Kohji Masuda, Ren Koda, Nobuyuki Watarai, Ryusuke Nakamoto, Yoshitaka Miyamoto and Toshio Chiba	Attempt for active control of microbubbles in blood flow by forming local acoustic field	Proc. of 9th Congress of Asian Federation of Societies for Ultrasound in Medicine and Biology	2010	90	2010
Kohji Masuda, Nobuyuki Watarai, Ryusuke Nakamoto, Yoshitaka Miyamoto, Keri Kim and Toshio Chiba	Study to prevent the Density of Microcapsules from diffusing in Blood Vessel by Local Acoustic Radiation Force	Proc. of 32nd Annual International Conference of the IEEE Engineering in Medicine and Biology Society (EMBS)	2010	402-405	2010
桝田晃司, 中元隆介, 江田廉, 渡會展之, 宮本義孝, 千葉敏雄	凝集体形成を利用した微小気泡の生体内超音波制御法の検討	日本超音波医学会第22回関東甲信越地方会学術集会抄録集	2010	60	2010
渡會展之, 江田廉, 中元隆介, 桝田晃司, 宮本義孝, 千葉敏雄	血管分岐部での凝集体形成によるマイクロカプセルの流路選択性向上のための実験的検討	日本超音波医学会 第83回学術集会論文集	37	S338	2010
Yazawa T, Kawabe S, Inaoka Y, Okada R, Mizutani T, Imamichi Y, Ju Y, Yamazaki Y, Usami Y, Kuribayashi M, Umezawa A, Miyamoto K.	Differentiation of mesenchymal stem cells and embryonic stem cells into steroidogenic cells using steroidogenic factor-1 and liver receptor homolog-1.	Mol Cell Endocrinol	336 (1-2)	127-132	2011
Inamura M, Kawabata K, Takayama K, Tashiro K, Sakurai F, Katayama K, Toyoda M, Akutsu H, Miyagawa Y, Okita H, Kiyokawa N, Umezawa A, Hayakawa T, Furue MK, Mizuguchi H.	Efficient generation of hepatoblasts from human ES cells and iPS cells by transient overexpression of homeobox gene HEX.	Mol Ther	19 (2)	400-407	2011
Okamoto A., Tachibana R., Yoshinaka K., Takagi S., Kataoka K., Matsumoto Y	A study of micro-bubble enhanced sonoporation	10th International Symposium on Therapeutic Ultrasound (AIP Conference Proceedings)			In press (2011)

D2-4

Minimally invasive fetal gene therapy using nonviral vector by ultrasound irradiation.

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Recent advances in prenatal diagnosis of genetic disease, fetoscopic technique, and fetal transplantation no longer pose significant technical hurdles for in utero clinical tests and have widened the door for minimally invasive delivery of therapeutic agents using ultrasound energy. In treating inborn errors of metabolism, two possible strategies, graft treatment and gene therapy, should be considered at the moment. Both procedures, however, have major disadvantages; relative lack of graft donors and inadvertent side effects associated with the use of virus vectors. Furthermore, if we miss the most effective timing of these procedures, an expensive and long-term treatment is likely to follow. Hence, we aim, in this research, at gene therapy using nonviral vector based on ultrasonic microbubble cavitations for inborn errors of metabolism treatment.

In our experiment, "Sonitron" gene transfection system (NEPAGENE Co.) was used for intracellular gene introduction in the liver. A test solution (5μl) consisting of nonviral vector (pEGF plasmid of 5 μgrms), microbubbles (25% in concentration) and saline solution was prepared. Then, laparotomy was performed on pregnant mice to inject the fetal liver with the solution. Immediately after, the injected microbubbles were destroyed by ultrasound irradiation creating microjet stream which allowed the target gene to be introduced intracellularly. Twenty-four hours later, the fetal liver was excised and the hepatic GFP expression was assessed using fluorescent microscope. In our experiment, the GFP fluorescence was definitely demonstrated in the fetal liver. On the other hand, the liver that had undergone an injection of the solution only without additional ultrasonic irradiation did not show any GFP expression. This outcome hopefully validates our goal that fetal gene transfection could be successfully achieved when ultrasound energy was co-irradiated.

Our preliminary results suggest that an assessment of the system feasibility might be the next step using fetal mice having inborn errors of metabolism.

D2-5

Time-reversal techniques in ultrasound-assisted convection-enhanced drug delivery to the brain: technology development and in vivo evaluation

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We describe a drug delivery method that combines time-reversal acoustics (TRA) with convection-enhanced delivery (CED) to improve the delivery of chemotherapeutics. CED has been used to treat a variety of neural disorders, including glioblastoma multiform, a malignancy that presents a very poor prognosis for patients. Preclinical and clinical trials have shown that the technical challenges in CED are to increase the penetration of drug into the tissue surrounding the cannula and control the spatial distribution of infused compounds.

Using a novel transducer-cannula assembly we infuse fluids into the rodent brain while simultaneously exposing the tissue to safe level of 1-MHz, low intensity, plane-wave ultrasound. Results show that exposure of the brain to ultrasound increases the distribution volume of infused compounds by 4-8x without significant damage to the treated tissue. Ultrasound-assisted CED (UCED) is believed to increase the tissue permeability, reduce mass-transfer resistance at the cannula-tissue interface, and enhance convection.

Although plane-wave UCED increases the penetration of tracers into tissues, it does little to control the spatial distribution of the infused material. To provide necessary spatial control, we are developing a system to implement time-reversal acoustics (TRA) in CED. The system includes a combined infusion needle-hydrophone, a 10-channel ultralow-output impedance amplifier, a broad-band ultrasound resonator, and MatLab-based TRA control and user interface. The tip of the infusion needle acts as a TRA beacon which allows accurate focusing of the ultrasound through the skull to the site where the drug is injected without complex phase-correction and array design. The smart targeting UCED system has been tested in vitro with brain-mimicking phantoms providing 1-mm spatial resolution. Results involving the use of the system in vivo will be described.

