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ORIGINAL ARTICLE

Transcriptional Profiles of Imprinted Genes in Human Embryonic Stem Cells During *In vitro* Differentiation

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Background and Objectives: Genomic imprinting is an inheritance phenomenon by which a subset of genes are expressed from one allele of two homologous chromosomes in a parent of origin-specific manner. Even though fine-tuned regulation of genomic imprinting process is essential for normal development, no other means are available to study genomic imprinting in human during embryonic development. In relation with this bottleneck, differentiation of human embryonic stem cells (hESCs) into specialized lineages may be considered as an alternative to mimic human development.

Methods and Results: In this study, hESCs were differentiated into three lineage cell types to analyze temporal and spatial expression of imprinted genes. Of 19 imprinted genes examined, 15 imprinted genes showed similar transcriptional level among two hESC lines and two human induced pluripotent stem cell (hiPSC) lines. Expressional patterns of most imprinted genes were varied in progenitors and fully differentiated cells which were derived from hESCs. Also, no consistence was observed in the expression pattern of imprinted genes within an imprinting domain during *in vitro* differentiation of hESCs into three lineage cell types.

Conclusions: Transcriptional expression of imprinted genes is regulated in a cell type- specific manner in hESCs during *in vitro* differentiation.

Keywords: Human embryonic stem cells, Genomic imprinting, *In vitro* differentiation, Transcriptional expression

Introduction

Genomic imprinting is an epigenetic event in which certain genes are mono-allelically expressed according to the parental origin. Most imprinted genes are clustered in a specific chromosomal loci that include protein-coding imprinted genes and non-coding RNA genes (1). The imprinted genes are regulated by highly ordered networks of epigenetic marks such as DNA methylation and histone modifications (2). In particular, imprinted control region (ICR) located in the imprinted cluster is differentially methylated to acquire mono-allelic expression. Differential methylation status of imprinted genes is resistant to the epigenetic reprogramming during preimplantation development in mammals (3). Instead, new methylation marks of the imprinted genes are acquired in the differentially methylated regions (DMRs) during gametogenesis. The newly acquired imprinted status is generally maintained throughout the life. Nonetheless, some imprinted genes show variable allelic expression patterns in a developmental stage- or tissue-specific manner (4). Studies about tissue-specific imprinting in human have mainly been per-

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formed in placenta, suggesting that tissue-specific expression of imprinted genes is crucial for fetus developments (5). Many imprinting disorders are closely related to abnormal loss- or gain-of imprinting in placental tissues (5). So far, approximately 50 to 100 imprinted genes have been identified in human and mouse, respectively (5). Recently, high-throughput sequencing in the mouse genome enables to identify differential methylation status of the newly identified imprinted loci in a base-level resolution (6). In spite of a small fraction in the whole genome, fine-tuned regulation of imprinted genes is crucial for mammalian development, growth, metabolism, and behaviors (7). Aberrant regulation of imprinted genes leads to various developmental disorders such as Prader-Willi /Angelman syndrome, Bechwith-Wiedemann syndrome, and Silver-Russell syndrome (7). Those imprinting disorders are attributed to genetic and epigenetic mutations in the imprinted genes or imprinting control region (ICR) (7). Thus, fine-tuned regulation of the imprinted gene expression is essential for mammalian development. Studies about genomic imprinting in mammals have been mainly performed in knock-out mice. The *in vivo* experiments are time-consuming and labor-intensive, and gene-targeting technique is not available in human *in vivo*. In addition, allelic expression status of some imprinted genes in mice is not concordant with human (8). Therefore, human embryonic stem cells (hESCs) can be alternative sources to study genomic imprinting underlying human development because they are able to differentiate to diverse cell types. In this study, we examined total transcriptional levels of 19 imprinted genes in hESCs during differentiation into specialized cell types. To compare expression levels of the imprinted genes between different developmental stages and lineages, hESCs were differentiated into ectodermal (neural progenitors and dopaminergic neurons), mesodermal (mesenchymal progenitors and osteoblasts), and endodermal lineage cell types (definitive endoderm cells and hepatocytes). Our results showed that respective imprinted genes were differently expressed in a cell type-specific manner during *in vitro* differentiation of hESCs.

Materials and Methods

Maintenance of human pluripotent stem cells (hPSCs)

Two hESC lines {H9-hESCs (passage No.: 36～70) and CHA4-hES (passage No.: 52～71)}, and two hiPSC lines {CRL-hiPSCs (passage No.: 32～40) and HDF-hiPSCs (passage No.: 35～40)} were maintained in the ESC mediun on mouse embryonic fibroblasts (MEFs) treated with Mitomycin-C (Sigma-Aldrich, MO, USA). The ESC mediun is DMEM/F12 supplemented with 20% KO-SR (Invitrogen, CA, USA), 100 IU/ml penicillin (Invitrogen), 1 mM L-glutamine, 100 μg/ml streptomycin (Invitrogen), 1% non-essential amino acids (NEAA; Invitrogen), 0.1 mM β-mercaptoethanol (Sigma-Aldrich), and 4～8 ng/ml basic fibroblast growth factor (bFGF; R&D Systems, MN, USA). The medium was daily changed, and passaging of hPSCs was performed at an interval of 5～6 days.

Differentiation of hESCs into dopaminergic neurons, osteoblasts, and hepatocytes

For differentiation of hESCs into dopaminergic (DA) neurons (9), undifferentiated H9-hESCs were seeded on MS5 stromal feeder cells and cultured on insulin/transferrin/selenium (ITS, Invitrogen) medium with 0.2 mM ascorbic acid (Sigma-Aldrich). After 7 days of culture, the cells were passaged onto MS5-SHH cells and cultured for 14 days. Then, cell clusters were further differentiated into neural precursor cells (NP) by culturing in ITS medium supplemented with 20 ng/ml bFGF and 0.2 mM AA, and re-plated on poly-L-ornithine (15 μg/ml; Sigma-Aldrich)/ fibronectin (1 μg/ml; Sigma-Aldrich)-coated dishes. hESC-NP cells were passaged every 7 days in medium supplemented with bFGF. DA neuron differentiation from hESC-NP cells was induced by withdrawing bFGF from the medium and adding brain-derived neurotrophic factor (BDNF, 20 ng/ml; R&D Systems), glial cell line-derived neurotrophic factor (20 ng/ml; R&D Systems), and dibutyryl cAMP (0.5 mM; Sigma-Aldrich).

