Conserved POU/OCT- and GATA-binding sites in 5'-flanking promoter region of mammalian *WNT8B* orthologs

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Abstract. WNT family members are secreted-type glycoproteins regulating cell fate, planar cell polarity, cell adhesion, and cell movement. WNT signals are context-dependently transduced to the canonical pathway for the transcriptional up-regulation of *MYC, CCND1, FGF20, JAG1, WISP1* and *DKK1* genes, and also to the non-canonical pathway for the activation of RHOA, JNK, PKC, NFAT and NLK signaling cascades. We cloned and characterized the wild-type human WNT8B, while another group the aberrant human WNT8B with Gly230Ala and Arg284Leu amino-acid substitutions. Although *WNT8B* is undetectable in normal adult tissues by using Northern blot analyses, *WNT8B* is expressed in gastric cancer, pancreatic cancer, colorectal cancer, breast cancer, and embryonal tumors. Here, comparative integromics on WNT8B orthologs were investigated by using bioinformatics (Techint) and human intelligence (Humint). Cow *Wnt8b* gene was identified within NW_001494361.1 genome sequence. Predicted sequence XM_582222.3 was an artificial cow Wnt8b with aberrant prediction for the first exon. Cow Wnt8b complete coding sequence was found to encode a 350-amino-acid protein, which showed 96.9% total-amino-acid identity with human WNT8B. Comparative proteomics revealed that N-terminal signal peptide, 22 Cys residues, two Asn-linked glycosylation sites, Gly230, and Arg284 of human WNT8B were conserved among mammalian WNT8B orthologs. Comparative genomics revealed that POU/OCT- and GATA-binding sites in the 5'-flanking promoter region were conserved among human, chimpanzee, cow, mouse, and rat *WNT8B* orthologs. *In silico* expression analyses revealed that human *WNT8B* was expressed in embryoid body derived from embryonic stem (ES) cells, hepatocyte progenitors derived from ES cells, fetal brain, diffuse-type gastric cancer, colorectal cancer, prostate cancer, and ovarian fibrotheoma. Based on the expression profiles of POU and GATA family transcription factors, it was revealed that *WNT8B* expression in hepatocyte progenitors derived from human ES cells is due to POU5F1 (OCT3/OCT4) and GATA3, and also that *WNT8B* expression in diffuse-type gastric cancer is due to POU5F1 and GATA6.

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Introduction

WNT family members are secreted-type glycoproteins regulating cell fate, planar cell polarity, cell adhesion, and cell movement (1-7). WNT signals are context-dependently transduced to the canonical signaling pathway through Frizzled receptors and LRP5/6 co-receptors for the transcriptional up-regulation of *MYC, CCND1, FGF20, JAG1, WISP1* and *DKK1* genes (8-16), and also to the non-canonical signaling pathway through Frizzled receptors and ROR2/PTK7/RYK co-receptors for the activation of RHOA, JNK, PKC, NFAT and NLK signaling cascades (15-19). Because WNT signaling cascades are components of the stem signaling network implicated in the embryogenesis and the maintenance of adult tissue homeostasis, dysregulation of human WNT signaling cascades leads to a variety of human diseases, such as obesity, metabolic syndrome, congestive heart failure, rheumatoid arthritis, and cancer (16,20,21).

We cloned and characterized the wild-type human WNT8B (22), while another group cloned the aberrant human WNT8B with Gly230Ala and Arg284Leu amino-acid substitutions (23). Although *WNT8B* is undetectable in normal adult tissues by using Northern blot analyses, *WNT8B* is expressed in gastric cancer, pancreatic cancer, colorectal cancer, breast cancer, and embryonal tumors (22,24).

We also identified and characterized the rat *Wnt8b* gene (25). GATA-binding site in the 5'-promoter region is conserved between human *WNT8B* and rat *Wnt8b* genes (25). Here, comparative integromics on WNT8B orthologs were investigated. Because XM_582222.3 was an artificial cow Wnt8b with aberrant prediction for the first exon, cow Wnt8b complete coding sequence (CDS) was determined in this study. Comparative proteomics revealed that Gly230 and Arg284 of human WNT8B cloned by us (22) were conserved among mammalian WNT8B orthologs. Comparative genomics revealed that POU/ OCT- and GATA-binding sites in the 5'-flanking promoter region were conserved among mammalian *WNT8B* orthologs. Based on the expression profiles of *POU* and *GATA* family transcription factors, mechanisms of

