SPANDIDOS PUBLICATIONS

CER1 is a common target of WNT and NODAL signaling pathways in human embryonic stem cells

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Abstract. Nodal and BMP signaling pathways network with WNT signaling pathway during embryogenesis and carcinogenesis. CER1 (Cerberus 1) and GREM3 (CKTSF1B3 or CER2) inhibit NODAL signaling through ACVR1B (ALK4) or ACVR1C (ALK7) to SMAD2 or SMAD3. GREM1 (CKTSF1B1) inhibits BMP signaling through BMPR1A (ALK3), BMPR1B (ALK6) or ACVR1 (ALK2) to SMAD1, SMAD5 or SMAD8. CER1, GREM1 and GREM3 are DAN domain (DAND) family members; however, transcriptional regulation of *DAND* family members by canonical WNT signaling pathway remains unclear. We searched for the TCF/LEF-binding site within the promoter region of *DAND* family genes, including *CER1, GREM1, GREM2, GREM3* and *NBL1*. Because triple TCF/LEF-binding sites were identified within human *CER1* promoter by using bioinformatics and human intelligence, comparative genomics analyses on *CER1* orthologs were further performed. Chimpanzee *CER1* gene, encoding 267-amino-acid protein, was identified within NW_111298.1 genome sequence. XM_528542.1 was not a correct coding sequence for chimpanzee CER1. Primate CER1 orthologs were significantly divergent from rodent Cer1 orthologs. Three TCF/LEF-binding sites within human *CER1* promoter were conserved in chimpanzee *CER1* promoter, two in cow and dog *Cer1* promoters, but not in rodent *Cer1* promoters. Binding sites for NODAL signaling effectors, SMAD3/SMAD4 and FOXH1, were also conserved among human, chimpanzee, cow and dog *CER1* promoters. *CER1* orthologs were evolutionarily conserved target of WNT and NODAL signaling pathways in non-rodent mammals. Human *CER1* mRNA was expressed in embryonic stem (ES) cells in the undifferentiated state and in the early endodermal lineage. CER1 upregulation in human ES cells leads to Nodal signaling inhibition associated with differentiation of human ES cells. Primate CER1 orthologs, playing a pivotal role during early embryogenesis, underwent protein evolution as well as promoter evolution. These facts indicate that molecular evolution of *CER1* orthologs contributes to the significantly divergent scenarios of early embryogenesis in primates and rodents.

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Introduction

TGFB1, TGFB2, TGFB3, NODAL, LEFTY1, LEFTY2, INHA, INHBA, INHBB, INHBC, INHBE, AMH, BMP2, BMP3, BMP4, BMP5, BMP6, BMP7, BMP8A, BMP8B, BMP10, BMP15, GDF1, GDF2, GDF3, GDF5, GDF6, GDF7, GDF8, GDF9, GDF10, GDF11, and *GDF15* are TGFß superfamily genes within the human genome (http://www.gene.ucl.ac.uk). TGFß signals are transduced through type I receptor TGFBR1 and type II receptor TGFBR2 to phosphorylate R-SMAD proteins, such as SMAD2 and SMAD3 (1-5). NODAL signals are transduced through type I receptor ACVR1B/ACVR1C and type II receptor ACVR2A/ACVR2B to phosphorylate SMAD2 or SMAD3 (6-8). BMP signals are transduced through type I receptor BMPR1A/BMPR1B/ACVR1 and type II receptor BMPR2 to phosphorylate R-SMAD proteins, such as SMAD1, SMAD5 and SMAD8 (9-11). R-SMADs, associated with SMAD4, are translocated to the nucleus to activate transcription of target genes.

CER1 (DAND4 or Cerberus 1), GREM1 (DAND2 or CKTSF1B1), GREM2 (DAND3 or CKTSF1B2), GREM3 (DAND5 or CKTSF1B3 or CER2) and NBL1 (DAND1) are secreted-type DAN domain (DAND) proteins (12-16). CER1 and GREM3 are Nodal antagonists, while GREM1 is a BMP antagonist.

