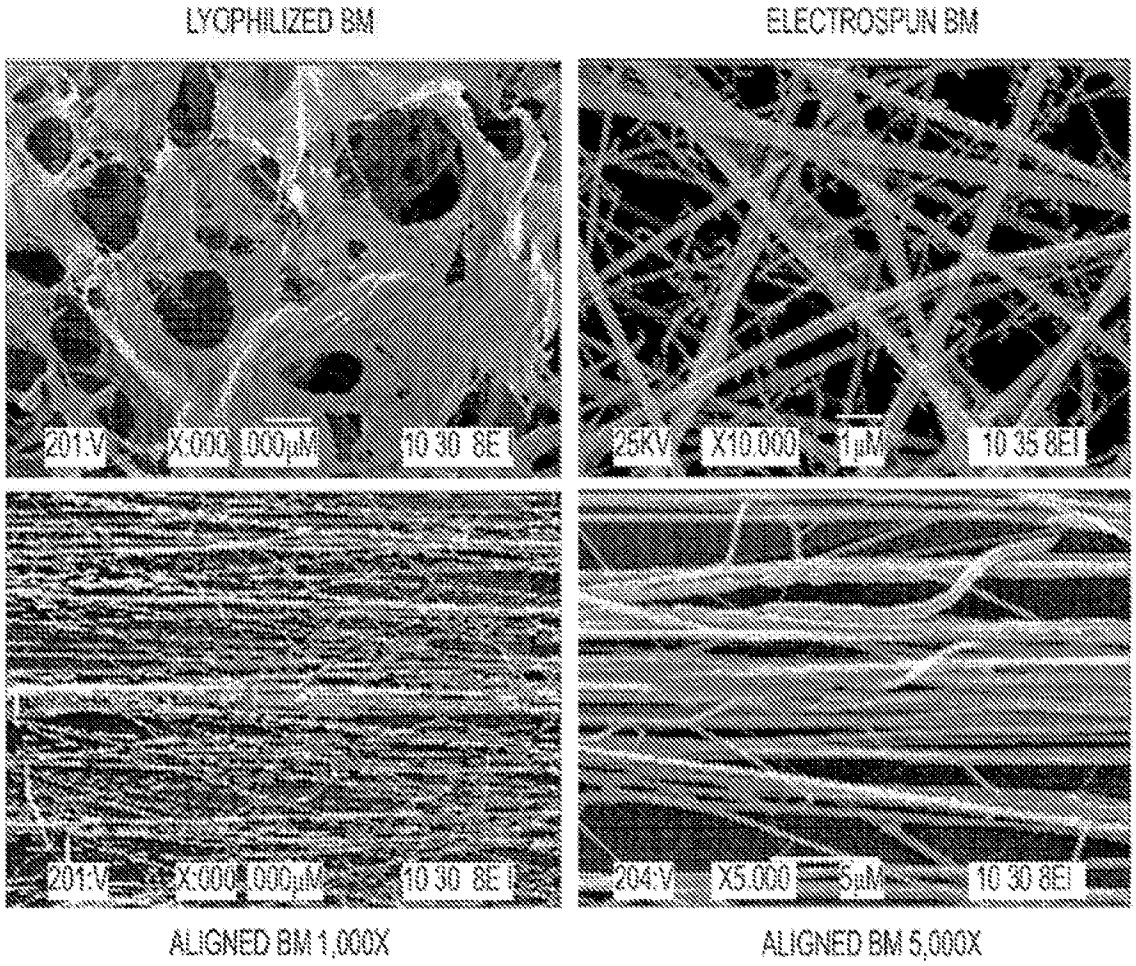


FIG. 1D



AVG DIAMETER (N=35, MEAN ± SD)	FIBER SIZE RANGE	AVG FIBER ANGLE (N=35, MEAN ± SD)	FIBER ANGLE RANGE
311 ± 71 NM	152-456 NM	92.340 ± 15.102 DEG	40-131 DEG

FIG. 2

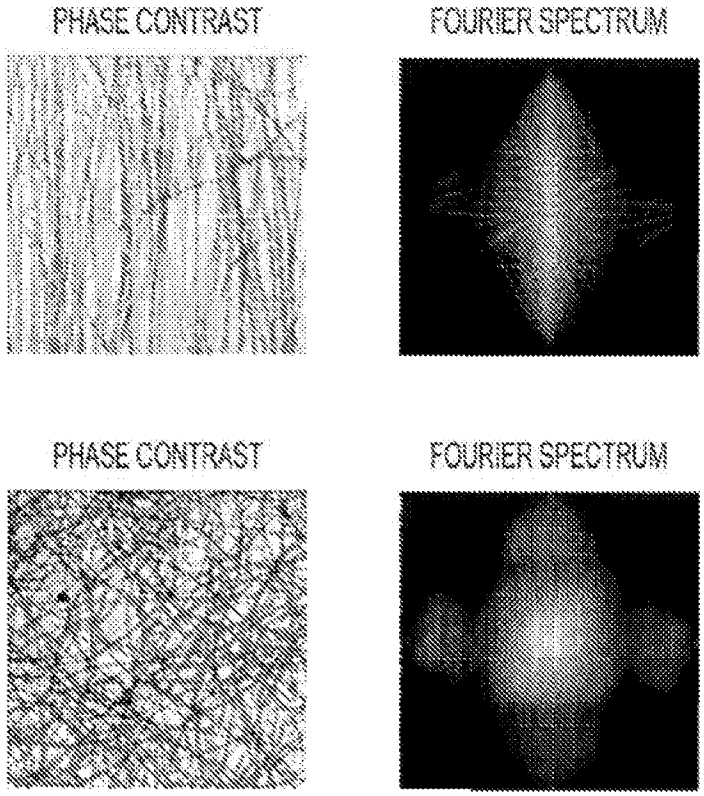

PHASE CONTRAST
FOURIER SPECTRUM
PHASE CONTRAST
FOURIER SPECTRUM

FIG. 3

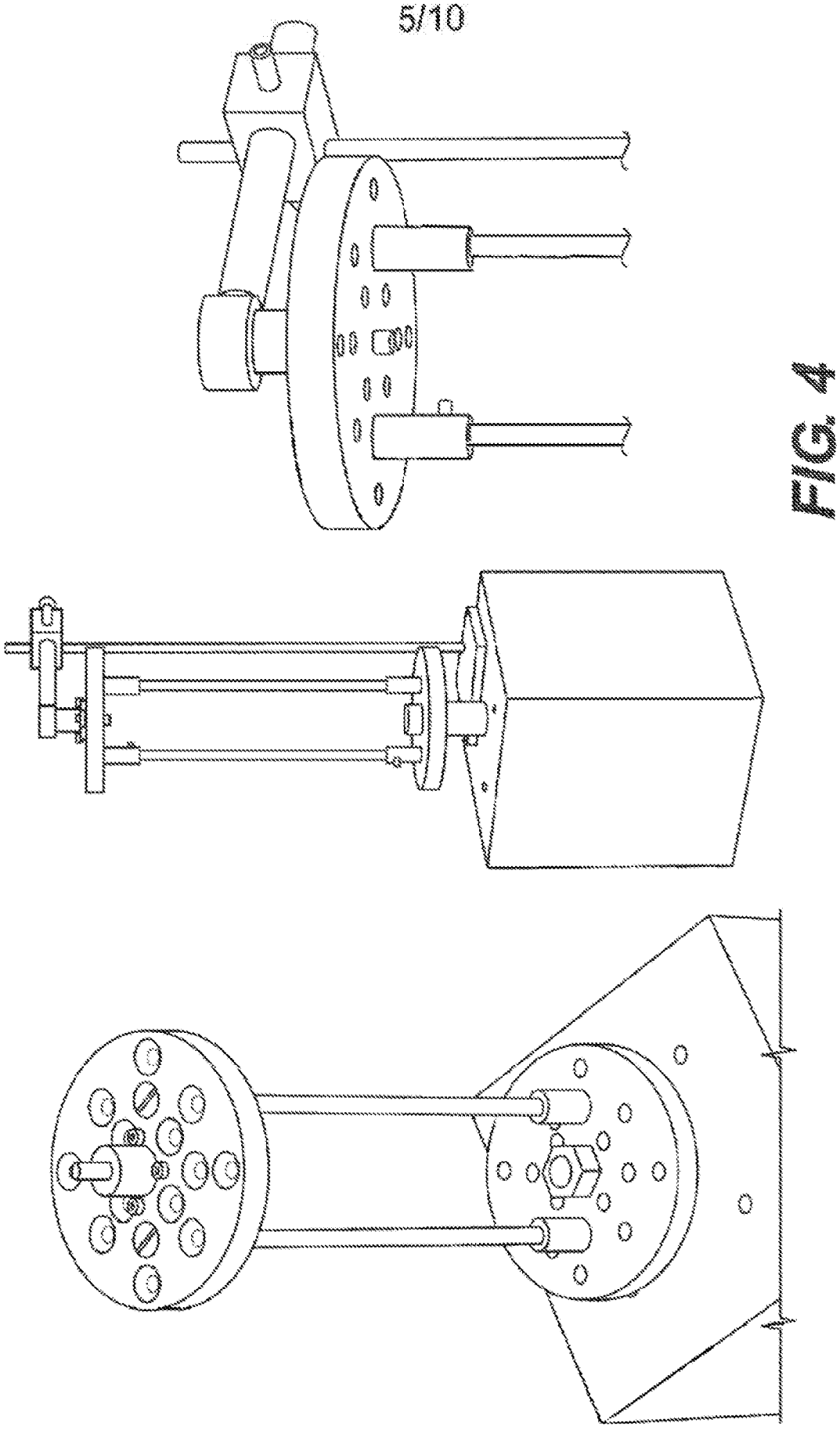


FIG. 4

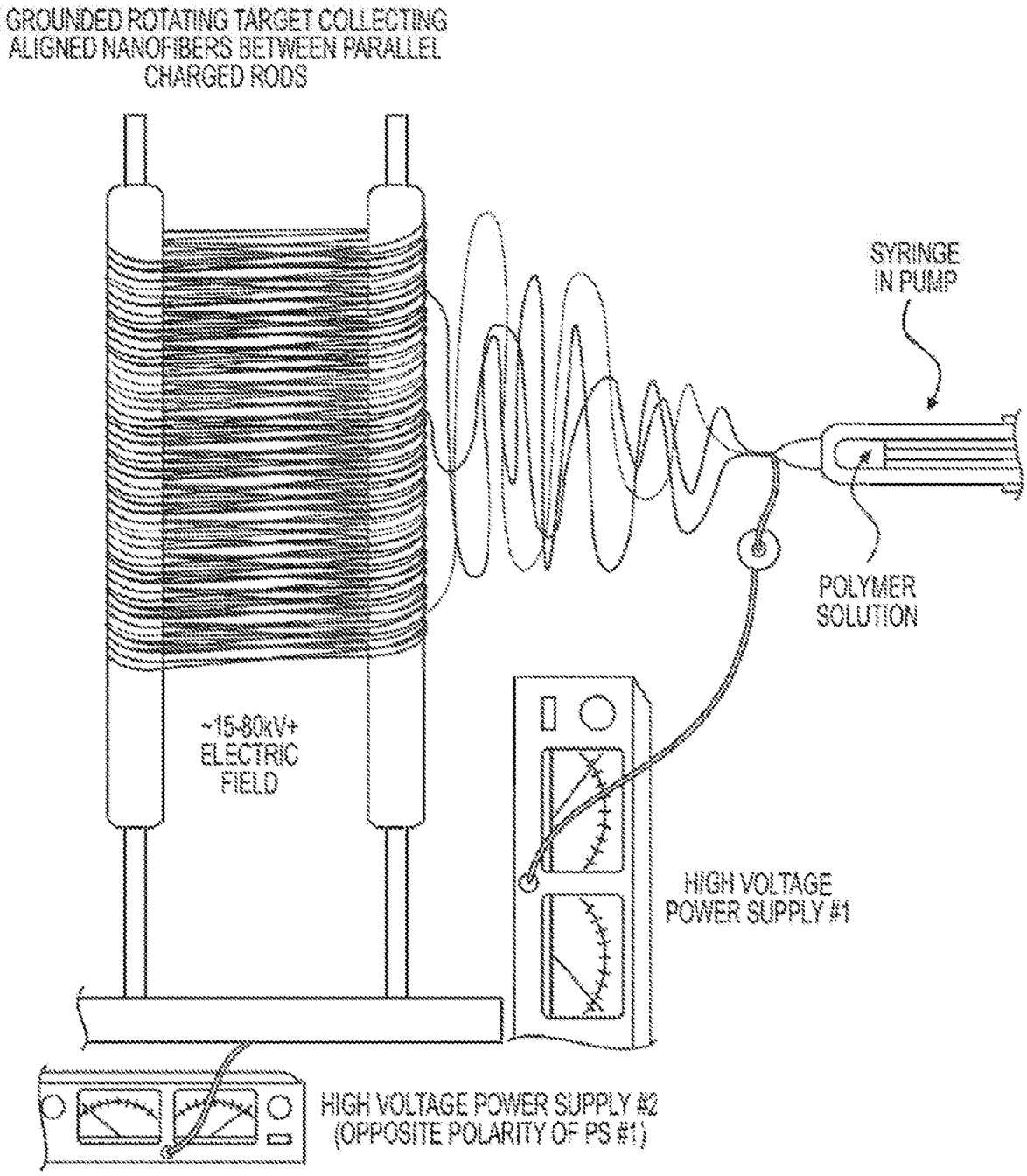
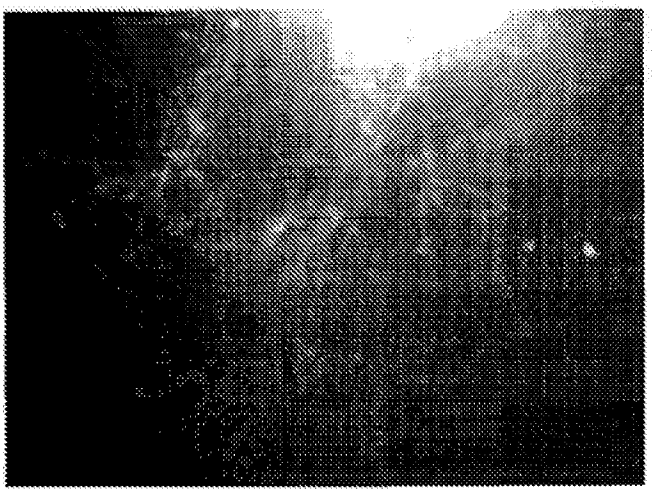

GROUNDED ROTATING TARGET COLLECTING ALIGNED NANOFIBERS BETWEEN PARALLEL CHARGED RODS
SYRINGE IN PUMP
POLYMER SOLUTION
~15-80kV+ ELECTRIC FIELD
HIGH VOLTAGE POWER SUPPLY #1
HIGH VOLTAGE POWER SUPPLY #2 (OPPOSITE POLARITY OF PS #1)

FIG. 5

DAY 2



DAY 4

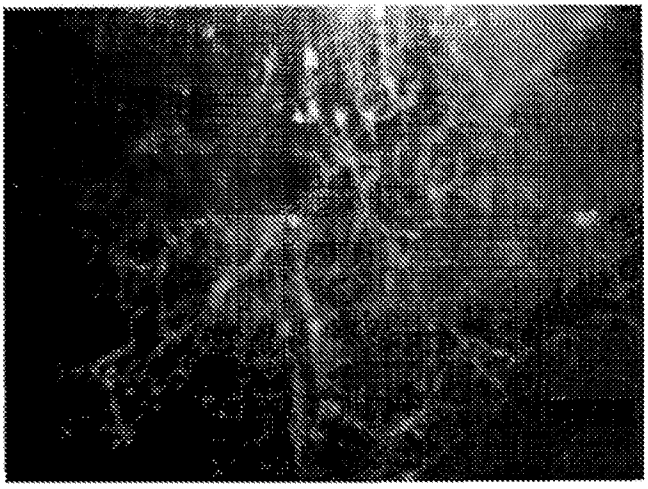


FIG. 6

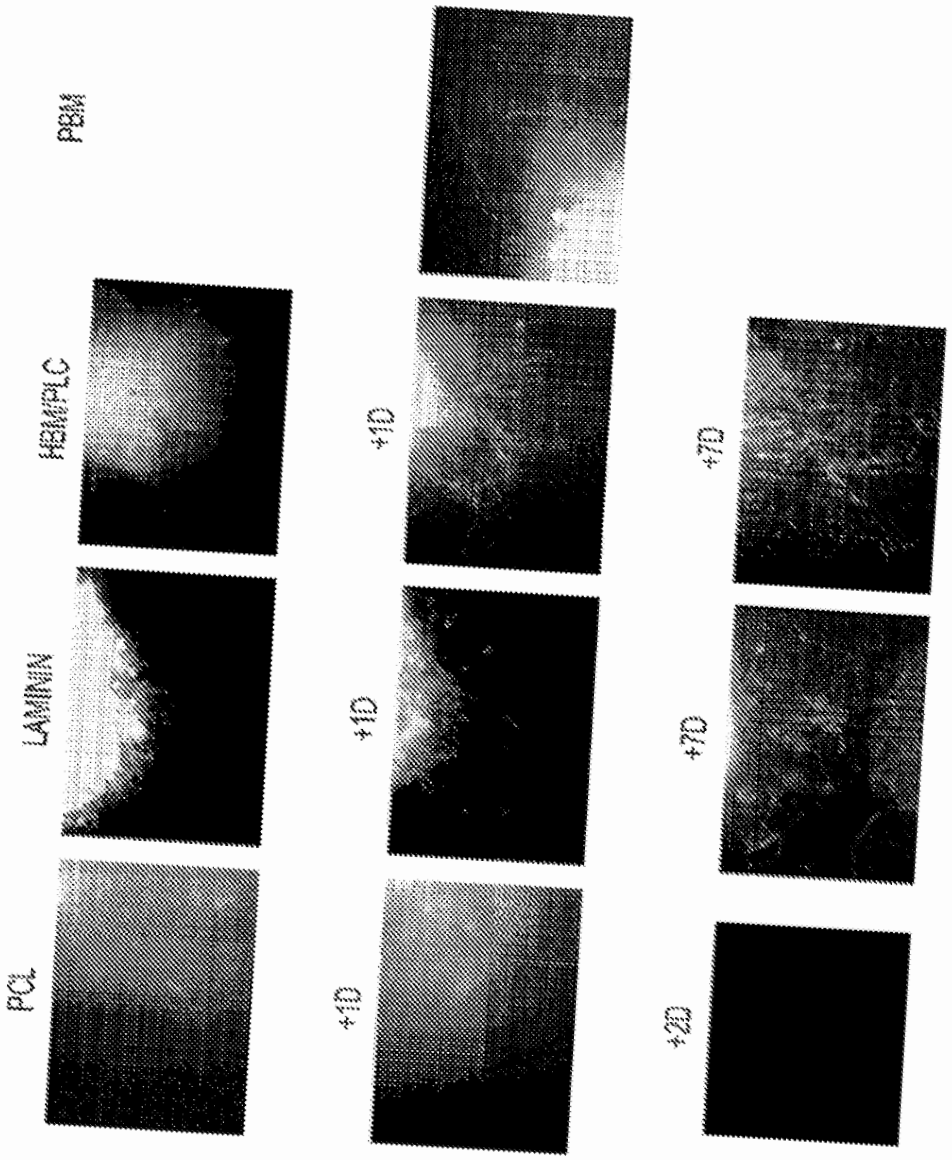


FIG. 7

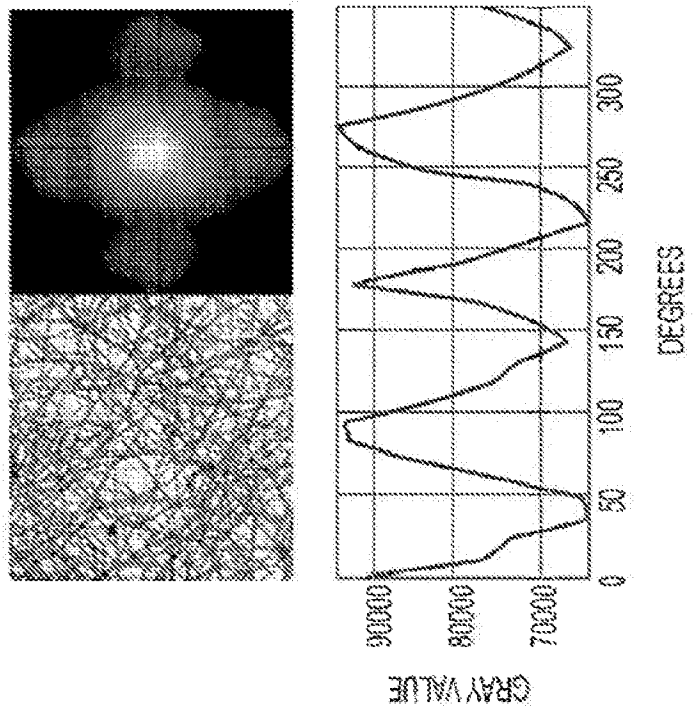

GRAY VALUE
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70000
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100
150
200
250
300
DEGREES

FIG. 8B

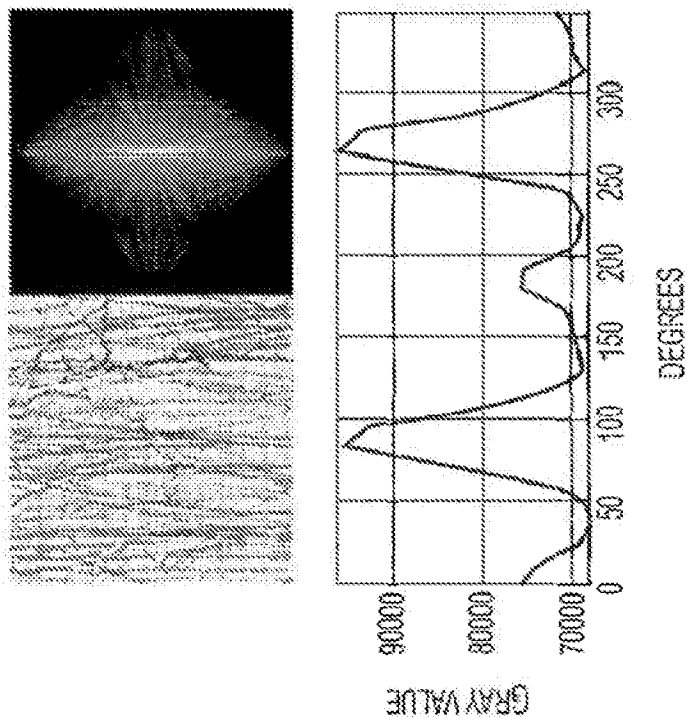

GRAY VALUE
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DEGREES

FIG. 8A

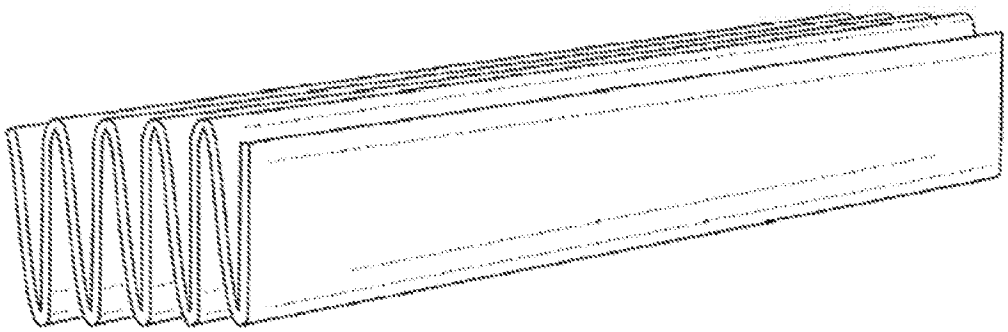


FIG. 9A

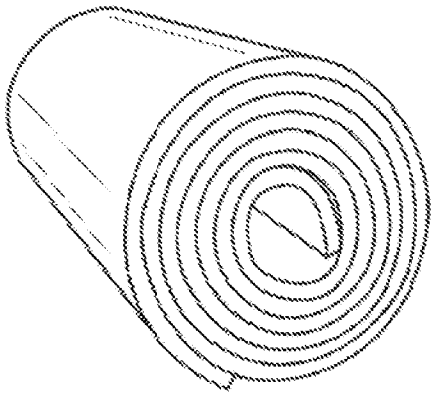


FIG. 9B

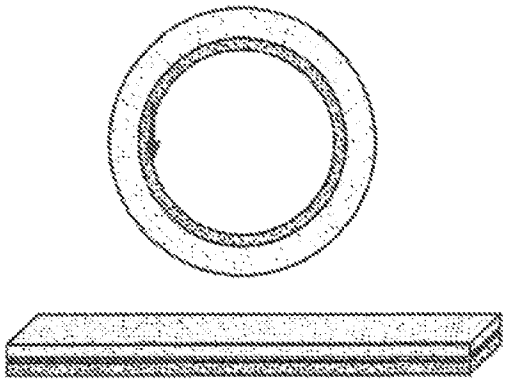


FIG. 9C

Electronic Patent Application Fee Transmittal

Application Number:	
Filing Date:	
Title of Invention:	ALIGNED FIBER AND METHOD OF USE THEREOF
First Named Inventor/Applicant Name:	Michael Francis
Filer:	Glenn E. Murphy/Nicole May
Attorney Docket Number:	LNH-152US1

Filed as Small Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
UTILITY FILING FEE (ELECTRONIC FILING)	4011	1	75	75
UTILITY SEARCH FEE	2111	1	330	330
UTILITY EXAMINATION FEE	2311	1	380	380
Pages:				
Claims:				
INDEPENDENT CLAIMS IN EXCESS OF 3	2201	1	230	230
Miscellaneous-Filing:				
Petition:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Miscellaneous:				
		Total in USD ($)		**1015**

Electronic Acknowledgement Receipt

EFS ID:	33279072
Application Number:	16045159
International Application Number:	
Confirmation Number:	5408
Title of Invention:	ALIGNED FIBER AND METHOD OF USE THEREOF
First Named Inventor/Applicant Name:	Michael Francis
Customer Number:	23122
Filer:	Glenn E. Murphy/Nicole May
Filer Authorized By:	Glenn E. Murphy
Attorney Docket Number:	LNH-152US1
Receipt Date:	25-JUL-2018
Filing Date:	
Time Stamp:	15:42:35
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	CARD
Payment was successfully received in RAM	$1015
RAM confirmation Number	072618INTEFSW15432700
Deposit Account	180350
Authorized User	Nicole May

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

37 CFR 1.17 (Patent application and reexamination processing fees)

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Application Data Sheet	ADS.pdf	447991 3845d4565d232815b920669929d2830b39d1a353	no	9
Warnings:					
Information:					
This is not an USPTO supplied ADS fillable form					
2	Oath or Declaration filed	DEC-Francis.pdf	732958 56a19308cd738cbfe54e8b6bc1531a18fdbceafa	no	2
Warnings:					
Information:					
3	Oath or Declaration filed	DEC-Ogle.pdf	483518 16684798095d05bf3952153454d11ea893ab09ff	no	2
Warnings:					
Information:					
4		Specification.pdf	16633291 2cc162ce10d158b0de84647c76e0b801db81dd77	yes	38

Multipart Description/PDF files in .zip description

Document Description	Start	End
Abstract	38	38
Claims	34	37
Specification	1	33

Warnings:

Information:

5	Preliminary Amendment	PreliminaryAmendment.pdf	654733 3a923ef39e5dbd7e32951cb93db2dd44baf15e9b	no	5
Warnings:					
Information:					
6	Drawings-only black and white line drawings	Drawings.pdf	2900907 98f58a5374e3b7ecccb163d6154cd019d21a5748	no	10
Warnings:					
Information:					
7	Fee Worksheet (SB06)	fee-info.pdf	36531 22bc979b739f75d8e5f8d481bc51800a7065f180	no	2
Warnings:					
Information:					
			Total Files Size (in bytes):	21889929	

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 a 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/AIA/14 (02-18)
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U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	LNH-152US1
	Application Number	
Title of Invention	ALIGNED FIBER AND METHOD OF USE THEREOF	

The application data sheet is part of the provisional or nonprovisional application for which it is being submitted. The following form contains the bibliographic data arranged in a format specified by the United States Patent and Trademark Office as outlined in 37 CFR 1.76.
This document may be completed electronically and submitted to the Office in electronic format using the Electronic Filing System (EFS) or the document may be printed and included in a paper filed application.

Secrecy Order 37 CFR 5.2:

☐ Portions or all of the application associated with this Application Data Sheet may fall under a Secrecy Order pursuant to 37 CFR 5.2 (Paper filers only. Applications that fall under Secrecy Order may not be filed electronically.)

Inventor Information:

Inventor 1 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Michael		Francis	

Residence Information (Select One) ◉ US Residency ○ Non US Residency ○ Active US Military Service

City	Virginia Beach	State/Province	VA	Country of Residence	US

Mailing Address of Inventor:

Address 1	1864 Concert Drive		
Address 2			
City	Virginia Beach	State/Province	VA
Postal Code	23453	Country	US

Inventor 2 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Roy		Ogle	

Residence Information (Select One) ◉ US Residency ○ Non US Residency ○ Active US Military Service

City	Virginia Beach	State/Province	VA	Country of Residence	US

Mailing Address of Inventor:

Address 1	1864 Concert Drive		
Address 2			
City	Virginia Beach	State/Province	VA
Postal Code	23453	Country	US

All Inventors Must Be Listed - Additional Inventor Information blocks may be generated within this form by selecting the **Add** button. Add

Correspondence Information:

Enter either Customer Number or complete the Correspondence Information section below.
For further information see 37 CFR 1.33(a).

PTO/AIA/14 (02-18)
Approved for use through 11/30/2020. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	LNH-152US1
	Application Number	
Title of Invention	ALIGNED FIBER AND METHOD OF USE THEREOF	

☐ An Address is being provided for the correspondence Information of this application.			
Customer Number	23122		
Email Address		Add Email	Remove Email

Application Information:

Title of the Invention	ALIGNED FIBER AND METHOD OF USE THEREOF		
Attorney Docket Number	LNH-152US1	**Small Entity Status Claimed**	☒
Application Type	Nonprovisional		
Subject Matter	Utility		
Total Number of Drawing Sheets (if any)	10	**Suggested Figure for Publication (if any)**	

Filing By Reference:

Only complete this section when filing an application by reference under 35 U.S.C. 111(c) and 37 CFR 1.57(a). Do not complete this section if application papers including a specification and any drawings are being filed. Any domestic benefit or foreign priority information must be provided in the appropriate section(s) below (i.e., "Domestic Benefit/National Stage Information" and "Foreign Priority Information").

For the purposes of a filing date under 37 CFR 1.53(b), the description and any drawings of the present application are replaced by this reference to the previously filed application, subject to conditions and requirements of 37 CFR 1.57(a).

Application number of the previously filed application	Filing date (YYYY-MM-DD)	Intellectual Property Authority or Country

Publication Information:

☐ Request Early Publication (Fee required at time of Request 37 CFR 1.219)

☐ **Request Not to Publish.** I hereby request that the attached application not be published under 35 U.S.C. 122(b) and certify that the invention disclosed in the attached application **has not and will not** be the subject of an application filed in another country, or under a multilateral international agreement, that requires publication at eighteen months after filing.

Representative Information:

Representative information should be provided for all practitioners having a power of attorney in the application. Providing this information in the Application Data Sheet does not constitute a power of attorney in the application (see 37 CFR 1.32). Either enter Customer Number or complete the Representative Name section below. If both sections are completed the customer Number will be used for the Representative Information during processing.

Please Select One:	◉ Customer Number	○ US Patent Practitioner	○ Limited Recognition (37 CFR 11.9)
Customer Number	23122		

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	LNH-152US1
	Application Number	
Title of Invention	ALIGNED FIBER AND METHOD OF USE THEREOF	

Domestic Benefit/National Stage Information:

This section allows for the applicant to either claim benefit under 35 U.S.C. 119(e), 120, 121, 365(c), or 386(c) or indicate National Stage entry from a PCT application. Providing benefit claim information in the Application Data Sheet constitutes the specific reference required by 35 U.S.C. 119(e) or 120, and 37 CFR 1.78.
When referring to the current application, please leave the "Application Number" field blank.

Prior Application Status			Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
	Division of	14776106	2015-09-14
Prior Application Status	Pending		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
14776106	a 371 of international	PCT/US2014/025636	2014-03-13
Prior Application Status	Expired		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
PCT/US2014/025636	Claims benefit of provisional	61785031	2013-03-14

Additional Domestic Benefit/National Stage Data may be generated within this form by selecting the **Add** button.

Foreign Priority Information:

This section allows for the applicant to claim priority to a foreign application. Providing this information in the application data sheet constitutes the claim for priority as required by 35 U.S.C. 119(b) and 37 CFR 1.55. When priority is claimed to a foreign application that is eligible for retrieval under the priority document exchange program (PDX)[i] the information will be used by the Office to automatically attempt retrieval pursuant to 37 CFR 1.55(i)(1) and (2). Under the PDX program, applicant bears the ultimate responsibility for ensuring that a copy of the foreign application is received by the Office from the participating foreign intellectual property office, or a certified copy of the foreign priority application is filed, within the time period specified in 37 CFR 1.55(g)(1).

			Remove
Application Number	Country[i]	Filing Date (YYYY-MM-DD)	Access Code[i] (if applicable)

Additional Foreign Priority Data may be generated within this form by selecting the **Add** button.

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications

PTO/AIA/14 (02-18)
Approved for use through 11/30/2020. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	LNH-152US1
	Application Number	
Title of Invention	ALIGNED FIBER AND METHOD OF USE THEREOF	

☐ This application (1) claims priority to or the benefit of an application filed before March 16, 2013 and (2) also contains, or contained at any time, a claim to a claimed invention that has an effective filing date on or after March 16, 2013.
NOTE: By providing this statement under 37 CFR 1.55 or 1.78, this application, with a filing date on or after March 16, 2013, will be examined under the first inventor to file provisions of the AIA.

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	LNH-152US1
	Application Number	
Title of Invention	ALIGNED FIBER AND METHOD OF USE THEREOF	

Authorization or Opt-Out of Authorization to Permit Access:

When this Application Data Sheet is properly signed and filed with the application, applicant has provided written authority to permit a participating foreign intellectual property (IP) office access to the instant application-as-filed (see paragraph A in subsection 1 below) and the European Patent Office (EPO) access to any search results from the instant application (see paragraph B in subsection 1 below).

Should applicant choose not to provide an authorization identified in subsection 1 below, applicant **must opt-out** of the authorization by checking the corresponding box A or B or both in subsection 2 below.

NOTE: This section of the Application Data Sheet is **ONLY** reviewed and processed with the **INITIAL** filing of an application. After the initial filing of an application, an Application Data Sheet cannot be used to provide or rescind authorization for access by a foreign IP office(s). Instead, Form PTO/SB/39 or PTO/SB/69 must be used as appropriate.

1. Authorization to Permit Access by a Foreign Intellectual Property Office(s)

A. Priority Document Exchange (PDX) - Unless box A in subsection 2 (opt-out of authorization) is checked, the undersigned hereby **grants the USPTO authority** to provide the European Patent Office (EPO), the Japan Patent Office (JPO), the Korean Intellectual Property Office (KIPO), the State Intellectual Property Office of the People's Republic of China (SIPO), the World Intellectual Property Organization (WIPO), and any other foreign intellectual property office participating with the USPTO in a bilateral or multilateral priority document exchange agreement in which a foreign application claiming priority to the instant patent application is filed, access to: (1) the instant patent application-as-filed and its related bibliographic data, (2) any foreign or domestic application to which priority or benefit is claimed by the instant application and its related bibliographic data, and (3) the date of filing of this Authorization. See 37 CFR 1.14(h)(1).

B. Search Results from U.S. Application to EPO - Unless box B in subsection 2 (opt-out of authorization) is checked, the undersigned hereby **grants the USPTO authority** to provide the EPO access to the bibliographic data and search results from the instant patent application when a European patent application claiming priority to the instant patent application is filed. See 37 CFR 1.14(h)(2).

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	LNH-152US1
	Application Number	
Title of Invention	ALIGNED FIBER AND METHOD OF USE THEREOF	

Applicant Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

Applicant 1

If the applicant is the inventor (or the remaining joint inventor or inventors under 37 CFR 1.45), this section should not be completed. The information to be provided in this section is the name and address of the legal representative who is the applicant under 37 CFR 1.43; or the name and address of the assignee, person to whom the inventor is under an obligation to assign the invention, or person who otherwise shows sufficient proprietary interest in the matter who is the applicant under 37 CFR 1.46. If the applicant is an applicant under 37 CFR 1.46 (assignee, person to whom the inventor is obligated to assign, or person who otherwise shows sufficient proprietary interest) together with one or more joint inventors, then the joint inventor or inventors who are also the applicant should be identified in this section.

Clear

◉ Assignee	○ Legal Representative under 35 U.S.C. 117	○ Joint Inventor
○ Person to whom the inventor is obligated to assign.	○ Person who shows sufficient proprietary interest	

If applicant is the legal representative, indicate the authority to file the patent application, the inventor is:

Name of the Deceased or Legally Incapacitated Inventor:

If the Applicant is an Organization check here. ☒

Organization Name	LifeNet Health		
Mailing Address Information For Applicant:			
Address 1	1864 Concert Drive		
Address 2			
City	Virginia Beach	**State/Province**	VA
Country	US	Postal Code	23453
Phone Number		Fax Number	
Email Address			

Additional Applicant Data may be generated within this form by selecting the Add button.

Assignee Information including Non-Applicant Assignee Information:

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	LNH-152US1
	Application Number	
Title of Invention	ALIGNED FIBER AND METHOD OF USE THEREOF	

Assignee 1

Complete this section if assignee information, including non-applicant assignee information, is desired to be included on the patent application publication. An assignee-applicant identified in the "Applicant Information" section will appear on the patent application publication as an applicant. For an assignee-applicant, complete this section only if identification as an assignee is also desired on the patent application publication.

If the Assignee or Non-Applicant Assignee is an Organization check here. ☐

Prefix	**Given Name**	Middle Name	**Family Name**	Suffix

Mailing Address Information For Assignee including Non-Applicant Assignee:

Address 1			
Address 2			
City		**State/Province**	
Country i		Postal Code	
Phone Number		Fax Number	
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Signature:

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This Application Data Sheet **must** be signed by a patent practitioner if one or more of the applicants is a **juristic entity** (e.g., corporation or association). If the applicant is two or more joint inventors, this form must be signed by a patent practitioner, **all** joint inventors who are the applicant, or one or more joint inventor-applicants who have been given power of attorney (e.g., see USPTO Form PTO/AIA/81) on behalf of **all** joint inventor-applicants.

See 37 CFR 1.4(d) for the manner of making signatures and certifications.

Signature				Date (YYYY-MM-DD)	2018-07-25
First Name	Glenn E. J.	Last Name	Murphy	Registration Number	33539

Additional Signature may be generated within this form by selecting the Add button.

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	LNH-152US1
	Application Number	
Title of Invention	ALIGNED FIBER AND METHOD OF USE THEREOF	

This collection of information is required by 37 CFR 1.76. 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 23 minutes to complete, including gathering, preparing, and submitting the completed application data sheet 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.**

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3 A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

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7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

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COMBINED DECLARATION/ASSIGNMENT - Patent Application	Docket Number: LNH-152US

Whereas, the Assignor, comprising the following named inventors:

ASSIGNOR(s)/ INVENTOR(s)	1. Michael Francis	2. Roy Ogle
	3.	4.
	5.	6.

made an invention entitled: ALIGNED FIBER AND METHOD OF USE THEREOF

for which an application for Letters Patent of the United States (the Application):
(Select one of the following):

☐ is submitted concurrently herewith
☒ was filed in the U.S. Patent and Trademark Office on 09/14/2015,
as U.S. Patent Application Serial No.: 14/776,106

ASSIGNEE (Full Name and Address)	LifeNet Health 1864 Concert Drive Virginia Beach, VA 23453

is desirous of acquiring the entire interest in and to said invention and the Letters Patent to be obtained therefor,

Now, therefore, in consideration of the payment by Assignee to Assignor of a sum corresponding to One Dollar ($1.00), and for other good and valuable consideration, the receipt of which is hereby acknowledged, Assignor, intending to be legally bound, hereby sells, assigns and transfers to Assignee, its successors and assigns, the full and exclusive right, title and interest in and to said invention, all applications for Letters Patent for said invention, including the Application, all divisions, continuations, and continuations-in-part thereof and non-provisional applications claiming priority thereto, all rights to claim priority based thereon, and all Letters Patent, including reissues, to be obtained therefor, including any and all foreign patent rights in this invention corresponding thereto and authorizes Assignee to file such applications for Letters Patent in the United States and any and all other countries.

Assignor hereby warrants that no assignment, sale, agreement or encumbrance has been or will be made or entered into which would conflict with this Assignment.

Assignor agrees it shall be legally bound, upon request of the Assignee or its successors or assigns or a legal representative thereof, to supply all information and evidence of which the Assignor has knowledge or possession, relating to the making and practice of said invention, to testify in any legal proceeding relating thereto, to execute all instruments proper to patent the invention in the United States of America and foreign countries in the name of the Assignee, and to execute all instruments proper to carry out the intent of this instrument.

If the invention requires a biological deposit, Assignor also grants to Assignee such control over any deposit made by Assignor as may be necessary to the validity of the patent rights assigned herein.

Assignor authorizes Assignee's attorney to insert the serial number and filing date of the aforesaid application for United States Letters Patent and/or the Attorney Docket or file designation for this application if not identified above.

If the Assignor includes more than one individual, these obligations shall apply to these individuals both individually and collectively.

In witness whereof, this Assignment is executed on the day indicated below.

COMBINED DECLARATION/ASSIGNMENT - Patent Application	Docket Number: LNH-152US

Declaration:

Note to Inventor: 37 C.F.R. § 1.63(c) states: "A person may not execute an oath or declaration for an application unless that person has reviewed and understands the contents of the application, including the claims, and is aware of the duty to disclose to the Office all information known to the person to be material to patentability as defined in § 1.56."

As a below named inventor, I declare that the Application was made or authorized to be made by me.

I believe I am the original inventor or an original joint inventor of a claimed invention in the application.

I hereby acknowledge that any willful false statement made in this declaration is punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.

	Typed or Printed Name	Signature	Date
SIGNATURE:	1. Michael Francis		9.6.16
		* Witness	
	2. Roy Ogle		
		* Witness	
	3.		
		* Witness	
	4		
		* Witness	
	5.		
		*Witness	
	6		
		* Witness	

Application for United States Letters Patent	
Serial No.:	14/776,109
Filed:	09/14/2015
Attorney Docket Number:	LNH-152US
Total Number of Assignment Pages	2

* Witness signature recommended, mandatory for some countries

COMBINED DECLARATION/ASSIGNMENT - Patent Application	Docket Number: LNH-152US

Whereas, the Assignor, comprising the following named inventors:

ASSIGNOR(s)/ INVENTOR(s)	1. Michael Francis	2. Roy Ogle
	3.	4.
	5.	6.

made an invention entitled: ALIGNED FIBER AND METHOD OF USE THEREOF

for which an application for Letters Patent of the United States (the Application):
(Select one of the following):

☐ is submitted concurrently herewith
☑ was filed in the U.S. Patent and Trademark Office on 09/14/2015,
as U.S. Patent Application Serial No.: 14/776,106

ASSIGNEE (Full Name and Address)	LifeNet Health 1864 Concert Drive Virginia Beach, VA 23453

is desirous of acquiring the entire interest in and to said invention and the Letters Patent to be obtained therefor.

Now, therefore, in consideration of the payment by Assignee to Assignor of a sum corresponding to One Dollar ($1.00), and for other good and valuable consideration, the receipt of which is hereby acknowledged, Assignor, intending to be legally bound, hereby sells, assigns and transfers to Assignee, its successors and assigns, the full and exclusive right, title and interest in and to said invention, all applications for Letters Patent for said invention, including the Application, all divisions, continuations, and continuations-in-part thereof and non-provisional applications claiming priority thereto, all rights to claim priority based thereon, and all Letters Patent, including reissues, to be obtained therefor, including any and all foreign patent rights in this invention corresponding thereto and authorizes Assignee to file such applications for Letters Patent in the United States and any and all other countries.

Assignor hereby warrants that no assignment, sale, agreement or encumbrance has been or will be made or entered into which would conflict with this Assignment.

Assignor agrees it shall be legally bound, upon request of the Assignee or its successors or assigns or a legal representative thereof, to supply all information and evidence of which the Assignor has knowledge or possession, relating to the making and practice of said invention, to testify in any legal proceeding relating thereto, to execute all instruments proper to patent the invention in the United States of America and foreign countries in the name of the Assignee, and to execute all instruments proper to carry out the intent of this instrument.

If the invention requires a biological deposit, Assignor also grants to Assignee such control over any deposit made by Assignor as may be necessary to the validity of the patent rights assigned herein.

Assignor authorizes Assignee's attorney to insert the serial number and filing date of the aforesaid application for United States Letters Patent and/or the Attorney Docket or file designation for this application if not identified above.

If the Assignor includes more than one individual, these obligations shall apply to these individuals both individually and collectively.

In witness whereof, this Assignment is executed on the day indicated below.

COMBINED DECLARATION/ASSIGNMENT - Patent Application	Docket Number: LNH-152US

Declaration:

Note to Inventor: 37 C.F.R. § 1.63(c) states: "A person may not execute an oath or declaration for an application unless that person has reviewed and understands the contents of the application, including the claims, and is aware of the duty to disclose to the Office all information known to the person to be material to patentability as defined in § 1.56."

As a below-named inventor, I declare that the Application was made or authorized to be made by me.

I believe I am the original inventor or an original joint inventor of a claimed invention in the application.

I hereby acknowledge that any willful false statement made in this declaration is punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.

	Typed or Printed Name	Signature	Date
SIGNATURE	1. Michael Francis		
		* Witness	
	2. Roy Ogle	Roy Ogle	10-5-16
		* Witness	
	3.		
		* Witness	
	4.		
		* Witness	
	5.		
		* Witness	
	6		
		* Witness	

Application for United States Letters Patent:	
Serial No.:	14/776,106
Filed:	09/14/2015
Attorney Docket Number:	LNH-152US
Total Number of Assignment Pages	2

* Witness signature recommended, mandatory for some countries

Abstract

A scaffold comprising an aligned fiber. Further, a scaffold comprising one or more electrospun fibers wherein a fast Fourier transform (FFT) analysis result of the fibers have adjacent major peaks with about 180° apart from each other. Also, methods for promoting differentiation of stem cells into osteoblasts, chondrocytes, ligament or tendon, the method comprising culturing the cells on the scaffold or aligned fiber in conditions suitable for the cell differentiation.

CLAIMS

1. A scaffold comprising one or more electrospun fibers, wherein a fast Fourier transform (FFT) analysis result of the fibers have adjacent major peaks with about 180° apart from each other.

2. The scaffold according to claim 1, wherein the scaffold essentially consists of the electrospun fibers.

3. The scaffold according to claim 1, wherein the scaffold consists of the electrospun fibers.

4. The scaffold according to any of claims 1-3, wherein the scaffold is biocompatible.

5. The scaffold according to any of claims 1-4, wherein the fiber comprise type I collagen in its native triple helical structure.

6. The scaffold according to any of claims 1-5, wherein the fiber has less than about 8 wt% residual calcium.

7. The scaffold according to any of claims 1-6, wherein the fiber has an average diameter from about 5 nm to about 1000 nm.

8. The scaffold according to any of claims 1-7, wherein the fiber has an average diameter of less than about 100 nm.

9. The scaffold according to any of claims 1-8, wherein the fiber has an average length from about 1 cm to about 50 cm.

10. The scaffold according to any of claims 1-9, wherein the fiber has an average length from about 10 cm to about 50 cm.

11. The scaffold according to any of claims 1-10, wherein the scaffold has an average porosity from about 60% to about 90%.

12. The scaffold according to any of claims 1-11, wherein the scaffold comprises collagen type I, bone matrix, adipose extracellular matrix, heart basement membrane extract, heart basement membrane extracellular matrix, placenta basement membrane extract, placenta basement membrane extracellular matrix, brain-derived extracellular matrix, polycaprolactone, an accessory polymer, or the mixture thereof.

13. The scaffold according to any of claims 1-11, wherein the scaffold comprises collagen type I, bone matrix, adipose extracellular matrix, heart basement membrane extract, heart basement membrane extracellular matrix, placenta basement membrane extract, placenta basement membrane extracellular matrix, brain-derived extracellular matrix, or polycaprolactone.

14. The scaffold according to any of claims 1-11, wherein the scaffold comprises heart basement membrane extract or heart basement membrane extracellular matrix.

15. The scaffold according to any of claims 1-11, wherein the scaffold comprises placenta basement membrane extract or placenta basement membrane extracellular matrix.

16. The scaffold according to any of claims 1-15, wherein the scaffold comprises an accessory polymer.

17. The scaffold according to claim 16, wherein the accessory polymer is selected from the group consisting of polycaprolactone, poly(glycolic acid), poly(lactic acid), polydioxanone, poly (lactide-co-glycolide) copolymers, polyesters polysaccharides, polyhydroxyalka noates , starch, polylactic acid, cellulose, proteins, agar, silks, alginate, collagen/gelatin, carrageenan, elastin, pectin, resilin, konjac, adhesives, gums, polyamino acids, polysaccharides, soy, zein, wheat gluten, casein, chitin/chitosan, serum albumin, hyaluronic acid, lipids/surfactants, xanthan, acetoglycerides, waxes, surfactants, dextran, emulsan, gelian, polyphenols, levan, lignin, curd, ian, tannin, polygalactosamine, humic acid, shellac, pullulan, poly-gamma-glutamic acid, elsinan, natural rubber, yeast glucans, synthetic polymers from natural fats and oils.

18. The scaffold according to any of claims 1-17, wherein the electrospun fibers are crosslinked.

19. The scaffold according to any of claims 1-18, wherein the scaffold comprises a bioactive factor.

20. The scaffold according to claim 19, wherein the bioactive factor is selected from the group consisting of an osteogenic growth factor, collagen, glycosaminoglycans, osteonectin, bone sialo protein, an osteoinductive factor, a chondrogenic factor, a cytokine, a mitogenic factor, a chemotactic factor, a transforming growth factor(TGF), a fibroblast growth factor (FGF), an angiogenic factor, an insulin-like growth factor (IGF), a platelet-derived growth factor (PDGF), an epidermal growth factor (EGF), a vascular endothelial growth factor (VEGF), a nerve growth

factor (NGF), a neurotrophin, a bone morphogenetic protein (BMP), osteogenin, osteopontin, osteocalcin, cementum attachment protein, erythropoietin, thrombopoietin, tumor necrosis factor (TNF), an interferon, a colony stimulating factor (CSF), stem cell derived factor-1 (SDF-1), and an interleukin.

21. The scaffold according to any of claims 1-20, wherein the scaffold comprises an extracellular matrix component.

22. The scaffold according to claim 21, wherein the extracellular matrix component is selected from the group consisting of collagen, glycosaminoglycans, osteocalcin, osteonectin, bone sialo protein, osteopontin, fibronectin, laminin, and mixtures thereof.

23. The scaffold according to any of claims 1-21, wherein the scaffold is in a form of one or more elongated sheet.

24. The scaffold according to any of claims 1-21, wherein the scaffold is in a form of one or more elongated roll.

25. A method of treating a tissue defect comprising applying the scaffold according to claim 23 to surround the tissue defect.

26. A method of treating a tissue defect or gap comprising applying the scaffold according to claim 24 between the tissue defect or gap.

27. A method of cell culture comprising culturing cells on the scaffold according to any of claims 1-24 in condition suitable for the cell culture.

28. The method of claim 27, wherein the cells are selected from the group consisting of stem cells, adipose derived stem cells, dental pulp stem cells, fibroblasts, and dorsal root ganglia.

29. The method of claim 27, wherein the cells are dorsal root ganglia.

30. The method of claim 27, wherein the cells are dorsal root ganglia, and the fiber comprise heart basement membrane extract, heart basement membrane extracellular matrix, placenta basement membrane extract, or placenta basement membrane extracellular matrix.

31. A method of promoting differentiation of stem cells into osteoblasts, chondrocytes, ligament or tendon, the method comprising culturing the cells on the scaffold according to any of claims 1-24 in conditions suitable for the cell differentiation.

32. The method of claim 31, wherein the stem cells are progenitor cells or adult stem cells.

33. The method of claim 32, wherein the progenitor cells or the adult stem cells are derived or isolated from placenta, bone marrow, adipose tissue, blood vessel, amniotic fluid, synovial fluid, synovial membrane, pericardium, periosteum, dura, peripheral blood, umbilical blood, menstrual blood, baby teeth, nucleus pulposus, brain, skin, hair follicle, intestinal crypt, neural tissue, or muscle.

34. The method of claim 31, wherein the stem cells are pluripotent stem cells.

35. The method of claim 34, wherein the stem cells are selected from the group consisting of adipose derived stem cells and dental pulp stem cells.

ALIGNED FIBER AND METHOD OF USE THEREOF

Inventor

Michael Francis and Roy Ogle

Background of the Invention

[0001] The invention relates to an aligned fiber and method of use thereof.

Summary of the Invention

[0002] The invention relates to a scaffold comprising an aligned fiber. The invention further relates to a scaffold comprising one or more electrospun fibers. In some embodiments, wherein a fast Fourier transform (FFT) analysis result of the fibers have adjacent major peaks with about 180° apart from each other.

[0003] The invention further relates to methods culturing cells on the scaffold or aligned fiber described herein.

[0004] The invention also relates to methods of promoting differentiation of stem cells into osteoblasts, chondrocytes, ligament or tendon, the method comprising culturing the cells on the scaffold or aligned fiber described herein in conditions suitable for the cell differentiation

Brief Description of the Drawings

[0005] FIGURE 1 depicts representative field lines that are modulated between a split point negative voltage sources and a single positive voltage source (A, D), aiding in imparting alignment in the electrospinning scheme. The change in field lines between a solid (B) and hollow (C) rod is also modeled.

[0006] FIGURE 2 depicts raw basement membrane (top left) next to it randomly electrospun architecture (to right), as compared to aligned basement membrane generated by parallel charged rod aligned electrospinning (bottom left and right).

[0007] FIGURE 3 depicts a Fourier spectrum from charged rod aligned and randomly electrospun fibers. The aligned pattern/texture recognition is typical to that of a regular, repeating line pattern, with the random fibers consistent with random noise generation spectra, as commonly seen in pattern recognition from FFT analysis.

[0008] FIGURE 4 depicts a modeling of a rotating parallel rod mandrel having a mercury coupled bearing inserted into the top of the device to split the electrical charge between the rods.

[0009] FIGURE 5 depicts the exemplary elements of the parallel electric charged rod electrospinning setup. Using two charged rods imparts alignment to the traveling fibers, optionally through the point charge splitting of the electric field.

[0010] FIGURE 6 depicts aligned heart base membrane fiber scaffold that allow dorsal root ganglia attachment and outgrowth.

[0011] FIGURE 7 depicts aligned placenta and heart base membrane fiber scaffolds that allow dorsal root ganglia attachment and outgrowth.

[0012] FIGURE 8 depicts exemplary FFT analysis results (a) of the fibers described herein and (b) a control sample.

[0013] FIGURES 9A, 9B and 9C depict exemplary scaffolds prepared into an elongated roll by folding an elongated sheet (9A), an elongated roll by rolling an elongated sheet (9B), and a composite of multiple layers of elongated sheets (9C).

Detailed Description of the Invention

[0014] The invention relates to an electrospinning apparatus comprising (i) at least one spinneret comprising an electrified tip; and (ii) a collector comprising two rods and a platform connected to the two rods, wherein the two rods are configured split an electric field between them. In one aspect, the spinneret comprising an electrified tip may have two, three, four, five or more electrified tips. In another aspect, the collector comprising two rods may have three, four, five or

more rods. In additional aspect, the platform connected to the two rods may have three, four, give or more rods connected to the platform. As defined herein, "comprising two rods" refers to comprising at least two rods, including, for example, two, three, four, and five rods.

[0015] The invention also relates to an electrospinning apparatus comprising (i) two spinnerets, each of which comprises a tip electrified with an opposite charge to one another; and (ii) a collector comprising two rods and a platform connecting the two rods, wherein the two rods are grounded.

[0016] Electrospray/electrospinning techniques can be used to form particles and fibers as small as one nanometer in a principal direction. The phenomenon of electrospray involves the formation of a droplet of polymer melt at an end of a needle, the electric charging of that droplet, and an expulsion of parts of the droplet because of the repulsive electric force due to the electric charges. In electrospraying, a solvent present in the parts of the droplet evaporates and small particles are formed but not fibers. The electrospinning technique is similar to the electrospray technique. In electrospinning and during the expulsion, however, fibers are formed from the liquid as the parts are expelled.

[0017] In particular, for example, the electrospinning typically involves a polymer solution (or melt) maintained at its surface tension on the tip of a nozzle via a syringe in a pump. When sufficiently high voltage is introduced (*e.g.* 15-40kV) to the polymer or oligomer in solution to create a charge imbalance, the solution may be drawn towards a grounded collector through the static electric field. As the polymer erupts from the needle and the assembling polymer whip through space, the fiber may be subjected to a series of stretching and bending instabilities, resulting in plastic stretching and elongation to minimize these instabilities generated by repulsive electrostatic forces. As the polymer or oligomer travels through space and rapidly thins into a fine stream, the solvent may evaporate and the polymer or oligomer assembles into fibers, leaving dry nano- to micro-scale fibers of tailorable physical attributes on the collector in the typical electrospinning scheme. Additionally, when high electrical potential is applied to a low viscosity polymer or oligomer melt or solution, or when improperly high or low voltage is applied, electrospraying may occur, which is typified by the polymer jet breaking down into fine droplets. It is therefore possible to produce particles or nano- to micro-sphere particles, fibers and bead-and-string type structures via this process from simply altering the solution or

electrospinning parameters. Polymer solution properties, applied electrical potential, polymer molecular weight, polymer solution flow rate, distance between spinner and collector, ambient parameters (*e.g.* humidity, air velocity, temperature) and motion of the collecting target can be altered to form fibers of controlled fiber distribution, diameter and alignment via electrospinning.

[0018] The electrospinning apparatus as described herein in one aspect may include one or more spinneret. The term "spinneret" used herein refers to a single- or multi-pored device through which a polymer or oligomer solution is extruded to form fibers. In one aspect, the spinneret is selected from the group consisting of a multiple nozzle spinneret, a single syringe or capillary spinneret, and a compound spinneret. In another aspect, the spinnerets of the electrospinning apparatus may be differently charged, resulting in differently charged polymer or oligomer solutions extruded from them. For example, different voltages may be applied to two or more polymer or oligomer sources whereby a positively charged solution is combined in space with a negatively charged solution, which is sent to the grounded rotating rods of the mandrel.

[0019] The electrospinning apparatus according to some embodiments of the present invention includes at least one spinneret comprising an electrified tip. The spinneret may have an electrified tip having a charge, and the tip may have one or more pores. In some embodiments, the electrospinning apparatus may include two or more tips having different charges. In particular, for example, the electrospinning apparatus may include two tips having opposite charges to each other.

[0020] In some embodiments, the spinneret described herein may be replaced with a different ejecting device comprising a drum configured to rotate in contact with a reservoir of a polymer or oligomer solution herein. In one aspect, this drum may pull the polymer or oligomer solution as a thin film on the surface of the drum. In another aspect, the drum may be charged, resulting in charging of the solution. In additional embodiments, the spinneret described herein may be replaced with a different ejecting device comprising a flat surface in contact with a polymer or oligomer solution, and the polymer or oligomer solution may form a polymer bubble(s) by applying air.

[0021] The electrospinning apparatus in one aspect may include a collector having at least two rods and a platform. In some embodiments, the collector may have, for example, three, four, five, six, seven, eight, nine, ten, twenty, thirty, or forty rods. Herein, the term "rod" used herein refers

to a bar of material having a shape including, but not limited to prism, cylinder, pentagonal rod, hexagonal rod, square rod, and triangular rod. The rod may comprise an electrical conducting material (*e.g.* a metal). In some embodiments, the rod may be electrified with a charge opposite to a charge of the electrified tip extruding a solution to the rod. In another aspect, the rod may be made of a non-electrical conducting material (*e.g.* insulating plastic). In further aspect, the rod may be made of both electrical and non-electrical conducting materials. For example, the electrical conducting material may be coated on the non-electrical conducting material.

[0022] The rod described herein may be directly or indirectly connected and/or fixed to a platform. The platform refers to any support for the rod. In one aspect, the rod comprises a proximal part and distal end, and the proximal part and/or distal end may be connected and/or fixed to the platform. In another aspect, the rod may go through the platform to which the proximal part of the rod is connected and/or fixed.

[0023] In one aspect, the platform may be a part of a machine frame. In another aspect, the electrospinning apparatus described herein may further include a chamber enclosing the spinneret and the collector. The chamber may comprise the platform described herein. In another aspect, the platform may consist of or comprise a rotating shaft or a bearing that is directly or indirectly connected and/or fixed to the rod.

[0024] The platform according to some embodiments includes a bearing connected to the rod described herein. The bearing used herein refers to a guide for rotating the rod. In one aspect, the bearing may be a rotating shaft. In another aspect, the platform or the bearing used herein may use plain bearing, rolling-element bearings, jewel bearings, fluid bearings, magnetic bearings, and/or flexure bearings. The bearing may include an electrical conductor configured to allow electrical conductance to the rods. For example, the bearing may have mercury.

[0025] In another aspect, the platform connected to the rod(s) may be configured to spin resulting in rotation of the rod(s) about the spinning axis of the platform. In some embodiments, the rods are configured to rotate at between about 0 and about 8000 RPM, between about 0 and about 7000 RPM, between about 0 and about 6000 RPM, between about 0 and about 5000 RPM, between about 0 and about 4000 RPM, between about 0 and about 3000 RPM, between about 0 and about 2000 RPM, between about 0 and about 1000 RPM, between about 0 and about 500 , between about 0 and about 300 RPM, between about 0 and about 100 RPM, between about 0 and

about 50, between about 1 and about 2000 RPM, between about 1 and about 1000 RPM, between about 1 and about 500 , between about 1 and about 300 RPM, between about 1 and about 100 RPM, between about 1 and about 50, between about 1000 and about 8000 RPM, between about 2000 and about 8000 RPM, between about 3000 and about 8000 RPM, between about 4000 and about 8000 RPM, between about 5000 and about 8000 RPM, between about 1000 and about 6000 RPM, between about 2000 and about 6000 RPM, or between about 3000 and about 6000 RPM. In additional embodiments, the rods are configured to rotate at about 50 RPM, about 500 RPM, about 1500 RPM, about 2500 RPM, about 3500 RPM, about 4500 RPM, about 5500 RPM, about 6500 RPM, about 7500 RPM or lower. In additional embodiments, the rods are configured to rotate at about 500 RPM, about 1500 RPM, about 2500 RPM, about 3500 RPM, about 4500 RPM, about 5500 RPM, about 6500 RPM, about 7500 RPM or higher. In some embodiments, when the rods are rotating, the fibers may spin around the rods. In other embodiments, when the rods are rotating, the fibers may also move or jump from one rod to another rod. In yet additional embodiments, the rods are stationary. In yet further embodiments, when the rods are stationary, the fibers may move or jump from one rod to another through an electric field.

[0026] In some embodiments, an air driven (pneumatic) motor and an electric (DC) motor may be employed in the electrospinning. In further embodiments, the pneumatic motor may generate less stray field anomalies and allow capturing of more nanofiber product on the rod(s) compared to the DC motor.

[0027] In one aspect, the fibers align on the rod (*e.g.* by spinning around the rods or jumping to one rod to another) in a perpendicular angle to the rods. In another aspect, the fibers align in an angle about 91, 92, 93, 94, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155 ° or less. In another aspect, the fibers align in an angle about 90, 91, 92, 93, 94, 95, 98, 103, 108, 113, 118, 123, 128, 133, 138, 143, 148, 153 ° or more. In another aspect, the fibers align in an angle between about 90 and about 93 °, between about 90 and about 95 °, between about 90 and about 100 °, between about 90 and about 105 °, between about 90 and about 110 °, between about 90 and about 115 °, between about 93 and about 95 °, between about 93 and about 100 °, between about 93 and about 105 °, between about 93 and about 110 °, or between about 93 and about 115 °.

[0028] As used herein, the term "about" modifying, for example, the quantity of an ingredient in a composition, concentrations, volumes, process temperature, process time, yields, flow rates, pressures, and like values, and ranges thereof, refers to variation in the numerical quantity that can occur, for example, through typical measuring and handling procedures used for making compounds, compositions, concentrates or use formulations; through inadvertent error in these procedures; through differences in the manufacture, source, or purity of starting materials or ingredients used to carry out the methods; and like considerations. The term "about" also encompasses amounts that differ due to aging of, for example, a composition, formulation, or cell culture with a particular initial concentration or mixture, and amounts that differ due to mixing or processing a composition or formulation with a particular initial concentration or mixture. Whether modified by the term "about" the claims appended hereto include equivalents to these quantities. The term "about" further may refer to a range of values that are similar to the stated reference value. In certain embodiments, the term "about" refers to a range of values that fall within 10, 9, 8,7, 6, 5,4, 3, 2, 1 percent or less of the stated reference value.

[0029] The electrospinning apparatus according to some embodiments of the present invention includes a collector comprising two or more rods and a platform connected to the rods, wherein the rods are configured split an electric field between them. In one aspect, operating the electrospinning apparatus described herein may result in forming a point charge splitting of an electric field, through which traveling polymer or oligomer solutions align. In another aspect, the rods may be grounded.

[0030] The split electric field in some embodiments is separated by an air insulator. In one aspect, with air as an insulator between electrically charged metal rods, electrospun fibers are collect between opposed metal rods in an aligned fashion. The electrospun fiber described herein may be a microfiber or a nanofiber.

[0031] The split electric field in some embodiments is insulated by non-conductive materials or environments placed between the rods (*e.g.* carbon fiber, PTFE [Teflon], mica, diamond, ceramic, rubbers, glass, vacuum, materials on the low ends of the triboelectric series in the high resistivity range, also including paper, cotton, wood, epoxy, plastics such as: ABS [acrylonitrile, butadiene, and styrene], polycarbonate, acetate, acrylic, delrin, fiberglass, FEP, high impact polystyrene [HIPS], kapton, kaptrex, kynan, macor, melamine, meldin 7001 unfilled polyimide, MICA,

neoprene, nomex, noryl PPO, PolyEtherEther-Ketone [PEEK], polyethylene terephthalate [P.E.T], P.E.T.G, phenolics such as micarta phenolics, perfluoroalkoxy [PFA], Polycarbonate, polyester [mylar], polyolefins, polystyrene, polysufone, polyurethane,), Rexolite® 1422 &220, polyphenylene sulfide [Ryton], silicone/fiberglass, silicone rubber, techtron, Ultem® 1000, Vespel® SP-1 [polyimide], electrically insulating papers such as vulcanized fibre, tapes from the above materials, and foams of these materials, including neoprene foam, polystyrene foam, polyurethane foam, silicone foam, vinyl foam.

[0032] The term microfiber as used herein means a fiber comprising a diameter of about 1000 μm or less. The term nanofiber as used herein means a fiber comprising a diameter of about 1000 nm or less. Relative to the parent bulk material, nano-features can impart many extraordinary properties to macrostructures, including superior mechanical, electrical, optical and magnetic properties, adding surface functionality and yielding high surface area. Matrices of nanofibers of varying diameters may show a range of variable surface properties (*e.g.* hydrophobicity and hydrophilicity), porosities, and usually superior mechanical properties (*e.g.* tensile strength, stiffness) relative to the material in other forms.

[0033] In some embodiments, the nanofiber described herein may have an average diameter of about 1000 nm or less, 900 nm or less, 800 nm or less, 700 nm or less, 600 nm or less, 500 nm or less, 400 nm or less, 300 nm or less, 200 nm or less, 100 nm or less, 50 nm or less, 20 nm or less, or 10 nm or less. In some embodiments, the nanofiber described herein may have an average diameter of about 1000 nm or more, 900 nm or more, 800 nm or more, 700 nm or more, 600 nm or more, 500 nm or more, 400 nm or more, 300 nm or more, 200 nm or more, 100 nm or more, 50 nm or more, 20 nm or more, 10 nm or more, or 1 nm or more.

[0034] In some embodiments, the microfiber described herein may have an average diameter of about 1000 μm or less, 900 μm or less, 800 μm or less, 700 μm or less, 600 μm or less, 500 μm or less, 400 μm or less, 300 μm or less, 200 μm or less, 100 μm or less, 50 μm or less, 20 μm or less, or 10 μm or less. In some embodiments, the microfiber described herein may have an average diameter of about 1000 μm or more, 900 μm or more, 800 μm or more, 700 μm or more, 600 μm or more, 500 μm or more, 400 μm or more, 300 μm or more, 200 μm or more, 100 μm or more, 50 μm or more, 20 μm or more, 10 μm or more, or 1 μm or more.

[0035] In some embodiments, the rods described herein may be set parallel to each other in the electrospinning apparatus. In other embodiments, the rods may not be set parallel to each other but at an angle, for example, at an angle smaller than 5°, 10°, 30°, 50°, 70°, or 90°. In other embodiments, the rods may not be set parallel to each other but at an angle, for example, at an angle bigger than 1°, 8°, 25°, 40°, 60°, or 75°. In additional embodiments, the rod described herein may be straight, curved or angled.

[0036] The rods described herein in some embodiments are separated from one another by an average distance of about 1 cm, about 2 cm, about 3 cm, about 5 cm, about 8 cm, about 10 cm, about 13 cm, about 15 cm, about 18 cm, about 20 cm, about 23 cm, about 25 cm, about 27 cm, about 30 cm or more. In one aspect, the rods are separated from one another by an average distance of about 1 cm, about 2 cm, about 3 cm, about 5 cm, about 8 cm, about 10 cm, about 13 cm, about 15 cm, about 18 cm, about 20 cm, about 23 cm, about 25 cm, about 27 cm, about 30 cm, about 40 cm, about 50 cm, about 60 cm or less. In another aspect, the rods are separated from one another by a distance from about 1 cm to about 25 cm, from about 6 cm to about 25 cm, from about 12 cm to about 25 cm, from about 18 cm to about 25 cm, from about 23 cm to about 25 cm, from about 1 cm to about 35 cm, from about 6 cm to about 35 cm, from about 12 cm to about 35 cm, from about 18 cm to about 35 cm, from about 24 cm to about 35 cm, or from about 10 cm to about 20 cm.

[0037] The collector may be biocompatible. In fact, any of the other elements of the electrospinning apparatus described herein may be biocompatible.

[0038] The electrospinning apparatus described herein may also include a control mechanism configured to control the electric potential of the electrified tip. The electrospinning apparatus described herein may also include a reservoir for a solution. The electrospinning apparatus described herein may also include an electric source connected to the electrified tip.

[0039] The invention further relates to methods of electrospinning using the electrospinning apparatus described herein and methods of preparing an electrospun fiber by the electrospinning. The methods of electrospinning and methods of preparing an electrospun fiber by the electrospinning may comprise (i) extruding a solution from an electrified tip of a spinneret, and (ii) collecting the extruded solution on at least a part of a collector. The methods of electrospinning or preparing an electrospun fiber by the electrospinning may further comprise

extruding two, three or more solutions from one, two, three or more electrified tips of a spinneret. The methods of electrospinning or preparing an electrospun fiber by the electrospinning may further comprise extruding two, three or more solutions from one, two, three or more electrified tips of one, two, three or more spinnerets. The methods of preparing an electrospun fiber by the electrospinning may further comprise coating the extruded solution with another electrospun fiber prepared by the methods described herein.

[0040] The solution that may be extruded using the electrospinning apparatus described herein may include, but is not limited to, solutions comprising collagen type I, adipose extracellular matrix, heart basement membrane extract or extracellular matrix, placenta basement membrane extract or extracellular matrix, and polycaprolactone. Additional materials that may be electrospun by the method described herein include, but are not limited to, poly(glycolic acid), poly(lactic acid), polydioxanone, poly (lactide-co-glycolide) copolymers, polyesters polysaccharides, polyhydroxyalka noates , starch, polylactic acid, cellulose, proteins, agar, silks, alginate, collagen/gelatin, carrageenan, elastin, pectin, resilin, konjac, adhesives, gums, polyamino acids, polysaccharides, soy, zein, wheat gluten, casein, chitin/chitosan, serum albumin, hyaluronic acid, lipids/s urfactants, xanthan, acetoglycerides, waxes, surfactants, dextran, emulsan, gelian, polyphenols, levan, lignin, curd, ian, tannin, polygalactosamine, humic acid, shellac, pullulan , poly-gamma-glutamic acid, elsinan, natural rubber, yeast glucans, and synthetic polymers from natural fats and oils, and the mixture thereof.

[0041] In one aspect, the collector of the electrospinning apparatus is a biocompatible support to be coated by the electrospun fiber on its surface and may be used as a part of the implant described herein. For example, the collector may comprise a bone matrix, and the electrospun fiber is collected on the surface of the bone matrix, resulting in coating of the bone matrix. The bone matrix may have two or more different electrospun fiber coatings on its surface.

[0042] The invention further relates to a method of coating a matrix on a surface comprising (i) extruding a solution from an electrified tip of a spinneret, and (ii) collecting the extruded solution on a portion of a collector, wherein the collector further comprises two rods and a platform connected to the two rods, the two rods are configured to split an electric field between them, and the portion of the collector comprises the matrix. The invention further relates to a method

of coating a matrix on a surface comprising (i) extruding one or more solutions from electrified trips of two spinnerets, each of which comprises at least one tip electrified with an opposite charge to a tip of the other spinnerets, and (ii) collecting the extruded solution on a portion of a collector, wherein the collector further comprises two rods and a platform connected to the two rods, the two rods are grounded, and the portion of the collector comprises the matrix. In some embodiments, the matrix is a bone matrix, and the solution comprises a bone matrix solution. In additional embodiments, the matrix may comprise a synthetic surface.

[0043] In some embodiments, the matrix is biocompatible. In further embodiments, the biocompatible matrix is an implantable biocompatible matrix that can function as a support system for the bone matrix described herein. A biocompatible matrix should be non-toxic, non-eliciting or stimulating severe inflammatory response or immunological rejections, and devoid of other undesired reactions at the implantation site. In one embodiment, the biocompatible matrix is bone matrix or cartilage or connective tissue.

[0044] In some embodiments, the biocompatible matrix is prepared into a form of an elongated roll, an elongated sheet, a composite of multiple layers of sheets, or a combination thereof before or after being coated with the electrospun fibers. For example, the biocompatible matrix may be prepared as shown in **figures 9A, 9B, and 9C**. In one aspect, the materials for the elongated roll and the elongated sheet may be different. In another aspect, the materials for each elongated sheet in the multiple layers of sheets may be different from each other. In another aspect, the electrospun fibers coated on the biocompatible matrix are aligned in one direction. For example, the electrospun fibers coated on the biocompatible matrix are aligned in the direction parallel or perpendicular to the length of the elongated sheet or the multiple layers. In another aspect, the biocompatible matrix is prepared by folding or rolling the sheet or sheets, along with the direction of nano fiber alignment to form an elongated roll implant. In one aspect, this biocompatible matrix in the form of an elongated sheet or a composite of multiple layers of sheets may be used to facilitate neuronal cell growth.

[0045] In some embodiments, the elongated roll implant may be prepared by combining a sheet or sheets to facilitate surgical implantation. The biomaterials to prepare the sheet and elongated roll may be the same, or different, as described previously.

[0046] In some embodiments, the elongated sheet described herein may have an average thickness of about 1000 μm or less, 900 μm or less, 800 μm or less, 700 μm or less, 600 μm or less, 500 μm or less, 400 μm or less, 300 μm or less, 200 μm or less, 100 μm or less, 50 μm or less, 20 μm or less, or 10 μm or less. In one aspect, the elongated sheet described herein may have an average thickness of about 1000 μm or more, 900 μm or more, 800 μm or more, 700 μm or more, 600 μm or more, 500 μm or more, 400 μm or more, 300 μm or more, 200 μm or more, 100 μm or more, 50 μm or more, 20 μm or more, 10 μm or more, or 1 μm or more.

[0047] In some embodiments, the length of the elongated sheet or roll implant is about 0.1 cm or more, about 0.3 cm or more, about 0.5 cm or more, about 0.7 cm or more, about 1 cm or more, about 2 cm or more, about 3 cm or more, about 5 cm or more, about 8 cm or more, about 10 cm or more, about 13 cm or more, about 15 cm or more, about 18 cm or more, about 20 cm or more, about 23 cm or more, about 25 cm or more, about 27 cm or more, or about 30 cm or more. In one aspect, the length of the elongated sheet or roll implant is about 1 cm or less, about 2 cm or less, about 3 cm or less, about 5 cm or less, about 8 cm or less, about 10 cm or less, about 13 cm or less, about 15 cm or less, about 18 cm or less, about 20 cm or less, about 23 cm or less, about 25 cm or less, about 27 cm or less, about 30 cm or less, about 40 cm or less, about 50 cm or less, or about 60 cm or less.

[0048] In some embodiments, the biocompatible matrix includes but is not limited to, bone graft implants, synthetic bone graft materials in forms of particulates, sheet, or blocks, tendon and/or ligament in bone tunnels, prosthetic implant, for example, for hip, shoulder, knee, or ankle, and trabecular metal. In further embodiments, the bone graft implants may include allograft or xeno-graft. In yet further embodiments, the bone graft implants may include structural bone implants including, but not limited to, monolithic or composite spinal implants (e.g., VERTIGRATs from LifeNet Health) and bone struts or blocks. In yet further embodiments, the bone graft implants may include demineralized, or non-demineralized bone particulates, including, but not limited to, cortical, cancellous, or cortical cancellous bone. In other embodiments, the electrospun fibers may be formed on cell or tissue culture surface.

[0049] Suitable biocompatible matrices include, but are not limited to, porous biocompatible scaffolds into which bone cells or progenitor cells may migrate. Osteogenic or chondrogenic

cells, i.e., cells involved in the process of deposition of new bone material or cartilagenous material, respectively, can often attach to such porous biocompatible matrices, which can then serve as scaffolding for bone and cartilage tissue growth. Cells involved in the process of deposition of new ligament or tendon material can also attach to such porous biocompatible matrices. For certain applications, the biocompatible matrix should have sufficient mechanical strength to maintain its three dimensional structure and help support the immobilization of the bone segments being united or grafted together. Porous biocompatible matrices which provide scaffolding for tissue growth can accelerate the deposition of new bone or the rate of bone growth and are said to be "osteoconductive." Osteoconductive biocompatible matrices are especially useful in the matrices described herein. Porous biocompatible matrices which provide scaffolding for tissue growth can accelerate the deposition of new cartilage or the rate of cartilage growth and are said to be "chondroconductive." Osteoconductive biocompatible matrices are especially useful in the matrices described herein. Chondroconductive biocompatible matrices are especially useful in the matrices described herein. Angiogenic (or vasculogenic) biocompatible matrices are especially useful in the pharmaceutical compositions described herein. The osteoinductive, angiogenic or chondroinductive activity of the surface coated with the electrospun fibers or may not be altered, including but not limited to, enhanced activity, relative to a surface without the electrospun fiber coating or a natural surface without any coating. Thus, the osteoconductive, angiogenic or chondroconductive activity of the biocompatible matrices treated with the electrospun fibers of the present invention may be enhanced compared to matrices not treated with the bone matrix described herein. Of course, the biocompatible matrices are considered to be osteoconductive, angiogenic or chondroconductive if cells within the biocompatible matrix begin to differentiate into more osteoblast-like or chondrocyte-like appearing or functional cells, respectively.

[0050] The biocompatible matrices according to some embodiments of the present invention can be derived from natural sources or they can be synthetic or a mixture of both. Biocompatible matrices from natural sources may also comprise natural polymers, including, but not limited to, collagen, hyaluronic acid, alginate, albumin, fibrinogen-fibrin, chitosan, elasin, laminin, connective tissues, intervertebral disc, cortical or cancellous bone, demineralized or mineralized bone, fascia lata, dermis, muscle, ligament, tendon, cartilage including articular/hyaline cartilage,

elastic cartilage, and fibrocartilage, a mixture thereof, and mixture of reconstituted tissue. Biocompatible matrices from synthetic sources refer to any material not produced by living organisms, which may include, not limited to, the synthetic material made up of organic components, inorganic components, or a mixture thereof. In some embodiments, a synthetic biocompatible matrix may comprise an organic synthetic polymer, such as poly(lactic-co-glycolic acid), polycaprolactone (PCL), polyglycolic acid (PGA), polylactic acid (PLA), polyhydroxybutyrate (PHB), Poly(ethylene glycol) (PEG), poly(ethylene oxide) (PEO)), and others. In some embodiments, a tissue, an organ, or biocompatible matrix comprising at least one of alginate, chitosan, collagen, gelatin, hyaluronic acid, a fibronectin, an elastin, a laminin, and a proteoglycan may be employed. In certain embodiments, a biocompatible matrix comprising inorganic components, such as hydroxyapatite, calcium sulfate, octacalcium phosphate, calcium phosphate, macroporous calcium metaphosphate ceramic, β-tricalcium phosphate, metal, metal alloy, and others, may be used. A biocompatible matrix used in certain embodiments of the present invention may be prepared by demineralizing, decellularizing or devitalizing a tissue or an organ and cells may be seeded onto the biocompatible matrix.

[0051] In some embodiments, the electrospun fibers described herein may be applied to a biocompatible matrix and may be incubated at conditions permitting the generation of a matrix that is partially or fully coated with the electrospun fibers. In some embodiments, incubation may be carried out at about 40 °C or lower, or between about 10 °C and about 37 °C, or about 20 °C and about 37 °C. Incubation may be carried out for between at least about 2 minutes and about 120 minutes, about 3 minutes and about 100 minutes, about 4 minutes and about 80 minutes, about 5 minutes and about 60 minutes, and about 5 minutes and about 30 minutes in certain embodiments. Incubation may be performed under static or dynamic conditions, such as with agitation, shaking, stirring, mixing, horizontal motion, rocking, and others.

[0052] In some embodiments of the present invention, a biocompatible matrix may be lyophilized before the electrospun fiber is applied to the biocompatible matrix. In certain embodiments, the electrospun fibers may be coated on the biocompatible matrix, and the coated matrix may be subsequently lyophilized. The lyophilized, coated matrix can then be rehydrated before it is used. Further, the cells can be seeded onto the matrix before implantation.

[0053] Examples of suitable osteoconductive or chondroconductive biocompatible matrices include but are not limited to, collagen (e.g., bovine dermal collagen), fibrin, calcium phosphate ceramics (e.g., hydroxyapatite and tricalcium phosphate), calcium sulfate, guanidine-extracted allogenic bone and combinations thereof. A number of suitable biocompatible matrices are commercially available, such as Collograft™ (Collagen Corporation), which is a mixture of hydroxyapatite, tricalcium phosphate and fibrillar collagen, and Interpore™ (Interpore International), which is a hydroxyapatite biomatrix formed by the conversion of marine coral calcium carbonate to crystalline hydroxyapatite.

[0054] A number of synthetic biodegradable polymers can serve as osteoconductive or chondroconductive biocompatible matrices with sustained release characteristics. Descriptions of these polymers can be found in Behravesh (1999) Clinical Orthopaedics 367, S118 and Lu (2000) Polymeric Delivery Vehicles for Bone Growth Factors in Controlled Drug Delivery: Designing Technologies for the Future, Park and Mrsny eds., American Chemical Society, which is incorporated herein in its entirety herein. Examples of these polymers include poly α-hydroxy esters such as polylactic acid/polyglycolic acid homopolymers and copolymers, polyphosphazenes (PPHOS), polyanhydrides and poly(propylene fumarates).

[0055] Polylactic acid/polyglycolic acid (PLGA) homo and copolymers are well known in the art as sustained release vehicles. The rate of release can be adjusted by the skilled artisan by variation of polylactic acid to polyglycolic acid ratio and the molecular weight of the polymer (see Anderson (1997) Adv. Drug Deliv. Rev. 28:5. The incorporation of PEG into the polymer as a blend to form microparticle matrices allows further alteration of the release profile of the active ingredient (see Cleek (1997) J. Control Release 48, 259). Ceramics such as calcium phosphate and hydroxyapatite can also be incorporated into the formulation to improve mechanical qualities.

[0056] In one embodiment, the biocompatible matrices used in the methods of the present invention are other types of bone matrices. For example, the electrospun fibers prepared by the methods described herein can be coated on a bone matrix. As used herein, the other types of the bone matrix may be a biocompatible matrix derived from or including elements of natural bone. In some embodiments, the natural bone is mineralized, partially demineralized, demineralized,

cancellous, cortical, or cortical cancellous bone. The bone matrices used herein may or may not include additional synthetic components not typically found in bone tissue. Other embodiments include methods utilizing a biocompatible matrix derived from cartilage, other soft tissues such as the dermis, connective tissue, fascia, small intestine submucosa, serous membrane, pericardium, tendon, ligament, muscle, adipose tissue, myelin, blood vessels, base membrane, amniotic membrane and others. A biocompatible matrix prepared from hyaline cartilage, fibrocartilage or elastic cartilage, may be employed in some embodiments. A biocompatible matrix may be prepared from hyaline cartilage found in the condyle, tibial plateau, femoral head, humeral head, costal cartilage, or fibrocartilage found in intervertebral discs, or elastic cartilage found in the epiglottis or ear. In certain embodiments, a biocompatible matrix derived from natural sources that has been optionally cleaned, disinfected, chemically modified, decellularized, particulated, homogenized, lyophilized, gamma ray irradiated, and/or plasticized may be used. Any of the biocompatible matrices used herein may or may not include additional synthetic components not typically found in such tissue.

[0057] In one specific embodiment, the bone or cartilage biocompatible matrices may be demineralized or decellularized, respectively. Examples of demineralized matrices and methods of making are described in U.S. Patent No. 6,189,537 and 6,305,379, which are incorporated herein in its entirety herein.

[0058] The biocompatible matrix, tissue, or organ used in certain embodiments of the present invention may be in the form of a powder, particulates, sheets, fibers, gels, putties, paste, blocks, cylinders, sponges, meshes, films, slices, curls, flakes, or wedges, among others. In certain embodiments of the present invention, the biocompatible matrix, tissue, or organ treated with the electrospun fibers by the methods described herein may be in the form of a powder, fibers, putty, or a sponge. In further embodiments, the sponge can include, for example, the implant having sponge-like structures disclosed in the co-pending, commonly-assigned patent application PCT/US09/04556 entitled "Composition for a Tissue Repair Implant and Methods of Making the Same" filed on August 7, 2009, which is incorporated herein in its entirety herein. The treated matrices can be used in any of the methods of the present invention.

[0059] The invention also related to a method of preparing an electrospun fiber by electrospinning comprising (i) extruding a solution from an electrified tip of a spinneret, and (ii) collecting the extruded solution on a portion of a collector comprising two rods and a platform connected to the two rods, wherein the two rods are configured to split an electric field between them. The invention further related to a method of preparing an electrospun fiber by electrospinning comprising (i) extruding one or more solutions from electrified trips of two spinnerets, each of which comprises at least one tip electrified with an opposite charge to a tip of the other spinnerets, and (ii) collecting the extruded solution on a part of a collector comprising two rods and a platform connecting the two rods, wherein the two rods are grounded. In some embodiments, the method of preparing an electrospun fiber further comprises applying an accessory polymer to the solution (*e.g.* as a copolymer, blend, or melt). An "accessory polymer" refers to a polymer that may be added to the electrospun fibers and have any effect on their physical, chemical, and/or biological properties (*e.g.* tensile strength, hydrophillicity, biocompatibility). For example, the accessory polymer may be selected from the group consisting of polycaprolactone, poly(glycolic acid), poly(lactic acid), polydioxanone, poly (lactide-co-glycolide) copolymers, polyesters polysaccharides, polyhydroxyalkanoates, starch, polylactic acid, cellulose, proteins, agar, silks, alginate, collagen/gelatin, carrageenan, elastin, pectin, resilin, konjac, adhesives, gums, polyamino acids, polysaccharides, soy, zein, wheat gluten, casein, chitin/chitosan, serum albumin, hyaluronic acid, lipids/s urfactants, xanthan, acetoglycerides, waxes, surfactants, dextran, emulsan, gelian, polyphenols, levan, lignin, curd, ian, tannin, polygalactosamine, humic acid, shellac, pullulan , poly-gamma-glutamic acid, elsinan, natural rubber, yeast glucans, and synthetic polymers from natural fats and oils.

[0060] In one aspect, the method of preparing the electrospun fiber(s) according to some embodiments of the present invention may further comprise crosslinking the electrospun fiber(s). In some embodiments, the crosslinking may be performed by any conventional chemical crosslinking method (*e.g.* chemical reagent-promoted, chemically reactive linker-promoted and/or enzyme-promoted) and/or dehydrothermal crosslinking method (*e.g.* heat-promoted condensation), forming the covalently crosslinked electrospun fiber(s). In additional embodiments, the crosslinking comprises applying a cross-linking agent to the polymer or oligomer solutions to be electrospun. For example, the cross-linking agent may be selected from

the group consisting of 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), EDC/hyaluronic acid, genipin, and glutaraldehyde.

[0061] In some embodiments, a method of preparing an electrospun fiber further comprises adding a bioactive factor to the solution. A "bioactive factor" refers to protein, carbohydrate, or mineral that has any effect on a cellular activity. Examples of bioactive factors include, but are not limited to, an osteogenic growth factor, collagen, glycosaminoglycans, osteonectin, bone sialo protein, an osteoinductive factor, a chondrogenic factor, a cytokine, a mitogenic factor, a chemotactic factor, a transforming growth factor (TGF), a fibroblast growth factor (FGF), an angiogenic factor, an insulin-like growth factor (IGF), a platelet-derived growth factor (PDGF), an epidermal growth factor (EGF), a vascular endothelial growth factor (VEGF), a nerve growth factor (NGF), a neurotrophin, a bone morphogenetic protein (BMP), osteogenin, osteopontin, osteocalcin, cementum attachment protein, erythropoietin, thrombopoietin, tumor necrosis factor (TNF), an interferon, a colony stimulating factor (CSF), stem cell derived factor-1 (SDF-1), or an interleukin, among others. The bioactive factor may be a BMP, PDGF, FGF, VEGF, TGF, insulin, among others. Examples of BMPs include but are not limited to BMP-2, BMP-3, BMP-4, BMP-5, BMP-6, BMP-7, BMP-8, BMP-9, BMP-10, BMP-11, BMP-12, BMP-13, BMP-14, BMP-15, truncated BMPs described in PCT/US2012/053584, which is incorporated by reference in its entirety herein, and a mixture thereof.

