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Variable allelic expression of imprinted genes in human pluripotent stem cells during differentiation into specialized cell types *in vitro*

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ABSTRACT

Genomic imprinting is an epigenetic phenomenon by which a subset of genes is asymmetrically expressed in a parent-of-origin manner. However, little is known regarding the epigenetic behaviors of imprinted genes during human development. Here, we show dynamic epigenetic changes in imprinted genes in hESCs during *in vitro* differentiation into specialized cell types. Out of 9 imprinted genes with single nucleotide polymorphisms, mono-allelic expression for three imprinted genes (*H19*, *KCNQ1OT1*, and *IPW*), and bi- or partial-allelic expression for three imprinted genes (*OSBPL5*, *PPP1R9A*, and *RTL1*) were stably retained in H9-hESCs throughout differentiation, representing imprinting stability. Three imprinted genes (*KCNK9*, *ATP10A*, and *SLC22A3*) showed a loss and a gain of imprinting in a lineage-specific manner during differentiation. Changes in allelic expression of imprinted genes were observed in another hESC line during *in vitro* differentiation. These findings indicate that the allelic expression of imprinted genes may be vulnerable in a lineage-specific manner in human pluripotent stem cells during differentiation.

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1. Introduction

Genomic imprinting is an inheritance process by which a set of genes are expressed in a parent-specific manner [1]. Imprinted genes are commonly found in clusters that are regulated by an imprinting control region (ICR) within the cluster [2]. In many cases, ICRs correspond to differentially methylated regions (DMRs) that acquire allele-specific DNA methylation in one of the parental germ lines, although there are a number of lone imprinted genes without regulatory DMRs [3–5]. The epigenetic states of both paternal and maternal genomes are reprogrammed on a genome-wide basis during the pre-implantation development and are then reset near the time of implantation [6]. However, imprinted genes are exceptionally refractory to epigenetic reprogramming during early embryonic development [7,8]. Demethylation and *de novo* methylation of imprinted genes occurs during germ cell development [6]. Abnormal regulation of imprinted genes is implicated in various human cancers and developmental defects [9]. For example, Beckwith–Wiedemann syndrome (BWS) is attributed to disruption of differential methylation in the IGF2/H19 and KCNQ1 domains on chromosome 11p15 [9]. Suppression of paternally-expressed *SNRPN* and maternally-expressed *UBE3A* in the PWS/AS imprinting domain (Ch.15q11-q13) is associated with Prader–Willi syndrome (PWS) and Angelman syndrome (AS), respectively [10]. Thus, imprinted genes are essential for fetal and placental development, even though imprinted genes represent a small fraction of the genome.

Human embryonic stem cells (hESCs) can be useful for studying epigenetic mechanisms in the process of human development. Allelic expression at imprinted loci has been used as indicators of epigenetic stability in hESCs [11,12]. The epigenetic status of imprinted genes is generally stable in both undifferentiated hESCs and hESC-derived embryoid bodies (EBs) [13,14], although mono-allelic expression of imprinted genes can be disrupted in hESCs and hiPSCs by differences between cell lines or in a gene-specific manner [15,16]. However, little is known regarding the epigenetic dynamics of imprinted genes during human development or

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differentiation into specialized cell types. In this context, a putative model for the molecular mechanisms of early human development can be provided by the *in vitro* differentiation of human pluripotent stem cells (hPSCs), such as hESCs and hiPSCs. In this study, we analyzed the allele-specific expression of the imprinted genes in hPSCs during *in vitro* differentiation into derivatives of the three germ-layers: ectoderm (dopaminergic neurons), mesoderm (osteoblasts), and endoderm (hepatocytes). As results, some imprinted genes were epigenetically stable in H9-hESCs throughout differentiation *in vitro*, whereas the other showed dynamic changes in the allelic expression. In particular, imprinted genes (*SLC22A2*, *SLC22A3*, and *IGF2R)* on an IGF2R imprinted cluster represented a gain or a loss of imprinting in derivatives derived from hESCs and hiPSCs, indicating that IGF2R domain would be epigenetically vulnerable in hPSCs during differentiation *in vitro*. Consequently, our findings give a hint that epigenetic imprinting may be changed in a developmental stage-specific manner during embryonic development in human.

