To the degree to which the Standard is adopted and complied with, issuance of the Standard will have a significant beneficial impact on the quality and validity of research based upon the use of human cells.

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Efficient Generation of Hepatoblasts From Human ES Cells and iPS Cells by Transient Overexpression of Homeobox Gene *HEX*

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Human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) have the potential to differentiate into all cell lineages, including hepatocytes, *in vitro*. Induced hepatocytes have a wide range of potential application in biomedical research, drug discovery, and the treatment of liver disease. However, the existing protocols for hepatic differentiation of PSCs are not very efficient. In this study, we developed an efficient method to induce hepatoblasts, which are progenitors of hepatocytes, from human ESCs and iPSCs by overexpression of the *HEX* gene, which is a homeotic gene and also essential for hepatic differentiation, using a HEX-expressing adenovirus (Ad) vector under serum/feeder cell-free chemically defined conditions. Ad-HEX-transduced cells expressed α-fetoprotein (AFP) at day 9 and then expressed albumin (ALB) at day 12. Furthermore, the Ad-HEX-transduced cells derived from human iPSCs also produced several cytochrome P450 (CYP) isozymes, and these P450 isozymes were capable of converting the substrates to metabolites and responding to the chemical stimulation. Our differentiation protocol using Ad vector-mediated transient HEX transduction under chemically defined conditions efficiently generates hepatoblasts from human ESCs and iPSCs. Thus, our methods would be useful for not only drug screening but also therapeutic applications.

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INTRODUCTION

Human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are able to replicate indefinitely and differentiate into most cell types of the body,[1–4] and thereby have the potential to provide an unlimited source of cells for a variety of applications.[5] Hepatocytes are useful cells for biomedical research, regenerative medicine, and drug discovery. They are particularly applicable to drug screenings, such as for the determination of metabolic and toxicological properties of drug compounds in *in vitro* models, because the liver is the main detoxification organ in the body.[6] For these applications, it is necessary to prepare a large number of functional hepatocytes from human ESCs and iPSCs. Many of the existing methods for cell differentiation of human ESCs and iPSCs into hepatocytes employ undefined, serum-containing medium and feeder cells.[7–9] Preparation of human ESC- and iPSC-derived hepatocytes for therapeutic applications and drug toxicity testing in humans should be done in nonxenogenic culture systems to avoid potential contamination with pathogens. Furthermore, the efficiency of the differentiation of the human ESCs and iPSCs into hepatocytes is not particularly high using these methods.[9–14]

In vertebrate development, the liver is derived from the primitive gut tube, which is formed by a flat sheet of cells called the definitive endoderm.[5,15] Shortly afterwards, the definitive endoderm is separated into endoderm derivatives containing the liver bud, the cells of which are referred to as hepatoblasts. The hepatoblasts have the potential to proliferate and differentiate into both hepatocytes and cholangiocytes. In the process of hepatic differentiation, the maturation is characterized by the expression of liver- and stage-specific genes. For example, α-fetoprotein (AFP) is an early hepatic marker, which is expressed in hepatoblasts in the liver bud until birth, and its expression is dramatically reduced after birth.[16] In contrast, albumin (ALB), which is the most abundant protein synthesized by hepatocytes, is initially expressed at lower levels in early fetal hepatocytes, but its expression level is increased as the hepatocytes mature, reaching a maximum in adult hepatocytes.[17] Furthermore, isoforms of cytochrome P450 (CYP) proteins also exhibit differential expression levels according to the developmental stages

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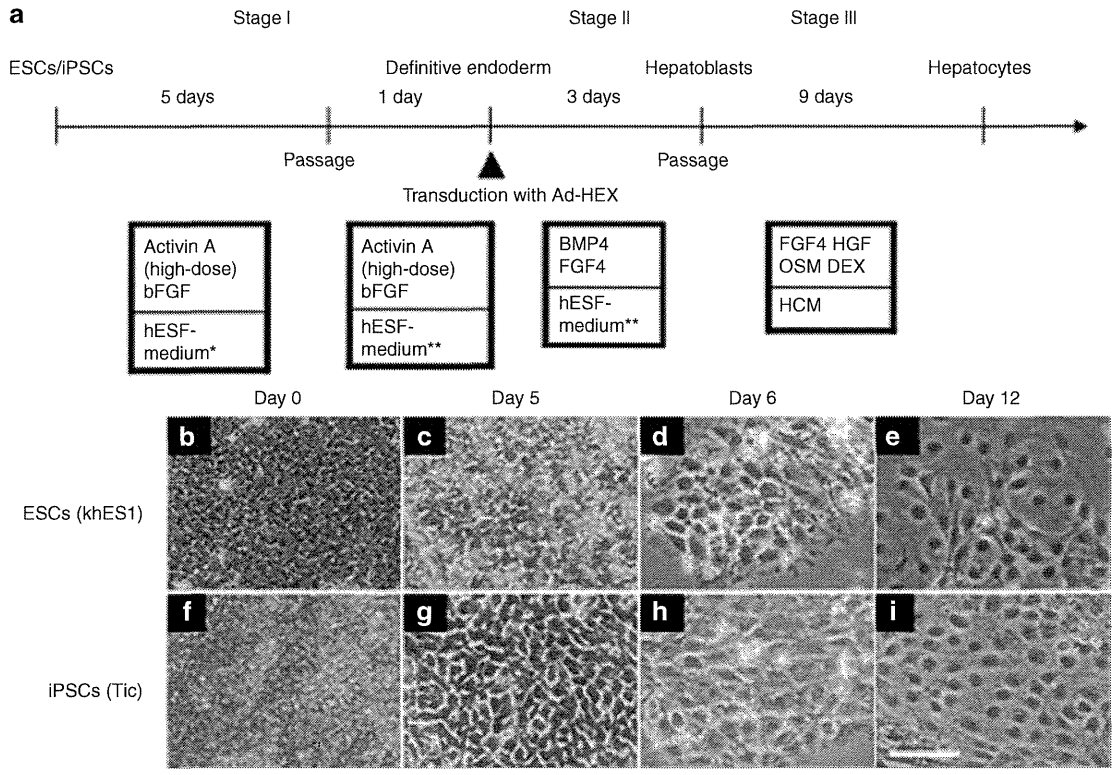

Figure 1 A strategy of differentiation of human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) to hepatoblasts and hepatocytes. (**a**) Schematic representation illustrating the procedure for differentiation of human ESCs (khES1) and iPSCs (Tic) to hepatocytes. (**b–i**) Phase contrast microscopy showing sequential morphological changes (day 0–12) from (**b–e**) human ESCs (khES1) and (**f–i**) iPSCs (Tic) to hepatoblasts via the definitive endoderm. Bar = 50 µm. bFGF, basic fibroblast growth factor; BMP4, bone morphogenetic protein 4; DEX, dexamethasone; FGF4, fibroblast growth factor 4; HGF, hepatocyte growth factor; OSM, Oncostatin M; HCM, hepatocytes culture medium; *, hESF-GRO medium that was 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 fatty acid free BSA; **, hESF-DIF medium that was supplemented with 10 µg/ml insulin, 5 µg/ml apotransferrin, 10 µmol/l 2-mercaptoethanol, 10 µmol/l ethanolamine, 10 µmol/l sodium selenite, 0.5 mg/ml BSA.

of the liver. Although most CYPs (including CYP3A4, CYP7A1, and CYP2D6) are only slightly expressed or not detected in the fetal liver tissue, the expression levels are dramatically increased after birth.[18]

For the development of hepatoblasts, numerous transcription factors are required, such as hematopoietically expressed homeobox (*HEX*), GATA-binding protein 6, prospero homeobox 1, and hepatocyte nuclear factor 4A.[15,19] Among them, *HEX* is suggested to function at the earliest stage of hepatic linage.[20] HEX is first expressed in the definitive endoderm and becomes restricted to the future hepatoblasts. Targeted deletion of the *HEX* gene in the mouse results in embryonic lethality and a dramatic loss of the fetal liver parenchyma.[19,21,22] The hepatic genes, including *ALB*, prospero homeobox1, and hepatocyte nuclear factor 4A, are transiently expressed in the definitive endoderm of *HEX*-null embryos, and further morphogenesis of the hepatoblasts does not occur.[23] In general, then, *HEX* is essential for the definitive endoderm to adopt a hepatic cell fate.

Adenovirus (Ad) vectors are one of the most efficient gene delivery vehicles and have been widely used in both experimental studies and clinical trials.[24] Ad vectors are attractive vehicles for gene transfer because they are easily constructed, can be prepared in high titers, and provide high transduction efficiency in both dividing and nondividing cells. We have developed efficient methods for Ad vector-mediated transient transduction into mouse ESCs and iPSCs.[25,26] We have also showed that the differentiations of mouse ESCs and iPSCs into adipocytes and osteoblasts were dramatically promoted by Ad vector-mediated peroxisome proliferator activated receptor γ and runt related transcription factor 2 transduction, respectively.[25,26]

In this study, we hypothesized that transient *HEX* transduction could efficiently induce hepatoblasts from human ESCs and iPSCs. A previous study demonstrated that *HEX* regulates the differentiation of hemangioblasts and endothelial cells from mouse ESCs,[27] whereas the role of *HEX* in the differentiation of hepatoblasts from human ESCs and iPSCs remains unknown. We found that differentiation of hepatoblasts from the human ESC- and iPSC-derived definitive endoderms, but not from undifferentiated human ESCs and iPSCs, could be facilitated by Ad vector-mediated transient transduction of a *HEX* gene. Furthermore, the Ad-HEX-transduced cells that were derived from human iPSCs were able to differentiate into functional hepatocytes *in vitro*. All the processes for cellular differentiation were performed under serum/feeder cell- free chemically defined conditions. Our culture systems and differentiation method based on Ad vector-mediated transient transduction under chemically defined conditions would provide a platform for drug screening as well as safe therapies.

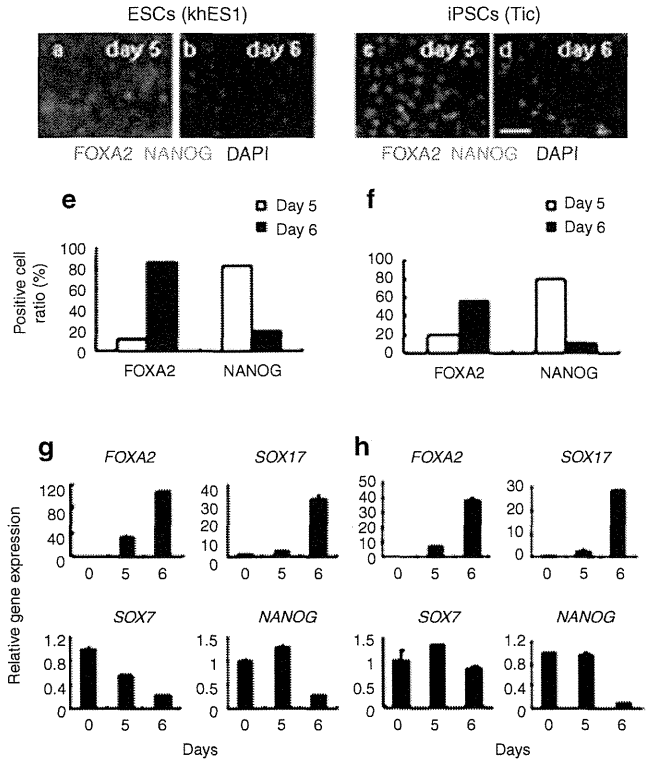

Figure 2 Characterization of the human ESC (khES1)- and iPSC (Tic) derived definitive endoderms. (**a–d**) The immunofluorescent staining of the human ESC (khES1)- and iPSC (Tic) derived differentiated cells before (**a** and **c**; day 5) and after passaging (**b** and **d**; day 6). The cells were immunostained with antibodies against FOXA2 and NANOG. Nuclei were stained with DAPI. (**e**,**f**) Semiquantitative analysis of the immunofluorescent staining in **a–d**. Data are presented as the mean of immunopositive cells counted in eight independent fields. (**g**,**h**) Real-time RT-PCR analysis of the level of definitive endoderm (*FOXA2* and *SOX17*), pluripotent (*NANOG*), and extra-embryonic endoderm (*SOX7*) gene expression at day 5 and 6. At day 5, the cells were passaged. Therefore, the data at day 5 and 6 show the levels of gene expression before (at day 5) or after the passage (at day 6). Data are presented as the mean ± SD from triplicate experiments. The graphs represent the relative gene expression level when the level of undifferentiated cells at day 0 was taken as 1. Bar = 50 μm. ESC, embryonic stem cells; iPSC, induced pluripotent stem cells.

