Integrative genomic analyses on HES/HEY family: Notch-independent *HES1, HES3* transcription in undifferentiated ES cells, and Notch-dependent *HES1, HES5, HEY1, HEY2, HEYL* transcription in fetal tissues, adult tissues, or cancer

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Abstract. Notch signaling pathway maintains stem cells through transcriptional activation of HES/HEY family members to repress tissue-specific transcription factors. Here, comparative integromic analyses on HES/HEY family members were carried out. *HES3* gene encodes two isoforms due to alternative promoters. Complete coding sequence of HES3 variant 2 was determined by curating CX755241.1 EST. Refined phylogenetic analysis using HES3 variant 2 instead of variant 1 revealed that mammalian bHLH transcription factors with Orange domain were grouped into HES subfamily (HES1, HES2, HES3, HES4, HES5, HES6, HES7) and HEY subfamily (HEY1, HEY2, HEYL, HESL/HELT, DEC1/BHLHB2, DEC2/BHLHB3). Eight amino-acid residues were added to the C-terminal WRPW motif in human HES3 due to lineage specific T to G nucleotide change at stop codon of chimpanzee, rat, and mouse *HES3* orthologs. *HES1* and *HES3* were expressed in undifferentiated embryonic stem (ES) cells. *HES1* was also expressed in fetal tissues, and regenerating liver. *HES1, HEY1* and *HEY2* were expressed in endothelial cells. *HES1, HES4* and *HES6* were expressed in gastric cancer, *HES1* and *DEC1* in pancreatic cancer, *HES1, HES2, HES4, HES6* and *DEC2* in colorectal cancer. *HES6* was also expressed in other tumors, such as brain tumors, melanoma, small cell lung cancer, retinoblastoma, ovarian cancer, and breast cancer. Double NANOG-binding sites, CSL/RBPSUH-binding site and TATA-box in *HES1* promoter, NANOG-, SOX2-, POU5F1/OCT3/OCT4-binding sites and TATA-box in *HES3* promoter, double CSL-binding sites in *HES5* promoter, SOX2-, POU-binding sites and TATA-box in *HES6* promoter, and CSL-binding site in *HEY1, HEY2* and *HEYL* promoters were evolutionarily conserved. However, double CSL-binding sites in mouse *Hes7* promoter were not conserved in human *HES7* promoter. Together these facts indicate that *HES1* and *HES3* were target genes of the ES cell-specific network of transcription factors, and that *HES1, HES5, HEY1, HEY2* and *HEYL* were target genes of Notch signaling pathway.

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Introduction

Notch signaling pathway is implicated in self-renewal of stem cells and cell-fate determination of progenitors (1-4). Notch signaling pathway constitutes the stem cell signaling network together with WNT signaling pathway (5-9), FGF signaling pathway (10-14), BMP signaling pathway (15-17), and Hedgehog signaling pathway (18-24). Stem cell signaling network is implicated in embryogenesis and maintenance of adult tissue homeostasis. Dysregulation of the stem cell signaling network leads to pathological conditions, such as congenital disorders, metabolic syndrome, and cancer (25-27).

JAG1, JAG2, DLL1, DLL3 and DLL4 are typical transmembrane-type Notch ligands sharing the common domain architecture with extracellular DSL domain and EGF-like repeats (28-31), while NOTCH1, NOTCH2, NOTCH3 and NOTCH4 are Notch family receptors sharing the common domain architecture with extracellular EGF-like repeats, Lin12/Notch repeats, cytolasmic RAM23 domain, Ankyrin repeats, and PEST domain (32,33). Lignand-binding induces the γ-secretase-mediated processing of Notch family receptors to release Notch intracellular domain (NICD) for its interaction with CSL (RBPSUH) transcription factor (34). MAML1, MAML2 and MAML3 are Mastermind family coactivators (35) associating with the CSL-NICD complex to activate the transcription of Notch target genes (1-4).

Notch target genes *HES1* and *HES5* encode HES/HEY family of transcription factors with basic helix-loop-helix (bHLH) domain and Orange domain (36-39). bHLH domain is implicated in the DNA-binding and dimerization, and Orange domain in the selection of bHLH heterodimer partner (39). Because HES1 and HES5 repress the transcription of tissue-specific transcription factors, canonical Notch signaling activation results in the maintenance of stem or progenitor cells through the inhibition of differentiation (1-4).