2. Materials and methods

2.1. Maintenance of hPSCs

H9-hESCs (passage No.: 36–70), CHA-hES4 (passage No.: 52–71) [17] and CRL-hiPSCs (passage No.: 32–40) were cultured on mitotically-inactivated mouse embryonic fibroblasts (MEFs) in DMEM/F12 medium (Invitrogen, CA) with 20% knockout serum replacement (KSR; Invitrogen), 100 IU/ml penicillin (Invitrogen), 1 mM l-glutamine, 100 ug/ml streptomycin (Invitrogen), 1% non-essential amino acids (NEAA; Invitrogen), 0.1 mM β-mercaptoethanol (Sigma–Aldrich, MO), and 4–8 ng/ml basic fibroblast growth factor (bFGF; R&D Systems, Minneapolis, MN). Medium was changed daily. Undifferentiated hPSCs were passaged at intervals of 5–7 days.

2.2. Differentiation of hPSCs into dopaminergic neurons, osteoblasts, and hepatocytes

Dopaminergic neurons were derived from hESCs as previously described [18]. Differentiation of hESCs into mesenchymal progenitors and osteoblasts was performed as previously described [19]. Derivation of hepatocytes from hPSCs was carried out as previously described [20]. Vascular endothelial cells could be differentiated from hESCs by using a method previously reported [21]. Detailed procedures for differentiation of hPSCs into three lineages are described in Supplementary materials and methods.

2.3. Genotyping

Single nucleotide polymorphisms (SNPs) of imprinted genes used for allelic expression were re-confirmed by direct sequencing of sample genomic DNA. Briefly, genomic DNAs were extracted from hESCs using a G-DEX™ II Genomic DNA Extraction Kit (iNtRON, Seoul, Korea). PCR was carried out in a 30 μl reaction volume containing 0.75–1 U h-Taq DNA polymerase (SolGent, Daejeon, Korea), 1X supplied reaction buffer, 0.3 μM primer, 1.5–2.5 mM $MgCl_2$, 0.2 mM dNTPs, and 50 ng DNA template. PCR conditions were 15 min at 95 °C (one cycle), 20 s at 95 °C, 40 s at 55–65 °C and 30–45 s at 72 °C (30–40 cycles), followed by 72 °C for 5 min. All primers and optimal PCR conditions are described in Supplementary Table 1. PCR products were purified using the NucleoSpin® Extract II Kit (MACHEREY-NAGEL, Germany), and sequenced using an ABI 3730XL Capillary DNA analyzer (Applied Biosystems). Chromatograms were analyzed using the Chromas software (Technelysium Pty, Australia). The information of the imprinted genes was obtained from a database http://igc.otago.ac.nz/home.html and a previous report [22].

2.4. Analysis of allele-specific expression in hPSCs

For allele-specific expression analysis, the QUASEP method was used [23,24]. This method, based on Pyrosequencing™ technology, can discriminate subtle differences in allele-specific transcripts by analyzing heterozygous SNPs. hPSCs, intermediate cell types, and terminally differentiated cells were used to analyze allele-specific expression. Total RNAs were extracted from hESCs and their derivatives by using TRIzol® (Invitrogen), followed by treatment with DNase I (Invitrogen). 2 μg of total RNA were reverse-transcribed into cDNA according to the manufacturer's procedure. cDNAs from each sample were amplified by PCR using one biotinylated primer per pair. Primers used for analyzing allele-specific expression were listed in Supplementary Table 2. Three independent experiments were run to calculate an arithmetic mean and standard deviation. Due to preferential allele amplification, the increased nucleotide signal and the baseline noise, the peak heights, and the resulting allele quantification were normalized in Pyrosequencing™. By changing the peak height adjustment factor and reanalyzing the sample using this software, the desired ratio (50:50) was adjusted from heterozygous genomic DNA. Then, the peak heights for each allele of a sample cDNA were compared with those of the heterozygous genomic DNA. Allelic expression ratios were determined by calculating the peak heights from pyrograms: the minor allele peak height was divided by the major allele peak height. Calculated allelic ratios between two alleles of the imprinted genes were indicated in Supplementary Table 3. In this study, expression profiles of imprinted genes were classified into three groups; mono-allelic (allelic ratio: less than 0.20), partial-allelic (allelic ratio: 0.21–0.70), and bi-allelic (allelic ratio: more than 0.71).

