Epigenetic states and expression of imprinted genes in human embryonic stem cells

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AIM: To investigate the epigenetic states and expression of imprinted genes in five human embryonic stem cell (hESC) lines derived in Taiwan.

METHODS: The heterozygous alleles of single nucleotide polymorphisms (SNPs) at imprinted genes were analyzed by sequencing genomic DNAs of hESC lines and the monoallelic expression of the imprinted genes were confirmed by sequencing the cDNAs. The expression profiles of 32 known imprinted genes of five hESC lines were determined using Affymetrix human genome U133 plus 2.0 DNA microarray.

RESULTS: The heterozygous alleles of SNPs at seven imprinted genes, IPW, PEG10, NESP55, KCNQ1, ATP10A, TCEB3C and IGF2, were identified and the monoallelic expression of these imprinted genes except IGF2 were confirmed. The IGF2 gene was found to be imprinted in hESC line T2 but partially imprinted in line T3 and not imprinted in line T4 embryoid bodies. Ten imprinted genes, namely GRB10, PEG10, SGCE, MEST, SDHD, SN-RPN, SNURF, NDN, IPW and NESP55, were found to be highly expressed in the undifferentiated hESC lines and down-regulated in differentiated derivatives. The UBE3A gene abundantly expressed in undifferentiated hESC lines and further up-regulated in differentiated tissues. The expression levels of other 21 imprinted genes were relatively low in undifferentiated hESC lines and five of these genes (TP73, COPG2, OSBPL5, IGF2 and ATP10A) were found to be up-regulated in differentiated tissues.

CONCLUSION: The epigenetic states and expression of imprinted genes in hESC lines should be thoroughly studied after extended culture and upon differentiation in order to understand epigenetic stability in hESC lines before their clinical applications.

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Key words: DNA microarray; Imprinting; Single nucleotide polymorphism; Human embryonic stem cell

INTRODUCTION

Genomic imprinting, the parent-of-origin-specific silencing of genomic sequences, is a characteristic feature of mammalian development and disease susceptibility. It is a mechanism that occurs on a subset of genes to influence phenotypic expression by maternally or paternally expressing particular genes and silencing the opposite allele. This process is highly conserved across species, and there are clear examples of imprinting in a wide range of species. The core components of the imprinting machinery, including the polycomb group proteins (e.g., SUZ12, EZH2, EZH1), the histone methyltransferase complex (e.g., H3K27me3), and the DNA methyltransferase (e.g., DNMT1) play a critical role in maintaining the epigenetic state of imprinted genes. The imprinted genes are typically expressed from one parental allele and silenced from the other, which is referred to as monoallelic expression. However, the exact mechanisms that drive this expression pattern remain unclear.
ing of genes, is an epigenetic modification that gives rise to differential expression of paternally and maternally inherited alleles of some genes. The imprinting is established afresh in the germ line in each generation and stably inherited throughout the somatic cell division\[1\]-\[3\]. Imprinted genes play important roles in human fetal and placental development and aberrant expression of imprinted genes is associated with human diseases including several cancers and a number of neurological disorders such as Prader-Willi syndrome\[4\]-\[8\].

Human embryonic stem cell (hESC) lines were derived from inner cell mass of blastocysts produced by in vitro fertilization using mitotically inactivated mouse embryonic fibroblast cells as feeder layer\[9\]. Thus far, many hESC lines have been derived and characterized (http://www.nih.gov/news/stemcell/). Because of the dual abilities to proliferate indefinitely and differentiate into various cell type derivatives of all three embryonic germ layers, ectoderm, mesoderm and endoderm, the hESC lines could potentially provide an unlimited supply of different cell types for transplantation therapy to treat a variety of degenerative diseases such as Parkinson's disease, spinal cord injury, diabetes and heart failure\[10\]-\[13\].

In vitro fertilization has been reported to increase human diseases caused by aberrant genomic imprinting\[14\] and abnormal imprinting has also been reported in mouse embryonic stem cells\[15\]. Furthermore, a large number of the imprinting genes show discordance of their imprinting states between human and mouse\[16\]. Therefore, it is important to monitor and maintain epigenetic stabilities in hESC lines for transplantation purposes\[17\]. However, little is known about the epigenetic states and the expression profiles of imprinted genes in hESC lines following extended culture and upon differentiation. In the present study, the allele-specific expressions of seven imprinted genes in five hESC lines derived in Taiwan\[17\] were investigated using single nucleotide polymorphism (SNP) markers. In addition, the expression profiles of 32 known imprinted genes\[18\] in undifferentiated state and some of differentiated derivatives of these five hESC lines were analyzed using DNA microarray.

