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Comparative integromics on FZD7 orthologs: Conserved binding sites for PU.1, SP1, CCAAT-box and TCF/LEF/SOX transcription factors within 5'-promoter region of mammalian *FZD7* orthologs

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Abstract. Canonical WNT signals are transduced through Frizzled (FZD) family receptor and LRP5/LRP6 co-receptor to upregulate *MYC, CCND1, FGF20, JAG1, WISP1* and *DKK1* genes, while non-canonical WNT signals are transduced through FZD family receptor and PTK7/ROR2/RYK co-receptor to activate RHOA/RHOU/RAC/CDC42, JNK, PKC, NFAT and NLK signaling cascades. *FZD7*, expressed in the normal gastrointestinal tract, is upregulated in esophageal cancer, gastric cancer, colorectal cancer, and hepatocellular carcinoma. Here, chimpanzee *FZD7* and cow *Fzd7* genes were identified and characterized by using bioinformatics (Techint) and human intelligence (Humint). Chimpanzee *FZD7* and cow *Fzd7* genes were identified within NW_001232110.1 and AC173037.2 genome sequences, respectively. Chimpanzee FZD7 and cow Fzd7 showed 100% and 97.2% total-amino-acid identity with human FZD7. All of the nine amino-acid residues substituted between human FZD7 and human FzE3 were identical to those of human FZD7 in chimpanzee, cow, mouse and rat FZD7 orthologs. Functional analyses using FzE3 with multiple cloning artifacts and/or sequencing errors are invalid. FZD7 orthologs were seven-transmembrane proteins with extracellular Frizzled domain, leucine zipper motif around the 5th transmembrane domain, and cytoplasmic DVL- and PDZ-binding motifs. Ser550 and Ser556 of FZD7 orthologs were putative aPKC phosphorylation sites. Dimerization and Ser550/556 phosphorylation were predicted as regulatory mechanisms for the signaling through FZD7. Transcriptional start site of human *FZD7* gene was 735-bp upstream of NM_003507.1 RefSeq 5'-end. In addition to gastrointestinal cancer, hepatocellular cancer and pancreatic cancer, human *FZD7* mRNAs were expressed in blastocysts, undifferentiated embryonic stem (ES) cells, ES-derived endodermal progenitors, ES-derived neural progenitors, fetal cochlea, retinal pigment epithelium, olfactory epithelium, regenerating liver, and multiple sclerosis. Comparative genomics analyses revealed that the binding sites for PU.1, SP1/Krüppel-like, CCAAT-box, and TCF/LEF/SOX transcription factors were conserved among 5'-promoter regions of mammalian *FZD7* orthologs.

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Introduction

Cross-talk of the WNT signaling pathway and FGF, Notch, Hedgehog and BMP/Nodal/TGFß signaling pathways constitute the stem-cell signaling network, which is implicated in embryogenesis and adult tissues homeostasis (1-13). Canonical WNT signals are transduced through Frizzled (FZD) family receptor and LRP5/LRP6 co-receptor to upregulate *MYC, CCND1, FGF20, JAG1, WISP1* and *DKK1* genes (14-24), while non-canonical WNT signals are transduced through the FZD family receptor and PTK7/ROR2/RYK co-receptor to activate RHOA/RHOU/RAC/CDC42, JNK, PKC, NFAT and NLK signaling cascades (25-30). WNT signals are context-dependently transduced to canonical and non-canonical signaling cascades.

We previously reported molecular cloning and characterization of human FZD7 (31), which showed six amino-acid substitutions with human FzE3 (32). We then identified and characterized rat *Fzd7* gene (33). *FZD7* is upregulated in gastric cancer (31,34), esophageal cancer (32), colorectal cancer (31,35), and hepatocellular carcinoma (36). Here, chimpanzee *FZD7* and cow *Fzd7* genes were identified and characterized by using bioinformatics (Techint) and human intelligence (Humint). Chimpanzee *FZD7* and cow *Fzd7* genes were identified within NW_001232110.1 and AC173037.2 genome sequences, respectively. Comparative proteomics analyses on FZD7 orthologs were then performed. *In silico* expression analyses revealed *FZD7* expression in human embryonic stem (ES) cells. In addition, comparative genomics analyses on *FZD7* promoter region revealed conserved transcription factor binding sites within 5'-promoter region of mammalian *FZD7* orthologs.

