SPANDIDOS PUBLICATIONS

Comparative integromics on non-canonical WNT or planar cell polarity signaling molecules: Transcriptional mechanism of PTK7 in colorectal cancer and that of SEMA6A in undifferentiated ES cells

MASUKO KATOH[1] and MASARU KATOH[2]

[1]M&M Medical BioInformatics, Hongo 113-0033; [2]Genetics and Cell Biology Section, National Cancer Center Research Institute, Tokyo 104-0045, Japan

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Abstract. Non-canonical WNT and planar cell polarity (PCP) are overlapping but distinct signaling pathways, which control convergent extension, neural tube closure, orientation of cilia and sensory hair cells, axon guidance, and cell motility. Non-canonical WNT signals, regulated by the interaction of WNT, WNT antagonist, Frizzled and ROR2, are transduced to JNK, ROCK, PKC, MAP3K7, and NFAT signaling cascades. PCP signals, regulated by the interaction of VANGL-PRICKLE complex, CELSR and Frizzled-DVL complex, are transduced to JNK, ROCK, and other uncharacterized signaling cascades. PTK7 signaling, regulated by SEMA6 and Plexin-A family members, affects PCP pathway through VANGL. Here, integrative genomic analyses on *WNT5A, WNT5B, WNT11, FZD3, FZD6, ROR1, ROR2, RYK, CELSR1, CELSR2, CELSR3, VANGL1, VANGL2, PRICKLE1, PRICKLE2, PTK7, SEMA6A, SEMA6B, SEMA6C* and *SEMA6D* were carried out. *PTK7* and *SEMA6A* were expressed in undifferentiated embryonic stem (ES) cells, *SEMA6A* in endodermal progenitors, *CELSR1, VANGL1* and *PTK7* in gastrointestinal tumors. *CELSR2, PRICKLE2* and *SEMA6C* were expressed in fetal brain, *CELSR2, PRICKLE1* and *SEMA6A* in adult brain, *WNT5A* and *CELSR3* in adult brain tumors. These facts indicate class switches of non-canonical WNT or PCP signaling molecules during embryogenesis and carcinogenesis. TCF/LEF-, SP1-, and 5 bHLH-binding sites within human *PTK7* promoter were conserved in chimpanzee, rhesus monkey, mouse, and rat *PTK7* orthologs, which explained the mechanism of *PTK7* upregulation in colorectal cancer. NANOG-, SOX2-, and POU5F1 (OCT3/OCT4)-binding sites within intron 1 of the human *SEMA6A* gene were conserved in chimpanzee, rhesus monkey, mouse, and rat *SEMA6A* orthologs, which explained the mechanism of *SEMA6A* upregulation in undifferentiated ES cells. Most of non-canonical WNT or PCP signaling molecules, except *PTK7* and *SEMA6A*, were not frequently expressed in undifferentiated human ES cells. Non-canonical WNT or PCP signaling pathway, activated to orchestrate gastrulation and neurulation, was relatively downregulated in undifferentiated ES cells derived from inner cell mass of blastocysts.

Correspondence to: Dr Masaru Katoh, Genetics and Cell Biology Section, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan
E-mail: mkatoh-kkr@umin.ac.jp