Functional and phenotypical analyses on HES/HEY family members in mouse and zebrafish experimental system have been reported (reviewed in ref. 39); however, biological function of human HES/HEY family members remained relatively unclear. Here, integrative genomic analyses on HES/HEY family members were carried out to elucidate the expression profile and transcriptional mechanisms of human HES/HEY family members.

Materials and methods

Comparative genomics analyses. Genome sequences of human and mouse HES/HEY family members were searched for using the BLAST programs as previously described (40-42). 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. Conserved transcription factor-binding sites within promoter regions were then searched for based on the Match program, Genetyx program, and manual curation as previously described (43-45).

Comparative proteomics analyses. The CLUSTALW program was used for phylogenetic analysis on human and mouse bHLH transcription factors with Orange domain. Amino-acid sequences of human HES1 (NP_005515.1), HES2 (NP_061962.2), HES3 (this study), HES4 (NP_066993.1), HES5 (NP_001010926.1), HES6 (NP_061115.2), HES7 (NP_115969.1), HEY1 (NP_036390.3), HEY2 (NP_036391.1), HEYL (NP_055386.1), HESL/HELT (46), DEC1/BHLHB2 (NP_003661.1), DEC2/BHLHB3 (NP_110389.1), mouse Hes1 (NP_032261.1), Hes2 (NP_032262.2), Hes3 (47), Hes5 (NP_034549.1), Hes6 (NP_062352.1), Hes7 (NP_149030.2), Hey1 (NP_034553.2), Hey2 (NP_038932.1), Heyl (NP_038933.2), Hesl (NP_038933.2), Dec1 (NP_035628.1), and Dec2 (NP_077789.1) were used for the phylogenetic analysis.

In silico expression analyses. Expressed sequence tags (ESTs) derived from human HES/HEY family members were searched for using the BLAST programs as previously described (48-50). Human HES1 RefSeq (NM_005524.2), HES2 RefSeq (NM_019089.3), HES3 EST (CX755241.1), HES4 RefSeq (NM_021170.2), HES5 RefSeq (NM_001010926.1), HES6 RefSeq (NM_018645.3), HES7 RefSeq (NM_032580.1), HEY1 RefSeq (NM_012258.3), HEY2 RefSeq (NM_012259.2), HEYL RefSeq (NM_014571.3), HESL cDNA (46), DEC1 RefSeq (NM_003670.1), and DEC2 RefSeq (NM_030762.1) were used as query sequences for the BLAST programs. Sources of human ESTs were then listed up for *in silico* expression analyses.

Results

Complete coding sequence of human HES3. We have previously reported the coding sequence of human HES3 (37); however, 5'-UTR and transcription start site of *HES3* remained unclear. Human HES3 RefSeq NM_001024598.1 was an artificial prediction with aberrant 5'-splicing site for exon 2 and aberrant splicing out within exon 4. We found that CX755241.1, CN370500.1, and CN413592.1 ESTs were derived from the human *HES3* gene. The first exon of human *HES3* transcript for CX755241.1, CN370500.1 and CN413592.1 ESTs was distinct from that of human HES3 RefSeq NM_001024598.1. Hirata *et al* reported that mouse *Hes3* gene encodes two splicing variants due to alternative promoters (47). Human HES3 RefSeq NM_001024598.1 corresponded to mouse Hes3 splicing variant 1 with exon 1a, while human HES3 ESTs CX755241.1, CN370500.1 and CN413592.1 corresponded to mouse Hes3 splicing variant 2 with exon 1b.

Three nucleotide substitutions were identified in CX755241.1 EST, compared with other human HES3 ESTs and human genome sequence. In addition, the transcription start site of the human *HES3* gene was predicted to be located at least 9-bp upstream position of CX755241.1 5'-end based on the comparison of human *HES3* and mouse *Hes3* genes. Complete coding sequence of human HES3 splicing variant 2 was determined by curating the points mentioned above (Fig. 1A). Nucleotide position 7-633 was the coding region of human HES3 splicing variant 2. Human HES3 variant 2 was a 208-amino-acid protein, which was longer than human HES3 variant 1 in the N-terminal region by 22 amino acids (Fig. 1A).