Materials and methods

Identification and characterization of chimpanzee and cow FZD7 orthologs. Chimpanzee and cow genome sequences

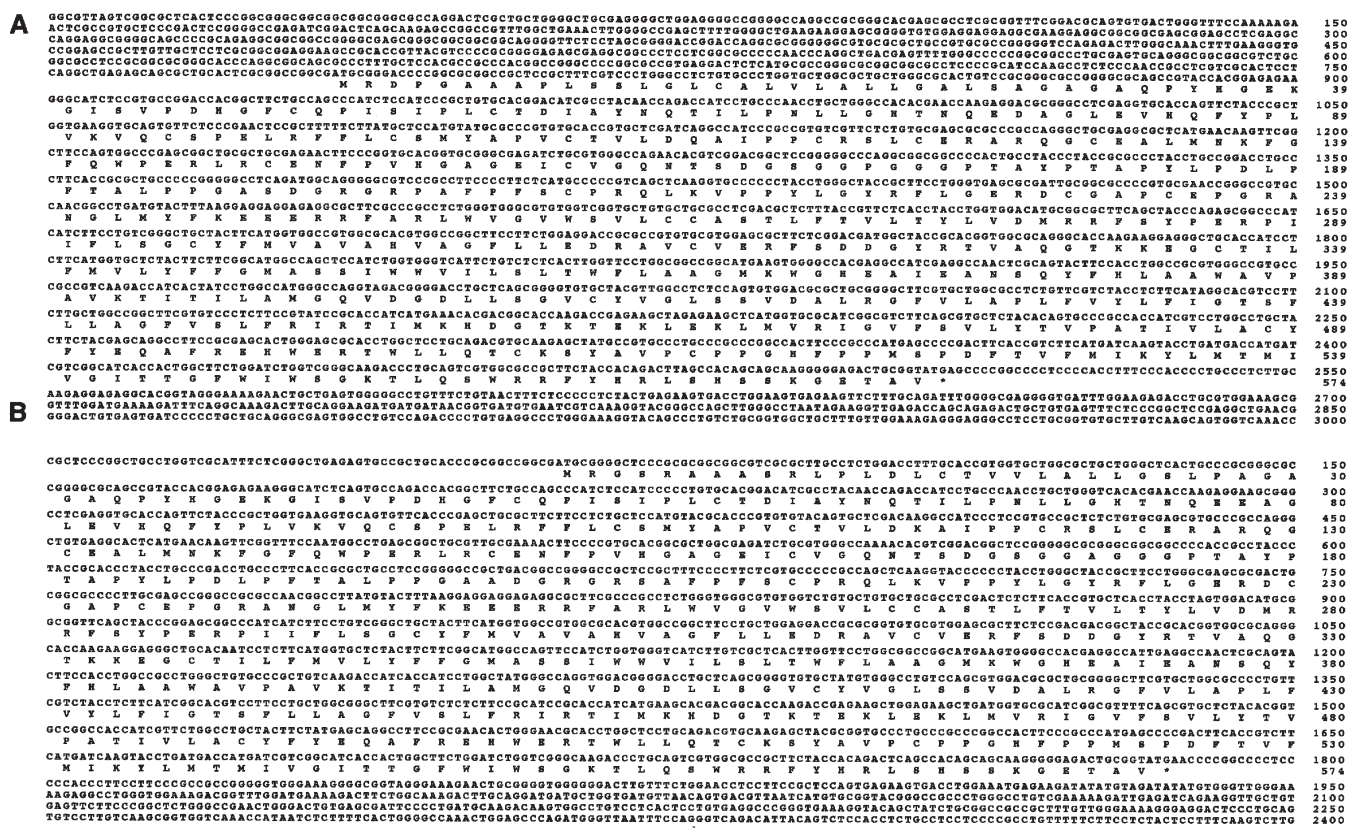

Figure 1. Chimpanzee and cow FZD7 orthologs. (A), Chimpanzee FZD7 complete CDS. Nucleotide position 1-3000 is shown. Nucleotides and amino-acid residues are numbered on the right. (B), Cow FZD7 CDS. Nucleotide position 1-2400 is shown. Nucleotides and amino-acid residues are numbered on the right.

homologous to human *FZD7* were searched for with BLAST programs as described previously (37-39). Exon-intron boundaries were determined based on the consensus sequence of exon-intron junctions ('gt ... ag' rule of intronic sequence) and codon usage within the coding region as described previously (40-42). Complete coding sequence (CDS) of chimpanzee FZD7 or cow Fzd7 was determined by assembling exonic region(s).

Comparative proteomics analyses. The domain architecture of FZD7 orthologs was analyzed by using RPS-BLAST and PSORT II programs.

In silico expression analyses. Expressed sequence tags (ESTs) derived from human *FZD7* were searched for by using the BLAST programs as described previously (43-45). The sources of human ESTs were listed up for *in silico* expression analyses.

Comparative genomics analyses. Human genome sequences around the *FZD7* gene was compared with chimpanzee, cow, mouse and rat genome sequences to identify evolutionarily conserved regions. Binding sites for transcription factors were then searched for as described previously.

Results