2.5. Statistical analysis

All statistical analyses in this study were performed using Prism 5.01 (GraphPad Software, USA). One-way ANOVA and Dunnett's post-hoc test were performed to confirm the statistical significance of pyrosequencing data. The differences between control and experimental groups were considered significant if the P value was less than 0.05.

3. Results

To examine the allele-specific expression of the imprinted genes, hESCs were differentiated into three germ layer cell types; dopaminergic neurons (ectoderm), osteoblasts (mesoderm), and hepatocytes (endoderm) (Fig. 1A). hESC-derived dopaminergic neurons (DA), osteoblasts (OB) and hepatocytes (HEP) were characterized by immunostaining or flow cytometric analysis for cell type specific markers, or functional assays (Fig. 2). hiPSC-derived osteoblasts or hepatocytes were also characterized by above methods (Supplementary Fig. 1). As a representative intermediate developmental stage of three germ layers, neuronal progenitors (NP), mesenchymal progenitors (MP), and definitive endoderm cells (DE) were subjected to the experiments for the allelic expression, respectively.

3.1. Identification of SNPs in imprinted genes in several hPSC lines

To identify SNPs of imprinted genes, genome-wide exome sequencing was performed in 7 hESC and 3 hiPSC lines. From the whole exome sequencing data, we identified 21 imprinted genes with informative SNPs within the exon (Supplementary Table 4).

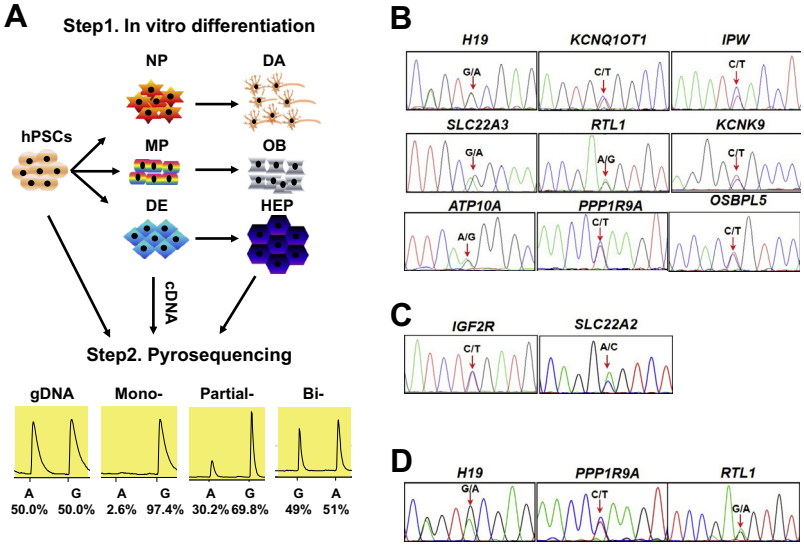

Fig. 1. Identification of SNPs and pyrosequencing for analyzing allele-specific expression of the imprinted genes. (A) Schematic steps for analyzing allele-specific expression by using the pyrosequencing. Step 1: *In vitro* differentiation of hPSCs into ectoderm, mesoderm and endoderm lineages. cDNAs were synthesized from respective progenitors and terminally differentiated cells. Step 2: Pyrosequencing of imprinted genes. Expression ratio between two alleles of an imprinted gene was shown in a pyrogram. Allelic expression patterns were classified into 3 groups (mono-, partial, and bi-allelic expression) (see Section 2). Abbreviation: hPSCs: human pluripotent stem cells, NP: neural progenitors, DA: dopaminergic neurons, MP: mesenchymal progenitors, OB: osteoblasts, DE: definitive endoderm, HEP: hepatocytes. (B–D) Identification of informative single nucleotide polymorphisms (SNP) in imprinted genes in H9-hESC (B), CHA-hES4 (C), and hiPSCs (D), respectively.