[0062] The invention further relates to a scaffold comprising an aligned fiber. The invention further relates to a scaffold comprising one or more electrospun fibers. In some embodiments, a fast Fourier transform (FFT) analysis result of the fibers have adjacent major peaks with about 180° apart from each other. The "major peaks" herein refer to peaks higher than the average of the peaks in the result.

[0063] In one aspect, the scaffold may essentially consist of the aligned fiber and/or electrospun fiber. In another aspect, the scaffold may consist of the aligned fiber and/or electrospun fiber. The scaffold described herein may be biocompatible. A biocompatible matrix scaffold may be non-toxic, non-eliciting or stimulating severe inflammatory response or immunological rejections, and/or devoid of other undesired reactions at the implantation site.

[0064] Alignment of the fibers in the scaffold may be measured by a fast Fourier transform (FFT) analysis. For example, the FFT analysis may be performed by the methods described in *Measuring fiber alignment in electrospun scaffolds: a user's guide to the 2D fast Fourier transform approach*, Ayres CE, Jha BS, Meredith H, Bowman JR, Bowlin GL, Henderson SC, Simpson DG. J Biomater Sci Polym Ed. 2008; 19(5):603-21, which is incorporated by reference in its entirety herein. In some embodiments, FFT result of the fibers described herein may have adjacent major peaks with about 180° apart from each other as shown in **figure 8**.

[0065] The aligned fiber, electrospun fiber, and/or scaffold of the present invention may comprise type I collagen. In some embodiments, total type I collagen may be present in the aligned fiber, electrospun fiber, and/or scaffold of the present invention in an amount from about 50% to about 95%, from about 60% to about 95%, from about 70% to about 95%, or from about 80% to about 95% by weight in it protein content. In additional embodiments, total type II collagen may be present in the aligned fiber, electrospun fiber, and/or scaffold of the present invention in an amount about 10% or less, about 5% or less, about 3% or less, or about 1% or less by weight in it protein content. In further embodiments, collagen type II may be absent from aligned fiber, electrospun fiber, and/or scaffold of the present invention. The types of collagen that are present or absent in the composition can be easily assessed using routine methods in the art. Methods of identifying and quantifying type of collagen are well known in the art, as disclosed, for example in Schnaper, H.W. and Kleinman, H.K., Pediatr. Neprol., 7:96-104 (1993), which is incorporated by reference.

[0066] In one aspect, the aligned fiber, electrospun fiber, and/or scaffold described herein may have less than about 8 wt%, 7 wt%, 6 wt%, 5 wt%, 4 wt%, 3 wt%, 2 wt%, or 1 wt% residual calcium. In another aspect, the average residual calcium amount in the aligned fiber, electrospun fiber, and/or scaffold described herein may be less than about 8 wt%, 7 wt%, 6 wt%, 5 wt%, 4 wt%, 3 wt%, 2 wt%, or 1 wt% .

[0067] In one aspect, the aligned fiber and/or electrospun fiber described herein may have a length from about 0.1 cm to about 10 cm, from about 1 cm to about 10 cm, from about 0.1 cm to about 20 cm, from about 1 cm to about 20 cm, from about 10 cm to about 20 cm, from about 0.1 cm to about 30 cm, from about 1 cm to about 30 cm, from about 10 cm to about 30 cm, from

about 20 cm to about 30 cm, from about 0.1 cm to about 40 cm, from about 1 cm to about 40 cm, from about 10 cm to about 40 cm, from about 20 cm to about 40 cm, from about 30 cm to about 40 cm, from about 0.1 cm to about 50 cm, from about 1 cm to about 50 cm, from about 10 cm to about 50 cm, from about 20 cm to about 50 cm, from about 30 cm to about 50 cm, from about 40 cm to about 50 cm, 0.1 cm to about 60 cm, from about 1 cm to about 60 cm, from about 10 cm to about 60 cm, from about 20 cm to about 60 cm, from about 30 cm to about 60 cm, or from about 40 cm to about 60 cm. In another aspect, the total the aligned fiber and/or electrospun fiber in the scaffold described herein may have an average length from about 0.1 cm to about 10 cm, from about 1 cm to about 10 cm, from about 0.1 cm to about 20 cm, from about 1 cm to about 20 cm, from about 10 cm to about 20 cm, from about 0.1 cm to about 30 cm, from about 1 cm to about 30 cm, from about 10 cm to about 30 cm, from about 20 cm to about 30 cm, from about 0.1 cm to about 40 cm, from about 1 cm to about 40 cm, from about 10 cm to about 40 cm, from about 20 cm to about 40 cm, from about 30 cm to about 40 cm, from about 0.1 cm to about 50 cm, from about 1 cm to about 50 cm, from about 10 cm to about 50 cm, from about 20 cm to about 50 cm, from about 30 cm to about 50 cm, from about 40 cm to about 50 cm, 0.1 cm to about 60 cm, from about 1 cm to about 60 cm, from about 10 cm to about 60 cm, from about 20 cm to about 60 cm, from about 30 cm to about 60 cm, or from about 40 cm to about 60 cm.

[0068] In one aspect, the scaffold has an average porosity from about 60% to about 70%, from about 60% to about 80%, from about 60% to about 90%, from about 60% to about 95%, from about 70% to about 80%, from about 70% to about 90%, from about 70% to about 95%, from about 80% to about 90%, or from about 80% to about 95%.

[0069] In some embodiments, the aligned fiber, electrospun fiber, and/or scaffold comprises collagen type I, bone matrix, adipose extracellular matrix, heart basement membrane extract, heart basement membrane extracellular matrix, placenta basement membrane extract, placenta basement membrane extracellular matrix, brain-derived extracellular matrix, polycaprolactone, a biodegradable polymer, an accessory polymer described herein, or the mixture thereof.

[0070] In one aspect, the aligned fiber, electrospun fiber, and/or scaffold may comprise a bone matrix fiber comprising bone. In some embodiments, the bone matrix fiber may be prepared by

the methods described in PCT/US2013/025226, which is incorporated herein in its entirety herein.

[0071] In another aspect, the aligned fiber, electrospun fiber, and/or scaffold may comprise heart basement membrane extract and/or heart basement membrane extracellular matrix. In some embodiments, the heart basement membrane extract and/or heart basement membrane extracellular matrix may be prepared by the methods described in PCT/US2011/49192, which is incorporated herein in its entirety herein. In an additional aspect, the placenta basement membrane extract and/or heart basement membrane extracellular matrix may be prepared by the same method to prepare the heart basement membrane extract and/or heart basement membrane extracellular matrix.

[0072] In another aspect, the aligned fiber, electrospun fiber, and/or scaffold may comprise a biodegradable polymer. The biodegradable, biocompatible polymers may include, but is not limited to, ethylene vinyl acetate, polyanhydricles, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. The biodegradable, biocompatible polymers may further include a number of synthetic biodegradable polymers described herein.

[0073] In some embodiments, the aligned fiber and/or electrospun fiber in the scaffold is crosslinked. In additional embodiments, the aligned fiber, electrospun fiber, and/or scaffold comprises a bioactive factor described herein.

[0074] In additional embodiments, the aligned fiber, electrospun fiber, and/or scaffold may comprise an extracellular matrix component. For example, the extracellular matrix component may include, but is not limited to, collagen, glycosaminoglycans, osteocalcin, osteonectin, bone sialo protein, osteopontin, fibronectin, laminin, vitronectin, elastin or mixtures thereof.

[0075] In some embodiments, the scaffold of the present invention may further comprise a support. In additional embodiments, the fibers described herein may be attached to and/or cover the support.

[0076] In some embodiments, the scaffold of the present invention may be in a form of an elongated sheet or a composite of multiple layers of sheets similarly to the biocompatible matrix

described herein. In additional embodiments, the scaffold may be prepared as shown in **figures 9A, 9B, and 9C**. In one aspect, the materials for the elongated roll and the elongated sheet may be different. In another aspect, the materials for each elongated sheet in the multiple layers of sheets may be different from each other. In another aspect, the electrospun fibers in the scaffold are aligned in one direction. For example, the electrospun fibers in the scaffold are aligned in the direction parallel or perpendicular to the length of the elongated sheet or the multiple layers. In another aspect, the scaffold is prepared by folding or rolling the sheet or sheets, along with the direction of nano fiber alignment to form an elongated roll implant.

[0077] In some embodiments, the elongated roll implant may be prepared by combining a sheet or sheets to facilitate surgical implantation. The biomaterials to prepare the sheet and elongated roll may be the same, or different, as described previously.

[0078] In some embodiments, the elongated sheet scaffold described herein may have an average thickness of about 1000 μm or less, 900 μm or less, 800 μm or less, 700 μm or less, 600 μm or less, 500 μm or less, 400 μm or less, 300 μm or less, 200 μm or less, 100 μm or less, 50 μm or less, 20 μm or less, or 10 μm or less. In one aspect, the elongated sheet scaffold described herein may have an average thickness of about 1000 μm or more, 900 μm or more, 800 μm or more, 700 μm or more, 600 μm or more, 500 μm or more, 400 μm or more, 300 μm or more, 200 μm or more, 100 μm or more, 50 μm or more, 20 μm or more, 10 μm or more, or 1 μm or more.

[0079] In some embodiments, the length of the elongated sheet or roll implant scaffold is about 0.1 cm or more, about 0.3 cm or more, about 0.5 cm or more, about 0.7 cm or more, about 1 cm or more, about 2 cm or more, about 3 cm or more, about 5 cm or more, about 8 cm or more, about 10 cm or more, about 13 cm or more, about 15 cm or more, about 18 cm or more, about 20 cm or more, about 23 cm or more, about 25 cm or more, about 27 cm or more, or about 30 cm or more. In one aspect, the length of the elongated sheet or roll implant scaffold is about 1 cm or less, about 2 cm or less, about 3 cm or less, about 5 cm or less, about 8 cm or less, about 10 cm or less, about 13 cm or less, about 15 cm or less, about 18 cm or less, about 20 cm or less, about 23 cm or less, about 25 cm or less, about 27 cm or less, about 30 cm or less, about 40 cm or less, about 50 cm or less, or about 60 cm or less.

[0080] The scaffold according to some embodiments of the present invention may be used as an implant. An "implant" refers to any object that is designed to be placed partially or wholly within a patient's body for one or more therapeutic or prophylactic purposes such as for tissue augmentation, contouring, restoring physiological function, repairing or restoring tissues damaged by disease or trauma, and/or delivering therapeutic agents to normal, damaged or diseased organs and tissues. A "bone matrix implant" refers to a medical device or implant that includes a volume replacement material for augmentation or reconstruction to replace a whole or part of a bone structure. A predetermined shape of the implant may be varied to fit the implant site.

[0081] The invention further relates to methods culturing cells on the scaffold or aligned fiber described herein. In some embodiments, the cells are selected from the group consisting of stem cells, adipose derived stem cells, dental pulp stem cells, fibroblasts, and dorsal root ganglia.

[0082] For example, the invention provides for growing and/or culturing cells on a scaffold produced by the method described herein *in vivo* or *in vitro*. "Growing and/or culturing cells on a scaffold" includes traditional cell culture methods as well as placing on a surface in any setting, such as in natural or synthetic biocompatible matrices or tissues. In some embodiments, the cells that are cultured on the scaffold are stem cells. In further embodiments, the scaffold can be used in *in vitro* methods for supporting cell growth and proliferation as well as for increasing osteogenesis, chondrogenesis, or ligament/tendon genesis in the stem cells cultured on the scaffold.

[0083] Any cell described herewith may be cultured on a scaffold produced by the method described herein for between about 15 minutes and about 4 weeks, about 2 hours and about 2 weeks, about 2 hours and about 1 week, about 2 hours and about 72 hours, about 24 hours and about 72 hours, or about 24 hours and about 96 hours, at between about 20 °C and about 40 °C or about 30°C and about 37°C, in an atmosphere containing between about 1% CO_2 and about 10% CO_2 or about 4% CO_2 and about 6% CO_2, in certain embodiments.

[0084] The invention also relates to methods of promoting nerve repair using the aligned fiber, electrospun fiber, and/or scaffold described herein. In some embodiments, the nerve repair

includes, but is not limited to, central and peripheral nerve repair. The methods of promoting nerve repair may comprise implanting the aligned fiber, electrospun fiber, and/or scaffold into a nerve damaged site of a subject in need thereof. The method may further comprise culturing dorsal root ganglia and/or stem cells on the aligned fiber, electrospun fiber, and/or scaffold prior to the implanting. The stem cells, prior to culture on the scaffold of the present invention, may be undifferentiated or partially differentiated cells. The nerve regenerating activity of the aligned fiber, electrospun fiber, and/or scaffold may or may not be altered, including but not limited to, enhanced activity, relative to other scaffolds without the aligned and/or electrospun fiber described herein. The scaffolds for promoting nerve repair according to some embodiments of the present invention are in a form of an elongated sheet or a composite of multiple layers of sheets as discussed herein.

[0085] The invention also relates to methods of promoting osteoinductivity, with the methods comprising culturing cells on an aligned fiber, electrospun fiber, and/or scaffold produced by the method described herein. The cells, prior to culture on the aligned fiber, electrospun fiber, and/or scaffold of the present invention, may be undifferentiated or partially differentiated cells. The osteoinductive activity of the aligned fiber, electrospun fiber, and/or scaffold may or may not be altered, including but not limited to, enhanced activity, relative to other scaffolds without the aligned and/or electrospun fiber described herein.

[0086] The invention also relates to methods of promoting chondroinductivity, with the methods comprising culturing cells on a aligned fiber, electrospun fiber, and/or scaffold produced by the method described herein. The cells, prior to culture on aligned fiber, electrospun fiber, and/or scaffold of the present invention, may be undifferentiated or partially differentiated cells. The chondroinductive activity of the aligned fiber, electrospun fiber, and/or scaffold may or may not be altered, including but not limited to, enhanced activity, relative to other scaffolds without the aligned and/or electrospun fiber described herein.

[0087] The invention also relates to methods of promoting ligament/tendon differentiation, with the methods comprising culturing cells on an aligned fiber, electrospun fiber, and/or scaffold produced by the method described herein. The cells, prior to culture on the aligned fiber, electrospun fiber, and/or scaffold of the present invention, may be undifferentiated or partially

differentiated cells. The ligament/tendon differentiation activity of the aligned fiber, electrospun fiber, and/or scaffold may or may not be altered, including but not limited to, enhanced activity, relative to other scaffolds without the aligned and/or electrospun fiber described herein.

[0088] In one aspect, *in vitro* alkaline phosphatase assays may be used to evaluate osteoinductivity in cells cultured on the scaffold prepared by the methods described herein. The ability of the aligned fiber, electrospun fiber, and/or scaffold prepared by the methods of the present invention to stimulate or induce the alkaline phosphatase expression in an otherwise non-bone forming cells, such as myoblast (C2C12 cells), would indicate that the aligned fiber, electrospun fiber, and/or scaffold of the present invention has osteoinductive activity. In these assays, cells cultured on other scaffolds without the aligned and/or electrospun fiber described herein are used as negative controls to show that the baseline alkaline phosphatase expression on non-bone forming cells. The baseline of the osteoblastic markers in the negative control need not be zero, meaning that the cells in the negative control group may have at least some level of phenotypic marker(s). Accordingly, an "osteoinductive" scaffold of the present invention would simply cause an increase in the osteoblastic markers in experimental cells over control grown on the other scaffolds without the aligned and/or electrospun fiber.

[0089] Moreover, osteoinductivity, chondroinductivity, and ligament/tendon differentiation may be determined in tissue culture by investigating the ability of the aligned fiber, electrospun fiber, and/or scaffold prepared by the methods of the present invention to differentiate or induce osteoblast phenotype, chondrocyte phenotype, ligament/tendon cell phenotype in cultured cells, such as primary cells, cell lines, or explants. For example, the cells may display increased production of a marker characteristic of osteoblasts and/or chondrocytes, such as alkaline phosphatase, etc. For example, the osteoinductive, chondroinductive, ligament/tendon differentiation potentials of the aligned fiber, electrospun fiber, and/or scaffold described herein may be more than 0.2, 0.4, 0.6, 0.8, 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 times greater than the fiber and/or scaffolds without the aligned and/or electrospun fiber. In another example, the osteoinductive, chondroinductive, ligament/tendon differentiation potentials of the culture on the scaffold and/or implant described herein may be more than 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 500 or even 1000 times greater than those of the aligned fiber, electrospun fiber, scaffold and/or implant without any aligned and/or electrospun fiber. Of course, this indicates that lower

concentrations of modified growth factor, compared to unmodified growth factor are required to achieve the same effects.

[0090] Osteoinductivity, chondroinductivity, ligament/tendon differentiation, for assessing the bone, cartilage, ligament or tendon forming potential induced by the scaffold of the present invention in a location such as muscle, may also be evaluated using a suitable animal model.

[0091] The invention also relates to methods of promoting cell attachment, proliferation or maintaining the differentiated state or preventing de-differentiation of osteoblasts, chondrocytes, ligament cells, tendon cells and/or any cell type disclosed herein with the methods comprising culturing the cells on a scaffold produced by the method described herein. The proliferative activity of the aligned fiber, electrospun fiber, and/or scaffold may or may not be altered, including but not limited to, enhanced activity, relative to a fiber and/or scaffold without any aligned and/or electrospun fiber.

[0092] Mitogenicity may be assessed by investigating cell proliferation induced by the aligned fiber, electrospun fiber, and/or scaffold prepared by the methods of the present invention using various in vitro assays that measure metabolic activity, such as MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, alamarBlue® assay, and others. The alamarBlue® assay uses a non-cytotoxic reduction-oxidation indicator to measure cell metabolic activity, making it a nondestructive assay for assessing the mitogenic activity of the aligned fiber, electrospun fiber, and/or scaffold described herein.

[0093] The invention also relates to methods of increasing or promoting osteogenesis, chondrogenesis, or ligament/tendon genesis in cells. The methods may comprise culturing the cells on an aligned fiber, electrospun fiber, and/or scaffold produced by the method described herein. The osteogenic, chondrogenic, ligament, or tendon inducing activity of the aligned fiber, electrospun fiber, and/or scaffold may or may not be altered, including but not limited to, enhanced activity, relative to a fiber and/or scaffold without aligned and/or electrospun fiber described herein.

[0094] The invention also relates to methods of treating a tissue or organ defect or injury, for example, a musculoskeletal, dental or soft-tissue defect or injury, in an animal comprising administering (1) cells cultured on the scaffold produced by the methods described herein and/or (2) the aligned fiber, electrospun fiber, scaffold and/or implant described herein to the tissue or organ defect (e.g. osseous defects, defects in cartilage, ligament, tendon, spinal disk, and tendon insertion site to bone).

[0095] The invention further relates to methods of treating a tissue or an organ defect or injury, for example, including, but not limited to a musculoskeletal, dental or soft-tissue defect, or a nerve defect in an animal by applying a aligned fiber, electrospun fiber, and/or scaffold prepared by the methods described herein to the defect, and application to the defect may be accomplished by injecting the scaffold into the defect, inserting the aligned fiber, electrospun fiber, and/or scaffold between tissue or organ, or placing the aligned fiber, electrospun fiber, and/or scaffold on top of the defect. The present invention is also directed to treating a defect or injury in an organ by applying an aligned fiber, electrospun fiber, and/or scaffold to the defect. In one embodiment, the tissue defect may be treated by applying an elongated sheet described herein to surround the defect, or by applying an elongated roll implant described herein between the tissue defect or gap. The tissue defect or gap may be from the tissues including, but not limited to, bone, tendon, ligament, blood vessel, skin, intestine, nerve, and cartilage. Optionally, the elongated sheet may be applied around the tissue defect, and/or the elongated roll implant may be applied to facilitate surgery and healing. For example, the elongated implant roll may be applied between the tissue defect or gap, then the ends of the elongated implant roll may be sutured to the tissue. In some embodiments, the elongated sheet may be applied or sutured around the tissue defect or gap, covering the elongate implant roll and/or the suture sites of the elongated implant roll with tissues. In additional embodiments, the elongated roll may facilitate the tissue growth through the defect gap. The elongated sheet can prevent adhesion of the elongated implant roll with surrounding tissue or prevent undesirable tissue ingrowth, for example, scar formation, to interfere the tissue regeneration or remodeling. The biomaterials used for preparing the enlongated sheet and the enlongated implant roll may be same or different, depending on the specific surgery and tissue regeneration needs. The tissue defect may occur in tendon, ligament, nerve and other tissue types enlisted previously.

[0096] In yet another embodiment, cells may be seeded onto an aligned fiber, electrospun fiber, and/or scaffold prepared by the methods provided herein. The cells seeded on the scaffold can be any cell, such as but not limited to, dorsal root ganglia, osteoblasts, chondrocytes, ligament cells, tendon cells, progenitor cells, and stem cells disclosed herein or otherwise known in the art. The seeded cells may be allowed to proliferate and possibly attach to the matrix. Methods of seeding cells onto matrices, such as collagen matrix coated with the aligned fiber, electrospun fiber, and/or scaffold prepared by the methods provided herein, are well known in the art.

[0097] While the invention has been described and illustrated herein by references to various specific materials, procedures and examples, it is understood that the invention is not restricted to the particular combinations of material and procedures selected for that purpose. Numerous variations of such details can be implied as will be appreciated by those skilled in the art. It is intended that the specification and examples be considered as exemplary, only, with the true scope and spirit of the invention being indicated by the following claims. All references, patents and patent applications referred to in this application are herein incorporated by reference in their entirety herein.

[0098] The following examples are illustrative and are not intended to limit the scope of the invention described herein.

[0099] Example 1:

[00100] 1,1,1,3,3,3-hexafluoro-2-propanol (HFP) (CAS# 920-66-1) was from Acros Organics (Geel, Belgium, acros.com). Polycaprolactone (PCL) (B6003-1, B6003-2) was from Durect Lactone (Pelham, AL, absorbables.com). The electrospinning apparatus was designed using an Aladdin Programmable Syringe Pump from World Precision Instruments, Inc. (Sarasota, FL, wpiinc.com) and two adjustable high voltage power supplies from Gamma High Voltage Research (Ormond Beach, FL). The aligned metal rods used in the rotating motor, or statically, may be of steel, stainless steel, copper, or other heavy metal. A Mercotac® connector (#110T and 110TS) was used as an electrically conductive bearing. The following items were purchased from McMaster-Carr:

8965K42 Ultra Conductive Copper (Alloy 101) Rod, 1/4" Diameter

1256T14	Multipurpose Stainless Steel (Type 304) Mirror Finish #8, 1/4" Diameter
88855K51	High-Strength Stainless Steel (Type 17-4 Ph) 1/4" Diameter
86985K31	High-Strength Aluminum (Alloy 2024) 1/4" Diameter
8965K86	Ultra Conductive Copper (Alloy 101) Tube, 1/4" OD, .186" ID, .032" Wall Thk
8457K536	Stainless Steel Shape Type 304/304L, Round Tube, 1/4" OD
1968T11	High-Strength Aluminum Tube (Alloy 2024) 1/4" OD, .180" ID, .035" Wall Thickness
2706A4	Tube Cutter 1/4" to 1-5/8" Tube OD, 6" Open/Closed Length
4575N3	Miniature Flange-Mounted SS Ball Bearing Shielded, for 1/4" Shaft Diameter
6384K352	Steel Ball Bearing Flanged Double Sealed for 1/4" Shaft Dia, 11/16" OD
8600N3	Miniature Alum Base-Mnt SS Ball Brng--ABEC-3 for 1/4" Shaft Diameter
7200K3	NEMA 34 Face-Mount Brushless DC Motor 1/3 hp, 3450 rpm, Integrated Speed Control
6099K41	Stainless Steel One-Piece Set-Screw Coupling 1/2" Bore, 1-1/2" Length, 1" OD, with Keyway
8774K33	Static-Dissipative Clear Cast Acrylic Sheet 1/4" Thick, 12" X 24"
97042A516	18-8 Stainless Steel One-End Threaded Stud 1/2"-13 x 5/8" X 1-1/2"
99223A067	Acetal Hex Nut 1/2"-13 Thread
94564A023	Nylon Flat Point Socket Set Screw 6-32 X 1/4"
9986K21	Black Delrin Rod 4" Diameter, 1/2" Length
8576K15	Black Delrin Rod 1/2" Diameter, 5' Length
94922A050	Nylon 6/6 Acorn Nut Off-White, 1/2"-13 Thread Size, 3/4" W, 13/16" H
8572K61	White Delrin Rod 1" Diameter, 1' length
8582K21	White Delrin Rod 4" Diameter, 1/2" Length
8572K55	White Delrin Rod 1/2" Diameter

93140A839 Polycarbonate Machine Screw Flat Head Slotted, 1/4"-20 X 3/4"

95868A148 Nylon 6/6 SHCS 6-32 Thread, 1/2" Length, Off-White

7643A421 1" x 30', Gray, .012" Thk, High-Temp Self-Fusing Silicone Rubber Tape

7586K12 Adhesive-Backed Cable Holder Press in, 1/4" Maximum Bundle

60015K42 Tear-Resistant Rubber Vibration Damping Pad 6" X 6" X 3/8" Thick, 45 PSI Max Load

4056K42 Oil-Resistant Nitrile Vibration Damping Pad 6" X 6" X 5/16" Thick, 100 PSI Max Load

94605A541 Nylon 82 Deg Flat Head Slotted Machine Screw 1/4"-20 X 3/4"

94564A080 Nylon Flat Point Socket Set Screw 1/4"-20 X 1/2"

5537K26 Tinned Copper Expandable Mesh Sleeving 1/4" ID, 3/16" to 5/16" Bundle Dia

8491A614 Steel Press-Fit Drill Bushing/Liner 0.257" (F) ID, 13/32" OD, 1/2" Length

3504T21 Push-on Round FDA Cap Fits 3/8" OD, 1/2" Inside Height

92805K22 Push-on High-Temperature Silicone Rubber Cap Fits .6" Outside Diameter, 1-1/2" Inside Height

[00101] A high voltage power supply source was used to apply a +10-40kV DC voltage to parallel metal rods, with a -5-40kV DC field applied to the syringe/needle containing a polymer solution, with syringe placed in a syringe pump. The metal posts can be hollow or solid, and have been tested as rods of stainless steel, steel, copper, and aluminum, both solid and hollow using thin-walled tubing. The absolute DC field strength ranged from 15kV to 60kV. A programmable syringe pump was set to dispense the solution at 0.50-9.50 mL/hr, ideally at 6.5ml/hr for pure PCL, and 3ml/hr for heart basement membrane (HBM) and HBM/PCL mixtures. After approximately 5 minutes to 1 hour of electrospinning, the aligned fibers are collected for imaging (SEM and DIC).

[00102] Metal rods were used to align electrospun PCL. PCL dissolved in HFP (100mg/ml) at a +15kV reference, set 15cm apart, was electrospun between the rods, with PCL pumped at 5ml/hr over 5 minutes to the form the aligned nanofibers seen collecting between the rods. **Figure 1 (a,**

d) shows the representative electric field lines and the know bending of these lines in a field with a split point charge (as simplified plane of point charges represented). This splitting of the electric charge field is believed to act on imparting alignment as they travel on the looping fibers in this warped electrostatic field. We have successfully aligned fibers with hollow and solid metal rods, and the modulation of the electric field lines by solid vs. hollow rods is also shown **(figure 1b and 1c)** (electric field diagrams created using the applet from cco.caltech.edu/~phys1/java/phys1/EField/EField.html).

[00103] For SEM images, aligned electrospun PCL and HBM/PCL blended samples were taken from the aligned rods and mounted on aluminum stubs using carbon stickers. The samples were then coated with gold at about 50nm thickness using a plasma-based sputter coater. Coated samples were imaged using a JOEL 6400 scanning electron microscope (SEM) with Orion image processing. Representative images of charged parallel rod aligned fibers, as compared to fibers generated in the traditional, randomly aligned fashion by collection onto a static piece of grounded foil, can be seen in **figure 2**. The average cross-sectional fiber diameter of the electrospun fibers generated from random and charged rod aligned basement membrane matrix was calculated by measuring 35 unique points in ImageJ64 (NIH shareware), with results shown in **figure 2**, along with the average angle of the fibers. A fast Fourier transform (FFT) was performed to indicate the degree of alignment as seen in **figure 3** using the ImageJ64 FFT tool, with patterns of aligned fibers shown to match with those of know aligned Fourier spectra, as commonly used for pattern recognition.

[00104] **Figures 4** illustrates a design for rotating the charged parallel rods using a mercury coupled bearing to allow electrical conductance with the rotating charged rods mounted in plastic platforms attaching to a high speed motor (0-4000RPMs). The actual implementation of this rotating rod design is shown in **figure 5**. In another aspect, this rotating charged parallel point charged rod fiber alignment process may be modified so that electrospun fibers of opposite charge are propelled towards each other in the static field, whereby the fibers combine in a "whirlpool" fashion roughly equidistant apart in space. In this arrangement, the fiber charges are effectively neutralized and the fibers are then collected on the grounded rotating rods of the mandrel, which allows a thicker layer of fibers to be collected due to less fiber-to-fiber charge repulsion. Equally charged fibers are believed to have a repulsive effect on each other in the

collected fibers. Charge neutralization of the fibers in this fashion theoretically negates this phenomenon to electrospin sheets that are up to about 1.5 times, 2.0 times, 3.0 times, or 3.4 times the thickness of conventionally electrospun fiber sheets. For example, two syringe pumps with basement membrane, each attached to a high voltage power supply may have one supply set to positive (+) voltage, and the other set to negative (-) voltage to generate the static electric field for electrospinning, with fibers collected on the rotating or static charged rods. The charges of equal voltage and opposite charge combine in space above the dual charged rod mandrel in a the vortex of the electric field, depositing as charge neutralized fibers on the rods, which allows for enhanced thickness of the collected aligned fibers over conventional methods.

[00105] By implementing a pneumatic motor in the conformations for electrospinning aligned (and random) nanofibers onto a mandrel as described above, on average 9.24% more material is collected on the mandrel(s) compared to using an electrical motor.

[00106] Example 2: Heart Base Membrane Fiber Scaffold

[00107] Heart basement membrane (HBM) and polycaprolactone (PCL) were dissolved separately in HFP at 100mg/ml, and mixed in a 10% ratio of HBM:PCL (0.100g HBM to 0.900g PCL), mixed at 4deg a total of 2hrs until fully into solution. The HBM:PCL was loaded into a glass 5ml syringe, loaded into a syringe pump set to expel at 1.5ml/ml, in a static +20kV electric field at the needle and -10kV to each of the parallel aligned collecting rods, then electrospun between the poles in a highly aligned fashion. The aligned fibers of HBM/PCL were collected and stored in a dessicator prior to use. To obtain dorsal root ganglion (DRGs), an 11 day old YFP-βIII Tubulin expressing transgenic mouse was sacrificed for collection of the spinal cord and removal of the DRGs, which were liberated from their capsule and briefly digested in trypsin, then placed upon the aligned HBM:PCL scaffold and cultured in basic neuron media containing B27 supplement. As shown in **figure 6**, outgrowths from the DRGs were visualized under light and fluorescent microscopy at 1 and 2 days post explantation, with aligned neurons seen erupting from the bundle, with the neurite outgrowths seen tracing the aligned fibers.

[00108] Example 3: Dorsal Root Ganglia Attachment and Outgrowth on Placenta and Heart Basement Membrane Nanofibers.

[00109] Heart basement membrane HBM and polycaprolactone (PCL) were dissolved separately in HFP at 100mg/ml, and mixed in a 10% ratio of HBM:PCL (0.100g HBM to 0.900g PCL), mixed at 4deg a total of 2hrs until fully into solution. Each biopolymer was individually loaded into a glass 5ml syringe, loaded into a syringe pump set to expel at 2.5ml/hr, in a static +20kV electric field at the needle, then electrospun onto 10mm glass coverslips backed by a grounded piece of foil. The randomly aligned fibers were collected and stored in a dessicator prior to use. To obtain dorsal root ganglion (DRGs), a 10 day old YFP-βIII Tubulin expressing transgenic mouse was sacrificed for collection of the spinal cord and removal of the DRGs, which were liberated from their capsule and briefly digested in trypsin, then placed upon the scaffolds and cultured in basic neuron media containing B27 supplement for 7 days. As shown in **figure 6**, Outgrowths from the DRGs were again visualized under light and fluorescent microscopy at 1-7 days post explantation. Neurite outgrowth was seen tracing the electrospun fibers on HBM/PCL coated coverslips.

[00110] Example 4: Dorsal Root Ganglia Attachment and Outgrowth on Polycaprolactone Nanofibers (Comparative Example)

[00111] Polycaprolactone (PCL) was dissolved in HFP at 100mg/ml and mixed by vortexing at room temperature for 2hrs until fully into solution. The PCL biopolymer was loaded into a glass 5ml syringe, loaded into a syringe pump set to expel at 5ml/h, in a static +20kV electric field at the needle, then electrospun onto 10mm glass coverslips backed by a grounded piece of foil. The randomly aligned fibers of each group were collected and stored in a dessicator prior to use. To obtain dorsal root ganglion (DRGs), an 10 day old YFP-βIII Tubulin expressing transgenic mouse was sacrificed for collection of the spinal cord and removal of the DRGs, which were liberated from their capsule and briefly digested in trypsin, then placed upon the scaffolds and cultured in basic neuron media containing B27 supplement for 7 days (n=3). Outgrowths from the DRGs were visualized under light and fluorescent microscopy at 1-7 days post explantation. DRGs did not grow out on PCL and were found to be non-viable after 2-3 days in repeat trials (n=6).

PTO/SB/08a (03-15)
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Application Number	16045159
Filing Date	2018-07-25
First Named Inventor	Michael Francis
Art Unit	TBD
Examiner Name	TBD
Attorney Docket Number	LNH-152US1

U.S.PATENTS

Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1	6189537	B1	2001-02-20	Wolfinbarger, L.	
	2	6305379	B1	2001-10-23	Wolfinbarger, L.	

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	1	20020090725	A1	2002-07-11	Simpson et al.	
	2	20040037813	A1	2004-02-26	Simpson et al.	
	3	20070225631	A1	2007-09-27	Bowlin et al.	
	4	20080213389	A1	2008-09-04	Lelkes et al.	

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	5	20090018643	A1	2009-01-15	Hashi et al.	
	6	20100233115	A1	2010-09-16	Patel et al.	
	7	20110236974	A1	2011-09-29	Ogle et al.	
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	1	2012027592	WO	A2	2012-03-01	LifeNet Health		☐
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	1	Anderson et al., "Biodegradation and Biocompatibility of PLA and PLGA Microspheres", Advanced Drug Delivery Reviews, 1997, Vol. 28, pp. 5-24	☐
	2	Ayres et al., "Measuring Fiber Alignment in Electrospun Scaffolds: A User's Guide to the 2D Fast Fourier Transform Approach," Journal of Biomaterials Science, Polymer Edition, (2008), Vol. 19, No. 5, pp. 603-621	☐
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	5	European Communication for European Application No. 14 773 677.1, dated November 6, 2017 - 8 pages	☐
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	10	Schnaper et al., "Regulation of Cell Function by Extracellular Matrix," Pediatr. Nephrol, 1993, Vol. 7 - pp. 96-104	☐
	11	Supplementary European Seach Report for European Application No. 14 773 677.1, dated October 25, 2016 - 3 pages	☐
	12	Zeugoils et al., "Electro-spinning of Pure Collagen Nano-fibres - Just an Expensive Way to Make Gelatin", Biomaterials, Vol. 29, 2008, pp. 2293-2305	☐
	13	Entire patent prosecution history of U.S. Patent Application No.14/776,106, filed September 14, 2015, entitled, "ALIGNED FIBER AND METHOD OF USE THEREOF."	☐

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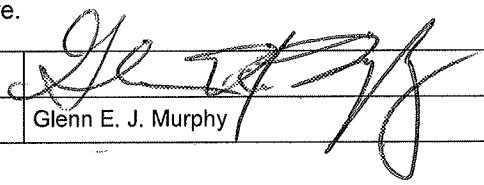
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(54) **Title**: COMPOSITION FOR A TISSUE REPAIR IMPLANT AND METHODS OF MAKING THE SAME

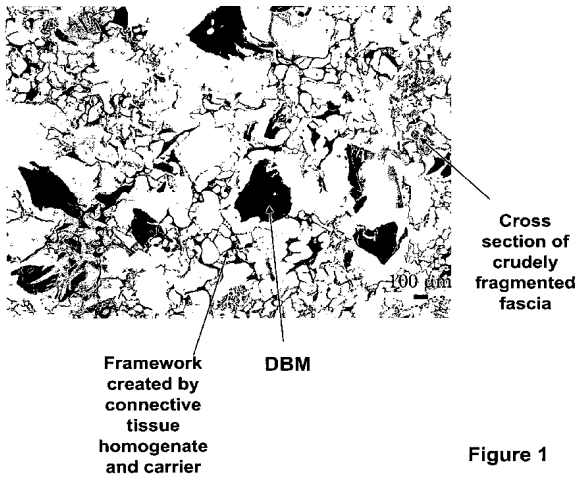


Figure 1

(57) **Abstract**: The invention is directed to a process for making a tissue repair implant having a porous sponge-like structure to repair bone, cartilage, or soft tissue defects by producing a connective tissue homogenate from one or more connective tissues, mixing the connective tissue homogenate with a carrier solution to produce a connective tissue carrier, optionally mixing one or more natural or synthetic bone fragments with said connective tissue carrier to produce a tissue repair mixture, freezing or freeze-drying the tissue repair mixture to produce a porous sponge-like structure and create a three-dimensional framework to entrap the natural or synthetic bone fragments, treating the frozen or freeze-dried porous sponge-like structure with one or more treatment solutions to produce a stabilized porous sponge-like structure A crudely fragmented connective tissue from one or more connective tissues is optionally mixed with the tissue repair mixture before freezing or freeze-drying.

WO 2010/016942 A1

Composition for a Tissue Repair Implant and Methods of Making the Same

Inventors: Silvia S. Chen, Jingsong Chen, and Lloyd Wolfinbarger

RELATED APPLICATION

[0001] The present invention claims priority to U.S. Application 12/188,127, filed August 7, 2009, which is incorporated herein in its entirety.

FIELD OF THE INVENTION

[0002] The present invention is related to a tissue repair implant having a porous sponge-like structure and to methods of preparing such an implant. The present invention is also related to methods of using the implant to repair defective tissues.

BACKGROUND OF THE INVENTION

[0003] The ability to promote tissue regrowth in vivo can facilitate wound healing and post-surgical recovery of patients who have suffered tissue damage or destruction. The need for such methods and compositions is readily apparent, when considering that in 1999, approximately 500,000 bone graft procedures were performed in the United States alone. Ideal bone graft materials for use in such procedures possess characteristics necessary to new bone growth, namely osteoconductivity and osteoinductivity.

[0004] Osteoconductivity refers to a graft's ability to support the attachment of new osteoblasts and osteoprogenitor cells. The osteoconductive components of a graft provide an interconnected structure through which new cells can migrate and new blood vessels can form. Osteoinductivity refers to the ability of a graft to induce nondifferentiated stem cells or osteoprogenitor cells to differentiate into osteoblasts.

[0005] In 1998, 9 of 10 bone graft procedures performed in the United States involved the use of either autograft or allograft bone tissue. Despite the benefits of autografts and allografts, the limitations of each have necessitated the pursuit of alternative graft materials. Using basic criteria necessary to a successful graft (e.g., osteoconduction and osteoinduction), investigators have developed several bone graft substitutes. These can contain a variety of materials, including natural and synthetic polymers, ceramics, and composites; and in some instances, production of bone graft

substitutes can involve biotechnological strategies (i.e., factor- and/or cell-based strategies).

[0006] Osteoinductive substances found in some bone graft substitutes are demineralized bone particles and/or powder. Contained in the extracellular matrix of bone tissue is a full cocktail of bone growth factors, proteins, and other bioactive materials necessary for osteoinduction and, ultimately, successful bone healing. To capitalize on this cocktail of proteins, bone tissue can be demineralized, leaving the osteoinductive agents in the demineralized bone matrix (DBM). Such osteoinductive DBM can be incorporated into a number of different bone graft substitutes.

[0007] While a number of different materials thought to enhance osteoconductivity (i.e., purified or partially purified polymers) have been used in DBM bone graft substitutes; new, more easily prepared, osteoconductive/structural materials for combining with DBM to produce a bone graft substitute are desirable.

SUMMARY OF THE INVENTION

[0008] The present invention provides a biologically functional tissue repair implant having a stable porous sponge-like structure. In one embodiment, the functionally active tissue repair implant of the present invention comprises a uniform composition containing a connective tissue homogenate and a carrier that are evenly distributed to form a three-dimensional framework. The carrier may be a stabilizer of the porous sponge-like structure. The carrier may be a composition containing one or more polysaccharides, polymers, salts, bioactive factors, and/or photoactive agents. The carrier may also be a solution including one or more polysaccharides, polymers, salts, bioactive factors, and/or photoactive agents, and the carrier may be a composition comprising one or more solutes of the carrier solution.

[0009] The tissue repair implant of the present invention may also consist essentially of evenly distributed connective tissue homogenate and carrier in a three-dimensional framework. In another embodiment, the functionally active tissue repair implant comprises a uniform composition further containing bone fragments interspersed in the connective tissue homogenate and carrier framework. The bone fragments are distributed throughout the framework. The tissue repair implant of the present

invention may consist essentially of evenly distributed connective tissue homogenate, carrier, and bone fragments in a three-dimensional framework.

[0010] The tissue repair implant of the present invention may be preshaped, elastic, resilient, and insoluble in water. The implant may maintain its shape in a dry or a wet state. In its dry state, the implant will quickly absorb fluid because it is porous. For example, in its wet state, the implant will maintain its porosity, cohesiveness, and integrity.

[0011] The connective tissue homogenate may contain one or more connective tissues. The bone fragments may be natural or synthetic bone fragments.

[0012] The present invention also discloses methods for producing a biologically functional tissue repair implant having a stable porous sponge-like structure. In one embodiment, the method comprises: (a) producing a connective tissue homogenate from one or more connective tissues; (b) mixing the connective tissue homogenate with a carrier solution to produce a connective tissue carrier; (c) mixing one or more natural or synthetic bone fragments with the connective tissue carrier to produce a tissue repair mixture; (d) freezing or freeze-drying the tissue repair mixture to produce a porous sponge-like structure and create a framework to entrap the natural or synthetic bone fragments; and (e) treating the frozen or freeze-dried porous sponge-like structure with one or more treatment solutions to produce a stabilized porous sponge-like structure. Optionally, a crudely fragmented connective tissue from one or more connective tissues may be mixed with the tissue repair mixture before freezing or freeze-drying.

[0013] In another embodiment, the method for preparing a tissue repair implant having a stable porous sponge-like structure comprises: (a) producing a connective tissue homogenate from one or more connective tissues; (b) mixing the connective tissue homogenate with a carrier solution to produce a tissue repair mixture; (c) freezing or freeze-drying the tissue repair mixture to produce a porous sponge-like structure and create a framework; and (d) treating the frozen or freeze-dried porous sponge-like structure with one or more treatment solutions to produce a stabilized porous sponge-like structure. Optionally, a crudely fragmented connective tissue

from one or more connective tissues may be mixed with the tissue repair mixture before freezing or freeze-drying.

[0014] In another embodiment, the method for preparing a tissue repair implant having a stable porous sponge-like structure comprises: (a) producing a connective tissue homogenate from one or more connective tissues; (b) mixing the connective tissue homogenate with one or more natural or synthetic bone fragments to produce a mixture; (c) adding a carrier solution to the mixture to produce a tissue repair mixture; (d) freezing or freeze-drying the tissue repair mixture to produce a porous sponge-like structure and create a three-dimensional framework to entrap the natural or synthetic bone fragments; and (e) treating the frozen or freeze-dried porous sponge-like structure with one or more treatment solutions to produce a stabilized porous sponge-like structure.

[0015] Moreover, the steps set forth above for preparing the tissue repair implant may be combined or the sequential order of the steps may be changed. As an example, the carrier solution may be added to the one or more connective tissues for making the connective tissue homogenate. The carrier solution may replace the water that is added to the connective tissues to make the homogenate.

[0016] The stabilized porous sponge-like structure may be treated with a water replacing agent such as glycerol (glycerin USP), adonitol, sorbitol, ribitol, galactitol, D-galactose, 1,3-dihydroxypropanol, ethylene glycol, triethylene glycol, propylene glycol, glucose, sucrose, mannitol, xylitol, meso-erythritol, adipic acid, proline, hydroxyproline, polyethylene glycol, alcohol, or lipids. The stabilized porous sponge-like structure may also be freeze-dried and/or sterilized. Sterilization may be carried out using gamma irradiation, supercritical solution, ethylene oxide, or electronic-beam. Sterilization may be carried out using supercritical fluid. As an example, sterilization may be carried out with supercritical fluid carbon dioxide.

[0017] The methods of the present invention may include combining the tissue repair implant with one or more bioactive supplements. The bioactive supplements may be growth factors, differentiation factors, cytokines, or anti-inflammatory agents. The growth or differentiation factors may be a growth factor of the the FGF-family or

TGF-family, IGF-1, PDGF, EGF, VEGF, HGF, PTHrP, Ihh, dexamethasone, insulin, transferrin, selenium, ITS, ascorbate, or a combination thereof. The cytokines may be GM-CSF, G-CSF, TNF-α, IL-1β, IL-4, IL-6, IL-8, IL-10, SLP1, MCP1, MIP-1α, MIP-2, IL-18, angiopoietin, KGF, endothelin, IFN-α, or IFN-β. The anti-inflammatory agents may be an IL-1αR antibody, TNF-a receptor antagonist, cyclooxygenase-2 specific inhibitors, MAP kinase inhibitors, NO synthase inhibitors, NF-κB inhibitors, or inhibitors of MMP. There are various fibroblast growth factors. As an example, the human FGF-family includes 22 members, FGF-1 through FGF-23. (There is no human FGF-15 because FGF-15 is the mouse ortholog of human FGF-19.) Examples of members of the TGF-family may include TGF-α and TGF-β superfamily. The TGF-β superfamily includes TGF-βs (such as TGF-β1, TGF-β2, TGF-β3), activins, inhibins, bone morphogenic factors (BMPs), anti-mullerian hormone (AMH), myostatins, and others.

[0018] BMPs are members of the transforming growth factor (TGF) superfamily, which are a phytogenetically conserved group of proteins. Structurally, the BMPs display extensive conservation among species having seven characteristic cysteine knot domains that participate in the formation of an interchain disulfide bond between monomers to form biologically active dimers. There are 20 isotypes of BMPs. They may be separated into four subfamilies, for example, (1) BMP2 and BMP4; (2) BMP3 and BMP3B (also known as growth/differentiation factor 10 (GDF10)); (3) BMPs 5, 6, 7 and 8; and (4) GDFs 5, 6, and 7.

[0019] The tissue repair implant may also be combined with one or more agents having bioactive supplement binding sites comprising hyaluronan, heparin, heparin sulfate, keratin sulfate, dermatan sulfate, chondroitin sulfate, betaglycan, heparan sulfate proteoglycan, syndecan, biglycan, or decorin. The one or more bioactive supplement binding sites on the agents may increase the affinity of growth factors, differentiation factors, cytokines, or anti-inflammatory agents to said tissue repair implant. Accordingly, the present invention also discloses a tissue repair implant comprising one or more bioactive supplements and one or more agents having bioactive supplement binding sites.

[0020] The methods of the present invention provide a tissue repair implant having a pore size that may be larger than about 50 microns and a void volume that may be from about 10% to about 95% or from about 30 to 80%. The tissue repair implant prepared by the present invention may be in the form of a rod, sheet, cube, tube, particle, sphere, ellipsoid, wedge, or ribbon.

[0021] The connective tissue homogenate used in the methods of the present invention may be made from one more connective tissues of human and/or animal origins. The connective tissue may comprise dermis, fascia, cartilage, tendon, ligament, pericardium, fat, muscle, urethra, small intestine, demineralized bone, non-demineralized bone, or a combination thereof.

[0022] The connective tissue in the connective tissue homogenate used in the methods of the present invention may be cleaned and/or disinfected. The connective tissue may also be devitalized to remove cellular components. The connective tissue may be freeze dried.

[0023] In the methods of the present invention, the connective tissue homogenate may be prepared by homogenizing the connective tissue at temperature from about 15 °C to about 100 °C for a period of time of about 0.5 minutes to about 30 minutes. The homogenization may be carried out in the presence of a solution. The weight percentage of the connective tissue homogenate in the tissue repair implant may be no more than 80% in the dry state, no more than 50% in the dry state, or no more than 20% in the dry state.

[0024] The carrier solution used in the processes of the present invention may comprise one or more polysaccharides selected from the group comprising alginate, propylene glycol alginate, native or crosslinked chitosan, starch, cellulose and its derivatives (such as cellulose acetate, carboxymethyl cellulose, and methyl cellulose), xanthan gum, dextran, carrageenan, hyaluronic acid, chondroitin sulfate, locust bean gum, gum tragacanth, gum arabic, curdlan, pullulan, scleroglucan, or lower methoxylpectin. The carrier solution may comprise salts of calcium, barium, aluminum, strontium, copper, zinc, magnesium, manganese, cobalt, or iron; glutaraldehyde, glyceraldehyde, genipin, glucose or ribose, poly(ethylene glycol)

diepoxide crosslinker, poly(ethylene glycol) diglycidyl ether, EDC and NHS, transglutaminase, ethylenediamine, lysyl oxidase family, hexamethylene diisocyanate (HMDIC); dimethyl suberimidate (DMS), dimethyl-3-3'-dithiobispropionimidate (DTBP), or acryl azide. The carrier solution may also contain natural and or synthetic polymers such as but limited to native or modified collagen, gelatin, agarose, modified hyaluronic acid, fibrin, chitin, biotin, avidin, MATRIGEL®, HUMAN EXTRACELLULAR MATRIX®, proteoglycans, laminin, fibronectin, elastin, heparin, glycerol, sucrose octasulfate, polyethylene glycol, polymethylmethacrylate, polyurethane, acryloilmorpholine, N,N-dimethyl acrylamide, N-vinyl pyrrolidone and tetrahydrofurfuryl methacrylate, hydroxyapatite, cross-linkage or functionalization of hyaluronan-based collagen and alginate, polyurethane, polylactic acid, or a combination comprising at least one of the foregoing polymers.

[0025] In one embodiment, the carrier solution used in the methods of the present invention to make the tissue repair implant may comprise bioactive supplements. The bioactive supplements may be growth factors, differentiation factors, cytokines, or anti-inflammatory agents. The growth or differentiation factors may include but are not limited to a growth factor of the FGF-family or TGF-family, IGF-1, PDGF, EGF, VEGF, HGF, PTHrP, Ihh, dexamethasone, insulin, transferrin, selenium, ITS, ascorbate, or a combination thereof. Examples of cytokines may include GM-CSF, G-CSF, TNF-α, IL-1β, IL-4, IL-6, IL-8, IL-10, SLP1, MCP1, MIP-1α, MIP-2, IL-18, angiopoietin, KGF, endothelin, IFN-α, or IFN-β. The anti-inflammatory agents may include but are not limited to an IL-1αR antibody, TNF-α receptor antagonist, cyclooxygenase-2 specific inhibitors, MAP kinase inhibitors, NO synthase inhibitors, NF-κB inhibitors, or inhibitors of MMP. There are various fibroblast growth factors. As an example, the human FGF-family includes 22 members, FGF-1 through FGF-23. (There is no human FGF-15 because FGF-15 is the mouse ortholog of human FGF-19.) Examples of members of the TGF-family may include TGF-α and TGF-β superfamily. The TGF-β superfamily includes TGF-βs (such as TGF-β1, TGF-β2, TGF-β3), activins, inhibins, bone morphogenic factors (BMPs), anti-mullerian hormone (AMH), myostatins, and others.

[0026] There are 20 isotypes of BMPs. They may be separated into four subfamilies, for example, (1) BMP2 and BMP4; (2) BMP3 and BMP3B (also known as growth/differentiation factor 10 (GDF10)); (3) BMPs 5, 6, 7 and 8; and (4) GDFs 5, 6, and 7.

[0027] The carrier solution may comprise bioactive supplements extracted from tissue selected from the group comprising demineralized bone matrix, basement membrane, or submucosa matrix. In another embodiment, the carrier solution may also comprise antioxidants selected from the group comprising sodium nitroprusside, cartilage matrix glycoprotein (CMGP), vitamins C, vitamin E, selenium, N-Acetylcysteine (NAC) estradiol, glutathione, melatonin, resveratrol, flavonoid, carotene, aminoguanidine, or lycopene.

[0028] Alternatively, the carrier solution used in the methods of the present invention may comprise photoactive agents selected from the group comprising a xanthene dye, naphthalimide compounds, riboflavin-5-phosphate, N-hydroxypyridine-2-(1H)-thione, N-(20-ethylaminoethyl)-4-amino-1,8-naphthalimide, bis-diazopyruvamide-N,N9-bis(3-diazopyruvoyl)-2,29-(ethylenedioxy)bis-(ethylamine) (DPD); diazopyruvoyl (DAP); methylene blue, erythrosin, phloxime, thionine, methylene green, rose Bengal, acridine orange, xanthine dye, and thioxanthine dyes, ethyl eosin, eosin Y, or a combination comprising at least one of the foregoing photoactive groups. The photoactive agent may be activated using a UV light or a laser. The weight percentage of the carrier in the tissue repair implant may be no more than 20% in the dry state or no more than 5% in the dry state.

[0029] The natural or synthetic bone fragments materials used in the methods of the present invention to prepare the implants may comprise non-demineralized bone, partially demineralized bone, demineralized bone, ceramics, hydroxyapatite, calcium phosphate, calcium sulfate, calcium carbonate, or a combination of two or more of the above. The natural bone fragments may be from a human or an animal. The natural or synthetic bone fragments may be in the form of particles, fibers, rods, or cubes. The particles may have an average particle size of about 125 micron to about 2000 micron or an average particle size of about 250 micron to about 710 micron. The fibers may have an average width of 0.1 to 2 mm and an average width of

between about 0.3 mm and about 2.5 mm. Fibers may be of varying lengths. An average length of the fiber may range from about 0.3 mm to about 100 mm. The rods may have an average width of 0.5 to 3 mm and an average length of about 1 mm to about 100 mm. The cubes may have an average volume of about 0.001 mm^3 to an average volume of about 1000 mm^3.

[0030] The demineralized bone used in the methods of the present invention may have a residual calcium content of less than 8% or from about 1% to about 4%. The tissue repair implant may include demineralized bone fragments having between about 0.5 wt % and about 4 wt % residual calcium, about 1 wt% and about 4 wt% residual calcium, or about 2 wt% and about 4 wt% residual calcium in certain embodiments.

[0031] The weight percentage of the natural or synthetic bone fragments used in the methods of the present invention to prepare the tissue repair implant is about 0.1% to about 95% in the dry state, or about 10% to about 90% in the dry state.

[0032] The crudely fragmented connective tissue used in the methods of the present invention may be obtained from tissues that are cleaned, disinfected, optionally devitalized to remove cellular components, and optionally freeze-dried. The crudely fragmented connective tissue may be from tissues comprising fascia, cartilage, ligament, tendon, pericardium, muscle, urethra, small intestine, demineralized bone, dermis, partial demineralized bone, or non-demineralized bone.

[0033] The average diameter of the crudely fragmented connective tissue may be from about 0.01 to about 5 mm. The weight percentage of said crudely fragmented connective tissue in said tissue repair implant is about 0.25% to about 95% in the dry state, or about 0.5% to about 90% in the dry state.

[0034] In the process of the present invention, the tissue repair mixture may be placed in a mold before the freezing or freeze-drying step. The tissue repair mixture may be optionally processed under negative hydrostatic pressure before being frozen to increase porosity.

[0035] The treatment solution used in the methods of the present invention may comprise ionic, enzymatic, or chemical crosslinking agents; or photoactive agents. The treatment solution may comprise salts of calcium, barium, aluminum, strontium, copper, zinc, magnesium, manganese, cobalt, or iron; glutaraldehyde, glyceraldehyde, genipin, glucose or ribose, poly(ethylene glycol) diepoxide crosslinker, poly(ethylene glycol) diglycidyl ether, EDC and NHS, transglutaminase, ethylenediamine, lysyl oxidase family, hexamethylene diisocyanate (HMDIC); dimethyl suberimidate (DMS), dimethyl-3-3'-dithiobispropionimidate (DTBP), or acryl azide. The treatment solution may comprise natural and or synthetic polymers selected from the group comprising native or modified collagen, gelatin, agarose, modified hyaluronic acid, fibrin, chitin, biotin, avidin, demineralized bone matrix, MATRIGEL®, HUMAN EXTRACELLULAR MATRIX®, proteoglycans, laminin, fibronectin, elastin, heparin, glycerol, sucrose octasulfate, polyethylene glycol, polymethylmethacrylate, polyurethane, acryloilmorpholine, N,N-dimethyl acrylamide, N-vinyl pyrrolidone and tetrahydrofurfuryl methacrylate, hydroxyapatite, cross-linkage or functionalization of hyaluronan-based collagen and alginate, polyurethane, or polylactic acid, or a combination comprising at least one of the foregoing polymers.

[0036] The treatment solution used in the process of the present invention may comprise one or more bioactive supplements. The bioactive supplements may be growth factors, differentiation factors, cytokines, or anti-inflammatory agents. The treatment solution may also contain an agent having one or more bioactive supplement binding sites. The treatment solution may comprise antioxidants to protect bioactive components from oxygen-radical-induced damage.

[0037] In the methods of the present invention, one or more treating steps with one or more treatment solutions may be conducted simultaneously or sequentially to stabilize the porous sponge-like structure and further entrap the natural or synthetic bone fragments. The treatment may be carried out for a duration of about 5 minutes to about 16 hours, about 15 minutes to about 120 minutes, or about 30 minutes to about 60 minutes.

[0038] The present invention also provides a process for repairing a defect in a tissue, for example, bone, cartilage, or soft tissue. In one aspect, the process of the present invention comprises implanting the tissue repair implant having a porous sponge-like structure of the present invention into the site of defect in the tissue without rehydration to allow the tissue repair implant to absorb blood or fluid as well as autologous cells in situ. In another aspect, the process includes rehydrating the tissue repair implant having a porous sponge-like structure of the present invention with a rehydrating solution; optionally seeding cells on the tissue repair implant to render the tissue repair implant vital; optionally culture the cell-seeded tissue repair implant before implantation; implanting the tissue repair implant into the defect. The cells seeded on the implant may be vital cells.

[0039] The rehydrating solution may contain blood or bone marrow aspirate, platelet rich plasma, enzymes, bioactive supplements , natural polymers, synthetic polymers, photoactive agents, antioxidants, crosslinking agents, antimicrobial agents, vital cells, one or more agents that have bioactive supplement binding site(s), or a mixture of two or more of the above. The vital cells may be one or more than one type of cells from autologous or allograft bone marrow aspirate; stromal cells from bone marrow, stromal cells from fat, synovium, periosteum, perichondrium, muscle, dermis, umbilical cord blood, and Warton's jelly; or pericytes. The vital cells may be cells from the placenta, amniotic fluid, peripheral blood, and neural tissue. The vital cells may be stem cells that are totipotent, multipotent, pluripotent, and oligopotent. Examples of stem cells include but not limited to embryonic stem cells, adult stem cells, amniotic stem cells, and induced pluripotent stem cells.

BRIEF DESCRIPTION OF THE DRAWINGS

[0040] Figure 1 shows H&E staining of a porous sponge-like structure containing DBM comprised of a framework created by connective tissue homogenate and alginate, crudely fragmented fascia, and DBM particles. The porous sponge-like structure was freeze-dried and treated with $CaCl_2$.

[0041] Figure 2 shows the amount of fluid that various types of porous sponge-like structures can absorb.

[0042] Figure 3 shows the time required to re-hydrate various types of porous sponge-like structures.

[0043] Figure 4 shows the remaining percentage of dry weight of various types of porous sponge-like structures after 24 hours of collagenase treatment relative to the pre-treatment dry weights.

[0044] Figure 5 shows cell viability study using alamar blue assay. Three porous sponge-like structures with DBM particles and various alginate content, fluid content, and duration of $CaCl_2$ treatment were used for the study.

[0045] Figure 6 shows a representative hematoxylin and eosin (H&E) staining of an explanted DBM porous sponge-like structure demonstrating new bone formation.

[0046] Figure 7 shows a representative soft x-ray radiograph of an explanted DBM porous sponge-like structure demonstrating new bone formation in an athymic rat calvaria critical size defect.

[0047] Figure 8 shows a representative soft x-ray radiograph of an explanted DBM porous sponge-like structure demonstrating new bone formation (white arrows) in an athymic rat posterolateral lumbar spine fusion model.

DESCRIPTION OF THE INVENTION

[0048] The invention discloses a biologically functional tissue repair implant having a porous sponge-like structure useful for repairing defects in tissues. The tissues may be bone tissues, cartilage, or soft tissues. Examples of soft tissues may include tendon, ligament, dermis, and rotator cuff.

[0049] The invention also provides processes for making a tissue repair implant having a porous sponge-like structure to repair bone, cartilage, or soft tissue defects by producing a connective tissue homogenate from one or more connective tissues; mixing the connective tissue homogenate with a carrier solution to produce a connective tissue carrier; optionally mixing one or more natural or synthetic bone fragments with said connective tissue carrier to produce a tissue repair mixture;

freezing or freeze-drying the tissue repair mixture to produce a porous sponge-like structure and create a framework to entrap the natural or synthetic bone fragments; treating the frozen or freeze-dried porous sponge-like structure with one or more treatment to produce a stabilized porous sponge-like structure. A crudely fragmented connective tissue from one or more connective tissues is optionally mixed with the tissue repair mixture before freezing or freeze drying. The tissue repair implant having a porous sponge-like structure is optionally combined with one or more bioactive supplement or one or more agents that have bioactive supplement binding site(s) to increase the affinity of growth factors, differentiation factors, cytokines, or anti-inflammatory agents to the tissue repair implant. The invention is further directed toward applying such tissue repair implant for tissue repair.

[0050] The invention also relates to a process for repairing a defect in a human or animal by implanting the tissue repair implant having a porous sponge-like structure into a defect without rehydration to allow the tissue repair implant to absorb blood or fluid as well as autologous cells in situ. Alternatively, implantation of a tissue repair implant into a human or animal can be conducted by re-hydrating the tissue repair implant with a rehydrating solution; optionally seeding vital cells on the tissue repair implant to render the tissue repair implant vital; optionally culture the cell-seeded tissue repair implant before implantation; and implanting the tissue repair implant into the defect.

[0051] Moreover, the invention is directed to producing tissue repair implants having a porous sponge-like structure using a connective tissue homogenate from one or more connective tissues, a carrier solution, and with or without one or more natural or synthetic bone fragments for tissue repair. The invention relates to a process for making a porous sponge-like structure and stabilizing the porous sponge-like structure through one or more freeze drying cycles and one or more treating steps using one or more treatment solutions.

[0052] In the present application, the term "connective tissue" refers to mesodermally and endodermally derived tissue that may be more or less specialized, and that may be, at least in part, made up of fibers. Most of the connective tissues contemplated in the present invention are less specialized tissues that are rich in extracellular matrix

(i.e., collagen, proteoglycan, among others), and that surround other more highly ordered tissues and organs. A relatively, more specialized tissue contemplated in the present invention is cartilage. Varieties of connective tissue that may be used in the present invention include but not limited to adipose; loose connective tissue; dense, regular, irregular, or elastic connective tissue; white fibrous connective tissue; and cartilage. Connective tissue may be classified according to concentration of fibers as loose (areolar) and dense, the latter having more abundant fibers than the former. Connective tissues may be obtained from vertebrates. In some embodiments, the tissues may have human, bovine, equine, porcine, ovine, caprine, or piscene origins, among others. Connective tissues may also be the product of biotechnological methods, for example, tissue engineered connective tissues produced using cell culture methods.

[0053] Specific examples of connective tissues that may be used in certain embodiments of the present invention include but are not limited by at least, fascia, dermis tendons, ligaments, pericardium, urethra, small intestine, muscle, bone, dermis, or cartilage. Examples of different types of fascia that may be used in certain embodiments of the present invention include: fascia lata, fascia adherens, fascia brachii, fascia axillaris, antebrachial fascia, abdominal fascia, internal fascia, fascia iliaca, fascia profunda, clavipectoral fascia, fascia cribosa, crucial fascia, deltoid fascia, dorsal deep fascia, pelvic fascia, fascia cruris, lumbar fascia, and pectoral fascia, among others. "Crudely fragmented connective tissue" refers to connective tissue that has been sliced, ground, carved, chipped, chopped, minced, cut, dissected, rent, ripped, sectioned, snipped, diced, shaved, comminuted, or trimmed into fragments. Such fragmented connective tissue may have an average diameter greater than about 50 microns and less than about 0.5 cm, for example, having cut dimensions of approximately 0.5 x 0.5 cm, and a thickness appropriate to the tissue being crudely fragmented. In some embodiments, the crude fragments may not be of uniform size. "Homogenized connective tissue" or "connective tissue homogenate" contains connective tissue that has been reduced to particles that are uniformly small and evenly distributed. Homogenized connective tissue may optionally include at least one of water, aqueous solutions, or water miscible polar organic solvents, in addition to the particles. The homogenized connective tissues used in methods of the present invention include particles having an average diameter of less than about 50

microns. In some embodiments, the homogenized connective tissue may be prepared by shear-induced shredding of a composition comprising connective tissue, and optionally, at least one of water, an aqueous solution and a water miscible polar organic solvent. A conventional blender may be used in preparing the homogenized connective tissue, in certain embodiments.

[0054] "Natural or synthetic bone" is meant to refer to bone tissue, tissue resembling bone, synthetic material resembling bone, and tissue capable of forming bone. The term "bone" or "bone tissue" is intended for the purposes of the invention to refer to for example, an autograft bone, an allograft bone, or an xenograft bone. Such bone includes any bone from any source, including but not limited to bone from a living human donor, bone from a human cadaveric donor, and bone from an animal. The bone may include cortical bone and/or cancellous bone and/or cortico-cancellous bone. The term "bone fragment," as used in the present application refers to ground bone, pulverized bone, bone cubes, bone chips, bone strips, bone particles, and bone fibers. Bone fragments may be "bone particles" or "bone fibers," in some embodiments of the present invention. "Bone particle" refers to a piece of bone having an average diameter of between about 125 microns and about 4 mm. "Bone fiber" refers to a filament or thread of bone having an average thickness of between about 0.1 mm and about 2 mm and an average width of between about 0.3 mm and about 2.5 mm. Fibers can be of varying lengths. In certain embodiments, a bone fiber can have an average length of between about 1.0 mm and about 100 mm. In certain embodiments, bone fiber contains lamellae in the shape of threads or filaments having a median length to median thickness ratio of about 10:1. Synthetic material resembling bone comprises ceramics, hydroxyapatite, calcium phosphate, or calcium sulfate.

[0055] "Demineralized bone," as used in the present application refers to bone having less than about 8 wt % residual calcium. Demineralization involves treating the surface of a bone tissue to remove a surface layer of its inorganic mineral hydroxyapatite material leaving the mechanical properties of the organic phase of the bone constructs substantially unchanged. The level of demineralization of a bone tissue is defined by the amount (wt %) of residual calcium found in the demineralized bone. In some embodiments, the demineralized bone may contain

physiologically active levels of growth and differentiation factors, for example, bone morphogenetic proteins (BMPs).

[0056] In some embodiments, the homogenized connective tissue may be prepared from allogenic or xenogenic tissue. Such connective tissue may be obtained from a human donor or an animal, such as bovine, porcine, or murine etc. Connective tissue may be obtained relatively economically. Varieties of connective tissue that may be used in certain embodiments of the present invention include but are not limited to areolar or loose connective tissue; adipose; dense, regular or irregular connective tissue; white fibrous; elastic connective tissue; and cartilage. Specific examples of connective tissues that may be used in certain embodiments of the present invention include, for example, fascia, dermis, tendons, ligaments, pericardium, urethra, small intestine, muscle, bone, and cartilage. Examples of different types of fascia that may be used in some embodiments of the present invention include: fascia lata, fascia adherens, fascia brachii, fascia axillaris, antebrachial fascia, abdominal fascia, internal fascia, fascia iliaca, fascia profunda, clavipectoral fascia, fascia cribosa, crucial fascia, deltoid fascia, dorsal deep fascia, pelvic fascia, fascia cruris, lumbar fascia, and pectoral fascia, among others. For practical reasons of availability during procurement and amount of fascia available, fascia lata from the anterior portion of the upper leg may be used in certain embodiments. Homogenized connective tissue may be prepared by methods involving, cleaning and disinfecting connective tissue, and removing extraneous tissues associated with the connective tissue. Connective tissues may be cut into small pieces to produce crudely fragmented connective tissue, and optionally triturated and washed with distilled/deionized endotoxin-free water and/or an aqueous solution, such as isotonic saline, among others. In processing, multiple "washes" may be affected using volumes of aqueous solution that are 10 times the approximated volume of the tissue being processed, in some embodiments. The use of three such processing steps may affect an approximate 1:1000 dilution of associated solubilizable elements rendering the tissue essentially free from such solubilizable elements. Connective tissue may be treated and homogenized at temperatures sufficient to produce a flowable homogenized connective tissue, in certain embodiments. The homogenized connective tissue may include connective tissue that has been reduced to particles that are uniformly small and evenly distributed. Homogenized connective tissue and/or the carrier may optionally

include at least one of water, aqueous solutions, for instance isotonic saline, and water miscible polar organic solvents in addition to the connective tissue particles. The homogenized connective tissues used in methods of the present invention may include particles having an average diameter of less than about 50 microns, less than about 20 microns, or less than about 50 microns and greater than about 5 microns. In some embodiments, the homogenized connective tissue and optionally, at least one of a water miscible polar organic solvent, water and an aqueous solution, may be prepared by shear-induced shredding of connective tissue. A conventional blender may be used in preparing the homogenized connective tissue, in certain embodiments.

[0057] Certain methods for producing the inventive tissue repair implants may include preparing a connective tissue homogenate. Prior to homogenization, connective tissues, such as dermis, fascia, tendons, ligaments, pericardium, muscle, bone, urethra, small intestine, and cartilage, among others, may be crudely fragmented. Connective tissue, which may be fresh or freeze-dried, may be sliced, ground, carved, chipped, chopped, minced, cut, dissected, rent, ripped, sectioned, snipped, diced, shaved, comminuted, or trimmed into crude fragments. In some embodiments, the crude fragments may have an average diameter greater than about 50 microns. The crude fragments may be of varying sizes, in some embodiments. Essentially intact connective tissue or crude fragments of connective tissue, which may be fresh or freeze-dried, may be homogenized at least one time to prepare the homogenate. The homogenization step(s) of certain inventive methods may involve shear-induced shredding of connective tissue. Connective tissue may be homogenized to have tissue fragments having an average diameter of less than about 50 microns, less than about 20 microns, or less than about 50 microns and more than about 5 microns. Water, at least one aqueous solution, such as isotonic saline, or other components may be combined with a connective tissue before homogenization.

[0058] Certain methods include at least one of (a) heating a connective tissue before it is homogenized, (b) heating a connective tissue while it is being homogenized, and (c) heating a connective tissue homogenate. In some embodiments the heating is done to a temperature of between about ambient temperature and about 100 °C, or between about 37 °C and about 100 °C. Heating may be carried out for between

about 0.5 minutes and about 30 minutes. Heating may be accomplished using sonication, microwave irradiation, or conventional heat transfer from a heating component, among other methods known in the art.

[0059] The tissue repair implant of the present invention has a "porous sponge-like structure". The term "porous sponge-like structure" refers to a three-dimensional structure that is porous, coherent, elastic, flexible, fibrous and resilient. In a dry state, the porous sponge-like implant of the present invention can quickly absorb fluid. In the wet state, the porous sponge-like implant of the present invention can maintain the porosity, cohesiveness, and integrity. The wet porous sponge-like structure can resist certain tensile stress, and bounce back and reabsorb fluid after being released from compression. The porous sponge-like implant can be twisted, folded, rolled, and inserted into the defect or wrapped around the defect of bone, cartilage or soft tissue.

[0060] In one embodiment, the present invention provides a process of making a tissue repair implant having a porous sponge-like structure. The process comprising preparing a connective tissue homogenate from one or more connective tissues; mixing the homogenate with a carrier solution to produce a connective tissue carrier; and mixing the connective tissue carrier with one or more natural or synthetic bone fragments to produce a tissue repair mixture. In another embodiment, the present invention provides a process of preparing a tissue repair implant having a porous sponge-like structure comprising obtaining a connective tissue homogenate from one or more connective tissues and mixing the connective tissue homogenate with a carrier solution, which does not contain natural or synthetic bone fragments, to produce a tissue repair mixture. A crudely fragmented connective tissue may be added into the tissue repair mixture to increase the integrity of the porous sponge-like structure. The connective tissue homogenate or the crudely fragmented connective tissue may be derived from one or more connective tissues of human or other animal(s). The natural or synthetic bone fragments may be in the form of particles, fibers, rods or cubes. The natural or synthetic bone fragments may have demineralized bone, partially demineralized bone, non-demineralized bone, ceramics, hydroxyapatite, calcium phosphate, calcium sulfate, calcium carbonate, or a combination of two or more of the above. In one embodiment of the invention, the

mixture can be distributed in a pre-designed mold and kept at a temperature between 0 °C to about 30 °C to allow initial gelation and form a non-flowable mixture. In another embodiment of the invention, in order to maintain the porosity and an even distribution of pores within the porous sponge-like structure, the tissue repair mixture can be frozen first to solidify the structure and then optionally freeze-dried. A three-dimensional framework in the form of a web (Figure 1), created by mixing the connective tissue homogenate with the carrier solution, may be treated with a treatment solution to further stabilize and increase the mechanical strength of the structure.

[0061] Certain embodiments of the present invention are directed to tissue repair implants comprising a plurality of demineralized bone fragments or non-demineralized bone fragment embedded in a porous three-dimensional framework. The framework may include at least one homogenized connective tissue and at least one carrier. In some embodiments, the tissue repair implant may be safely used in repairing damaged tissue, for instance bone in a patient. The tissue repair implant may, in some embodiments, be biocompatible, osteoinductive, and/or osteoconductive, such that it may ultimately be remodeled to a mineralized, hard tissue at the application site in vivo. In certain embodiments, the tissue repair implants may further include at least water, an aqueous solution, or a water miscible polar organic solvent.. The tissue repair implant may include materials that improve handling or functional characteristics post-implantation. In some embodiments, the bone and connective tissue may be obtained from the same donor source (i.e., a single human cadaver donor). In certain embodiments, the tissue repair implant may be aseptic or sterile.

[0062] Each of the demineralized bone fragments and/or non-demineralized bone fragments and the homogenized connective tissue of the inventive implant may include materials derived from allogenic or xenogenic sources. In certain embodiments, bone and connective tissues obtained from vertebrate species, for example human, bovine, porcine, ovine, caprine, and piscene sources may be used to prepare demineralized bone fragments and carrier. The plurality of bone fragments may include more than one type of bone tissue (e.g., cancellous, cortical, or cortico-cancellous bone), and the homogenized connective tissue may include more than one

type of connective tissue (i.e., fascia and tendon). The plurality of demineralized bone fragments and/or non-demineralized bone fragments may include bone from a single donor source, or from multiple donor sources, and the homogenized connective tissue may also include tissue from a single donor source, or from multiple donor sources.

[0063] In one embodiment, the water insoluble porous sponge-like implant of the present invention comprises a uniform composition containing evenly distributed connective tissue homogenate and carrier that forms a three-dimensional framework. In another embodiment, the implant of the present invention comprises a uniform composition containing a connective tissue homogenate and carrier framework interspersed with bone fragments. The connective tissue homogenate and the carrier form a uniform framework which traps or embraces the bone fragments.

[0064] The present invention provides an implant having a porous sponge-like structure and is insoluble in water. The implant may include connective tissue, bone, and one or more carriers. The connective tissue may be a homogenized connective tissue, crudely fragmented connective tissue, or a combination of both. Thus, the connective tissue in the implant may be a homogenate, and the implant may comprise homogenized connective tissue and crudely fragmented connective tissue. The bone may be demineralized, non-demineralized, or a combination of both. The bone may be synthetic, naturally occurring, or a combination thereof. The bone may be bone fragments, bone powder, bone fiber, or bone particles. The bone may also be frozen or freeze dried. The carrier may be a solution including one or more polysaccharides such as alginate, one or more polymers, one or more salts, or a combination thereof. The carrier may also be a composition including one or more of the solutes of the carrier composition.

[0065] The following are examples of implants of the present invention. The implant may include homogenized connective tissue and a polysaccharide. The implant may include or consist essentially of homogenized connective tissue and a polysaccharide. The implant may include homogenized connective tissue, a polysaccharide, and demineralized bone. The implant may include homogenized connective tissue, a polysaccharide, and non-demineralized bone. The implant may include homogenized

connective tissue, a polysaccharide, demineralized bone, and non-demineralized bone. The homogenized connective tissue may be fascia. The implant may also include crudely fragmented fascia in addition to homogenized connective tissue. The polysaccharide may be an alginate. The non-demineralized bone may be ground cortical bone. The implant may also include polymers and salts.

[0066] Various factors and agents may be added to the implants of the present invention. In one embodiment, bioactive factors such as growth factors may be added to the implants. In another embodiment, cells may be seeded onto the implants. The implants of the present invention may be used as a vehicle for delivering bioactive factors and vital cells to damaged tissues. Accordingly, the implants of the present invention may be used as a delivery vehicle. As an example, the implants may be used for delivering bioactive factors and vital cells to injured or damaged sites in a subject. The implant of the present invention having homogenized connective tissue and a polysaccharide may act as a non-bone delivery vehicle.

[0067] The connective tissue homogenate may have excellent histocompatibility and elicit minimal antibody formation or immunological rejection, in certain embodiments. The homogenized connective tissue may be made acellular by treating it with detergents, endonucleases and decontaminating agents.

[0068] A devitalized process used to prepare homogenized connective tissue of the present invention may be performed without damage to matrix and/or tissue structure, in some embodiments and may employ detergents, sarcosinates, and decontaminating agents. The matrix structure may include collagens, hyaluronins, elastins, mucopolysaccharides and proteoglycans, among other components. Connective tissue that is devitalized may have a thickness that does not exceed about 8 mm, about 6 mm, or about 4 mm, in certain embodiments. Devitalization processing may be altered to accommodate the thicker tissues.

[0069] In certain aspects, the tissue repair implant may include no more than about 80 wt %, no more than about 50 wt %, or no more than about 20 wt % of the connective tissue homogenate in the dry state. The amount of connective tissue

homogenate added in a tissue repair implant may be varied to adjust the viscosity and gelation characteristics of the tissue repair mixture as well as the re-hydration characteristics of the porous sponge-like structure. Moreover, incorporating connective tissue homogenate in the tissue repair mixture, specifically, in the connective tissue carrier, may strengthen the three-dimensional framework and increase the integrity of the porous sponge-like structure. Incorporating connective tissue homogenate in the tissue repair mixture also may increase the cellular response towards the framework of the porous sponge-like structure by facilitating cellular attachment, migration, and proliferation since the connective tissue homogenate comprises natural or heat-denatured collagen.

[0070] In certain embodiments, the tissue repair implant may include between about 0.25 wt % and about 95 wt %, or between about 0.5 wt % and about 90 wt % of the crudely fragmented connective tissue in the dry state. The amount of crudely fragmented connective tissue added in a tissue repair mixture may be varied to adjust the gelation characteristics and prevent shrinkage of the tissue repair mixture during initial gelation, freeze-drying, and/or one or more treating steps with treatment solution. The crudely fragmented connective tissue may also provide structure reinforcement and further increase the integrity and mechanical strength of the porous sponge-like structure. Moreover, incorporating crudely fragmented connective tissue in the tissue repair implant may further increase the cellular response by facilitating cellular attachment, proliferation, and migration since the crudely fragmented connective tissue comprises collagen.

[0071] In certain embodiments, the tissue repair implant may include demineralized bone fragments having less than about 8 wt % or less than about 4 wt % residual calcium. The tissue repair implant may include demineralized bone fragments having between about 0.5 wt % and about 4 wt % residual calcium, about 1 wt% and about 4 wt% residual calcium, or about 2 wt% and about 4 wt% residual calcium in certain embodiments.

[0072] The plurality of demineralized bone fragments may include at least one of demineralized bone particles and demineralized bone fibers, in some embodiments. The demineralized bone fragments may include materials derived from allogenic or

xenogenic sources. The demineralized bone fragments may be derived from cortical bone or cancellous bone. In certain embodiments, the plurality of demineralized bone fragments may include at least one of demineralized allogenic cortical bone particles, demineralized xenogenic cortical bone particles, demineralized allogenic cancellous bone particles, or demineralized xenogenic cancellous bone particles.

[0073] Certain tissue repair implants of the present invention may include demineralized bone particles. Demineralized bone particles may be prepared from cleaned and disinfected bone fragments that have been freeze-dried and ground/fractured into bone particles. Bone particles may be selected by, for example, using sieving devices (i.e., mesh sieves) commercially available for obtaining particles within a desired size range. Such demineralized bone particles may have an average diameter of between about 125 microns and about 4 mm; between about 710 microns and about 2 mm; between about 125 microns and about 500 microns; between about 125 microns and about 850 microns; between about 125 microns and about 710 microns; or between about 250 microns and about 710 microns. Certain embodiments of the present invention may include demineralized bone particle that is commercially available. For example, a suitable demineralized bone particle that is widely and reliably available is produced by LifeNet Health, Virginia Beach, Virginia.

[0074] Some tissue repair implants of the present invention may include demineralized bone fibers. In certain embodiments, the demineralized bone fibers may have an average thickness of between about 0.1 mm and about 0.3 mm and an average width of between about 0.3 mm and about 1.0 mm. The length of the fibers may vary. In some embodiments, the demineralization process begins by producing bone particles having an average diameter size range of between about 1 mm and about 2 mm or bone fibers having an average dimension of 0.1 mm to 0.3 mm thickness and an average width of about 0.3 mm to about 1 mm. The fragments may then be treated with cleaning solutions. If the bone to be processed into fragments has not been previously cleaned and/or disinfected, they may be cleaned and/or disinfected by the use of detergents, hydrogen peroxides, antibiotics, and/or alcohols to affect a removal of associated tissues such as bone marrow and cellular elements. Following cleaning and disinfection, these fragments (i.e., particles and fibers) may

be demineralized by exposure to dilute hydrochloric acid to affect a removal/reduction of the mineral component of the bone fragments (i.e., particles and fibers). Such additional processing may, in some instances, inactivate potential viral contamination (i.e., HIV and hepatitis viruses, among others).

[0075] Certain embodiments of the present invention are directed to methods for the preparation of inventive tissue repair implant having a porous sponge-like structure, as described above. Such methods include combining a homogenized connective tissue, a carrier solution, a plurality of demineralized or non-demineralized bone fragments, and crudely fragmented connective tissue. The tissue repair implant having a porous sponge-like structure can contain between about 0.1 wt % and about 95 wt % or between about 10 wt % and about 90 wt % demineralized or non-demineralized bone fragments. Bone fragments may include at least one of bone particles and bone fibers from bone tissue. Some methods of preparing the tissue repair implants of the present invention may include the production of particles or fibers from bone tissue, as discussed above. Bone fragments may be demineralized, as described above, in certain embodiments. The fragments may be demineralized to have less than about 8 wt % residual calcium, less than about 4 wt % residual calcium, between about 0.5 wt % and about 4 wt % residual calcium, between about 1 wt% and about 4 wt% residual calcium, or between about 2 wt% and about 4 wt% residual calcium, in some methods of the present invention. Certain methods of the present invention may include freeze-drying demineralized bone fragments. In some embodiments, the demineralized bone fragments may be freeze-dried to a point such that the freeze-dried fragments have an average residual moisture of less than about 10 wt %, or less than about 5 wt %. In some embodiments, freeze-dried demineralized bone fragments may be rehydrated before use in preparing the tissue repair implants having a porous sponge-like structure of the present invention. Rehydrated freeze-dried demineralized bone particles may have a residual moisture content of less than about 80 wt %, less than about 50 wt %, less than about 25 wt %, or between about 10 wt% to about 25 wt %, in certain embodiments.

[0076] In some embodiments of the present invention, the connective tissue carrier will contain a carrier solution in addition to the connective tissue homogenate. Due to the hydrophilic nature of the connective tissue homogenate and the carrier

solution, these two components can penetrate each other and fully integrate so that the connective tissue carrier can form a three-dimensional framework to entrap the natural or synthetic bone fragments. The carrier solution can function as a diluent for the connective tissue homogenate to increase the porosity in the final porous sponge-like structure as well as to change the thickness of the web wall of the three-dimensional framework after one or more freeze-dry cycles. Addition of the carrier solution may change the viscosity of the tissue repair mixture so that the natural or synthetic bone fragments, such as demineralized or non-demineralized bone fragments, will remain "afloat" and evenly distributed in the wet state of tissue repair mixture. The weight percentage of the carrier in the tissue repair implant may be no more than 20% or may be no more than 5% in the dry state.

[0077] The carrier solution may comprise alginate, propylene glycol alginate, native or crosslinked chitosan, starch, polyethylene glycol, cellulose and its derivatives (such as cellulose acetate, carboxymethyl cellulose, and methyl cellulose), xanthan gum, dextran, hyaluronic acid, chondroitin sulfate, locust bean gum, gum tragacanth, gum arabic, curdlan, pullulan, scleroglucan, lower methoxylpectin, or carrageenan. The carrier solution may comprise salts of calcium, barium, aluminum, strontium, copper, zinc, magnesium, manganese, cobalt, or iron; glutaraldehyde, glyceraldehyde, genipin, glucose or ribose, poly(ethylene glycol) diepoxide crosslinker, poly(ethylene glycol) diglycidyl ether, EDC and NHS, transglutaminase, ethylenediamine, lysyl oxidase family, hexamethylene diisocyanate (HMDIC); dimethyl suberimidate (DMS), dimethyl-3-3'-dithiobispropionimidate (DTBP), or acryl azide. The carrier solution may also comprise natural and or synthetic polymers selected from the group comprising native or modified collagen, gelatin, agarose, modified hyaluronic acid, fibrin, chitin, biotin, avidin, MATRIGEL®, HUMAN EXTRACELLULAR MATRIX®, proteoglycans, laminin, fibronectin, elastin, heparin, glycerol, polymethylmethacrylate, polyurethane, acryloilmorpholine, N,N-dimethyl acrylamide, N-vinyl pyrrolidone and tetrahydrofurfuryl methacrylate, hydroxyapatite, cross-linkage or functionalization of hyaluronan-based collagen and alginate, polyurethane, polylactic acid, or a combination comprising at least one of the foregoing polymers.

[0078] In one embodiment of the present invention, a water replacing agent, such as glycerol (glycerin USP), adonitol, sorbitol, ribitol, galactitol, D-galactose, 1,3-dihydroxypropanol, ethylene glycol, triethylene glycol, propylene glycol, glucose, sucrose, mannitol, xylitol, meso-erythritol, adipic acid, proline, hydroxyproline, polyethylene glycol, alcohol, or lipids, can be included in the carrier solution to increase the flexibility of the porous sponge-like structure in the dry state and act as a moisture barrier. In another embodiment of the present invention, the carrier solution can also include one or more agents that have bioactive supplement binding site(s) so that when the porous sponge-like structure is implanted in vivo or exposed to a biological environment, it can attract cells or bioactive agents from the environment to the porous sponge-like structure to facilitate the healing process. Agents that have one or more bioactive supplement binding sites include but are not limited to hyaluronan, heparin, heparin sulfate, keratin sulfate, dermatan sulfate, chondroitin sulfate, betaglycan, heparan sulfate proteoglycan, syndecan, biglycan, or decorin.

[0079] In one embodiment of the present invention, the carrier solution may also include one or more bioactive supplements to directly stimulate the healing process. The bioactive supplements may be growth factors, differentiation factors, cytokines, or anti-inflammatory agents. The growth or differentiation factors may be for example, a growth factor of the FGF-family or TGF-family, IGF-1, PDGF, EGF, VEGF, HGF, PTHrP, Ihh (Indian Hedgehog Homolog), dexamethasone, insulin, transferrin, selenium, ITS supplement, ascorbate, or a combination thereof. The cytokines may include GM-CSF, G-CSF, TNF-α, IL-1β, IL-4, IL-6, IL-8, IL-10, SLP1, MCP1, MIP-1α, MIP-2, IL-18, angiopoietin, KGF, endothelin, IFN-α, or IFN-β. Examples of anti-inflammatory agents may include an IL-1αR antibody, TNF-α receptor antagonist, cyclooxygenase-2 specific inhibitors, MAP kinase inhibitors, NO synthase inhibitors, NF-κB inhibitors, or inhibitors of MMP. There are various fibroblast growth factors. As an example, the human FGF-family includes 22 members, FGF-1 through FGF-23. (There is no human FGF-15 because FGF-15 is the mouse ortholog of human FGF-19.) Examples of members of the TGF-family may include TGF-α and TGF-β superfamily. The TGF-β superfamily includes TGF-βs (such as TGF-β1, TGF-β2, TGF-β3), activins, inhibins, bone morphogenic factors (BMPs), anti-mullerian hormone (AMH), myostatins, and others. There are 20

isotypes of BMPs. They may be separated into four subfamilies, for example, (1) BMP2 and BMP4; (2) BMP3 and BMP3B (also known as growth/differentiation factor 10 (GDF10)); (3) BMPs 5, 6, 7 and 8; and (4) GDFs 5, 6, and 7.

[0080] The carrier solution may include bioactive supplements extracted from tissue comprising demineralized bone matrix, basement membrane, or submucosa matrix. The carrier solution may also include antioxidants, for instance, sodium nitroprusside, cartilage matrix glycoprotein (CMGP), vitamins C, vitamin E, selenium, N-Acetylcysteine (NAC) estradiol, glutathione, melatonin, resveratrol, flavonoid, carotene, aminoguanidine, or lycopene to protect bioactive components from oxygen-radical-induced damage antioxidants.

[0081] In certain methods, the tissue repair implant may be cast in a mold. In some embodiments, a method may further include freeze-drying a cast composition or cross-linking a cast composition using ionic, enzymatic, chemical; or photoactive agents known in the art. In one preferred embodiment, a cast tissue repair implant can be freeze-dried first to obtain the porosity, allow even distribution of pores within the porous sponge-like structure, and at the same time to entrap natural or synthetic bone fragments within the three-dimensional framework created by the connective tissue carrier. In order to stabilize the framework of the porous sponge-like structure, further entrap the natural or synthetic bone fragments, and increase the mechanical strength of the structure, the freeze-dried porous sponge-like structure can be treated with a treatment solution. The treatment solution may comprise ionic, enzymatic, chemical, or photoactive crosslinking agents. Examples of ionic crosslinking agents include but are not limited to metal ions selected from the group consisting of calcium, barium, aluminum, strontium, copper, zinc, magnesium, manganese, cobalt, and iron. Other ionic crosslinking agents include compounds containing phosphate-bearing groups, or compounds that are sulfates or citrates. Examples of enzymatic crosslinking agents include but are not limited to transglutaminase, ethylenediamine, lysyl oxidase family, hexamethylene diisocyanate (HMDIC), dimethyl suberimidate (DMS), or dimethyl-3-3'-dithiobispropionimidate (DTBP). Examples of chemical crosslinking agents include but are not limited to glutaraldehyde, glyceraldehyde, genipin, glucose or ribose, poly(ethylene glycol)

diepoxide crosslinker, poly(ethylene glycol) diglycidyl ether, EDC and NHS, or acryl azide.