RESULTS

Differentiation of human ESC- and iPSC-derived definitive endoderms

Our three-step differentiation protocol is illustrated in Figure 1a. After treatment with 50 ng/ml of Activin A (high-dose) and basic fibroblast growth factor (bFGF) for 5 days on a laminin-coated plate, morphologically, the human ESCs and iPSCs were gradually transformed from typical, defined, tight human ESC, and iPSC colonies (day 0) into less dense, flatter cells containing prominent nuclei (day 5), even though the majority of the cells had a morphology resembling that of undifferentiated cells (Figure 1b,c,f,g). FACS analysis showed that ~46% of human iPSC-derived differentiated cells expressed CXCR4 (expressed in the definitive endoderm but not the primitive endoderm) (**Supplementary Figure S1a**). Human ESC- and iPSC-derived differentiated cells were immunostained with the definitive endoderm marker, FOXA2 (Figure 2a,c). However, the majority of the cells expressed the pluripotent marker NANOG, indicating that undifferentiated cells remain in the induced cultures at day 5. After the cells were passaged with trypsin-EDTA and seeded on a laminin-coated plate a second time, the resultant cells were found to be more homogeneous and flatter at day 6 (Figure 1d,h). Semiquantitative analysis by counting immunopositive cells revealed that the number of FOXA2-positive cells was increased and, in turn, the number of NANOG-positive cells was decreased at day 6 after passaging (Figure 2e,f). Real-time reverse transcriptase (RT)-PCR analysis showed that the definitive endoderm markers *FOXA2* and *SOX17* mRNA were upregulated, whereas the pluripotent marker *NANOG* mRNA was downregulated at day 6 (Figure 2g,h). These results were consistent with the immunofluorescence results (Figure 2a–d). The expression levels of the mesoderm marker *FLK1* mRNA and ectoderm marker *PAX6* mRNA were downregulated or unchanged at day 6 (**Supplementary Figure S1b–e**). Importantly, the expression of *SOX7* mRNA (expressed in the extra-embryonic endoderm but not the definitive endoderm) was downregulated (Figure 2g,h). These results indicate that the definitive endoderm is induced or selected from human ESCs and iPSCs after passaging. We obtained the same results using another human iPSC line (**Supplementary Figure S2a–d**).

HEX induces hepatoblasts from the human ESC- and iPSC-derived definitive endoderms

To investigate whether forced expression of transcription factors could promote hepatic differentiation, the human ESC- and iPSC-derived definitive endoderms were transduced with Ad vectors. We used a fiber-modified Ad vector containing the elongation factor-1α promoter and a stretch of lysine residue (K7) peptides in the C-terminal region of the fiber knob to examine the transduction efficiency in the human ESC- and iPSC-derived definitive endoderms. The elongation factor-1α promoter was found to be highly active in human ESCs.[28] The K7 peptide targets heparan sulfates on the cellular surface, and the fiber-modified Ad vector containing K7 peptides was shown to be efficient for transduction into many kinds of cells.[29,30] The human ESC- and iPSC-derived definitive endoderms were transduced with a LacZ-expressing Ad vector (Ad-LacZ) at 3,000 vector particle/cell. X-Gal staining showed that the Ad-LacZ-transduced human ESC- and iPSC-derived definitive endoderms successfully expressed LacZ (Figure 3). Nearly 100% of the cells transduced with Ad-LacZ were strongly X-gal positive. The transduction efficiency in the human ESC- and iPSC-derived definitive endoderms transduced with the conventional Ad vector containing the wild-type capsid at 3,000 vector particle/cell was ~80% and X-gal staining was much weaker than that in the cells transduced with fiber-modified Ad vectors (**Supplementary Figure S6**).

Next, the human ESC- and iPSC-derived definitive endoderms were transduced with a HEX-expressing fiber-modified Ad vector (Ad-HEX). Although HEX is known to be a transcription factor that is essential for liver development, it remains unclear what the effect of transient *HEX* overexpression is on differentiation from human ESCs and iPSCs or their derivatives *in vitro*. We confirmed the overexpression of *HEX* in the human ESC- and iPSC-derived definitive endoderms transduced with Ad-HEX (**Supplementary Figure S3a–f**). Gene expression analysis revealed the upregulation of *AFP* mRNA, which was expressed by hepatoblasts or early hepatocytes, in Ad-HEX-transduced cells as

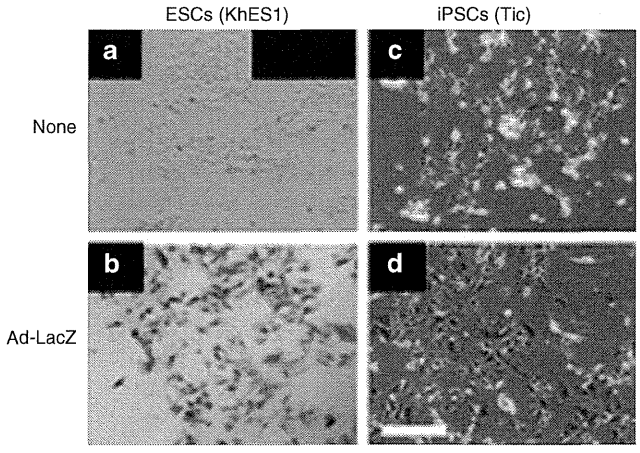

Figure 3 Efficient transgene expression in the human ESC (khES1)- and iPSC (Tic) derived definitive endoderms by using a fiber-modified Ad vector containing the EF-1α promoter. (**a,b**) Human ESC (khES1)-derived and (**c,d**) iPSC (Tic) derived definitive endoderms were transduced with 3,000VP/cell of Ad-LacZ for 1.5 hours. The next day after transduction, X-gal staining was performed as described in the Materials and Methods section. Similar results were obtained in two independent experiments. Scale = 50μm. Ad, adenovirus; EF-1α, elongation factor-1α; ESC, embryonic stem cells; iPSC, induced pluripotent stem cells; LacZ, Ad-LacZ-transduced cells; None, nontransduced cells.

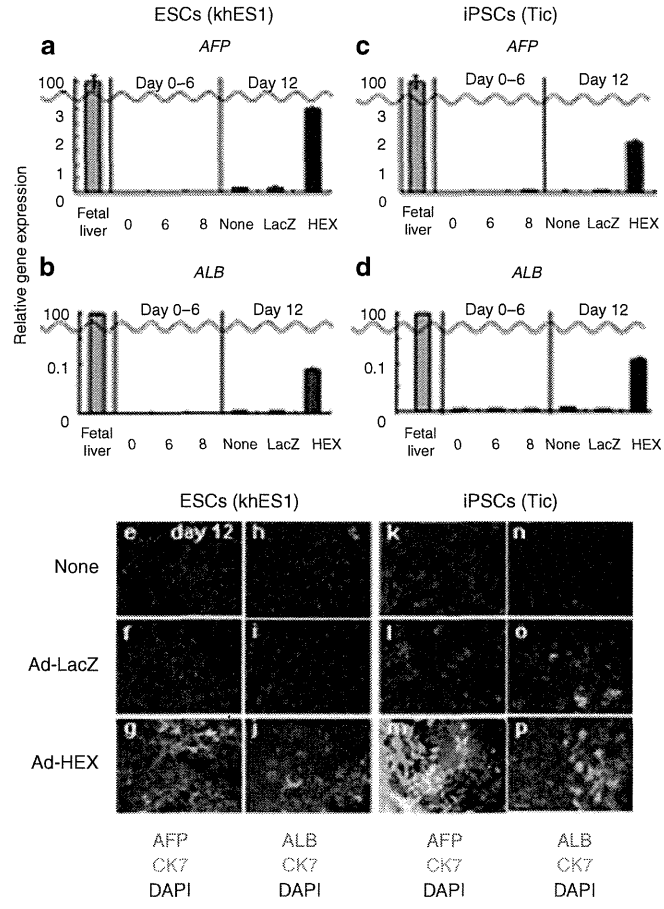

Figure 4 Efficient hepatoblast differentiation from the human ESC (khES1)- and iPSC (Tic) derived definitive endoderms by transduction of the *HEX* gene. (**a–d**) Real-time RT-PCR analysis of the level of (**a,c**) *AFP* and (**b,d**) *ALB* expression in nontransduced cells, Ad-LacZ-transduced cells, and Ad-HEX-transduced cells, all of which were induced from the human ESC (khES1)- and iPSC (Tic) derived definitive endoderms (day 0, 5, 6, and 12). The cells were transduced with Ad-LacZ or Ad-HEX at day 6 as described in **Figure 1a**. The data at day 6 was obtained before the transduction with Ad-HEX. The graphs represent the relative gene expression levels when the level in the fetal liver was taken as 100. (**e–p**) Immunocytochemistry of AFP, ALB, and CK7 expression in nontransduced cells (**e,h,k,** and **n**), Ad-LacZ-transduced cells (**f,i,l,** and **o**), and Ad-HEX-transduced cells (**g,j,m,** and **p**) at day 12, all of which were induced from the human ESC (khES1)- and iPSC (Tic) derived definitive endoderms. Nuclei were stained with DAPI. Bar = 50μm. Ad, adenovirus; AFP, α-fetoprotein; ALB, albumin; CK7, cytokeratin 7; HEX, Ad-HEX-transduced cells; ESC, embryonic stem cells; iPSC, induced pluripotent stem cell; LacZ, Ad-LacZ-transduced cells; None, nontransduced cells.

compared with nontransduced cells or Ad-LacZ-transduced cells (**Figure 4a,c**). Expression of *ALB* mRNA, which is the most abundant protein in liver, was also observed in Ad-HEX-transduced cells (**Figure 4b,d**).

During liver development, both hepatocytes and cholangiocytes were differentiated from the hepatoblasts. We examined the protein expression of AFP, ALB, and the cholangiocyte marker cytokeratin 7 (CK7) in Ad-HEX-transduced cells by immunostaining (**Figure 4e–p**). The AFP-positive populations were detected in Ad-HEX-transduced cells (**Figure 4g,m**). ALB-positive cells were also detected, although the detection efficiency was very low (**Figure 4j,p**). CK7-positive cells were observed among the Ad-HEX-transduced cells, and all CK7-positive cells were found near the AFP- and ALB-positive cells, suggesting that hepatoblasts are generated by the transient overexpression of a *HEX* gene. Semiquantitative RT-PCR analysis showed that the expression levels of the liver-enriched transcription factors hepatocyte nuclear factor 1A, hepatocyte nuclear factor 1B, hepatocyte nuclear factor 4A, and hepatocyte nuclear factor 6 mRNA were upregulated in Ad-HEX-transduced cells (**Supplementary Figure S4a,b**). The expressions of CCAAT/enhancer binding protein α and prospero homeobox 1 mRNA, two transcription factors known to play a pivotal role in the establishment of the hepatoblasts, were also induced in Ad-HEX-transduced cells (**Supplementary Figure S4a, b**). Taken together, these findings indicate that *HEX* enhances the specification of hepatoblasts from the human ESC- and iPSC-derived definitive endoderms. Similar results were obtained with another human iPSC line (**Supplementary Figure S2e–g**).

