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STEM CELL BIOLOGY

Dynamic expression patterns of imprinted genes in human embryonic stem cells following prolonged passaging and differentiation

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Abstract
Purpose To evaluate the overall expression patterns of imprinted genes in human embryonic stem cells following long term culture and differentiation.
Materials and methods Expression levels of 65 imprinted genes determined by PCR array were analyzed in one human embryonic stem cell line (cHES1) following prolonged passaging and differentiation.
Results Transcripts of 63 imprinted genes were detected in cHES1 cells. Expression levels of all but 5 imprinted genes did not correlate with passage numbers or differ in cells after passage 50 compared with those before passage 50. SLC22A2, SLC22A3, CPA, H19, COPG2IT1 and IGF2 expression were significantly increased in embryoid bodies compared with undifferentiated cells.
Conclusions The global expression profiles of imprinted genes are generally stable in human embryonic stem cells after prolonged passaging and differentiation.

Xiuyun Mai and Qingyun Mai contributed equally to this work.

Capsule Transcriptional levels of imprinted genes are generally stable in human embryonic stem cells following extended culture and upon differentiation.

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Keywords Embryoid bodies · Epigenetic · Human embryonic stem cells · Imprinted gene

Introduction

Human embryonic stem (hES) cells carry the potential to differentiate into all known types of somatic cells in living organisms and capacity to self renew indefinitely [1, 2]. Thus hES hold great promise for cell replacement therapies. Embryonic stem (ES) cells are derived from pre-implantation embryos, a stage characterized by global epigenetic remodeling [3]. Genomic imprinting is a unique epigenetic phenomenon, which refers to silencing of one parental allele in somatic nucleus depending upon the parent of origin, eventually resulting in allele specific expression. Although imprinted genes comprise a small subset of the human genome, they have been shown to have profound effects on cell behavior and play important roles in fetal and behavioral development [4]. Differential epigenetic/chromatin modifications occur during gametogenesis and preimplantation development. Thus the epigenetic status of ES cells may be disturbed due to embryo manipulation by assisted reproductive technology (ART) or derivation and in vitro culture of ES cells. Inappropriate expression of imprinted genes correlates with several human pathologies, including cancers [5–7]. Therefore investigation on imprinting status of hES cells is crucial for their use in regenerative medicine.

It has been reported that ART actually destroys the imprinting status of some genes in mammals such as human oocytes [8], sheep [9] and mouse [10] embryos. Furthermore, imprinted genes in mouse and rhesus monkey ES

cells were documented to be perturbed [11, 12]. Research on a small number of imprinted genes showed that hES cells have a more stable imprinting status compared with mouse ES cells [13]. Kim examined allele specific expression of 22 imprinted genes in hES cells and found that 14 imprinted genes showed monoallelic while 8 genes showed biallelic expression in the majority of hES cell lines. This result suggests that there are specific genes which are vulnerable to imprinting variability in hES cells [14]. However, the dynamic alteration ofexpression of these genes during prolonged passage was not recorded in Kim's study.

ES cells could form embryoid bodies (EBs) in vitro, attachment of EBs to the adhesive substrate is accompanied by subsequent differentiation into various somatic tissues [15]. Since EBs represent the common initial stage of lineage specific differentiation in hES cells, the investigation on imprinted genes in EBs would be essential to the potential use of hES cells in cell therapies. Additionally, ES cells are derived from inner cell mass of blastocyst, their differentiation into EBs, which comprise three embryonic germ layers resembles events that occur in vivo shortly before and after the embryonic implantation [16]. Thus, study on imprinted genes of EBs will also help to understand the epigenetic regulatory mechanisms in early human development. Whereas little is known about epigenetic factors accompanying proliferation and differentiation of hES cells, a recent data reported expression levels of a limited amount of imprinted genes in hES cells derived EBs [17], However, the mRNA levels of the majority of imprinted genes in EBs still remain obscure.

Our study selected 65 genes (27 maternally and 38 paternally expressed genes) which cover the majority of currently known imprinted genes in humans. Expression levels of imprinted genes were examined simultaneously by real-time quantitative reverse transcriptase-polymerase chain reaction (Q-RT-PCR) using PCR array in the human embryonic stem cell line cHES1. Changes in dynamic expression of imprinted genes were tested during long-term culture of hES cells to evaluate the disruption of imprinted genes by in vitro culture. Expression profiles of imprinted genes were also determined in EBs at day 14 to investigate the epigenetic status in the early stage of hES cells differentiation.

