bidirectional differentiation; heterogeneous populations, which contain the hepatocytes and pancreas cells or hepatocytes and cholangiocytes, were obtained, respectively (**Figure 1**), consistent with a previous report that HNF4α plays an important role not only in the liver but also in the pancreas.[12] Therefore, we concluded that HNF4α plays a significant stage-specific role in the differentiation of human ESC- and iPSC-derived hepatoblasts to hepatocytes (**Figure 5e**).

We found that the expression levels of the hepatic functional genes were upregulated by HNF4α transduction (**Figure 3a,b**, and **Supplementary Figures S7** and **S8**). Although the c/EBPα and GATA4 expression levels of the three factors-transduced cells were higher than those of primary human hepatocytes, the FOXA1, FOXA2, FOXA3, and HNF1α, which are known to be important for hepatic direct reprogramming and hepatic differentiation,[35,36] expression levels of three factors-transduced cells were slightly lower than those of primary human hepatocytes (**Supplementary Figure S8**). Therefore, additional transduction of FOXA1, FOXA2, FOXA3, and HNF1α might promote further hepatic maturation. Some previous hepatic differentiation protocols that utilized growth factors without gene transfer led to the appearance only of heterogeneous hepatocyte populations.[4–6] The HNF4α transduction led not only to the upregulation of expression levels of several hepatic markers but also to an almost homogeneous hepatocyte population; the differentiation efficacy based on CYPs, ASGR1, or ALB expression was ~80% (**Figure 3c–e**). The efficient hepatic maturation in this study might be attributable to the activation of many hepatocyte-associated genes by the transduction of HNF4α, which binds to the promoters of nearly half of the genes expressed in the liver.[12] In the later stage of hepatic maturation, hepatocyte-associated genes would be strongly upregulated by endogenous transcription factors but not exogenous HNF4α because transgene expression by Ad vectors was almost disappeared on day 18 (**Supplementary Figure S5**). Another reason for the efficient hepatic maturation would be that sequential transduction of SOX17, HEX, and HNF4α could mimic hepatic differentiation in early embryogenesis.

Next, we examined whether or not the hepatocyte-like cells had hepatic functions. The activity of many kinds of CYPs was upregulated by HNF4α transduction (**Figure 4b**). Ad-HNF4α-transduced cells exhibit many characteristics of hepatocytes: uptake of LDL, uptake and excretion of ICG, and storage of glycogen (**Figure 4a,c,d**). Many conventional tests of hepatic characteristics have shown that the hepatocyte-like cells have mature hepatocyte functions. Furthermore, the hepatocyte-like cells can catalyze the toxication of several compounds (**Figure 4e**). Although the activities to catalyze the toxication of test compounds in primary human hepatocytes are slightly higher than those in the hepatocyte-like cells, the handling of primary human hepatocytes is difficult for a number of reasons: since their source is limited, large-scale primary human hepatocytes are difficult to prepare as a homogeneous population. Therefore, the hepatocyte-like cells derived from human ESCs and iPSCs would be a valuable tool for predicting drug toxicity. To utilize the hepatocyte-like cells in a drug toxicity study, further investigation of the drug metabolism capacity and CYP induction potency will be needed.

We also investigated the mechanisms underlying efficient hepatic maturation by HNF4α transduction. Although the number of cholangiocyte populations did not change by HNF4α transduction, we found that the number of hepatoblast populations decreased and that of hepatocyte populations increased, indicating that HNF4α promotes selective hepatic differentiation from hepatoblasts (**Figure 5a**). As previously reported, HNF4α regulates the expression of a broad range of genes that code for cell adhesion molecules,[13] extracellular matrix components, and cytoskeletal proteins, which determine the main morphological characteristics of epithelial cells.[14,35,37] In this study, we elucidated that MET was promoted by HNF4α transduction (**Figure 5b,d**). Thus, we conclude that HNF4α overexpression in hepatoblasts promotes hepatic differentiation by activating MET (**Figure 5e**).

Using human iPSCs as well as human ESCs, we confirmed that the stage-specific overexpression of HNF4α could promote hepatic maturation (**Supplementary Figure S9**). Interestingly, the differentiation efficacies differed among human iPS cell lines: two of the human iPS cell lines (Dotcom and Tic) were more committed to the hepatic lineage than another human iPS cell line (201B7) (**Supplementary Figure S7**). Therefore, it would be necessary to select a human iPS cell line that is suitable for hepatic maturation in the case of medical applications, such as drug screening and liver transplantation. The difference of hepatic differentiation efficacy among the three iPSC lines might be due to the difference of epigenetic memory of original cells or the difference of the inserted position of the foreign genes for the reprogramming.

To control hepatic differentiation mimicking embryogenesis, we employed Ad vectors, which are one of the most efficient transient gene delivery vehicles and have been widely used in both experimental studies and clinical trials.[38] We used a fiber-modified Ad vector containing the EF-1α promoter and a stretch of lysine residue (KKKKKKK, K7) peptides in the C-terminal region of the fiber knob.[19] The K7 peptide targets heparan sulfates on the cellular surface, and the fiber-modified Ad vector containing the K7 peptides was shown to be efficient for transduction into many kinds of cells including human ESCs and human ESC-derived cells.[7–8,19] Thus, Ad vector-mediated transient gene transfer should be a powerful tool for regulating cellular differentiation.

In summary, the findings described here demonstrate that transcription factor HNF4α plays a crucial role in the hepatic differentiation from human ESC-derived hepatoblasts by activating MET (**Figure 5e**). In the present study, both human ESCs and iPSCs (three lines) were used and all cell lines showed efficient hepatic maturation, indicating that our protocol would be a universal tool for cell line-independent differentiation into functional hepatocytes. Moreover, the hepatocyte-like cells can catalyze the toxication of several compounds as primary human hepatocytes. Therefore, our technology, by sequential transduction of SOX17, HEX, and HNF4α, would be a valuable tool for the efficient generation of functional hepatocytes derived from human ESCs and iPSCs, and the hepatocyte-like cells could be used for the prediction of drug toxicity.

MATERIALS AND METHODS

Human ESC and iPSC culture. A human ES cell line, H9 (WiCell Research Institute, Madison, HI), was maintained on a feeder layer of mitomycin C-treated mouse embryonic fibroblasts (Millipore, Billerica, MA) with Repro Stem (Repro CELL, Tokyo, Japan) supplemented with 5 ng/ml fibroblast

growth factor 2 (FGF2) (Sigma, St Louis, MO). Human ESCs were dissociated with 0.1 mg/ml dispase (Roche Diagnostics, Indianapolis, IN) into small clumps and then were subcultured every 4 or 5 days. H9 was used following the Guidelines for Derivation and Utilization of Human Embryonic Stem Cells of the Ministry of Education, Culture, Sports, Science and Technology of Japan. Two human iPS cell lines generated from the human embryonic lung fibroblast cell line MCR5 were provided from the JCRB Cell Bank (Tic, JCRB Number: JCRB1331; and Dotcom, JCRB Number: JCRB1327).[39,40] These human iPS cell lines were maintained on a feeder layer of mitomycin C-treated mouse embryonic fibroblasts with iPSellon (Cardio, Kobe, Japan) supplemented with 10 ng/ml FGF2. Another human iPS cell line, 201B7, generated from human dermal fibroblasts was kindly provided by Dr S. Yamanaka (Kyoto University).[2] The human iPS cell line 201B7 was maintained on a feeder layer of mitomycin C-treated mouse embryonic fibroblasts with Repro Stem (Repro CELL) supplemented with 5 ng/ml FGF2 (Sigma). Human iPSCs were dissociated with 0.1 mg/ml dispase (Roche Diagnostics) into small clumps and were then subcultured every 5 or 6 days.

In vitro differentiation. Before the initiation of cellular differentiation, the medium of human ESCs and iPSCs was exchanged for a defined serum-free medium, hESF9, and cultured as we previously reported.[41] hESF9 consists of hESF-GRO medium (Cell Science & Technology Institute, Sendai, Japan) supplemented with 10 µg/ml human recombinant insulin, 5 µg/ml human apotransferrin, 10 µmol/l 2-mercaptoethanol, 10 µmol/l ethanolamine, 10 µmol/l sodium selenite, oleic acid conjugated with fatty-acid-free bovine albumin (BSA), 10 ng/ml FGF2, and 100 ng/ml heparin (all from Sigma).

The differentiation protocol for the induction of DE cells, hepatoblasts, and hepatocytes was based on our previous report with some modifications.[7] Briefly, in mesendoderm differentiation, human ESCs and iPSCs were dissociated into single cells and cultured for 3 days on Matrigel (Becton, Dickinson and Company, Tokyo, Japan) in hESF-DIF medium (Cell Science & Technology Institute) supplemented with 10 µg/ml human recombinant insulin, 5 µg/ml human apotransferrin, 10 µmol/l 2-mercaptoethanol, 10 µmol/l ethanolamine, 10 µmol/l sodium selenite, 0.5 mg/ml BSA, and 100 ng/ml Activin A (R&D Systems, Minneapolis, MN). To generate mesendoderm cells and DE cells, human ESC-derived cells were transduced with 3,000 vector particles (VP)/cell of Ad-SOX17 for 1.5 hours on day 3 and cultured until day 6 on Matrigel (BD) in hESF-DIF medium (Cell Science & Technology Institute) supplemented with 10 µg/ml human recombinant insulin, 5 µg/ml human apotransferrin, 10 µmol/l 2-mercaptoethanol, 10 µmol/l ethanolamine, 10 µmol/l sodium selenite, 0.5 mg/ml BSA, and 100 ng/ml Activin A (R&D Systems). For induction of hepatoblasts, the DE cells were transduced with 3,000 VP/cell of Ad-HEX for 1.5 hours on day 6 and cultured for 3 days on a Matrigel (BD) in hESF-DIF (Cell Science & Technology Institute) medium supplemented with the 10 µg/ml human recombinant insulin, 5 µg/ml human apotransferrin, 10 µmol/l 2-mercaptoethanol, 10 µmol/l ethanolamine, 10 µmol/l sodium selenite, 0.5 mg/ml BSA, 20 ng/ml bone morphogenetic protein 4 (R&D Systems), and 20 ng/ml FGF4 (R&D Systems). In hepatic differentiation, hepatoblasts were transduced with 3,000 VP/cell of Ad-LacZ or Ad-HNF4α for 1.5 hr on day 9 and were cultured for 11 days on Matrigel (BD) in L15 medium (Invitrogen, Carlsbad, CA) supplemented with 8.3% tryptose phosphate broth (BD), 8.3% fetal bovine serum (Vita, Chiba, Japan), 10 µmol/l hydrocortisone 21-hemisuccinate (Sigma), 1 µmol/l insulin, 25 mmol/l NaHCO$_3$ (Wako, Osaka, Japan), 20 ng/ml hepatocyte growth factor (R&D Systems), 20 ng/ml Oncostatin M (R&D Systems), and 10^{-6} mol/l Dexamethasone (Sigma).

