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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.

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).