Osteoblasts were differentiated from hESC-derived mesenchymal progenitors (MPs) as previously described (10). Briefly, hESCs were dissected into small clumps, transferred to a petri dish, and cultured in embryoid body (EB) medium (EBM; DMEM/F12, 10% SR without bFGF and 0.1 mM β-mercaptoethanol) supplemented with 10 μM SB431542 (SB) (Sigma-Aldrich). After 10 days of culture, hEBs were transferred to fibronectin-coated dishes in serum-free medium (DMEM/F12 supplemented with 1% ITS, 2% B27, and 1% concentrated lipids (Invitrogen)) containing 1 μM SB431542 (SB1-treated cells). The attached EBs were cultured to confluence, followed by expansion over three or four passages. To induce MPs, SB1-treated cells were seeded on FN-coated dishes at a density of 10^4 cells/cm^2 in α-MEM medium containing 10% FBS. Medium was refreshed every other day. After 20 days in culture, induced cells showing homogeneous morphology were characterized. For osteogenesis, MPs were seeded at a density of 0.5×10^4 cells/cm^2 into culture vessels containing α-MEM medium supplemented with 10% FBS. Cells were incubated at 37°C, 5% CO_2 for 1 day.

Table 1. Information for Quantitative real-time PCR primer sets

Gene name	Sense primer (5′-3′)	Antisense primer (5′-3′)	Acc. Number	Length (bp)
GAPDH	GACTTCAACAGCGACACCCACTC	AGGGTCTCTCTCTTCCTCTTGTGC	NM_002046	208
IGF2	ATGCTGGTGCTTCTCACCTTC	CGGAAACAGCACTCCTCAAC	NM_000612	194
H19	TAGCTTGGAAATGAATATGCTGC	GGGGGTGGCCATGAAGATGGA	NR_002196	180
SLC22A18	GGGCTCTTCATGGTCATGTT	AGTGGAAGACGCTGGACATC	NM_002555.5	229
KCNQ1OT1	GATCCTCACATCCCCATTTC	TGGAGTACACCACTTAGCAAGC	NR_002728	198
KCNQ1	CATGAGAACCAACAGCTTCG	TCTTGGCCACAAAGTACTGC	NM_000218	143
CDKN1C	CTCTGATCTCCGATTTCTTCG	TGGGCTCTAAATTGGCTCAC	NM_000076	159
PHLDA2	GCTCATCGATTTCCAGAACC	CGACTCGTCCAGCGTATG	NM_003311	200
OSBPL5	GCCAAGTCCAGGTTTTATGG	GCCATACAGGATTCCTTTGC	NM_020896	132
NDN	GCTTTCGCTTTTGTGCTACC	GCTGGTGACTTCTTCCAAACTC	NM_002487	157
SNRPN	ATTTCCAGGCTGAACTGAGG	TGGGCAGAAGCTTCAAAGAG	NM_005678	131
IPW	TCTTTCCTGTGTTGCAGTGG	TTGAGTCAAGTGGCAGCAAG	U12897	132
MAGEL2	AGTTTGGCCTTCTGATGGTG	TCTGCCTGACAAACACTTCG	NM_019066	168
UBE3A	TTGATGAGGGAGGTGTTTCCAAAG	GCTTCCTGTAGACAACCATGGGA	NM_130838	228
ATP10A	AGAAGCCAAACAACGACCTG	AGCCTTGGTTTCATGTCCTG	NM_024490	164
SGCE	GAAATGTTGGCCAGTGAGGT	ATAAACGCCCTCCTTCAGGT	NM_001099401.1	153
PEG10	ATCGATTCTGGTGCTTCTGG	TCAAATGACAGCACCTCTCG	NM_001040152	194
PPP1R9A	TGGAACTTTTCCCAGTGGAG	TCCCAGCTTTTCAAGTCCAG	NM_017650	101
DIO3	TCGAGCGTCTCTATGTCATCC	ATAGCGTTCCAACCAAGTGC	NM_001362.3	104
RTL1	GACGTGTTTAACCCGAAGGA	GGTGGAGGGACACTCGTAAA	NM_001134888.2	150

When the cells reached to 50～60% confluence, medium was replaced by pre-warmed osteogenesis complete medium containing STEMPRO Osteogenesis Supplement (Invitrogen). Medium was refreshed every 3 days. After 2～3 weeks, cells were analyzed by Alizarin Red staining and Von Kossa staining.

Derivation of hepatocytes from hESCs was carried out as previously described (11), Briefly, hESCs were cultured to confluence in conditioned medium on Matrigel for 2 days. To induce definitive endoderm (DE), hESCs were cultured in RPMI-1640 (HyClone, UT) containing 1% B27 (GIBCO) and 50 ng/ml activin A (Peprotech) for 5 days. To further differentiate into hepatocytes, DE cells were cultured in hepatocyte culture medium (HCM, Lonza) containing 30 ng/ml FGF4 (Peprotech) and 20 ng/ml BMP2 (Peprotech) for 5 days and further incubated in HCM supplemented with 20 ng/ml hepatocyte growth factor (Peprotech) for 5 days. Finally, hESC-derived hepatocytes were matured by culturing in HCM supplemented with 10 ng/ml oncostatin M (R&D Systems) and 0.1 μM dexamethasone (Sigma-Aldrich) for 5 days.