TGFß superfamily signaling pathways network with WNT signaling pathway upregulating target genes based on the TCF/LEF transcriptional complex (17-28); however, WNT-dependent transcriptional regulation of *DAND* family members remains unclear. Here, we searched for TCF/LEF-binding site within the promoter region of *DAND* family genes. Because triple TCF/LEF-binding sites were identified within human *CER1* promoter, comparative genomics analyses on *CER1* orthologs were further performed.

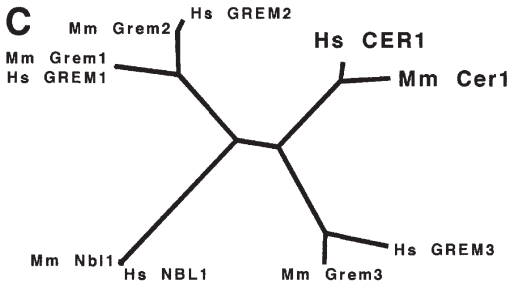
Materials and methods

WNT target gene screening. Genome sequences corresponding to human *CER1, GREM1, GREM2, GREM3* and *NBL1* genes

A

Human gene	Alias	Chromosomal localization	RefSeq ID	Genome clone	TCF/LEF-binding sites
CER1	*DAND4*	9p22.3	NM_005454.2	AL390732.10	Triple sites
GREM1	*DAND2*	15q13.3	NM_013372.5	AC090877.4	Double sites
GREM2	*DAND3*	1q43	NM_022469.3	AL358176.22	
GREM3	*DAND5*	19p13.13	NM_152654.2	AC092069.2	
NBL1	*DAND1*	1p36.13	NM_182744.1	AL031727.43	

C



B

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                                                                                    TCF/LEF
                                                                                    =======
TCAGTTTTCTGGACGTGTTTCTTTTTCGTATCCTAGGCTGATTCATGATAGTTTTGAATGATAGTTATTTTTCAATTTTTCCTTTGATCCCCGAAGACTCTTATTTGTGTCAATCTTTATTTAGC
CTGGCCATCAATTAGTGTAAAACAAGGGGAGATGATGTGGATTCAATAAGGTAAGAAACTGACTAGCCAGCGTCACACACAAAGTTATCAAACCTGGTTCTTAGAATCCACGTTCTCATGTCTGG
  TCF/LEF
  =======
CTCTTTGTTCAAAGGGAAAAAAAAAATCAATAGGATGGCTGGAGGGAGACATTTCCCTATCCTCATTGAAGACTGATGAGCTGTCAGTCTATTACGTGGTATCCTGTTGAGGAACATGTGAACAAT
TGGAAGAGTTTATCTGATGACTGACCAGTTGTGAAATTTAGCACCCATTAATTGATTCTTAATGTGAGAGGATTAACTTGGGAATTGTGAGTTTCTACGTGTGGAAAGGAAAACATATTCCCTTT
                                                                                                                    TCF/LEF
                                                                                                                    =======
ACTCAGTTACTGTGCAAAAATCAATAACCCCAATTTGCATGTAAGTAAGAAACAATATGTCCCTTGCTCAAAAAAATTAACATCCTTCTAGCTCTTCCTTTCTTCTCCTTACAACTTTGATATAC
TTCTTCGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGAATAGCCTTCCTAAAGGCCTGGGCTTTTTCTAAATGTATGCCTTTAATTTTTATCCAAAAACTTCTCTTACA
GCAACATGTCTTGTCTATTCTATTCCAGCACAAGGCAGATGCACAGCAAATGTGAGCTGACTCTAGTCCTTCTTCTGAAAACAGCCATGGGAAATTTAGGCAAAGAATGTGTTGTCTTTGCTAAT
                SMADs                                                                                                FOXH1
                ======                                                                                               ========
ACTGCTCTTTAAGCCCCAGACATAGCTAAACTCTTAGCTAATTACCCCCTGGGTCCCAGGCTTTCACTGGGGCCTTTTAAAATACACAAAACCAAAGTGACGGCAGGAGGCCATTAGCACTACAT
AATTCAAGCAAACAATAAATGTGTTTATTCTGCCTGGCTACTGACCACCTGCCTTCCCATCCCGCCAGGCAGGTATCTATATATACGATTTCCTTTTTCCCAGTCCTGCAGAGAATGAGCCTCTC
CTTTGGGCCTCATCATTTACAAAAGAAGCTTGGGCCCCTGACAGCATGCATCTCCTCTTATTTCAGCTGCTGGTACTCCTGCCTCTAGGAAAGACCACACGGCACCAGGATGGCCGCCAGAATCA
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Figure 1. (A), *DAND* gene family. *CER1, GREM1, GREM2, GREM3* and *NBL1* constitute the *DAND* gene family. (B), Human *CER1* promoter. The region corresponding to exon 1 is boxed. Three TCF/LEF-binding sites as well as SMAD- and FOXH1-binding sites are shown by over-lines. (C), Phylogenetic analyses on DAND family. Human CER1 and mouse Cer1 are significantly divergent.

