

***Frizzled-10*, up-regulated in primary colorectal cancer, is a positive regulator of the WNT - β -catenin - TCF signaling pathway**

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Abstract. WNT signaling pathway is implicated in embryogenesis as well as in carcinogenesis. We have previously cloned and characterized *Frizzled-1* (*FZD1*), *FZD2*, *FZD3*, *FZD4*, *FZD5*, *FZD6*, *FZD7*, *FZD8*, and *FZD10*, encoding seven-transmembrane-type WNT receptors. Here, expression of *FZD10* mRNA in various types of human cancer and effects of *FZD10* mRNA microinjection into *Xenopus* early embryos were investigated. Northern blot analyses revealed relatively high-level expression of 4.0-kb *FZD10* mRNA in cervical cancer cell lines HeLa S3, SKG-I, SKG-IIIa, and in a glioblastoma cell line A-172. Matched tumor/normal expression array analysis revealed significant up-regulation of *FZD10* mRNA in 2 cases of primary colon cancer. Function of *FZD10* was next investigated by using *Xenopus* axis duplication assay, in which positive regulators of the WNT - β -catenin - TCF signaling pathway induce axis duplication. Injection of wild-type *FZD10* mRNA into the ventral marginal zone of 4-cell-stage *Xenopus* embryos induced partial axis duplication in 40% of embryos. Ventral injection of Thr579Ala *FZD10* mRNA or Val581Leu *FZD10* mRNA with mutations in the C-terminal Ser/Thr-X-Val motif also induced partial axis duplication in about 40% of embryos. Furthermore, ventral injection of *FZD10* mRNA significantly augmented the potential of co-injected *Xenopus wnt-8* (*Xwnt-8*) mRNA to induce complete axis duplication. These results suggest that up-regulation of *FZD10* mRNA in several types of human cells might lead to carcinogenesis through activation of the β -catenin - TCF signaling pathway synergistically with some class of WNTs.

Introduction

Secreted-type glycoprotein WNTs play important roles in carcinogenesis as well as in embryogenesis. Seven-transmembrane type WNT receptors, encoded by *Frizzled* (*FZD*)

genes, transduce WNT signal into the β -catenin - TCF pathway, the c-JUN - N-terminal kinase (JNK) pathway, or the Ca^{2+} -releasing pathway. We have previously cloned and characterized the following human *FZDs* and *WNTs*: *FZD1* (1), *FZD2* (1), *FZD3* (2), *FZD4* (3), *FZD5* (4), *FZD6* (5), *FZD7* (1,6), *FZD8* (7), *FZD10* (8-10), *WNT2B/WNT13* (11-14), *WNT3* (15), *WNT3A* (16), *WNT5B* (17), *WNT6* (18), *WNT7B* (19), *WNT8A* (20), *WNT10A* (18,21,22), *WNT10B* (23), *WNT11* (24), *WNT14* (16,25), and *WNT14B* (25,26).

Genetic alterations of WNT signaling molecules, such as *Wnt1* (27), *WNT2* (28,29), *Wnt10b* (30), *Frat1* (31), *APC* (32), *AXIN1* (33), or β -catenin (34), lead to carcinogenesis through transcriptional activation of WNT target genes induced by stabilization and nuclear translocation of β -catenin.

FZD10 gene on human chromosome 12q24.33 encodes a seven-transmembrane-type WNT receptor with the extracellular cysteine-rich domain and the cytoplasmic C-terminal Ser/Thr-X-Val motif (8). *FZD10* mRNA is relatively highly expressed in a cervical cancer cell line HeLa S3 (8). Here, we investigated expression of *FZD10* mRNA in various types of human cancer as well as effects of *FZD10* mRNA microinjection into *Xenopus* early embryos. *FZD10* mRNA was significantly up-regulated in 2 cases of primary colon cancer. Ventral injection of *FZD10* mRNA induced *Xenopus* axis duplication by itself, and also significantly augmented the axis-duplication potential of co-injected *Xenopus wnt-8* (*Xwnt-8*) mRNA. This is the first report on expression and functional analyses on *FZD10*.