Materials and methods

Culture and in vitro differentiation of hES cells

One hES cell line, which was derived in our center was cHES1, 46XX. Methods of in vitro passage and differentiation into EBs at day 14 were performed as previously reported [18]. Undifferentiated cells at different passages (p27, p37, p47, p57, p67 and p77) and hES (p27, p37 and p47) derived EBs at day 14 were collected for mRNA level examination by Q-RT-PCR using PCR array.

Quantitative real time reverse transcription-PCR

Samples were derived into 3 groups (Undifferentiated hES cells before passage 50, undifferentiated hES cells after passage 50, hES cells derived EBs at day 14). Each group has 3 independent samples (biological replicates). Each sample was repeated twice. For undifferentiated cHES1 cells and cHES1 derived EBs, total RNA was extracted and Dnase digested using the RNeasy Mini Kit (Qiagen). 1.5 μg of total RNA was reverse transcribed (RT) into cDNA using SuperScript III Reverse Transcriptase (Invitrogen), and oligo(dT) 18(Promega). For quantitative real-time PCR (qPCR, we used RT^2 Profiler Custom PCR Array from SuperArray Bioscience to simultaneously assay the mRNA levels of 69 genes including four housekeeping genes (B2M, RPL13A, GAPDH and HPRT1) according to the recommendations of the manufacturer. The gene list focused on those with affirmative imprinting status (27 maternally and 38 paternally expressed genes), excluding predicted, conflicting, provisional and unknown data (listed at least on one of the two web sites: http://WWW.geneimprint.com; http://WWW.otago.ac.nz, Table 1). The selected genes cover the majority of currently known imprinted genes in humans. Thermal cycling was carried out with a 10 min denaturation step at 95°C, followed by 40 two-step cycles: 15 s at 95°C and 60 s at 60°C. Each reaction included 40 ng of total RNA and the proper negative controls (no reverse transcription, no template), positive PCR control and genomic DNA control. Amplification data was collected by the ABI PRISM 7900 and analyzed by the Sequence Detection System 2.0 software (ABI). After completion of these cycles, melting–curve data were then collected to verify PCR specificity. Array results showed that expressions of 4 candidate housekeeping genes (HKG) did not change significantly in hES cells under the experimental conditions (in vitro culture and differentiation); see supplementary Fig. 1. Thus, GAPDH, B2M, HPRT1 and RPL13A were all chosen as suitable reference genes for quantitative gene expression analysis. Threshold cycle numbers (Ct) for each imprinted gene from different samples were determined and standardized to the average HKG value ($\triangle$Ct = Gene Ct- average HKG Ct), The values were then compared between groups using the $2^{-\triangle\triangle Ct}$method [19]. RT-qPCR was also carried out on total RNA extracted from the mouse feeders to confirm the absence of reactivity with mouse cDNA.

