AP1- and NF-κB-binding sites conserved among mammalian *WNT10B* orthologs elucidate the TNFα-WNT10B signaling loop implicated in carcinogenesis and adipogenesis

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Abstract. WNT signals are context-dependently transduced to canonical and non-canonical signaling cascades. We cloned and characterized wild-type human WNT10B, while another group cloned aberrant human WNT10B with Gly60Asp amino-acid substitution. Proto-oncogene *WNT10B* is expressed in gastric cancer, pancreatic cancer, breast cancer, esophageal cancer, and cervical cancer. Because WNT10B blocks adipocyte differentiation, coding SNP of *WNT10B* gene is associated with familial obesity. In 2001, we reported *WNT10B* up-regulation by TNFα. Here, comparative integromics analyses on WNT10B orthologs were performed to elucidate the transcriptional mechanism of *WNT10B*. Chimpanzee *WNT10B* and cow *Wnt10b* genes were identified within NW_001223159.1 and AC150975.2 genome sequences, respectively, by using bioinformatics (Techint) and human intelligence (Humint). Chimpanzee WNT10B and cow Wnt10b showed 98.7% and 95.1% total-amino-acid identity with human WNT10B, respectively. N-terminal signal peptide, 24 Cys residues, two Asn-linked glycosylation sites, and Gly60 of human WNT10B were conserved among mammalian WNT10B orthologs. Transcription start site of human *WNT10B* gene was 106-bp upstream of NM_003394.2 RefSeq 5'-end. Number of GC di-nucleotide repeats just down-stream of *WNT10B* transcription start site varied among primates and human population. Comparative genomics analyses revealed that double AP1-binding sites in the 5'-flanking promoter region and NF-κB-binding site in intron 3 were conserved among human, chimpanzee, cow, mouse, and rat *WNT10B* orthologs. Because TNFα signaling through TNFR1 and TRADD/RIP/TRAF2 complex activates JUN kinase (JNK) and IκB kinase (IKK) signaling cascades, conserved AP1- and NF-κB-binding sites explain the mechanism of TNFα-induced *WNT10B* up-regulation. TNFα-WNT10B signaling loop is the negative feedback mechanism of adipogenesis to prevent obesity and metabolic syndrome. On the other hand, TNFα-WNT10B signaling loop is implicated in carcinogenesis. Inhibitors of TNFα-WNT10B signaling loop could be utilized for the prevention or treatment of cancer associated with chronic inflammation, such as gastric, liver, breast and pancreatic cancer.

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Introduction

WNT family members with conserved 22 or 24 Cys residues are implicated in embryogenesis and adult tissue homeostasis (1-7). WNT signals are context-dependently transduced through Frizzled (FZD) receptors to canonical and non-canonical signaling cascades (8-11). Canonical WNT signal transduction leads to the upregulation of *MYC, CCND1, FGF20, JAG1,* and *DKK1* genes for the cell fate determination (12-16), while non-canonical WNT signal transduction leads to the activation of RHOA, JUN kinase (JNK), PKC, NFAT and NLK signaling cascades for the regulation of tissue polarity, cell adhesion, and cell movement (17-19). Dysregulation of human WNT signaling cascades results in pathological conditions, such as cancer, rheumatoid arthritis, obesity, metabolic syndrome, and congestive heart failure (11).

We cloned and characterized wild-type human WNT10B (20), while another group cloned aberrant human WNT10B with Gly60Asp amino-acid substitution (21). We then identified and characterized rat Wnt10b (22). Human *WNT10B* is expressed in a variety of cancer, such as gastric, pancreatic, breast, esophageal and cervical cancer (20,23,24). Mouse *Wnt10b* upregulation due to the MMTV proviral integration results in the mammary carcinogenesis (25). Aberrant upregulation of *WNT10B* leads to carcinogenesis through the canonical WNT signaling activation. *WNT10B* is a proto-oncogene.