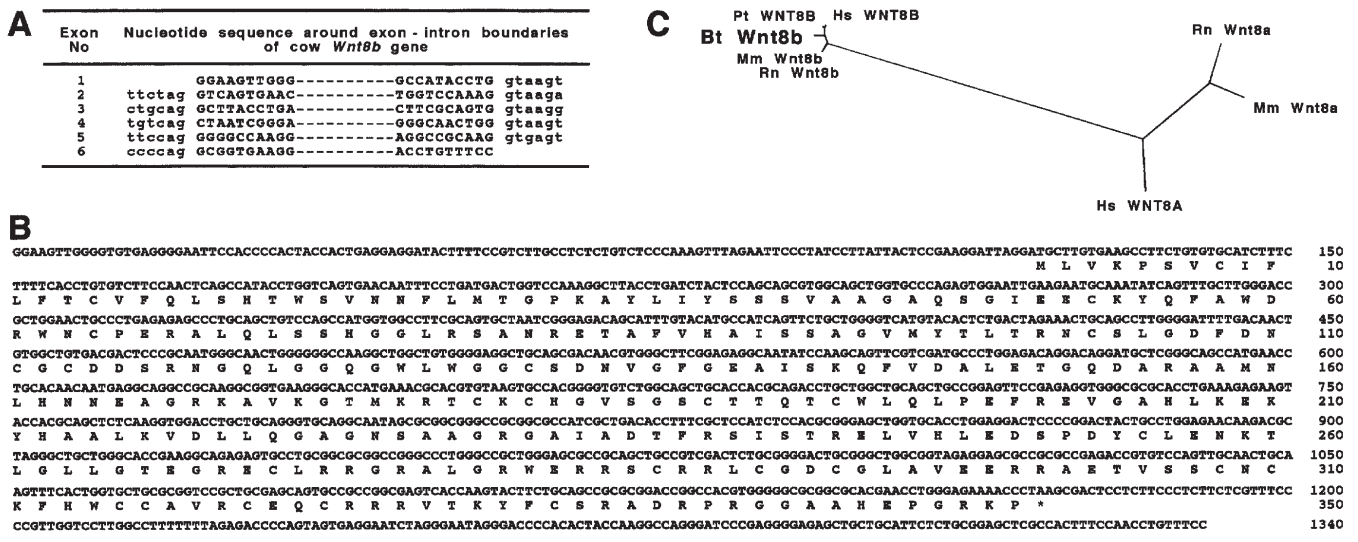

Figure 1. Cow Wnt8b gene and phylogenetic analysis. (A), Exon-intron structure of cow *Wnt8b* gene. (B), Cow Wnt8b complete CDS. Nucleotides and amino-acid residues are numbered on the right. (C), Phylogenetic analyses on WNT8B homologs. Hs, human; Pt, chimpanzee; Bt, cow; Mm, mouse; Rn, rat.

WNT8B expression in hepatocyte progenitors derived from human embryonic stem (ES) cells and diffuse-type gastric cancer were then elucidated.

Materials and methods

Identification and characterization of cow Wnt8b gene. Cow genome sequence homologous to human *WNT8B* was searched for with BLAST programs as described previously (26-29). 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 proteomics. Mammalian WNT8B orthologs were aligned for the comparative integromic analyses.

Comparative genomics. Human genome sequence around the *WNT8B* 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 (30-33).

In silico expression analyses. Expressed sequence tags (ESTs) derived from human *WNT8B, POU* family members, and *GATA* family members were searched for by using the BLAST programs as described previously (34-37). The sources of human ESTs were listed up for *in silico* expression analyses.

Results

Cow Wnt8b gene. BLAST programs revealed that cow *Wnt8b* gene was located within NW_001494361.1 genome sequence. Exon-intron boundaries of cow *Wnt8b* gene were determined based on the consensus sequence of exon-intron junctions. Cow *Wnt8b* gene was found consisting of six exons (Fig. 1A). Predicted sequence XM_582222.3 was an artificial cow Wnt8b with aberrant prediction for the first exon. Because cow *Wnt8b* intron 1 of about 22-kb in size was relatively large, an aberrant 11-bp nucleotide sequence near exon 2 was incorporated into predicted sequence XM_582222.3 instead of the real exon 1.