Table 1 Homo sapiens imprinted genes included in PCR array

Gene symbol	RefSeq[a]	Description[a]	Expressed allele[b]
TP73	NM_005427	Tumor protein p73	maternal
SLC22A2	NM_003058	solute carrier family 22 (organic cation transporter), member 2	maternal
SLC22A3	NM_021977	solute carrier family 22 (extraneuronal monoamine transporter), member 3	maternal
TFPI2	NM_006528	tissue factor pathway inhibitor 2	maternal
PPP1R9A	NM_017650	protein phosphatase 1, regulatory (inhibitor) subunit 9A	maternal
DLX5	NM_005221	distal-less homeobox 5	maternal
CPA4	NM_016352	carboxypeptidase A4	maternal
KLF14	NM_138693	Kruppel-like factor 14	maternal
KCNK9	NM_016601	potassium channel, subfamily K, member 9	maternal
H19	NR_002196	imprinted maternally expressed transcript (non-protein coding)	maternal
KCNQ1	NM_000218	potassium voltage-gated channel, KQT-like subfamily, member 1	maternal
KCNQ1DN	XR_039806	KCNQ1 downstream neighbor	maternal
CDKN1C	NM_000076	cyclin-dependent kinase inhibitor 1C	maternal
SLC22A18	NM_183233	solute carrier family 22, member 18	maternal
PHLDA2	NM_003311	pleckstrin homology-like domain, family A, member 2	maternal
OSBPL5	NM_020896	oxysterol binding protein-like 5	maternal
MEG3	NR_002766	maternally expressed 3 (non-protein coding)	maternal
UBE3A	NM_000462	ubiquitin protein ligase E3A	maternal
ATP10A	NM_024490	ATPase, class V, type 10A	maternal
TCEB3C	NM_145653	transcription elongation factor B polypeptide 3C	maternal
ZNF264	NM_003417	zinc finger protein 264	maternal
GNAS	NM_000516	GNAS complex locus	maternal
BLCAP	NM_006698	bladder cancer associated protein	maternal
CALCR	NM_001742	calcitonin receptor	maternal
PRIM2	NM_000947	primase, DNA, polypeptide 2 (58 kDa)	maternal
SLC22A18AS	XR_041459	solute carrier family 22 (organic cation transporter), member 18 antisense	maternal
ZNF597	NM_152457	zinc finger protein 597	maternal
DIRAS3	NM_004675	DIRAS family, GTP-binding RAS-like 3	paternal
NAP1L5	NM_153757	nucleosome assembly protein 1-like 5	paternal
PLAGL1	NM_002656	pleiomorphic adenoma gene-like 1	paternal
HYMAI	NR_002768	hydatidiform mole associated and imprinted	paternal
MESTIT1	NR_004382	MEST intronic transcript 1 (non-protein coding)	paternal
COPG2IT1	NR_024086	COPG2 imprinted transcript 1 (non-protein coding)	paternal
DDC	NM_000790	dopa decarboxylase (aromatic L-amino acid decarboxylase)	paternal
GRB10	NM_005311	growth factor receptor-bound protein 10	paternal
SGCE	NM_003919	sarcoglycan, epsilon	paternal
PEG10	NM_001040152	paternally expressed 10	paternal
MEST	NM_177525	mesoderm specific transcript homolog	paternal
DLGAP2	NM_004745	discs, large (Drosophila) homolog-associated protein 2	paternal
INPP5F	NM_014937	inositol polyphosphate-5-phosphatase F (INPP5F)	paternal
KCNQ1OT1	NR_002728	KCNQ1 overlapping transcript 1 (non-protein coding)	paternal
IGF2	NM_000612	insulin-like growth factor 2 (somatomedin A)	paternal
IGF2AS	NM_016412	insulin-like growth factor 2 antisense	paternal
INS	NM_000207	insulin	paternal
WT1	NM_024425	Wilms tumor 1	paternal
DLK1	NM_003836	delta-like 1 homolog (Drosophila)	paternal
SNORD109A	NR_001295	small nucleolar RNA, C/D box 109A	paternal
SNRPN	NM_022808	small nuclear ribonucleoprotein polypeptide N	paternal
MKRN3	NM_005664	makorin ring finger protein 3	paternal

Table 1 (continued)

Gene symbol	RefSeq[a]	Description[a]	Expressed allele[b]
MAGEL2	NM_019066	MAGE-like 2	paternal
NDN	NM_002487	necdin homolog (mouse)	paternal
SNURF	NM_005678	SNRPN upstream reading frame	paternal
SNORD107	NR_001293	small nucleolar RNA, C/D box 107	paternal
SNORD64	NR_001294	small nucleolar RNA, C/D box 64	paternal
SNORD108	NR_001292	small nucleolar RNA, C/D box 108	paternal
SNORD109B	NR_001289	small nucleolar RNA, C/D box 109B	paternal
ZIM2	NM_015363	zinc finger, imprinted 2	paternal
PEG3	NM_006210	paternally expressed 3	paternal
SANG	NR_002785	GNAS antisense RNA (non-protein coding)	paternal
NNAT	NM_181689	neuronatin	paternal
L3MBTL	NM_015478	l(3)mbt-like (Drosophila)	paternal
IPW	NR_023915	imprinted in Prader-Willi syndrome (non-protein coding)	paternal
PSIMCT-1	NR_003677	MCTS1 pseudogene	paternal
LRRTM1	NM_178839	leucine rich repeat transmembrane neuronal 1	paternal
WIF1	NM_007191	WNT inhibitory factor 1	paternal

Refseq NCBI reference sequences

[a] Pattern was shown according to www.ncbi.nlm.nih.gov/gene

[b] Expressed allele of the specific gene was shown according to WWW.geneimprint.com; WWW.otago.ac.nz

Statistical analysis

Pearson's or Spearman's correlation coefficient test was used to analyze the correlations among levels of gene expression and passage numbers. One way ANOVA or Student's *t* test was used for the analysis of gene expression. A *P* value of <0.05 was considered significant. All statistical analyses were performed with Excel X and SPSS15.0.