[0082] Accordingly, the treatment solution may comprise agents such as but not limited to salts of calcium, barium, aluminum, strontium, copper, zinc, magnesium, manganese, cobalt, or iron; glutaraldehyde, glyceraldehyde, genipin, glucose or ribose, poly(ethylene glycol) diepoxide crosslinker, poly(ethylene glycol) diglycidyl ether, EDC and NHS, transglutaminase, ethylenediamine, lysyl oxidase family, hexamethylene diisocyanate (HMDIC); dimethyl suberimidate (DMS), dimethyl-3-3'-dithiobispropionimidate (DTBP), or acryl azide. The treatment solution may also comprise natural and or synthetic polymers selected from the group comprising native or modified collagen, gelatin, agarose, modified hyaluronic acid, fibrin, chitin, biotin, avidin, demineralized bone matrix, MATRIGEL®, HUMAN EXTRACELLULAR MATRIX®, proteoglycans, laminin, fibronectin, elastin, heparin, glycerol, sucrose octasulfate, polyethylene glycol, polymethylmethacrylate, polyurethane, acryloilmorpholine, N,N-dimethyl acrylamide, N-vinyl pyrrolidone and tetrahydrofurfuryl methacrylate, hydroxyapatite, cross-linkage or functionalization of hyaluronan-based collagen and alginate, polyurethane, or polylactic acid, or a combination comprising at least one of the foregoing polymers. The treatment solution may also include bioactive supplements. The bioactive supplements may be growth factors, differentiation factors, cytokines, or anti-inflammatory agents. Examples of growth or differentiation factors may include the FGF-family, TGF-family, IGF-1, PDGF, EGF, VEGF, HGF, PTHrP, Ihh, dexamethasone, insulin, transferrin, selenium, ITS, ascorbate, or a combination thereof. Cytokines may include, for example, GM-CSF, G-CSF, TNF-α, IL-1β, IL-4, IL-6, IL-8, IL-10, SLP1, MCP1, MIP-1α, MIP-2, IL-18, angiopoietin, KGF, endothelin, IFN-α, or IFN-β. The anti-inflammatory agents may include but are not limited to an IL-1αR antibody, TNF-α receptor antagonist, cyclooxygenase-2 specific inhibitors, MAP kinase inhibitors, NO synthase inhibitors, NF-κB inhibitors, or inhibitors of MMP. There are various fibroblast growth factors. As an example, the human FGF-family includes 22 members, FGF-1 through FGF-23. Examples of members of the TGF-family may include TGF-α and TGF-β superfamily. The TGF-β superfamily includes TGF-βs (such as TGF-β1, TGF-β2, TGF-β3), activins,

inhibins, bone morphogenic factors (BMPs), anti-mullerian hormone (AMH), myostatins, and others. There are 20 isotypes of BMPs. They may be separated into four subfamilies, for example, (1) BMP2 and BMP4; (2) BMP3 and BMP3B (also known as growth/differentiation factor 10 (GDF10)); (3) BMPs 5, 6, 7 and 8; and (4) GDFs 5, 6, and 7.

[0083] The treatment solution may also include agents having bioactive supplement binding site(s) comprising hyaluronan, heparin, heparin sulfate, keratin sulfate, dermatan sulfate, chondroitin sulfate, betaglycan, heparan sulfate proteoglycan, syndecan, biglycan, or decorin.

[0084] Since the porous sponge-like structure in the tissue repair implant has been created by freeze-drying before the application of one or more treating steps, the treatment solution can easily penetrate and evenly distribute into the entire porous sponge-like structure, which can significantly shorten the duration of treatment to about one or two hours. Shortening the treatment is vitally important for maintaining the biological function of the bioactive component present in the bone particles, such as BMPs in the demineralized bone matrix, which are prone to be inactivated by hydration. After treating with the treatment solution, the stabilized porous sponge-like structure may be washed with isotonic, hypertonic, or hypotonic solution. Then the porous sponge-like structure can be freeze-dried again and stored dry. In one embodiment of the present invention, a water replacing agent such as glycerol (glycerin USP), adonitol, sorbitol, ribitol, galactitol, D-galactose, 1,3-dihydroxypropanol, ethylene glycol, triethylene glycol, propylene glycol, glucose, sucrose, mannitol, xylitol, meso-erythritol, adipic acid, proline, hydroxyproline, polyethylene glycol, alcohol, or lipids may be applied to increase the flexibility of the porous sponge-like structure in the dry state and act as a moisture barrier. The porous sponge-like structure may be treated with a water replacing agent and then stored wet. The porous sponge-like structure can also be treated with a water replacing agent and then freeze-dried and stored.

[0085] In one embodiment of the present invention, the functionally active tissue repair implant can be combined with one or more bioactive supplements. Examples of bioactive supplements include growth factors, differentiation factors, cytokines,

and anti-inflammatory agents. In another embodiment of the present invention, the tissue repair implant can be combined with one or more agents that have bioactive supplement binding sites through covalent coupling or adsorption to increase the affinity of a bioactive supplement to the tissue repair implant. Agents that have bioactive supplement binding sites may be one or a combination of extracellular matrix proteins. Agents that have one or more bioactive supplement binding sites may be a natural or synthetic molecule comprising hyaluronan, heparin, heparin sulfate, keratin sulfate, dermatan sulfate, chondroitin sulfate, betaglycan, heparan sulfate proteoglycan, syndecan, biglycan, or decorin. Moreover, an extra functional group, such as but not limited to, COOH, NH_2, or OH may be added to the natural or synthetic proteins or peptides to facilitate the coating. The extra functional group may change the hydrophilicity or charge. In one embodiment, one or more bioactive supplements or one or more agent(s) that have bioactive supplement binding sites can be incorporated in one or more treatment solutions. In another embodiment, coating with one or more bioactive supplements or one or more agent(s) that have bioactive supplement binding sites can be conducted after one or more treating steps with the treatment solution. Yet, in another embodiment, coating with one or more bioactive supplements or one or more agent(s) that have bioactive supplement binding sites can be conducted during or right before clinical application.

[0086] In one embodiment, one or more polysaccharides are included in the carrier solution in the tissue repair mixture. The preferred polysaccharide comprises alginate, propylene glycol alginate, native or crosslinked chitosan, starch, cellulose and its derivatives (such as cellulose acetate, carboxymethyl cellulose, and methyl cellulose), xanthan gum, dextran, locust bean gum, gum tragacanth, gum arabic, curdlan, pullulan, scleroglucan, lower methoxylpectin, or carrageenan. Due to the hydrophilic nature of the connective tissue homogenate and the polysaccharide solution, these two components can penetrate each other and fully integrate so that the connective tissue carrier can form a three-dimensional framework to entrap solid components such as the natural or synthetic bone fragments. In addition, due to the ionic nature of the polysaccharides, by adjusting the pH of the mixture, it is possible to control the ionic interaction between the carrier solution and the collagen components in the crudely fragmented connective tissue as well as in the connective tissue homogenate so that an enhancement of the gelation occurs in the tissue repair

mixture and a non-flowable mixture can be obtained before freezing or freeze-drying. In one embodiment of the present invention, the tissue repair mixture can be freeze-dried first to produce a three-dimensional framework created by the mixture of connective tissue homogenate and the polysaccharide solution, allow even distribution of pores within the porous sponge-like structure, and at the same time entrap natural or synthetic bone fragments within the framework. The dried polysaccharide(s) present in the porous sponge-like structure may be further treated with covalent and/or ionic-crosslinking agents. An ionic-crosslinking agent may be employed because it is easy to apply and is not likely to cause modification of the bioactive components in the bone particles, such as BMPs in the demineralized bone matrix. In one embodiment, sodium alginate with viscosity from about 20 to about 200 and G/M ratio larger than 1 may be mixed with the connective tissue homogenate and freeze-dried to produce a stable three-dimensional framework of the porous sponge-like structure. The porous sponge like structure may then be treated with a solution of divalent salt such as calcium salt and/or a positively charged solution such as poly-L-lysine or chitosan. The treatment step may be conducted for a period of time of about 5 minutes to about 16 hours, from 15 minutes to about 120 minutes, or between 30 minutes to 60 minutes. Treatment of calcium salt on freeze dried porous sponge-like structure instead of the gel-like tissue repair mixture before freeze drying has multiple advantages. For example, treatment of calcium salt on freeze dried porous sponge-like structure does not limit the diffusion of the calcium salt through out the entire porous sponge-like structure and may produce (a) a stronger and evenly distributed cross-links within the porous sponge-like structure; (b) a porous sponge-like structure that can absorb more fluid in less time; and (c) a larger pore area that can accommodate more natural or synthetic bone fragments within a three-dimensional framework of web. Moreover, treatment of calcium salt on freeze-dried porous sponge-like structure will not crosslink the surface first and form a highly crosslinked surface and an unreacted core (inhomogeneous), which will likely occur if treatment of calcium salt is conducted before freeze-drying. The crosslinked surface can decrease and even inhibit the diffusion of the calcium salt towards the core of the structure. In another embodiment of the present invention, application of the calcium salt not only may crosslink the polysaccharide such as sodium alginate, but also may become a source of mineralization in subsequently induced new bone formation. Moreover, comparing to the framework formed by

alginate only, versus one that incorporates the connective tissue homogenate in the alginate solution, the latter can increase the strength and integrity of the three-dimensional framework and produce a porous sponge-like structure that is stronger, more resilient, and can better entrap bone fragments. The weight percentage of the polysaccharide incorporated in the final dry state of the porous sponge-like structure is less than about 20%, less than about 10%, or less than about 5%.

[0087] Three-dimensional (3-D) macro-porous sponge-like structure in the present invention is designed to provide support for the cells until they are organized into a functioning tissue. After implantation, the architecture of the macro-porous sponge-like structure can control the extent of vascularization and tissue ingrowth. The pores of the porous sponge-like structure in the present invention will be highly interconnected, between about 10 to about 800 μm, or between about 50 to about 300 μm. The pores can occupy about 10 to about 95% of the volume in a dry state. The pore size and volume can be adjusted by adding porogens, application of inert gas, or application of a negative hydrostatic pressure before freeze-drying the tissue repair mixture.

[0088] Methods of the current invention may include sterilization of the functional tissue repair implants having a porous sponge-like structure, components of tissue repair implants, and/or sterilization of packaged tissue repair implants having a porous sponge-like structure. Sterilization may involve the use of ionizing radiation, in some embodiments. In other embodiments, the absorbed dose of ionizing radiation may be between about 8.0 KGy and about 50 KGy, between about 8.0 KGy and about 25 KGy, or between about 8.0 KGy and about 18 KGy. In some embodiments, the sterilizing step may include placing the packaged tissue repair implants having a porous sponge-like structure on dry ice and irradiating the packaged composition. In certain embodiments, sterilization may be performed at a temperature of between about -20 °C. and -50 °C. The implants of the present invention may be sterilized using gamma irradiation, supercritical carbon dioxide, ethylene oxide, or electronic-beam.

[0089] Certain tissue repair implants of the present invention may include other elements. Additional elements may include but are not limited to antibiotics (i.e., penicillin), antiviral agents (i.e., Triton X-100, Nonidet P-40, N-lauroyl sarcosinate, Brij-35, and peroxide generating agents), antitumor agents, analgesics, immunosuppressive agents (i.e., bovine intestinal alkaline phosphatase), permeation enhancers (i.e., fatty acid esters, such as the laurate, myristate and stearate monoesters of polyethylene glycol), or nucleic acids.

[0090] The present invention also provides a method of repairing a damaged tissue such as but not limited to a bone, cartilage, or soft tissue. The method comprises implanting the tissue repair implant at the site of the damaged tissue to promote repair or regeneration of the tissue. The present invention also provides a method of inducing tissue formation comprising providing the tissue repair implant to the site of defect to induce tissue formation or regeneration. The implant may also be a vehicle for delivering needed agents such as bioactive factors and vital cells to injured or damaged tissues.

[0091] In some embodiments, for on-site preparation, the tissue repair implant having a porous sponge-like structure can be applied directly without re-hydration to allow the tissue repair implant to absorb blood or fluid as well as autologous cells in situ. On-site preparation has the advantage that permits the addition of optional components at the discretion of the clinician. Prior to implantation, the freeze-dried tissue repair implant can be re-hydrated with one or a cocktail of bioactive supplements or autologous or allogenic bone marrow aspirate, blood, or platelet rich plasma. Alternatively, the tissue repair implant can be combined with one or more bioactive supplements or one or more agent(s) that has bioactive supplement binding sites through covalent coupling or adsorption prior to the clinical application to increase the affinity of exogenous or endogenous bioactive supplement or cytokine to the porous sponge-like structure.

[0092] In some embodiments, the tissue repair implant may be seeded with vital cells to render the tissue repair implant vital just prior to implantation. Alternatively, the tissue repair implant may be seeded with vital cells and cultured in vitro for certain duration and then implanted. The vital cells may comprise one or more than one type

of cells from autologous or allograft bone marrow aspirate; stromal cells from bone marrow, fat, synovium, periostieum, perichondrium, muscle, dermis, umbilical cord blood, and Warton's jelly; or pericytes.

[0093] Various cells may be seeded on the implants of the present invention. The cells may be derived from autologous or allogeneic sources. The cells may be differentiated cells including chondrocytes, osteoblasts, osteoclasts, endothelial cells, epithelial cells, fibroblasts. Additionally, the cells may be totipotent, pluripotent, multipotent, progenitor, or adult somatic stem cells. The stem cells may be derived from embryos, placenta, bone marrow, adipose tissue, blood vessel, amniotic fluid, synovial fluid, synovial membrane, pericardium, dura, peripheral blood, umbilical blood, menstrual blood, baby teeth, nucleus pulposus, brain, skin, hair follicle, intestinal crypt, neural tissue, muscle. The stem cells may be derived from skeletal muscle, smooth muscle, and cardiac muscle. The stem cells may be derived from genetic reprogramming of mature cells, such as induced pluripotent stem cells (iPSCs). The cells may be an isolated population or a mixture of differentiated cells and/or stem cells. The cells may be applied to the implants with or without culture expansion.

[0094] In some embodiments, the tissue repair implant may be provided in a wet state where a water replacing agent can be present. The tissue repair implant may be sufficiently flexible to allow easy insertion into or wrapping around the defect. The presence of water replacing agent will also allow fast fluid exchange and absorption of the fluid such as blood into the tissue repair implant.

[0095] In some embodiments, the tissue repair implant may be provided in a unitary kit that also include a re-hydration agent, a bioactive agent, a bioactive cocktails, or one or more agents that has bioactive supplement binding sites. The kit may include the implant, one or more types of vital cells, and one or more bioactive factors. The kit may be used to treat or repair damaged or injured tissue.

[0096] In other embodiments, the present invention provides a kit for making the tissue repair implant. The kit may include homogenized connective tissue, one or more bone fragments, a carrier solution, treatment solution and a combination

thereof. The homogenized connective tissue may be fascia. The bone fragments may be demineralized or non-demineralized bone fragments or a combination thereof. The bone fragments may be synthetic or naturally occurring bone fragments or a combination of the two. The carrier solution may comprise a polysaccharide such as alginate.

[0097] In certain embodiments, the tissue repair implant may be provided together with other scaffolds or devices, such as a surgical suture, a decellularized or non-decellularized tissue, a synthetic polymer or metallic cage, for treating or repairing injured soft tissues, osseus defects, or spinal injuries. Examples of soft tissue injuries include rotator cuff tears and injuries and ligament tears and injuries. As an example, the implants of the present invention may be combined with dermis to repair or treat rotator cuff tears or injuries. As an example, the implant of the present invention may also be used in spinal fusion procedures to immobilize vertebrae in subjects in need thereof. The procedure eliminates pain caused by the abnormal motion of vertebrae in a subject. The implants may be used with a graft such as a bone allograft to immobilize vertebrae in subjects in need thereof.

[0098] The tissue repair implants of the present invention may also be used to treat or repair ligament tears or injuries. The implants may be used to treat or repair achilles tendon and anterior cruciate ligament injuries and tears. For example, the implant may be in the form of a sheet and wrapped around a ligament to promote repair of tears or injuries.

[0099] The present invention provides methods of promoting bone repair or regeneration involving applying the tissue repair implant of the present invention to a damaged bone tissue to promote repair or regeneration of the bone tissue. The implant having bioactive factors and/or vital cells may promote repair or regeneration of bone tissue. Some embodiments of the present invention are directed to methods of inducing bone formation involving applying an implant of the present invention to an implant site to induce bone formation.

[0100] The present invention also provides methods for repairing injured or damaged tissues. The method comprises applying the tissue repair implant of the present

invention to a damaged or injured tissue and allowing the implant to promote repair or regeneration of the damaged or injured tissue.

[0101] Without further description, it is believed that one of ordinary skill in the art can, using the preceding description and the following illustrative examples, make and utilize the claimed invention. The following working examples therefore, specifically point out preferred embodiments of the present invention, and are not to be construed as limiting in any way the remainder of the disclosure.

EXAMPLES

Example 1. Preparation of tissue repair implants: porous sponge-like structure with DMB particles.

[0102] Fascia lata and bone from a human cadaver were procured and returned to the processing facility under sterile conditions. Donor histories, personal and medical, were obtained following accepted standards of the American Association of Tissue Banks. Microbiological tests were performed following FDA guidelines for testing sterility of products.

[0103] The bone and fascia were cleaned of unwanted tissues and freeze-dried. The freeze-dried fascia was cut into small (about 1.5 cm by 1.5 cm) pieces (e.g., crude fragments). Sterile water in a volume (1 cm^3 of isotonic saline corresponds to about 1 g) approximating 20 times the weight of tissue, was added to a container containing the cut fascia. The ingredients were heated to a temperature of 100 degree C using a hot plate, and maintained at this temperature for about 5 minutes. Optionally, water was added to the heated composition to replace the liquid lost to evaporation. The heated composition was transferred into a conventional blender and mechanically homogenized (e.g., blended) for about 1 to 3 minutes. The homogenized connective tissue was re-heated for an additional 3 minutes on a hot plate, and mechanical homogenization was repeated for an additional 4 minutes (e.g., until the mixture was liquefied and homogeneous).

[0104] Ground demineralized bone powder was prepared by impact fragmentation of bone, followed by freeze-drying. The freeze-dried particles were sized using mesh

sieves. Ground demineralized bone particles having a size in the range of about 250 to 710 microns and demineralized to an average weight percent residual calcium of 2 ±1% were used.

[0105] Crudely fragmented fascia was made by blending them in a small blender until the fascia fiber diameter is less than 2 mm. Alginate solution was prepared by dissolving alginate powder (Protanal, LF 10/60, FMC Biopolymer, Dramen, Norway) in sterile water to a final concentration of 0.5 to 2%. The solution was filter sterilized and stored at 2°C - 10°C until use. $CaCl_2$ solution was made by dissolving $CaCl_2$ powder (FW=147.02, Mallinckrodt, Hazelwood, MO) in sterile water to a final concentration of 100 mM. The solution was filter sterilized and stored at ambient temperature (16°C – 30°C) until use.

[0106] To prepare the tissue repair implant, the homogenized fascia was mixed with a sodium alginate solution first to produce a connective tissue carrier that was mixed further with crudely fragmented fascia and sized, freeze-dried demineralized bone powder. After mixing all the components, the mixture was distributed into molds with pre-determined shapes and sizes, freeze-dried for 48 to 72 hours, treated with $CaCl_2$ for 0.25, 0.5, 1, or 2 hours, washed with water, and freeze-dried again for 48 to 96 hours. Before the first freeze-drying cycle, some samples were exposed to a negative hydrostatic pressure to allow the expansion of the DBM mixture to a preset thickness. The resultant porous DBM structure was then sent out for sterilization by gamma irradiation.

[0107] In one of the tissue repair implants, the porous DBM structure contained about 91% DBM particle, 3% alginate, 5% homogenized fascia, and 1% fascia fiber by dry weight. In another tissue repair implant, the porous DBM structure contained about 70% DBM particle, 3% alginate, 12% homogenized fascia, and 15% fascia fiber by dry weight. The freeze-dried, molded, tissue repair implants demonstrated high mechanical strength and maintained the shape of their mold.

Example 2. Preparation of tissue repair implants: porous sponge-like structure without bone fragment.

[0108] The homogenized fascia, sodium alginate solution, crudely fragmented fascia, and $CaCl_2$ solution were prepared as described in Example 1.

[0109] To prepare the tissue repair implant, the homogenized fascia was mixed with a sodium alginate solution first to produce a connective tissue carrier, which was mixed further with crudely fragmented fascia. After mixing all the components, the mixture was distributed into molds with pre-determined shapes and sizes, freeze-dried, treated with $CaCl_2$, washed, and freeze-dried again. Before the first freeze-drying cycle, some samples were exposed to a negative hydrostatic pressure to allow the expansion of the mixture to a preset thickness. The resultant porous sponge-like structure was then sent out for sterilization by gamma irradiation.

[0110] In one of the tissue repair implants, the porous sponge-like structure contained about 20% alginate, 60% homogenized fascia, and 20% fascia fiber by dry weight. The freeze-dried, molded, tissue repair implants demonstrated high mechanical strength and maintained the shape of their mold.

Example 3. Preparation of tissue repair implants: porous sponge-like structure with DBM particles and DMB fiber bone.

[0111] The homogenized fascia, sodium alginate solution, crudely fragmented fascia, demineralized bone powder, and $CaCl_2$ solution were prepared as described in Example 1.

[0112] Demineralized fiber bone was prepared by cutting cortical bone to produce cut fiber bone having an average length from about 1 mm to about 100 mm. Then the cut fiber bone was cleaned and disinfected, and a total of 463 grams of bone materials were dermineralized to 2.5% residual calcium using 2 cycles of 0.5 N HCl and acid volumes of 4.0 liters/cycle and 3.0 liters/cycle, 1 cycle of ultrapure water of 3.0 liters/cycle, and 2 cycles ultrapure water plus buffer of 3.0 liters/cycle to terminate the demineralization process. The bone fibers were finally washed in 3.0 liters of ultrapure water, stored frozen at -80 degree C in a sterile container, and freeze-dried for 96 hrs.

[0113] To prepare the tissue repair implant, the homogenized fascia was mixed with a sodium alginate solution first to produce a connective tissue carrier, which was mixed further with demineralized fiber bone and sized, freeze-dried demineralized bone powder. After mixing all the components, the mixture was distributed into molds with pre-determined shapes and sizes, freeze-dried, treated with $CaCl_2$, washed with water, and freeze-dried again. The resultant porous sponge-like structure was then sent out for sterilization by gamma irradiation. Before the first freeze dry cycle, some samples were exposed to a negative hydrostatic pressure to allow the expansion of the mixture to a preset thickness. In one of the tissue repair implants, the porous sponge-like structure contains about 70% DBM particle, 17% demineralized fiber bone, 3 % alginate, and 10% homogenized fascia by dry weight. In another tissue repair implant, the porous sponge-like structure contains about 56% DBM particle, 35% demineralized fiber bone, 2.5 % alginate, and 6.5% homogenized fascia by dry weight. The freeze-dried, molded, tissue repair implants demonstrated high mechanical strength and maintained the shape of their mold.

Example 4. Preparation of tissue repair implants: porous sponge-like structure with DBM fiber bone.

[0114] The homogenized fascia, sodium alginate solution, demineralized fiber bone, and $CaCl_2$ solution were prepared as described in Example 1 and Example 3.

[0115] To prepare a tissue repair implant, the homogenized fascia was mixed with a sodium alginate solution first to produce a connective tissue carrier, which was mixed further with demineralized fiber bone. After mixing all the components, the mixture was distributed into molds with pre-determined shapes and sizes, freeze-dried, treated with $CaCl_2$, washed with water, and freeze-dried again. The resultant porous sponge-like structure was then sent out for sterilization by gamma irradiation.

[0116] In one of the tissue repair implants, the porous sponge-like structure contained about 87% demineralized fiber bone, 3 % alginate, and 10% homogenized fascia by dry weight. The freeze-dried, molded, tissue repair implants demonstrated high mechanical strength and maintained the shape of their mold.

Example 5. Preparation of tissue repair implants: flexible porous sponge-like structure with DBM particles.

[0117] The homogenized fascia, sodium alginate solution, demineralized bone powder, and $CaCl_2$ solution were prepared as described in Example 1.

[0118] To prepare the tissue repair implant, the homogenized fascia was mixed with a sodium alginate solution first to produce a connective tissue carrier, which was mixed further with sized, freeze-dried demineralized bone powder. After mixing all the components, the mixture was freeze-dried, treated with $CaCl_2$, washed with water. Then the porous sponge-like structure was soaked in about 99.5% glycerol solution for about 1 hr. The excessive glycerol solution was removed and the porous sponge-like structure was freeze-dried again. The resultant flexible porous sponge-like structure was then sent out for sterilization by gamma irradiation.

[0119] In one of the tissue repair implants, the porous sponge-like structure contained about 26% DBM particle, 1 % alginate, 3 % homogenized fascia, and 70% of glycerol by dry weight. The freeze-dried, molded, tissue repair implants demonstrated high mechanical strength and maintained the shape of their mold.

Example 6. Preparation of tissue repair implants: porous sponge-like structure with DBM particles and high or low dose of heparin.

[0120] The homogenized fascia, sodium alginate solution, crudely fragmented fascia, demineralized bone powder, and $CaCl_2$ solution were prepared as described in Example 1. Heparin stock solution was prepared by dissolving heparin sodium salt grade I-A from porcine intestinal mucosa (Sigma, St. Louis, MO) in sterile water to a final concentration of 10 mg/mL. The solution was filter sterilized and stored at 2°C – 10°C until use.

[0121] To prepare the tissue repair implant, the homogenized fascia was mixed with a sodium alginate solution, and various amount of heparin solution to produce a connective tissue carrier, which was mixed further with crudely fragmented fascia and the sized, freeze-dried demineralized bone powder. After mixing all the

components, the mixture was distributed into molds with pre-determined shapes and sizes, freeze-dried, treated with $CaCl_2$, washed with water, and freeze-dried again for. The resultant porous sponge-like structure was then sent out for sterilization by gamma irradiation.

[0122] In one of the tissue repair implants, the porous sponge-like structure contained about 70% DBM particle, 2.9 % alginate, 12% homogenized fascia, and 15% fascia fiber, and 0.1% heparin by dry weight. In another tissue repair implants, the porous sponge-like structure contained about 70% DBM particle, 2.6 % alginate, 12% homogenized fascia, and 15% fascia fiber, and 0.4% heparin by dry weight. The freeze-dried, molded, tissue repair implants demonstrated high mechanical strength and maintained the shape of their mold.

Example 7. Preparation of tissue repair implants: porous sponge-like structure with ground cortical bone particles.

[0123] The homogenized fascia, sodium alginate solution, crudely fragmented fascia, and $CaCl_2$ solution were prepared as described in Example 1.

[0124] Ground cortical (GC) bone powder was prepared by impact fragmentation of bone, followed by freeze-drying. The freeze-dried particles were sized using mesh sieves. Ground cortical bone particles having a size in the range of about 250 to 1000 microns were used.

[0125] To prepare the tissue repair implant, the homogenized fascia was mixed with a sodium alginate solution to produce a connective tissue carrier, which was mixed further with crudely fragmented fascia, and the sized, freeze-dried ground cortical bone powder. After mixing all the components, the mixture was freeze-dried, treated with $CaCl_2$, washed with water, and freeze-dried again. The porous sponge-like structure was then sent out for sterilization by gamma irradiation.

[0126] In one of the tissue repair implants, the porous sponge-like structure contained about 85% GC particles, 3 % alginate, 6% homogenized fascia, and 6% fascia fiber

by dry weight. The freeze-dried, molded, tissue repair implants demonstrated high mechanical strength and maintained the shape of their mold.

Example 8. Preparation of tissue repair implants: porous sponge-like structure with GC particles crosslinked with genipin.

[0127] The homogenized fascia, sodium alginate solution, crudely fragmented fascia, and ground cortical bone powder were prepared as described in Example 1 and Example 7. Genipin stock solution was made by dissolving genipin (MW= 226.2, Sigma) in 40% molecular grade ethanol to a final concentration of 100 mg/ml. The solution was filter sterilized and used right away.

[0128] To prepare the tissue repair implant, the homogenized fascia was mixed with various amounts of genipin solution to produce a connective tissue carrier. The wet weight percentage of genipin was 1%, 0.5%, or 0.25% in the tissue repair mixture. Then the connective tissue carrier was mixed further with crudely fragmented fascia and the sized, freeze-dried ground cortical bone powder. After mixing all the components, the mixture was distributed into molds with pre-determined shapes and sizes and kept in dark for 4 to 16 hours at room temperature to allow crosslinking by genipin to occur. The structure turned dark blue after crosslinking. Then the crosslinked and molded structure was washed to remove unbound genipin and freeze-dried. The resultant porous sponge-like structure was then sent out for sterilization by gamma irradiation. The freeze-dried, molded, tissue repair implants with 1% genipin in the wet mixture demonstrated higher mechanical strength than the ones with 0.5 or 0.25 % genipin in the wet mixture.

Example 9. Preparation of tissue repair implants: porous sponge-like structure with GC particles crosslinked with genipin and stabilized with CaCl2.

[0129] The homogenized fascia, sodium alginate solution, crudely fragmented fascia, ground cortical bone powder, $CaCl_2$ solution, and genipin were prepared as described in Example 1 and Example 8.

[0130] To prepare the tissue repair implant, the homogenized fascia was mixed with a sodium alginate solution and 1% genipin solution to produce a connective tissue carrier. Then the connective tissue carrier was mixed further with crudely fragmented fascia and the ground cortical bone powder. After mixing all the components, the mixture was distributed into molds with pre-determined shapes and sizes and kept in the dark for 4 to 16 hours at room temperature to allow crosslinking by genipin to occur. The structure turned dark blue after crosslinking. Then the crosslinked and molded structure was washed to remove unbound genipin, freeze-dried, treated with $CaCl_2$, washed with water, and freeze-dried again. The resultant porous sponge-like structure was then sent out for sterilization by gamma irradiation.

[0131] In one of the tissue repair implants, the porous sponge-like structure contained about 75% ground cortical particle, 2 % alginate, 13% homogenized fascia, and 10% fascia fiber by dry weight. The freeze-dried, molded, tissue repair implants demonstrated high mechanical strength and maintained the shape of their mold.

Example 10. Preparation of tissue repair implants: porous sponge-like structure with GC particles absorbed with BMP-2.

[0132] The homogenized fascia, sodium alginate solution, crudely fragmented facia, ground cortical bone powder, and $CaCl_2$ solution were prepared as described in Example 1 and Example 7. Sterile recombinant human BMP-2 (rh BMP-2) produced by expressing BMP-2 gene in *E. coli* (355-BEC, R&D systems) was reconstituted in sterile 4 mM HCl containing 0.2% bovine serum albumin to a stock concentration of 0.2 μg/μL. The aliquots of rhBMP2 were stored at -10°C to -30°C until use.

[0133] To prepare the tissue repair implant, the homogenized fascia was mixed with a sodium alginate solution to produce a connective tissue carrier, which was mixed further with crudely fragmented fascia and the sized, freeze-dried ground cortical bone powder. After mixing all the components, the mixture was freeze-dried, treated with $CaCl_2$, washed water, and freeze-dried again. The porous sponge-like structure was then sent out for sterilization by gamma irradiation.

[0134] In one of the tissue repair implants, the porous sponge-like structure contained about 70% GC particles, 3 % alginate, 11% homogenized fascia, and 16% fascia fiber by dry weight. The rh BMP-2 stock solution was added onto the freeze-dried and molded porous sponge-like structure and allowed to absorb for about 0.5 to 2 hours at room temperature. The amount of rh-BMP-2 that was absorbed on the porous sponge-like structure was about 1 micro gram to about 500 microgram per milligram of dry porous sponge-like structure. Then the structure was freeze dried again.

Example 11. Preparation of tissue repair implants: porous sponge-like structure with GC absorbed with DBM digest.

[0135] The homogenized fascia, sodium alginate solution, crudely fragmented fascia, ground cortical bone powder, and $CaCl_2$ solution were prepared as described in Example 1 and Example 7. The collagenase digested DBM was prepared by digesting weighed DBM with 0.5 to 1 mg/mL collagenase. The digested DBM was centrifuged and the supernatant of the digested DBM was transferred to a new sterile tube and freeze-dried. The freeze-dried DBM collagenase digest was stored at -10°C to -30°C until use.

[0136] To prepare the tissue repair implant, the homogenized fascia was mixed with a sodium alginate solution to produce a connective tissue carrier, which was mixed further with crudely fragmented fascia, and the sized, freeze-dried ground cortical bone powder. After mixing all the components, the mixture was freeze-dried, treated with $CaCl_2$, washed with water, and freeze-dried again. The porous sponge-like structure was then sent out for sterilization by gamma irradiation.

[0137] In one of the tissue repair implants, the porous sponge-like structure contained about 70% GC particles, 3 % alginate, 11% homogenized fascia, and 16% fascia fiber by dry weight. The lyophilized collagenase digested DBM was reconstituted with sterile normal saline, added onto the freeze-dried and molded porous sponge-like structure, and allowed to absorb for about 0.5 to 6 hours at room temperature. The amount of DBM that was absorbed on the porous sponge-like structure was

about 0.1 to 100 milligram on about one milligram of dry porous sponge-like structure. Then the structure was freeze dried again.

Example 12. Characterization of the porous sponge-like structure.

[0138] Tissue repair implants having a porous sponge-like structures were prepared according to the processing steps described in Example 1, Example 2, and Example 3.

[0139] Porosity and DBM distribution in the porous sponge-like structure: Various prototypes of tissue repair implants having a porous sponge-like structures containing DBM were analyzed. H&E staining of one of the representative porous sponge-like structure was shown in Figure 1. It was found the framework of the porous sponge-like structure created by the connective tissue carrier (mixture of connective tissue homogenate and alginate, purple color) form web structures with large pores. The average pore size is larger than 100 μm. DBM particles and crudely fragmented fascia were distributed within the framework.

[0140] Hydration experiment: Each porous sponge-like structure containing DBM was cut and the dry weight of each sample was determined. Then, the sample was placed in a plastic weigh boat and 1 mL of 0.9% saline was added to each sample. The time needed for hydration was measured using a timer until the porous sponge-like structure became completely saturated and visibly changed color from white to beige. The hydrated sample was blotted lightly on a paper towel, and the wet weight was determined for each sample.

[0141] Regardless of the application of negative hydrostatic pressure before freeze-drying, the porous sponge-like structures without DBM were able to absorb more fluid than the porous sponge-like structure containing DBM (Figure 2). However, the amount and the type of DBM (particle or fiber) entrapped in the porous sponge-like structure did not change the amount of fluid that the porous sponge-like structure can absorb. The porous sponge-like structure containing DBM can absorb the amount of fluid that is about 80% of its wet weight or about four times of its dry weight. In addition, the application of negative hydrostatic pressure before freeze-

drying tends to decrease the time required to rehydrate the porous sponge-like structure especially in the porous DBM structure containing 69% of DBM particles (Figure 3).

[0142] Collagenase degradation assay: Three replicates of each porous sponge-like structure were weighed and placed in microcentrifuge tubes. For the samples that contained DBM, 30 mg of the porous sponge-like structure were digested with 600 micro-liters of 0.5 mg/mL collagenase in DMEM supplemented with 1% FBS. Only 10 mg of porous sponge-like structures containing no DBM were used for the collagenase degradation assay. All of the degradation samples were digested at 37° C for 16 hours without shaking and an additional 8 hrs with shaking at 1000 r.p.m. The supernatant was separated from the pellet by centrifuging at 10000 g for 10 minutes. The pellets were freeze dried in microcentrifuge tubes, and the dry weight of each pellet was determined. Some porous sponge-like structures containing DBM that were not treated with calcium chloride were used as controls.

[0143] Over 50% (dry weight) of the porous sponge-like structure without DBM was digested, while over 80% of the porous sponge-like structures containing DBM were digested after 24 hrs (Figure 4). Neither the calcium chloride treatment nor the application of negative pressure before freeze-drying changed the degradation. The amount of digested porous sponge-like structure was not different for different amount or type of DBM present within the porous sponge-like structure. Therefore, the encapsulation of the DBM in the porous sponge-like structure did not change the accessibility of the DBM.

Example 13. In vitro biocompatibility

[0144] Mouse fibroblast L929 cells were seeded directly on the surface of the porous sponge-like structure at the density of 0.1×10^6 cells/cm^2 and cultured. Cells seeded on the tissue culture plate were used as a control. Same seeding density was maintained for the porous sponge-like structure group and the control group. AlamarBlue reagent (10%) was added into the culture media on day 3 of the culture and incubated for 20 hrs. The fluorescent intensity of the culture media with AlamarBlue reagent was measured. The intensity of the fluorescence is

correspondent to the viability/metabolism of the cells, i.e., the higher the intensity the more viable/metabolic active cells. As shown in Figure 5, regardless of the concentration of alginate or the fluid content or $CaCl_2$ treatment duration, all the porous sponge-like structure tested not only maintained the cell viability but also support cell growth as the cell number in the porous sponge-like structure group was higher than that in the control groups after three day of culture. In addition, the porous sponge-like structure maintained their integrity during the culture.

Example 14. Determination of new bone formation.

[0145]Tissue repair implants having a porous DBM structures were prepared according to the processing steps described in Example 1.

[0146] The prepared tissue repair implants and demineralized bone matrix (DBM) controls (without homogenized connective tissue or carrier) were implanted heterotopically (e.g., into muscle pouches) in the hind quarters of athymic (e.g., nude) mice.

[0147] The amounts of materials implanted were varied to always implant 20 mg of DBM. For example, 30 mg of the tissue repair implant that contains about 69% DBM composition was implanted to reach about 20 mg of DBM. Two to three mice with two implants per mouse were used for each of the tissue repair implant groups and DBM control group.

[0148] After 28 days, the implants were explanted, and one explant from each mouse was fixed. At least one histological section was cut from the center of each of these explants. Samples were fixed in 10% buffered formalin. Standard dehydration, embedding and sectioning protocols were used to produce light microscopy slides that were subsequently stained with hematoxylin and eosin. Using histomorphometric analysis, the percent new bone formed was calculated as a cross-sectional area of newly formed bone (mm^2) divided by the total cross-sectional area (mm^2) for a representative microscopic view of a histology slide multiplied by 100. Every other field of view with at least 50% bone content was used as a representative view with about 10 representative views being analyzed per slide.

[0149] A representative H&E staining of an explanted porous sponge-like structure containing DBM showed new bone formation (Figure 6). All the experimental groups and the controls had at least 9% new bone formation.

Example 15. Assessment of new bone formation in an athymic rat calvaria critical size defect model.

[0150] Tissue repair implants having a porous DBM structures were prepared according to the processing steps described in Example 1.

[0151] Athymic rats were anesthetized with intraperitoneal injection of xylazine (10 mg/kg) and ketamine (50 mg/kg). Using aseptic surgical techniques, an anteroposterior midline skin incision (4-5cm) was made over the cranial vault. The periosteum was elevated and retracted to expose the cranial bone. An 8-mm diameter circular opening was created in the skull of rat using a drill bit. Prior to implantation, each DBM sponge implant was rehydrated. Following implantation of DBM sponge into defect, the skin wound was closed with suture. After recovery from anesthesia, animals were returned to their respective housing area. Six weeks after implantation, rats were euthanized by carbon dioxide asphyexiation and cavarial bones were harvested and fixed in 10% neutral buffered formalin. Soft x-ray was performed for assessment of new bone formation. A representative radiograph of an explant showed new bone formation in the defect (Figure 7, white arrow).

Example 16. Assessment of new bone formation in an athymic rat posterolateral lumbar spine fusion model.

[0152] Tissue repair implants having a porous DBM structures were prepared according to the processing steps described in Example 1.

[0153] Athymic rats were anesthetized with intraperitoneal injection of xylazine (10 mg/kg) and ketamine (50 mg/kg). The posterior spine was approached through a single midline skin incision. The paraspinal muscles were reflected using a scalpel and dissector to expose the spinous and transverse processes of lumbar segments 4-5

(L4 and L5). An electric burr was used to decorticate the transverse processes and exposed cancellous bone. DBM sponge was implanted to each lateral gutter. Six weeks after implantation, rats were sacrificed and new bone formation was demonstrated by radiography (Figure 8, white arrows).

Example 17. Preparation of Tissue Repair Implants: Porous Sponge-Like Structure with DBM and Ground Cortical (GC) Bone Fragment.

[0154] The homogenized fascia, sodium alginate solution, DBM, and ground cortical (GC) bone powder were prepared as described in Example 1 and Example 7.

[0155] To prepare the tissue repair implant, the homogenized fascia was mixed with sized, freeze-dried demineralized bone powder, and GC powder to produce a mixture. Then a sodium alginate solution was added and mixed further to produce a tissue repair mixture. The mixture was distributed into molds with pre-determined shapes and sizes, freeze-dried for 48 to 72 hours, treated with $CaCl_2$ for 0.25, 0.5, 1, or 2 hours, washed with water, and freeze-dried again for 48 to 96 hours. Before the first freeze-drying cycle, some samples were exposed to a negative hydrostatic pressure to allow the expansion of the DBM mixture to a preset thickness. The resultant porous DBM/GC structure was then sent out for sterilization by gamma irradiation.

[0156] In one of the tissue repair implants, the porous DBM/GC structure contained about 60% DBM particle, 30% GC particles, 1% alginate, and 9% homogenized fascia by dry weight. In another tissue repair implant, the porous DBM/GC structure contained about 75% DBM particle, 10% GC particles, 3% alginate, 12% homogenized fascia by dry weight. The freeze-dried, molded, tissue repair implants demonstrated high mechanical strength and maintained the shape of their mold.

CLAIMS:

Claim 1: A biologically functional tissue repair implant having a porous sponge-like structure comprising a connective tissue homogenate and a carrier, wherein the implant is insoluble in water.

Claim 2: The tissue repair implant of claim 1, further comprising one or more natural or synthetic bone fragments.

Claim 3: A process for preparing a biologically functional tissue repair implant having a porous sponge-like structure comprising:

a. producing a connective tissue homogenate from one or more connective tissues;
b. mixing said connective tissue homogenate with a carrier solution to produce a connective tissue carrier;
c. mixing one or more natural or synthetic bone fragments with said connective tissue carrier to produce a tissue repair mixture;
d. freezing or freeze-drying said tissue repair mixture to produce a porous sponge-like structure and create a three-dimensional framework to entrap said natural or synthetic bone fragments; and
e. treating said frozen or freeze-dried porous sponge-like structure with one or more treatment solutions to produce a stabilized porous sponge-like structure.

Claim 4: The process of claim 3, wherein a crudely fragmented connective tissue from one or more connective tissues is mixed with said tissue repair mixture before freezing or freeze-drying.

Claim 5: A process for preparing a biologically functional tissue repair implant having a porous sponge-like structure comprising:

a. producing a connective tissue homogenate from one or more connective tissues;
b. mixing said connective tissue homogenate with a carrier solution to produce a tissue repair mixture;

c. freezing or freeze-drying said tissue repair mixture to produce a porous sponge-like structure and create a three-dimensional framework; and
d. treating said frozen or freeze-dried porous sponge-like structure with one or more treatment solutions to produce a stabilized porous sponge-like structure.

Claim 6: The process of claim 5, wherein a crudely fragmented connective tissue from one or more connective tissues is mixed with said tissue repair mixture before freezing or freeze-drying.

Claim 7: The process of claim 3, wherein said stabilized porous sponge-like structure is treated with a water replacing agent.

Claim 8: The process of claim 3, wherein said stabilized porous sponge-like structure is freeze-dried.

Claim 9: The process of claim 3, wherein said stabilized porous sponge-like structure is sterilized.

Claim 10: The process of claim 3, wherein said framework of said porous sponge-like structure is created by said connective tissue carrier.

Claim 11: The process of claim 3, wherein said tissue repair implant is combined with one or more bioactive supplements .

Claim 12: The process of claim 3, wherein said tissue repair implant is combined with one or more agents that have bioactive supplement binding site(s).

Claim 13: The process of claim 12, wherein the bioactive supplement binding site(s) increases the affinity of growth factors, differentiation factors, cytokines, or anti-inflammatory agents to said tissue repair implant.

Claim 14: A method of repairing defects in bone, cartilage, or soft tissue comprising implanting the tissue repair implant of claim 3 at the site of defect.

Claim 15: A tissue repair implant having a porous sponge-like structure made according to claim 3, wherein the pore size is larger than about 50 microns.

Claim 16: A tissue repair implant having a porous sponge-like structure made according to claim 3, wherein the void volume is from about 10% to about 95%.

Claim 17: A tissue repair implant having a porous sponge-like structure made according to claim 3, wherein the void volume is from about 30% to about 80%.

Claim 18: he process of claim 3, wherein said tissue repair implant having a porous sponge-like structure is in the form of rod, sheet, cube, tube, particle, sphere, ellipsoid, wedge, or ribbon.

Claim 19: The process of claim 3, wherein said connective tissue homogenate is made from one more connective tissue(s) of human and/or animal origins.

Claim 20: The process of claim 3, wherein said connective tissue comprises fascia, cartilage, tendon, ligament, pericardium, fat, muscle, urethra, small intestine, dermis, demineralized bone, non- demineralized bone or a mixture of two or more of the above.

Claim 21: The process of claim 3, wherein said connective tissue in the connective tissue homogenate is cleaned and disinfected.

Claim 22: The process of claim 20, wherein said connective tissue is devitalized to remove cellular components.

Claim 23: The process of claim 21, wherein said connective tissue is freeze-dried.

Claim 24: The process of claim 3, wherein producing said connective tissue homogenate is carried out by homogenizing said connective tissue at temperature from about 15 °C to about 100 °C for a period of time of about 0.5 minutes to about 30 minutes.

Claim 25: The process of claim 24, wherein said homogenizing is carried out in the presence of a solution.

Claim 26: The process of claim 3, wherein the weight percentage of said connective tissue homogenate in said tissue repair implant is no more than 80% in the dry state.

Claim 27: The process of claim 3, wherein the weight percentage of said connective tissue homogenate in said tissue repair implant is no more than 50% in the dry state.

Claim 28: The process of claim 3, wherein the weight percentage of said connective tissue homogenate in said tissue repair implant is no more than 20% in the dry state.

Claim 29: The process of claim 3, wherein said carrier solution comprises one or more polysaccharides selected from the group comprising alginate, propylene glycol alginate, native or crosslinked chitosan, starch, cellulose and its derivatives (such as cellulose acetate, carboxymethyl cellulose, and methyl cellulose), xanthan gum, dextran, carrageenan, hyaluronic acid, condroitin sulfate, locust bean gum, gum tragacanth, gum arabic, curdlan, pullulan, scleroglucan, or lower methoxylpectin.

Claim 30: The process of claim 3, wherein said carrier solution comprises salts of calcium, barium, aluminum, strontium, copper, zinc, magnesium, manganese, cobalt, or iron; glutaraldehyde, glyceraldehyde, genipin, glucose or ribose, poly(ethylene glycol) diepoxide crosslinker, poly(ethylene glycol) diglycidyl ether, EDC and NHS, transglutaminase, ethylenediamine, lysyl oxidase family,

hexamethylene diisocyanate (HMDIC); dimethyl suberimidate (DMS), dimethyl-3-3'-dithiobispropionimidate (DTBP), or acryl azide.

Claim 31: The process of claim 3, wherein said carrier solution comprises natural and or synthetic polymers selected from the group comprising native or modified collagen, gelatin, agarose, modified hyaluronic acid, fibrin, chitin, biotin, avidin, MATRIGEL®, HUMAN EXTRACELLULAR MATRIX®, proteoglycans, laminin, fibronectin, elastin, heparin, glycerol, sucrose octasulfate, polyethylene glycol, polymethylmethacrylate, polyurethane, acryloilmorpholine, N,N-dimethyl acrylamide, N-vinyl pyrrolidone and tetrahydrofurfuryl methacrylate, hydroxyapatite, cross-linkage or functionalization of hyaluronan-based collagen and alginate, polyurethane, polylactic acid, or a combination comprising at least one of the foregoing polymers.

Claim 32: The process of claim 3, wherein said carrier solution comprises bioactive supplements selected from the group comprising a growth or differentiation factor of the FGF family, TGF-family, IGF-1, PDGF, EGF, VEGF, HGF, PTHrP, Ihh, dexamethasone, insulin, transferrin, selenium, ITS, or ascorbate.

Claim 33: The process of claim 3, wherein said carrier solution comprises bioactive supplements selected from the group comprising cytokine of GM-CSF, G-CSF, TNF-α, IL-1β, IL-4, IL-6, IL-8, IL-10, SLP1, MCP1, MIP-1α, MIP-2, IL-18, angiopoietin, KGF, endothelin, IFN-α, or IFN-β.

Claim 34: The process of claim 3, wherein said carrier solution comprises bioactive supplements selected from the group comprising anti-inflammatory agent of an IL-1αR antibody, TNF-α receptor antagonist, cyclooxygenase-2 specific inhibitors, MAP kinase inhibitors, NO synthase inhibitors, NF-κB inhibitors, or inhibitors of MMP.

Claim 35: The process of claim 3, wherein said carrier solution comprises bioactive supplements extracted from tissue selected from the group comprising demineralized bone matrix, basement membrane, or submucosa matrix.

Claim 36: The process of claim 3, wherein said carrier solution comprises antioxidants selected from the group comprising sodium nitroprusside, cartilage matrix glycoprotein (CMGP), vitamins C, vitamin E, selenium, N-Acetylcysteine (NAC) estradiol, glutathione, melatonin, resveratrol, flavonoid, carotene, aminoguanidine, or lycopene.

Claim 37: The process of claim 3, wherein said carrier solution comprises photoactive agents selected from the group comprising a xanthene dye, naphthalimide compounds, riboflavin-5-phosphate, N-hydroxypyridine-2-(1H)-thione, N-(20-ethylaminoethyl)-4-amino-1,8-naphthalimide, bis-diazopyruvamide—N,N9-bis(3-diazopyruvoyl)-2,29-(ethylenedioxy)bis-(ethylamine) (DPD); diazopyruvoyl (DAP); methylene blue, erythrosin, phloxime, thionine, methylene green, rose Bengal, acridine orange, xanthine dye, and thioxanthine dyes, ethyl eosin, eosin Y, or a combination comprising at least one of the foregoing photoactive groups.

Claim 38: The process according to 37, wherein said photoactive agent is activated by UV light or a laser.

Claim 39: The process of claim 3, wherein the weight percentage of said carrier in said tissue repair implant is no more than 20% in the dry state.

Claim 40: The process of claim 3, wherein the weight percentage said carrier in said tissue repair implant is no more than 5% in the dry state.

Claim 41: The process of claim 3, wherein said natural or synthetic bone fragments materials comprise non-demineralized bone, partially demineralized bone, demineralized bone, ceramics, hydroxyapatite, calcium phosphate, calcium sulfate, calcium carbonate, or a combination of two or more of the above.

Claim 42: The process of claim 3, said natural bone fragments are from human or animals.

Claim 43: The process of claim 3, wherein said natural or synthetic bone fragments are in the form of particles, fibers, rods, or cubes.

Claim 44: The process of claim 40, wherein said particles have an average particle size of about 125 micron to about 2000 micron.

Claim 45: The process of claim 40, wherein said fibers have an average width of 0.1 to 2 mm and an average length of about 0.3 mm to about 100 mm.

Claim 46: The process of claim 40, wherein said rods have average width of 0.5 to 5 mm and an average length of about 1 mm to about 100 mm.

Claim 47: The process of claim 40, wherein said cubes have an average volume of about 0.001 mm^3 to an average volume of about 1000 mm^3.

Claim 48: The process of claim 37, wherein said demineralized bone have a residual calcium content from about 1% to about 4%.

Claim 49: The process of claim 3, wherein the weight percentage of said natural or synthetic bone fragments in said tissue repair implant is about 0.1% to about 95% in the dry state.

Claim 50: The process of claim 3, wherein the weight percentage of said natural or synthetic bone fragments in said tissue repair implant is about 10% to about 90% in the dry state.

Claim 51: The process of claim 3, wherein the weight percentage of said natural or synthetic bone fragments in said tissue repair implant is about 30% to about 80% in the dry state.

Claim 52: The process of claim 3, wherein said crudely fragmented connective tissue is from tissues that are cleaned, disinfected, optionally devitalized to remove cellular components, and optionally freeze dried

Claim 53: The process of claim 3, wherein said crudely fragmented connective tissue is from tissues comprising fascia, cartilage, ligament, tendon, pericardium, muscle, urethra, small intestine, dermis, demineralized bone, partial demineralized bone, or non demineralized bone.

Claim 54: The process of claim 3, wherein the average diameter of said crudely fragmented connective tissue is from about 0.01 to about 5 mm.

Claim 55: The process of claim 3, wherein the weight percentage of said crudely fragmented connective tissue in said tissue repair implant is about 0.25% to about 95% in the dry state.

Claim 56: The process of claim 3, wherein the weight percentage of said crudely fragmented connective tissue in said tissue repair implant is about 0.5% to about 90% in the dry state.

Claim 57: The process of claim 3, wherein said tissue repair mixture is placed in a mold before said freezing or freeze-drying step.

Claim 58: The process of claim 3, wherein said tissue repair mixture is optionally processed under negative hydrostatic pressure before being frozen to increase porosity.

Claim 59: The process of claim 3, wherein said treatment solution comprises ionic, enzymatic, or chemical crosslinking agents; or photoactive agents.

Claim 60: The process of claim 59, wherein said ionic crosslinking agents comprise one or more metal ions selected from the group consisting of calcium, barium, aluminum, strontium, copper, zinc, magnesium, manganese, cobalt, or iron.

Claim 61: The process of claim 59, wherein said enzymatic crosslinking agents comprise transglutaminase, ethylenediamine, lysyl oxidase family, hexamethylene diisocyanate (HMDIC), dimethyl suberimidate (DMS), or dimethyl-3-3'-dithiobispropionimidate (DTBP).

Claim 62: The process of claim 59, wherein said chemical crosslinking agents comprise glutaraldehyde, glyceraldehyde, genipin, glucose or ribose, poly(ethylene glycol) diepoxide crosslinker, poly(ethylene glycol) diglycidyl ether, EDC and NHS, or acryl azide.

Claim 63: The process of claim 59, wherein said treatment solution comprises natural and or synthetic polymers selected from the group comprising native or modified collagen, gelatin, agarose, modified hyaluronic acid, fibrin, chitin, biotin, avidin, demineralized bone matrix, MATRIGEL®, HUMAN EXTRACELLULAR MATRIX®, proteoglycans, laminin, fibronectin, elastin, heparin, glycerol, sucrose octasulfate, polyethylene glycol, polymethylmethacrylate, polyurethane, acryloilmorpholine, N,N-dimethyl acrylamide, N-vinyl pyrrolidone and tetrahydrofurfuryl methacrylate, hydroxyapatite, cross-linkage or functionalization of hyaluronan-based collagen and alginate, polyurethane, or polylactic acid, or a combination comprising at least one of the foregoing polymers.

Claim 64: The process of claim 11, wherein said one or more bioactive supplements are incorporated in the treatment solution.

Claim 65: The process of claim 12, wherein said one or more agents that have bioactive supplement binding site(s) is incorporated in the treatment solution.

Claim 66: The process of claim 3, wherein said treatment solution comprises antioxidants to protect bioactive components from oxygen-radical-induced damage.

Claim 67: The process of claim 3, wherein said treating step comprising one or more treatment solutions are conducted simultaneously or sequentially to stabilize the porous sponge-like structure and further entrap said natural or synthetic bone fragments.

Claim 68: The process of claim 3, wherein said treatment is carried out for a duration of about 5 minutes to about 16 hours.

Claim 69: The process of claim 3, wherein said treatment is carried out for a duration of about 15 minutes to about 120 minutes.

Claim 70: The process of claim 3, wherein said treatment is carried out for a duration of about 30 minutes to about 60 minutes.

Claim 71: The process of claim 9, wherein said sterilization is carried out using gamma irradiation, super critical solution, ethylene oxide, or electronic-beam.

Claim 72: A process for repairing a defect comprising
implanting said tissue repair implant having a porous sponge-like structure according to claim 3 or 5 into a defect without rehydration to allow said tissue repair implant to absorb blood or fluid as well as autologous cells in situ.

Claim 73: A process for repairing a defect comprising:
rehydrating said tissue repair implant having a porous sponge-like structure according to claim 3 or 5 with a rehydrating solution;
optionally seeding vital cells on said tissue repair implant to render said tissue repair implant vital;
optionally culture said cell-seeded tissue repair implant before implantation;
implanting said tissue repair implant into said defect.

Claim 74: The process of claim 73, wherein said rehydrating solution comprises blood or bone marrow aspirate, platelet rich plasma, enzymes, bioactive supplements , natural polymers, synthetic polymers, photoactive agents, antioxidants, crosslinking agents, antimicrobial agents, vital cells, one or more agents that have bioactive supplement binding site(s), or a mixture of two or more of the above.

Claim 75: The process of claim 73, wherein said vital cells comprise one or more than one type of cells from autologous or allograft bone marrow aspirate; stromal cells from bone marrow, stromal cells from fat, synovium, periostieum, perichondrium, muscle, dermis, umbilical cord blood, and Warton's jelly; or pericytes.

Claim 76: A process for preparing a biologically functional tissue repair implant having a porous sponge-like structure comprising:

a. producing a connective tissue homogenate from one or more connective tissues;

b. mixing said connective tissue homogenate with one or more natural or synthetic bone fragments to produce a mixture;

c. adding a carrier solution to said mixture to produce a tissue repair mixture;

d. freezing or freeze-drying said tissue repair mixture to produce a porous sponge-like structure and create a three-dimensional framework to entrap said natural or synthetic bone fragments; and

e. treating said frozen or freeze-dried porous sponge-like structure with one or more treatment solutions to produce a stabilized porous sponge-like structure.

Claim 77: A method of repairing an injured tissue in a subject comprising applying the implant of claim 1 to the injured tissue to promote repair or regeneration of the tissue.

Claim 78: The process according to claim 3, 5, or 76, wherein the process further comprises seeding cells on the implant.

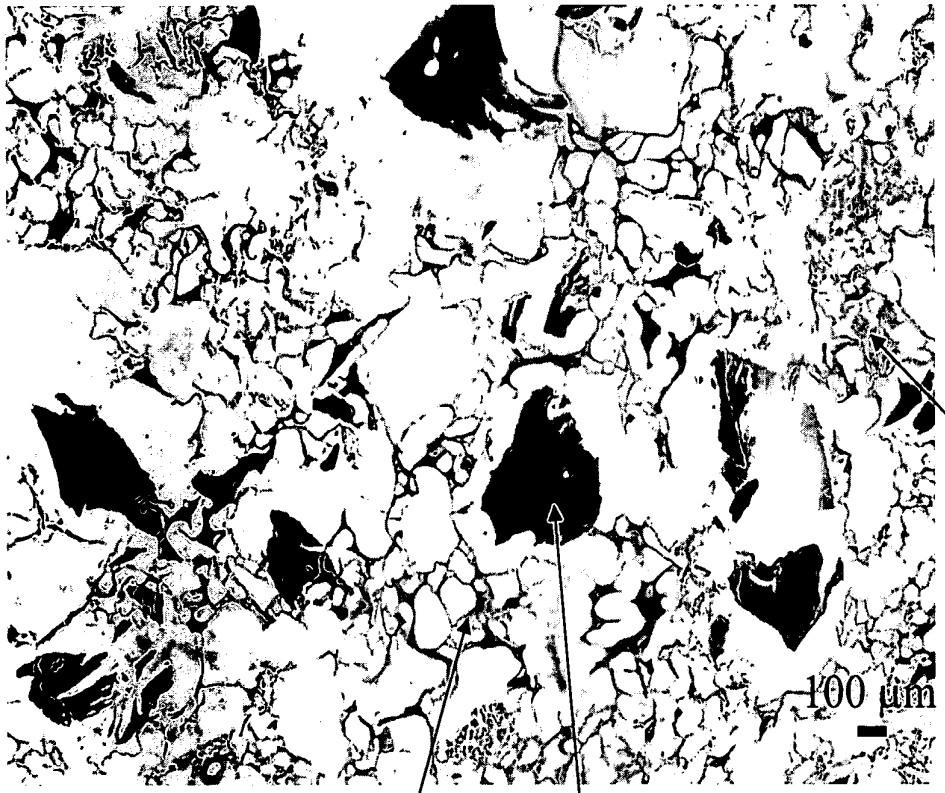

100 µm
Cross section of crudely fragmented fascia
Framework created by connective tissue homogenate and carrier
DBM

Figure 1

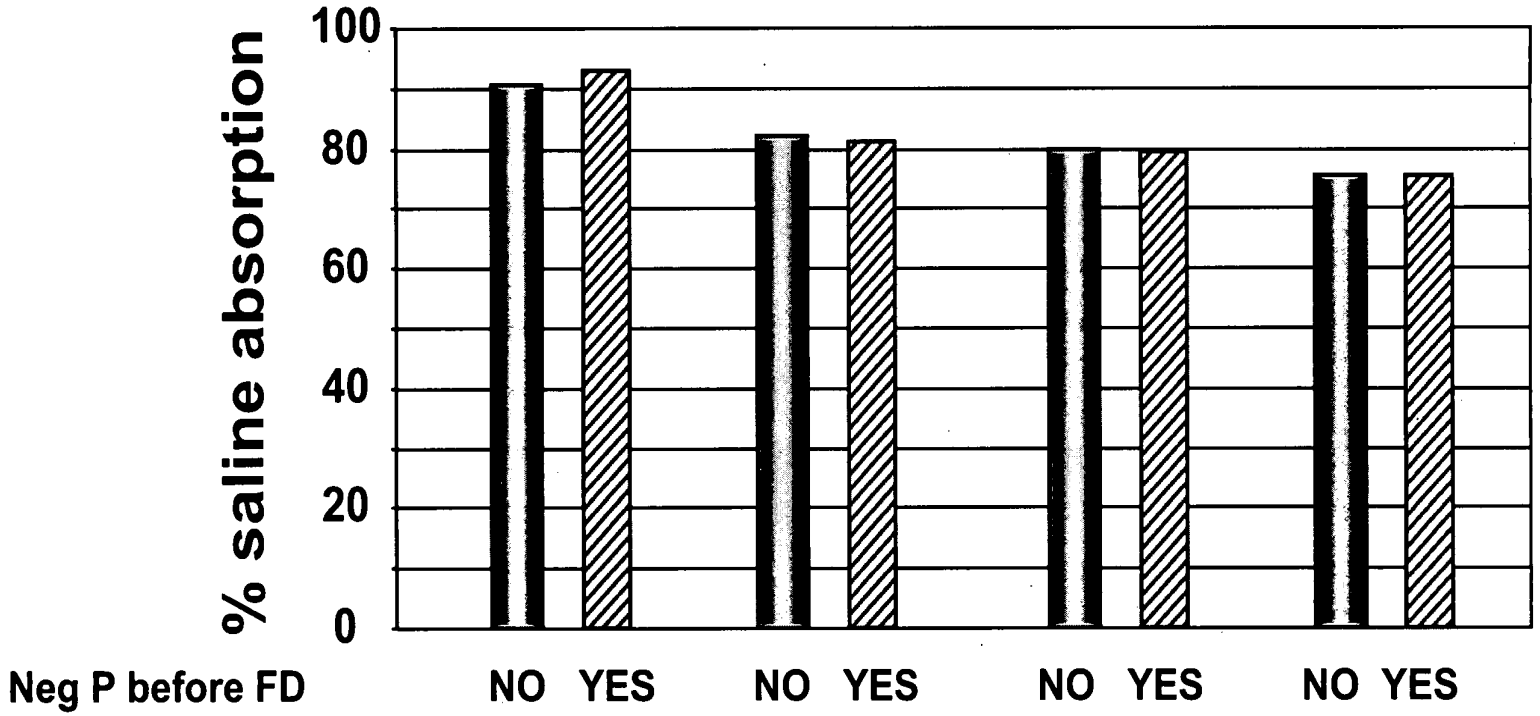

% saline absorption
100
80
60
40
20
0
Neg P before FD
NO YES NO YES NO YES NO YES
% of DBM particle
0 69 70 56
% of DBM fiber
0 0 17 35

Figure 2

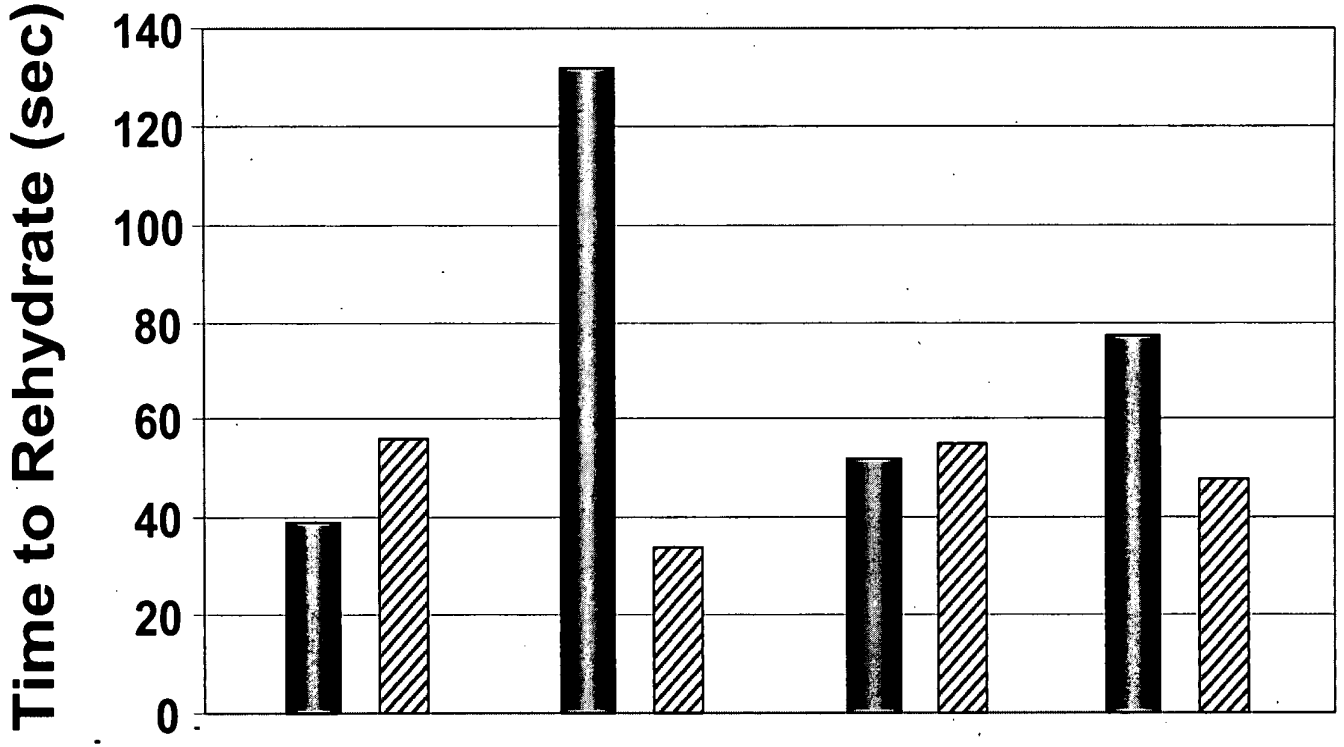
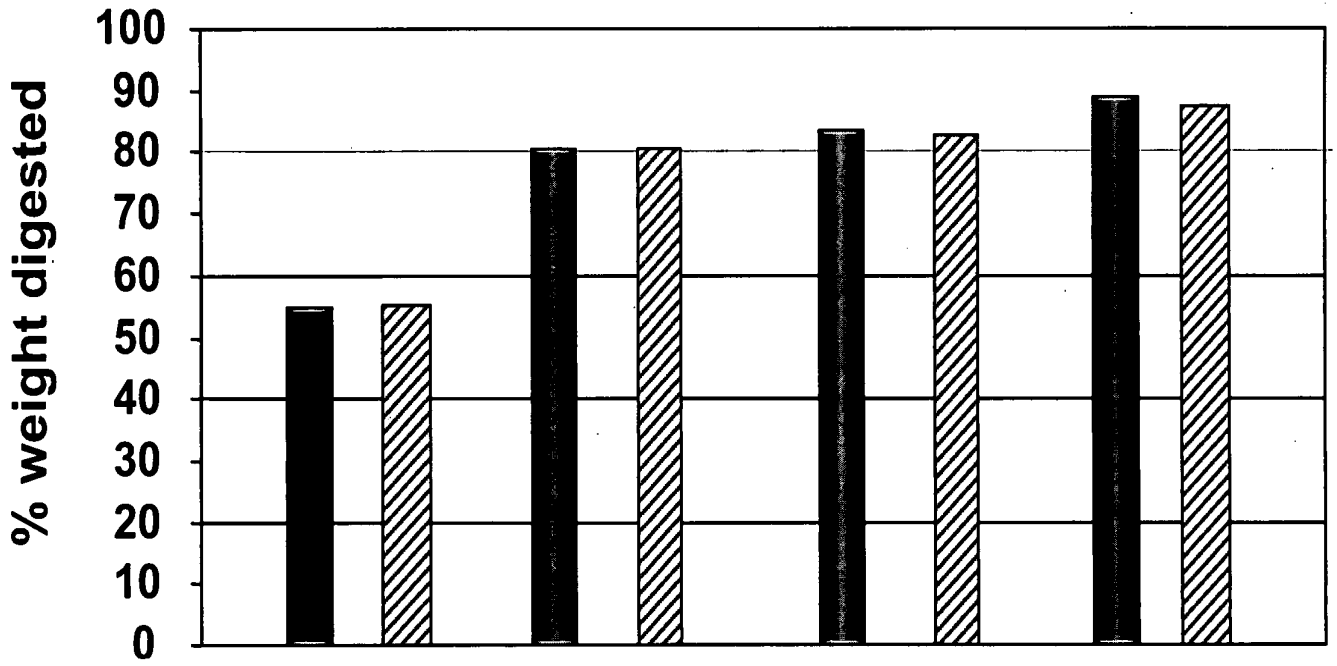


Figure 3

WO 2010/016942
PCT/US2009/004556

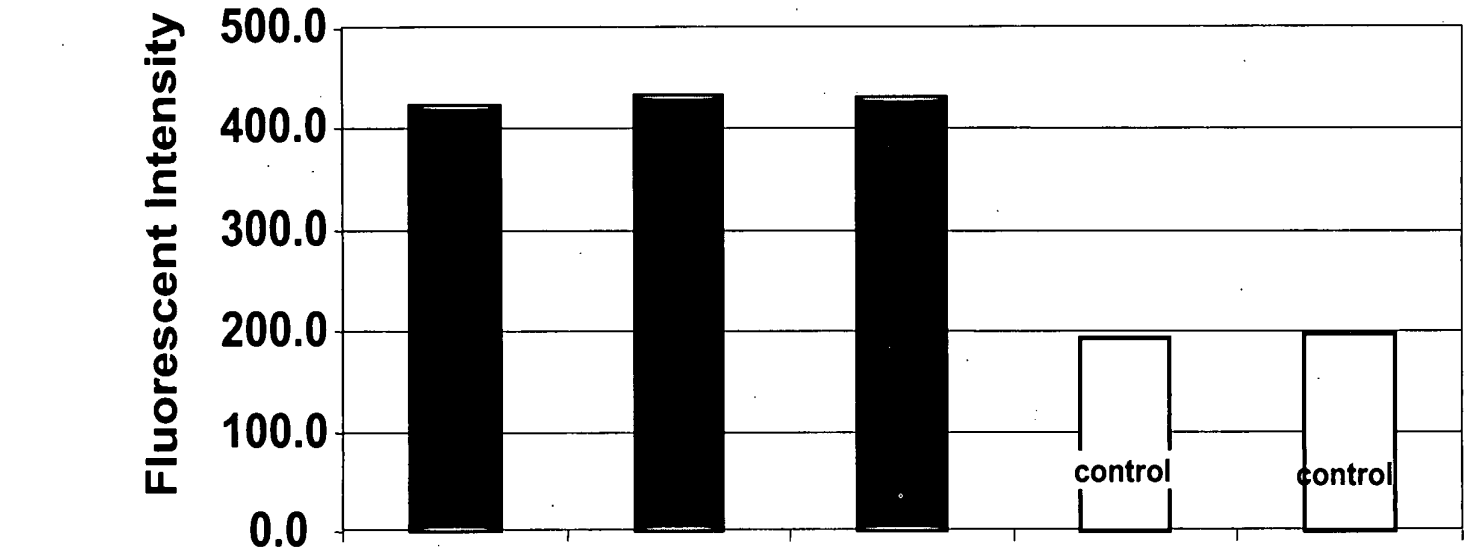
EMBZIM EX1002



$CaCl_2$ Treatment	YES	YES	YES	YES	YES	YES	NO	NO
Neg P before FD	NO	YES	NO	YES	NO	YES	NO	YES
% of DBM particle	0		69		70		69	
% of DBM fiber	0		0		17		0	

Figure 4

WO 2010/016942 PCT/US2009/004556



% of Alginate	0.5	0.5	1		
% Fluid content During Fabrication	60	70	60		
Duration of CaCl2 Treatment	15 min	60 min	15 min		

Figure 5



Figure 6

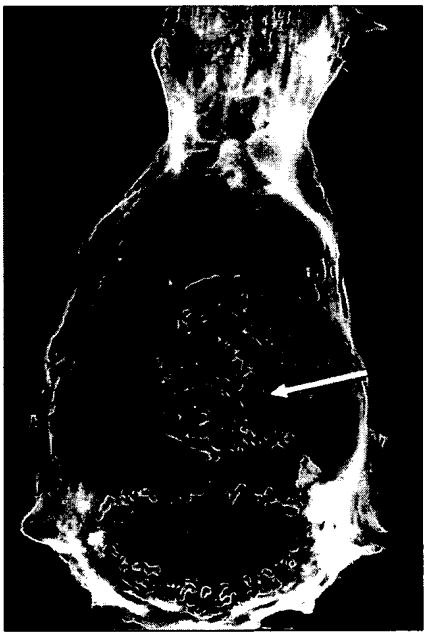


Figure 7

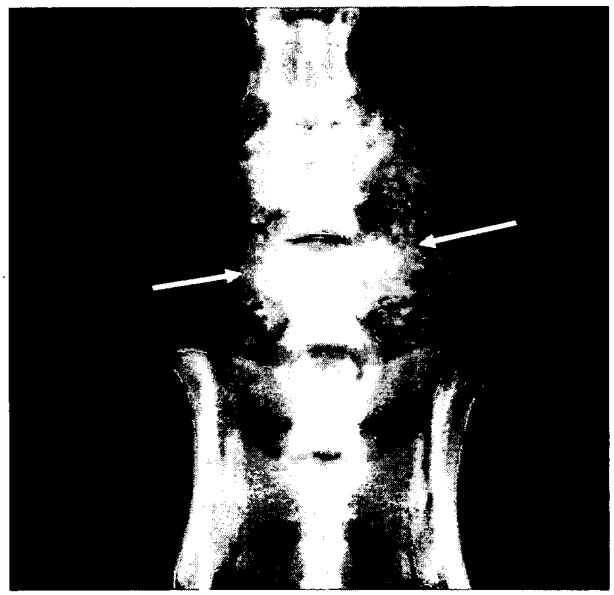


Figure 8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2009/004556

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 35/32 (2009.01)
USPC - 424/549
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)
IPC(8) - A61K 35/32 (2009.01)
USPC - 424/549

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 practicable, search terms used)
MicroPatent

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	US 2008/0069893 A1 (MASINAEI et al) 20 March 2008 (20.03.2008) entire document	3-6, 8-14, 19-21, 23-28, 35, 39-46, 49-51, 53-57, 59, 64-65, 67, 71, 76 --- 1-2, 7, 15-18, 22, 29-34, 36-38, 47-48, 52, 58, 60-63, 66, 68-70, 72-75, 77-78
Y	US 2004/0059364 A1 (GASKINS et al) 25 March 2004 (25.03.2004) entire document	1-2, 77
Y	US 5,607,474 A (ATHANASIOU et al) 04 March 1997 (04.03.1997) entire document	15-17, 58
Y	US 2008/0077251 A1 (CHEN et al) 27 March 2008 (27.03.2008) entire document	7, 18, 22, 29-34, 36-38, 47-48, 52, 61-63, 66, 68-70, 72-75, 78
Y	US 2008/0132991 A1 (PINCHUK et al) 05 June 2008 (05.06.2008) entire document	60

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
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"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
23 September 2008	01 OCT 2009
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. 571-273-3201	Authorized officer: Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

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Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

WO 2012/027592 A2

(54) Title: BASEMENT MEMBRANE COMPOSITIONS AND APPLICATIONS THEREOF

(57) Abstract: The invention relates to cell support compositions comprising a basement membrane extract isolated from cardiac or smooth muscle. The invention also relates to methods of using the cell support compositions for supporting cellular functions.

Basement Membrane Compositions and Applications Thereof

Inventor:

Roy Ogle

Cross-Reference to Related Applications

[0001] This application claims priority to U.S. Provisional Application Serial No. 61/376,966, filed 25 August 2010, which is incorporated by reference in its entirety.

Background of the Invention

Field of the Invention

[0002] The invention relates to cell support compositions comprising a basement membrane extract isolated from cardiac or smooth muscle. The invention also relates to methods of using the cell support compositions for supporting cellular functions.

Background

[0003] Basement membranes are thin, continuous sheets that separate an epithelium from adjacent stroma and surround nerves, muscle fibers, smooth muscle cells and adipose cells. As shown in many species, basement membranes appear early during embryogenesis and cover the inner cell mass, suggesting a critical role for basement membrane in early development. All basement membranes contain members of 3 extracellular matrix families: laminins, collagen IV and proteoglycans, along with the glycoprotein, nidogen/entactin. The basement membrane is a nanoscale network of filaments that interacts to form a lattice. This network, in part, results from the assembly of independent networks of laminins and collagen IVs, joined together by the nidogens and other associated proteins.

[0004] Basement membrane preparations have been used for cell biology research to provide a physiological environment promoting cell attachment, growth, and differentiation. Thus for culturing embryonic and other pluripotent stem cells *in vitro*, the basement membrane promotes cell growth and can be used to promote the differentiation of certain cell types.

[0005] Among the most broadly employed basement membrane preparations is the basement membrane-rich matrix from Engelbreth Holm-Swarm (EHS) murine sarcoma, known by its trade name MATRIGEL™ (BD Biosciences). MATRIGEL™ has been a useful product to facilitate cell growth,

development and differentiation for a broad assortment of cell types including stem cells, epithelial cells, endothelial cells, adipocytes, neurons, all forms of muscle cells and their precursors. MATRIGEL™ has been used commonly as an alternative to mouse embryonic fibroblast feeder layer to propagate pluripotent stem cells.

[0006] However, MATRIGEL™ has limited utility in many applications that arises from its origin as a mouse tumor or limits in the biological activity of the components that comprise it. Many products and processes for making these products require that the products or processes used to make the products be free of animal products. Clearly mouse-derived products are not suitable, for example, for the culture of human cells where the cells are grown to generate biologics products for human use such as antibodies or other proteins. The mouse-derived products are unsuitable for fabrication of tissue engineering scaffolds or other forms of implantation in humans due to concerns of passage of murine pathogens or elicitation of unwanted immune response. Beyond the concern of species difference is that of the composition itself. For example, MATRIGEL™ contains only laminin type I and alpha 1 and alpha 2 chains of collagen type IV. There are 6 chains of type IV collagen and 15 chains of laminin found in human cells, each assumed to impart specific biological functions to the basement membranes in which they occur.

[0007] There are undoubtedly components of the basement membrane of each tissue that are unique to that given tissue, although this area of research still requires much greater elucidation. For example, the heart is known not to contain a specific population of stem cells that give rise to cardiomyocytes. Rather, the cardiac tissue contains specific signaling molecules like the chemokines, SDF-1, which recruits circulating stem cells to the heart upon damage. Likewise, the basement membrane composition of tissues like smooth muscle or cardiac muscle constitute a unique signature for each tissue.

Summary of the Invention

[0008] The invention relates to cell supports compositions comprising a basement membrane extract isolated from cardiac or smooth muscle tissue. The compositions comprise molecules from extracellular matrices including, but not limited laminin, collagen, perlecan, and nidogen/entactin. In some embodiments, the composition may further comprise additional heparan sulfate proteoglycans.

[0009] The invention also relates to methods of using the novel compositions for supporting cellular functions, including but not limited to promotion of cell attachment, proliferation, differentiation, or maintenance of the phenotype of differentiated cells, or maintenance of the totipotency, pluripotency, multipotency of stem cells or progenitor cells through multiple culture passages *in vitro*

[0010] The invention also provides methods of applying or injecting the cell support compositions of the present invention in a subject in need of treatment such treatment.

[0011] The invention also relates to articles of manufacture comprising the cell support compositions of the present invention. For example, the invention provides for cell culture surfaces comprising the cell support compositions of the present invention.

Brief Description of the Drawings

[0012] Figure 1 depicts the protein content of the cell support compositions of the present invention. Equivalent amounts of protein from 4 preparations of human heart basement membrane extract (HBME) from individual donors and MATRIGEL™ (MG) were resolved by electrophoresis on 5% SDS-polyacrylamide gels. The pattern of polypeptides in HBME was very reproducible and distinct from that of MATRIGEL™. The majority of the protein in MATRIGEL™ is present as laminin 1—the alpha 1 chain migrates at 400 kD and the beta 1 and gamma 1 chains co-migrate at about 200 kD. A number of the polypeptide bands in the region of 180-400 kD in the HBME samples reacted positively with monospecific antibodies against known laminin chains. Similarly, specific antibodies to type IV collagen (alpha 1 and alpha 2 chains), type I collagen, nidogen/entactin and perlecan were able to bind to the HBME. Nidogen was also present. Tandem mass spec sequencing analysis provided further confirmation of the identity of these polypeptides and identified the presence of several non-basement membrane cytoskeletal proteins, including titin protein, myosin 7 and actinin.

[0013] Figure 2 depicts the attachment of cells to the cell support composition of the present invention. 20,000 of each cell type was allowed to attach to a surface coating of 10 $\mu G/cm^2$ for 1 hour at 37°C in serum free media. The Table in this figure shows the ratio of attachment to the cell support compositions of the present invention relative to MATRIGEL™. For stem cells and fibroblasts, the cell support compositions of the present invention promote roughly twice the level of attachment as compared to MATRIGEL™.

[0014] Figure 3 depicts the growth and maintenance of plasticity of pluripotent stem cells cultured on the cell support compositions of the present invention and on MATRIGEL™. Either the cell support compositions of the present invention or MATRIGEL™ was coated at 17 μG/cm^2 were tested for the ability to support the growth and prevent the unwanted differentiation of embryonic and induced pluripotent stem cells in serum-free, chemically defined medium. Mouse embryonic stem cells (D3), mouse induced pluripotent stem cells (W5) or human induced pluripotent stem cells were plated and passaged 2 times on the indicated substrates. Proliferation rates and colony morphology was comparable. When the human induced pluripotent stem cells were stained at second passage using a live cell marker for pluripotency (Tra-1-60, stain alive, Stemgent) the green FITC signal indicates the cells were still in a pluripotent state.

Detailed Description of the Invention

[0015] The invention relates to a cell support composition comprising a basement membrane extract isolated from human cardiac or smooth muscle tissues. The compositions comprise molecules from extracellular matrices including, but not limited laminin, collagen, perlecan, and nidogen/entactin. In some embodiments, the compositions may further comprise additional heparan sulfate proteoglycans. The cell support compositions can be obtained from any mammal, such as but not limited to dogs, cats, horses, pigs, cows, non-human and human primates. It is understood that the components of the cell support compositions of the present invention will be species-specific base on the source of the tissue. For example, if the cell surface compositions of the present invention are extracted from human cardiac basement membrane, then the core components of the composition, *e.g.,* laminin, will be human laminin.

[0016] If the tissue from which the compositions are extracted is human tissue, the human tissue can, but need not, be obtained from certified tissue banks. The starting material, regardless of the animal source, can also be screened for bioburden, endotoxins and/or the presence of known pathogens. If the material is obtained from a tissue bank, it is likely that the tissue bank would perform the screening process to ensure safety and efficacy. Accordingly, the methods of preparing the cell support compositions of the present invention as disclosed below may or may not include steps of screening the material for bioburden, endotoxins and/or known pathogens. The screening steps may be performed on the starting material or the screening steps may be performed after extraction of the cell support compositions.

[0017] The compositions of the present invention comprise laminin type 2 and laminin type 10. In some embodiments, total laminin is present in the cell support compositions of the present invention in an amount from about 5% to about 50% by weight. As used herein, laminin type 2 is well understood in the art and is understood to mean a laminin molecule with an alpha 2 chain, a beta 1 chain and a gamma 1 chain. Laminin 10 is also well understood in the art and is understood to mean a laminin molecule with an alpha 5 chain, a beta 1 chain and a gamma 1 chain. In some embodiments, the compositions of the present invention also comprise one or more additional types of laminins, with these additional types of laminins containing an alpha 2, 3, 4 or 5 chain. Examples of laminins containing alpha chains 2, 3, 4 or 5 include but are not limited to, laminin type 2, laminin type 4, laminin type 5, laminin type 6, laminin type 7, laminin type 8, laminin type 9, laminin type 10, laminin type 11, laminin type 12, laminin type 13, laminin type 14, and laminin type 15. The molecular structure of laminin present in the compositions can be elucidated by using monoclonal antibodies specific for one of the chains the laminin type to isolate the laminin, which can then be analyzed using native gels or chromatography followed by mass spectroscopy and/or SDS-PAGE. One of skill in the art would easily be able to identify the laminin types based on well-known procedures in the art.

[0018] The compositions of the present invention do not contain any detectable levels of laminin type 1. In one embodiment, the compositions of the present invention lack detectable levels of laminin type 3. In another embodiment, the compositions of the present invention lack detectable levels of both laminin type 1 and laminin type 3. As used herein, laminin type 1 is well understood in the art and is understood to mean a laminin molecule with an alpha 1 chain, a beta 1 chain and a gamma 1 chain. Laminin type 3 is also well known in the art and is understood to mean a laminin molecule with an alpha 1 chain, a beta 2 chain and a gamma 1 chain. In another embodiment, the compositions of the present invention lack any detectable levels of any laminin that normally contains an alpha 1 chain.

[0019] In yet another embodiment, the compositions of the present invention do not contain any detectable levels of laminin type 5. In still another embodiment, the compositions of the present invention lack detectable levels of both laminin type 1 and laminin type 5. In still another embodiment, the compositions of the present invention lack detectable levels of both laminin type 3 and laminin type 5. In still another embodiment, the compositions of the present invention lack detectable levels of laminin type 1, laminin type 3 and laminin type 5. As used herein, laminin type 5 is well understood in the art and is understood to mean a laminin molecule with an alpha 3 chain, a beta 3 chain and a

gamma 2 chain. In another embodiment, the compositions of the present invention lack any detectable levels of any type of laminin that normally contains a beta 3 chain and/or a gamma 2 chain.

[0020] The compositions of the present invention comprise at least one type of collagen. In one embodiment, the composition comprises type IV collagen. In some embodiments, total collagen is present in the cell support compositions of the present invention in an amount from about 5% to about 50% by weight. In another embodiment, the composition comprises type I collagen. In yet another embodiment, the composition comprises type III collagen. In another embodiment, the composition comprises type XIII collagen. In another embodiment, the composition comprises type XVII collagen. In yet another embodiment, collagen type II is absent from the cell support compositions of the present invention. The types of collagen that are present or absent in the composition can be easily assessed using routine methods in the art. The type IV collagen was identified by its unique molecular mass (180 kD) and reaction with multiple monospecific antibodies. Methods of identifying and quantifying type of collagen are well known in the art, as disclosed, for example in Schnaper, H.W. and Kleinman, H.K., *Pediatr. Neprol.,* 7:96-104 (1993), which is incorporated by reference.

[0021] The compositions of the present invention comprise perlecan. Perlecan is a well known protein in the art that is also known as basement membrane-specific heparan sulfate proteoglycan core protein. In some embodiments, perlecan is present in the cell support compositions of the present invention in an amount from about 5% to about 60% by weight. Perlecan is essential for normal vascularization, critical for normal heart development and critical for regulating vascular responses to injuries.

[0022] The compositions of the present invention comprise entactin, which is also known as nidogen 1 protein. In some embodiments, entactin is present in the cell support compositions of the present invention in an amount from about 5% to about 50% by weight.

[0023] In some embodiments, the compositions of the present invention further comprise titin protein, which is also known as connectin protein. In some embodiments, the amount of titin protein, by weight, present in the cell support compositions of the present invention can be up to about 15%.

[0024] In some embodiments, the compositions of the present invention further comprise myosin 7 protein. In some embodiments, the amount of myosin protein, by weight, present in the cell support compositions of the present invention can be up to about 15%.

[0025] In some embodiments, the compositions of the present invention further comprise actinin protein. In some embodiments, the amount of actinin protein, by weight, present in the cell support compositions of the present invention can be up to about 7.5%. In still additional embodiments, the compositions of the present invention may comprise one or more of titin, myosin 7 and actinin, including all three molecules.

[0026] In select embodiments the compositions optionally comprise signaling molecules. In one embodiment, one or more signaling molecules are naturally present in cardiac or smooth muscle, such that extract composition contains one or more signaling molecules normally present in the natural tissue, *i.e.,* "endogenous signaling molecules." In another embodiment, one or more signaling molecules can be added to the extract such that the one or more signaling molecules present in the composition are not normally present in the natural tissue, *i.e.,* exogenous signaling molecules. In yet another embodiment, additional levels of one or more signaling molecules that are normally present in the natural tissue can be added to the extract such that the levels of a particular signaling molecule may be higher in the composition than would normally be after the extraction process, i.e., "additional endogenous signaling molecules." The exogenous signaling molecules or the additional endogenous signaling molecules added to the extracts of the invention need not be from the same animal source as the source of the extracted tissue, *e.g.,* mouse VEGF may be added to human-derived cardiac basement membrane extract. In addition, the exogenous signaling molecules and the additional endogenous signaling molecules added to the extract can be recombinant or isolated from an organism, cell or tissue. As used herein, the term "natural tissue" is used to mean heart muscle or smooth muscle that is either present in an organism or that has been physically removed from the organism, but prior to any processing steps, *e.g.,* enzyme digestion. Tissue that is removed and frozen and has not been subjected to any processing steps, *e.g.,* enzyme digestion and the like, is considered to be "natural tissue" for the purposes of the present invention.