Comparative proteomics on mammalian HES/HEY family members. Orthologs of human *HES/HEY* family genes, except *HES4*, were identified within the mouse genome. Human HES/HEY family consists of 13 members, while mouse Hes/Hey family consists of 12 members. We previously reported phylogenetic analysis on HES/HEY family members using human HES3 variant 1 and mouse Hes3 variant 1 (46). Here, refined phylogenetic analysis on HES/HEY family members was carried out using human HES3 variant 2 and mouse Hes3 variant 2 (Fig. 2A). HES1, HES2, HES3, HES4, HES5, HES6 and HES7 were classified as the HES subfamily, while HEY1, HEY2, HEYL, HESL, DEC and DEC2 were classified as the HEY subfamily. HES subfamily members share the common domain architecture of bHLH domain, Orange domain and WRPW motif. HEY subfamily members share the common domain architecture of bHLH domain and Orange domain; however, the WRPW motif was absent in HEY subfamily members. HES1, HEY1 and HEYL orthologs were well conserved between human and mouse, while HES2 and HES3 orthologs were divergent (Fig. 2A).

Alignment of human HES3 and mouse Hes3 revealed that eight amino-acid residues were added to the C-terminal WRPW motif in human HES3 (Fig. 1B). Coding region of human *HES3* transcript was elongated in the 3'-position by 24 bp compared to that of mouse *Hes3* transcript due to the T to G nucleotide substitution at the position corresponding to the stop codon of mouse *Hes3* transcript. The T to G

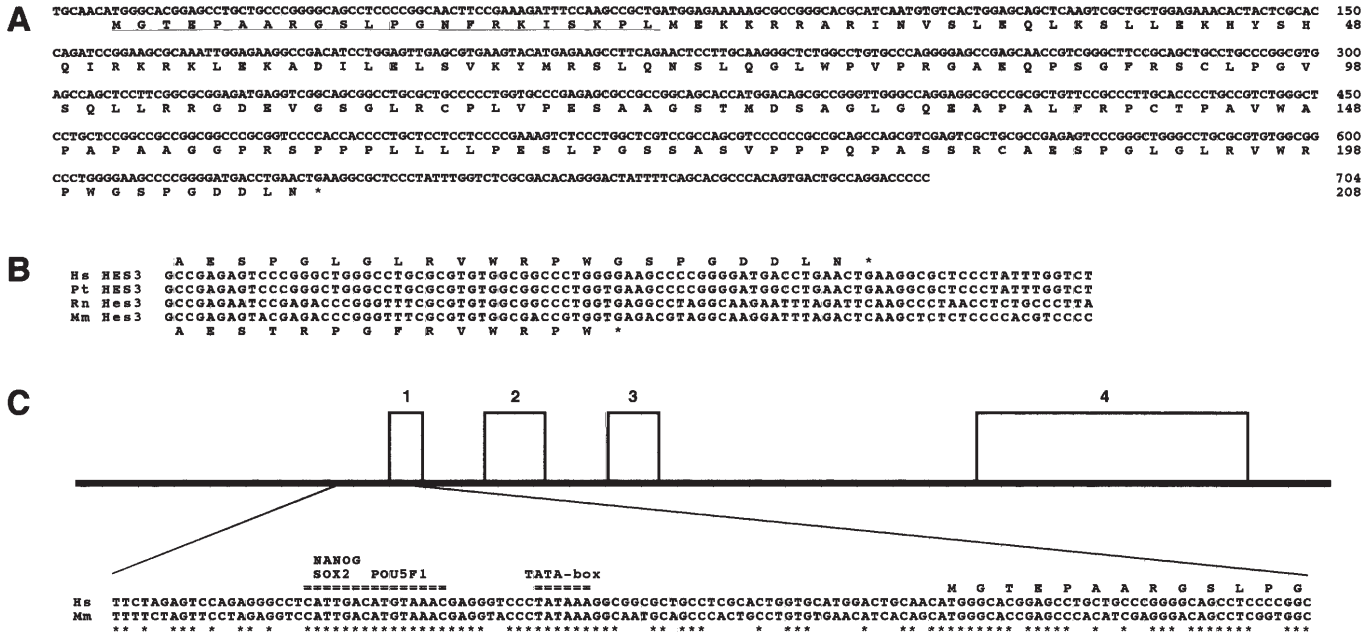
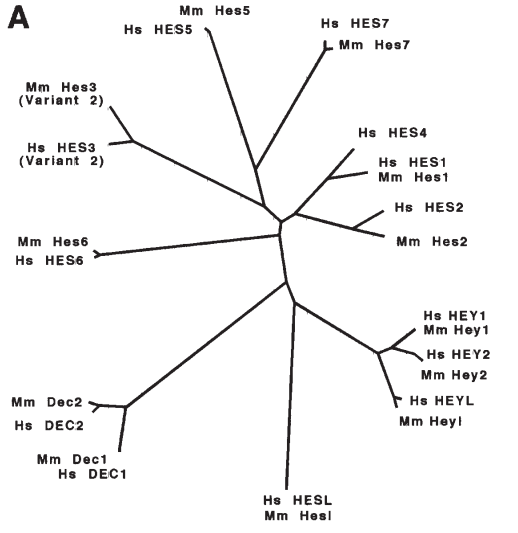