Results

Imprinted genes detected in undifferentiated hES cells

We showed that paternally expressed genes INS and MKRN3 were not detected in all the passages of hES cells. The other 63 (96.9%) imprinted genes were present in hES cells (Table 2). 27(100%) maternally and 36 (94.7%) paternally expressed genes were detected in hES cells. All the examined genes were not detected in MEFs, confirming no reaction of feeder cDNA (Data not shown).

Transcriptional levels of imprinted genes in undifferentiated hES cells during long term culture

Transcriptional levels of INPP5F (r^2=0.8506, P<0.01) and KCNQ1OT1 (r^2=0.9504, P<0.01) showed negative correlation with passage numbers, while NNAT (r^2=0.6995, P<0.05) showed positive correlation with passage numbers (Fig. 1a, b, c). The other 62 imprinted genes did not correlate with passage numbers of hES cells (Table 2). Furthermore, expression levels of five genes including INPP5F, KCNQ1OT1 and NNAT changed dramatically with no alteration of other 60 genes in hES cells after passage 50 compared with those before passage 50 (Fig. 1d). INPP5F expression levels decreased 2.9 fold (P<0.05); KCNQ1OT1 expression levels decreased 2.9 fold (P<0.05); NNAT levels increased 6.7 fold (P<0.01). Additionally, expression of CALCR and PEG10 levels decreased 2.5 and 3.6 fold respectively.

Transcriptional levels of imprinted genes in differentiated hES cells

We examined the expression levels of imprinted genes in EBs at day 14 from hES cells of different passages (passage 27, 37 and 47) compared with their undifferentiated counterparts by RT-qPCR and found that in hES cells derived EBs, expression levels of 5 maternally (SLC22A2, SLC22A3, COPG2IT1, H19 and CPA4) and 1 paternally (IGF2) expressed genes were markedly increased (Fig. 2). SLC22A2 levels increased 4.7 fold, P<0.01; SLC22A3 levels increased 3.7 fold, P<0.01; CPA4 levels increased 3.7 fold; P<0.01; H19 levels increased 2.2 fold, P<0.05; COPG2IT1 levels increased 3.0 fold, P<0.05; IGF2 levels increased 2.3 fold P<0.05. The expression levels of other 59 imprinted genes did not change significantly following EB formation in hES cells (Table 3).

