SPANDIDOS PUBLICATIONS

WNT antagonist, DKK2, is a Notch signaling target in intestinal stem cells: Augmentation of a negative regulation system for canonical WNT signaling pathway by the Notch-DKK2 signaling loop in primates

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Received August 2, 2006; Accepted October 23, 2006

Abstract. Notch and WNT signaling pathways are key components of the stem cell signaling network. Canonical WNT signaling to intestinal progenitor cells leads to transcriptional activation of the *JAG1* gene, encoding Serrate-type Notch ligand. JAG1 then binds to the Notch receptor on adjacent stem cells to induce Notch receptor proteolyses for the release of Notch intracellular domain (NICD). NICD is associated with CSL/RBPSUH and Mastermind (MAML1, MAML2, or MAML3) to activate Notch target genes, such as *HES1* and *HES5*. Although WNT-dependent Notch signaling activation in intestinal stem cells is clarified, the effects of Notch signaling activation on WNT signaling in progenitor cells remain unclear. We searched for Notch-response element (NRE) in the promoter region of genes encoding secreted WNT signaling inhibitors, including *DKK1, DKK2, DKK3, DKK4, SFRP1, SFRP2, SFRP3, SFRP4, SFRP5* and *WIF1*. Double NREs were identified within human *DKK2* promoter by bioinformatics and human intelligence (Humint). The human *DKK2* gene was characterized as Notch signaling target in intestinal stem cells. Because DKK2 is a key player in the stem cell signaling network, the *DKK2* gene at human chromosome 4q25 is a candidate tumor suppressor gene inactivated due to epigenetic silencing and/or deletion. The chimpanzee *DKK2* gene was identified within the NW_105990.1 genome sequence, while the cow *Dkk2* gene was identified within the AC156664.2 and AC158038.2 genome sequences. Chimpanzee DKK2 and cow Dkk2 showed 98.5% and 95.8% total-amino-acid identity with human DKK2, respectively. Double NREs in human *DKK2* promoter were conserved in chimpanzee *DKK2* promoter, partially in rat *Dkk2* promoter, but not in cow and mouse *Dkk2* promoters. The Notch-DKK2 signaling loop, created or potentiated in primates, was complementary to WNT-DKK1 and BMP-IHH-SFRP1 signaling loops for negative regulation of canonical WNT signaling pathway. Together, these facts indicate that *DKK2* promoter evolution resulted in the augmentation of a WNT negative regulation system in primates.

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Key words: Notch, WNT, FGF, stem cell signaling network, integrome network, systems biology

Introduction

Interaction among Notch (1-3), WNT (4-6), FGF (7-9), BMP (10-12) and Hedgehog (13-15) signaling pathways constitutes the stem cell signaling network. Canonical Notch signaling in intestinal stem cells leads to Notch receptor proteolyses for the release of Notch intracellular domain (NICD), which is associated with CSL/RBPSUH and Mastermind to activate Notch target genes, such as *HES1* and *HES5* (16,17).

Canonical WNT signaling to intestinal progenitor cells leads to transcriptional activation of the *JAG1* gene, encoding Serrate-type Notch ligand (18). JAG1 then binds to the Notch receptor on adjacent stem cells to induce Notch signaling activation. Because canonical WNT signaling is implicated in cell-fate determination, dysregulation of the canonical WNT signaling pathway leads to carcinogenesis. The WNT-DKK1 signaling loop (19) and BMP-IHH-SFRP1 signaling loop (20) are known as components of a negative regulation system for canonical WNT signaling pathway.

Although WNT-dependent Notch signaling activation in stem cells is clarified, the effects of Notch signaling activation in stem cells on WNT signaling progenitor cells remain unclear. Here, we searched for Notch-response element (NRE) in the promoter region of human genes encoding secreted WNT signaling inhibitors. Double NREs were identified within human *DKK2* promoter. Based on promoter and expression domain analyses, human *DKK2* was characterized as the target of Notch signaling pathway in intestinal stem cells. Chimpanzee *DKK2* and cow *Dkk2* genes were then identified. Comparative genomics analyses revealed *DKK2* promoter evolution in primates. The Notch-DKK2 signaling loop, created or potentiated in primates, was complementary to WNT-DKK1