Figure 1. Comparative integromics on *HES3* orthologs. (A) Nucleotide and amino-acid sequences of human HES3 variant 2. N-terminal region of human HES3 variant 2 (underline) is missing in human HES3 variant 1. (B) Alignment of nucleotide sequence around the stop codon of mammalian *HES3* orthologs. Hs, human; Pt, chimpanzee; Rn, rat; Mm, mouse. T to G nucleotide change at the stop codon of other mammalian *HES3* orthologs results in human-specific elongation of *HES3* coding region. (C) Alignment of promoter regions for human HES3 variant 2 and mouse Hes3 variant 2. NANOG-, SOX2-, and POU5F1-binding sites, and TATA-box in human *HES3* promoter are conserved in the mouse *Hes3* promoter.



B

	Human ES cells and their derivatives				Gastric cancer	Pancreatic cancer	Colorectal cancer
	Undifferentiated ES cells	Embryoid body	Neural progenitors	Hepatocyte progenitors			
HES1	11	0	3	2	11	3	4
HES2	0	0	1	0	0	0	4
HES3	5	0	0	0	0	0	0
HES4	0	0	0	0	4	2	3
HES5	0	0	0	0	0	0	1
HES6	0	2	1	0	6	1	10
HES7	0	0	0	0	0	0	0
HEY1	0	0	5	2	0	0	2
HEY2	0	0	0	0	0	0	1
HEYL	0	0	0	0	1	2	0
HESL	0	0	0	0	0	0	0
DEC1	2	5	3	0	1	5	0
DEC2	0	0	0	0	0	1	6

Figure 2. Comparative proteomics and transcriptomics on HES/HEY family members. (A) Phylogenetic tree of human and mouse HES/HEY family proteins. (B) Expression profile of human *HES/HEY* family transcripts.

nucleotide substitution in the human *HES3* gene was not detected in the chimpanzee, mouse, and rat *HES3* orthologs (Fig. 1B). These facts indicate that the 8-amino-acid elongation at the C-terminus of human HES3 was caused by a nucleotide substitution specific to the human lineage.

Expression profile of human HES/HEY family members. *In silico* expression analyses were carried out to investigate the expression profile of human *HES/HEY* family transcripts (Fig. 2B). *HES1* mRNA was expressed in undifferentiated ES cells, fetal tissues, retina, endothelial cells, pancreatic islet, regenerating liver, and gastroenterological tumors, such as gastric, pancreatic, and colorectal cancer. *HES2* mRNA was expressed in placenta, trachea, tongue tumor, and colorectal cancer. *HES3* mRNA was preferentially expressed in undifferentiated ES cells. *HES4* mRNA was expressed in fetal brain, hypothalamus, and gastroenterological tumors. *HES5* mRNA was expressed in hypothalamus, and brain tumors. *HES6* mRNA was expressed in a variety of tumors, such as brain tumors, melanoma, small cell lung cancer, retinoblastoma, ovarian, gastric, colorectal, and breast cancer. *HES7* mRNA was expressed in cervical cancer. *HEY1* mRNA was expressed in a variety of neural tissues, such as hippocampus, amygdala, substantia nigra, pineal gland, and

