## Author contributions

T.Y. performed cellular and biochemical analyses; T.Y. and N.S. established the ES cell differentiation system; M.T., N.K., H.O., Y.M., H.A. and A.U. established human iPS Toe cell line; Y.S., K.K. and S.K. provided technical advice, designed the experiments and wrote the paper. All authors discussed the results and commented on the manuscript.

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Regular Article

# Differentiation of Human Induced Pluripotent Stem Cells into Functional Enterocyte-like Cells Using a Simple Method

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**Summary:** Human induced pluripotent stem (iPS) cells were differentiated into the endoderm using activin A and were then treated with fibroblast growth factor 2 (FGF2) for differentiation into intestinal stem cell-like cells. These immature cells were then differentiated into enterocyte-like cells using epidermal growth factor (EGF) in 2% fetal bovine serum (FBS). At the early stage of differentiation, mRNA expression of caudal type homeobox 2 (CDX2), a major transcription factor related to intestinal development and differentiation, and leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5), an intestinal stem cell marker, was markedly increased by treatment with FGF2. When cells were cultured in medium containing EGF and a low concentration of FBS, mRNAs of specific markers of intestinal epithelial cells, including sucrase–isomaltase, the intestinal oligopeptide transporter SLC15A1/peptide transporter 1 (PEPT1), and the major metabolizing enzyme CYP3A4, were expressed. In addition, sucrase–isomaltase protein expression and uptake of β-Ala-Lys-N-7-amino-4-methylcoumarin-3-acetic acid (β-Ala-Lys-AMCA), a fluorescence-labeled substrate of the oligopeptide transporter, were detected. These results demonstrate a simple and direct method for differentiating human iPS cells into functional enterocyte-like cells.

**Keywords: human iPS cells; intestinal differentiation; enterocytes; pharmacokinetics; drug metabolizing enzymes; drug transporters**

## Introduction

The small intestine and liver play important roles in all aspects of pharmacokinetics, including drug disposition, drug metabolism, drug transport, drug interactions, and bioavailability. Because drug-metabolizing enzymes such as cytochrome P450 (CYP) and UDP-glucuronyltransferase (UGT) and drug transporters such as ATP-binding cassette (ABC) and solute carrier (SLC) transporters are appreciably expressed in the small intestinal epithelia,[1,2] it is necessary to estimate intestinal metabolism and absorption during the early stages of drug development. To this end, various *in vivo* and *in vitro* systems have been employed to assess the intestinal first-pass effect. However, extrapolation of experimental animal data to humans is often hampered by species differences, and primary human intestinal cells are rarely available. Therefore, a system that accurately and easily estimates intestinal membrane permeability and metabolism is urgently required.

Human induced pluripotent stem (iPS) cells can be generated by transducing reprogramming factors (OCT3/4, SOX2, KLF4, c-MYC) into somatic cells[3] and these cells share many characteristics of embryonic stem (ES) cells.[4] Human iPS cells are expected to be useful not only in regenerative medicine but also in pharmacokinetic and toxicokinetic drug development studies because their use is not as ethically regulated as that of human ES cells.

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Therefore, human iPS cells have been differentiated into various cell types, including pancreatic cells,[5,6] neuron cells,[7] cardiomyocytes,[8] and hepatocytes.[9-13]

A few studies report the differentiation of iPS cells into enterocytes. In particular, mouse iPS cells were differentiated into a gut-like organ following the formation of embryoid bodies (EBs),[14] and human iPS cells were differentiated into intestinal tissue using a culture method for intestinal crypt stem cells.[15] However, functional characteristics of drug transporters and drug-metabolizing enzymes of differentiated cells are almost entirely unexplored in these reports. Thus, whether differentiated intestinal tissue or organoids can be used in drug development studies, particularly studies of the absorbability and metabolic capacity of drugs, remains unclear.

The small intestinal epithelium comprises absorptive cells, goblet cells, endocrine cells, and Paneth cells. Several signaling pathways such as Notch, Wnt, phosphoinositide 3-kinase, and bone morphogenic protein signaling are associated with intestinal development.[16] Leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5) has been identified as an intestinal stem cell marker.[17] Indeed, this was also observed in mouse LGR5-positive cells that formed a crypt–villus structure *in vitro*.[18] Improvements in this technique have enabled long-term culture of human epithelial cells isolated from the small intestine,[19] leading to advances in intestinal stem cell research. However, mechanisms of intestinal development are not sufficiently understood, and it is difficult to control differentiation into all four cell types.

In this study, we established a functional enterocyte-like cell line from human iPS cells for use in drug development studies. We propose a simple and direct differentiation method by two-dimensional culture. Our data may facilitate the development of an intestinal pharmacokinetic analysis system to identify safe drugs with favorable pharmacokinetic characteristics.

## Materials and Methods

**Materials:** FGF2, FGF4, activin A, and epidermal growth factor (EGF) were purchased from PeproTech Inc. (Rocky Hill, NJ). Wnt3a was purchased from R&D Systems, Inc. (Minneapolis, MN). BD Matrigel matrix Growth Factor Reduced (Matrigel) was purchased from BD Biosciences (Bedford, MA). Affinity-isolated rabbit polyclonal antihuman sucrase–isomaltase antibody and intestinal recombinant protein epitope signature tags were purchased from Sigma-Aldrich Co. (St. Louis, MO). The purified IgG fraction of polyclonal goat antiserum against rabbit IgG conjugated with Alexa Fluor 568 and KnockOut Serum Replacement (KSR) were purchased from Invitrogen Life Technologies Co. (Carlsbad, CA). β-Ala-Lys-N-7-amino-4-methylcoumarin-3-acetic acid (β-Ala-Lys-AMCA) was purchased from BIOTREND Chemicals (Destin, FL), and (+)-(*R*)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl)cyclohexanecarboxamide dihydrochloride (Y-27632) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Human adult small intestine total RNA from a 66-year-old male donor was purchased from BioChain Institute Inc. (Newark, CA). Murine embryonic fibroblasts (MEFs) were obtained from Oriental Yeast Co. (Tokyo, Japan). The RNeasy Mini Kit was purchased from Qiagen (Valencia, CA). The PrimeScript RT Reagent Kit and TaKaRa SYBR Premix EX Taq II were purchased from Takara Bio Inc. (Otsu, Japan). All other reagents were of the highest quality available.

**Human iPS cell cultures:** A human iPS cell line (Windy) was provided by Dr. Akihiro Umezawa of the National Center for Child Health and Development. Human iPS cells were maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's nutrient mixture F-12 (DMEM/F12) containing 20% KSR, 2 mM L-glutamine, 1% MEM nonessential amino acid solution (NEAA), 0.1 mM 2-mercaptoethanol, and 5 ng/ml FGF2 at 37°C in humidified air with 5% $CO_2$. The human iPS cells were cultured on a feeder layer of mitomycin C-treated MEFs, and the medium was changed every day.

**Differentiation into enterocyte-like cells:** The human iPS cells were used for differentiation studies between passages 30 and 50. When the cells reached approximately 70% confluence, differentiation was initiated by replacing the medium with Rosewell Park Memorial Institute (RPMI) 1640 medium containing 2 mM GlutaMAX, 0.5% fetal bovine serum (FBS), 100 ng/ml activin A (a member of the transforming growth factor-β family that is known to efficiently induce differentiation into the definitive endoderm),[20,21] 100 units/ml penicillin, and 100 μg/ml streptomycin. After 48 h, the medium was replaced with RPMI 1640 containing 2 mM GlutaMAX, 2% FBS, 100 ng/ml activin A, 100 units/ml penicillin, and 100 μg/ml streptomycin, and the cells were cultured for 24 h. Subsequently, the culture medium was replaced with DMEM/F12 containing 2% FBS, 2 mM GlutaMAX, and 250 ng/ml FGF2 or FGF4 with or without 50 ng/ml Wnt3a for 96 h. The cells were then treated for 1 h with the selective Rho-associated kinase inhibitor Y-27632 at 10 μM.[22,23] The cells were then passaged on Matrigel-coated 24-well plates and cultured in DMEM/F12 containing 2% or 10% FBS, 2% B-27 supplement, 1% N2 supplement, 1% NEAA, 2 mM L-glutamine, antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin), and 20 ng/ml EGF for 1, 4, 13, or 19 days. Y-27632 was added at 10 μM during the initial 24 h of culture. The medium was changed every 3 days (**Fig. 1**).

**RNA extraction and reverse transcription reaction:** Total RNA was isolated from differentiated iPS cells using the RNeasy Mini Kit. First-strand cDNA was prepared from 500 ng of total RNA. The reverse transcription reaction was performed using the PrimeScript RT Reagent Kit according to the manufacturer's instructions.

**Real-time polymerase chain reaction (PCR) analysis:** Relative mRNA expression levels were determined using SYBR Green real-time quantitative reverse transcription-PCR (RT-PCR). Real-time PCR analysis was performed on the Applied Biosystems 7300 Real Time PCR System using 7300 System SDS software version 1.4 (Applied Biosystems, Carlsbad, CA). PCR was performed with the primer pairs listed in **Table 1** using SYBR Premix EX Taq II. mRNA expression levels were normalized relative to that of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

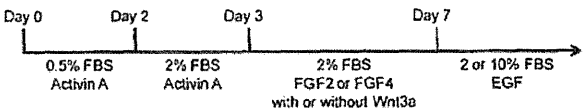


**Fig. 1. Schematic of the protocol for the differentiation of human iPS cells into enterocytes**
Human iPS cells were cultured in the presence of activin A (100 ng/ml) for 3 days. The cells were further cultured in medium containing FGF2 (250 ng/ml) or FGF4 (250 ng/ml) with or without Wnt3a (50 ng/ml) for 4 days. After 7 days of differentiation, the cells were treated with Y-27632 (10 μM), passaged, and subsequently cultured in the presence of 2% or 10% FBS and EGF (20 ng/ml) for 19 days.