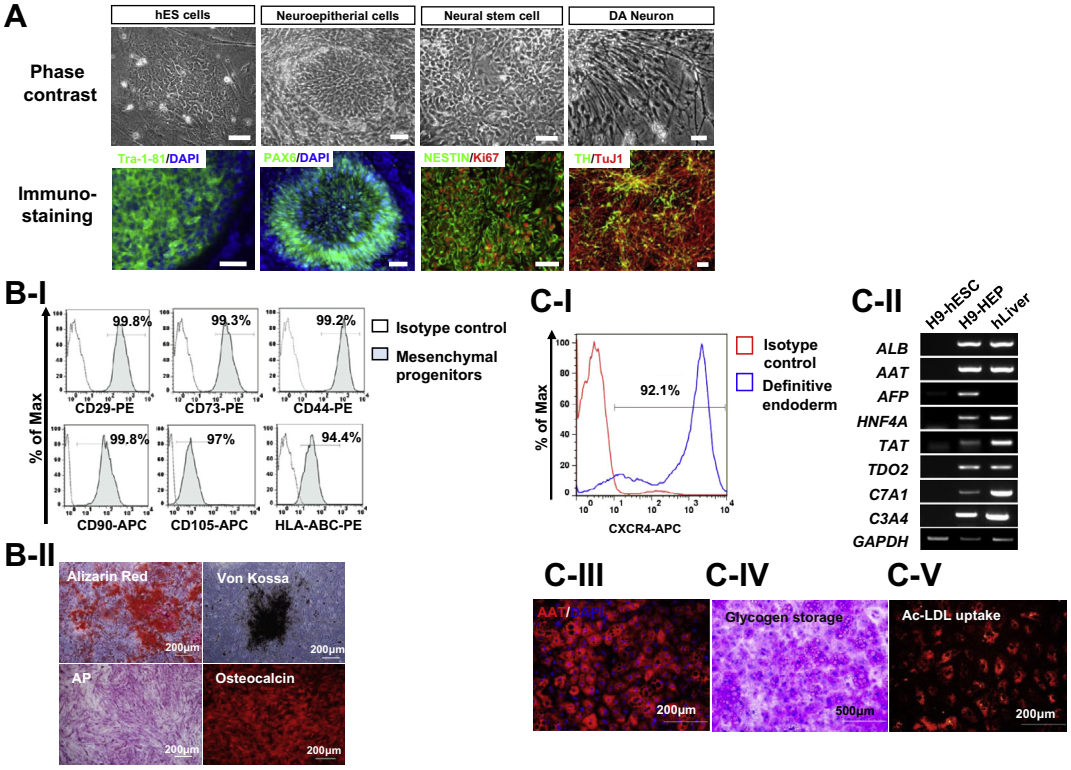

Fig. 2. Differentiation of H9-hESCs into various cell types. (A) Differentiation of H9-hESCs into dopaminergic neurons. In NP stage cells, most cells (~90%) were positive for both NESTIN (NP marker) and Ki67 (cell proliferation marker). Also, large population (~50%) of TuJ1 + neurons co-expressed TH (tyrosine hydroxylase), a DA neuronal marker. Scale bar is 30 μm. (B) Differentiation of H9-hESCs into osteoblasts. (B–I) Derivation of MPs from hESCs. hESC-derived MPs expressed mesenchymal stem cell-specific CD markers, such as CD29, CD73, CD44, CD90, CD105, and HLA-ABC with higher efficiency (above 90%). (B-II) Differentiation of hESC-derived MPs into osteoblasts (OB). Alizarin Red staining (red) and Von Kossa staining (black) were detected in hESC-derived osteoblasts. Alkaine phosphatase activity and osteocalcin expression were also detected in hESC-derived OBs. (C) Differentiation of hESCs into hepatocytes. (C-I) Flow cytometric analysis of hESC-derived DE cells. Most (~92%) of hESCs were differentiated into CXCR4-expressing DE cells. (C-II) RT-PCR analysis of hepatocyte-specific genes in hESC-derived hepatocytes. cDNAs of H9-hESCs and human primary liver cells (hLiver) were loaded as negative and positive controls, respectively. Various hepatocyte-specific genes were highly transcribed in hESC-derived hepatocytes. (C-III) Immunostaining of 1-antitrypsin (AAT) in hESC-derived hepatocytes. (C-IV–V) Functionality of hESC-derived hepatocytes. hESC-derived hepatocytes had potentials for glycogen storage(C-IV) and Ac-LDL uptake (C–V).

SNPs of *H19, KCNQ1OT1* and *IPW* which were previously reported [14] were confirmed in H9-hESCs and CRL-hiPSCs by direct sequencing (Fig. 1B and 1D). SNPs of the other imprinted genes used for analysis of allelic expression in this study were confirmed again by direct sequencing (Fig. 1B, C, and D).