P2-29

Ultrasound -assisted gene transfer to adipose tissue-derived stem/progenitor cells (ASCs)

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In recent years, multilineage adipose tissue-derived stem cells (ASCs) have become increasingly expected as a promising source for cell transplantation as well as regenerative medicine. It is especially becoming likely to make tissue stem cells such as ASCs and marrow stromal cells (MSCs) differentiate by gene transfection. Gene transfection using highly efficient viral vectors such as adeno- and Sendai viruses have been preferably adopted for this purpose. Meanwhile, sonoporation, a sort of ultrasound (US) -assisted gene transfer is a gene manipulation technique which employs creation of jet stream by ultrasonic microbubble cavitation. The sonoporation using non-viral vectors has been expected much safer, although less efficient, tool for prospective clinical gene therapy. In this report, we assessed the efficacy of sonoporation technique for gene transfer to ASCs.
We isolated and cultured adipocyets from mouse adipose tissue. ASCs that potentially differentiate with transformation to adipocytes or osteoblasts were obtained. Using the US -assisted system, plasmid DNA containing beta-galactosidase (beta-Gal) and green fluorescent protein (GFP) genes were transferred to the ASCs. For this purpose, Sonitron 4000 (NEPAGENE Co.) and Sonazoid (Daiichi Sankyo Co.) were used in combination. ASCs were irradiated with US (3.1 MHz, duty 50%, burst rate 2.0 Hz, duration 15-120 sec.) of different intensities (approximately 1.2 W/cm2). The gene was more efficiently transferred with increased concentrations of plasmid DNA (5-100 μg/mL). However, further optimization of the US parameters should be required as the gene transfer efficiency was not high enough. In conclusion, it was shown that gene could be transferred to ASCs using our US - assisted system. In regenerative medicine, this system might hopefully work better replacing current procedures in terms of safety, but not efficiency.

P2-30

Ultrasound lithotripsy for pet kidney stones

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It has been investigated that cavitating microbubbles can erode kidney stones. In this study, collapse of cloud cavitation by High Intensity Focused Ultrasound (HIFU) is used to fragment kidney stones. Cloud cavitation is potentially the most destructive form of cavitation. When the cloud cavitation is acoustically forced into a collapse, it has the potential to concentrate a very high pressure.
For the control of the cloud cavitation collapse, a two-frequency wave was used; an ultrasound pulse at a frequency of 3.4 MHz to create the cloud cavitation and a trailing pulse at a frequency of 1.1 MHz following the high-frequency pulse to force the cloud into collapse. An air-backed ultrasound transducer consisting of a spherical PZT ceramic element was placed in a water tank and the two-frequency ultrasound was focused to struvite stones extracted from pets. Both diameter and focal length of the PZT element were 72 mm. The duration time of the high-frequency and low-frequency ultrasound was 30 us and 5 us, respectively. The pulse repetition frequency of the two-frequency ultrasound was 20 Hz. The stones were dipped in degassed water more than 24 hours before the experiment.
The struvite stones were eroded and most of the resulting fragments were much less than 1 mm in diameter. The method has the potential to provide a novel lithotripsy system with small fragments which would be applicable to pets as well as human.

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Differentiation of mesenchymal stem cells and embryonic stem cells into steroidogenic cells using steroidogenic factor-1 and liver receptor homolog-1☆

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ABSTRACT

Previously, we have demonstrated that mesenchymal stem cells could be differentiated into steroidogenic cells through steroidogenic factor-1 and 8bromo-cAMP treatment. Use of liver receptor homolog-1, another of the nuclear receptor 5A family nuclear receptors, with 8bromo-cAMP also resulted in the differentiation of human mesenchymal stem cells into steroid hormone-producing cells. The same approaches could not be applied to other undifferentiated cells such as embryonic stem cells or embryonal carcinoma cells, because the over-expression of the nuclear receptor 5A family is cytotoxic to these cells. We established embryonic stem cells carrying tetracycline-regulated steroidogenic factor-1 gene at the *ROSA26* locus. The embryonic stem cells were first differentiated into a mesenchymal cell lineage by culturing on collagen IV-coated dishes and treating with pulse exposures of retinoic acid before expression of steroidogenic factor-1. Although the untreated embryonic stem cells could not be converted into steroidogenic cells by expression of steroidogenic factor-1 in the absence of leukemia inhibitory factor due to inability of the cells to survive, the differentiated cells could be successfully converted into steroidogenic cells when expression of steroidogenic factor-1 was induced. They exhibited characteristics of adrenocortical-like cells and produced a large amount of corticosterone. These results indicated that pluripotent stem cells could be differentiated into steroidogenic cells by the nuclear receptor 5A family of protein *via* the mesenchymal cell lineage. This approach may provide a source of cells for future gene therapy for diseases caused by steroidogenesis deficiencies.

1. Introduction

Steroidogenic factor-1 (SF-1), also known as Ad4BP, and liver receptor homolog-1 (LRH-1) belong to the NR5A subfamily of nuclear receptors (Krylova et al., 2005). SF-1 is essential for adrenal and gonadal development, and SF-1 knockout mice exhibit adrenal and gonadal agenesis and impaired gonadotropin expression, resulting in postnatal death due to severe adrenal insufficiency (Luo et al., 1994; Sadovsky et al., 1995). SF-1 is expressed in the adrenal cortex, testicular Leydig and Sertoli cells, ovarian theca and granulosa cells, pituitary gonadotropes and hypothalamus (Parker and Schimmer, 1997; Morohashi, 1999; Schimmer and White, 2010). It regulates the cell-specific expression of a variety of different genes involved in steroidogenesis, including a number of steroid hydroxylases (Lala et al., 1992; Morohashi et al., 1992). With the aid of cAMP, it can induce the differentiation of bone marrow-derived mesenchymal stem cells (MSCs) into steroidogenic cells such as testicular Leydig cells and adrenocortical cells (Yazawa et al., 2006, 2008). However, the same approaches were inappropriate for other undifferentiated cells such as embryonic stem (ES) cells or embryonal carcinomas, because they barely survived after expression of SF-1. LRH-1 is mainly expressed in tissues of endodermal origin in adults (Fayard et al., 2004; Lee and Moore, 2008). Recently, elevated expression of LRH-1 has been demonstrated in gonads, suggesting the involvement of LRH-1 in steroidogenesis (Volle et al., 2007; Duggavathi et al., 2008).

In this study, we showed the differentiation of steroidogenic cells from MSCs and ES cells by SF-1 and LRH-1. Treatment with LRH-1 and 8br-cAMP resulted in the differentiation of human MSCs (hMSCs) into steroidogenic cells, with similar results exhibited using SF-1. This method could not be applied to ES cells. In this

Abbreviations: SF-1, steroidogenic factor-1; LRH-1, liver receptor homolog-1; MSC, mesenchymal stem cell; ES cells, embryonic stem cells; Tc, tetracycline; RA, retinoic acid; Gapdh, glyceraldehyde-3-phosphate dehydrogenase.