Total RNA extraction and qRT-PCR

Total RNAs were extracted from undifferentiated hPSCs and hESC-derived cells using TRIzol® (Invitrogen) according to the manufacturer's procedure. Then, extracted RNAs were treated with DNase I to eliminate genomic DNA. cDNAs were synthesized by using M-MLV reverse transcriptase (Enzynomics, Korea), and diluted 1:5 ratio with RNase- and DNase-free water. The cDNAs were analyzed by real-time quantitative PCR (qPCR) on a Bio-RAD iQ5 cycler real-time PCR Detection System (Bio-Rad, CA, USA) using the Primer Q-Master Mix (GenetBio, Chungnam, Korea). PCR conditions were 10 min at 94°C (one cycle), 30 sec at 94°C, 30 sec at 60°C and 30 sec at 72°C (50 cycles). Melting-curve analysis was performed to ensure specific amplification. All sets of reactions were conducted in triplicate. Expression levels of imprinted genes were normalized to that of *GAPDH* as an internal control, and calculated using the comparative C_T method ($\Delta\Delta C_T$). The primers used in this study were designed using Primer3Plus and are listed in Supplemental Table 1.

Statistics

Statistical analyses were performed using Prism 5.01 (GraphPad Software, USA). Also, one-way ANOVA and Dunnett's post-hoc test were used to confirm the statistical significance. The differences of gene expression between control and experimental groups were considered significant if the p value was less than 0.05.

Results

Conservative expression of imprinted genes in undifferentiated hPSC lines

To check whether imprinted genes are expressed in undifferentiated hPSCs, 24 imprinted genes which are

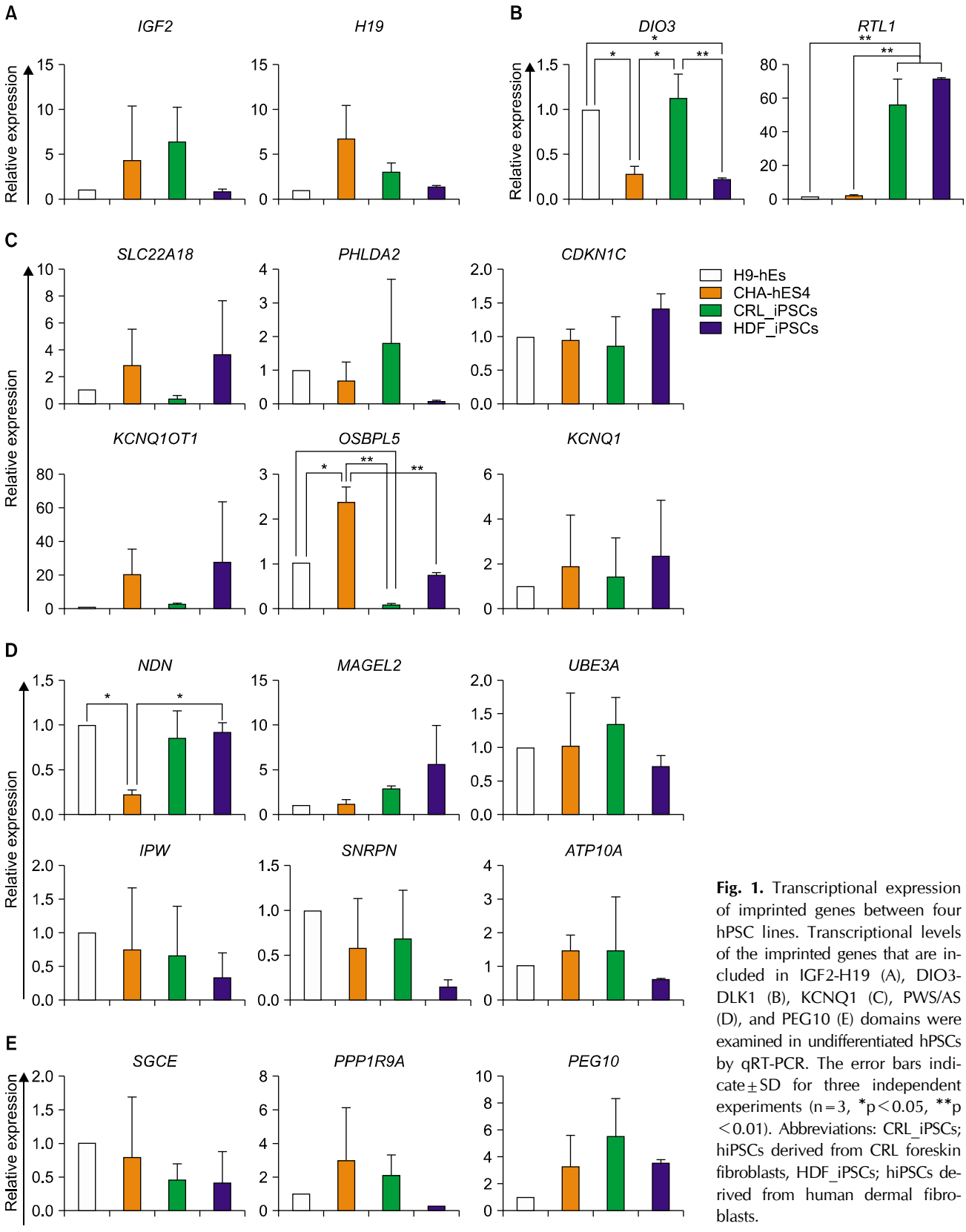

Fig. 1. Transcriptional expression of imprinted genes between four hPSC lines. Transcriptional levels of the imprinted genes that are included in IGF2-H19 (A), DIO3-DLK1 (B), KCNQ1 (C), PWS/AS (D), and PEG10 (E) domains were examined in undifferentiated hPSCs by qRT-PCR. The error bars indicate ± SD for three independent experiments (n = 3, *$p < 0.05$, **$p < 0.01$). Abbreviations: CRL_iPSCs; hiPSCs derived from CRL foreskin fibroblasts, HDF_iPSCs; hiPSCs derived from human dermal fibroblasts.

known to be mono-allelically expressed in human tissues (http://igc.otago.ac.nz/home.html) were tested in two hESC lines and two hiPSC lines. Of 24 imprinted genes, 19 imprinted genes were expressed in H9-hESCs (data was not shown). Transcriptional expression of 19 imprinted genes was also observed in other hPSCs (Fig. 1). The relative expression levels of imprinted genes did not show distinct difference between hPSC lines (2 hESC lines and 2 hiPSC lines) except some genes such as *DIO3*, *RTL1*, *OSBPL5*, and *NDN*. These results indicate that the relative expression levels of imprinted genes are generally conserved in undifferentiated hPSC lines.