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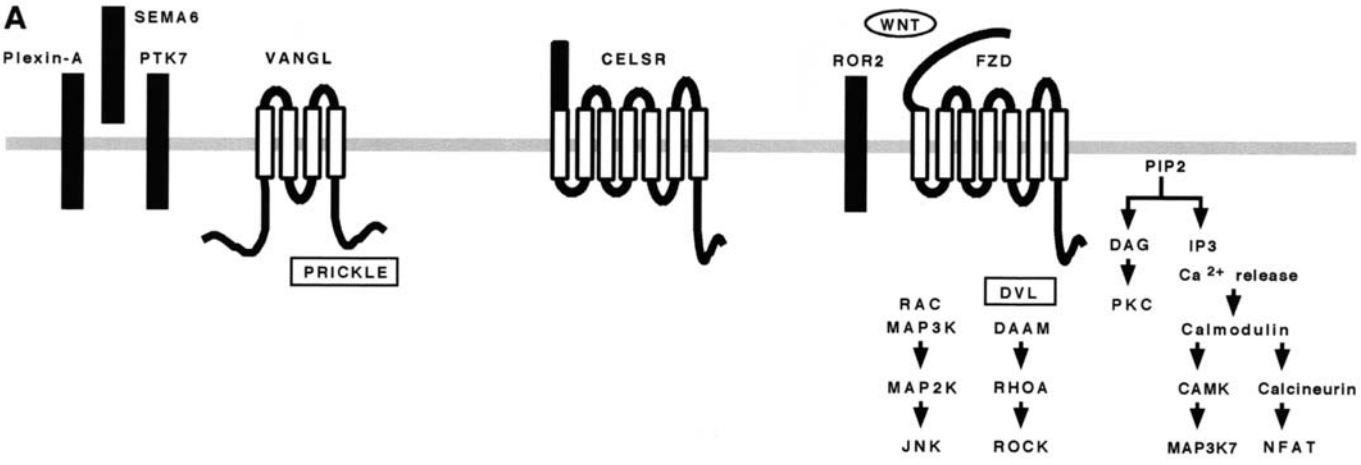
Introduction

WNT signals are transduced to the canonical pathway for the cell fate determination, and to the non-canonical pathway for the regulation of planar cell polarity (PCP), cell adhesion and motility in a context-dependent manner (1-3). Transcription of *MYC, CCND1, FGF20, WISP1, JAG1, DKK1,* and *GCG* genes are activated by the canonical WNT signals (4-11), while JNK, ROCK, PKC, MAP3K7 and NFAT signaling cascades are activated by the non-canonical WNT signals (12-14).

PCP signaling pathway is overlapping with the non-canonical WNT signaling pathway (Fig. 1A); however, PCP and non-canonical WNT are distinct signaling pathways. PCP signals, regulated by the interaction of VANGL-PRICKLE complex, CELSR and Frizzled-DVL complex, are transduced to JNK, ROCK, and other uncharacterized signaling cascades (14-17). PTK7 signaling, regulated by SEMA6 and Plexin-A family members, affects the PCP pathway through VANGL (18,19). Non-canonical WNT or PCP signaling pathway controls convergent extension, neural tube closure, orientation of cilia and sensory hair cells, axon guidance, and cell motility (15-17,20-25).

We cloned and characterized *WNT5B, WNT11, FZD3, FZD6, VANGL1,* and *VANGL2* using molecular biology techniques (26-32). We then identified and characterized *PRICKLE1* and *PRICKLE2* using bioinformatics/techint and human intelligence/humint (33). Other groups cloned and characterized *WNT5A, ROR1, ROR2, RYK, CELSR1, CELSR2, CELSR3, PTK7, SEMA6A, SEMA6B, SEMA6C,* and *SEMA6D* (34-41).



B

	Human ES cells	Embryoid body	Neural progenitors	Fetal brain	Infant brain	Adult brain	Brain tumors	Endodermal progenitors	Gastric cancer	Pancreatic cancer	Colorectal cancer
WNT5A	1	7	2	0	1	2	10	0	1	0	2
WNT5B	0	0	0	1	0	2	2	0	2	1	4
WNT11	0	0	0	7	0	1	0	0	1	0	1
FZD3	0	3	2	7	0	9	1	3	1	1	1
FZD6	4	3	0	2	0	11	0	2	2	0	1
ROR1	3	3	1	0	5	5	1	1	0	1	3
ROR2	2	1	0	0	0	1	1	3	2	1	1
RYK	1	2	2	0	6	3	3	2	3	1	2
CELSR1	6	1	3	5	1	0	0	2	1	6	2
CELSR2	2	4	1	17	3	84	5	0	0	1	0
CELSR3	0	1	0	6	1	7	10	1	2	1	1
VANGL1	1	1	0	1	0	4	1	0	4	3	4
VANGL2	6	7	4	9	2	3	6	0	0	1	1
PRICKLE1	3	1	4	9	6	27	3	0	0	0	1
PRICKLE2	1	4	1	27	13	9	4	0	2	1	0
PTK7	13	11	4	5	0	9	2	6	1	3	7
SEMA6A	27	1	5	9	2	31	1	13	1	0	0
SEMA6B	2	2	0	1	2	2	4	0	1	3	0
SEMA6C	1	1	2	13	1	6	3	1	0	1	3
SEMA6D	1	1	0	8	0	12	0	0	0	0	0