3.2. Allelic expression of mono-allelically expressing imprinted genes in hPSCs during differentiation in vitro

Out of 9 imprinted genes with informative SNPs, three imprinted genes (*H19*, *KCNQ1OT1*, and *IPW*) were mono-allelically expressed in undifferentiated H9-hESCs (Fig. 3A). *KCNQ1OT1* and *IPW* showed robust mono-allelic expression in H9-hESCs during *in vitro* differentiation. However, mono-allelic expression ratio (0.084 ± 0.102) of *H19* in undifferentiated hESCs was slightly changed (0.1745 ± 0.019) in DE and then restored in HEP (0.02775 ± 0.019) (Fig. 3A). We also examined allelic expression patterns during hiPSC generation and its differentiation (Fig. 3B). Mono-allelic expression of *H19* and *RTL1* in human fibroblasts was stably conserved in hiPSCs. Robust establishment of mono-allelic expression in a *H19* gene was also observed in hiPSCs during *in vitro* differentiation (Fig. 3B). Allelic expression (average ratio; 0.1795 ± 0.004) of *H19* in hiPSCs exhibited robust mono-allelic expression patterns in MP (0.0235 ± 0.003) and OB (0.0185 ± 0.001) (Fig. 3B). Like in H9-hESCs, a transient instability of allelic expression in *H19* in hiPSC-derived DE (0.208 ± 0.004) was also stabilized in hiPSC-derived HEP (0.0245 ± 0.005). These results imply that mono-allelically expressing imprinted genes tend to be more stabilized in hPSCs during *in vitro* differentiation.

3.3. Allelic expression of bi- and partial-allelically expressing imprinted genes in hPSCs during in vitro differentiation

Several imprinted genes were bi- and partial-allelically expressed in undifferentiated hPSCs (Fig. 4). Among them, *PPP1R9A* and *OSBPL5* are bi-allelically expressed in diverse fetal tissues except the placenta [25,26]. The imprinted genes showed bi-allelic expression in three cell types which were differentiated from hESCs and hiPSCs (Fig. 4A). Another imprinted gene *RTL1*, which is predominantly expressed from the maternal allele in human fibroblasts [27], maintained its partial-expression pattern in H9-hESCs during *in vitro* differentiation (Fig. 4A). Thus, some imprinted genes represented conservation in the allelic expression during *in vitro* differentiation of hPSCs.

Intriguingly, we found dynamic changes of some imprinted genes in the allelic expression during *in vitro* differentiation of hESCs into specialized cell types (Fig. 4B). *KCNK9*, which is predominantly expressed from the maternal allele in the human fetal brain [23], was partial-allelically expressed in hESCs (0.419 ± 0.131) and showed a loss of imprinting in MP (0.891 ± 0.077) during osteoblastic differentiation. However, loss of imprinting status of *KCNK9* in hESC-derived MPs was restored to partial allelic expression in differentiated OBs (0.4875 ± 0.109). *ATP10A*, which is maternally imprinted in the adult human brain [28], was bi-allelically expressed in hESCs. Bi-allelic expression of *ATP10A* in hESCs (0.8225 ± 0.027) was maintained during neuronal differentiation, whereas it was slightly shifted to partial-alleic expression in MP (0.652 ± 0.0312) and OB (0.5838 ± 0.072) during osteoblastic differentiation. Interestingly, *ATP10A* showed a gain of imprinting at the intermediate stage (DE; 0.2648 ± 0.047) and then recovered its allelic expression in hepatocytes (0.8778 ± 0.142). *SLC22A3* was bi-allelically expressed in undifferentiated H9-hESCs (1.042 ± 0.097) and their mesodermal (MP, OB) and endodermal (DE, HEP) lineage cells, but it showed a gain of imprinting status during neuronal differentiation (NP; 0.4343 ± 0.083, DA; 0.35 ± 0.063). These results indicate that a gain or a loss of imprinting occurs in a lineage-specific manner during differentiation of hPSCs into specialized cell types *in vitro*. In particular, imprinted genes (*IGF2R, SLC22A2,* and *SLC22A3*) within human IGF2R domain appear to be more vulnerable to changes of allelic expression in hESCs during *in vitro* differentiation (Fig. 4B and C). It has been known that *SLC22A3* located in the IGF2R domain is maternally expressed in mice [29], whereas it shows polymorphic imprinted status in human placenta [5]. The other imprinted genes (*IGF2R* and *SLC22A2*) in the IGF2R domain also showed a gain and a loss of imprinting status in the CHA-hES4 line during differentiation into endothelial cells and hepatocytes, respectively (Fig. 4C). Partial-allelically expressed *IGF2R* (0.6563 ± 0.0577) in CHA-hES4 showed a gain of imprinting during endothelial cell differentiation (VP; 0.0695 ± 0.081, EC; 0.13 ± 0.090), and *SLC22A2* (0.5243 ± 0.165) represented a loss of imprinting during hepatocyte differentiation (DE; 0.7798 ± 0.081, HEP; 0.7803 ± 0.105). This domain-specific variation gives