were searched for with BLAST programs (http://www.ncbi.nlm.nih.gov) as described previously (29-33). TCF/LEF-binding sites within the 5'-flanking promoter region of the above genes were searched for based on bioinformatics and manual inspection as described previously (34-38).

Identification of chimpanzee CER1 ortholog. Chimpanzee genome sequences homologous to human *CER1* were searched for with BLAST programs as described previously (39-42). 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 (43-46). Coding sequence of chimpanzee CER1 was determined by assembling exonic regions.

Comparative proteomics analysis. Phylogenetic analyses on mammalian DAND family members were performed by using the CLUSTALW program.

Comparative genomics analyses. Promoter region of mammalian *CER1* orthologs were aligned by using the Genetyx program and manual curation. TCF/LEF-binding sites within the promoter region were determined as mentioned above.

In silico expression analysis. Expressed sequence tags (ESTs) derived from human *CER1* genes were searched for by using the BLAST programs. The sources of *CER1* ESTs were listed up for *in silico* expression analysis.

Results

Screening of the TCF/LEF-binding site within promoter region of DAND family genes. Human CER1 RefSeq (NM_005454.2), GREM1 RefSeq (NM_013372.5), GREM2 RefSeq (NM_022469.3), GREM3 RefSeq (NM_152654.2) and NBL1 RefSeq (NM_182744.1) were used as query sequences for the BLAST programs to identify genome clones corresponding to *DAND* family genes. The 5'-flanking promoter region of human *CER1, GREM1, GREM2, GREM3* and *NBL1* genes were identified within AL390732.10, AC090877.4, AL358176.22, AC092069.2 and AL031727.43 genome sequences, respectively (Fig. 1A). TCF/LEF-binding sites within the 5'-promoter region of human *CER1, GREM1, GREM2, GREM3* and *NBL1* genes were then searched for based on manual inspection as described previously (34-38). Triple TCF/LEF-binding sites were identified within human *CER1* promoter (Fig. 1B).

Identification of the chimpanzee CER1 gene. BLAST programs using human CER1 RefSeq revealed that chimpanzee *CER1* gene was located within NW_111298.1 genome sequence. Exon-intron boundaries of chimpanzee *CER1* gene were determined based on the consensus sequence of exon-intron junctions. Exon 1 corresponded to nucleotide position 289994-289443 of NW_111298.1 genome sequence, while exon 2 corresponded to nucleotide position 287557-286907. Chimpanzee *CER1* gene was found consisting of two exons.

LOC473172 predicted sequence (XM_528542.1), corresponding to 5'-flanking regions, intron 1 and exon 2 of chimpanzee *CER1* gene, was not the correct chimpanzee CER1 sequence. Complete coding sequence (CDS) of chimpanzee CER1 was determined by assembling nucleotide sequences of two exons in this study (Fig. 2A).

Genetyx program revealed that nucleotide position 46-849 was the coding region of chimpanzee CER1 complete CDS (Fig. 2A). Chimpanzee *CER1* gene was found to encode a 267-amino-acid protein.