Materials and methods

Cell lines and poly(A)⁺ RNA extraction. Poly(A)⁺ RNAs of human fetal brain, fetal kidney, and adult brain were purchased from Clontech Laboratories. Poly(A)⁺ RNAs of cervical cancer cell lines HeLa S3, HeLa, SKG-I, SKG-II, SKG-IIIa, esophageal cancer cell lines TE1, TE2, TE3, TE4, TE5, TE6, TE7, TE8, TE10, TE11, TE12, TE13, and brain tumor cell lines DBTRG-05MG, T98G, U-373MG, SW1088, SW1783, A-172, and Hs683 were extracted with the FastTrack 2.0 kit (Invitrogen) as described previously (35).

Northern blot analyses. Two μg of poly(A)⁺ RNAs, separated by denaturing agarose-gel electrophoresis, were transferred onto nitrocellulose membrane, and fixed as described

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Table I. Oligonucleotide primers.

Primer	Orientation	Nucleotide sequence	Nucleotide position
P10-101	Sense	CTCTGCAGTCCTGGCAGCAGGTG	1597-1619 of <i>FZD10</i>
P10-102	Anti-sense	CCGAAATTCACACGCAGGTGGGCGACTGG	1769-1742 of <i>FZD10</i>
P10-104	Anti-sense	CCGAAATTCACACGCAGGCGGGCGACTGG	1769-1742 of <i>FZD1</i>
P10-106	Anti-sense	CCGAAATTCACAGGCAGGTGGGCGACTGG	1769-1742 of <i>FZD10</i>
P10-107	Sense	ACACGTCCAACGCCAGCATG	1-20 of <i>FZD10</i>
P10-108	Anti-sense	ACGAGTCATGTTGTAGCCGATG	170-149 of <i>FZD10</i>
BACT1	Sense	GACTACCTCATGAAGATCCT	632-651 of β -actin
BACT2	Anti-sense	GCGGATGTCCACGTCACACT	943-924 of β -actin

previously (36). After pre-hybridized for 1 h at 68°C in QuikHyb solution (Stratagene), Northern blot filters were hybridized with a [α - ^{32} P]dCTP-labeled probe for 1 h at 68°C in QuikHyb solution. After washing twice in 2X SSC buffer and 0.1% SDS at room temperature for 15 min, and once in 0.1X SSC buffer and 0.1% SDS at 60°C for 30 min, Northern blot filters were exposed to XAR-5 film (Kodak) as described previously (37). The TEN-S probe corresponds to nucleotide position 1751-2673 of the *FZD10* cDNA (8).

One-step cDNA-PCR. Ten ng of poly(A)⁺ RNAs were used as templates for cDNA-PCR with One-step RT-PCR kit (Qiagen) as described previously (38). After reverse-transcription reaction at 50°C for 30 min, and activation of HotStarTaq DNA polymerase by heating at 95°C for 15 min, *FZD10* cDNA was amplified by 30 cycles of PCR (94°C for 0.5 min, 60°C for 0.5 min, 72°C for 2 min), and β -actin cDNA by 17 cycles of cDNA-PCR. Nucleotide sequences of PCR primers are listed in Table I.

Matched tumor/normal expression array analysis. After pre-hybridization for 30 min at 65°C in ExpressHyb solution (Clontech Laboratories), a matched tumor/normal expression array filter (Clontech Laboratories) was hybridized with [α - ^{32} P]dCTP-labeled TEN-S probe at 65°C overnight. After washing twice in 2X SSC and 1% SDS at 65°C for 30 min, once in 0.1X SSC and 0.5% SDS at 65°C for 30 min, and once in 2X SSC at room temperature for 5 min, expression array filter was exposed to the Imaging Plate (Fuji) for image analysis in the Storm system (Molecular Dynamics) as described previously (29).

Construction of *FZD10* expression plasmids. The C-terminal region of wild-type *FZD10*, Thr579Ala *FZD10*, or Val581Leu *FZD10* was synthesized from *FZD10*/pUC118 plasmid by PCR using KOD Plus DNA polymerase (Toyobo) with P10-101 and P10-102 primers, P10-101 and P10-104 primers, P10-101 and P10-106 primers, respectively. Nucleotide sequences of these PCR primers are also listed in Table I. P10-101 primer contains an internal *Pst*I site within the coding region of *FZD10* mRNA. P10-102, P10-104, and P10-106 primers contain an *Eco*RI linker. After digestion with *Pst*I and *Eco*RI, PCR products were ligated into the *FZD10*/

pUC118 plasmid digested with *Pst*I and *Eco*RI. Wild-type, Thr579Ala, and Val581Leu *FZD10* cDNAs were excised with *Eco*RI digestion, and then subcloned into pCS2+ vector. Thr579Ala and Val581Leu *FZD10* cDNAs were confirmed to be mutated in the C-terminal Ser/Thr-X-Val motif by nucleotide sequence analysis.