Mesenchymal stem cells give rise to a variety of cell lineages, such as adipocyte, osteocyte, chondrocyte, fibroblast, myocyte, and hepatocyte. WNT10B activates the canonical

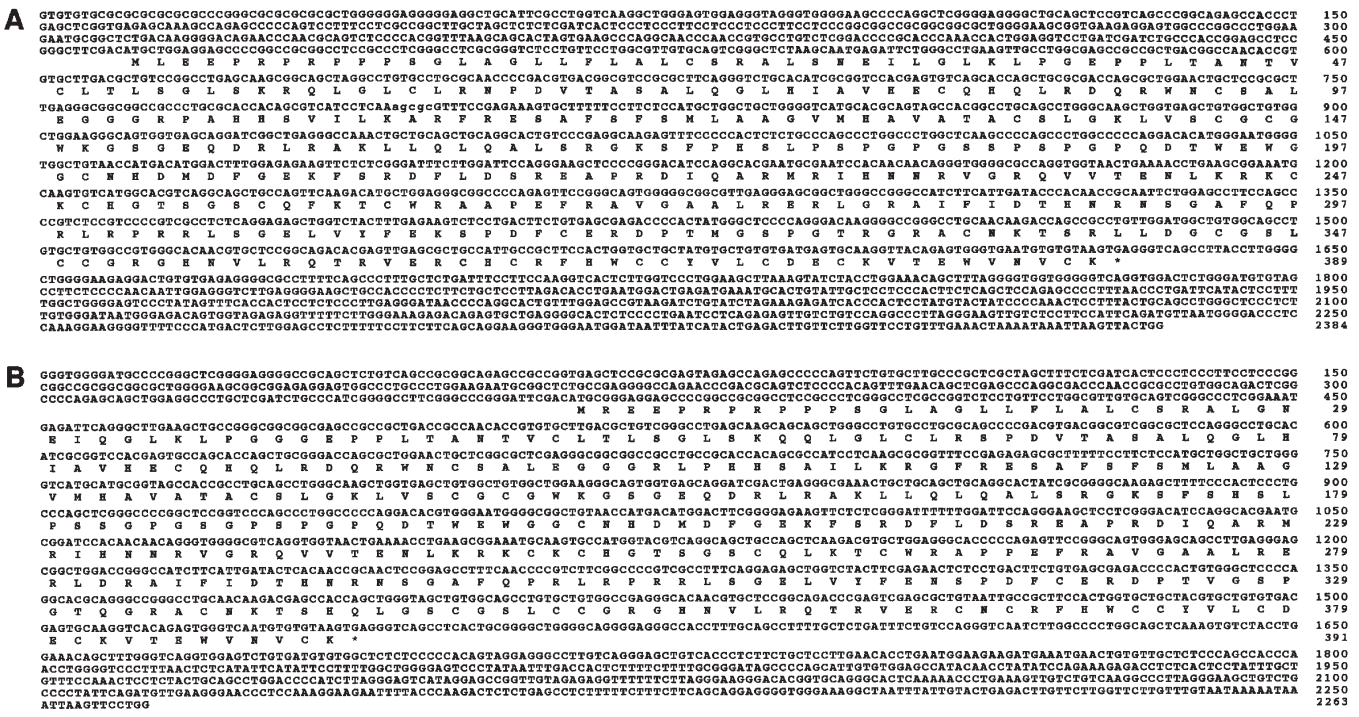

Figure 1. Chimpanzee and cow WNT10B orthologs. (A), Chimpanzee WNT10B complete CDS. (B), Cow Wnt10b complete CDS. Nucleotides and amino-acid residues are numbered on the right.

WNT signaling cascade to block adipocyte differentiation (26). Coding SNP of *WNT10B* gene to introduce missense mutation on one of conserved Cys residues is associated with familial obesity (27). Because loss-of-function coding SNP of *WNT10B* leads to obesity, *WNT10B* is a suppressor gene for metabolic syndrome.