Ad vectors. Ad vectors were constructed by an improved *in vitro* ligation method.[42,43] The human HNF4α gene (accession number NM_000457) was amplified by PCR using primers designed to incorporate the 5′ Not I and 3′ Xba I restriction enzyme sites: Fwd 5′-ggcctctagatggaggcagggagaatg-3′ and Rev 5′-ccccgcggccgcagcggcttgctagataac-3′. The human HNF4α gene was inserted into pBSKII (Invitrogen), resulting in pBSKII-HNF4α, and

then the human HNF4α gene was inserted into pHMEF5,[44] which contains the human elongation factor-1α (EF-1α) promoter, resulting in pHMEF-HNF4α. The pHMEF-HNF4α was digested with I-CeuI/PI-SceI and ligated into I-Ceu I/PI-SceI-digested pAdHM41-K7,[19] resulting in pAd-HNF4α. The human EF-1α promoter-driven LacZ-, SOX17-, or HEX-expressing Ad vectors, Ad-LacZ, Ad-SOX17, or Ad-HEX, were constructed previously.[7,8,45] Ad-LacZ, Ad-SOX17, Ad-HEX, and Ad-HNF4α, each of which contains a stretch of lysine residue (K7) peptides in the C-terminal region of the fiber knob for more efficient transduction of human ESCs, iPSCs, and DE cells, were generated and purified as described previously.[7] The VP titer was determined by using a spectrophotometric method.[46]

LacZ assay. Human ESC- and iPSC-derived cells were transduced with Ad-LacZ at 3,000 VP/cell for 1.5 hours. After culturing for the indicated number of days, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) staining was performed as described previously.[44]

Flow cytometry. Single-cell suspensions of human ESCs, iPSCs, and their derivatives were fixed with methanol at 4 °C for 20 minutes and then incubated with the primary antibody, followed by the secondary antibody. Flow cytometry analysis was performed using a FACS LSR Fortessa flow cytometer (BD).

RNA isolation and reverse transcription-PCR. Total RNA was isolated from human ESCs, iPSCs, and their derivatives using ISOGENE (Nippon Gene) according to the manufacturer's instructions. Primary human hepatocytes were purchased from CellzDirect, Durham, NC. complementary DNA was synthesized using 500 ng of total RNA with a Superscript VILO cDNA synthesis kit (Invitrogen). Real-time reverse transcription-PCR was performed with Taqman gene expression assays (Applied Biosystems, Foster City, CA) or SYBR Premix Ex Taq (TaKaRa) using an ABI PRISM 7000 Sequence Detector (Applied Biosystems). Relative quantification was performed against a standard curve and the values were normalized against the input determined for the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase. The primer sequences used in this study are described in **Supplementary Table S1**.

Immunohistochemistry. The cells were fixed with methanol or 4% paraformaldehyde (Wako). After blocking with phosphate-buffered saline containing 2% BSA (Sigma) and 0.2% Triton X-100 (Sigma), the cells were incubated with primary antibody at 4 °C for 16 hours, followed by incubation with a secondary antibody that was labeled with Alexa Fluor 488 (Invitrogen) or Alexa Fluor 594 (Invitrogen) at room temperature for 1 hour. All the antibodies are listed in **Supplementary Table S2**.

Assay for CYP activity. To measure cytochrome P450 3A4, 2C9, and 1A2 activity, we performed Lytic assays by using a P450-GloTM CYP3A4 Assay Kit (Promega, Madison, WI). For the CYP3A4 and 2C9 activity assay, undifferentiated human ESCs, the hepatocyte-like cells, and primary human hepatocytes were treated with rifampicin (Sigma), which is the substrate for CYP3A4 and CYP2C9, at a final concentration of 25 µmol/l or DMSO (0.1%) for 48 hours. For the CYP1A2 activity assay, undifferentiated human ESCs, the hepatocyte-like cells, and primary human hepatocytes were treated with omeprazole (Sigma), which is the substrate for CYP1A2, at a final concentration of 10 µM or DMSO (0.1%) for 48 hours. We measured the fluorescence activity with a luminometer (Lumat LB 9507; Berthold, Oak Ridge, TN) according to the manufacturer's instructions.

Pyronin Y/Hoechst Staining. Human ESC-derived cells were stained with Hoechst33342 (Sigma) and Pyronin Y (PY) (Sigma) in Dulbecco's modified Eagle medium (Wako) supplemented with 0.2 mmol/l HEPES and 5% FCS (Invitrogen). Samples were then placed on ice for 15 minutes, and 7-AAD was added to a final concentration of 0.5 mg/ml for exclusion of dead cells. Fluorescence-activated cell-sorting analysis of these cells was

performed on a FACS LSR Fortessa flow cytometer (Becton Dickinson) equipped with a UV-laser.

Cellular uptake and excretion of ICG. ICG (Sigma) was dissolved in DMSO at 100 mg/ml, then added to a culture medium of the hepatocyte-like cells to a final concentration of 1 mg/ml on day 20 of differentiation. After incubation at 37 °C for 60 minutes, the medium with ICG was discarded and the cells were washed with phosphate-buffered saline. The cellular uptake of ICG was then examined by microscopy. Phosphate-buffered saline was then replaced by the culture medium and the cells were incubated at 37 °C for 6 hours. The excretion of ICG was examined by microscopy.

Periodic Acid-Schiff assay for glycogen. The hepatocyte-like cells were fixed with 4% paraformaldehyde and stained using a Periodic Acid-Schiff staining system (Sigma) on day 20 of differentiation according to the manufacturer's instructions.

Cell viability tests. Cell viability was assessed by Alamar Blue assay kit (Invitrogen). After treatment with test compounds[47–50] (troglitazone, acetaminophen, cyclophosphamide, and carbamazepine) (all from Wako) for 2 days, the culture medium was replaced with 0.5 mg/ml solution of Alamar Blue in culturing medium and cells were incubated for 3 hours at 37 °C. The supernatants of the cells were measured at a wavelength of 570 nm with background subtraction at 600 nm in a plate reader. Control refers to incubations in the absence of test compounds and was considered as 100% viability value.

Uptake of LDL. The hepatocyte-like cells were cultured with medium containing Alexa-488-labeled LDL (Invitrogen) for 1 hour, and then the cells that could uptake LDL were assessed by immunohistochemistry and flow cytometry.

Primary human hepatocytes. Cryopreserved human hepatocytes were purchased from CellzDirect (lot Hu8072). The vials of hepatocytes were rapidly thawed in a shaking water bath at 37 °C; the contents of the vial were emptied into prewarmed Cryopreserved Hepatocyte Recovery Medium (CellzDirect) and the suspension was centrifuged at 100g for 10 minutes at room temperature. The hepatocytes were seeded at 1.25×10^5 cells/cm^2 in hepatocyte culture medium (Lonza, Walkersville, MD) containing 10% FCS (GIBCO-BRL) onto type I collagen-coated 12-well plates. The medium was replaced with hepatocyte culture medium containing 10% FCS (GIBCO-BRL) 6 hours after seeding. The hepatocytes, which were cultured 48 hours after plating the cells, were used in the experiments.

SUPPLEMENTARY MATERIAL
Figure S1. Genome-wide screening of transcription factors involved in hepatic differentiation emphasizes the importance of the transcription factor HNF4α.
Figure S2. Summary of specific markers for DE cells, hepatoblasts, hepatocytes, cholangiocytes, and pancreas cells.
Figure S3. The formation of DE cells, hepatoblasts, hepatocytes, and cholangiocytes from human ESCs.
Figure S4. Overexpression of HNF4α mRNA in hepatoblasts by Ad-HNF4α transduction.
Figure S5. Time course of LacZ expression in hepatoblasts transduced with Ad-LacZ.
Figure S6. The morphology of the hepatocyte-like cells.
Figure S7. Upregulation of the expression levels of conjugating enzymes and hepatic transporters by HNF4α transduction.
Figure S8. Upregulation of the expression levels of hepatic transcription factors by HNF4α transduction.
Figure S9. Generation of hepatocytes from various human ES or iPS cell lines.
Figure S10. Promotion of MET by HNF4α transduction.

Figure S11. Arrest of cell growth by HNF4α transduction.
Table S1. List of Taqman probes and primers used in this study.
Table S2. List of antibodies used in this study.

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REVIEW

Investigating cellular identity and manipulating cell fate using induced pluripotent stem cells

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Abstract

Induced pluripotent stem (iPS) cells, obtained from reprogramming somatic cells by ectopic expression of a defined set of transcription factors or chemicals, are expected to be used as differentiated cells for drug screening or evaluations of drug toxicity and cell replacement therapies. As pluripotent stem cells, iPS cells are similar to embryonic stem (ES) cells in morphology and marker expression. Several types of iPS cells have been generated using combinations of reprogramming molecules and/or small chemical compounds from different types of tissues. A comprehensive approach, such as global gene or microRNA expression analysis and whole genomic DNA methylation profiling, has demonstrated that iPS cells are similar to their embryonic counterparts. Considering the substantial variation among iPS cell lines reported to date, the safety and therapeutic implications of these differences should be thoroughly evaluated before they are used in cell therapies. Here, we review recent research defining the concept of standardization for iPS cells, their ability to differentiate and the identity of the differentiated cells.

The potential of stem cells and reprogramming

During mammalian development, cells in the developing fetus gradually become more committed to their specific lineage. The cellular differentiation process specializes to achieve a particular biological function in the adult, and the potential to differentiate is lost. Cellular differentiation has traditionally been thought of as a unidirectional process, during which a totipotent fertilized zygote becomes pluripotent, multipotent, and terminally differentiated, losing phenotypic plasticity (Figure 1). However,

recent cloning experiments using nuclear transplantation have demonstrated that the epigenetic constraints imposed upon differentiation in mammalian oocytes can be released and the adult somatic nucleus restored to a totipotent embryonic state [1]. This process, a rewinding of the developmental clock, is termed nuclear reprogramming.

Embryonic stem (ES) cells derived from the inner cell mass of the mammalian blastocyst, an early-stage embryo, were first established from mice by Evans and Kaufman in 1981 [2]. Approximately two decades later, a human ES (hES) cell line was established by Thomson and colleagues [3]. ES cells possess a nearly unlimited capacity for self-renewal and pluripotency: the ability to differentiate into cells of three germ layers. This unique property might be useful to generate a sufficient amount of any differentiated cell type for drug screening or evaluations of drug toxicity and for cell replacement therapy. In addition, pluripotent stem cells provide us with an opportunity to understand early human embryonic development and cellular differentiation. Pluripotent ES cells are spun off directly from pre-implantation embryos [2-5]. To induce the somatic cell back to a pluripotent state, a strategy such as nuclear transplantation is fraught with technical complications and ethical issues. Thus, the direct generation of pluripotent cells without the use of embryonic material has been deemed a more suitable approach that lends itself well to mechanistic analysis and has fewer ethical implications [6].