Transcriptional start site of human FZD7. *FZD7* gene at human chromosome 2q33.1 is located within human genome sequence AC069148.6 as previously reported (33). BLAST programs using human genome sequence around the *FZD7* gene revealed that several FZD7 ESTs were transcribed from more upstream position than the 5'-end of FZD7 RefSeq (NM_003507.1). CD673704.1 EST was transcribed from 735-bp upstream position, CN288787.1 EST from 706-bp upstream position, CN370065.1 EST from 699-bp upstream position, and CN370066.1 EST from 654-bp upstream position. Based on these facts, it was concluded that the transcriptional start site of human *FZD7* gene was 735-bp upstream of NM_003507.1 RefSeq 5'-end.

Chimpanzee FZD7 and cow Fzd7 genes. BLAST programs using human FZD7 complete CDS revealed that chimpanzee *FZD7* gene was located within NW_001232110.1 genome sequence. *FZD7* gene without intron corresponded to the nucleotide position 33170705-33175279 of NW_001232110.1. Complete CDS of chimpanzee FZD7 was then determined. Genetyx program revealed that nucleotide position 785-2509 was the coding region. Chimpanzee *FZD7* gene was found to encode a 574-amino-acid protein (Fig. 1A).

BLAST programs revealed that cow *Fzd7* gene was located within AC173037.2 genome sequence. CDS of cow Fzd7 was next determined. Genetyx program revealed that nucleotide position 62-1786 was the coding region. Cow *Fzd7* gene was found to encode a 574-amino-acid protein (Fig. 1B).

Comparative proteomics analyses on FZD7 orthologs. Chimpanzee FZD7 and cow Fzd7 showed 100% and 97.2% total-amino-acid identity with human FZD7, respectively. Among nine amino-acid substitutions between human FZD7 (31) and human FzE3 (32), Ala8, Leu15, Arg201, Leu308,