**Table 1. Sequences of primers for real-time PCR analysis**

| Gene name | Sense (5′→3′) | Antisense (5′→3′) | Product length (bp) |
|---|---|---|---|
| CDX2 | ACCTGTGCGAGTGGATGC | TCCTTTGCTCTGCGGTTCT | 232 |
| LGR5 | TGCTCTTCACCAACTGCATC | CTCAGGCTCACCAGATCCTC | 193 |
| DPP4 | CAAATTGAAGCAGCCAGACA | GGAGTTGGGAGACCCATGTA | 212 |
| Sucrase-isomaltase | GGTAAGGAGAAACCGGGAAG | GCACGTCGACCTATGGAAAT | 195 |
| Villin 1 | AGCCAGATCACTGCTGAGGT | TGGACAGGTGTTCCTCCTTC | 169 |
| ISX | CAGGAAGGAAGGAAGAGCAA | TGGGTAGTGGGTAAAGTGGAA | 96 |
| CYP3A4 | CTGTGTGTTTCCAAGAGAAGTTAC | TGCATCAATTTCCTCCTGCAG | 298 |
| SLC15A1/PEPT1 | CACCTCCTTGAAGAAGATGGCA | GGGAAGACTGGAAGAGTTTTATCG | 105 |
| SLC46A1/PCFT | GGTCTTTGCCTTTGCCACTA | AGAGTTTAGCCCGGATGACA | 98 |
| GAPDH | GAGTCAACGGATTTGGTCGT | GACAAGCTTCCCGTTCTCAG | 185 |

**Immunofluorescence staining:** The cells differentiated with FGF2 and 2% FBS were washed three times with phosphate-buffered saline (PBS) without calcium or magnesium, fixed for 30 min at room temperature in 4% paraformaldehyde, and permeabilized in PBS containing 0.1% Triton X-100 for 5 min at room temperature. After being washed three times with PBS, the cells were blocked in PBS with 2% skim milk for 20 min at room temperature and were incubated with antisucrase–isomaltase antibody diluted at 1:200 for 60 min at room temperature. Rabbit serum was used as a negative control. The cells were washed three times with PBS and incubated with a 1:500 dilution of Alexa Fluor 568-labeled secondary antibody for 60 min at room temperature. After being washed three times with PBS, the cells were incubated with 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) for 5 min at room temperature and washed with PBS. The cells were mounted on a glass slide using a 9:1 mixture of glycerol and PBS and viewed using an LSM 510Meta confocal microscope (Carl Zeiss Inc., Oberkochen, Germany).

**Uptake study of β-Ala-Lys-AMCA:** The cells differentiated with FGF2 and 2% FBS were rinsed several times with PBS and incubated with DMEM/F12 containing 25 μM β-Ala-Lys-AMCA for 4 h at 37°C. After incubation, uptake of β-Ala-Lys-AMCA was stopped by washing with ice-cold PBS. The cells were fixed for 30 min at room temperature in 4% paraformaldehyde, and immunofluorescence staining was performed using the primary and secondary antibodies as described above. The cells were then mounted using a 9:1 mixture of glycerol and PBS and viewed using an LSM 510Meta confocal microscope.

**Statistical analysis:** Levels of statistical significance were assessed using Student's *t*-test, and multiple comparisons were performed using analysis of variance (ANOVA) followed by Tukey's test.

## Results

**Early stages of differentiation into intestinal cells:** For efficient, selective, and direct differentiation, a protocol designed to mimic intestinal development is desirable. We attempted differentiation into enterocytes that mediate the formation of the definitive endoderm. Because the intestine is an endoderm-derived organ, the human iPS cells were initially differentiated into the endoderm using a high concentration of activin A (100 ng/ml). Subsequently, we investigated the effects of FGF2, FGF4, and Wnt3a, which promote the development of mid- and hindgut lineages,[24,25] during differentiation from the definitive endoderm to intestinal stem cells. In these experiments, mRNA expression of caudal type homeobox 2 (CDX2), a major transcription factor of intestinal development and cell differentiation,[26,27] was slightly higher in FGF4-treated cells than that in FGF2-treated cells (**Fig. 2A**). In contrast, mRNA expression of LGR5 in FGF4-treated cells was significantly lower than that in FGF2-treated cells (**Fig. 2B**). Under all conditions, these mRNA expression levels were higher than those in undifferentiated human iPS cells. No effects of Wnt3a were observed on mRNA expression of CDX2 or LGR5 during the early stages of differentiation.

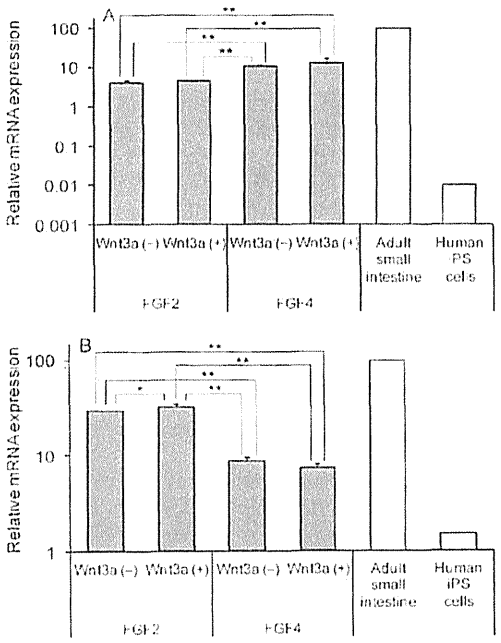


**Fig. 2. Relative mRNA expression levels of CDX2 (A) and LGR5 (B) in differentiated intestinal stem cell-like cells**
Human iPS cells were cultured in the presence of activin A for 3 days. The cells were further cultured in medium containing FGF2 or FGF4 with or without Wnt3a for 4 days and then in the presence of EGF for 1 day. After 8 days of differentiation, total RNA was extracted and mRNAs were analyzed by SYBR Green real-time RT-PCR. mRNA expression levels were normalized relative to that of GAPDH. Gene expression levels are represented relative to the level in the adult small intestine, which is set as 100. The adult small intestine and undifferentiated human iPS cells (shown as open columns) were used as positive and negative controls, respectively. Data are presented as the mean ± S.D. ($n = 3$), except for the adult small intestine and human iPS cells. Levels of statistical significance were compared among all groups: $^{**}p < 0.01$, $^{*}p < 0.05$.

**Differentiation into enterocyte-like cells:** To effectively differentiate human iPS cells into enterocyte-like cells, we examined

the effects of FBS concentration in the differentiation medium. Expression of LGR5 in differentiated human iPS cells did not differ in the presence of 2% or 10% FBS (**Fig. 3A**). However, mRNA expression of sucrase–isomaltase was 3.5-fold higher in 2% FBS than that in 10% FBS (**Fig. 3B**). In addition, mRNA expression levels of SLC15A1/peptide transporter 1 (PEPT1) and CYP3A4 were higher in the presence of 2% FBS (**Figs. 3C** and **3D**). In differentiated enterocyte-like cells, sucrase–isomaltase and CYP3A4, which were not detected in undifferentiated human iPS cells, were expressed, and mRNA expression levels of LGR5 and SLC15A1/PEPT1 were 30–40-fold higher than those in undifferentiated human iPS cells. Morphological changes in differentiating human iPS cells are shown in **Figure 4**. Similar to ES cells, undifferentiated human iPS cells had little cytoplasm and were small in size (**Fig. 4A**). When human iPS cells were cultured in the presence of activin A and FGF2, the cells gradually exhibited morphological changes such as enlargement and acquisition of spiky shapes (**Fig. 4B**). At the final stage of differentiation with EGF and 2% FBS, a number of dome-like structures formed and were assumed to contain liquids and cells (**Figs. 4C** and **4D**). In cells differentiated with activin A, FGF2, EGF, and 2% FBS, villin 1[28,29] and intestine specific homeobox (ISX)[30] were expressed, whereas intestinal fatty acid-binding protein (IFABP) was not expressed. Interestingly, mRNA expression levels of CDX2, dipeptidyl peptidase 4 (DPP4), and SLC46A1/proton-coupled folate transporter (PCFT) were similar to those in the adult small intestine, which was used as a positive control (**Fig. 5**). However, gene expression levels of UGT1A1 and ABCB1/multidrug resistance 1 (MDR1) were similar to those in undifferentiated human iPS cells (data not shown).

To determine the optimal duration of differentiation, we examined time-dependent variations in expression levels of specific small intestine genes such as LGR5, sucrase–isomaltase, and SLC15A1/PEPT1. After short-term culture (11 days), mRNA expression levels of sucrase isomaltase and SLC15A1/PEPT1 were very low but gradually increased with differentiation until day 26. Similarly, CYP3A4 mRNA was not expressed after 11 days of differentiation but was expressed after 20 days (**Fig. 6**). LGR5 mRNA did not change with the duration of differentiation.

**Immunofluorescence staining of sucrase–isomaltase in enterocyte-like cells:** Sucrase–isomaltase is an essential carbohydrate digestion enzyme that is specifically expressed in brush border membranes of mature enterocytes. Therefore, sucrase–isomaltase expression is thought to be an indicator of differentiation into enterocytes. Indeed, protein expression of sucrase–isomaltase was confirmed in differentiated cells using immunofluorescence staining, in particular, in dense clusters of cells (**Fig. 7**).

**Uptake of β-Ala-Lys-AMCA in enterocyte-like cells:** Oligopeptide transporters are expressed in the brush border membrane and participate in peptide absorption from the intestinal lumen.[2] As shown in **Figure 3C**, expression of SLC15A1/PEPT1 mRNA in differentiated enterocyte-like cells was more than 30-fold higher than that in undifferentiated human iPS cells. To determine whether this leads to active peptide transport in differentiated cells, we performed peptide uptake assays using β-Ala-Lys-AMCA, a fluorescence-labeled substrate of the oligopeptide transporter.[31] As shown in **Figure 8**, intracellular uptake of β-Ala-Lys-AMCA was observed in cells expressing the sucrase–isomaltase proteins. However, uptake of β-Ala-Lys-AMCA at 4°C was low compared with that at 37°C (data not shown).