Figure 1. Non-canonical WNT or PCP signaling pathway. (A) Schematic representation of non-canonical WNT or PCP signaling molecules. Non-canonical WNT signals, regulated by the interaction of WNT, Frizzled and ROR2, are transduced to JNK, ROCK, PKC, MAP3K7, and NFAT signaling cascades. PCP signals, regulated by the interaction of VANGL-PRICKLE complex, CELSR and Frizzled-DVL complex, are transduced to JNK, ROCK, and other uncharacterized signaling cascades. PTK7 signaling, regulated by SEMA6 and Plexin-A family members, affects PCP pathway through VANGL. Non-canonical WNT or PCP signaling pathway controls convergent extension, neural tube closure, orientation of cilia and sensory hair cells, axon guidance, and cell motility. (B) Expression profile. Number of ESTs derived from human genes encoding non-canonical WNT or PCP signaling molecules are listed up. *PTK7* and *SEMA6A* are relatively frequently expressed in undifferentiated ES cells.

Here, expression analyses on *WNT5A, WNT5B, WNT11, FZD3, FZD6, ROR1, ROR2, RYK, CELSR1, CELSR2, CELSR3, VANGL1, VANGL2, PRICKLE1, PRICKLE2, PTK7, SEMA6A, SEMA6B, SEMA6C,* and *SEMA6D* were carried out. Most of these genes were preferentially expressed in fetal tissues, adult tissues, and tumors, while *PTK7* and *SEMA6A* in undifferentiated human embryonic stem (ES) cells. Comparative genomic analyses on *PTK7* and *SEMA6A* genes were further carried out to elucidate the mechanisms of *PTK7* and *SEMA6A* expression.

Materials and methods

In silico expression analyses. Expressed sequence tags (ESTs) derived from human genes encoding non-canonical WNT or PCP signaling molecules were searched for using the BLAST programs as described previously (42-44). WNT5A RefSeq (NM_003392.3), WNT5B RefSeq (NM_030775.2), WNT11 RefSeq (NM_004626.2), FZD3 RefSeq (NM_017412.2), FZD6 RefSeq (NM_003506.2), ROR1 RefSeq (NM_005012.2), ROR2 RefSeq (NM_004560.2), RYK RefSeq (NM_001005861.2), CELSR1 RefSeq (NM_014246.1), CELSR2 RefSeq (NM_001408.1), CELSR3 RefSeq (NM_001407.2), VANGL1 cDNA (45), VANGL2 cDNA (45), PRICKLE1 RefSeq (NM_153026.1), PRICKLE2 RefSeq (NM_198859.2), PTK7 RefSeq (NM_002821.3), SEMA6A RefSeq (NM_020796.3), SEMA6B RefSeq (NM_032108.2), SEMA6C RefSeq (NM_030913.3), and SEMA6D RefSeq (NM_024966.2) were used as query sequences for the BLAST programs. The sources of human ESTs were listed up for *in silico* expression analyses.

Exon-intron structure of the human PTK7 and SEMA6A genes. Human genome sequences corresponding to *PTK7* and *SEMA6A* genes were searched for with the BLAST programs as described previously (46-48). 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.