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ATCATTTACAAAAGAAGCTTGGGCCCCTGACAGCATGCATCTCCTCTTCTTTCAGCTGCTGGTACTCCTGCCTCTAGGAAAGACCACACGGCACCAGGATGGCCGCCAGAATCAGAGTTCTCTTTCCCCCGTACTCCTG   150
                                                    M  H  L  L  F  F  Q  L  L  V  L  L  P  L  G  K  T  T  R  H  Q  D  G  R  Q  N  Q  S  S  L  S  P  V  L  L    35

CCAAGGAATCAAAGAGAGCTTCCCACAGGCAACCATGAGGAAGCTGAGGAGAAGCCAGATCTGTTTGTCGCAGTGCCACACCTTGTAGGCACCAGCCCTGCAGGGGAAGGCCAGAGGCAGAGAGAGAAGATGCTGTCCAGATTTGGCAGG   300
 P  R  N  Q  R  E  L  P  T  G  N  H  E  E  A  E  E  K  P  D  L  F  V  A  V  P  H  L  V  G  T  S  P  A  G  E  G  Q  R  Q  R  E  K  M  L  S  R  F  G  R    85

TTCTGGAAGAAGCCTGAGAGAGAAGTGCATCCATCCAGGGACTCAGATAGTGAGCCCTTCCCACCTGGGACCCAGTCCCTCATCCAGCCGATAGATGGAATGAAAATGGAGAAATCTCCTCTTCGGGAAGAAGCCAAGAAATTCTGGCAC   450
 F  W  K  K  P  E  R  E  V  H  P  S  R  D  S  D  S  E  P  F  P  P  G  T  Q  S  L  I  Q  P  I  D  G  M  K  M  E  K  S  P  L  R  E  E  A  K  K  F  W  H   135

CACTTCATGTTCAGAAAAAGTCCGGCTTCTCAGGGGGTCATCTTGCCCATCAAAAGCCATGAAGTACATTGGGAGACCTGCAGGACAGTGCCCTTCAGCCAGACGATAACCCACGAAGGCTGTGAAAAACTAGTTGTTCAGAACAACCTT   600
 H  F  M  F  R  K  S  P  A  S  Q  G  G  V  I  L  P  I  K  S  H  E  V  H  W  E  T  C  R  T  V  P  F  S  Q  T  I  T  H  E  G  C  E  K  L  V  V  Q  N  N  L   185

TGCTTTGGGAAATGCGGGTCTGTTCATTTTCCTGGAGCTGCGCAGCACTCCCATACCTTCTGCTCTCACTGTTTGCCTGCCAAGTTCACCACGATGCACTTGCCACTGAACTGCACTGAACTTTCCTCCGTGATCAAGGTGGTGATGCTG   750
 C  F  G  K  C  G  S  V  H  F  P  G  A  A  Q  H  S  H  T  F  C  S  H  C  L  P  A  K  F  T  T  M  H  L  P  L  N  C  T  E  L  S  S  V  I  K  V  V  M  L   235

GTGGAGGAGTGCCAGTGCAAGGTGAAGACGGAGCATGAAGATGGACACATCCTACATGCTGGCTCCCAGGATTCCTTTATCCCAGGAGTTTCAGCTTGAAGAGCTATCCCACTATTACCTTTGAAAAGCAAAACCACAACAGCAAAGATG   900
 V  E  E  C  Q  C  K  V  K  T  E  H  E  D  G  H  I  L  H  A  G  S  Q  D  S  F  I  P  G  V  S  A  *                                                          267

CTGATTATTCAGTCTGAAAATGTTAAGTGGGTACATAACATTTTCAGGGAAAGGTGACTTGAAAAGTAGTTTTAAATTAGAACGATAGAGGAAATGATATTAGTCTAGTTATTGGCACACGTTTGAGACCTTGTCTCAGCTCTGCCACTA  1050
ACTAGCCGTAGAATGTTAAGTTGTAAAACCTTTCTCCATCTAAAGATTTTCATCTATAAATGACGGACCCGACCTAGATGATTGCTAAAATCCTTTCCACTACTAATATTCCGTGATGCATTTTCTCCAAGTTTGGGTAAAAGCCCTCCA  1200
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B