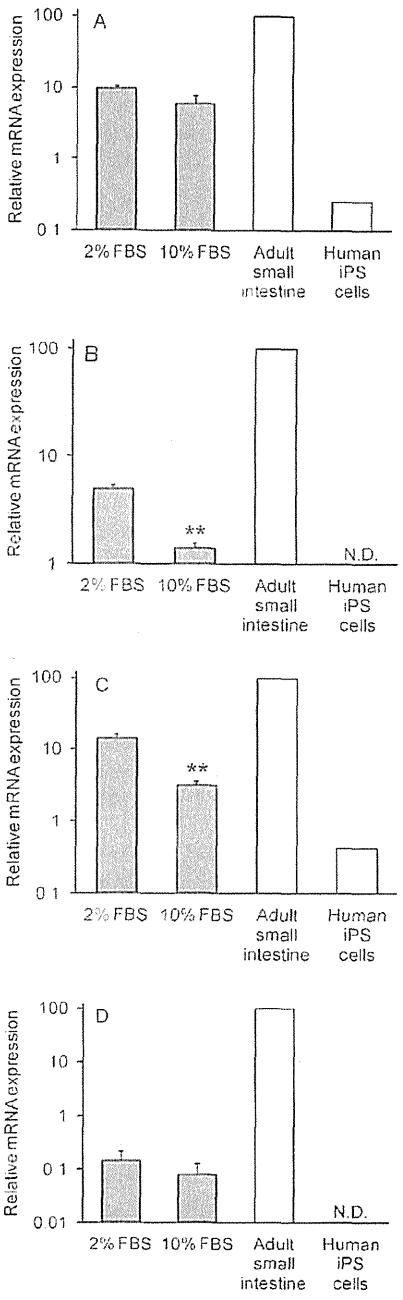


**Fig. 3. Relative mRNA expression levels of LGR5 (A), sucrase–isomaltase (B), SLC15A1/PEPT1 (C), and CYP3A4 (D) in differentiated enterocyte-like cells cultured in 2% or 10% FBS**

Human iPS cells were cultured in the presence of activin A for 3 days. The cells were further cultured in medium containing FGF2 for 4 days and then in the presence of 2% or 10% FBS and EGF for 17 days. After 24 days of differentiation, total RNA was extracted and mRNAs were analyzed by SYBR Green real-time RT-PCR. mRNA expression levels were normalized relative to that of GAPDH. Gene expression levels are represented relative to the level in the adult small intestine, which is set as 100. The adult small intestine and undifferentiated human iPS cells (shown as open columns) were used as positive and negative controls, respectively. Data are presented as the mean ± S.D. ($n = 3$), except for the adult small intestine and human iPS cells. N.D., not detected. Levels of statistical significance were compared with the 2% FBS group: $^{**}p < 0.01$.

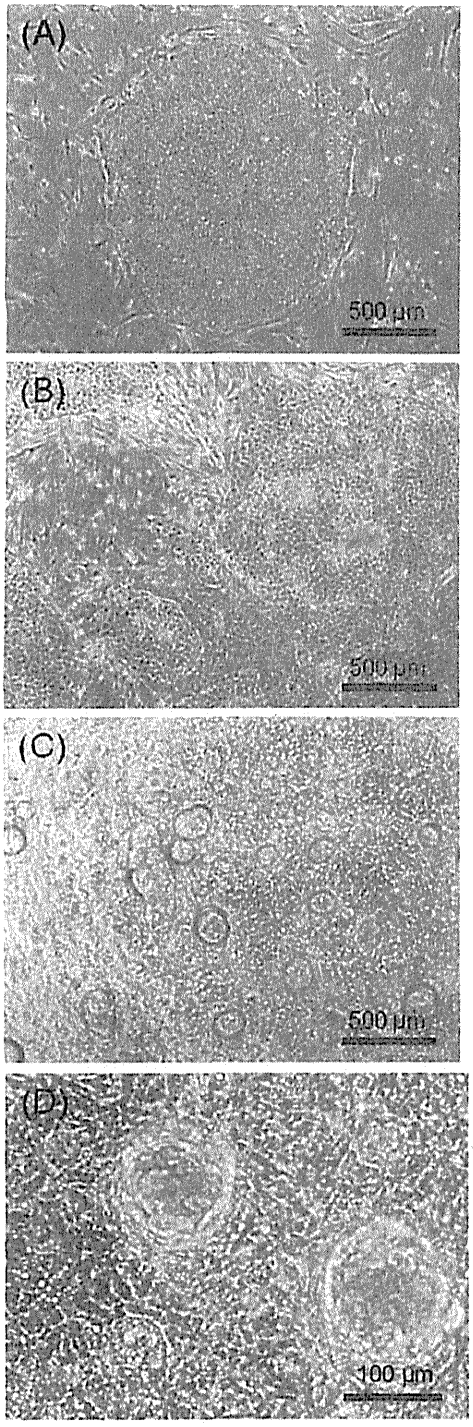


**Fig. 4. Morphological changes in human iPS cells during differentiation into enterocyte-like cells**
Human iPS cells were cultured in medium containing activin A for 3 days, FGF2 for 4 days, and 2% FBS and EGF for 17 days. (A) Undifferentiated human iPS cells; (B) midgut lineage cell-like cells after 7 days of differentiation; (C, D) enterocyte-like cells after 24 days of differentiation. Scale bar, 500 μm (A–C), 100 μm (D).

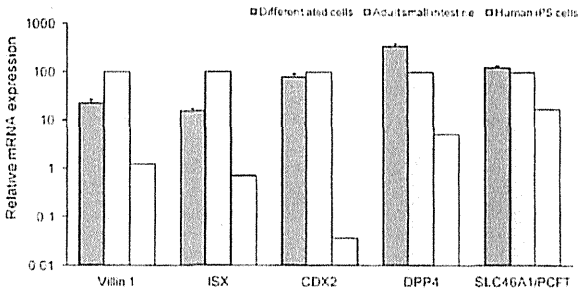


**Fig. 5. Relative mRNA expression levels of the intestinal markers villin 1, ISX, CDX2, DPP4, and SLC46A1/PCFT in differentiated enterocyte-like cells**
Human iPS cells were cultured in the presence of activin A for 3 days. The cells were further cultured in medium containing FGF2 for 4 days and then in the presence of 2% FBS and EGF for 19 days. After 26 days of differentiation, total RNA was extracted and mRNAs were analyzed by SYBR Green real-time RT-PCR. mRNA expression levels were normalized relative to that of GAPDH. Gene expression levels are represented relative to the level in the adult small intestine, which is set as 100. The adult small intestine and undifferentiated human iPS cells (shown as open columns) were used as positive and negative controls, respectively. Data are presented as the mean ± S.D. ($n = 3$), except for the adult small intestine and human iPS cells.

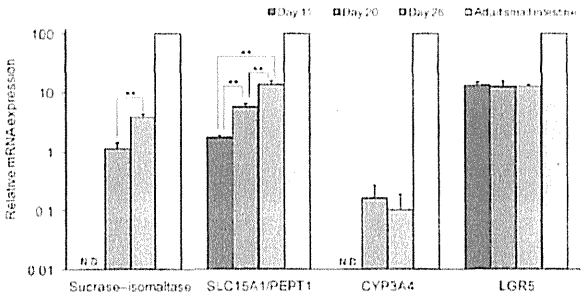


**Fig. 6. Time-dependent variation in mRNA expression levels of sucrase-isomaltase, SLC15A1/PEPT1, CYP3A4, and LGR5 in differentiated enterocyte-like cells**
Human iPS cells were cultured in the presence of activin A for 3 days. The cells were further cultured in medium containing FGF2 for 4 days and then in the presence of 2% FBS and EGF for 4, 13, or 19 days. After 11, 20, or 26 days of differentiation, total RNA was extracted and mRNAs were analyzed by SYBR Green real-time RT-PCR. mRNA expression levels were normalized relative to that of GAPDH. Gene expression levels are represented relative to the level in the adult small intestine, which is set as 100. The adult small intestine was used as a positive control. Data are presented as the mean ± S.D. ($n = 4$) except for the adult small intestine. N.D., not detected. Levels of statistical significance were compared among all groups: $^{**}p < 0.01$.

## Discussion

Ueda *et al.*[14] reported the synthesis of gut-like organs from mouse iPS cells using the EB formulation technique with mouse ES cells. However, this hanging drop culture technique is hampered by its high requirement of skill, low EB formulation efficiency, unstable EB quality, and differing differentiation efficiencies between EBs. Spence *et al.*[15] reported the direct differentiation of human iPS cells into three-dimensional intestinal organoids. These organoids contained various cell types, including enterocytes, endocrine cells, goblet cells, and Paneth cells, although expression of drug-metabolizing enzymes and transporters, which are central to drug absorption and metabolism, was not examined. In their study, the intestinal crypt culture system, in which spheroids