Time course of differentiation of the definitive endoderm to hepatoblasts

Next, we examined the time course of AFP and CK7 expression during differentiation of human iPSCs to hepatoblasts in Ad-HEX-transduced cells and nontransduced cells. At day 7 (the day after transduction), the expression of AFP was not detectable in Ad-HEX-transduced or nontransduced cells (**Supplementary Figure S5a,d**). At day 8–9, morphological changes to hepatocyte-like cells were observed in Ad-HEX-transduced cells (**Supplementary Figure S5h,i**). We also observed homogeneous AFP-positive cells at day 9 (**Supplementary Figure S5e**). At day 10, CK7-positive cells appeared, indicating that hepatoblasts started to differentiate into hepatocytes and cholangiocytes at day 9–10 (**Supplementary Figure S5f**). At day 12, ALB-positive cells appeared, indicating that hepatocytes were differentiated from Ad-HEX-transduced cells (**Figure 4p**). These results showed that *HEX* induces the hepatoblasts from the

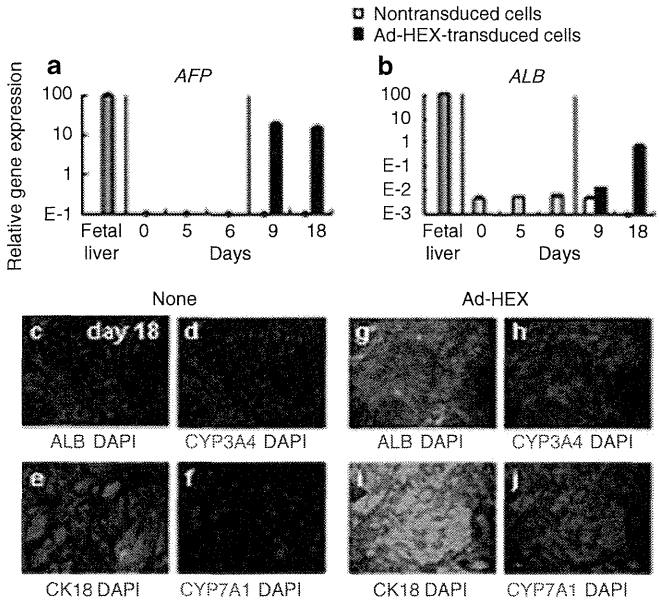

Figure 5 Efficient differentiation of Ad-HEX-transduced hepatoblasts into hepatocytes. (**a**,**b**) Real-time RT-PCR analysis of (**a**) *AFP* and (**b**) *ALB* expression in nontransduced cells and Ad-HEX-transduced cells, both of which were induced from the human iPSC (Tic) derived definitive endoderm (day 0, 5, 6, and 12). The cells were transduced with Ad-HEX at day 6 as described in **Figure 1a**. The data at day 6 were obtained before the transduction with Ad-HEX. The graphs represent the relative gene expression level when the level in the fetal liver was taken as 100. (**c–j**) Immunocytochemistry of ALB, CYP3A4, CYP7A1, and CK18 expression in (**c–f**) nontransduced cells and (**g–j**) Ad-HEX-transduced cells, all of which were induced from the human iPSC (Tic) derived definitive endoderm at day 18. Nuclei were stained with DAPI. Bar = 50 μm. Ad, adenovirus; AFP, α-fetoprotein; ALB, albumin; CK18, cytokeratin 18; ESC, embryonic stem cells; HEX, Ad-HEX-transduced cells; iPSC, induced pluripotent stem cell; None, nontransduced cells; RT-PCR, reverse transcriptase-PCR.

definitive endoderm, and the Ad-HEX-transduced cells could differentiate into both hepatocytes and cholangiocytes.

Directed hepatic differentiation from hepatoblasts

With the protocol described above, heterogeneous populations containing CK7-positive cholangiocytes were observed at day 12 (**Figure 4p**). To promote the differentiation of hepatoblasts to hepatocytes, the human iPSC-derived differentiated cells at day 9 (**Supplementary Figure S5e**) were dislodged with trypsin-EDTA and plated on collagen I-coated dishes as previously reported.[11] After 8–11 days in culture with medium containing FGF4, HGF, OSM, and DEX, the Ad-HEX-transduced cells became more flattened (**Supplementary Figure S5m**), whereas the nontransduced cells became fibroblast-like cells (**Supplementary Figure S5i**). Gene expression analysis showed the upregulation of *ALB* mRNA in Ad-HEX-transduced cells under this culture condition, whereas the expression of *ALB* mRNA was reduced in the nontransduced cells at day 18 (**Figure 5b**). Immunostaining showed that only a small percentage of Ad-HEX-transduced cells expressed ALB at day 12 (**Figure 4p**), whereas most of the Ad-HEX-transduced cells were ALB-positive at day 18 (**Figure 5g**). Most of the Ad-HEX-transduced cells also expressed CYP3A4 at day 18 (**Figure 5h**). More importantly, in the Ad-HEX-transduced cells, CYP7A1 and cytokeratin 18 were detected and these proteins are known

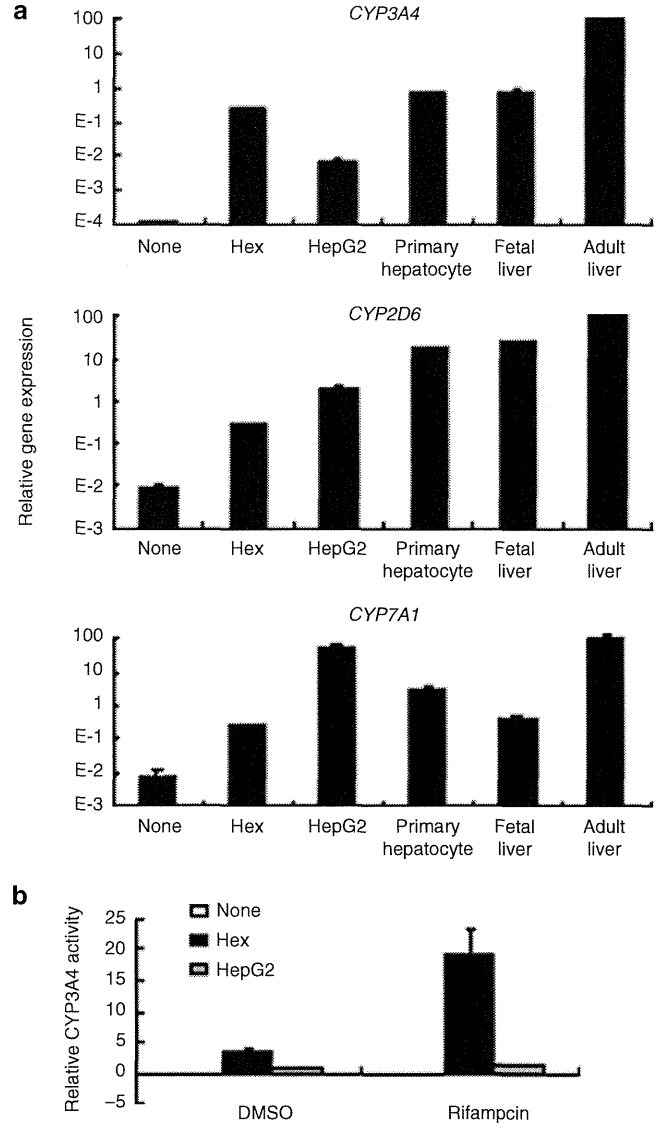

Figure 6 Cytochrome P450 isozymes in human iPSC (Tic) derived hepatocytes. (**a**) Real-time RT-PCR analysis of *CYP3A4*, *CYP7A1*, and *CYP2D6* expression in iPSC (Tic) derived nontransduced cells, Ad-HEX-transduced cells, and fetal and adult liver tissues. (**b**) Induction of CYP3A4 by rifampicin in human iPSC (Tic) derived nontransduced cells, Ad-HEX-transduced cells, the HepG2 cell line and primary human hepatocytes, which were cultured 48 hours after plating the cells. Data are presented as the mean ± SD from triplicate experiments. The graphs represent the relative gene expression level when the level in the adult liver was taken as 100. AFP, α-fetoprotein; ALB, albumin; DMSO, dimethyl sulfoxide; ESC, embryonic stem cells; HEX, Ad-HEX-transduced cells; iPSC, induced pluripotent stem cell; LacZ, Ad-LacZ-transduced cells; None, nontransduced cells.

to be detected in hepatocytes but not in extra-embryonic cells[31,32] (**Figure 5i,j**). Quantitative analysis showed that ~84, 80, 88, and 92% of Ad-HEX-transduced cells expressed ALB, CYP3A4, CYP7A1, and cytokeratin 18, respectively. These results indicate that Ad-HEX-transduced cells could differentiate to hepatic cells. However, the expression level of *ALB* mRNA in Ad-HEX-transduced cells was lower than that in fetal liver tissue and in turn, the expression of *AFP* mRNA was maintained (**Figure 5a**). Therefore, Ad-HEX-transduced cells are committed to the hepatic linage, but are not yet mature hepatocytes.

Ad-HEX-transduced cells exhibit hepatic functions

To test the hepatic function in the Ad-HEX-transduced cells, we investigated the liver metabolism, because P450 cytochrome enzymes play a critical role in this function. We examined the expression level of several members of this multigene family, *i.e.*, *CYP3A4*, *CYP7A1*, mRNA and *CYP2D6* in Ad-HEX-transduced cells by real-time RT-PCR. The real-time RT-PCR analysis showed that the mRNAs for *CYP3A4*, *CYP7A1*, and *CYP2D6* were expressed in Ad-HEX-transduced cells, whereas none of these mRNAs were expressed in the nontransduced cells (**Figure 6a**). The expression levels of *CYP3A4* in Ad-HEX-transduced cells were similar to those observed in primary human hepatocytes, which were cultured 48 hours after plating the cells, or fetal liver tissues but lower than those in adult liver. The *CYP2D6* and *CYP7A1* mRNA expressions in Ad-HEX-transduced cells were lower than those in primary hepatocytes or adult tissues. Next, we investigated the metabolism of the P450 3A4 substrates by measuring the activity of P450 isozymes. The metabolites were detected in Ad-HEX-transduced cells, and their activity was 3.4-fold higher than that in the most commonly used human hepatocyte cell line, HepG2 (**Figure 6b**; DMSO column). This result was consistent with the real-time RT-PCR data (**Figure 6a**). We further tested the induction of CYP3A4 upon chemical stimulation, because CYP3A4 is the most prevalent P450 isozyme in the liver and is involved in the metabolism of a significant proportion of the currently available commercial drugs. Because CYP3A4 can be induced with rifampicin, both Ad-HEX-transduced cells and HepG2 cells were treated with rifampicin, followed by treatment with CYP3A4 substrate. Ad-HEX-transduced cells produced 5.4-fold higher levels of metabolites in response to rifampicin treatment (**Figure 6b**; rifampicin column). This result indicates that P450 isozymes are active in Ad-HEX-transduced cells.

DISCUSSION

The object of this study was to develop an efficient method for generating hepatoblasts and hepatocytes from human ESCs and iPSCs for application to drug toxicity screening tests as well as therapeutics such as regenerative medicine. We found that transient HEX transduction in the definitive endoderm together with a culture under chemically defined conditions was useful for this purpose.

It has been reported that a high concentration of Activin A induces differentiation of human ESCs into the definitive endoderm.[8,33,34] On the other hand, undifferentiated human ESCs are maintained by a low concentration of Activin A.[35] Several studies have shown that bFGF promotes the differentiation of ESCs into the definitive endoderm and inhibits the differentiation of ESCs into the extra-embryonic endoderm.[35–38] bFGF has been reported to inhibit the BMP signaling, which can promote the extra-embryonic lineage differentiation.[39] The extra-embryonic endoderm expresses most of the hepatocyte markers, such as AFP.[40] Contamination of the extra-embryonic endoderm makes it difficult to estimate the hepatic differentiation from human ESCs and iPSCs.[11,14,40] In this study, we showed that both Activin A and bFGF induce definitive endoderm populations, while they repress the extra-embryonic endoderm differentiation (**Figure 2g,h**). Interestingly, after the differentiated cells that were cultured on laminin-coated plates with Activin A and bFGF were passaged at day 5, FOXA2-positive cells (definitive endoderm) were enriched in the resultant cells at day 6 (**Figure 2a–f**). This may have been because FOXA2-positive cells efficiently adhered to the laminin-coated plate and/or because trypsinized, single undifferentiated ESCs/iPSCs cannot survive. The passaging of differentiated cells might be attributed to the reduction in the number of not only the extra-embryonic endoderm cells but also the undifferentiated cells. However, the efficiency of the definitive endoderm differentiation in this study was not as efficient as that reported by other groups.[8,33,34] Other cell lineages, such as the mesoderm and extra-embryonic endoderm, might remain at day 6 (**Figure 2g,h** and **Supplementary Figure S1**). Further improvement of the culture conditions will thus be needed in order to enhance the definitive endoderm differentiation.

Hepatoblasts and hepatocytes were differentiated from the human ESC- and iPSC-derived definitive endoderms by transient overexpression of the homeobox gene *HEX*. A fiber-modified Ad vector containing K7 peptides mediated much higher gene expression than conventional Ad vectors in the human ESC- and iPSC-derived definitive endoderms (**Supplementary Figure S6**). This new hepatic differentiation protocol shows that *HEX* induces AFP-positive hepatoblasts at day 9 and ALB-positive hepatocytes at day 12 from human ESCs and iPSCs, whereas the previous protocols require a few weeks or months to induce AFP- and ALB-positive hepatocytes from PSCs.[9–11] Previous studies suggested that *HEX* could regulate liver-enriched transcription factors such as hepatocyte nuclear factor 4A and hepatocyte nuclear factor 6.[19,23] Overexpression of the *HEX* gene under the conditions employed in the present study could activate several transcription factors that are required for hepatic differentiation (**Supplementary Figure S4a,b**). However, the Ad-HEX-transduced cells showed a low level of expression of *ALB* and some CYP450 species, as well as a high level of *AFP* expression, indicating that the cells were still immature. To promote further hepatic differentiation or maturation, it may be effective to culture the hepatic cells in a 3D environment or on feeder cells such as cardiomyocyte- or endothelium-derived cells.[41,42] In addition, the function of our hepatic cells was still limited. Further analysis of the other functions of our hepatic cells, such as glycogen storage, uptake of indocyanine green and organic anion low-density lipoprotein, and transplantation of Ad-HEX-transduced cells into the liver of immunodeficient mice, is clearly needed for the appreciation to drug screening and therapeutic treatment modalities.

