FGF signaling inhibitor, SPRY4, is evolutionarily conserved target of WNT signaling pathway in progenitor cells

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Abstract. WNT, FGF and Hedgehog signaling pathways network together during embryogenesis, tissue regeneration, and carcinogenesis. *FGF16, FGF18*, and *FGF20* genes are targets of WNT-mediated TCF/LEF-ß-catenin-BCL9/BCL9L-PYGO transcriptional complex. *SPROUTY (SPRY)* and *SPRED* family genes encode inhibitors for receptor tyrosine kinase signaling cascades, such as those of FGF receptor family members and EGF receptor family members. Here, transcriptional regulation of *SPRY1, SPRY2, SPRY3, SPRY4, SPRED1, SPRED2*, and *SPRED3* genes by WNT/ß-catenin signaling cascade was investigated by using bioinformatics and human intelligence (humint). Because double TCF/LEF-binding sites were identified within the 5'-promoter region of human *SPRY4* gene, comparative genomics analyses on *SPRY4* orthologs were further performed. *SPRY4-FGF1* locus at human chromosome 5q31.3 and *FGF2-NUDT6-SPATA5-SPRY1* locus at human chromosome 4q27-q28.1 were paralogous regions within the human genome. Chimpanzee *SPRY4* gene was identified within NW_107083.1 genome sequence. Human, chimpanzee, rat and mouse *SPRY4* orthologs, consisting of three exons, were well conserved. *SPRY4* gene was identified as the evolutionarily conserved target of WNT/ß-catenin signaling pathway based on the conservation of double TCF/LEF-binding sites within 5'-promoter region of mammalian *SPRY4* orthologs. Human *SPRY4* mRNA was expressed in embryonic stem (ES) cells, brain, pancreatic islet, colon cancer, head and neck tumor, melanoma, and pancreatic cancer. WNT signaling activation in progenitor cells leads to the growth regulation of progenitor cells themselves through SPRY4 induction, and also to the growth stimulation of proliferating cells through FGF secretion. Epigenetic silencing and loss-of-function mutations of *SPRY4* gene in progenitor cells could lead to carcinogenesis. SPRY4 is the pharmacogenomics target in the fields of oncology and regenerative medicine.

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Introduction

WNT, FGF, and Hedgehog signaling pathways network together in a variety of cellular processes, such as stem cell differentiation cascade, body axis formation, angiogenesis, organogenesis during embryogenesis, adult tissue regeneration during chronic persistent inflammation, cell fate determination and cancer cell proliferation during carcinogenesis (1-11).

Canonical WNT signals are transduced through Frizzled receptors and LRP5/6 co-receptors to activate transcription of target genes, such as *FGF18, FGF20, CCND1* and *MYC*, based on the transcriptional complex consisting of TCF/LEF, ß-catenin, BCL9/BCL9L, and PYGO1/PYGO2 (12-21). FGF signals are transduced through FGF receptors and FRS2 docking protein to activate RAS-RAF-MAPKK-MAPK signaling cascade as well as PI3K-AKT signaling cascade (4,5,22-24). Hedgehog signals are transduced through Patched receptors and Smoothened signal transducers to activate transcription of target genes, such as *PTCH1, SFRP1*, *CCND2* and *FOXM1* genes, based on active form of GLI family transcription factors (6-9,25-28). WNT, FGF, and Hedgehog signaling pathways network together during embryogenesis, tissue regeneration, and carcinogenesis.

SPROUTY (SPRY) and *SPRED* family genes encode inhibitors for receptor tyrosine kinase signaling cascades, such as those of FGF receptor family members and EGF receptor family members (29-35). Here, transcriptional regulation of *SPRY1, SPRY2, SPRY3, SPRY4, SPRED1, SPRED2*, and *SPRED3* genes by WNT/ß-catenin signaling pathway was investigated by using bioinformatics and human intelligence. *SPRY4* gene was identified as the evolutionarily conserved target of the WNT/ß-catenin signaling pathway in progenitor cells.