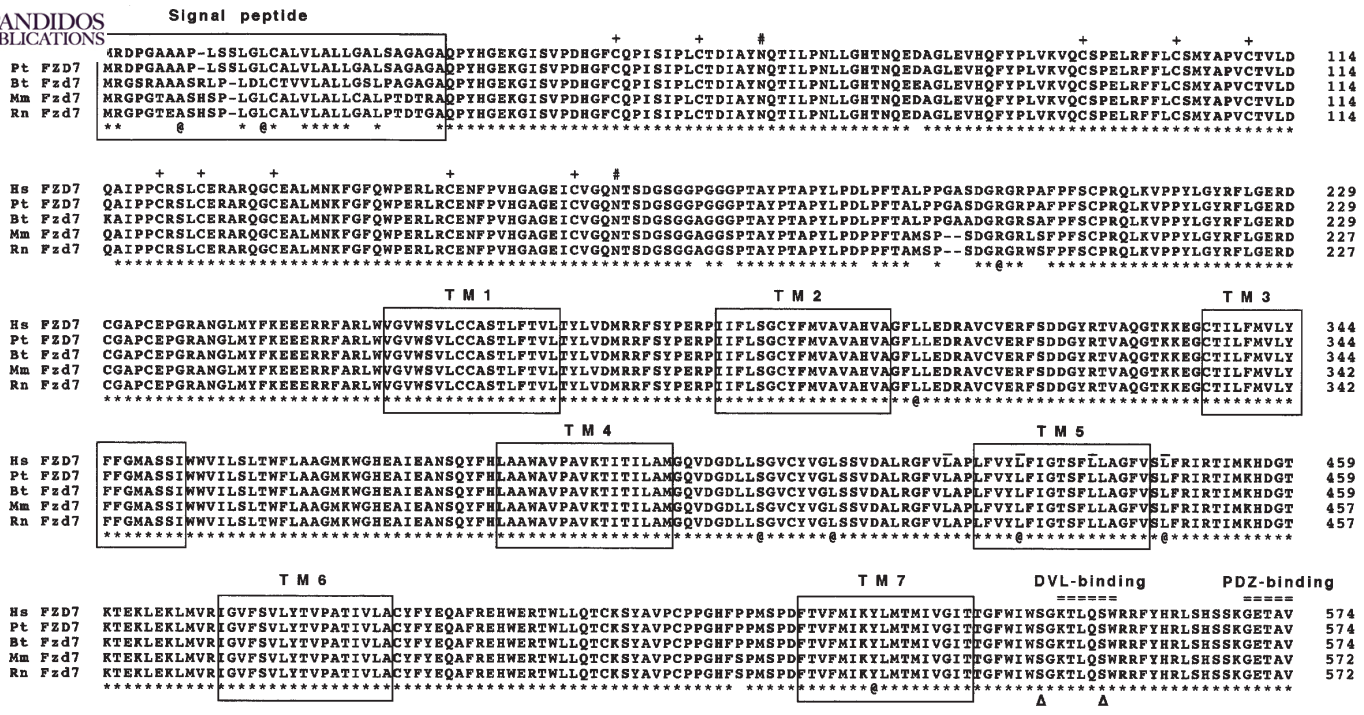

Figure 2. Mammalian FZD7 orthologs. Hs, human; Pt, chimpanzee; Bt, cow; Mm, mouse; Rn, rat. Signal peptide and seven-transmembrane domains (TM1-TM7) are boxed. Amino-acid residues are numbered on the right. Conserved Cys residues (cross) and Asn-linked glycosylation sites (sharp) within the N-terminal extracellular Frizzled region, leucine zipper motif around the TM5 domain (over line), DVL-binding and PDZ-binding motifs within the C-terminal cytoplasmic region (double over line) are shown above the alignment. Ser550 and Ser556 around the DVL-binding motif (open arrow head) and conserved amino-acid residues (* or @) are shown below the alignment. Locations of nine amino-acid substitutions between human FZD7 and human FzE3 (@) are also shown.