Quantitative expression of the imprinted genes in hESCs during *in vitro* differentiation

To investigate the expression level of imprinted genes during lineage-specific differentiation, H9-hESCs were differentiated into dopaminergic neurons (ectoderm), osteoblasts (mesoderm) and hepatocytes (endoderm) as representative cell types of 3 germ layers, respectively. Characterization and functionality of differentiated cell types from hESCs were previously reported (12). In this study, we analyzed the transcriptional expression of 19 imprinted genes that were commonly expressed in 4 different hPSC lines. These 19 imprinted genes were categorized in well-known imprinted domains, including IGF2/H19 domain (Fig. 1A), DIO3-DLK domain (Fig. 1B), KCNQ1 domain (Fig. 1C), PWS/AS domain (Fig. 1D), and PEG10 domain (Fig. 1E). Total transcriptional levels of the imprinted genes were investigated in three developmental stage cell types such as pluripotent stem cells, intermediate progenitors, and terminally-differentiated cells, respectively.

IGF2/H19 domain: IGF2/H19 (chr.11p15) imprinting domain contains paternally-expressing *IGF2* gene and maternally-expressing non-coding RNA gene *H19*. The expression of these imprinted genes is regulated by an in-

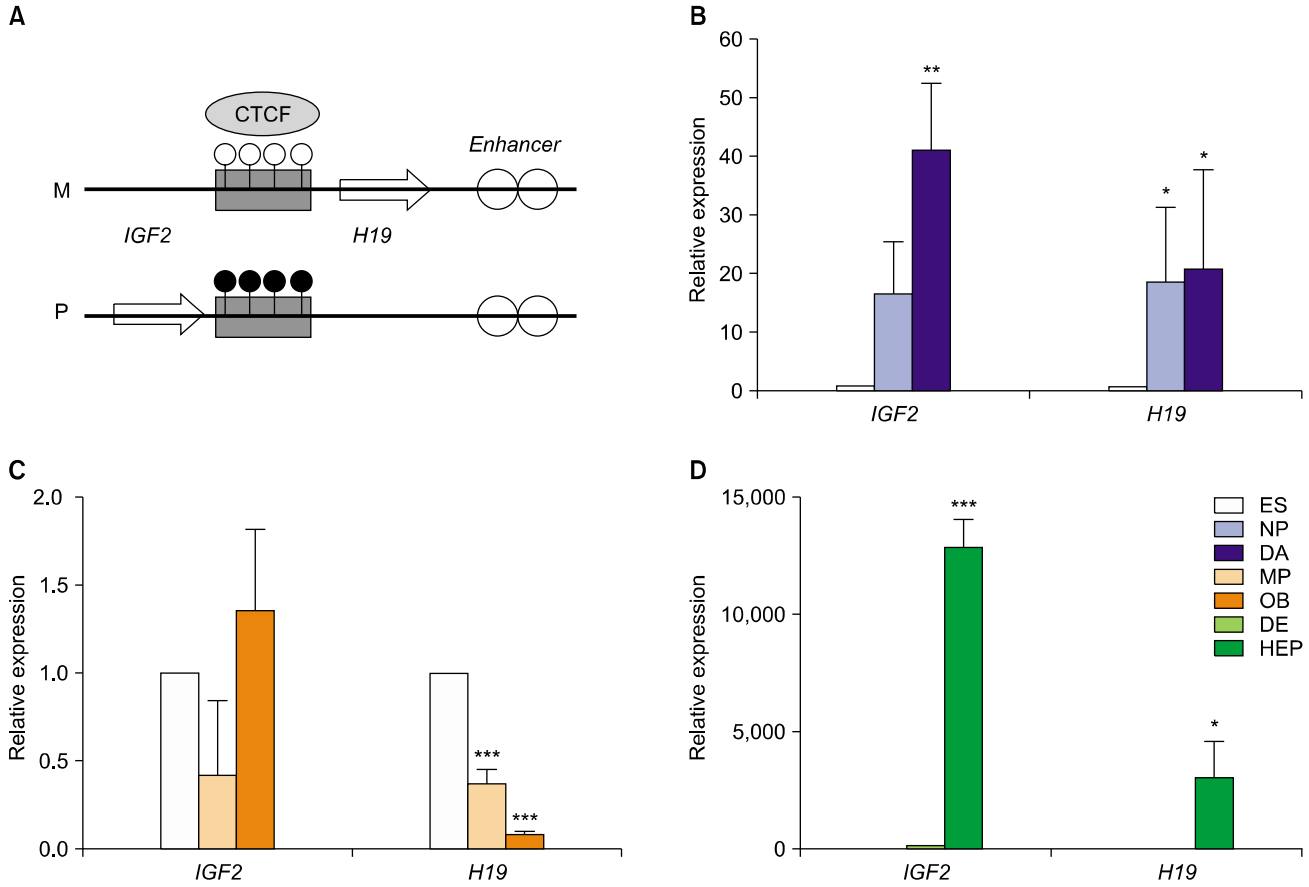

Fig. 2. Relative expression levels of imprinted genes in IGF2/H19 domain in hESCs during *in vitro* differentiation. (A) A schematic diagram of IGF2/H19 domain (Ch 11p15). Relative expression level of *IGF* and *H19* imprinted genes in hESCs during *in vitro* differentiation to DA neurons (B), osteoblasts (C), and hepatocytes (D). The error bars indicate±SD for three independent experiments (n=3, *$p<0.05$, **$p<0.01$, ***$p<0.001$). Abbreviations, ES: H9-hESCs; NP: neuronal progenitors; DA: dopaminergic neurons; MP: mesenchymal progenitors; OB: osteoblasts; DE: definitive endoderm; HEP: hepatocytes.

