Transcriptional mechanisms of *WNT5A* based on NF-κB, Hedgehog, TGFß, and Notch signaling cascades

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Abstract. *WNT5A* is a cancer-associated gene involved in invasion and metastasis of melanoma, breast cancer, pancreatic cancer, and gastric cancer. WNT5A transduces signals through Frizzled, ROR1, ROR2 or RYK receptors to ß-catenin-TCF/LEF, DVL-RhoA-ROCK, DVL-RhoB-Rab4, DVL-Rac-JNK, DVL-aPKC, Calcineurin-NFAT, MAP3K7-NLK, MAP3K7-NF-κB, and DAG-PKC signaling cascades in a context-dependent manner. *SNAI1 (Snail), CD44, G3BP2,* and *YAP1* are *WNT5A* target genes. We and other groups previously reported that IL6- or LIF-induced signaling through JAK-STAT3 signaling cascade is involved in *WNT5A* upregulation (STAT3-WNT5A signaling loop). Here, refined integrative genomic analyses of *WNT5A* were carried out to elucidate other mechanisms of *WNT5A* transcription. The *WNT5A* gene was found to encode two isoforms by using alternative first exons 1A and 1B. Quadruple Smad-binding elements (SBEs), single Sp1-binding site (GC-box), PPARγ-binding site, C/EBP-binding site and bHLH-binding site within the promoter A region, 5'-adjacent to exon 1A, were conserved in human *WNT5A*, chimpanzee *WNT5A*, mouse *Wnt5a*, and rat *Wnt5a*. NF-κB-binding site, CUX1-binding site, double SBEs and double GC-boxes within the promoter B region, 5'-adjacent to exon 1B, were conserved in mammalian *WNT5A* orthologs. Quadruple FOX-binding sites and double SBEs within ultra-conserved intron 1 were also conserved in mammalian *WNT5A* orthologs. Conserved NF-κB-binding site within the *WNT5A* promoter B region elucidated the mechanisms that TNFα and toll-like receptor (TLR) signals upregulate *WNT5A* via MAP3K7. Quadruple FOX-binding sites rather than GLI-binding site revealed that Hedgehog signals induce *WNT5A* upregulation indirectly via FOX family members, such as FOXA2, FOXC2, FOXE1, FOXF1 and FOXL1. TGFß signals were found to upregulate *WNT5A* expression directly through the Smad complex, and also indirectly through Smad-induced CUX1 and MAP3K7-mediated NF-κB. Together these facts indicate that *WNT5A* is transcribed based on multiple mechanisms, such as NF-κB, Hedgehog, TGFß, and Notch signaling cascades.

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Introduction

WNT signaling cascades are involved in a variety of cellular processes during embryogenesis and carcinogenesis (1-4). Because biological functions of human genes and those of model-animal orthologs are not always conserved due to protein evolution and promoter evolution, we have carried out the human WNTome project for the comprehensive characterization of human genes encoding WNT signaling molecules during the genomic era before 2003 (5). Nineteen *WNT* family genes in the human genome are conserved in the mammalian genomes (6).

The *WNT5A* gene at human chromosome 3p14.3 (7,8) is the paralog of the *WNT5B* gene at human chromosome 12p13.33 (9). Human WNT5A shows 98.7% total amino-acid identity with rodent Wnt5a (10,11), indicating that mammalian WNT5A orthologs are ultra-conserved. Frizzled family members (12-14), ROR1 (15), ROR2 (16), and RYK (17) are WNT5A receptors. WNT5A signals are transduced to the ß-catenin-TCF/LEF (12), DVL-RhoA-ROCK (18), DVL-RhoB-Rab4 (19), DVL-Rac-JNK (16), DVL-aPKC (20), Ca^{2+}-Calcineurin-NFAT (21), Ca^{2+}-MAP3K7-NLK (18,22), Ca^{2+}-MAP3K7-NF-κB (15), and DAG-PKC (23) signaling cascades in a context-dependent manner (13,24,25).

WNT5A is expressed in a variety of human tumors (Table I), including breast cancer (8,26-28), lung cancer (26,29), melanoma (26,30), osteosarcoma (26), prostate cancer (26, 31,32), endometrial uterine cancer (33,34), colorectal cancer (34-36), pancreatic cancer (37,38), gastric cancer (34), esophageal cancer (39), embryonal tumor (39), Ewing sarcoma (40), neuroblastoma (41), skin basal cell carcinoma (BCC) (42), skin squamous cell carcinoma (SCC) (43), and leukemia (44,45). *SNAI1 (Snail), CD44, G3BP2,* and *YAP1* are WNT5A target genes (23). WNT5A is involved in invasion, peritoneal dissemination, and distant metastasis of tumor cells via RhoB and Snail (18,19,23,46).