During the preparation of this article, Kubo *et al.* have reported that *HEX* could promote hepatoblast differentiation from mouse ESCs.[43] Their report is consistent with our data, suggesting that *HEX* plays a pivotal regulatory role in not only mouse but also human hepatic differentiation. They also showed that the overexpression of *HEX* at the definitive endoderm stage is critical for hepatic specification of the mouse ESCs. We also confirmed that forced expression of *HEX* in the undifferentiated human ESCs and iPSCs did not elevate the expression of *ALB* and *CK7* (**Supplementary Figure S7**), indicating that *HEX* enhances the hepatic differentiation not from the undifferentiated cells but from the definitive endoderm. However, Kubo *et al.* used recombinant mouse ESCs (tet-HEX ESCs), in which the tetracycline-regulated *HEX* expression cassette

is integrated into the host cell genome to induce *HEX* in a stage-specific manner. Their system would not be appropriate for clinical use because the transgene is randomly integrated into the host cell genome and this leads to a risk of mutagenesis.[44] On the other hand, we generated human hepatoblasts by Ad vector-mediated transient *HEX* transduction, method which avoids the integration of exogenous DNA into the host chromosome.

Touboul *et al.* reported that human ESCs and iPSCs can differentiate into functional hepatocytes under chemically defined conditions.[34] In the present study, hepatoblasts were generated in a chemically defined serum-free medium, which minimized exposure to animal cells and proteins, and on a defined extracellular matrix, such as laminin or collagen, which do not contain undefined growth factors. To generate hepatocytes, hepatocyte culture medium, which is serum-free but not defined, was used in the stage III. When defined hESF-medium was used in the stage III, the expression levels of *ALB* and *CYP3A4* mRNA were half the levels seen in the cells cultured with hepatocyte culture medium in the preliminary experiment (data not shown). Human ESCs and iPSCs were also grown for maintaining the undifferentiated state on a feeder layer, which contains xenoantigen such as bovine apolipoprotein B-100. Bovine apolipoprotein B-100 is known to be a dominant xenoantigen for cell-based therapies.[45] Human ESC- and iPSC-derived hepatocytes should be generated and cultured under chemically defined conditions not only to avoid potential contamination with pathogens for the safer therapeutic application, but also to obtain reproducible results using the differentiation protocols.[34,46] Development of differentiation protocols using other genes of transcription factors as well as *HEX* genes based on a chemically defined medium is under way. Overall, our strategy should provide a novel protocol for hepatic differentiation from human ESCs and iPSCs, which could be useful for regenerative medicine and drug screening.

MATERIALS AND METHODS

Ad vectors. Ad vectors were constructed by an improved *in vitro* ligation method.[47,48] The human *HEX* complementary DNA derived from pDNR-LIB-*HEX* (Invitrogen, Carlsbad, CA) was inserted into pHMEF5,[29] which contains the human elongation factor-1α promoter, resulting in pHMEF-HEX. The pHMEF-HEX was digested with I-CeuI/PI-SceI and ligated into I-CeuI/PI-SceI-digested pAdHM41-K7,[30] resulting in pAd-HEX. Ad-HEX and Ad-LacZ, both of which contain the elongation factor-1α promoter and a stretch of lysine residues (K7) peptides in the C-terminal region of the fiber knob, were generated and purified as described previously.[26,29] The vector particle titer was determined by using a spectrophotometric method.[49]

Human ESCs and iPSCs culture. A human ESC line, khES1, was obtained from Kyoto University (Kyoto, Japan).[50] khES1 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 after approval by the review board at Kyoto University. Human ESCs were maintained on a feeder layer of mitomycin-inactivated mouse embryonic fibroblasts (ICR; ReproCELL Incorporated, Tokyo, Japan) with Dulbecco's modified Eagle's medium/F-12 (Sigma, St Louis, MO) supplemented with 0.1 mmol/l 2-mercaptoethanol, 0.1 mmol/l nonessential amino acids, 2 mmol/l L-glutamine, 20% GIBCO knockout serum replacement (Invitrogen), and 5 ng/ml bFGF (Sigma) in a humidified atmosphere of 3% CO_2 and 97% air at 37 °C. Human ESCs were dissociated with 0.1 mg/ml dispase (Roche Diagnostics, Burgess Hill, UK) into small clumps, and subcultured every 5 or 6 days.

Two human iPS clones derived from the embryonic human lung fibroblast cell line MCR5 were provided from JCRB Cell Bank (Tic, JCRB Number: JCRB1331; and Dotcom, JCRB Number: JCRB1327).[3,4] In the present study, we mainly used the Tic cell line, but similar results were obtained using the Dotcom cell line, and these are shown in the supplementary figures. Human iPSCs were maintained on a feeder layer of mitomycin-inactivated mouse embryonic fibroblasts (Hygro Resistant Strain C57/BL6; Hygro, Millipore, MA) on a gelatin-coated flask in human iPS medium. Human iPS medium consists of knockout Dulbecco's modified Eagle's medium/F12 (Invitrogen), supplemented with 0.1 mmol/l 2-mercaptoethanol, 0.1 mmol/l nonessential amino acids, 2 mmol/l L-glutamine, 20% knockout serum replacement, and 10 ng/ml bFGF in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. Human iPSCs were dissociated with 0.1 mg/ml dispase (Roche) into small clumps and subcultured every 7 or 8 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 in a humidified atmosphere of 10% CO_2 and 90% air at 37 °C.[46] hESF9 consists of hESF-GRO medium (Cell Science & Technology Institute, Sendai, Japan) supplemented with five factors (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 ALB, 10 ng/ml bFGF, and 100 ng/ml heparin (all from Sigma). For induction of definitive endoderm, human ESCs and iPSCs were dissociated into single cells with Accutase (Invitrogen) and cultured for 5 days on a mouse laminin-coated tissue 12-well plate (6.0×10^4 cells/cm^2) in hESF-GRO medium (Cell Science & Technology Institute) supplemented with the five factors, 0.5 mg/ml fatty acid free bovine ALB (BSA) (Sigma), 10 ng/ml bFGF, and 50 ng/ml Activin A (R&D Systems, Minneapolis, MN) in a humidified atmosphere of 10% CO_2 and 90% air at 37 °C. The medium was refreshed every day.

For induction of hepatoblasts, the human ESC- and iPSC-derived definitive endoderms (day 5) were dissociated with 0.0125% trypsin-0.01325 mmol/l EDTA, and then the trypsin was inactivated with 0.1% soybean trypsin inhibitor (Sigma). The cells were seeded at 1.2×10^5 cells/cm^2 on a laminin-coated 12-well plate with hESF-DIF (Cell Science & Technology Institute) medium supplemented with the five factors, 0.5 mg/ml BSA, 10 ng/ml bFGF, and 50 ng/ml Activin A in a humidified atmosphere of 10% CO_2 and 90% air at 37 °C. The next day, the cells were transduced with 3,000 vector particle/cell of Ad vectors (Ad-HEX and Ad-LacZ) for 1.5 hours in hESF-DIF medium supplemented with the five factors, BSA, 10 ng/ml FGF4 (R&D Systems) and 10 ng/ml BMP4 (R&D Systems).[10] The medium was refreshed every day.

For induction of hepatocytes, human iPSC-derived hepatoblasts in one well (day 9) were passaged onto two wells with 0.0125% trypsin-0.01325mmol/l EDTA and 0.1% trypsin inhibitor, on type I collagen-coated tissue 12-well plate (15 μg/cm^2) (Nitta Gelatin, Osaka, Japan). The cells were cultured in hepatocyte culture medium supplemented with SingleQuots (Lonza, Walkersville, MD), 10 ng/ml FGF4, 10 ng/ml HGF (R&D Systems), 10 ng/ml Oncostatin M (R&D Systems), and 0.392 ng/ml dexamethasone (Sigma).[11] The medium was refreshed every 2 days.

RNA isolation, RT-PCR, immunostaining, flow cytometry, lacz assay, and assay for cytochrome P4503A4 activity. For details of these procedures, See **Supplementary Materials and Methods, Supplementary Tables S1 and S2.**

SUPPLEMENTARY MATERIAL

Figure S1. Characterization of the human ESC (khES1)- and iPSC (Tic)-derived definitive endoderms.

Figure S2. Efficient differentiation of another human iPSC line (Dotcom) into hepatoblasts by overexpression of the *HEX* gene.

Figure S3. Overexpression of *HEX* in the human ESC (khES1)- and iPSC (Tic)-derived definitive endoderms.

Figure S4. Characterization of Ad-HEX-transduced hepatoblasts.
Figure S5. Progression of differentiation of the definitive endoderm to hepatoblasts.
Figure S6. X-gal staining of human iPSC (Tic)-derived definitive endoderms transduced with a conventional or a fiber-modified Ad vector containing the EF-1α promoter.
Figure S7. *HEX* promotes the differentiation into the hepatic lineage, not from undifferentiated iPSCs (Tic), but from iPSC (Tic)-derived definitive endoderm.
Table S1. List of Taqman gene expression assays and primers.
Table S2. List of antibodies used.
Materials and Methods.

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Reduction of N-Glycolylneuraminic Acid in Human Induced Pluripotent Stem Cells Generated or Cultured under Feeder- and Serum-Free Defined Conditions

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Abstract

Background: The successful establishment of human induced pluripotent stem cells (hiPSCs) has increased the possible applications of stem cell research in biology and medicine. In particular, hiPSCs are a promising source of cells for regenerative medicine and pharmacology. However, one of the major obstacles to such uses for hiPSCs is the risk of contamination from undefined pathogens in conventional culture conditions that use serum replacement and mouse embryonic fibroblasts as feeder cells.

Methodology/Principal Findings: Here we report a simple method for generating or culturing hiPSCs under feeder- and serum-free defined culture conditions that we developed previously for human embryonic stem cells. The defined culture condition comprises a basal medium with a minimal number of defined components including five highly purified proteins and fibronectin as a substrate. First, hiPSCs, which were generated using Yamanaka's four factors and conventional undefined culture conditions, adapted to the defined culture conditions. These adapted cells retained the property of self renewal as evaluated morphologically, the expression of self-renewal marker proteins, standard growth rates, and pluripotency as evaluated by differentiation into derivatives of all three primary germ layers *in vitro* and *in vivo* (teratoma formation in immunodeficient mice). Moreover, levels of nonhuman N-glycolylneuraminic acid (Neu5Gc), which is a xenoantigenic indicator of pathogen contamination in human iPS cell cultures, were markedly decreased in hiPSCs cultured under the defined conditions. Second, we successfully generated hiPSCs using adult dermal fibroblast under the defined culture conditions from the reprogramming step. For a long therm culture, the generated cells also had the property of self renewal and pluripotency, they carried a normal karyotype, and they were Neu5Gc negative.

Conclusion/Significance: This study suggested that generation or adaption culturing under defined culture conditions can eliminate the risk posed by undefined pathogens. This success in generating hiPSCs using adult fibroblast would be beneficial for clinical application.

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Introduction

Human induced pluripotent cells (hiPSCs) generated by the introduction of defined factors from somatic cells exhibit pluripotency similar to human embryonic stem cells (hESCs) [1,2]. The broad developmental potential of hiPSCs makes them a possible source of cells for the regenerative medical transplantation of various tissues. However, before hiPSC-derived cells can be used in human transplantation, a number of safety concerns need to be overcome. One such concern is the risk of contamination by undefined pathogens or immunoreactive materials from undefined components used in the culturing of hiPSCs [3]. N-Glycolylneur-

aminic acid (Neu5Gc) has been identified as an immunoreactive material that contaminates cells in culture. Neu5Gc, a sialic acid found on the cell surface, is considered a xenoantigen for humans because human cells cannot produce Neu5Gc genetically [4], although it can be taken up from the culture environment [5,6]. Furthermore, most humans have circulating antibodies specific for Neu5Gc. Contamination of hESCs by Neu5Gc was confirmed following culturing under conventional conditions with mouse embryonic fibroblast (MEF)-derived feeder cells and knockout serum replacement (KSR)-supplemented medium [7,8]. Neu5Gc could therefore be a useful indicator of pathogen contamination in pluripotent stem cell cultures.