In vitro RNA synthesis. Capped mRNAs were synthesized from linearized expression constructs in pCS2+ plasmid by using the MEGAscript SP6 Kit (Ambion) as described previously (39).

Xenopus axis duplication assay. Twelve hours after injection of Gonatropin (Teikoku Zouki) into female *Xenopus laevis*, eggs were manually ovulated and artificially fertilized with *Xenopus laevis* sperma. Fertilized eggs were dejellied, and were transferred to culture dishes containing Steinberg's solution. Wild-type *FZD10* mRNA, Thr579Ala *FZD10* mRNA, Val581Leu *FZD10* mRNA, *Xwnt-8* mRNA, or β -globin mRNA were injected into the ventral marginal zone of *Xenopus* embryos at the 4-cell stage as described previously (40). Phenotypes of *Xenopus* embryos were evaluated 24 h after mRNA injection.

Results

Expression of *FZD10* mRNA in human cancer cell lines. We examined expression of *FZD10* mRNA in 24 human cancer cell lines derived from various tissues by using Northern blot analyses with the *FZD10* specific probe, TEN-S. The 4.0-kb *FZD10* mRNA was relatively highly expressed in cervical cancer cell lines HeLa S3, SKG-I, SKG-IIIa (Fig. 1A), and a glioblastoma cell line A-172 (Fig. 1B), and also faintly expressed in esophageal cancer cell lines TE5, and TE11 (Fig. 1C).

By using cDNA-PCR, expression levels of *FZD10* mRNA in HeLa S3, SKG-I, SKG-II, SKG-IIIa, TE5, and A-172 cells were compared with that in normal human fetal kidney, in which *FZD10* mRNA is moderately expressed (8). β -actin mRNA was almost equally detected in HeLa S3, SKG-I, SKG-II, SKG-IIIa, TE5, and A-172 cells and fetal kidney. *FZD10* mRNA was more strongly expressed in HeLa S3, SKG-I, SKG-IIIa, and A-172 than in fetal kidney (Fig. 1D).