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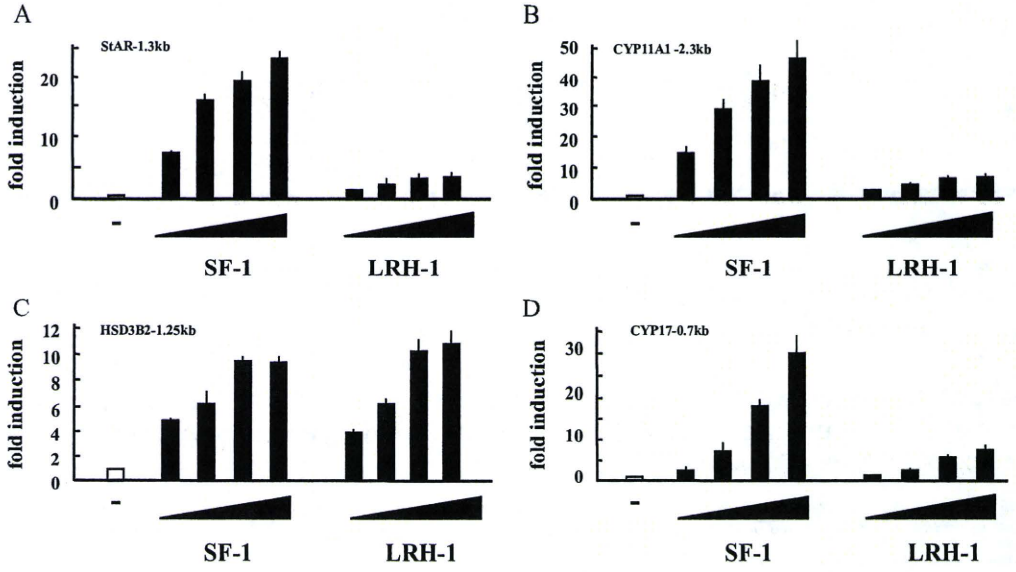


Fig. 1. Activation of the promoter activities of steroidogenic genes by SF-1 and LRH-1 (0, 1.25, 2.5, 5 ng). HEK293 cells were transiently transfected with each reporter and with 0, 1.25, 2.5, 5 ng of expression vector as indicated. Luciferase activities were measured after 48 h and relative activities are shown. Data are expressed as the mean ± SEM of at least four independent experiments.

study we developed a method for differentiation of ES cells into steroidogenic cells using a tetracycline (Tc)-regulated gene expression system for SF-1 (Masui et al., 2005).

2. Materials and methods

2.1. Cell culture, transfection and luciferase assays

The hMSCs (Okamoto et al., 2002; Mori et al., 2005), HEK293 and Phoenix cells were cultured in DMEM with 10% fetal calf serum (FCS). HEK293 cells were transfected using Lipofectamine plus (Invitrogen, Carlsbad, CA, USA). Luciferase assays were performed as described previously (Yazawa et al., 2003). Each data point represents the mean of at least four independent experiments. The murine ES cells, EBRTcH3, were cultured as described before (Masui et al., 2005). Briefly, they were cultured in the absence of feeder cells in Glasgow minimal essential medium (GMEM; Sigma–Aldrich; St. Louis, MO, USA) supplemented with 10% FCS, 1 mM sodium pyruvate (Invitrogen), 10^{-4} M 2-mercaptoethanol (Nacalai Tesque; Kyoto, Japan), 1× nonessential amino acids (Invitrogen) and 1000 U/ml of leukemia inhibitory factor (LIF) on gelatin-coated dishes. Culture methods for the induction of MSCs have been described elsewhere (Takashima et al., 2007). Culture media were collected for the measurement of steroid hormone production by enzyme-linked immunosorbent (ELISA) assays (Cayman Chemical Co., Ann Arbor, MI, USA) as described previously (Yazawa et al., 2008, 2009, 2010).

2.2. Plasmids

The pGL2-StAR1.3kb vector was kindly provided by Dr. Teruo Sugawara (Hokkaido University Graduate School of Medicine, Sapporo, Japan). To introduce the *SF-1* gene into *ROSA-TET* locus, the exchange vector was created by the insertion of the *XhoI-NotI* fragment of human SF-1 cDNA into pPthC (Masui et al., 2005), which had been cleaved by *XhoI–NotI*. Exchange vector is necessary for introduction of genes of interest into mouse genomic *ROSA-TET* locus by homologous recombination. The pCAGGS-Cre plasmid has been described elsewhere (Araki et al., 1997). Each vector containing the entire coding region for SF-1 and LRH-1 was generated by RT-PCR and subcloned into pQCXIP (Clontech, Palo Alto, CA, supplied by Takara Bio Inc., Shiga, Japan). The other vectors have been described before (Yazawa et al., 2009, 2010).

2.3. RT-PCR

Total RNA from the cultured cells was extracted using Trizol reagent (Invitrogen). RT-PCR was performed as described previously (Yazawa et al., 2006). The RT-PCR products were subjected to electrophoresis on 1.5% (w/v) agarose gels, and the resulting bands were visualized by staining with ethidium bromide. The primers used have been described previously (Yazawa et al., 2006, 2009; Takashima et al., 2007).

2.4. Retrovirus preparation and infection

The Phoenix packaging cell line was transiently transfected with the retroviral plasmids using the Lipofectamine Plus reagent (Invitrogen). The supernatant was concentrated by centrifugation and the virus solution stored at −80 °C until required. MSCs were infected with the retrovirus in the presence of 8 μg/ml polybrene (Sigma) for 48 h. The cells were then replated and selected using puromycin.

2.5. Exchange reaction of the Tc-regulated unit

EBRTcH3 cells were seeded onto gelatin-coated 6-well plates in medium containing 1 μg/ml Tc (Tc^+ medium; Sigma–Aldrich). The circular plasmid DNA of the exchange vector, pCAGGS-Cre and Lipofectamine 2000 (Invitrogen) was separately mixed with GMEM and combined to make the transfection mixture. This was added and incubated for 3–5 h, and then re-plated onto 10 cm dishes containing Tc^+ medium. After two days, the medium was changed to Tc^+ medium with 1.5 μg/ml puromycin (Sigma).

2.6. Western blot analysis

The extraction of protein from cultured cells and subsequent quantification was performed as described previously (Yazawa et al., 2003, 2008). Equal amounts of protein (50 μg) were analyzed by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. Western blot analyses of SF-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were carried out with antiserum directed against Ad4BP (kindly provided by Dr. Ken-ichirou Morohashi, University of Kyushu, Fukuoka, Japan) and GAPDH (6C5; Santa Cruz Biotechnology, Santa Cruz, CA). ECL Western blot reagents (Amersham Pharmacia Biotech, Piscataway, NJ) were used to visualize protein bands.