Table 2 Change of relative mRNA levels during prolonged passages of hES cells

Gene symbol	Before passage 50[a]	After passage 50[a]	R^{2b}
TP73	3.60E-03	4.64E-03	0.271
SLC22A2	3.24E-03	3.06E-03	−0.417
SLC22A3	3.45E-02	2.62E-02	−0.471
TFPI2	4.52E-02	4.03E-02	−0.455
PPP1R9A	1.15E-02	9.47E-03	−0.065
DLX5	1.19E-02	7.21E-03	−0.408
CPA4	4.70E-02	2.99E-02	−0.535
KLF14	2.94E-04	6.98E-04	0.370
KCNK9	2.66E-03	1.57E-03	0.447
H19	3.33E+00	4.32E+00	−0.732
KCNQ1	1.24E-03	4.77E-03	0.776
KCNQ1DN	8.77E-05	5.15E-05	−0.261
CDKN1C	5.08E-03	3.13E-03	−0.418
SLC22A18	3.16E-02	2.33E-02	−0.461
PHLDA2	6.82E-03	1.13E-02	0.169
OSBPL5	1.05E-02	1.14E-02	−0.156
MEG3	6.47E-01	5.28E-01	−0.010
UBE3A	3.01E-01	2.04E-01	−0.323
ATP10A	9.95E-04	1.11E-03	0.526
TCEB3C	4.28E-03	1.94E-03	−0.125
ZNF264	3.24E-03	2.68E-03	0.069
GNAS	5.12E+00	3.89E+00	−0.273
BLCAP	1.54E-01	1.42E-01	−0.304
CALCR	**7.50E-03**	**3.04E-03***	−0.785
PRIM2	2.27E-01	1.90E-01	0.102
SLC22A18AS	9.73E-05	4.99E-05	−0.347
ZNF597	1.26E-02	7.79E-03	−0.337
DIRAS3	1.14E-03	2.58E-03	−0.678
NAP1L5	1.20E-02	6.02E-03	−0.372
PLAGL1	2.64E-02	3.23E-02	−0.518
HYMAI	3.38E-03	3.30E-03	−0.032
MESTIT1	6.94E-03	3.86E-03	−0.212
COPG2IT1	2.61E-02	7.97E-02	−0.796
DDC	1.24E-03	6.32E-04	−0.495
GRB10	3.74E-03	4.42E-03	0.391
SGCE	1.17E-01	1.20E-01	0.086
PEG10	**1.88E-01**	**5.28E-02***	−0.621
MEST	2.43E+00	9.33E-01	−0.668
DLGAP2	1.66E-03	1.49E-03	−0.233
INPP5F	**6.10E-01**	**2.08E-01***	**−0.922****
KCNQ1OT1	**4.08E-03**	**1.42E-03***	**−0.975****
IGF2	1.33E-01	3.38E-01	−0.695
IGF2AS	4.29E-03	1.34E-03	−0.401
INS	ND	ND	
WT1	8.72E-04	1.46E-03	0.403
DLK1	5.05E-02	5.73E-02	0.194
SNORD109A	1.87E+00	2.60E+00	0.318
SNRPN	2.08E+00	2.93E+00	0.671
MKRN3	ND	ND	
MAGEL2	2.46E-02	2.31E-02	−0.172
NDN	6.31E-02	3.31E-02	−0.572
SNURF	1.17E+00	1.92E+00	0.366
SNORD107	4.84E-02	7.22E-02	0.724
SNORD64	9.89E-03	4.06E-03	−0.322
SNORD108	2.73E-04	2.25E-04	0.047
SNORD109B	8.86E-02	4.18E-02	−0.353
ZIM2	4.72E-04	1.04E-03	0.604
PEG3	3.44E-02	3.00E-02	0.053
SANG	3.28E-02	4.08E-02	0.369
NNAT	**2.04E-02**	**1.36E-01****	**0.836***
L3MBTL	1.19E-02	1.40E-02	0.321
IPW	7.04E-02	3.10E-02	−0.659
PSIMCT-1	4.69E-02	3.40E-02	0.055
LRRTM1	1.36E-02	2.21E-03	−0.454
WIF1	3.42E-03	2.62E-03	−0.086

R correlation coefficient, *ND* not detected

[a] Comparison of mRNA levels of imprinted genes between cells before and after passage 50. The relative expression levels of each imprinted genes as determined by comparison with the average expression level of house-keeping controls (B2M, RPL13A, HPRT1 and GAPDH); each group has 3 independent samples

[b] Correlation analysis between passage numbers and mRNA levels

*$P<0.05$, **$P<0.01$

Discussion

This study assayed the mRNA levels of imprinted genes in undifferentiated and differentiated hES cells to describe the global transcriptional status of imprinted genes comprehensively and investigate the dynamic transcript alteration of imprinted genes in hES cells following prolonged passages and upon differentiation. To our knowledge, the widest range of imprinted genes in hES cells was investigated in this research to date. Sun [17] and Abeyta [20] detected 19, 28, 22 and 32 imprinted genes in 4 different hES cell lines by Affymetrix Oligo micro-array. Actually, we found that not only 19 ~ 32 but nearly all the imprinted genes (63/65, 96.9%) were detected in cHES1 cells. The fact that there were more imprinted genes detected in our study than previously reported might be due to the different methods used to examine imprinted genes. We used PCR array which is more sensitive and accurate than Affymetrix Oligo micro-array used by previous studies. INS and MKRN3 were not detected in all the passages of cHES1 cell line, while somatic cells showed significant expression of INS and MKRN3 tested on the same arrays (unpublished data