Complete CDS of cow Wnt8b was determined by assembling exonic regions (Fig. 1B). Genetyx program revealed that nucleotide position 120-1172 was the coding region of cow Wnt8b complete CDS. Cow *Wnt8b* gene was found to encode a 350-amino-acid protein (Fig. 1B).

Comparative proteomics on WNT8B orthologs. Cow Wnt8b showed 96.9% total-amino-acid identity with human WNT8B. Alignment of human, chimpanzee, cow, mouse, and rat WNT8B orthologs revealed that N-terminal signal peptide, 22 Cys residues and two Asn-linked glycosylation sites were conserved among mammalian WNT8B orthologs (Fig. 2). Gly230 and Arg284 of human WNT8B reported by us (22) were conserved among mammalian WNT8B orthologs.

Comparative proteomics next revealed that WNT8B orthologs were more evolutionarily conserved than WNT8A orthologs (Fig. 1C).

Comparative genomics on WNT8B orthologs. Human *WNT8B* gene is located within AL359759.19 and AL133352.12 genome sequences, while rat *Wnt8b* gene is located within AC105487.6 and AC103018.7 genome sequences as previously reported (25). Cow Wnt8b gene was located within NW_001494361.1 genome sequence as mentioned above. BLAST programs revealed that chimpanzee *WNT8B* gene and mouse *Wnt8b* gene were located within NW_001220741.1 and AC124401.3 genome sequences, respectively.

Conserved regions among the *WNT8B* orthologs were searched for. BLAST programs revealed that the 5'-flanking promoter region, six exonic regions, and parts of large intron 1 were well conserved between human *WNT8B* and mouse *Wnt8b* genes (Fig. 3A).

Transcription factor-binding sites within the 5'-flanking promoter region and intron 1 were next searched for. GATA-

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Hs WNT8B  MFLSKPSVYICLFTCVLQL-SHSWSVNNFLMTGPKAYLIYSSSVAAGAQSGIEECKYQFAWDRWNCPERALQLSSHGGLRSANRETAFVHAISSAGVMYTLTRNCSLGDFDNCGCDDSRN  119
Pt WNT8B  MFLSKPSVYISLFTCVLQL-SHSWSVNNFLMTGPKAYLIYSSSVAAGAQSGIEECKYQFAWDRWNCPERALQLSSHGGLRSANRETAFVHAISSAGVMYTLTRNCSLGDFDNCGCDDSRN  119
Bt Wnt8b  M-LVKPSVCIFLFTCVFQL-SHTWSVNNFLMTGPKAYLIYSSSVAAGAQSGIEECKYQFAWDRWNCPERALQLSSHGGLRSANRETAFVHAISSAGVMYTLTRNCSLGDFDNCGCDDSRN  118
Mm Wnt8b  MFLMKP-VCVLLVTCVLHR-SHAWSVNNFLMTGPKAYLVYSSSVAAGAQSGIEECKYQFAWDRWNCPERALQLSSHGGLRSANRETAFVHAISSAGVMYTLTRNCSLGDFDNCGCDDSRN  118
Rn Wnt8b  MFLMKP-VCVLLVTCVLHR-SHAWSVNNFLMTGPKAYLVYSSSVAAGAQSGIEECKYQFAWDRWNCPERALQLSSHGGLRSANRETAFVHAISSAGVMYTLTRNCSLGDFDNCGCDDSRN  118
Hs WNT8A  M-GNLFMLWAALGICCAAFSASAWSVNNFLITGPKAYLTYTTSVALGAQSGIEECKFQFAWERWNCPENALQLSTHNRLRSATRETSFIHAISSAGVMYIITKNCSMGDFENCGCDGSNN  119
Mm Wnt8a  M-GHLLMLWVAAGMCYPALGASAWSVNNFLITGPKAYLTYTASVALGAQIGIEECKFQFAWERWNCPEHAFQFSTHNRLRAATRETSFIHAIRSAAIMYAVTKNCSMGDLENCGCDESQN  119
Rn Wnt8a  M-GHFFMLWVAAGMCYTAFGASAWSVNNFLITGPKAYVTYTASVALGAQMGMEECKFQFAWERWNCPEHAFQFSTHTRPRGATRETSFIHAIRSAAVMYAVTKNCSMGDLETCGCDESNN  119