A

	Representative cDNA	Genome sequence	Exon No	Transcription factor-binding sites and TATA-box within 5'-promoter
HES1	NM_005524.2	AC080129.26	4	NANOG – NANOG – CSL – TATA
HES2	NM_019089.3	AL031848.11	4	
HES3	CX755241.1	AL031847.17	4	NANOG/SOX2 – POU5F1 – TATA
HES4	NM_021170.2	AL645608.30	4	
HES5	NM_001010926.1	AL139246.21	3	CSL – CSL
HES6	NM_018645.3	AC016757.10	4	SOX2 – POU5F1 – TATA
HES7	NM_032580.1	AC129492.6	4	
HEY1	NM_012258.3	AC016240.23	5	CSL
HEY2	NM_012259.2	AL078594.36	5	CSL
HEYL	NM_014571.3	AL035404.20	5	CSL
HESL	Reference 46	AC093824.3	4	
DEC1	NM_003670.1	AC090955.3	5	
DEC2	NM_030762.1	AC022509.21	5	

B

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                    NANOG
                    =======
Hs HES1  TCAGGCGCGCGCCATTGGCCGCCAGACCTTGTGCCTGGCGGCCAATGGGGGGGCGCGGTCCACGAGCGGTGCCGCGTGTC
         ||||||||||||||||||||||||||||||||||| |||||||||||||||||||| |||||||||||||||||||||||
Mm Hes1  TCAGGCGCGCGCCATTGGCCGCCAGACCTTGTGCCTAGCGGCCAATGGGGGGGCGCAGTCCACGAGCGGTGCCGCGTGTC

                NANOG                CSL
                =======              =======
Hs HES1  TCCTCCTCCCATTGGCTGAAAGTTACTGTGGGAAAGAAAGTTTGGGAAGTTTCACACGAGCCGTTCGCGTGCAGTCCCAG
         || |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Mm Hes1  TCTTCCTCCCATTGGCTGAAAGTTACTGTGGGAAAGAAAGTTTGGGAAGTTTCACACGAGCCGTTCGCGTGCAGTCCCAG

          TATA-box
          =======
Hs HES1  ATATATATAGAGGCCGCCAGGGCCTAGGGATCACACAGGATCCGGAGCTGGTGCTGATAACAGCGGAATCCCCCGTCTAC
         |||||||||||||||||||||||||  ||||||||||||||| |||||||||||||||||||||||||||||| ||||||
Mm Hes1  ATATATATAGAGGCCGCCAGGGCCTGCGGATCACACAGGATCTGGAGCTGGTGCTGATAACAGCGGAATCCCCTGTCTAC
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C

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           CSL
           =======
Hs HES5  GCGTTCCCACAGCCCGGACATTGTGCCGCGCGGCCCACCTGCTCCTTGGGGAGCGCCCATTGTGCCCGCGCCAATTCACA
         |||||||||||||||||||||||||||||||||||||||||||||||||||||||| |||||||| ||||||||||||||||||||||
Mm Hes5  GCGTTCCCACAGCCCGGACATTGTGCCGCGCGGCCCACCTGCTCCTCGGGGAGCGACCATTGTGCCCGCGCCAATTCACA

                                                                          CSL
                                                                          =======
Hs HES5  GGCAATTTAGCGTGCGCTAATGGGCCGGCGCCTTTGTGCGGCCCGCCCGGCCATTGGCCGCGGAGTGTGGGAACGGCCGC
         |||||||||||||||||||||||||||||||||||||||||||||||||| || | |||||||||||| ||||||||||||||||||
Mm Hes5  GGCAATTTAGCGTGCGCTAATGGGCCGGCGCCTTTGTGCGGCCGGCGCCGCCATTGGCCGCCGAGTGTGGGAACGGCCGC

Hs HES5  GGCGCCCGGACTCCAGGCGCCAGGCCGCCGCCCGCGCCTATATAGGGCGTCGGCGCGCGGGGCCGGTGTCCGCGCCAGCC
         |||||||||||| ||||||||| |||||| |||||||||||||||||||     | ||  |||| |    ||  ||||||||||
Mm Hes5  GGCGCCCGGACCCCAGGCGCCGGGCCGCTGCCCGCGCCTATATAGGGCTGGCGTGC-TGGGGTCCAGGT-CGCGCCAGTC
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D