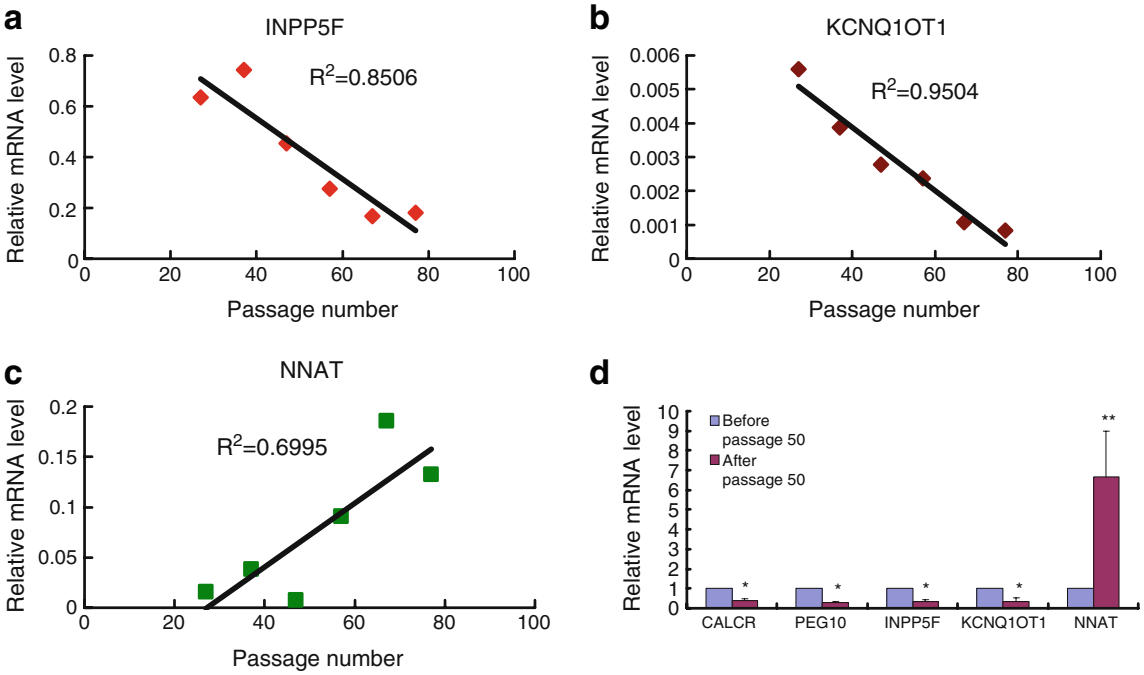

Fig. 1 **a**, **b**, **c** High correlation between relative expression level of imprinted genes (INPP5F, KCNQ1OT1, NNAT) and passage numbers for hES cells (n=6). **d** Comparison of relative expression of imprinted genes between hES cells before and after passage 50. Y-value is expressed as relative fold change in mRNA levels in cells after passage 50 when compared with those in cells before passage 50, defined as 1. Bars indicate mean ± SEM (n=3). *P<0.05, **P<0.01

from our lab), indicating that these genes are still silenced in human blastocysts and hES cells. So far, the expression status of 22 imprinted genes in hES cells have been reported previously. Most of them have been identified as monoallelic expression (establishment of imprint). Our study expanded the scope of imprinted genes and demonstrated that 96.9% imprinted genes were expressed in hES cells, suggesting that the majority of imprinted genes have been activated at least at blastocyst stage or during derivation of hES cells, and hES cells offer an excellent model for studying epigenetic regulation of imprinting.

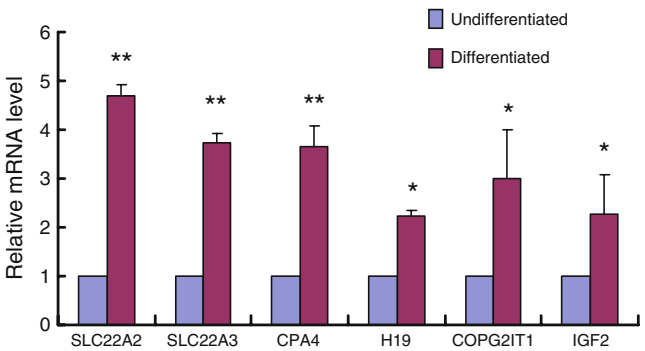

Fig. 2 Comparison of relative mRNA levels between hES cells before and after differentiation. Y-value is expressed as relative fold change in mRNA levels at EB formation when compared with mRNA level of undifferentiated ES cells, defined as 1. Bars indicate mean ± SEM (n=3). *P<0.05, **P<0.01

Recent work focused on monoallelic or biallelic expression status of imprinted genes in hES cells [13, 14, 17]. However, the transcriptional levels of imprinted genes are largely unknown. Taking into account that the mRNA levels influence the amount of protein directly, resulting in different biological effects, we first analyzed the expression levels of 65 imprinted genes in hES cells from passage 27 to passage 77. We showed that transcriptional levels of INPP5F, KCNQ1OT1 and NNAT gradually reduced or increased with passage numbers, indicating that transcript of these three imprinted genes might closely relate with duration of in vitro culture of hES cells. This study also showed that under the same culture conditions, the variation trends of expression levels for imprinted genes were different. INPP5F and KCNQ1OT1 showed a decrease while NNAT showed an increase. The reason for these results is not understood and may relate with epigenetic property of individual imprinted genes. Expression of 5 genes (CALCR, PEG10, INPP5F, KCNQ1OT1 and NNAT) varied greatly in hES cells after passage 50 compared with those before passage 50, suggesting that in vitro culture actually affect the expression of a few imprinted genes in hES cells.