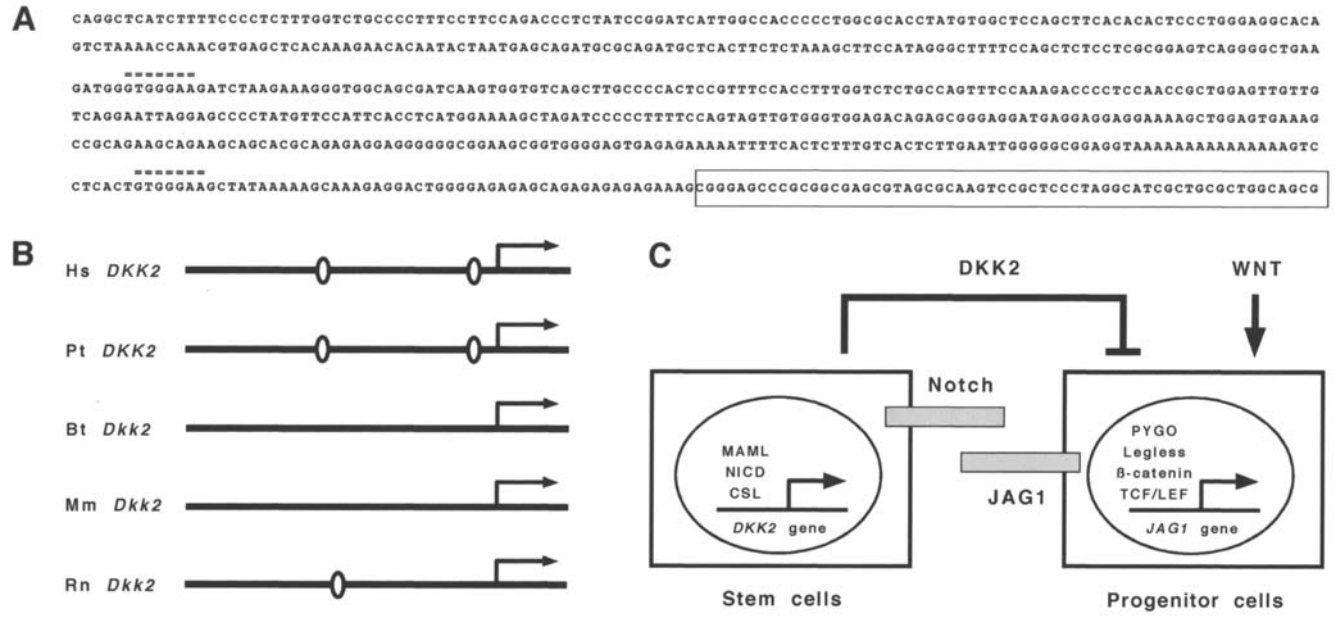

Figure 1. Promoters of mammalian *DKK2* orthologs. (A) Human *DKK2* promoter. Double Notch-response elements (NREs) are shown by double over-lines. Region corresponding to exon 1 of the human *DKK2* gene is boxed. (B) Schematic representation of promoters of mammalian *DKK2* orthologs. Hs, human; Pt, chimpanzee; Bt, cow; Mm, mouse; Rn, rat. NRE is shown by open oval. (C) Notch-DKK2 signaling loop. Canonical WNT signaling in progenitor cells activates *JAG1* expression for Notch signaling activation in stem cells. Notch signaling in stem cells then activates *DKK2* expression for canonical WNT signaling downregulation in progenitor cells. The Notch-DKK2 signaling loop functions as a negative feedback loop for the canonical WNT signaling pathway.

and BMP-IHH-SFRP1 signaling loops for negative regulation of the canonical WNT signaling pathway.

Materials and methods

Promoter regions of genes encoding secreted WNT signaling inhibitors. DKK1, DKK2, DKK3, DKK4, SFRP1, SFRP2, SFRP3, SFRP4, SFRP5 and *WIF1* genes encode secreted-type WNT signaling inhibitors. Promoter regions of human *DKK1, DKK2, DKK3, DKK4, SFRP1, SFRP2, SFRP3, SFRP4, SFRP5* and *WIF1* genes are located within the AC009986.10, AP001819.2, AC124276.5, AF170802.6, AC016868.8, AC020703.7, AC108514.3, AC018634.3, AL358938.8, and AC026124.35 genome sequences, respectively, as previously described (20).