Materials and methods

Screening for WNT target gene. Genome sequences corresponding to human *SPRY1, SPRY2, SPRY3, SPRY4, SPRED1, SPRED2*, and *SPRED3* genes were searched for with BLAST programs (http://www.ncbi.nlm.nih.gov) as described previously (36,37). TCF/LEF-binding sites within

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Human gene	Chromosomal localization	RefSeq ID	Genome clone containing promoter region	TCF/LEF-binding sites
SPRY1	4q28.1	NM_199327.1	AC026402.3	
SPRY2	13q31.1	NM_005842.2	AL354668.13	
SPRY3	Xq28	NM_005840.1	AC025226.4	
SPRY4	5q31.3	NM_030964.2	AC091825.4	Double sites
SPRED1	15q14	NM_152594.1	AC069381.8	
SPRED2	2p14	NM_181784.1	AC012370.8	
SPRED3	19q13.2	XM_371165.2	AC005789.1	

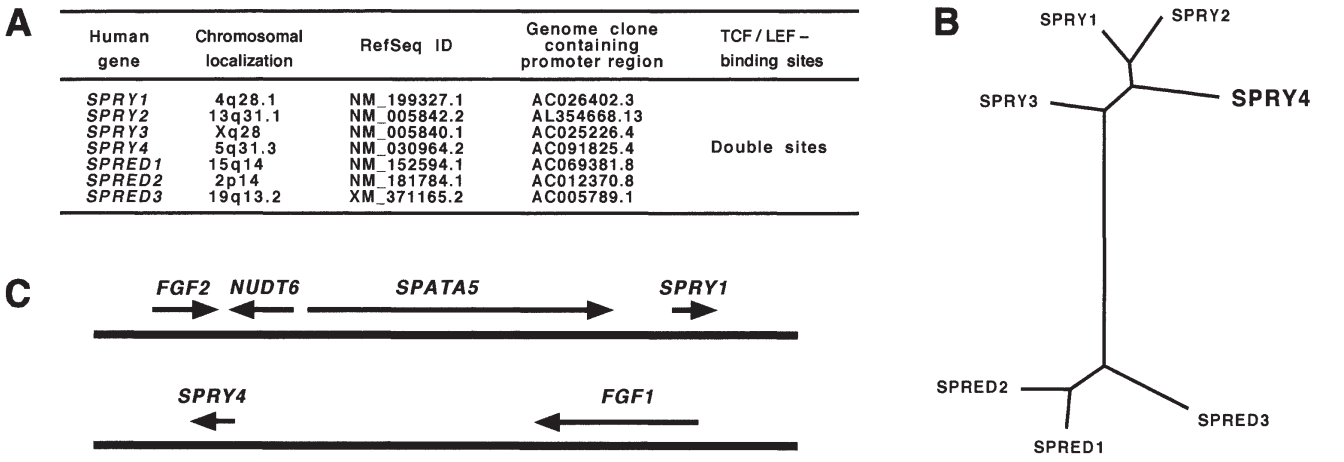

Figure 1. (A), *SPRY/SPRED* gene family. (B), Phylogenetic analyses on SPRY/SPRED family. (C), *SPRY4-FGF1* locus and *SPRY1-FGF2* locus within the human genome.

the 5'-flanking promoter region of the above genes were searched for based on bioinformatics and human intelligence.

Identification of chimpanzee ortholog. Chimpanzee genome sequence homologous to human *SPRY4* was searched for with BLAST programs as described previously (38,39). Exon-intron boundaries were determined by examining the consensus sequence of exon-intron junctions ('gt .... ag' rule of intronic sequence) and the codon usage within the coding region as described previously (40,41). Coding sequence of chimpanzee SPRY4 was determined by assembling exonic regions.

Comparative genomics analyses. Promoter region of human, chimpanzee, rat and mouse *SPRY4* orthologs were aligned by using the Genetyx program and manual curation. TCF/LEF-binding sites within the promoter regions were determined as mentioned above. Other transcription factor-binding sites were searched for by using the Match program as described previously (42,43).