In a breakthrough experiment, Takahashi and Yamanaka [7] identified reprogramming factors normally expressed in ES cells, Oct3/4, Sox2, c-Myc, and Klf4, that were sufficient to reprogram mouse fibroblasts to become pluripotent stem cells closely resembling ES cells. Because they were induced by the expression of defined factors, these cells were termed induced pluripotent stem (iPS) cells [7]. Since this landmark report in 2006, the technology has been rapidly confirmed among a number of species, including humans [8,9], rhesus monkeys [10], rats [11,12], rabbits [13], pigs [14] and two endangered primates [15]. In addition, mouse iPS (miPS) cells can be derived from various cell types, including fibroblasts [7,16], neural cells [17,18], liver cells [19], pancreatic β

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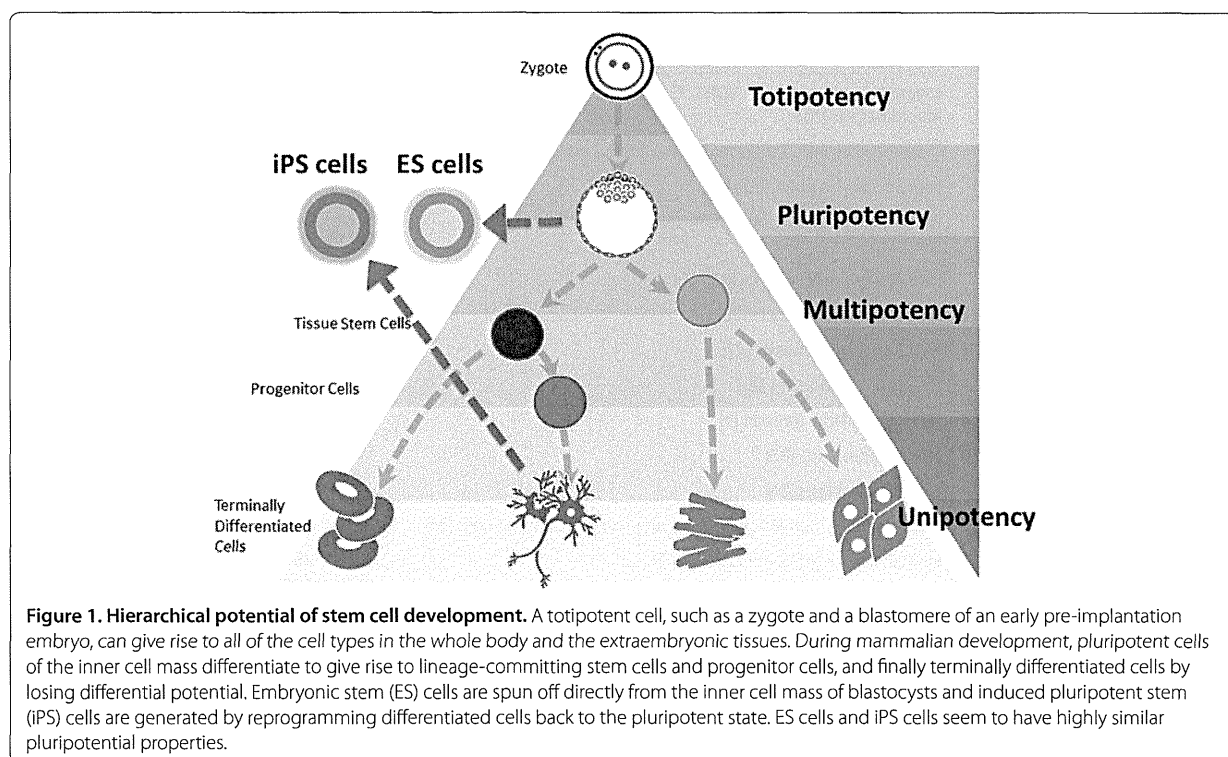Full list of author information is available at the end of the article

Figure 1. Hierarchical potential of stem cell development. A totipotent cell, such as a zygote and a blastomere of an early pre-implantation embryo, can give rise to all of the cell types in the whole body and the extraembryonic tissues. During mammalian development, pluripotent cells of the inner cell mass differentiate to give rise to lineage-committing stem cells and progenitor cells, and finally terminally differentiated cells by losing differential potential. Embryonic stem (ES) cells are spun off directly from the inner cell mass of blastocysts and induced pluripotent stem (iPS) cells are generated by reprogramming differentiated cells back to the pluripotent state. ES cells and iPS cells seem to have highly similar pluripotential properties.

cells [20], and terminally differentiated lymphocytes [21,22]. Subsequently, human iPS (hiPS) cells have been derived from various readily accessible cell types, including skin fibroblasts [8,9], keratinocytes [23], gingival fibroblasts [24], peripheral blood cells [25,26], cord blood cells [27,28] and hair follicle cells [29].

These products and systems for this state-of-the art technology provide useful platforms for disease modeling and drug discovery, and could enable autologous cell transplantation in the future. Given the methodologies for studying disease mechanisms, disease- and patient-specific iPS cells can be derived from patients. For applying novel reprogramming technologies to biomedical fields, we need to determine the essential features of iPS cells. In this review, we summarize the functional and molecular properties of iPS cells in comparison to ES cells in the undifferentiated state and with regard to differentiation efficiency. We also review evaluation for the types of differentiated cells derived from of iPS and ES cells and compare the functions of these.

Reprogramming methods and factors

Although the establishment of iPS cells from somatic cells is technically easier and simpler compared with nuclear transplantation, several variables should be considered due to variations in the reprogramming process, including the reprogramming factors used, the combinations of factors and the types of donor-parent cells. Each method has advantages and disadvantages, such as efficiency of reprogramming, safety, and complexity, with the process used affecting the quality of the resultant iPS cells. Initial generations of miPS and hiPS cells employed retroviral and lentiviral vectors [7-9] (Table 1), carrying the risk of both insertional mutagenesis and oncogenesis due to misexpression of the exogenous reprogramming factors, Oct3/4, Sox2, c-Myc, and Klf4. In particular, reactivation of c-Myc increases tumorigenicity in the chimeras and progeny mice, hindering clinical applications.

Since the initial report of iPS cell generation, modifications to the reprogramming process have been made in order to decrease the risk of tumorigenicity and increase reprogramming efficiency [30-32]. Several small molecules and additional factors have been reported to enhance the reprogramming process and/or functionally replace the role of some of the transcription factors (Table 1). Small molecules are easy to use and do not result in permanent genome modifications, although iPS generation using only a set of small molecules has not been reported. Combining small molecule compounds with reprogramming factors would enhance reprogramming efficiency. Integration-free hiPS cells have been established using Sendai virus [33,34], episomal plasmid vectors [35,36], minicircle vectors [37], and direct protein

Table 1. Various methods used for reprogramming

Method	Factors[a]	Sources	Enhancement factors
Adenovirus	OSKM	Mouse fibroblast and liver cells [77], human embryonic fibroblast cells [78]	
Bacteriophage	OSKM	Mouse embryonic fibroblasts, human amniocytes [79]	
Episomal vector	OSKMNL	Human foreskin fibroblasts [36]	SV40LT
		Human fibroblasts, adipose stem cells, cod blood cells [80]	SV40LT, LIF, MEK/GSK3b/TGFBR inhibitor, HA-100/human
	OSKM*L	Human dermal fibroblasts [81]	p53 shRNA
Lentivirus	OSKM	Mouse pancreatic b cells [20]	
		Human adult fibroblasts [82]	p53 siRNA, UTF1
		Mouse B lymphocytes [21]	C/EBPa or Pax5 shRNA
	OSNL	Human newborn foreskin [9]	
		Human fibroblasts [83]	SV40LT
	OSKMNL	Human fibroblasts [84]	
	OSN	Gut mesentery-derived cells [85], human amnion-derived cells [86]	
	O	Human epidermal keratinocytes [87]	TGFBR/MEK1 inhibitor, PDK1 activator, sodium butyrate
Minicircle vector	OSNL	Human adipose stromal cells [37]	
microRNA	miR-200c, 302a/b/c/d, 369-3p/5p	Human and mouse adipose stromal cells [64]	
mRNA	OSNL	Human fibroblasts [88]	
	OSKM(L)	Primary human neonatal epidermal keratinocytes [40]	
piggyBAC	OSKM	Human and mouse embryonic fibroblasts [89,90]	
Plasmid	OSKM	Mouse embryonic fibroblasts [35,91]	
	OSNL	Human foreskin fibroblasts [92]	MEK inhibitor
Protein	OSKM	Mouse embryonic fibroblasts [38]	VPA
	OSKM	Human fibroblasts [39]	
Retrovirus	OSKM	Human fibroblasts [8], mouse fibroblasts [7], human keratinocytes [23], human peripheral blood cells [25]	
		Human fibroblasts, adipose stem cells [93]	Vitamin C, VPA
	OSK	Adult human dermal fibroblasts [30]	
		Mouse embryonic fibroblasts [94]	Wnt3a
		Rat liver progenitor cells [11]	MEK/ALK5/GSK3b inhibitor
		Mouse embryonic fibroblasts [93]	Vitamin C
		Mouse and human fibroblasts [32]	GLIS1
		Mouse embryonic fibroblasts [95]	mmu-miR-106a/18b/20b/19b/92a/363 or 302a/302b/302c/302d/367
		Human fibroblasts [96]	hsa-miR-302b or 372
	OK	Mouse embryonic fibroblasts [97]	BIX01294, BayK8644
		Neonatal human epidermal keratinocytes [98]	GSK3b inhibitor
	O	Mouse neural stem cells [99]	
		Mouse fibroblasts [100]	GSK3b inhibitor, vitamin C, BMP4
	hsa-miR-302a/b/c/d	Human skin cancer cells [101]	
Sendai virus	OSKM	Human fibroblasts [33], human cord blood [102]	

[a]O, OCT3/4; S, SOX2; K, KLF4; M, C-MYC; M*, L-MYC; N, NANOG; L, LIN28. ALK, anaplastic lymphoma kinase; BayK8644, L-type calcium channel agonist; BIX01294, histone methyltransferase inhibitor; BMP, bone morphogenetic protein; GSK, glycogen synthase kinase; GLIS, GLI (MIM 165220)-related Kruppel-like zinc finger; LIF, leukemia inhibitory factor; PDK, pyruvate dehydrogenase kinase; shRNA, short hairpin RNA; siRNA, small interfering RNA; TGFBR, transforming growth factor beta receptor; UTF, undifferentiated transcription factor; VPA, valproic acid (histone deacetylase inhibitor).