Screening for Notch target genes. NRE within the 5'-flanking promoter region was searched for based on bioinformatics and human intelligence (Humint) as described previously for the search of canonical WNT target genes (21-23).

Identification of chimpanzee and cow DKK2 orthologs. Chimpanzee and cow genome sequences homologous to human *DKK2* were searched for with the BLAST programs as previously described (24-26). 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 previously described (27-29). Coding sequences of chimpanzee DKK2 and cow Dkk2 were determined by assembling exonic regions.

Comparative integromics analyses. Promoters and expression domains of mammalian DKK2 orthologs were compared as previously described (20,30).

Results

Screening of NRE within promoter regions of genes encoding secreted WNT signaling inhibitors. Among 5'-promoter regions of *DKK1, DKK2, DKK3, DKK4, SFRP1, SFRP2, SFRP3, SFRP4, SFRP5* and *WIF1* genes, double NREs were identified within the promoter region of the human *DKK2* gene (Fig. 1A). NREs within *DKK2* promoter were located approximately 400 and 50 bp upstream of the transcriptional start site. Human *DKK2* was characterized as the Notch target gene.

Comparative genomics on DKK2 orthologs. We previously reported the identification and characterization of the rat *Dkk2* gene *in silico* (29). Other mammalian *DKK2* othologs were further searched for in this study. The chimpanzee *DKK2* gene was identified within the NW_105990.1 genome sequence, while the cow *Dkk2* gene was identified within AC156664.2 and AC158038.2 genome sequences. Exon-intron boundaries of chimpanzee *DKK2* and cow *Dkk2* genes were determined based on the consensus sequence of exon-intron junctions. Both the chimpanzee *DKK2* gene (Fig. 2A) and cow *Dkk2* gene (Fig. 2B) were found to consist of four exons. The complete coding sequence (CDS) of chimpanzee DKK2 (Fig. 2C) and that of cow Dkk2 (Fig. 2D) were determined by assembling nucleotide sequences of four exons. The chimpanzee *DKK2* gene as well as cow *Dkk2* gene were found to encode 259-amino-acid protein. Chimpanzee DKK2 and cow Dkk2 showed 98.5% and 95.8% total-amino-acid identity with human DKK2, respectively.

NRE within the promoter region of mammalian *DKK2* orthologs was next searched for. Double NREs were identified within chimpanzee *DKK2* promoter, which were approximately 400 and 50 bp upstream of the putative transcriptional start site. Single NRE was identified within rat *Dkk2* promoter,

	Nucleotide sequence of chimpanzee *DKK2* gene around exon - intron boundaries	Nucleotide position of exon in genome sequence
1	CGGGAG------GGGCAG gtagga	11321317 - 11320391 of NW_105990.1
2	tgacag GCCTAC------ATAATG gtaaaa	11215721 - 11215571 of NW_105990.1
3	ctccag GCATCT------TAAAAG gtaggg	11214472 - 11214317 of NW_105990.1
4	ctgcag GGCATG------AAACTG	11213981 - 11211581 of NW_105990.1

B

Exon No.	Nucleotide sequence of cow *Dkk2* gene around exon - intron boundaries	Nucleotide position of exon in genome sequence
1	GGGCAA------GGGCAG gtagga	176464 - 177031 of AC156664.2
2	tgacag GCCTAC------ATAATG gtaaag	99617 - 99767 of AC158038.2
3	ctccag GCATCT------TAAAAG gtgggg	101072 - 101227 of AC158038.2
4	ctttag GACATG------GGTGGA	103780 - 104099 of AC158038.2