Results

Screening of the TCF/LEF-binding site within promoter region of SPRY/SPRED family genes. By using human RefSeqs as query sequences for the BLAST programs, the 5'-flanking promoter region of human *SPRY1, SPRY2, SPRY3, SPRY4, SPRED1, SPRED2* and *SPRED3* genes were identified within AC026402.3, AL354668.13, AC025226.4, AC091825.4, AC069381.8, AC012370.8 and AC005789.1 genome sequences, respectively. TEF/LEF-binding site within the 5'-promoter region of human *SPRY1, SPRY2, SPRY3, SPRY4, SPRED1, SPRED2* and *SPRED3* genes were then searched for based on manual inspection. Double TEF/LEF-binding sites were identified within the 5'-flanking promoter region of human *SPRY4* gene (Fig. 1A).

Comparative integromics analyses. Phylogenetic analyses on SYRY/SPRED family members revealed that SPRY4 was more homologous to SPRY1 and SYRY2 (Fig. 1B). Intra-species comparative genomics analyses revealed that *SPRY4* gene at human chromosome 5q31.3 was linked to *FGF1* gene, and that *SPRY1* gene at human chromosome 4q28.1 was linked to *FGF2* gene (Fig. 1C). These facts indicate that *SPRY4-FGF1* locus at human chromosome 5q31.3 and *FGF2-NUDT6-SPATA5-SPRY1* locus at human chromosome 4q27-q28.1 were paralogous regions within the human genome.

Identification and characterization of chimpanzee SPRY4 gene. BLAST programs using human SPRY4 RefSeq revealed that chimpanzee *SPRY4* gene was located within NW_107083.1 genome sequence. Exon-intron boundaries of chimpanzee *SPRY4* gene were determined based on the consensus sequence of exon-intron junctions. Exon 1 corresponded to nucleotide position 1968269-1968113 of chimpanzee genome sequence NW_107083.1, exon 2 to 1962961-1962911, and exon 3 to 1958281-1953557. Complete coding sequence of chimpanzee SPRY4 was determined by assembling nucleotide sequences of three exons (Fig. 2A).

Genetyx program revealed that nucleotide position 187-1155 of chimpanzee SPRY4 complete coding sequence was the coding region, which indicated that chimpanzee *SPRY4* gene encodes a 322-amino-acid protein (Fig. 2A). Chimpanzee SPRY4 and human SPRY4 with 6-amino-acid substitutions showed 98.1% total-amino-acid identity.

Comparative genomics analyses on SPRY4 promoters. Human and chimpanzee *SPRY4* promoters were located within AC091825.4 and NW_107083.1 genome sequences, respectively, as mentioned above. BLAST programs next revealed that rat and mouse *Spry4* promoters were located within AC099211.8 and AC151580.2 genome sequences, respectively. GC content of human *SPRY4* promoter, chimpanzee *SPRY4* promoter, rat *Spry4* promoter, and mouse *Spry4* promoter was 63.3%, 63.3%, 60.7%, and 58.9% respectively.

Human *SPRY4* promoter, chimpanzee *SPRY4* promoter, rat *Spry4* promoter and mouse *Spry4* promoter were aligned by using the Genetyx program and manual curation. Human *SPRY4* promoter showed 99.0% nucleotide identity with chimpanzee *SPRY4* promoter, and 80.6% nucleotide identity with rat *Spry4* promoter. Double TCF/LEF-binding sites were conserved among 5'-promoter regions of mammalian *SPRY4* orthologs (Fig. 2B). These facts indicate that *SPRY4* is the evolutionarily conserved target of the WNT/ß-catenin signaling pathway.