Hs WNT8B  GQLGGQGWLWGGCSDNVGFGEAISKQFVDALETGQDARAAMNLHNNEAGRKAVKGTMKRTCKCHGVSGSCTTQTCWLQLPEFREVGAHLKEKYHAALKVDLLQG---AGNSAAGRGAIAD  236
Pt WNT8B  GQLGGQGWLWGGCSDNVGFGEAISKQFVDALETGQDARAAMNLHNNEAGRKAVKGTMKRTCKCHGVSGSCTTQTCWLQLPEFREVGAHLKEKYHAALKVDLLQG---AGNSAAGRGAIAD  236
Bt Wnt8b  GQLGGQGWLWGGCSDNVGFGEAISKQFVDALETGQDARAAMNLHNNEAGRKAVKGTMKRTCKCHGVSGSCTTQTCWLQLPEFREVGAHLKEKYHAALKVDLLQG---AGNSAAGRGAIAD  235
Mm Wnt8b  GQLGGQGWLWGGCSDNVGFGEAISKQFVDALETGQDARAAMNLHNNEAGRKAVKGTMKRTCKCHGVSGSCTTQTCWLQLPEFREVGAHLKEKYHAALKVDLLQG---AGNSAAGRGAIAD  235
Rn Wnt8b  GQLGGQGWLWGGCSDNVGFGEAISKQFVDALETGQDARAAMNLHNNEAGRKAVKGTMKRTCKCHGVSGSCTTQTCWLQLPEFREVGAHLKEKYHAALKVDLLQG---AGNSAAGRGAIAD  235
Hs WNT8A  GKTGGHGWIWGGCSDNVEFGERISKLFVDSLEKGKDARALMNLHNNRAGRLAVRATMKRTCKCHGISGSCSIQTCWLQLAEFREMGDYLKAKYDQALKIEMDKRQLRAGNSAEGHWVPAE  239
Mm Wnt8a  GKTGGHGWIWGGCSDNVEFGEKISRLFVDSLEKGKDARALVNLHNNRAGRLAVRASTKRTCKCHGISGSCSIQTCWLQLADFRQMGNYLKAKYDRALKIEMDKRQLRAGNRAEGRWALTE  239
Rn Wnt8a  GKAGGHGWIWGGCSDNVEFGEKISRLFVDSLEKGKDARALMNLHNNRAGRLAVRASMKRTCKCHGISGSCSIQTCWLQLADFRQMGNYLKAKYDRALKIETDKRQLRAGNRAEGRWAPIE  239