Defined culture conditions are therefore required when using hiPSC to avoid contamination from undefined pathogens or immunoreactive materials [7]. KSR-supplemented medium is not defined and thus may contain a variety of contaminating factors [9,10,11]. Based on previous findings indicating that the phenotypes of hiPSCs are similar to those of hESCs [1,2], we hypothesized that hESC culture conditions could also be used for hiPSCs. Previously, we developed a defined serum-free medium, namely hESF9, for culturing hESCs on a type I collagen substrate without feeders [12]. Although several defined culture conditions without feeders for hESCs have been reported, difficulties remain in propagating the undifferentiated hESCs [13,14,15,16]. Recently, we found that adding activin A to hESF9 medium supports robust propagation of hES cells and enhances the stable attachment of these cells to fibronectin [16]. We modified our medium accordingly and subsequently cultured our hESCs on a fibronectin substrate without feeders. The modified medium (hESF9a) comprises a basal medium supplemented with heparin sulphate and five highly purified proteins: bovine pancreatic insulin, human apotransferrin, fatty acid-free bovine serum albumin conjugated with oleic acid, human recombinant fibroblast growth factor (FGF)-2, and human recombinant activin [16].

In the present study, we generated hiPSCs from skin keratinocytes using conventional culture conditions with KSR and feeder cells [17]. The cells were then moved into defined culture conditions in hESF9a medium on fibronectin without feeders. We confirmed that the hiPSCs cultured under defined conditions were pluripotent stem cells on the basis of cell morphology, growth rate, the expression of self-renewal genes, cell differentiation *in vitro*, and teratoma formation *in vivo*. Furthermore, we observed that levels of Neu5Gc decreased steadily in hiPSCs cultured under defined conditions. Finally, we generated hiPSCs from adult dermal fibroblast under defined conditions. For long-term culture, these hiPSCs maintained their pluripotency and normal karyotype.

Results and Discussion

Generation of the hiPSC line and adaptation to defined culture conditions

The hiPSC cell line was established from neonatal skin keratinocytes by the infection of amphotropic retroviruses carrying the *OCT4*, *SOX2*, *KLF4*, and C-*MYC* genes using conventional culture conditions with KSR medium and mitomycin C-treated MEF feeder cells (KSR-based conditions) [1,17,18]. Under the KSR-based conditions, cells maintained their undifferentiated morphology and expressed markers of pluripotency, namely ALP (Figure 1B), NANOG (Figure 1E), OCT3/4 (Figure 1H), SSEA4 (Figure 1N), and TRA1-60 (Figure 1Q), shown by immunocytochemistry or substrate staining for ALP. However, the cells did not express stage-specific embryonic antigen (SSEA)-1, which is a self-renewal marker of murine ES cells and a differentiation marker of hESCs (Figure 1K). The cell line was designated UTA1.

At passage 18, the UTA1 cells were transferred into the defined culture conditions with hESF9a medium on fibronectin-coated dishes without feeder cells (hESF9a-based conditions) [12,16]. After a further five passages, the UTA1 cells also expressed the self-renewal markers, ALP (Figure 1C), NANOG (Figure 1F), OCT3/4 (Figure 1I), SSEA4 (Figure 1O), and TRA1-60 (Figure 1R); however, there was no detectable expression of SSEA1 under either culture condition (Figure 1L), suggesting that hiPSCs grown in the hESF9a-based conditions maintained their undifferentiated characteristics. The UTA1 cells steadily proliferated under hESF9a-based conditions for a prolonged culture period, as in the conventional KSR-based conditions (Figure S1). We continued to culture the UTA1 cells in hESF9a-based condition up to 27 passages. These results suggested that the UTA1 cells cultured under the hESF9a-based conditions retained the property of self-renewal.

In vitro and in vivo differentiation of hiPSCs under defined culture conditions

Differentiation potential in the UTA1 cells grown under KSR- and hESF9a-based conditions was assessed using *in vitro* differentiation assays involving embryoid body generation. After 24 days in differentiation culture conditions, the embryoid bodies contained various types of differentiated cells characterized by germ-layer markers as follows: MAP2 (Figure 2A, B) and TUJ1 (Figure 2C, D) as ectoderm markers; FLK1 (Figure 2E, F) and vimentin (Figure 2G, H) as mesoderm markers; and PDX1 as an endoderm marker (Figure 2I, J). We also validated the pluripotency of UTA1 cells under KSR-based conditions (17 passages) and hESF9a-based conditions (18 passage in KSR-based condition and 12 passage in ESF9a-based condition) by teratoma formation in the testes of severe combined immunodeficient (SCID) mice injected with hiPSCs. Eight weeks after injection, histological analysis demonstrated that the formed teratomas were derived from all three primary germ layers. Neural tissues (ectoderm), muscle (mesoderm), cartilage (mesoderm), and intestinal epithelia (endoderm) were all identified histologically in the hiPSC-derived teratomas (Figure 3). These results suggested that UTA1 cells remained pluripotent to differentiate into all three germ layers when grown under hESF9a-based conditions.

The level of xenoantigen Neu5Gc in the hiPSC under defined culture conditions

Our data showed that hESF9a culture conditions maintain the pluripotency of hiPSCs. Conventional culture conditions currently use KSR for human ES/iPS cells, and it is accepted that these commercially supplied components may contain undefined animal-derived xenoantigens and pathogens. Because human cells cannot produce Neu5Gc genetically [4], it becomes a useful indicator of xenogenic contamination in human pluripotent stem cells [7]. We therefore examined the expression of Neu5Gc in UTA1 cells grown under KSR- and hESF9a-based conditions by flow cytometry using an antibody against Neu5Gc. The level of Neu5Gc was high in UTA1 cells cultured under the KSR-based conditions (23 passages) and was comparable with that in Chinese hamster ovary (CHO) cells cultured in fetal calf serum (FCS)-containing medium (Figure 4A, B). The Neu5Gc expression decreased almost to negative control levels (with control antibody or no primary antibody) in UTA1 cells cultured under the hESF9a-based conditions (18 passages in KSR-based condition and 27 passages in ESF9a-based condition) (Figure 4C). The conventional culture conditions contain animal-derived compo-

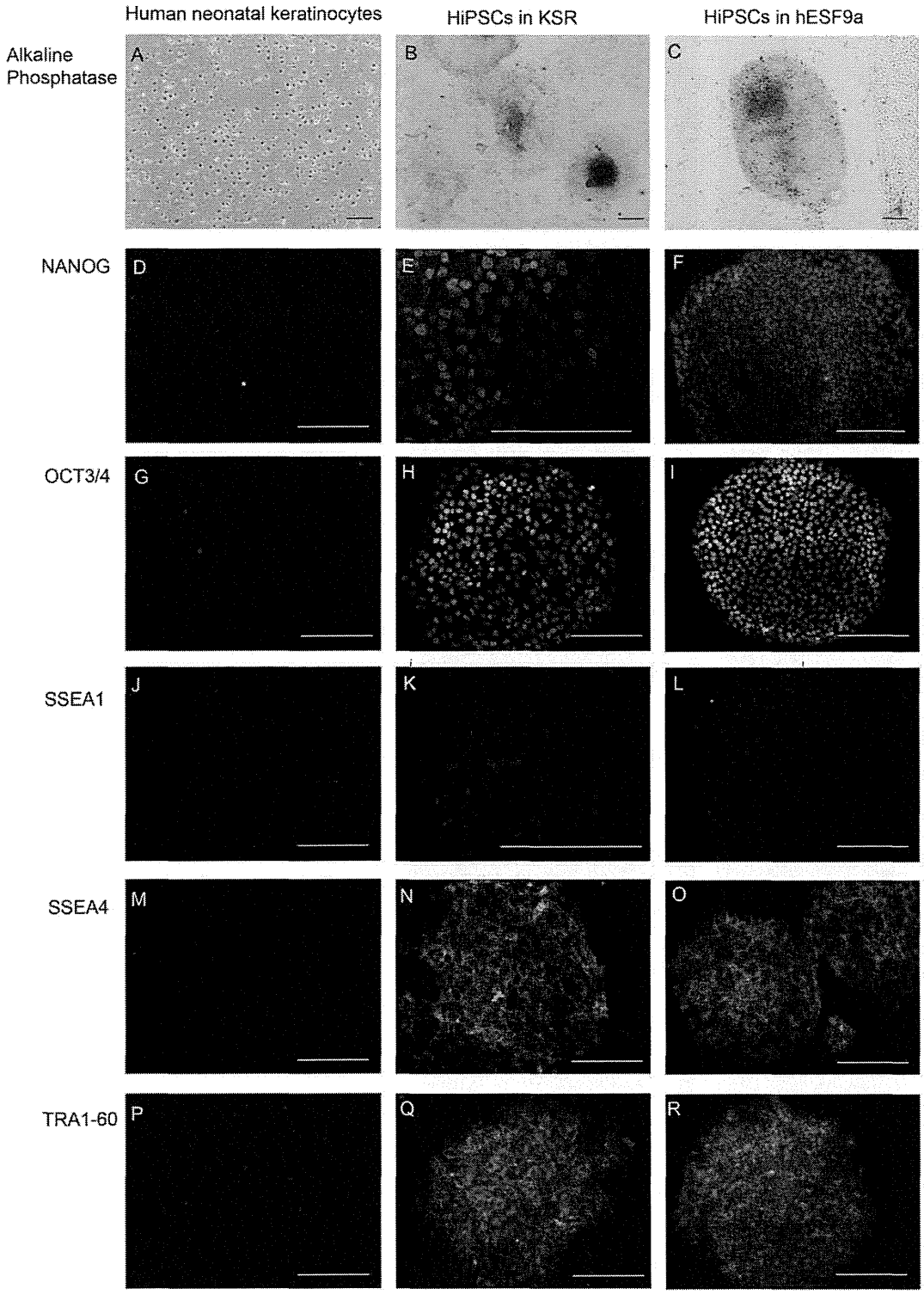

Figure 1. Self-renewal marker expression of pluripotent stem cells in hiPSCs adapted in defined culture conditions. Parental human neonatal keratinocytes (A, D, G, J, M, and P), hiPSC line, UTA1, grown under KSR-based conditions (B, E, H, K, N, and Q), and UTA1 grown under hESF9a-based culture conditions for 5 passages (C, F, I, L, O, and R) were fixed and reacted with antibodies (or stained with alkaline phosphatase substrate, Fast Red). (A–C): Alkaline phosphatase staining. (D–F): Immunocytochemistry of NANOG protein. (G–I): Immunocytochemistry of OCT3/4 protein. (J–L): Immunocytochemistry of SSEA1 antigen. (M–O): Immunocytochemistry of SSEA4 antigen. (P–R): Immunocytochemistry of TRA1-60 antigen. Binding of these antibodies was visualized with AlexaFluor 488-conjugated secondary antibodies (green). Nuclei were stained with DAPI (blue). Scale bars represent 50 μm.
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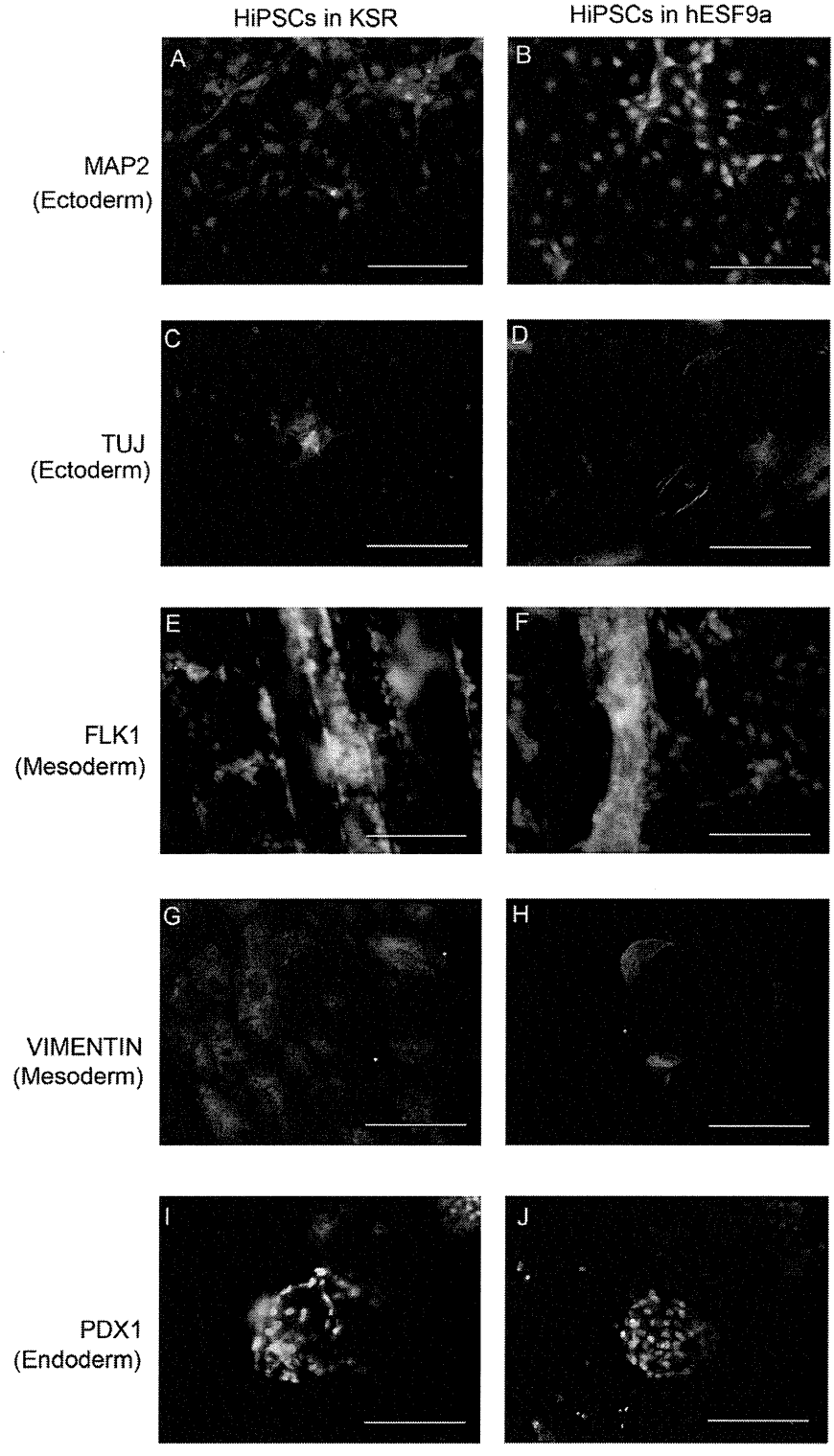