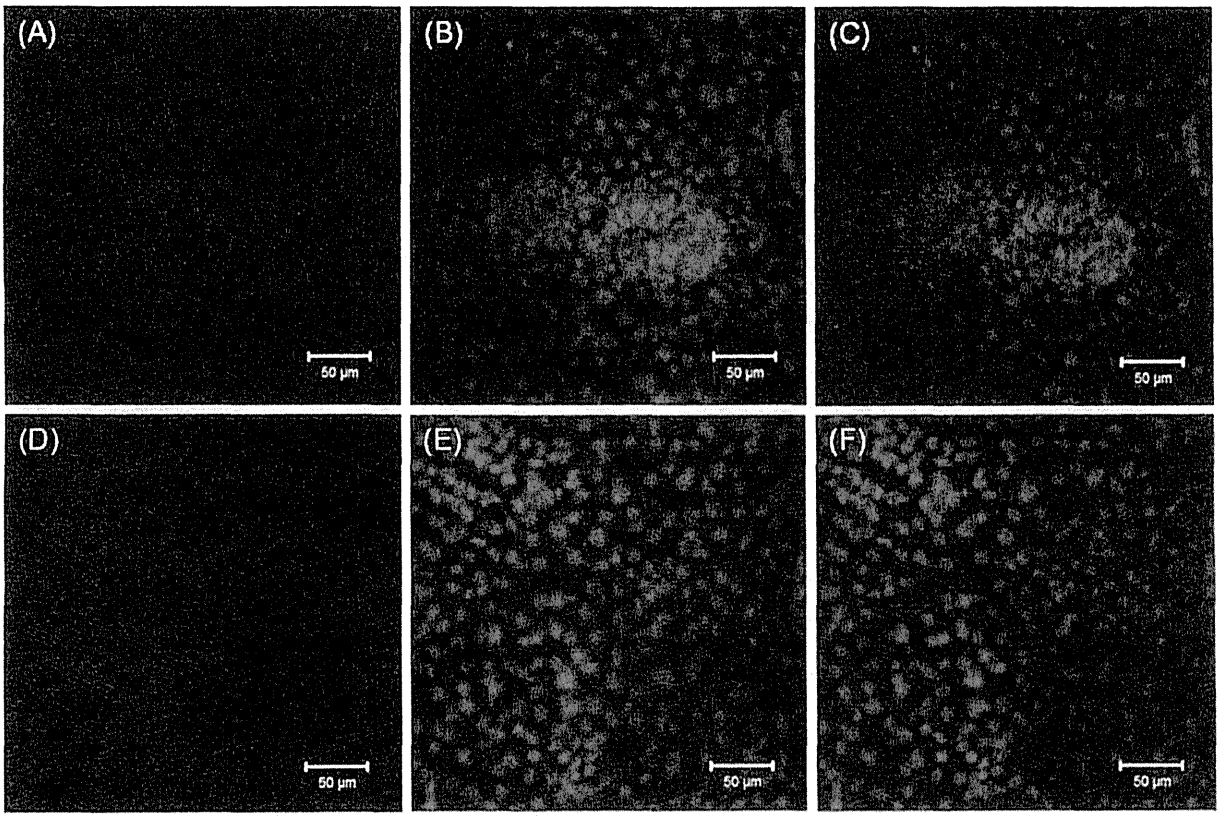


**Fig. 7. Immunofluorescence analysis of sucrase–isomaltase in differentiated enterocyte-like cells**
Human iPS cells were cultured in the presence of activin A for 3 days. The cells were further cultured in medium containing FGF2 for 4 days and then in the presence of 2% FBS and EGF for 19 days. After 26 days of differentiation, differentiated cells were stained with antisucrase–isomaltase antibody (A–C) or nonimmune rabbit serum as a negative control (D–F). Nuclei were counterstained with DAPI. (A) Immunofluorescence staining of sucrase–isomaltase (red); (D) immunofluorescence staining of rabbit serum as a negative control; (B, E) DAPI-stained DNA (blue); (C, F) overlay (Merge) image of sucrase–isomaltase and DAPI. Scale bar, 50 μm.

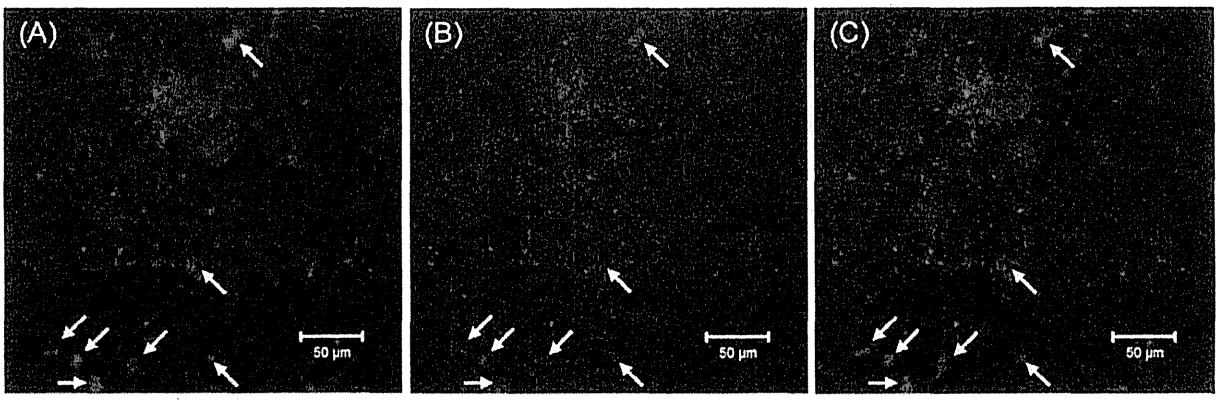


**Fig. 8. Uptake of β-Ala-Lys-AMCA into differentiated enterocyte-like cells**
Human iPS cells were cultured in the presence of activin A for 3 days. The cells were further cultured in medium containing FGF2 for 4 days and then in the presence of 2% FBS and EGF for 19 days. After 26 days of differentiation, the differentiated cells were incubated with β-Ala-Lys-AMCA (25 μM) for 4 h at 37°C. After uptake was stopped, the differentiated cells were fixed and stained with antisucrase–isomaltase antibody. Typical images from β-Ala-Lys-AMCA uptake experiments. Arrows indicate co-localization of β-Ala-Lys-AMCA and sucrase–isomaltase protein. (A) Intracellular uptake of β-Ala-Lys-AMCA (blue); (B) immunofluorescence staining of sucrase–isomaltase (red); (C) overlay (Merge) image of β-Ala-Lys-AMCA and sucrase–isomaltase. Scale bar, 50 μm.

are formed by three-dimensional culture, was applied during differentiation from the endoderm to intestinal organoids, and several factors were added in large quantities to induce differentiation. This method may be suitable for the culture of intestinal stem cells but not for selective differentiation into enterocytes, and it is complicated and costly. Therefore, we generated enterocyte-like cells using a simple two-dimensional culture method.

Spence *et al.*[15] also reported that the combination of FGF4 (500 ng/ml) and Wnt3a (500 ng/ml) effectively posteriorized the endoderm. Ameri *et al.*[32] reported the induction of CDX2 with FGF2 (256 ng/ml) in a concentration-dependent manner in the human ES cell-derived endoderm. In the present study, we examined the effects of FGF2, FGF4, and Wnt3a on the induction of the midgut endoderm lineage. Our results showed that the expression level of CDX2 was comparable in the FGF2 and FGF4 treatment groups, whereas no effect of Wnt3a was observed. These results were inconsistent with those of Spence *et al.*,[15] possibly because of the lower concentrations of Wnt3a used in our study (50 ng/ml). Sherwood *et al.*[33] demonstrated that β-catenin-dependent Wnt signaling, activated by the glycogen synthase kinase-3 (GSK3) inhibitor, induced the expression of the intestinal master regulator Cdx2 and induced intestinal differentiation of the ES cell-derived endoderm and large intestinal gene expression. They also indicated that because of poor bioactivity, the induction level of Cdx2 expression by Wnt3a was lower than that by the GSK3 inhibitor. Therefore, superior stability and bioactivity of small-molecule compounds may lead to more effective experimental regulation of these signaling pathways. However, the striking activation of Wnt signaling also induced large intestinal gene expression, and further investigations of the extent of activation of Wnt signaling during small intestinal differentiation may be required. The expression of LGR5[17] was higher in the FGF2 treatment group than that in the FGF4 treatment group. Therefore, subsequent differentiation experiments were performed using FGF2.

We have demonstrated that enterocyte-like cells, which express the specific intestinal markers sucrase–isomaltase,[34,35] villin 1, ISX, and pharmacokinetics-related genes, were differentiated from an intestinal stem cell-like cell population by two-dimensional culture with EGF and a low serum concentration. Sucrase–isomaltase and SLC15A1/PEPT1 mRNA gradually increased with the duration of differentiation, indicating that longer duration may be necessary to efficiently obtain mature enterocytes. In contrast, LGR5 mRNA expression remained unchanged, suggesting that enterocytes matured during intestinal stem cell proliferation. DPP4 (serine protease) and SLC46A1/PCFT (folate transporter) are known to be abundantly expressed in epithelial cells of the small intestine.[36,37] Expression of DPP4 and SLC46A1/PCFT in differentiated cells was higher than that in adult small intestine samples. Intestinal differentiation was promoted by low FBS concentrations in this study. Potentially, the growth of extra-enterocytic cells such as fibroblasts may be suppressed with decreasing FBS concentrations, and some differentiation and growth-inhibiting factors may be present in FBS, although the mechanisms underlying these effects remain unclear.

At present, human intestinal epithelial cells are difficult to obtain, and no appropriate model cell system exists. Instead, other tissue cell-derived cell lines, including Caco-2 cells (human colon carcinoma cell line) and Madin–Darby canine kidney (MDCK) cells, have been used as intestinal models in drug absorption studies.[2,38] However, drug transporter expression patterns in these cells considerably differ from those in enterocytes. In particular, CYP3A4 is expressed at very low levels in these cell lines. In the present study, cells expressing the sucrase–isomaltase protein showed uptake of β-Ala-Lys-AMCA, the substrate of the oligopeptide transporter (**Fig. 8**). Differentiated cells also expressed CYP3A4 mRNA, albeit at levels lower than those in the adult small intestine (**Fig. 3**), suggesting that enterocyte-like cells differentiated from human iPS cells have peptide-transporting activity and may be useful in the study of drug absorbability.

In this study, we used a human iPS cell line, which is easily differentiated into hepatocytes of an endodermal lineage, because the intestine is also an endoderm-derived tissue. Differentiation propensity is known to be markedly different among human ES and iPS cell lines.[39,40] Thus, there may be a difference in the degree of intestinal differentiation depending on the human iPS cell line being tested. Regarding this point, we believe it is necessary to perform studies comparing intestinal differentiation among human iPS cell lines in the future.

In conclusion, because the intestine is an endoderm-derived tissue, human iPS cells were directly differentiated into the endoderm using activin A. Subsequently, we devised a simple method for differentiation into enterocyte-like cells with functional peptide transport by two-dimensional culture and the addition of several growth factors. These data suggest that human iPS-derived enterocytes may facilitate future drug development studies. If enterocyte-like cells, which have functional features similar to those of enterocytes, can be generated from human iPS cells, it may be possible to construct systems for easy estimation of overall intestinal function, including absorption and metabolism.