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TCF/LEF
=======
ATTTTTCCTTTGATCCCCGAAGACTCTTATTTGTGTCAATCTTTATTTAGCCTGGCCATCAATTAGTGTAAAACAAGGGGAGATGATGTGGATTCAATAAGGTAAGAAACTGACTAGCCAGCGTCACACCCAAAGTTATCAAACCTGGTT

                TCF/LEF
                =======
CTTAGAATCCGCGTTCTCATGTCTGGCTCTTTGTTTAAAGGGAAAAAAAAATCAATAGGATGGCTGGAGGGAGACATTTCCCTATCCTCATTGAAGACTGATGAGCTATCAGTCTATTACGTGGTATCCTGTTGAGAAACGTGAACAATT
GGAAGAGTTTATCTGATGACTGACCAGTTGTGAAATTTAACACCCATTAATTGATTCTTAATGTGAGAGGATTAACTTGGGAATTGTGAGTTTCTATGTGTGGAAAGGAAAACATATTCCCTTTACTCAGTTACCATGCAAAAATCAATA

                                                                TCF/LEF
                                                                =======
ACCCCAGTTCGCATGTAAGTAAGCAACAATATGTCCCTTGCTCAAAAAAATTAACATCCTTCTAGCTCTTCCTTTCTTCTCCTTACAACTTTGATATACTTCTTCGTGTGTCTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTG
TGAATAGCCTTCCTAAAAGCCTGGGCTTTTTCTAAATGTATGCCTTTAATTTTTATCCAATATACTTCTCTTACAGCAACATGTCTTGTCTATTCTATTCCAGCACAAGGCAGATGCGCAGCAAATGTGAGCTGACTCTAGTCCTTCTTC