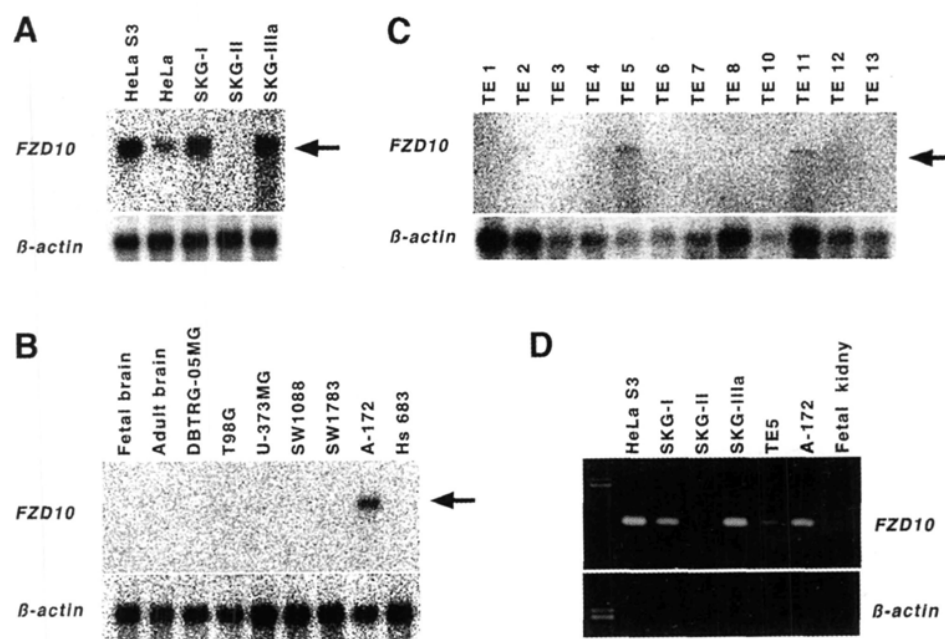


Figure 1. Expression of *FZD10* mRNA in human cancer cell lines. A, Cervical cancer; B, Brain tumor; C, Esophageal cancer. Northern blot filters were hybridized with [α - ^{32} P]dCTP-labeled *FZD10* specific probe, TEN-S (nucleotide position 1751-2673 of the *FZD10* cDNA). The 4.0-kb *FZD10* mRNA (arrow) was relatively highly expressed in cervical cancer cell lines HeLa S3, SKG-I, SKG-IIIa, and a glioblastoma cell line A-172. D, cDNA-PCR analysis on *FZD10* mRNA. Ten ng of poly(A)⁺ RNAs were used as templates for cDNA-PCR. *FZD10* mRNA was detected by 30 cycles of cDNA-PCR with P10-107 and P10-108 primers, and β -actin mRNA by 17 cycles of cDNA-PCR with BACT1 and BACT2 primers. β -actin mRNA was almost equally detected. *FZD10* mRNA was relatively highly expressed in HeLa S3, SKG-I, SKG-IIIa, and A-172 cells than in fetal kidney.

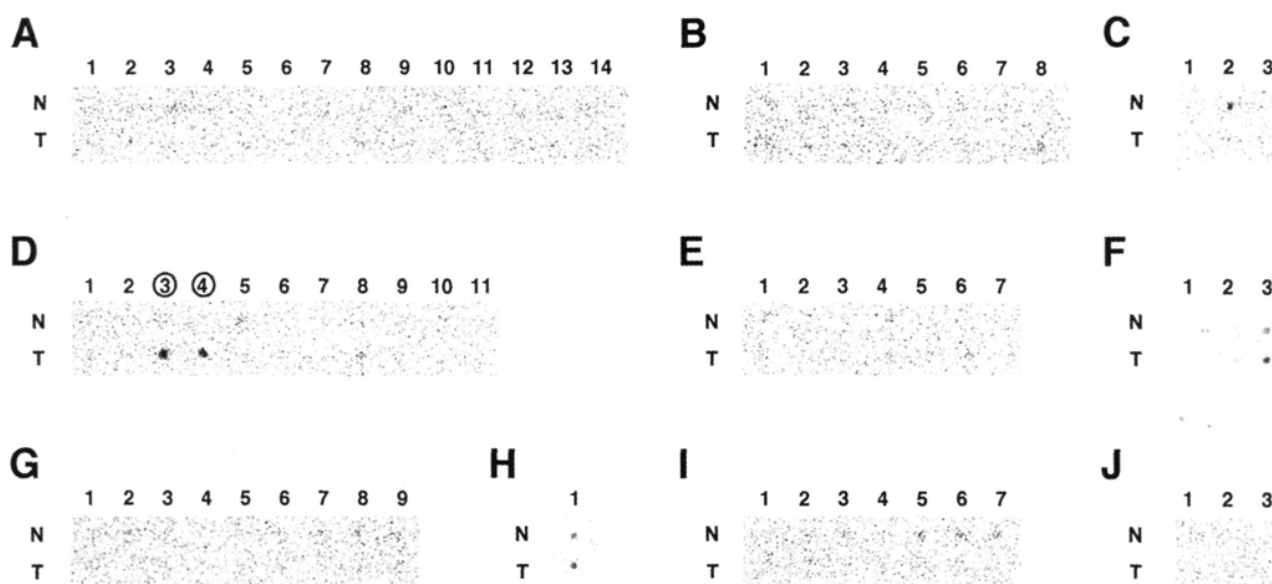


Figure 2. Expression of *FZD10* mRNA in various types of human primary cancer. A, Kidney tumors; B, Gastric cancer; C, Lung cancer; D, Colon tumors; E, Rectal cancer; F, Prostate cancer; G, Breast cancer; H, Cervical cancer; I, Uterus tumors; J, Ovarian cancer. Matched tumor/normal expression array filter was hybridized with [α - ^{32} P]dCTP-labeled TEN-S probe. N, Normal. T, Tumor. Case number is shown on each panel. Case number in small open circle indicates that *FZD10* mRNA is up-regulated in such a case of primary tumor. *FZD10* mRNA was significantly up-regulated in 2 cases of primary colon cancer out of 66 primary tumors.