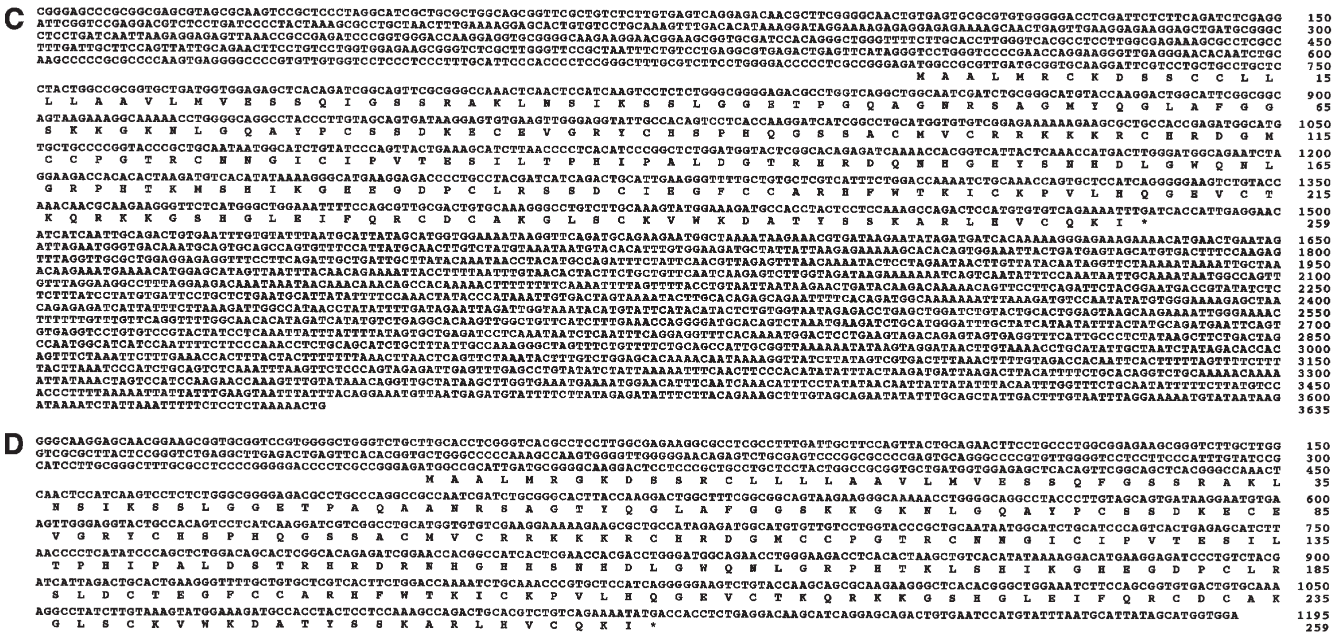

Figure 2. Chimpanzee *DKK2* and cow *Dkk2* genes. (A) Exon-intron structure of the chimpanzee *DKK2* gene. (B) Nucleotide and amino-acid sequences of chimpanzee DKK2 complete CDS. (C) Exon-intron structure of the cow *Dkk2* gene. (D) Nucleotide and amino-acid sequences of cow Dkk2 complete CDS.

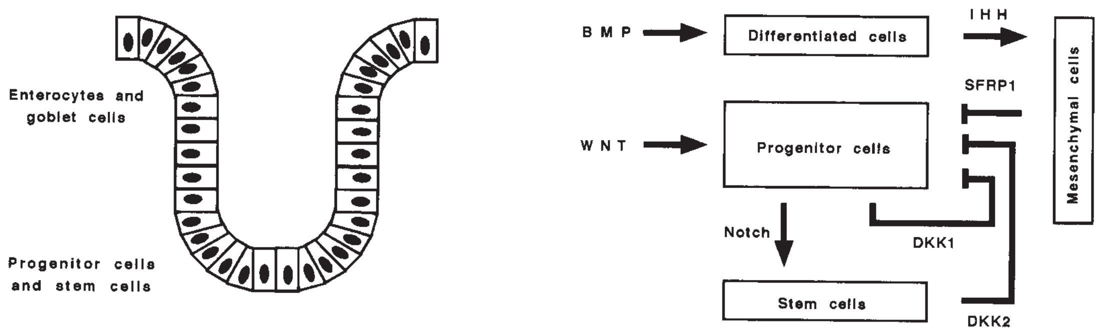

Figure 3. Multi-layer regulation system for the canonical WNT signaling pathway in human intestine. (Left) Location of stem, progenitor, and differentiated cells in the human intestine. (Right) Diagram of WNT signaling regulation system. Notch-DKK2, WNT-DKK1, and BMP-IHH-SFRP1 signaling loops constitute the multi-layer regulation system for the canonical WNT signaling pathway in human intestine.

which was approximately 400 bp upstream of the putative transcriptional start site. NRE was not identified within cow and mouse *Dkk2* promoters. Double NREs within human *DKK2* promoter were conserved in chimpanzee *DKK2* promoter, partially in rat *Dkk2* promoter, but not in cow and mouse *Dkk2* promoters (Fig. 1B). Primate *DKK2* orthologs were characterized as the target of Notch signaling pathway.