[0027] As used herein, a "signaling molecule" is a molecule that either initiates an intracellular signal or signaling pathway or is an intermediate molecule in the signaling or pathway process. A "signal" is a stimulus that creates a cellular response thereto. Signaling molecules can include any molecule that either initiates or participates in the signaling pathway. For example, signaling molecules include but are not limited to growth factors, cytokines and chemokines that may or may not be present in the natural tissue.

[0028] Examples of signaling molecules include but are not limited to fibroblast growth factors (FGF), *e.g.,* FGF1, FGF2, FGF3, FGF4, FGF5, FGF6, FGF7, FGF8, FGF9, FGF10, FGF11, FGF12, FGF13, FGF14, FGF15, FGF16, FGF17, FGF18, FGF19, FGF20, FGF21, FGF22, FGF23, epidermal growth factor (EGF), nerve growth factor (NGF), platelet derived growth factor (PDGF-AA, AB and BB), insulin-like growth factors, *e.g.*, IGF-1 and 2, transforming growth factor beta, *e.g.,* TGF-beta 1,2 or 3, vascular endothelial cell growth factor, *e.g.,* VEGF-A, VEGF-B,VEGF-C,VEGF-D, placental growth factor, tumor necrosis factor alpha (TNF-alpha), TNF-beta, bone morphogenetic factors *e.g.,* BMP1, BMP2, BMP3, BMP4, BMP5, BMP6, BMP7, BMP8, BMP9, BMP10, BMP11, BMP12, BMP13, BMP14,and BMP15, Leukemia inhibitory factor (LIF-1), endostatin, angiostatin, thrombospondin, osteogenic protein-1, osteonectin, somatomedin-like peptide, osteocalcin, interferons (IFN), such as but not limited to IFN Type I, IFN Type II and IFN Type III, examples of all of which include but are not limited to IFN-alpha, IFN, beta, IFN-gamma and IFN-omega, interleukins (IL), *e.g.,* IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, IL-19, IL-20, IL-21, IL-22, IL-23, IL-24, IL-25, IL-26, IL-27, IL-28, IL-29, IL-30, IL-31, IL-32, IL-33 and IL-35 and stromal-derived factor (SDF-1) to name a few.

[0029] The compositions of the present invention may or may not be polymerized in any setting used. The cell support compositions of the present invention are liquid at temperatures of between about 0°C and about 8°C at normal atmospheric pressure. The compositions, however, will polymerize within about 2-10 hours when the temperature is raised to at least about 22°C or within about 20 minutes to about 1 hours at 37°C. As used herein, the term “polymerize” or “gelation” when used in conjunction with the cell support compositions of the present invention means that the compositions form a gel. The term gel is well understood in the art and, in general, means a cross-linked network of molecules dispersed in a liquid that generally exhibits little to no flow. As used herein, the terms “gelation” and “polymerization” are interchangeable when used in conjunction with the cell support compositions of the present invention.

[0030] In some embodiments, the cell support compositions further comprise materials that are added to the compositions. The additional materials may be natural or synthetic, or a combination thereof. Examples of natural materials include, but are not limited to, amino acids, peptides, polypeptides, proteins, carbohydrates, lipids, nucleic acids, glycoproteins, glycosaminoglycans, and proteoglycans. Examples of synthetic materials include, but are not limited to, polymers such as poly (lactic acid) (PLA), polyglycolic acid (PGA), copolymers of PLA and PGA, polycaprolactone, poly (ethylene-

co-vinyl acetate), (EVOH), poly (vinyl acetate) (PVA), polyethylene glycol (PEG) and poly (ethylene oxide) (PEO).

[0031] The cell support compositions of the present invention can be used in cell culturing techniques. As used herein, cell culture refers to the maintenance of cells in an artificial environment, commonly referred to as an *in vitro* environment. 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, organs, organ systems or whole organisms. The cells used in the culture methods disclosed herein can be any prokaryotic or eukaryotic cell. The cell type used in the culture methods disclosed herein need not be from the same species from which the cell support compositions derive. In addition, the cells may be from an established cell line, or they may be primary cells or genetically engineered cells.

[0032] The cell support compositions of the present invention can be used in *in vitro* methods for supporting cell growth and proliferation as well as for sustaining or maintaining the plasticity of stem cells in culture. Accordingly, the invention provides methods of maintaining the plasticity of stem cells with the methods comprising culturing stem cells on a cell culture surface that comprises the cell support compositions of the present invention. In one embodiment, the cell support composition is polymerized prior to culturing the stem cells on the cell surface.

[0033] As used herein, a stem cell is used as it is in the art and means a cell that has the ability to divide and give rise to one daughter cell that may be at least partially differentiated and to another daughter cell that retains the developmental potential of the mother cell. As used herein, stem cells can be adult stem cells (ASCs), embryonic stem cells (ESCs), committed progenitor cells, and/or induced pluripotent stem cells (iPSCs).

[0034] The terms “cell” and “cell line” may be used interchangeably herein. In one embodiment, stem cells that are attached to cell culture surfaces coated with the cell support compositions of the present invention and are able to maintain their state of plasticity after attachment. Specifically, cells attached on surfaces coated the cell support compositions of the present invention can maintain their state of plasticity for up to at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100 passages or even more. As used herein, “state of plasticity” is used to mean the development potential that the cells have, such as, but not limited to, pluripotent, totitpotent, multipotent or unipotent cells. Maintaining a state of plasticity indicates that cell daughter cells, after division, are equally as plastic as the parent cells, *e.g.,* the daughter cells are pluripotent like the

pluripotent parent cell. In another embodiment, the cells that are attached to surfaces coated with the cell support compositions of the present invention are able to maintain at least a partial state of plasticity after attachment. Specifically, the cells attached on surfaces coated with the cell support compositions of the present invention can maintain at least a partial state of plascticity for up to at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100 passages or even more. As used herein, "partial state of plasticity" is used to mean the development potential that the daughter cells have, such as, but not limited to, pluripotent, totitpotent or unipotent cells. Maintaining at least a partial state of plasticity indicates that cell daughter cells, after division, are not as plastic as the parent cells but nonetheless are still in an undifferentiated state, *e.g.,* the daughter cells are multipotent whereas the parent cell was considered to be pluripotent.

[0035] The terms pluripotent, totipotent, multipotent and unipotent are used as they are in the art. Namely, a pluripotent cell is a cell that, depending on environment, has the potential to develop into any type of mature cell found in the organism from which it derived, except germ cells. A totipotent cell is a cell that, depending on environment, has the potential to develop into any type of mature cell found in the organism from which it derived, including germ cells. A multipotent cell is a cell that, depending on environment, has the potential to develop into several different types of mature cell found in the organism from which it derived, usually 2 or more. One example of a multipotent cell is a hematopoietic stem cell. A unipotent cell is a cell that, depending on environment, has the potential to develop into one type of mature cell found in the organism from which it derived. Unipotent cells are not completely differentiated cells, but may be partially differentiated or completely undifferentiated.

[0036] The cell support compositions of the present invention can be used in methods for supporting cell growth and proliferation as well as for sustaining or maintaining the state of differentiation of differentiated or partially differentiated cells, with the methods comprising culturing the differentiated or partially differentiated cells on a cell culture surface that comprises the cell support compositions of the present invention. In one embodiment, the cell support composition is polymerized prior to culturing the differentiated or partially differentiated cells on the cell surface In one embodiment, the differentiated or partially differentiated cells that are attached to cell culture surfaces coated with the cell support compositions of the present invention and are able to maintain their state of differentiation after attachment. Specifically, differentiated or partially differentiated cells attached on surfaces coated the cell support compositions of the present invention can maintain their state of differentiation for up to at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85,

90, 95 or 100 passages or even more. As used herein, "state of differentiation" is used to mean the development state of the cells after division. Maintaining a state of differentiation means that the daughter cells are at least as differentiated, and perhaps more so, than the parent cell. The differentiated or partially differentiated cells may or may not divide when cultured on surfaces coated with the cell support compositions of the present invention.

[0037] In one embodiment, neuronal precursors are seeded onto cell culture surfaces comprising the cell support compositions of the present invention. After adhesion, the cells will extend neurites and express neuron-specific genes in the process of neuronal differentiation. In another embodiment, endothelial cells are seeded are seeded onto cell culture surfaces comprising the cell support compositions of the present invention. After adhesion, the cells will associate to form branching tubular structures with lumens resembling blood vessels. These processes may or may not be inhibited in the presence of excess free peptides derived from the laminin binding sites like IKVAV.

[0038] In one embodiment, neuronal precursors are seeded onto cell culture surfaces comprising the cell support compositions of the present invention. After adhesion, the cells will extend neurites and express neuron-specific genes in the process of neuronal differentiation. In another embodiment, endothelial cells are seeded are seeded onto cell culture surfaces comprising the cell support compositions of the present invention. After adhesion, the cells will associate to form branching tubular structures with lumens resembling blood vessels. These processes may or may not be inhibited in the presence of excess free peptides derived from the laminin binding sites like IKVAV.

[0039] The cells may be from an established cell line, or they may be primary cells or genetically engineered cells. For example, neovascularization can be stimulated by angiogenic and growth-promoting factors, administered as peptides, proteins or as gene therapy. Angiogenic agents can be incorporated into the cell support compositions by culturing on the cell support compositions (or adding genetically engineered cells to unpolymerized cell support compositions) genetically engineered cells that are engineered to produce, for example, VEGF. Alternatively, where neovascularization is not desired, antiangiogenic agents can be incorporated into the cell support compositions by culturing on the cell support compositions (or adding genetically engineered cells to unpolymerized cell support compositions) genetically engineered cells that are engineered to produce, for example, angiostatin.

[0040] The term "culture surface" as used herein means a surface on which cells or tissue can be cultured. The culture surface may, for example, be glass or plastic, or polymers such as alginate. Other

surfaces include but are not limited to, biopolymers such as PLAGA (polylactic acid glycolic acid copolymer), PLA (polylactic acid), PCL (polycaprolactone), PDO (polydione), PEO (polyester), metals such as but not limited to, stainless steel or titanium, glass, other forms of plastic besides polystyrene, such as but not limited to polycarbonate, polyallomer, polyethylene, and any composition of calcium phosphate crystal including but not limited to hyaluronic acid. Culture surfaces include, but are not limited to, single and multiwall culture plates, chambered and multi-chambered culture slides, cover-slips, cups, flasks, tubes, bottles, roller bottles, spinner bottles, perfusion chambers, bioreactors, fermenters and the like.

[0041] In one embodiment, the cell support compositions of the present invention can be fabricated into a scaffold, for cell culture or tissue engineering applications. For example, the cell support compositions of the present invention can be fabricated into nanofibers and mesh through electrospinning technologies as described in US2010/0120115, which is incorporated by reference. The term nanofiber as used herein means a fiber comprising a diameter of about 1000 nanometers or less. Once fabricated into a scaffold, the cell support compositions of the present invention can be placed into cell culture environment as disclosed herein.

[0042] The cell support compositions of the present invention can be used in *in vivo* methods as well. For example, in one embodiment of the present invention, the cell support composition can be used as a tissue regenerative composition for repair or replacement of tissues in an *in vivo* setting. Thus the present invention provides therapeutic methods, with the methods comprising administering the cell support compositions of the present invention to a subject in need of such therapy. The cell support compositions can be used in a variety of tissues or organs, including but not limited to, cardiac tissue, bone tissue, ligament tissue, tendon tissue, skin, muscle tissue, vasculature, liver tissue, lung tissue, and the like. The tissue to which the cell support compositions can be applied may be diseased, normal, damaged or even dead. The tissues or organs to which the cell support compositions can be applied would include all compartments or subdivisions of the tissue or organ. For example, cardiac tissue includes, but is not limited to, diseased, damaged, or missing heart tissue including myocardium, epicardium, endocardium, pericardium. For example, the cell support compositions of the present invention can be fluidized or maintained as a fluid, powderized, or pulverized and applied to or injected into or adjacent to diseased or defective cardiac tissue to promote tissue repair.

[0043] As used herein, the term "administer" and "administering" are used to mean introducing at least one compound or composition into a subject. When administration is for the purpose of treatment, the substance is provided at, or after the diagnosis of an abnormal condition, such as an infarction.

[0044] The cell support compositions may also be coadministered with other compounds or compositions. As used herein, the term "coadminister" is used to mean that each of at least two compounds are administered during a time frame wherein the respective periods of biological activity overlap. Thus the term includes sequential as well as coextensive administration of the compositions of the present invention. If more than one substance is coadministered, the routes of administration of the two or more substances need not be the same. The scope of the invention is not limited by the identity of the substance which may be coadministered with the compositions of the present invention.

[0045] For embodiments in which the cell support compositions are used *in vivo*, the cell support compositions can further comprise one or more therapeutic molecules including, without limitation, any pharmaceutical or drug. Examples of pharmaceuticals include, but are not limited to, anesthetics, hypnotics, sedatives and sleep inducers, antipsychotics, antidepressants, antiallergics, antianginals, antiarthritics, antiasthmatics, antidiabetics, antidiarrheal drugs, anticonvulsants, antigout drugs, antihistamines, antipruritics, emetics, antiemetics, antispasmodics, appetite suppressants, neuroactive substances, neurotransmitter agonists, antagonists, receptor blockers and reuptake modulators, beta-adrenergic blockers, calcium channel blockers, disulfuram and disulfuram-like drugs, muscle relaxants, analgesics, antipyretics, stimulants, anticholinesterase agents, parasympathomimetic agents, hormones, anticoagulants, antithrombotics, thrombolytics, immunoglobulins, immunosuppressants, hormone agonists/antagonists, vitamins, antimicrobial agents, antineoplastics, antacids, digestants, laxatives, cathartics, antiseptics, diuretics, disinfectants, fungicides, ectoparasiticides, antiparasitics, heavy metals, heavy metal antagonists, chelating agents, gases and vapors, alkaloids, salts, ions, autacoids, digitalis, cardiac glycosides, antiarrhythmics, antihypertensives, vasodilators, vasoconstrictors, antimuscarinics, ganglionic stimulating agents, ganglionic blocking agents, neuromuscular blocking agents, adrenergic nerve inhibitors, anti-oxidants, vitamins, cosmetics, anti-inflammatories, wound care products, antithrombogenic agents, antitumoral agents, antiangiogenic agents, anesthetics, antigenic agents, wound healing agents, plant extracts, growth factors, emollients, humectants, rejection/anti-rejection drugs, spermicides, conditioners, antibacterial agents, antifungal agents, antiviral agents, antibiotics, tranquilizers, cholesterol-reducing drugs, antitussives, histamine-blocking drugs, monoamine oxidase

inhibitor. All substances listed by the U.S. Pharmacopeia are also included within the substances of the present invention.

[0046] The cell support compositions of the present invention may or may not further comprise objects. Examples of objects include, but are not limited to, tablets, vesicles, liposomes, capsules, nanoparticles, and other structures that could enclose molecules. In some embodiments, the additional objects comprise vesicles, liposomes, capsules, or other enclosures that contain compounds that are released at a time such as at the time of implantation or upon later stimulation or interaction. In one illustrative embodiment, transfection agents such as liposomes contain desired nucleotide sequences that can be incorporated into cells that are located in or on the cell support compositions.

[0047] The cell support compositions of the present invention may or may not further comprise additional amino acids, proteins or peptides that are not necessarily signaling molecules. Examples include, but are not limited to, structural proteins, enzymes, and peptide hormones. These additional proteins and peptides compounds can serve a variety of functions. In some embodiments, the matrix may contain peptides containing a sequence that suppresses enzyme activity through competition for the active site. In other applications, antigenic agents that promote an immune response and invoke immunity can be incorporated into a construct.

[0048] The cell support compositions of the present invention may or may not further comprise additional nucleic acids. Examples of nucleic acids include, but are not limited to deoxyribonucleic acid (DNA), ent-DNA, oligonucleotides, aptamers, and ribonucleic acid (RNA). Embodiments involving DNA include, but are not limited to, cDNA sequences, natural DNA sequences from any source, and sense or anti-sense oligonucleotides. For example, DNA can be naked (e.g., U.S. Pat. Nos. 5,580,859; 5,910,488) or complexed or encapsulated (e.g., U.S. Pat. Nos. 5,908,777; 5,787,567). DNA can be present in vectors of any kind, for example in a viral or plasmid vector. In some embodiments, nucleic acids used will serve to promote or to inhibit the expression of genes in cells inside and/or outside the present composition. The nucleic acids can be in any form that is effective to enhance uptake into cells.

[0049] The present invention also provides methods of preparing basement membrane extracts that has been isolated from cardiac or smooth muscle tissue from an organism. As used herein, isolated means that the material has been removed from its native environment, regardless of the level of impurities that may or may not be present. For example, cardiac basement membrane has been isolated when it has been removed or extracted from cardiac tissue. As used herein, the isolated

material can be placed back into a setting that resembles, substantially resembles or is identical to the original environment from which the material was isolated. For example, the isolated cardiac basement membrane extract may be inserted or introduced back into cardiac tissue of another organism. Any degree of purification or concentration greater than that which occurs in the natural setting of the compound, including, but not limited to, (1) purification from other associated structures or compounds or (2) association with structures or compounds to which the material is not normally associated is included in the term purified.

[0050] The following examples are illustrative and are not intended to limit the scope of the invention described herein.

Examples

[0051] *Example 1: Process for isolating basement membrane components from cardiac muscle*

[0052] Methods for preparing the isolated human basement membrane extract (HBME) can be conducted at a temperature of about 0°C, 4°C, 15°C, 24°C, 27°C, 30°C, or 37°C. The solutions used for the isolation process can be chilled or warmed to the appropriate temperature before starting the process.

[0053] Frozen, thinly sliced or minced pieces of cardiac tissue were washed or not washed with a buffer such as phosphate or potassium or calcium chloride at a concentration such as 0.4M to 0.05M for a period of time such a 1,2,3,4,...24 hour. After washing ice cold 3.0 M, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4.0 NaCl or KCl buffer is added to tissue including protease inhibitors such as PMSF, Aprotinin, leupeptin, pepstatin A, NEM, phenanthroline, Benzamidine, AEBSF, Bestatin, E-64 (volume to weight 2:1, 3:1, 4:1, 5:1, 6:1, 7:1) and homogenized.

[0054] The homogenized slurry was then centrifuged and the supernatant was poured off and the pellets collected in a tripour beaker. Another aliquot of 3.0 M, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4.0NaCl or KCl buffer was added as above and the pellets were washed and homogenized. After homogenization, the slurry was again centrifuged. These washes and homogenizations were repeated 3-5 times.

[0055] After repeated washing of the processed tissue, 2 M Urea Buffer was added at a concentration of 1 ml/gm starting weight of tissue. The mixture was stirred overnight. The mixture was then

centrifuged and decanted. The supernatant was saved for later use. The pellet was recovered and homogenized again in 2 M urea buffer (half the volume as the first use of the Urea Buffer) stirred at least 2 hours.

[0056] The mixture was centrifuged again and the supernatant was recovered and added to the first volume of supernatant recovered above. The mixture was dialysed against a buffer such as phosphate, TEA, Bicine, MOPS, Tris, Hepes, TAPSO, PIPES, MES, or other Good's buffers. The last buffer used can be DMEM, DMEM/F12, HEPES, Hanks or other cell culture media. Antibiotics can be added to the extract and stored at 4°C or long term at -80°C. The concentration of the mixture is determined by dialyzing the mixture against dH_2O and lyophilizing in tared containers and weighing when dry.

[0057] *Example 2: Coating Cell Culture Surfaces with Isolated HMBE*

[0058] The protein needed to coat the plate at each concentration is calculated at about 10-20 $\mu g/cm^2$. Other supplemental purified matrix molecules are coated in the range of 5-20 $\mu g/cm^2$. MATRIGEL™ is coated at 17.5$\mu g /cm^2$ according to the WiCell protocol and the cell support composition product may be used at this concentration or less for some applications or at higher concentration for other applications.

[0059] For dry coating, 100µl of the cell support composition was pipetted into each well of plate at the desired concentration and allowed to dry uncovered in biosafety cabinet. After coating, the plates were washed very gently with 1-2 volumes of a typical cell culture buffer such as culture media, PBS or Hanks balanced salt solution.

[0060] After washing, 200µl of sterile buffer containing 2% albumin can be added to each well and incubated for 15 min room temperature. After incubation, the albumin mixture is removed and media is added to wash the plate.

[0061] *Example 3: Cell Attachment Assay*

[0062] Plates were coated at various concentrations of the cell support compositions of the present invention (see Figure 2) to test cell attachment using 3T3, BHK and adipose stem cells. Cells were allowed to attach to plates coated with the indicated amounts of the cell support compositions of the present invention for 60 minutes in the absence of serum. After attachment, the plates were gently

rinsed to remove unattached cells and attached cells were counted. Similar results were observed with human mesenchymal stem cells.

[0063] *Example 4: Differentiation Assays of Attached Cells*

[0064] Adipose derived stem cells are isolated using well known procedures and are plated at a density of about 5,000 cells/100 mm dish. The dishes have been coated with isolated the cell support compositions of the present invention, and the cells are cultured for a few days after plating. After successive rounds of cell division, some clones are picked with a cloning ring and transferred to wells in a 48 well plate, where the wells have also been coated with isolated the cell support compositions of the present invention. These cells are cultured for several weeks, changing the medium twice weekly, until they are about 80% to about 90% confluent (at 37°C, in about 5% CO_2 in 2/3 F_{12} medium (with 20% fetal bovine serum) and 1/3 standard medium). Thereafter, each culture is transferred to a 35 mm dish (coated with the cell support compositions of the present invention) and grown, and then retransferred to a 100 mm dish (coated with isolated the cell support compositions of the present invention) and grown until close to confluence. Following this, one cell population is frozen, and the remaining populations were plated on 12 well plates, at 1000 cells/well.

[0065] The cells are cultured for more than 15 passages in medium and on surfaces coated with the cell support compositions of the present invention and monitored for signs of differentiation. The undifferentiated state of each clone remains true after successive rounds of division.

[0066] Populations of the clones are then established on culture surfaces not coated with the cell support compositions of the present invention and then exposed to adipogenic, chondrogenic, myogenic, and osteogenic medium, or other medium or factors known to promote differentiation of the stem cells. The clones are able to differentiate into bone, fat, cartilage, and muscle when exposed to the respective media as determined by phonotypic assays of protein expression and visual inspection of cell types.

[0067] *Example 5: SDS-PAGE Analysis of HMBE*

[0068] An SDS-PAGE was performed on the cell support compositions of the present invention using standard procedures. The gel showed that the cell support compositions of the present invention is an aggregate of macromolecules purified from human cardiac tissue. These polypeptides range in size

between 350 and 100 killodaltons (kDa) and cross-react with antibodies against mouse and human laminin, type IV collagen and heparan sulfate proteoglycan.

[0069] *Example 6: Process for isolating basement membrane components from smooth muscle*

[0070] Frozen, thinly sliced or minced pieces of smooth muscle tissue are washed with buffers such as Phosphate or potassium or Calcium Chloride then added to cold 3.4M NaCl or KCl buffer (3X volume to weight) including protease inhibitors such asPMSF, Aprotinin, leupeptin, pepstatin A, NEM, phenanthroline, Benzamidine, AEBSF, Bestatin, E-64 and homogenized.

[0071] The homogenized slurry is then centrifuged and the supernatant is poured off and the pellets collected. Another aliquot of 3.4M NaCl buffer is added as above and the pellets are homogenized. After homogenization, the slurry is again centrifuged. These homogenizations are repeated 4-5 times.

[0072] After repeated washing by homogenization of the processed tissue, 2 M Urea Buffer is added at a concentration of 1 ml/gm starting weight of tissue. The mixture is stirred overnight. The mixture is then centrifuged and decanted. The supernatant is saved for later use. The pellet is recovered and homogenized again in 2 M urea buffer (half the volume as the first use of the Urea Buffer) stirred at least 2 hours.

[0073] The mixture is centrifuged again and the supernatant is recovered and added to the first volume of supernatant recovered above. Antibiotics can be added to the combined supernatant and stored at 4°C or long term at -80°C.

[0074] *Example 7 – Direct Injection of the Cell Support Composition into a Subject*

[0075] The cell support composition as prepared according to Example 1 is prepared for injection into damaged cardiac tissue. A procedure is administered to mice to induce myocardial infarction as disclosed in Salto-Tellez, M. *et al., Cardiovascular Path.,* 13(2):91-97 (2004). Specifically, a coronary artery is ligated to cause infarction. After reperfusion of the vessel, a cell support composition of the present invention is injected into the damaged cardiac tissue.

[0076] The injections can be single injections or multiple injections over time. A single injection of the cell support composition of the present invention improves heart function as measured by normal parameters, such as ejection volume, end systolic volume, stroke volume and echocardiagraphy. In as

little as 7 days, the mice receiving the cell support composition of the present invention show improvement over control animals not receiving the compositions of the present invention.

What is Claimed is:

1. A cell support composition comprising in parts by weight 5-50% laminin, wherein laminin type 1 and laminin type 3 are not present in any amount, 5-50% collagen, 1-10% nidogen, 5-60% heparan sulfate proteoglycan, and 1-10% entactin.

2. The cell support composition of claim 1, wherein laminin type 5 is not present in any amount.

3. The cell support composition of claim 1, wherein collagen type IV is present.

4. The cell support composition of claim 1, wherein collagen type I is present.

5. The cell support composition of claim 1, wherein collagen type VI is present.

6. The cell support composition of claim 3, wherein collagen type VI is present.

7. The cell support composition of claim 4, wherein collagen type VI is present.

8. The cell support composition of claim 7, wherein collagen type VI is present.

9. The cell support composition of claim 1, wherein the heparan sulfate proteoglycan comprises perlecan, wherein the perlecan is present in the cell support composition in an amount from about 5% to about 60% by weight.

10. The cell support composition of claim 1, further comprising titan protein.

11. The cell support composition of claim 1, further comprising myosin 7.

12. The cell support composition of claim 1, further comprising actinin protein.

13. The cell support composition of claim 1, wherein the cell support composition promotes the maintenance of plasticity of stem cells in cell culture.

14. The cell support composition of claim 1, wherein the cell support composition polymerizes at a temperature of at least about 37°C within about 1 hour.

15. The cell support composition of claim 1, wherein the cell support composition is polymerized.

16. The cell support composition of claim 15, wherein the polymerized cell support composition is in form of nanofibers.

17. A cell culture surface comprising the cell support composition of claim 15.

18. A method of maintaining the plasticity of stem cells, comprising culturing the stem cells on a cell culture surface comprising the cell support composition of claim 1, wherein the cell support composition has been polymerized prior to culturing the stem cells thereon .

19. The method of claim 18, wherein the stem cells are adult stem cells (ASCs).

20. The method of claim 18, wherein the stem cells are embryonic stem cells (ESCs).

21. The method of claim 18, wherein the stem cells are induced pluripotent cells (iPCs).

22. The method of claim 18, wherein the cell support composition is in the form of nanofibers.

23. A method of treating tissue comprising administering to a subject in need of treatment the cell support composition of claim 1.

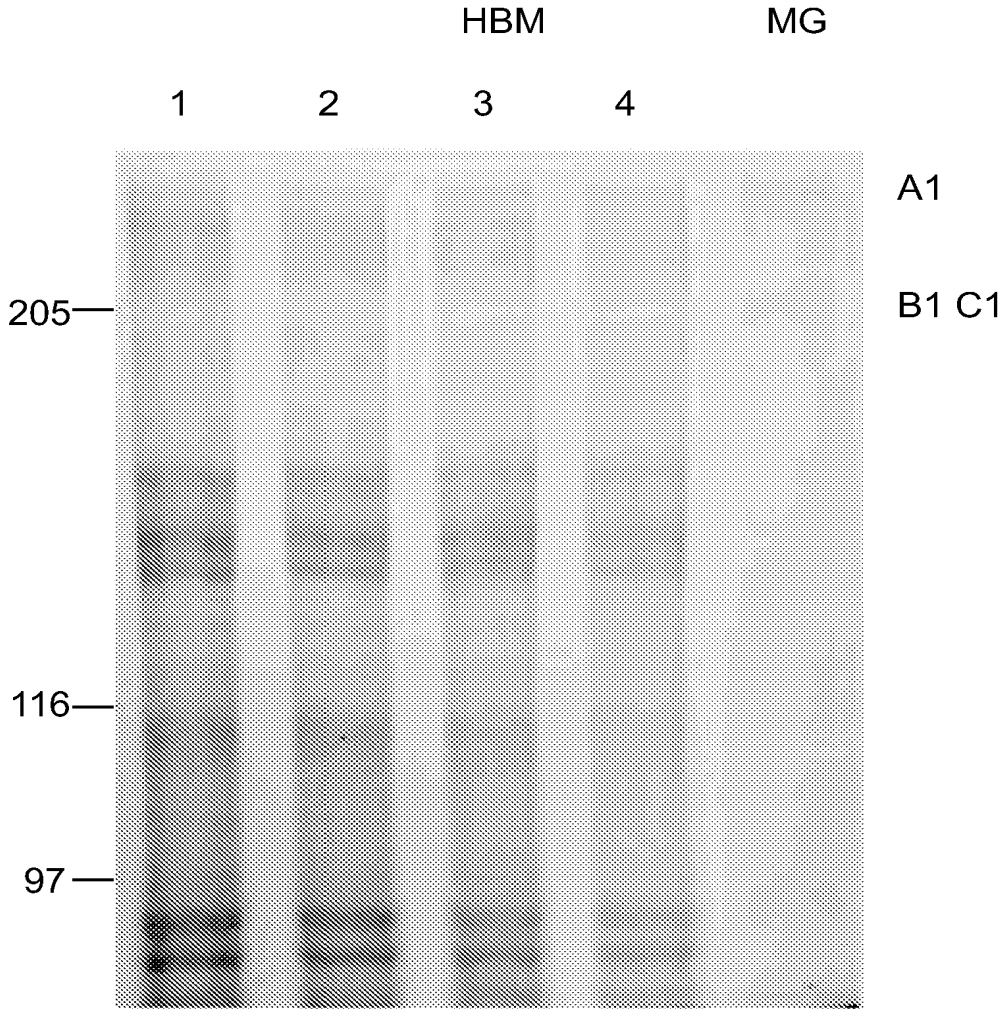
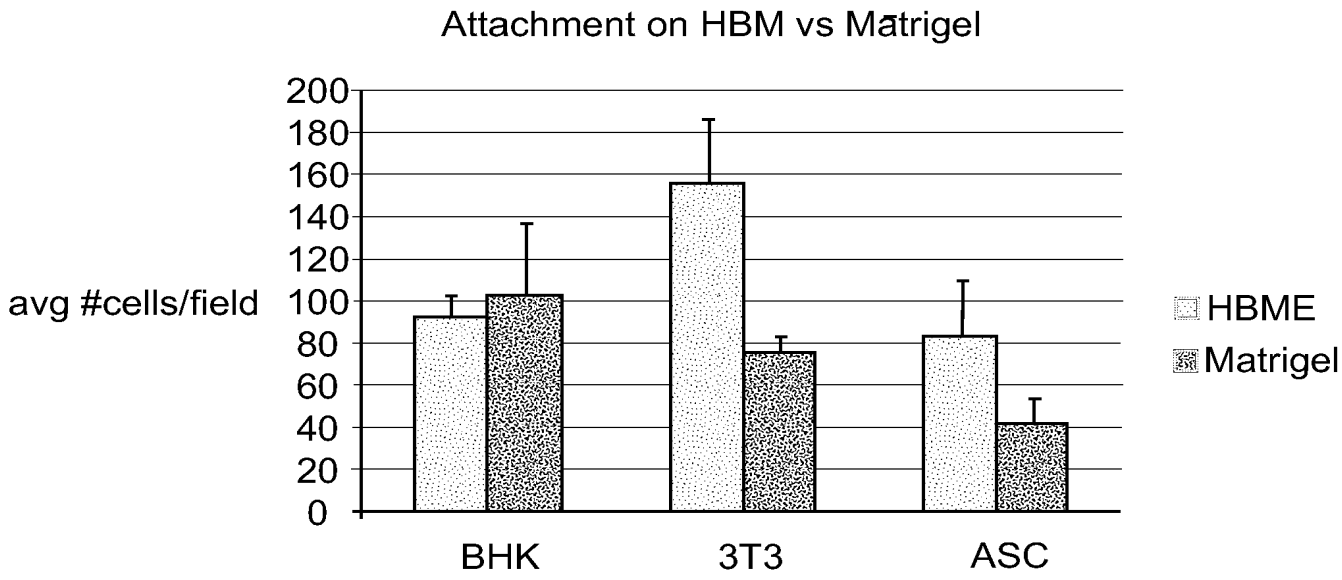


FIG. 1



CELL NAME	CELL TYPE	SPECIES	HBM/MG ATTACHMENT
3T3	FIBROBLAST-LIKE	MOUSE	2.08
BHK	KIDNEY EPITHELIAL	HAMSTER	0.90
ASC	MULTIPOTENT SC	HUMAN	1.76
hIPSc	PLURIPOTENT SC	HUMAN	2.30

FIG. 2

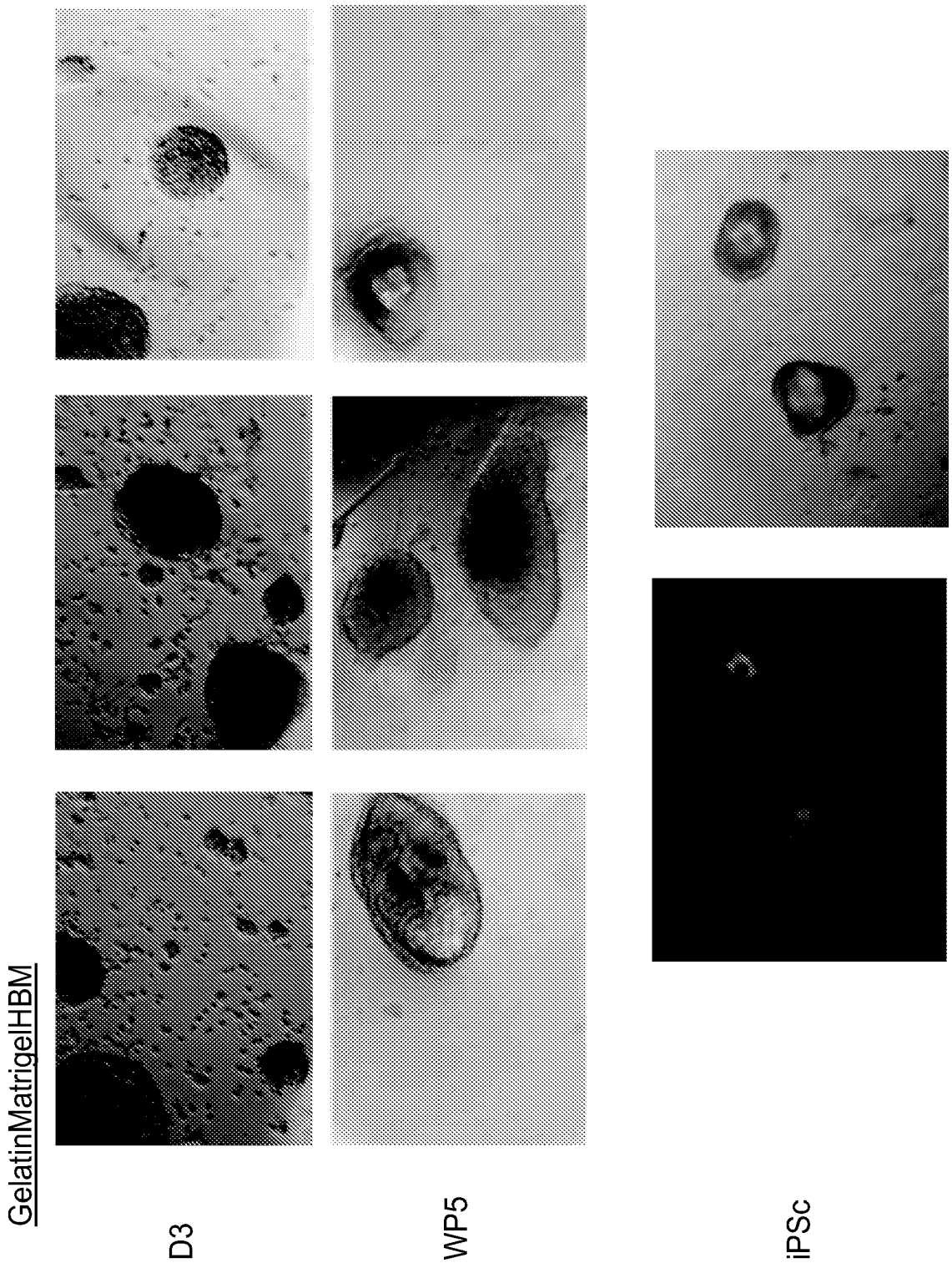


FIG. 3

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(54) **Title**: BMP PEPTIDES & METHODS OF USE

(57) **Abstract**: The invention relates to truncated growth factors and variants thereof. The invention also relates to methods of making and using the truncated growth factors. The invention further relates to compositions including a protease and a growth factor comprising a bone morphogenic protein (BMP) or a variant thereof. The invention also relates to methods of using the composition.

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BMP PEPTIDES & METHODS OF USE

Cross-Reference to Related Applications

[0001] This application claims priority to U.S. Provisional Application Nos. 61/530,706, 61/530,715, 61/530,727, and 61/536,035, filed September 2, 2011, September 2, 2011, September 2, 2011, and September 18, 2011, respectively, all of which are incorporated herein by reference in their entireties.

Background of the Invention

[0002] The invention relates to truncated growth factors and variants thereof. The invention also relates to methods of making and using the truncated growth factors.

[0003] The invention relates to compositions including a protease and a growth factor comprising a bone morphogenic protein (BMP) or a variant thereof. The invention also relates to methods of using the composition.

Summary of the Invention

[0004] The invention relates to isolated peptides of 137 residues or less, with the peptide comprising an amino acid sequence at least 95% identical to the amino acid sequence of SEQ ID NO:20, 21, 22, 23, 24, 25, 26, 27 or 28.

[0005] The invention relates to isolated peptides of 119 residues or less, with the peptide comprising an amino acid sequence at least 95% identical to the amino acid sequence of SEQ ID NO:34.

[0006] The invention relates to isolated peptides of 119 residues or less, with the peptide comprising an amino acid sequence at least 95% identical to the amino acid sequence of SEQ ID NO:35.

[0007] The invention also relates to fusion proteins comprising the inventive peptides described herein fused to a second peptide, wherein the second peptide comprises an amino acid sequence that is less than 70% identical to the amino acid sequence of SEQ ID NO:40, 41, 42, 43, 44, 45, 46, 47, 48, 54, or 55.

[0008] The invention also relates to methods of promoting osteoinductive, chondroinductive, ligament differentiating, and/or tendon differentiating activity, with the methods comprising contacting cells with the inventive peptides described herein.

[0009] The invention also relates to methods of administering the inventive peptides described herein to matrices.

[0010] The invention also relates to methods of increasing osteogenesis, chondrogenesis, or ligament/tendon genesis in cells comprising administering to the cells the inventive peptides described herein.

[0011] The invention also relates to methods of increasing a cellular growth factor activity comprising administering to the cells at least one protease and the growth factor comprising SEQ ID NO: 20, 21, 22, 23, 24, 25, 26, 27, 28, 34 or 35. In some embodiments, the composition comprises two or more proteases.

[0012] The invention relates to compositions comprising at least one protease and a growth factor, wherein the growth factor is (i) a bone morphogenic protein (BMP) or (ii) an isolated peptide comprising an amino acid sequence at least 92% identical to any of amino acid sequences of SEQ ID NO: 16-35, wherein the peptide has osteoinductive, chondroinductive, or ligament/tendon differentiating activity. In some embodiments, the composition comprises two or more proteases.

[0013] The invention also relates to methods of increasing a growth factor activity comprising administering to the cells the composition described herein.

Brief Description of the Drawings

[0014] FIGURE 1 depicts myoblast expression of alkaline phosphatase after being cultured with rhBMP-2 modified with trypsin. Myoblasts treated with rhBMP-2 modified with trypsin showed significantly greater alkaline phosphatase activity over myoblasts treated with unmodified rhBMP-2.

[0015] FIGURE 2 depicts myoblast expression of alkaline phosphatase after being cultured with rhBMP-2 modified with collagenase. Myoblasts treated with rhBMP-2 modified with collagenase showed significantly greater alkaline phosphatase activity over myoblasts treated with unmodified rhBMP-2. When fractionated via HPLC, fractions A42-44, A45-46 and A47-49 induced the highest levels of alkaline phosphatase in myoblasts.

[0016] FIGURE 3 depicts myoblast expression of alkaline phosphatase after being cultured with rhBMP-2 control, purified modified rhBMP-2 and non-purified modified rhBMP-2.

[0017] FIGURE 4 depicts myoblast expression of alkaline phosphatase after being cultured with rhBMP-2 control and purified modified rhBMP-2 in the presence of no additional protease, dispase, inactive dispase, collagenase, or inactive collagenase.

[0018] FIGURE 5 depicts myoblast expression of alkaline phosphatase after being cultured with modified rhBMP-4. At a concentration of 30 ng/mL, cells treated with modified rhBMP4 expressed significantly higher AP activities than those treated with unmodified mature rhBMP-4.

[0019] FIGURE 6 depicts myoblast expression of alkaline phosphatase after being cultured with different concentrations of unmodified or dispase-modified rhBMP-5. Myoblasts treated with dispase modified rhBMP-5 showed significantly greater alkaline phosphatase activity over myoblasts treated with unmodified rhBMP-5.

[0020] FIGURE 7 depicts myoblast expression of alkaline phosphatase after being cultured with different concentrations of unmodified or collagenase-modified rhBMP-5. Myoblasts treated with collagenase modified rhBMP-5 showed significantly greater alkaline phosphatase activity over myoblasts treated with unmodified rhBMP-5.

[0021] FIGURE 8 depicts the expression of alkaline phosphatase in myoblast after being cultured with purified modified rhBMP-5 alone, or the same concentration of purified modified rhBMP-5 with the addition of dispase or collagenase.

[0022] FIGURE 9 depicts myoblast expression of alkaline phosphatase after being cultured with modified rhBMP-7. Myoblasts treated with modified rhBMP-7 showed significantly greater alkaline phosphatase activity over myoblasts treated with unmodified rhBMP-7. When fractionated via HPLC, fractions 43 induced the highest levels of alkaline phosphatase in myoblasts.

[0023] FIGURE 10 depicts expression of alkaline phosphatase in myoblasts after being cultured with various concentrations of modified rhBMP-7.

[0024] FIGURE 11 depicts expression of alkaline phosphatase in myoblasts after being cultured with unmodified rhBMP-7 or trypsin-modified rhBMP-7.

[0025] FIGURE 12 depicts in vitro alkaline phosphatase expressions by myoblast cells cultured with non-treated rhBMP-7 control, dispase modified rhBMP-7, or dispase modified rhBMP-7 with the addition of collagenase in culture media.

[0026] FIGURE 13 depicts the expression of alkaline phosphatase in C2C12 cells after being cultured with various concentrations of unmodified or dispase modified rhBMP-13 (rhGDF-6). The alkaline phosphatase activity induced by dispase-modified mature rhBMP-13 was about 300 - 350 times higher than that induced by unmodified mature rhBMP-13control.

[0027] FIGURE 14 depicts the expression of alkaline phosphatase in C2C12 cells after being cultured with various concentrations of unmodified or collagenase modified mature rhBMP-13 (rhGDF-6). The alkaline phosphatase activity induced by collagenase-modified mature rhBMP-13 was about 0.7 - 20 times higher than that induced by unmodified mature rhBMP-13 control.

[0028] FIGURE 15 depicts myoblast expression of alkaline phosphatase after being cultured with various concentrations of unmodified or modified rhBMP-14 (rhGDF-5). Myoblasts treated with dispase modified rhBMP-14 showed significantly greater alkaline phosphatase activity over myoblasts treated with unmodified rhBMP-14 control. The level of alkaline phosphatase activity increase was dose-dependent upon rhBMP-14 concentation.

[0029] FIGURE 16 depicts the expression of alkaline phosphatase in C2C12 cells after being cultured with purified modified rhBMP-14 alone, or the same concentration of purified modified rhBMP-14 with the addition of dispase. The alkaline phosphatase activity in the cells induced by the purified modified rhBMP-14 in combination with dispase was about 235% higher than that induced by the purified modified rhBMP-14 alone.

[0030] FIGURE 17 depicts the expression of alkaline phosphatase in C2C12 cells after being cultured with non-purified modified rhBMP-14, or the same concentration of non-purified modified rhBMP-14 with addition of dispase or collagenase. The alkaline phosphatase activity in the cells induced by the non-purified modified rhBMP-14 in combination with dispase or collagenase were about 120% and 45% higher than that induced by the non-purified modified rhBMP-14 alone, respectively.

Detailed Description of the Invention

[0031] The invention relates to compositions comprising at least one protease and a growth factor comprising a bone morphogenic protein (BMP) or a variant thereof. In some embodiments, the growth factors are bone morphogenic proteins ("BMP," "hBMP," or "rhBMP"). The term "bone morphogenetic protein" refers to a protein belonging to the BMP family of the TGF-β superfamily of proteins (BMP family) based on DNA and amino acid sequence homology. A protein belongs to the BMP family according to this invention when it has at least 50% amino acid sequence identity with at least one known BMP family member within the conserved C-terminal cysteine-rich domain which characterizes the BMP protein family. Members of the BMP family may have less than 50% DNA or amino acid sequence identity overall. Examples of BMPs include, but are not limited to, BMP-2, BMP-3, BMP-4, BMP-5, BMP-6, BMP-7, BMP-8, BMP-9, BMP-10, BMP-11, BMP-12, BMP-13, BMP-14, BMP-15, and BMP-16, *e.g.*, amino acid sequences of SEQ ID NO:1-15, respectively. In further embodiments, the growth factors are isolated peptides comprising mature peptide sequences of the BMPs. In additional embodiments, the growth factors are isolated peptides comprising modified growth factors described below. In yet additional embodiments, the growth factors are fusion proteins comprising the modified growth factors described herein fused to a second peptide, wherein the second peptide comprises an amino acid sequence that is less than 70% identical to the amino acid sequences of SEQ ID NO: 36-55.

[0032] In some embodiments, the BMPs used in the present invention expressly exclude BMP-2, BMP-4, BMP-5, BMP-7, BMP-13, and BMP-14. In some embodiments, the BMPs used in the present invention expressly exclude BMP-2, BMP-4 and BMP-7.

[0033] In one specific embodiment, the composition of the present invention comprises at least one protease and a growth factor, wherein the growth factor is a bone morphogenic protein (BMP), wherein the BMP is not BMP-2, BMP-4, BMP-5, BMP-7, BMP-13, or BMP-14. In further embodiments, the method of the present invention comprise treating a cell with the composition comprising at least one protease and a growth factor, wherein the growth factor is a bone morphogenic protein (BMP), wherein the BMP is not BMP-2, BMP-4, BMP-5, BMP-7, BMP-13, or BMP-14. In the embodiments where the BMP is not BMP-2, BMP-4, BMP-5, BMP-7, BMP-13, or BMP-14, the composition optionally comprises two or more proteases, the protease is optionally selected from the group consisting of collagenase, clostripain, dispase, trypsin, cathepsin, BMP-1 (bone morphogenetic protein-1), MMP-13 (matrix metalloproteinase-13), and a mixture thereof, the protease is optionally collagenase or dispase.

[0034] In another specific embodiment, the composition of the present invention comprises at least one protease and a growth factor, wherein the growth factor is a bone morphogenic protein (BMP), wherein the BMP is not BMP-2, BMP-4 or BMP-7. In further embodiments, the method of the present invention comprise treating a cell with the composition comprising at least one protease and a growth factor, wherein the growth factor is a bone morphogenic protein (BMP), wherein the BMP is not BMP-2, BMP-4 or BMP-7. In the embodiments where the BMP is not BMP-2, BMP-4 or BMP-7, the composition optionally comprises two or more proteases, the protease is optionally selected from the group consisting of collagenase, clostripain, dispase, trypsin, cathepsin, BMP-1 (bone morphogenetic protein-1), MMP-13 (matrix metalloproteinase-13), and a mixture thereof, the protease is optionally collagenase or dispase.

[0035] In another specific embodiment, the composition of the present invention comprises at least one protease and a growth factor, wherein the growth factor is a bone morphogenic protein (BMP), wherein the BMP does not have any amino acid sequence of residue 283-396 of SEQ ID NO: 1, residue 293-408 of SEQ ID NO: 3, residue 317-454 of SEQ ID NO: 4, residue 293-431 of SEQ ID NO: 6, and residue 336-455 of SEQ ID NO: 12, and residue 382-501 of SEQ ID NO: 13. In further embodiments, the method of the present invention comprise treating a cell with the composition comprising at least one protease and a growth factor, wherein the growth factor is a bone morphogenic protein (BMP), wherein the BMP does not have any amino acid sequence of residue 283-396 of SEQ ID NO: 1, residue 293-408 of SEQ ID NO: 3, residue 317-454 of SEQ ID NO: 4, residue 293-431 of SEQ ID NO: 6, residue 336-455 of SEQ ID NO: 12, and residue 382-501 of SEQ

ID NO: 13. In the embodiments where the BMP does not have any amino acid sequence of 283-396 of SEQ ID NO: 1, residue 293-408 of SEQ ID NO: 3, residue 317-454 of SEQ ID NO: 4, residue 293-431 of SEQ ID NO: 6, residue 336-455 of SEQ ID NO: 12, and residue 382-501 of SEQ ID NO: 13, the composition optionally comprises two or more proteases, the protease is optionally selected from the group consisting of collagenase, clostripain, dispase, trypsin, cathepsin, BMP-1 (bone morphogenetic protein-1), MMP-13 (matrix metalloproteinase-13), and a mixture thereof, the protease is optionally collagenase or dispase.

[0036] In another specific embodiment, the composition of the present invention comprises at least one protease and a growth factor, wherein the growth factor is a bone morphogenic protein (BMP), wherein the BMP does not have any amino acid sequence of residue 283-396 of SEQ ID NO: 1, residue 293-408 of SEQ ID NO: 3, and residue 293-431 of SEQ ID NO: 6. In further embodiments, the method of the present invention comprise treating a cell with the composition comprising at least one protease and a growth factor, wherein the growth factor is a bone morphogenic protein (BMP), wherein the BMP does not have any amino acid sequence of residue 283-396 of SEQ ID NO: 1, residue 293-408 of SEQ ID NO: 3, and residue 293-431 of SEQ ID NO: 6. In the embodiments where the BMP does not have any amino acid sequence of residue 283-396 of SEQ ID NO: 1, residue 293-408 of SEQ ID NO: 3, and residue 293-431 of SEQ ID NO: 6, the composition optionally comprises two or more proteases, the protease is optionally selected from the group consisting of collagenase, clostripain, dispase, trypsin, cathepsin, BMP-1 (bone morphogenetic protein-1), MMP-13 (matrix metalloproteinase-13), and a mixture thereof, the protease is optionally collagenase or dispase.

[0037] In another specific embodiment, the composition of the present invention comprises at least one protease and a growth factor, wherein the growth factor is a bone morphogenic protein (BMP), wherein the BMP does not have any amino acid sequence of SEQ ID NO: 1, 3, 4, 6, 12, and 13. In further embodiments, the method of the present invention comprise treating a cell with the composition comprising at least one protease and a growth factor, wherein the growth factor is a bone morphogenic protein (BMP), wherein the BMP does not have any amino acid sequence of SEQ ID NO: 1, 3, 4, 6, 12, and 13. In the embodiments where the BMP does not have any amino acid sequence of SEQ ID NO: 1, 3, 4, 6, 12, and 13, the composition optionally comprises two or more proteases, the protease is optionally selected from the group consisting of collagenase, clostripain, dispase, trypsin, cathepsin, BMP-1 (bone morphogenetic protein-1), MMP-13 (matrix metalloproteinase-13), and a mixture thereof, the protease is optionally collagenase or dispase.

[0038] In another specific embodiment, the composition of the present invention comprises at least one protease and a growth factor, wherein the growth factor is a bone morphogenic protein (BMP), wherein the BMP does not have any amino acid sequence of SEQ ID NO: 1, 3 and 6. In further

embodiments, the method of the present invention comprise treating a cell with the composition comprising at least one protease and a growth factor, wherein the growth factor is a bone morphogenic protein (BMP), wherein the BMP does not have any amino acid sequence of SEQ ID NO: 1, 3 and 6. In the embodiments where the BMP does not have any amino acid sequence of SEQ ID NO: 1, 3 and 6, the composition optionally comprises two or more proteases, the protease is optionally selected from the group consisting of collagenase, clostripain, dispase, trypsin, cathepsin, BMP-1 (bone morphogenetic protein-1), MMP-13 (matrix metalloproteinase-13), and a mixture thereof, the protease is optionally collagenase or dispase.

[0039] In another specific embodiment, the composition of the present invention comprises at least one protease and a growth factor, wherein the growth factor is a bone morphogenic protein (BMP), wherein the BMP does not have any amino acid sequence of SEQ ID NO: 16-28 and 30-35. In further embodiments, the method of the present invention comprise treating a cell with the composition comprising at least one protease and a growth factor, wherein the growth factor is a bone morphogenic protein (BMP), wherein the BMP does not have any amino acid sequence of SEQ ID NO: 16-28 and 30-35. In the embodiments where the BMP does not have any amino acid sequence of SEQ ID NO: 16-28 and 30-35, the composition optionally comprises two or more proteases, the protease is optionally selected from the group consisting of collagenase, clostripain, dispase, trypsin, cathepsin, BMP-1 (bone morphogenetic protein-1), MMP-13 (matrix metalloproteinase-13), and a mixture thereof, the protease is optionally collagenase or dispase.

[0040] In another specific embodiment, the composition of the present invention comprises at least one protease and a growth factor, wherein the growth factor is a bone morphogenic protein (BMP), wherein the BMP does not have any amino acid sequence of SEQ ID NO: 16-19 and 30-33. In further embodiments, the method of the present invention comprise treating a cell with the composition comprising at least one protease and a growth factor, wherein the growth factor is a bone morphogenic protein (BMP), wherein the BMP does not have any amino acid sequence of SEQ ID NO: 16-19 and 30-33. In the embodiments where the BMP does not have any amino acid sequence of SEQ ID NO: 16-19 and 30-33, the composition optionally comprises two or more proteases, the protease is optionally selected from the group consisting of collagenase, clostripain, dispase, trypsin, cathepsin, BMP-1 (bone morphogenetic protein-1), MMP-13 (matrix metalloproteinase-13), and a mixture thereof, the protease is optionally collagenase or dispase.

[0041] The terms "peptide," "polypeptide" and "protein" are used interchangeably herein. As used herein, an "isolated polypeptide" is intended to mean a polypeptide that has been completely or partially removed from its native environment. For example, polypeptides that have been removed or purified from cells are considered isolated. In addition, recombinantly produced polypeptides

molecules contained in host cells are considered isolated for the purposes of the present invention. Moreover, a peptide that is found in a cell, tissue or matrix in which it is not normally expressed or found is also considered as "isolated" for the purposes of the present invention. Similarly, polypeptides that have been synthesized are considered to be isolated polypeptides. "Purified," on the other hand is well understood in the art and generally means that the peptides are substantially free of cellular material, cellular components, chemical precursors or other chemicals beyond, perhaps, buffer or solvent. "Substantially free" is not intended to mean that other components beyond the novel peptides are undetectable. The peptides of the present invention may be isolated or purified.

[0042] The amino acid sequences of SEQ ID NO:1-15 represent the full length "prepropeptide" of some exemplary BMPs, BMP-2-16, respectively. In some embodiments, the BMP is formed as a full length prepropeptide, which usually contains, from N-terminus to C-terminus, a signal sequence, a propeptide domain and the "mature" peptide. Table 1 shows exemplary BMP sequences and the "mature" peptide sequences in the sequences.

[0043] Table 1. Mature peptide sequences in various types of BMPs.

Type of BMP	Genbank Accession No. or UniProt Accession No.	Mature Peptide
BMP-2	NP_001191	residue 283-396 of SEQ ID NO: 1
BMP-3	P12645	residue 363-472 of SEQ ID NO: 2
BMP-4	P12644	residue 293-408 of SEQ ID NO: 3
BMP-5	NP_066551	residue 317-454 of SEQ ID NO: 4
BMP-6	P22004	residue 375-513 of SEQ ID NO: 5
BMP-7	P18075	residue 293-431 of SEQ ID NO: 6
BMP-8	P34820	residue 243-402 of SEQ ID NO: 7
BMP-9	Q9UK05	residue 319-429 of SEQ ID NO: 8
BMP-10	O95393	residue 316-424 of SEQ ID NO: 9
BMP-11	O95390	residue 298-407 of SEQ ID NO: 10
BMP-12	P43029	residue 316-461 of SEQ ID NO: 11
BMP-13	NP_001001557	residue 336-455 of SEQ ID NO: 12
BMP-14	NP_000548	residue 382-501 of SEQ ID NO: 13
BMP-15	O95972	residue 267-392 of SEQ ID NO: 14
BMP-16	AAO31471	residue 171-280 of SEQ ID NO: 15

[0044] Normally, the BMP family of growth factors is translated into the full length prepropeptide. Through subsequent post-translational processing, the signal peptide and the propeptide domains are cleaved with the remaining portion of the peptide recognized as the "mature" form of the growth factor. After processing, the "mature" form of the protein normally dimerizes and active homodimeric or even heterodimeric bone morphogenetic proteins are secreted from cells and this dimer, in general, binds to its heteromeric receptor complex composed of BMP receptor type I and

BMP receptor type II, to initiate the cell signaling cascade typically associated with the specific BMP activity.

[0045] As used herein, "modified growth factor," "modified BMP," "truncated growth factor," or "truncated BMP" refers to truncations of any form of a wild type BMP disclosed herein, *i.e.* BMP 2-16, including the full length prepropeptide, the propeptide (the full length peptide without the signaling sequence) and the mature form of the disclosed BMP. The invention therefore provides the composition comprising a protease and an isolated peptide, with the peptide comprising an amino acid sequence at least about 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to any of the amino acid sequences of these modified growth factors. In one specific embodiment, the isolated peptide comprises an amino acid sequence at least 92% identical to any of the amino acid sequences of the modified growth factors. In further embodiments, the peptide according to some embodiments of the present invention comprise an amino acid sequence 100% identical to any of the amino acid sequences of the modified growth factors. In still even more embodiments, the peptides consist of any of the amino acid sequences of the modified growth factors.

[0046] The modified growth factors in accordance with some embodiments of the present invention include, but are not limited to, truncations of the mature BMPs. Specifically, Table 2 below shows exemplary truncations of various mature BMPs.

[0047] Table 2. Exemplary truncations of various mature BMPs

Type of BMP	Truncated Sequence
BMP-2	SEQ ID NO: 16-18
BMP-4	SEQ ID NO: 19
BMP-5	SEQ ID NO: 20-28
BMP-6	SEQ ID NO: 29
BMP-7	SEQ ID NO: 30-33
BMP-13	SEQ ID NO: 34
BMP-14	SEQ ID NO: 35

[0048] In one embodiment, the isolated peptide is 113,112, 111, 110, 109, 108, 107, 106, 105, 104 or 103 residues or less with each peptide independently comprising an amino acid sequence at least 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequences of SEQ ID NO:16, 17, or 18.

[0049] In another embodiment, the isolated peptide is 115, 114, 113, 112, 111, 110, 109, 108, 107 or 106 residues or less with each peptide independently comprising an amino acid sequence at least

75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO:19.

[0050] In another embodiment, the isolated peptide is 137, 136, 135, 134, 133, 132, 131, 130, 129, 128, 127, 126, 125, 124, 123, 122, 121, 120, 119, 118, 117, 116, 115, 114, 113, 112, 111, 110, 109 residues or less with each peptide independently comprising an amino acid sequence at least 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO:20, 21, 22, 23, 24, 25, 26, 27 or 28.

[0051] In another embodiment, the isolated peptide is 138, 137, 136, 135, 134, 133, 132, 131, 130, 129, 128, 127, 126, 125, 124, 123, 122, 121, 120, 119, 118, or 117 residues or less with each peptide independently comprising an amino acid sequence at least 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO:29.

[0052] In another embodiment, the isolated peptide is 138, 137, 136, 135, 134, 133, 132, 131, 130, 129, 128, 127, 126, 125, 124, 123, 122, 121, 120, 119, 118, 117, 116, 115, 114, 113 or 112 residues or less with each peptide independently comprising an amino acid sequence at least 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO:30, 31, 32 or 33.

[0053] In another embodiment, the isolated peptide is 119, 118, 117, 116, 115, 114, 113, 112, 111, 110, 109 residues or less with each peptide independently comprising an amino acid sequence at least 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO:34.

[0054] In another embodiment, the isolated peptide is 119, 118, 117, 116, 115, 114, 113, 112, 111, 110, 109, 108, 107, 106 residues or less with each peptide independently comprising an amino acid sequence at least 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO:35.

[0055] In some embodiments, the peptide variants described herein still retain their ability to specifically interact, at least partially, with its corresponding bone morphogenic protein receptor (BMPR). In additional embodiments, the peptide variants described herein are functional and capable of promoting osteoinductivity, stimulating proliferation of osteoblasts and/or promoting osteogenesis. In further embodiments, the peptide variants described herein are functional and capable of promoting chondroinductivity, stimulating proliferation of chondrocytes and/or promoting chondrogenesis. In even more embodiments, the modified growth factors of the present invention have enhanced activity

compared to the unmodified growth factors. In further embodiments, the compositions comprising a protease and the growth factor described herein have enhanced activity compared to compositions comprising the growth factor without the protease.

[0056] A polypeptide having an amino acid sequence at least, for example, about 95% "identical" to a reference an amino acid sequence, *e.g.*, SEQ ID NO:16-35, is understood to mean that the amino acid sequence of the polypeptide is identical to the reference sequence except that the amino acid sequence may include up to about five modifications per each 100 amino acids of the reference amino acid sequence. In other words, to obtain a peptide having an amino acid sequence at least about 95% identical to a reference amino acid sequence, up to about 5% of the amino acid residues of the reference sequence may be deleted or substituted with another amino acid or a number of amino acids up to about 5% of the total amino acids in the reference sequence may be inserted into the reference sequence. These modifications of the reference sequence may occur at the N- terminus or C-terminus positions of the reference amino acid sequence or anywhere between those terminal positions, interspersed either individually among amino acids in the reference sequence or in one or more contiguous groups within the reference sequence.

[0057] As used herein, "identity" is a measure of the identity of nucleotide sequences or amino acid sequences compared to a reference nucleotide or amino acid sequence. In general, the sequences are aligned so that the highest order match is obtained. "Identity" per se has an art-recognized meaning and can be calculated using well known techniques. While there are several methods to measure identity between two polynucleotide or polypeptide sequences, the term "identity" is well known to skilled artisans (Carillo (1988) J. Applied Math. 48, 1073). Examples of computer program methods to determine identity and similarity between two sequences include, but are not limited to, GCG program package (Devereux (1984) Nucleic Acids Research 12, 387), BLASTP, ExPASy, BLASTN, FASTA (Atschul (1990) J. Mol. Biol. 215, 403) and FASTDB. Examples of methods to determine identity and similarity are discussed in Michaels (2011) Current Protocols in Protein Science, Vol. 1, John Wiley & Sons.

[0058] In one embodiment of the present invention, the algorithm used to determine identity between two or more polypeptides is BLASTP. In another embodiment of the present invention, the algorithm used to determine identity between two or more polypeptides is FASTDB, which is based upon the algorithm of Brutlag (1990) Comp. App. Biosci. 6, 237-245). In a FASTDB sequence alignment, the query and reference sequences are amino sequences. The result of sequence alignment is in percent identity. In one embodiment, parameters that may be used in a FASTDB alignment of amino acid sequences to calculate percent identity include, but are not limited to: Matrix=PAM, k-tuple=2, Mismatch Penalty=1, Joining Penalty=20, Randomization Group Length=0, Cutoff Score=1,

Gap Penalty=5, Gap Size Penalty 0.05, Window Size=500 or the length of the subject amino sequence, whichever is shorter.

[0059] If the reference sequence is shorter or longer than the query sequence because of N-terminus or C-terminus additions or deletions, but not because of internal additions or deletions, a manual correction can be made, because the FASTDB program does not account for N-terminus and C-terminus truncations or additions of the reference sequence when calculating percent identity. For query sequences truncated at the N- or C- termini, relative to the reference sequence, the percent identity is corrected by calculating the number of residues of the query sequence that are N-and C-terminus to the reference sequence that are not matched/aligned, as a percent of the total bases of the query sequence. The results of the FASTDB sequence alignment determine matching/alignment. The alignment percentage is then subtracted from the percent identity, calculated by the above FASTDB program using the specified parameters, to arrive at a final percent identity score. This corrected score can be used for the purposes of determining how alignments "correspond" to each other, as well as percentage identity. Residues of the reference sequence that extend past the N- or C-termini of the query sequence may be considered for the purposes of manually adjusting the percent identity score. That is, residues that are not matched/aligned with the N- or C-termini of the comparison sequence may be counted when manually adjusting the percent identity score or alignment numbering.

[0060] For example, a 90 amino acid residue query sequence is aligned with a 100 residue reference sequence to determine percent identity. The deletion occurs at the N-terminus of the query sequence and therefore, the FASTDB alignment does not show a match/alignment of the first 10 residues at the N-terminus. The 10 unpaired residues represent 10% of the reference sequence (number of residues at the N- and C-termini not matched/total number of residues in the reference sequence) so 10% is subtracted from the percent identity score calculated by the FASTDB program. If the remaining 90 residues were perfectly matched (100% alignment) the final percent identity would be 90% (100% alignment – 10% unmatched overhang). In another example, a 90 residue query sequence is compared with a 100 reference sequence, except that the deletions are internal deletions. In this case the percent identity calculated by FASTDB is not manually corrected, since there are no residues at the N- or C-termini of the subject sequence that are not matched/aligned with the query. In still another example, a 110 amino acid query sequence is aligned with a 100 residue reference sequence to determine percent identity. The addition in the query occurs at the N-terminus of the query sequence and therefore, the FASTDB alignment may not show a match/alignment of the first 10 residues at the N-terminus. If the remaining 100 amino acid residues of the query sequence have 95% identity to the entire length of the reference sequence, the N-terminal addition of the query would be ignored and the percent identity of the query to the reference sequence would be 95%.

[0061] The invention further embraces other species, preferably mammalian, homologs with amino acid sequences that correspond to the modified growth factors of the present invention. Species homologs, sometimes referred to as "orthologs," in general, share at least 35%, 40%, 45%, 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence identity with the human version of the growth factors. Such corresponding sequences account for the modified growth factor from across a variety of species, such as canine, feline, mouse, rat, rabbit, monkey, etc. of BMP. In another embodiment, the invention provides novel peptides whose sequences correspond to the sequence of SEQ ID NO:16-35 and retain at least some minimal function.

[0062] Modified growth factor products with an additional methionine residue at position -1 (Met^{-1}-peptide) are contemplated, as are variants with additional methionine and lysine residues at positions -2 and -1 (Met^{-2}-Lys^{-1}-peptide). Variants of the modified growth factor with additional Met, Met-Lys, or Lys residues (or one or more basic residues in general) are particularly useful for enhanced recombinant protein production in bacterial host cells.

[0063] Variants resulting from insertion of the polynucleotide encoding the modified growth factor into an expression vector system are also contemplated. For example, variants (usually insertions) may arise from when the amino terminus and/or the carboxy terminus of modified growth factor is/are fused to another polypeptide.

[0064] In another aspect, the invention provides deletion variants wherein one or more amino acid residues in the modified growth factor peptide are removed. Deletions can be effected at one or both termini of the modified growth factor peptide, or with removal of one or more non-terminal amino acid residues of the modified growth factor peptide. Deletion variants, therefore, include all fragments of the modified growth factor peptide.

[0065] Within the confines of the disclosed percent identity, the invention also relates to substitution variants of disclosed polypeptides of the invention. Substitution variants include those polypeptides wherein one or more amino acid residues of truncated growth factor are removed and replaced with alternative residues. In one aspect, the substitutions are conservative in nature; however, the invention embraces substitutions that are also non-conservative. Conservative substitutions for this purpose may be defined as set out in the tables below. Amino acids can be classified according to physical properties and contribution to secondary and tertiary protein structure. A conservative substitution is recognized in the art as a substitution of one amino acid for another amino acid that has similar properties. Exemplary conservative substitutions are set out in below.

[0066] Table 3: Conservative Substitutions

Side Chain Characteristic	Amino Acid
Aliphatic	
Non-polar	Gly, Ala, Pro, Iso, Leu, Val
Polar-uncharged	Cys, Ser, Thr, Met, Asn, Gln
Polar-charged	Asp, Glu, Lys, Arg
Aromatic	His, Phe, Trp, Tyr
Other	Asn, Gln, Asp, Glu

[0067] Alternatively, conservative amino acids can be grouped as described in Lehninger (1975) Biochemistry, Second Edition; Worth Publishers, pp. 71-77, as set forth below.

[0068] Table 4: Conservative Substitutions

Side Chain Characteristic	Amino Acid
Non-polar (hydrophobic)	
Aliphatic:	Ala, Leu, Iso, Val, Pro
Aromatic:	Phe, Trp
Sulfur-containing:	Met
Borderline:	Gly
Uncharged-polar	
Hydroxyl:	Ser, Thr, Tyr
Amides:	Asn, Gln
Sulfhydryl:	Cys
Borderline:	Gly
Positively Charged (Basic):	Lys, Arg, His
Negatively Charged (Acidic)	Asp, Glu

[0069] And still other alternative, exemplary conservative substitutions are set out below.

[0070] Table 5: Conservative Substitutions

Original Residue	Exemplary Substitution
Ala (A)	Val, Leu, Ile
Arg (R)	Lys, Gln, Asn
Asn (N)	Gln, His, Lys, Arg
Asp (D)	Glu
Cys (C)	Ser
Gln (Q)	Asn
Glu (E)	Asp
His (H)	Asn, Gln, Lys, Arg
Ile (I)	Leu, Val, Met, Ala, Phe
Leu (L)	Ile, Val, Met, Ala, Phe
Lys (K)	Arg, Gln, Asn
Met (M)	Leu, Phe, Ile
Phe (F)	Leu, Val, Ile, Ala
Pro (P)	Gly
Ser (S)	Thr
Thr (T)	Ser
Trp (W)	Tyr
Tyr (Y)	Trp, Phe, Thr, Ser
Val (V)	Ile, Leu, Met, Phe, Ala

[0071] The polypeptides according to the present invention may or may not be involved in a dimer. In one embodiment, the invention provides dimers, wherein the dimers comprise at least one of the novel, modified growth factors described herein. In one specific embodiment, the dimers are homodimers of the novel modified growth factors. In another embodiment, the dimers are heterodimers comprising at least one of the novel modified growth factors. As used herein, a "heterodimer" means a dimer of two peptides, wherein the amino acid sequences of the peptides are

not 100% identical to each other. Thus, a heterodimer may include a modified growth factor peptide of the present invention dimerized with a normal, "mature" version of the same growth factor.

[0072] As used herein in the specification and in the claims, the phrase "at least one," in reference to a list of one or more elements, should be understood to mean at least one element selected from any one or more of the elements in the list of elements, but not necessarily including at least one of each and every element specifically listed within the list of elements and not excluding any combinations of elements in the list of elements. In some embodiments, at least one refers, for example, to 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10.

[0073] It should be understood that the definition of peptides or polypeptides of the invention is intended to include polypeptides bearing modifications other than insertion, deletion, or substitution of amino acid residues. By way of example, the modifications may be covalent in nature, and include for example, chemical bonding with polymers, lipids, other organic and inorganic moieties. Such derivatives may be prepared to increase circulating half-life of a polypeptide, or may be designed to improve the targeting capacity of the polypeptide for desired cells, tissues or organs. Similarly, the invention further embraces modified growth factor peptides that have been covalently modified to include one or more water-soluble polymer attachments such as polyethylene glycol, polyoxyethylene glycol or polypropylene glycol.

[0074] Modified growth factor compositions in which the modified growth factor is linked to a polymer are included within the scope of the present invention. The polymer may be water soluble to prevent precipitation of the protein in an aqueous environment, such as a physiological environment. Suitable water-soluble polymers may be selected from the group consisting of, for example, polyethylene glycol (PEG), monomethoxypolyethylene glycol, dextran, cellulose, or other carbohydrate based polymers, poly-(N-vinyl pyrrolidone) polyethylene glycol, polypropylene glycol homopolymers, a polypropylene oxide/ethylene oxide copolymer polyoxyethylated polyols (*e.g.*, glycerol) and polyvinyl alcohol. The selected polymer is usually modified to have a single reactive group, such as an active ester for acylation or an aldehyde for alkylation, so that the degree of polymerization may be controlled. Polymers may be of any molecular weight, and may be branched or unbranched, and mixtures of such polymers may also be used. When the chemically modified NgR polymer is destined for therapeutic use, pharmaceutically acceptable polymers will be selected for use.

[0075] Pegylation of modified growth factor peptides may be carried out by any of the pegylation reactions known in the art. For example, the pegylation is carried out via an acylation reaction or an alkylation reaction with a reactive polyethylene glycol molecule (or an analogous reactive water-soluble polymer). One water-soluble polymer for pegylation of polypeptides is polyethylene glycol (PEG), including, but not limited to bi-functional PEGs. As used herein, "polyethylene glycol" is

meant to encompass any of the forms of PEG that have been used to derivatize other proteins, such as mono (Cl-ClO) alkoxy- or aryloxy-polyethylene glycol.

[0076] Chemical derivatization of modified growth factor may be performed under any suitable conditions used to react with a biologically active substance with an activated polymer molecule. Methods for preparing pegylated modified growth factor will generally comprise the steps of (a) reacting the polypeptide with polyethylene glycol, such as a reactive ester or aldehyde derivative of PEG, under conditions whereby modified growth factor polypeptide becomes attached to one or more PEG groups, and (b) obtaining the reaction products. It will be apparent to one of ordinary skill in the art to select the optimal reaction conditions or the acylation reactions based on known parameters and the desired result.

[0077] Pegylated and other polymer modified growth factor polypeptides may generally be used to treat conditions that may be alleviated or modulated by administration of the modified growth factor polypeptides described herein. However, the chemically-derivatized polymer: modified growth factor polypeptide molecules disclosed herein may have additional activities, enhanced or reduced biological activity, or other characteristics, such as increased or decreased half-life, as compared to the nonderivatized molecules. The modified growth factor polypeptides, fragments thereof, variants and derivatives, may be employed alone, together, or in combination with other pharmaceutical compositions. For example, cytokines, growth factors, antibiotics, anti-inflammatories and/or chemotherapeutic agents may be co-administered as is appropriate for the indication being treated.

[0078] The present invention provides compositions comprising purified polypeptides of the invention. Preferred compositions comprise, in addition to the polypeptide of the invention, a pharmaceutically acceptable (*i.e.*, sterile and non-toxic) liquid, semisolid, or solid diluent that serves as a pharmaceutical vehicle, excipient or medium. Any diluent known in the art may be used. Exemplary diluents include, but are not limited to, water, saline solutions, polyoxyethylene sorbitan monolaurate, magnesium stearate, methyl- and propylhydroxybenzoate, talc, alginates, starches, lactose, sucrose, dextrose, sorbitol, mannitol, glycerol, calcium phosphate, mineral oil and cocoa butter.

[0079] In one embodiment, the growth factors according to the present invention are fusion proteins that comprise at least a first and a second fusion peptides. The fusion partners are, generally speaking, covalently bonded to one another via a typical amine bond between the fusion peptides, thus creating one contiguous amino acid chain. In some embodiments, the first peptide of the fusion protein comprises the modified growth factor described herein. In further embodiments, the second fusion peptide comprises an amino acid sequence that does not include the amino acid sequence of SEQ ID NO:36-55.

[0080] In one embodiment, the first fusion peptide comprises a modified growth factor from BMP-2, and the second fusion peptide comprises an amino acid sequence that does not include the amino acid sequence of SEQ ID NO:36, 37 or 38. In more specific embodiments, the second fusion of the present invention comprises an amino acid sequence that is less than 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% identical to SEQ ID NO:36, 37 or 38.

[0081] In another embodiment, the first fusion peptide comprises a modified growth factor from BMP-4, and the second fusion peptide comprises an amino acid sequence that does not include the amino acid sequence of SEQ ID NO:39. In more specific embodiments, the second fusion of the present invention comprises an amino acid sequence that is less than 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% identical to SEQ ID NO:39.

[0082] In another embodiment, the first fusion peptide comprises a modified growth factor from BMP-5, and the second fusion peptide comprises an amino acid sequence that does not include the amino acid sequence of SEQ ID NO:40, 41, 42, 43, 44, 45, 46, 47 or 48. In more specific embodiments, the second fusion of the present invention comprises an amino acid sequence that is less than 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% identical to SEQ ID NO:40, 41, 42, 43, 44, 45, 46, 47 or 48.

[0083] In another embodiment, the first fusion peptide comprises a modified growth factor from BMP-6, and the second fusion peptide comprises an amino acid sequence that does not include the amino acid sequence of SEQ ID NO:49. In more specific embodiments, the second fusion of the present invention comprises an amino acid sequence that is less than 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% identical to SEQ ID NO:49.

[0084] In another embodiment, the first fusion peptide comprises a modified growth factor from BMP-7, and the second fusion peptide comprises an amino acid sequence that does not include the amino acid sequence of SEQ ID NO:50, 51, 52 or 53. In more specific embodiments, the second fusion of the present invention comprises an amino acid sequence that is less than 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% identical to SEQ ID NO:50, 51, 52 or 53.

[0085] In another embodiment, the first fusion peptide comprises a modified growth factor from BMP-13, and the second fusion peptide comprises an amino acid sequence that does not include the amino acid sequence of SEQ ID NO:54. In more specific embodiments, the second fusion of the present invention comprises an amino acid sequence that is less than 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% identical to SEQ ID NO:54.

[0086] In another embodiment, the first fusion peptide comprises a modified growth factor from BMP-14, and the second fusion peptide comprises an amino acid sequence that does not include the amino acid sequence of SEQ ID NO:55. In more specific embodiments, the second fusion of the present invention comprises an amino acid sequence that is less than 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% identical to SEQ ID NO:55.

[0087] Other types of fusion proteins provided by the present invention include but are not limited to, fusions with secretion signals and other heterologous functional regions. Thus, for instance, a region of additional amino acids, particularly charged amino acids, may be added to the N-terminus of the protein to improve stability and persistence in the host cell, during purification or during subsequent handling and storage.

[0088] Additional fusion proteins include fusions for enhancing translocation of the protein across cell membranes. For example, Tat is an 86-amino acid protein involved in the replication of human immunodeficiency virus type 1 (HIV-1). The HIV-1 Tat transactivation protein is efficiently taken up by cells, and it has been demonstrated that low concentrations (nM) are sufficient to transactivate a reporter gene expressed from the HIV-1 promoter. Exogenous Tat protein is able to translocate through the plasma membrane and reach the nucleus to transactivate the viral genome. Tat peptide-mediated cellular uptake and nuclear translocation have been demonstrated in several systems. Chemically coupling a Tat-derived peptide (residues 37-72 of Tat) to several proteins results in their internalization in several cell lines or tissues (Fawell (1994) Proc. Natl. Acad. Sci. USA 91, 664-668).

[0089] It is well-known that a region of the Tat protein centered on a cluster of basic amino acids is responsible for this translocation activity. A synthetic peptide consisting of the Tat basic amino acids 48-60 with a cysteine residue at the C-terminus coupled to fluorescein maleimide translocates to the cell nucleus as determined by fluorescence microscopy. In addition, a fusion protein (Tat-NLS-β-Gal) consisting of Tat amino acids 48-59 fused by their amino-terminus to β-galactosidase amino acids 9-1023 translocates to the cell nucleus in an ATP-dependent, cytosolic factor-independent manner. Accordingly, the fusion proteins of the present invention may comprise all or a portion of HIV-Tat, such as any sequential residues of the Tat protein basic peptide motif 37-72 (37-CFITKALGISYGRKKRRQRRRPPQGSQTHQVSLSKQ-72 (SEQ ID NO:56). The minimum number of amino acid residues can be in the range of from about three to about six. In one embodiment, the Tat portion of the fusion protein is from about three to about five contiguous amino acids in length. In another embodiment, the Tat portion of the fusion protein is about four amino acids in length, *i.e.*, the minimal requirement for one alpha helical turn. In another embodiment, the Tat portion of the fusion protein comprises Tat protein residues 48-57 (GRKKRRQRRR) (SEQ ID NO:57).

[0090] In additional embodiments of fusion proteins, a region may be added to facilitate purification. For example, "histidine tags" ("his tags") or "lysine tags" (the second fusion peptide) may be appended to the first fusion peptide. Examples of histidine tags include, but are not limited to hexaH, heptaH and hexaHN. Examples of lysine tags include, but are not limited to pentaL, heptaL and FLAG. Such regions may be removed prior to final preparation of the protein. Other examples of a second fusion peptide include, but are not limited to, glutathione S-transferase (GST) and alkaline phosphatase (AP).

[0091] The addition of peptide moieties to proteins, whether to engender secretion or excretion, to improve stability and to facilitate purification or translocation, among others, is a familiar and routine technique in the art and may include modifying amino acids at the terminus to accommodate the tags. For example in any of SEQ ID NO: 16-35, the N-terminus amino acid may be modified to, for example, arginine and/or serine to accommodate a tag. Of course, the amino acid residues of the C-terminus may also be modified to accommodate tags. One particularly useful fusion protein comprises a heterologous region from immunoglobulin that can be used solubilize proteins. For example, EP A0464 533 discloses fusion proteins comprising various portions of constant region of immunoglobin molecules together with another human protein or part thereof. In many cases, the Fc part in a fusion protein is thoroughly advantageous for use in therapy and diagnosis and thereby results, for example, in improved pharmacokinetic properties (EP A0232 262). On the other hand, for some uses, it would be desirable to be able to delete the Fc part after the fusion protein has been expressed, detected and purified in the advantageous manner described.

[0092] The fusion proteins of the current invention can be recovered and purified from recombinant cell cultures by well-known methods including, but not limited to, ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, *e.g.*, immobilized metal affinity chromatography (IMAC), hydroxylapatite chromatography and lectin chromatography. High performance liquid chromatography ("HPLC") may also be employed for purification. Well-known techniques for refolding protein may be employed to regenerate active conformation when the fusion protein is denatured during isolation and/or purification.

[0093] Fusion proteins of the present invention include, but are not limited to, products of chemical synthetic procedures and products produced by recombinant techniques from a prokaryotic or eukaryotic host, including, for example, bacterial, yeast, higher plant, insect and mammalian cells. Depending upon the host employed in a recombinant production procedure, the fusion proteins of the present invention may be glycosylated or may be non-glycosylated. In addition, fusion proteins of the invention may also include an initial modified methionine residue, in some cases as a result of host-mediated processes.

[0094] The invention also relates to isolated nucleic acids and to constructs comprising these nucleic acids. The nucleic acids of the invention can be DNA or RNA, for example, mRNA. The nucleic acid molecules can be double-stranded or single-stranded; single stranded RNA or DNA can be the coding, or sense, strand or the non-coding, or antisense, strand. In particular, the nucleic acids may encode any polypeptide of the invention, including, but not limited to, the fusion proteins of the present invention. For example, the nucleic acids of the invention include polynucleotide sequences that encode glutathione-S-transferase (GST) fusion protein, poly-histidine (*e.g.*, His_6), poly-HN, poly-lysine, hemagglutinin, HSV-Tag and at least a portion of HIV-Tat. If desired, the nucleotide sequence of the isolated nucleic acid can include additional non-coding sequences such as non-coding 3' and 5' sequences (including regulatory sequences, for example).

[0095] In some embodiments, the isolated nucleic acid of the present invention further relates to the nucleic acids consisting of a nucleic acid sequence at least 95% identical to any of the nucleic acid sequences of SEQ ID NO: 58-77. In further embodiments, the invention therefore provides the nucleic acids consisting of a nucleic acid sequence at least about 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to any of the nucleic acid sequences of SEQ ID NO: 58-77.

[0096] The nucleic acid molecules of the invention can be "isolated." As used herein, an "isolated" nucleic acid molecule or nucleotide sequence is intended to mean a nucleic acid molecule or nucleotide sequence that is not flanked by nucleotide sequences normally flanking the gene or nucleotide sequence (as in genomic sequences) and/or has been completely or partially removed from its native environment (*e.g.*, a cell, tissue). For example, nucleic acid molecules that have been removed or purified from cells are considered isolated. In some instances, the isolated material will form part of a composition (for example, a crude extract containing other substances), buffer system or reagent mix. In other circumstances, the material may be purified to near homogeneity, for example as determined by PAGE or column chromatography such as HPLC. Thus, an isolated nucleic acid molecule or nucleotide sequence can includes a nucleic acid molecule or nucleotide sequence which is synthesized chemically, using recombinant DNA technology or using any other suitable method. To be clear, a nucleic acid contained in a vector would be included in the definition of "isolated" as used herein. Also, isolated nucleotide sequences include recombinant nucleic acid molecules (*e.g.*, DNA, RNA) in heterologous organisms, as well as partially or substantially purified nucleic acids in solution. "Purified," on the other hand is well understood in the art and generally means that the nucleic acid molecules are substantially free of cellular material, cellular components, chemical precursors or other chemicals beyond, perhaps, buffer or solvent. "Substantially free" is not intended to mean that other components beyond the novel nucleic acid molecules are undetectable. The nucleic acid molecules of the present invention may be isolated or purified. Both in vivo and in

vitro RNA transcripts of a DNA molecule of the present invention are also encompassed by "isolated" nucleotide sequences.

[0097] The invention also encompasses variations of the nucleotide sequences of the invention, such as those encoding functional fragments or variants of the polypeptides as described above. Such variants can be naturally-occurring, or non-naturally-occurring, such as those induced by various mutagens and mutagenic processes. Intended variations include, but are not limited to, addition, deletion and substitution of one or more nucleotides which can result in conservative or non-conservative amino acid changes, including additions and deletions.

[0098] The invention described herein also relates to fragments of the isolated nucleic acid molecules described herein. The term "fragment" is intended to encompass a portion of a nucleotide sequence described herein which is from at least about 20 contiguous nucleotides to at least about 50 contiguous nucleotides or longer in length. Such fragments may be useful as probes and primers. In particular, primers and probes may selectively hybridize to the nucleic acid molecule encoding the polypeptides described herein. For example, fragments which encode polypeptides that retain activity, as described below, are particularly useful.

[0099] The invention also provides nucleic acid molecules that hybridize under high stringency hybridization conditions, such as for selective hybridization, to the nucleotide sequences described herein (*e.g.*, nucleic acid molecules which specifically hybridize to a nucleotide sequence encoding polypeptides described herein and encode a modified growth factor). Hybridization probes include synthetic oligonucleotides which bind in a base-specific manner to a complementary strand of nucleic acid.

[00100] Such nucleic acid molecules can be detected and/or isolated by specific hybridization *e.g.*, under high stringency conditions. "Stringency conditions" for hybridization is a term of art that refers to the incubation and wash conditions, *e.g.*, conditions of temperature and buffer concentration, which permit hybridization of a particular nucleic acid to a second nucleic acid; the first nucleic acid may be perfectly complementary, *i.e.*, 100%, to the second, or the first and second may share some degree of complementarity, which is less than perfect, *e.g.*, 60%, 75%, 85%, 95% or more. For example, certain high stringency conditions can be used which distinguish perfectly complementary nucleic acids from those of less complementarity.

[00101] "High stringency conditions," "moderate stringency conditions" and "low stringency conditions" for nucleic acid hybridizations are explained in Current Protocols in Molecular Biology, John Wiley & Sons). The exact conditions which determine the stringency of hybridization depend not only on ionic strength, *e.g.*, 0.2× SSC, 0.1× SSC of the wash buffers, temperature, *e.g.*, room temperature, 42°C., 68°C., etc., and the concentration of destabilizing agents such as formamide or denaturing agents such as SDS, but also on factors such as the length of the nucleic acid sequence,

base composition, percent mismatch between hybridizing sequences and the frequency of occurrence of subsets of that sequence within other non-identical sequences. Thus, high, moderate or low stringency conditions may be determined empirically.

[00102] By varying hybridization conditions from a level of stringency at which no hybridization occurs to a level at which hybridization is first observed, conditions which will allow a given sequence to hybridize with the most similar sequences in the sample can be determined. Exemplary conditions are described in Krause (1991) Methods in Enzymology, 200:546-556. Washing is the step in which conditions are usually set so as to determine a minimum level of complementarity of the hybrids. Generally, starting from the lowest temperature at which only homologous hybridization occurs, each degree (°C) by which the final wash temperature is reduced, while holding SSC concentration constant, allows an increase by 1% in the maximum extent of mismatching among the sequences that hybridize. Generally, doubling the concentration of SSC results in an increase in Tm. Using these guidelines, the washing temperature can be determined empirically for high, moderate or low stringency, depending on the level of mismatch sought. Exemplary high stringency conditions include, but are not limited to, hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 0.1× SSC at 60°C. Example of progressively higher stringency conditions include, after hybridization, washing with 0.2 X SSC and 0.1% SDS at about room temperature (low stringency conditions); washing with 0.2× SSC, and 0.1% SDS at about 42°C (moderate stringency conditions); and washing with 0.1× SSC at about 68°C (high stringency conditions). Washing can be carried out using only one of these conditions, *e.g.*, high stringency conditions, washing may encompass two or more of the stringency conditions in order of increasing stringency. Optimal conditions will vary, depending on the particular hybridization reaction involved, and can be determined empirically.

[00103] Equivalent conditions can be determined by varying one or more of the parameters given as an example, as known in the art, while maintaining a similar degree of identity or similarity between the target nucleic acid molecule and the primer or probe used. Hybridizable nucleotide sequences are useful as probes and primers for identification of organisms comprising a nucleic acid of the invention and/or to isolate a nucleic acid of the invention, for example. The term "primer" is used herein as it is in the art and refers to a single-stranded oligonucleotide which acts as a point of initiation of template-directed DNA synthesis under appropriate conditions in an appropriate buffer and at a suitable temperature. The appropriate length of a primer depends on the intended use of the primer, but typically ranges from about 15 to about 30 nucleotides. Short primer molecules generally require cooler temperatures to form sufficiently stable hybrid complexes with the template. A primer need not reflect the exact sequence of the template, but must be sufficiently complementary to hybridize with a template. The term "primer site" refers to the area of the target DNA to which a primer hybridizes. The term "primer pair" refers to a set of primers including a 5' (upstream) primer that

hybridizes with the 5' end of the DNA sequence to be amplified and a 3' (downstream) primer that hybridizes with the complement of the 3' end of the sequence to be amplified.