We and other groups previously reported that IL6- or LIF-induced signaling through JAK-STAT3 signaling cascade

Table I. WNT5A expression in human cancer.

Type of cancer	Notes	Author/(Refs.)
Breast cancer	Upregulation in primary tumors	Lejeune *et al* (8)
	Upregulation in primary tumors	Iozzo *et al* (26)
	Downregulation in primary tumors	Leris *et al* (27)
	Stromal expression	Pukrop *et al* (28)
Lung cancer	Upregulation in primary tumors	Iozzo *et al* (26)
	Upregulation in primary NSCLC	Huang *et al* (29)
Melanoma	Expression in a tumor cell line	Iozzo *et al* (26)
	Upregulation in primary tumors	Weeraratna *et al* (30)
Osteosarcoma	Expression in a tumor cell line	Iozzo *et al* (26)
Prostate cancer	Expression in primary tumors	Iozzo *et al* (26)
	Downregulation by BMP	Kumagai *et al* (31)
	Promoter hypomethylation	Wang *et al* (32)
Uterine cancer	Downregulation in primary tumors	Bui *et al* (33)
	Some up, others down	Saitoh *et al* (34)
Colorectal cancer	Stromal expression	Smith *et al* (35)
	Some up, others down	Saitoh *et al* (34)
	Epigenetic silencing in primary tumors	Ying *et al* (36)
Pancreatic cancer	Downregulation in primary tumors	Crnogorac-Jurcevic *et al* (37)
	Stromal expression	Pilarsky *et al* (38)
Gastric cancer	Upregulation in primary tumors	Saitoh *et al* (34)
Esophageal cancer	Upregulation in tumor cell lines	Saitoh and Katoh (39)
Embryonal tumor	Upregulation in tumor cell lines	Saitoh and Katoh (39)
Ewing sarcoma	Expression in tumor cell lines	Uren *et al* (40)
Neuroblastoma	Downregulation in primary tumors	Blanc *et al* (41)
Skin BCC	Upregulation in primary tumors	Bonifas *et al* (42)
Skin SCC	Upregulation in primary tumors	Haider *et al* (43)
Leukemia	Expression in B-ALL	Khan *et al* (44)
	Epigenetic silencing in ALL	Roman-Gomez *et al* (45)

is involved in *WNT5A* upregulation (STAT3-WNT5A signaling loop) (46-48). Here, refined integrative genomic analyses of *WNT5A* were carried out to elucidate the mechanisms of *WNT5A* transcription other than the STAT3-WNT5A signaling loop.

Materials and methods

Comparative genomic analyses. Human genome sequences corresponding to human WNT5A RefSeq (NM_003392.3) were searched for by using BLAST programs, as previously described (49,50). *WNT5A* expressed sequence tags (ESTs) were also searched for to identify *WNT5A* splicing variants (51,52). Conserved transcription factor-binding sites within *WNT5A* promoters were then searched for based on manual inspection, as previously described (53,54).

Regulatory network analyses. Literature on WNT5A, Hedgehog, TGFß, Notch and inflammatory signaling molecules in PubMed and Medline databases was critically evaluated to