**MATERIALS AND METHODS**

**Allele-specific expression of imprinted genes**

Genomic DNAs (gDNA) were isolated using the Wizard SV Genomic DNA Purification System (Promega) and total RNAs were extracted using the Absolutely RNA NanoPrep Kit (Stratagene) from undifferentiated cells, embryoid bodies and/or teratomas of hESC lines. The cDNAs were synthesized using the 'Microarray' Target Amplification Kit and purified with 'Microarray' Target Purification Kit (Roche Applied Science). Polymerase chain reaction (PCR) amplification of genomic DNA and cDNA was carried out in a 25 µL reaction volume with 2 units of the Go Taq Flexi DNA polymerase (Promega), 1x supplied reaction buffer, 0.12 umol/L of each primer, 0.75 mmol/L MgCl₂, 0.2 mmol/L of dNTPs and 10-200 ng DNA template. Cycle conditions are as follows: initial denaturation at 95°C for 2 min then 30 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 3 min followed by a final extension at 72°C for 5 min. Primer sequences are given in Table 1. Amplified DNA was purified using the Wizard SV Gel and PCR Clean-up System (Promega) and sequenced with the BigDye terminator cycle Sequencing Kit (3.1 version) and ABI 3730 DNA sequencer.

**Expression profiling of imprinted genes in human embryonic stem cell lines**

Five hESC lines were derived with IRB approval from surplus blastocysts in Taiwan and continuously cultured on mitotically inactivated mouse embryonic fibroblast feeder layer for more than 44 passages. hESC lines T1 and T3 possess normal female karyotypes whereas lines T4 and T5 are normal male but line T2 is male trisonomy 12 (47,XY,+12). hESC lines T1, T2, T3 and T5 were able to produce teratomas in SCID mice and line T4 could only form embryoid bodies in vitro. Expression profiles of imprinted genes from undifferentiated hESC lines, embryoid bodies and teratoma were analyzed using Affymetrix human genome U133 plus 2.0 GeneChip containing 54675 probe sets for 47,400 transcripts and variants including 38,500 well-characterized human genes, as previously reported\[17\]. It may be noted that Affymetrix GeneChip expression analysis can be used as a stand-alone quantitative comparison since the correlation between Affymetrix GeneChip results and TagMan RT-qPCR results was shown in a good linearity of R² = 0.95 by the MicroArray Quality Control Study, a collaborative effort of 137 scientists led by the US-FDA\[16\].

**RESULTS**

**Allele-specific expression of imprinted genes**

In order to distinguish mRNA transcripts from each parental allele, the potential SNPs of the 32 known imprinted genes\[18\] were researched from the literature\[19\] and SNP database of NCBI. The heterozygous alleles of SNPs at seven genes, IPW, Peg10, Nesp55, Kcnq1, Atp10a, Tceb3c and Igf2 genes, were found by sequencing gDNA of hESC lines (Table 2). The gDNA of hESC lines T1 and T2 exhibited T and C alleles of IPW gene whereas the cDNA sequencing of undifferentiated cells and teratomas from hESC lines T1 and T2 showed only T allele of IPW gene (Figure 1). The genomic DNA from hESC lines T2 and T3 exhibited C and T alleles of Peg10 gene whereas the cDNA sequencing of undifferentiated differentiated hESC T2 and T3 cells, as well as hESC line T2 teratoma, showed only C allele of Peg10 gene. The T and C alleles of Nesp55 gene were identified in the genomic DNA of hESC line T1 whereas the cDNA from hESC line T1 teratoma (TT1) showed only T allele of Nesp55 gene. The G and A alleles of Kcnq1 gene were identified in genomic DNA of hESC line T3 whereas only G allele of Kcnq1 was found in the sequencing cDNA from undifferentiated hESC line T3 cells. The C and G alleles of Atp10a gene
Table 1  Polymerase chain reaction primer sequences and polymorphisms