Rugg-Gunn revealed that H19 was biallelically expressed with elevated expression levels after prolonged passages (passage66 ~ passage101) in H9 line, while this phenomenon did not exist in three other hES cell lines [13]. In our study, H19 did not vary significantly in cHES1 cells line after extended culture, which might suggest that

Table 3 Comparison of expression levels of imprinted genes between undifferentiated and differentiated hES cells

Gene symbol	Undifferentiated[a]	Differentiated[a]
TP73	3.60E-03	6.67E-03
SLC22A2	**3.24E-03**	**1.52E-02****
SLC22A3	**3.45E-02**	**1.28E-01****
TFPI2	4.52E-02	2.48E-02
PPP1R9A	1.15E-02	1.06E-02
DLX5	1.19E-02	2.45E-02
CPA4	**4.70E-02**	**1.72E-01****
KLF14	2.94E-04	4.61E-04
KCNK9	2.66E-03	3.73E-03
H19	**3.33E+00**	**7.41E+00***
KCNQ1	1.24E-03	1.08E-03
KCNQ1DN	8.77E-05	8.08E-05
CDKN1C	5.08E-03	1.96E-03
SLC22A18	3.16E-02	2.98E-02
PHLDA2	6.82E-03	3.48E-03
OSBPL5	1.05E-02	1.46E-02
MEG3	6.47E-01	5.83E-01
UBE3A	3.01E-01	3.09E-01
ATP10A	9.95E-04	1.05E-03
TCEB3C	4.28E-03	2.75E-03
ZNF264	3.24E-03	2.48E-03
GNAS	5.12E+00	5.58E+00
BLCAP	1.54E-01	2.59E-01
CALCR	7.50E-03	7.10E-03
PRIM2	2.27E-01	1.27E-01
SLC22A18AS	9.73E-05	5.27E-05
ZNF597	1.26E-02	4.74E-03
DIRAS3	1.14E-03	8.56E-04
NAP1L5	1.20E-02	1.39E-02
PLAGL1	2.64E-02	3.11E-02
HYMAI	3.38E-03	2.46E-03
MESTIT1	6.94E-03	5.86E-03
COPG2IT1	**2.61E-02**	**7.82E-02***
DDC	1.24E-03	7.81E-04
GRB10	3.74E-03	2.54E-03
SGCE	1.17E-01	9.96E-02
PEG10	1.88E-01	1.09E-01
MEST	2.43E+00	4.99E+00
DLGAP2	1.66E-03	1.12E-03
INPP5F	6.10E-01	4.81E-01
KCNQ1OT1	4.08E-03	1.92E-03
IGF2	**1.33E-01**	**3.03E-01***
IGF2AS	4.29E-03	4.73E-03
INS	ND	1.54E-05
WT1	8.72E-04	5.64E-04
DLK1	5.05E-02	3.95E-02
SNORD109A	1.87E+00	1.07E+00
SNRPN	2.08E+00	2.06E+00
MKRN3	ND	1.81E-05
MAGEL2	2.46E-02	3.73E-02
NDN	6.31E-02	8.02E-02
SNURF	1.17E+00	7.08E-01
SNORD107	4.84E-02	3.96E-02
SNORD64	9.89E-03	1.72E-03
SNORD108	2.73E-04	2.29E-04
SNORD109B	8.86E-02	4.53E-02
ZIM2	4.72E-04	2.48E-04
PEG3	3.44E-02	1.67E-02
SANG	3.28E-02	1.88E-02
NNAT	2.04E-02	1.12E-02
L3MBTL	1.19E-02	1.61E-02
IPW	7.04E-02	5.66E-02
PSIMCT-1	4.69E-02	3.30E-02
LRRTM1	1.36E-02	3.27E-02
WIF1	3.42E-03	2.73E-03

ND not detected

[a] The relative expression levels of each imprinted gene as determined by comparison with the average expression level of housekeeping controls (B2M, RPL13A, HPRT1 and GAPDH). Each group has 3 independent samples