In 2001, we reported upregulation of *WNT10B* by TNFα (20). TNFα signal is transduced through TNFR1 and TRADD/RIP/TRAF2 complex to JNK and IκB kinase (IKK) signaling cascades, which leads to the activation of AP1 and NF-κB transcriptional complexes, respectively (28). Here, comparative integromics analyses on WNT10B orthologs were performed to elucidate the mechanism of TNFα-induced *WNT10B* up-regulation. Chimpanzee *WNT10B* and cow *Wnt10b* genes were identified and characterized at first. AP1- and NF-κB-binding sites in the promoter and introns of *WNT10B* orthologs were then searched for. Evolutionarily conserved AP1-binding sites in the 5'-flanking promoter region and NF-κB-binding site in intron 3 of mammalian *WNT10B* orthologs were successfully identified in this study. Based on these facts, we propose that the TNFα-WNT10B signaling loop is implicated in carcinogenesis and adipogenesis.

Materials and methods

Identification and characterization of chimpanzee and cow WNT10B orthologs. Chimpanzee and cow genome sequences homologous to human *WNT10B* were searched for with BLAST programs as described previously (30-33). 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 (34-37). Complete coding sequence (CDS) of chimpanzee WNT10B and cow Wnt10b were determined by assembling exonic regions.

Comparative proteomics analyses. Complete CDS of chimpanzee and cow WNT10B orthologs were translated into amino-acid sequence by using Genetyx program. Mammalian WNT10B orthologs were then aligned for the comparative integromics analyses.

Comparative genomics analyses. Human genome sequence around the *WNT10B* gene was compared with chimpanzee, cow, mouse and rat genome sequences to identify evolutionarily conserved regions by using the BLAST programs. Transcription factor-binding sites within the evolutionarily conserved regions were then searched for by using Match program, Genetyx program, and the manual curation as described previously (38-41).

Results

Transcription start site of human WNT10B. BLAST programs using human WNT10B RefSeq (NM_003394.2) revealed that *WNT10B* gene at human chromosome 12q13 was located within human genome sequence AC025256.33. BLAST programs using human genome sequence around the *WNT10B* gene next revealed that several WNT10B ESTs were transcribed from more upstream position than the 5'-end of NM_003394.2 RefSeq. DA359350.1 and DA530656.1 ESTs were transcribed from the 106-bp upstream position. Number of GC di-nucleotide repeats just down-stream of *WNT10B* transcription start site was 9 in DA359350.1 and DA530656.1 ESTs, but was 11 in AC025256.33 genome sequence. Based on these facts, it was concluded that the transcription start site of