| Gene     | Primer sequence (5′→3′)                                                                 | Size (bp) | SNP       | Acc. No | Location     | Ref. |
|----------|-----------------------------------------------------------------------------------------|-----------|-----------|---------|--------------|------|
| IGF2     | F CTTGACCTTTGACTCAAAATTCG; R CCCTTCTGTTCTTTACGGG                                                                 | 235       | G→A      | T07866  |              | [16] |
| IPW      | F GGGAGACTTCTGAGTAACTTTCA; R TGAGGAGAAGGGGGTGGTT                                                                 | 1550      | C→T      | U12897  | Pos. 820     | [16] |
| NESP55 gDNA | F TCATTTTCCTGCCTGGTTG; R CAGAAGATGGAGGAGTCG                                                                 | 868       | T→C      | M21741  | Pos. 1670    | [16] |
| NESP55 cDNA | F TCAGAGATGACGAGTAAG; Seq. GCACCGAAAGGGGGTGAGTCG                                                                 | 233       |          | 2.929   |              | [16] |
| PEG10 gDNA  | F TCATTTCCTCTGGCTGTTG; R TGAGGAGAAGGGGGTGGTT                                                                 | 405       | C→T      | XM_496707 | Pos. 4404    | [16] |
| KCNQ1 gDNA | F CACTGCCTGCAACGGCC; R GTGAGGAGAAGGGGGTGGTT                                                                 | 282       | G→A      | AJ006345 | Pos. 333010  | [16] |
| KCNQ1 cDNA | F GCAGCTGAGAGGAGGAGACT; R GGAGCCTCTTGACCTTTCT                                                                 | 282       |          |         |              | [16] |
| ATP10A    | F AAAGACACCACCGACAGGAA; R TTCGGATCTGACCACGCGCA                                                                 | 318       | G→C      | BC052251 | Pos. 4006    | [16] |
| TCEB3C    | F CCAAGCTGAGAGGAGATTG; R TTTCCGCGGAGACGAGTTG                                                                 | 249       | C→G      | NM_145653 | Pos. 772     | This study |

were found in the genomic DNA of hESC line T2 whereas the sequencing cDNA products from undifferentiated cells and teratoma of hESC line T2 showed only C allele of ATP10A gene. The G and C alleles of TCEB3C gene were found in the gDNA of hESC lines T1 whereas only G allele of TCEB3C gene was identified in the cDNAs of hESC line TT1. These results clearly demonstrated the monoallelic expression of six imprinted genes, IPW, PEG10, NESP55, KCNQ1, ATP10A and TCEB3C, in undifferentiated hESC lines and/or differentiated derivatives. As to IGF2 gene, the A and G alleles were identified by sequencing genomic DNA of hESC lines T2 and T3 whereas the cDNA sequencing of undifferentiated cells and teratoma from hESC line T2 showed only A allele. However, the cDNA of undifferentiated cells from hESC line T3 detected the full expression of G allele and partial expression of A allele, indicating the partially relaxed imprinting of IGF2 gene. Furthermore, the embryoid bodies of hESC line T4 (EB4) showed equal expression of both A and G alleles, indicating no imprinting of IGF2 gene.

Expression profiles of imprinted genes in human embryonic stem cell lines

Expression profiles of the 32 known imprinted genes [16] from five undifferentiated hESC lines, T4 embryoid bodies (EB4) and TT1 were analyzed using Affymetrix human genome U133 plus 2.0 GeneChip and the results are given in Table 3. Ten imprinted genes, namely GRB10, PEG10, SGCE, MEST, SDHD, SNRPN, SNURF, NDN, IPW and NESP55, were found to be highly expressed in the undifferentiated hESC lines and down-regulated in differentiated derivatives (EB4 and TT1) (Table 3 top). The UBE3A gene abundantly expressed in undifferentiated hESC lines and further up-regulated in differentiated tissues (EB4 and TT1). The expression levels of other 21 imprinted genes were relatively low in undifferentiated hESC lines and six of them (TP73, COP2G, O9BLPL5, IGF2, ATP10A and PEG) were found to be up-regulated in differentiated tissues (EB4 and TT1) (Table 3 bottom).

