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Clone- and Gene-Specific Aberrations of Parental Imprinting in Human Induced Pluripotent Stem Cells

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ABSTRACT

Genomic imprinting is an epigenetic phenomenon whereby genes are expressed in a monoallelic manner, which is inherited either maternally or paternally. Expression of imprinted genes has been examined in human embryonic stem (ES) cells, and the cells show a substantial degree of genomic imprinting stability. Recently, human somatic cells were reprogrammed to a pluripotent state using various defined factors. These induced pluripotent stem (iPS) cells are thought to have a great potential for studying genetic diseases and to be a source of patient-specific stem cells. Thus, studying the expression of imprinted genes in these cells is important. We examined the allelic expression of various imprinted genes in several iPS cell lines and found polymorphisms in four genes. After analyzing parent-specific expression of these genes, we observed overall normal monoallelic expression in the iPS cell lines. However, we found biallelic expression of the *H19* gene in one iPS cell line and biallelic expression of the *KCNQ10T1* gene in another iPS cell line. We further analyzed the DNA methylation levels of the promoter region of the *H19* gene and found that the cell line that showed biallelic expression had undergone extensive DNA demethylation. Additionally we studied the imprinting gene expression pattern of multiple human iPS cell lines via DNA microarray analyses and divided the pattern of expression into three groups: (a) genes that showed significantly stable levels of expression in iPS cells, (b) genes that showed a substantial degree of variability in expression in both human ES and iPS cells, and (c) genes that showed aberrant expression levels in some human iPS cell lines, as compared with human ES cells. In general, iPS cells have a rather stable expression of their imprinted genes. However, we found a significant number of cell lines with abnormal expression of imprinted genes, and thus we believe that imprinted genes should be examined for each cell line if it is to be used for studying genetic diseases or for the purpose of regenerative medicine. STEM CELLS *2009;27:2686–2690*

Disclosure of potential conflicts of interest is found at the end of this article.

Genomic imprinting is an epigenetic phenomenon through which gene expression is regulated in a parent-of-origin monoallelic manner. This process is essential for normal mammalian development, and abnormal expression of imprinted genes has been correlated with various human genetic disorders and malignancies [1]. Thus far, expression of imprinted genes has been examined in embryonic stem (ES) cells, and in general, human ES cells show a substantial degree of genomic imprinting stability [2–4]. Recently, human somatic cells were reprogrammed to a pluripotent state using various defined factors [5, 6]. The establishment of these induced pluripotent stem (iPS) cells is both a slow and inefficient process consisting largely of unknown events [5, 6]. To date, experiments using mouse iPS cells as a source of cells in tetraploid complementation assays, although successful, are inefficient [7, 8]. Furthermore, reprogramming of somatic cells by nuclear transfer may also result in abnormal embryogenesis that is partially linked to abnormal imprinting [9]. Because iPS cells are thought to have a great potential for studying many genetic diseases and to be a source of patient-specific stem cells for therapy, it is important to study the expression of imprinted genes in these cells. Given that imprinted gene expression is determined during early embryogenesis, human ES cells, derived from blastocyst stage embryos, should show parental monoallelic expression of their imprinted genes. Nonetheless, iPS cells, which are reprogrammed from human somatic cells, may demonstrate aberrant expression of these genes.

We therefore examined the monoallelic expression of seven different imprinted genes (*H19*, *IPW*, *KCNQ10T1*,

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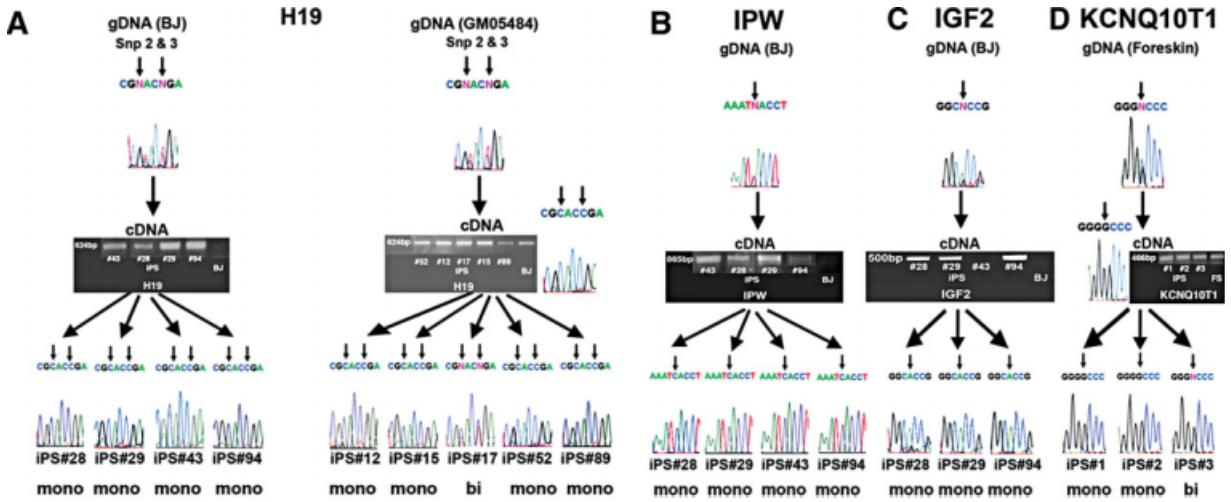