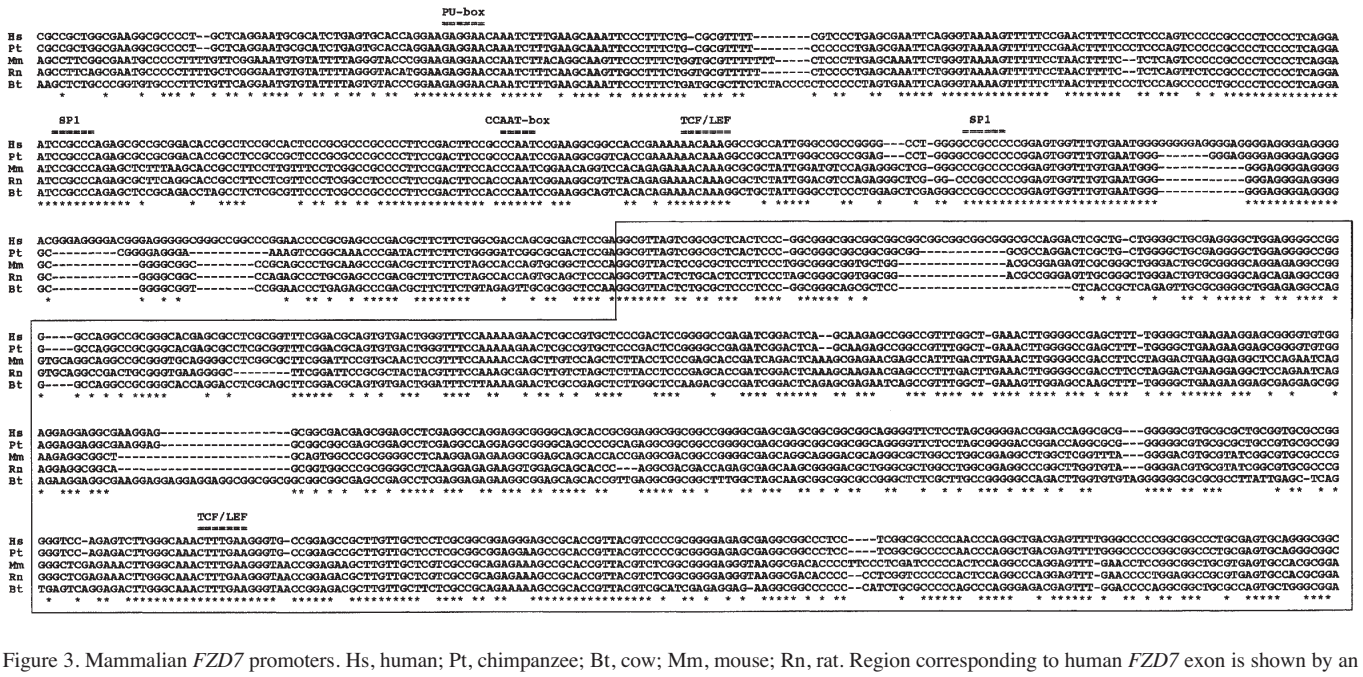

Figure 3. Mammalian *FZD7* promoters. Hs, human; Pt, chimpanzee; Bt, cow; Mm, mouse; Rn, rat. Region corresponding to human *FZD7* exon is shown by an open box. Conserved PU.1-, SP1-, CCAAT box-, and TCF/LEF/SOX-binding sites are shown by double overlines.

Ser408, Leu415, Leu433, Leu447 and Tyr534 corresponding to human FZD7 were conserved among mammalian FZD7 orthologs (Fig. 2). These facts indicate that 9 amino-acid substitutions in FzE3 are caused by sequencing errors and/or cloning artifacts.

FZD7 orthologs were seven-transmembrane proteins with extracellular Frizzled domain and, leucine zipper motif around the 5th transmembrane domain, and cytoplasmic DVL- and PDZ-binding motifs (Fig. 2). Asn63 and Asn164 within the N-terminal extracellular region of FZD7 orthologs were Asn-linked glycosylation sites. Ser550 and Ser556 around the DVL-binding motif of FZD7 orthologs were very similar to Ser554 and Ser560 of *Drosophila* Frizzled, which are phosphorylated by human aPKC (46).