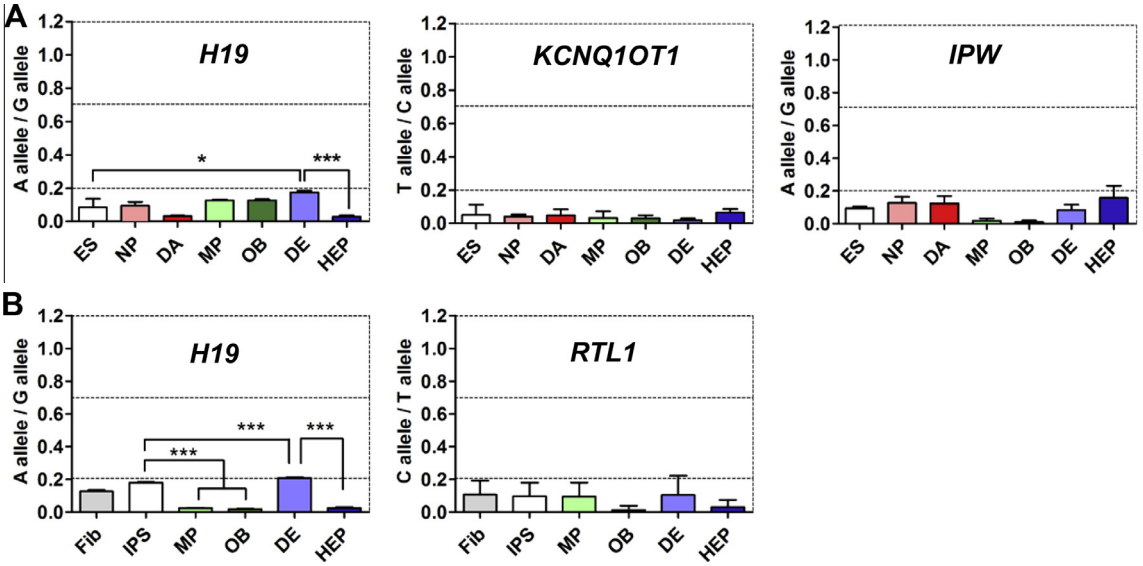

Fig. 3. Expression patterns of mono-allelically expressed imprinted genes in H9-hESCs (A) and hiPSCs (B) during *in vitro* differentiation. Allele-specific expression ratios between two alleles in hPSCs and hPSC-derivatives were calculated from pyrogram; the minor allele peak height was divided by the major allele peak height. The data obtained in triplicates were shown as the mean ± SD ($n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