Figure 1. Allele-specific expression of various imprinted genes in induced pluripotent stem (iPS) cell lines. To analyze allelic expression of various genes, polymorphism in the genetic sequence of the genomic DNA (gDNA) was identified by sequencing, and the complimentary DNA (cDNA) of the genes was sequenced to examine monoallelic or biallelic expression. **(A):** A single nucleotide polymorphism (SNP) in the *H19* imprinted gene was observed in both the hTERT-BJ1 and GM05484 fibroblast cell lines. Monoallelic expression was observed in eight of nine iPS cell lines (one GM05484 iPS line showed biallelic expression). **(B):** A SNP was found in the *IPW* gDNA from hTERT-BJ1 cells, with all four iPS cell lines showing normal monoallelic expression. **(C):** A polymorphism in *IGF2* was detected in hTERT-BJ1 cells, with monoallelic expression seen in the iPS cell lines; however, there was an absence of expression in clone #43. **(D):** A SNP in *KCNQ10T1* was found in primary foreskin fibroblasts and monoallelic expression was seen in the somatic cells and two of the three iPS cell lines; the third line showed biallelic expression of the SNP.

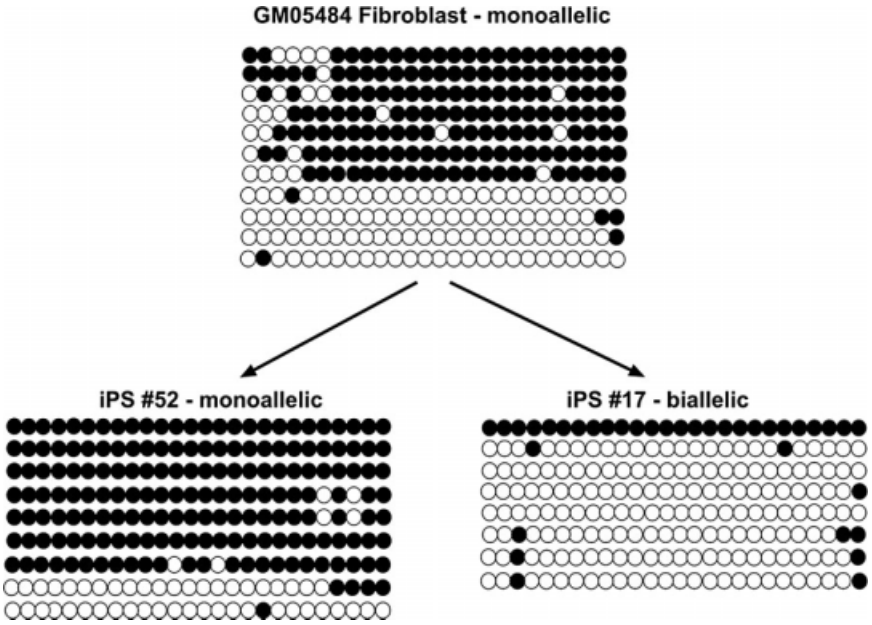

Figure 2. Bisulphite sequencing of differentially DNA methylated regions of the *H19* gene. Circles represent the position and DNA methylation sites of 26 individual CpGs present in the genomic DNA sequence (filled, methylated; open, unmethylated). Abbreviations: iPS, induced pluripotent stem.