sulator binding protein CTCF (CCCTC-binding factor) (13). CTCF binds to unmethylated DMRs on maternal allele, and blocks the expression of *IGF2*. In contrast, expression of *H19* is modulated by downstream enhancer. Since CFCF does not bind to methylated DMRs, the enhancers activates *IGF2* gene in the paternal allele, not *H19* gene (Fig. 2A). Transcription of both *IGF2* and *H19* was up-regulated in hESCs during differentiation to neurons (Fig. 2B) and hepatocytes (Fig. 2D). However, transcriptional expression of *H19* reduced in hESCs during osteoblastic differentiation (Fig. 2C).

KCNQ1 domain: KCNQ1 domain (ch.11p15) has 6 maternally-expressing genes and a paternally-expressing *KCNQ1OT1* (Fig. 3A). The genomic imprinting at this domain is controlled by maternally-expressing non-coding RNA gene *KCNQ1OT1* (Fig. 3A). Quantitative expression level of imprinted genes in this domain showed distinct patterns in a cell-type dependent manner during *in vitro* differentiation (Fig. 3B~D). During neuronal differentiation of hESCs, expression levels of *OSBPL5*, *PHLDA2*, and *KCNQ1OT1* did not changed in neural progenitors and DA neuron (Fig. 3B). However, relative expression level of *CDKN1C* and *KCNQ1* was enhanced in neural progenitor (NPs) and DA neurons, respectively. Besides, all imprinted genes in KCNQ1 domain significantly decreased in hESCs during differentiation to osteoblasts (Fig. 3C). In contrast, most of the imprinted genes showed enhanced expression in hESC-derived hepatocytes (Fig. 3D). These results indicate that transcriptional expression of the imprinted genes in KCNQ1 domain may be regulated in a cell type-dependent manner.

PWS/AS domain: PWS/AS domain (chr. 15q11-q13) contains four paternally-expressing genes and three maternally-expressing genes (Fig. 4A). In this domain, several imprinted genes represented distinct expression patterns in hESCs during *in vitro* differentiation. During differentiation of hESCs into DA neurons and hepatocytes, two genes (*MAGEL2*, *NDN*) were up-regulated and three genes