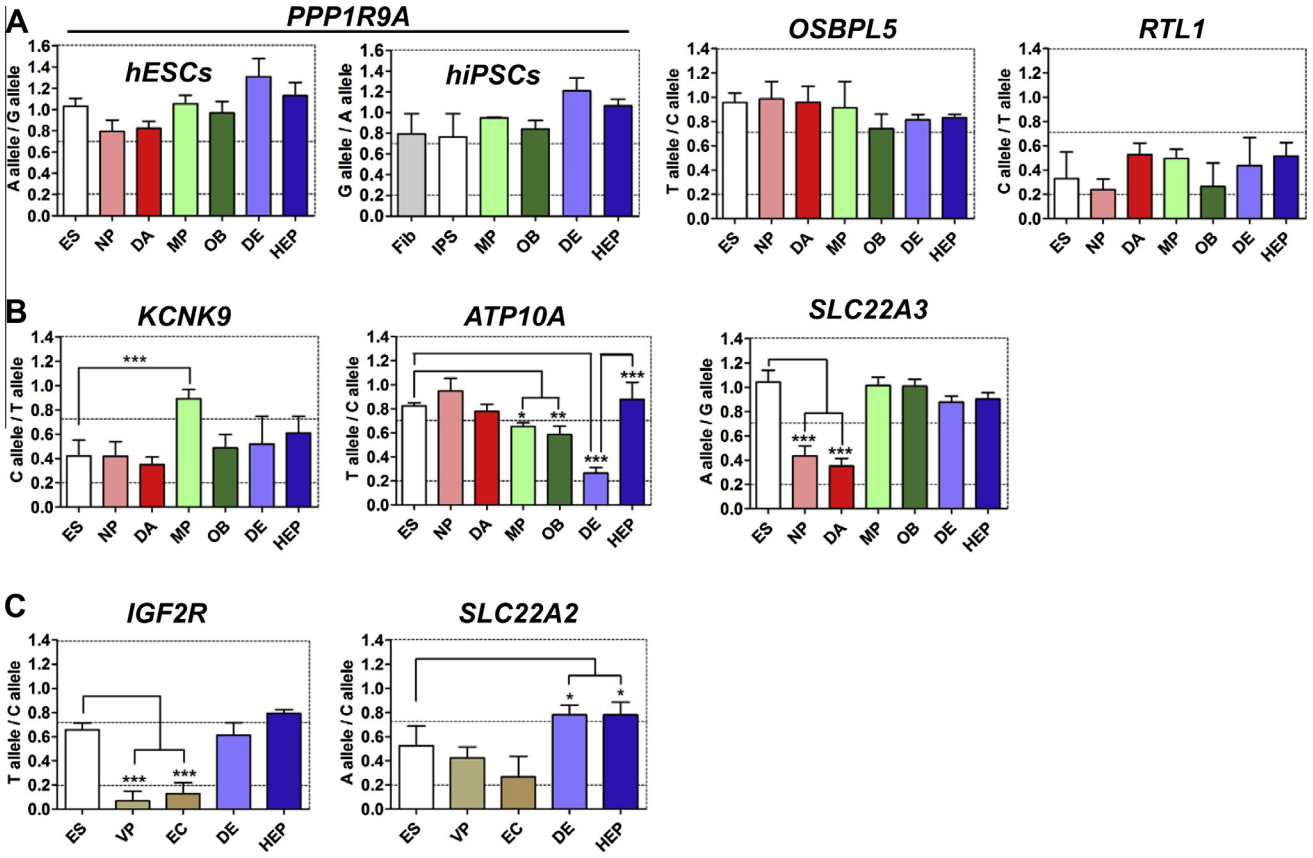

Fig. 4. Expression patterns of bi- or partial-allelically expressed imprinted genes in hPSCs during *in vitro* differentiation. (A) Stable allele-specific expression patterns of *PPP1R9A*, *OSBPL5*, and *RTL1* during *in vitro* differentiation. (B) Changes of allelic expression in some imprinted genes during *in vitro* differentiation. (C) Allelic expression of imprinted genes within the IGF2R domain in CHA-hES4 during *in vitro* differentiation. Abbreviations: ES, undifferentiated CHA-hES4; VP, vascular progenitors; EC, endothelial cells. The data obtained in triplicates were shown as the mean ± SD ($n = 3$, $^{*}p < 0.05$, $^{***}p < 0.001$).

a hint that some imprinted loci may be vulnerable to changes in the allelic expression during human development.