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# Stability of genomic imprinting in human induced pluripotent stem cells

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## Abstract

**Background:** hiPSCs are generated through epigenetic reprogramming of somatic tissue. Genomic imprinting is an epigenetic phenomenon through which monoallelic gene expression is regulated in a parent-of-origin-specific manner. Reprogramming relies on the successful erasure of marks of differentiation while maintaining those required for genomic imprinting. Loss of imprinting (LOI), which occurs in many types of malignant tumors, would hinder the clinical application of hiPSCs.

**Results:** We examined the imprinting status, expression levels and DNA methylation status of eight imprinted genes in five independently generated hiPSCs. We found a low frequency of LOI in some lines. Where LOI was identified in an early passage cell line, we found that this was maintained through subsequent passages of the cells. Just as normal imprints are maintained in long-term culture, this work suggests that abnormal imprints are also stable in culture.

**Conclusions:** Analysis of genomic imprints in hiPSCs is a necessary safety step in regenerative medicine, with relevance both to the differentiation potential of these stem cells and also their potential tumorigenic properties.

**Keywords:** Genomic imprinting, Loss of imprinting (LOI), DNA methylation, Histone modification, Human induced pluripotent cells

## Background

Human induced pluripotent stem cells (hiPSCs) represent a promising therapeutic tool for many diseases, and might be useful for regenerating aged tissues and organs at high risk of failure [1,2]. However, the intrinsic self-renewal and pluripotency of hiPSCs potentially make them tumorigenic, hindering their clinical application [3-5]. hiPSCs are generated through epigenetic reprogramming of somatic tissue. It was initially thought that hiPSCs and human embryonic stem cells (hESCs) shared a high degree of epigenetic similarity [6,7]. However, recent reports have indicated that substantial differences exist between hiPSCs and hESCs with regard to gene expression, miRNA expression and DNA methylation [8-10]. Cell-of–origin-specific genetic and epigenetic differences exist in hiPSCs [11] and some of these stem cell lines spontaneously differentiate during serial passage [12]. Extensive evaluation of hiPSCs is consequently an essential component of the process required for their safe use in regenerative medicine.

Many types of malignant tumors are characterized by complex genetic and epigenetic alterations, including loss of heterozygosity (LOH) and loss of imprinting (LOI) [13,14]. Such alterations are presumed to represent the second hit, according to Knudson's two-hit hypothesis (OMIM #167000) [15]. However, alterations in DNA methylation can also occur as the first hit during human carcinogenesis [16]. Alterations in the expression of imprinted genes represent one of the most common changes seen in cancer [17,18]. Some imprinted genes, including *H19* [19], *GTL2* [20], *PEG1, PEG3* [21], *LIT1* (*KCNQ1OT1*) [22] and *ZAC* [23] are known to act, or are strongly implicated to act, as

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**Table 1 LOI and MOI in hiPSCs**

| | | H19 | | IGF2 | | PEG3 | | PEG1 | | GTL2 | | KCNQ1 | | NDN | | LIT1 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | (RsaI) | | (ApaI) | | (MnlI) | | (AflIII) | | (TaaI) | | (SmaI) | | (MboI) | | (RsaI) | |
| Cell | Passage | gDNA | cDNA | gDNA | cDNA | gDNA | cDNA | gDNA | cDNA | gDNA | cDNA | gDNA | cDNA | gDNA | cDNA | gDNA | cDNA |
| AM936EP | P9 | a/b | a | b | - | a | - | b | - | a/b | a | a | - | b | - | a/b | a |
| AM-iPS -2 | P13 | a/b | a | b | - | a | - | b | - | a/b | a | a | - | b | - | a/b | a |
| AM-iPS -2 | P19 | a/b | a | b | - | a | - | b | - | a/b | a | a | - | b | - | a/b | a |
| AM-iPS -2 | P35 | a/b | a | b | - | a | - | b | - | a/b | a | a | - | b | - | a/b | a |
| AM-iPS -3 | P9 | a/b | a | b | - | a | - | b | - | a/b | a | a | - | b | - | a/b | a |
| AM-iPS -3 | P21 | a/b | a | b | - | a | - | b | - | a/b | a | a | - | b | - | a/b | a |
| AM-iPS -3 | P29 | a/b | b | b | - | a | - | b | - | a/b | a | a | - | b | - | a/b | a |
| AM-iPS -3 | P36 | a/b | a | b | - | a | - | b | - | a/b | a | a | - | b | - | a/b | a |
| AM-iPS -7 | P12 | a/b | a | b | - | a | - | b | - | a/b | a/b | a | - | b | - | a/b | a |
| AM-iPS -7 | P22 | a/b | a | b | - | a | - | b | - | a/b | a/b | a | - | b | - | a/b | a |
| AM-iPS -7 | P32 | a/b | a | b | - | a | - | b | - | a/b | a/b | a | - | b | - | a/b | a |
| AM-iPS -8 | P13 | a/b | a | b | - | a | - | b | - | a/b | a | a | - | b | - | a/b | a |
| AM-iPS -8 | P20 | a/b | a | b | - | a | - | b | - | a/b | a | a | - | b | - | a/b | a |
| AM-iPS -8 | P37 | a/b | a | b | - | a | - | b | - | a/b | a | a | - | b | - | a/b | a |
| AM-iPS -20 | P8 | N.T. | a | N.T. | - | N.T. | - | N.T. | - | N.T. | a | N.T. | - | N.T. | - | N.T. | a |
| AM-iPS -20 | P11 | a/b | a | b | - | a | - | b | - | a/b | a | a | - | b | - | a/b | a |
| AM-iPS -20 | P14 | a/b | a | b | - | a | - | b | - | a/b | a | a | - | b | - | a/b | a |
| AM-iPS -20 | P16 | a/b | N.D. | b | - | a | - | b | - | a/b | a | a | - | b | - | a/b | a |
| AM-iPS -20 | P32 | a/b | N.D. | b | - | a | - | b | - | a/b | a | a | - | b | - | a/b | a |
| PL551Ar | P16 | a/b | a | a/b | a | b | - | a/b | N.D. | b | - | b | N.D. | a | - | a | - |
| PAE-iPS -05 | P19 | a/b | a | a/b | N.D. | b | - | a/b | N.D. | b | - | b | - | a | - | a | - |
| PAE-iPS -05 | P31 | a/b | a | a/b | N.D. | b | - | a/b | N.D. | b | - | b | - | a | - | a | - |
| PAE-iPS -11 | P14 | a/b | a | a/b | N.D. | b | - | a/b | N.D. | b | - | b | - | a | - | a | - |
| PAE-iPS -11 | P18 | a/b | a | a/b | N.D. | b | - | a/b | N.D. | b | - | b | - | a | - | a | - |
| PAE-iPS -11 | P30 | a/b | a | a/b | N.D. | b | - | a/b | N.D. | b | - | b | - | a | - | a | - |
| MRC-5 | - | a/b | N.D. | b | - | a | - | b | - | a/b | a/b | a/b | N.D. | a/b | N.D. | a | - |
| MRC-iPS -16 | P30 | a/b | N.D. | b | - | a | - | b | - | a/b | ND | a/b | b | a/b | a | a | - |
| MRC-iPS -25 | P6 | a/b | N.D. | b | - | a | - | b | - | a/b | ND | a/b | b | a/b | a | a | - |
| MRC-iPS -25 | P30 | a/b | N.D. | b | - | a | - | b | - | a/b | ND | a/b | b | a/b | a | a | - |
| MRC-iPS -40 | P11 | a/b | N.D. | b | - | a | - | b | - | a/b | ND | a/b | b | a/b | a | a | - |
| MRC-iPS -40 | P30 | a/b | N.D. | b | - | a | - | b | - | a/b | ND | a/b | b | a/b | a | a | - |
| UtE1104 | P9 | a/b | N.D. | a | - | a/b | a/b | a/b | b | a/b | a/b | b | N.D. | b | - | a | - |
| UtE-iPS -6 | P20 | a/b | N.D. | a | - | a/b | a/b | a/b | a/b | a/b | a/b | b | - | b | - | a | - |
| UtE-iPS -6 | P31 | a/b | b | a | - | a/b | a/b | a/b | a/b | a/b | a/b | b | - | b | - | a | - |
| UtE-iPS -11 | P13 | a/b | N.D. | a | - | a/b | N.D. | a/b | a/b | a/b | a | b | N.D. | b | - | a | - |
| UtE-iPS -11 | P20 | a/b | N.D. | a | - | a/b | N.D. | a/b | a/b | a/b | a/b | b | N.D. | b | - | a | - |
| UtE-iPS -11 | P30 | a/b | N.D. | a | - | a/b | N.D. | a/b | a/b | a/b | a | b | - | b | - | a | - |
| Edom22 | P5 | b | - | a/b | a/b | a | - | a/b | b | b | - | a/b | a | a | - | a | - |
| Edom-iPS -1 | P27 | b | - | a/b | N.D. | a | - | a/b | b | b | - | a/b | a | a | - | a | - |

**Table 1 LOI and MOI in hiPSCs** *(Continued)*

| | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| hES 3 | P29 | a/b | a | b | - | a/b | a | a/b | b | b | - | a | - | a/b | b | a | - |
| SEES 1 | P10 | a/b | a | a/b | a | a | - | b | - | a/b | b | a | - | a | - | a | - |
| SEES 4 | P9 | a/b | b | a/b | a | a | - | b | - | b | - | a | - | a/b | b | a | - |

A summary of LOI and MOI RFLP data for the 8 imprinted genes analyzed in 22 hiPSCs and 3 control hES cell lines. hiPSCs derived from extraembryonic amniotic membrane (AM-iPS), embryonic lung tissue (MRC-iPS), uterine endometrium (UtE-iPS), adult menstrual blood (Edom-iPS) and extraembryonic placental tissue (PAE-iPS). Samples were analyzed at the specified passage number. (-): not informative.

tumor suppressor genes (TSGs). Furthermore, imprinted genes play key roles in regulating growth and differentiation [24]. Thus the aberrant expression of imprinted genes may contribute to tumorigenesis or alter the differentiation potential of stem cells.