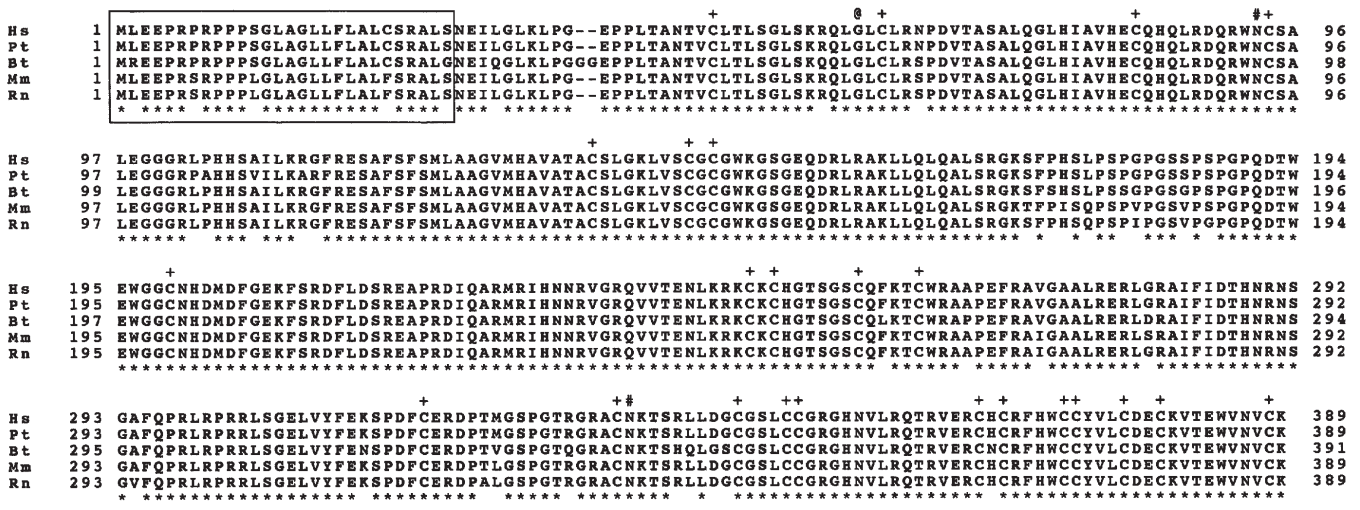

Figure 2. Mammalian WNT10B orthologs. Hs, human; Pt, chimpanzee; Bt, cow; Mm, mouse; Rn, rat. Amino-acid residues are numbered on the right. Signal peptide is boxed. Conserved Cys residues (cross) and Asn-linked glycosylation sites (sharp) are shown above the alignment. Location of Gly60Asp amino-acid substitution (@) is also shown. Gly60 of wild-type human WNT10B cloned by us (20) is conserved among mammalian WNT10B orthologs.

human *WNT10B* gene was 106-bp upstream of NM_003394.2 RefSeq 5'-end.

Chimpanzee WNT10B and cow Wnt10b genes. BLAST programs using human WNT10B complete CDS revealed that chimpanzee *WNT10B* gene was located within NW_001223159.1 genome sequence. Exon-intron boundaries of chimpanzee *WNT10B* gene were determined based on the consensus sequence of exon-intron junctions. Chimpanzee *WNT10B* gene was found consisting of five exons. Complete CDS of chimpanzee WNT10B was determined by assembling exonic regions (Fig. 1A). Number of GC di-nucleotide repeats just down-stream of *WNT10B* transcription start site was 15 in chimpanzee NW_001223159.1 genome sequence. Genetyx program revealed that nucleotide position 461-1630 was the coding region. Chimpanzee *WNT10B* gene was found to encode a 389-amino-acid protein (Fig. 1A).

BLAST programs next revealed that cow *Wnt10b* gene was located within AC150975.2 genome sequence. Complete CDS of cow Wnt10b was determined by assembling five exonic regions (Fig. 1B). Genetyx program revealed that nucleotide position 364-1539 was the coding region. Cow *Wnt10b* gene was found to encode a 391-amino-acid protein (Fig. 1B).

Comparative proteomics analyses on WNT10B orthologs. Chimpanzee WNT10B and cow Wnt10b showed 98.7% and 95.1% total-amino-acid identity with human WNT10B, respectively. N-terminal signal peptide, 24 Cys residues and two Asn-linked glycosylation sites were conserved among human, chimpanzee, cow, mouse, and rat WNT10B orthologs. In addition, Gly60 rather than Asp60 of human WNT10B were conserved among mammalian WNT10B orthologs (Fig. 2).

Comparative genomics analyses on WNT10B orthologs. Human *WNT10B*, chimpanzee *WNT10B* and cow *Wnt10b* genes are located within AC025256.33, NW_001223159.1 and AC150975.2 genome sequences, respectively, as mentioned above. Rat *Wnt10b* gene is located within AC118760.4 genome sequence as reported previously (22). BLAST programs using mouse Wnt10b RefSeq (NM_011718.1) revealed that mouse *Wnt10b* gene was located within AC156543.10 genome sequence.

Conserved regions around the *WNT10B* gene were next searched for. BLAST programs revealed that the 5'-flanking promoter region, five exonic regions, and intron 3 were well conserved between human *WNT10B* and mouse *Wnt10b* genes (Fig. 3A). Transcription factor-binding sites within the 5'-flanking promoter region and intron 3 were next searched for. Double AP1-binding sites located at about 2.2- and 0.9-kb upstream of the transcriptional start site of human *WNT10B* gene were conserved in chimpanzee, cow, mouse, and rat *WNT10B* orthologs (Fig. 3A). In addition, NF-κB-binding site in intron 3 of human *WNT10B* gene was conserved in chimpanzee, cow, mouse, and rat *WNT10B* orthologs (Fig. 3A).