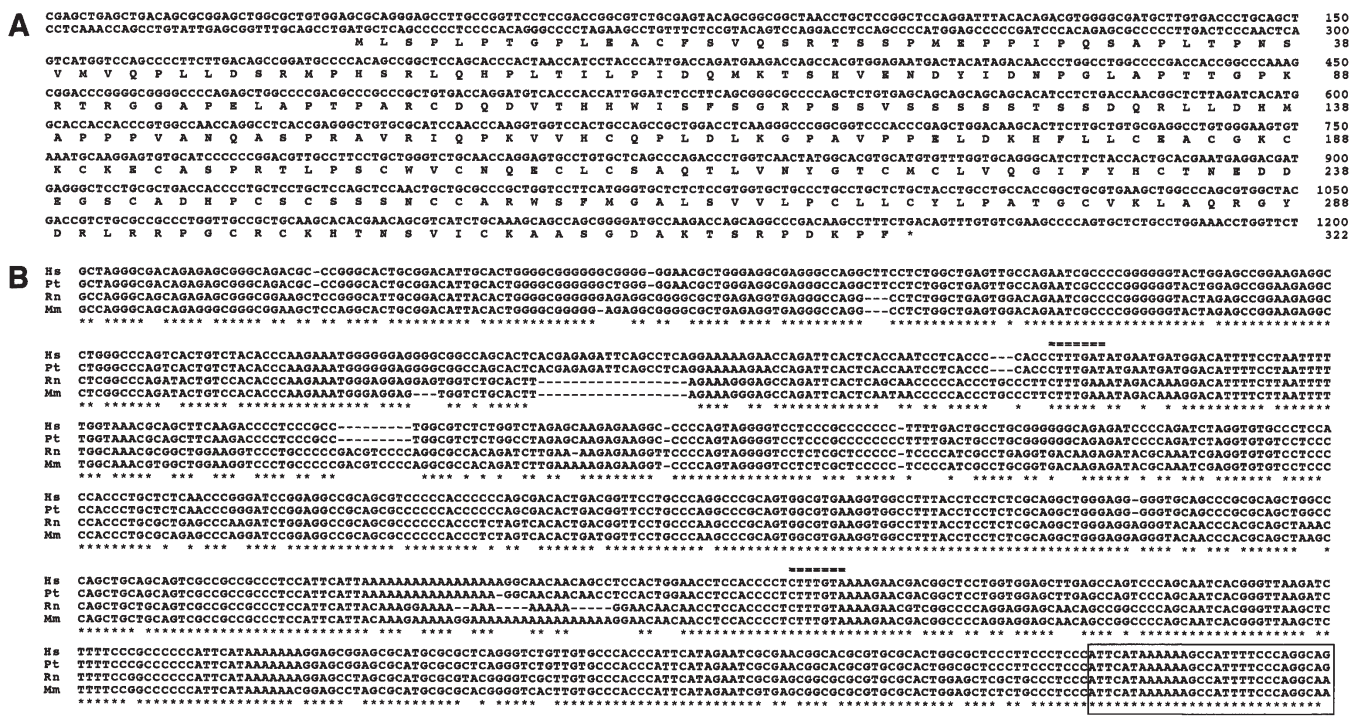

Figure 2. (A), Nucleotide and amino-acid sequences of chimpanzee SPRY4. (B), Double TCF/LEF-binding sites conserved among *SPRY4* orthologs. Hs, human; Pt, chimpanzee; Rn, rat; Mm, mouse. Region corresponding to exon 1 of human *SPRY4* gene is boxed. TCF/LEF-binding sites are shown by double over-lines.