Expression of FZD10 mRNA in various types of human primary tumor. Expression of *FZD10* mRNAs in 66 human primary tumors was investigated by using matched tumor/normal expression array filter. Among kidney tumors (14 cases), gastric cancer (8 cases), lung cancer (3 cases), colon tumors

(11 cases), rectal cancer (7 cases), prostate cancer (3 cases), breast cancer (9 cases), cervical cancer (1 case), uterus tumors (7 cases), and ovarian cancer (3 cases), expression of *FZD10* mRNA was significantly up-regulated in 2 cases of primary colon cancer (Fig. 2). These results indicate that expression

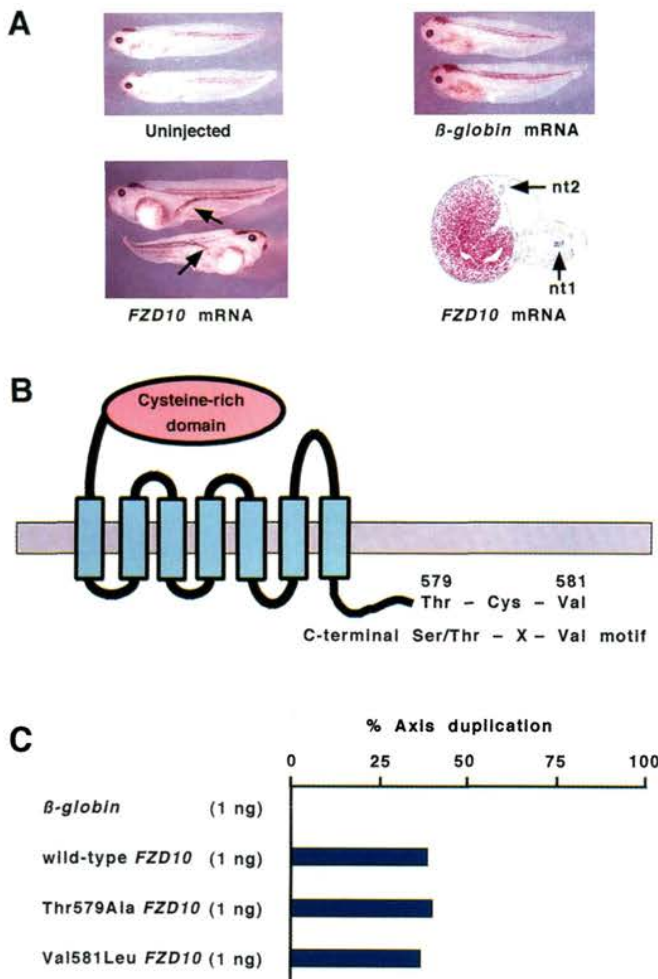


Figure 3. FZD10 mRNA induces partial axis duplication in *Xenopus* embryos. **A**, Picture of uninjected *Xenopus* embryos, *Xenopus* embryos injected with β -globin mRNA (1 ng), or FZD10 mRNA (1 ng). Synthetic mRNAs were injected into the ventral marginal zone of fertilized *Xenopus* eggs at the 4-cell stage. Ventral injection of wild-type FZD10 mRNA, but not that of β -globin mRNA, induced partial axis duplication (arrow) in *Xenopus* embryos. Cross section of *Xenopus* embryo ventrally injected with FZD10 mRNA shows the existence of original notocord (nt1) and an additionally-formed (secondary) notocord (nt2). **B**, Schematic presentation of human FZD10, consisting of the extracellular cysteine-rich domain, seven transmembrane domains, and the cytoplasmic C-terminal Ser/Thr-X-Val motif. **C**, Summary of *Xenopus* axis duplication assay. Partial axis duplication is shown by the blue bar. Ventral injection of wild-type FZD10 mRNA (1 ng) induced partial axis duplication in 40% of embryos. Ventral injection of Thr579Ala FZD10 mRNA (1 ng) or Val581Leu FZD10 mRNA (1 ng) with mutation in the C-terminal Ser/Thr-X-Val motif also induced partial axis duplication in 42% or 37% of embryos, respectively.

of FZD10 mRNA is up-regulated not only in human cancer cell lines but also in human primary tumors.