3. Results

3.1. Differentiation of MSCs into steroidogenic cells using SF-1 and LRH-1

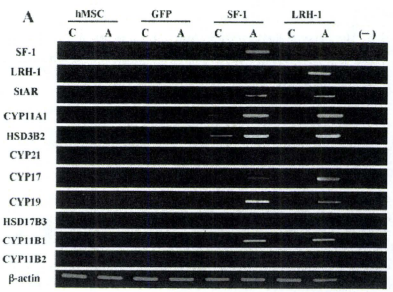
We investigated the effects of SF-1 and LRH-1 on activation of steroidogenesis-related gene promoters in HEK293 cells (Fig. 1). Consistent with many previous reports, SF-1 activated the promoters of steroidogenesis-related genes, such as StAR, CYP11A1, HSD3B2 and CYP17, in a dose-dependent manner. LRH-1 could also activate these promoters, although the extent was much lower than that of SF-1 except in the case of HSD3B2.

To examine abilities of SF-1 and LRH-1 to induce the differentiation of hMSCs into steroidogenic cells, hMSCs were transduced with SF-1 or LRH-1 by retrovirus-mediated transfection. Trans-

duction of SF-1 into hMSCs induced expression of the type II 3β-hydroxysteroid dehydrogenase (HSD3B2) gene (Fig. 2A), with these cells also producing progesterone (Fig. 2B). As we reported previously (Yazawa et al., 2006), 8br-cAMP treatment further induced various steroidogenic enzymes. Concomitantly, 8br-cAMP treatment markedly increased the production of progesterone. Similar results were obtained in LRH-1-transduced hMSCs. Expression of SF-1 was never induced in LRH-1-transduced cells, and *vice versa*. These results demonstrate that LRH-1 had similar potential as SF-1 for the induction of MSC differentiation into steroidogenic cells.

3.2. Differentiation of ES cells into steroidogenic cells using tetracycline

Our studies clearly indicate that the NR5A family can direct the differentiation of stem cells into steroidogenic cells. However, this approach was not appropriate for pluripotent stem cells such as ES cells (Yazawa et al., 2006), as they barely survive the expression of the NR5A family in the absence of LIF. To circumvent these problems, we used the ES cell line EBRTcH3, carrying a Tc-repressible transgene at the *ROSA26* locus (Masui et al., 2005). The SF-1 cDNA along with a gene encoding the yellow fluorescent protein, Venus, was integrated into the *ROSA-TET* locus by a knock-in method, and puromycin resistant clones were selected (Fig. 3A). Induction of the genes from the *ROSA-TET* locus was checked by RT-PCR and fluorescence of the reporter protein, Venus. Withdrawal of Tc from the culture medium resulted in the induction of Venus fluorescence in virtually all cells within 48 h (Fig. 3B), whereas no fluorescence was detected in cells cultured in the presence of Tc. Although SF-1 mRNA and proteins were also induced in ES cells in



B

Cells	8BrcAMP(-)	8BrcAMP(+) (fmol/cell/hour)
GFP-hMSC	N.D.	N.D.
SF-1-hMSC	0.04±0.01	1.17±0.26
LRH-1-hMSC	0.07±0.02	1.31±0.17

Fig. 2. Differentiation of human BM-MSCs into steroidogenic cells by SF-1 and LRH-1. (A) RT-PCR analysis of each gene in each clone cultured with (lane A) or without (lane C) 8-br-cAMP for two days. (B) Production of progesterone by BM-MSCs stably expressing GFP, SF-1 or LRH-1 in the presence (+) or absence (−) of 8br-cAMP (1 mM). Means and SEM values of at least three independent experiments. N.D. indicates no detectable values.

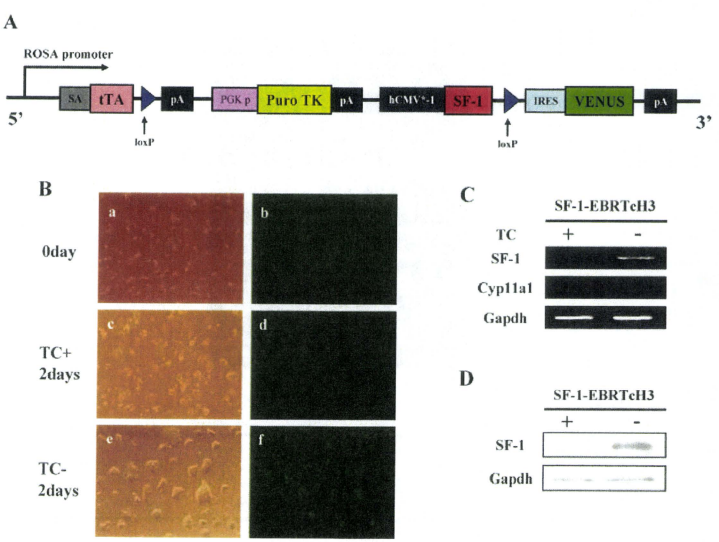


Fig. 3. Induction of SF-1 expression in the *ROSA-TET* system. (A) Schematic representation of the *ROSA-TET* locus exchanged with the SF-1-expression cassette in EBRTcH3 cells. (B) Induction of Venus fluorescence. Cells were observed before (panels a and b) or 48 h after induction (panels c–f) cultured with or without Tc. Bright field (panels a, c and e) and fluorescence images (panels b, d and f) are presented. (C and D) Induction of SF-1 expression in SF-1-EBRTcH3 cells. The mRNA (C) and protein (D) samples were prepared 48 h after the removal of Tc.

the absence of Tc, the cells were maintained in the undifferentiated state and never expressed any steroidogenic marker genes, including Cyp11a1 (Fig. 3Cand D). On the other hand, ES cells ceased to proliferate and died after several days when LIF was removed from culture medium. These results were in agreement with our previous observations in which steroidogenic cells could not be induced directly from ES cells (Yazawa et al., 2006).

We induced the expression of SF-1 after differentiation of the ES cells into MSCs. For differentiation of ES cells into MSCs, they were cultured on collagen IV-coated dishes and treated with pulse exposures to RA as described by Nishikawa and colleagues (Takashima et al., 2007) (Fig. 4A). Consistent with previous reports, molecular markers of the mesenchymal cell lineage such as PDGFRα, PDGFRβ and OB-CAD were robustly induced by RA treatment (Fig. 4B), indicating that the ES cells were successfully differentiated into mesenchymal cells. The cells were further cultured in the absence of RA and Tc for three days. In contrast to undifferentiated ES cells, the differentiated cells were able to survive following SF-1 expression in the absence of LIF.