Hs WNT8B  TFRSISTRELVHLEDSPDYCLENKTLGLLGTEGRECLRRGRALGRWERRSCRRLCGDCGLAVEERRAETVSSCNCKFHWCCAVRCEQCRRRVTKYFCSRAERPRGGAAHKPGRKP  351
Pt WNT8B  TFRSISTRELVHLEDSPDYCLENKTLGLLGTEGRECLRRGRALGRWERRSCRRLCGDCGLAVEERRAETVSSCNCKFHWCCAVRCEQCRRRVTKYFCSRAERPRGGAAHKPGRKP  351
Bt WNT8b  TFRSISTRELVHLEDSPDYCLENKTLGLLGTEGRECLRRGRALGRWERRSCRRLCGDCGLAVEERRAETVSSCNCKFHWCCAVRCEQCRRRVTKYFCSRADRPRGGAAHEPGRKP  350
Mm Wnt8b  TFRSISTRELVHLEDSPDYCLENKTLGLLGTEGRECLRRGRALGRWERRSCRRLCGDCGLAVEERRAETVSSCNCKFHWCCAVRCEQCRRRVTKYFCSRAERPPRGAAHKPGKNS  350
Rn Wnt8b  TFRSISTRELVHLEDSPDYCLENKTLGLLGTEGRECLRRGRALGRWERRSCRRLCGDCGLAVEERRAETVSSCNCKFHWCCAVRCEQCRRRVTKYFCSRAERPPRGAAHKPGKNP  350
Hs WNT8A  AFLPSAEAELIFLEESPDYCTCNSSLGIYGTEGRECLQNSHNTSRWERRSCGRLCTECGLQVEERKTEVISSCNCKFQWCCTVKCDQCRHVVSKYYCARSP----GSAQSLGKGSA  351
Mm Wnt8a  AFLPSTEAELIFLEGSPDYCNRNASLSIQGTEGRECLQNARSASRREQRSCGRLCTECGLQVEERRAEAVSSCDCNFQWCCTVKCGQCRRVVSRYYCTRPV----GSARPRGRGKDSAW  354
Rn Wnt8a  AFLPSAEAELIFLEGSPDYCNRNASLGIYGTEGRECLQNARSASRWEQRSCGRLCTECGLQVEERRTEAVSSCDCNFQWCCTVKCGQCRRVVNRYYCTRPA----GSAGPRGTTTKSLVA  355
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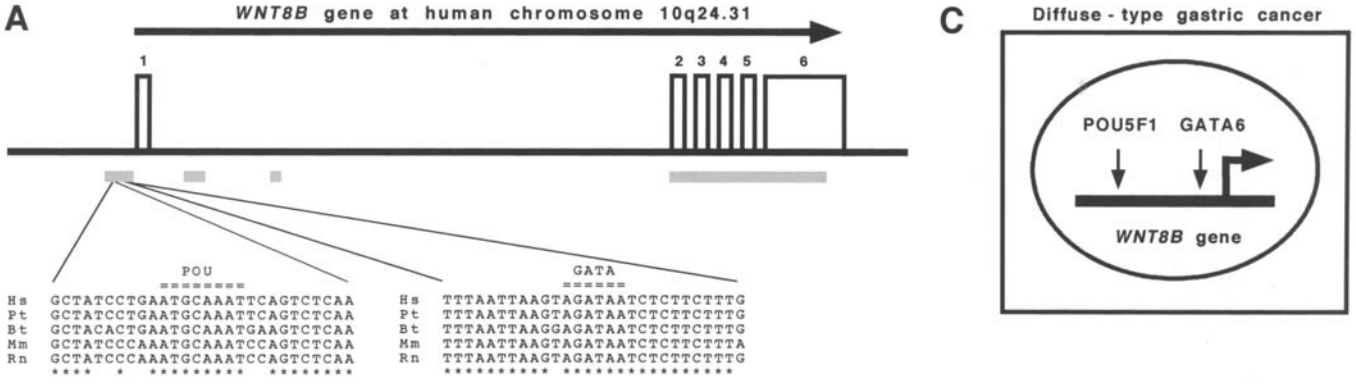
Figure 2. Mammalian WNT8B and WNT8A orthologs. Amino-acid residues are numbered on the right. Signal peptide is boxed. Conserved Cys residues (cross) and Asn-linked glycosylation sites (sharp) are shown above the alignment. Location of Gly230Ala and Arg284Leu amino-acid substitutions (@) are also shown. Gly230 and Arg284 of wild-type human WNT8B cloned by us (22) are conserved among mammalian WNT8B orthologs.



B

Cell population or tissue	Number of human ESTs														
	WNT8B	POU2F1	POU2F2	POU2F3	POU3F1	POU3F2	POU3F3	POU3F4	POU5F1	GATA1	GATA2	GATA3	GATA4	GATA5	GATA6
Embryoid body derived from ES cells	2	2	0	0	0	1	0	0	0	0	2	2	3	1	1
Hepatocyte progenitors derived from ES cells	1	1	0	0	0	0	0	0	2 *	0	0	8 *	0	0	2
Diffuse-type gastric cancer	1	1	0	1	0	0	0	0	6 *	0	1	0	2	0	5 *

Figure 3. Transcriptional mechanisms of *WNT8B*. (A), POU- and GATA-binding sites within mammalian *WNT8B* orthologs. Human *WNT8B* gene consists of six exons. Regions conserved between human *WNT8B* and mouse *Wnt8b* genes are shown by gray bars. Hs, human; Pt, chimpanzee; Bt, cow; Mm, mouse; Rn, rat. POU- and GATA-binding sites in the 5'-flanking promoter region are conserved among human, chimpanzee, cow, mouse, and rat *WNT8B* orthologs. (B), Expression profile of human *WNT8B* as well as *POU* and *GATA* family members. Expression profile of *POU* family members in diffuse-type gastric cancer is cited from our previous report on *FZD5* (36). *POU5F1* is preferentially expressed in hepatocyte progenitors derived from human ES cells and diffuse-type gastric cancer. *GATA3* is preferentially expressed in hepatocyte progenitors derived from human ES cells, while *GATA6* in diffuse-type gastric cancer. (C), Schematic representation of the *WNT8B* transcription in diffuse-type gastric cancer. *WNT8B* expression in diffuse-type gastric cancer is due to POU5F1 and GATA6 transcription factors.