IGF2, *NESP55*, *PEG3*, and *SNRPN*) in 12 different iPS cell lines. These iPS cell lines were generated from three independent fibroblast lines: hTERT-BJ1, a line of neonatal foreskin fibroblasts (four iPS cell lines); neonatal primary foreskin fibroblasts (three iPS cell lines); and GM05484, a line of fibroblasts from a 4-year-old child (five iPS cell lines). Generated iPS cell lines showed typical human ES cell-like morphology, expressed stem cell markers such as alkaline phosphatase, Tra-1-60, and Oct3/4, silenced their reprogramming transgenes, and generated teratomas containing all three embryonic germ layers (see supplemental online data, supplemental online Figs. 1, 2, and Urbach A, Bar-Nur O, Daley GQ et al., submitted manuscript). DNA was extracted from low-passage iPS cells and their sequence was analyzed for single nucleotide polymorphisms (SNPs). We found SNPs in three paternally expressed genes—*IPW*, *IGF2*, and *KCNQ10T1*, and in the maternally expressed *H19* gene (Fig. 1). To determine whether iPS cells express these genes in a bi- or monoallelic fashion, cDNA from the cells was sequenced. *H19* was not expressed in the parental, hTERT-BJ1 fibroblasts (passage [p]13); however, its expression in four iPS cell lines (p7) generated from these fibroblasts showed proper monoallelic expression (Fig. 1A). On the other hand, in another fibroblast cell line, GM05484 (p11),