As shown in Fig. 5A, expression of SF-1 in the differentiated cells resulted in the expression of various steroidogenesis-related genes, such as Cyp11a1, Hsd3b1, Cyp17, Cyp21 Cyp11b1 and Acthr. The gene expression pattern was quite similar to that in adrenocortical cells, especially fasciculata cells. Consistent with the gene expression profile, corticosterone was the most secreted steroid hormone from these cells (Fig. 5B), with Cyp17 expression barely detectable in the adult murine adrenal gland. Cortisol was also produced in these cells, although it was markedly lesser than corticosterone. These results indicate that ES cells could also be differentiated into steroidogenic cells by SF-1 *via* the mesenchymal cell lineage.

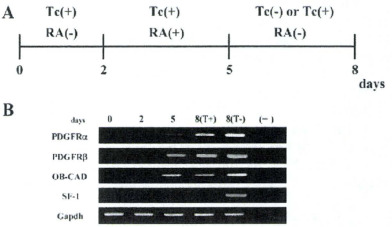
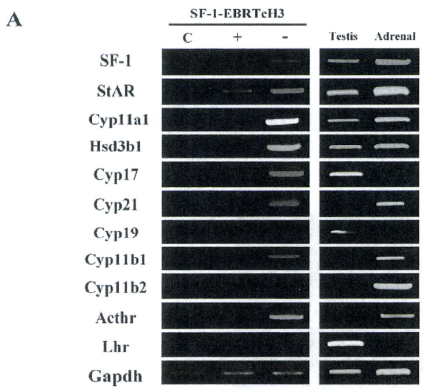


Fig. 4. Protocols for inducing steroidogenic cells from ES cells *via* the differentiation of ES cells into MSCs. Cells were cultured on collagen IV-coated dishes with differentiation medium containing Tc, with 10^{-7} M RA added to the medium from days 2 to 5. The medium was replaced with a RA- and Tc-free medium on day five. (B) RT-PCR analysis of marker genes for the MSC lineage and SF-1 genes at each time point.

4. Discussion

SF-1 has been clearly demonstrated to be a master regulator of steroidogenic organs. SF-1 knockout mice show agenesis of the primary steroidogenic organs, including the adrenal glands and gonads (Parker and Schimmer, 1997; Morohashi, 1999). SF-1 can induce the differentiation of MSCs into steroidogenic cells (Yazawa et al., 2006, 2008). In this study, we demonstrated that LRH-1 also has the capability to differentiate MSCs into steroidogenic cells. Consistent with our results, it has been reported that LRH-1 and SF-



B

Cells (Tet 1μg/ml)	progesterone	testosterone	estradiol	corticosterone	aldosterone	(ng/ml)
SF-1-EBRTcH3(+)	N.D.	N.D.	N.D.	N.D.	N.D.	
SF-1-EBRTcH3-MSCs(−)	N.D.	N.D.	N.D.	N.D.	N.D.	
SF-1-EBRTcH3-MSCs(-)	638.2±65.98	65.1±5.33	0.05±0.04	896.7±90.25	12.4±2.19	

1. N.D. means for no detectable values.
2. Data are means and SEM values of at least two independent experiments.

Fig. 5. Differentiation of ES cells into adrenocortical-like cells. (A) RT-PCR analysis of each gene in SF-1 induced (−) and uninduced (+) cells. Testis and adrenal represent a testis and an adrenal gland from an adult mouse. (B) Production of steroid hormones by each treatment. Means and SEM values of at least two independent experiments. N.D. means no detectable values.

1 could play similar roles in steroidogenesis in certain cells (Wang et al., 2001; Saxena et al., 2007; Yazawa et al., 2010). On the other hand, differentiated cells such as HEK293 cells stably transformed with SF-1 or LRH-1 did not express steroidogenic enzymes nor did they produce steroid hormones, suggesting that the expression of these genes in situ are controlled by additional factors.

LRH-1 is abundantly expressed in ES cells and is necessary for Oct-3/4 expression (an essential gene for maintenance of the inner cell mass and pluripotency of ES cells) at the epiblast stage (Gu et al., 2005). In addition, Oct-3/4 can be replaced by LRH-1 for the reprogramming of murine somatic cells into induced pluripotent stem (iPS) cells (Heng et al., 2010). As the differentiation of MSCs into steroidogenic cells, SF-1 and LRH-1 have similar potential for the regulation of Oct-3/4 expression (Barnea and Bergman, 2000; Gu et al., 2005), and therefore the same potential for induction of somatic cells into iPS cells (Heng et al., 2010). Niwa et al. (2000) demonstrated that quantitative expression of Oct-3/4 defines the fate of ES cells. A less than twofold increase in Oct-3/4 expression causes differentiation of ES cells into primitive endoderm and mesoderm, whereas repression of Oct-3/4 expression induces loss of pluripotency and causes de-differentiation of cells into the trophectoderm. It has been shown that DAX-1, a common transcriptional inhibitor of Oct-3/4, SF-1 and LRH-1 are also important for the pluripotency and survival of ES cells (Yu et al., 1998; Sun et al., 2009; Khalfallah et al., 2009). DAX-1 expression is detectable in ES cells and its expression is reduced upon differentiation of the cells into each germ layer. DAX-1 knockdown induces loss of pluripotency even under culture conditions for maintaining the undifferentiated state (Sun et al., 2009; Khalfallah et al., 2009), whereas complete deletion of DAX-1 by gene targeting results in cell death (Yu et al., 1998). Over-expression of the NR5A family was also cytotoxic to ES cells. These facts strongly suggest that regulated and coordinated expression of NR5A genes is essential for the pluripotency and survival of ES cells. These properties of the NR5A family are likely to cause difficulties in the induction of steroidogenic cells by NR5A members directly from ES cells.

In a previous study (Crawford et al., 1997), stable expression of SF-1 was shown to direct ES cells towards the steroidogenic lineage. However, the steroidogenic capacity of the cells was limited since a membrane-permeable substrate, 20α-hydroxycholesterol, was necessary to produce progesterone, the only steroid produced from the cells (Crawford et al., 1997). We demonstrated that regulated expression of SF-1 by the *ROSA-TET* system made it possible to derive steroidogenic cells from ES cells, with a capacity for autonomously secreting various steroid hormones.