binding site located at 77-bp upstream of the transcriptional start site of human *WNT8B* gene previously identified by us (25) was conserved in chimpanzee, cow, mouse, and rat *WNT8B* orthologs (Fig. 3A). In addition, POU-binding site located at 460-bp upstream of the transcriptional start site of human *WNT8B* gene was also conserved in chimpanzee, cow, mouse, and rat *WNT8B* orthologs (Fig. 3A).

In silico expression analysis on human WNT8B. Expression of human *WNT8B* mRNA was detected in embryoid body derived from ES cells, hepatocyte progenitors derived from ES cells, fetal brain, diffuse-type gastric cancer, colorectal cancer, prostate cancer, and ovarian fibrotheoma by using *in silico* expression analysis.

Transcriptional mechanism of WNT8B. To elucidate the mechanisms of *WNT8B* transcription in hepatocyte progenitors and diffuse-type gastric cancer, expression profiles of *POU* and *GATA* family members were investigated.

POU5F1 (OCT3/OCT) was preferentially expressed in undifferentiated human ES cells and diffuse-type gastric cancer as previously reported by us (36). *POU5F1* was also preferentially expressed in hepatocyte progenitors derived from human ES cells (Fig. 3B).

GATA6 was preferentially expressed in diffuse-type gastric cancer as previously reported by us (25). *GATA3* was preferentially expressed in hepatocyte progenitors derived from human ES cells (Fig. 3B).

Discussion

Comparative integromics on WNT8B orthologs were investigated in this study. Cow *Wnt8b* gene was identified within NW_001494361.1 genome sequence (Fig. 1A). Predicted sequence XM_582222.3 was an artificial cow Wnt8b with aberrant prediction for the first exon. Cow Wnt8b complete coding sequence was found to encode a 350-amino-acid protein (Fig. 1B), which showed 96.9% total-amino-acid identity with human WNT8B. Comparative proteomics revealed that N-terminal signal peptide, 22 Cys residues, two Asn-linked glycosylation sites, Gly230, and Arg284 of human WNT8B cloned by us (22) were conserved among mammalian WNT8B orthologs (Fig. 2). Human WNT8B reported by another group (23) is an aberrant WNT8B with two amino-acid substitutions due to nucleotide changes during cDNA library construction or nucleotide sequencing errors.

We have previously reported *WNT8B* expression in gastric cancer, pancreatic cancer, colorectal cancer, breast cancer, and embryonal tumors (22,24). In this study, *WNT8B* mRNA was detected in embryoid body derived from human ES cells, hepatocyte progenitors derived from human ES cells, fetal brain, diffuse-type gastric cancer, colorectal cancer, prostate cancer, and ovarian fibrotheoma by using *in silico* expression analysis.

Single nucleotide polymorphism (SNP) of *WNT8B* gene might be associated with genetic predisposition for diffuse-type gastric cancer, pancreatic cancer, colorectal cancer, breast cancer, and prostate cancer. In addition, WNT8B oncofetal protein is a potential diagnostic marker for a variety of human cancers mentioned above.

Because WNT8B activates the canonical WNT signaling cascade for the cell fate determination, *WNT8B* mRNA expression in hepatocyte progenitors indicates that WNT8B is a potential inducer of hepatocyte differentiation. Mesenchymal stem cells are more promising sources for hepatocytes in the field of regenerative medicine. WNT8B mimetic compounds activating the canonical WNT signaling pathway should be developed for hepatocyte induction from human ES cells or mesenchymal stem cells.

Comparative genomics revealed that POU- and GATA-binding sites in the 5'-flanking promoter region were conserved among human, chimpanzee, cow, mouse, and rat *WNT8B* orthologs (Fig. 3A). Based on the expression profiles of *POU* and *GATA* family transcription factors, it was revealed that *WNT8B* expression in hepatocyte progenitors derived from human ES cells is due to POU5F1 and GATA3 (Fig. 3B), and also that *WNT8B* expression in diffuse-type gastric cancer is due to POU5F1 and GATA6 (Fig. 3C).

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