[00104] The present invention also relates to vectors that include nucleic acid molecules of the present invention, host cells that are genetically engineered with vectors of the invention and the production of proteins of the invention by recombinant techniques.

[00105] In accordance with this aspect of the invention, the vector may be, for example, a plasmid vector, a single-or double-stranded phage vector, or a single-or double-stranded RNA or DNA viral vector. Such vectors may be introduced into cells as polynucleotides, for example DNA, by well-known techniques for introducing DNA and RNA into cells. Viral vectors may be replication competent or replication defective. In the latter, case viral propagation generally will occur only in complementing host cells.

[00106] In certain respects, the vectors to be used are those for expression of polynucleotides and proteins of the present invention. Generally, such vectors comprise cis-acting control regions effective for expression in a host operatively linked to the polynucleotide to be expressed. Appropriate trans-acting factors are supplied by the host, supplied by a complementing vector or supplied by the vector itself upon introduction into the host.

[00107] A great variety of expression vectors can be used to express the proteins of the invention. Such vectors include chromosomal, episomal and virus-derived vectors, *e.g.*, vectors derived from bacterial plasmids, from bacteriophage, from yeast episomes, from yeast chromosomal elements, from viruses such as adeno-associated virus, lentivirus, baculoviruses, papova viruses, such as SV40, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses and retroviruses, and vectors derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, such as cosmids and phagemids. All may be used for expression in accordance with this aspect of the present invention. Generally, any vector suitable to maintain, propagate or express polynucleotides or proteins in a host may be used for expression in this regard.

[00108] The DNA sequence in the expression vector is operatively linked to appropriate expression control sequence(s) including, for instance, a promoter to direct mRNA transcription. Representatives of such promoters include, but are not limited to, the phage lambda PL promoter, the E. coli lac, trp and tac promoters, HIV promoters, the SV40 early and late promoters and promoters of retroviral LTRs, to name just a few of the well-known promoters. In general, expression constructs will contain sites for transcription, initiation and termination and, in the transcribed region, a ribosome binding site for translation. The coding portion of the mature transcripts expressed by the constructs will include a translation initiating AUG at the beginning and a termination codon (UAA, UGA or UAG) appropriately positioned at the end of the polypeptide to be translated.

[00109] In addition, the constructs may contain control regions that regulate, as well as engender expression. Generally, such regions will operate by controlling transcription, such as repressor binding sites and enhancers, among others.

[00110] Vectors for propagation and expression generally will include selectable markers. Such markers also may be suitable for amplification or the vectors may contain additional markers for this purpose. In this regard, the expression vectors may contain one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells. Preferred markers include dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, and tetracycline, kanamycin or ampicillin resistance genes for culturing E. coli and other bacteria.

[00111] The vector containing the appropriate DNA sequence, as well as an appropriate promoter, and other appropriate control sequences, may be introduced into an appropriate host using a variety of well-known techniques suitable to expression therein of a desired polypeptide. Representative examples of appropriate hosts include bacterial cells, such as, but not limited to, E. coli, Streptomyces, Bacillus, and Salmonella cells; fungal cells, such as yeast cells; insect cells such as Drosophila S2 and Spodoptera Sf9 cells; animal cells such as CHO, COS and Bowes melanoma cells; and plant cells. Hosts for of a great variety of expression constructs are well known, and those of skill in the art will be enabled by the present disclosure to select an appropriate host for expressing one of the proteins of the present invention.

[00112] Examples of vectors that may be useful for fusion proteins include, but are not limited to, pGEX (Pharmacia), pMAL (New England Biolabs) and pRIT5 (Pharmacia) that fuse glutathione-S-transferase (GST), maltose E binding protein, or protein A, respectively, to the target recombinant protein. Often, in fusion expression vectors, a proteolytic cleavage site is introduced at the junction of the fusion moiety and the recombinant protein to enable separation of the recombinant protein from the fusion moiety subsequent to purification of the fusion protein. Such enzymes, and their cognate recognition sequences, include Factor Xa, thrombin and enterokinase.

[00113] Examples of vectors for expression in yeast S. cerevisiae include pYepSecl (Baldari (1987) EMBO J. 6, 229-234), pMFa (Kurjan (1982) Cell 30, 933-943), pJRY88 (Schultz (1987) Gene 54, 137-123), pYES2 (Invitrogen) and picZ (Invitrogen).

[00114] Alternatively, the modified growth factors can be expressed in insect cells using baculovirus expression vectors. Baculovirus vectors available for expression of proteins in cultured insect cells (*e.g.*, SF9 cells) include the pAc series (Smith (1983) Mol. Cell. Biol. 3, 2156 2165) and the pVL series (Lucklow (1989) Virology 170, 31-39).

[00115] In yet another embodiment, a nucleic acid of the invention is expressed in mammalian cells using a mammalian expression vector. Examples of mammalian expression vectors include pCDM8

(Seed (1987) Nature 329, 840) and pMT2PC (Kaufman (1987) EMBO J. 6, 187 195). When used in mammalian cells, the expression vector's control functions are often provided by viral regulatory elements. For example, commonly used promoters are derived from polyoma, adenovirus 2, cytomegalovirus and Simian Virus 40. For other suitable expression systems for both prokaryotic and eukaryotic cells, see Sambrook (2010) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press.

[00116] In another embodiment, the recombinant mammalian expression vector is capable of directing expression of the nucleic acid preferentially in a particular cell type (*e.g.*, tissue-specific regulatory elements are used to express the nucleic acid). Tissue-specific regulatory elements are known in the art. Non-limiting examples of suitable tissue-specific promoters include liver-specific promoters (*e.g.*, albumin promoter), lymphoid-specific promoters such as, but not limited to, T cell receptors and immunoglobulins, neuron-specific promoters (*e.g.*, neurofilament promoter), pancreas-specific promoters, mammary gland-specific promoters (*e.g.*, milk whey promoter), bone-specific promoters (*e.g.*, osteocalcin, osteopontin or bone sialoprotein, promoter regions), cartilage specific promoters (*e.g.*, WARP) and muscle specific promoters (Desmin, myglobin, etc) just to name a few. Developmentally-regulated promoters are also encompassed, *e.g.*, the murine hox promoters (Kessel and Gruss (1990) Science 249, 374 379) and the α-fetoprotein promoter (Campes and Tilghman (1989) Genes Dev. 3, 537 546).

[00117] The present invention also relates to host cells containing the above-described constructs. The host cell can be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic cell, such as a yeast cell, or the host cell can be a prokaryotic cell, such as a bacterial cell. The host cell can be stably or transiently transfected with the construct. The polynucleotides may be introduced alone or with other polynucleotides. Such other polynucleotides may be introduced independently, co-introduced or introduced joined to the polynucleotides of the invention. As used herein, a "host cell" is a cell that normally does not contain any of the nucleotides of the present invention and contains at least one copy of the nucleotides of the present invention. Thus, a host cell as used herein can be a cell in a culture setting or the host cell can be in an organism setting where the host cell is part of an organism, organ or tissue.

[00118] Suitable host cells for expression of the polypeptides of the invention include, but are not limited to, prokaryotes, yeast, and eukaryotes. If a prokaryotic expression vector is employed, then the appropriate host cell would be any prokaryotic cell capable of expressing the cloned sequences. Suitable prokaryotic cells include, but are not limited to, bacteria of the genera Escherichia, Bacillus, Pseudomonas, Staphylococcus, and Streptomyces.

[00119] If a eukaryotic expression vector is employed, then the appropriate host cell would be any eukaryotic cell capable of expressing the cloned sequence. In one embodiment, eukaryotic cells are

cells of higher eukaryotes. Suitable eukaryotic cells include, but are not limited to, non-human mammalian tissue culture cells and human tissue culture cells. Other host cells include, but are not limited to, insect cells, HeLa cells, Chinese hamster ovary cells (CHO cells), African green monkey kidney cells (COS cells), human 293 cells, and murine 3T3 fibroblasts.

[00120] In addition, a yeast cell may be employed as a host cell. Yeast cells include, but are not limited to, the genera Saccharomyces, Pichia and Kluveromyces. In one embodiment, the yeast hosts are S. cerevisiae or P. pastoris. Yeast vectors may contain an origin of replication sequence from a 2T yeast plasmid, an autonomously replication sequence (ARS), a promoter region, sequences for polyadenylation, sequences for transcription termination and a selectable marker gene. Shuttle vectors for replication in both yeast and E. coli are also included herein.

[00121] Introduction of a construct into the host cell can be affected by calcium phosphate transfection, DEAE-dextran mediated transfection, cationic lipid-mediated transfection, electroporation, transduction, infection or other methods.

[00122] For stable transfection of mammalian cells, it is known that, depending upon the expression vector and transfection technique used, only a small fraction of cells may integrate the foreign DNA into their genome. To identify and select these integrants, a gene that encodes a selectable marker (*e.g.*, resistance to antibiotics) is generally introduced into the host cells along with the gene of interest. Various selectable markers include those that confer resistance to drugs, such as G418, hygromycin, dihydrofolate reductase (DHFR) and methotrexate. Nucleic acid encoding a selectable marker can be introduced into a host cell on the same vector as that encoding modified growth factor or can be introduced on a separate vector. Cells stably transfected with the introduced nucleic acid can be identified by drug selection (*e.g.*, cells that have incorporated the selectable marker gene will survive, while the other cells die). In one embodiment, the polypeptides of the invention are expressed in Chinese Hamster Ovary (CHO) cells.

[00123] The modified growth factor expression vectors prepared as above are introduced into CHO cells by any known method, including, but not limited to the calcium phosphate method and electroporation.

[00124] Transformants carrying the expression vectors are selected based on the above-mentioned selectable markers. Repeated clonal selection of the transformants using the selectable markers allows selection of stable cell lines expressing the modified growth factor constructs. Increased concentrations in the selection medium allows gene amplification and greater expression of the desired modified growth factor. The host cells, for example CHO cells, containing the recombinant modified growth factor can be produced by cultivating the CHO cells containing the modified growth factor expression vectors constitutively expressing the modified growth factor constructs.

[00125] Accordingly, the current invention also relates to methods of producing a modified growth factor comprising culturing the host cells of the invention under conditions such that the modified growth factor is expressed, and recovering said protein. The culture conditions required to express the proteins of the current invention are dependent upon the host cells that are harboring the polynucleotides of the current invention. The culture conditions for each cell type are well-known in the art and can be easily optimized, if necessary. For example, a nucleic acid encoding a polypeptide of the invention, or a construct comprising such nucleic acid, can be introduced into a suitable host cell by a method appropriate to the host cell selected, *e.g.*, transformation, transfection, electroporation, infection, such that the nucleic acid is operably linked to one or more expression control elements as described herein. Host cells can be maintained under conditions suitable for expression in vitro or in vivo, whereby the encoded polypeptide is produced. For example host cells may be maintained in the presence of an inducer, suitable media supplemented with appropriate salts, growth factors, antibiotic, nutritional supplements, etc., which may facilitate protein expression. In additional embodiments, the modified growth factors of the invention can be produced by in vitro translation of a nucleic acid that encodes the modified growth factor, by chemical synthesis or by any other suitable method. If desired, the modified growth factor can be isolated from the host cell or other environment in which the protein is produced or secreted. It should therefore be appreciated that the methods of producing the modified growth factors encompass expression of the polypeptides in a host cell of a transgenic animal or plant. See U.S. Patent Nos. 6,013,857, 5,990,385, and 5,994,616.

[00126] In situations where the modified BMP will be found primarily intracellularly, intracellular material (including inclusion bodies for Gram-negative bacteria) can be extracted from the host cell using any standard technique known to one of ordinary skill in the art. Such methods would encompass, by way of example and not by way of limitation, lysing the host cells to release the contents of the periplasm/cytoplasm by French press, homogenization and/or sonication followed by centrifugation.

[00127] If the modified BMP has formed inclusion bodies in the cytosol, such inclusion bodies may frequently bind to the inner and/or outer cellular membranes. Upon centrifugation, the inclusion bodies will be found primarily in the pellet material. The pellet material can then be treated at pH extremes or with one or more chaotropic agents such as a detergent, guanidine, guanidine derivatives, urea, or urea derivatives in the presence of a reducing agent such as dithiothreitol at alkaline pH or tris-carboxyethyl phosphine at acid pH to release, break apart and solubilize the inclusion bodies. Once solubilized, the modified growth factor peptide can be analyzed using gel electrophoresis, immunoprecipitation or the like. Various methods of isolating the modified growth factor peptide would be apparent to one of ordinary skill in the art, for example, isolation may be accomplished

using standard methods such as those set forth below and in Marston et al (1990) Meth. Enzymol. 182, 264-275.

[00128] If the modified growth factor peptide is not biologically active following the isolation procedure employed, various methods for "refolding" or converting the polypeptide to its tertiary structure and generating disulfide linkages, can be used to restore biological activity. Methods known to one of ordinary skill in the art include adjusting the pH of the solubilized polypeptide to a certain pH, usually above 7, and in the presence of a particular concentration of a chaotrope. The selection of chaotrope is very similar to the choices used for inclusion body solubilization, but usually at a lower concentration, and is not necessarily the same chaotrope as used for the solubilization. It may be required to employ a reducing agent or the reducing agent plus its oxidized form in a specific ratio, to generate a particular redox potential allowing for disulfide shuffling during the formation of the protein's cysteine bridge(s). Some of the commonly used redox couples include cysteine/cysteine, glutathione (GSH)/dithiobis GSH, cupric chloride, dithiothreitol (DTT)/dithiane DTT, 2-mercaptoethanol (bME)/dithio-b(ME). To increase the efficiency of the refolding, it may be necessary to employ a cosolvent, such as glycerol, polyethylene glycol of various molecular weights and arginine.

[00129] Other methods of preparing the modified growth factors of the present invention include, but are not limited to, contacting a form of BMP with a protease. In one embodiment, the methods comprise contacting the mature form of BMP with a protease to produce the modified growth factors of the present invention.

[00130] As used herein, the term "protease" refers to any of various enzymes that catalyze the hydrolytic breakdown of proteins into smaller segments, including polypeptides and/or amino acids. Accordingly, included in the definition are proteinases and peptidases. The term "proteinase" refers to a protease that catalyzes the hydrolytic breakdown of proteins by splitting them into smaller peptide fractions, *i.e.*, polypeptides or amino acids. The term "peptidase" refers to a protease that catalyzes the hydrolytic breakdown of peptides via cleavage of an individual amino acid from the peptide chain.

[00131] In one embodiment, the protease is a serine protease, a threonine protease, a metalloproteinase, a cysteine protease, an aspartate protease or a glutamic acid protease. Examples of such proteases are well known in the art and include proteases from both eukaryotic and prokaryotic sources. Examples of serine proteases include, but are not limited to, chymotrypsin, trypsin, thrombin, elastase, subtilisin and alpha/beta hydrolases. Examples of cysteine proteases include, but are not limited to, actinidain, bromelain, calpains, some cathepsins, clostripain and papain. Examples of aspartate proteases include, but are not limited to, some cathepsins, chyomsin, renin, pepsin and HIV-1 protease. Examples of metalloproteinases include, but are not limited to, the matrix

metalloproteinase (MMP) family of proteases that include MMP-1, MMP-2, MMP-3, MMP-4, MMP-5, MMP-6, MMP-7, MMP-8, MMP-9, MMP-10, MMP-11, MMP-12, MMP-13, MMP-14, MMP-15, MMP-16, MMP-17, MMP-18 and membrane-type MMP (MT-MMP), to name a few. Many of these MMPs are known by other names in the art and one of skill will be informed of synonyms of the various enzymes. For example, some of the "collagenases" are MMP-1, MMP-8, MMP-13, MMP-14, MMP-18 and dispases, and some of the "gelatinases" are MMP-2, MMP-9 and MMP-12; some of the "stromelysins" are MMP-3, MMP-10 and MMP-11, and some of the membrane-type matrix metalloproteinases are MMP-14, MMP-15, MMP-16, MMP-17, MMP-24, and MMP-25. Collagenases are examples of metalloproteinases that break down native collagen or gelatin. A variety of microorganisms and many different types of animal cells produce collagenases. Bacterial collagenases are often used in the laboratory to digest tissues and isolate individual cells. Collagenase produced by Clostridium histolyticum (C. histolyticum) is a zinc metalloproteinase that degrades various types of collagen and gelatin. Additional examples of metalloproteinases are the astacin family, such as, but not limited to, astacin, tolloid, xolloid, mammalian tolloid like (mTLL), BMP-1, meprin A and B, and matrilysin and the aggrecanase (ADAMTS) family of metalloproteinase, such as, but not limited to, ADAMTS-1, ADAMTS-2, ADAMTS-3, ADAMTS-4, ADAMTS-5.

[00132] Of course, the methods include contacting a form of BMP with more than one protease either sequentially or simultaneously. In addition, the proteases used herein can be recombinant or isolated from various sources. The proteases used herein need not be isolated from the same cellular or animal source as the mature form of the BMP. For example, many proteases are found only in prokaryotes, yet these proteases are functional against proteins from eukaryotes. Factors affecting enzyme activity include, but are not limited to, salt concentrations of buffer, pH and temperature. One of skill in the art will readily understand the conditions necessary to promote peptide cleave based upon the enzyme used.

[00133] In some embodiments, the ratio of the amount of protease(s) to the amount of growth factor(s) may be between about 1 mol protease:1000 mol growth factor and about 1000 mol:1 mol, between about 1 mol protease:1000 mol growth factor and about 100 mol:1 mol, between about 1 mol:100 mol and about 100 mol:1 mol, between about 1 mol:100 mol and about 10 mol:1 mol, between about 1 mol:10 mol and about 10 mol:1 mol, or between about 1 mol:10 mol and about 1 mol:1 mol. In further embodiments, the ratio of the amount of collagenase(s) to the amount of growth factor(s) may be between about 1 mol collagenase:100 mol growth factor and about 1000 mol:1 mol, between about 1 mol:10 mol and about 1000 mol:1 mol, between about 1 mol:10 mol and about 100 mol:1 mol, or between about 1 mol:1 mol and about 10 mol:1 mol. The ratio of the amount of trypsin to the amount of growth factor(s) may be between about 1 mol trypsin: 1000 mol growth factor and about 1000 mol:1 mol, between about 1 mol:1000 mol and about 100 mol:1 mol, between about 1 mol:100 mol

and about 10 mol:1 mol, or between about 1 mol:10 mol and about 10 mol:1 mol. The ratio of the amount of clostripain to the amount of growth factor(s) may be between about 1 mol clostripain:1000 mol growth factor and about 1000 mol:1 mol, between about 1 mol:100 mol and about 1000 mol:1 mol, between about 1 mol:10 mol and about 100 mol:1 mol, or between about 1 mol:1 mol and about 10 mol:1 mol. The ratio of the amount of dispase to the amount of growth factor(s) may be between about 1 mol dispase: 1000 mol growth factor and about 1000 mol:1 mol, between about 1 mol:1000 mol and about 100 mol:1 mol, between about 1 mol:100 mol and about 10 mol:1 mol, or between about 1 mol:10 mol and about 10 mol:1 mol.

[00134] The treatment of growth factor(s) with proteases may be performed at a temperature between about 0°C and about 40°C, about 4°C and about 40°C, or at about 37°C or lower, in some embodiments. A growth factor may be treated with collagenase for between about 30 minutes and 3 days, about 1 hour and about 48 hours, about 2 hours and about 48 hours, or about 2 hours and about 24 hours, in certain embodiments. A growth factor may be treated with trypsin or clostripain for between about 5 minutes and 3 days, about 5 minutes and about 48 hours, about 5 minutes and about 24 hours, or about 5 minutes and about 8 hours. A growth factor may be treated with dispase for between about 5 minutes and 2 days, about 5 minutes and about 24 hours, about 5 minutes and about 18 hours, or about 5 minutes and about 8 hours. All these conditions do not count the extra protein or peptide substrate in the treatment mixture. Treatment may include mixing and/or incubation. Incubation may be performed under static or dynamic conditions, such as with agitation, shaking, stirring, mixing, horizontal motion, rocking, and others.

[00135] In some embodiments, the protease may be a recombinant protein or peptide fragment, a chemically synthesized protein or peptide fragment, or it may be extracted from a natural source and, optionally, modified, for example by being cleaved, inactivated, chemically-modified, or other methods. In other embodiments, certain aspects of the enzymatic activity of a protease may be inhibited or altered, and the protease having altered activity may be added to one or more growth factors to change the biological activity of the growth factor.

[00136] In additional embodiments, the activity of a protease may be modulated before, during, or after treatment of at least one growth factor with the protease. For example, the protease activity of a collagenase may be significantly reduced before it is used to treat a growth factor. In another example, the activity of a protease may be modulated before, during, or after administering to cells. In certain embodiments, the activity of a protease may be modulated by methods comprising heat inactivation, radiation inactivation, protease substrate neutralization, or chemical inhibition, among others. The chemical inhibitor used to modulate the activity of metalloproteinases may be selected from metal chelating agents, such as EDTA; cysteine and serine protease inhibitors, such as N-ethylmaleimide, phenylmethylsulfonyl fluoride (PMSF), and leupeptin; classical metalloproteinase inhibitor, such as

phosphoramidon,and bestatin; general protein inhibitor, such as α2-macroglobuli, or natural or synthetic tissue inhibitors of metalloproteinases (TIMPs), such as TIMP-1, TIMP-2, TIMP-3, TIMP-4; and other inhibitors.

[00137] The form of BMP used in these methods can also be recombinant or can be isolated from a variety of cellular or animal sources. In one embodiment of the present invention, the BMP, in any form, that is contacted with the at least one protease is a recombinant BMP. In one specific embodiment, the recombinant BMP is contacted with a metalloproteinase to generate the modified BMP peptides of the present invention. In a more specific embodiment, the recombinant BMP is contacted with a collagenase to generate the novel, truncated BMP peptides of the present invention. In an even more specific embodiment, the recombinant BMP that is contacted with a collagenase is a recombinant mature form of BMP. In a still more specific embodiment, recombinant mature BMP is contacted with a collagenase and/or clostripain to generate the novel, truncated BMP peptides of the present invention. In another specific embodiment, recombinant mature BMP is contacted with a trypsin or dispase to generate the novel, truncated BMP peptides of the present invention.

[00138] Once treated with at least one protease, in some embodiments, the resulting peptides can be isolated and purified using routine methods in the art. Examples of purification methods include, but are not limited to size exclusion chromatography, high-performance liquid chromatography, ion exchange chromatography, electrophoresis, Western blotting and subsequent processing of the membrane. In one embodiment of the present invention, the resulting peptide that is purified after protease treatment comprises any of the amino acid sequence of SEQ ID NO:16-35. In other embodiments, after being treated with a protease, the resulting peptides are not isolated or purified and are administered to a cell in the presence of the protease. In another embodiment, the resulting peptide is isolated or purified and then is administered to a cell with a protease. In again another embodiment, after being treated with a protease, without being isolated or purified, the resulting peptides are administered to a cell in the presence of the protease, then another protease is administered together.

[00139] The modified growth factor peptides of the present invention may also be prepared by synthetic methods using solid-phase synthetic techniques. The synthesized polypeptides may be re-natured into correct folding pattern under appropriate conditions and bioactive dimmers can be induced from monomers using appropriate buffers and conditions. The correctly folded dimers maybe further separated from monomers using appropriate column to increase the yield of functional bioactive factors.

[00140] Regardless of method of preparation, be it recombinantly, enzymatically or synthetically, the novel modified growth factors, as monomers or dimers, can be prepared as a composition. In one embodiment, the composition is a pharmaceutical composition. For example, one or more cofactors

may be added to the truncated mature bioactive factor of the present invention to form a composition. Cofactors that may be added include, but are not limited to, heparin, hyaluronic acid, a fibronectin, an elastin, a laminin, albumin, a proteoglycan, collagen, gelatin, a divalent cation, calcium chloride, zinc sulfate, magnesium chloride, sodium bicarbonate, sodium chloride, sodium acetate, or sodium phosphate. In some embodiments, a protein or a protein fragment may be added as a cofactor to the modified growth factor peptides of the present invention.

[00141] The compositions, or pharmaceutical compositions, comprising the nucleic acid molecules or polypeptides typically comprise the nucleic acid molecule or protein and a pharmaceutically acceptable carrier. As used herein, "pharmaceutically acceptable carrier" or "pharmaceutical carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The nature of the pharmaceutical carrier or other ingredients will depend on the specific route of administration and particular embodiment of the invention to be administered. Examples of techniques and protocols that are useful in this context are, inter alia, found in Remington: The Science and Practice of Pharmacy (2010), Lippincott, Williams & Wilkins. Examples of such pharmaceutical carriers or diluents include, but are not limited to, water, saline, Ringer's solution, dextrose solution and 5% human serum albumin. Liposomes and non-aqueous vehicles such as fixed oils may also be used. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

[00142] A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Examples of routes of administration include oral and parenteral (*e.g.*, intravenous, intradermal, subcutaneous, inhalation, transdermal (topical), transmucosal and rectal administration). Solutions or suspensions used for parenteral, intradermal or subcutaneous application can include, but are not limited to, a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents, antibacterial agents such as benzyl alcohol or methyl parabens, antioxidants such as ascorbic acid or sodium bisulfite, chelating agents such as ethylenediaminetetraacetic acid, buffers such as acetates, citrates or phosphates, and agents 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. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

[00143] Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile

injectable solutions or dispersion. For intravenous administration, suitable pharmaceutical carriers include physiological saline, bacteriostatic water, Cremophor EL™ (BASF) or phosphate buffered saline (PBS). In all cases, the compositions must be sterile and should be fluid to the extent that easy syringeability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The pharmaceutical carrier can be 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 may be desirable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

[00144] Sterile injectable solutions can be prepared by incorporating the active compound (*e.g.*, the modified growth factor) 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.

[00145] Oral compositions generally include an inert diluent or an edible pharmaceutical carrier. They can be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches or capsules. Oral compositions can also be prepared using a fluid pharmaceutical carrier for use as a mouthwash, wherein the compound in the fluid pharmaceutical carrier is applied orally and swished and expectorated or swallowed. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like may contain any of the following ingredients, or compounds of a similar nature, such as but not limited to a binder, such as microcrystalline cellulose, gum tragacanth or gelatin, an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel or corn starch, a lubricant such as magnesium stearate or Sterotes, a glidant such as colloidal silicon dioxide, a sweetening agent such as sucrose or saccharin, or a flavoring agent such as peppermint, methyl salicylate or orange flavoring.

[00146] In one embodiment, the active compounds are prepared with pharmaceutical carriers that will protect the compound 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 from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These compositions can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811. It is especially advantageous to formulate oral or parenteral 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 compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on the unique characteristics of the active compound and the particular therapeutic effect to be achieved.

[00147] The pharmaceutical compositions can be included in a container, pack or dispenser together with instructions for administration.

[00148] The dosage of BMP peptides or the composition comprising the BMP peptides and a protease will depend on the disease state or condition to be treated and other clinical factors such as weight and condition of the human or animal and the route of administration of the compound. For treating human or animals, between approximately 0.005 mg/kg of body weight to 500 mg/kg of body weight of the compound can be administered. Therapy is typically administered at lower dosages and is continued until the desired therapeutic outcome is observed.

[00149] Methods of determining the dosages of compounds to be administered to a patient and modes of administering compounds to an organism are disclosed in, for example, WO 96/22976. Those skilled in the art will appreciate that such descriptions are applicable to the present invention and can be easily adapted to it.

[00150] The proper dosage depends on various factors such as the type of disease being treated, the particular composition being used and the size and physiological condition of the patient. Therapeutically effective doses for the compounds described herein can be estimated initially from cell culture and animal models. For example, a dose can be formulated in animal models to achieve a circulating concentration range that initially takes into account the IC_{50} as determined in cell culture assays. The animal model data can be used to more accurately determine useful doses in humans.

[00151] The invention also relates to methods of altering intracellular signaling of a cell, comprising contacting the cells with at least one of the modified peptides or the composition comprising the modified peptide and a protease, wherein the cell possesses a receptor that specifically binds to the modified growth factors of the present invention. The peptides utilized in the methods can be in monomeric, homodimeric or heterodimeric form. The specific binding of the modified growth factors to its receptor will, in turn, initiate the intracellular signaling cascade that is normally associated with the unmodified forms of the growth factor. For example, the "mature" form of BMP normally binds to its receptor complex including type I and type II receptors. The receptor phosphorylates cytoplasmic targets that include, but is not limited to, the Smad family of proteins. Smads are a class of proteins that function as intracellular signaling effectors for the TGF-β superfamily of secreted polypeptides. The activated BMP type I receptors will phosphorylate Smad1, Smad 5, and/or Smad 8. Phosphorylated Smad 1, 5, and 8 proteins, in turn, form a complex with Smad 4 and then translocate into the nucleus and interact with the transcription factors that regulate the expression of target genes.

[00152] Accordingly, the present invention provides methods of stimulating phosphorylation of Smad 1, Smad 5 and/or Smad 8 in a cell comprising contacting the cell(s) with at least one modified growth factor of the invention. The peptides utilized in the methods can be in monomeric, homodimeric or heterodimeric form. The activity of the novel peptides with respect to phosphorylating the Smad proteins may or may not be altered relative to the normal, mature growth factor. For example, the novel modified growth factors may increase or decrease phosphorylation of Smad 1, 5 and/or 8 compared to receptor binding of normal, mature growth factor. As another example, the novel modified growth factors may bind tighter or looser to its receptor (lower Kd) compared to receptor binding of normal, mature growth factor. One of skill in the art can readily determine if a particular protein is more or less phosphoylated over control groups using well known techniques such as transcription of reporter genes, ELISA assays, etc. Additional methods of the present invention comprise assessing the levels of Smad phosphorylation, for example, Smad 1, Smad 5 or Smad 8, both before and after contacting the cell(s) with the novel peptides of the present invention and determining the increase or decrease of Smad phosphorylation in response to the novel peptides of the present invention.

[00153] In another embodiment, the present invention provides methods of stimulating promoter activity in a cell or population of cells, where the promoter is responsive to activated Smad complexes, with the methods comprising contacting the cell(s) with at least one modified growth factor peptide of the present invention. The peptides utilized in the methods can be in monomeric, homodimeric or heterodimeric form. One of skill in the art would be aware of promoters that respond to activated Smad complexes. See, for example, Massague (2000) Cell, 103:295-309. The activity of the novel peptides with respect to stimulating Smad-responsive promoters may or may not be altered relative to the normal, mature growth factor. For example, the novel modified growth factors may

increase or decrease activation of Smad-responsive promoters compared to receptor binding of normal, mature growth factor. One of skill in the art can readily determine if a promoter is more or less activated over control groups using well known techniques such as transcription of reporter genes, ELISA assays, etc. Additional methods of the present invention comprise assessing the activity of a Smad-responsive promoter both before and after contacting the cell(s) with the novel peptides of the present invention and determining the increase or decrease of the promoter in response to the novel peptides of the present invention.

[00154] As used herein, "contacting," when used in connection with the methods of the present invention means bringing the novel peptides, in monomeric, homodimeric or heterodimeric form, in proximity to the target cells such that a specific binding event or a biological effect is possible. Thus, contacting can include adding the novel peptides in culture medium and applying the culture medium to cells in culture. Contacting also encompasses transfecting a cell with at least one vector described herein and allowing the cell to produce the modified growth factor. Of course, contacting would also include administration of the modified growth factor peptides, or pharmaceutical compositions thereof, of the present invention to cells in an intact organism. Compositions for administering the novel peptides of the present invention have been described herein.

[00155] As used herein, "administering," and "administer" are used to mean introducing at least one compound comprising at least one novel modified growth factor peptide into a subject. The peptides utilized in the administration methods can be in monomeric, homodimeric or heterodimeric form. When administration is for the purpose of treatment, the substance is provided at, or after the onset of, a symptom or condition in need of treatment. The therapeutic administration of this substance serves to attenuate any symptom, or prevent additional symptoms from arising. When administration is for the purposes of preventing a condition from arising ("prophylactic administration"), the substance is provided in advance of any visible or detectable symptom. The prophylactic administration of the substance serves to attenuate subsequently arising symptoms or prevent symptoms from arising altogether. The route of administration of the compound includes, but is not limited to, topical, transdermal, intranasal, vaginal, rectal, oral, subcutaneous intravenous, intraarterial, intramuscular, intraosseous, intraperitoneal, epidural and intrathecal as previously disclosed herein.

[00156] Furthermore, the methods would also include coadministering one or more substances in addition to the novel peptides of the present invention. The term "coadminister" indicates that each of at least two compounds is administered during a time frame wherein the respective periods of biological activity or effects overlap. Thus the term includes sequential as well as coextensive administration of the compounds of the present invention. And similar to administering compounds, coadministration of more than one substance can be for therapeutic and/or prophylactic purposes. If

more than one substance is coadministered, the routes of administration of the two or more substances need not be the same.

[00157] The invention also relates to methods of promoting osteoinductivity, with the methods comprising contacting cells with at least one modified growth factor peptide of the present invention, or compositions described herein. As used herein, "osteoinductivity" can refer to causing cells to differentiate into cells that are more osteoblast-like in phenotype, or the term can refer to increasing the proliferation of osteoblasts, or both. The cells, prior to contact with the modified growth factor peptide(s) of the present invention, may be undifferentiated or partially differentiated cells. The cells may be present in culture or in a tissue, organ or portion thereof or in an organism. The osteoinductive activity of the novel peptides may or may not be altered, including but not limited to, enhanced activity, relative to the normal, mature growth factor.

[00158] The invention also relates to methods of promoting chondroinductivity, with the methods comprising contacting cells with at least one modified growth factor peptide of the present invention, or compositions described herein. As used herein, "chondroinductivity" can refer to causing cells to differentiate into cells that are more chondrocyte-like in phenotype, or the term can refer to increasing the proliferation of chondrocytes, or both. The cells, prior to contact with the modified growth factor peptide(s) of the present invention, may be undifferentiated or partially differentiated cells. The cells may be present in culture or in a tissue, organ or portion thereof or in an organism. The chondroinductive activity of the novel peptides may or may not be altered, including but not limited to, enhanced activity, relative to the normal, mature growth factor.

[00159] The invention also relates to methods of promoting ligament/tendon differentiation, with the methods comprising contacting cells with at least one modified growth factor peptide of the present invention, or compositions described herein. As used herein, "ligament/tendon differentiation" can refer to causing cells to differentiate into cells that are more ligament and/or tendon-like in phenotype, or the term can refer to increasing the proliferation of ligament and/or tendon, or both. The cells, prior to contact with the modified growth factor peptide(s) of the present invention, may be undifferentiated or partially differentiated cells. The cells may be present in culture or in a tissue, organ or portion thereof or in an organism. The ligament/tendon differentiation activity of the novel peptides may or may not be altered, including but not limited to, enhanced activity, relative to the normal, mature growth factor.

[00160] For example, the invention provides for growing and/or culturing cells in the presence of one or more modified growth factor peptides, or compositions described herein. "Growing and/or culturing cells in the presence of" includes traditional cell culture methods as well as placing cells in the presence of the modified growth factors in any setting, such as in natural or synthetic matrices or tissues. The cells may be mammalian, such as but not limited to human, bovine, porcine, murine,

ovine, equine, canine, feline and others. In some embodiments, the cells may be mesenchymal stem cells, such as adipose-derived stem cells, embryonic stem cells, progenitor cells, differentiated cells, undifferentiated cells, and/or pluripotent stem cells. Appropriate cells may also include, but are not limited to cells of the ectodermal lineage, cells of the mesodermal lineage, and cells of the endodermal lineage. Examples of cells of the ectodermal lineage include but are not limited to keratinocytes, osteoblasts, chondrocytes, neurons. Examples of cells of the mesodermal lineage include but are not limited to myoblasts, adipocytes, fibroblasts, endothelial cells, or stromal cells. Examples of cells of the endodermal lineage include but not limited to epithelial cells of the auditory tube, the respiratory tract, such as trachea, bronchi, and alveoli of the lungs, the gastrointestinal tract, the urinary bladder and epithelial cells lining all glands. The cells may also be primary cells derived from tissues or organs. Appropriate cell lines used in the present invention may include but are not limited to mesenchymal cell lines, preosteoblastic cell lines, osteoblastic cell lines, and chondroblastic cell lines. The cells to which the modified growth factor peptides have been administered may be placed directly into a tissue, organism or other setting such as a matrix, including, but not limited to, bone matrices.

[00161] In some embodiments, the cells may be derived from autologous or allogeneic sources. The cells may be differentiated cells including chondrocytes, osteoblasts, osteoclasts, endothelial cells, epithelial cells, fibroblasts, and periosteal cells. Additionally, the cells may be totipotent, pluripotent, multipotent, progenitor, or adult somatic stem cells. The stem cells may be derived from embryos, placenta, bone marrow, adipose tissue, blood vessel, amniotic fluid, synovial fluid, synovial membrane, pericardium, periosteum, dura, peripheral blood, umbilical blood, menstrual blood, teeth, nucleus pulposus, brain, skin, hair follicle, intestinal crypt, neural tissue, muscle. The stem cells may be derived from skeletal muscle, smooth muscle, and cardiac muscle. The stem cells may be derived from genetic reprogramming of mature cells, such as induced pluripotent stem cells (iPSCs).

[00162] Any cell described herewith may be cultured with one or more modified growth factorss or compositions described herein for between about 15 minutes and about 4 weeks, about 2 hours and about 2 weeks, about 2 hours and about 1 week, about 2 hours and about 72 hours, about 24 hours and about 72 hours, or about 24 hours and about 96 hours, at between about 20°C and about 40°C or about 30°C and about 37°C, in an atmosphere containing between about 1% CO_2 and about 10% CO_2 or about 4% CO_2 and about 6% CO_2, in certain embodiments. In some embodiments of the present invention, cells may be cultured in the presence of one or more modified growth factors and/or (1) a tissue or an organ, (2) a matrix, or (3) a combination thereof. Cells that have been cultured in the presence of one or more modified growth factors in a cell culture medium may subsequently be applied to a matrix, a tissue, an organ or a combination thereof, in certain embodiments.

[00163] In some embodiments, treated cells are cryopreserved. Cryopreservation agents may be used to preserve treated cells, in certain embodiments. In some embodiments, treated cells are seeded onto

a matrix, and the cell-seeded matrix may be preserved using at least one cryopreservation agent. At least one cofactor may be added to treated cells, in some embodiments. Cofactors that may optionally be used are heparin, hyaluronic acid, a fibronectin, an elastin, a laminin, a proteoglycan, collagen, or gelatin, among others. In certain embodiments, a divalent cation, calcium chloride, zinc sulfate, magnesium chloride, sodium bicarbonate, sodium chloride, or sodium phosphate may be added as a cofactor to treated cells. In some embodiments, a protein or a protein fragment may be a cofactor that is added to treated cells. In further embodiments, a protease described herein may be a cofactor that is added to treated cells. Alternatively, the treated cells can be kept at a low temperature (*e.g.* 4 °C) or room temperature for 24-48 hours without cryopreservation.

[00164] In certain embodiments, treated cells that have been cryopreserved are optionally revived at a temperature between about 10°C and about 37°C before being applied to a tissue or an organ defect, a tissue, an organ, a matrix, or a mixture of two or more of these. In certain embodiments, treated cells may be combined with one or more body fluid, for example blood, platelet-rich plasma, platelet-poor plasma, plasma, bone marrow, and cord blood, among others, and an isotonic, hypotonic, or hypertonic solution, for instance saline, or Lactated ringer solution, and others, before they are used to treat a tissue or an organ defect. In some embodiments, treated cells may be applied to a tissue or an organ defect by injecting or inserting the cells between tissues or organs, or placing the cells on top of the defect. Modified growth factors may be administered to cells in vitro, in vivo, or in situ, in some embodiments. The modified growth factors may be administered to cells that are in tissue or organ or that have been isolated from tissues or organs, in some embodiments. In certain embodiments, at least one modified growth factor or compositions described herein may be administered to cells in (1) a cell culture medium, (2) a tissue or an organ, (3) a matrix, or (4) a combination of two or more of these.

[00165] There are a variety of osteoblast, chondrocyte, ligament/tendon differentiation markers that can be measured to assess osteoinductivity, chondroinductivity, or ligament/tendon differentiation, respectively. For example, cells express alkaline phosphatases during the early stages of differentiation toward osteoblast lineages. Therefore, in vitro alkaline phosphatase assays may be used to evaluate osteoinductivity in cells contacted with the modified growth factor peptide(s) of the present invention. The ability of the modified growth factor peptide(s) of the present invention to stimulate or induce the alkaline phosphatase expression in an otherwise non-bone forming cells, such as myoblast (C2C12 cells), would indicate that the modified growth factor peptide(s) of the present invention has osteoinductive activity. In these assays, cells cultured without added growth factors of any kind and without added modified growth factor peptide(s) of the present invention are used as negative controls to show that the baseline alkaline phosphatase expression on non-bone forming cells. The baseline of the osteoblastic markers in the negative control need not be zero, meaning that the cells in the negative control group may have at least some level of phenotypic marker(s). Accordingly, an "osteoinductive" peptide of the present invention would simply cause an increase in

the osteoblastic markers in experimental cells over control. Similarly, chondrocyte markers, including but not limited to type X collagen, type II collagen, Sox 9, Aggrecan. Matrilin-1 and CEP-68, to name a few, can be used to assess chondroinductive potential. Moreover, ligament/tendon markers, including but not limited to scleraxis, can be used to assess ligament/tendon differentiation potential.

[00166] Moreover, osteoinductivity, chondroinductivity, and ligament/tendon differentiation may be determined in tissue culture by investigating the ability of the modified growth factor peptide(s) of the present invention to differentiate or induce osteoblast phenotype, chondrocyte phenotype, ligament/tendon cell phenotype in cultured cells, such as primary cells, cell lines, or explants. For example, the cells may display increased production of a marker characteristic of osteoblasts and/or chondrocytes, such as alkaline phosphatase, etc. For example, the osteoinductive, chondroinductive, ligament/tendon differentiation potentials of the modified growth factors may be more than 0.2, 0.4, 0.6, 0.8, 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 times greater than those of the unmodified growth factors. In another example, the osteoinductive, chondroinductive, ligament/tendon differentiation potentials of the modified growth factors may be more than 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 500 or even 1000 times greater compared to those of the unmodified growth factors. Of course, this indicates that lower concentrations of modified growth factor, compared to unmodified growth factor are required to achieve the same effects.

[00167] Osteoinductivity,chondroinductivity, ligament/tendon differentiation, for assessing the bone, cartilage, ligament or tendon forming potential induced by the modified growth factor peptide(s) of the present invention in a location such as muscle, may also be evaluated using a suitable animal model. For example, intramuscular implantation into a rodent has been used as a model to assess osteoinductive activity of bioactive factors.

[00168] The invention also relates to methods of promoting proliferation or maintaining the differentiated state or preventing de-differentiation of osteoblasts, chondrocytes, ligament cells, tendon cells and/or any cell type disclosed herein comprising administering to the osteoblasts, chondrocytes, ligament cells, or tendon cells the modified growth factor peptides or compositions described herein. The proliferative activity of the novel peptides may or may not be altered, including but not limited to, enhanced activity, relative to the normal, mature growth factor.

[00169] Mitogenicity may be assessed by investigating cell proliferation induced by the modified growth factor peptides using various in vitro assays that measure metabolic activity, such as MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, alamarBlue® assay, and others. The alamarBlue® assay uses a non-cytotoxic reduction-oxidation indicator to measure cell metabolic activity, making it a nondestructive assay for assessing the mitogenic activity of the modified growth factors. Proliferation can also be assessed by measuring DNA quantification, such as by using a PicoGreen™ DNA assay, radioactive labeling of DNA synthesis, such as [^{3H}]thymidine labeling or

BrdU incorporation. Proliferation can also be assessed via manual cell counting, such as using a trypan blue hemacytometer.

[00170] The invention also relates to methods of increasing a cellular growth factor activity comprising administering to the cell at least one protease and the growth factor described herein. The protease is a protease described herein, including, but not limited to, collagenase, clostripain, dispase, trypsin, cathepsin, MMP-1, MMP-13, and a mixture thereof. In some embodiments, the growth factor comprises the mature growth factor and/or the modified growth factor described herein. The cell is a cell described herein, including, but not limited to, differentiated cells, adult stem cells, progenitor cells, and pluripotent stem cells. As explained previously, the modification of the growth factors as disclosed herein also enhances the activity of BMP. In certain embodiments, administering the protease along with the modified growth factor described herein further enhances the activity of the modified growth factor, such as an osteoinductive, chondroinductive activity, ligament/tendon differentiation potential.

[00171] In some embodiments, the addition of the protease described herein into the composition enhances stability of the growth factor in the same composition. As described previously, the modification of the growth factors as disclosed herein also enhances the stability of the growth factor. In certain embodiments, the addition of the protease into the composition comprising the modified growth factor further enhances the stability of the modified growth factor.

[00172] In additional embodiments, the composition administered to a cell in increasing a cellular growth factor activity comprises two or more protease. In some embodiments, a second protease can be mixed into the composition comprising the protease and the growth factor or can be administered separately from the composition comprising the protease and the growth factor.

[00173] The invention further relates to tissue or organ repair or regeneration compositions comprising (i) at least one modified growth factor peptide or the compositions of the present invention and (ii) a tissue, an organ, a matrix and/or a mixture of two or more thereof. The tissue or organ repair composition may comprise –bone, cartilage, and/or connective tissue.

[00174] An implantable biocompatible matrix for use with the compositions described herein can function as a suitable delivery or support system for the modified growth factor peptides. A biocompatible matrix should be non-toxic, non-eliciting or stimulating severe inflammatory response or immunological rejections, and devoid of other undesired reactions at the implantation site. Suitable matrices may also provide for release of the modified growth factor peptides, for example, to promote a slow, sustained release over time at the implantation site. In one embodiment, the matrix is a bone matrix or cartilage or connective tissue. In addition to its common, ordinary meaning, "administering to the matrix" includes embedding into the matrix or matrices host cells capable of producing at least one modified growth factors peptide. In another embodiment, the invention provides for methods of

treating cells with at least one modified growth factor peptide or compositions described herein and implanting these treated cells into a matrix, such as, but not limited to a bone matrix or cartilage.

[00175] Suitable matrices include, but are not limited to, porous scaffolds into which bone cells or progenitor cells may migrate. Osteogenic or chondrogenic cells, *i.e.*, cells involved in the process of deposition of new bone material or cartilagenous material, respectively, can often attach to such porous matrices, which can then serve as scaffolding for bone and cartilage tissue growth. Cells involved in the process of deposition of new ligament or tendon material can also attach to such porous matrices. For certain applications, the matrix should have sufficient mechanical strength to maintain its three dimensional structure and help support the immobilization of the bone segments being united or grafted together. Porous matrices which provide scaffolding for tissue growth can accelerate the deposition of new bone or the rate of bone growth and are said to be "osteoconductive." Osteoconductive matrices are especially useful in the pharmaceutical compositions described herein. Porous matrices which provide scaffolding for tissue growth can accelerate the deposition of new cartilage or the rate of cartilage growth and are said to be "chondroconductive." Osteoconductive matrices are especially useful in the pharmaceutical compositions described herein. Chondroconductive matrices are especially useful in the pharmaceutical compositions described herein. The osteoinductive or chondroinductive activity of the novel peptides may or may not be altered, including but not limited to, enhanced activity, relative to the normal, mature growth factor. Thus, the osteoconductive or chondroconductive activity of the treated matrices of the present invention may be enhanced compared to matrices not treated with modified growth factor. Of course, the matrices are considered to be osteoconductive or chondroconductive if cells within the matrix begin to differentiate into more osteoblast-like or chondrocyte-like appearing or functional cells, respectively.

[00176] Matrices can be derived from natural sources or they can be synthetic or a mixture of both. Matrices from natural sources may also comprise natural polymers, including, but not limited to, collagen, hyaluronic acid, alginate, albumin, fibrinogen-fibrin, chitosan, elasin, laminin, connective tissues, intervertebral disc, cortical or cancellous bone, demineralized or mineralized bone, fascia lata, dermis, muscle, ligament, tendon, cartilage including articular/hyaline cartilage, elastic cartilage, and fibrocartilage, a mixture thereof, and mixture of reconstituted tissue. Matrices from synthetic sources refer to any material not produced by living organisms, which may include, not limited to, the synthetic material made up of organic components, inorganic components, or a mixture thereof. In some embodiments, a synthetic matrix may comprise an organic synthetic polymer, such as poly(lactic-co-glycolic acid), polycaprolactone (PCL), polyglycolic acid (PGA), polylactic acid (PLA), polyhydroxybutyrate (PHB), Poly(ethylene glycol) (PEG), poly(ethylene oxide) (PEO)), and others. In some embodiments, a tissue, an organ, or matrix comprising at least one of alginate, chitosan, collagen, gelatin, hyaluronic acid, a fibronectin, an elastin, a laminin, and a proteoglycan

may be employed. In certain embodiments, a matrix comprising inorganic components, such as hydroxyapatite, calcium sulfate, octacalcium phosphate, calcium phosphate, macroporous calcium metaphosphate ceramic, β-tricalcium phosphate, metal, metal alloy, and others, may be used. A matrix used in certain embodiments of the present invention may be prepared by demineralizing, decellularizing or devitalizing a tissue or an organ and cells may be seeded onto the matrix.

[00177] In some embodiments, at least one modified growth factor compositions described herein may be applied to the matrix and may be incubated at conditions permitting the generation of a treated matrix. In some embodiments, incubation may be carried out at about 40°C or lower, or between about 10°C and about 37°C, or about 20°C and about 37°C. Incubation may be carried out for between at least about 2 minutes and about 120 minutes, about 3 minutes and about 100 minutes, about 4 minutes and about 80 minutes, about 5 minutes and about 60 minutes, and about 5 minutes and about 30 minutes in certain embodiments. Incubation may be performed under static or dynamic conditions, such as with agitation, shaking, stirring, mixing, horizontal motion, rocking, and others.

[00178] In some embodiments of the present invention, a matrix may be lyophilized before at least one modified growth factor or compositions described herein are administered to it. In certain embodiments, one or more modified growth factors may be administered to a matrix, and the treated matrix may be subsequently lyophilized. The lyophilized, treated matrix can then be rehydrated before it is used. Further, the cells can be seeded onto the matrix before implantation.

[00179] Examples of suitable osteoconductive, chondroconductive matrices include but are not limited to, collagen (*e.g.*, bovine dermal collagen), fibrin, calcium phosphate ceramics (*e.g.*, hydroxyapatite and tricalcium phosphate), calcium sulfate, guanidine-extracted allogenic bone and combinations thereof. A number of suitable matrices are commercially available, such as Collograft™ (Collagen Corporation), which is a mixture of hydroxyapatite, tricalcium phosphate and fibrillar collagen, and Interpore™ (Interpore International), which is a hydroxyapatite biomatrix formed by the conversion of marine coral calcium carbonate to crystalline hydroxyapatite.

[00180] A number of synthetic biodegradable polymers can serve as osteoconductive or chondroconductive matrices with sustained release characteristics. Descriptions of these polymers can be found in Behravesh (1999) Clinical Orthopaedics 367, S118 and Lu (2000) Polymeric Delivery Vehicles for Bone Growth Factors in Controlled Drug Delivery: Designing Technologies for the Future, Park and Mrsny eds., American Chemical Society. Examples of these polymers include polyα -hydroxy esters such as polylactic acid/polyglycolic acid homopolymers and copolymers, polyphosphazenes (PPHOS), polyanhydrides and poly(propylene fumarates).

[00181] Polylactic acid/polyglycolic acid (PLGA) homo and copolymers are well known in the art as sustained release vehicles. The rate of release can be adjusted by the skilled artisan by variation of polylactic acid to polyglycolic acid ratio and the molecular weight of the polymer (see Anderson

(1997) Adv. Drug Deliv. Rev. 28:5. The incorporation of PEG into the polymer as a blend to form microparticle matrices allows further alteration of the release profile of the active ingredient (see Cleek (1997) J. Control Release 48, 259). Ceramics such as calcium phosphate and hydroxyapatite can also be incorporated into the formulation to improve mechanical qualities.

[00182] In one embodiment, the matrices used in the compositions and methods of the present invention are bone matrices. As used herein, a bone matrix is a matrix derived from or including elements of natural bone. In some embodiments, the natural bone is mineralized, partially demineralized, demineralized, cancellous, cortical, or cortical cancellous bone. The bone matrices used herein may or may not include additional synthetic components not typically found in bone tissue. Other embodiments include compositions and methods utilizing a matrix derived from cartilage, other soft tissues such as the dermis, connective tissue, fascia, small intestine submucosa, serous membrane, pericardium, tendon, ligament, muscle, adipose tissue, myelin, blood vessels, base membrane, amniotic membrane and others. A matrix prepared from hyaline cartilage, fibrocartilage or elastic cartilage, may be employed in some embodiments. A matrix may be prepared from hyaline cartilage found in the condyle, tibial plateau, femoral head, humeral head, costal cartilage, or fibrocartilage found in intervertebral discs, or elastic cartilage found in the epiglottis or ear. In certain embodiments, a matrix derived from natural sources that has been optionally cleaned, disinfected, chemically modified, decellularized, particulated, homogenized, lyophilized, gamma irradiated, and/or plasticized may be used. Any of the matrices used herein may or may not include additional synthetic components not typically found in such tissue.

[00183] In one specific embodiment, the bone matrices or cartilage matrices may be demineralized or decellularized, respectively. Examples of demineralized matrices and methods of making are described in U.S. Patent No. 6,189,537 and 6,305,379.

[00184] The matrix, tissue, or organ used in certain embodiments of the present invention may be in the form of a powder, particulates, sheets, fibers, gels, putties, paste, blocks, cylinders, sponges, meshes, films, slices, curls, flakes, or wedges, among others. In certain embodiments of the present invention the matrix, tissue, or organ comprising the modified growth factor peptide(s) may be in the form of a powder, fibers, putty, or a sponge. In further embodiments, the sponge can include, for example, the implant having sponge-like structures disclosed in the co-pending, commonly-assigned patent application PCT/US09/04556 entitled "Composition for a Tissue Repair Implant and Methods of Making the Same" filed on August 7, 2009. The treated matrices can be used in any of the methods of the present invention.

[00185] The invention also relates to methods of increasing or promoting osteogenesis, chondrogenesis, or ligament/tendon genesis in cells. The methods may comprise treating the cells in matrices with at least one of the modified growth factor peptides, or compositions described herein.

As used herein, "osteogenesis" is the deposition new bone material or formation of new bone, including, but not limited to, intramembranous osteogenesis and endochondral osteogenesis. As used herein, "chondrogenesis" is the deposition new cartilage material or formation of new cartilage. As used herein, "ligament/tendon genesis" is the deposition new ligament and/or tendon material or formation of new ligament and/or tendon. The osteogenic, chondrogenic, ligament, or tendon inducing activity of the novel peptides may or may not be altered, including but not limited to, enhanced activity, relative to the normal, mature growth factor. The cells to which the modified growth factors may be administered include cells in any tissue in which bone, cartilage, ligament, or tendon formation is desired, such as, but not limited to, bone, cartilage, ligament, muscle, tendon, etc.

[00186] The invention also relates to methods of treating a tissue or organ defect or injury, for example, a musculoskeletal, dental or soft-tissue defect or injury, in an animal comprising administering (1) cells cultured in the presence of one or more modified growth factors, or compositions described herein and/or (2) one or more modified growth factors, or compositions described herein to the tissue or organ defect (*e.g.* osseous defects, defects in cartilage, ligament, tendon, spine disk, and tendon insertion site to bone).

[00187] The invention further relates to methods of treating a tissue or an organ defect or injury, for example a musculoskeletal, dental or soft-tissue defect, in an animal by applying a treated matrix to the defect, and application to the defect may be accomplished by injecting the treated matrix into the defect, inserting the treated matrix between tissue or organ, or placing the treated matrix on top of the defect. The present invention is also directed to treating a defect or injury in an organ in a similar manner. At least one cofactor may be added to a treated matrix, such as a matrix comprising a modified growth factor, in some embodiments.

[00188] In yet another embodiment, cells may be seeded onto a treated matrix. The cells seeded on the treated matrix can be any cell, such as but not limited to, osteoblasts, chondrocytes, ligament cells, tendon cells, progenitor cells, and stem cells disclosed herein or otherwise known in the art. The seeded cells may be allowed to proliferate and possibly attach to the matrix. Methods of seeding cells onto matrices, such as collagen, are well known in the art. Alternatively, cells may first be treated with at least one modified growth factor or compositions described herein and the treated cells may then be seeded onto a treated or untreated matrix.

[00189] Any of the methods of the present invention can be performed in virtually any setting, such as an in vivo, ex vivo, in situ or in vitro setting. For example, methods of promoting osteogenesis, chondrogenesis, or tendon/ligament inducing activities in cells may be performed in cell culture, may be performed in seeded cells on matrix, or may be performed in an intact organism. Moreover, any combination of any two or more of any of the embodiments described herein are contemplated.

[00190] While the invention has been described and illustrated herein by references to various specific materials, procedures and examples, it is understood that the invention is not restricted to the particular combinations of material and procedures selected for that purpose. Numerous variations of such details can be implied as will be appreciated by those skilled in the art. It is intended that the specification and examples be considered as exemplary, only, with the true scope and spirit of the invention being indicated by the following claims. All references, patents and patent applications referred to in this application are herein incorporated by reference in their entirety.

[00191] The following examples are illustrative and are not intended to limit the scope of the invention described herein.

[00192] Example 1 – Preparation and Sequencing of Modified BMP-2

[00193] Collagenase (Sigma Aldrich) was linked to sepharose beads and the un-bound collagenase was removed by extensive washing. Collagenase linked-sepharose beads were used to treat recombinant human BMP-2 (rhBMP-2) with incubation at 37°C overnight with gentle mixing. The rhBMP-2 had the amino acid sequence of amino acids 283-396 of SEQ ID NO: 1 prior to treatment. The modified mature rhBMP-2 and unmodified mature rhBMP-2 control were separated by SDS-PAGE gel electrophoresis and stained by silver staining. The related bands were cut and sequenced using MALDI-TOF mass spectrometry.

[00194] The results of MALDI-TOF mass spectrometry of the collagenase-treated rhBMP-2 sequencing revealed the same 13 amino acids on the C-terminus as the last 13 amino acids of carboxyl-terminus of untreated mature rhBMP-2. The MALDI-TOF sequencing confirms that the collagenase treatment did not truncate the C-terminus of the rhBMP-2.

[00195] Example 2 – Preparation and Sequencing of Modified BMP-2

[00196] The mature rhBMP-2 was treated with trypsin or collagenase in a microcentrifuge tube at a molarity ratio of between about 1:10 and about 100:1 and incubated overnight at 37 °C. The treated mature rhBMP-2 was further purified by reverse phase HPLC using a C-18 column. The collected fractions from HPLC were tested for osteoinductive potential using an in vitro alkaline phosphatase assay. The fractions that showed significantly high alkaline phosphatase activities were further separated by SDS-PAGE gel electrophoresis and blotted onto PVDF membrane. Proteins on the PVDF membrane were stained by coomassie blue and the related bands were cut and sent for N-terminal sequencing..

[00197] In the Coomassie blue stained PVDF membrane, the fraction B43-44 sample from the trypsin-treated rhBMP-2 and the fraction A42-44 sample from the collagenase-treated rhBMP-2 showed clean bands at around 14-15 kDa.

[00198] Nine out of ten amino acids of the N-terminus of the trypsin-treated mature rhBMP-2 and eight out of the ten amino acids of the N-terminus of the collagenase-treated mature rhBMP-2 were detected using Edman degradation chemistry with an ABI Procise® 494 sequencer. The deduced amino acid sequence of the N-terminus of the trypsin-treated rhBMP-2 was SSCKRHPLYV, which is the first 10 amino acids of SEQ ID NO:16 herein, and the deduced amino acid sequence of the N-terminus of the collagenase-treated rhBMP-2 was LKSSCKRHPL, which is the first 10 amino acids of SEQ ID NO:18 herein. The N-terminus of the trypsin-treated rhBMP-2 matches the untreated mature rhBMP-2 sequence after the 11th amino acid of the N-terminus, indicating that the trypsin cleaved the mature BMP-2 after the first 11 amino acids. Similarly, the N-terminus of the collagenase-treated rhBMP-2 matches the untreated mature rhBMP-2 sequence after the 9th amino acid of the N-terminus, indicating that the collagenase cleaved the mature BMP-2 after the first 9 amino acids. Western blot detected untreated and trypsin or collagenase-treated mature rhBMP-2, and the treated rhBMP-2 was about 1-2 kDa smaller than the untreated rhBMP-2.

[00199] Example 3 – Osteoinductive Potential of Modified rhBMP-2

[00200] Alkaline phosphatase is one of the distinctive biological or biologically-derived indicators of osteoinductivity. The AP assay measures the product para-nitrophenol (pNP) at a wavelength of 405 nm after 60 minutes of incubation of the substrate para-nitrophenyl phosphate (pNPP) with a cell lysate at 37 °C. The alkaline phosphatase activities of the HPLC fractions from Example 2 are shown in Figures 1 and 2. The fraction B43-44 from the trypsin-treated rhBMP-2 and the fraction A42-44 from the collagenase treated rhBMP-2 showed significantly higher alkaline phosphatase activity compared to other HPLC fractions. These results along with Figure 3 described below demonstrate that trypsin or collagenase-treated mature rhBMP-2, having an N-terminal truncation, facilitates an increase in alkaline phosphatase activity in vitro.

[00201] Example 4 – Osteoinductive Potential of Modified rhBMP-2 with or without a Protease

[00202] C2C12 cells (ATCC CRL-1772), which are from a mouse myoblast cell line, were seeded at a density of 25,000 cells/cm^2 in 24-well plates on day one. Collagenase and rhBMP-2 were mixed in microcentrifuge tubes at a molarity ratio of between about 1:10 and about 100:1 and incubated overnight at 37 °C. The treated mature rhBMP-2 was aliquoted with or without further HPLC purification described above. The aliquot with further HPLC purification (purified modified BMP2) was quantified by ELISA. The aliquot without further HPLC purification (non-purified modified

BMP2) retained the collagenase in the aliquot. The aliquots were used to test the effect of treated mature rhBMP-2 on osteogenic potential of C2C12 myoblasts.

[00203] Each of the rhBMP-2 control, and the purified modified rhBMP-2, and the non-purified modified rhBMP-2 at the same concentration was introduced into wells of the C2C12 cell seeded 24-well plate. C2C12 cells cultured in media alone (without addition of rhBMP-2 or modified rhBMP-2) were used as a negative control.

[00204] After 3 days incubation at 37 ℃, 5% CO_2, the AP assay as described above was performed, and the results are shown in Figure 3. The alkaline phosphatase activity of cells cultured with the purified modified rhBMP-2 or the non-purified modified rhBMP-2 were about 39 times and 103 times higher than that of cells cultured with non-treated rhBMP-2 control, respectively. The alkaline phosphatase activity of cells cultured with non-purified modified rhBMP-2 was about 1.6 times higher than that of cells cultured with purified modified rhBMP-2. Thus, the results demonstrate a significant increase in the alkaline phosphatase activity by administering the non-purified modified rhBMP-2 compared to administering the purified modified rhBMP-2.

[00205] Further, with another set of C2C12 cells cultured as described above, the purified modified rhBMP-2 was introduced into wells of the C2C12 cell along with 138 ng/mL of active dispase or inactive dispase, or 100 ug/mL collagenase or inactive collagenase. The AP assay as described above was performed, and the results are demonstrated in Figure 4. The results showed that adding active or inactive dispase or collagenase to the purified modified rhBMP-2 samples also increased the alkaline phosphatase activity.

[00206] In addition, rhBMP-2 treated by clostripain, dispase, and the mixture thereof, without further purification, also increased the alkaline phosphatase activity compared to non-modified rhBMP-2 control. In addition, rhBMP-2 treated by BMP1 or MMP13, without further purification, also increased the alkaline phosphatase activity compared to non-modified rhBMP-2 control.

[00207] Example 5 – Osteoinductive Potential of Modified rhBMP-2 with a Protease

[00208] C2C12 cells (ATCC CRL-1772) were cultured as described above. Collagenase and rhBMP-2 were mixed in microcentrifuge tubes at a molarity ratio of between about 1:10 and about 100:1 and incubated overnight at 37 ℃ with agitation. The treated mature rhBMP-2 was aliquoted, and the aliquots were used to test the effect of treated mature rhBMP-2 on osteogenic potential of C2C12 myoblasts as follows.

[00209] For the osteoinductive potential assessment, base media of DMEM containing 1% FBS, 50 μg/mL of ascorbic acid and 10 mM of β-glycerolphosphate was used as a control group. The base

media control with the addition of 50 ng/mL of rhBMP-2 was used as the BMP-2 positive control group. The base media control with the addition of 50 ng/mL of modified rhBMP-2 without the purification was used as the test group. The media were added into C2C12 cells seeded on chamber slides and changed every 3-4 days.

[00210] After 4 days incubation at 37 ºC, 5% CO_2, photographs were also taken for cell cultures in each group. The nodule formation of C2C12 cells appeared only in modified rhBMP-2 group, but not in the other two control groups. After 6 or 12 days of incubation, the media was removed from chamber slides and the slides were stained with Alizarin Red S. Alizarin red S has been used to identify calcium-rich deposits by cells in culture or tissue sections, which indicates the effect of osteoinductive material on osteogenic potential of cells.

[00211] For the base media control group, no positive Alizarin red S staining was found at any tested time points. For the rhBMP-2 control group, some Alizarin red S staining was detected after 6 or 12 days of incubation. For the modified rhBMP-2 group, after 6 days of incubation, significant amount of nodules stained positive by Alizarin red S. After 12 days of incubation, more Alizarin red S positively stained nodules of larger size were observed in C2C12 cells cultured with modified rhBMP-2 than that in C2C12 cells cultured with unmodified rhBMP-2. This data suggests that modified rhBMP-2 has a significantly greater effect on osteogenic potential of C2C12 myoblasts as compared to the unmodified mature rhBMP-2 control.

[00212] Example 6 – Alkaline Phosphatase Assay for Cells Cultured with Modified rhBMP-2

[00213] Alkaline phosphatase is one of the distinctive indicators of osteoinductivity. C2C12 cells (ATCC CRL-1772), which are from a mouse myoblast cell line, were seeded at a density of 25,000 cells/cm^2 in 24-well plates on day one. Collagenase and rhBMP-2 were mixed in microcentrifuge tubes at a molarity ratio of between about 1:10 and about 100:1 and incubated overnight at 37ºC with agitation. On day two, the collagenase-treated rhBMP-2, untreated rhBMP-2 at the same concentration as treated rhBMP-2, or collagenase alone at the same concentration of collagenase used to treat rhBMP-2 was introduced into each well of the C2C12 cell seeded 24-well plate. C2C12 cells cultured without the addition of rhBMP-2 or collagenase were used as a negative control. After 3 days of incubation at 37ºC, 5% CO_2, cells were collected from the culture plates, and cell lysates were prepared for AP assays and bicinchoninic acid (BCA) total protein assays (BCA reagent available from Pierce Biotechnology). The AP assay was performed as described above. The BCA assay is a detergent-compatible protein assay used for colorimetric detection and quantitation of total protein. The results of the in vitro AP assay are shown in Table IV. The alkaline phosphatase activity of cells cultured with collagenase-treated rhBMP-2 was about 87 times higher than that of cells cultured with untreated rhBMP-2.

[00214] Table 6. Alkaline phosphatase activity of cells

Groups	Negative Control	Unmodified rhBMP-2	Collagenase Control	Modified rhBMP-2
Mean AP activity (nmol pNP/min/mg protein)	0.86 ± 0.08	9.93 ± 0.08	1.02 ± 0.29	862.31 ± 40.71

[00215] Example 7 – Osteoinductive potential of mature rhBMP-2 with the addition of protease

[00216] C2C12 mouse myoblast cell line (ATCC CRL-1772), were cultured as described above. The mature rhBMP-2 (50 ng/mL, R&D Systems, Inc.), or the same concentration of mature rhBMP-2 with the addition of dispase or collagenase (molarity ratio between about 1:100 and about 100:1) was introduced into wells of the C2C12 cells. C2C12 cells cultured in media alone (without addition of rhBMP-2) were used as a negative control.

[00217] After 3 days incubation at 37 °C, 5% CO_2, an AP assay was performed as described above. The alkaline phosphatase activities in cells induced by the mature rhBMP-2 in combination with either dispase or collagenase were about 24% and 293% higher than that induced by the mature rhBMP-2 without addition of protease, respectively.

[00218] Example 8 – Preparation and Sequencing of Modified BMP-4

[00219] Collagenase (Sigma Aldrich) was linked to sepharose beads and the un-bound collagenase was removed by extensive washing. Collagenase linked-sepharose beads were used to treat mature rhBMP-4 by incubation at 37 °C overnight with gentle mixing. The treated mature rhBMP-4 was separated by SDS-PAGE gel electrophoresis followed by protein transferring onto PVDF membrane. The proteins on PVDF membrane were stained by Coomassie blue and the related bands were cut for N-terminal sequencing using Edman degradation chemistry with an ABI Procise 494 sequencer. Proteins on PVDF membrane were also detected by Western Blot using antibody against C-terminus of rhBMP-4.

[00220] By comparing the collagenase treated mature rhBMP-4 bands with the untreated mature rhBMP-4 control bands, extra bands at around 17-22 kDa were cut for N-terminal sequencing. Ten amino acids of the N-terminus of the collagenase treated mature rhBMP-4 were detected using Edman degradation chemistry with an ABI Procise® 494 sequencer. The deduced amino acid sequence of the N-terminus of the treated rhBMP-4 was KKNKNCRRHS, which is the first 10 amino acids of SEQ ID NO:19 herein. The N-terminus of the treated rhBMP-4 matched the untreated mature rhBMP-4 sequence, indicating that the collagenase cleaved the mature BMP-4 after the first 10 amino

acids. The Western blot detected a band for collagenase treated rhBMP-4 group, which is about 1-2 kDa smaller than the band for untreated mature rhBMP-4.

[00221] Example 9 – Osteoinductive Potential of Modified rhBMP-4

[00222] Alkaline phosphatase is one of the distinctive biological or biologically-derived indicators of osteoinductivity. The AP assay measures the product para-nitrophenol (pNP) at a wavelength of 405 nm after 60 minutes of incubation of the substrate para-nitrophenyl phosphate (pNPP) with a cell lysate at 37 °C. C2C12 cells (ATCC CRL-1772) were seeded at a density of about 25,000 cells/cm^2 in 24-well plates on day one. Collagenase and various concentrations of mature rhBMP4 were mixed in microcentrifuge tubes at molarity ratios between about 1:10 and about 1000:1 and incubated at 37 °C overnight. The collagenase-treated rhBMP-4 or untreated rhBMP4 was introduced into each well of the C2C12 cell seeded 24-well plate on day two. The final concentrations of rhBMP4 were 1, 5, 10, and 30 ng/mL. After 3 days of incubation at 37 °C and 5% CO_2, cells were collected from culture plates and cell lysates were prepared for AP assays and BCA total protein assays.

[00223] The results of the in vitro AP assay are shown in Figure 5. When cells were cultured with treated or untreated mature rhBMP-4 at a concentration of 10 ng/mL or less, the differences in alkaline phosphatase activity were negligible. At a concentration of 30 ng/mL, cells treated with collagenase-treated rhBMP-4 expressed significantly higher AP activities than those treated with untreated mature rhBMP-4.

[00224] Example 10 – Osteoinductive Potential of Modified rhBMP-4 with or without a Protease

[00225] C2C12 cells (ATCC CRL-1772), which are from a mouse myoblast cell line, are seeded at a density of 25,000 cells/cm^2 in 24-well plates on day one. Collagenase and rhBMP-4 are mixed in microcentrifuge tubes at a molarity ratio of between about 1:10 and about 100:1 and are incubated overnight at 37 °C. The treated mature rhBMP-4 is aliquoted with or without further HPLC purification described above. The aliquot with further HPLC purification (purified modified BMP-4) is quantified by ELISA. The aliquot without further HPLC purification (non-purified modified BMP-4) retained the collagenase in the aliquot. The aliquots are used to test the effect of treated mature rhBMP-4 on osteogenic potential of C2C12 myoblasts.

[00226] Each of the rhBMP-4 control, and the purified modified rhBMP-4, and the non-purified modified rhBMP-4 at the same concentration is introduced into wells of the C2C12 cell seeded 24-well plate. C2C12 cells cultured in media alone (without addition of rhBMP-4 or modified rhBMP-4) are used as a negative control.

[00227] After 3 days incubation at 37 °C, 5% CO_2, the AP assay as described above is performed. The alkaline phosphatase activity of cells cultured with the purified modified rhBMP-4 or the non-purified modified rhBMP-4 are significantly higher than that of cells cultured with non-treated rhBMP-4

control. The alkaline phosphatase activity of cells cultured with non-purified modified rhBMP-4 is also significantly higher than that of cells cultured with purified modified rhBMP-4. The results demonstrate a significant increase in the alkaline phosphatase activity by administering the non-purified modified rhBMP-4 compared to administering the purified modified rhBMP-4.

[00228] Example 11 – Preparation and Sequencing of Modified BMP-5

[00229] Modified recombinant human BMP-5 (rhBMP-5) was obtained by treating mature rhBMP-5 with dispase (Worthington), collagenase (Sigma-Aldrich), or cathepsin K. The mature rhBMP-5 had the sequence of amino acids 317-454 of SEQ ID NO:4 prior to modification. Aliquots of the modified mature rhBMP-5 and mature rhBMP-5 control were separated by SDS-PAGE gel electrophoresis and the peptides were transferred onto PVDF membrane. The proteins on PVDF membrane were stained by Coomassie blue or detected with Western blot using antibody against amino acids 407-450 of rhBMP-5.

[00230] The related bands from Coomassie blue stained PVDF membrane were cut and used for N-terminal sequencing. Ten amino acids of the N-terminus of the dispase-modified or collagenase-modified mature rhBMP-5 were detected using Edman degradation chemistry with an ABI Procise® 494 sequencer. The deduced amino acid sequence of the N-terminus of the modified rhBMP-5 was taken to be YNTSEQKQAC, which is the first 10 amino acids of SEQ ID NO:21 herein. Another deduced amino acid sequence of the N-terminus of the modified rhBMP-5 was taken to be SVGDYNTSEQ, which is the first 10 amino acids of SEQ ID NO:20 herein. Another deduced amino acid sequence of the N-terminus of the treated rhBMP-5 was taken to be TSEQKQACKK, which is the first 10 amino acids of SEQ ID NO:22 herein. Another deduced amino acid sequence of the N-terminus of the treated rhBMP-5 was taken to be DYNTSEQKQA, which is the first 10 amino acids of SEQ ID NO:23 herein. Another deduced amino acid sequence of the N-terminus of the treated rhBMP-5 was taken to be MSSVGDYNTS, which is the first 10 amino acids of SEQ ID NO:24 herein. Another deduced amino acid sequence of the N-terminus of the treated rhBMP-5 was taken to be VGDYNTSEQK, which is the first 10 amino acids of SEQ ID NO:25 herein. Another deduced amino acid sequence of the N-terminus of the treated rhBMP-5 was taken to be QA(C)KKHELYV, which is the first 10 amino acids of SEQ ID NO:26 herein. One more deduced amino acid sequence of the N-terminus of the treated rhBMP-5 was taken to be EQKQA(C)KKHE, which is the first 10 amino acids of SEQ ID NO:27 herein. One more deduced amino acid sequence of the N-terminus of the treated rhBMP-5 was taken to be SEQKQACKKH, which is the first 10 amino acids of SEQ ID NO:28 herein.

[00231] Example 12 – Osteoinductive Potential of Modified rhBMP-5

[00232] C2C12 cells (ATCC CRL-1772) were seeded at a density of 25,000 cells/cm^2 in 24-well plates on day one. Dispase or collagenase and rhBMP-5 were mixed in microcentrifuge tubes at molarity ratios between about 1:100 and about 1000 :1 and incubated at 37°C for 10-60 minutes or 18-20hours. The modified rhBMP5 or unmodified rhBMP5 was introduced into each well of the C2C12 cell seeded 24-well plate on day two. The final concentrations of rhBMP5 were 0.25 ug/mL, 0.5 ug/mL, and 1 ug/mL in each well of control and modified groups. After 3 days of incubation at 37°C and 5% CO_2, cells were collected from culture plates and cell lysates were prepared for AP assays and BCA total protein assays. Alkaline phosphatase is one of the distinctive biological or biologically-derived indicators of osteoinductivity. The AP assay measures the product para-nitrophenol (pNP) at a wavelength of 405 nm after 60 minutes of incubation of the substrate para-nitrophenyl phosphate (pNPP) with a cell lysate at 37°C.

[00233] The results of the in vitro AP assay are shown in Figures 6 and 7. When cells were cultured with dispase-modified or unmodified rhBMP5 at a concentration of 0.25 ug/mL, 0.5 ug/mL, or 1 ug/mL, the alkaline phosphatase activity induced by dispase-modified rhBMP5 was about 400 times, 120 times, or 35 times higher than that induced by unmodified rhBMP5, respectively (Fig. 6). When cells were cultured with collagenase-modified or unmodified rhBMP5, significant increases on alkaline phosphatase activity were found in all concentration groups (Fig. 7). At 0.25 ug/mL, 0.5 ug/mL, or 1 ug/mL in cell culture, the alkaline phosphatase activity induced by collagenase-modified rhBMP5 was about 1000 times, 300 times, or 50 times higher than that induced by unmodified rhBMP5, respectively.

[00234] Example 13 – Osteoinductive Potential of Modified rhBMP-5 with or without a Protease

[00235] Dispase and rhBMP-5 were mixed in microcentrifuge tubes at a molarity ratio of between about 1:1000 and about 100:1 and incubated at 20 to37 °C for 10-60 minutes. The treated mature rhBMP-5 was further purified with reverse phase HPLC using a C-18 column. The collected fractions from HPLC were lyophilized, quantified and used to test the effect of modified rhBMP-5 on osteoinductive potential of C2C12 myoblasts.

[00236] C2C12 cells (ATCC CRL-1772) were seeded at a density of 25,000 cells/cm^2 in 24-well plates on day one. The purified modified rhBMP-5 alone, or the same concentration of purified modified rhBMP-5 with the addition of dispase or collagnease was introduced into wells of C2C12 cell. C2C12 cells cultured in media alone (without addition of modified rhBMP-5) were used as a negative control.

[00237] After 3 days incubation at 37 °C, 5% CO_2, an AP assay was performed as described above. As shown in Figure 8, Alkaline phosphatase activity in the cells induced by the purified modified

rhBMP-5 in combination with dispase was about 76% higher than that induced by the purified modified rhBMP-5 alone. Alkaline phosphatase activity in the cells induced by the purified modified rhBMP-5 in combination with collagenase was about 176% higher than that induced by the purified modified rhBMP-5 alone.

[00238] Example 14 – Chondrogenic Potential of Modified rhBMP-5

[00239] The ATDC5 murine chondrosarcoma cell line (Riken Cell Bank) that have been widely used to study chondrocyte differentiation were seeded at 2,000 cells/cm^2 in 6-well plates. After 5 days of culture, the culture media was changed to the differentiation media that contains 5% fetal bovine serum, 10 ug/mL bovine insulin, and 50 ug/mL ascorbic acid (differentiation media control), or the differentiation media with the addition of rhBMP-5 or dispase-modified rhBMP-5 prepared as shown above. The cells were cultured in the differentiation media for a total of 13 days and the media was changed every 3 days. Cells were collected at different time points and total RNA was extracted and followed by reverse transcription using Applied Biosystems RNA-to-cDNA kit. The chondrogenic-specific genes, collagen type 2a (COL2a1), aggrecan (ACAN), cartilage oligomeric matrix protein (COMP) and transcription factor SOX9, were quantified using TaqMan Gene Expression Assay kit from Applied Biosystems, Inc. The relative expression (ΔCt) of the target gene was normalized to GAPDH endogenous gene expression and the fold change ($2^{-\Delta\Delta Ct}$) was calculated relative to the differentiation media control. As shown in tables 7-10 below, the COL2a1, ACAN, COMP, SOX9 gene expressions in cells cultured with dispase-modified rhBMP-5 were all higher than that in cells cultured with rhBMP-5 control.

Table 7. Expression of COL2a1

	rhBMP-5 Control	Modified rhBMP-5	
Incubation Time (days)	Fold change relative to differenetiated media control	Fold change relative to differenetiated media control	% of increase
3	1.30 ±0.21	2.08 ±0.19	60.58
5	1.23 ±0.09	1.07 ±0.08	-12.94
7	0.70 ±0.06	1.85 ±0.57	163.29
9	0.84 ±0.05	1.17 ±0.10	39.80
11	1.52 ±0.19	3.40 ±0.43	123.97
13	1.38 ±0.05	3.38 ±0.19	145.09

[00240] Table 8. Expression of ACAN

	rhBMP-5 Control	Modified rhBMP-5	
Incubation Time (days)	Fold change relative to differenetiated media control	Fold change relative to differenetiated media control	% of increase
3	0.85 ±0.08	1.94 ±0.10	127.10
5	0.78 ±0.05	2.71 ±0.22	249.03

7	0.83 ±0.04	3.46 ±1.06	316.02
9	0.35 ±0.01	3.90 ±0.16	1000.43
11	1.97 ±0.22	4.50 ±0.56	128.15
13	4.16 ±0.18	13.15 ±0.82	216.02

[00241] Table 9. Expression of COMP

	rhBMP-5 Control	Modified rhBMP-5	
Incubation Time (days)	Fold change relative to differenetiated media control	Fold change relative to differenetiated media control	% of increase
3	0.55 ±0.06	1.27 ±0.06	130.80
5	0.36 ±0.03	0.23 ±0.03	-37.58
7	0.37 ±0.03	0.92 ±0.28	146.80
9	0.16 ±0.08	0.89 ±0.18	459.19
11	0.47 ±0.06	1.46 ±0.17	210.23
13	0.91 ±0.06	1.77 ±0.17	94.08

Table 10. Expression of SOX9

	rhBMP-5 Control	Modified rhBMP-5	
Incubation Time (days)	Fold change relative to differenetiated media control	Fold change relative to differenetiated media control	% of increase
3	0.60 ±0.05	0.67 ±0.07	10.70
5	0.59 ±0.18	0.89 ±0.06	50.18
7	0.80 ±0.05	0.71 ±0.21	-11.32
9	0.61 ±0.04	0.83 ±0.07	35.97
11	1.08 ±0.13	1.18 ±0.16	8.92
13	1.44 ±0.26	2.84 ±0.16	97.25

[00242] Example 15-Osteoinductive potential of mature rhBMP-5 with the addition of protease

[00243] C2C12 cells (ATCC CRL-1772) were cultured as described above. The mature rhBMP-5 control (200 ng/mL, R&D Systems, Inc.), or the same concentration of mature rhBMP-5 with the addition of collagenase or dispase (molarity ratio between about 1:100 and about 100:1) was introduced into wells of the C2C12 cells. C2C12 cells cultured in media alone (without addition of rhBMP-5) were used as a negative control.

[00244] After 3 days incubation at 37 ºC, 5% CO_2, an AP assay was performed as described above. The results showed that the alkaline phosphatase activities in cells induced by the mature rhBMP-5 in

combination with either collagenase or dispase were 21 and 42 times higher than that induced by the mature rhBMP-5 without addition of protease, respectively.

[00245] Example 16 – Preparation and Sequencing of Collagenase-Modified BMP-7

[00246] The mature rhBMP-7 was treated with collagenase in a microcentrifuge tube at a molarity ratio of between about 1:10 and about 100:1 and incubated overnight at 37 °C. The treated mature rhBMP-7 was further purified by reverse phase HPLC using a C-18 column. The collected fractions from HPLC were tested for osteoinductive potential using an in vitro alkaline phosphatase assay. The fractions that showed significantly high alkaline phosphatase activities were further separated by SDS-PAGE gel electrophoresis and blotted onto PVDF membrane. Proteins on the PVDF membrane were stained by coomassie blue and the related bands were cut and sent for N-terminal sequencing.

[00247] In the Coomassie blue stained PVDF membrane, the fraction 43 sample from the collagenase-treated rhBMP-7 showed clean bands at around 15-16 kDa.

[00248] Ten amino acids of the N-terminus of the collagenase treated mature rhBMP-7 were detected using Edman degradation chemistry with an ABI Procise® 494 sequencer. The deduced amino acid sequence of the N-terminus of the treated rhBMP-7 was taken to be ENSSSDQRQA, which is the first 10 amino acids of SEQ ID NO:31 herein. This deduced N-terminus of the treated rhBMP-7 matches the untreated mature rhBMP-7 sequence except, indicating that the collagenase cleaved the mature BMP-7 after the first 27 amino acids. Western blot detected untreated and collagenase-treated mature rhBMP-7, and the treated rhBMP-7 was about 2-3 kDa smaller than the untreated rhBMP-7.

[00249] Example 17 – Osteoinductive Potential of Collagenase-Modified BMP-7

[00250] Alkaline phosphatase is one of the distinctive biological or biologically-derived indicators of osteoinductivity. The AP assay measures the product para-nitrophenol (pNP) at a wavelength of 405 nm after 60 minutes of incubation of the substrate para-nitrophenyl phosphate (pNPP) with a cell lysate at 37 °C. The alkaline phosphatase activities induced by the HPLC fractions from Example 16 are shown in Figure 9. The alkaline phosphatase activity induced by the fraction 43 from the collagen-treated rhBMP-7 was significantly higher than the alkaline phosphatase activity induced by other HPLC fractions.

[00251] Example 18 – Dose Responsiveness of Modified rhBMP-7

[00252] C2C12 cells (ATCC CRL-1772) were seeded at a density of 25,000 cells/cm^2 in 24-well plates on day one. Collagenase and various concentrations of rhBMP7 were mixed in microcentrifuge tubes at molarity ratios between about 1:10 and about 1000 :1 and incubated at 37°C overnight. The

collagenase-treated rhBMP7 or untreated rhBMP7 was introduced into each well of the C2C12 cell seeded 24-well plate on day two. The final concentrations of rhBMP7 were 5 ng/mL, 10 ng/mL, 25 ng/mL, 50 ng/mL, 100 ng/mL, and 200 ng/mL in each well. After 3 days of incubation at 37°C and 5% CO_2, cells were collected from culture plates and cell lysates were prepared for AP assays and BCA total protein assays.

[00253] The results of the in vitro AP assay are shown in Figure 10. When cells were cultured with treated or untreated rhBMP7 at a concentration of 10 ng/mL or less, the differences in alkaline phosphatase activity were negligible. At concentrations of 25 ng/mL or higher, significant differences were found between collagenase-treated rhBMP7 groups and untreated rhBMP7 groups. At 50 ng/mL in cell culture, the alkaline phosphatase activity induced by collagenase-treated rhBMP7 was about 80 times higher than that induced by untreated rhBMP7.

[00254] Example 19 – Preparation and Sequencing of Trypsin-Modified BMP-7

[00255] rhBMP-7 was incubated with trypsin overnight at 37°C. Trypsin and rhBMP-7 were used at a molarity ratio between about 1:50 and about 100:1. The treated mature rhBMP-7 was separated by SDS-PAGE gel electrophoresis followed by protein transferring onto PVDF membrane. The proteins on PVDF membrane were stained by Coomassie blue and the related bands were cut for N-terminal sequencing using Edman degradation chemistry with an ABI Procise 494 sequencer. Proteins on PVDF membrane were also detected by Western Blot using antibody against C-terminus of rhBMP-7.

[00256] By comparing the trypsin treated mature rhBMP-7 bands with the untreated mature rhBMP-7 control bands, extra band at around 14-16 kDa were cut for N-terminal sequencing. Ten amino acids of the N-terminus of the trypsin treated mature rhBMP-7 were detected using Edman degradation chemistry with an ABI Procise® 494 sequencer. The deduced amino acid sequence of the N-terminus of the treated rhBMP-7 was QACKKHELYV, which is the first 10 amino acids of SEQ ID NO:30 herein. The N-terminus of the trypsin-treated rhBMP-7 matches the untreated mature rhBMP-7 sequence, indicating that the trypsin cleaved the mature BMP-7 after the first 35 amino acids. The Western blot detected a band for trypsin treated rhBMP-7 group, which is about 2-3 kDa smaller than the band for untreated mature rhBMP-7.

[00257] Example 20 – Osteoinductive Potential of Trypsin-Modified BMP-7

[00258] C2C12 cells were cultured as described above. Trypsin and rhBMP7 were mixed in a microcentrifuge tube at molarity ratios between about 1:50 and about 100 :1 and incubated at 37°C overnight. The trypsin-modified rhBMP7 or unmodified rhBMP7 was introduced into each well of the C2C12 cell seeded 24-well plate on day two. After 3 days of incubation at 37 °C and 5% CO_2,

cells were collected from culture plates and cell lysates were prepared for AP assays and BCA total protein assays.

[00259] The results of the in vitro AP assay are shown in Figure 11. The alkaline phosphatase activity induced by trypsin-modified rhBMP7 was about 1.5 times higher than that induced by unmodified rhBMP7.

[00260] Example 21 – Osteoinductive Potential of Modified rhBMP-7 with or without a Protease

[00261] C2C12 cells were cultured as described above. Collagenase and rhBMP-7 are mixed in microcentrifuge tubes at a molarity ratio of between about 1:10 and about 100:1 and are incubated overnight at 37 ºC. The treated mature rhBMP-7 is aliquoted with or without further HPLC purification described above. The aliquot with further HPLC purification (purified modified BMP-7) is quantified by ELISA. The aliquot without further HPLC purification (non-purified modified BMP-7) retained the collagenase in the aliquot. The aliquots are used to test the effect of treated mature rhBMP-7 on osteogenic potential of C2C12 myoblasts.

[00262] Each of the rhBMP-7 control, and the purified modified rhBMP-7, and the non-purified modified rhBMP-7 at the same concentration is introduced into wells of the C2C12 cell seeded 24-well plate. C2C12 cells cultured in media alone (without addition of rhBMP-7 or modified rhBMP-7) are used as a negative control.

[00263] After 3 days incubation at 37 ºC, 5% CO_2, the AP assay as described above is performed. The alkaline phosphatase activity of cells cultured with the purified modified rhBMP-7 or the non-purified modified rhBMP-7 are significantly higher than that of cells cultured with non-treated rhBMP-7 control. The alkaline phosphatase activity of cells cultured with non-purified modified rhBMP-7 is also significantly higher than that of cells cultured with purified modified rhBMP-7. The results demonstrate a significant increase in the alkaline phosphatase activity by administering the non-purified modified rhBMP-7 compared to administering the purified modified rhBMP-7.

[00264] Example 22 – Osteoinductive Potential of Modified rhBMP-7 with or without an additional Protease

[00265] Dispase and rhBMP-7 were mixed in microcentrifuge tubes at a molarity ratio between about 1:100 and about 1000:1 and incubated at 37ºC from about 15 minutes to about 24 hours.