[38,39] or mRNA [40] delivery (Table 1). However, direct delivery of proteins or RNA requires multiple transfection steps with reprogramming factors compared to other viral integration methods.

iPS cells appear indistinguishable from ES cells

The key to generating iPS cells is to revert somatic cells to a pluripotent state that is molecularly and functionally equivalent to ES cells derived from blastocysts (Table 2). Reprogrammed iPS cells express endogenous transcription factors that are required for self-renewal and maintenance of pluripotency, such as OCT3/4, SOX2, and NANOG, and for unlimited proliferation potential, such as TERT [8,9]. Telomeres were elongated in iPS cells compared to the parental differentiated cells in both humans and mice [41,42]. In addition, cellular organelles such as mitochondria within hiPS cells were morphologically and functionally similar to those within ES cells [43]. The establishment of an ES cell-like epigenetic state is a critical step during the reprogramming of somatic cells to iPS cells and occurs through activation of endogenous pluripotency related genes. Bisulfite genomic sequencing has shown that the promoter regions of the pluripotency markers NANOG and OCT3/4 are significantly demethylated in both hiPS and hES cells [8,44], and the heterogeneity of X chromosome inactivation in hiPS cells is similar to that in ES cells [45].

In terms of multilineage differentiation capacity, miPS cells from various tissue types have been shown to be competent for germline chimeras [19,32,46]. It was shown that miPS cells generated viable mice via tetraploid complementation [47,48]. In the mouse system, iPS cells retain a developmental pluripotency highly similar to that of mouse ES cells according to the most stringent tests. Although it has been generally assumed that autologous cells should be immune-tolerated by the recipient from whom the iPS cells were derived, Zhao and colleagues [49] reported that the transplantation of immature miPS cells induced a T-cell-dependent immune response even in a syngeneic mouse. This is an unexpected result but some issues need to be considered: the influence of the cell type of origin on the immunogenic properties of resultant iPS cells must be explored; undifferentiated iPSCs should never be used for medical applications; and the mechanism of aberrant gene expression should be determined [50].

To functionally assay hiPS cells, teratoma formation and histological analysis to confirm the presence of structures derived from all three germ layers are currently regarded as the most rigorous ways to prove pluripotency of human stem cells. Recently, Müller and colleagues [51] proposed the use of PluriTest, a bioinformatics assay for the prediction of stem cell pluripotency using microarray data. Such microarray-based gene expression and DNA methylation assays are low cost, save time and have been used to evaluate the differentiation efficiency of individual cell lines [52].

ES and iPS cells differ in their epigenetic signatures

Epigenetic modification of the genome ensures proper gene activation for maintaining the pluripotency of stem cells and also differentiation into proper functional cells [1]. It will be important to assess the epigenetic state of hiPS cells compared to donor parent cells and embryo-derived hES cells. Analyzing epigenetic states, such as histone modifications and DNA methylation of selected key pluripotency genes, showed the chromatin state of iPS cells to be identical to that of ES cells upon reprogramming (reviewed in [53]).

Genome-wide analyses of histone methylation patterns have demonstrated that iPS cells were clearly distinguished from their origin and similar to ES cells in the mouse [54]. All of these analyses, however, reported some differentially methylated regions (DMRs) between ES and iPS cells. Recent studies found that miPS cell lines retained the residual signatures of DNA methylation of the parental cells [55,56]. Additionally, some of the hyper-methylated regions in hiPS cells are also hyper-methylated in the original cells, meaning that an epigenetic memory is inherited during the reprogramming process through early passaging [57]. Parental cell-related DMRs and incomplete promoter DNA methylation contributed to aberrant gene expression profiles in iPS cells to some extent [58]. The other remaining DMRs appeared to be aberrantly methylated regions established in iPS cells during reprogramming that differ from both the parental cells and the ES cells. Nishino and colleagues [57] compared methylation profiles of six hiPS cell lines and two hES cell lines and reported that approximately 60% of DMRs were inherited and 40% were iPS-specific. Interestingly, most aberrant DMRs were hyper-methylated in iPS cell lines [57,59]. Lister and colleagues [60] also compared methylation profiles in five hiPS cell lines and two hES cell lines and found that the hiPS cells shared megabase-scale DMRs proximal to centromeres and telomeres that display incomplete reprogramming of non-CpG methylation, and differences in CpG methylation and histone modifications in over a thousand DMRs between hES and hiPS cells. Although lots of studies have detected several DMRs shared between iPS and ES cells, no DMRs were found in all iPS cell lines.

microRNAs (miRNAs), which are also epigenetically regulated, play critical roles in gene regulation by targeting specific mRNAs for degradation or by suppressing their translation. Several studies recently reported the presence of unique clusters of miRNAs, such as the human and mouse miR-302 cluster in ES and iPS cells [61,62]. These miRNAs enhance the transcription factor-mediated

Table 2. Characteristics of human induced pluripotent stem cells compared to human embryonic stem cells

Variable factor	Characteristics	Characteristics of hiPS cells
Cell source		Without the use of embryonic material Enable autologous cell transplantation
Technique for the generation of iPS cells		Simply trans-activating several transcription factors and/or exposure to several chemical components Variables due to reprogramming methods and/or donor-parental cells
Morphology		Flat and tightly packed colony identical to hES cells
Proliferation potency		Unlimited self-renewal identical to hES cells
Pluripotency	Genes	OCT3/4, NANOG, SOX2 expression identical to hES cells
	Gene promoter	OCT3/4, NANOG demethylation identical to hES cells
	Cell surface antigens	SSEA3, SSEA4, TRA-1-60, TRA-1-81 positive identical to hES cells
	Teratoma formation	Differentiation into three germ layers similar to hES cells
X chromosome inactivation (XCI)		Heterogeneity (complete XCI, partial XCI, pre-XCI) similar to hES cells
Mitochondria	Genome	Accumulated mtDNA mutations transmitted from parental cells Genetic mutations during reprogramming
	Morphology	Globular shape with only small christae similar to hES cells and ES cell-like distribution
	Function	Expression of nuclear factors involved in mitochondrial biogenesis
Telomere		Telomere elongation and ES cell-like telomerase activity
Epigenetic profile		Retention of somatic memory and aberrant methylation during the reprogramming process
microRNAs		Up-regulation of miR-302 cluster identical to hES cells

ES, embryonic stem; hES, human embryonic stem; hiPS, human induced pluripotent stem; iPS, induced pluripotent stem; mtDNA, mitochondrial DNA; XCI, X chromosome inactivation.

reprogramming process (Table 1). Furthermore, two independent groups generated human and mouse iPS cells by adding only miRNAs in the absence of any additional protein factors [63,64]. Two reports have described a small number of differences in miRNA expression patterns between hiPS and hES cells [62,65], although our preliminary analysis showed that miR-372 and miR-373 are expressed at similar levels in both hiPS and hES cells and they were not detected in parental cells.

Changes of epigenetic profiles in iPS cells during culture

It is possible that iPS cells vary in their epigenetic profiles and degree of pluripotency due to differential levels of reprogramming. Nishino and colleagues [66] investigated the effect of continuous passaging on DNA methylation profiles of seven hiPS cell lines derived from five cell types. Although *de novo* DMRs that differ between hES and hiPS cells appeared at each passage, their number decreased and they disappeared with passaging; therefore, the total number of DMRs that differ between ES and iPS cells decreased with passaging. Thus, continuous passaging of the iPS cells diminished the epigenetic differences between iPS and ES cells, implying that iPS cells lose the characteristics inherited from the parental cells and develop to very closely resemble ES cells over

time [66]. They also confirmed that the transgenes were silenced at each passage examined, indicating that the number of DMRs that differed between ES and iPS cells decreased during the transgene-independent phase. This is consistent with a study by Chin and colleagues [67], who found that the gene expression profile of hiPS cells appeared to become more similar to that of hES cells upon extended passaging. Although comprehensive DNA methylation profiles have recently been generated for hiPS cells, it seems harder to determine common DMR sites during iPS reprogramming. There are three possible explanations for the many inconsistent results regarding iPS cell-specific DMRs: hiPS cells have only been analyzed at a single point of passage in almost all studies; inherited methylation from parental cells is non-synchronous and stochastic, much like aberrant methylation, rather than deterministic [66]; and the aberrant hypermethylation at DMRs in iPS cells occurs 'stochastically' throughout the genome during passaging [66].

Genetic changes during reprogramming and extended culture

Genomic stability is critical for the clinical use of hiPS cells. The occurrence of genetic changes in hES cells is now well known as well as that the karyotypic changes observed are nonrandom and commonly affect only a few chromosomes [68]. Recent studies revealed that the

reprogramming process and subsequent culture of iPS cells *in vitro* can induce genetic changes. Three types of genomic abnormalities were seen: aberrations of somatic cell origin, aberrations present in early passages but not of apparent somatic cell origin, and aberrations acquired during passaging. Notably, the high incidence of chromosome 12 duplications observed by Mayshar and colleagues [69] caused significant enrichment for cell cycle-related genes, such as *NANOG* and *GDF3*. Another study reported that regions close to pluripotency-associated genes were duplicated in multiple samples [70]. Selection during hiPS cell reprogramming, colony picking and subsequent culturing may be factors contributing to the accumulation of mutations.