DISCUSSION

The five hESC lines were previously derived with IRB approval in Taiwan and hESC lines T1, T2, T3 and T5 were able to produce teratomas in SCID mice while hESC line T4 could only form embryoid bodies in vitro [17]. In this investigation, the monoallelic expression of six imprinted genes (IPW, PEG10, NESP55, KCNQ1, ATP10A and TCEB3C) was confirmed in undifferentiated hESC lines and/or differentiated teratomas. The monoallelic expression of PEG10, NESP55 and KCNQ1 genes was also reported previously in hESC lines [18,19]. However, the IGF2
gene was found to be imprinted in hESC line T2 but partially imprinted in hESC line T3 and not imprinted in hESC line EB4. The different extents of \( \text{IGF2} \) imprinting among different hESC lines might be due to different developmental stages of blastocysts at which hESC lines were derived. The molecular mechanism responsible for
this variability of imprinting remains to be elucidated. The IGF2, as well as H19 in the same chromosomal region 11p15.5, was also reported to be more variable and thus could potentially provide a sensitive indication of epigenetic status of hESC lines \[23\]. The IGF2 gene was also shown to be only partially imprinted in human germ cell-derived lines \[23\]. It may be noted that the IGF2 gene was found to be highly expressed in differentiated derivatives, namely hESC lines EB4 and TT1 (see EB4 and TT1 in Table 3).

The expression of imprinted genes plays important roles during early embryo development \[6,8\]. hESC lines and their differentiated derivatives offer an opportunity for studying the expression of different imprinted genes shortly before and after the embryonic implantation. In this investigation, using DNA microarray we analyzed the expression profiles of 32 known imprinted genes \[14\] in five undifferentiated hESC lines derived in Taiwan and some of their differentiated derivatives. The expression levels of these 32 imprinted genes were relatively consistent among five hESC lines. It may be noted that five (SNRPN, SNURF, NDN, IPW and UBE3A) of eleven highly expressed imprinted genes in undifferentiated hESC lines are located on chromosomal region 15q11-q13 (Table 3) and that abnormal expression of SNRPN and NDN genes results in the neurogenetic disorder known as Prader-Willi Syndrome \[23\]. In short, the epigenetic states and expression of imprinted genes in hESC lines should be thoroughly studied after extended culture and upon differentiation in order to understand epigenetic stability in hESC lines before their clinical applications \[24\].

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**COMMENTS**

**Background**

Human embryonic stem cell (hESC) lines possess the dual abilities to proliferate indefinitely and differentiate into various cell types in the body. Thus, hESC lines could potentially provide an unlimited supply of different cell types for transplantation therapy. Genomic imprinting is established anew in the germ cells in each generation and stably inherited throughout the somatic cell divisions.

**Research frontiers**

The imprinted genes play important roles in human fetal and placental development and aberrant expression of imprinted genes is associated with human diseases. Therefore, it is important to monitor and maintain epigenetic