                                                SMADs                                                                                   FOXH1
                                                ======                                                                                  ========
TGAAAACAGCCATGGGAAATTTAGGCAAAGAATGTGTTGTCTTTGCTAATACTACTCTTTAAGCCCCAGACATAGCTAAACTCTTAGCTAATTACCCCCTGGGTCCCAGGCTTTCACTGGGGCCTTTTAAAATACACAAAACCAAAGTGA
CGGCAGGAGGCCATTAGCACTACATAATTCAAGCAAACAATAAATGTGTTTATTCTGCCTGGCTACTGACCACCTGCCTTCCCATCCCGCCAGGCAGGTATCTATATATACGATTTCCTTTTTCCCAGTCCTGCAGAGAATGAGCCTCTC
CTTTGGGCCTCATCATTTACAAAAGAAGCTTGGGCCCCTGACAGCATGCATCTCCTCTTCTTTCAGCTGCTGGTACTCCTGCCTCTAGGAAAGACCACACGGCACCAGGATGGCCGCCAGAATCAGAGTTCTCTTTCCCCCGTACTCCTG
```

Figure 2. (A), Nucleotide and amino-acid sequences of chimpanzee CER1. Nucleotides and amino-acid residues are numbered on the right. (B), Chimpanzee *CER1* promoter. The region corresponding to exon 1 is boxed. Three TCF/LEF-binding sites as well as SMAD- and FOXH1-binding sites are shown by over-lines.

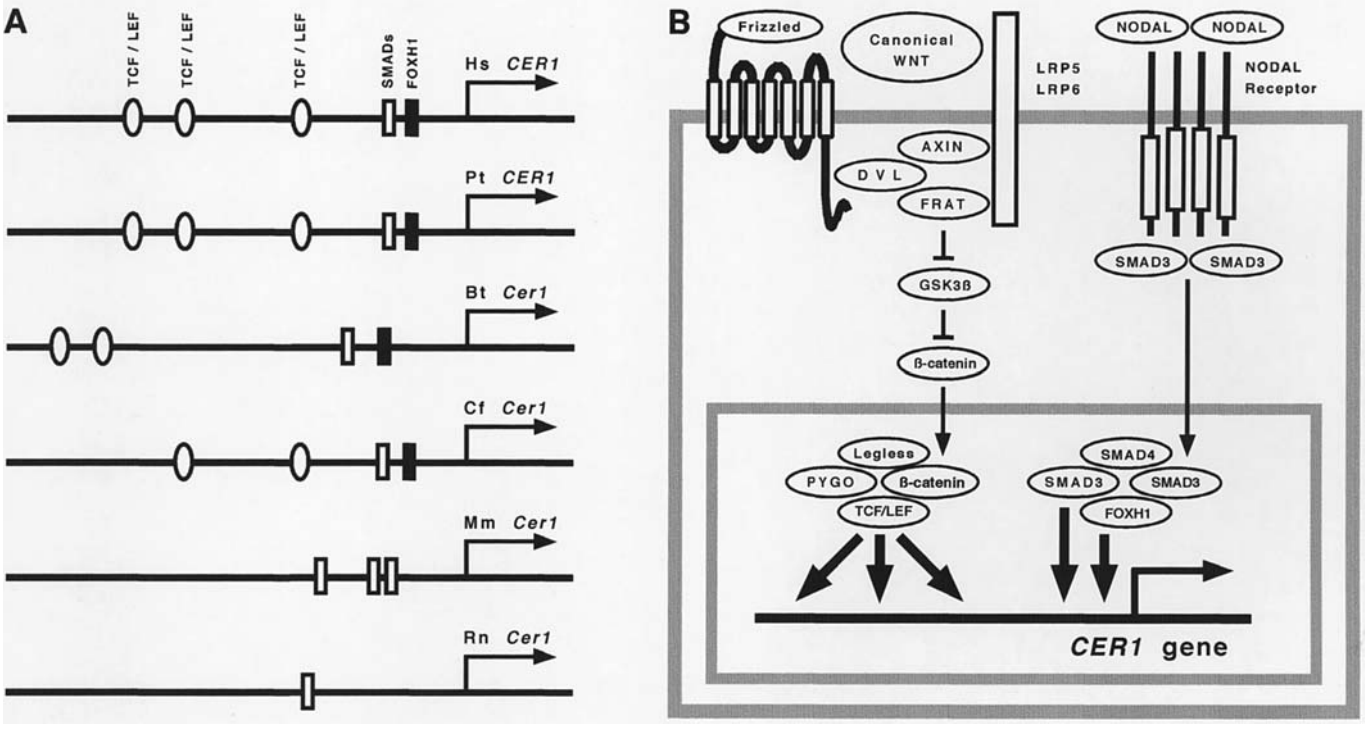

Figure 3. (A), Schematic representation of 5'-promoter region of mammalian *CER1* orthologs. Hs, human; Pt, chimpanzee; Bt, cow; Cf, dog; Mm, mouse; Rn, rat. TCF/LEF-binding site (oval), SMAD-binding site (open box) and FOXH1-binding site (closed box) are shown. Human, chimpanzee, cow and dog *CER1* orthologs are evolutionarily conserved target of WNT and NODAL signaling pathways. (B), Schematic representation of WNT and NODAL signaling pathways in human ES cells. WNT and NODAL signaling pathways are necessary for the maintenance of human ES cells, and CER1 is the common target of WNT and NODAL signaling pathways.

Comparative proteomics analysis on mammalian DAND family members. Phylogenetic analysis revealed that GREM1, GREM2 and NBL1 orthologs were more related to each other than the CER1 and GREM3 orthologs (Fig. 1C). Chimpanzee CER1 showed 97.8% and 68.5% total-amino-acid identity with human CER1 and mouse Cer1, respectively. These facts indicate that primate CER1 orthologs were significantly divergent from rodent Cer1 orthologs.

Expression of human CER1 mRNA. In silico expression analyses were performed to investigate expression of human *CER1*. Human *CER1* mRNA was expressed in embryonic stem (ES) cells in the undifferentiated state and in the early endodermal lineage.

Comparative genomics analyses on CER1 promoters. Human *CER1* promoter and chimpanzee *CER1* promoter were located

within AL390732.10 and NW_111298.1 genome sequences, respectively, as mentioned above. BLAST programs revealed that the cow *Cer1*, dog *Cer1*, mouse *Cer1* and rat *Cer1* promoters were located within AC173174.3, NW_876253.1, AL670958.4 and AC091341.6 genome sequences, respectively. Phylogenetic analysis on the 5'-promoter region of mammalian *CER1* orthologs revealed that human, chimpanzee, cow and dog *CER1* promoters were significantly divergent from mouse and rat *Cer1* promoters.

GC content of human *CER1* promoter was 42.8%, that of chimpanzee *CER1* promoter was 42.9%, that of cow *Cer1* promoter was 45.5%, that of dog *Cer1* promoter was 41.2%, that of mouse *Cer1* promoter was 47.0%, and that of rat *Cer1* promoter were 47.7%. GC content of human, chimpanzee, cow and dog *CER1* promoters were lower than those of mouse and rat *Cer1* promoters.