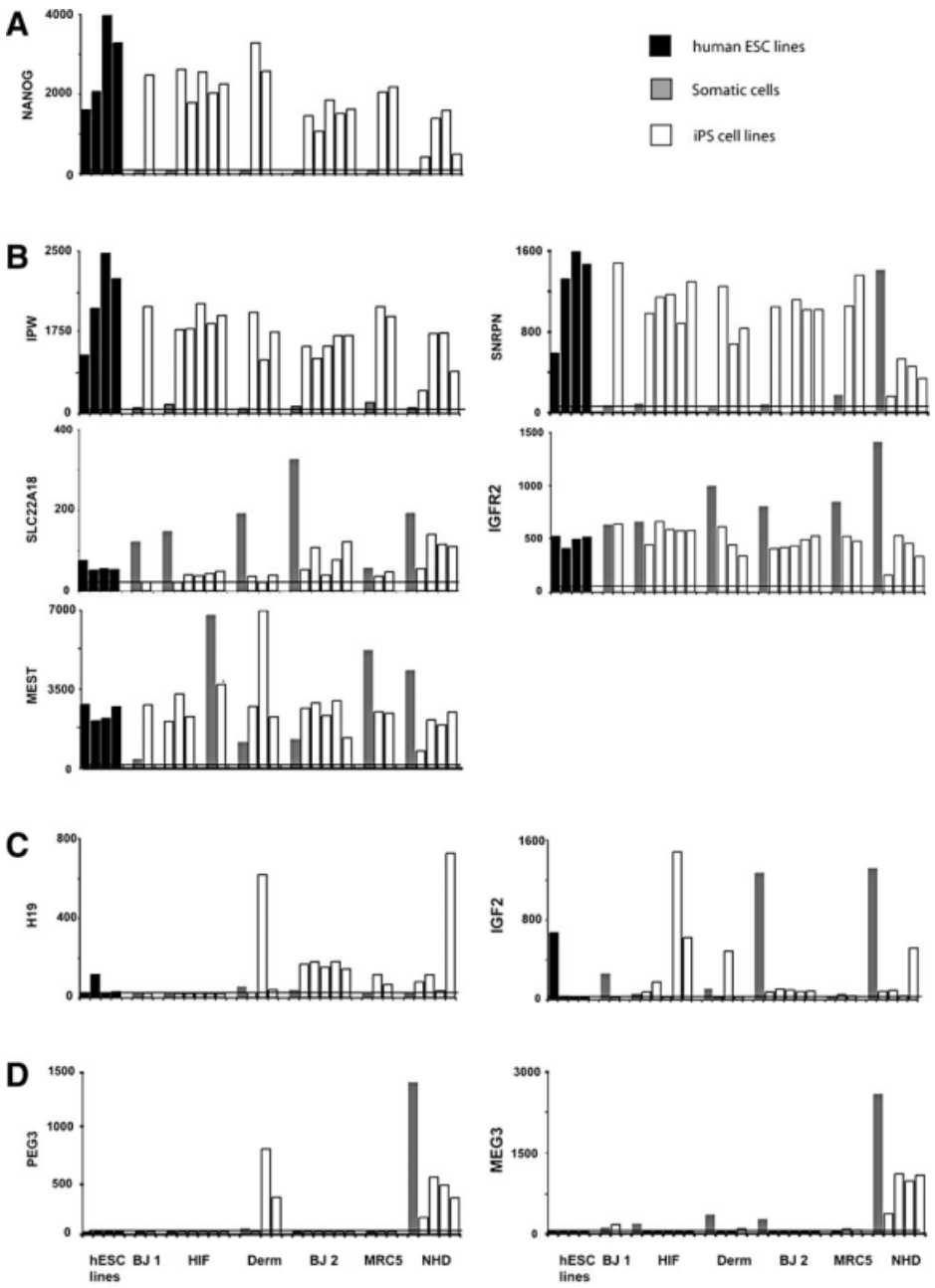

Figure 3. DNA microarray analysis of nine imprinted genes. The expression levels of nine imprinted genes were analyzed for four different human embryonic stem cell (hESC) lines (black bars) and compared with six different fibroblast lines (gray bars) and their derived induced pluripotent stem (iPS) cell lines (open bars). **(A):** Reprogramming of a stem cell-specific gene. **(B):** Imprinted genes that are epigenetically stable during reprogramming. **(C):** Imprinted genes that show variable expressed in pluripotent cells. **(D):** Imprinted genes that are partially epigenetically stable during reprogramming. The *y*-axis represents the relative expression level of each gene. The x-axis groups the data by cell origin. The horizontal line represents negligible expression levels for each gene. BJ1 and BJ2 are two different foreskin lines containing the telomerase gene; HIF indicates human embryonic fibroblasts; Derm indicates human neonatal dermal fibroblasts; MRC5 are primary fetal cells; NHD indicates normal human dermal fibroblasts. The hESC lines where HI-OGN, HUES 8, HSF1, and H9. The BJ1 cell line was originally designated BJ48 and its iPS cell line is BJ-iPS1. The HIF line was originally designated Hif Fib 44 and its iPS cell lines are HIF iPS3, HIF iPS10, HIF iPS20, HIF iPS30, and HIF iPS32, consecutively. Derm was originally designated Derm Fib and its iPS cells lines are hips clone 1, hips clone 2, and hips clone 3. BJ2 was originally designated BJ and its iPS cell lines are BJ hiPS 12, BJ hiPS 5, BJ hiPS 6, BJ hiPS 8, and BJ hiPS AFP 12. MRC5 was originally designated MRC5 40 and its iPS cell lines are MRC5 iPS 2 and MRC5 iPS 22. The NHD fibroblasts were originally designated NHDF and its iPS cell lines are HiPS 1, HiPS 5, HiPS 2, and HiPS 7.

in which *H19* expression was monoallelic in the fibroblasts, one of the five iPS cell lines (p9) showed biallelic expression, suggesting that regulation of *H19* is vulnerable to perturbation (Fig. 1A). In the paternally expressed gene *IPW*, we observed consistent monoallelic expression (Fig. 1B). Interestingly, we observed that the expression of *IGF2* in one of the four iPS cell lines (p7) made from hTERT-BJ1 fibroblasts (p13) was undetectable, which also hints at aberrations in genetic imprinting (Fig. 1C). As for the paternally expressed *KCNQ10T1*, we found relaxation in the genetic imprinting in one iPS cell line (p10), which showed partial biallelic expression (25% minor allele) (Fig. 1D). The heterogeneity observed in *H19* gene expression in the different parental fibroblast cell lines is caused by differences in the somatic cells source of origin and is possibly a result of the variability in transcription and the intrinsic differences among different fibroblast cell lines. This is a cell-specific phenomenon that explains the absence or presence of *H19* gene expression between the BJ-TERT1 and GM05484 fibroblast cells lines.

We further analyzed the DNA methylation levels of the promoter of the *H19* gene and found that the somatic fibroblast cell line GM05484 had both methylated and unmethylated alleles, consistent with the notion of monoallelic expression (Fig. 2). We then analyzed the iPS clone, derived from GM05484, that showed biallelic expression and compared it with an iPS clone that had stable monoallelic expression and found significant DNA demethylation in the *H19* promoter in the biallelic clone (Fig. 2).

To further study the regulation of imprinted genes in iPS cells, the expression patterns of nine imprinted genes were examined by a meta-analysis of previously published genomic expression arrays of 26 iPS cell lines reprogrammed with OCT4, SOX2, KLF4, and c-MYC and generated from six different fibroblast lines in four different laboratories [10–13].