Xenopus axis duplication induced by ventral injection of FZD10 mRNAs. Ectopic activation of the WNT - β -catenin - TCF signaling pathway in the ventral marginal zone of *Xenopus* early embryo leads to axis duplication (39-41). We, therefore, investigated the effects of FZD10 mRNA micro-injection into the ventral marginal zone of *Xenopus* embryos. Ventral injection of wild-type FZD10 mRNA (1 ng), but not that of β -globin mRNA (1 ng), induced partial axis duplication in 40% of *Xenopus* embryos (Fig. 3). By cross-section analyses,

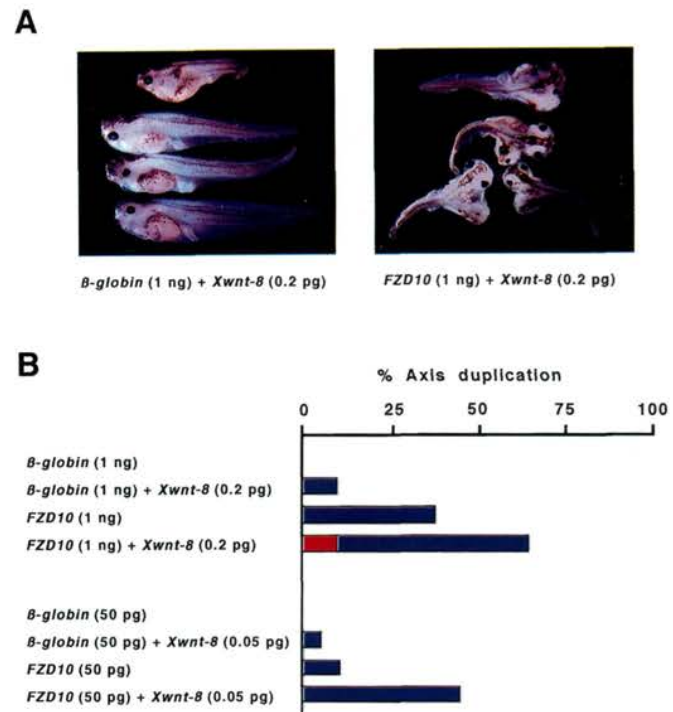


Figure 4. Ventral injection of FZD10 mRNA augments axis-duplication potential of co-injected Xwnt-8 mRNA. **A**, Picture of *Xenopus* embryos ventrally co-injected with β -globin mRNA and submaximal-dose Xwnt-8 mRNA (left), or that with FZD10 mRNA and submaximal-dose Xwnt-8 mRNAs (right). **B**, Summary of *Xenopus* axis duplication assay. Complete axis duplication (additional head structure with eyes) is shown by the red bar, and partial axis duplication by the blue bar. Ventral injection of FZD10 mRNA significantly augmented the axis-duplication potential of co-injected Xwnt-8 mRNA.

existence of an additional notocord was demonstrated in *Xenopus* embryos with partial axis duplication induced by ventral injection of wild-type FZD10 mRNA itself (Fig. 3A).

We also investigated the role of the C-terminal Ser/Thr-X-Val motif of FZD10 in the WNT - β -catenin - TCF signaling pathway by using Thr579Ala FZD10 mRNA and Val581Leu FZD10 mRNA with artificial mutation in the C-terminal Ser/Thr-X-Val motif. Ventral injection of Thr579Ala FZD10 mRNA (1 ng) and Val581Leu FZD10 mRNA (1 ng) induced partial axis duplication just like the wild-type FZD10 mRNA (Fig. 3C). These results indicate that the C-terminal Ser/Thr-X-Val motif of FZD10 is not necessary to induce axis duplication in *Xenopus* embryos.

Augmentation of axis-duplication potential of FZD10 by co-injection of Xwnt-8. We investigated the synergism between ventral injection of FZD10 mRNA and that of Xwnt-8 mRNA. Ventral injection of FZD10 mRNA (1 ng) induced partial axis duplication in 40% *Xenopus* embryos, which was consistent with the result of experiment in Fig. 3. Ventral co-injection of β -globin mRNA (1 ng) and submaximal-dose Xwnt-8 mRNA (0.2 pg) induced partial axis duplication in 10% of *Xenopus* embryos. When FZD10 mRNA (1 ng) was ventrally co-injected with submaximal-dose Xwnt-8 mRNA (0.2 pg), the incidence of axis duplication was increased up to 65%, which included 10% of complete axis duplication with head structure (Fig. 4).