The monoallelic expression of imprinted genes is reliant on epigenetic mechanisms, most notably DNA methylation, which is established in the male and female germlines at discrete locations termed germline or gametic differentially methylated regions (gDMRs) [25]. Imprinted domains generally contain several genes displaying allele-specific expression and gDMRs within these domains act as imprinting centers or imprint control regions for the domain [26]. The majority of imprinted genes reside within these complex domains [27]. Although gametic DMRs are maintained throughout the life of the organism,

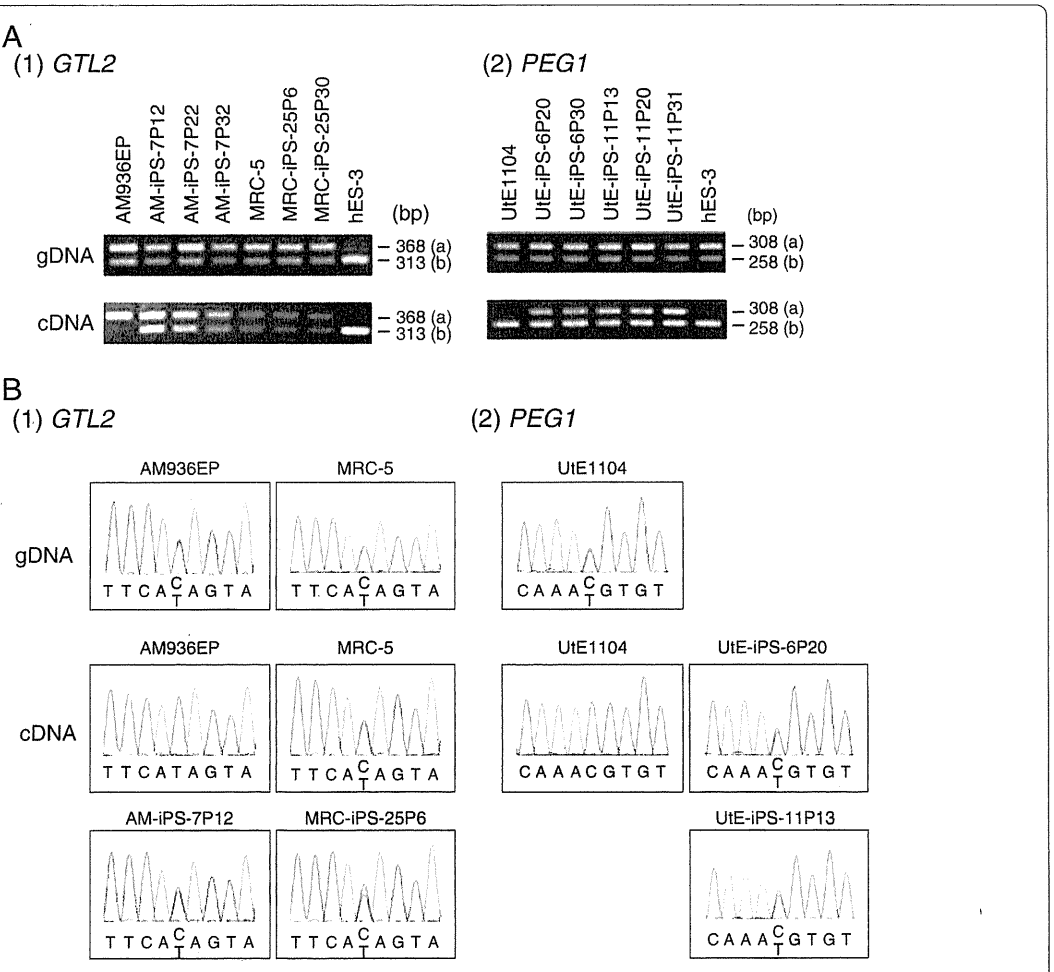


**Figure 1 Loss of imprinting in hiPSCs.** Loss of allelic expression of *GTL2* in cell line AM-iPS-7 (1) and *PEG1* in cell lines UtE-iPS-6 and 11 (2). PCR products amplified from paired genomic DNA and cDNA samples were digested with the specified restriction enzymes (**A**). Results were confirmed by direct sequencing (**B**). AM936EP and UtE1104 are primary culture cells made from amniotic membrane and uterine endometrium cells, respectively. Band 'a': without a restriction site. Band 'b': with a restriction site.

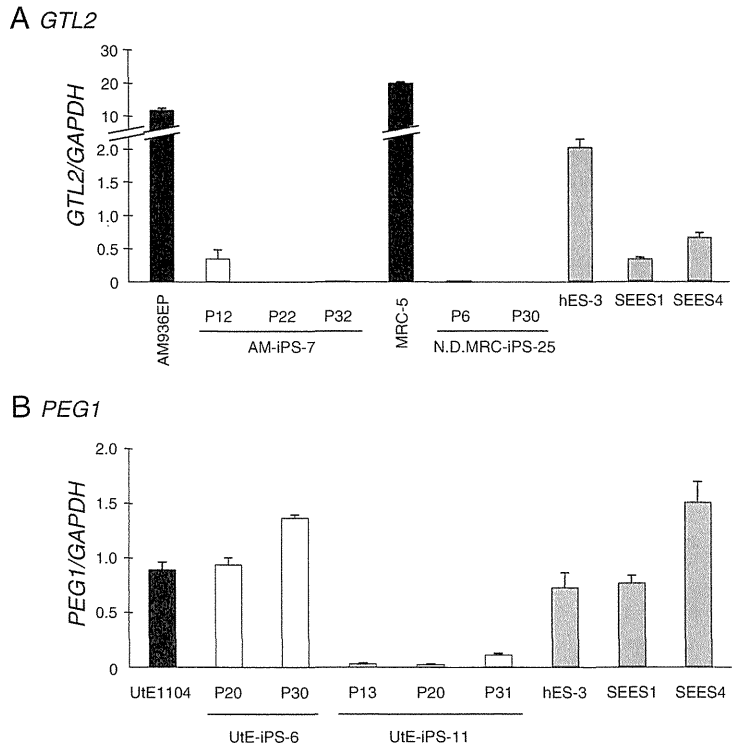


**Figure 2 Reduced expression of hiPSCs with LOI.** Gene expression levels of *GTL2* (**A**) and *PEG1* (**B**) in the original and the passaged hiPSCs were compared to that of hESCs. The *GAPDH* ratio was calculated. The bars indicate the means ± standard deviation (SD) from two replicates. N.D.: not determined.

genes within the domain can be imprinted in tissue- and developmentally specific manners [28].

In a recent paper, we demonstrated that hiPSCs exhibit epigenetic patterns distinct from hESCs [29]. After continuous passaging of the hiPSCs, these differences diminished such that over time the hiPSCs more closely resembled hESCs. However, we found that the imprinted DMRs showing abnormal methylation in early passage hiPSCs did not resolve during passaging. In this study we focused on the expression of imprinted genes in hiPSCs. Several reports on imprinted gene expression in hESCs demonstrate a substantial degree of instability [30]. Less is known regarding the stability of imprints in hiPSCs, although some work has begun [31]. We are particularly concerned with the stability of imprints in pluripotent stem cells during prolonged culture. Here, we examined the imprinting status and expression levels of eight imprinted genes and the methylation status of their DMRs in five independently derived hiPSCs. We found that the frequency LOI was very low in the early passaged lines. We also found that, in contrast, the epigenetic changes that took place at non-imprinted loci during prolonged culture for both normal and aberrant imprints were stably inherited despite prolonged passaging of the lines.

## Results

### Loss of heterozygosity (LOH) and loss of imprinting (LOI) in hiPSCs

We first determined whether hiPSCs showed LOH by comparing the restriction fragment length polymorphism (RFLP) patterns of the original tissue DNA with those of the hiPSC DNA samples. Samples in which RFLPs were present in the original DNA sample but absent or with an altered ratio in the hiPSC samples were considered to exhibit LOH. We found no evidence for LOH at the 8 loci tested (*H19*, *IGF2*, *KCNQ1*, *LIT1*, *GTL2*, *PEG1*, *PEG3* and *NDN*).

We next performed RT-PCR and RFLP analyses to identify samples that demonstrated loss of imprinting (LOI). Where expression of genes was low in undifferentiated cells, it was not possible to determine their imprinting status (*H19* in MRC-iPS and UtE-iPS, *IGF2* in PAE-iPS and *GTL2* in Edom-iPS). Of the 16 informative loci, we identified LOI at three loci in hiPSCs, *GTL2*, *PEG1* and *PEG3*, but we did not detect any LOI in hESCs (Table 1). Of particular interest, we observed loss of imprinting during the