Figure 2. *In vitro* differentiation using embryoid bodies from hiPSCs adapted in defined culture conditions. Immunocytochemistry of MAP2 (A, B), TUJ (C, D), FLK1 (E, F), vimentin (G, H), and PDX1 (I, J) in the differentiated hiPSC line, UTA1, grown under KSR-based conditions (A, C, E, G, I) or hESF9a-based conditions (B, D, F, H, J). Differentiation was performed using embryoid body formation, and the differentiated cells were fixed and reacted with antibodies. Binding of these antibodies was visualized with AlexaFluor 488-conjugated secondary antibodies (green). Nuclei were stained with DAPI (blue). Scale bars represent 50 μm.
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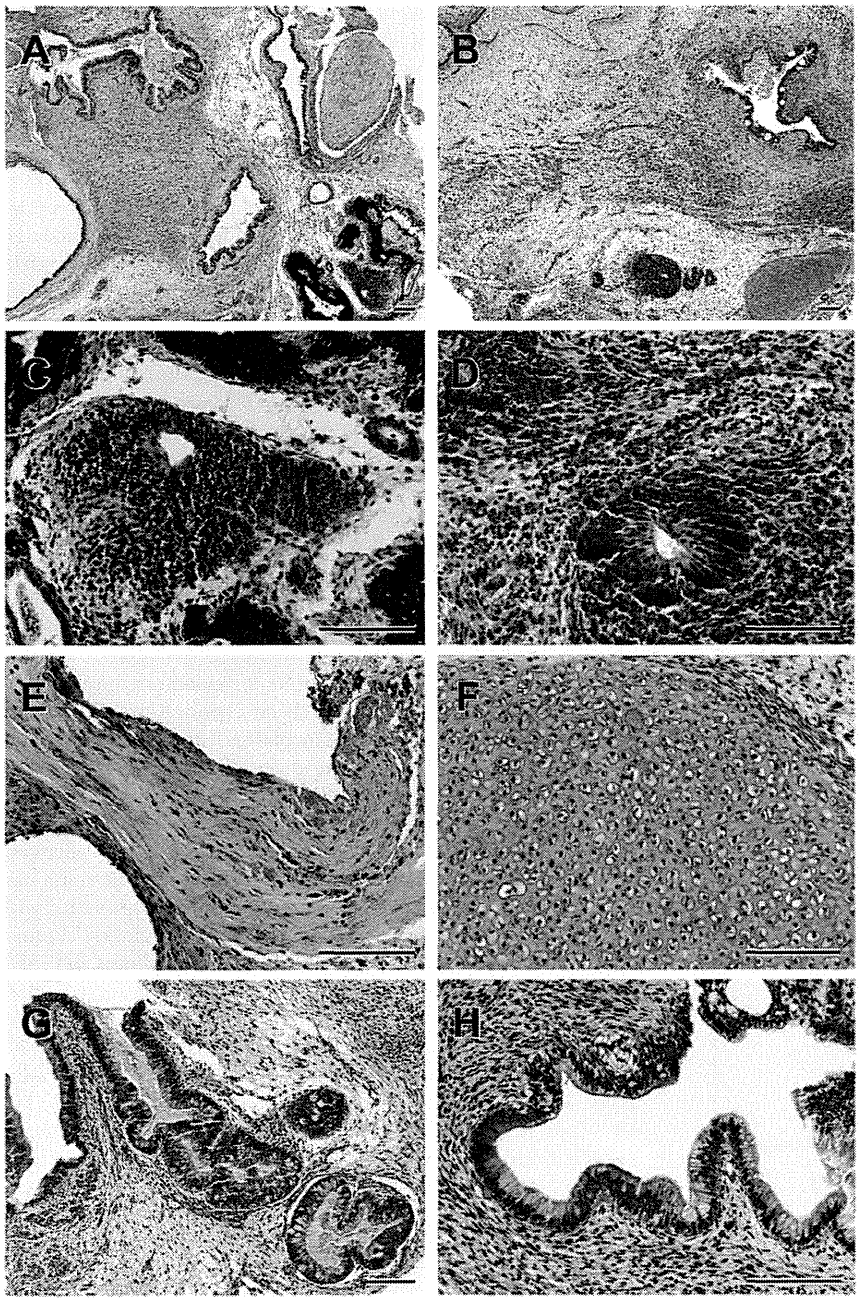

Figure 3. The *in vivo* differentiation using teratoma formation of hiPSCs adapted in defined culture conditions. Teratoma were generated in SCID mice from UTA1 grown under KSR-based and hESF9a-based conditions. Histological analysis with HE staining demonstrated that teratoma formed by the UTA1 cells cultured in both KSR-based (A, C, E, G) and hESF9a-based conditions (B, D, F, H) contained derivatives of all three germ layers. Histology of teratoma derived from UTA1 cultured under KSR-based (A) or hESF9a-based conditions (B). Neural tissues in teratoma derived from UTA1 cultured under KSR-based (C) or hESF9a-based conditions (D). (E): Muscle. (F): Cartilage. (G and H): Intestinal epithelia. Scale bars represent 100 μm.
doi:10.1371/journal.pone.0014099.g003

nents such as MEF, FCS in which the MEFs were cultured, and porcine gelatin. Further, the KSR used for the conventional culture conditions also includes animal-derived components, such as lipid-enriched bovine serum albumin [9,10,11]. Hence, the UTA1 cells might metabolically incorporate substantial amounts of Neu5Gc from these factors. However, although the hESF9a-based conditions also contain animal-derived components such as bovine insulin, bovine serum albumin, porcine heparin, and bovine fibronectin, the UTA1 cells incorporate only low amounts of Neu5Gc. These results suggested that culturing of hiPSCs under the defined conditions with purified components could decrease the risk of xenogenic and human-derived pathogens.

The generation of hiPSC lines using adult dermal fibroblasts under feeder- and serum-free, defined culture conditions from the reprogramming step

Next, we examined whether hiPSCs were generated from the reprogramming step under our feeder-free, defined culture conditions. For comparison, we used another defined xeno-free

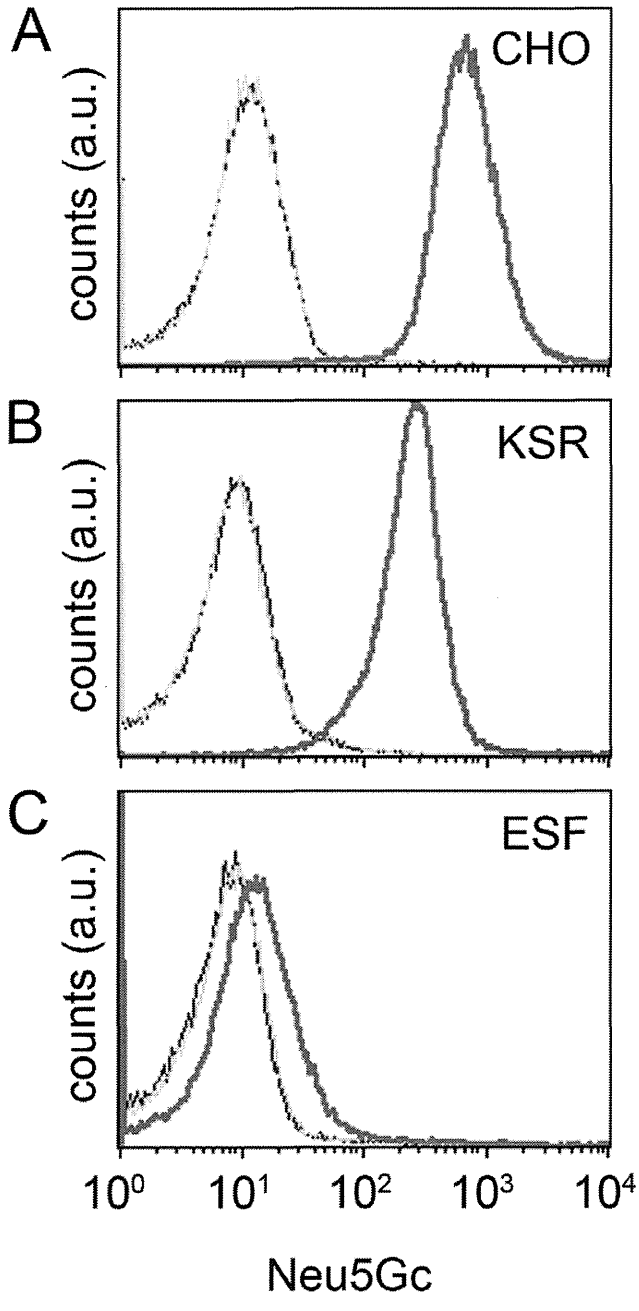

Figure 4. Decreased expression of xenoantigen Neu5Gc in hiPSCs adapted under defined culture conditions. Flow cytometry analysis of Neu5Gc expression. CHO cells were grown in FCS-containing medium (A) and hiPSCs were grown under KSR-based conditions (B) or hESF9a-based conditions (C). The cells were exposed to anti-Neu5Gc antibody (red), control antibody (green), or blocking buffer (blue), and then stained with a secondary antibody for analysis by flow cytometry.
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TeSR2 media, which was modified from mTeSR1 medium [13], for culturing human pluripotent stem cells. Adult human dermal fibroblasts (HDF) were infected with amphotropic retroviruses carrying the *OCT4*, *SOX2*, *KLF4*, and C-*MYC* genes and were cultured under hESF9a-based conditions or under TeSR2-based conditions (with TeSR2 medium on matrigel-coated dishes). After 26 days of culture, we detected hiPSC-like colonies by staining for ALP substrate or by their morphologies (Figure 5A). While no hiPSC-like colonies were detected under the TeSR2-based conditions, eight hiPSC-like colonies were detected under the hESF9a-based condition. We confirmed these results by independent multiple experiments (0 ALP-positive colonies/2 experiments in TeSR2-based conditions and 3 ALP-positive colonies/2 experiments in hESF9a-based conditions) (Figure 5B and C). In another experiment of the hiPSCs induction under hESF9a-based conditions, we picked up hiPS-like colonies for expansion under the same culture conditions. The ALP activity was maintained at 5 passages (Figure 5D). After 25–26 passages, we confirmed self-renewal marker expression and differentiation potential in these cell lines by immunocytochemistry (Figure 6, 7). One of the cell lines, designated UTA-SF2-2, retained proper proliferation rates for human pluripotent stem cells (Figure 8A; population doubling time: 28.2±4.9 h). Karyotype analysis revealed that UTA-SF2-2 cells at passage 31 was 46XX (Figure 8B). Finally, we examined Neu5Gc expression in the UTASF2-2 line by flow cytometry and showed that the levels of Neu5Gc in passage-30 cells were almost negative, as in negative control cells (Figure 9). Our established cell lines therefore showed little or no Neu5Gc contamination, suggesting that the hESF9a-based culture conditions generated hiPSCs steadily from reprogramming step using adult HDF and supported their pluripotency for long-term culture with less contamination of pathogens.