We then performed ventral co-injection experiments at the lowered dosage of *FZD10* mRNA and *Xwnt-8* mRNA. Ventral injection of submaximal-dose *FZD10* mRNA (50 pg) induced partial axis duplication in 11% of *Xenopus* embryos. However, when submaximal-dose *FZD10* mRNA (50 pg) was ventrally co-injected with submaximal-dose *Xwnt-8* mRNA (0.05 pg), the incidence of partial axis duplication was increased up to 45% (Fig. 4). These results clearly indicate that axis-duplication potential of *FZD10* mRNA was augmented by co-injection of *Xwnt-8* mRNA.

Discussion

Expression profile of *FZD10* mRNA in human cancer cell lines and human primary tumors was investigated in this study. The 4.0-kb *FZD10* mRNA was relatively highly expressed in cervical cancer cell lines HeLa S3, SKG-I, SKG-IIIa, and a glioblastoma cell line A-172 among 24 human cancer cell lines (Fig. 1), and in 2 cases of primary colon cancer among 66 cases of primary tumors derived from various organs (Fig. 2). In addition, we have previously reported up-regulation of *FZD10* mRNA in gastric cancer cell lines TMK1 and MKN74 (9). Thus, we conclude that *FZD10* mRNA is up-regulated in several types of human cancer cell lines and in human primary tumors.

WNT - β -catenin - TCF signaling pathway plays a pivotal role in the body axis formation during *Xenopus* early development, and ventral injection of positive regulators of WNT - β -catenin - TCF signaling pathway induces the secondary-axis formation (39-41). Ventral injection of wild-type *FZD10* mRNA induced partial axis duplication in 40% of *Xenopus* embryos (Fig. 3). Furthermore, ventral injection of *FZD10* mRNA significantly augmented the axis-duplication potential of co-injected *Xwnt-8* mRNA (Fig. 4). These results indicate that human *FZD10* positively regulates of the *Xenopus* WNT - β -catenin - TCF signaling pathway. Though in a heterogeneous system, this is the first report demonstrating the biological roles of mammalian *FZD10*.

The C-terminal Ser/Thr-X-Val motif of *FZD10* is not necessary to induce axis duplication in *Xenopus* embryos, because ventral injection of Thr579Ala *FZD10* mRNA or Val581Leu *FZD10* mRNA induced partial axis duplication just like that of the wild-type *FZD10* mRNA (Fig. 3A). GOPC/Fzip-1, consisting of one PDZ domain, two coiled-coil motifs and two evolutionally conserved regions, binds to C-terminal Ser/Thr-X-Val motif of FZDs (42). Interaction between the C-terminal Ser/Thr-X-Val motif of *FZD10* and GOPC might be necessary for the vesicle transport of *FZD10* from the Golgi apparatus to the plasma membrane, but might not be necessary for signaling from WNT receptor to the β -catenin - TCF pathway.

Lys-Thr-X-X-X-Trp motif in the cytoplasmic tail region of FZDs is required for β -catenin - TCF signaling (43). Lys-Thr-X-X-X-Trp motif, initially identified in *Xenopus* frizzled-3 (Xfz3), Xfz4, and Xfz7, was also identified in human *FZD10* as well as in other human FZDs (Katoh, unpublished data). Although signaling molecules binding to the Lys-Thr-X-X-X-Trp motif of FZDs are not identified yet, *FZD10* with the Lys-Thr-X-X-X-Trp motif might transduce signals to the β -catenin - TCF pathway in cooperation with WNTs, which is consistent with our observation (Figs. 3 and 4).

Genetic alterations of WNT signaling molecules, resulting in activation of the WNT - β -catenin - TCF signaling pathway, lead to carcinogenesis through transcriptional activation of WNT target genes (27-34). *FZD10* mRNA was up-regulated in primary colon cancer as well as in several human cancer cell lines (Figs. 1 and 2), and *FZD10* was found to be a positive regulator of the WNT - β -catenin - TCF signaling pathway (Figs. 3 and 4). Axis-duplication potential of *FZD10* mRNA was significantly augmented by co-injection of *Xwnt-8* (Fig. 4), but not by that of mouse *Wnt-4* or *Xwnt-11* (unpublished data). Therefore, up-regulation of *FZD10* mRNA in several types of human cells might lead to carcinogenesis through activation of the WNT - β -catenin - TCF signaling pathway synergistically with some class of WNTs.

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