*$P<0.05$, **$P<0.01$

imprinted genes which are vulnerable to disruption varied among hES cell lines. All the 65 imprinted genes except CALCR, PEG10, INPP5F, KCNQ1OT1 and NNAT did not change dramatically after long term culture of cHES1, indicating that mRNA levels of the majority of imprinted genes are quite stable in hES cells, in accordance with previous data that imprinted genes could generally maintain monoallelic expression after long term culture of hES cells [13, 14, 17]. Since many more imprinted genes were included in our study, we provide more evidence to show that hES cells posses substantial imprinting stability during prolonged passaging at least in the cell line we examined. This is an encouraging fact when considering the safety of transplanting hES cell derived tissues. However, differential expression of a few imprinted genes was identified in subculture of cHES1. Evidence has been presented that prenatal growth retardation may be due to deletion of PEG10 [21]. Defects of KCNQ1OT1 were found to be associated with children born small for gestational age when they are conceived by intracytoplasmic sperm injection [22]. NNAT is frequently overexpressed in a variety of human cancers [23–25]. Thus whether these aberrantly expressed genes influence the cellular function, cause phenotypic features and eventually affect the therapeutic use of hES cells needs further investigation.

It was reported that Expression of IGF2 and H19 increased significantly in SHhES1 and HUES-7 derived EBs [17]. We showed that mRNA levels of H19 and IGF2 increased remarkably in cHES1 derived EBs at day 14. H19 and IGF2 are known to be involved in embryonic growth and development [26]. Additionally, we found that expression levels of 4 imprinted genes (COPG2IT1, CPA4, SLC22A2 and SLC22A3) were elevated in EBs. SLC22A2, SLC22A3 and SLC22A18 are highly expressed in tissues with metabolite transport functions [27–29]. Dosage regulation of the metabolite transporter genes by imprinting may regulate placental and fetal growth [29]. CPA4 has a potential role in cell proliferation and differentiation [30]. Our study is the first to note that expression levels of SLC22A2, SLC22A3 and CPA4 increased during EB formation of hES cells. This suggests that these genes are developmentally regulated. SLC22A2/SLC22A3, COPG2IT1/CPA4 and H19/IGF2 are located nearby on chromosome 6q26 [31], 7q32.2 [32] and 11p15.5 [33] respectively. Therefore these results raise important questions about mechanistic and functional relationships between imprinted genes in a cluster. Previous evidence showed that in the imprinted IGF2/H19 domain. IGF2 and H19 are co-expressed in endoderm- and mesoderm-derived tissues during embryogenesis, co-regulate embryonic growth and development. H19 influences growth by way of a cis control on IGF2 expression [34]. Transcripts of both genes are controlled by a common imprinted control region, (ICR) [35]. However, such interactions and mechanisms remain unclear in the imprinted domain 6q26 and 7q32. Our study showed that imprinted domain at 6q26, 7q32.2 and 11p15 might be involved in formation of the 3 germ layers with differentiation of hES cells, and might play an important role in human early development. Though how these physically linked imprinted genes interact with each other to exert their effects needs to be further elucidated.

The other 59 imprinted genes did not differ significantly in EBs compared with undifferentiated cells This may indicate that only a small amount of imprinted genes are involved in early differentiation of hES cells, while the transcriptional levels of other imprinted genes, which are irrelevant to differentiation, remain stable.

During the differentially expressed genes identified in our study, PEG10 and KCNQ1OT1 were reported to show monoallelic expression in most examined hES cells lines [14]. H19 showed monoallelic expression when its expression increased dramatically in EBs [17]. Combined with previous reports, these phenomenons might suggest that expression level is not always consistent with mono or biallelic expression status in imprinted genes. Transcriptional level might reflect imprinting stability of imprinted genes more sensitively. However, the relationship between mRNA level and allelic expression of imprinted genes and their regulatory mechanism need to be further studied.

Conclusions

hES cells expressed the majority of imprinted genes (63/65,96.9%). Long-term culture in vitro did not affect transcriptional levels of the majority of (60/65, 92.3%) imprinted genes. H19, IGF2, COPG2IT1, CPA4, SLC22A and SLC22A3 might play a role in early differentiation of hES cells. Whether the differential expression of a few imprinted genes identified in subculture of hES cells affect the cellular function and cause phenotypic consequences needs to be established.

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