Three TCF/LEF-binding sites within human *CER1* promoter were conserved in chimpanzee *CER1* promoter, two in cow and dog *Cer1* promoters, but not in rodent *Cer1* promoters (Fig. 3A).

Because WNT and NODAL signaling pathways play a key role in the maintenance of human ES cells (47), we next investigated the binding sites for NODAL signaling effectors, SMAD3/SMAD4 and FOXH1. SMAD3/SMAD4-binding site was conserved among mammalian *CER1* promoters. On the other hand, FOXH1-binding site was conserved only among human, chimpanzee, cow and dog *CER1* promoters, but not in mouse and rat *Cer1* promoters (Fig. 3A).

These facts indicate that *CER1* orthologs were evolutionarily conserved target of WNT and NODAL signaling pathways in human, chimpanzee, cow and dog.

Discussion

TCF/LEF-binding site within the promoter region of *DAND* family genes, including *CER1, GREM1, GREM2, GREM3* and *NBL1,* were searched for by using bioinformatics and human intelligence in this study. Because triple TCF/LEF-binding sites were identified within human *CER1* promoter (Fig. 1B), comparative genomics analyses on *CER1* orthologs were further performed.

Chimpanzee *CER1* gene, consisting of two exons, was identified within NW_111298.1 genome sequence. Because XM_528542.1 was not a correct coding sequence for chimpanzee CER1, complete CDS of chimpanzee CER1 was determined by assembling exonic regions (Fig. 2A). Chimpanzee *CER1* gene was found to encode a 267-amino-acid protein showing 97.8% and 68.5% total-amino-acid identity with human CER1 and mouse Cer1, respectively. Phylogenetic analysis on human and mouse DAND family members next revealed that human CER1 and mouse Cer1 were significantly divergent (Fig. 1C). These facts clearly indicate that the CER1 protein evolution has occurred during mammalian evolution.

Three TCF/LEF-binding sites within human *CER1* promoter were conserved in chimpanzee *CER1* promoter, two sites in cow and dog *Cer1* promoters, but no site in rodent *Cer1* promoters (Fig. 3A). Binding sites for NODAL signaling effectors, SMAD3/SMAD4 and FOXH1, were also conserved among human, chimpanzee, cow and dog *CER1* promoters (Fig. 3A). Based on these facts, non-rodent mammalian *CER1* orthologs were identified as the evolutionarily conserved target of WNT and NODAL signaling pathways.

CER1 mRNA was expressed in human ES cells in the undifferentiated state and in the early endodermal lineage as mentioned in the Results. WNT and NODAL signaling pathways are indispensable for human ES cells (47), and CER1 is the common target of WNT and NODAL signaling pathways (Fig. 3B). Because CER1 upregulation in human ES cells leads to Nodal signaling inhibition associated with endodermal differentiation, CER1 is a key molecule for the maintenance of human ES cells. CER1 is the pharmacogenomics target in the field of regenerative medicine.

Primate CER1 orthologs, playing a pivotal role during early embryogenesis, underwent protein evolution as well as promoter evolution. These facts indicate that molecular evolution of *CER1* orthologs contributes to the significantly divergent scenarios of early embryogenesis in primates and rodents.

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