[00266] C2C12 cells were cultured as described above. The non-treated rhBMP-7 control, dispase modified rhBMP-7 without further purification, or dispase modified rhBMP-7 with the addition of 100ug/mL collagenase in culture media were introduced into wells of the C2C12 cell seeded 24-well plate on day two. C2C12 cells cultured in media alone (without addition of rhBMP-7 or treated rhBMP-7) were used as a negative control. After 3 days of incubation at 37°C/5% CO_2, cells were

collected from culture plates and cell lysates were prepared for AP assays and BCA total protein assays as described above.

[00267] The results of the in vitro AP assay are shown in Figure 12. The alkaline phosphatase activity of cells cultured with dispase modified rhBMP-7 was about 141% higher than that of cells cultured with non-treated rhBMP-7 control, while the alkaline phosphatase activity of cells cultured with dispase modified rhBMP-7 with the addition of collagenase in media further increased 116% compared to the dispase modified rhBMP-7 group. The alkaline phosphatase activity of cells cultured with dispase modified rhBMP-7 with the addition of collagenase in media was about 421% higher than that of cells cultured with non-treated rhBMP-7 control.

[00268] Example 23 – Osteoinductive Potential of Modified rhBMP-7 with a Protease

[00269] C2C12 cells (ATCC CRL-1772) are cultured as described above. Collagenase and rhBMP-7 are mixed in microcentrifuge tubes at a molarity ratio of between about 1:10 and about 100:1 and incubated overnight at 37 °C with agitation. The treated mature rhBMP-7 is aliquoted, and the aliquots are used to test the effect of treated mature rhBMP-7 on osteogenic potential of C2C12 myoblasts as follows.

[00270] For the osteoinductive potential assessment, base media of DMEM containing 1% FBS, 50 μg/mL of ascorbic acid and 10 mM of β-glycerolphosphate is used as a control group. The base media control with the addition of 50 ng/mL of rhBMP-7 is used as the BMP-7 positive control group. The base media control with the addition of 50 ng/mL of modified rhBMP-7 without the purification is used as the test group. The media are added into C2C12 cells seeded on chamber slides and changed every 3-4 days.

[00271] After several days of incubation at 37 °C, 5% CO_2, photographs are also taken for cell cultures in each group. The nodule formation of C2C12 cells appear only in modified rhBMP-7 group, but not in the other two control groups. After more days of incubation, the media is removed from chamber slides and the slides are stained with Alizarin Red S. Alizarin red S is used to identify calcium-rich deposits by cells in culture or tissue sections, which indicates the effect of osteoinductive material on osteogenic potential of cells.

[00272] For the base media control group, no positive Alizarin red S staining is found at any tested time points. For the rhBMP-7 control group, some Alizarin red S staining is detected after 6 or 12 days of incubation. For the modified rhBMP-7 group, after 6 days of incubation, significant amount of nodules stain positive by Alizarin red S. After 12 days of incubation, more Alizarin red S positively stain nodules of larger size are observed in C2C12 cells cultured with modified rhBMP-7 than that in C2C12 cells cultured with unmodified rhBMP-7. This data suggests that modified

rhBMP-7 has a significantly greater effect on osteogenic potential of C2C12 myoblasts as compared to the unmodified mature rhBMP-7 control.

[00273] Example 24 – Preparation and Sequencing of Modified BMP-13

[00274] Modified recombinant human BMP-13 (rhBMP-13 or rhGDF-6) was obtained by treating mature rhBMP-13 with dispase (Worthington) or collagenase (Sigma-Aldrich). The mature rhBMP-13 had the sequence of amino acids 336-455 of SEQ ID NO:12 prior to modification. Aliquots of the modified mature rhBMP-13 and unmodified mature rhBMP-13 control were separated by SDS-PAGE gel electrophoresis and the peptides were transferred onto PVDF membrane. The proteins on PVDF membrane were stained by Coomassie blue or detected with Western blot using antibody against C-terminal region of mature rhBMP-13.

[00275] The related bands from Coomassie blue stained PVDF membrane were cut and used for N-terminal sequencing. Ten amino acids of the N-terminus of the dispase modified mature rhBMP-13 were detected using Edman degradation chemistry with an ABI Procise® 494 sequencer. The deduced amino acid sequence of the N-terminus of the modified mature rhBMP-13 was taken to be GKKSRLRCSK, which is the first 10 amino acids of SEQ ID NO:34 herein.

[00276] Example 25 – Improvement of Osteoinductive Potential of Modified BMP-13

[00277] C2C12 cells (ATCC CRL-1772) were seeded at a density of 25,000 cells/cm^2 in 24-well plates on day one. Dispase and mature rhBMP-13 (rhGDF-6) were mixed in microcentrifuge tubes at molarity ratios between about 1:100 and about 1000 :1 and incubated at 37°C for 5-60 minutes. The dispase-modified mature rhGDF-6 or unmodified mature rhGDF-6 control was introduced into each well of the C2C12 cell seeded 24-well plate on day two. The final concentrations of rhBMP-13 were 1 ug/mL, 2 ug/mL, and 3.67 ug/mL for both control and modified groups. After 3 days of incubation at 37°C and 5% CO_2, cells were collected from culture plates and cell lysates were prepared for Alkaline phosphatase (AP) assays and BCA total protein assays. Alkaline phosphatase is one of the distinctive biological or biologically-derived indicators of osteoinductivity. The AP assay measures the product para-nitrophenol (pNP) at a wavelength of 405 nm after 60 minutes of incubation of the substrate para-nitrophenyl phosphate (pNPP) with a cell lysate at 37°C.

[00278] The results of the in vitro AP assay are shown in Figure 13. When cells were cultured with dispase-modified or unmodified rhBMP-13 at a concentration of 1 ug/mL or higher, significant enhancements of AP activity were found in cells treated with dispase-modified mature rhBMP-13 compared to the cells treated with unmodified mature rhBMP-13 (Fig.13). The alkaline phosphatase activity induced by dispase-modified mature rhBMP-13 was about 300 - 350 times higher than that induced by unmodified mature rhBMP-13 control.

[00279] Example 26 – Improvement of Osteoinductive Potential of Modified BMP-13

[00280] C2C12 cells (ATCC CRL-1772) were seeded at a density of 25,000 cells/cm^2 in 24-well plates on day one. Collagenase (Sigma) and mature rhBMP-13 were mixed in microcentrifuge tubes at molarity ratios between about 1:100 and about 1000 :1 and incubated at 37°C for 0.5-24 hours. The collagenase-modified mature rhBMP-13 or unmodified mature rhBMP-13 control was introduced into each well of the C2C12 cell seeded 24-well plate on day two. The final concentrations of rhBMP-13 were 0.5 ug/mL, 1 ug/mL, and 2 ug/mL for both control and modified groups. After 3 days of incubation at 37°C and 5% CO_2, cells were collected from culture plates and cell lysates were prepared for AP assays and BCA total protein assays.

[00281] The results of the in vitro AP assay are shown in Figure 14. When cells were cultured with collagenase-modified or unmodified rhBMP-13 at a concentration of 0.5 ug/mL or higher, significant enhancements of AP activity were found in cells treated with collagenase-modified mature rhBMP-13 compared to the cells treated with unmodified mature rhGDF-6 (Fig.14). The alkaline phosphatase activity induced by collagenase-modified mature rhBMP-13 was about 0.7 - 20 times higher than that induced by unmodified mature rhBMP-13 control.

[00282] Example 27 – Osteoinductive Potential of purified Modified rhBMP-13 with or without a Protease

[00283] Dispase and rhBMP-13 (PeproTech, Inc.) were mixed in microcentrifuge tubes at a molarity ratio of between about 1:100 and about 100:1 and incubated at 35-40 °C for 10-60 minutes. The mixture was purified with reverse phase HPLC using a C-18 column. The HPLC purified modified rhBMP-13 was used to test the effect of modified rhBMP-13 on osteogenic potential of C2C12 myoblasts.

[00284] C2C12 cells (ATCC CRL-1772) were seeded at a density of 25,000 cells/cm^2 in 24-well plates on day one. The purified modified rhBMP-13, or the same concentration of purified modified rhBMP-13 with the addition of dispase or collagenase (molarity ratio between about 1:100 and about 100:1), were introduced into wells of the C2C12 cells on day two. C2C12 cells cultured in media alone (without addition of modified rhBMP-13) were used as a negative control.

[00285] After 3 days of incubation at 37 °C, 5% CO_2, the AP assay was performed as described above. The alkaline phosphatase activity in the cells induced by the purified modified rhBMP-13 in combination with either dispase or collagenase was about of 25 times and 50% higher than that induced by purified modified rhBMP-13 alone, respectively.

[00286] Example 28 – Osteoinductive Potential of Modified rhBMP-13 with or without an additional Protease

[00287] C2C12 cells were cultured as described above. Non-purified modified rhBMP-13(1 ug/mL), or an equal concentration of non-purified modified rhBMP-13 with the addition of either dispase or collagenase was introduced into wells of the C2C12 cells. The AP assay was performed as described above. The alkaline phosphatase activity in the cells induced by the non-purified modified rhBMP-13 in combination with either dispase or collagenase were about 18% and 81% higher than that induced by the non-purified modified rhBMP-13 alone, respectively.

[00288] Example 29 – Osteoinductive potential of mature rhBMP-13 with the addition of protease

C2C12 cells (ATCC CRL-1772) were seeded at a density of 25,000 cells/cm^2 in 24-well plates on day one. The mature rhBMP-13 (1μg/mL, PeproTech, Inc.), or the same concentration of mature rhBMP-13 with the addition of dispase or collagenase (molarity ratio between about 1:100 and about 100:1) was introduced into wells of the C2C12 cells. C2C12 cells cultured in media alone (without addition of rhBMP-13) were used as a negative control.

After 3 days incubation at 37 °C, 5% CO_2, an AP assay was performed as described above. The results showed that the alkaline phosphatase activities in cells induced by the mature rhBMP-13 in combination with either dispase or collagenase were about 50-180 times and 235% higher than that induced by the mature rhBMP-13 without addition of protease, respectively.

[00289] Example 30 – Effect of Modified rhBMP-13 on tendon-specific gene expression of human adipose-derived stem cells

[00290] Human adipose-derived stem cells (hASC) were isolated from fat tissue of a human donor with research consent. Cells were propagated until the 5th passage and seeded at5,000 cells/cm^2 in 6-well plates. After one day, the culture media was changed to the differentiation media that contains rhBMP-13 control (100ng/mL) or dispase-modified rhBMP-13 (100ng/mL) prepared as shown above. The cells were cultured in differentiation media for a total of 12 days and the media was changed every 3 days. Cells were collected at different time points and total RNA was extracted and followed by reverse transcription using Applied Biosystems RNA-to-cDNA kit. The target tendon-specific genes, scleraxis (SCX) and tenomodulin (TMND), were amplified and quantified using TaqMan Gene Expression Assay kit from Applied Biosystems, Inc. The relative expression (ΔCt) of the target gene was normalized to GAPDH endogenous gene expression and the fold change ($2^{-\Delta\Delta Ct}$) was calculated relative to the hASC control.

[00291] As shown in table IV below, the SCX gene expression in cells cultured with dispase-modified rhBMP-13 was about 18% to 40% higher than that in cells cultured with rhBMP-13 control for the first 6 days of culture. On day 9 and day 12, the SCX expression in cells cultured with dispase-modified rhBMP-13 was about 63% and 97% higher than that in cells cultured with rhBMP-13 control. The TMND gene expression in cells cultured with dispase-modified rhBMP-13 was about 84% higher than that in cells cultured with the rhBMP-13 control on day 6.

[00292] Table 11. Expression of Scleraxis (SCX)

	rhBMP-13 Control	Modified rhBMP-13	
Incubation Time (days)	Fold change relative to differenetiated media control	Fold change relative to differenetiated media control	% of increase
1	1.34 ±0.42	1.62 ±0.71	20.86
2	1.16 ±0.16	1.67 ±0.38	44.73
3	2.05 ±0.67	2.49 ±0.93	21.42
6	1.16 ±0.27	1.37 ±0.13	18.1
9	0.98 ±0.15	1.60 ±0.19	63.58
12	1.52 ±0.24	2.99 ±0.41	97.25

[00293] Example 31 – Preparation and Sequencing of Modified BMP-14

[00294] Modified recombinant human BMP-14 (rhGDF-5 or rhBMP-14) was obtained by treating mature rhBMP-14 with dispase (Worthington) or collagenase (Sigma-Aldrich). The rhBMP-14 had the sequence of amino acids 382-501 of SEQ ID NO:13 prior to modification. Aliquots of the modified mature rhBMP-14 and unmodified mature rhBMP-14 control were separated by SDS-PAGE gel electrophoresis and the peptides were transferred onto PVDF membrane. The proteins on PVDF membrane were stained by Coomassie blue or detected with Western blot using antibody against internal region of mature rhBMP-14.

[00295] The related bands from Coomassie blue stained PVDF membrane were cut and used for N-terminal sequencing. Ten amino acids of the N-terminus of the dispase modified mature rhBMP-14 were detected using Edman degradation chemistry with an ABI Procise® 494 sequencer. The deduced amino acid sequence of the N-terminus of the modified rhBMP-14 was taken to be LKARCSRKAL, which is the first 10 amino acids of SEQ ID NO:35 herein.

[00296] Example 32 – Dose-dependent Change of Osteoinductive Potential of Modified BMP-14

[00297] C2C12 cells (ATCC CRL-1772) were seeded at a density of 25,000 cells/cm^2 in 24-well plates on day one. Dispase and rhBMP-14 were mixed in microcentrifuge tubes at molarity ratios between about 1:100 and about 1000 :1 and incubated at 37ºC for 5-60 minutes. The dispase-modified rhBMP-14 or unmodified rhBMP-14 control was introduced into each well of the C2C12 cell seeded 24-well plate on day two. The final concentrations of rhBMP-14 were 0.2 ug/mL, 0.5 ug/mL, and 1 ug/mL in each well for both control and modified groups. After 3 days of incubation at 37°C and 5% CO_2, cells were collected from culture plates and cell lysates were prepared for AP assays and BCA total protein assays. Alkaline phosphatase is one of the distinctive biological or biologically-derived indicators of osteoinductivity. The AP assay measures the product para-

nitrophenol (pNP) at a wavelength of 405 nm after 60 minutes of incubation of the substrate para-nitrophenyl phosphate (pNPP) with a cell lysate at 37°C.

[00298] The results of the in vitro AP assay are shown in Figure 15. When cells were cultured with dispase-modified or unmodified rhBMP-14 at a concentration of 0.2 ug/mL or higher, significant enhancements were found in dispase-modified rhBMP-14 groups compared to the unmodified rhBMP-14 control groups (Fig.15). At 0.2 ug/mL, 0.5 ug/mL, or 1 ug/mL in cell culture, the alkaline phosphatase activity induced by dispase-modified rhBMP-14 was about 1.5 times, 9 times, or 19 times higher than that induced by unmodified rhBMP-14 control, respectively.

[00299] Example 33 – Osteoinductive Potential of Modified rhBMP-14 with or without a Protease

[00300] Dispase and rhBMP-14 (PeproTech, Inc.) were mixed in microcentrifuge tubes at a molarity ratio of between about 1:100 and about 100:1 and incubated at 37 °C for 10-60 minutes. The mixture was purified with reverse phase HPLC using a C-18 column. The HPLC purified modified rhBMP-14 was used to test the effect of modified rhBMP-14 on osteogenic potential of C2C12 myoblasts.

[00301] C2C12 cells (ATCC CRL-1772) were seeded at a density of 25,000 cells/cm^2 in 24-well plates on day one. The purified modified rhBMP-14 alone, or the same concentration of purified modified rhBMP-14 with the addition of dispase (molarity ratio between about 1:100 and about 100:1) was introduced into wells of the C2C12 cells. C2C12 cells cultured in media alone (without addition of modified rhBMP-14) were used as a negative control.

[00302] After 3 days incubation at 37 °C, 5% CO_2, the AP assay was performed as described above. As shown in Figure 16, the alkaline phosphatase activity in the cells induced by the purified modified rhBMP-14 in combination with dispase was about 235% higher than that induced by the purified modified rhBMP-14 alone.

[00303] Example 34 – Osteoinductive Potential of Modified rhBMP-14 with or without an additional Protease

[00304] C2C12 cells were cultured as described above. Non-purified modified rhBMP-14 (1 ug/mL), or the same concentration of non-purified modified rhBMP-14 with addition of dispase or collagenase were introduced into wells of the C2C12 cell. The AP assay was performed as described above. As shown in Figure 17, the alkaline phosphatase activity in the cells induced by the non-purified modified rhBMP-14 in combination with dispase or collagenase were about 120% and 45% higher than that induced by the non-purified modified rhBMP-14 alone, respectively.

[00305] Example 35 – Osteoinductive potential of mature rhBMP-14 with the addition of protease

[00306] C2C12 cells (ATCC CRL-1772) were seeded at a density of 25,000 cells/cm^2 in 24-well plates on day one. The mature rhBMP-14 (1μg/mL, PeproTech, Inc.), or the same concentration of mature rhBMP-14 with the addition of dispase or collagenase (molarity ratio between about 1:100 and about 100:1) was introduced into wells of the C2C12 cells. C2C12 cells cultured in media alone (without addition of rhBMP-14) were used as a negative control.

[00307] After 3 days incubation at 37 ºC, 5% CO_2, the AP assay was performed as described above. The results showed that the alkaline phosphatase activities in cells induced by the mature rhBMP-14 in combination with either dispase or collagenase was about 8-80 times and 53 – 150% higher than that induced by the mature rhBMP-14 without addition of protease, respectively.

[00308] Example 36 – Effect of Modified rhBMP-14 on tendon-specific gene expression of human adipose-derived stem cells

[00309] Human adipose-derived stem cells (hASC) were isolated from fat tissue of a human donor with research consent. Cells were propagated until the 5th passage and seeded at5,000 cells/cm^2 in 6-well plates . After one day, the culture media was changed to the differentiation media that contains rhBMP-14 control (100ng/mL) or dispase-modified rhBMP-14 (100ng/mL) prepared as shown above.

[00310] The cells were cultured in differentiation media for a total of 12 days and the media was changed every 3 days. Cells were collected at different time points and total RNA was extracted and followed by reverse transcription using Applied Biosystems RNA-to-cDNA kit. The tendon-specific gene, scleraxis (SCX), was amplified and quantified using TaqMan Gene Expression Assay kit from Applied Biosystems, Inc. The relative expression (ΔCt) of the target gene was normalized to GAPDH endogenous gene expression and the fold change ($2^{-\Delta\Delta Ct}$) was calculated relative to the hASC control. The SCX gene expressions in cells cultured with dispase-modified rhBMP-14 were about 30% and 39% higher than that in cells cultured with the rhBMP-14 control on day 9 and day 12, respectively.

[00311] Example 37 – Stability of Modified rhBMP-2 with or without a Protease in Cell Culture

[00312] C2C12 cells were cultured, and purified and non-purified modified rhBMP-2 were prepared as described herein. The purified modified rhBMP-2, the non-purified modified rhBMP-2, or the untreated mature rhBMP-2 at the same concentration was introduced into each well of the C2C12 cell seeded 24-well plate on day two. C2C12 cells cultured without addition of rhBMP-2 or modified rhBMP-2 were used as a negative control. After incubation at 37 ºC, 5% CO_2 for different durations (24 hr, 48 hr, or 72 hr), spent culture media were collected from these original 24-well plates and transferred into new 24-well plates containing fresh C2C12 cells seeded the previous day, and the new 24-well plates were incubated at 37ºC, 5% CO_2 for another 3 days. Cells from the original 24-well

plates and new 24-well plates were collected at the end of each culture period and cell lysates were prepared for AP assays and BCA total protein assays.

[00313] For the control groups wherein C2C12 cells were cultured for three days in untreated rhBMP-2 spent media collected after 24 hrs, 48 hrs, and 72 hrs, the alkaline phosphatase activity reduced 96%, 98%, and 99% respectively as compared to that of cells cultured for three days with fresh rhBMP-2 media. For the experimental groups in which C2C12 cells were cultured for three days in purified modified rhBMP-2 spent media collected after 24 hrs, 48 hrs, and 72 hrs, the alkaline phosphatase activity reduced 41%, 75%, and 99% respectively as compared to that of cells cultured for three days with fresh purified modified rhBMP-2 media. For the experimental groups in which C2C12 cells were cultured for three days in non-purified modified rhBMP-2 spent media collected after 24 hrs, 48 hrs, and 72 hrs, the alkaline phosphatase activity was maintained at a similar or higher level as compared to that of cells cultured for three days with fresh non-purified modified rhBMP-2 media. This demonstrates that the presence of a protease can further enhance the stability of modified BMP-2 in culture media.

[00314] Example 38 – Stability of Modified rhBMP-5 with or without a Protease in Cell Culture

[00315] C2C12 cells were cultured. Purified dispase-modified rhBMP-5 and purified collagenase-modified rhBMP-5 were prepared as described above. The purified dispase-modified rhBMP-5, the purified collagenase-modified rhBMP-5, the purified modified rhBMP-5 with the addition of either dispase or collagenase, or the untreated mature rhBMP-5 at the same concentration were introduced into each well of the C2C12 cell seeded 24-well plate on day two. C2C12 cells cultured without addition of rhBMP-5 or modified rhBMP-5 were used as a negative control. Cells cultured with either dispase or collagenase were used as enzyme negative controls. After incubation at 37 °C, 5% CO_2 for different durations (24 hr, 48 hr, or 72 hr), spent culture media were collected from these original 24-well plates and transferred into new 24-well plates containing fresh C2C12 cells seeded the previous day, and the new 24-well plates were incubated at 37°C, 5% CO_2 for another 3 days. Cells from the original 24-well plates and new 24-well plates were collected at the end of each culture period, and cell lysates were prepared for AP assays and BCA total protein assays.

[00316] The enzyme negative control groups containing either dispase or collagenase showed similar alkaline phosphatase activity as the negative control without any addition of rhBMP5 or modified rhBMP5. For the control groups wherein C2C12 cells were cultured for three days in untreated mature rhBMP-5 spent media collected after 24 hrs, 48 hrs, and 72 hrs, the alkaline phosphatase activity was reduced as compared to that of cells cultured for three days with fresh rhBMP-5 media by 82%, 92% and 94%, respectively.

[00317] For the experimental groups in which C2C12 cells were cultured for three days with the spent media of purified dispase-modified rhBMP-5 collected after 24, 48, and 72hrs, the alkaline phosphatase activity increased about 14%, reduced 10%, and 14% respectively as compared to that of cells cultured for three days with fresh purified dispase-modified BMP5 media.

[00318] For the experimental groups in which C2C12 cells were cultured for three days with the spent media of purified dispase-modified rhBMP-5 and additional dispase collected after 24 hrs, 48 hrs, and 72 hrs, the alkaline phosphatase activity reduced 4%, 16%, and 4% respectively as compared to that of cells cultured for three days with fresh purified dispase-modified rhBMP-5 with the addition of dispase media.

[00319] For the experimental groups in which C2C12 cells were cultured for three days in purified collagenase-modified rhBMP-5 spent media collected after 24 hrs, 48 hrs, and 72 hrs, the alkaline phosphatase activity was maintained at similar or higher level as compared to that of cells cultured for three days with fresh purified collagenase-modified rhBMP-5 media. For the experimental groups in which C2C12 cells were cultured for three days in purified collagenase-modified rhBMP-5 with the addition of dispase or collagenase spent media collected after 24 hrs, 48 hrs, and 72 hrs, the alkaline phosphatase activity was also maintained at similar or higher level as compared to that of cells cultured for three days with fresh purified collagenase-modified rhBMP-5 with the addition of dispase or collagenase media.

[00320] For the experimental groups in which C2C12 cells were cultured for three days with the spent media of purified dispase-modified rhBMP-5 and additional collagenase collected after 24 hrs, 48 hrs, and 72 hrs, the alkaline phosphatase activity reduced 19%, 11%, and 37% respectively as compared to that of cells cultured for three days with fresh purified dispase-modified rhBMP-5 with addition of collagenase media. Therefore, the modified rhBMP-5 showed higher stability than the unmodified rhBMP-5.

[00321] Example 39 – Stability of Modified rhBMP-13 with or without a Protease in Cell Culture

[00322] C2C12 cells were seeded and cultured for one day. The same quantity of non-modified mature rhBMP-13 or dispase-modified rhBMP-13 without purification was introduced into each well of the C2C12 cell seeded 24-well plate on day two. C2C12 cells cultured without addition of the non-modified rhBMP-13 or modified rhBMP-13 was used as a negative control. After incubation at 37 °C, 5% CO_2 for different durations (24 hr, 48 hr, or 72 hr), spent culture media were collected from these original 24-well plates and transferred into new 24-well plates containing fresh C2C12 cells seeded the previous day, and the new 24-well plates were incubated at 37°C, 5% CO_2 for 3 days. Cells from the original 24-well plates and new 24-well plates were collected at the end of each culture period, and cell lysates were prepared for AP assays and BCA total protein assays.

[00323] For the experimental groups in which C2C12 cells were cultured for three days in non-purified dispase-modified rhBMP-13 spent media collected after 24 hrs, 48 hrs, and 72 hrs, the alkaline phosphatase activities were 44, 46, and 30 times higher than those of cells cultured for three days with non-modified rhBMP-13 spent media collected after 24 hrs, 48 hrs, and 72 hrs, respectively.

[00324] Example 40 – Stability of Modified rhBMP-14 with or without a Protease in Cell Culture

[00325] C2C12 cells were seeded and cultured for one day. The same quantity of non-modified mature rhBMP-14 or dispase-modified rhBMP-14 without purification was introduced into each well of the C2C12 cell seeded 24-well plate on day two. C2C12 cells cultured without addition of the non-modified rhBMP-14 or modified rhBMP-14 was used as a negative control. After incubation at 37 °C, 5% CO_2 for different durations (24 hr or 48 hr), spent culture media were collected from these original 24-well plates and transferred into new 24-well plates containing fresh C2C12 cells seeded the previous day, and the new 24-well plates were incubated at 37°C, 5% CO_2 for 3 days. Cells from the original 24-well plates and new 24-well plates were collected at the end of each culture period, and cell lysates were prepared for AP assays and BCA total protein assays.

[00326] For the experimental groups in which C2C12 cells were cultured for three days in non-purified dispase modified rhBMP-14 spent media collected after 24 hrs or 48 hrs, the alkaline phosphatase activities were 7.5% and 103% higher than those of cells cultured for three days with non-modified rhBMP-14 spent media collected after 24 hrs and 48 hrs, respectively.

[00327] Example 41 – Stability of Modified rhBMPs- 4 and 7 with or without a Protease in Cell Culture

[00328] C2C12 cells are cultured, and purified and non-purified modified rhBMP-4 and 7 are prepared as described above. Each of the purified modified rhBMP-4 and 7, the non-purified modified rhBMP-4 and 7, and the untreated mature rhBMP-4 and 7 at the same concentration is introduced into each well of the C2C12 cell seeded 24-well plate on day two. C2C12 cells cultured without addition of rhBMP- 4 and 7 or modified rhBMP-4 and 7 are used as a negative control. After incubation at 37 °C, 5% CO_2 for different durations (24 hr, 48 hr, or 72 hr), spent culture media are collected from these original 24-well plates and transferred into new 24-well plates containing fresh C2C12 cells seeded the previous day, and the new 24-well plates are incubated at 37°C, 5% CO_2 for another 3 days. Cells from the original 24-well plates and new 24-well plates are collected at the end of each culture period, and cell lysates are prepared for AP assays and BCA total protein assays.

[00329] For the control groups wherein C2C12 cells are cultured for three days in untreated rhBMP-4 or 7 spent media collected after 24 hrs, 48 hrs, and 72 hrs, the alkaline phosphatase activity is reduced as compared to that of cells cultured for three days with fresh rhBMP-4 or 7 media, respectively. For the experimental groups in which C2C12 cells are cultured for three days in purified modified

rhBMP-4 or 7 spent media collected after 24 hrs, 48 hrs, and 72 hrs, the alkaline phosphatase activity is also reduced less than for the control group. For the experimental groups in which C2C12 cells are cultured for three days in non-purified modified rhBMP-4 or 7 spent media collected after 24 hrs, 48 hrs, and 72 hrs, however, the alkaline phosphatase activity is maintained at a similar or higher level as compared to that of cells cultured for three days with fresh non-purified modified rhBMP-4 or 7 media, respectively. This demonstrates that the presence of a protease can further enhance the stability of modified BMP-4 or 7 in culture media.

[00330] Example 42 – Stability of Modified rhBMP-2 with or without a Protease on Shelf

[00331] Purified and non-purified modified rhBMP-2 were prepared as described herein. Aliquots of the purified and non-purified modified rhBMP-2 at the same volume and concentration were incubated at 37ºC for different durations (2 days to 2 weeks). After each time point, one aliquot from each group was transferred into -20ºC freezer until the last time point was completed. The aliquots of purified modified rhBMP-2 or non-purified modified rhBMP-2 from different time points were introduced into each well of the C2C12 cell cultured as described above. C2C12 cells cultured without the addition of rhBMP-2 or collagenase were used as a negative control. After 3 days incubation at 37 ºC, 5% CO_2, the AP assay as described above was performed.

[00332] The alkaline phosphatase activity of cells cultured with purified modified rhBMP-2 on shelf (37 ºC) for 7 days or 14 days reduced 94% and 98% respectively as compared to that of cells cultured with purified modified rhBMP-2 on shelf (37 ºC) for 2 days. The alkaline phosphatase activity of cells cultured with non-purified modified rhBMP-2 on shelf (37 ºC) for 7 days or 14 days reduced 7% and 6% respectively as compared to that of cells cultured with non-purified modified rhBMP-2 on shelf (37 ºC) for 2 days. This demonstrates that the presence of a protease can further enhance the stability of modified BMP-2 on shelf.

[00333] Example 43 – Stability of Modified rhBMP-5 with or without a Protease on Shelf

[00334] Purified modified rhBMP-5 was prepared as described in Example 11 above. Aliquots of the purified dispase-modified rhBMP-5, the purified dispase-modified rhBMP-5 with the addition of dispase or collagenase, the purified collagenase-modified rhBMP-5, or the untreated mature rhBMP-5 at the same volume and concentration were incubated at 37ºC for different durations (1 day to 7 days). After each time point, one aliquot from each group was transferred into -20ºC freezer until the last time point was completed. All the aliquots were thawed at the same time and were introduced into each well of the C2C12 cell cultured as described above. C2C12 cells cultured without the addition of rhBMP-5 or modified rhBMP-5 were used as a negative control. After 3 days incubation at 37 ºC, 5% CO_2, the AP assay as described above was performed.

[00335] The alkaline phosphatase activity of cells cultured with purified dispase-modified rhBMP-5 on shelf (37 °C) for 1 day was reduced about 99% as compared to that of cells cultured with fresh purified modified rhBMP-5, but was about 99 times higher than the alkaline phosphatase activity of the untreated mature rhBMP-5 control after 1 day of incubation. The alkaline phosphatase activity of cells cultured with purified collagenase-modified rhBMP-5 on shelf (37 °C) for 3 days or 7 days was reduced 27% and 97% respectively as compared to that of cells cultured with fresh purified collagenase-modified rhBMP-5. However, the alkaline phosphatase activity of cells cultured with purified dispase-modified rhBMP-5 with the addition of collagenase on shelf (37 °C) for 1 day, 3 days, or 7 days was reduced 17%, 36%, and 61% respectively as compared to that of cells cultured with fresh purified dispase-modified rhBMP-5 with the addition of collagenase. This demonstrates that the presence of a protease can further enhance the stability of modified BMP-5 on shelf.

[00336] In addition, the alkaline phosphatase activity of cells cultured with purified dispase-modified rhBMP-5 with the addition of collagenase on shelf (37 °C) for 1 day, 3 days, or 7 days was 1460 times, 990 times, and 603 times higher than that of cells cultured with non-modified mature rhBMP-5 after 1day, 3 day, and 7 day shelf-life, respectively.

[00337] Example 44 – Stability of Modified rhBMPs- 4 and 7 with or without a Protease

[00338] Purified and non-purified modified rhBMP-4 and 7 are prepared as described in Examples above. Aliquots of each of the purified and non-purified modified rhBMP-4 or 7 at the same volume and concentration are incubated at 37°C for different durations (2 days to 2 weeks). After each time point, one aliquot from each group is transferred into -20°C freezer until the last time point is completed. The aliquots of each of the purified modified rhBMP-4 or 7, or non-purified modified rhBMP-4 or 7 from different time points are introduced into each well of the C2C12 cell cultured as described above. C2C12 cells cultured without the addition of rhBMP-4 or 7 or collagenase are used as a negative control. After 3 days incubation at 37 °C, 5% CO_2, the AP assay as described above is performed.

[00339] The alkaline phosphatase activity of cells cultured with purified modified rhBMP-4 or 7 on shelf (37 °C) for 7 days or 14 days is reduced as compared to that of cells cultured with purified modified rhBMP-4 or 7 on shelf (37 °C) for 2 days. The alkaline phosphatase activity of cells cultured with non-purified modified rhBMP-4 or 7 on shelf (37 °C) for 7 days or 14 days is reduced less than that of cells cultured with purified modified rhBMP-4 or 7, respectively. This demonstrates that the presence of a protease can further enhance the stability of modified BMP-4 and 7 on shelf.

[00340] Example 45 – Stability of Modified rhBMP13 with or without a Protease on shelf

[00341] The same amount of rhBMP13 without treatment and rhBMP13 with dispase or collagenase treatment but without purification were prepared and incubated at 37°C for different durations (1 day

to 10days). After each time point, one aliquot from each group was transferred into -20°C freezer until the last time point was completed. All aliquots were thawed at the same time and were introduced into each well of the C2C12 cell cultured as described above. C2C12 cells cultured without the addition of rhBMP-13 or modified rhBMP-13 were used as a negative control. After 3 days incubation at 37 °C, 5% CO_2, the AP assay as described above was performed.

[00342] The alkaline phosphatase activity in cells cultured with non-purified collagenase modified rhBMP-13 stored at 37 °C for 3 days or 10 days reduced 32% and 52%, respectively, as compared to that in cells cultured with fresh non-purified collagenase modified rhBMP-13 without storage; however, they were still 291% and 174% higher than that of cells cultured with non-modified rhBMP-13 stored at 37 °C for 3 days or 10 days. The alkaline phosphatase activity in cells cultured with non-purified dispase modified rhBMP-13 (stored at 37°C) for 3 days or 10 days were 79% and 27% higher than that of cells cultured with non-purified rhBMP-13 (stored at 37°C) for 3 days or 10 days.

[00343] Example 46 – Stability of Modified rhBMP14 with or without a Protease on shelf

[00344] The same amount of rhBMP14 without treatment and rhBMP14 with collagenase treatment but without purification were prepared and incubated at 37°C for different durations (1 day to 10days). After each time point, one aliquot from each group was transferred into -20°C freezer until the last time point was completed. The aliquots of non-modified rhBMP-14 or non-purified collagenase modified rhBMP-14 from different time points were introduced into each well of the C2C12 cell cultured as described above. C2C12 cells cultured without the addition of rhBMP-14 or modified rhBMP-14 were used as a negative control. After 3 days incubation at 37 °C, 5% CO_2, the AP assay as described above was performed.

[00345] The alkaline phosphatase activity of cells cultured with non-purified collagenase modified rhBMP-14 on shelf (37 °C) for 3 days or 7 days only reduced 11% and 12.7% respectively as compared to that of cells cultured with fresh non-purified collagenase modified rhBMP-14, while the alkaline phosphatase activity of cells cultured with non- modified rhBMP-14 on shelf (37 °C) for 3 days or 7 days reduced 25% and 39% respectively as compared to that of cells cultured with fresh non-modified rhBMP-14.

What is Claimed is:

1. A method of promoting differentiation of cells into osteoblasts, chondrocytes, ligament or tendon, the method comprising treating the cells with a peptide selected from the group consisting of

   a) a peptide of 137 residues or less, said peptide comprising an amino acid sequence at least 92% identical to the amino acid sequence of SEQ ID NO:20, 21, 22, 23, 24, 25, 26, 27, or 28

   b) a peptide of 119 residues or less, said peptide comprising an amino acid sequence at least 92% identical to the amino acid sequence of SEQ ID NO:34 or 35,

   c) a peptide of 137 residues or less, said peptide comprising the amino acid sequence of SEQ ID NO:20, 21, 22, 23, 24, 25, 26, 27, or 28,

   d) a peptide of 119 residues or less, said peptide comprising the amino acid sequence of SEQ ID NO:34 or 35, and

   e) a peptide with an amino acid sequence consisting of the amino acid sequence of SEQ ID NO:20, 21, 22, 23, 24, 25, 26, 27, 28, 34 or 35,

   wherein each of peptides (a)-(e) has osteoinductive, chondroinductive, or ligament/tendon differentiating activity.

2. The method of claim 1, wherein the cells are progenitor cells or adult stem cells.

3. The method of claim 2, wherein the progenitor cells or the adult stem cells are derived from placenta, bone marrow, adipose tissue, blood vessel, amniotic fluid, synovial fluid, synovial membrane, pericardium, periosteum, dura, peripheral blood, umbilical blood, menstrual blood, teeth, nucleus pulposus, brain, skin, hair follicle, intestinal crypt, neural tissue, or muscle.

4. The method of claim 1, wherein the cells are pluripotent stem cells.

5. A method of promoting osteogenesis, chondrogenic, or ligament/tendon differentiation of cells in a tissue, the method comprising treating the tissue with a peptide selected from the group consisting of

   a) a peptide of 137 residues or less, said peptide comprising an amino acid sequence at least 92% identical to the amino acid sequence of SEQ ID NO:20, 21, 22, 23, 24, 25, 26, 27 or 28,

b) a peptide of 119 residues or less, said peptide comprising an amino acid sequence at least 92% identical to the amino acid sequence of SEQ ID NO:34 or 35,

c) a peptide of 137 residues or less, said peptide comprising the amino acid sequence of SEQ ID NO:20, 21, 22, 23, 24, 25, 26, 27 or 28,

d) a peptide of 119 residues or less, said peptide comprising the amino acid sequence of SEQ ID NO:34 or 35, and

e) a peptide with an amino acid sequence consisting of the amino acid sequence of SEQ ID NO:20, 21, 22, 23, 24, 25, 26, 27, 28, 34 or 35,

wherein each of peptides (a)-(e) has osteoinductive, chondroinductive, or ligament/tendon differentiating activity.

6. The method of claim 5, wherein the osteogenic activity of cells in the treated tissue is greater than the osteogenic activity of cells in untreated tissue.

7. The method of claim 5, wherein the tissue is bone, cartilage, or connective tissue.

8. An isolated peptide of 137 residues or less, said peptide comprising an amino acid sequence at least 92% identical to the amino acid sequence of SEQ ID NO:20, 21, 22, 23, 24, 25, 26, 27 or 28, wherein the peptide has osteoinductive, chondroinductive, or ligament/tendon differentiating activity.

9. The isolated peptide of claims 8, wherein the amino acid sequence comprises the amino acid sequence of SEQ ID NO:20, 21, 22, 23, 24, 25, 26, 27 or 28.

10. The isolated peptide of claim 9, wherein the peptide consists of the amino acid sequence of SEQ ID NO:20, 21, 22, 23, 24, 25, 26, 27 or 28.

11. A fusion protein comprising (a) the peptide of any of claims 8-10, and (b) a second peptide, wherein the second peptide comprises an amino acid sequence that is less than 70% identical to the amino acid sequence of SEQ ID NO:40, 41, 42, 43, 44, 45, 46, 47 or 48.

12. A composition comprising the peptide of claim 8 and at least one protease.

13. The composition according to claim 12, wherein the at least one protease is selected from the group consisting of collagenase, clostripain, dispase, trypsin, cathepsin, BMP-1 (bone morphogenetic protein-1), MMP-13 (matrix metalloproteinase-13), and a mixture thereof.

14. A method of making the peptide of any one of claims 8-10 comprising contacting mature BMP-5 with a protease under conditions that promote protein cleavage and harvesting the peptide.

15. The method of claim 14, wherein the at least one protease is selected from the group consisting of collagenase, clostripain, dispase, trypsin, cathepsin, BMP-1 (bone morphogenetic protein-1), MMP-13 (matrix metalloproteinase-13), and a mixture thereof.

16. A vector encoding the peptide of any one of claims 8-10.

17. A host cell comprising the vector of claim 16.

18. A method of making the peptide of any one of claims 8-10, comprising culturing a host cell under conditions suitable for protein expression and harvesting the protein, wherein the host cell comprises a vector that encodes said peptide.

19. A pharmaceutical composition comprising the peptide of any one of claims 8-10.

20. A method of increasing a growth factor activity, the method comprising treating a cell with at least one protease and the growth factor, wherein the growth factor is a peptide comprising an amino acid sequence of SEQ ID NO: 20, 21, 22, 23, 24, 25, 26, 27 or 28, wherein the peptide has osteoinductive, chondroinductive, or ligament/tendon differentiating activity.

21. The method according to claim 20, wherein the peptide is selected from the group consisting of

a) a peptide of 137 residues or less, said peptide comprising an amino acid sequence at least 92% identical to the amino acid sequence of SEQ ID NO:20, 21, 22, 23, 24, 25, 26, 27 or 28,

b) a peptide of 137 residues or less, said peptide comprising the amino acid sequence of SEQ ID NO:20, 21, 22, 23, 24, 25, 26, 27 or 28, and

c) a peptide with an amino acid sequence consisting of the amino acid sequence of SEQ ID NO:20, 21, 22, 23, 24, 25, 26, 27 or 28.

22. The method according to claim 20, wherein the peptide is a fusion protein comprising (a) the peptide comprising the amino acid sequence of SEQ ID NO: 20, 21, 22, 23, 24, 25, 26, 27 or 28, and (b) a second peptide, wherein the second peptide comprises an amino acid sequence that is less than 70% identical to the amino acid sequence of SEQ ID NO: 45.

23. The method according to claim 20, wherein the composition comprises two or more proteases.

24. The method according to claim 20, wherein the at least one protease is selected from the group consisting of collagenase, clostripain, dispase, trypsin, cathepsin, BMP-1 (bone morphogenetic protein-1), MMP-13 (matrix metalloproteinase-13), and a mixture thereof.

25. The method according to claim 24, wherein the at least one protease is collagenase, dispase or cathepsin.

26. The method according to claim 20, wherein the cells are progenitor cells or adult stem cells.

27. The method according to claim 26, wherein the progenitor cells or the adult stem cells are derived from placenta, bone marrow, adipose tissue, blood vessel, amniotic fluid, synovial fluid, synovial membrane, pericardium, periosteum, dura, peripheral blood, umbilical blood, menstrual blood, teeth, nucleus pulposus, brain, skin, hair follicle, intestinal crypt, neural tissue, or muscle.

28. The method according to claim 26, wherein the progenitor cells or the adult stem cells are derived from pluripotent stem cells.

29. The method according to claim 20, wherein the cells are pluripotent stem cells.

30. An isolated peptide of 119 residues or less, said peptide comprising an amino acid sequence at least 92% identical to the amino acid sequence of SEQ ID NO:34, wherein the peptide has osteoinductive, chondroinductive, or ligament/tendon differentiating activity.

31. The isolated peptide of claims 30, wherein the amino acid sequence comprises the amino acid sequence of SEQ ID NO:34.

32. The isolated peptide of claim 31, wherein the peptide consists of the amino acid sequence of SEQ ID NO:34.

33. A fusion protein comprising (a) the peptide of any of claims 30-32, and (b) a second peptide, wherein the second peptide comprises an amino acid sequence that is less than 70% identical to the amino acid sequence of SEQ ID NO:54.

34. A composition comprising the peptide of claim 30 and at least one protease.

35. The composition according to claim 34, wherein the at least one protease is selected from the group consisting of collagenase, clostripain, dispase, trypsin, cathepsin, BMP-1 (bone morphogenetic protein-1), MMP-13 (matrix metalloproteinase-13), and a mixture thereof.

36. A method of making the peptide of any one of claims 30-32 comprising contacting mature BMP-13 with a protease under conditions that promote protein cleavage and harvesting the peptide.

37. The method of claim 36, wherein the at least one protease is selected from the group consisting of collagenase, clostripain, dispase, trypsin, cathepsin, BMP-1 (bone morphogenetic protein-1), MMP-13 (matrix metalloproteinase-13), and a mixture thereof.

38. A vector encoding the peptide of any one of claims 30-32.

39. A host cell comprising the vector of claim 38.

40. A method of making the peptide of any one of claims 30-32, comprising culturing a host cell under conditions suitable for protein expression and harvesting the protein, wherein the host cell comprises a vector that encodes said peptide.

41. A pharmaceutical composition comprising the peptide of any one of claims 30-32.

42. A method of increasing a growth factor activity, the method comprising treating a cell with at least one protease and the growth factor, wherein the growth factor is a peptide comprising an amino acid sequence of SEQ ID NO: 34, wherein the peptide has osteoinductive, chondroinductive, or ligament/tendon differentiating activity.

43. The method according to claim 42, wherein the peptide is selected from the group consisting of

a) a peptide of 119 residues or less, said peptide comprising an amino acid sequence at least 92% identical to the amino acid sequence of SEQ ID NO: 34,

b) a peptide of 119 residues or less, said peptide comprising an amino acid sequence of SEQ ID NO: 34, and

c) a peptide with an amino acid sequence consisting of the amino acid sequence of SEQ ID NO: 34.

44. The method according to claim 42, wherein the peptide is a fusion protein comprising (a) the peptide comprising the amino acid sequence of SEQ ID NO: 33, and (b) a second peptide, wherein the second peptide comprises an amino acid sequence that is less than 70% identical to the amino acid sequence of SEQ ID NO: 54.

45. The method according to claim 42, wherein the composition comprises two or more proteases.

46. The method according to claim 42, wherein the at least one protease is selected from the group consisting of collagenase, clostripain, dispase, trypsin, cathepsin, BMP-1 (bone morphogenetic protein-1), MMP-13 (matrix metalloproteinase-13), and a mixture thereof.

47. The method according to claim 46, wherein the at least one protease is collagenase, dispase or cathepsin.

48. The method according to claim 42, wherein the cells are progenitor cells or adult stem cells.

49. The method according to claim 48, wherein the progenitor cells or the adult stem cells are derived from placenta, bone marrow, adipose tissue, blood vessel, amniotic fluid, synovial fluid, synovial membrane, pericardium, periosteum, dura, peripheral blood, umbilical blood, menstrual blood, baby teeth, nucleus pulposus, brain, skin, hair follicle, intestinal crypt, neural tissue, or muscle

50. The method according to claim 48, wherein the progenitor cells or the adult stem cells are derived from pluripotent stem cells.

51. The method according to claim 42, wherein the cells are pluripotent stem cells.

52. An isolated peptide of 119 residues or less, said peptide comprising an amino acid sequence at least 92% identical to the amino acid sequence of SEQ ID NO:35, wherein the peptide has osteoinductive, chondroinductive, or ligament/tendon differentiating activity.

53. The isolated peptide of claims 52, wherein the amino acid sequence comprises the amino acid sequence of SEQ ID NO:35.

54. The isolated peptide of claim 53, wherein the peptide consists of the amino acid sequence of SEQ ID NO:35.

55. A fusion protein comprising (a) the peptide of any of claims 52-54, and (b) a second peptide, wherein the second peptide comprises an amino acid sequence that is less than 70% identical to the amino acid sequence of SEQ ID NO:55.

56. A composition comprising the peptide of claim 52 and at least one protease.

57. The composition according to claim 56, wherein the at least one protease is selected from the group consisting of collagenase, clostripain, dispase, trypsin, cathepsin, BMP-1 (bone morphogenetic protein-1), MMP-13 (matrix metalloproteinase-13), and a mixture thereof.

58. A method of making the peptide of any one of claims 52-54 comprising contacting mature BMP-14 with a protease under conditions that promote protein cleavage and harvesting the peptide.

59. The method of claim 58, wherein the at least one protease is selected from the group consisting of collagenase, clostripain, dispase, trypsin, cathepsin, BMP-1 (bone morphogenetic protein-1), MMP-13 (matrix metalloproteinase-13), and a mixture thereof.

60. A vector encoding the peptide of any one of claims 52-54.

61. A host cell comprising the vector of claim 60.

62. A method of making the peptide of any one of claims 52-54, comprising culturing a host cell under conditions suitable for protein expression and harvesting the protein, wherein the host cell comprises a vector that encodes said peptide.

63. A pharmaceutical composition comprising the peptide of any one of claims 52-54.

64. A method of increasing a growth factor activity, the method comprising treating a cell with at least one protease and the growth factor, wherein the growth factor is a peptide comprising an amino acid sequence of SEQ ID NO: 35, wherein the peptide has osteoinductive, chondroinductive, or ligament/tendon differentiating activity.

65. The method according to claim 64, wherein the peptide is selected from the group consisting of

d) a peptide of 119 residues or less, said peptide comprising an amino acid sequence at least 92% identical to the amino acid sequence of SEQ ID NO: 35,

e) a peptide of 119 residues or less, said peptide comprising an amino acid sequence of SEQ ID NO: 35, and

f) a peptide with an amino acid sequence consisting of the amino acid sequence of SEQ ID NO: 35.

66. The method according to claim 64, wherein the peptide is a fusion protein comprising (a) the peptide comprising the amino acid sequence of SEQ ID NO: 35, and (b) a second peptide,

wherein the second peptide comprises an amino acid sequence that is less than 70% identical to the amino acid sequence of SEQ ID NO: 55.

67. The method according to claim 64, wherein the composition comprises two or more proteases.

68. The method according to claim 64, wherein the at least one protease is selected from the group consisting of collagenase, clostripain, dispase, trypsin, cathepsin, BMP-1 (bone morphogenetic protein-1), MMP-13 (matrix metalloproteinase-13), and a mixture thereof.

69. The method according to claim 68, wherein the at least one protease is collagenase, dispase or cathepsin.

70. The method according to claim 64, wherein the cells are progenitor cells or adult stem cells.

71. The method according to claim 70, wherein the progenitor cells or the adult stem cells are derived from placenta, bone marrow, adipose tissue, blood vessel, amniotic fluid, synovial fluid, synovial membrane, pericardium, periosteum, dura, peripheral blood, umbilical blood, menstrual blood, baby teeth, nucleus pulposus, brain, skin, hair follicle, intestinal crypt, neural tissue, or muscle

72. The method according to claim 70, wherein the progenitor cells or the adult stem cells are derived from pluripotent stem cells.

73. The method according to claim 64, wherein the cells are pluripotent stem cells.

74. A composition comprising at least one protease and a growth factor, wherein the growth factor is (i) a bone morphogenic protein (BMP) or (ii) an isolated peptide comprising an amino acid sequence at least 92% identical to any of amino acid sequences of SEQ ID NO: 16-35, wherein the peptide has osteoinductive, chondroinductive, or ligament/tendon differentiating activity.

75. The composition according to claim 74, wherein the growth factor is BMP-2, BMP-3, BMP-4, BMP-5, BMP-6, BMP-7, BMP-8, BMP-9, BMP-10, BMP-11, BMP-12, BMP-13, BMP-14, BMP-15, or BMP-16.

76. The composition according to claim 74, wherein the growth factor comprises an amino acid sequence selected from the group consisting of residue 283-396 of SEQ ID NO: 1, residue 363-472 of SEQ ID NO: 2, residue 293-408 of SEQ ID NO: 3, residue 317-454 of SEQ ID NO: 4, residue 375-513 of SEQ ID NO: 5, residue 293-431 of SEQ ID NO: 6, residue 243-

402 of SEQ ID NO: 7, residue 319-429 of SEQ ID NO: 8, residue 316-424 of SEQ ID NO: 9, residue 298-407 of SEQ ID NO: 10, residue 316-461 of SEQ ID NO: 11, residue 336-455 of SEQ ID NO: 12, residue 382-501 of SEQ ID NO: 13, residue 267-392 of SEQ ID NO: 14, and residue 171-280 of SEQ ID NO: 15.

77. The composition according to claim 74, wherein the growth factor comprises an amino acid sequence at least 92% identical to any of amino acid sequences of SEQ ID NO: 16-35.

78. The composition according to claim 76, wherein the peptide is selected from the group consisting of

a) a first peptide of an isolated peptide of 113 residues or less comprising an amino acid sequence at least 92% identical to the amino acid sequence of SEQ ID NO: 16, 17, or 18;

b) a second peptide of an isolated peptides of 115 residues or less comprising an amino acid sequence at least 95% identical to the amino acid sequence of SEQ ID NO: 19;

c) a third peptide of an isolated peptides of 137 residues or less comprising an amino acid sequence at least 95% identical to the amino acid sequence of SEQ ID NO: 20, 21, 22, 23, 24, 25, 26, 27 or 28;

d) a fourth peptide of an isolated peptides of 138 residues or less comprising an amino acid sequence at least 95% identical to the amino acid sequence of SEQ ID NO: 29;

e) a fifth peptide of an isolated peptides of 138 residues or less comprising an amino acid sequence at least 92% identical to the amino acid sequence of SEQ ID NO: 30, 31, 32 or 33;

f) a sixth peptide of an isolated peptides of 119 residues or less comprising an amino acid sequence at least 92% identical to the amino acid sequence of SEQ ID NO: 34; and

g) a seventh peptide of an isolated peptides of 119 residues or less comprising an amino acid sequence at least 92% identical to the amino acid sequence of SEQ ID NO: 35.

79. The composition according to claim 76, wherein the peptide is selected from the group consisting of

a) a first peptide of an isolated peptide of 113 residues or less comprising the amino acid sequence of SEQ ID NO: 16, 17, or 18;

b) a second peptide of an isolated peptides of 115 residues or less comprising the amino acid sequence of SEQ ID NO: 19;

c) a third peptide of an isolated peptides of 137 residues or less comprising the amino acid sequence of SEQ ID NO: 20, 21, 22, 23, 24, 25, 26, 27 or 28;

d) a fourth peptide of an isolated peptides of 138 residues or less comprising the amino acid sequence of SEQ ID NO: 29;

e) a fifth peptide of an isolated peptides of 138 residues or less comprising the amino acid sequence of SEQ ID NO: 30, 31, 32 or 33;

f) a sixth peptide of an isolated peptides of 119 residues or less comprising the amino acid sequence of SEQ ID NO: 34; and

g) a seventh peptide of an isolated peptides of 119 residues or less comprising the amino acid sequence of SEQ ID NO: 35.

80. The composition according to claim 74, wherein the peptide is selected from the group consisting any of amino acid sequences of SEQ ID NO: 16-35.

81. The composition according to claim 74, wherein the composition comprises two or more proteases.

82. The composition according to claim 74, wherein the at least one protease is selected from the group consisting of collagenase, clostripain, dispase, trypsin, cathepsin, BMP-1 (bone morphogenetic protein-1), MMP-13 (matrix metalloproteinase-13), and a mixture thereof.

83. The composition according to claim 74, wherein the at least one protease is collagenase, dispase or cathepsin.

84. A method of increasing a growth factor activity, the method comprising treating a cell with the composition of claims 80-83.

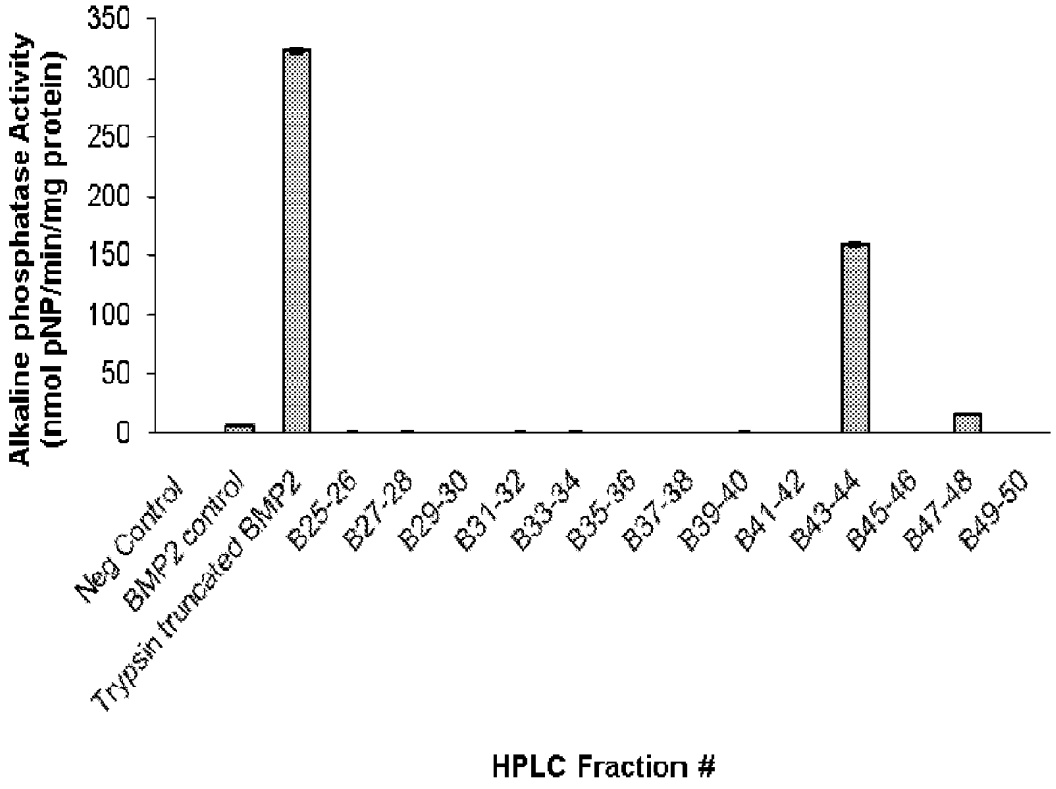
85. The method according to claim 84, wherein the cells are progenitor cells or adult stem cells.

86. The method according to claim 85, wherein the progenitor cells or the adult stem cells are derived from placenta, bone marrow, adipose tissue, blood vessel, amniotic fluid, synovial fluid, synovial membrane, pericardium, periosteum, dura, peripheral blood, umbilical blood, menstrual blood, teeth, nucleus pulposus, brain, skin, hair follicle, intestinal crypt, neural tissue, or muscle.

87. The method according to claim 85, wherein the progenitor cells or the adult stem cells are derived from pluripotent stem cells.

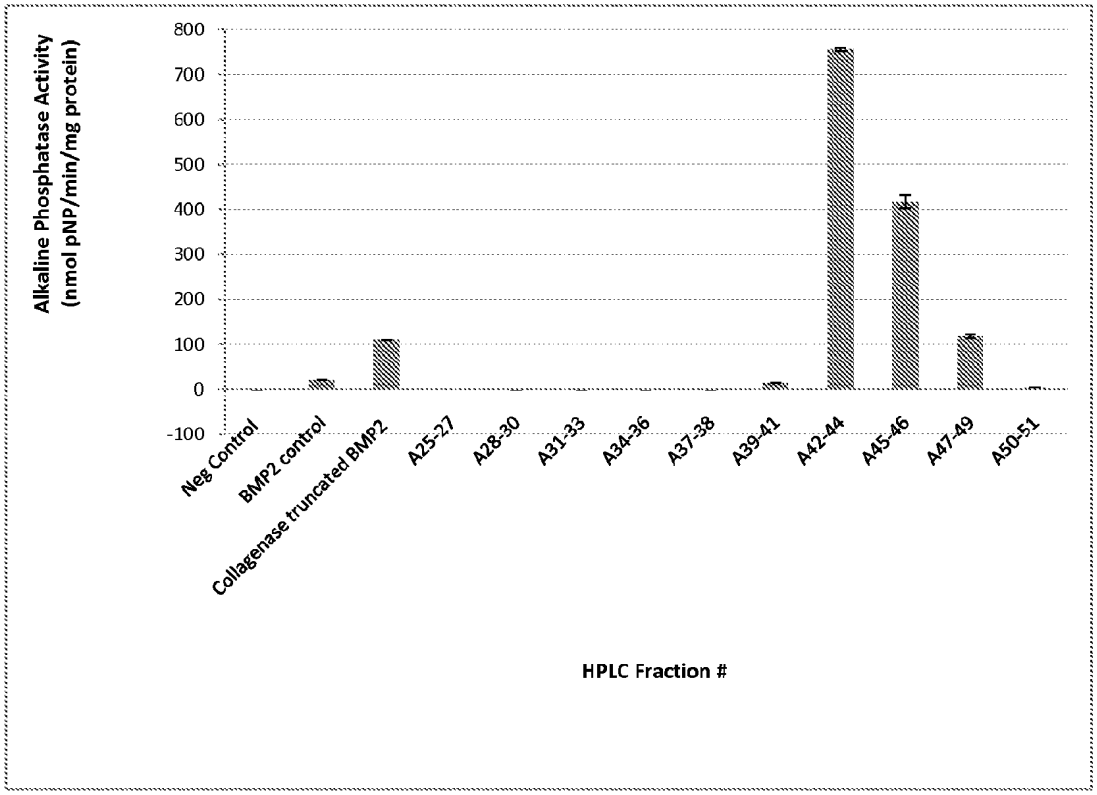
88. The method according to claim 84, wherein the cells are pluripotent stem cells.

Figure 1:


Alkaline phosphatase Activity (nmol pNP/min/mg protein)
350
300
250
200
150
100
50
0
Neg Control
BMP2 control
Trypsin truncated BMP2
B25-26
B27-28
B29-30
B31-32
B33-34
B35-36
B37-38
B39-40
B41-42
B43-44
B45-46
B47-48
B49-50
HPLC Fraction #

EMBZIM EX1002

Figure 2:



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Figure 3:

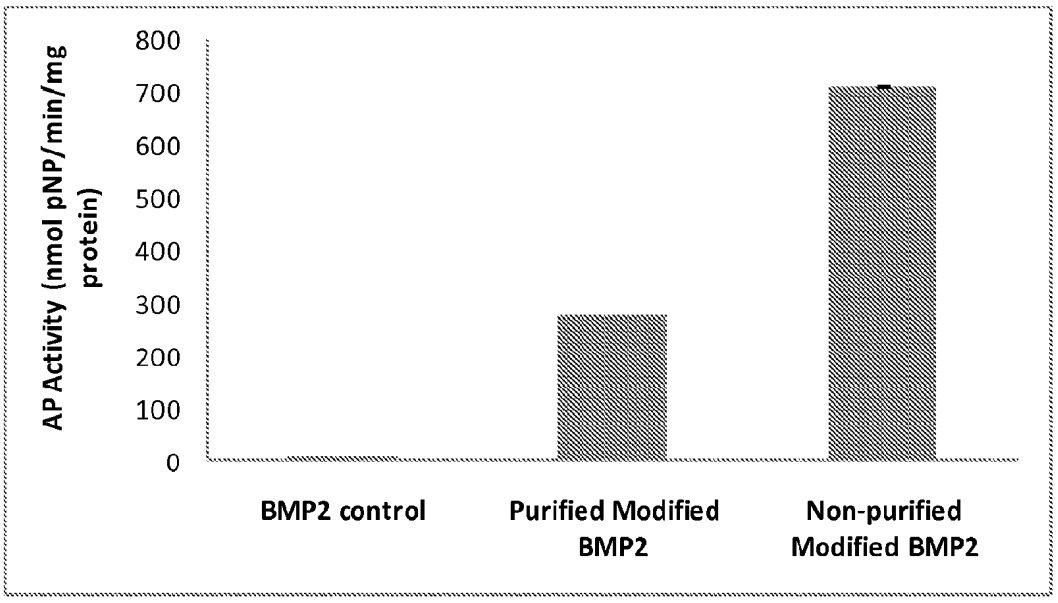

AP Activity (nmol pNP/min/mg protein)
800
700
600
500
400
300
200
100
0
BMP2 control
Purified Modified BMP2
Non-purified Modified BMP2

Figure 4:

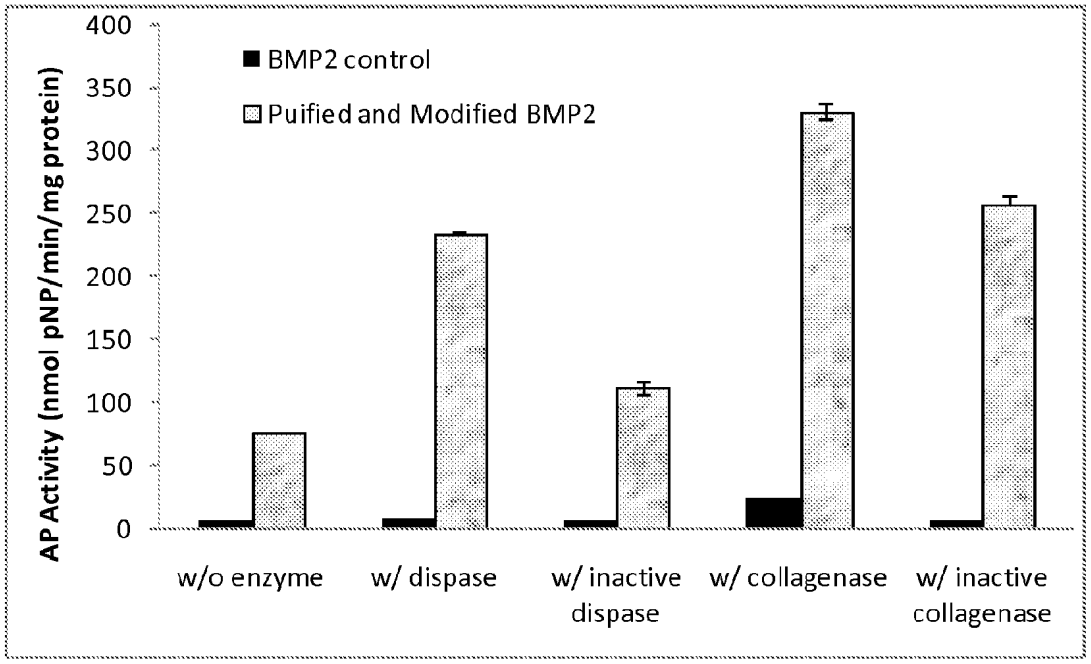

AP Activity (nmol pNP/min/mg protein)
400
350
300
250
200
150
100
50
0
BMP2 control
Puified and Modified BMP2
w/o enzyme
w/ dispase
w/ inactive dispase
w/ collagenase
w/ inactive collagenase

Figure 5:

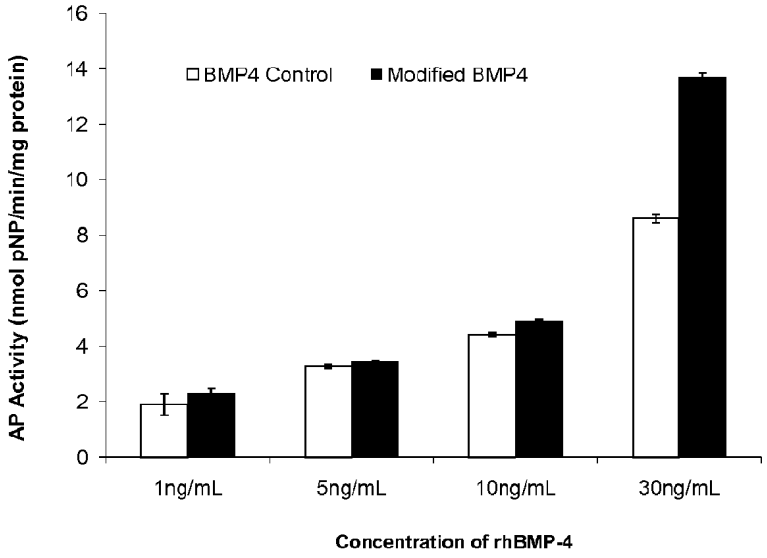

16
14
12
10
8
6
4
2
0
AP Activity (nmol pNP/min/mg protein)
BMP4 Control
Modified BMP4
1ng/mL
5ng/mL
10ng/mL
30ng/mL
Concentration of rhBMP-4

Figure 6:

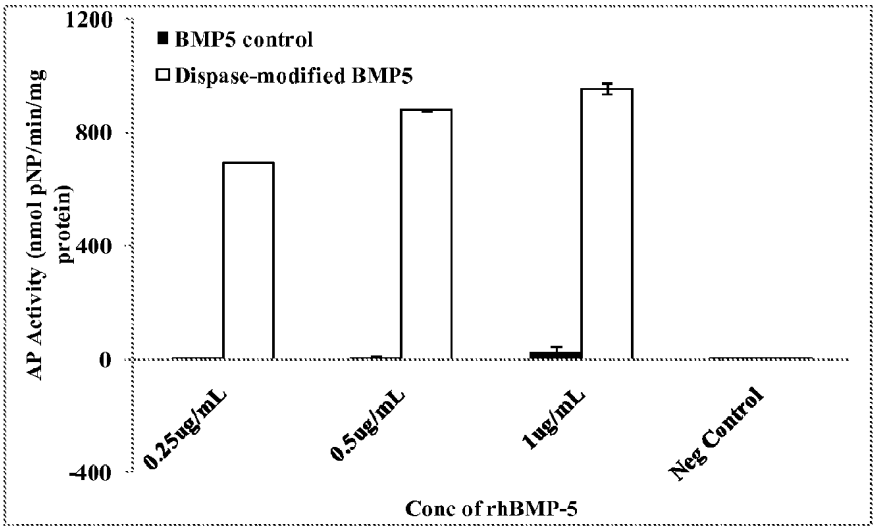

1200
800
400
0
-400
AP Activity (nmol pNP/min/mg protein)
BMP5 control
Dispase-modified BMP5
0.25ug/mL
0.5ug/mL
1ug/mL
Neg Control
Conc of rhBMP-5

Figure 7:

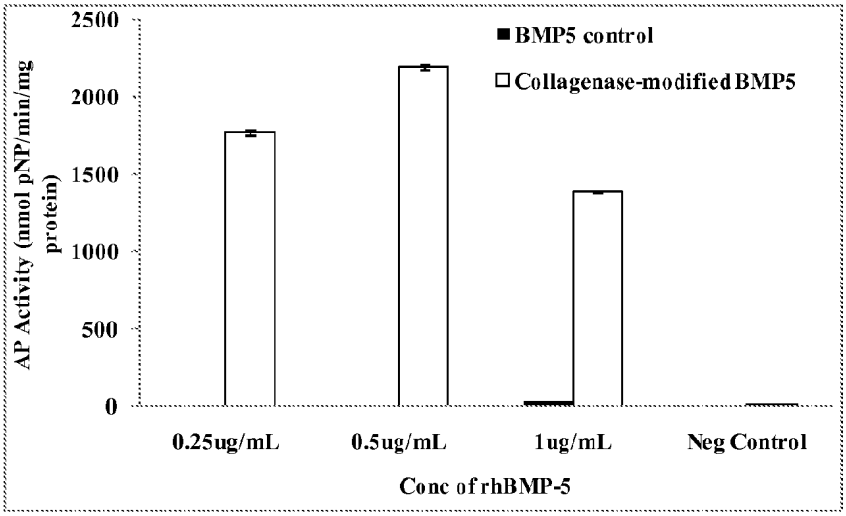

2500
2000
1500
1000
500
0
AP Activity (nmol pNP/min/mg protein)
BMP5 control
Collagenase-modified BMP5
0.25ug/mL
0.5ug/mL
1ug/mL
Neg Control
Conc of rhBMP-5

Figure 8:

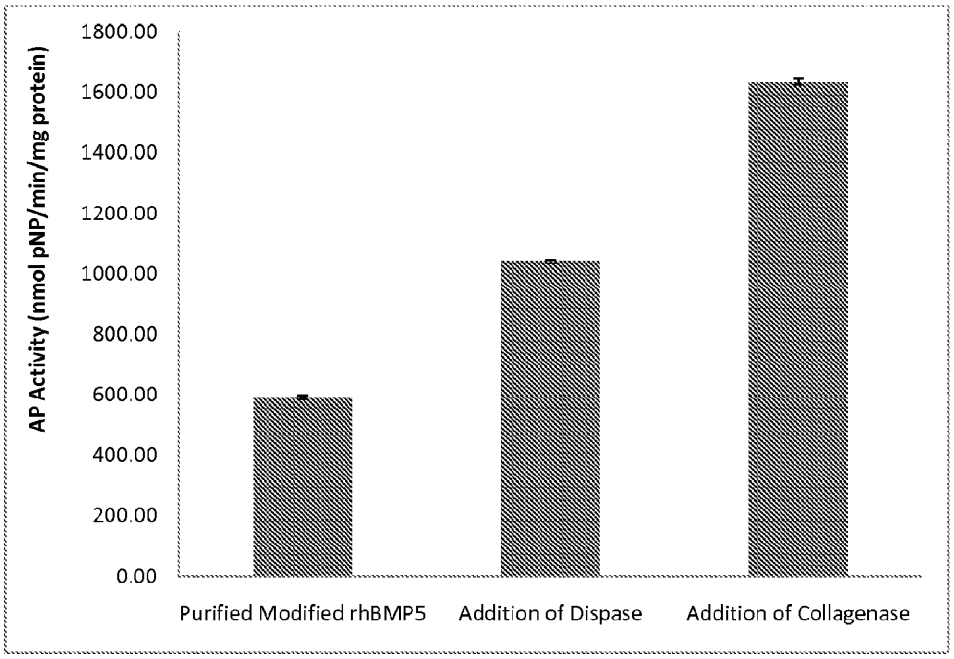

AP Activity (nmol pNP/min/mg protein)
1800.00
1600.00
1400.00
1200.00
1000.00
800.00
600.00
400.00
200.00
0.00
Purified Modified rhBMP5
Addition of Dispase
Addition of Collagenase

Figure 9:

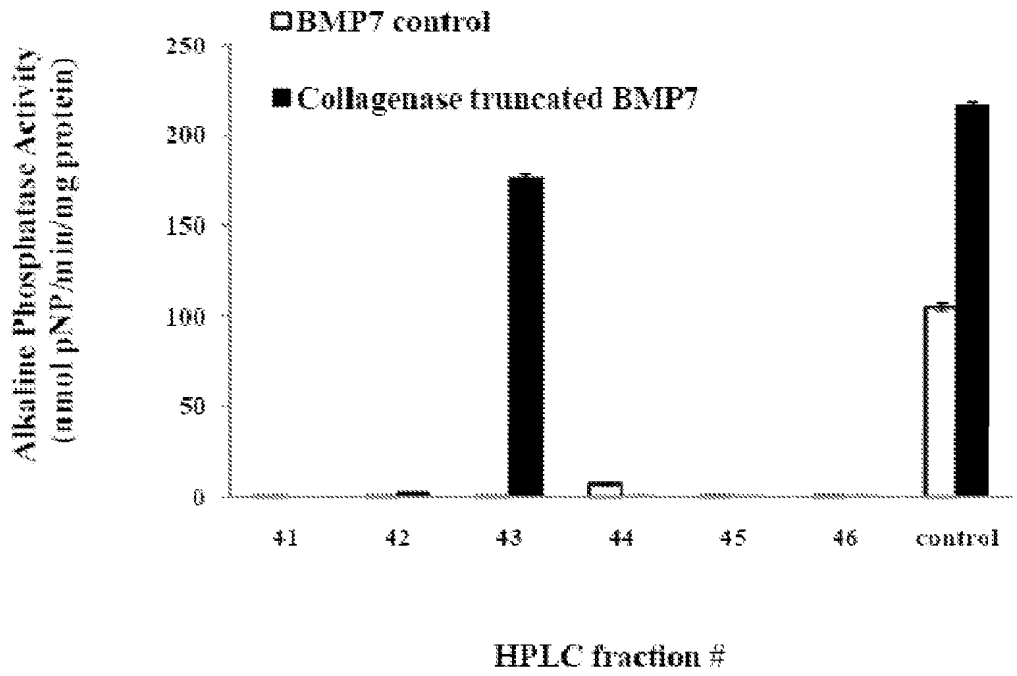

BMP7 control
Collagenase truncated BMP7
Alkaline Phosphatase Activity (nmol pNP/min/mg protein)
250
200
150
100
50
0
41
42
43
44
45
46
control
HPLC fraction #

Figure 10:

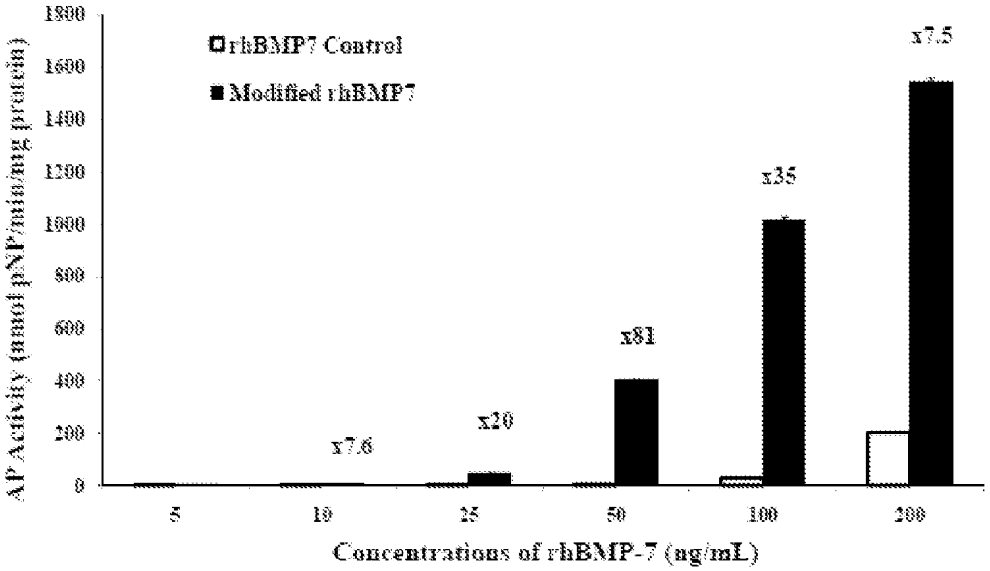

rhBMP7 Control
Modified rhBMP7
AP Activity (nmol pNP/min/mg protein)
1800
1600
1400
1200
1000
800
600
400
200
0
x7.6
x20
x81
x35
x7.5
5
10
25
50
100
200
Concentrations of rhBMP-7 (ng/mL)

Figure 11:

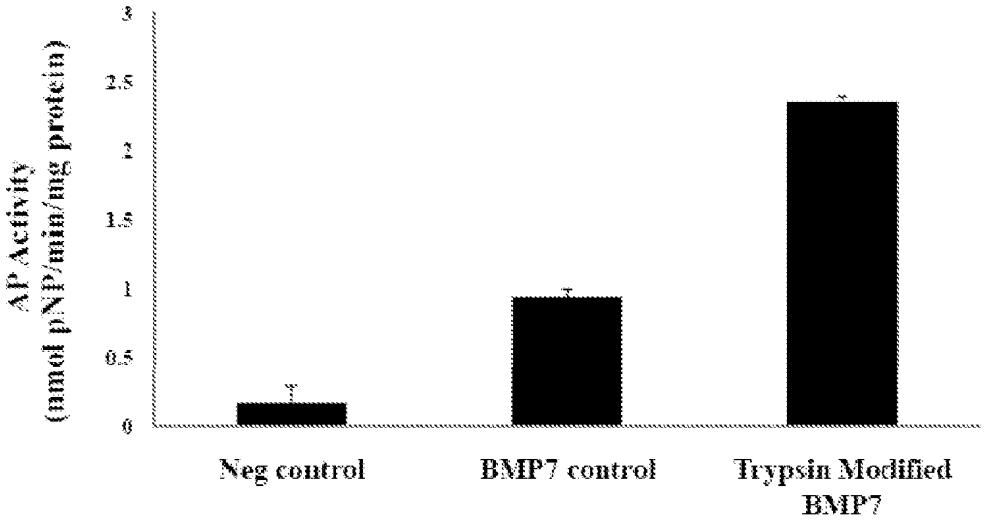

AP Activity
(nmol pNP/min/mg protein)
3
2.5
2
1.5
1
0.5
0
Neg control
BMP7 control
Trypsin Modified BMP7

Figure 12:

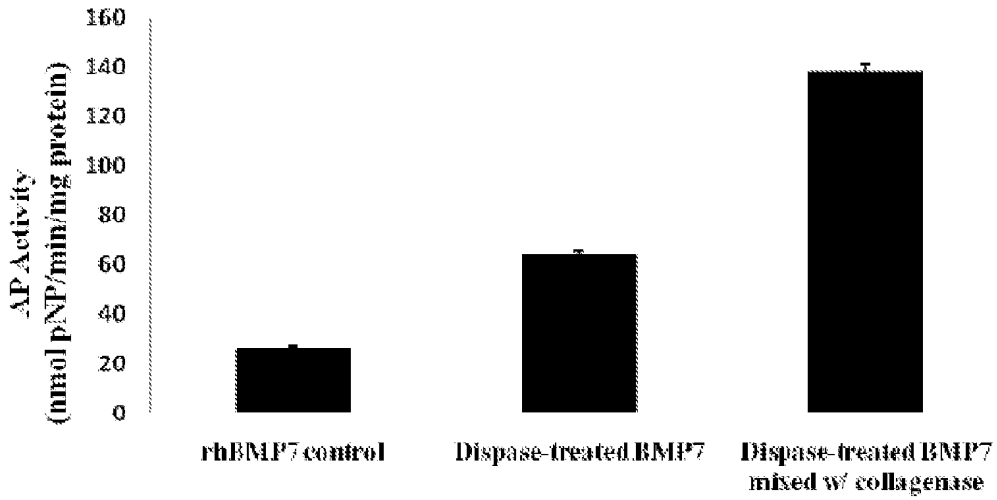

AP Activity
(nmol pNP/min/mg protein)
160
140
120
100
80
60
40
20
0
rhBMP7 control
Dispase-treated BMP7
Dispase-treated BMP7
mixed w/ collagenase

Figure 13:

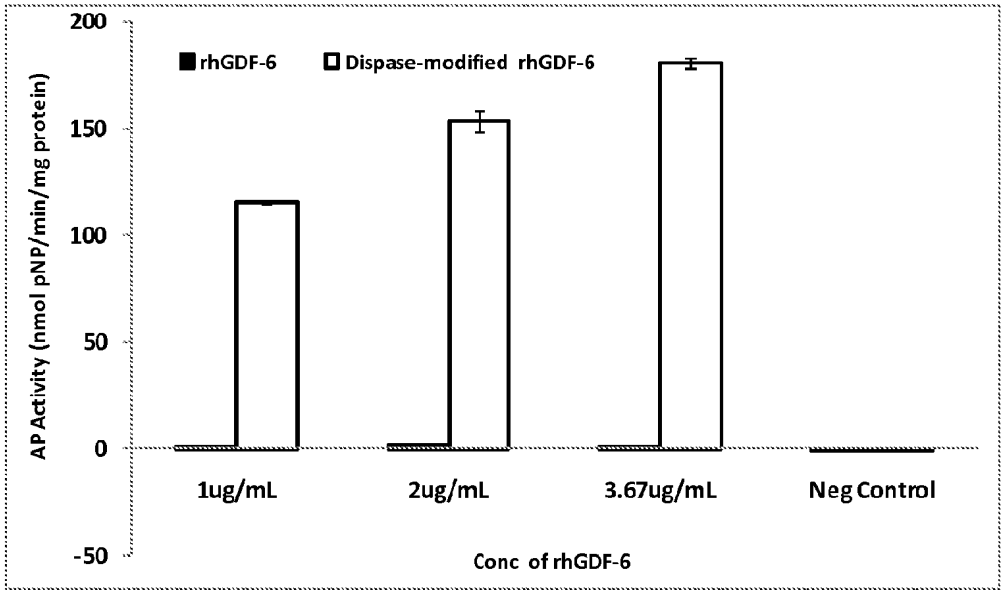

200
150
100
50
0
-50
AP Activity (nmol pNP/min/mg protein)
rhGDF-6
Dispase-modified rhGDF-6
1ug/mL
2ug/mL
3.67ug/mL
Neg Control
Conc of rhGDF-6

Figure 14:

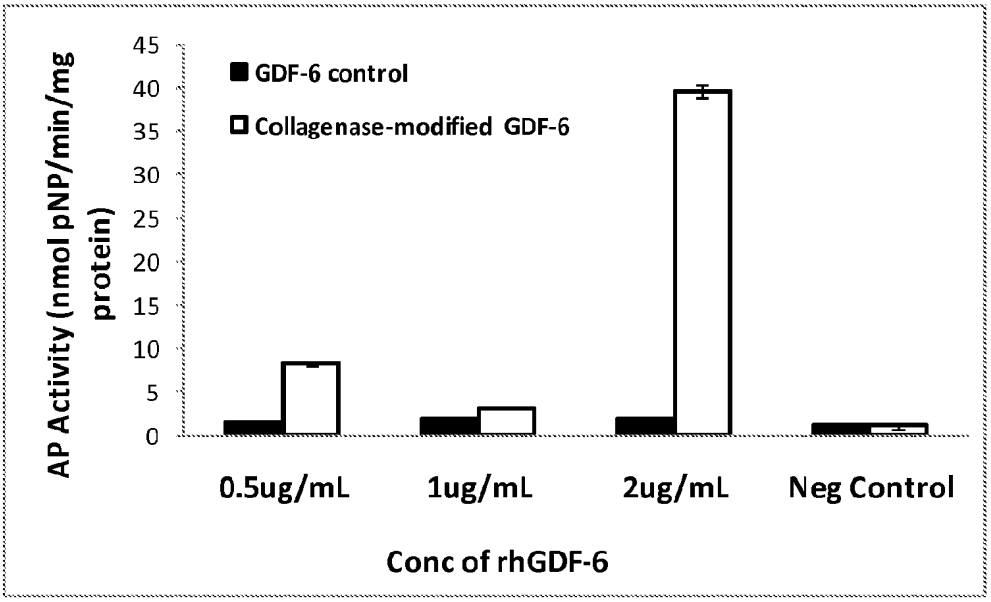

AP Activity (nmol pNP/min/mg protein)
45
40
35
30
25
20
15
10
5
0
GDF-6 control
Collagenase-modified GDF-6
0.5ug/mL
1ug/mL
2ug/mL
Neg Control
Conc of rhGDF-6

Figure 15:

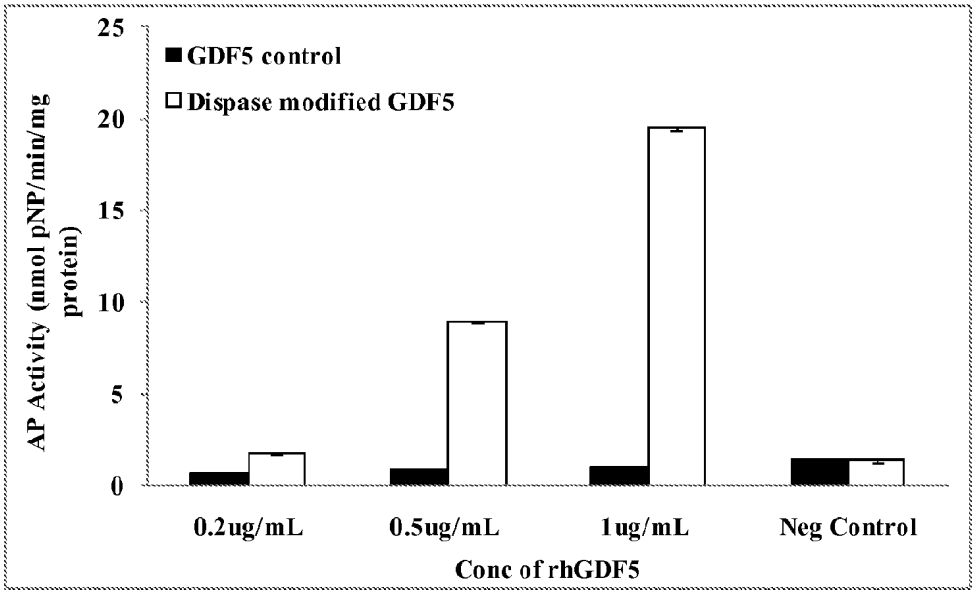

25
20
15
10
5
0
AP Activity (nmol pNP/min/mg protein)
GDF5 control
Dispase modified GDF5
0.2ug/mL
0.5ug/mL
1ug/mL
Neg Control
Conc of rhGDF5

Figure 16:

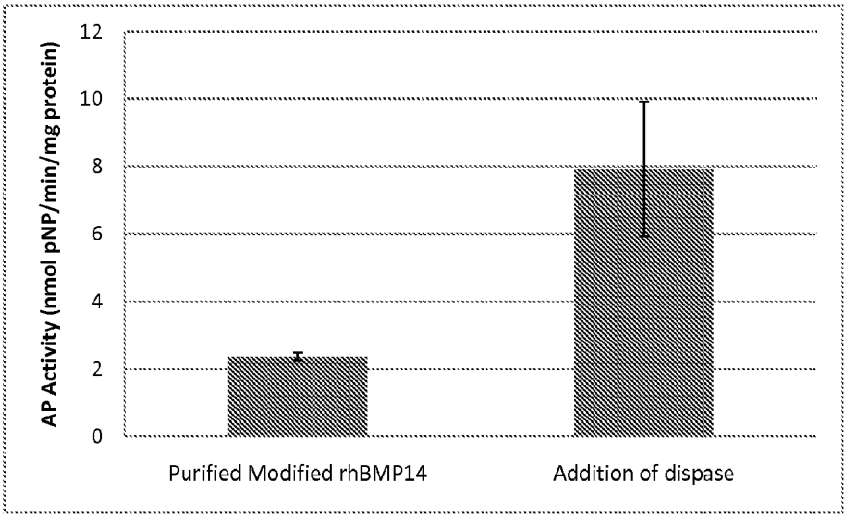
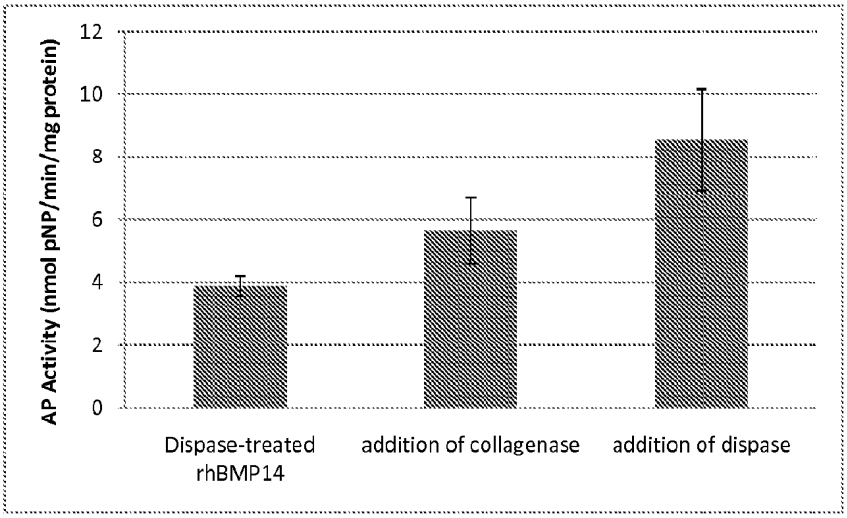

AP Activity (nmol pNP/min/mg protein)
12
10
8
6
4
2
0
Purified Modified rhBMP14
Addition of dispase

Figure 17:


AP Activity (nmol pNP/min/mg protein)
12
10
8
6
4
2
0
Dispase-treated rhBMP14
addition of collagenase
addition of dispase

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 12/53584

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12N 5/077, A61P 19/00 (2012.01)
USPC - 435/377, 435/405, 514/8.8
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)
USPC: 435/377, 435/405, 514/8.8

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC: 424/93.21; 435/325, 375, 377, 404, 405; 514/8.8, 16.7, 17.1
(keyword limited; terms below)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatBase; Google; PubMed
Search terms: BMP-5, osteoblast, chondrocyte, ligament, tendon, differentiation, pluripotent, stem cells, SEQ ID NO:20

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2009/0298761 A1 (ENGELMAN) 3 December 2009 (03.12.2009) para [0013], [0025], [0044], [0073], [0083], [0088], [0092], [0103]; SEQ ID NO:13	1-7
Y	US 2001/0018509 A1 (LEE et al.) 30 August 2001 (30.08.2001) para [0004], [0094]; SEQ ID NO:13	1-7
Y	US 2005/0136042 A1 (BETZ et al.) 23 June 2005 (23.06.2005) para [0079], [0091], [0180], [0188]	4
A	WU et al., Proteolysis involving matrix metalloproteinase 13 (collagenase-3) is required for chondrocyte differentiation that is associated with matrix mineralization. J. Bone Miner. Res. April 2002, Vol. 17, No. 4, pages 639-651	1-7
A	US 5,770,444 A (LEE et al.) 23 June 1998 (23.06.1998) entire document	1-7
A	US 7,198,790 A (LEE et al.) 3 April 2007 (03.04.2007) entire document	1-7

☐ Further documents are listed in the continuation of Box C. ☐

* 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	Date of mailing of the international search report
22 January 2013 (22.01.2013)	05 FEB 2013
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. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 12/53584

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.:
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:
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 1-7, drawn to a method of promoting osteogenesis, chondrogenic, or ligament/tendon differentiation comprising treating the cells with a peptide comprising an amino acid having identity to the amino acid sequence of SEQ ID NO:20.