Impact of epigenetic differences on pluripotency

One of the goals of using hiPS cells is to generate functional target cells for medical screening and therapeutic applications. For these applications, it must be evaluated thoroughly whether small DMRs among ES and iPS cells affect the competency, differentiation propensities, stability and safety of iPS cells. It remains to be elucidated how the degree of these differences contributes to the variance in pluripotency among ES and iPS cells. Analysis of iPS cells obtained from mouse fibroblasts and hematopoietic and myogenic cells demonstrated that cellular origin influences the potential of miPS cells to differentiate into embryoid bodies and different cell types *in vitro*. In a related study, Kim and colleagues [56] compared the ability to differentiate to blood lineages of iPS cells derived from fibroblasts, neural cells, hematopoietic cells and ES cells in the mouse system, and demonstrated consistent differences in blood-forming ability - that is, blood derivatives showed more robust hematopoiesis *in vitro* than neural derivatives. Therefore, low-passage iPS cells derived from different tissues harbor residual DNA methylation signatures characteristic of their somatic tissue of origin, which favors their differentiation along lineages related to the parental cell, while restricting alternative cell fates. Similarly, Miura and colleagues [71] demonstrated that differences in gene expression in miPS cells derived from different types of parental cells result in variations in teratoma formation. These studies demonstrate that reprogramming to generate iPS cells is a gradual process that modifies epigenetic profiles beyond the acquisition of a pluripotent state.
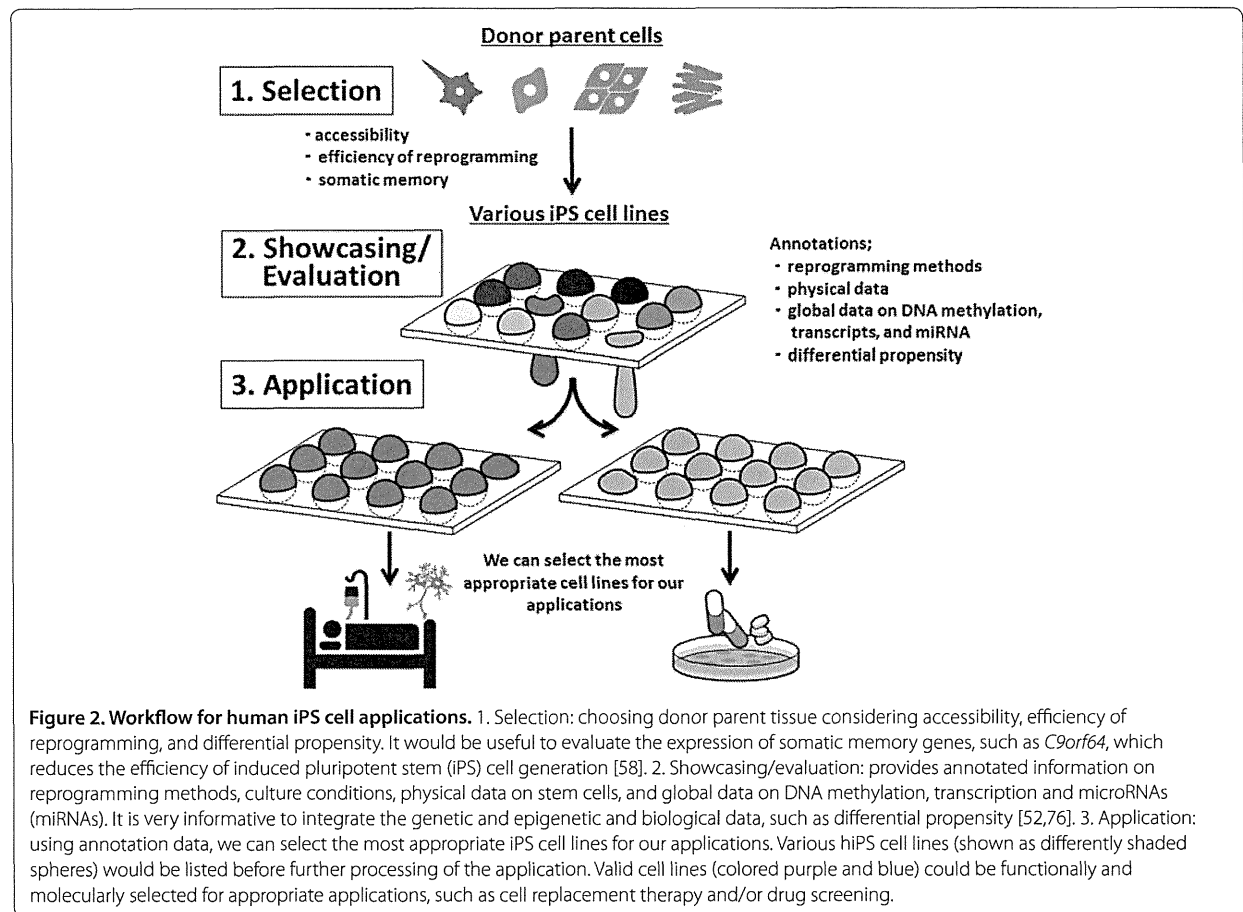
Prediction for pluripotency and differentiation preference

Significant variation has been also observed in the differentiation efficiency of various hES cell lines [72]. Incomplete DNA methylation of somatic cells regulates the efficiency of hiPS cell generation [58], and selection of parental cell types influences the propensity for differentiation [73,74]. Such differences must be better understood before hES and hiPS cell lines can be confidently used for translational research. To predict a cell line's propensity to differentiate into the three germ layers, Bock and colleagues [52] performed DNA methylation mapping by genome-scale bisulfite sequencing and gene expression profiling using microarrays and quantified the propensity to form multiple lineages by utilizing a non-directed embryoid bodies formation assay and high-throughput transcript counting of 500 lineage marker genes in embryoid bodies using 20 hES cells lines and 12 hiPS cell lines over passages 15 to 30. They bioinformatically integrated these genomic assays into a scorecard that measures the quality and utility of any human pluripotent cell line. The resulting lineage scorecard pinpoints quantitative differences among cell-line-specific differentiation propensities. For example, one hES cell line that received a high score for endoderm differentiation performed well in directed endoderm differentiation, and other hES cell lines that received high scores for neural lineage differentiation efficiently differentiated into motor neurons. In addition, two hiPS lines that the scorecard predicted to have a low propensity to differentiate into the neural lineage were impaired in motor neuron-directed differentiation. On the other hand, other hiPS lines that the scorecard predicted to have a high propensity to differentiate into ectodermal and neural lineages were found to differentiate well into motor neurons. Therefore, the scorecard can detect lineage-specific differences in the differentiation propensities of a given cell line [52].

Functional assay for differentiated cells from iPS and ES cells

Although the propensity for differentiation could be predicted, it remains to be elucidated whether iPS cell-derived cells are functionally and molecularly the same as ES cell-derived cells. To address this issue, two studies conducted functional assays comparing differentiated neural cells derived from iPS cells to those derived from ES cells by marker gene expression and action potential measurements [75,76]. There was some variation in efficiency and quantitative differences in motor neuron generation among the lines, but the treatment of neuroepithelial cells from pluripotent stem cells with retinoic acid and sonic hedgehog resulted in the generation of iPS and ES cell lines with a neuronal morphology that expressed TUJ1. In addition, electrophysiological recordings using whole-cell patch clamping showed inward and outward currents, and it was concluded that ES cell- and iPS cell-derived neurons are similarly functional at a physiological level. These studies demonstrated that the temporal course and gene-expression pattern during

Figure 2. Workflow for human iPS cell applications. 1. Selection: choosing donor parent tissue considering accessibility, efficiency of reprogramming, and differential propensity. It would be useful to evaluate the expression of somatic memory genes, such as *C9orf64*, which reduces the efficiency of induced pluripotent stem (iPS) cell generation [58]. 2. Showcasing/evaluation: provides annotated information on reprogramming methods, culture conditions, physical data on stem cells, and global data on DNA methylation, transcription and microRNAs (miRNAs). It is very informative to integrate the genetic and epigenetic and biological data, such as differential propensity [52,76]. 3. Application: using annotation data, we can select the most appropriate iPS cell lines for our applications. Various hiPS cell lines (shown as differently shaded spheres) would be listed before further processing of the application. Valid cell lines (colored purple and blue) could be functionally and molecularly selected for appropriate applications, such as cell replacement therapy and/or drug screening.

neuroepithelial cell differentiation and production of functional neurons were nearly identical between ES and iPS cells, regardless of the reprogramming method, cellular origin, and differences between iPS and ES cells. These findings raise hopes of applying human iPS cells to the modeling of diseases and potential autologous cell transplantation.

It is important to acquire scientific information on pluripotential stem cells for further applications, such as industrial and clinical uses. Pluripotent stem cells, including disease-specific stem cells, could be showcased with useful annotation data and the most appropriate cell lines could be selected (Figure 2).

Conclusion

Many issues have yet to be resolved before the results of stem cell research can benefit the public in the form of medical treatments. In this review, we have discussed the substantial variation observed among pluripotent stem cells, including transcriptional and epigenetic profiles in the undifferentiated state, the ability to differentiate into various types of cells, and the functional and molecular nature of embryoid body or stem cell-derived differentiated

cells. These results suggest that most, but not all, iPS cell lines are indistinguishable from ES cell lines, even though there is a difference between the average ES cell and the average iPS cell. Thus, ES and iPS cells should not be regarded as one or two well-defined points in the cellular space but rather as two partially overlapping point clouds with inherent variability among both ES and iPS cell lines [52,76]. Notably, human iPS cells seemed to be more variable than human ES cells. No single stem cell line may be equally powerful for deriving all cell types *in vitro*, implying that researchers would benefit from identifying the best cell lines for each application. Furthermore, for clinical use in the future, it is important to use both ES and iPS cells in research, and to standardize reprogramming methods, culture equipment and techniques and to optimize differentiation methods and evaluate the functions and tumorigenicity of differentiated cells.

Abbreviations

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Establishment of Functioning Human Corneal Endothelial Cell Line with High Growth Potential

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Abstract

Hexagonal-shaped human corneal endothelial cells (HCEC) form a monolayer by adhering tightly through their intercellular adhesion molecules. Located at the posterior corneal surface, they maintain corneal translucency by dehydrating the corneal stroma, mainly through the Na^+- and K^+-dependent ATPase (Na^+/K^+-ATPase). Because HCEC proliferative activity is low *in vivo*, once HCEC are damaged and their numbers decrease, the cornea begins to show opacity due to overhydration, resulting in loss of vision. HCEC cell cycle arrest occurs at the G1 phase and is partly regulated by cyclin-dependent kinase inhibitors (CKIs) in the Rb pathway (p16-CDK4/CyclinD1-pRb). In this study, we tried to activate proliferation of HCEC by inhibiting CKIs. Retroviral transduction was used to generate two new HCEC lines: transduced human corneal endothelial cell by human papillomavirus type E6/E7 (THCEC (E6/E7)) and transduced human corneal endothelial cell by Cdk4R24C/ CyclinD1 (THCEH (Cyclin)). Reverse transcriptase polymerase chain reaction analysis of gene expression revealed little difference between THCEC (E6/E7), THCEH (Cyclin) and non-transduced HCEC, but cell cycle-related genes were up-regulated in THCEC (E6/E7) and THCEH (Cyclin). THCEH (Cyclin) expressed intercellular molecules including ZO-1 and N-cadherin and showed similar Na^+/K^+-ATPase pump function to HCEC, which was not demonstrated in THCEC (E6/E7). This study shows that HCEC cell cycle activation can be achieved by inhibiting CKIs even while maintaining critical pump function and morphology.

Introduction

Human corneal endothelial cells (HCEC) are hexagonal in shape and form a fragile monolayer lying posterior to the surface of the cornea. These cells maintain corneal transparency by their tight intercellular barrier and perform an ion transport pump function through Na^+/K^+-ATPase, which regulates the hydration of the corneal stroma [1,2]. If HCEC sustain damage, excessive hydration and opacity of the cornea occur, resulting in decreased vision.

Corneal endothelia are believed not to increase in adult humans and in fact gradually decrease by approximately 0.5% per year [3,4,5]. Damage, injury or HCEC disease such as Fuchs' corneal dystrophy [6], diabetes [7], trauma [8], cataract surgery [9] or elevation of intraocular pressure [10] does not lead to increased proliferation but rather to an increase in cell size to compensate for the wounded area [11]. Once the cell number falls below 1,000 cells/mm², the monolayer of enlarged HCEC cannot maintain corneal translucency [12] and surgical treatment is required to restore vision.

Penetrating keratoplasty has long been the surgical treatment of choice, involving replacement of a total layer of cornea by donor material. However, it can also result in adverse effects such as astigmatism and severe rejection requiring long term usage of immunosuppressive drugs [13]. Recently, alternative transplantation strategies, including modified posterior lamellar keratoplasty techniques such as deep lamellar endothelial keratoplasty (DLEK) [14], Descemet's stripping with endothelial keratoplasty (DSEK) [15] and Descemet membrane endothelial keratoplasty (DMEK) [16] have been introduced to overcome these problems. Despite these advances, an increasingly aging population requiring corneal transplants and inadequate tissue quality limit the availability of donor corneas, such that alternative ways of preparing endothelial cell monolayers need to be explored.