Comparative genomics on the PTK7 and SEMA6A genes. Human genome sequences around the *PTK7* and *SEMA6A* genes were compared with chimpanzee, rhesus monkey,

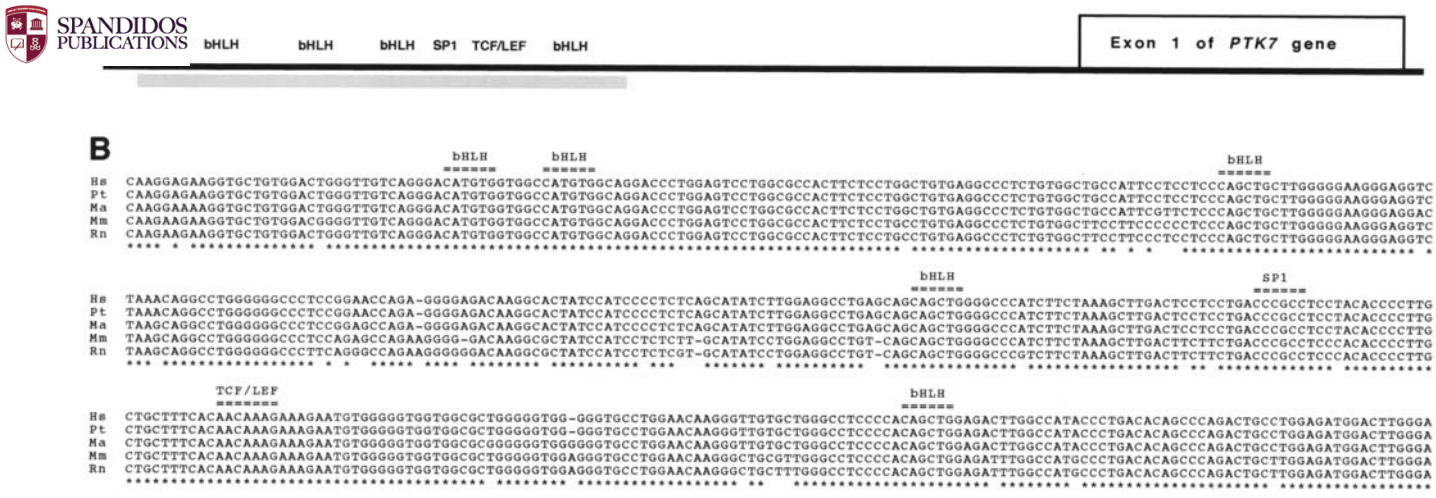


Figure 2. Comparative genomics on the *PTK7* promoter. (A) Schematic representation of human *PTK7* promoter region. Exon 1 is shown by an open box. Region highly conserved in mouse *Ptk7* promoter is shown by a gray under bar. Conserved transcription factor-binding sites are also shown. (B) Alignment of conserved promoter region of the *PTK7* orthologs. Hs, human; Pt, chimpanzee; Ma, rhesus monkey; Mm, mouse; Rn, rat. Conserved nucleotides are shown by asterisks below the alignment. Conserved bHLH-, SP1-, and TCF/LEF-binding sites are shown by a double overline above the alignment.