Group II+: Claims 1-7, drawn to a method of promoting osteogenesis, chondrogenic, or ligament/tendon differentiation comprising treating the cells with a peptide comprising an amino acid sequence having identity to the amino acid sequence of SEQ ID NO:21, 22, 23, 24, 25, 26, 27, 28, 34, or 35. If Applicant elects to have this group searched, Applicant must specify the specific amino acid sequence to be searched. Each unique amino acid sequence constitutes an inventive concept.

---please see continuation on extra sheet---

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.:
1-7, limited to SEQ ID NO:20

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.

Continuation of: Box No III Observations where unity of invention is lacking

Group III+: Claim 8-19, drawn to an isolated peptide of 137 residues or less, said peptide comprising an amino acid sequence at least 92% identical to the amino acid sequence of SEQ ID NO:20, 21, 22, 23, 24, 25, 26, 27 or 28. If Applicant elects to have this group searched, Applicant must specify the specific amino acid sequence to be searched. Each unique amino acid sequence constitutes an inventive concept.

Group IV+: Claims 20-29, drawn to a method of increasing a growth factor activity, wherein the growth factor is a peptide comprising an amino acid sequence of SEQ ID NO: 20, 21,22,23, 24, 25, 26, 27 or 28. If Applicant elects to have this group searched, Applicant must specify the specific amino acid sequence to be searched. Each unique amino acid sequence constitutes an inventive concept.

Group V: Claims 30-41, drawn to an isolated peptide of 119 residues or less, said peptide comprising an amino acid sequence at least 92% identical to the amino acid sequence of SEQ ID NO:34.

Group VI: Claims 42-51, drawn to a method of increasing a growth factor activity, wherein the growth factor is a peptide comprising an amino acid sequence of SEQ ID NO: 34.

Group VII: Claims 52-63, drawn to an isolated peptide of 119 residues or less, said peptide comprising an amino acid sequence at least 92% identical to the amino acid sequence of SEQ ID NO:35.

Group VIII: Claims 64-73, drawn to a method of increasing a growth factor activity, wherein the growth factor is a peptide comprising an amino acid sequence of SEQ ID NO: 35.

Group IX: Claims 74-88, drawn to a composition comprising at least one protease and a growth factor

The inventions listed as Groups I, II+, III+, IV+, V, VI, VII, VIII, and IX do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The special technical feature of the claims of each of the Groups are disclosed in the Group descriptions, above.

The shared technical feature of the inventions listed as Groups I and II+ is the method steps comprising treating cells with a peptide set forth in claim 1, wherein each of peptides has osteoinductive, chondroinductive, or ligament/tendon differentiating activity. This shared technical feature fails to provide a contribution over the prior art, as evidenced by US 2009/0298761 A1 to Engelman in view of US 2001/0018509 A1 to Lee et al. (hereinafter 'Lee' 509'). Engelman discloses a method of promoting differentiation of cells into chondrocytes, ligament, or tendon (para [0013] - "A class of proteins known as "osteogenic proteins" or "morphogenic proteins" or "morphogens," are competent to act as true bone and cartilage tissue morphogens, able, on their own, to induce the proliferation and differentiation of progenitor cells into functional bone, cartilage, tendon, and/or ligamentous tissue"; para [0073] - "The term "cartilage" refers to a type of connective tissue that contains chondrocytes or chondrocyte-like cells"), the method comprising treating the cells (para [0092] - "contacting the cartilage tissue with the soluble morphogenic protein complex") with a peptide with an amino acid sequence comprising of the amino acid sequence of SEQ ID NO:20 (para [0025] - BMP-5, GDF-6; para [0044] - BMP-5 sequence set forth as SEQ ID NO:13, which is 100% identical to claimed SEQ ID NO:20), wherein the peptide has chondroinductive or ligament/tendon differentiating activity (para [0013]). Although Engelman does not disclose wherein the amino acid sequence comprising SEQ ID NO:20 is 137 residues in length or less, Lee '509 teaches a peptide comprising the amino acid sequence of SEQ ID NO:20 that has a length of 137 residues or less (SEQ ID NO:13 is 118 residues in length and exhibits 100% identity to SEQ ID NO:20). It would have been obvious to one of ordinary skill in the art that the peptide of Lee '509 can be used in the method of Engelman because the peptide in the method of Engelman is a BMP-5 peptide (para [0025]), and the sequence disclosed by Lee '509 is that of the C-terminal region of BMP-5 (para [0094]) and Lee '509 teaches that BMPs can induce de novo cartilage and bone formation (para [0004]). In the absence of a contribution over the prior art, the shared technical feature is not a shared special technical feature.

A further shared technical feature of the inventions listed as Groups I and II+ is the specific amino acid sequences recited therein. Significant structural similarities cannot readily be ascertained among the amino acid sequences. Without significant structural similarities, the amino acid sequences do not have a shared special technical feature. In the absence of a shared special technical feature, the inventions lack unity with one another.

The shared technical feature of the inventions listed as Groups III+ is an isolated peptide of 137 residues or less, said peptide comprising an amino acid sequence at least 92% identical to the amino acid sequence of SEQ ID NO:20, 21, 22, 23, 24, 25, 26, 27 or 28. This shared technical feature fails to provide a contribution over Lee '509, which discloses a peptide comprising the amino acid sequence of SEQ ID NO:20 that has a length of 137 residues or less (SEQ ID NO:13 is 118 residues in length and exhibits 100% identity to SEQ ID NO:20). Furthermore, significant structural similarities cannot readily be ascertained among the amino acid sequences. Without significant structural similarities, the amino acid sequences do not have a shared special technical feature. In the absence of a shared special technical feature, the inventions lack unity with one another.

The shared technical feature of the inventions listed as Groups II+, V, and VI is a peptide comprising SEQ ID NO:34. The shared technical feature of the inventions listed as Groups II+ and V is a peptide of 119 residues or less, said peptide comprising an amino acid sequence at least 92% identical to the amino acid sequence of SEQ ID NO:34, wherein the peptide has osteoinductive, chondroinductive, or ligament/tendon differentiating activity. This shared technical feature fails to provide a contribution over the prior art, as evidenced by US 5770444 A to Lee et al. (hereinafter 'Lee '444'), which discloses a peptide of 119 residues or less, said peptide comprising an amino acid sequence at least 92% identical to the amino acid sequence of SEQ ID NO:34 (SEQ ID NO:7 is 119 residues in length and exhibits 100% identity to SEQ ID NO:34), wherein the peptide has osteoinductive, chondroinductive, or ligament/tendon differentiating activity (col 14, ln 9-10 - "SEQ ID NO:7 is the amino acid for the C-terminal sequence of GDF-6"; col 3, ln 19-25 - "The BMP's can induce new bone growth and are effective for the treatment of fractures and other skeletal defects . . . GDF-6 may have similar activities and may be useful in repair of tissue injury caused by trauma or burns for example"). In the absence of a shared special technical feature, the inventions lack unity with one another.

---please see continuation on next extra sheet---

Continuation of: Previous extra sheet (Box No III Observations where unity of invention is lacking)

The shared technical feature of the inventions listed as Groups II+, VII and VIII is a peptide comprising SEQ ID NO: 35. The shared technical feature of the inventions listed as Groups II+ and VII is a peptide of 119 residues or less, said peptide comprising an amino acid sequence at least 92% identical to the amino acid sequence of SEQ ID NO:35, wherein the peptide has osteoinductive, chondroinductive, or ligament/tendon differentiating activity. This shared technical feature fails to provide a contribution over the prior art, as evidenced by US 7198790 A to Lee et al. (hereinafter 'Lee '790'), which discloses a peptide of 119 residues or less, said peptide comprising an amino acid sequence at least 92% identical to the amino acid sequence of SEQ ID NO:35 (SEQ ID NO:13 is 119 residues in length and exhibits 100% identity to SEQ ID NO:35), wherein the peptide has osteoinductive, chondroinductive, or ligament/tendon differentiating activity (col 2, ln 27-28 - "C-terminal sequences of GDF-5 (SEQ ID NO:13)"; col 3, ln 28-33 - "The expression of GDF-5 during embryogenesis and specifically in the precartilaginous mesenchyme associated with early bone formation in the limbs, suggests a variety of applications using the polypeptide, polynucleotide, and antibodies of the invention, related to skeletal development, cartilage differentiation, and cell proliferative diseases."). In the absence of a shared special technical feature, the inventions lack unity with one another.

The shared technical feature of the inventions listed as Groups IV+, VI, VIII and IX is a composition or method comprising at least one protease and a growth factor. The shared technical feature of the inventions listed as Groups IV+, VI, and VIII is method of increasing a growth factor activity, the method comprising treating a cell with at least one protease and a growth factor, wherein the growth factor is a peptide having osteoinductive, chondroinductive, or ligament/tendon differentiating activity. This shared technical feature fails to provide a contribution over Engelman in view of the article entitled "Proteolysis involving matrix metalloproteinase 13 (collagenase-3) is required for chondrocyte differentiation that is associated with matrix mineralization" by Wu et al. (published in J. Bone Miner. Res. April 2002, Vol. 17, No. 4, pages 639-651; hereinafter 'Wu'). Engelman teaches a method comprising treating a cell with at least one growth factor (para [0092] - "contacting the cartilage tissue with the soluble morphogenic protein complex"), wherein the growth factor is a peptide comprising an amino acid sequence of SEQ ID NO:20 (para [0025] - BMP-5; para [0044] - BMP-5 sequence set forth as SEQ ID NO:13, which is 100% identical to claimed SEQ ID NO:20), wherein the peptide has chondroinductive or ligament/tendon differentiating activity (para [0013] - "A class of proteins known as "osteogenic proteins" or "morphogenic proteins" or "morphogens," are competent to act as true bone and cartilage tissue morphogens, able, on their own, to induce the proliferation and differentiation of progenitor cells into functional bone, cartilage, tendon, and/or ligamentous tissue"; para [0073] - "The term "cartilage" refers to a type of connective tissue that contains chondrocytes or chondrocyte-like cells"). Engelman does not teach that the method further comprises treating the cell with at least one protease. Wu teaches that the protease MMP-13 is required for chondrocyte differentiation (abstract). Accordingly, it would have been obvious to one of skill in the art that it would be beneficial to additionally treat the cells with MMP-13 in the method of Engelman, in order to ensure optimal chondrocyte differentiation (and which would inherently increase the moprhogenic activity of the growth factor). In the absence of a contribution over the prior art, the shared technical feature is not a shared special technical feature.

A further shared technical feature of the inventions listed as Groups IV+ is the specific amino acid sequences recited therein. Significant structural similarities cannot readily be ascertained among the amino acid sequences. Without significant structural similarities, the amino acid sequences do not have a shared special technical feature. In the absence of a shared special technical feature, the inventions lack unity with one another.

Therefore, the inventions of Groups I, II+, III+, IV+, V, VI, VII, VIII, and IX lack unity with one another because they fail to share a same or corresponding special technical feature.

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(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, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

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Published:

— *with international search report (Art. 21(3))*

WO 2013/119873 A1

(54) **Title**: LIQUEFACTION OF BONE MATRIX

(57) **Abstract**: The invention relates to methods of preparing a bone matrix solution, a bone matrix implant, and variants thereof. The invention also relates to methods of cell culture using the same. The invention further relates to bone matrix scaffolds comprising one or more bone matrix nanofibers, methods of preparing, and methods of use thereof. The invention also relates to methods of culturing cells and promoting differentiation of stem cells using the same.

LIQUEFACTION OF BONE MATRIX

Background of the Invention

[0001] The invention relates to methods of preparing a bone matrix solution, a bone matrix implant, and variants thereof. The invention also relates to methods of cell culture using the same.

[0002] The invention further relates to bone matrix scaffolds comprising one or more bone matrix nanofibers, methods of preparing, and methods of use thereof. The invention also relates to methods of culturing cells and promoting differentiation of stem cells using the same.

Summary

[0003] The invention relates to methods of preparing a bone matrix solution comprising dissolving bone in an amount of an anhydrous solvent comprising (i) hexafluoroisopropanol (HFP) or 2, 2, 2-trifluoroethanol (TFE) and (ii) at least about 5 wt% trifluoroacetic acid (TFA) sufficient to form the bone matrix solution.

[0004] The invention also relates to methods of preparing a bone matrix implant with a predetermined shape comprising: dissolving a bone in an amount of an anhydrous solvent comprising (i) hexafluoroisopropanol (HFP) or 2, 2, 2-trifluoroethanol (TFE) and (ii) at least about 5 wt% trifluoroacetic acid (TFA) sufficient to form the bone matrix solution; exposing the bone solution to conditions sufficient to evaporate an amount of the solvent to form a colloidal gel; and allowing the colloidal gel to harden into the predetermined shape to form the bone matrix implant.

[0005] The invention also relates to methods of preparing a film of bone matrix on a surface comprising: dissolving a bone preparation in an amount of an anhydrous solvent comprising (i) hexafluoroisopropanol (HFP) or 2, 2, 2-trifluoroethanol (TFE), and (ii) at least about 5 wt% trifluoroacetic acid (TFA) sufficient to form the bone matrix solution; exposing the bone solution to conditions sufficient to evaporate an amount of the solvent to form a colloidal gel; coating the surface with the colloidal gel; and exposing the

coated surface to conditions sufficient to further evaporate an amount of solvent to form a film. In particular, the bone may be demineralized bone or non-demineralized bone.

[0006] The invention also relates to methods of promoting osteogenesis, chondrogenic, or ligament/tendon differentiation of cells, comprising contacting the cells on a surface coated with the bone matrix produced by the method described herein.

[0007] The invention further relates to bone matrix scaffolds comprising one or more bone matrix nanofibers.

[0008] The invention also relates to methods of preparing the bone matrix scaffold, the method comprising dissolving bone in an amount of an anhydrous solvent comprising (i) hexafluoroisopropanol (HFP) or 2, 2, 2-trifluoroethanol (TFE) and (ii) at least about 5 wt% trifluoroacetic acid (TFA) sufficient to form a bone matrix solution at a temperature between about 0 °C and about 22 °C, and electrospinning the bone matrix solution to form the bone matrix scaffold.

[0009] The invention also relates to methods of preparing the bone matrix scaffold, the method comprising dissolving bone in an amount of an anhydrous solvent comprising (i) hexafluoroisopropanol (HFP) or 2, 2, 2-trifluoroethanol (TFE) and (ii) at least about 5 wt% trifluoroacetic acid (TFA) sufficient to form a bone matrix solution at a temperature between about 0 °C and about 22 °C, and neutralizing the bone matrix solution to form the bone matrix scaffold.

[0010] The invention also relates to bone matrix implants produced by the methods of preparing the bone matrix scaffold.

[0011] The invention further relates to methods of cell culture comprising culturing cells on the bone matrix scaffold in condition suitable for the cell culture. The invention also relates to methods of promoting differentiation of stem cells into osteoblasts, chondrocytes, ligament or tendon, the method comprising culturing the cells on the bone matrix scaffold in conditions suitable for the cell differentiation.

Brief Description of the Drawings

[0012] FIGURE 1 depicts BMP-2 ELISA assay results. An ELISA for BMP-2 confirms that high quantities of BMP-2 are present in the DBM that was dissolved in TFA/HFP and later slectrospun. An estimated amount of rhBMP2 was added to the assay based on a serial dilution, with the calculated amount by the ELISA confirming the precision of the assay. The average amount of Gn-HCL extracted

from DBM by established protocols is shown (purple bar) next to the amount extracted from our tested donor (teal bar) using the established guanidine hydrochloride method. Data was normalized and compared to standard controls, with statistical significance determined by a one-way ANOVE with $p<0.05$.

[0013] FIGURE 2 depicts an exemplary electrospinning apparatus.

[0014] FIGURE 3 depicts electrospun bone nano-features. Representative scanning electron micrographs of electrospun demineralized bone matrix. The architecture of the large demineralized bone matrix chips are shown relative to the pure electrospun demineralized bone matrix (note scale bar size differences), both as uncross-linked and as cross-linked with 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), and as blended with various ratios of DBM/PCL.

[0015] FIGURE 4 depicts average electrospun bone matrix fiber size. This graph depicts demineralized bone matrix diameter from raw powder and electrospun nanofibers, with variable amounts of polycaprolactone added to the liquefied bone for electrospinning. "Powder" indicates raw ground bone measured across the particle width. The amount of demineralized bone in electrospun fibers are shown as a percent of DBM relative to the amount of PCL in the copolymer blend as a percentage (100=100% DBM, 10=10% DBM and 90% PCL, etc.). Error bars indicate standard deviation and significance is denoted as $*p<0.05$, as determined by a one way ANOVA followed by Tukey's test for pairwise comparisons. Note: log scale is used on the ordinate axis.

[0016] FIGURE 5 depicts Fourier Transform Infrared Spectroscopy (FTIR). FTIR analysis of demineralized bone matrix and PCL co-polymer blends was performed, confirming homology of electrospun DMB nanofibers to its native DMB powder form. The peaks noted by red arrows have been shown to associate with an intact alpha-helical structure as present in type I collagen.

[0017] FIGURE 6 depicts collagen structural analysis of electrospun demineralized bone. SDS gel show the band pattern of DBM as electrospun alone or with PCL, as compared with pure type I collagen, either with or without pepsin treatment. Size markers (red bands labeled), collagen type I an electrospun DMB are show before and after treatment with pepsin, with the collagen alpha 1 and alpha 2 helices (noted) shown unchanged under the electrospinning conditions.

[0018] FIGURE 7 depicts porosity of electrospun bone matrix scaffolds. The average scaffold porosity was determined by calculating void fraction of dry materials relative to wet material with respect to material density as shown. We show the average scaffold porosity of electrospun DBM in its native/un-

crosslinked form, or as cross-linked by blending with either PCL or EDC, or as cross-linked with EDC with the scaffold weighted to prevent material shrinkage. Overall, electrospun DBMs had average porosity above that of demineralized bone matrix powder.

[0019] FIGURE 8 depicts mesenchymal stem cell attachment on bone matrix scaffolds. Significantly more cells are seen to attach to electrospun DBM as cross-linked with EDC or as blended with PCL as compared to cell left to attach on glass coverslips alone in a cell attachment assay. Cell were grown in serum-free media over the course of 15, 30, 60 and 120 minutes with cell counts of crystal violet stained cells that were quantitated with image J, and verified by optical densitometry of released stain (not shown). The DBM film dissolved rapidly and negatively biased the attachment assay at all time points. Cell attachment was shown to be statistically significantly higher on DBM-EDC, DBM/PCL and PCL relative to attachment on the coverslip alone with cells grown in serum free media as determined by a one-way ANOVA ($P<0.05$).

[0020] FIGURE 9 depicts i*n vivo* implants of bone matrix scaffolds. A four-week animal study with implanted EDC cross-linked DBM and DBM/PCL scaffolds was performed using 0.1cc of material implanted into a rat intramuscularmuscle pouch assay. H&E stained sections of implanted electrospun DBM constructs indicate minimal immunoreactivity and high cell recruitment and infiltration into electrospun materials. Abundant, dense, loosely-organized layers of mesenchymal-like-cells are seen throughout the electrospun constructs, with a high degree of original scaffold turnover observed, particularly in DBM/PCL sample. Adipogenic differentiation is potentially seen in DBM/EDC samples, with unique interdigitations observed in most DBM/PCL samples at the scaffold/muscle boundary, evocative of a tendon-type structural interface forming.

[0021] FIGURE 10 depicts Alizarin Red S and Toluidine Blue Assay of bone matrix scaffolds. Alizarin red S histology stains confirmed the presence of calcium in adipose stem cell seeded electrospun DBM crosslinked with EDC (A-EDC), with DBM/PCL (A-PCL), with DBM/EDC without cells (A-EDC no ASCs) and of the unprocessed DBM granules (A-RAW) (A). Toluidine blue suggests the formation of new bone from ASCs that are grown in the presence of electrospun DBM (C) vs grown in control ASC media for 3 weeks (B).

[0022] FIGURE 11 depicts osteoinduction capability of bone matrix scaffolds. Uncrosslinked electrospun DBM was cultured with adipose derived stem cells (ASCs) and dental pulp stem cells (DPSCs) to assay the osteoinductive potential of this material. Both ASCs and DPSCs showed expression of alexa488 labeled bone sialo protein I (aka osteopontin) and bone sialo protein II, as seen in

the many cell mounds or nodules that formed throughout the cultures. Cell nuclei are shown counter-labeled with DAPI.

Detailed Description of the Invention

[0023] The invention relates to methods of preparing a bone matrix solution. The method of preparing a bone matrix solution according to some embodiments of the present invention comprises dissolving bone in an anhydrous solvent. The bone includes, but is not limited to, autograft bone, allograft bone, and xenograft bone. Such bone includes any bone from any source, including, but not limited to, bone from a living human donor, bone from a human cadaveric donor, and bone from an animal. The bone may include cortical bone and/or cancellous bone and/or cortico-cancellous bone.

[0024] The bone may be demineralized bone or non-demineralized bone. "Demineralized bone matrix (DBM)" as used herein refers to bone having less than about 8 wt % residual calcium. Demineralization involves treating a bone tissue to remove its inorganic mineral hydroxyapatite material. The level of demineralization of a bone tissue is defined by the amount (wt %) of residual calcium found in the demineralized bone. In some embodiments, the demineralized bone may still contain physiologically active levels of growth and differentiation factors (e.g., osteogenic growth factors, such as bone morphogenetic proteins (BMPs)) remaining from the initial bone even after the demineralization treatment. In further embodiments, the demineralized bone may contain collagen, glycosaminoglycans, osteocalcin, osteonectin, bone sialo protein, osteopontin, and mixtures thereof. "Non-demineralized bone" as used in the present application refers to bone that has not been treated to remove minerals present such as, for example, hydroxyapatite.

[0025] The extracellular matrix of bone is comprised primarily of collagen type I and inorganic bone mineral hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$). "Collagen type I" refers to a heterotrimer of two alpha1(I) and one alpha2(I) chains, which is the most common form of fibrillar collagen in a body. Collagen type I is present in the form of elongated fibers greater than 500 μm in length and 500 nm in diameter. These building blocks of the collagen are rod-like triple helices that are stabilized by intramolecular hydrogen bonds between Gly and Hyp in adjacent chains. Dissolving the collagen type I in a solvent and maintaining its native biological activities, however, has been challenging because its native conformation can be degraded by a disruption in the intermolecular crosslinks of the aldimine or keto-imine type and/or a disruption in the intramolecular bonds of the triple helical structure. For example, dilute acidic solvents have been used to dissolve collagen by breaking the intermolecular crosslinks of its aldimine type, while proteolytic enzymes, such as pepsin, have been used to cleave the more stable crosslinks of its keto-imine

type. Moreover, dissolving and electro-spinning collagen in fluoroalcohol solvents, such as 1, 1, 1, 3, 3, 3-hexafluoro-2-propanol (HFP) and 2, 2, 2-trifluoroethanol (TFE), have been shown to result in the significant disruption in the intramolecular bonds of the collagen triple helices (Zeugolis et al., Biomaterials 29, 2008, 2293-2305). For the same reasons, it has been challenging to dissolve the extracellular matrix of bone comprised primarily of collagen type I in a solvent while maintaining its native biological activities.

[0026] Moreover, the extracellular matrix of bone is comprised of a complex mixture of the collagen type I, the inorganic bone mineral hydroxyapatite, other proteins (e.g. laminins, fibronectin, elastin, and growth factors), other minerals (e.g. calcium phosphate, magnesium, carbonate, and fluoride ions), and carbohydrates (e.g. parts of glycoproteins). Accordingly, dissolving bone has been even more challenging in view of the complex mixture of the various components in the bone.

[0027] Fluoroalcohol solvents (e.g. HFP and TFE), have been shown to disrupt the intramolecular bonds of the collagen triple helices and thus were not considered as a desirable candidate for dissolving collagen type I. The present invention surprisingly shows that dissolving bone in an anhydrous solvent comprising a highly volatile fluoroalcohols (e.g. HFP, TFE, and their variants) and trifluoroacetic acid (TFA), would still maintain the native structure and/or triple collagen structure of the collagen type I in the bone. In some embodiments, collagen type I in the bone matrix solution prepared according to the methods described herein consists of collagen in its native structure or in its native triple helical structure. In other embodiments, collagen type I in the bone matrix solution prepared according to the methods described herein essentially consists of collagen in its native structure or its native triple helical structure. In additional embodiments, more than about 99 %, 98 %, 97 %, 96 %, 95 %, 90 %, 80 %, 70 %, 60 %, 50 %, 40 %, 30 %, 20 %, 10 %, 5 %, 3% or 1% of collagen type I in the bone matrix solution prepared according to the methods described herein is collagen in its native structure or in its native triple helical structure. In yet additional embodiments, less than about 100%, 99 %, 98 %, 97 %, 96 %, 95 %, 90 %, 80 %, 70 %, 60 %, 50 %, 40 %, 30 %, 20 %, 10 %, 5 %, or 3% collagen type I in the bone matrix solution prepared according to the methods described herein is collagen in its native structure. In further embodiments, between about 100% and about 95%, between about 100% and about 85%, between about 95% and about 85%, between about 85% and about 75%, between about 85% and about 65%, between about 75% and about 65%, between about 65% and about 45%, between about 65% and about 55%, between about 55% and about 35%, between about 55% and about 45%, between about 45% and about 25%, between about 45% and about 35%, between about 35% and about 15%, between about 35% and about 25%, between about 25% and about 5%, or between about 25% and about 15% of collagen type I in

the bone matrix solution prepared according to the methods described herein is collagen in its native structure or in its native triple helical structure.

[0028] The solvents of the present invention may be anhydrous solvents or non-anhydrous solvents with a protectant. The protectant prevents heat or solvent related degradation or denaturation of proteins in the non-anhydrous solvents. The protectants include, but are not limited to, bovine serum albumin and human serum albumin, which has been shown to enhance the long term stability of proteins dissolved in organic solvents.

[0029] Anhydrous trifluoroacetic acid (TFA) has been used in the peptide synthesis to cleave an N-terminal amino acid of a purified synthetic peptide, such as polypeptide models of collagen (Guantieri and Tamburro, Int. J. Peptide Protein Res., 2, 1984, 274-278; Fields et al., Biopolymers, 33, 11, 1993, 1695-1707). The extracellular matrix of bone, however, is comprised of a complex mixture of carbohydrates, proteins, and minerals and thus is difficult to be dissolved in the same solution as the purified synthetic peptide. For example, as shown in the comparative examples herein, a solvent having 50% TFA and 50% dimethylformamide (DMF) was not able to dissolve the bone even after an extended period of stirring on the order of 14-28 days at 4°C or 22°C. The present invention surprisingly shows an anhydrous solvent comprising TFA and a volatile fluoroalcohol (e.g. HFP, TFE, and their variants) effectively dissolves bone having complex mixtures of carbohydrates, proteins, and minerals. In some embodiments of the present invention, a secondary solvent may be added to the TFA and fluoroalcohol solvent. Specifically, the secondary solvent may be selected from the group consisting of the family of hexa-flouro-iso-propanol (HFP), N-methyl morpholine N-oxide, calcium nitrate-methanol, or related organic solvents.

[0030] The anhydrous solvent to dissolve the bone according to some embodiments of the present invention may comprise (i) hexafluoroisopropanol (HFP), 2, 2, 2-trifluoroethanol (TFE) or their variants, and (ii) trifluoroacetic acid (TFA). As used herein, the variants of HFP and TFE refers to other fluoroalcohol solvents, including, but not limited to, 2,3-difluorobenzyl alcohol, 1,3-difluoro-2-propanol, trifluoroethanol, 2,2,3,3,4,4,4-heptafluoro-1-butanol, 3,3,4,4,5,5,5-heptafluoro-2-pentanol, 2,2,3,3,4,4,5,5-octafluoro-1-pentanol, 1H,1H,2H,2H-perfluoro-1-decanol, 1H,1H,2H,3H,3H-perfluorononane-1,2-diol, 1H,1H,9H-perfluoro-1-nonanol, 1H,1H-perfluorooctanol, 1H,1H,2H,2H-perfluorooctanol, 1H, 1H, 2H, 3H, 3H-perfluoroundecane-1,2-diol, trifluorobutanol, 1,1,1-trifluoro-2-propanol, 3,3,3-trifluoro-1-propanol, 1,1,1,3,3,3-hexafluoro-2-propanol, 1,1,1,3,3,3-hexafluoro-2-methyl-2-propanol, 2,2,3,4,4,4-hexafluoro-1-butanol, 2-trifluoromethyl-2-propanol, 2,2,3,3-tetrafluoro-1-propanol, 3,3,3-trifluoro-1-propanol, 4,4,4-trifluoro-1-butanol, and a mixture thereof. In further embodiments, the anhydrous solvent to dissolve the bone may consist of (i) HFP, TFE or their variants, and (ii) trifluoroacetic acid (TFA). In

additional embodiments, the anhydrous solvent to dissolve the bone may exclude dimethylformamide (DMF).

[0031] In one aspect, the amount of HFP, TFE, their variants in the solvent may be about 95 wt%, 90 wt%, 80 wt%, 70 wt%, 60 wt%, 50 wt%, 40 wt%, 30 wt%, 20 wt%, 10 wt%, 5 wt%, between about 95 wt% and about 5 wt%, between about 95 wt% and about 10 wt%, between about 95 wt% and about 25 wt%, between about 95 wt% and about 45 wt%, between about 95 wt% and about 65 wt%, between about 95 wt% and about 85 wt%, between about 95 wt% and about 90 wt%, between about 90 wt% and about 5 wt%, between about 90 wt% and about 10 wt%, between about 90 wt% and about 25 wt%, between about 90 wt% and about 45 wt%, between about 90 wt% and about 65 wt%, between about 90 wt% and about 85 wt%, between about 80 wt% and about 5 wt%, between about 80 wt% and about 10 wt%, between about 80 wt% and about 25 wt%, between about 80 wt% and about 45 wt%, between about 80 wt% and about 65 wt%, and between about 80 wt% and about 75 wt%. In another aspect, the amount of TFA in the solvent may be at least about 1, 2, 3, 4, or 5 wt%, 6 wt%, 7 wt%, 8 wt%, 9 wt%, 10 wt%, 13 wt%, 15 wt%, 20 wt%, 25 wt%, 30 wt%, 40 wt%, 50 wt%, 60 wt%, 70%, 80 wt%, or 90 wt% between about 95 wt% and about 5 wt%, between about 95 wt% and about 10 wt%, between about 95 wt% and about 25 wt%, between about 95 wt% and about 45 wt%, between about 95 wt% and about 65 wt%, between about 95 wt% and about 85 wt%, between about 95 wt% and about 90 wt%, between about 90 wt% and about 5 wt%, between about 90 wt% and about 10 wt%, between about 90 wt% and about 25 wt%, between about 90 wt% and about 45 wt%, between about 90 wt% and about 65 wt%, between about 90 wt% and about 85 wt%, between about 80 wt% and about 5 wt%, between about 80 wt% and about 10 wt%, between about 80 wt% and about 25 wt%, between about 80 wt% and about 45 wt%, between about 80 wt% and about 65 wt%, and between about 80 wt% and about 75 wt%.

[0032] As used herein, the term "about" modifying, for example, the quantity of an ingredient in a composition, concentrations, volumes, process temperature, process time, yields, flow rates, pressures, and like values, and ranges thereof, refers to variation in the numerical quantity that can occur, for example, through typical measuring and handling procedures used for making compounds, compositions, concentrates or use formulations; through inadvertent error in these procedures; through differences in the manufacture, source, or purity of starting materials or ingredients used to carry out the methods; and like considerations. The term "about" also encompasses amounts that differ due to aging of, for example, a composition, formulation, or cell culture with a particular initial concentration or mixture, and amounts that differ due to mixing or processing a composition or formulation with a particular initial concentration or mixture. Whether modified by the term "about" the claims appended hereto include equivalents to

these quantities. The term "about" further may refer to a range of values that are similar to the stated reference value. In certain embodiments, the term "about" refers to a range of values that fall within 10, 9, 8,7, 6, 5,4, 3, 2, 1 percent or less of the stated reference value.

[0033] The amount of the anhydrous solvent used in preparing the bone matrix solution according to some embodiments of the present invention is the amount sufficient to dissolve the bone and thus sufficient to form the bone matrix solution at a temperature described as follows. In some aspects, the bone may be dissolved in the solvent described herein at about 37 °C, 36 °C, 35 °C, 34 °C, 33 °C, 32 °C, 31 °C, 30 °C, 29 °C, 28 °C, 27 °C, 26 °C, 25 °C, 24 °C, 23 °C, 22 °C, 21 °C, 20 °C, 19 °C, 18 °C, 17 °C, 16 °C, 15 °C, 14 °C, 13 °C, 12 °C, 11 °C, 10 °C, 9 °C, 8 °C, 7 °C, 6 °C, 5 °C, 4 °C or lower. In other aspects, the bone may be dissolved in the solvent described herein at a temperature lower than about 37 °C, 36 °C, 35 °C, 34 °C, 33 °C, 32 °C, 31 °C, 30 °C, 29 °C, 28 °C, 27 °C, 26 °C, 25 °C, 24 °C, 23 °C, 22 °C, 21 °C, 20 °C, 19 °C, 18 °C, 17 °C, 16 °C, 15 °C, 14 °C, 13 °C, 12 °C, 11 °C, 10 °C, 9 °C, 8 °C, 7 °C, 6 °C, 5 °C, or 4 °C. In further aspects, the bone is dissolved in the solvent described herein at a temperature high enough to effectively break the bonds in a solid. In some embodiments, the bone may be dissolved in the solvent described herein at a temperature higher than about 20 °C, 19 °C, 18 °C, 17 °C, 16 °C, 15 °C, 14 °C, 13 °C, 12 °C, 11 °C, 10 °C, 9 °C, 8 °C, 7 °C, 6 °C, 5 °C, 4 °C, 3 °C, 2 °C, 1 °C, or 0 °C. It has been found by the inventors that the temperature in which the bone is dissolved has an effect on the native structure and/or the native triple helical structure of collagen in the bone. In some embodiments, dissolving bone at temperatures below about room temperature, which is defined as 25 °C, has provided an improved collagen product in its native structure and/or in its native triple helical structure compared to dissolving bone at higher temperatures. In some embodiments, bone is dissolved at a temperature below 22 °C. In other embodiments, bone is dissolved at a temperature between about 4 °C and about 21 °C.

[0034] The amount of the bone dissolved in the solvent according to some embodiments of the present invention may be between about 0.001 mg/mL and about 300 mg/mL, between about 1 mg/mL and about 300 mg/mL, between about 10 mg/mL and about 300 mg/mL, between about 20 mg/mL and about 300 mg/mL, between about 0.001 mg/mL and about 200 mg/mL, between about 1 mg/mL and about 320 mg/mL, between about 10 mg/mL and about 200 mg/mL, between about 20 mg/mL and about 200 mg/mL, between about 30 mg/mL and about 150 mg/mL, between about 50 mg/mL and about 150 mg/mL, between about 100 mg/mL and about 150 mg/mL, between about 130 mg/mL and about 150 mg/mL, between about 30 mg/mL and about 100 mg/mL, or between about 30 mg/mL and about 50 mg/mL. According to additional embodiments of the present invention, the bone may be dissolved by

stirring the bone in the solvent described herein for more than about 3 hours, 6 hours, 12 hours, 24 hours, 36 hours, 48 hours, 3 days, 4 days, 5 days or 6 days. In another aspect, the bone may be dissolved by stirring the bone in the solvent described herein for less than about 5 hours, 10 hours, 15, hours, 20 hours, 25 hours, 30 hours, 40 hours, 50 hours, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, or 14 days. In another aspect, the bone may be dissolved by stirring the bone in the solvent described herein for about 3 hours, 5 hours, 6 hours, 10 hours, 12 hours, 15, hours, 20 hours, 24 hours, 25 hours, 30 hours, 40 hours, 50 hours, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 14 days, 20 days, 30 days, 40 days, or 50 days. According to yet additional embodiments of the present invention, the bone to be dissolved in the solvent described herein may be in the forms of particles and/or powder. Demineralized bone particles may be prepared from cleaned and disinfected bone fragments that have been freeze-dried and ground/fractured into bone particles. Bone particles may be selected by, for example, using sieving devices (i.e., mesh sieves) commercially available to obtain particles within a desired size range. Such demineralized bone particles may have an average diameter of between about 125 microns and about 4 mm; between about 710 microns and about 2 mm; between about 125 microns and about 500 microns; between about 125 microns and about 850 microns; or between about 250 microns and about 710 microns. Certain embodiments of the present invention may include demineralized bone powder that is commercially available. For example, a suitable demineralized bone powder that is widely and reliably available is produced by LifeNet Health, Virginia Beach, VA.

[0035] The invention also relates to methods of preparing a bone matrix implant with a predetermined shape. An “implant” refers to any object that is designed to be placed partially or wholly within a patient's body for one or more therapeutic or prophylactic purposes such as for tissue augmentation, contouring, restoring physiological function, repairing or restoring tissues damaged by disease or trauma, and/or delivering therapeutic agents to normal, damaged or diseased organs and tissues. A “bone matrix implant” refers to a medical device or implant that includes a volume replacement material for augmentation or reconstruction to replace a whole or part of a bone structure. A predetermined shape of the bone matrix implant may be varied to fit the implant site. A “bone matrix” refers to any mixture of the volume replacement materials for augmentation or reconstruction to replace a whole or part of a bone structure.

[0036] The methods of preparing a bone matrix implant with a predetermined shape according to some embodiments of the present invention comprises dissolving a bone in an amount of an anhydrous solvent comprising (i) hexafluoroisopropanol (HFP), 2, 2, 2-trifluoroethanol (TFE) or isopropanol and (ii) at least

about 5 wt% trifluoroacetic acid (TFA) sufficient to form a bone matrix solution; exposing the bone matrix solution to conditions sufficient to evaporate an amount of the solvent to form a colloidal gel; and allowing the colloidal gel to harden into the predetermined shape to form the bone matrix implant. In some embodiments, the colloidal gel may be placed in a mold to form the predetermined shape, formed with or without particles of bone in the suspension. As used herein, a "colloidal gel" refers to a colloid that has developed resistance to flow and thus is in a more solid form than a solution. A "colloid" refers to a system composed of a continuous medium throughout which are distributed small particles, e.g., 1 to 1000 nm in size or larger, that do not settle out under the influence of gravity; the particles may be in emulsion or in suspension. The invention further relates to a bone matrix implant produced by the method described herein.

[0037] Methods to evaporate the solvent to form a colloidal gel from the bone matrix solution according to some embodiments of the present invention may include, but are not limited to, air drying, vacuum drying, heat drying, and freeze drying. The amount of the solvent to form a colloidal gel from the bone matrix solution according to some embodiments of the present invention may be about 98 wt%, 95 wt%, 90 wt%, 85 wt%, 80 wt%, 75 wt%, 70 wt%, 65 wt%, 60 wt%, 55 wt%, 50 wt%, 45 wt%, 40 wt%, 35 wt%, 30 wt%, 25 wt%, 20 wt%, 15 wt%, 10 wt%, 5 wt%, or 0 wt%. In one aspect, the amount of the solvent to form a colloidal gel from the bone matrix solution may be less than about 98 wt%, 95 wt%, 90 wt%, 85 wt%, 80 wt%, 75 wt%, 70 wt%, 65 wt%, 60 wt%, 55 wt%, 50 wt%, 45 wt%, 40 wt%, 35 wt%, 30 wt%, 25 wt%, 20 wt%, 15 wt%, 10 wt%, or 5 wt%. In another aspect, the amount of the solvent to form a colloidal gel from the bone matrix solution may be more than about 98 wt%, 95 wt%, 90 wt%, 85 wt%, 80 wt%, 75 wt%, 70 wt%, 65 wt%, 60 wt%, 55 wt%, 50 wt%, 45 wt%, 40 wt%, 35 wt%, 30 wt%, 25 wt%, 20 wt%, 15 wt%, 10 wt%, 5 wt%, or 0 wt%.

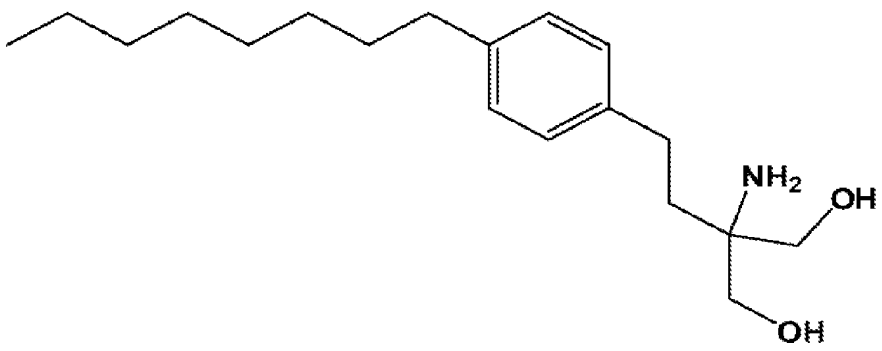
[0038] The methods of preparing a bone matrix implant with a predetermined shape according to some embodiments of the present invention may further comprise adding a bioactive factor to the bone matrix solution described herein. A "bioactive factor" refers to protein, carbohydrate, or mineral that has any effect on a cellular activity. Examples of bioactive factors include, but are not limited to, an osteogenic growth factor, collagen, glycosaminoglycans, osteonectin, bone sialo protein, an osteoinductive factor, a chondrogenic factor, a cytokine, a mitogenic factor, a chemotactic factor, a transforming growth factor(TGF), a fibroblast growth factor (FGF), an angiogenic factor, an insulin-like growth factor (IGF), a platelet-derived growth factor (PDGF), an epidermal growth factor (EGF), a vascular endothelial growth factor (VEGF), a nerve growth factor (NGF), a neurotrophin, a bone morphogenetic protein (BMP), osteogenin, osteopontin, osteocalcin, cementum attachment protein, erythropoietin, thrombopoietin,

tumor necrosis factor (TNF), an interferon, a colony stimulating factor (CSF), or an interleukin, among others. The bioactive factor may be a BMP, PDGF, FGF, VEGF, TGF, insulin, among others. Examples of BMPs include but are not limited to BMP-2, BMP-3, BMP-4, BMP-5, BMP-6, BMP-7, BMP-8, BMP-9, BMP-10, BMP-11, BMP-12, BMP-13, BMP-14, BMP-15, and a mixture thereof.

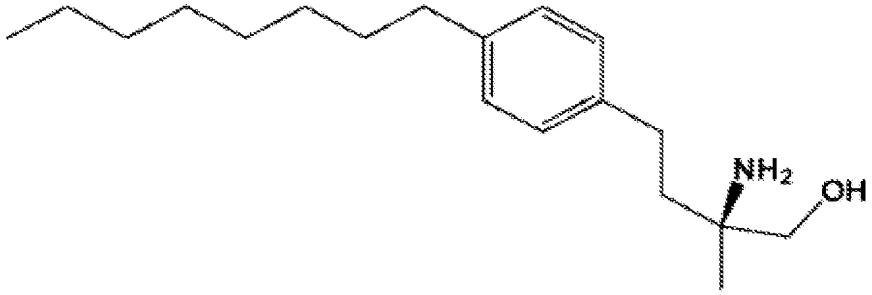
[0039] In some embodiments, the bioactive factor may include chemokines. Chemokines refers to a family of small proteins secreted from cells that promote the movement or chemotaxis of nearby cells. Some chemokines are considered pro-inflammatory and can be induced during an immune response while others are considered homeostatic. Typically, chemokines exert their chemoattractant function and other functions by binding to one or more chemokine receptors. Chemokines include proteins isolated from natural sources as well as those made synthetically, by recombinant means or by chemical synthesis. Exemplary chemokines include, but are not limited to, MCP-1, Eotaxin, SDF-1β, GRO-α, MIP-1β, IL-8, IP-10, MCP-3, MIP-3α, MDC, MIP-1α, BCA-1, GCP-2, ENA-78, PBP, MIG, PF-4, PF-4-var1, SDF-2, MCP-2, MCP-4, MIP-4, MIP-3β, MIP-2α, MIP-2β, MIP-5, HCC-1, RANTES, Eotaxin-2, TARC, I-309, Lymphotactin, Lungkine, C10, MIP-1γ, MCP-5, LEC, Exodus-2, MIP-3, TECK, Eotaxin-3, CTACK, MEC, SCM-1β, I-TAC, BRAK, SR-PSOX, Fractalkine, LD78-β, MIP-1b2, and others known to those of skill in the art. References to chemokines typically include monomeric forms of such chemokines. Chemokines also include dimeric or other multimeric forms.

[0040] In additional embodiments, the bioactive factor may also include small molecules drugs. Small molecule drugs include a molecule, whether naturally-occurring or artificially created (e.g., via chemical synthesis) that has a relatively low molecular weight and that is not a protein, a nucleic acid, or a carbohydrate. In one aspect, though not necessarily, small molecule drugs are monomeric and have a molecular weight of less than about 1500 g/mol. In another aspect, the drug is one that has already been deemed safe and effective for use by the appropriate governmental agency or body. For example, drugs for human use listed by the FDA under 21 C.F.R. §§ 330.5, 331 through 361 and 440 through 460; drugs for veterinary use listed by the FDA under 21 C.F.R. §§ 500 through 589, incorporated herein by reference, are all considered acceptable for use in inventive conjugates. In another aspect, the small molecule drugs may include agonists of a Sphingosine-1 -phosphate (SlP) agonist, such as fingolimod (FTY720), which is a synthetic compound that acts as an agonist of the SlPl, S1P3, S1P4, and S1P5 receptors when phosphorylated into FTY720P. For example, the small molecule drugs may include the following molecules:

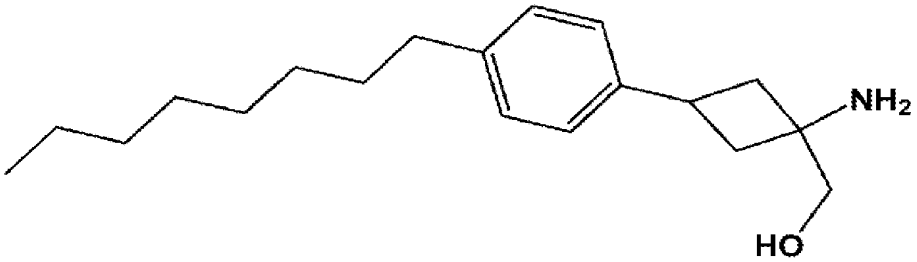
FTY720 (fingolimod)



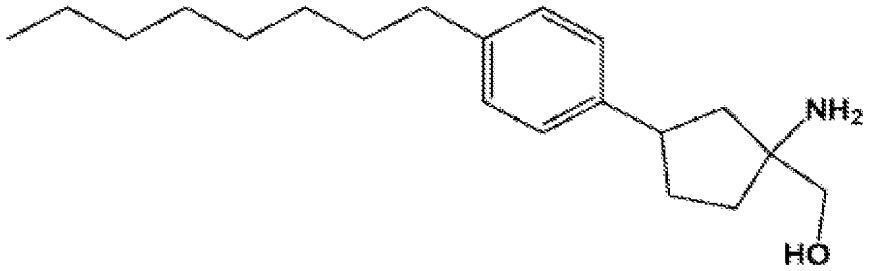
2-amino-2-(4-octylphenethyl)propane-l,3-diol,



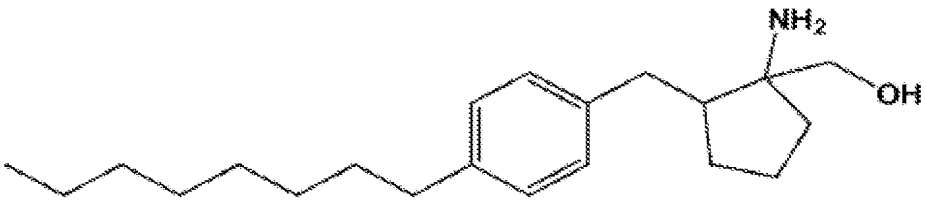
(5')-2-amino-2-methyl-4-(4-octylphenyl)butan-1-ol,



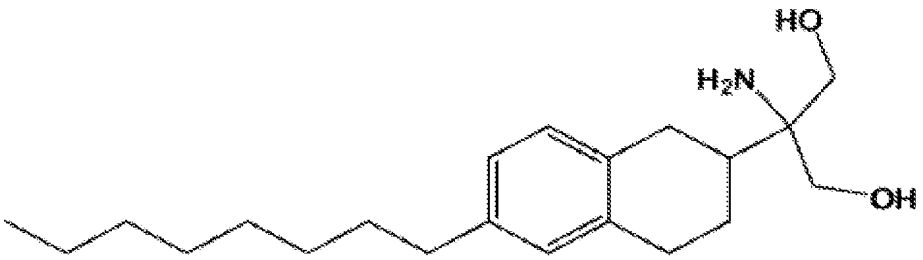
(1 -amino-3-(4-octylphenyl)cyclobutyl)methanol,



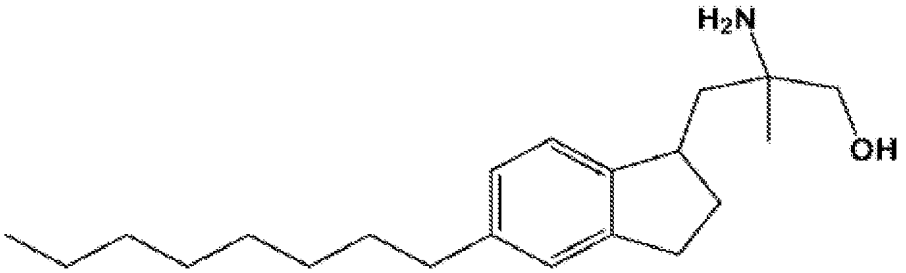
(1 -amino-3-(4-octylphenyl)cyclopentyl)methanol,



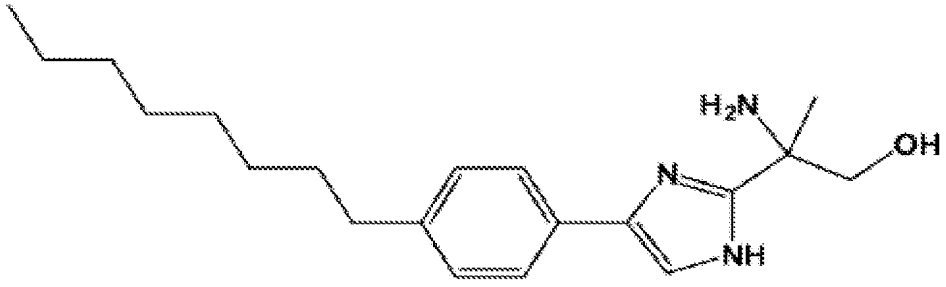
(1 -amino-2-(4-octylbenzyl)cyclopentyl)methanol,



2-amino-2-(6-octyl- 1 ,2,3 ,4-tetrahydronaphthalen-2-yl)propane-1,3-diol,



2-amino-2-methyl-3-(5-octyl-2,3-dihydro-li/-inden-1-yl)propan-1-ol, and



2-amino-2-(4-(4-octylphenyl)-lH-imidazol-2-yl)propan-l-ol.

[0041] The bone matrix implants may further comprise a tissue, an organ, a biocompatible matrix and/or a mixture of two or more thereof. The bone matrix implants may comprise bone, cartilage, and/or connective tissue.

[0042] The invention also relates to methods of preparing a film of bone matrix on a surface. The method of preparing a film of bone matrix on a surface according to some embodiments of the present invention comprises dissolving a bone in an amount of an anhydrous solvent comprising (i) hexafluoroisopropanol (HFP), 2, 2, 2-trifluoroethanol (TFE) or isopropanol, and (ii) at least about 5 wt% trifluoroacetic acid (TFA) sufficient to form a bone matrix solution; exposing the bone matrix solution to conditions sufficient to evaporate an amount of the solvent to form a colloidal gel; coating the surface with the colloidal gel, and exposing the coated surface to conditions sufficient to further evaporate an amount of solvent to form a film. The invention further relates to a synthetic surface coated with the bone matrix produced by the method described herein.

[0043] In some embodiments, the film of the bone matrix may be formed on a surface of another biocompatible matrix. In further embodiments, the biocompatible matrix is an implantable biocompatible matrix that can function as a support system for the bone matrix described herein. A biocompatible matrix should be non-toxic, non-eliciting or stimulating severe inflammatory response or immunological rejections, and devoid of other undesired reactions at the implantation site. In one embodiment, the biocompatible matrix is another bone matrix or cartilage or connective tissue.

[0044] In some embodiments, the film of the bone matrix may be formed on a surface of biocompatible matrix including, but not limited to, bone graft implants, synthetic bone graft materials in forms of particulates, sheet, or blocks, tendon and/or ligament in bone tunnels, prosthetic implant, for example, for hip, shoulder, knee, or ankle, and trabecular metal. In further embodiments, the bone graft implants may include allograft or xeno-graft. In yet further embodiments, the bone graft implants may include structural bone implants including, but not limited to, monolithic or composite spinal implants (e.g., VERTIGRATs from LifeNet Health) and bone struts or blocks. In yet further embodiments, the bone graft implants may include demineralized, or non-demineralized bone particulates, including, but not limited to, cortical, cancellous, or cortical cancellous bone. In other embodiments, the film of the bone matrix may be formed on cell or tissue culture surface.

[0045] Suitable biocompatible matrices include, but are not limited to, porous biocompatible scaffolds into which bone cells or progenitor cells may migrate. Osteogenic or chondrogenic cells, i.e., cells involved in the process of deposition of new bone material or cartilagenous material, respectively, can often attach to such porous biocompatible matrices, which can then serve as scaffolding for bone and cartilage tissue growth. Cells involved in the process of deposition of new ligament or tendon material can also attach to such porous biocompatible matrices. For certain applications, the biocompatible matrix should have sufficient mechanical strength to maintain its three dimensional structure and help support

the immobilization of the bone segments being united or grafted together. Porous biocompatible matrices which provide scaffolding for tissue growth can accelerate the deposition of new bone or the rate of bone growth and are said to be "osteoconductive." Osteoconductive biocompatible matrices are especially useful in the pharmaceutical compositions described herein. Porous biocompatible matrices which provide scaffolding for tissue growth can accelerate the deposition of new cartilage or the rate of cartilage growth and are said to be "chondroconductive." Osteoconductive biocompatible matrices are especially useful in the pharmaceutical compositions described herein. Chondroconductive biocompatible matrices are especially useful in the pharmaceutical compositions described herein. The osteoinductive or chondroinductive activity of the surface coated with the bone matrix may or may not be altered, including but not limited to, enhanced activity, relative to a surface without the bone matrix coating or a natural surface without any coating. Thus, the osteoconductive or chondroconductive activity of the biocompatible matrices treated with the bone matrix of the present invention may be enhanced compared to matrices not treated with the bone matrix described herein. Of course, the biocompatible matrices are considered to be osteoconductive or chondroconductive if cells within the biocompatible matrix begin to differentiate into more osteoblast-like or chondrocyte-like appearing or functional cells, respectively.

[0046] The biocompatible matrices according to some embodiments of the present invention can be derived from natural sources or they can be synthetic or a mixture of both. Biocompatible matrices from natural sources may also comprise natural polymers, including, but not limited to, collagen, hyaluronic acid, alginate, albumin, fibrinogen-fibrin, chitosan, elasin, laminin, connective tissues, intervertebral disc, cortical or cancellous bone, demineralized or mineralized bone, fascia lata, dermis, muscle, ligament, tendon, cartilage including articular/hyaline cartilage, elastic cartilage, and fibrocartilage, a mixture thereof, and mixture of reconstituted tissue. Biocompatible matrices from synthetic sources refer to any material not produced by living organisms, which may include, not limited to, the synthetic material made up of organic components, inorganic components, or a mixture thereof. In some embodiments, a synthetic biocompatible matrix may comprise an organic synthetic polymer, such as poly(lactic-co-glycolic acid), polycaprolactone (PCL), polyglycolic acid (PGA), polylactic acid (PLA), polyhydroxybutyrate (PHB), Poly(ethylene glycol) (PEG), poly(ethylene oxide) (PEO)), and others. In some embodiments, a tissue, an organ, or biocompatible matrix comprising at least one of alginate, chitosan, collagen, gelatin, hyaluronic acid, a fibronectin, an elastin, a laminin, and a proteoglycan may be employed. In certain embodiments, a biocompatible matrix comprising inorganic components, such as hydroxyapatite, calcium sulfate, octacalcium phosphate, calcium phosphate, macroporous calcium metaphosphate ceramic, β-tricalcium phosphate, metal, metal alloy, and others, may be used. A biocompatible matrix used in certain embodiments of the present invention may be prepared by

demineralizing, decellularizing or devitalizing a tissue or an organ and cells may be seeded onto the biocompatible matrix.

[0047] In some embodiments, the bone matrix described herein may be applied to another biocompatible matrix and may be incubated at conditions permitting the generation of a matrix that is partially or fully coated with the bone matrix. In some embodiments, incubation may be carried out at about 40 °C or lower, or between about 10 ºC and about 37 ºC, or about 20 ºC and about 37 ºC. Incubation may be carried out for between at least about 2 minutes and about 120 minutes, about 3 minutes and about 100 minutes, about 4 minutes and about 80 minutes, about 5 minutes and about 60 minutes, and about 5 minutes and about 30 minutes in certain embodiments. Incubation may be performed under static or dynamic conditions, such as with agitation, shaking, stirring, mixing, horizontal motion, rocking, and others.

[0048] In some embodiments of the present invention, a biocompatible matrix may be lyophilized before the bone matrix is applied to the biocompatible matrix. In certain embodiments, the bone matrix may be coated on the biocompatible matrix, and the coated matrix may be subsequently lyophilized. The lyophilized, coated matrix can then be rehydrated before it is used. Further, the cells can be seeded onto the matrix before implantation.

[0049] Examples of suitable osteoconductive or chondroconductive biocompatible matrices include but are not limited to, collagen (e.g., bovine dermal collagen), fibrin, calcium phosphate ceramics (e.g., hydroxyapatite and tricalcium phosphate), calcium sulfate, guanidine-extracted allogenic bone and combinations thereof. A number of suitable biocompatible matrices are commercially available, such as Collograft™ (Collagen Corporation), which is a mixture of hydroxyapatite, tricalcium phosphate and fibrillar collagen, and Interpore™ (Interpore International), which is a hydroxyapatite biomatrix formed by the conversion of marine coral calcium carbonate to crystalline hydroxyapatite.

[0050] A number of synthetic biodegradable polymers can serve as osteoconductive or chondroconductive biocompatible matrices with sustained release characteristics. Descriptions of these polymers can be found in Behravesh (1999) Clinical Orthopaedics 367, S118 and Lu (2000) Polymeric Delivery Vehicles for Bone Growth Factors in Controlled Drug Delivery: Designing Technologies for the Future, Park and Mrsny eds., American Chemical Society, which is incorporated herein in its entirety. Examples of these polymers include polyα -hydroxy esters such as polylactic acid/polyglycolic acid homopolymers and copolymers, polyphosphazenes (PPHOS), polyanhydrides and poly(propylene fumarates).

[0051] Polylactic acid/polyglycolic acid (PLGA) homo and copolymers are well known in the art as sustained release vehicles. The rate of release can be adjusted by the skilled artisan by variation of polylactic acid to polyglycolic acid ratio and the molecular weight of the polymer (see Anderson (1997) Adv. Drug Deliv. Rev. 28:5. The incorporation of PEG into the polymer as a blend to form microparticle matrices allows further alteration of the release profile of the active ingredient (see Cleek (1997) J. Control Release 48, 259). Ceramics such as calcium phosphate and hydroxyapatite can also be incorporated into the formulation to improve mechanical qualities.

[0052] In one embodiment, the biocompatible matrices used in the methods of the present invention are other types of bone matrices. For example, the bone matrix prepared by the methods described herein can be coated on another type of bone matrix. As used herein, the other types of the bone matrix may be a biocompatible matrix derived from or including elements of natural bone. In some embodiments, the natural bone is mineralized, partially demineralized, demineralized, cancellous, cortical, or cortical cancellous bone. The bone matrices used herein may or may not include additional synthetic components not typically found in bone tissue. Other embodiments include methods utilizing a biocompatible matrix derived from cartilage, other soft tissues such as the dermis, connective tissue, fascia, small intestine submucosa, serous membrane, pericardium, tendon, ligament, muscle, adipose tissue, myelin, blood vessels, base membrane, amniotic membrane and others. A biocompatible matrix prepared from hyaline cartilage, fibrocartilage or elastic cartilage, may be employed in some embodiments. A biocompatible matrix may be prepared from hyaline cartilage found in the condyle, tibial plateau, femoral head, humeral head, costal cartilage, or fibrocartilage found in intervertebral discs, or elastic cartilage found in the epiglottis or ear. In certain embodiments, a biocompatible matrix derived from natural sources that has been optionally cleaned, disinfected, chemically modified, decellularized, particulated, homogenized, lyophilized, gamma ray irradiated, and/or plasticized may be used. Any of the biocompatible matrices used herein may or may not include additional synthetic components not typically found in such tissue.

[0053] In one specific embodiment, the bone or cartilage biocompatible matrices may be demineralized or decellularized, respectively. Examples of demineralized matrices and methods of making are described in U.S. Patent No. 6,189,537 and 6,305,379, which are incorporated herein in its entirety.

[0054] The biocompatible matrix, tissue, or organ used in certain embodiments of the present invention may be in the form of a powder, particulates, sheets, fibers, gels, putties, paste, blocks, cylinders, sponges, meshes, films, slices, curls, flakes, or wedges, among others. In certain embodiments of the present invention, the biocompatible matrix, tissue, or organ treated with the bone matrix by the methods described herein may be in the form of a powder, fibers, putty, or a sponge. In further embodiments, the

sponge can include, for example, the implant having sponge-like structures disclosed in the co-pending, commonly-assigned patent application PCT/US09/04556 entitled "Composition for a Tissue Repair Implant and Methods of Making the Same" filed on August 7, 2009, which is incorporated herein in its entirety. The treated matrices can be used in any of the methods of the present invention.

[0055] The invention also relates to methods of cell culture comprising culturing cells on a cell culture surface coated with the bone matrix produced by the method described herein. As used herein, cell culture refers to the maintenance of cells in an artificial environment, commonly referred to as an *in vitro* environment. 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, organs, organ systems or whole organisms. The cells used in the culture methods disclosed herein can be any prokaryotic or eukaryotic cell. The cell type used in the culture methods disclosed herein need not be from the same species from which the cell support compositions derive. In addition, the cells may be from an established cell line, or they may be primary cells or genetically engineered cells.

[0056] For example, the invention provides for growing and/or culturing cells on a cell culture surface coated with the bone matrix produced by the method described herein. "Growing and/or culturing cells cells on a cell culture surface" includes traditional cell culture methods as well as placing on a surface in any setting, such as in natural or synthetic biocompatible matrices or tissues. The cells may be mammalian, such as but not limited to human, bovine, porcine, murine, ovine, equine, canine, feline and others. In some embodiments, the cells that are cultured on the substrate coated with the bone matrix are stem cells. As used herein, a stem cell is used as it is in the art and means a cell that has the ability to divide and give rise to one daughter cell that may be at least partially differentiated and to another daughter cell that retains the developmental potential of the mother cell. As used herein, stem cells can be adipose derived stem cells, dental pulp stem cells, adult stem cells (ASCs), embryonic stem cells (ESCs), committed progenitor cells, and/or induced pluripotent stem cells (iPSCs). In further embodiments, the bone matrix coated surface can be used in *in vitro* methods for supporting cell growth and proliferation as well as for increasing osteogenesis, chondrogenesis, or ligament/tendon genesis in the stem cells cultured on the bone matrix coated surface.

[0057] In some embodiments, the cells may be mesenchymal stem cells, such as adipose-derived stem cells, embryonic stem cells, progenitor cells, differentiated cells, undifferentiated cells, and/or -pluripotent stem cells. Appropriate cells may also include, but are not limited to cells of the ectodermal lineage, cells of the mesodermal lineage, and cells of the endodermal lineage. Examples of cells of the ectodermal lineage include but are not limited to keratinocytes, osteoblasts, chondrocytes, neurons. Examples of cells

of the mesodermal lineage include but are not limited to myoblasts, adipocytes, fibroblasts, endothelial cells, or stromal cells. Examples of cells of the endodermal lineage include but not limited to epithelial cells of the auditory tube, the respiratory tract, such as trachea, bronchi, and alveoli of the lungs, the gastrointestinal tract, the urinary bladder and epithelial cells lining all glands. The cells may also be primary cells derived from tissues or organs. Appropriate cell lines used in the present invention may include but are not limited to mesenchymal cell lines, preosteoblastic cell lines, osteoblastic cell lines, and chondroblastic cell lines. The bone matrix may be coated directly into a tissue, organism or other setting such as a matrix, including, but not limited to, bone matrices.

[0058] In some embodiments, the cells may be derived from autologous or allogeneic sources. The cells may be differentiated cells including chondrocytes, osteoblasts, osteoclasts, endothelial cells, epithelial cells, fibroblasts, and periosteal cells. Additionally, the cells may be totipotent, pluripotent, multipotent, progenitor, or adult somatic stem cells. The stem cells may be derived from embryos, placenta, bone marrow, adipose tissue, blood vessel, amniotic fluid, synovial fluid, synovial membrane, pericardium, periosteum, dura, peripheral blood, umbilical blood, menstrual blood, baby teeth, nucleus pulposus, brain, skin, hair follicle, intestinal crypt, neural tissue, muscle. The stem cells may be derived from skeletal muscle, smooth muscle, and cardiac muscle. The stem cells may be derived from genetic reprogramming of mature cells, such as induced pluripotent stem cells (iPSCs). All cells may further be derived from living or recently deceased donors.

[0059] Any cell described herewith may be cultured on a cell culture surface coated with the bone matrix produced by the method described herein for between about 15 minutes and about 4 weeks, about 2 hours and about 2 weeks, about 2 hours and about 1 week, about 2 hours and about 72 hours, about 24 hours and about 72 hours, or about 24 hours and about 96 hours, at between about 20 ºC and about 40 ºC or about 30°C and about 37°C, in an atmosphere containing between about 1% CO_2 and about 10% CO_2 or about 4% CO_2 and about 6% CO_2, in certain embodiments. In some embodiments of the present invention, cells may be cultured in the presence of one or more modified growth factors and (1) a tissue or an organ, (2) a matrix, or (3) a combination thereof. Cells that have been cultured in the presence of one or more modified growth factors in a cell culture medium may subsequently be applied to a matrix, a tissue, an organ or a combination thereof, in certain embodiments.

[0060] The invention also relates to methods of promoting osteoinductivity, with the methods comprising culturing cells on a cell culture surface coated with the bone matrix produced by the method described herein. As used herein, “osteoinductivity” can refer to causing cells to differentiate into cells that are more osteoblast-like in phenotype, or the term can refer to increasing the proliferation of

osteoblasts, or both. The cells, prior to culture on the bone matrix of the present invention, may be undifferentiated or partially differentiated cells. The cells may be present in culture or in a tissue, organ or portion thereof or in an organism. The osteoinductive activity of the surface coated with the bone matrix may or may not be altered, including but not limited to, enhanced activity, relative to a surface without the bone matrix coating or a natural surface without any coating.

[0061] The invention also relates to methods of promoting chondroinductivity, with the methods comprising culturing cells on a cell culture surface coated with the bone matrix produced by the method described herein. As used herein, "chondroinductivity" can refer to causing cells to differentiate into cells that are more chondrocyte-like in phenotype, or the term can refer to increasing the proliferation of chondrocytes, or both. The cells, prior to culture on the bone matrix of the present invention, may be undifferentiated or partially differentiated cells. The cells may be present in culture or in a tissue, organ or portion thereof or in an organism. The chondroinductive activity of the surface coated with the bone matrix may or may not be altered, including but not limited to, enhanced activity, relative to a surface without the bone matrix coating or a natural surface without any coating.

[0062] The invention also relates to methods of promoting ligament/tendon differentiation, with the methods comprising culturing cells on a cell culture surface coated with the bone matrix produced by the method described herein. As used herein, "ligament/tendon differentiation" can refer to causing cells to differentiate into cells that are more ligament and/or tendon-like in phenotype, or the term can refer to increasing the proliferation of ligament and/or tendon, or both. The cells, prior to culture on the bone matrix of the present invention, may be undifferentiated or partially differentiated cells. The cells may be present in culture or in a tissue, organ or portion thereof or in an organism. The ligament/tendon differentiation activity of the surface coated with the bone matrix may or may not be altered, including but not limited to, enhanced activity, relative to a surface without the bone matrix coating or a natural surface without any coating.

[0063] There are variety of osteoblast, chondrocyte, ligament/tendon differentiation markers that can be measured to assess osteoinductivity, chondroinductivity, or ligament/tendon differentiation, respectively. For example, cells express alkaline phosphatases during the early stages of differentiation toward osteoblast lineages. Therefore, *in vitro* alkaline phosphatase assays may be used to evaluate osteoinductivity in cells cultured on the surface coated by the bone matrix by the methods described herein. The ability of the bone matrix prepared by the methods of the present invention to stimulate or induce the alkaline phosphatase expression in an otherwise non-bone forming cells, such as myoblast (C2C12 cells), would indicate that the bone matrix of the present invention has osteoinductive activity. In

these assays, cells cultured on a surface not coated by the bone matrix of the present invention and on a natural surface without any coating are used as negative controls to show that the baseline alkaline phosphatase expression on non-bone forming cells. The baseline of the osteoblastic markers in the negative control need not be zero, meaning that the cells in the negative control group may have at least some level of phenotypic marker(s). Accordingly, an "osteoinductive" surface of the present invention would simply cause an increase in the osteoblastic markers in experimental cells over control grown on the bone matrix prepared by the methods described herein. Similarly, chondrocyte markers, including but not limited to type X collagen, type II collagen, Sox 9, Aggrecan. Matrilin-1 and CEP-68, to name a few, can be used to assess chondroinductive potential. Moreover, ligament/tendon markers, including but not limited to scleraxis, can be used to assess ligament/tendon differentiation potential.

[0064] Moreover, osteoinductivity, chondroinductivity, and ligament/tendon differentiation may be determined in tissue culture by investigating the ability of the bone matrix prepared by the methods of the present invention to differentiate or induce osteoblast phenotype, chondrocyte phenotype, ligament/tendon cell phenotype in cultured cells, such as primary cells, cell lines, or explants. For example, the cells may display increased production of a marker characteristic of osteoblasts and/or chondrocytes, such as alkaline phosphatase, etc. For example, the osteoinductive, chondroinductive, ligament/tendon differentiation potentials of the surface coated with bone matrix may be more than 0.2, 0.4, 0.6, 0.8, 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 times greater than those not coated with the bone matrix. In another example, the osteoinductive, chondroinductive, ligament/tendon differentiation potentials of the culture on the bone matrix implant described herein may be more than 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 500 or even 1000 times greater than those of the unmodified growth factors. Of course, this indicates that lower concentrations of modified growth factor, compared to unmodified growth factor are required to achieve the same effects.

[0065] Osteoinductivity, chondroinductivity, ligament/tendon differentiation, for assessing the bone, cartilage, ligament or tendon forming potential induced by the bone matrix of the present invention in a location such as muscle, may also be evaluated using a suitable animal model. For example, intramuscular implantation into a rodent has been used as a model to assess osteoinductive activity of bioactive factors.

[0066] The invention also relates to methods of promoting cell attachment, proliferation or maintaining the differentiated state or preventing de-differentiation of osteoblasts, chondrocytes, ligament cells, tendon cells and/or any cell type disclosed herein with the methods comprising culturing the cells on a cell culture surface coated with the bone matrix produced by the method described herein. The

proliferative activity of the surface coated with the bone matrix may or may not be altered, including but not limited to, enhanced activity, relative to a surface without the bone matrix coating or a natural surface without any coating.

[0067] Mitogenicity may be assessed by investigating cell proliferation induced by the bone matrix prepared by the methods of the presentinvention using various in vitro assays that measure metabolic activity, such as MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, alamarBlue® assay, and others. The alamarBlue® assay uses a non-cytotoxic reduction-oxidation indicator to measure cell metabolic activity, making it a nondestructive assay for assessing the mitogenic activity of the bone solution described herein. Proliferation can also be assessed by measuring DNA quantification, such as by using a PicoGreen™ DNA assay, radioactive labeling of DNA synthesis, such as [^{3H}]thymidine labeling or BrdU incorporation. Proliferation can also be assessed via manual cell counting, such as staining cells with trypan blue and counting with a hemacytometer.

[0068] The invention also relates to methods of increasing or promoting osteogenesis, chondrogenesis, or ligament/tendon genesis in cells. The methods may comprise culturing the cells on a cell culture surface coated with the bone matrix produced by the method described herein. As used herein, "osteogenesis" is the deposition of new bone material or formation of new bone, including, but not limited to, intramembranous osteogenesis and endochondral osteogenesis. As used herein, "chondrogenesis" is the deposition new cartilage material or formation of new cartilage. As used herein, "ligament/tendon genesis" is the deposition new ligament and/or tendon material or formation of new ligament and/or tendon. The osteogenic, chondrogenic, ligament, or tendon inducing activity of the surface coated with the bone matrix may or may not be altered, including but not limited to, enhanced activity, relative to a surface without the bone matrix coating or a natural surface without any coating. The cells may include cells in any tissue in which bone, cartilage, ligament, or tendon formation is desired, such as, but not limited to, bone, cartilage, ligament, muscle, tendon, etc.

[0069] The invention also relates to methods of treating a tissue or organ defect or injury, for example, a musculoskeletal, dental or soft-tissue defect or injury, in an animal comprising administering (1) cells cultured on the surface coated with the bone matrix produced by the methods described herein and/or (2) the bone matrix implant described herein to the tissue or organ defect (e.g. osseous defects, defects in cartilage, ligament, tendon, spinal disk, and tendon insertion site to bone).

[0070] The invention further relates to methods of treating a tissue or an organ defect or injury, for example a musculoskeletal, dental or soft-tissue defect, in an animal by applying a biocompatible matrix

partially or fully coated with the bone matrix by the methods described herein to the defect, and application to the defect may be accomplished by injecting the coated biocompatible matrix into the defect, inserting the coated biocompatible matrix between tissue or organ, or placing the coated biocompatible matrix on top of the defect. The present invention is also directed to treating a defect or injury in an organ by applying a coated biocompatible matrix to the defect.

[0071] In yet another embodiment, cells may be seeded onto a biocompatible matrix coated with the bone matrix prepared by the methods provided herein. The cells seeded on the coated matrix can be any cell, such as but not limited to, osteoblasts, chondrocytes, ligament cells, tendon cells, progenitor cells, and stem cells disclosed herein or otherwise known in the art. The seeded cells may be allowed to proliferate and possibly attach to the matrix. Methods of seeding cells onto matrices, such as collagen matrix coated with the bone matrix prepared by the methods provided herein, are well known in the art.

[0072] Any of the methods of the present invention can be performed in virtually any setting, such as an in vivo, ex vivo, in situ or in vitro setting. For example, methods of promoting osteogenesis, chondrogenesis, or tendon/ligament inducing activities in cells may be performed in cell culture, may be performed in seeded cells on matrix, or may be performed in an intact organism. Moreover, any combination of any two or more of any of the embodiments described herein are contemplated.

[0073] The invention further relates to bone matrix scaffolds comprising one or more bone matrix nanofibers. In one aspect, the bone matrix scaffold may essentially consist of the bone matrix nanofibers. In another aspect, the bone matrix scaffold may consist of the bone matrix nanofibers. The bone matrix scaffold described herein may be biocompatible. A biocompatible bone matrix scaffold may be non-toxic, non-eliciting or stimulating severe inflammatory response or immunological rejections, and/or devoid of other undesired reactions at the implantation site. The bone matrix nanofiber described herein comprises bone.

[0074] The bone matrix scaffold and bone matrix nanofiber of the present invention may type I collagen. In some embodiments, total type I collagen may be present in the bone matrix scaffold and/or bone matrix nanofiber of the present invention in an amount from about 50% to about 95%, from about 60% to about 95%, from about 70% to about 95%, or from about 80% to about 95% by weight in it protein content. In additional embodiments, total type II collagen may be present in the bone matrix scaffold and/or bone matrix nanofiber of the present invention in an amount about 10% or less, about 5% or less, about 3% or less, or about 1% or less by weight in it protein content. In further embodiments, collagen type II may be absent from the bone matrix scaffold and/or bone matrix nanofiber of the present invention. The types of

collagen that are present or absent in the composition can be easily assessed using routine methods in the art. Methods of identifying and quantifying type of collagen are well known in the art, as disclosed, for example in Schnaper, H.W. and Kleinman, H.K., Pediatr. Neprol., 7:96-104 (1993), which is incorporated by reference.

[0075] In one aspect, the bone matrix nanofiber described herein may have less than about 8 wt%, 7 wt%, 6 wt%, 5 wt%, 4 wt%, or 3 wt% residual calcium. In another aspect, the average residual calcium amount in the bone matrix scaffold described herein may be less than about 8 wt%, 7 wt%, 6 wt%, 5 wt%, 4 wt%, or 3 wt%.

[0076] The term nanofiber as used herein means a fiber comprising a diameter of about 1000 nm or less. Relative to the parent bulk material, nano-features can impart many extraordinary properties to macrostructures, including superior mechanical, electrical, optical and magnetic properties, adding surface functionality and yielding high surface area. Matrices of nanofibers of varying diameters may show a range of variable surface properties (*e.g.* hydrophobicity and hydrophilicity), porosities, and usually superior mechanical properties (*e.g.* tensile strength, stiffness) relative to the material in other forms.

[0077] Nanofibers as matrices recently found applications for orchestrating regeneration and cellular repair processes, whereby the nano-architecture itself provides the specific developmental or morphogenetic cues. Along with proteins, growth factors, and therapeutic agents, nanofibers matrices may influence the cellular response. Further, scaffold fiber alignment plays an important biological role, such that, for example, aligned nanofibers guide cell migration, having been applied to regenerating injured neurons by guiding outgrowth. The matrix of electrospun nanofibers may mimic the natural ECM, including properties such as high porosity and high surface to volume ratios, which facilitate oxygen transport and cell waste removal, assist in cell migration across the implanted scaffold, and make delivery of therapeutic agents possible. In implanted structures for clinical use, nanofiber matrices may have further advantages of improved biocompatibility and reduced friction, and thus reduced irritation, upon delivery and as implanted.

[0078] In some embodiments, the nanofiber described herein may have an average diameter of about 1000 nm or less, 900 nm or less, 800 nm or less, 700 nm or less, 600 nm or less, 500 nm or less, 400 nm or less, 300 nm or less, 200 nm or less, 100 nm or less, 50 nm or less, 20 nm or less, or 10 nm or less. In additional embodiments, the total nanofibers in the bone matrix scaffolds described herein may have an average diameter of about 1000 nm or less, 900 nm or less, 800 nm or less, 700 nm or less, 600 nm or less,

500 nm or less, 400 nm or less, 300 nm or less, 200 nm or less, 100 nm or less, 50 nm or less, 20 nm or less, or 10 nm or less.

[0079] In one aspect, the bone matrix nanofiber described herein may have a length from about 0.1 cm to about 10 cm, from about 1 cm to about 10 cm, from about 0.1 cm to about 20 cm, from about 1 cm to about 20 cm, from about 10 cm to about 20 cm, from about 0.1 cm to about 30 cm, from about 1 cm to about 30 cm, from about 10 cm to about 30 cm, from about 20 cm to about 30 cm, from about 0.1 cm to about 40 cm, from about 1 cm to about 40 cm, from about 10 cm to about 40 cm, from about 20 cm to about 40 cm, from about 30 cm to about 40 cm, from about 0.1 cm to about 50 cm, from about 1 cm to about 50 cm, from about 10 cm to about 50 cm, from about 20 cm to about 50 cm, from about 30 cm to about 50 cm, from about 40 cm to about 50 cm, 0.1 cm to about 60 cm, from about 1 cm to about 60 cm, from about 10 cm to about 60 cm, from about 20 cm to about 60 cm, from about 30 cm to about 60 cm, or from about 40 cm to about 60 cm. In another aspect the total nanofibers in the bone matrix scaffolds described herein may have an average length from about 0.1 cm to about 10 cm, from about 1 cm to about 10 cm, from about 0.1 cm to about 20 cm, from about 1 cm to about 20 cm, from about 10 cm to about 20 cm, from about 0.1 cm to about 30 cm, from about 1 cm to about 30 cm, from about 10 cm to about 30 cm, from about 20 cm to about 30 cm, from about 0.1 cm to about 40 cm, from about 1 cm to about 40 cm, from about 10 cm to about 40 cm, from about 20 cm to about 40 cm, from about 30 cm to about 40 cm, from about 0.1 cm to about 50 cm, from about 1 cm to about 50 cm, from about 10 cm to about 50 cm, from about 20 cm to about 50 cm, from about 30 cm to about 50 cm, from about 40 cm to about 50 cm, 0.1 cm to about 60 cm, from about 1 cm to about 60 cm, from about 10 cm to about 60 cm, from about 20 cm to about 60 cm, from about 30 cm to about 60 cm, or from about 40 cm to about 60 cm.

[0080] In one aspect, the bone matrix scaffold has an average porosity from about 60% to about 70%, from about 60% to about 80%, from about 60% to about 90%, from about 60% to about 95%, from about 70% to about 80%, from about 70% to about 90%, from about 70% to about 95%, from about 80% to about 90%, or from about 80% to about 95%.

[0081] In some embodiments, the bone matrix nanofiber described herein may be prepared, for example, by electrospinning, meltblowing, bicomponent spinning, forcespinning, flash-spinning, extrusion, core-sheath electrospinning with or without copolymer, or self-assembly.

[0082] Alignment of the bone matrix nanofibers in the bone matrix scaffold may be measured by a fast Fourier transform (FFT) analysis. For example, the FFT analysis may be performed by the methods described in *Measuring fiber alignment in electrospun scaffolds: a user's guide to the 2D fast Fourier*

transform approach, Ayres CE, Jha BS, Meredith H, Bowman JR, Bowlin GL, Henderson SC, Simpson DG. J Biomater Sci Polym Ed. 2008; 19(5):603-21, which is incorporated by reference in its entirety. In some embodiments, FFT result of the bone matrix nanofibers described herein may have adjacent major peaks with about 180° apart from each other.

[0083] In one aspect, the bone matrix scaffold and/or the bone matrix nanofibers may further comprise an accessory polymer. An "accessory polymer" refers to a polymer that may be added to the bone matrix nanofibers and have any effect on their physical, chemical, and/or biological properties (*e.g.* tensile strength, hydrophillicity, biocompatibility). For example, the accessory polymer may be selected from the group consisting of polycaprolactone, poly(glycolic acid), poly(lactic acid), polydioxanone, poly (lactide-co-glycolide) copolymers, polyesters polysaccharides, polyhydroxyalkanoates , starch, polylactic acid, cellulose, proteins, agar, silks, alginate, collagen/gelatin, carrageenan, elastin, pectin, resilin, konjac, adhesives, gums, polyamino acids, polysaccharides, soy, zein, wheat gluten, casein, chitin/chitosan, serum albumin, hyaluronic acid, lipids/s urfactants, xanthan, acetoglycerides, waxes, surfactants, dextran, emulsan, gelian, polyphenols, levan, lignin, curd, ian, tannin, polygalactosamine, humic acid, shellac, pullulan , poly-gamma-glutamic acid, elsinan, natural rubber, yeast glucans, and synthetic polymers from natural fats and oils.

[0084] In another aspect, the bone matrix scaffold and/or the bone matrix nanofibers may further comprise one or more biodegradable, biocompatible polymers. The biodegradable, biocompatible polymers may include, but is not limited to, ethylene vinyl acetate, polyanhydricles, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. The biodegradable, biocompatible polymers may further include a number of synthetic biodegradable polymers that can serve as osteoconductive or chondroconductive biocompatible matrices with sustained release characteristics. Descriptions of these polymers can be found in Behravesh (1999) Clinical Orthopaedics 367, S118 and Lu (2000) Polymeric Delivery Vehicles for Bone Growth Factors in Controlled Drug Delivery: Designing Technologies for the Future, Park and Mrsny eds., American Chemical Society, which is incorporated herein in its entirety. Examples of these polymers include polyα -hydroxy esters such as polylactic acid/polyglycolic acid homopolymers and copolymers, polyphosphazenes (PPHOS), polyanhydrides and poly(propylene fumarates).

[0085] Polylactic acid/polyglycolic acid (PLGA) homo and copolymers are well known in the art as sustained release vehicles. The rate of release can be adjusted by the skilled artisan by variation of polylactic acid to polyglycolic acid ratio and the molecular weight of the polymer (see Anderson (1997) Adv. Drug Deliv. Rev. 28:5. The incorporation of PEG into the polymer as a blend to form microparticle

matrices allows further alteration of the release profile of the active ingredient (see Cleek (1997) J. Control Release 48, 259). Ceramics such as calcium phosphate and hydroxyapatite can also be incorporated into the formulation to improve mechanical qualities.

[0086] In another aspect, the bone matrix scaffold and/or the bone matrix nanofibers may further comprise an extracellular matrix component. For example, the extracellular matrix component may include, but is not limited to, collagen, glycosaminoglycans, osteocalcin, osteonectin, bone sialo protein, osteopontin, or mixtures thereof.

[0087] In another aspect, the bone matrix scaffold and/or the bone matrix nanofibers may further comprise a bioactive factor.

[0088] In another aspect, the bone matrix scaffold and/or the bone matrix nanofibers may further comprise one or more biocompatible material selected from the group consisting of collagen, polycaprolactone, poly(glycolic acid), poly(lactic acid), polydioxanone, poly (lactide-co-glycolide) copolymers, polyesters polysaccharides, polyhydroxyalka noates, starch, polylactic acid, cellulose, proteins, agar, silks, alginate, collagen, gelatin, carrageenan, elastin, pectin, resilin, konjac, adhesives, various gums, polyamino acids, polysaccharides, soy, zein, wheat gluten, casein, chitin, chitosan, serum albumin, hyaluronic acid, lipids, surfactants, xanthan, acetoglycerides, waxes, dextran, emulsan, gelian, polyphenols, levan, lignin, curd, ian, tannin, polygalactosamine, humic acid, cellulose, shellac, pullulan, poly-gamma-glutamic acid, elsinan, natural rubber, yeast glucans, synthetic polymers from natural fats and oils.

[0089] In some embodiments, the bone matrix scaffold of the present invention may further comprise a support. In additional embodiments, the bone matrix nanofibers described herein may be attached to and/or cover the support.

[0090] The invention also relates to methods of preparing the bone matrix scaffold. The method of preparing the bone matrix scaffold according to some embodiments of the present invention comprises preparing a bone matrix solution. The method of preparing a bone matrix solution according to some embodiments of the present invention comprises dissolving bone in an anhydrous solvent as described herein.

[0091] In one aspect, once the bone matrix solution is prepared, the bone matrix solution may be fabricated into nanofibers and mesh through electrospinning technologies as described in

US2010/0120115, which is incorporated by reference. Once fabricated into a scaffold, the bone matrix can be placed into cell culture environment as disclosed herein.

[0092] In another aspect, the bone matrix solution described herein may be fabricated into nanofibers and mesh using the exemplary electrospinning setup as shown in FIGURE 2. Electrospinning typically involves a polymer solution (or melt) maintained at its surface tension on the tip of a nozzle via a syringe in a pump. For example, when sufficiently high voltage is introduced (*e.g.* 15-40kV) to the polymer in solution to create a charge imbalance, the solution is drawn towards a grounded collector through the static electric field. As the polymer erupts from the needle and the assembling polymer whip through space, it may be subjected to a series of stretching and bending instabilities, resulting in plastic stretching and elongation to minimize these instabilities generated by repulsive electrostatic forces. As the polymer travels through space and rapidly thins into a fine stream, the solvent may evaporate, and the polymer may assemble into fibers, leaving dry nano- to micro-scale fibers of tailorable physical attributes on the collector.

[0093] Additionally, when high electrical potential is applied to a low viscosity polymer melt or solution, electrospraying may occur, which is typified by the polymer jet breaking down into fine droplets. It is therefore possible to produce particles or nano- to micro-sphere particles, fibers and bead-and-string type structures via this process from simply altering the solution or electrospinning apparatus setup variables. Among the variable in electrospinning that can be changed to tailor the final product, such parameters as polymer solution properties, applied electrical potential, polymer molecular weight, polymer solution flow rate, distance between spinner and collector, ambient parameters (humidity, air velocity, temperature) and motion of the collecting target may be altered to form defect-free nanofibers of controlled fiber distribution, diameter and alignment.

[0094] In some embodiments, the method of preparing the bone matrix scaffold according to some embodiments of the present invention comprises preparing a bone matrix solution by dissolving bone in an amount of an anhydrous solvent comprising (i) hexafluoroisopropanol (HFP) or 2, 2, 2-trifluoroethanol (TFE) and (ii) at least about 5 wt% trifluoroacetic acid (TFA) sufficient to form a bone matrix solution at a temperature between about 0 °C and about 22 °C, and electrospinning the bone matrix solution as described herein to form the bone matrix scaffold.