It was reported that ES cells cultured on collagen-IV coated plates and treated with RA undergo differentiation into the mesenchymal cell lineage including MSCs, and that steroidogenic cells could be induced by SF-1 from MSCs (Takashima et al., 2007; Yazawa et al., 2006, 2008). Although we did not fully characterize the differentiated cells derived from the ES cells by RA treatment in this study, it is conceivable that the ES cell-derived steroidogenic cells must be produced *via* multipotent MSCs. Steroidogenic cells could be induced by SF-1 from MSCs, but not from differentiated cells such as fibroblasts, preadipocytes and HEK293 cells (Yazawa et al., 2006; Yanase et al., 2006). In support of this hypothesis, the ES cells could not be converted into steroidogenic cells *via* Tc-induced expression of SF-1 under culture conditions that induced differentiation of ES cells into preadipocytes, which also expressed PDGFRα as MSCs (data not shown). However, the exact origin of ES cell-derived steroidogenic cells should further investigated.

The ES cells-derived steroidogenic cells exhibited the very similar gene expression patterns to that of adrenocortical cells and produced a large amount of corticosterone, despite with Cyp17 expression was detectable. As in the case of other steroid hydroxylases, it is well-known that the expression of the CYP17/Cyp17 gene is regulated by SF-1 and LRH-1 (Zhang and Mellon, 1996; Lin et al., 2001; Yazawa et al., 2009). Hence, it is conceivable that the human CYP17 gene is expressed in both in gonadal and adrenal steroidogenic cells. In contrast, the murine Cyp17 gene is expressed only in gonadal cells in adults. However, it was shown that Cyp17 is expressed in the murine fetal adrenal gland (Heikkilä et al., 2002). Therefore, it is possible that steroidogenic cells derived from murine ES cells might reflect the fetal adrenal phenotype. Further studies are necessary for the determination of steroidogenic lineage and the regulation of Cyp17 expression.

In summary, we have shown that, as in the case of SF-1, LRH-1 could drive the differentiation of MSCs into steroidogenic cells. In addition, ES cells could also be differentiated into steroidogenic cells through the regulated expression of SF-1 using the *ROSA-TET* system. This approach might also provide the opportunity, through the use of MSCs, for the development of cell and gene therapy treatments in steroidogenesis deficiencies. Additionally, this approach could be a powerful tool for studies on the differentiation of steroidogenic cell lineages.

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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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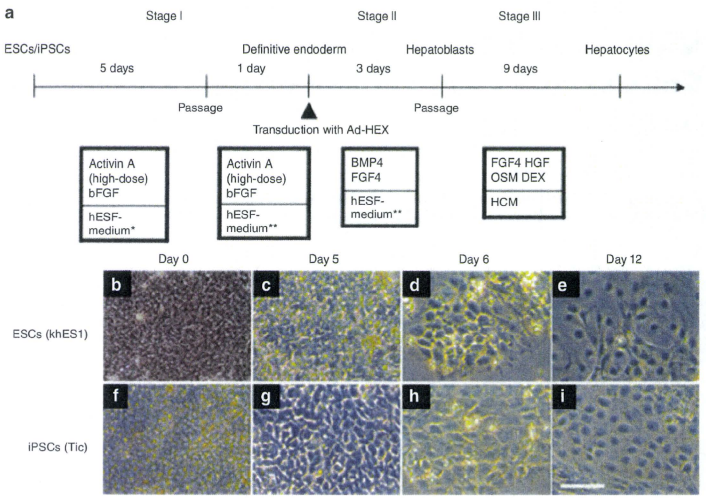

Figure 1 A strategy of differentiation of human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) to hepatoblasts and hepatocytes. (**a**) Schematic representation illustrating the procedure for differentiation of human ESCs (khES1) and iPSCs (Tic) to hepatocytes. (**b–i**) Phase 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.

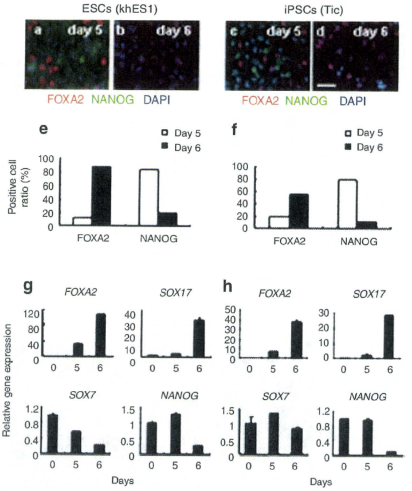

Figure 2 Characterization of the human ESC (khES1)- and iPSC (Tic) derived definitive endoderms. (**a–d**) The immunofluorescent staining of the human ESC (khES1)- and iPSC (Tic) derived differentiated cells before (**a** and **c**; day 5) and after passaging (**b** and **d**; day 6). The cells were 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 = 50μm. 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 50ng/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

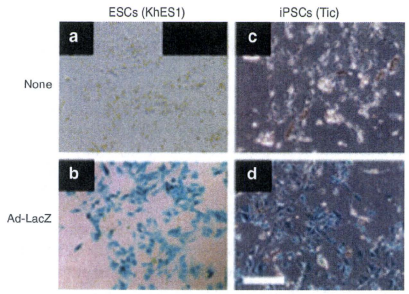

Figure 3 Efficient transgene expression in the human ESC (khES1)- and iPSC (Tic) derived definitive endoderms by using a fiber-modified Ad vector containing the EF-1α promoter. (**a,b**) Human ESC (khES1)-derived and (**c,d**) iPSC (Tic) derived definitive endoderms were transduced with 3,000VP/cell 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 = 50 μm. Ad, adenovirus; EF-1α, elongation factor-1α; ESC, embryonic stem cells; iPSC, induced pluripotent stem cells; LacZ, Ad-LacZ-transduced cells; None, nontransduced cells.