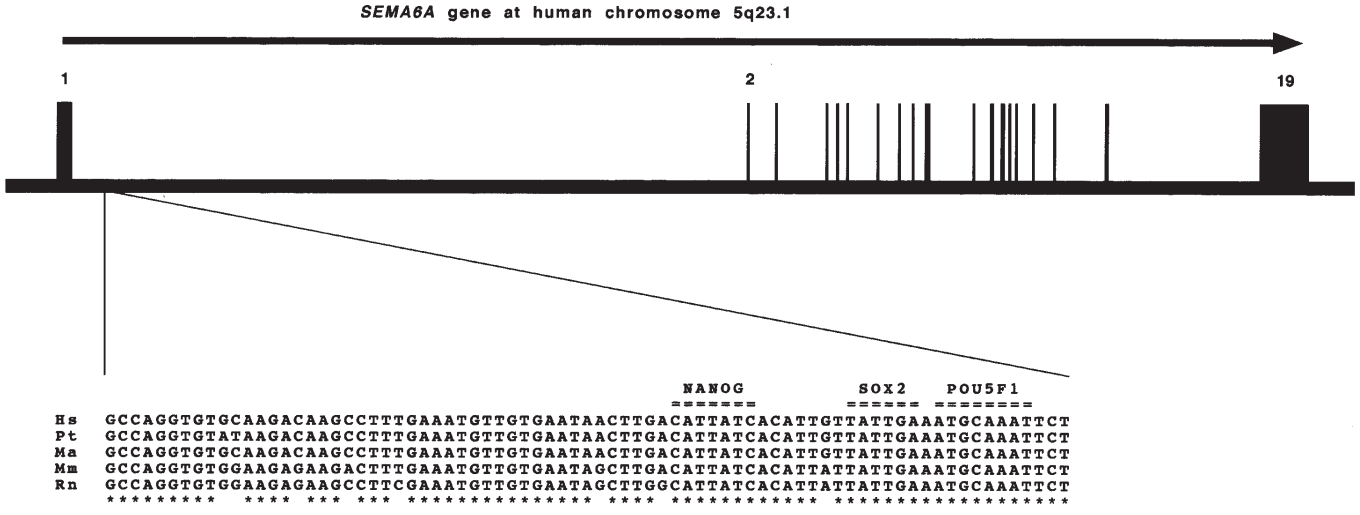


Figure 3. Comparative genomics on the *SEMA6A* gene. Schematic representation of human *SEMA6A* gene. Exons are shown by a closed box. Alignment of conserved region within intron 2 is also shown below the gene structure. Hs, human; Pt, chimpanzee; Ma, rhesus monkey; Mm, mouse; Rn, rat. Conserved nucleotides are shown by asterisks below the alignment. Conserved NANOG-, SOX2-, and POU5F1-binding sites are shown by a double overline above the alignment.

mouse and rat genome sequences using the BLAST programs. Transcription factor-binding sites within the evolutionarily conserved regions were then searched for using the Match program, Genetyx program, and the manual curation as described previously (49-51).

Results

Expression profile of non-canonical WNT or PCP signaling molecules. Sources of ESTs derived from human *WNT5A, WNT5B, WNT11, FZD3, FZD6, ROR1, ROR2, RYK, CELSR1, CELSR2, CELSR3, VANGL1, VANGL2, PRICKLE1, PRICKLE2, PTK7, SEMA6A, SEMA6B, SEMA6C,* and *SEMA6D* mRNAs were listed up for the *in silico* expression analyses (Fig. 1B). *PTK7* and *SEMA6A* were expressed in undifferentiated ES cells, *SEMA6A* in endodermal progenitors, *CELSR1, VANGL1* and *PTK7* in gastrointestinal tumors. *CELSR2, PRICKLE2* and *SEMA6C* were expressed in fetal brain, *CELSR2, PRICKLE1* and *SEMA6A* in adult brain, *WNT5A* and *CELSR3* in adult brain tumors.

Because we have been working on the signaling network in ES cells and cancer stem/progenitor cells (50-53), comparative genomic analyses on *PTK7* and *SEMA6A* genes were further carried out.

Comparative genomic analyses on PTK7 orthologs. BLAST programs revealed that the human *PTK7* gene was located within AL355385.15 genome sequence, chimpanzee *PTK7* gene within NW_001236528.1 genome sequence, rhesus monkey *PTK7* gene within AC197857.4 genome sequence, mouse *Ptk7* gene within AC165445.1 genome sequence, and rat *Ptk7* gene within AC131887.3 genome sequence.

Comparative genomic analyses revealed that the 5'-promoter region of the human *PTK7* gene, corresponding to nucleotide position -991 to -559, were well conserved in chimpanzee, rhesus monkey, mouse, and rat *PTK7* orthologs (Fig. 2A).

Transcription factor-binding sites within the conserved region of *PTK7* 5'-flanking promoter region were next searched for. Five bHLH-binding sites, SP1-binding site, and TCF/LEF-binding site within human, chimpanzee, rhesus monkey, mouse, and rat *PTK7* promoters were completely conserved (Fig. 2B).