[0095] In one aspect, once the bone matrix solution is prepared, the bone matrix solution may be fabricated into scaffolds through neutralizing the bone matrix solution. For example, the neutralizing the bone matrix solution may comprise titrating sodium hydroxide from the bone matrix solution into a

solution. In some embodiments, the neutralization may be performed using a neutralizing solution. The "neutralizing solution" described herein may be an acid-neutralizing solution present in an amount sufficient to raise the pH of the acidic bone matrix solution to about 7.0 to about 7.5 after mixing and/or rinsing. In another aspect, the acid-neutralizing solution may be present in an amount sufficient to raise the pH of the acidic bone matrix solution to about 6.5 to about 7.45 after mixing and/or rinsing. In another aspect, the acid-neutralizing solution may be present in an amount sufficient to raise the pH of the bone matrix solution to about 7.0 to about 7.4 after mixing and/or rinsing. In some embodiments, the acid-neutralizing solution is selected from the group consisting of a bicarbonate solution, a phosphate solution, a carbonate solution, a sodium hydroxide solution, a potassium hydroxide solution, a calcium hydroxide solution, an aluminum hydroxide solution, and any combination of these solutions.

[0096] In another aspect, the method of preparing the bone matrix scaffold described herein may further comprise transferring the bone matrix solution into an acid-resistant mold and drying the bone matrix solution. The drying may be performed by, for example, air drying, vacuum drying, heat drying, or freeze drying. In another aspect, the dried bone matrix solution may be neutralized by rinsing it with a neutralizing solution described herein. In another aspect, the method of preparing the bone matrix scaffold described herein may further comprise rinsing the dried bone matrix solution with water. In some embodiments, the bone matrix solution may comprise sodium chloride granulates.

[0097] In one aspect, the method of preparing the bone matrix scaffold according to some embodiments of the present invention may further comprise crosslinking the bone matrix solution. In some embodiments, the crosslinking may be performed by any conventional chemical crosslinking method (*e.g.* chemical reagent-promoted, chemically reactive linker-promoted and/or enzyme-promoted) and/or dehydrothermal crosslinking method (*e.g.* heat-promoted condensation), forming the covalently crosslinked bone matrix. In additional embodiments, the crosslinking comprises applying a cross-linking agent to the bone matrix solution. For example, the cross-linking agent may be selected from the group consisting of 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), EDC/hyaluronic acid, genipin, and glutaraldehyde.

[0098] In another aspect, the method of preparing the bone matrix scaffold according to some embodiments of the present invention may further comprise applying an accessory polymer described herein to the bone matrix solution. In another aspect, the method of preparing the bone matrix scaffold according to some embodiments of the present invention may further comprise adding a bioactive factor described herein to the bone matrix scaffold.

[0099] The invention also relates to bone matrix implants produced by the methods of preparing the bone matrix scaffold. An "implant" refers to any object that is designed to be placed partially or wholly within a patient's body for one or more therapeutic or prophylactic purposes such as for tissue augmentation, contouring, restoring physiological function, repairing or restoring tissues damaged by disease or trauma, and/or delivering therapeutic agents to normal, damaged or diseased organs and tissues. A "bone matrix implant" refers to a medical device or implant that includes a volume replacement material for augmentation or reconstruction to replace a whole or part of a bone structure. A predetermined shape of the bone matrix implant may be varied to fit the implant site. A "bone matrix" refers to any mixture of the volume replacement materials for augmentation or reconstruction to replace a whole or part of a bone structure.

[00100] The invention also relates to methods of cell culture comprising culturing cells on a bone matrix scaffold produced by the method described herein.

[00101] For example, the invention provides for growing and/or culturing cells on a bone matrix scaffold produced by the method described herein. "Growing and/or culturing cells on a bone matrix scaffold" includes traditional cell culture methods as well as placing on a surface in any setting, such as in natural or synthetic biocompatible matrices or tissues. In some embodiments, the cells that are cultured on the bone matrix scaffold are stem cells. In further embodiments, the bone matrix scaffold can be used in *in vitro* methods for supporting cell growth and proliferation as well as for increasing osteogenesis, chondrogenesis, or ligament/tendon genesis in the stem cells cultured on the bone matrix scaffold.

[00102] Any cell described herewith may be cultured on a bone matrix scaffold produced by the method described herein for between about 15 minutes and about 4 weeks, about 2 hours and about 2 weeks, about 2 hours and about 1 week, about 2 hours and about 72 hours, about 24 hours and about 72 hours, or about 24 hours and about 96 hours, at between about 20 ºC and about 40 ºC or about 30°C and about 37°C, in an atmosphere containing between about 1% CO_2 and about 10% CO_2 or about 4% CO_2 and about 6% CO_2, in certain embodiments.

[00103] The invention also relates to methods of promoting osteoinductivity, with the methods comprising culturing cells on a bone matrix scaffold produced by the method described herein. The cells, prior to culture on the bone matrix scaffold of the present invention, may be undifferentiated or partially differentiated cells. The osteoinductive activity of the bone matrix scaffold may or may not be altered, including but not limited to, enhanced activity, relative to other scaffolds without the bone matrix nanofiber described herein.

[00104] The invention also relates to methods of promoting chondroinductivity, with the methods comprising culturing cells on a bone matrix scaffold produced by the method described herein. The cells, prior to culture on the bone matrix of the present invention, may be undifferentiated or partially differentiated cells. The chondroinductive activity of the bone matrix scaffold may or may not be altered, including but not limited to, enhanced activity, relative to other scaffolds without the bone matrix nanofiber described herein.

[00105] The invention also relates to methods of promoting ligament/tendon differentiation, with the methods comprising culturing cells on a bone matrix scaffold produced by the method described herein. The cells, prior to culture on the bone matrix of the present invention, may be undifferentiated or partially differentiated cells. The ligament/tendon differentiation activity of the bone matrix scaffold may or may not be altered, including but not limited to, enhanced activity, relative to other scaffolds without the bone matrix nanofiber described herein.

[00106] In one aspect, *in vitro* alkaline phosphatase assays may be used to evaluate osteoinductivity in cells cultured on the bone matrix scaffold prepared by the methods described herein. The ability of the bone matrix prepared by the methods of the present invention to stimulate or induce the alkaline phosphatase expression in an otherwise non-bone forming cells, such as myoblast (C2C12 cells), would indicate that the bone matrix of the present invention has osteoinductive activity. In these assays, cells cultured on other scaffolds without the bone matrix nanofiber described herein are used as negative controls to show that the baseline alkaline phosphatase expression on non-bone forming cells. The baseline of the osteoblastic markers in the negative control need not be zero, meaning that the cells in the negative control group may have at least some level of phenotypic marker(s). Accordingly, an "osteoinductive" bone matrix scaffold of the present invention would simply cause an increase in the osteoblastic markers in experimental cells over control grown on the other scaffolds without the bone matrix nanofiber.

[00107] Moreover, osteoinductivity, chondroinductivity, and ligament/tendon differentiation may be determined in tissue culture by investigating the ability of the bone matrix prepared by the methods of the present invention to differentiate or induce osteoblast phenotype, chondrocyte phenotype, ligament/tendon cell phenotype in cultured cells, such as primary cells, cell lines, or explants. For example, the cells may display increased production of a marker characteristic of osteoblasts and/or chondrocytes, such as alkaline phosphatase, etc. For example, the osteoinductive, chondroinductive, ligament/tendon differentiation potentials of the bone matrix scaffold having a bone matrix nanofiber described herein may be more than 0.2, 0.4, 0.6, 0.8, 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 times greater than the

scaffolds without the bone matrix nanofiber. In another example, the osteoinductive, chondroinductive, ligament/tendon differentiation potentials of the culture on the bone matrix scaffold and/or implant described herein may be more than 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 500 or even 1000 times greater than those of the scaffold and/or implant without any bone matrix nanofiber. Of course, this indicates that lower concentrations of modified growth factor, compared to unmodified growth factor are required to achieve the same effects.

[00108] Osteoinductivity, chondroinductivity, ligament/tendon differentiation, for assessing the bone, cartilage, ligament or tendon forming potential induced by the bone matrix scaffold of the present invention in a location such as muscle, may also be evaluated using a suitable animal model.

[00109] The invention also relates to methods of promoting cell attachment, proliferation or maintaining the differentiated state or preventing de-differentiation of osteoblasts, chondrocytes, ligament cells, tendon cells and/or any cell type disclosed herein with the methods comprising culturing the cells on a bone matrix scaffold produced by the method described herein. The proliferative activity of the bone matrix scaffold may or may not be altered, including but not limited to, enhanced activity, relative to a scaffold without any bone matrix nanofiber.

[00110] Mitogenicity may be assessed by investigating cell proliferation induced by the bone matrix prepared by the methods of the present invention using various in vitro assays that measure metabolic activity, such as MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, alamarBlue® assay, and others. The alamarBlue® assay uses a non-cytotoxic reduction-oxidation indicator to measure cell metabolic activity, making it a nondestructive assay for assessing the mitogenic activity of the bone matrix scaffold and/or bone matrix nanofiber described herein.

[00111] The invention also relates to methods of increasing or promoting osteogenesis, chondrogenesis, or ligament/tendon genesis in cells. The methods may comprise culturing the cells on a bone matrix scaffold produced by the method described herein. The osteogenic, chondrogenic, ligament, or tendon inducing activity of the bone matrix scaffold may or may not be altered, including but not limited to, enhanced activity, relative to a scaffold without bone matrix nanofiber described herein.

[00112] The invention also relates to methods of treating a tissue or organ defect or injury, for example, a musculoskeletal, dental or soft-tissue defect or injury, in an animal comprising administering (1) cells cultured on the bone matrix scaffold produced by the methods described herein and/or (2) the bone matrix

implant described herein to the tissue or organ defect (e.g. osseous defects, defects in cartilage, ligament, tendon, spinal disk, and tendon insertion site to bone).

[00113] The invention further relates to methods of treating a tissue or an organ defect or injury, for example a musculoskeletal, dental or soft-tissue defect, in an animal by applying a bone matrix scaffold prepared by the methods described herein to the defect, and application to the defect may be accomplished by injecting the bone matrix scaffold into the defect, inserting the bone matrix scaffold between tissue or organ, or placing the bone matrix scaffold on top of the defect. The present invention is also directed to treating a defect or injury in an organ by applying a bone matrix scaffold to the defect.

[00114] In yet another embodiment, cells may be seeded onto a bone matrix scaffold prepared by the methods provided herein. The cells seeded on the bone matrix scaffold can be any cell, such as but not limited to, osteoblasts, chondrocytes, ligament cells, tendon cells, progenitor cells, and stem cells disclosed herein or otherwise known in the art. The seeded cells may be allowed to proliferate and possibly attach to the matrix. Methods of seeding cells onto matrices, such as collagen matrix coated with the bone matrix prepared by the methods provided herein, are well known in the art.

[00115] While the invention has been described and illustrated herein by references to various specific materials, procedures and examples, it is understood that the invention is not restricted to the particular combinations of material and procedures selected for that purpose. Numerous variations of such details can be implied as will be appreciated by those skilled in the art. It is intended that the specification and examples be considered as exemplary, only, with the true scope and spirit of the invention being indicated by the following claims. All references, patents and patent applications referred to in this application are herein incorporated by reference in their entirety.

[00116] The following examples are illustrative and are not intended to limit the scope of the invention described herein.

EXAMPLES

[00117] Example 1: Dissolving bone in various solvents

[00118] Demineralized bone matrix (DBM) was prepared by methods as shown in U.S. Patent No. 6,189,537 and 6,305,379, which are incorporated herein in its entirety. The demineralized bone powder was dissolved at 100mg/ml in a volatile solvent or mixture of solvents.

[00119] Demineralized bone powder was initially dissolved in TFA or from a 5/95, 10/90, 20/80, 50/50 and 100/0 ratio of organic solvents TFA/HFP, respectively, at a starting concentration of 30-150 mg/ml demineralized bone powder, with final ratios set at 20/80 TFA/HFA and 100 mg/ml for all subsequent experiments, apart from solubility assays. The bone was allowed to vigorously stir for 3-4 days at 4 ºC up to 21 ºC to maintain intact alpha-helical collagen, or at room temp to 37 ºC to more rapidly dissolve the DBM with apparent increase in protein denaturation. This resulting brownish liquid was then further assayed for the ability to form a gel or film though evaporation of the volatile organic solvents with or without subsequent addition of water with the suspension placed at either 4 ºC, room temp, or 37 ºC, or through neutralizing the acidity of the liquid through strong base addition, such as sodium hydroxide, with or without a buffer system for physiological pH. DBM was found to dissolve in TFA and TFA/HPF at 4ºC after an extended period of stirring, on the order of 14-28+ days.

[00120] DBM powder was dissolved in TFA/HFP, TFA/HFP/Water, and TFA/HFP. DBM was dissolved in TFA/HFP, and the solvent subsequently fully evaporated off to leave behind a semisolid colloidal suspension, or gel, or a thin film coating. The protein banding pattern present in this dissolved DBM in cooled TFA/HFP was also observed for reference with intact bands for the alpha chains of collagen prominently seen, indicating that proteins are maintained in structure and composition between the DBM powder (un-dissolved) and the DBM dissolved in the organic solvent system.

[00121] Other systems that were found to dissolve DBM entirely were a combination of TFA with isopropanol, and TFA with 2,2,2-trifluoroethanol (TFE), for which HFP is a precursor molecule. The DBM was dissolved for 2-6 days. While DBM was dissolved in a mixture of trifluoroacetic acid and isopropanol and in trifluoroacetic acid with 2,2,2-trifluoroetanol, but DBM was not dissolved in 2,2,2-trifluoroethanol and hexafluoroisopropanol even after 2 months of mixing.

[00122] A longer period on the order of 10-14 days suggested a degradation of collagen and other matrix proteins as seen on SDS gel analysis. SDS gel analysis was performed on various DBM compositions. The results suggest high homology of proteins present between dissolved DBM (20% TFA/80 %HFP; 50% TFA/50% HFP) and unprocessed DBM powder. The degradation of the DBM was observed when an equal volume of distilled water is added to the TFA/HFP mix. The results also showed the degradation of the DBM in 100% TFA solution.

[00123] Moreover, TFA/HFP/DBM mixture stirred for three days formed a gel from a fully evaporated solution of the dissolved gel re-suspended when placed in water at 37 ºC, whereas TFA/HFP/DBM solution stirred for 14 days was only able to form a gel at 4 ºC when the DBM was likewise evaporated

and rehydrated at placed at 4 °C, room temp, and 37 °C. TFA/HFP/DBM solution stirred over a 2-6 day period was stirred at 16-21 °C, as temperatures above room temp, as can be generated by the stirring plate generating and transferring a slight amount of heat, can potentially degrade the intact proteins. The addition of water into the solvent to dissolve the DBM resulted in radical protein degradation.

[00124] Example 2: ELISA study for growth factor detection

An ELISA assay was performed to detect BMP-2 in the dissolved DBM solution (DBM dissolved in TFA/HFP as shown in Example 2), as compared to controls and to the amount of BMP-2 released by the standard BMP-2 isolation technique of guanidine hydrochloride extraction (FIGURE 1). The DBM solution prepared as shown in Example 2 was shown to have significantly more BMP-2 released compared to the conventional extraction methods (e.g. Pietrzak et al., J Craniofac Surg., 17, 1, 2006, 84-90) with nearly 4-fold more BMP-2 released per gram of DBM.

[00125] Example 3: Preparation of Bone Matrix Scaffolds

[00126] 1,1,1,3,3,3-hexafluoro-2-propanol (HFP) (CAS# 920-66-1) was from Acros Organics (Geel, Belgium), trifluoroacetic acid was obtained from Sigma-Aldrich (T6508, batch # 61496MK). Polycaprolactone (PCL) (B6003-1, B6003-2) was from Durect Lactone (Pelham, AL, http:www.absorbables.com). Bone powder was isolated from human donor bone procured by LifeNet Health, with ECM isolation procedures carried out in the laboratory of Dr. Roy Ogle. The electrospinning apparatus was designed using an Aladdin Programmable Syringe Pump from World Precision Instruments, Inc. (Sarasota, FL) and one or two adjustable high voltage power supply from Gamma High Voltage Research (Ormond Beach, FL). N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) was purchased from Thermo Scientific (Pittsburg, PA). Genipin was purchased from Wako (Richmond, VA).

[00127] Demineralized bone powder was dissolved in TFA or a TFA/HFP blend, tested as workable from a 10/90, 20/80, 50/50 and 100/0 ratio of organic solvents, respectively, at 30-150mg/ml bone powder concentrations, with final ideal paramaters set at 20/80 TFA/HFP at 100mg/ml and used for the subsequent experiments. DBM was also co-electrospun with an equal volume of polycaprolactone (PCL) that was dissolved in HPF at 100mg/ml. The bone is allowed to vigorously stir for 3-4 days at below room temp (<~21°C). This resulting brown colored solution was then loaded into the glass syringe with an 18ga stainless steel needle and secured into the syringe pump, in the device design shown in FIGURE 2. A stainless steel plate coated with aluminum foil or number 1 glass coverslips on top of the collector plate were used to collect randomly aligned nanofibers, or the fibers were collected between earthed

stainless steel posts 3-10mm apart to impart fiber alignment, with the stainless steel posts either statically mounted or rotating at around 4,000RPMs to enhance alignment and maximal thickness of the scaffold. The voltage source was used to apply a +20kV DC voltage to the positive lead, attached to the metal syringe needle, with the collector either earthed or with a 15kV DC voltage applied from a second power supply set to opposite polarity. The programmable syringe pump was set to dispense the solution at 0.50-3.50 mL/hr, ideally at 2.5ml/hr for pure DBM and 4ml/hr for DBM/PCL mixtures. After approximately 1 hour of electrospinning, the collector was replaced with a new aluminum sheet and the flow rate was changed. Concentrations of 10%, 50% , 90% and 100% demineralized bone blended with PCL were examined, as well as flow rates of 0.5mL/hr-5.75mL/hr to identify the ideal parameters (which can be affected day-to-day by relative humidity, ambient temperature, etc.). Further modifications to this arrangement have been made based on intended use, including multiple spinnerets, rotating targets of various shapes and sizes, focusing of the polymer jet by electrode modifications, and additional power source and grounding schemas.

[00128] Electrospun demineralized bone matrix and DBM/PCL blended samples were taken from each collector and mounted on aluminum stubs using carbon stickers. The samples were then coated with gold at about 50nm thickness using a sputter coater. Coated samples were imaged using a JOEL 6400 Scanning electron microscope (SEM) with Orion image processing. Representative images can be seen in FIGURE 3.

[00129] Scaffolds of pure demineralized bone nanofibers were found to dissolve when placed in aqueous solutions, including culture media, PBS and milliQ water, so 10mm scaffold punches were tested for stability after crosslinking with various chemical agents under a wide array of conditions. A solution of 0.01% genipin dissolved in ethanol at 37°C for 24-48hrs was found to be a suitable cross-linker, maintaining scaffold stability in aqueous solution. A 50 molar excess of EDC with 18hrs of cross-linking time was also found to sufficiently cross-link demineralized bone scaffold. Additionally, glutaraldehyde vapors also provided stability by lightly cross-linking the electrospun DBM nanofiber scaffold. These so called “zero length cross-linkers” altered the fibrous morphology of the electrospun scaffolds as shown in FIGURE 3, however, linkers or spacers commonly used with these cross-linkers will retain the native porous topology of the scaffolds. The average size of the electrospun fibers generated from pure demineralized bone matrix, and as blended with PCL, and as compared to the native demineralized bone power size was calculated by measuring 50 unique points in ImageJ64 (NIH shareware), with results shown in FIGURE 4.

[00130] To confirm co-polymerization of demineralized bone and PCL in the forming blended nanofibers of the two materials, FTIR analysis was used to assess the chemical bond structure and to quantitatively determine the presence of each polymer in the different blends. FTIR also was used to show a high degree of similarity between the unprocessed demineralized bone powder and the electrospun nanofibrous mesh as shown in FIGURE 5, where peaks/bonds were highly conserved between raw and electrospun DBM, including putative peaks that suggest a maintained alpha helical structure in the collagen present. Lastly, to assess the structure of collagen to be unchanged from the native DBM powder state we compared native DBM and electrospun DBM with pure collagen type I that was either treated or not treated overnight with pepsin to show the maintenance of the alpha 1 and alpha 2 bands of collagen type 1 between samples (FIGURE 6). The porosity of the electrospun scaffolds was characterized as shown in FIGURE 7.

[00131] Example 4: Cell Culture on Bone Matrix Scaffolds

[00132] Excellent and rapid mesenchymal stem cell attachment was also demonstrated on electrospun DBM as cross-linked with EDC or blended with PCL as compared to controls are also shown FIGURE 8.

[00133] Example 5: *In Vivo* Study

[00134] Animal studies with implanted EDC cross-linked DBM and DBM/PCL indicated new bone formation along with high cellularity in electrospun DBM-EDC cross-linked samples with a mild inflammatory response at 4 weeks of gestation of 0.1cc of material in a rat pouch assay (FIGURE 9).

[00135] Example 6: Cell Differentiation on Bone Matrix Scaffolds

[00136] Alizarin Red S histological stains of electrospun DBM/PCL, PCL and DBM indicated that trace calcium in the native DBM remains present in the electrospun variants of DBM and DBM/PCL (FIGURE 10), with the trace calcium believed critical for new bone formation. Toluidine blue histology, along with apparent bone nodule formation, confirmed osteoblastogenesis on samples of electrospun DBM that was dissolved in contact with stem cells grown on plastic (FIGURE 10).

[0001] The osteoinductive capacity of the electrospun DBM was further confirmed through showing adipose derived stem cells (ASCs) and dental pulp stem cells (DPSCs) were induced to osteoblasts from their contact with the electrospun DBM. Osteoblastogenesis was confirmed using fluorescent microscopy to visualize expression of bone sialo protein I (aka osteopontin) and bone sialo protein II using the respective monoclonal antibodies conjugated to alexa-488 secondary antibodies, with a DAPI stain for the

nucleus (FIGURE 11) all seen in cells on DBM scaffolds only. The alexa488 fluorescence was absent in control samples and on no-secondary-antibody controls.

CLAIMS

1. A method of preparing a bone matrix solution, the method comprising

dissolving bone in an amount of an anhydrous solvent comprising (i) hexafluoroisopropanol (HFP) or 2, 2, 2-trifluoroethanol (TFE) and (ii) at least about 5 wt% trifluoroacetic acid (TFA) sufficient to form the bone matrix solution at a temperature lower than about 25 °C.

2. The method according to claim 1, wherein the bone is demineralized bone (DBM).

3. The method according to any of claims 1-2, wherein the bone comprises an osteogenic growth factor.

4. The method according to any of claims 1-3, wherein the bone comprises an extracellular matrix selected from the group consisting of collagen, glycosaminoglycans, osteocalcin, osteonectin, bone sialo protein, osteopontin, and mixtures thereof.

5. The method according to any of claims 1-4, wherein the bone comprises collagen type I.

6. The method according to any of claims 1-5, wherein the bone matrix solution comprises collagen in its native triple helical structure.

7. The method according to any of claims 1-6, wherein the solvent consists of (i) the HFP, the TFE, or the isopropanol, and (ii) the TFA.

8. The method according to any of claims 1-7, wherein the solvent comprises at least about 20 wt% TFA.

9. The method according to any of claims 1-8, wherein the solvent comprises at least about 50 wt% TFA.

10. The method according to any of claims 1-9, wherein the bone is dissolved at a temperature lower than about 24 °C.

11. The method according to any of claims 1-10, wherein the bone is dissolved at a temperature lower than about 22 °C.

12. The method according to any of claims 1-11, wherein the bone is dissolved at a temperature lower than about 15 °C.

13. The method according to any of claims 1-12, wherein the bone is dissolved at a temperature lower than about 10 °C.

14. The method according to any of claims 1-13, wherein the bone is dissolved in a concentration between about 30 mg/mL and about 150 mg/mL.

15. The method according to any of claims 1-14, wherein the bone is dissolved by stirring for more than about 24 hours.

16. The method according to any of claims 1-15, wherein the bone is in the form of particles and/or powder.

17. A method of coating bone matrix on a surface, the method comprising:

dissolving a bone at a temperature lower than about 25 °C in an amount of an anhydrous solvent comprising (i) hexafluoroisopropanol (HFP) or 2, 2, 2-trifluoroethanol (TFE), and (ii) at least about 5 wt% trifluoroacetic acid (TFA) sufficient to form a bone matrix solution,

exposing the bone matrix solution to conditions sufficient to evaporate a portion of the solvent to form a colloidal gel,

coating the surface with the colloidal gel, and

exposing the coated surface to conditions sufficient to further evaporate an amount of solvent.

18. The method according to claim 17, wherein the surface is of a second biocompatible matrix.

19. The method according to any of claims 17-18, wherein the bone comprises an osteogenic growth factor.

20. The method according to any of claims 17-19, wherein the bone comprises an extracellular matrix selected from the group consisting of collagen, glycosaminoglycans, osteocalcin, osteonectin, bone sialo protein, osteopontin, and mixtures thereof.

21. The method according to any of claims 17-20, wherein the bone comprises collagen type I.

22. The method according to any of claims 17-21, wherein the dissolved bone comprises collagen in its native triple helical structure.

23. The method according to any of claims 17-22, wherein the solvent consists of (i) HFP or TFE, and (ii) TFA.

24. The method according to any of claims 17-23, wherein the solvent comprises at least about 20 wt% TFA.

25. The method according to any of claims 17-24, wherein the solvent comprises at least about 50 wt% TFA.

26. The method according to any of claims 17-25, wherein the bone is dissolved at a temperature lower than about 24 °C.

27. The method according to any of claims 17-26, wherein the bone is dissolved at a temperature lower than about 22 °C.

28. The method according to any of claims 17-27, wherein the bone is dissolved at a temperature lower than about 15 °C.

29. The method according to any of claims 17-28, wherein the bone is dissolved at a temperature lower than about 10 °C.

30. The method according to any of claims 17-29, wherein the bone is dissolved in a concentration between about 30 mg/mL and about 150 mg/mL.

31. The method according to any of claims 17-30, wherein the bone is dissolved by stirring for more than about 24 hours.

32. The method according to any of claims 17-31, wherein the bone is in the form of particles and/or powder.

33. The method according to any of claims 17-32, wherein the colloidal gel is placed in a mold to form the predetermined shape.

34. The method according to any of claims 17-33, wherein the evaporation is performed by air drying, vacuum drying, heat drying, or freeze drying.

35. The method according to any of claims 17-34, wherein the amount of the solvent to form a colloidal gel from the bone matrix solution according to some embodiments of the present invention may be between about 95 wt% and about 5 wt%.

36. The method according to any of claims 17-35, further comprising adding a bioactive factor to the bone matrix solution.

37. The method of claim 36, wherein the bioactive factor is selected from the group consisting of an osteogenic growth factor, collagen, glycosaminoglycans, osteonectin, bone sialo protein, an osteoinductive factor, a chondrogenic factor, a cytokine, a mitogenic factor, a chemotactic factor, a transforming growth factor(TGF), a fibroblast growth factor (FGF), an angiogenic factor, an insulin-like growth factor (IGF), a platelet-derived growth factor (PDGF), an epidermal growth factor (EGF), a vascular endothelial growth factor (VEGF), a nerve growth factor (NGF), a neurotrophin, a bone morphogenetic protein (BMP), osteogenin, osteopontin, osteocalcin, cementum attachment protein, erythropoietin, thrombopoietin, tumor necrosis factor (TNF), an interferon, a colony stimulating factor (CSF), or an interleukin, among others. The bioactive factor may be a BMP, PDGF, FGF, VEGF, TGF, insulin, among others. Examples of BMPs include but are not limited to BMP-2, BMP-3, BMP-4, BMP-5, BMP-6, BMP-7, BMP-8, BMP-9, BMP-10, BMP-11, BMP-12, BMP-13, BMP-14, BMP-15, and a mixture thereof.

38. A bone matrix implant produced by the method according to any of claims 17-37.

39. A synthetic surface coated with the bone matrix produced by the method according to any of claims 17-37.

40. A method of cell culture comprising culturing cells on a cell culture surface coated with the bone matrix produced by the method according to any of claims 17-37.

41. The method of claim 40, wherein the cells are selected from the group consisting of stem cells, adipose derived stem cells and dental pulp stem cells.

42. A method of promoting differentiation of stem cells into osteoblasts, chondrocytes, ligament or tendon, the method comprising culturing the cells on a surface coated with the bone matrix produced by the method according to any of claims 17-37.

43. The method of claim 42, wherein the cells are progenitor cells or adult stem cells.

44. The method of claim 43, wherein the progenitor cells or the adult stem cells are derived from placenta, bone marrow, adipose tissue, blood vessel, amniotic fluid, synovial fluid, synovial membrane, pericardium, periosteum, dura, peripheral blood, umbilical blood, menstrual blood, baby teeth, nucleus pulposus, brain, skin, hair follicle, intestinal crypt, neural tissue, or muscle.

45. The method of claim 42, wherein the stem cells are pluripotent stem cells.

46. The method of claim 42, wherein the stem cells are selected from the group consisting of adipose derived stem cells and dental pulp stem cells.

47. A bone matrix scaffold comprising one or more bone matrix nanofibers.

48. The bone matrix scaffold of claim 47, wherein the bone matrix scaffold essentially consists of the bone matrix nanofibers.

49. The bone matrix scaffold according to any of claims 47-48, wherein the bone matrix scaffold consists of the bone matrix nanofibers.

50. The bone matrix scaffold according to any of claims 47-49, wherein the bone matrix scaffold is biocompatible.

51. The bone matrix scaffold according to any of claims 47-50, wherein the bone matrix nanofibers comprise type I collagen in its native triple helical structure.

52. The bone matrix scaffold according to any of claims 47-51, wherein the bone matrix nanofibers has less than about 8 wt% residual calcium.

53. The bone matrix scaffold according to any of claims 47-52, wherein the bone matrix nanofibers have an average diameter from about 5 nm to about 1000 nm.

54. The bone matrix scaffold according to any of claims 47-53, wherein the bone matrix nanofiber have an average diameter of less than about 100 nm.

55. The bone matrix scaffold according to any of claims 47-54, wherein the bone matrix nanofibers have an average length from about 1 cm to about 50 cm.

56. The bone matrix scaffold according to any of claims 47-55, wherein the bone matrix scaffold has an average porosity from about 60% to about 90%.

57. The bone matrix scaffold according to any of claims 47-56, wherein the bone matrix nanofibers are electrospun.

58. The bone matrix scaffold according to any of claims 47-57, wherein a fast Fourier transform (FFT) analysis result of the bone matrix nanofibers have adjacent major peaks with about 180° apart from each other.

59. The bone matrix scaffold according to any of claims 47-58, wherein the bone matrix nanofibers further comprise an accessory polymer.

60. The bone matrix scaffold of claim 59, wherein the accessory polymer is selected from the group consisting of polycaprolactone, poly(glycolic acid), poly(lactic acid), polydioxanone, poly (lactide-co-glycolide) copolymers, polyesters polysaccharides, polyhydroxyalka noates , starch, polylactic acid, cellulose, proteins, agar, silks, alginate, collagen/gelatin, carrageenan, elastin, pectin, resilin, konjac, adhesives, gums, polyamino acids, polysaccharides, soy, zein, wheat gluten, casein, chitin/chitosan, serum albumin, hyaluronic acid, lipids/s urfactants, xanthan, acetoglycerides, waxes, surfactants, dextran, emulsan, gelian, polyphenols, levan, lignin, curd, ian, tannin, polygalactosamine, humic acid, shellac, pullulan , poly-gamma-glutamic acid, elsinan, natural rubber, yeast glucans, and synthetic polymers from natural fats and oils.

61. The bone matrix scaffold according to any of claims 47-60, wherein the bone matrix nanofibers further comprise one or more biodegradable, biocompatible polymers selected from the group consisting of ethylene vinyl acetate, polyanhydricles, polyglycolic acid, collagen, polyorthoesters, and polylactic acid.

62. The bone matrix scaffold according to any of claims 47-61, wherein the bone matrix nanofibers further comprise an extracellular matrix component selected from the group consisting of collagen, glycosaminoglycans, osteocalcin, osteonectin, bone sialo protein, osteopontin, and mixtures thereof.

63. The bone matrix scaffold according to any of claims 47-62, wherein the bone matrix nanofibers further comprise a bioactive factor.

64. The bone matrix scaffold of claim 63, wherein the bioactive factor is selected from the group consisting of an osteogenic growth factor, collagen, glycosaminoglycans, osteonectin, bone sialo protein, an osteoinductive factor, a chondrogenic factor, a cytokine, a mitogenic factor, a chemotactic factor, a transforming growth factor(TGF), a fibroblast growth factor (FGF), an angiogenic factor, an insulin-like growth factor (IGF), a platelet-derived growth factor (PDGF), an epidermal growth factor (EGF), a vascular endothelial growth factor (VEGF), a nerve growth factor (NGF), a neurotrophin, a bone morphogenetic protein (BMP), osteogenin, osteopontin, osteocalcin, cementum attachment protein, erythropoietin, thrombopoietin, tumor necrosis factor (TNF), an interferon, a colony stimulating factor (CSF), and an interleukin.

65. The bone matrix scaffold of claim 63, wherein the bioactive factor is an osteogenic growth factor.

66. The bone matrix scaffold according to any of claims 47-65, wherein the bone matrix nanofibers further comprise one or more biocompatible material selected from the group consisting of collagen, polycaprolactone, poly(glycolic acid), poly(lactic acid), polydioxanone, poly (lactide-co-glycolide) copolymers, polyesters polysaccharides, polyhydroxyalka noates, starch, polylactic acid, cellulose, proteins, agar, silks, alginate, collagen, gelatin, carrageenan, elastin, pectin, resilin, konjac, adhesives, various gums, polyamino acids, polysaccharides, soy, zein, wheat gluten, casein, chitin, chitosan, serum

albumin, hyaluronic acid, lipids, surfactants, xanthan, acetoglycerides, waxes, dextran, emulsan, gelian, polyphenols, levan, lignin, curd, ian, tannin, polygalactosamine, humic acid, cellulose, shellac, pullulan, poly-gamma-glutamic acid, elsinan, natural rubber, yeast glucans, synthetic polymers from natural fats and oils.

67. The bone matrix scaffold according to any of claims 47-66, further comprising a support, wherein the bone matrix nanofibers cover the support.

68. A method of preparing a bone matrix scaffold, the method comprising

dissolving bone in an amount of an anhydrous solvent comprising (i) hexafluoroisopropanol (HFP) or 2, 2, 2-trifluoroethanol (TFE) and (ii) at least about 5 wt% trifluoroacetic acid (TFA) sufficient to form a bone matrix solution, and

electrospinning the bone matrix solution to form the bone matrix scaffold.

69. The method of claim 68, further comprising crosslinking the bone matrix solution.

70. The method of claim 69, wherein the crosslinking is performed by dehydrothermal crosslinking.

71. The method of claim 69, wherein the crosslinking comprises applying a cross-linking agent to the bone matrix solution.

72. The method of claim 71, wherein the cross-linking agent is selected from the group consisting of 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), EDC/hyaluronic acid, genipin, and glutaraldehyde.

73. The method according to any of claims 68-72, further comprising applying an accessory polymer to the bone matrix solution.

74. The method of claim 73, wherein the accessory polymer is selected from the group consisting of polycaprolactone, poly(glycolic acid), poly(lactic acid), polydioxanone, poly (lactide-co-glycolide) copolymers, polyesters polysaccharides, polyhydroxyalka noates , starch, polylactic acid, cellulose, proteins, agar, silks, alginate, collagen/gelatin, carrageenan, elastin, pectin, resilin, konjac, adhesives, gums, polyamino acids, polysaccharides, soy, zein, wheat gluten, casein, chitin/chitosan, serum albumin, hyaluronic acid, lipids/s urfactants, xanthan, acetoglycerides, waxes, surfactants, dextran, emulsan, gelian, polyphenols, levan, lignin, curd, ian, tannin, polygalactosamine, humic acid, shellac, pullulan , poly-gamma-glutamic acid, elsinan, natural rubber, yeast glucans, and synthetic polymers from natural fats and oils.

75. The method according to any of claims 68-74, wherein the bone is demineralized bone (DBM).

76. The method according to any of claims 68-75, wherein the bone comprises an osteogenic growth factor.

77. The method according to any of claims 68-76, wherein the bone comprises an extracellular matrix selected from the group consisting of collagen, glycosaminoglycans, osteocalcin, osteonectin, bone sialo protein, osteopontin, and mixtures thereof.

78. The method according to any of claims 68-77, wherein the bone comprises collagen type I.

79. The method according to any of claims 68-78, wherein the bone matrix solution comprises collagen in its native triple helical structure.

80. The method according to any of claims 68-79, wherein the anhydrous solvent consists of (i) the HFP, the TFE, or the isopropanol, and (ii) the TFA.

81. The method according to any of claims 68-80, wherein the anhydrous solvent comprises at least about 20 wt% TFA.

82. The method according to any of claims 68-81, wherein the anhydrous solvent comprises at least about 50 wt% TFA.

83. The method according to any of claims 68-82, wherein the bone is dissolved at a temperature lower than about 25 °C.

84. The method according to any of claims 68-83, wherein the bone is dissolved at a temperature lower than about 24 °C.

85. The method according to any of claims 68-84, wherein the bone is dissolved at a temperature lower than about 22 °C.

86. The method according to any of claims 68-85, wherein the bone is dissolved at a temperature lower than about 15 °C.

87. The method according to any of claims 68-86, wherein the bone is dissolved at a temperature lower than about 10 °C.

88. The method according to any of claims 68-87, wherein the bone is dissolved in a concentration between about 30 mg/mL and about 150 mg/mL.

89. The method according to any of claims 68-88, wherein the bone is dissolved by stirring for more than about 24 hours.

90. The method according to any of claims 68-89, wherein the bone is in the form of particles and/or powder.

91. The method according to any of claims 68-90, further comprising adding a bioactive factor to the bone matrix scaffold.

92. The method of claim 91, wherein the bioactive factor is selected from the group consisting of an osteogenic growth factor, collagen, glycosaminoglycans, osteonectin, bone sialo protein, an osteoinductive factor, a chondrogenic factor, a cytokine, a mitogenic factor, a chemotactic factor, a transforming growth factor(TGF), a fibroblast growth factor (FGF), an angiogenic factor, an insulin-like growth factor (IGF), a platelet-derived growth factor (PDGF), an epidermal growth factor (EGF), a vascular endothelial growth factor (VEGF), a nerve growth factor (NGF), a neurotrophin, a bone morphogenetic protein (BMP), osteogenin, osteopontin, osteocalcin, cementum attachment protein,

erythropoietin, thrombopoietin, tumor necrosis factor (TNF), an interferon, a colony stimulating factor (CSF), or an interleukin, among others. The bioactive factor may be a BMP, PDGF, FGF, VEGF, TGF, insulin, among others. Examples of BMPs include but are not limited to BMP-2, BMP-3, BMP-4, BMP-5, BMP-6, BMP-7, BMP-8, BMP-9, BMP-10, BMP-11, BMP-12, BMP-13, BMP-14, BMP-15, and a mixture thereof.

93. A bone matrix implant produced by the method according to any of claims 68-92.

94. A method of preparing a bone matrix scaffold, the method comprising

dissolving bone in an amount of an anhydrous solvent comprising (i) hexafluoroisopropanol (HFP) or 2, 2, 2-trifluoroethanol (TFE) and (ii) at least about 5 wt% trifluoroacetic acid (TFA) sufficient to form the bone matrix solution, and

neutralizing the bone matrix solution to form the bone matrix scaffold.

95. The method of claim 94, further comprising crosslinking the bone matrix solution.

96. The method of claim 95, wherein the crosslinking is performed by dehydrothermal crosslinking.

97. The method of claim 96, wherein the crosslinking comprises applying a cross-linking agent to the bone matrix solution.

98. The method of claim 97, wherein the cross-linking agent is selected from the group consisting of 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), EDC/hyaluronic acid, genipin, and glutaraldehyde.

99. The method according to any of claims 94-98, further comprising applying an accessory polymer to the bone matrix solution.

100. The method of claim 99, wherein the accessory polymer is selected from the group consisting of polycaprolactone, poly(glycolic acid), poly(lactic acid), polydioxanone, poly (lactide-co-glycolide) copolymers, polyesters polysaccharides, polyhydroxyalka noates , starch, polylactic acid, cellulose, proteins, agar, silks, alginate, collagen/gelatin, carrageenan, elastin, pectin, resilin, konjac, adhesives, gums, polyamino acids, polysaccharides, soy, zein, wheat gluten, casein, chitin/chitosan, serum albumin, hyaluronic acid, lipids/s urfactants, xanthan, acetoglycerides, waxes, surfactants, dextran, emulsan, gelian, polyphenols, levan, lignin, curd, ian, tannin, polygalactosamine, humic acid, shellac, pullulan , poly-gamma-glutamic acid, elsinan, natural rubber, yeast glucans, and synthetic polymers from natural fats and oils.

101. The method according to any of claims 94-100, wherein the bone is demineralized bone (DBM).

102. The method according to any of claims 94-101, wherein the bone comprises an osteogenic growth factor.

103. The method according to any of claims 94-102, wherein the bone comprises an extracellular matrix selected from the group consisting of collagen, glycosaminoglycans, osteocalcin, osteonectin, bone sialo protein, osteopontin, and mixtures thereof.

104. The method according to any of claims 94-103, wherein the bone comprises collagen type I.

105. The method according to any of claims 94-104, wherein the bone matrix solution comprises collagen in its native triple helical structure.

106. The method according to any of claims 94-105, wherein the anhydrous solvent consists of (i) the HFP, the TFE, or the isopropanol, and (ii) the TFA.

107. The method according to any of claims 94-106, wherein the anhydrous solvent comprises at least about 20 wt% TFA.

108. The method according to any of claims 94-107, wherein the anhydrous solvent comprises at least about 50 wt% TFA.

109. The method according to any of claims 94-108, wherein the bone is dissolved at a temperature lower than about 25 °C.

110. The method according to any of claims 94-109, wherein the bone is dissolved at a temperature lower than about 24 °C.

111. The method according to any of claims 94-110, wherein the bone is dissolved at a temperature lower than about 22 °C.

112. The method according to any of claims 94-111, wherein the bone is dissolved at a temperature lower than about 15 °C.

113. The method according to any of claims 94-112, wherein the bone is dissolved at a temperature lower than about 10 °C.

114. The method according to any of claims 94-113, wherein the bone is dissolved in a concentration between about 30 mg/mL and about 150 mg/mL.

115. The method according to any of claims 94-114, wherein the bone is dissolved by stirring for more than about 24 hours.

116. The method according to any of claims 94-115, wherein the bone is in the form of particles and/or powder.

117. The method according to any of claims 94-116, further comprising adding a bioactive factor to the bone matrix scaffold.

118. The method of claim 117, wherein the bioactive factor is selected from the group consisting of an osteogenic growth factor, collagen, glycosaminoglycans, osteonectin, bone sialo protein, an osteoinductive factor, a chondrogenic factor, a cytokine, a mitogenic factor, a chemotactic factor, a transforming growth factor(TGF), a fibroblast growth factor (FGF), an angiogenic factor, an insulin-like growth factor (IGF), a platelet-derived growth factor (PDGF), an epidermal growth factor (EGF), a vascular endothelial growth factor (VEGF), a nerve growth factor (NGF), a neurotrophin, a bone morphogenetic protein (BMP), osteogenin, osteopontin, osteocalcin, cementum attachment protein,

erythropoietin, thrombopoietin, tumor necrosis factor (TNF), an interferon, a colony stimulating factor (CSF), or an interleukin, among others. The bioactive factor may be a BMP, PDGF, FGF, VEGF, TGF, insulin, among others. Examples of BMPs include but are not limited to BMP-2, BMP-3, BMP-4, BMP-5, BMP-6, BMP-7, BMP-8, BMP-9, BMP-10, BMP-11, BMP-12, BMP-13, BMP-14, BMP-15, and a mixture thereof.

119. The method according to any of claims 94-118, wherein the neutralizing the bone matrix solution comprises titrating sodium hydroxide from the bone matrix solution into a solution.

120. The method according to any of claims 94-119, further comprising

transferring the bone matrix solution into an acid-resistant mold,

drying the bone matrix solution, and

neutralizing the dried bone matrix solution by rinsing with a neutralizing solution.

121. The method of claim 120, further comprising rinsing the dried bone matrix solution with water.

122. The method of claim 121, wherein the bone matrix solution comprises sodium chloride granulates.

123. The method according to any of claims 120-122, wherein the drying is performed by air drying.

124. A bone matrix implant produced by the method according to any of claims 94-123.

125. A method of cell culture comprising culturing cells on the bone matrix scaffold according to any of claims 47-67 in condition suitable for the cell culture.

126. The method of claim 125, wherein the cells are selected from the group consisting of stem cells, adipose derived stem cells and dental pulp stem cells.

127. A method of promoting differentiation of stem cells into osteoblasts, chondrocytes, ligament or tendon, the method comprising culturing the cells on the bone matrix scaffold according to any of claims 47-67 in conditions suitable for the cell differentiation.

128. The method of claim 127, wherein the stem cells are progenitor cells or adult stem cells.

129. The method of claim 128, wherein the progenitor cells or the adult stem cells are derived or isolated from placenta, bone marrow, adipose tissue, blood vessel, amniotic fluid, synovial fluid, synovial membrane, pericardium, periosteum, dura, peripheral blood, umbilical blood, menstrual blood, baby teeth, nucleus pulposus, brain, skin, hair follicle, intestinal crypt, neural tissue, or muscle.

130. The method of claim 127, wherein the stem cells are pluripotent stem cells.

131. The method of claim 127, wherein the stem cells are selected from the group consisting of adipose derived stem cells and dental pulp stem cells.

FIGURE 1.

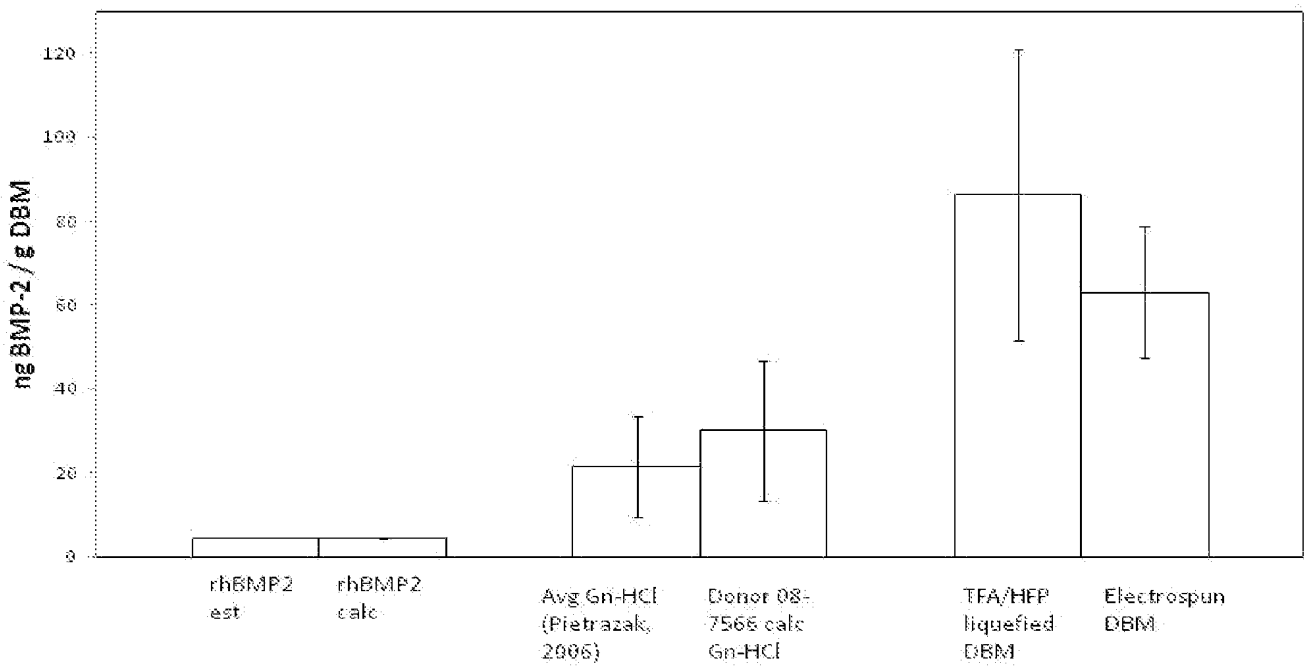

ng BMP-2 / g DBM
120
100
80
60
40
20
0
rhBMP2 est
rhBMP2 calc
Avg Gn-HCl (Pietrazak, 2008)
Donor 08-7566 calc Gn-HCl
TFA/HFP liquefied DBM
Electrospun DBM

FIGURE 2.

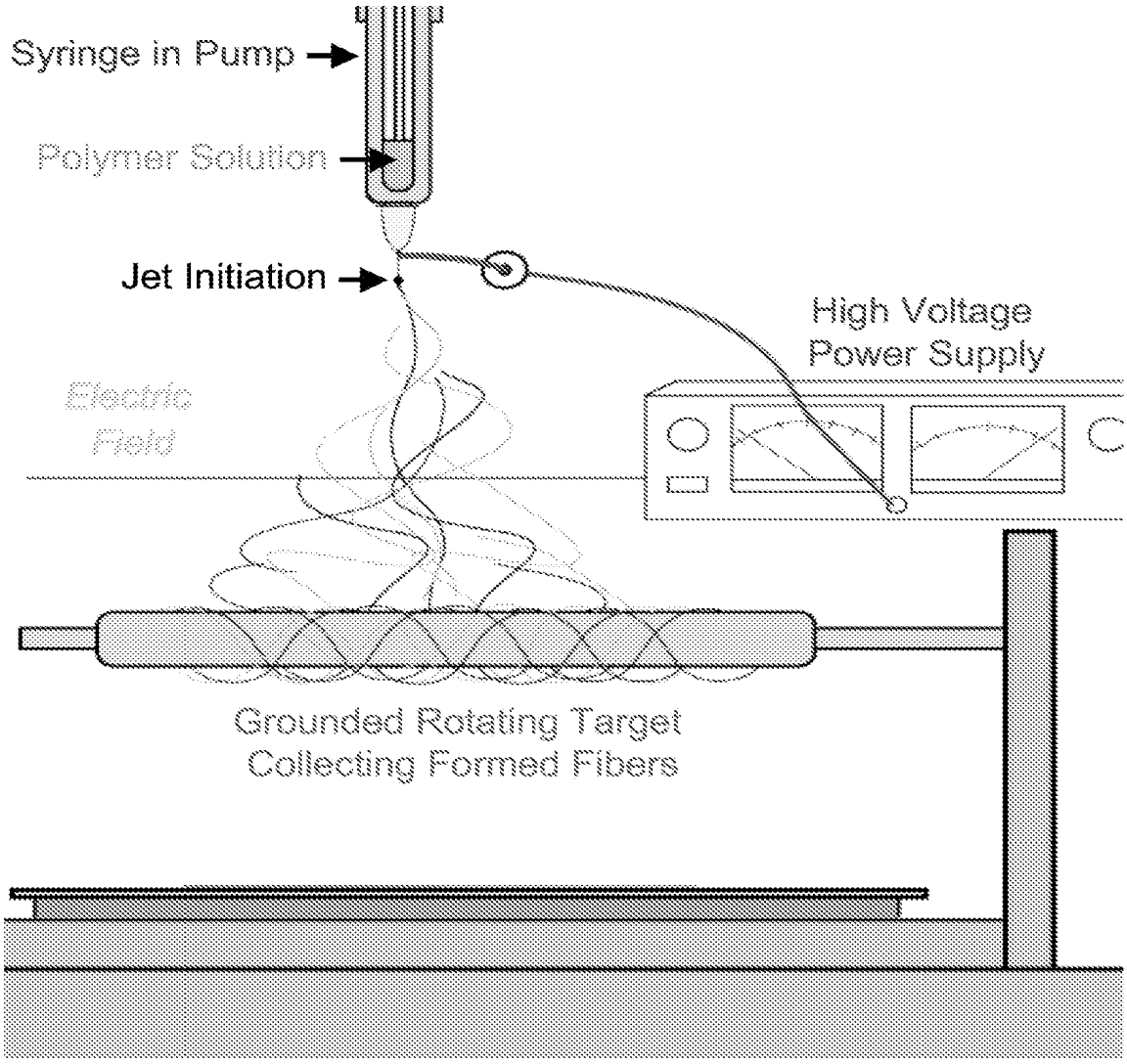

Syringe in Pump
Polymer Solution
Jet Initiation
High Voltage
Power Supply
Electric
Field
Grounded Rotating Target
Collecting Formed Fibers

FIGURE 3.

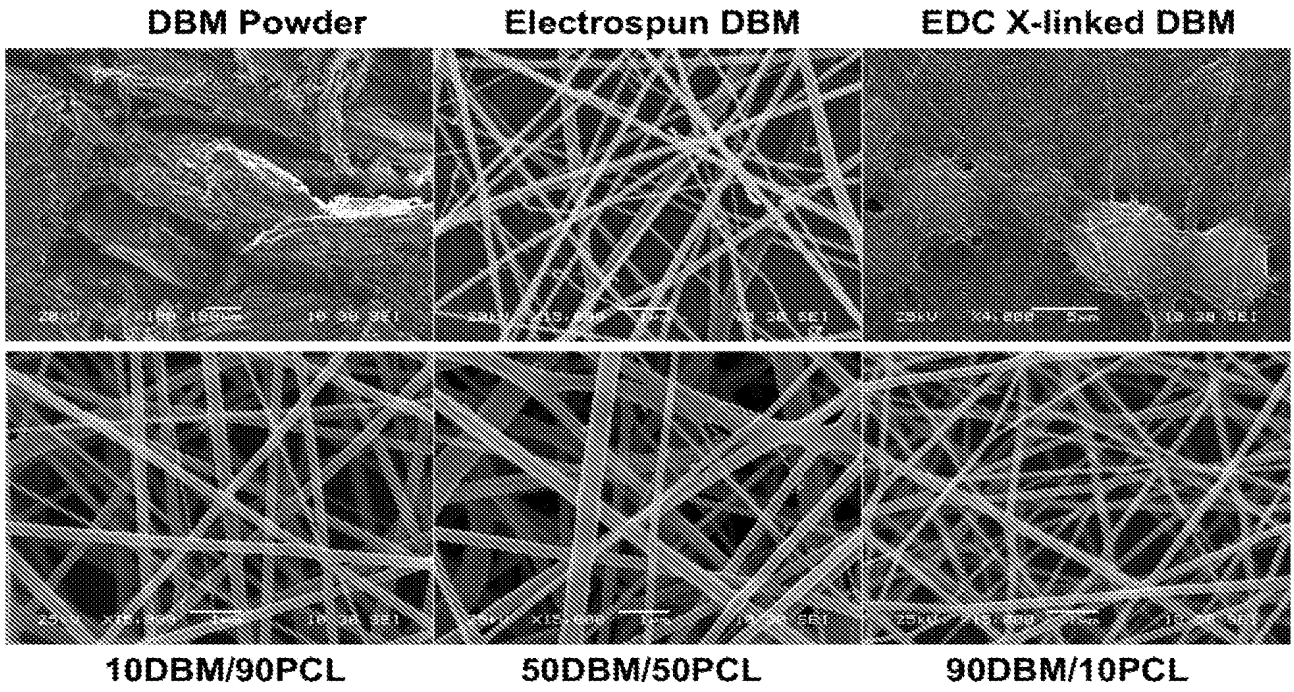

DBM Powder
Electrospun DBM
EDC X-linked DBM
10DBM/90PCL
50DBM/50PCL
90DBM/10PCL

FIGURE 4.

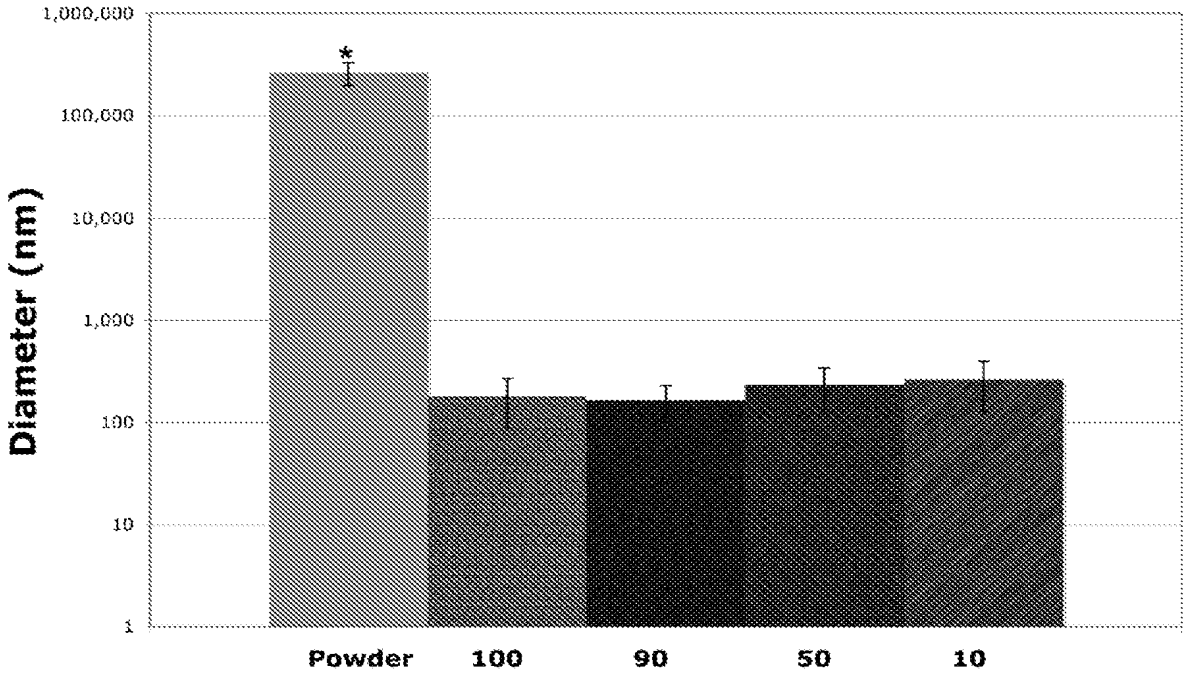

1,000,000
100,000
10,000
1,000
100
10
1
Diameter (nm)
*
Powder
100
90
50
10

FIGURE 5.

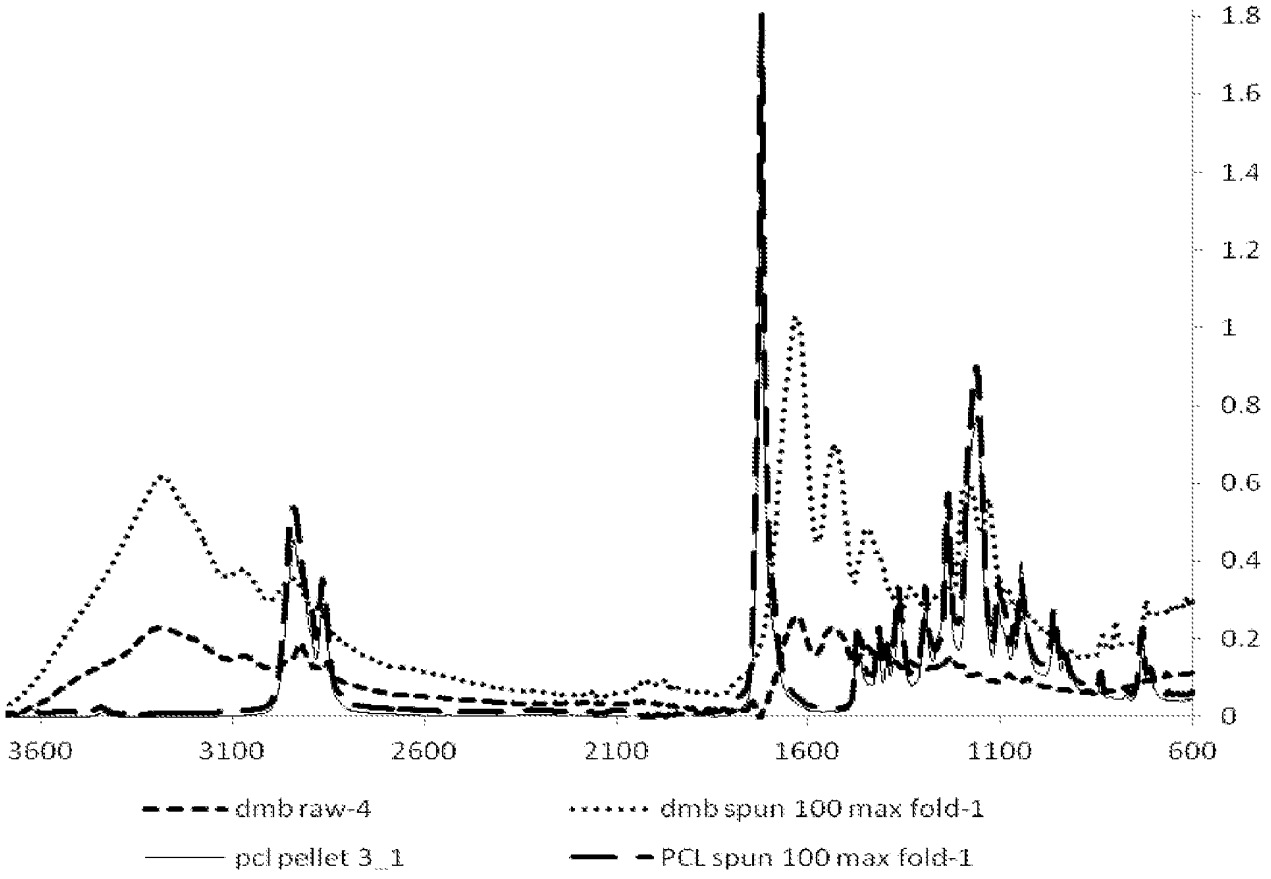

1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
3600
3100
2600
2100
1600
1100
600
dmb raw-4
dmb spun 100 max fold-1
pcl pellet 3_1
PCL spun 100 max fold-1

FIGURE 6.

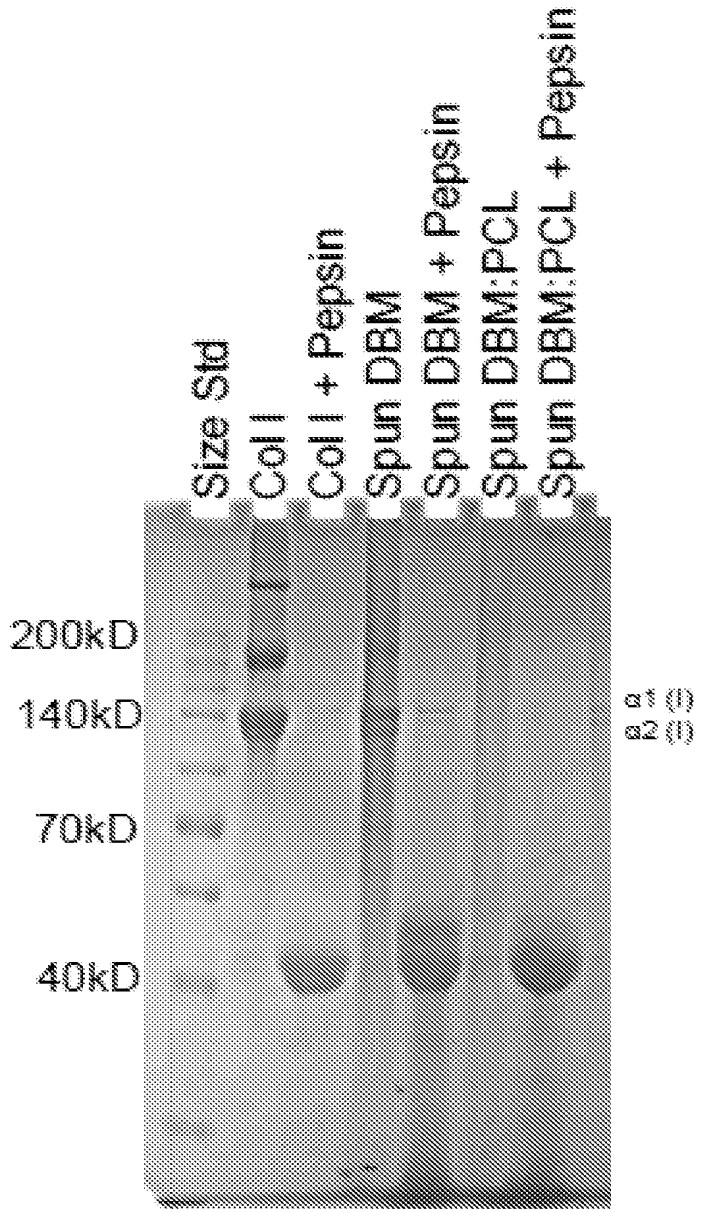

Size Std
Col I
Col I + Pepsin
Spun DBM
Spun DBM + Pepsin
Spun DBM:PCL
Spun DBM:PCL + Pepsin
200kD
140kD
70kD
40kD
α1 (I)
α2 (I)

FIGURE 7.

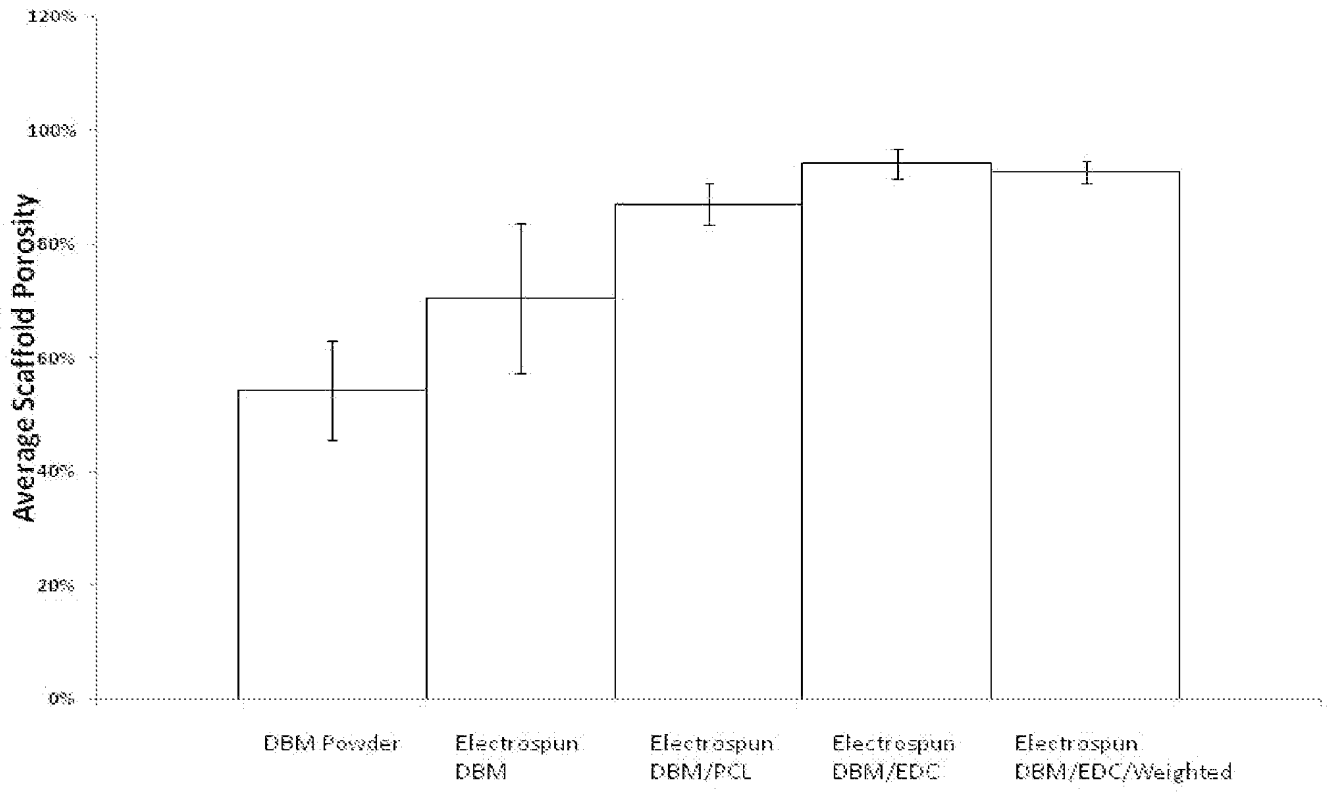

120%
100%
80%
60%
40%
20%
0%
Average Scaffold Porosity
DBM Powder
Electrospun DBM
Electrospun DBM/PCL
Electrospun DBM/EDC
Electrospun DBM/EDC/Weighted

FIGURE 8.

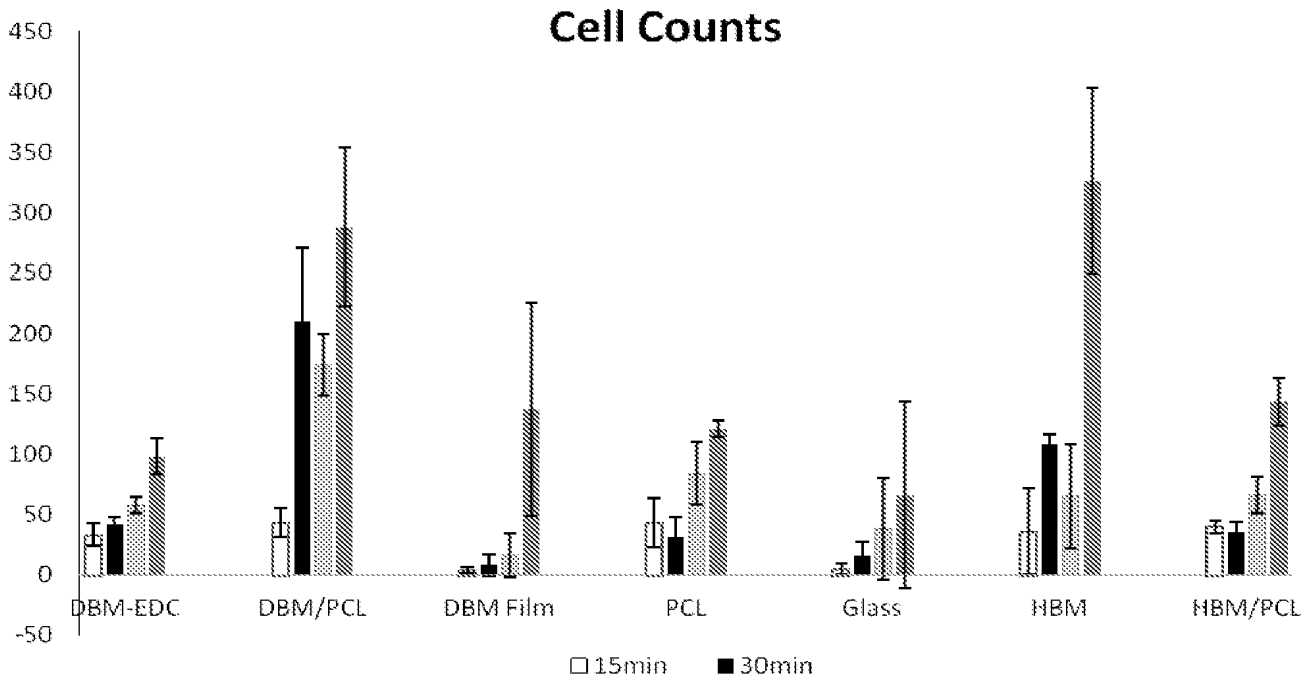

Cell Counts
450
400
350
300
250
200
150
100
50
0
-50
DBM-EDC
DBM/PCL
DBM Film
PCL
Glass
HBM
HBM/PCL
15min
30min

FIGURE 9.

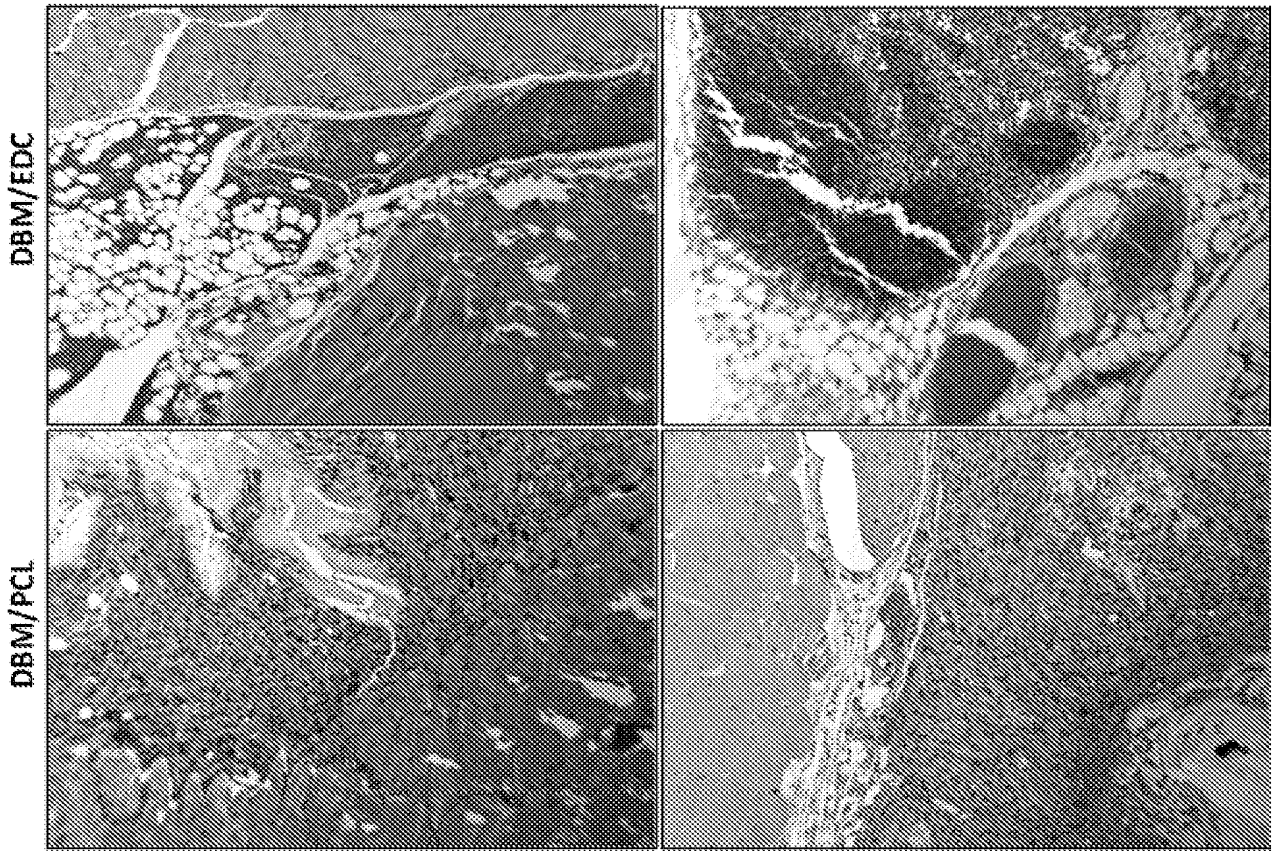


FIGURE 10.

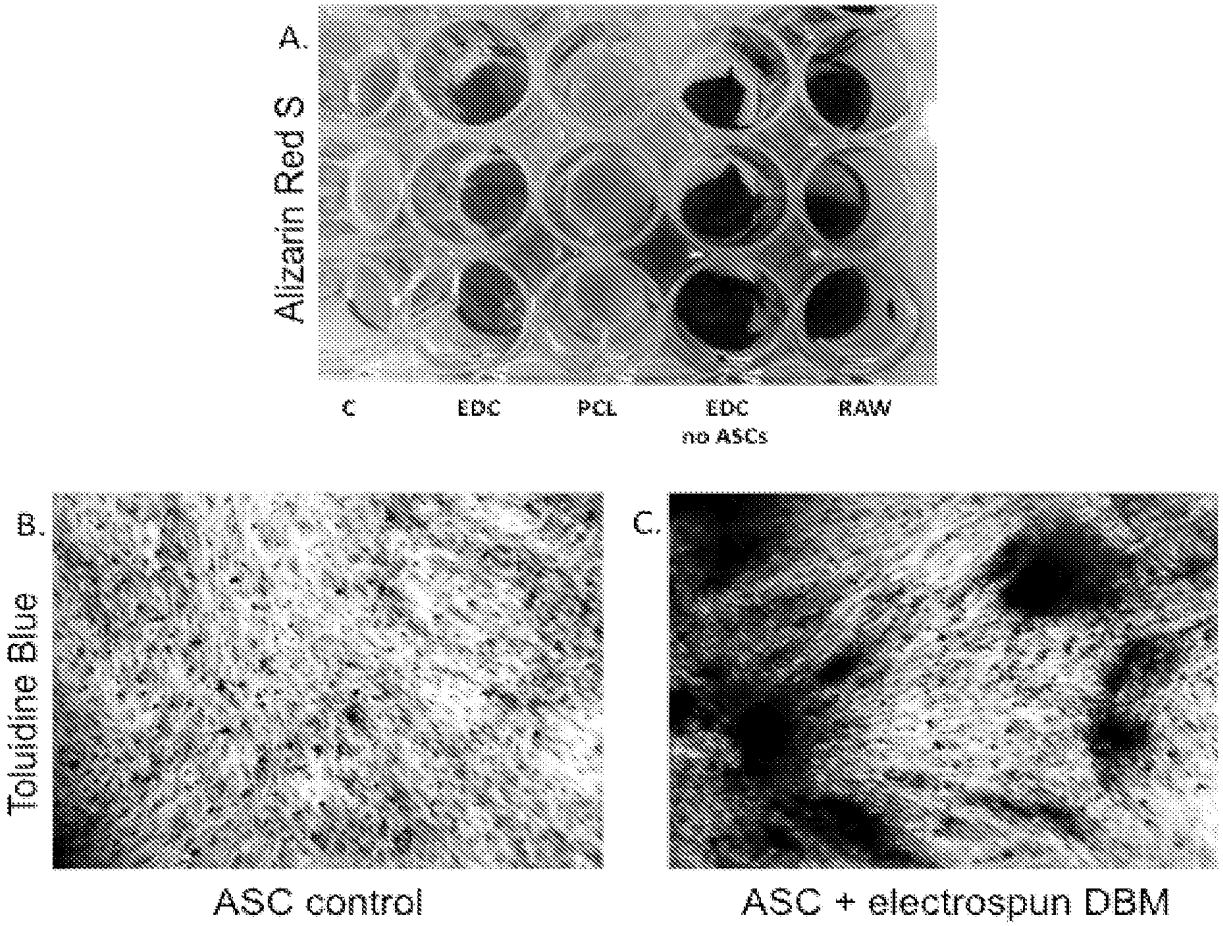
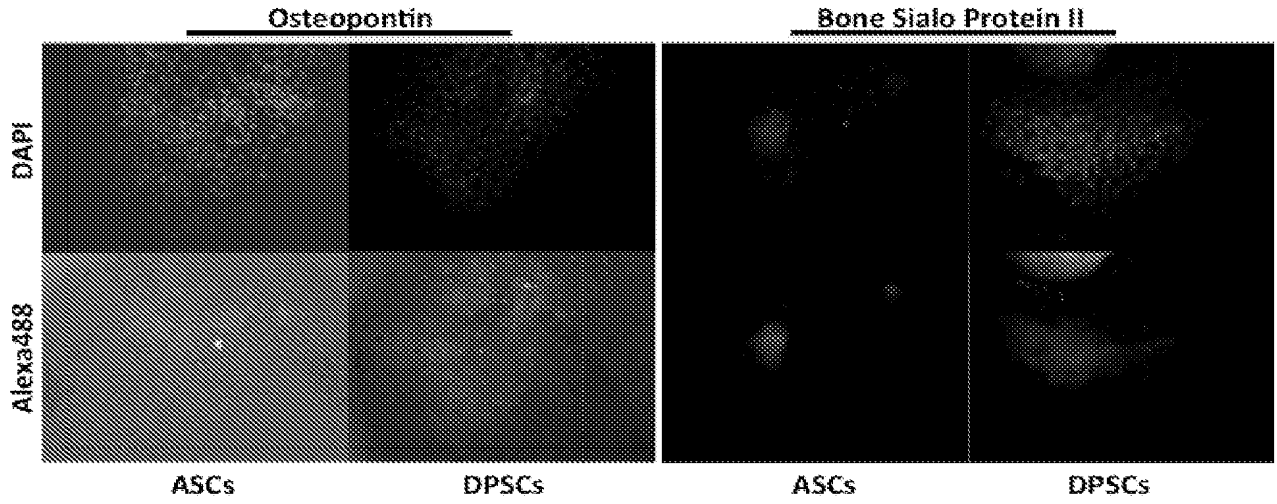

A.
Alizarin Red S
C
EDC
PCL
EDC
no ASCs
RAW
B.
Toluidine Blue
ASC control
C.
ASC + electrospun DBM

FIGURE 11.


Osteopontin
Bone Sialo Protein II
DAPI
Alexa488
ASCs
DPSCs
ASCs
DPSCs

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 13/25226

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 35/32 (2013.01)
USPC - 424/549
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)
USPC: 424/549

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC: 424/549, 426, 602 (see search terms below)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST,Google Patents, Google Scholar, Freepatentsonline, PatBase: Bone Matrix, Dissolving bone, demineralized bone, DBM, Liquefaction, Anhydrous fluoroalcohols, HFIP, HFP, TFE, Hexafluoroisopropanol, Trifluoroethanol, TFA, Dehydrothermal crosslinking, Nanofibers, implant$, Demineral$, colloid$, colloid#, dissol#.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- A	WO 2009/082554 A2 (Wei et al.) 02 July 2009 (02.07.2009) para [0008]-[0009], [0033], [0049], [0077]-[0079], [0105]-[0107], [0119]-[0121].	47-49 --- 17-19
A	US 6340477 B1 (Anderson) 22 January 2002 (22.01.2002) col. 1, ln 34-65; col. 3, ln 5-25.	1-3
A	US 2011/0218646 A1 (Pertici) 08 September 2011 (08.09.2011) para [0012], [0031], [0039]-[0040], [0060]-[0061].	68-75, 94-100

☐ Further documents are listed in the continuation of Box C. ☐

* 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	Date of mailing of the international search report
20 March 2013 (20.03.2013)	23 APR 2013
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. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 13/25226

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.: 4-16, 20-46, 50-67, 76-93, 101-131
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:

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.:

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.

Form PCT/ISA/210 (continuation of first sheet (2)) (July 2009)

Electronic Acknowledgement Receipt

EFS ID:	33290860
Application Number:	16045159
International Application Number:	
Confirmation Number:	5408
Title of Invention:	ALIGNED FIBER AND METHOD OF USE THEREOF
First Named Inventor/Applicant Name:	Michael Francis
Customer Number:	23122
Filer:	Glenn E. Murphy/Nicole May
Filer Authorized By:	Glenn E. Murphy
Attorney Docket Number:	LNH-152US1
Receipt Date:	26-JUL-2018
Filing Date:	
Time Stamp:	15:53:07
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Transmittal Letter	IDS.pdf	64646 e57469550ebd8a91d7ae1e201d011b869b3629b7	no	2

Warnings:

Information:					
2	Information Disclosure Statement (IDS) Form (SB08)	PTOSB08a.pdf	270071 82a744ae327b6bab43536b14e8aa3acfe07669d3	no	6
Warnings:					
Information:					
This is not an USPTO supplied IDS fillable form					
3	Foreign Reference	WO2010016942A1.pdf	3275961 1ba265c7985aedd981236e8897dbfe3a61850c44	no	70
Warnings:					
Information:					
4	Foreign Reference	WO2012027592A2.pdf	1300880 142220fdf5827e50abf8dfd8cdb69261ed0fff7c	no	25
Warnings:					
Information:					
5	Foreign Reference	WO2013033680A1.pdf	5289669 3926f8f77405485cf30a5258fad580c21c5c7130	no	106
Warnings:					
Information:					
6	Foreign Reference	WO2013119873A1.pdf	3841700 cffd479e90cece27d45f29053c887a68b1165c9c	no	63
Warnings:					
Information:					
7	Non Patent Literature	Anderson_Biodegradation.pdf	1154129 0e21431f25645e2da5c2860e46fe8978fcba1e32	no	20
Warnings:					
Information:					
8	Non Patent Literature	Ayres_Measuring.pdf	9908173 96731e6c573d19ec842aaef5f77e25cabeecb11e	no	20
Warnings:					
Information:					

9	Non Patent Literature	Cleek_Microparticles.pdf	452496 4957b8f72f5cafc944998b826b075f81d19dcf5a	no	10
Warnings:					
Information:					
10	Non Patent Literature	Schnaper_Regulation.pdf	1716032 f68112aaa5d8bb9e47649ccb6d031f3c6389173a	no	9
Warnings:					
Information:					
11	Non Patent Literature	Behravesh_Synthetic.pdf	2631526 104d914f3ce6d3091eb28ab89e67db9fc49eb43b	no	12
Warnings:					
Information:					
12	Non Patent Literature	Lu_Polymeric.pdf	3201013 b93b0fc4ac6a4cead7009a4796da4d73d7da4dc2	no	15
Warnings:					
Information:					
		Total Files Size (in bytes):	33106296		

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 a 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.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Appln. No:	16/045,159
Applicant:	LifeNet Health
Filed:	July 25, 2018
Title:	ALIGNED FIBER AND METHOD OF USE THEREOF
TC/A.U.:	TBD
Examiner:	TBD
Confirmation No.:	5408

INFORMATION DISCLOSURE STATEMENT TRANSMITTAL

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Commissioner:

Pursuant to 37 C.F.R. §§ 1.97 and 1.98 and to the duty of disclosure set forth in 37 C.F.R. § 1.56, Applicants submit the information listed on the attached form PTO/SB/08a and ask that it be considered and made of record in this application. Although the Examiner may determine the information submitted here to be material to examining this application, this submission is not an admission that any such information is prior art to the claimed invention. Further, in accordance with 37 C.F.R. § 1.97(g), the Office may not construe the filing of this Information Disclosure Statement to mean that a search has been made.

Under 37 C.F.R. § 1.98(d), copies of certain patents and publications listed on the enclosed PTO/SB/08a are not required to be provided, because they are of record in prior application Serial No. 14/776,106, filed September 14, 2015, to which this application claims priority under 35 U.S.C. § 120. Copies of the following cited documents, which are not of record in the prior application, are being provided with this Statement:

WO 2010/016942

WO 2012/027592

WO 2013/033680

WO 2013/119873

Anderson et al., "Biodegradation and Biocompatibility of PLA and PLGA Microspheres", Advanced Drug Delivery Reviews, 1997, pp. 5-24

Ayres et al., "Measuring Fiber Alignment in Electrospun Scaffolds: A User's Guide to the 2D Fast Fourier Transform Approach," Journal of Biomaterials Science, Polymer Edition, 2008, Vol. 19, No. 5, pp. 603-621

Behravesh et al., "Synthetic Biodegradable Polymers for Orthopaedic Applications," Clinical Orthopaedics and Related Research, No. 367S, pp. S118-S125

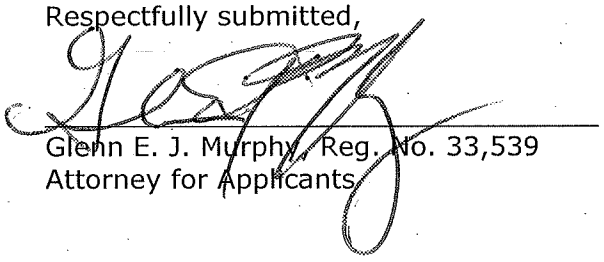
Cleek et al., "Microparticles of Poly(DL-lactic-co-glycolic acid)/poly(ethylene glycol) Blends for Controlled Drug Delivery," Journal of Controlled Release, Vol. 48 1997 pp. 259-268

Lu et al., "Polymeric Delivery Vehicles for Bone Growth Factors," (2000), Park & Mrsny; Controlled Drug Delivery, ACS Symposium Series; American Chemical Society, pp. 124-138

Schnaper et al., "Regulation of Cell Function by Extracellular Matrix," Pediatr. Nephrol, (1993), Vol. 7, pp. 96-104

This Information Disclosure Statement is being filed within three months of the filing date of this application. Accordingly, no fee or statement under 37 C.F.R. § 1.97(e) is required. 37 C.F.R. § 1.97(b).

Respectfully submitted,



Glenn E. J. Murphy, Reg. No. 33,539
Attorney for Applicants

GEM/nm

Enclosures: PTO/SB/08a
(10) References

Dated: July 26, 2018

2200 Renaissance Blvd
Suite 350
King of Prussia, PA 19406
(610) 407-0700

The Commissioner for Patents is hereby authorized to charge payment to Deposit Account No. **18-0350** of any fees associated with this communication.

4079335

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number: 16/045,159

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY FEE($)
BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A	75		N/A	
SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A	330		N/A	
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A	380		N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	11 minus 20 =	*	x 50 =	0.00	OR		
INDEPENDENT CLAIMS (37 CFR 1.16(h))	4 minus 3 =	* 1	x 230 =	230			
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).			0.00			
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))				0.00			
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL	1015		TOTAL	

APPLICATION AS AMENDED - PART II

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
AMENDMENT A	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
AMENDMENT B	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** 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 found in the appropriate box in column 1.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
16/045,159	07/25/2018		1015	LNH-152US1	11	4

CONFIRMATION NO. 5408

FILING RECEIPT

23122
RATNERPRESTIA
2200 Renaissance Blvd
Suite 350
King of Prussia, PA 19406

OC000000101876053

Date Mailed: 08/28/2018

Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Inventor(s)

Michael Francis, Virginia Beach, VA;
Roy Ogle, Virginia Beach, VA;

Applicant(s)

LifeNet Health, Virginia Beach, VA;

Power of Attorney: None

Domestic Priority data as claimed by applicant

This application is a DIV of 14/776,106 09/14/2015
which is a 371 of PCT/US2014/025636 03/13/2014
which claims benefit of 61/785,031 03/14/2013

Foreign Applications for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.) - None.
Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.

Permission to Access Application via Priority Document Exchange: Yes

Permission to Access Search Results: Yes

Applicant may provide or rescind an authorization for access using Form PTO/SB/39 or Form PTO/SB/69 as appropriate.

If Required, Foreign Filing License Granted: 08/27/2018

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 16/045,159**

Projected Publication Date: To Be Determined - pending completion of Corrected Papers

Non-Publication Request: No

Early Publication Request: No
**** SMALL ENTITY ****
Title

ALIGNED FIBER AND METHOD OF USE THEREOF

Preliminary Class

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

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Title 37, Code of Federal Regulations, 5.11 & 5.15

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