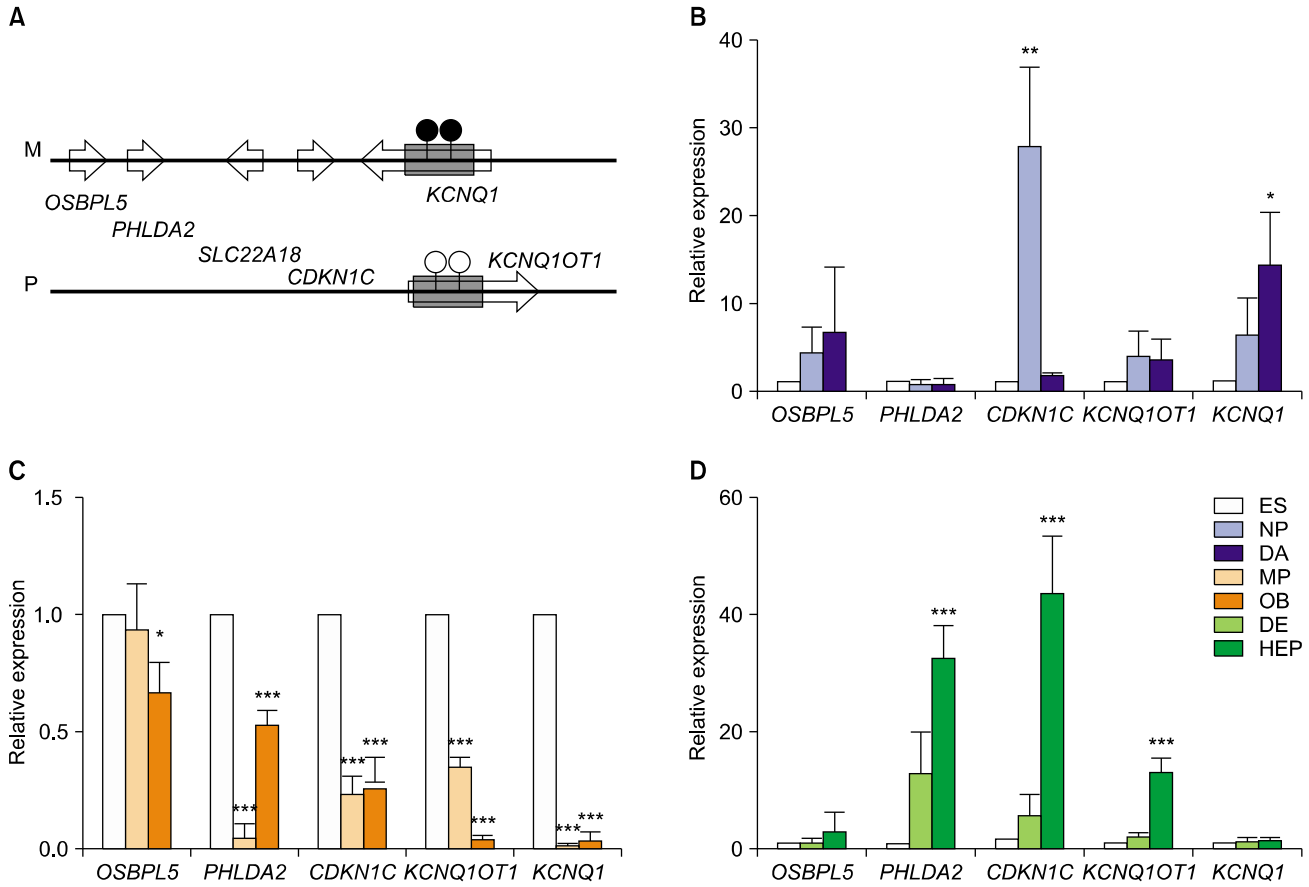

Fig. 3. Relative expression levels of imprinted genes in KCNQ1 domain in hESCs during *in vitro* differentiation. (A) A schematic diagram of KCNQ1 domain (Ch 11p15). This KCNQ1 domain contains several imprinted genes, including *OSBPL5*, *PHLDA2*, *CDKN1C*, *KCNQ1*, and *KCNQ1OT1*. Relative expression level of several imprinted genes in hESCs during *in vitro* differentiation to DA neurons (B), osteoblasts (C), and hepatocytes (D). The error bars indicate±SD for three independent experiments (n=3, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$).

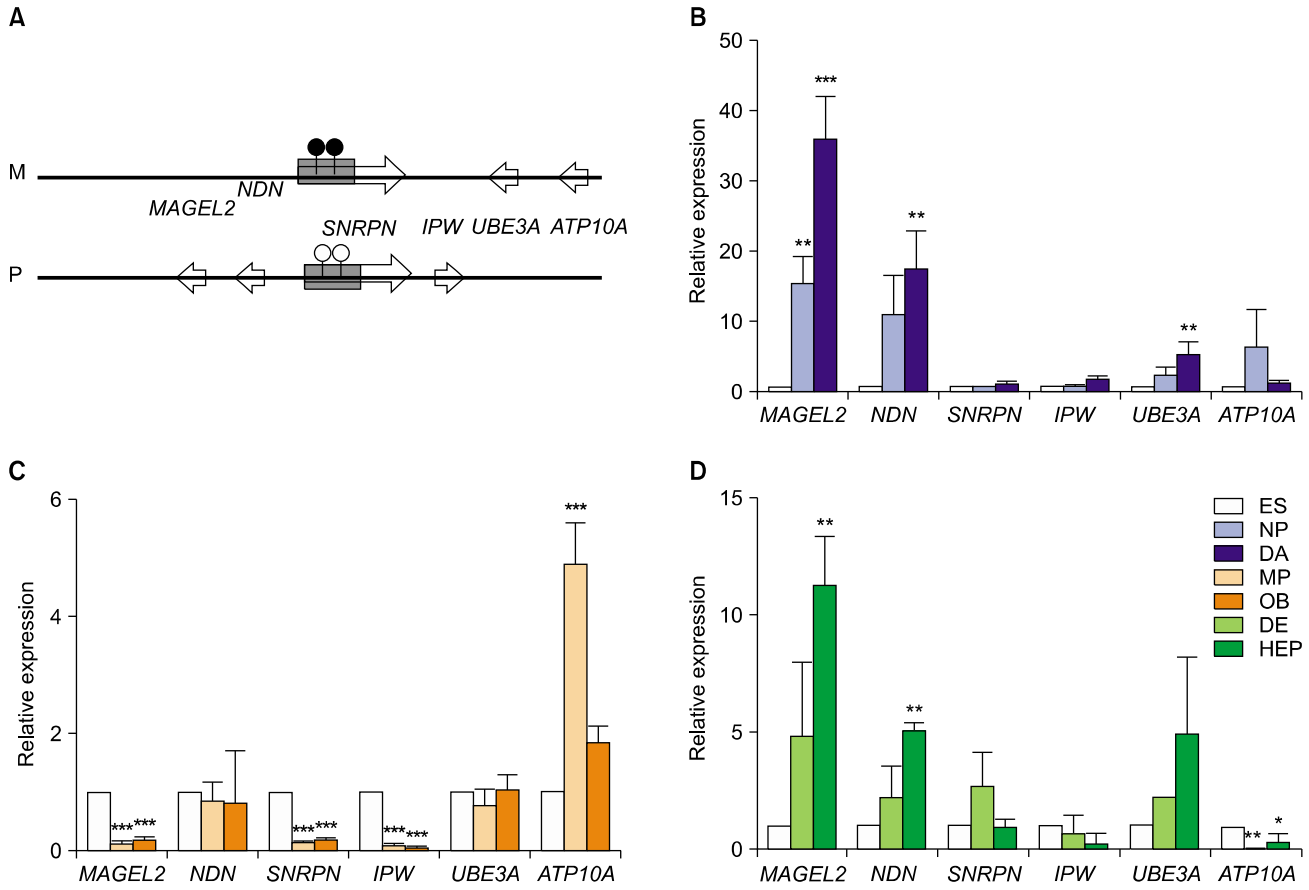

Fig. 4. Relative expression levels of imprinted genes in PWS/AS domain in hESCs during *in vitro* differentiation. (A) A schematic diagram of PWS/AS domain (Ch. 15q11-q13). This domain contains several imprinted genes, including *MAGEL2*, *NDN*, *SNRPN*, *IPW*, *UBE3A*, and *ATP10A*. Relative expression level of several imprinted genes in hESCs during *in vitro* differentiation to DA neurons (B), osteoblasts (C), and hepatocytes (D). The error bars indicate ±SD for three independent experiments (n=3, *p<0.05, **p<0.01, ***p<0.001).

(*SNRPN*, *IPW*, and *UBE3A*) revealed constant expression levels (Fig. 4B, D). However, *MAGEL2*, *SNRPN*, and *IPW* were significantly down-regulated in hESCs during osteoblastic differentiation (Fig. 4C).