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                    SOX2    POU5F1          TATA-box
                    ======  ========        =======
Hs HES6  CAATCGGCGCATTGAGATGCAAATAAGCGGCTATAAAAGGGGCGGGACCGCGGCGGGCCGGAAGCCGCGAGGAGCGCGGA
         |||||||  |||||||  |||  ||||||||  |||||||||  |||||||   | |  ||||||||||||  | |  |||| ||
Mm Hes6  CAATCGGTGCATTGACATGTAAATAAGTTGCTATAAAATGGGCGGGGTC-CCGCGGGCCGGAAGACTCC-GGAGAGCTAG
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Figure 3. Comparative genomics on 5'-promoter region of mammalian *HES/HEY* family genes. (A) List of conserved transcription factor-binding sites in the promoter region of *HES/HEY* family genes. (B) Alignment of human *HES1* and mouse *Hes1* promoters. Double NANOG-binding sites, CSL-binding site, and TATA-box in human *HES1* promoter are conserved in mouse *Hes1* promoter. (C) Alignment of human *HES5* and mouse *Hes5* promoters. Double CSL-binding sites in human *HES5* promoter are conserved in mouse *Hes5* promoter. (D) Alignment of human *HES6* and mouse *Hes6* promoters. SOX2-, POU5F1-binding sites, and TATA-box in human *HES6* promoter are conserved in the mouse *Hes6* promoter.

also in endothelial cells, and brain tumors. *HEY2* mRNA was expressed in neural tissues, and endothelial cells. *HEYL* mRNA was expressed in neural tissues, pancreatic islet, and rhabdomyosarcoma. *HESL* mRNA was expressed in ovarian fibrotheoma. *DEC1* mRNA was expressed in ES cell-derived embryoid body, neural tissues, pancreatic islet, pancreatic cancer, melanoma, cervical cancer, head and neck cancer. *DEC2* mRNA was expressed in neural tissues, germinal center B cells, multiple sclerosis, brain tumors, parathyroid tumors, and colorectal cancer.

Comparative genomics on mammalian HES/HEY family members. The human *HES1, HES2, HES3, HES4, HES5, HES6, HES7, HEY1, HEY2, HEYL, HESL, DEC1* and *DEC2* genes were located within AC080129.26, AL031848.11, AL031847.17, AL645608.30, AL139246.21, AC016757.10, AC129492.6, AC016240.23, AL078594.36, AL035404.20, AC093824.3, AC090955.3, AC022509.21 genome sequences, respectively (Fig. 3A). Mouse *Hes1, Hes2, Hes3, Hes5, Hes6, Hes7, Hey1, Hey2, Heyl, Hesl, Dec1* and *Dec2* genes were located within CT030736.8, AL772240.7, AL611985.22, BX004788.7, AC110510.6, AL645527.20, AC132225.3, AC125532.3, AL606934.12, AC156551.5, AC153593.4, and AC144936.4 genome sequences, respectively.

Transcription factor-binding sites conserved between human and mouse *HES/HEY* orthologs were then searched for (Fig. 3A). Double NANOG-binding sites, CSL-binding site, and TATA-box in human *HES1* promoter were conserved in mouse *Hes1* promoter (Fig. 3B). NANOG/SOX2-binding site, POU5F1 (OCT3/4)-binding site, and TATA-box in human *HES3* promoter were conserved in mouse *Hes3* promoter (Fig. 1C). Double CSL-binding sites in human *HES5* promoter were conserved in mouse *Hes5* promoter (Fig. 3C). SOX2-binding site, POU5F1-binding site and TATA-box in human *HES6* promoter were conserved in mouse *Hes6* promoter (Fig. 3D). Although double CSL-binding sites in mouse *Hes7* promoter was not conserved in human *HES7* promoter, CSL-binding site in human *HEY1, HEY2,* and *HEYL* promoters was evolutionarily conserved (Fig. 3A).