In silico expression analysis on human FZD7. Expression of human *FZD7* mRNAs were detected in blastocysts, ES cells in undifferentiated state, ES cells differentiated to endodermal progenitors, ES cells differentiated to neural progenitors, fetal cochlea, retinal pigment epithelium, olfactory epithelium, regenerating liver, multiple sclerosis, and a variety of cancer, such as gastric cancer, colorectal cancer, pancreatic cancer, head/neck tumors, adrenal cortex carcinoma, lymphoma, osteosarcoma, melanoma and germ cell tumors.

Comparative genomics analyses on FZD7 orthologs. Human *FZD7*, chimpanzee *FZD7* and cow *Fzd7* genes are located within AC069148.6, NW_001232110.1 and AC173037.2 genome sequences, respectively, as mentioned above. Mouse *Fzd7* and rat *Fzd7* genes are located within AC132574.3 and AC136379.2 genome sequences, respectively, as previously reported (33). The 5'-promoter regions of mammalian *FZD7* orthologs were aligned to search for the conserved transcription factor-binding sites. PU.1-, SP1-, CCAAT box-, and TCF/LEF/SOX-binding sites within 5'-promoter regions of mammalian *FZD7* orthologs were evolutionarily conserved (Fig. 3).

Discussion

Comparative integromics analyses on FZD7 orthologs were performed in this study. Chimpanzee *FZD7* was identified within NW_001232110.1 genome sequence, while cow *Fzd7* gene within AC173037.2 genome sequence. Chimpanzee *FZD7* and cow *Fzd7* genes were found to encode 574-amino-acid protein showing 100% and 97.2% total-amino-acid identity with human FZD7, respectively (Fig. 1).

FZD7 orthologs were seven-transmembrane proteins with extracellular Frizzled domain, leucine zipper motif around the 5th transmembrane domain, and cytoplasmic DVL- and PDZ-binding motifs. Ser550 and Ser556 of FZD7 orthologs were putative aPKC phosphorylation sites (Fig. 2). Dimerization is necessary for the functional activation of seven-transmembrane G-protein-coupled receptors (47). Cytoplasmic C-terminal phosphorylation on *Drosophila* Frizzled by human aPKC is implicated in the inhibition of Frizzled signaling to the non-canonical WNT signaling pathway or planar cell polarity (PCP) signaling pathway (46). Together, these facts indicate that dimerization and Ser550/556 phosphorylation are important for the regulation of the signaling through FZD7.

All of the nine amino-acid residues substituted between human FZD7 and human FzE3 were identical to those of human FZD7 in chimpanzee, cow, mouse and rat FZD7 orthologs (Fig. 2), which clearly indicates that FzE3 is an aberrant cDNA with multiple sequencing errors and/or cloning artifacts. Because Leu433 and Leu447 are substituted to Phe433 and Phe447 in FzE3, leucine zipper motif around the 5th transmembrane domain is disrupted in FzE3 as previously pointed out (33,34). Therefore, functional analyses using FzE3 are invalid.

Transcriptional start site of human *FZD7* gene was 735-bp upstream of NM_003507.1 RefSeq 5'-end. *In silico* expression analyses revealed that human *FZD7* mRNAs were expressed in blastocysts, undifferentiated ES cells, ES-derived endodermal progenitors, ES-derived neural progenitors, fetal cochlea, retinal pigment epithelium, olfactory epithelium, regenerating liver, and multiple sclerosis. Comparative genomics analyses revealed that the binding sites for PU.1, SP1/Krüppel-like, CCAAT-box, and TCF/LEF/SOX transcription factors were conserved among 5'-promoter region of mammalian *FZD7* orthologs (Fig. 3). Human *FZD7* mRNA is expressed in gastrointestinal tract and gastroenterological cancer (31-36), and mouse *Fzd7* mRNA is expressed in stem/progenitor cells in colonic epithelium (48). Together, these facts indicate that FZD7 plays a key role for ES cells and gastrointestinal stem/progenitor cells to orchestrate the scenario of embryogenesis and tissue homeostasis, respectively.

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