According to the original studies, all iPS cell lines fulfilled the criteria of true reprogrammed human ES-like cells: mainly typical morphology, expression of stem cell markers, differentiation into the various embryonic germ layers, and gene expression profiles similar to those of human ES cells. Four human ES cell lines were used to determine the expected expression levels of these genes and represented the expected expression. NANOG, a stem cell marker, was used to determine typical changes in expression levels during reprogramming (Fig. 3A). One group of genes (*IPW*, *SNRPN*, *SLC22A18*, *IGFR2*, and *MEST*) showed significant epigenetic stability in the levels of expression in human iPS cells, comparable with that observed in human ES cells, whereas their somatic cell counterparts showed varying levels of expression of these genes (Fig. 3B). *H19* and *IGF2* are two imprinted genes sharing the same regulatory region in chromosome 11p15.5. Analyzed data demonstrated a substantial degree of variability in expression in these genes in both human ES cells and iPS cells (Fig. 3C), suggesting that the mechanisms controlling the cluster of imprinted genes on chromosome 11p15.5 might be subject to perturbation. Lastly, and most significantly, we found two imprinted genes, *PEG3* and *MEG3*, to have aberrant expression levels in several iPS cell lines, compared with human ES cells; whereby no expression was detected and thus they were classified as having partial epigenetic instability of imprinted genes (Fig. 3D). We also studied the DNA microarray analysis of fibroblast cells reprogrammed using OCT4, SOX2, LIN28, and NANOG alone or with the addition of KLF4 and c-MYC [14, 15]. These iPS cell lines also showed clone-specific aberrant expression of the *MEG3* gene (supplemental online data, supplemental online Fig. 3).

During their reprogramming, iPS cells do not pass through the normal stages of embryonic development that human ES cells undergo. Nonetheless, most of the iPS cell lines we studied showed normal and stable imprinted gene expression. However, we saw two types of aberrant parental imprinting in iPS cell lines: (a) biallelic expression, which correlated with changes in DNA methylation and was observed in specific iPS cell clones (Fig. 1A, 1D), and (b) abnormally high levels of expression in the imprinted genes *PEG3* and *MEG3*, as compared with human ES cells, present in iPS cell lines generated from particular fibroblasts (Fig. 3D). Aberrant expression of *PEG3* and *MEG3* has been implicated in the onset of various cancers [16–19]. If these iPS cell lines were to be used for regenerative medicine purposes, generation of tumors in the recipient patients could occur. Although stem cell markers, such as NANOG and OCT4, overcome extensive DNA demethylation during the establishment of iPS cells, in general, the expression of imprinted genes remained monoallelic and escaped extensive demethylation. Many iPS cell lines have stable expression of the imprinted genes. This implies that their parental fibroblast cells have the correct signals to facilitate stable monoallelic expression during reprogramming, and that the imprinted gene signals were present in the fibroblasts, even if the imprinted genes themselves were not expressed. Abnormal allelic expression of imprinted genes was previously shown to be correlated with high passage numbers in human ES cells [3]. We observed this abnormal allelic expression in very early passages of iPS cell lines, and thus believe that they are more vulnerable to parental imprinting perturbation than human ES cells. We assume that, in later passages, the iPS cell lines will become even more aberrant, as was seen by Rugg-Gunn et al. [4], whereby human ES cells showed increased biallelic expression in later passages.

In conclusion, our systematic analysis of expression of imprinted genes in iPS cell lines showed aberrations at various levels: (a) abnormal expression in most clones produced during reprogramming of specific fibroblast cell lines (Fig. 3D), (b) clone-specific aberrations (Fig. 1), and (c) gene-specific aberrations, such that some imprinted genes were more sensitive to perturbation of expression than others. We believe that analysis of imprinted genes should be performed in all iPS cell lines because several published iPS cell lines that passed necessary reprogramming criteria showed aberrations in imprinted gene expression (Fig. 3). The analysis of imprinted genes will be vital if iPS cell lines are to be used for regenerative medicine, because aberrations in imprinted genes could cause problems with cell differentiation and perhaps even cause transformation. The analysis of imprinted genes is also essential for modeling of genetic diseases in iPS cell lines because abnormal imprinting can seriously confuse the disease phenotype with that of parental imprinting disorders. We suggest that each cell line should be examined for at least the expression levels of imprinted genes and that this imprinted gene analysis should be included as one of the gold standards to establish true iPS cell lines.

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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