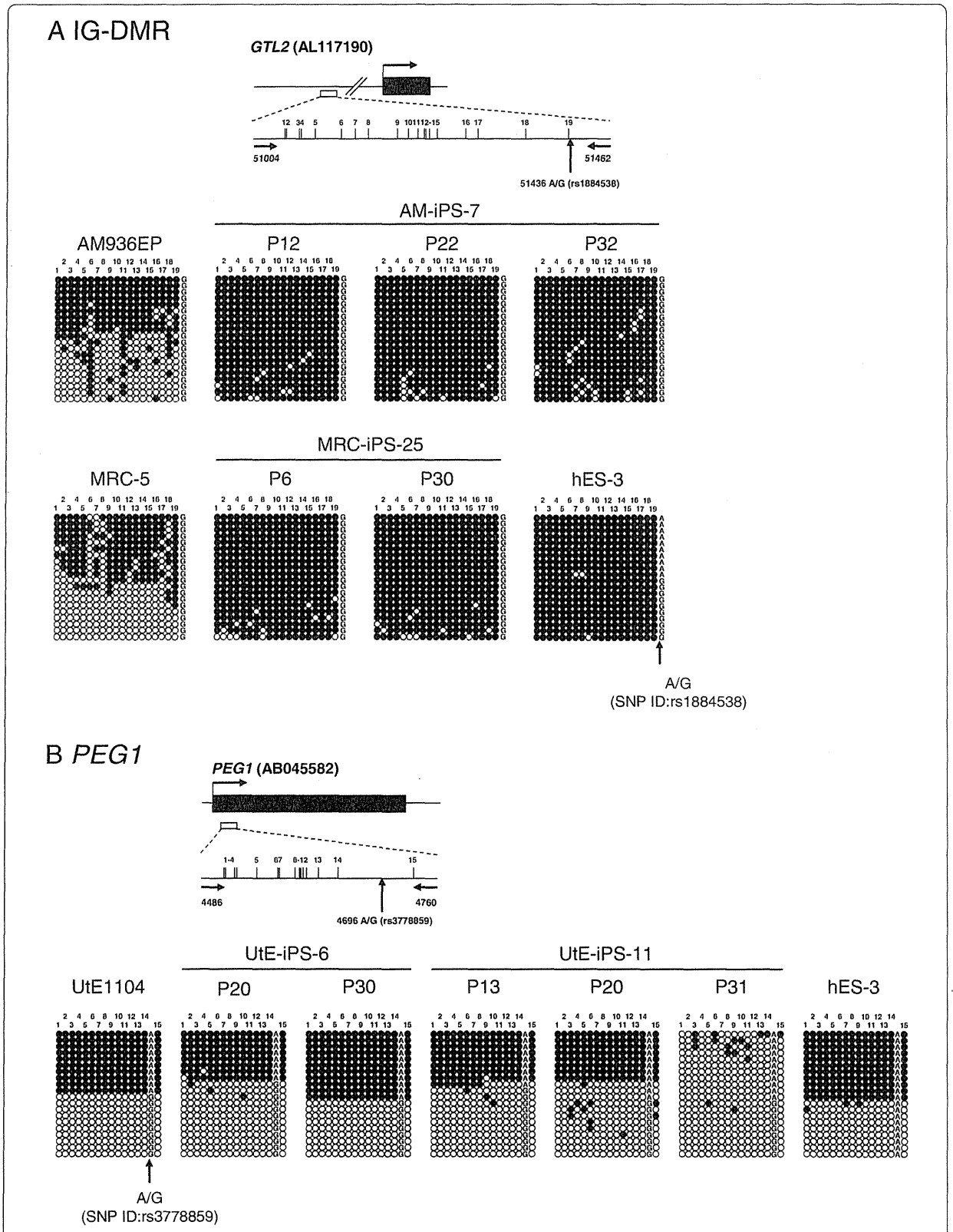


**Figure 3** (See legend on next page.)

(See figure on previous page.)
**Figure 3 Aberrant DNA methylation of hiPSCs with LOI.** Bisulfite PCR sequencing methylation assay of genomic DNA prepared from AM-iPS-7 and MRC-iPS-25 at the IG-DMR (*GTL2*-DMR) (**A**) and UtE-iPS-6 and 11 at *PEG1* (**B**). Each row represents a unique methylation profile within the pool of 20 clones sequenced. Closed and open circles represent methylated and unmethylated CpGs, respectively. The numbers represent the percentages of methylation by bisulfite sequencing. SNPs are shown by arrows.

process of establishing the AM-iPSC (*GTL2*) and UtE-iPSC (*PEG1*) lines (Figure 1A). Where LOI was observed in early passage cells, this was maintained even after 30 or more passages (Figure 1).

### Expression level of the imprinted genes in hiPSCs

LOI can refer to silencing of an originally active allele or expression of a normally silent allele. Therefore, we compared the expression levels of the three genes that displayed LOI in hiPSCs and hESCs (Table 1). The expression of *IGF2* and *GTL2* was decreased in almost all the hiPSC lines in comparison with the hES cells (Additional files 1 and 2). *GTL2* in cell line AM-iPS-7 and *PEG1* in line UtE-iPS-11 showed apparent biallelic expression but their expression levels were relatively low in comparison to hESCs with reduced expression maintained stably through to late passages (Figure 2). In contrast, expression of *PEG1* in cell line UtE-iPS-6 was not significantly different from that of hESCs. These results were in accordance with the DNA microarray analysis data we already reported [29]. Since, in two cases, LOI correlated with reduced gene expression, this has potential functional implications due to loss of function.

### Analysis of the DNA methylation status and the histone modification of GTL2 and PEG1 DMRs in hiPSC lines

We determined the allele-specific methylation status of the *GTL2* (IG-DMR) and *PEG1* imprinted DMRs using polymorphic bisulfite-PCR sequencing (Figure 3). In cell line AM-iPS-7, which showed LOI and reduced expression of *GTL2*, we observed hypermethylation of IG-DMR, which was maintained during continuous passaging. IG-DMR methylation is normally present on the silent allele of *GTL2* [32], which suggests aberrant signaling between this DMR and *GTL2* expression. In cell line UtE-iPS-11, in which there was LOI and reduced expression of *PEG1*, abnormal methylation was detected in passage 31 cells but not earlier passages. In cell line UtE-iPS-6, in which there was LOI but not reduced expression of *PEG1*, abnormal methylation was not detected. Allele-specific expression of some genes has been reported to be regulated by histone modification rather than direct DNA methylation [33-35]. We therefore analyzed histone modifications in the hiPS cell line by chromatin immunoprecipitation (ChIP) analyses using the following antibodies: dimethylated H3-Lys4 (H3K4me2), acetylated H3-Lys9 (H3K9ac), H3K9me2, and H3K27me3. H3K4me2 and H3K9ac mark active genes and H3K9me2 and H3K27me3 are repressive marks. In the *GTL2* promoter region, H3K9me2 and H3K27me3 were enriched in AM-iPS-7 and MRC-iPS-25 cells (Figure 4D).

#### *Reactivation of the imprinted genes by the HDACi treatment*

Previous reports demonstrated that the *GTL2* gene was aberrantly silenced in most mouse iPSC lines but that expression could be restored through HDACi treatment [36,37]. In our study, AM-iPS and MRC-iPS cells showed LOI of *GTL2*, with a reduction in gene expression and hypermethylation of the IG-DMR. To assess whether *GTL2* expression could be restored, AM-iPS and MRC-iPS cells were treated with the HDAC inhibitor VPA (sodium valproate). VPA-treated cells did achieve a 3.0–5.8-fold increase in *GTL2* expression levels (Figure 4A) and H3K4me2 and H3K9ac were enriched in its promoter region (Figure 4D). However, the DNA methylation pattern was stable under VPA treatment and the imprinting status of *GTL2* was not changed, with cells maintaining biallelic expression of the gene (Figure 4B). These results suggested that the aberrant DNA methylation and imprinting that were established and maintained in early passages (Figure 4C) were not sustainably reversed by the treatment.

## Discussion

Most hES and hiPS cell lines possess stable imprinted gene expression, at least in undifferentiated cells [30,31 and findings in this study]. This implies that imprints withstand the process of reprogramming and the rigors of growing in culture. In our study, we found that only three of the 22 hiPS cell lines we derived from a variety of somatic cell types showed LOI, and at only a few sites. The majority of cases had normal imprinting status. While LOI was rare in our hiPS cell lines, we found that it was maintained during prolonged passage, and resistant to VPA treatment. These abnormalities would preclude the use of these cell lines for therapeutic applications but might provide a mechanistic insight relevant to imprinting and reprogramming.

We previously reported that abnormal DNA methylation detected in early passage iPSCs diminished after continued passaging, such that these cells ultimately more resembled ESCs. However, abnormal DNA methylation at imprinted loci in ESCs occurs in response to continuous passaging [29]. Rugg-Gunn et al. suggested three possible explanations for LOI in hESCs [30]. First, the developmental onset of transcription might influence imprinted gene expression. Second, a particular imprinted gene's expression might