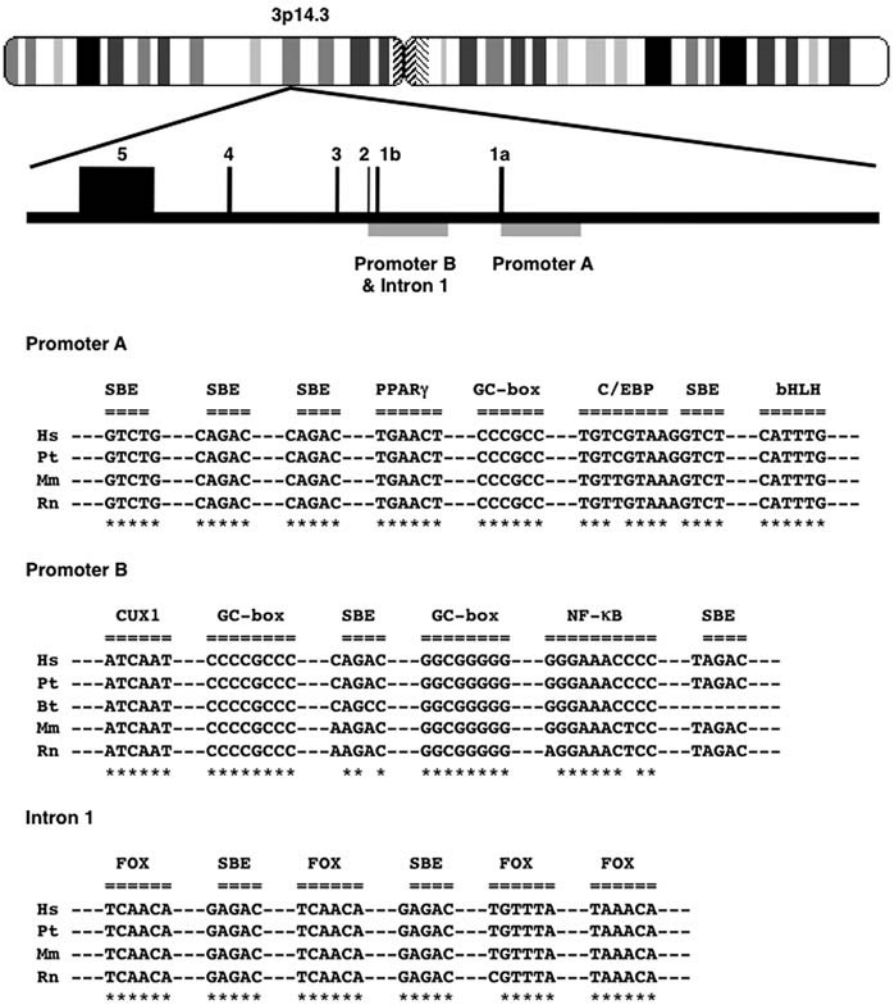

Figure 1. Integrative genomic analyses of *WNT5A*. Schematic representation of *WNT5A* gene at human chromosome 3p14.3 is shown in the upper part. *WNT5A* gene encodes two transcripts by using alternative first exons 1A and 1B. Conserved promoter and enhancer regions (gray bar) are shown. Conserved transcription factor-binding sites within *WNT5A* regulatory regions are shown in the lower part.

extract knowledge on the regulation of GLI, FOX, SMAD, and NF-κB transcription factors. The mechanisms of *WNT5A* transcription were then investigated based on our data of conserved transcription factor-binding sites within *WNT5A* promoters and in-house knowledgebase of transcription factors regulated by the stem-cell signaling network.

Results

WNT5A splicing variants transcribed by using alternative promoters. Human *WNT5A* gene is located within human genome sequence AC121764.2, as previously described (11). BLAST programs using the *WNT5A* genome sequence revealed that human ESTs CB988958.1, CF994133.1, DA459745.1, DA874427.1 and DB276659.1 were transcribed from exon 1, and that human ESTs DA030580.1, DA650006.1 and DB224740.1 were transcribed from alternative first exon located between exon 1 and exon 2. To distinguish two alternative first exons of human *WNT5A* gene, exon 1 was renamed exon 1a, and alternative first exon was designated exon 1b (Fig. 1). WNT5A isoform A (NM_003392.3 RefSeq) consists of exons 1a, 2, 3, 4 and 5, whereas WNT5A isoform B (AK290869.1 cDNA) consists of exons 1b, 2, 3, 4 and 5.

Comparative genomics on mammalian WNT5A orthologs. Rat *Wnt5a* gene is located within AC095764.5 incomplete genome sequence with gaps, as previously described (11). To our surprise, AC095764.5 draft sequence had not been replaced by complete genome sequence since September 22, 2002. BLAST programs next revealed that chimpanzee *WNT5A* gene and mouse *Wnt5a* gene were located within NW_001232823.1 draft sequence and CT025649.7 complete sequence, respectively (data not shown).

Comparative genomic analyses of mammalian *WNT5A* orthologs revealed that the *WNT5A* promoter A region located at the 5'-adjacent position of exon 1a, the *WNT5A* promoter B region located at the 5'-adjacent position of exon 1b and *WNT5A* intron 1 located between exon 1b and exon 2 were well conserved in mammalian *WNT5A* orthologs (Fig. 1).

Conserved transcription factor-binding sites within WNT5A regulatory regions. We previously identified the evolutionary conservation between the promoter A region of human *WNT5A* gene and that of rat *Wnt5a* gene (11), whereas Danielson *et al* identified putative transcription factor-binding sites within the promoter B of human *WNT5A* gene without any information on evolutionary conservation (7). Because database of

mammalian genome sequences and knowledgebase of transcription factor-binding sites have been quantitatively and qualitatively improved during the past three years, refined integrative genomic analyses of *WNT5A* regulatory regions were next carried out.