HCEC were originally believed to be incapable of expanding *in vitro*, but have been successfully isolated and cultured by introducing stimulating agents such as epidermal growth factor, platelet-derived growth factor-BB, bovine pituitary extract and fetal bovine serum [17,18]. However, the number of cells with proliferative activity and the ability to respond to such agents is relatively low, and much variation in proliferative activity exists between donors of different ages [19,20]. Thus, there is a requirement to achieve a stable and effective culture of cells in terms of both cell proliferation and physiologic function.

The HCEC cell cycle is mainly regulated by the p53 and pRB pathways, both of which have been inactivated by human papilloma virus (HPV) type 16 E6/E7 to successfully immortalize cells. Kim et al. reported the establishment of an immortalized HCEC line using HPV type 16 E6/E7 on lyophilized human amniotic membrane [21]. However, several studies have reported carcinogenesis of the cell line established by viral oncogenes including HPV type 16 E6/E7 or SV40 large T antigen [22,23]. Therefore a corneal endothelial cell line developed in this way does not appear to be suitable for the treatment of human corneal diseases. To resolve this problem, we expressed mutant cyclin-dependent kinase (Cdk) 4 and CyclinD1 to inactivate the pRB pathway and generate corneal endothelial cell lines without transducing viral oncogenes.

Results

HCEC with Descemet's membranes were proliferated slowly in a culture dish coated in type IV collagen. After two passages, the cells were transferred into 24-well dishes and transfected with a retroviral vector carrying E6/E7 or mutant Cdk4 and CyclinD1. Three cell lines were successfully generated, as shown in Fig. 1A, with obvious differences in growth (Fig. 1B). Protein expression from the transduced gene was confirmed by western blotting (Fig. 1C). As previously reported [21], THCEC (E6/E7) was immortalized, and THCEC (Cyclin) demonstrated the same proliferative capacity as THCEC (E6/E7), while primary cells grew more slowly even when cultured in 10% fetal bovine serum. These results indicate that induction of mutant Cdk4 and CyclinD1 is sufficient to generate a HCEC line that proliferates at a faster rate than the primary cell line.

Proliferation capacity was also confirmed by immunohisto-chemistry of Ki-67 (Fig. 2A). Expression of downstream genes of CyclinD1 which are associated with cell proliferation was analyzed by real-time polymerase chain reaction (PCR) (Fig. 2B). Positive staining of Ki-67, which is detected in the nucleus, was confirmed in both THCEC (Cyclin) and THCEC (E6/E7). Real-time PCR also revealed that CDC2 and PCNA, target genes of E2F (an upstream transcriptional factor), that are activated by CyclinD1, were up-regulated in THCEC (E6/E7) and especially in THCEC (Cyclin).

Expression of genes involved in active transmembrane transporter activity, including Na^+/K^+-ATPase, or cell adhesion, including ZO-1 and N-cadherin, were assessed by semi-quantitative reverse transcriptase (RT)-PCR (Fig. 3A). Expression of intercellular adhesion molecules was confirmed by immunohisto-chemistry (Fig. 3B–J). Semi-quantitative RT-PCR showed that there was no significant difference between the three cell lines regarding the expression of genes associated with several molecules of cell adhesion or of ion transporter channels, which are characteristically expressed by HCEC [21,24]. This was also confirmed by real-time PCR (data not shown).

ZO-1 and N-cadherin, key HCEC adhesion molecules [24], demonstrated positive staining at the intercellular junction in HCEH (Fig. 3F, I) and THCEC (Cyclin) (Fig. 3E, H), while neither ZO-1 nor N-cadherin was detected in THCEC (E6/E7) despite sufficient cellular density (Fig. 3G, J). Although positive staining of ZO-1 and N-cadherin was observed at the intercellular junction in THCEC (Cyclin), ZO-1 staining also occurred around the nucleus (Fig. 3E), indicating the immature distribution of the ZO-1 protein. In THCEC (Cyclin) and HCEC, hexagonal morphology was identified both by phase-contrast micrography (Fig. 3B, C) and immunocytochemistry, while the structure of hexagonal cell shape was not maintained in THCEC (E6/E7)

(Fig. 3D). These data indicate that THCEC (Cyclin) and HCEC, but not THCEC (E6/E7), maintain contact inhibition which is crucial for preserving the monolayer.

Scanning electron microscopy was performed to reveal detailed information on the cellular junction (Fig. 4). THCEC (Cyclin) and HCEC showed a clear cellular junction including a tight junction, whereas THCEC (E6/E7) grew as a multilayer without forming a cellular junction, which confirms the immunohistochemistry result.

Representative traces of circuit current driven by the Na^+/K^+-ATPase were of similar shapes in both HCEC and THCEC (Cyclin) (Fig. 5A). These circuit currents maintain corneal translucency and their levels in both cell lines were clearly reduced by the presence of the Na^+/K^+-ATPase inhibitor ouabain, which confirms that the origin of the current is Na^+/K^+-ATPase. Meanwhile, the pump function in THCEC (Cyclin), detected in both earlier and later passages of cells, was more variable than that in HCEC (Fig. 5B), possibly indicating incomplete Na^+/K^+-ATPase activity or the presence of an intercellular barrier that regulates ion permeability. No regular circuit current was detected in THCEC (E6/E7) (Fig. 5A, B), which probably reflects the absence of intercellular adhesion preventing free ion transport across the membrane. This experiment clearly showed that the THCEC (Cyclin) monolayer has similar Na^+/K^+-ATPase activity to that of HCEC.

A tumorigenesis assay of nude mice detected no solid tumor in either THCEC (Cyclin) or THCEC (E6/E7), while HeLa cells formed a solid tumor in all mice (Table 1). Since THCEC (Cyclin) has a similar morphology and pump function to HCEC, THCEC (Cyclin) could be suitable for HCEC studies.

Discussion

THCEC (E6/E7) was shown to achieve immortalization with a highly activated proliferative capacity, as previously described [21]. However, the cell lines did not show normal intercellular contact or normal pump function, probably because contact inhibition in the cell line was not achieved. Meanwhile, THCEC (Cyclin) was demonstrated to have normal physiologic function with a greater proliferative capacity than primary cells, but slightly lower than that of THCEC (E6/E7).

In expanding the cellular life span, E7 has been shown to play a role in the inactivation of pRB, while E6 activates telomerase [25] and accelerates p53 degradation, which induces the Cdk inhibitor p21 [26]. However, little is known about the effector sites of the viral oncogene that may be related to genetic instability of immortalized cells. In the present study, expression of genes specific to HCEC was not drastically different between the three cell lines. However, key proteins including ZO-1 and N-cadherin that are important in forming intercellular contacts were detected, probably because of the unknown influence of viral oncogenes on post-translational modification, posttranslational import or protein stability/degradation.

We recently established genetically stable, non-transformed immortalized ovarian surface epithelium (OSE) cell lines without viral oncogenes by expressing mutant Cdk 4, CyclinD1 and hTERT, based on the hypothesis that inactivation of the pRb pathway and activation of telomerase are sufficient for OSE immortalization [27]. Meanwhile, Rane et al. demonstrated that mutant Cdk 4 (Cdk4R24C) is sufficient to induce carcinogenesis in several other tissues including those of the pancreas, pituitary and brain [28], and Joyce and colleagues showed that HCEC are arrested in the G1 phase and regulated by CKIs, p16INK4a and p21WAF1/Cip1 [29]. Considering the importance of maintaining
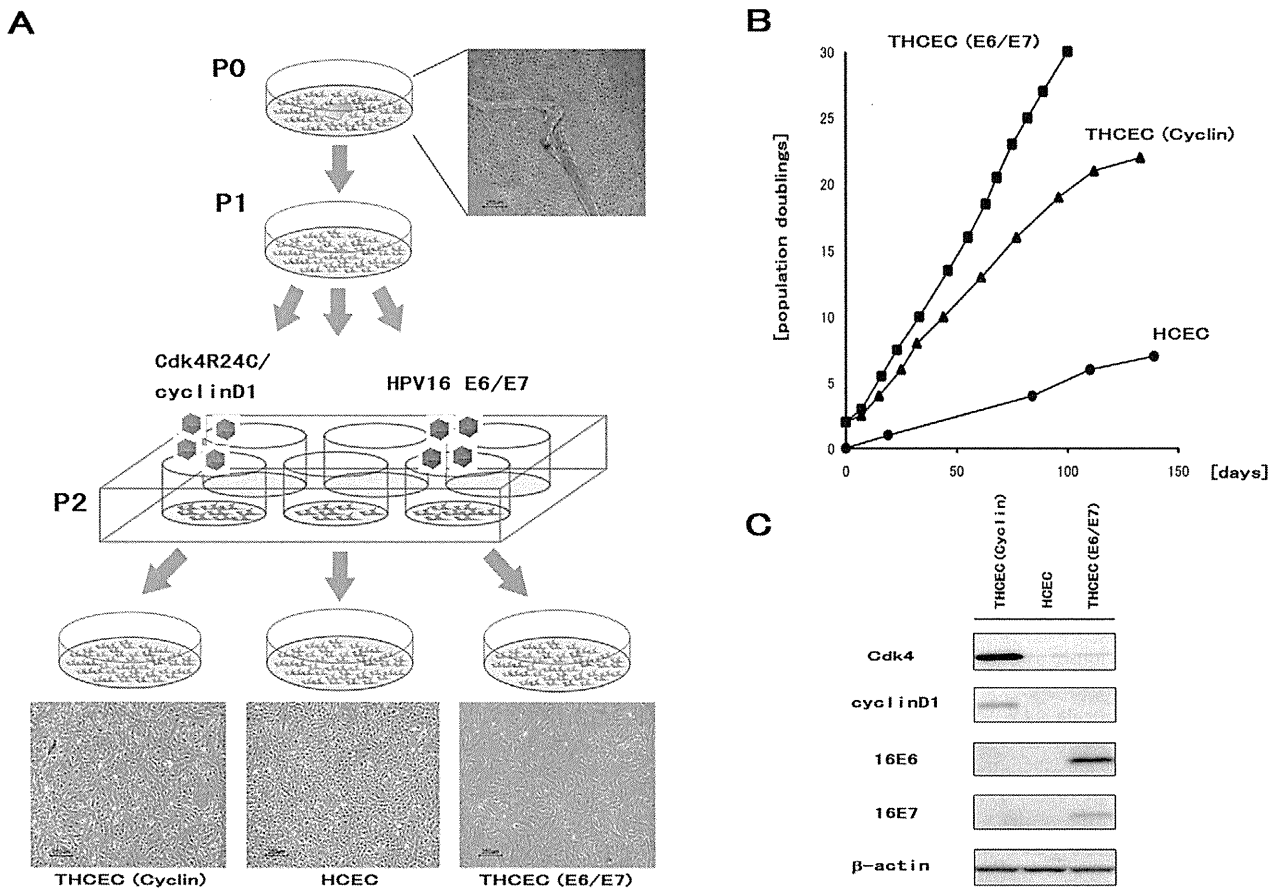
Figure 1. Establishment of THCEC (E6/E7), THCEC (Cyclin) and HCEC. (A) HCEC with Descemet's membrane were placed on Type IV collagen-coated 35 mm cell culture dishes with growth medium (P0). After one passage (P1), retroviral infection was conducted in 6-well cell culture dishes at P2. THCEC (E6/E7) and THCEC (Cyclin) were infected by retroviral vectors carrying HPV16 E6/E7 and both CyclinD1 and Cdk4R24C, respectively. (B) Growth curves of THCEC (E6/E7), THCEC (Cyclin) and HCEC cell lines. THCEC (E6/E7) was immortalized as reported previously, and THCEC (Cyclin) obtained the same proliferative activity as that of THCEC (E6/E7). Transfection was performed on day 0 for THCEC (E6/E7) and THCEC (Cyclin), with population doublings of 2. For HCEC, primary culture commenced on day 0. (C) Western blotting confirmed the expression of the following transgenes: E6 and E7 in THCEC (E6/E7), and CyclinD1 and Cdk4R24C in THCEC (Cyclin).
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morphology and physiologic function in HCEC, we only transduced mutant Cdk 4 and CyclinD1, not hTERT, in the present study. We believe that our careful method enabled THCEC (Cyclin) to form a fragile and regularly arranged monolayer complete with physiologic function.