Integromics analyses on human DKK2 and mouse Dkk2. Notch signals are activated in the intestinal stem cell region, and double NREs were identified within human *DKK2* promoter, but not within mouse promoter, as mentioned above. Byun *et al* reported that human *DKK2* is expressed in colonic stem cell region (31). Gregorieff *et al* reported that mouse *Dkk2* is not expressed in normal colonic mucosa (32). Together, these facts indicate that human *DKK2*, but not mouse *Dkk2*, was the Notch target gene in intestinal stem cells (Fig. 1C).

Multi-layer system for canonical WNT signaling inhibition. In addition to WNT-DKK1 and BMP-IHH-SFRP1 signaling loops (19,20), Notch-DKK2 signaling loops were identified as the novel mechanism for canonical WNT signaling inhibition in primates (Fig. 1C). These facts indicate that the negative regulation system for the canonical WNT signaling pathway consists of multiple signaling loops, such as Notch-DKK2, WNT-DKK1, and BMP-IHH-SFRP1 (Fig. 3).

Discussion

NRE in the promoter region of human genes encoding secreted WNT signaling inhibitors was searched for in this study,

because the effects of Notch signaling activation in intestinal stem cells on WNT signaling in intestinal progenitor cells remain unclear. Among 10 human genes encoding secreted WNT signaling inhibitor, double NREs were identified within human *DKK2* promoter (Fig. 1A). On the other hand, NRE was not identified within mouse *Dkk2* promoter (Fig. 1B). Human *DKK2* is expressed in colonic stem cell region (31), while mouse *Dkk2* is not expressed in normal colonic mucosa (32). Although mouse *Dkk2* was not the target gene of Notch signaling pathway, human *DKK2* was characterized as the Notch target gene, especially in the intestinal stem cells (Fig. 1C).

Secreted WNT signaling inhibitors are implicated in the regulation of WNT signaling pathway, which plays key roles in a variety of processes, such as embryogenesis, maintenance of adult tissue homeostasis, tissue repair during chronic persistent inflammation, and carcinogenesis (4-6,33-35). Because aberrant WNT signaling activation leads to carcinogenesis, *SFRP1, DKK1* and *WIF1* genes, encoding secreted WNT signaling inhibitors, are claimed as tumor suppressor genes inactivated due to CpG hypermethylation. The *DKK2* gene is located at the human chromosome 4q25 region, which is deleted in colorectal cancer, breast cancer, lung cancer, and cervical cancer (36-39). Epigenetic changes occur at the early stage during multi-stage carcinogenesis associated with chronic persistent inflammation and/or aging, and then genetic alterations occur to augment the malignant potential of cancer cells (40,41). Therefore, *DKK2* is a candidate tumor suppressor gene inactivated due to epigenetic silencing and/or deletion.

We characterized rat *Dkk2* (29), chimpanzee *DKK2* (Fig. 2B), and cow *Dkk2* (Fig. 2D). Krupnik *et al* characterized human *DKK2* (42). Monaghan *et al* characterized mouse *Dkk2* (43). Human DKK2 showed 98.5%, 95.8%, 95.8 and 95.4% total-amino-acid identity with chimpanzee DKK2, cow Dkk2, rat Dkk2 and mouse Dkk2, respectively. Amino-acid sequences of DKK2 orthologs were well-conserved among mammals.

Double NREs in human *DKK2* promoter were conserved in chimpanzee *DKK2* promoter, partially in rat *Dkk2* promoter, but not in cow and mouse *Dkk2* promoters (Fig. 1B). Comparative genomics analyses on the promoter region of *DKK2* orthologs revealed that the Notch-DKK2 signaling loop was created or potentiated in primates. The Notch-DKK2 signaling loop identified in this study was complementary to WNT-DKK1 and BMP-IHH-SFRP1 signaling loops for negative regulation of the canonical WNT signaling pathway (Fig. 3). Together, these facts indicate that *DKK2* promoter evolution resulted in the augmentation of a WNT negative regulation system in primates.

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