PEG10 domain: PEG10 domain (chr.7q21.3) has two paternally-expressing imrinted genes and one maternally-expressing gene (Fig. 5A). The paternally-expressing gene 10 (*PEG10*) is a retrotransposon-derived gene that is important to placental development (14). This imprinting locus is controlled by DMR located in *PEG10* promoter region (Fig. 5A). The imprinted genes in PEG10 domain also exhibited distinct expression patterns between different cell types derived from hESCs. Even though *PPP1R9A* showed invariable expression levels in all three cell types, *SGCE* and *PEG10* were differently regulated in all differentiated cell types. Although transcription of *SGCE* was relatively constant in the neuronal lineage, transcripts of *SGCE* were significantly enhanced in osteoblasts and hepatocytes, respectively (Fig. 5B~D). Expression of *PEG10* was significantly upregulated in hESCs during differentiation to DA neurons and hepatocytes, but down-regulated during osteoblastic differentiation (Fig. 5B~D).

DIO3-DLK1 domain: In addition to above imprinted genes, we also examined the quantitative expression patterns of *DIO3*, *RTL1*, *MEG3* and *DLK1* that are clustered in chromosome 14q32.2 region (Fig. 6A). Of these imprinted genes, only *DIO3* and *RTL1* were expressed in undifferentiated hESCs. Transcription of *DIO3* was activated in hESCs during differentiation to neuroectodermal and endodermal lineages, not mesodermal lineage, respectively (Fig. 6B~D). Interestingly, expression of *RTL1* was enhanced in hESC-derived differentiated cells such as DA neurons, osteoblasts, and hepatocytes (Fig. 6B~D).

Discussion

We have previously reported that allele-specific expression of imprinted genes is changed in a cell-type specific

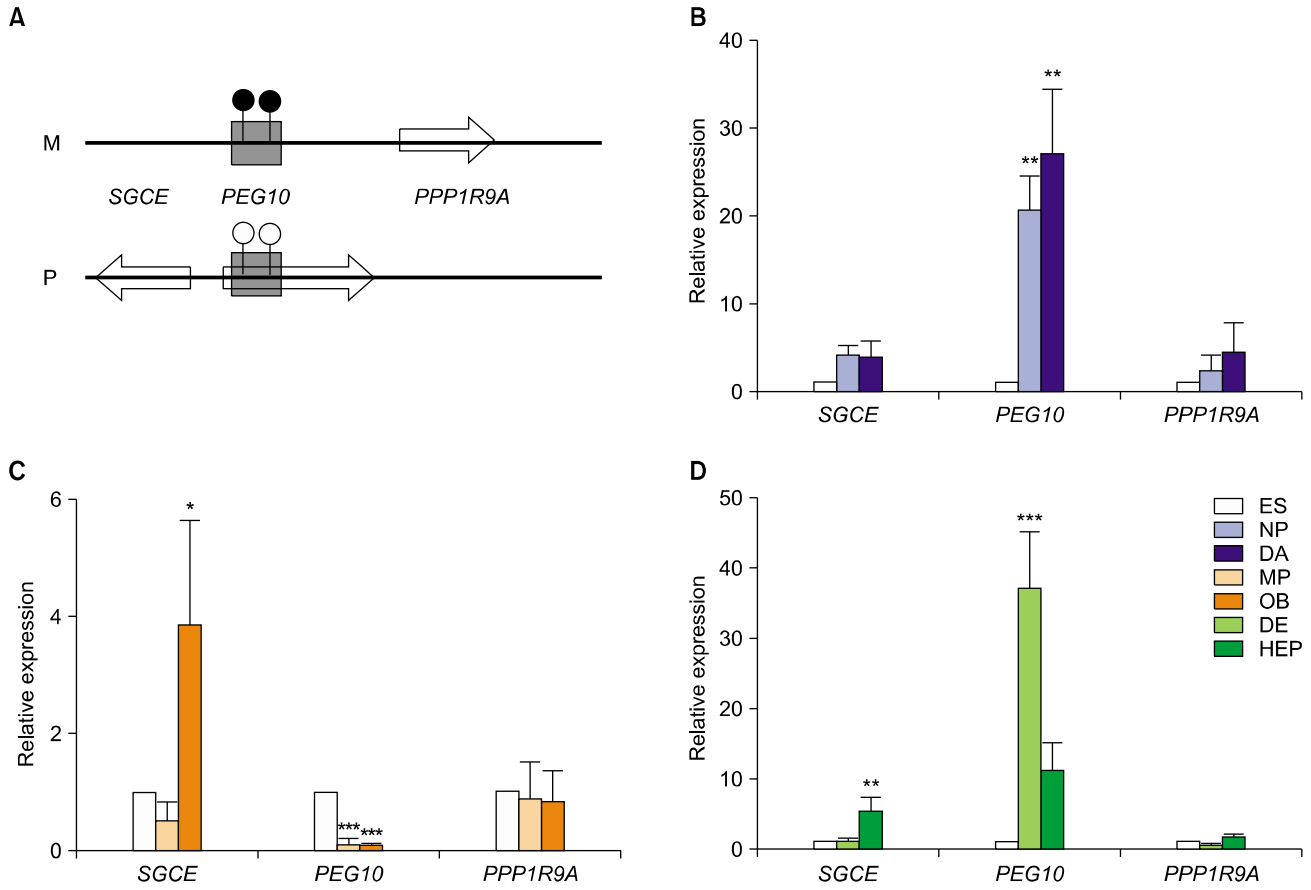

Fig. 5. Relative expression levels of imprinted genes in PEG10 domain in hESCs during *in vitro* differentiation. (A) A schematic diagram of PEG10 domain (Ch. 7q21.3). This domain contains three imprinted genes such as *SGCE*, *PEG10* and *PPP1R9A*. Relative expression level of these imprinted genes in hESCs during *in vitro* differentiation to DA neurons (B), osteoblasts (C), and hepatocytes (D). The error bars indicate±SD for three independent experiments (n=3, *p<0.05, **p<0.01, ***p<0.001).