Although THCEC (Cyclin) has similar characteristics to primary HCEC, immunohistochemistry and the Ussing chamber assay also highlighted the differences between the cells. ZO-1 protein was expressed around the nucleus of THCEC (Cyclin) but not in primary cells. Since semi-quantitative PCR detected almost the same level of mRNA expression between the cell lines, staining around the nucleus in THCEC (Cyclin) probably reflects an error in posttranslational import of ZO-1 protein. The Ussing chamber assay detected a similar pump function between THCEC (Cyclin) and primary cells, but the current in THCEC (Cyclin) was more variable than that of the primary cells, which might have been caused by reduced Na^+/K^+-ATPase activity, immature intercellular adhesion allowing irregular intercellular ion transport or differences in cellular density.

Cells established by a retrovirus carry a potential risk of promoting carcinogenesis [30], and direct transplantation to humans of cell sheets composed of such cells may lead to complex problems. Recently, to resolve this problem, several studies have reported the establishment of untransfected corneal endothelial cell lines [31,32,33], which are the most ideal cell lines for the treatment of human corneal disease. Meanwhile, alternative bioengineering approaches, including lipofection of p27kip1 siRNA [34], proteomics technology analyzing the difference between younger and older HCEC [35] and drug usage of promyelocytic leukemia zinc finger protein, a cell cycle transcriptional repressor and negative regulator [36], have also been introduced. The present findings support the idea that targeting the interaction between p16INK4a and Cdk4 using such methods is a promising strategy to generate HCEC with sufficient proliferative capacity and physiologic function.

Materials and Methods

Isolation and cell culture of human corneal cells

Ethics Statement. A cornea was excised from the surgically enucleated eye of a 2-year-old infant undergoing therapy for retinoblastoma, with the approval (approval number, #156) of the
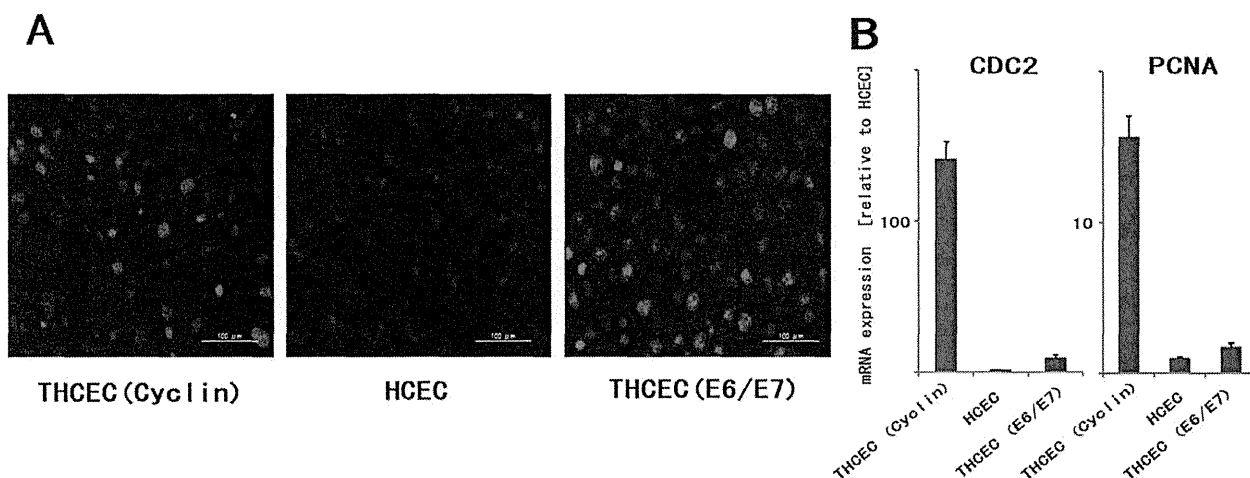
A

THCEC (Cyclin)　　　　HCEC　　　　THCEC (E6/E7)

B

Figure 2. Evaluation of proliferative capacity. (A) Immunohistochemistry of Ki-67 in three cell lines. Positive staining of Ki-67, located in the nucleus, was obviously identified in THCEC (Cyclin) and THCEC (E6/E7), but rarely detected in HCEC. (B) Real-time PCR of downstream genes of cyclinD1 associated with proliferation. Gene expression levels of both CDC2 and PCAN were clearly higher than that of HCEC. The gene expression was much more activated in THCEC (Cyclin) in which the expression of E2F, an upstream transcriptional factor of two genes, was constitutively activated by transduced mutant Cdk4 and CyclinD1.
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Ethics Committee of the National Institute for Child and Health Development, Tokyo, Japan. Signed informed consent was obtained from the donor's parents, and the surgical specimens were irreversibly de-identified. All experiments handling human cells and tissues were performed in line with the tenets of the Declaration of Helsinki.

The corneal piece, which was grossly normal with no pathological lesions, was cut 1.5 mm from the corneal limbus,
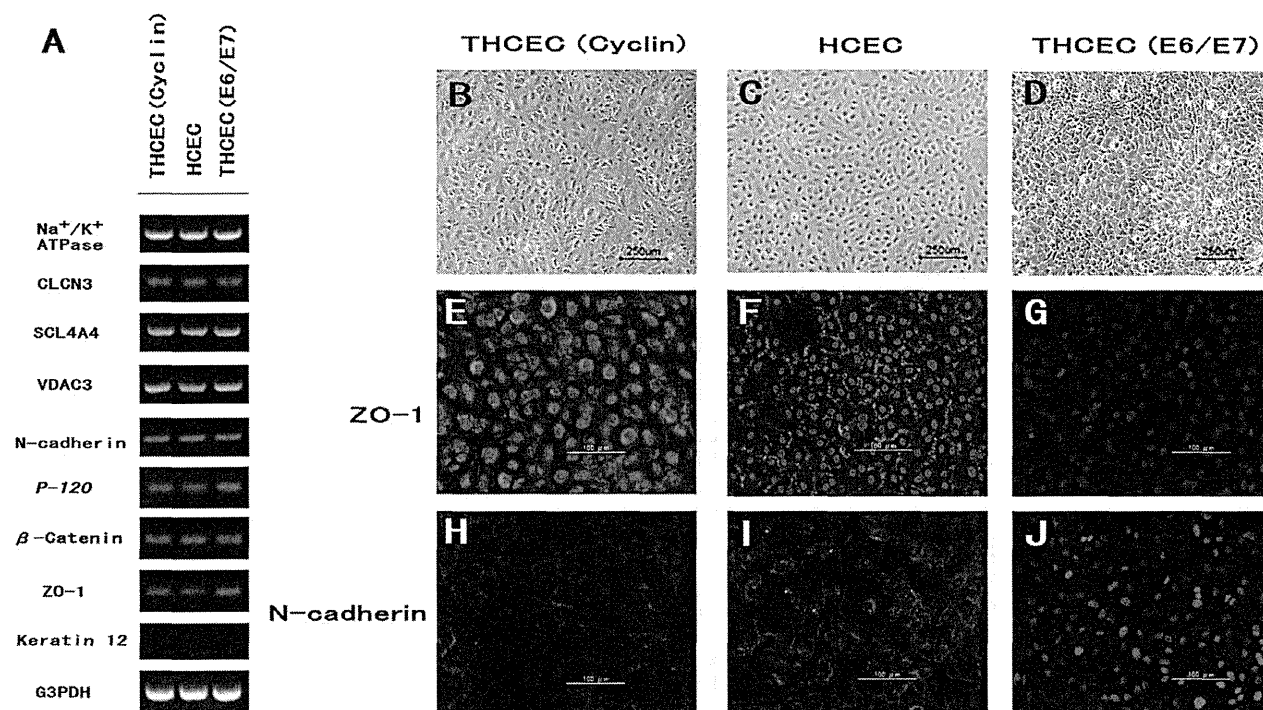

Figure 3. HCEC-associated genes and cytolocalization of junctional components expressed by cell lines. (A) Semi-quantitative reverse transcriptase polymerase chain reaction for HCEC-associated genes. Total RNA was prepared from cultured cells seven days after reaching confluency. No significant difference in mRNA expression was observed between the three cell lines. Compared with phase-contrast micrographs of (B) THCEC (Cyclin), (C) HCEC and (D) THCEC (E6/E7), cytolocalization was examined by immunofluorescence staining of ZO-1 (E, F,G) and N-cadherin (H, I, J). THCEC (E6/E7) did not stain positive for intercellular junctional molecules, while ZO-1 and N-cadherin stained positive at the junction in THCEC (Cyclin) and HCEC.
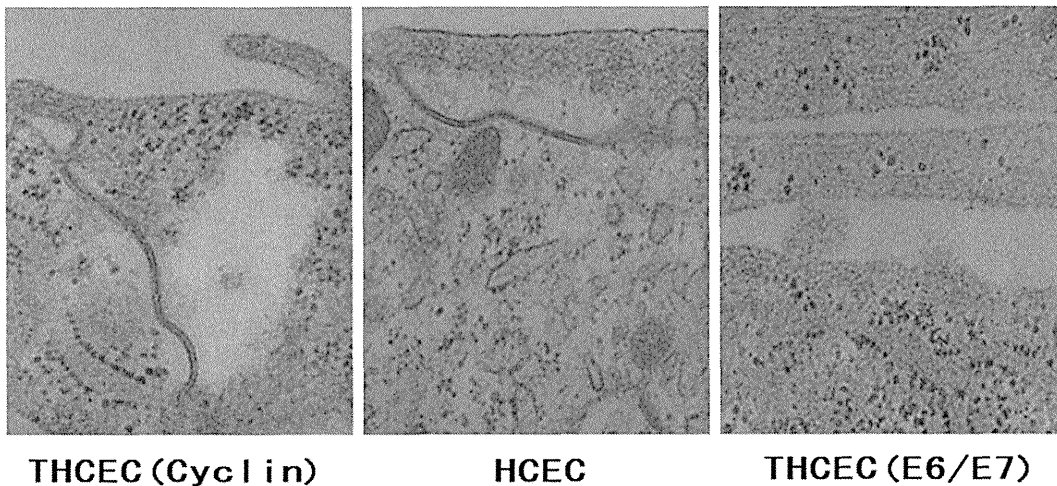doi:10.1371/journal.pone.0029677.g003