Comparative genomic analyses on SEMA6A orthologs. BLAST programs revealed that the human *SEMA6A* gene was located within AC027304.4 and AC008524.6 genome sequences, chimpanzee *SEMA6A* gene within NW_001235419.1 genome sequence, rhesus monkey *SEMA6A* gene within AC169874.1 genome sequence, mouse *Sema6a* gene within AC121783.2 genome sequence, and rat *Sema6a* gene within AC094771.5 genome sequence.

Exon-intron structure of the human *SEMA6A* gene was determined based on the consensus sequence of exon-intron junctions. The human *SEMA6A* gene, consisting of 19 exons, was about 130 kb in size (Fig. 3). The first and last exons were larger than the other exons. Intron 1 was about 70 kb in size. Comparative genomic analyses revealed that the 5'-promoter region and intron 1 of human *SEMA6A* gene was relatively well conserved in mouse *Sema6a* gene.

Transcription factor-binding sites within the conserved promoter regions and intron 1 of *SEMA6A* orthologs were next searched for. NANOG-, SOX2-, and POU5F1 (OCT3/OCT4)-binding sites within intron 1 of human *SEMA6A* gene were completely conserved in chimpanzee, rhesus monkey, mouse, and rat *SEMA6A* orthologs (Fig. 3).

Discussion

Integrative genomic analyses on non-canonical WNT or PCP signaling molecules were carried out in this study. *PTK7* and *SEMA6A* were expressed in undifferentiated ES cells, *SEMA6A* in endodermal progenitors, *CELSR1, VANGL1* and *PTK7* in gastrointestinal tumors (Fig. 1B). *CELSR2, PRICKLE2* and *SEMA6C* were expressed in fetal brain, *CELSR2, PRICKLE1* and *SEMA6A* in adult brain, *WNT5A* and *CELSR3* in adult brain tumors (Fig. 1B). These facts indicate class switches of non-canonical WNT or PCP signaling molecules during embryogenesis and carcinogenesis.

Comparative genomic analyses on the *PTK7* and *SEMA6A* genes were further carried out to elucidate the mechanisms of their expression in undifferentiated ES cells. Five bHLH-binding sites, SP1-binding site, and TCF/LEF-binding site within the human *PTK7* 5'-promoter region were conserved in chimpanzee, rhesus monkey, mouse, and rat *PTK7* genes (Fig. 2). NANOG-, SOX2-, and POU5F1-binding sites within intron 1 of human *SEMA6A* gene were conserved in chimpanzee, rhesus monkey, mouse, and rat *SEMA6A* genes (Fig. 3).

PTK7 is a transmembrane protein with extracellular immunoglobulin-like domains and a cytoplasmic tyrosine kinase-like domain (39). PTK7 is homologous to ROR1, ROR2 and RYK receptor tyrosine kinases; however, PTK7 is a pseudokinase without detectable catalytic tyrosine kinase activity due to amino-acid substitution within the catalytic domain (54). PTK7, also known as CCK4, is upregulated in colorectal cancer. Because the canonical WNT signaling pathway is frequently activated in colorectal cancer, conserved TCF/LEF-binding site within the *PTK7* promoter region explains the mechanism of *PTK7* upregulation in colorectal cancer.

SEMA6 family members are transmembrane-type ligands for Plexin-A family receptors to regulate axon guidance and cell motility (18,19). SEMA6A is reported to bind to Plexin-A2, Plexin-A4, while SEMA6C and SEMA6D to Plexin-A1. We also investigated the expression profile of Plexin-A family members to identify that Plexin-A1 was expressed in undifferentiated human ES cells (Katoh and Katoh, unpublished data). SEMA6D signal is transduced through the Plexin-A1 and PTK7 receptor complex for the activation of Rac signaling cascade rather than Rho signaling cascade (18). SEMA6A signal transduction through Plexin-A1 and PTK7 receptor complex in undifferentiated human ES cells should be investigated in the future.

Most of non-canonical WNT or PCP signaling molecules, except *PTK7* and *SEMA6A*, were not frequently expressed in undifferentiated human ES cells. Non-canonical WNT or PCP signaling pathway, activated to orchestrate gastrulation and neurulation, was relatively downregulated in undifferentiated ES cells derived from the inner cell mass of blastocysts.

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