Discussion

Complete coding sequence of HES3 variant 2 was determined by curating CX755241.1 EST (Fig. 1A). Human HES3 variant 2 was longer than human HES3 variant 1 in the N-terminal region by 22 amino acids (Fig. 1A). Mouse Hes3 variant 2 with the basic region binds to genomic DNA, but mouse Hes3 variant 1 without the basic region does not bind to genomic DNA (47). *HES3* orthologs encode two isoforms with N-terminal divergence due to alternative promoters.

Refined phylogenetic analysis using HES3 variant 2 instead of variant 1 revealed that mammalian bHLH transcription factors with Orange domain were grouped into HES subfamily (HES1, HES2, HES3, HES4, HES5, HES6, HES7) and HEY subfamily (HEY1, HEY2, HEYL, HESL/HELT, DEC1/BHLHB2, DEC2/BHLHB3) (Fig. 2A).

Phylogenetic analysis also indicated that HES3 orthologs were relatively divergent among the HES/HEY family (Fig. 2A). Comparative genomics revealed that eight amino-acid residues were added to the C-terminal WRPW motif in human HES3 due to lineage specific T to G nucleotide change at the position corresponding to the stop codon of chimpanzee, rat, and mouse *HES3* orthologs (Fig. 1B). These facts indicated that protein evolution occurred in human HES3.

HES3 expression was restricted to undifferentiated ES cells, while *HES1* expression was detected in undifferentiated ES cells as well as in fetal tissues, adult tissues, and gastrointestinal tumors (Fig. 2B). NANOG-, SOX2-, POU5F1-binding sites, and TATA-box in human *HES3* promoter were conserved in the mouse *Hes3* promoter (Fig. 1C). Double NANOG-binding sites, CSL-binding site, and TATA-box in human *HES1* promoter were conserved in the mouse *Hes1* promoter (Fig. 3B). *HES1* and *HES3* were transcribed in undifferentiated human ES cells due to ES cell-specific network of transcription factors, while *HES1* was also transcribed in fetal and adult tissues due to Notch signaling activation.

HES1, HES4, HES5, HES6, HEY1, HEY2, HEYL, DEC1, and *DEC2* were expressed in neural tissues. *HES4, HES5,* and *HES6* were preferentially expressed in hypothalamus, while *HEY1* and *DEC2* in hippocampus. Regional preferentiality of *HES/HEY* family members within human brain was clarified using *in silico* expression analyses in this study.

CSL-binding sites within *HES1, HES5, HEY1, HEY2,* and *HEYL* promoters were evolutionarily conserved; however, those within *HES7* promoter were not conserved (Fig. 3A). Because canonical Notch signaling induces the transcriptional activation of target genes through the MAML-NICD-CSL transcriptional complex (1-4), expression of *HES1, HEY1* and *HEY2* in endothelial cells indicate the implication of the Notch signaling pathway in angiogenesis.

HES1 transcription is oscillated in many cell types, such as fibroblasts, myoblasts, neuroblasts, and mesenchymal stem cells (51,52). Human *HES1* is oscillated with a period of 5 h, while mouse *Hes1* is oscillated with a period of 2 h (52). After *HES1* transcription and translation, HES1 protein represses the transcription of *HES1* mRNA in cell autonomous manner based on the negative autoregulation (39).

HES1, HES4 and *HES6* were expressed in gastric cancer; *HES1* and *DEC1* in pancreatic cancer; *HES1, HES2, HES4, HES6* and *DEC2* in colorectal cancer (Fig. 2B). *HES6* was also expressed in other tumors, such as brain tumors, melanoma, small cell lung cancer, retinoblastoma, ovarian cancer, and breast cancer (Fig. 2B). *HES1* expression in gastroenterological tumors is due to Notch signaling activation, and *HES6* expression in tumors is partly due to SOX-POU transctiptional complex (Fig. 3). Because *HES6* was expressed in a variety of tumors, further investigation on HES6 could lead to better understanding of carcinogenesis, and development of novel diagnostics or therapeutics for human cancer in various tissues.

Integrative genomic analyses on the HES/HEY family revealed that *HES1* and *HES3* were target genes of the ES cell-specific network of transcription factors, and that *HES1, HES5, HEY1, HEY2* and *HEYL* were target genes of the Notch signaling pathway.

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