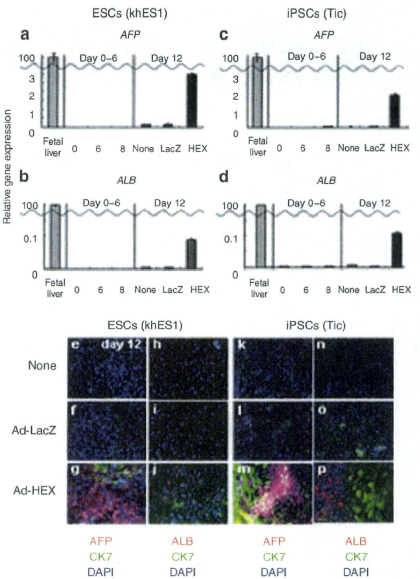

Figure 4 Efficient hepatoblast differentiation from the human ESC (khES1)- and iPSC (Tic) derived definitive endoderms by transduction of the *HEX* gene. (**a–d**) Real-time RT-PCR analysis of the level of (**a,c**) *AFP* and (**b,d**) *ALB* expression in nontransduced cells, Ad-LacZ-transduced cells, 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 = 50 μm. 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

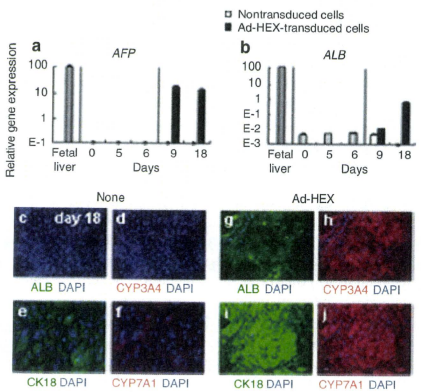

Figure 5 Efficient differentiation of Ad-HEX-transduced hepatoblasts into hepatocytes. (**a**,**b**) Real-time RT-PCR analysis of (**a**) *AFP* and (**b**) *ALB* expression in nontransduced cells and Ad-HEX-transduced cells, both of which were induced from the human iPSC (Tic) derived definitive endoderm (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

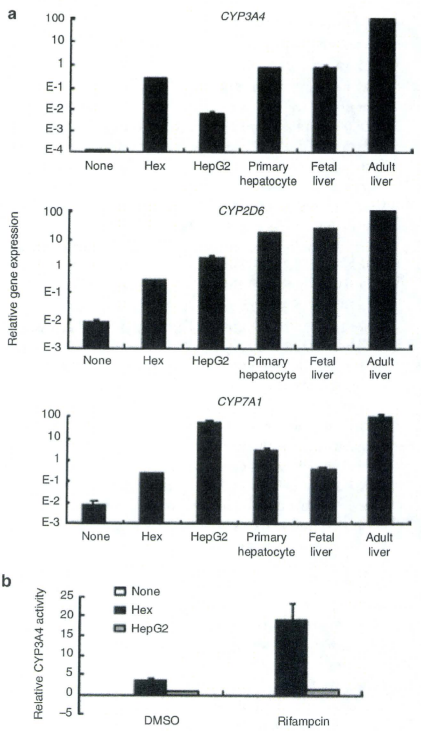

Figure 6 Cytochrome P450 isozymes in human iPSC (Tic) derived hepatocytes. (**a**) Real-time RT-PCR analysis of *CYP3A4*, *CYP7A1*, and *CYP2D6* expression in iPSC (Tic) derived nontransduced cells, Ad-HEX-transduced cells, and fetal and adult liver tissues. (**b**) Induction of CYP3A4 by rifampicin 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-0.01325 mmol/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.

ACKNOWLEDGMENTS

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Development of a New Assay System for Evaluating the Permeability of Various Substances Through Three-Dimensional Tissue

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A novel assay system with cell-dense three-dimensional (3D) tissue was developed for measuring the permeability of substances. In this paper, the permeabilities of various molecules containing nutrients, a cytokine, and a chemokine were examined and analyzed. A single-layered cell sheet was approximately 20 μm thick, and as the number of layers of these cell sheets increased, so did the total thickness of the tissue. The diffusion rates of glucose and pyruvic acid were reduced to approximately 30–40% by a single-layered cell sheet compared with the control without the cell sheet, and the diffusion of both substances were completely inhibited by a quadruple-layered cell sheet. The diffusion rate of creatinin was reduced to approximately 50% and 15–20% by a single-layered and by a quintuplet-layered cell sheet, respectively. On the other hand, the diffusion rate of stromal cell–derived factor 1α, vascular endothelial growth factor, β2-microglobulin, and transferrin was reduced to approximately 10%, 5%, 20%, and 10%, by only a single-layered cell sheet, respectively. The diffusion of these substances were completely inhibited by a double-layered cell sheet. These results show that the permeability of substances through 3D tissue significantly decreased with the increase of the molecular weight. Therefore, the system could give a simulated living-tissue condition for measuring the permeability of substances. To our knowledge, this is the first report about measuring the permeability of substances through cell-dense 3D tissues without scaffolds. The assay system is believed to contribute to the progress of physiology, metabolony, biochemistry, and pharmacokinetics. Further, the system may give some hints for developing a new dialysis membrane technology for an artificial kidney.

Introduction

THERE ARE MANY REPORTS that described the adverse effects, the permeability, and the uptakes of various substances, including nutrients and drugs, by *in vitro* cell assay systems, which were designed and used model systems for the heart tissue, the small intestinal mucosa, the oral mucosa, the blood–brain barrier, the blood–retinal barrier, and so on.[1–6] These assay systems are essential in the field of pharmacokinetics as well as in the understanding of biochemistry, physiology, and metabolony of tissues and organs. An adequate assay system has a clear advantage as *in vitro* models that could require no animal experiments. To date, experiments in these fields have relied on assays using two-dimensional (2D) single-layered cell cultures. Two-dimensional culture system is too simple in comparison with actual living tissues or organs. Cells of 2D culture system are significantly different from that of three-dimensional (3D) culture system in terms of their morphology, cell-to-cell interactions, surrounding extracellular matrix, proliferation rates, and differentiation.[7–9] These differences may affect their gene expression and other biological activities. It is believed that 3D culture system can simulate *in vivo* situations.[7,10,11] An *in vitro* assay system using 3D tissues would, therefore, be clearly desirable in the fields described above.

Three-dimensional tissues can be re-constructed *in vitro* using tissue engineering techniques.[12] Conventional tissue engineering has employed 3D scaffolds (e.g., polyglycolic acid, collagen gel, and gelatin) that are useful as alternatives for extracellular matrix, and cells are seeded into the scaffolds. However, 3D tissues fabricated by using the scaffolds are extremely cell-sparse tissues because of insufficient cell migration into the scaffolds.[13,14] In an attempt to improve this situation, our laboratory has created and utilized an original

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technology called cell sheet engineering,[15] which can prepare 3D tissues without the scaffolds by layering cell sheets harvested from temperature-responsive culture dishes.[16–19] This method allows cell harvest to require no proteolytic treatments and to preserve cell-to-cell connections completely.[20,21] The technique also can control the thickness of re-constructed 3D tissues by manipulating the number of cell layers. Therefore, 3D tissues fabricated by the cell sheet engineering are suitable as an *in vitro* cell-dense 3D tissue model.

In this study, we develop a new assay system that uses cell-dense 3D tissues made by the cell sheet engineering in conjunction with a modified cell culture insert, and measured the permeability of various substances, including nutrients, a cytokine, and a chemokine, through the cell-dense 3D tissues for verifying the usefulness of the system.