WNT5A promoter A without TATA-box is predicted to show 'broad peak' pattern of transcription start sites (TSSs). We utilized the most 5'-TSS of *WNT5A* in our previous study (11); however, TSS of NM_003392.3 RefSeq was 338-bp 3'-position compared with the most 5'-TSS. Based on manual inspection of the promoter A region, we found that PPARγ-binding site, Sp1-binding site (GC-box), C/EBP-binding site, bHLH-binding site and quadruple Smad-binding elements (SBEs) within the promoter A region were completely conserved in human *WNT5A*, chimpanzee *WNT5A*, mouse *Wnt5a*, and rat *Wnt5a* genes (Fig. 1).

WNT5A promoter B was less conserved compared with *WNT5A* promoter A (data not shown). CUX1 (CUTL1)-binding site, double SBEs, and double GC-boxes within the promoter B region were completely conserved in human *WNT5A*, chimpanzee *WNT5A*, mouse *Wnt5a*, and rat *Wnt5a* genes (Fig. 1). NF-κB-binding site within the promoter B region was completely conserved in human *WNT5A*, chimpanzee *WNT5A*, and mouse *Wnt5a* genes, and was almost conserved in rat *Wnt5a* gene except one-base substitution (Fig. 1). Because rat genome sequence AC095764.5 was incomplete as mentioned above, we next searched for the existence of the NF-κB-binding site within other mammalian *WNT5A* orthologs, and found that the NF-κB-binding site was also conserved in the cow *WNT5A* ortholog (Fig. 1).

WNT5A intron 1 was ultra-conserved among mammals. For example, inton 1 of human *WNT5A* gene showed 87.9% total nucleotide identity with that of mouse *Wnt5a* gene (data not shown). Manual inspection of transcription factor-binding sites then revealed that quadruple FOX-binding sites and double SBEs were completely conserved in human *WNT5A*, chimpanzee *WNT5A*, and mouse *Wnt5a* genes (Fig. 1). Most of these transcription factor-binding sites, except the third FOX-binding site, were also completely conserved in the rat *Wnt5a* gene (Fig. 1).

Conserved NF-κB-binding site, FOX-binding sites, SBEs and CUX1-binding site within the regulatory regions of *WNT5A* orthologs were novel findings in this study (Fig. 1).

NF-κB signaling cascades and WNT5A. Chronic inflammation is involved in tumor progression via activation of STAT3 and NF-κB transcription factors (46,48,55-59). We have previously reported the mechanisms of IL6- or LIF-induced *WNT5A* upregulation via STAT3 (46), and McCall *et al* reported the involvement of the IL6-STAT3 signaling loop in *WNT5A* upregulation in papillary thyroid carcinoma (48). Mechanisms of NF-κB-mediated *WNT5A* upregulation will be described in this section.

TNF receptors (TNFRs), Toll-like receptors (TLRs), IL1 receptors (IL1Rs), and TGFß receptors (TGFßRs) are involved in MAP3K7-mediated IKK activation (58). IKK then phosphorylates IκB to induce ubiquitin-mediated degradation, which results in stabilization and activation of NF-κB (56). We reported TNFα-induced *WNT5A* upregulation in gastric cancer cells in 2002 (39). Blumenthal *et al* reported TLR-mediated NF-κB activation and *WNT5A* upregulation in macrophages in 2006 (60). Because NF-κB-binding site within *WNT5A* promoter B was conserved in mammals (Fig. 1), it was concluded that *WNT5A* is upregulated by TNFα, TLR, IL1, and TGFß signaling activation via NF-κB (Fig. 2).

Hedgehog signaling cascades and WNT5A. Hedgehog signals are transduced through Patched family receptors and Smoothened signal transducer to GLI transcriptional activators (61-63). Hedgehog signals are activated in gastric cancer (64,65), basal cell carcinoma (66), and other tumors. *WNT5A* is expressed in gastric cancer (34), basal cell carcinoma (42), and other tumors, as mentioned above. Due to the co-existence of Hedgehog signaling activation and *WNT5A* expression in human tumors, causal relationship between Hedgehog signaling activation and *WNT5A* expression was investigated.