manner in human pluripotent stem cells (hPSCs) during *in vitro* differentiation (12). However, total expression levels of imprinted genes were not still reported in hPSCs during *in vitro* differentiation. Here we demonstrate that total transcript levels of imprinted genes are varied in hPSCs during *in vitro* differentiation. In general, transcription of imprinted genes is down-regulated in somatic cells (15). In contrast, expression of some imprinted genes (*PEG10*, *NDNL1*, *IGF2*, *GNAS*, *DCN*, and *H19*) is up-regulated in hESCs during the formation of embryoid body (EB) (16). However, there is a limitation to understand cell type- or tissue-specific imprinting because EBs consist of mixed cell populations, which include different cell types (ectoderm, endoderm, and mesoderm) as well as undifferentiated cells. Imprinting of some imprinted genes is changed in a developmental stage- or tissue-specific manner (17). Therefore, hESCs undergoing differentiation into specialized cell types, rather than EBs derived from hESCs, would be better *in vitro* models for studying tissue-specific imprinting. In this study, the transcriptional expression of imprinted genes was varied in a cell type-dependent or a domain- independent manner in hESCs during *in vitro* differentiation. Transcriptional expression of imprinted genes within *IGF2/H19* and *KCNQ1* domain was enhanced in hESC- derived neuronal progenitors (18). Similarly, transcript levels of some imprinted genes (*IGF2* and *H19* in IGF2/H19 domain, *CDKN1C* and *KCNQ1* in KCNQ1 domain) were significantly increased during differentiation of hESCs into the neuronal lineage (Fig. 2B, 3B). Consequently, this study provides a possibility that hESCs can be employed as a valuable source for *in vitro* modeling to understand the behavior of imprinted genes in the embryonic development and/or cellular differentiation in human.

Acknowledgments

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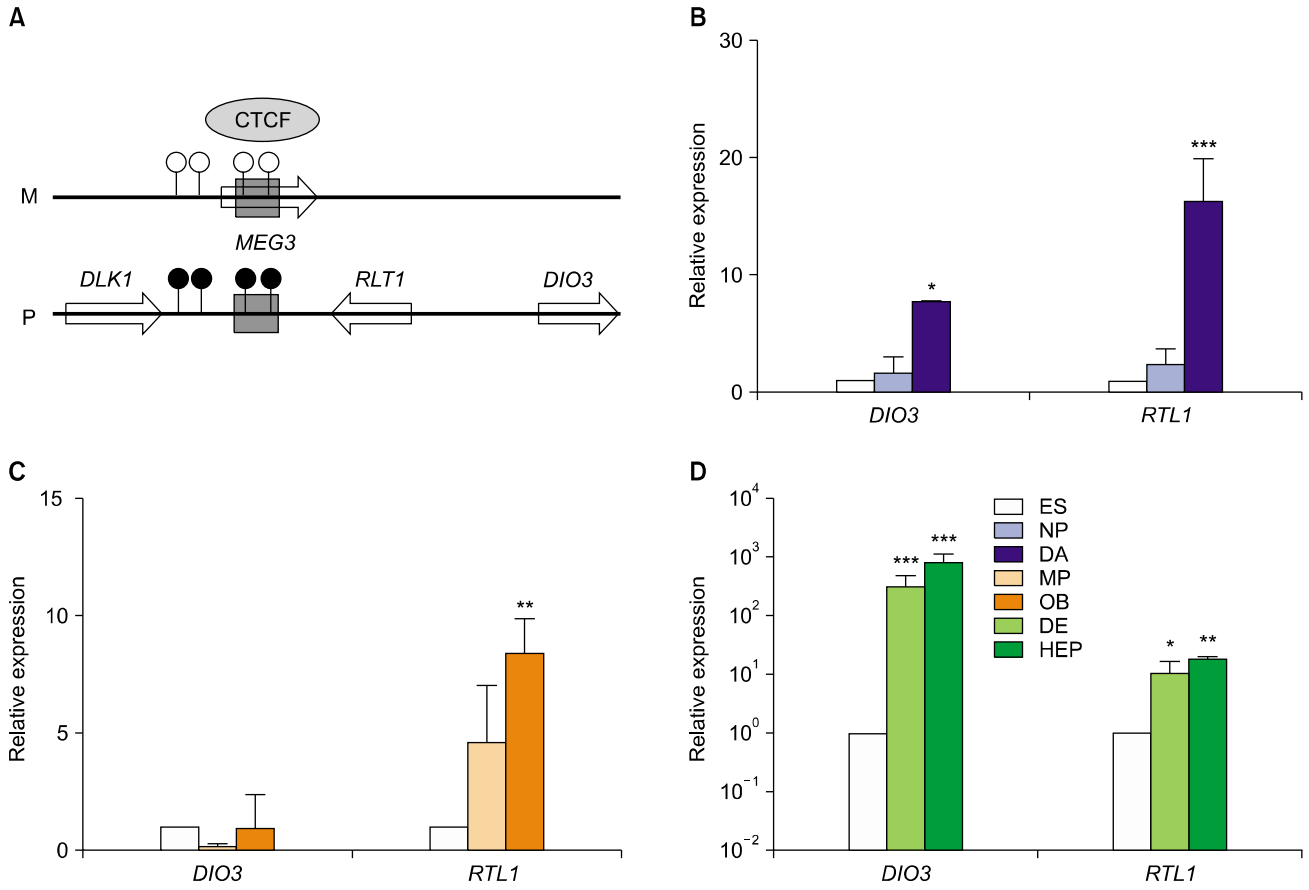

Fig. 6. Relative expression levels of imprinted genes in DIO3-DLK1 domain in hESCs during *in vitro* differentiation. (A) A schematic diagram of DIO3-DLK1 domain (Ch. 14q32.2). This domain contains four imprinted genes such as *DLK1*, *MEG3*, *DIO3* and *RTL1*. Relative expression level of *DIO3* and *RTL1* imprinted genes in hESCs during *in vitro* differentiation to DA neurons (B), osteoblasts (C), and hepatocytes (D). The error bars indicate±SD for three independent experiments (n=3, *p<0.05, **p<0.01, ***p<0.001).

Potential conflict of interest

The authors have no conflicting financial interest.

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