4. Discussion

A series of system for differentiation of hPSCs into a specialized lineage can provide an *in vitro* model system for understanding the genomic imprinting that occurs during human development. Here, we demonstrated that the allelic expression of imprinted genes could be changed in a lineage-specific manner during differentiation of hPSCs into specialized cell types. There are some possible explanations for the vulnerability of allelic expression of imprinted genes in hPSCs during *in vitro* differentiation. Firstly, the epigenetic error may be intrinsic at the imprinting loci in hESCs. The epigenetic variations could be induced by non-physiological culture conditions in hESCs [11,16,30]. Allelic expression and DNA methylation status in the imprinted genes are recurrently aberrant in undifferentiated hESCs and hiPSCs, and their epigenetic states are kept in differentiated hPSC-derivatives such as neural cells [31]. This vulnerable epigenetic status of imprinted genes in hPSCs may impact to cell therapy or *in vitro* modeling for human development. Therefore, various *in vitro* culture conditions, including derivation methods, growth supplements, passage method and/or differentiation protocols, are needed to be standardized to diminish epigenetic variations in hPSCs. In particular, monoallelic expression of *H19* gene is established in peri-implantation stage [11]. Because hESCs are derived from blastocysts, allelic expression pattern of *H19* can be vulnerable in undifferentiated hESCs. In this study, mono-allelic expression pattern of *H19* in undifferentiated hESCs and hiPSCs was loosed in early developmental stage such as definitive endoderm (DE), and then robustly established in differentiated hepatocytes (Fig. 3). Also, DNA methylation states of the H19 DMR were changed in H9-hESCs during hepatic differentiation (Supplementary Fig. 2). In this study, the presence of polymorphisms in the H19 DMR enabled us to distinguish between maternal and paternal alleles. Undifferentiated hESCs or hESC-derived DE cells showed aberrant methylation patterns in which the paternal allele was fully methylated and the maternal allele was predominantly demethylated. However, partial methylation states of the maternal allele were demethylated in hESC-derived hepatocytes. This result indicates that partial disruption of differential DNA methylation status at the H19 DMR may be responsible for loss-of-imprinting of the *H19* gene in DE cells (Fig. 3A). Fluctuations of the allelic expression in early developmental cell types were also observed in *KCNK9* and *ATP10A* in the process of osteoblasts and hepatocytes differentiation, respectively (Fig. 4B). These results indicate that allelic expression of imprinted genes may be vulnerable at intermediate stages in hESCs during *in vitro* differentiation. Secondly, partial gain or loss of imprinting may be responsible for tissue-specific imprinting. Genes are mono-allelically or partial-allelically expressed at certain stages of development in a tissue-specific manner, thereby resulting in determination of the cell fate [32]. In this study, partial gain- and loss-of-imprinting phenomena was also observed in several imprinted genes in a lineage- or tissue-specific manner during *in vitro* differentiation of hPSCs. *OSBPL5* and *PPP1R9A* were paternally imprinted in human placenta, but other tissues such as brain, heart, and liver showed bi-allelic expression [25,26]. In this study, these genes were also bi-allelically expressed in hPSC-derived specialized cell types,

including DA neurons, osteoblasts, and hepatocytes. *KCNQ1OT1* and *IPW*, which are known to be imprinted in a majority of fetal tissues and placenta [5,33], maintained mono-allelic expression patterns in H9 ESCs during *in vitro* differentiation (Fig. 3A). Although allelic expression profiles of some imprinted genes in hPSC-derivatives were coincident with previous results derived from human tissues, other imprinted genes exhibited different allelic expression patterns. *KCNK9*, which is expressed from the maternal allele in the human fetal brain [23], retained partial-allelic expression in H9 hESCs during DA neuronal differentiation. *RTL1*, which is known to be maternally expressed in human fibroblasts [27], represented robust mono-allelic expression in fibroblasts, hiPSCs and the hiPSC-derivatives (Fig. 3B), whereas showed partial-allelic expression patterns in H9 hESCs and their derivatives (Fig. 4A). Thus, we found differences in the allelic expression of *RTL1* between hESCs and hiPSCs. This difference would be due to the intrinsic imprinting property between cell sources or individuals. Allelic expression patterns of certain imprinted genes are still controversial in human tissues. Imprinted genes within IGF2R imprinting domain (*IGF2R*, *SLC22A2*, and *SLC22A3*) are bi-allelically expressed in human placenta and fetal tissues [5]. In this study, imprinted genes within IGF2R domain exhibited bi- or partial-allelic expression patterns in undifferentiated hPSCs (Fig. 4B and C). These results imply that allelic expression of imprinted genes may be different in a cell-type or domain-specific manner. Thus, human pluripotent stem cells would be useful for studying epigenetic mechanisms of imprinting genes during *in vitro* differentiation even though it does not exclude the possibility that the epigenetic vulnerability might be attributed by the cell line variation and/or *in vitro* culture condition.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.02.141.

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