Consensus GLI-binding site was not located within *WNT5A* promoters and *WNT5A* gene (data not shown). *FOXA2, FOXC2, FOXE1, FOXF1* and *FOXL1* are direct target genes of Hedgehog-GLI signaling cascade (63), and quadruple FOX-binding sites within human *WNT5A* intron 1 were conserved in chimpanzee *WNT5A*, cow *WNT5A*, and mouse *Wnt2b* (Fig. 1). Together these facts indicate that Hedgehog signals induce *WNT5A* upregulation indirectly through FOX family members (Fig. 2).

TGFß signaling cascades and WNT5A. TGFß signals are transduced through receptor-type serine/threonine kinases to Smad2/3-Smad4 transcriptional complex and MAP3K7-NF-κB signaling cascade (67,68). Smad2/3-Smad4 complex, binding to the SBE of target genes, is associated with other transcription factors, such as Sp1 and ETS for transcriptional regulation of TGFß-target genes. TGFß-induced upregulation of *CDNK1A (P16)* and *CDKN2B (P15)* is involved in growth inhibition of tumor cells, whereas TGFß-induced upregulation of EMT regulators is involved in invasion, peritoneal dissemination, and distant metastasis of tumor cells. Roarty and Serra reported that TGFß-induced *Wnt5a* upregulation is required for proper mammary gland development (69). Ripka *et al* reported that TGFß induces *WNT5A* upregulation via CUX1 (70); however, CUT1-binding site within the *WNT5A* regulatory regions remained unclear. Causal link between TGFß signaling activation and *WNT5A* expression was next investigated.

Quadruple SBEs within the promoter A region, double SBEs within the promoter B region, and double SBEs within intron 1 were conserved in mammalian *WNT5A* orthologs; single GC-box within the promoter A region and double GC-boxes within the promoter B region were conserved in mammalian *WNT5A* orthologs; single CUX1-binding site within the promoter B region was conserved in mammalian *WNT5A* orthologs (Fig. 1). In addition, NF-κB-binding site within the promoter B region was conserved in mammalian *WNT5A* orthologs, as discussed above. Together these facts indicate that TGFß signals upregulate *WNT5A* expression directly through the Smad complex, and also indirectly through Smad-induced CUX1 and MAP3K7-mediated NF-κB (Fig. 2).

Notch signaling cascades and WNT5A. Notch ligands-induced Notch signaling activation leads to transcriptional activation of Notch target genes via NICD-CSL complex or NICD-