Recently, the generation of induced pluripotent stem cells under xeno-free culture conditions has been reported [19,20]. These culture conditions contain human plasma or xeno-free KSR on irradiated human fibroblasts. However, the components of KSR or xeno-free KSR are not publicly available. For clinical application, all the components used should be traceable and also widely reviewed. Our defined culture methods without feeder cells make it easier to track all the components because the minimum essential components, only five highly purified proteins with heparin, are added into the basal medium. Previously, human iPSCs were also generated using a defined medium, mTeSR1 from not adult but neonatal HDF (ADA) [21] or adult adipose stem cells [22]. Sun *et al.* also reported that no iPS-like colonies were generated from neonatal HDF (IMR90) using mTeSR1 medium [22]. Together with our results that hiPSCs from adult HDF were derived under defined culture conditions for the first time and steadily, our hESF9a medium is suitable to generate hiPSCs from adult HDF. Compared with our hESF9a medium, mTeSR1 and TeSR2 medium contain a variety of chemicals (inorganic chemicals, trace minerals, lipids, surfactants, and amino acid derivatives) that regulate signal transduction and metabolism [13]. Although these chemicals may enhance the self-renewal of human pluripotent stem cells, some may inhibit the reprogramming steps during human iPSC generation.

Conclusions

This study demonstrated that defined culture conditions that were developed for hESCs are also applicable for hiPSCs. The hiPSCs were generated under conventional KSR-based conditions and then adapted to the defined hESF9a-based condition. Moreover, we succeeded to generate the hiPSCs from adult HDF under hESF9a-based conditions. The levels of xenoantigen Neu5Gc were markedly decreased in the hiPSCs which were either adapted or generated in the hESF9a-based condition. The hESF9a media consists of a basal medium and known components, reducing the risk of contamination from undefined pathogens and antigens. Taken together, our findings suggested that the defined culture conditions described herein are suitable for culturing hiPSCs with the added benefit of eliminating contamination risks caused by undefined factors. The defined

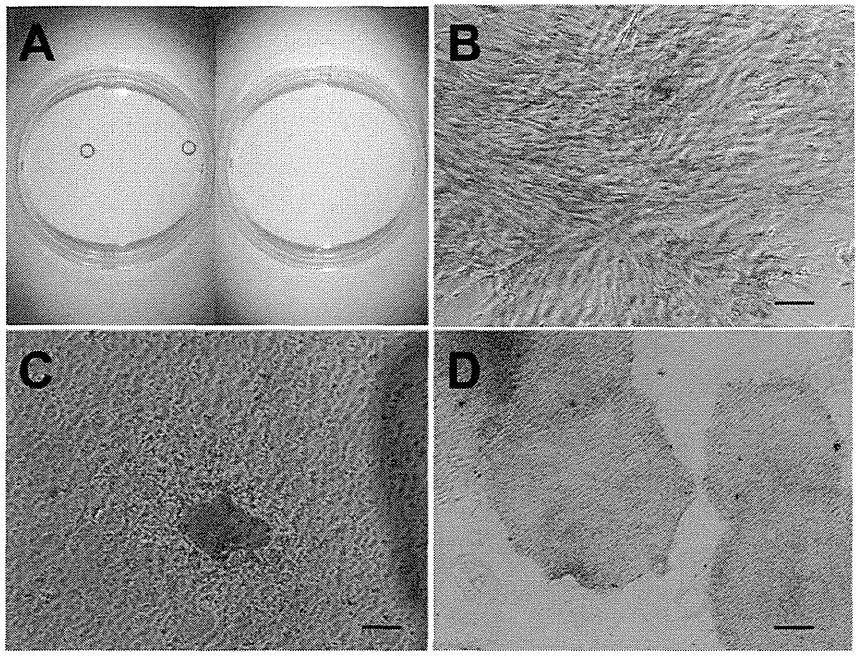

Figure 5. Generation of hiPSC lines under defined culture conditions. (A):ALP staining of HDFs cultured in hESF9a (left) and in TeSR2 (right) at 26 days after virus transduction. Red arrows indicate the ALP-positive hiPSC-like colonies. (B and C): The appearances of transduced HDFs cultured in TeSR2 (B) and in hESF9a (C). Cells were fixed and stained with ALP substrate BM purple at 26 days after virus transduction. (D): ALP staining of the hiPSC-like colony picked up and cultured under hESF9a conditions at five passages. Scale bars represent 50 μm.
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culture condition provides a safer source of hiPSCs for potential clinical applications.

Materials and Methods

hiPSCs induction and cell culture

Human neonatal keratinocytes were purchased from Invitrogen (Carlsbad, CA). An hiPS cell line was generated using the VSV-G-pseudotyped retroviral vector system carrying *OCT4*, *SOX2*, *KLF4*, and *C-MYC* as described previously [18]; the line was designated UTA1. UTA1 cells were maintained in DMEM-F12 medium (Invitrogen) supplemented with 20% KSR (Invitrogen), 0.1 mM 2-mercaptoethanol (Sigma, St. Louis, MO), MEM non-essential amino acids (Invitrogen), and 5–10 ng/mL recombinant human basic FGF (Peprotech, Rocky Hill, NJ) on mitomycin C-treated mouse embryo fibroblast feeder cells. For subculturing, the cells were detached from the culture dish using CTK medium [17]. The mouse embryonic fibroblasts were cultured in fibroblast medium (DMEM medium supplemented with 10% fetal calf serum, 1% penicillin, and 1% streptomycin) on gelatin-coated dishes.

UTA1 cells at passage number 18 were transferred into a defined feeder- and serum-free culture condition, the hESF9a-based condition (with hESF9a medium and fibronectin coat), and passaged a further 5 times at least before assaying. The hESF9a medium comprises hESF-Grow medium (Cell Science & Technology Institute, Miyagi, Japan) supplemented with 10 μg/mL of bovine pancreas insulin (Sigma I-5500), 5 μg/mL human apotransferrin (Sigma T-1147), 10 μM 2-mercaptoethanol (Sigma M-7522), 10 μM ethanolamine (Sigma E-0135), 20 nM sodium selenite (Sigma S-9133), 4.7 μg/mL of oleic acid conjugated with 0.5 mg/mL of fraction V fatty acid-free bovine serum albumin (Sigma O-3008), 100 ng/mL L-ascorbic acid-2-phosphate (Wako, Osaka, Japan, 013-196411), 100 ng/mL heparin sodium salt from porcine intestinal mucosa (Sigma H-3149), 10 ng/mL human recombinant fibroblast growth factor 2 (FGF-2, Peprotech 100-18B), and 10 ng/mL human recombinant activin A (Ajinomoto Pharmaceuticals, Japan) [12,16]. The culture dishes were coated with 2 μg/cm^2 fibronectin from bovine plasma (Sigma F-1141) in PBS for at least 30 min at 37°C, and then excess solution was removed. For subculturing, the cells were detached from the culture dish using 50~300 μg/mL dispase (Invitrogen 17105-041) in hESF9a medium and replated in hESF9a medium with 5 μM ROCK inhibitor (Y-27632; Wako Pure Chemical Industries, Ltd., Japan). Medium changes were made every day with hESF9a medium.

Chinese hamster ovary (CHO) cells (No. 85050302, European Collection of Cell Culture) were maintained in DMEM-F12 (Invitrogen) supplemented with 10% fetal calf serum, 1% penicillin, and 1% streptomycin.

Generation and maintenance of hiPSCs in feeder- and serum-free defined culture condition from reprogramming step

Four hiPSC lines, UTA-SF2-1, UTA-SF2-2, UTA-SF3-1, and UTA-SF3-2, were generated from primary adult human dermal fibroblasts (female of 41, 46, or 51 yr old; 106-05a, cell applications, inc. San Diego, CA) under hESF9a-based conditions. The HDFs were seeded at 8×10^5 cells/dish in 10-cm petri dishes coated with gelatin in the fibroblast medium. The VSV-G-pseudotyped retroviral vector system carrying OCT4, SOX2, KLF4, and C-MYC was added to HDF cultures. After two 24-hour-exposures of virus, the transduced HDFs were replated at 6×10^4 cells/dish onto 10-cm petri dishes coated with fibronectin or matrigel in the fibroblast medium. On the next day, the medium was changed to hESF9a (on fibronectin-coated dishes) or TeSR2 (on matrigel-coated dishes), and thereafter changed daily.