Discussion

Comparative integromics analyses on WNT10B orthologs were performed. Chimpanzee *WNT10B* gene was identified within NW_001223159.1 genome sequence (Fig. 1A), while cow *Wnt10b* gene within AC150975.2 genome sequence (Fig. 1B). Chimpanzee WNT10B and cow Wnt10b showed 98.7% and 95.1% total-amino-acid identity with human WNT10B, respectively. N-terminal signal peptide, 24 Cys residues, two Asn-linked glycosylation sites, and Gly60 of human WNT10B were conserved among mammalian WNT10B orthologs (Fig. 2).

Transcription start site of human *WNT10B* gene was located at 106-bp upstream of NM_003394.2 RefSeq 5'-end. GC di-nucleotide repeats just down-stream of the transcriptional start site was identified in human and chimpanzee *WNT10B* genes, but not in cow, mouse, and rat *Wnt10b* gene. Number of GC di-nucleotide repeats just down-stream of *WNT10B* transcription start site was 9 in human *WNT10B* ESTs DA359350.1 and DA530656.1, 11 in human genome sequence AC025256.33, and 15 in chimpanzee genome sequence

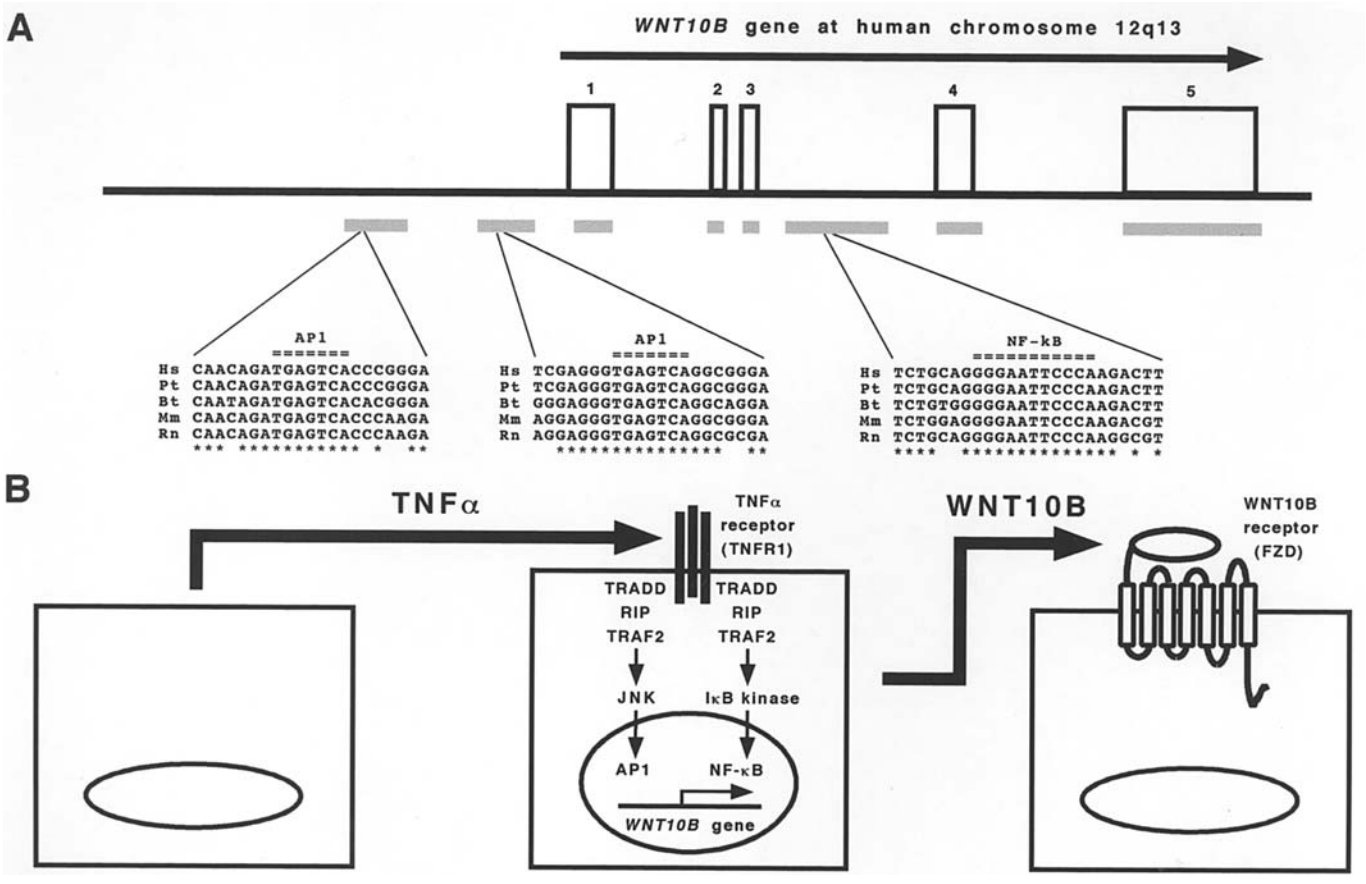

Figure 3. The TNFα-WNT10B signaling loop. (A), AP1- and NF-κB-binding sites within mammalian *WNT10B* orthologs. Human *WNT10B* gene consists of five exons. Regions conserved between human *WNT10B* and mouse *Wnt10b* genes are shown by gray bars. Hs, human; Pt, chimpanzee; Bt, cow; Mm, mouse; Rn, rat. Double AP1-binding sites in the 5'-flanking promoter region and NF-κB-binding site in intron 3 are conserved among human, chimpanzee, cow, mouse, and rat *WNT10B* orthologs. *WNT10B* gene is the evolutionarily conserved target of TNFα signaling cascade. (B), Schematic presentation of the TNFα-WNT10B signaling loop. TNFα-WNT10B signaling loop is the negative feedback mechanism of adipogenesis to prevent obesity and metabolic syndrome. On the other hand, TNFα-WNT10B signaling loop is implicated in the carcinogenesis associated with chronic inflammation.

NW_001223159.1. These facts indicate that the number of GC di-nucleotide repeats just down-stream of *WNT10B* transcription start site varied among primates as well as among human population.