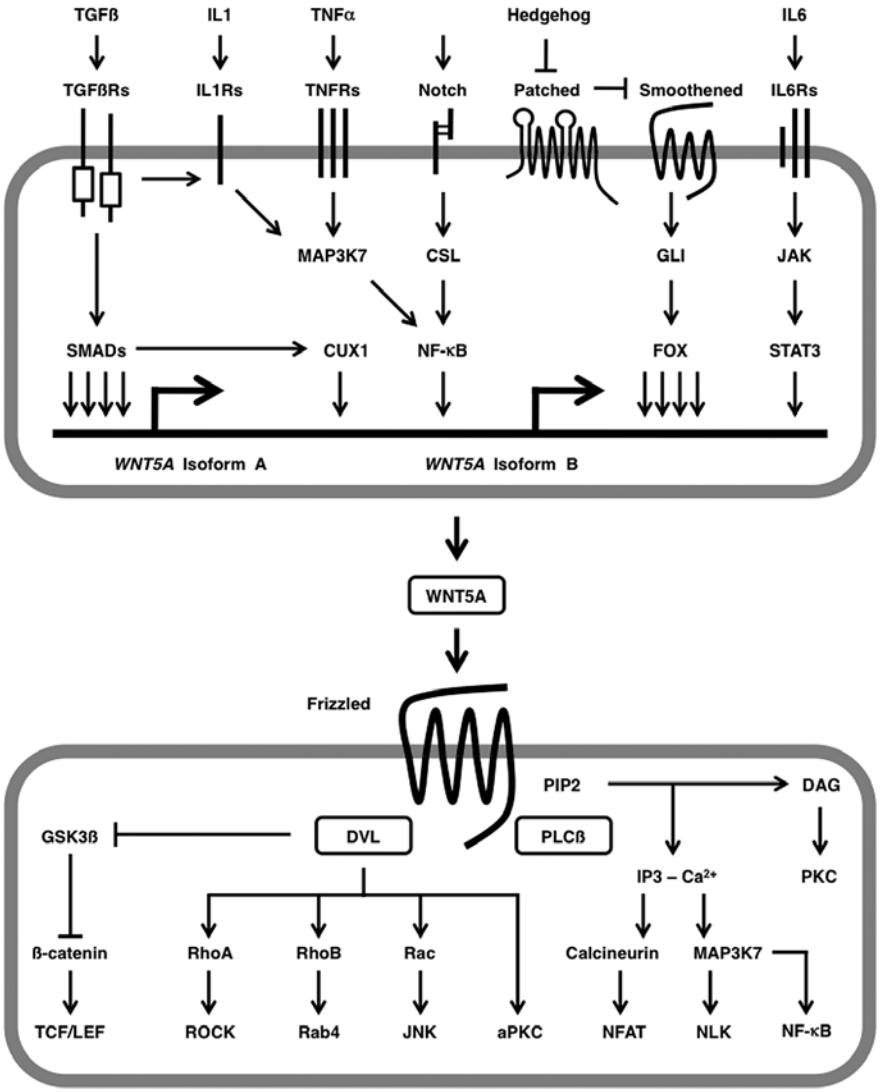

Figure 2. WNT5A at the cross roads of regulatory signaling cascades. Transcriptional mechanisms of *WNT5A* are shown in the upper part, and WNT5A signaling cascades are shown in the lower part.

NF-κB complex (71-73). Koyanagi *et al* reported Notch ligand-induced WNT5A upregulation in endothelial progenitor cells (74); however, the mechanism of Notch-mediated *WNT5A* upregulation remained to be elucidated. Manual inspection in this study revealed that consensus CSL-binding site within *WNT5A* regulatory regions was not conserved in mammals (data not shown). On the other hand, NF-κB-binding site within the promoter B region was conserved in mammalian *WNT5A* orthologs, as discussed above. Together these facts indicate that Notch signals upregulate *WNT5A* expression via the NICD-NF-κB complex (Fig. 2).

Discussion

Refined integrative genomic analyses of *WNT5A* orthologs were carried out in this study. The *WNT5A* gene was found to encode two transcripts by using alternative first exons 1A and 1B (Fig. 1). Comparative genomic analyses revealed that the promoter A region, promoter B region, and intron 1 were well conserved in mammalian *WNT5A* orthologs (Fig. 1).

Transcription factor-binding sites within the conserved *WNT5A* regulatory regions were searched for in this study. Quadruple SBEs, single GC-box, PPARγ-binding site, C/EBP-binding site, and bHLH-binding site within the promoter A region were conserved in human *WNT5A*, chimpanzee *WNT5A*, mouse *Wnt5a*, and rat *Wnt5a*; NF-κB-binding site, CUX1-binding site, double SBEs and double GC-boxes within the promoter B region were conserved in mammalian *WNT5A* orthologs; quadruple FOX-binding sites and double SBEs within ultra-conserved intron 1 were also conserved in the mammalian *WNT5A* orthologs (Fig. 1).

Conserved NF-κB-binding site within *WNT5A* promoter B region elucidated the mechanisms that TNFα and TLR signals upregulate *WNT5A* via MAP3K7. Quadruple FOX-binding sites within the *WNT5A* enhancer region and absence of GLI-binding site around and within the *WNT5A* gene revealed that Hedgehog signals induce *WNT5A* upregulation indirectly via FOX family members rather than directly via GLI activators. TGFß signals were found to upregulate *WNT5A* expression directly through the Smad complex, and

also indirectly through Smad-induced CUX1 and MAP3K7-mediated NF-κB. Together these facts indicate that *WNT5A* is transcribed due to multiple mechanisms, such as NF-κB, Hedgehog, TGFß, and Notch signaling activation.

WNT signaling cascades cross-talk with FGF, Notch, Hedgehog, and TGFß/BMP signaling cascades constituting the stem cell signaling network (75-77). Chronic inflammation is involved in carcinogenesis through tumor-stromal interaction activating STAT3 and NF-κB signaling cascades, and also through epigenetic alterations of the stem-cell signaling molecules (55-57). Results obtained in this study clearly indicate that WNT5A is the key molecule at the crossroads of inflammation and carcinogenesis. Because next-generation sequence technology and peta-scale supercomputer are emerging to open up the frontier of personalized medicine (78), large scale analyses of single nucleotide polymorphisms (SNPs) and copy number variations (CNVs) of the *WNT5A* gene in melanoma, breast cancer, lung cancer, pancreatic cancer and gastric cancer should be carried out with the stratification based on the co-existence of environmental insults, such as ultraviolet exposure, tobacco smoking, and chronic infection.

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