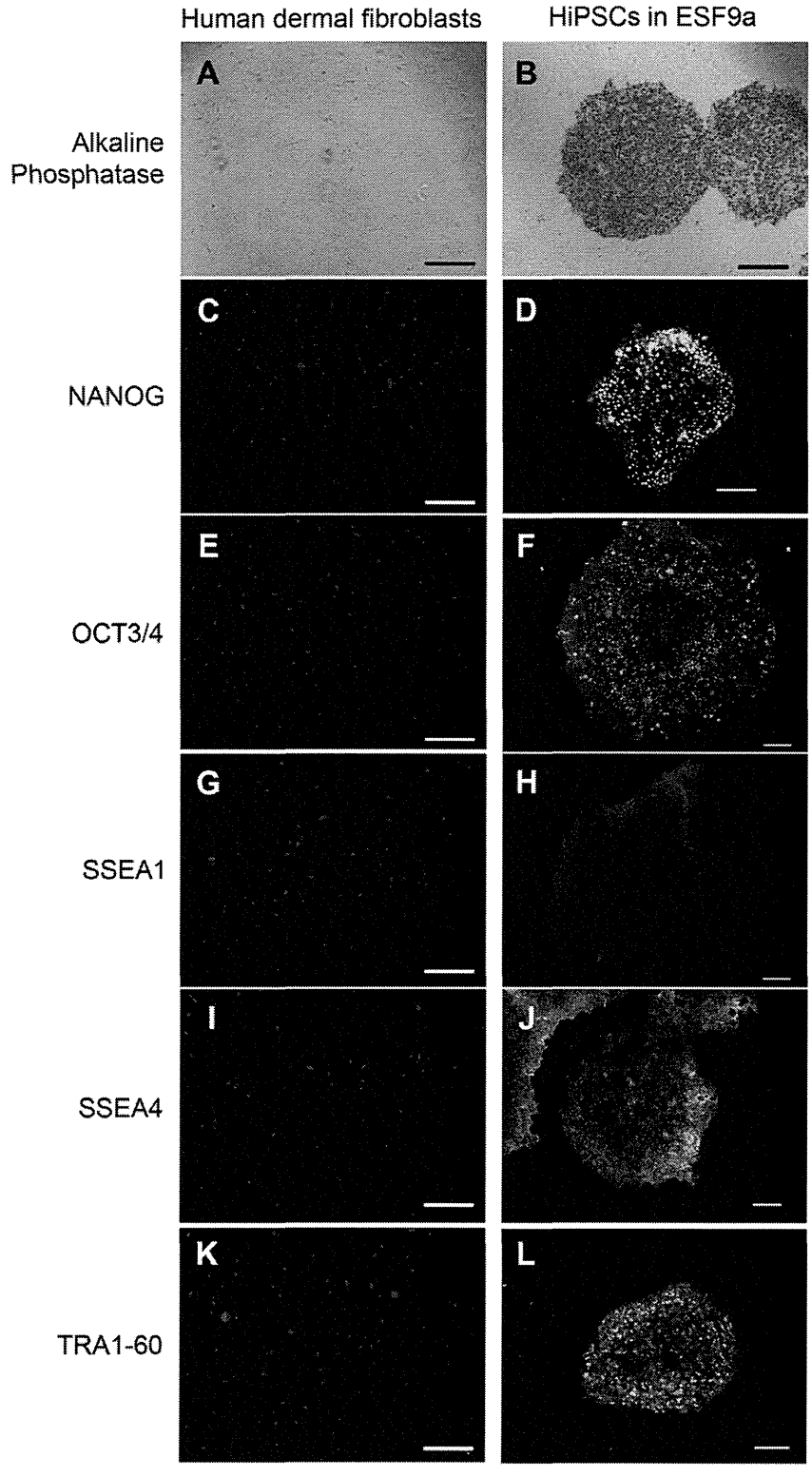

Figure 6. Self-renewal marker expression of pluripotent stem cells in hiPSCs generated and maintained in defined culture conditions. Parental human dermal fibroblasts (A, C, E, G, I, and K), and hiPSC line, SF2-2 generated and maintained under the ESF9-based conditions (B, D, F, H, J, and L) were fixed and reacted with antibodies (or stained with alkaline phosphatase substrate, BM Purple). (A and B): Alkaline phosphatase staining. (C and D): Immunocytochemistry of NANOG protein. (E and F): Immunocytochemistry of OCT3/4 protein. (G and H): Immunocytochemistry of SSEA1 antigen. (I and J): Immunocytochemistry of SSEA4 antigen. (K and L): Immunocytochemistry of TRA1-60 antigen. Binding of these antibodies was visualized with Alexafluor 488-conjugated secondary antibodies (green). Nuclei were stained with DAPI (blue). Scale bars are 50 μm.
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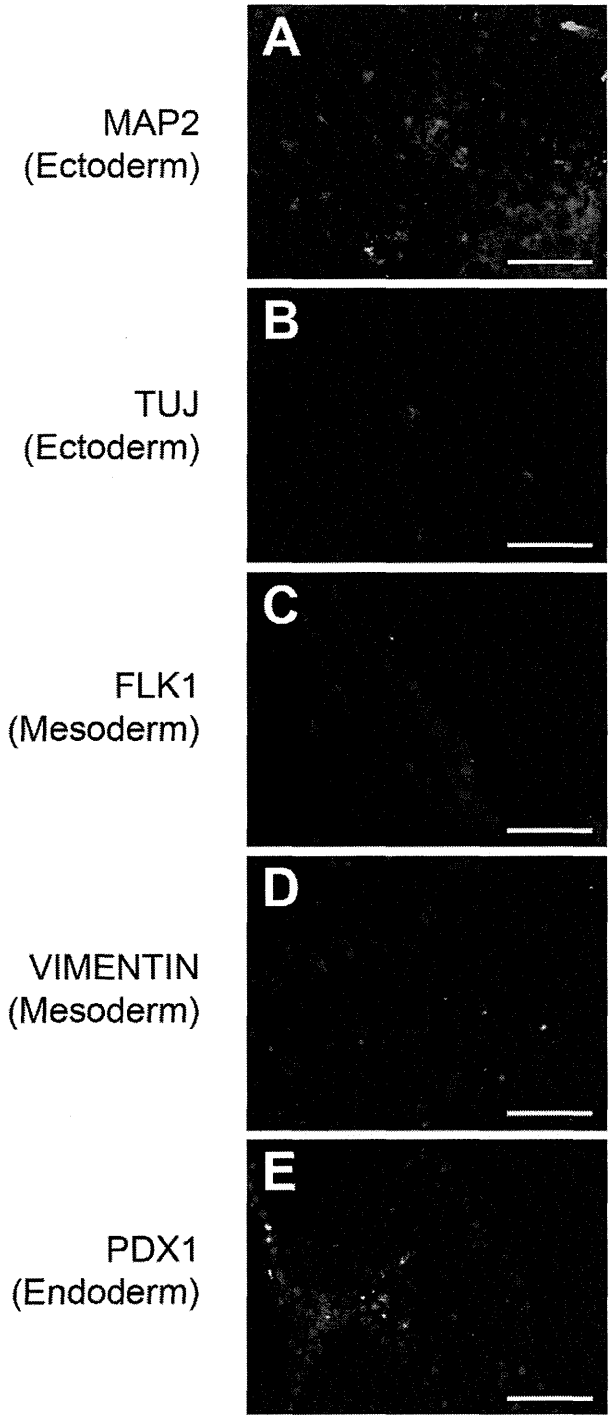

Figure 7. *In vitro* differentiation using embryoid bodies from hiPSCs generated and maintained in defined culture conditions. Immunohistochemistry of MAP2 (A), TUJ (B), FLK1 (C), VIMENTIN (D), and PDX1 (E) in the differentiated hiPSC line, UTA-SF-2-2, grown under hESF9a-based conditions. Differentiation was performed using embryoid body formation. The tissues in embryoid bodies were fixed and reacted with antibodies. Binding of these antibodies was visualized with AlexaFluor 488-conjugated secondary antibodies (green). Nuclei were stained with DAPI (blue). Scale bars are 50 μm.
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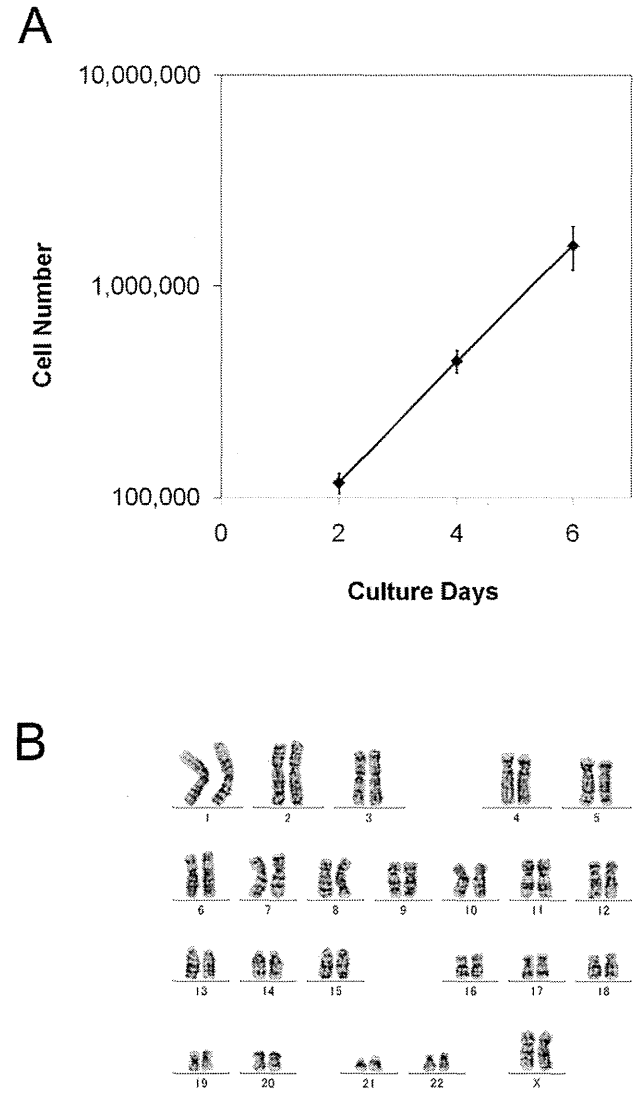

Figure 8. Cell growth and karyotype of hiPSCs generated and maintained in defined culture conditions. (A): Growth curves for the hiPSC line. UTA-SF2-2 hiPSCs cultured under hESF9a-based conditions at passages 29, 30, and 31 were seeded in a 6-well plate coated with fibronectin and counted every 48 h. The values are the mean ± SEM (n = 3). (B): Representative G-banded karyotype of chromosome in a UTASF2-2 hiPSC at passage 31 maintained under hESF91-based conditions.
doi:10.1371/journal.pone.0014099.g008

At around 30 days after transduction, hiPSC-like colonies were picked up and cultured in the same defined culture conditions as described for the hiPSC UTA1 cell lines.

Alkaline phosphatase (ALP) staining

Cells were fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) at room temperature for 10 minutes. The fixed cells were washed with PBS three times and then twice with alkaline phosphatase (ALP) buffer. Finally, these samples were stained with ALP substrate Fast-Red (Nichirei, Japan) or BM purple (Roche, Switzerland) for 30 minutes at room temperature.

Embryoid body formation

In vitro differentiation was induced by the formation of embryoid bodies as described previously [12]. Briefly, undifferentiated

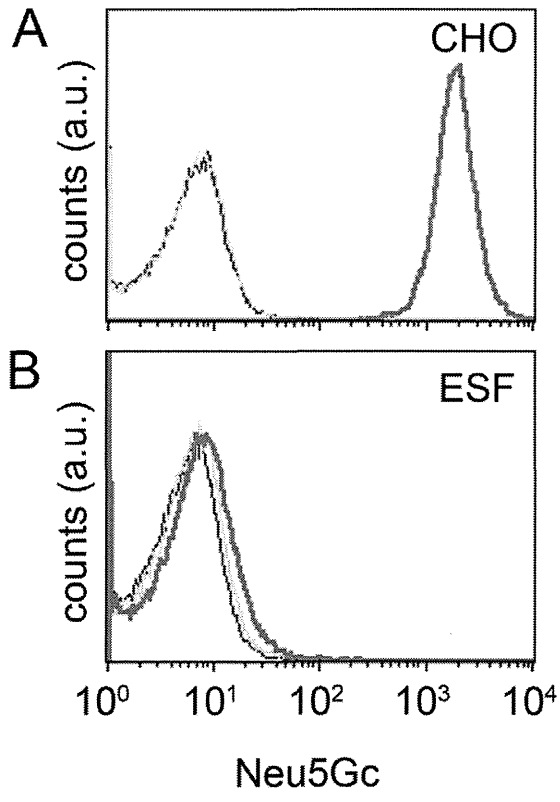

Figure 9. Flow cytometry analysis of xenoantigen Neu5Gc expression in hiPSCs generated and maintained in defined culture conditions. CHO cells grown in FCS-containing medium (A) and UTA-SF2-2 hiPSCs established under hESF9a-based conditions (B) were exposed to anti-Neu5Gc (red) or control antibody (green), or blocking buffer (blue). Then stained with a secondary antibody for analysis by flow cytometry.
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hiPSCs were cultured in DMEM with 10% FCS for 8 days in low-attachment plates (Corning, Corning, NY). Then, floating embryoid bodies were replated onto gelatin-coated dishes in the same culture medium for 16 days.

Immunocytochemistry

Immunocytochemistry was performed as described previously [23,24,25]. Briefly, hiPSCs were fixed in 4% PFA, permeabilized with 0.1% Triton X-100, blocked with 1% BSA, and then reacted with primary antibodies. The primary antibody binding was visualized with AlexaFluor 488-conjugated anti-rabbit, anti-mouse, and anti-goat IgG or AlexaFluor 594-conjugated donkey anti-mouse, anti-rabbit, or anti-goat IgG (Invitrogen). The following primary antibodies were used: anti-FLK1 antibody (Chemicon, Billerica, MA; 1:100), anti-MAP2 antibody (Chemicon; 1:200), anti-PDX1 antibody (Chemicon; 1:100), anti-NANOG antibody (Reprocell, Tokyo, Japan; 1:200), anti-OCT3/4 antibody (Santa Cruz Biotechnology, Santa Cruz, CA 1:100), anti-SSEA1 antibody (Kyowa, Tokyo, Japan; 1:100), anti-SSEA4 antibody (eBiosciences, San Diego, CA; 1:100), anti-TUJ antibody (Chemicon; 1:100), and anti-vimentin antibody (Santa Cruz Biotechnology; 1:200).

Teratoma formation assay

For teratoma formation assays, approximately 3 million hiPSCs were suspended in 60 μL of PBS and injected into the testes of anesthetized severe combined immunodeficient (SCID) mice. The tumors were excised 8 weeks after injection, fixed in 4% PFA, embedded in paraffin, and then sectioned at 8 μm. The histology of formed teratomas was analyzed using hematoxylin-eosin (HE) staining. The Institutional Animal Care and Use Committee of the Institute of Medical Science, University of Tokyo approved the use of experimental animals (the permit number was PA09-4).

Flow cytometry

Flow cytometry for Neu5Gc was performed as described previously [7]. Briefly, all cells were removed from culture dishes using 0.02% (w/v) EDTA-4Na in PBS. The hiPSCs cultured under KSR-based conditions were then replated on plastic dishes and incubated for 1 hour to remove the MEFs, and then 0.5–2.5×10^6 cells were incubated with a chicken anti-Neu5Gc (Gc-Free, 1:100 dilution) antibody or a control antibody (Gc-Free, 1:100 dilution) in PBS containing blocking buffer (Gc-Free), but without Neu5Gc. The cells were finally incubated with a donkey anti-chicken IgY secondary antibody conjugated to Cy5 (Jackson; diluted 1:500 in PBS containing blocking buffer). A FACS-Calibur (BD) was used for data acquisition.

Supporting Information

Figure S1 Cell growth of hiPSCs cultured under defined culture conditions. Growth curves for the hiPSC line, UTA1, cultured under KSR-based or hESF9a-based conditions. Growth curves were calculated from each passage split ratio. The relative cell number was set as 1 when the hiPSCs were cultured at passage 18 for conventional feeder conditions or at passage 5 for defined culture conditions.
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Author Contributions

Conceived and designed the experiments: YH TC MKF TM KO MA. Performed the experiments: YH TC MW MF TA NT MO MKF KO. Analyzed the data: YH TC TA KO MKF TM KO. Contributed reagents/materials/analysis tools: MW MF KO KE HN. Wrote the paper: YH TC MKF TM KO MA.

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