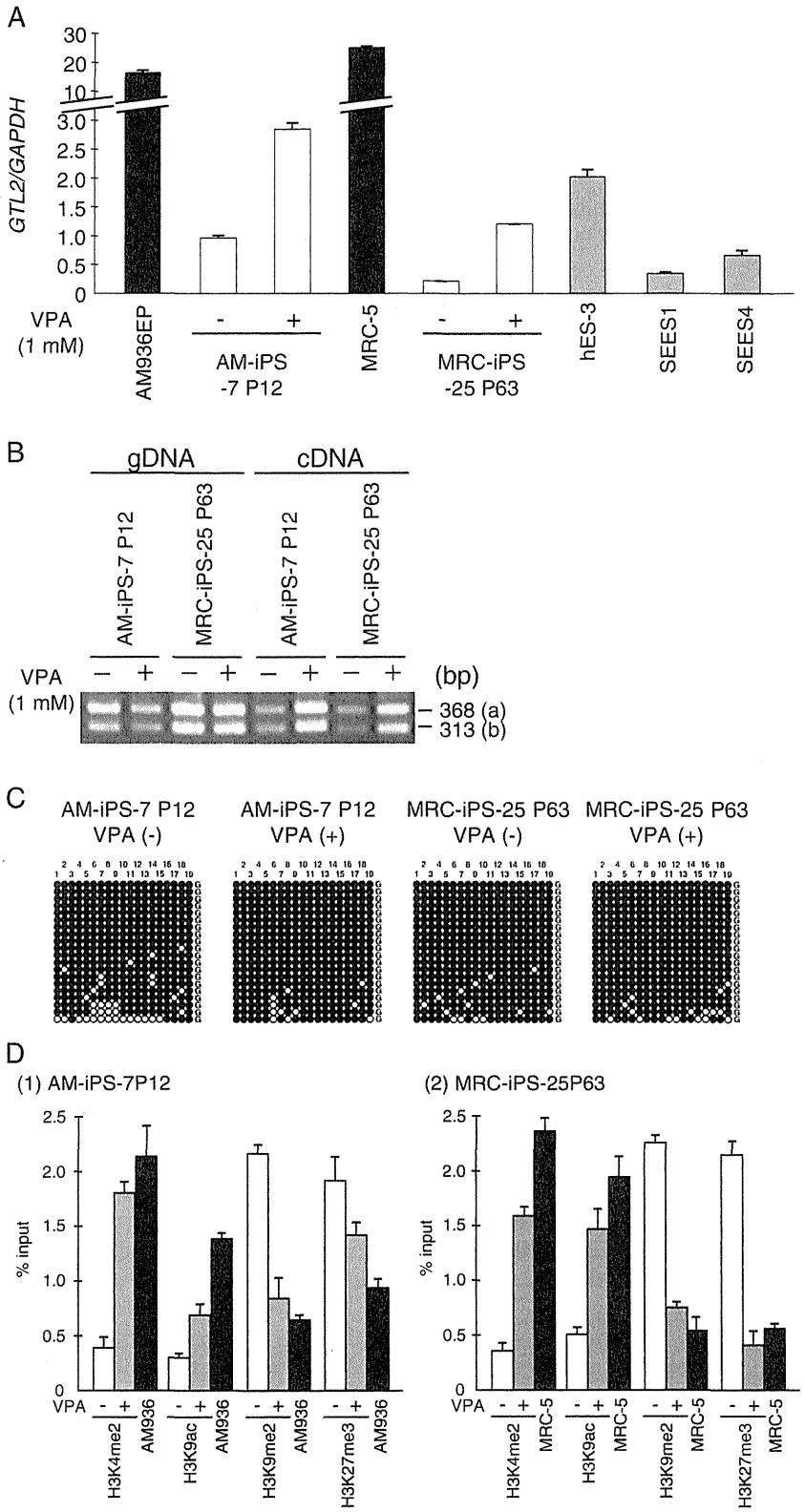


**Figure 4** (See legend on next page.)

(See figure on previous page.)
**Figure 4 Reactivation of *GTL2* expression by treatment with VPA.** Reactivation of *GTL2* expression by treatment with VPA (**A**). The expression level of *GTL2* mRNA was restored by VPA treatment. Gene expression of the original cells and of the hiPSCs was compared to that of hESCs. The *GAPDH* ratio was calculated. The bars indicate the means ± SD from two replicates. The imprinting status of *GTL2* was stable in response to VPA (**B**). Methylation status in bisulfite-PCR sequencing analyses of IG-DMR is unchanged (**C**). Histone modifications of the *GTL2* promoter were changed by VPA (D). The immunoprecipitation/input ratio was calculated. The bars indicate the means ± SD from three replicates.

differ depending on whether it is regulated by maternally or paternally inherited methylation. Third, the pattern of imprinted gene expression might depend on whether the gene provides a growth advantage to hESCs. These possibilities might also apply to hiPSCs.

There are two caveats that apply to this work. First, we examined expression in undifferentiated cells. Consequently, we may have missed changes in imprinted gene expression where genes are expressed only in differentiated cells or where imprinting is tissue specific. Second, we examined total levels of expression and total methylation patterns of populations of cells. Therefore we cannot exclude the possibility that a small population within our samples could behave in a different manner from the general population. Nonetheless, our data are encouraging in suggesting that imprinting errors in iPSCs are derived from a variety of human somatic cell types.

One of the key advantages of iPSCs is that they can be derived from patients, supporting the further investigation of certain diseases, as well as the replacement of degenerated and damaged tissues. Careful analysis of imprinted genes should therefore be performed on all iPS cell lines since several published iPS cell lines that passed the necessary reprogramming criteria also showed aberrations in imprinted gene expression and DNA methylation of DMRs. This is particularly critical if these hiPSs are to be used for regenerative medicine since aberrations in imprinted genes could cause problems with cell differentiation and perhaps even cause tumors [38]. The analysis of imprinted genes is also essential for modeling of genetic diseases because abnormal imprinting can seriously confuse the disease phenotyping.

Recent advances in high-throughput technologies for gene expression analysis and DNA methylation analysis indicate the possibility that all newly generated stem cell lines can be characterized at the epigenetic level rapidly and precisely. However, our work and that of others suggest that certain imprinted loci may be more susceptible to LOI. This means that it might be possible to design targeted assays for specific loci as the first step in the characterization of newly generated cell lines, and also those that have been extensively passaged.

## Conclusions

In conclusion, while imprinting errors may be rare in iPSCs, they are resistant to reversal strategies. The aberrant expression of imprinted genes in these lines is likely to hamper their use both for the understanding of certain pathologies and regenerative medicine.

## Methods

### Ethics statement

All experiments handling human cells and tissues were performed in line with the tenets of the Declaration of Helsinki. This study was approved by the Institutional Review Board of the National Institute for Child Health and Development and the Ethics Committee of Tohoku University School of Medicine.

### DNA/RNA preparation of iPSCs

We generated 22 hiPSCs from extraembryonic amniotic membrane (AM-iPS), embryonic lung tissue (MRC-iPS), uterine endometrium (UtE-iPS), adult menstrual blood (Edom-iPS), and extraembryonic placental tissue (PAE-iPS) and characterized the pluripotent nature using culture methods described previously [39-41]. Prior to RNA and DNA preparation, feeder layers were removed from the undifferentiated cells by panning for 20 minutes.

### Loss of heterozygosity (LOH) and loss of imprinting (LOI) analyses

PCR was performed on parental tissue and the genomic DNA of hiPSCs using the primer sequences summarized in Additional file 3. A PCR reaction mix containing 0.5 μM concentrations of each primer set, 200 μM dNTPs, 1× PCR buffer, and 1.25U of EX *Taq* Hot Start DNA Polymerase (Takara Bio, Tokyo, Japan) in a total volume of 20 μl was used. The following PCR program was used: 1 minute of denaturation at 94°C followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 50-70°C, 30 seconds at 72°C and a final extension for 5 minutes at 72°C. PCR products were digested by unique polymorphic enzymes to identify samples that were heterozygous for a single nucleotide polymorphism (SNP). For samples found to be heterozygous for a SNP, RNA was prepared from matched hiPSCs, followed by reverse transcription-PCR (RT-PCR) and restriction digestion (Additional file 3) [42-49]. The digested PCR products were electrophoresed on 3% agarose gel.

### Gene expression analysis

RNA expression levels of 8 imprinted genes were also analyzed by microarray and the real-time PCR. Microarray analysis was performed using an Agilent Whole Human

Genome Microarray chip (G4112F, Agilent, Santa Clara, CA). Raw data were normalized and analyzed using GeneSpringGX11 software (Silicon Genetics, Redwood City, CA). The microarray data have been deposited in Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/). Real-time PCR reaction was done with SYBR Premix Ex Taq II (Takara Bio). In the case of PEG3 expression analysis, TaqMan Gene Expression Assay (Assay ID: Hs00300418-s1, Applied Biosystems, Foster City, CA) was carried out according to the manufacturer's protocol using a StepOne Real-time PCR System (Applied Biosystems). The relative expression levels of the detected genes from these cells were estimated visually by comparing relative band intensities with the expression level of the housekeeping gene *GAPDH*.

### Polymorphic bisulfite PCR methylation assay

We performed standard methylation assays using the SNPs and bisulfite sequencing [50]. The primary DMRs of eight imprinted genes (*H19, GTL2, ZDBF2, PEG1, KCNQ1OT1, ZAC, PEG3* and *SNRPN*) were analyzed as described previously [50,51]. Each DNA sample was treated with sodium bisulfite using the EZ methylation kit (Zymo Research, Orange, CA) and amplified by PCR. PCR products were purified, cloned into pGEM-T (Promega, Medison, WI) and an average of 20 clones per individual were sequenced using reverse primer M13 and an automated ABI Prism 3130xl Genetic Analyzer (Applied Biosystems). To avoid any allelic bias, we used specific polymorphic sites. Sodium bisulfite modification treatments were carried out in duplicate for each DNA sample and at least three independent amplification experiments were performed for each individual examined.

### *Chromatin immunoprecipitation (ChIP) assay*

ChIP analysis was performed using the Magna ChIP G Chromatin Immunoprecipitation Kit (Millipore, Temecula, CA) according to the manufacturer's protocol. We used the following antibodies: dimethylated H3-Lys4, acetylated H3-Lys9, dimethylated H3-Lys9 and trimethylated H3-Lys27 (Millipore). The histone modifications were analyzed by real-time PCR. Real-time PCR reaction was done with SYBR Premix Ex Taq II (Takara Bio). The amount of precipitated DNA was determined as percentage relative to input DNA. Primers used are listed in Additional file 3.

### *Treatment of cells with sodium valproate*

hiPSCs were plated at a density of $5 \times 10^5$ cells/60mm$^2$ dish. Twenty-four hours later, they were treated with 1 mM sodium valproate (Wako, Tokyo, Japan) for the times stated. Total RNA was prepared and analyzed by the RT-PCR method. The methylation status of the DMRs was examined using the bisulfite PCR sequencing methylation assay described previously [51].

## Additional files

**Additional file 1: Microarray analysis.** Scatter plots of MRC-iPS-25P22 versus hES3 (**A**) and UtE-iPS-6P30 versus hES3 (**B**). Scatter plot comparing the spot intensities in hybridization with probes from hiPSCs (*y* axis) and hESCs (*x* axis). The magenta plots indicate the imprinted genes.

**Additional file 2: Gene expression analysis of the imprinted genes.** *H19* (**A**), *IGF2* (**B**), *PEG3* (**C**), *PEG1* (**D**), *GTL2* (**E**), *KCNQ1* (**F**), *NDN* (**G**) and *LIT1* (**H**). Gene expression of the original and hiPSCs was compared to that of hESCs. The *GAPDH* ratio was calculated. The bars indicate the means ± SD from two replicates.

**Additional file 3: PCR primers and conditions.**

### Abbreviations

DMR: Differentially methylated region; hESCs: Human embryonic stem cells; hiPSCs: Human induced pluripotent cells; LOH: Loss of heterozygosity; LOI: Loss of imprinting; MOI: Maintenance of imprinting; N.D: Not determined; N.T: Not tested; PCR: Polymerase chain reaction; RFLP: Restriction fragment length polymorphism; RT-PCR: Reverse transcription-PCR; SNP: Single nucleotide polymorphism; SD: Standard deviation.

### Competing interest

The authors declare that they have no competing interests.

### Authors' contributions

AU and TA conceived and designed the study and wrote the manuscript. AU generated hiPSCs and hESCs. TA analyzed genomic imprinting. HH carried out the molecular study and data analysis and wrote the manuscript. H Okae, MS, NM and AS performed the molecular study and contributed to data analysis. MT, NK, H Okita, YM and HA prepared cell materials and contributed to data analysis. KN performed the transcriptome data analysis. All authors reviewed the results from the data analysis and approved the final manuscript.

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