THCEC (Cyc I i n)   HCEC   THCEC (E6/E7)

Figure 4. Transmission electron microscopy of cell line intercellular junctions. The junctional complex was detected at the intercellular junction in THCEC (Cyclin) and HCEC. No component of the intercellular junction was found in THCEC (E6/E7), in which cells grew in multilayers without being inhibited by cellular contact (scale bar = 200 nm).
doi:10.1371/journal.pone.0029677.g004

avoiding contamination of the trabecular meshwork tissue. HCEC with Descemet's membrane were stripped from the posterior surface of the corneal tissue with sterile surgical forceps under a dissecting microscope. They were cut into two pieces and cultured in a cell culture dish covered with Type IV collagen in a growth medium (GM); Dulbecco's modified Eagle's medium (DMEM)/Nutrient mixture F12 (1:1) with high glucose supplemented with 10% fetal bovine serum, insulin-transferrin-selenium and MEM-NEAA (Gibco, Auckland, NZ). Cells were subcultured after reaching confluency by treating with trypsin/EDTA and seeded at a density of 5×10^5 cells/well in 6-well dishes.

Viral vector construction and viral transduction

Lentiviral vector plasmids, CSII-CMV-cyclin D1 and -CDK4R24C were constructed by recombination using the

Gateway system (Invitrogen, Carlsbad, CA) as described previously [37]. Briefly, cDNAs of human cyclinD1 and a mutant form of Cdk4 (Cdk4R24C: an inhibitor resistant form of Cdk4, generously provided by Dr Hara) were recombined with a lentiviral vector, CSII-CMV-RfA (a gift from Dr Miyoshi), by LR reaction to create a Gateway expression plasmid (Invitrogen) according to the manufacturer's instructions.

Previous work has described the production of recombinant lentiviruses with the vesicular stomatitis virus G glycoprotein [37], the recombinant retrovirus vector plasmid, pCLXSN-16E6E7 encoding HPV16 E6/E7 (16E6E7) [38] and recombinant retroviruses [39]. Following the addition of recombinant viral fluid to cells seeded in 24-well dishes in the presence of 4 μg/ml polybrene, the cells were infected by the viruses. Stably transduced cells with an expanded life span were designated transduced
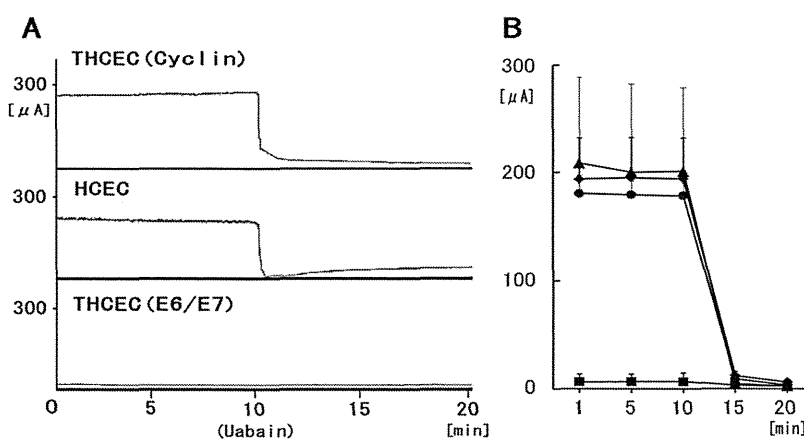

Figure 5. The pump function of cell lines. Short-circuit currents representing $Na^+/K+$-ATPase activity from corneal cell monolayers on the insert well area of 4.67 cm² were calculated before and after addition of the $Na^+/K+$-ATPase inhibitor ouabain. (A) Representative tracings of short-circuit current (μA/well) obtained with cell monolayers of THCEC (Cyclin) (upper panel), HCEC (middle panel) and THCEC (E6/E7) (lower panel). THCEC (Cyclin) possessed equal transport activity to HCEC, whereas no pump function was detected in THCEC (E6/E7). (B) Time-course changes in the average short circuit current of cultured monolayers of cell lines at 1, 5, 10 and 20 min. Data shown are for (▲) THCEC (Cyclin) at PD8, (♦) THCEC (Cyclin) at PD 21, (•) HCEC and (■) THCEC (E6/E7); all data are expressed as mean±SD of four replicate experiments of each cell line.
doi:10.1371/journal.pone.0029677.g005

Table 1. Tumorigenesis assay of cell lines in BALB/C nude mice.

Inoculated cells	Total dose (cell/mouse)	Number of mice (% mortality)	Number of mice with tumor
THCEC (Cyclin)	1.7×10^6	3(0)	0
THCEC (E6/E7)	1.7×10^6	3(0)	0
HeLa cells	2.0×10^6	3(0)	3

doi:10.1371/journal.pone.0029677.t001

human corneal endothelial cell by E6/E7 (THCEC (E6/E7)) and transduced human corneal endothelial cell by Cdk4R24C/cyclinD1 (THCEH (Cyclin)).

Culture of transfected cell lines and growth curve

When the cultures reached subconfluence, the cells were harvested with 0.25% trypsin and 1 mM EDTA, collected into tubes, and centrifuged. The cells were counted using a cell viability analyzer (Vi-CELL Cell Viability Analyzer, Beckman Coulter, Brea, CA), and population doubling (PD) was calculated. The pellets were suspended in growth medium, and the cells were passaged at a density of 5×10^5 cells/well in a 100-mm dish. The original cells were regarded as PD 2 (day 0).

Western blot analysis

Western blotting was conducted as described previously [40]. Antibodies against Cdk4 (ser473; Cell Signaling Technology, Danvers, MA), CyclinD1 (clone G124-326; BD Biosciences, Franklin Lakes, NJ), β-actin (sc-1616; Santa Cruz Biotechnology, Santa Cruz, CA) were used as probes, and horseradish peroxidase-conjugated anti-mouse, anti-rabbit (Jackson Immunoresearch Laboratories, West Grove, PA) or anti-goat (sc-2033; Santa Cruz Biotechnology, Santa Cruz, CA) immunoglobulins were employed as secondary antibodies.

Immunocytochemistry

Cell lines were grown on Type IV collagen-coated glass dishes 14 days after reaching confluency and were fixed with 4% formaldehyde (pH 7.0) for 15 min at room temperature. Cell lines were then rehydrated in phosphate buffered saline (PBS), incubated with 0.2% Triton X-100 for 15 min and rinsed three times with PBS for 5 min each. After incubation with 2% BSA to block nonspecific staining for 30 min, cell lines were incubated with anti-ZO-1 (1:50; sc-8146; Santa Cruz Biotechnology, Santa Cruz, CA), anti-N-cadherin (1:50; sc-7939; Santa Cruz Biotechnology) and anti-Ki67 (1:100; ab15580; Abcam, Cambridge, UK) for 16 h at 4°C. After three washes with PBS, cell lines were incubated with the secondary antibody for 60 min, followed by counterstaining with 4',6-diamidino-2-phenylindole (1:200; sc-3598; Santa Cruz Biotechnology) for 10 min.

Semi-quantitative RT-PCR

Total RNA was extracted from 1×10^6 cultured HCEC using the RNeasy Plus mini-kitH (Qiagen, Germantown/Gaithersburg, MA) according to the manufacturer's instructions and quantified by absorption at 260 nm. Total RNA was then reverse-transcribed into cDNA using Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) with oligo random hexamers. cDNAs of each component were amplified by PCR using specific primers and DNA polymerase. The reaction was first incubated at 95°C for 10 min, followed by 39 cycles at 98°C for 30 s, 58°C for 30 s and 74°C for 30 s. PCR primers are listed in Table 2.

Quantitative real-time RT-PCR

Total RNA extraction and reverse transcription into cDNA was carried out as above. Each quantitative real-time RT-PCR for target genes, including Cell Division Cycle 2 (*CDC2*) and proliferating cell nuclear antigen (*PCNA*), was performed using the Chromo4 real time detection system (Bio-Rad, Hercules, CA). For a 20 ml PCR, the cDNA template was mixed with the primers to final concentrations of 200 nM and 10 µl of SsoFast EvaGreen Supermix (BIO-RAD), respectively. The reaction was first incubated at 95°C for 10 min, followed by 45 cycles at 95°C for 10 s, 57°C for 15 s, and 72°C for 20 s.

Transmission Electron Microscopy

Cell lines cultured on Type IV collagen-coated dishes were fixed in HEPES buffered 2% glutaraldehyde and subsequently post-fixed in 2% osmium tetroxide for 3 h on ice. Specimens were then dehydrated in graded ethanol and embedded in the epoxy resin. Ultrathin sections were obtained by ultramicrotomy and stained with uranyl acetate for 10 min and modified Sato's lead solution for 5 min then submitted to TEM observation (JEM-2000EX, JEOL).

Measurement of pump function

The pump function of confluent monolayers of HCEC was measured using an Ussing chamber as described previously [41]. Cells cultured on Snapwell inserts coated with Type IV collagen were placed in the Ussing chamber EM-CSYS-2 (Physiologic Instruments, San Diego, CA) with the endothelial cell surface side in contact with one chamber and the Snapwell membrane side in contact with another chamber. The chambers were carefully filled with Krebs-Ringer bicarbonate (120.7 mM NaCl, 24 mM NaHCO₃, 4.6 mM KCl, 0.5 mM MgCl₂, 0.7 mM Na₂HPO₄, 1.5 mM NaH₂PO₄ and 10 mM glucose bubbled with a mixture of 5% CO₂, 7% O₂ and 88% N₂ to pH 7.4). The chambers were maintained at 37°C using an attached heater.

The short-circuit current was sensed by narrow polyethylene tubes positioned close to either side of the Snapwell, filled with 3 M KCl and 4% agar gel and connected to silver electrodes. These electrodes were connected to the computer through the Ussing system VCC-MC2 (Physiologic Instruments) and an iWorx 118 Research Grade Recorder (iWorx Systems, Dover, NH), and the short-circuit current was recorded by Labscribe2 Software for Research (iWorx). After the short-circuit current had reached a steady state, ouabain (final concentration, 1 mM) was added to the chamber, and the short-circuit current was re-measured. The pump function attributable to Na⁺/K⁺-ATPase activity was calculated as the difference in short-circuit current measured before and after the addition of ouabain.

Tumorigenesis assay

Cells were harvested by Trypsin/EDTA treatment, collected into tubes, and centrifuged, and the pellets were suspended in