| Genes       | T1  | T2  | T3  | T4  | T5  | EB4 | TT1 | Probe ID | Chromosome | Exp. allele |
|-------------|-----|-----|-----|-----|-----|-----|-----|---------|------------|-------------|
| GRB10       | 2543| 3249| 3128| 3778| 5927| 68  | 96  | 209409_at| 7p12-p11.2 | P/M         |
| PEG10       | 1602| 1080| 1710| 2033| 3255| 1330| 683 | 212094_at| 7q21-q22   | P           |
| SGCE        | 1256| 1620| 1725| 751 | 501 | 250 | 737 | 204688_at| 7q21-q22   | P           |
| MEST        | 7066| 7416| 2609| 8727| 581 | 48  | 28  | 202016_at| 7q32       | P           |
| SDHD        | 2702| 4707| 2324| 4849| 4079| 104 | 235 | 202026_at| 11q23      | P           |
| SNRPN       | 4819| 3228| 6721| 5534| 5904| 2895| 1571| 228670_at| 15q11.2-q12| P           |
| SNURF       | 42361| 58257| 2721| 64033| 57468| 11 | 159 | 201522_at| 15q11.2-q12| P           |
| NDN         | 458 | 932 | 735 | 734 | 334 | 8   | 49  | 209350_at| 15q11.2-q12| P           |
| IPW         | 2653| 2848| 1842| 4685| 8969| 113 | 328 | 213447_at| 15q11.2-q12| P           |
| GNAT-NESP5S | 30303| 49688| 45911| 55643| 46465| 66  | 905 | 212273_x_at| 15q11.2-q12| P           |
| UBE3A       | 4119| 2012| 3125| 2362| 4491| 7812| 6646| 211285_s_at| 15q11.2-q12| P           |
| HYMAI       | 60  | 196 | 76  | 16  | 110 | 113 | 401 | 215513_at| 6q24       | P           |
| PLAC1       | 35  | 12  | 22  | 5   | 18  | 79  | 235 | 1559028_at| 6q24       | P           |
| WTI         | 90  | 25  | 56  | 9   | 126 | 31  | 129 | 206067_s_at| 11p13      | P           |
| KCNQ4DN     | 89  | 78  | 31  | 17  | 62  | 15  | 13  | 220829_at| 11p15.4     | P           |
| KCNQ1       | 232 | 552 | 25  | 170 | 28  | 89  | 159 | 204487_at| 11p15.5     | M           |
| SLC22A18    | 274 | 31  | 39  | 519 | 72  | 13  | 134 | 204981_at| 11p15.5     | M           |
| PHLDA2      | 203 | 131 | 149 | 189 | 131 | 66  | 117 | 208082_at| 11p15.5     | M           |
| H19         | 189 | 130 | 22  | 95  | 12  | 3   | 107 | 224997_x_at| 11p15.5     | M           |
| CDKN1C      | 89  | 30  | 17  | 125 | 19  | 119 | 938 | 213183_s_at| 11p15.5     | M           |
| DLK1        | 13  | 461 | 38  | 1049| 8   | 23  | 32  | 209560_s_at| 14q32       | P           |
| MECP3       | 38  | 125 | 215 | 87  | 12  | 40  | 10  | 226213_at| 14q32       | P           |
| HBB1-A3T    | 97  | 101 | 60  | 128 | 168 | 25  | 1091| 214134_at| 15q11.2-q12| P           |
| MAGEL2      | 22  | 26  | 7   | 22  | 17  | 40  | 125 | 219894_at| 15q11.2-q12| P           |
| MKRN3       | 82  | 71  | 55  | 168 | 148 | 85  | 304 | 206585_at| 15q11.2-q12| P           |
| TCEB3C      | 15  | 135 | 13  | 5   | 144 | 49  | 548 | 1552860_at| 18q21.1     | M           |
| TP73        | 136 | 62  | 13  | 62  | 78  | 1241| 1058| 223546_at| 1p36.3      | M           |
| COGP2       | 50  | 75  | 15  | 45  | 106 | 1040| 737 | 222298_at| 7q32        | P           |
| OSBPL5      | 240 | 160 | 170 | 509 | 526 | 1210| 1895| 230734_at| 11p15.4     | P           |
| IGF2        | 20  | 17  | 67  | 12  | 20  | 2596| 2014| 210881_at| 11p15.5     | P           |
| ATPI10A     | 138 | 248 | 105 | 198 | 98  | 134 | 1081| 212456_at| 15q11.2     | M           |
| PEG3        | 72  | 44  | 57  | 14  | 134 | 808 | 479 | 209242_at| 19q13.4     | P           |
stabilities in hESC lines before their clinical applications.

Innovations and breakthroughs
Six of seven imprinting genes were shown to be fully imprinted but the extent of IGF2 imprinting was found to be varied between different hESC lines. The observed variability of IGF2 imprinting adds to the overall picture of genomic stability of imprinting genes among hESC lines. The IGF2 gene was further found to be highly expressed in differentiated derivatives.

Applications
The IGF2 could potentially provide a sensitive indication of epigenetic status of hESC lines. The epigenetic stability of hESC lines should be fully understood before their medical applications.

Terminology
Genomic imprinting: genomic imprinting is an epigenetic modification that gives rise to differential expression of paternally and maternally inherited alleles of some genes. hESC lines: hESC lines were derived from inner cell mass of blastocysts before their medical applications.

Peer review
Imprinting and epigenetic stability of hESCs is an important issue in the field and, as such, the research performed is important. Although imprinting has previously been studied in hESCs, the observed variability between different hESC lines adds to the overall picture of variations in imprinting amongst hESC lines.

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