We previously reported *WNT10B* upregulation by TNFα (20); however, the mechanism of TNFα-induced WNT10B upregulation remained unclear. TNFα signaling through TNFR1 and TRADD/RIP/TRAF2 complex to JNK and IKK signaling cascades results in the activation of AP1 and NF-κB transcriptional complex, respectively (28). In this study, we identified that double AP1-binding sites in the 5'-flanking promoter region and NF-κB-binding site in intron 3 were conserved among human, chimpanzee, cow, mouse, and rat *WNT10B* orthologs (Fig. 3A). Based on these facts, it was demonstrated that TNFα signaling to AP1 and NF-κB nuclear complex induces transcriptional upregulation of *WNT10B* (Fig. 3B).

TNFα is secreted from mature adipocytes, and WNT10B blocks adipocytic differentiation. Because *WNT10B* is a target gene of TNFα signaling cascade (Fig. 3), it is clear that the TNFα-WNT10B signaling loop functions as the negative feedback loop to maintain the homeostasis of adipose tissue. Therefore, inactivation of the TNFα-WNT10B signaling loop leads to obesity and metabolic syndrome.

Helicobacter pylori is a causative agent for peptic ulcer diseases, chronic gastritis, and gastric cancer (42-45). TNFα concentration is significantly elevated in patients with active gastritis than in those with inactive gastritis, while interleukin-6 is elevated in those with active and inactive gastritis (46). HBV and HCV are causative agents for hepatitis, liver cirrhosis, and liver cancer, and TNFα concentration is also elevated during hepatocarcinogenesis (28). TNFα then induces upregulation of *WNT10B* proto-oncogene during chronic inflammation (Fig. 3B). Because the TNFα-WNT10B signaling loop plays a key role during carcinogenesis, inhibitors of the TNFα-WNT10B signaling loop could be utilized for the prevention or treatment of cancer associated with chronic inflammation, such as gastric, liver, breast and pancreatic cancer.

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