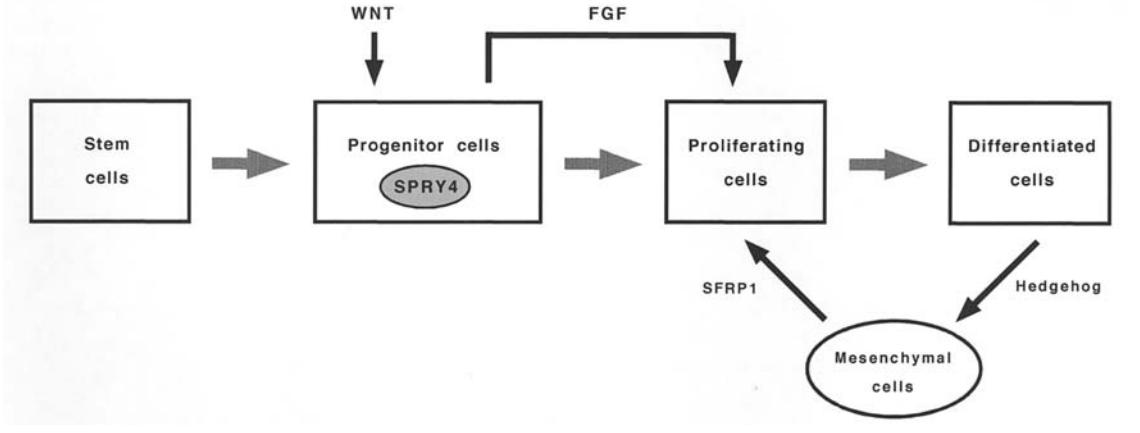

Figure 3. WNT, FGF and Hedgehog signaling network in stem cells, progenitor cells, proliferating cells, and differentiated cells. Canonical WNT signaling activation in progenitor cells leads to transcriptional activation of *SPRY4* and *FGFs*, which results in FGF signaling down-regulation in progenitor cells themselves as well as FGF signaling activation in proliferating cells. Hedgehog secreted from differentiated cells induces SFRP1 expression in mesenchymal cells to down-regulate canonical WNT signaling in proliferating cells. Canonical WNT signaling pathway is activated in progenitor cells, and FGF signaling pathway is activated in proliferating cells.

Expression of SPRY4 mRNA. Human expressed sequence tags (ESTs) derived from *SPRY4* gene were searched for with the BLAST programs. Sources of SPRY4 ESTs were then listed up. *In silico* expression analyses revealed that human *SPRY4* mRNA was expressed in embryonic stem cells, brain, pancreatic islet, colon cancer, head and neck tumor, melanoma, and pancreatic cancer.

Discussion

Transcriptional regulation of *SPRY1, SPRY2, SPRY3, SPRY4, SPRED1, SPRED2*, and *SPRED3* genes by the WNT/ß-catenin signaling cascade was investigated in this study. Because double TCF/LEF-binding sites were identified within the 5'-promoter region of human *SPRY4* gene (Fig. 1A), comparative genomics analyses on *SPRY4* orthologs were further performed. *SPRY4-FGF1* locus at human chromosome 5q31.3 and *FGF2-NUDT6-SPATA5-SPRY1* locus at human chromosome 4q27-q28.1 were paralogous regions within the human genome (Fig. 1C). Human *SPRY4* mRNA was expressed in embryonic stem cells, brain, pancreatic islet, colon cancer, head and neck tumor, melanoma, and pancreatic cancer.

Chimpanzee *SPRY4* gene, encoding a 322-amino-acid protein, was identified within NW_107083.1 genome sequence

(Fig. 2A). Chimpanzee SPRY4 showed 98.1% total-amino-acid identity with human SPRY4. Human, chimpanzee, rat and mouse *SPRY4* orthologs, consisting of three exons, were well conserved.

Based on the comparative genomics analyses, we successfully identified evolutionarily conserved TCF/LEF-binding sites within the 5'-promoter region of mammalian *SPRY4* orthologs (Fig. 2B). Although Ding *et al* reported conservation of human *SPRY4* and mouse *Spry4* promoters, they failed to identify TCF/LEF-binding sites (44). Therefore, this is the first report on the characterization of *SPRY4* as the target gene of WNT/ß-catenin signaling pathway.

WNT/ß-catenin signaling pathway is activated in progenitor cells (45), and *SPRY4* (Fig. 2B) as well as *FGFs* (18-20) are its target genes. WNT signaling activation in progenitor cells leads to the growth regulation of progenitor cells themselves through SPRY4 induction, and also to the growth stimulation of proliferating cells through FGF induction (Fig. 3). Epigenetic silencing and loss-of-function mutations of *SPRY4* gene in progenitor cells could lead to carcinogenesis. Therefore, SPRY4 is the pharmacogenomics target in the fields of oncology and regenerative medicine.

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