Materials and Methods

Culture of C2C12 mouse skeletal myoblast cell lines and human endometrial gland–derived mesenchymal cells

C2C12 mouse skeletal myoblast cell lines were purchased from Dainippon Sumitomo Pharma (Osaka, Japan). C2C12 cells and human endometrial gland–derived mesenchymal cells (EMCs)[22] were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich Japan, Tokyo) supplemented with 10% fetal bovine serum (Japan Bio Serum, Nagoya, Japan) and 1% penicillin and streptomycin (Invitrogen, Carlsbad, CA). These cells were cultured at 37°C in a humidified atmosphere with 5% CO_2.

A device for permeability experiments

A device was developed by modifying a cell culture insert (the membrane pore size: 3 μm; Becton, Dickinson and Company, Franklin Lakes, NJ) as shown in Fig. 1. Briefly, a round polyethylene terephthalate film (diameter: 30 mm) having a hole in its center (diameter: 5 mm) was glued to the outside-bottom of the membrane of the cell culture insert with cyanoacrylate adhesive (Toagosei, Tokyo, Japan) (Fig. 1). A single cell sheet or a several layered cell sheet was then placed on the bottom of the device, covering the permeance hole, as described in detail below (Fig. 2A–E). Thus, substances in the upper medium could diffuse only through the cell sheets and the permeance hole.

Preparation of cell sheets and the manipulation of the cell sheets into layered constructs

Cell suspensions were plated onto a 35 mm temperature-responsive culture dish (Upcell; CellSeed, Tokyo, Japan) at 6×10^5 cells/dish (for C2C12 cells) or 1×10^6 cells/dish (for EMCs). After 3 days (C2C12 cells) or 4 days (EMCs), the culture dishes were placed in a separate CO_2 incubator set at 20°C. Each cell sheet with its medium was gently aspirated into a tip of a pipette and put on the bottom membrane of the device one at a time. An additional medium was then poured into the upper part of the device, having the cell sheet for spreading out any folded portions. Once the cell sheet was spread out, the medium was aspirated away, and the device was incubated for 60 min at 37°C to allow the cell sheet to fully adhere to the bottom membrane of the device. Cell sheets were layered by repeating the following procedure: detaching another cell sheet from a temperature-responsive culture dish and stacking it onto the first cell sheet. In this manner, triple-, quadruple-, and quintuple-layered constructs were created. After 60 min incubation, a fresh medium was added to the device's upper and lower parts, and the device was incubated at 37°C for 24 h. Upon the end of the incubation, the culture media of the upper and lower parts were separately collected and were used for chemical and protein analyses, and enzyme-linked immunosorbent assay (ELISA). The volumes of the upper and lower media were also measured.

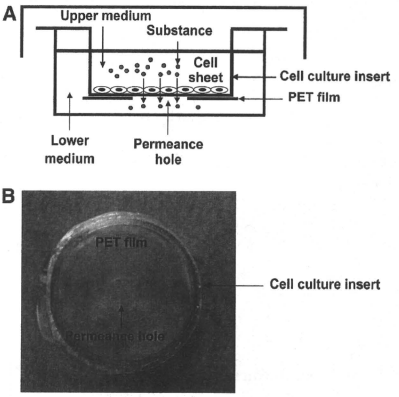

FIG. 1. A device to measure the permeability of substances. A schematic illustration of the device as viewed from the side is shown in (**A**). (**B**) is a photograph as viewed from the bottom.

Histological analysis

Cell sheets on the device were fixed with 4% paraformaldehyde. Specimens were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Prepared specimens were examined by a microscope (Elipse TE2000-U; Nikon, Tokyo, Japan).

Medium, chemical, and proteins

Glucose/pyruvic acid–deficient DMEM was purchased from Invitrogen; creatinin, human β2-microglobulin, and human transferrin were from Wako Pure Chemicals (Tokyo, Japan); human vascular endothelial growth factor (VEGF) and human stromal–derived factor 1α (SDF-1α) were from Funakoshi (Tokyo, Japan).

Chemical and protein analyses, and ELISA

Concentrations of glucose, pyruvic acid, and creatinin were measured by the hexokinase UV method,[23] the pyruvate oxidase method,[24] and an enzymatic method by SRL (Tokyo, Japan), respectively. Concentrations of β2-microglobulin and transferrin were measured by a latex agglutination test (SRL).

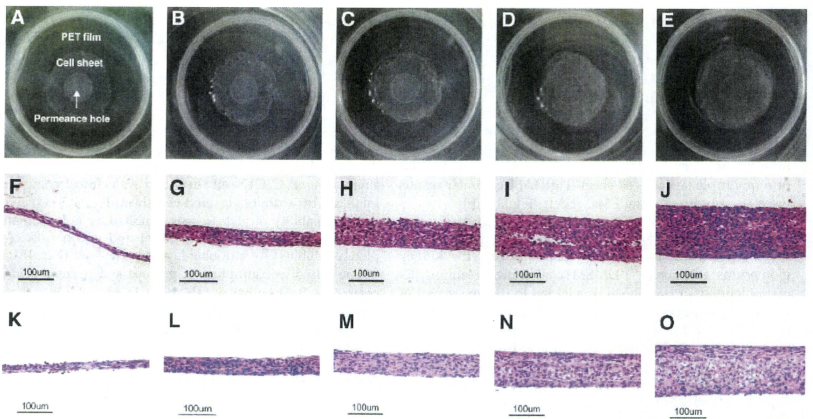

FIG. 2. Morphological and histological observation of single cell sheets and of cell sheets with several layers. The photograph (**A**) shows a monolayer EMC sheet on a new device; (**B**), a double-layered EMC sheet; (**C**), a triple-layered EMC sheet; (**D**), a quadruple-layered EMC sheet; and (**E**), a quintuplet-layered EMC sheet. Cross-sectional observation of layered C2C12 cell sheets (**F–J**) and EMC sheets (**K–O**). Photographs (**F**) and (**K**) show single-layered cell sheets; (**G**) and (**L**), double-layered cell sheets; (**H**) and (**M**), triple-layered cell sheets; (**I**) and (**N**), quartet-layered cell sheets; (**J**) and (**O**), quintet-layered cell sheets. Scale bars indicate 100 μm.

Amounts of human VEGF and SDF-1α were quantitated by a commercially available ELISA kit (Funakoshi).

Relative permeability and relative residual amount of each substance

Permeability of each substance was calculated by the following equation:

$$\text{Permeability} = \frac{\text{The concentration of a substance in the lower medium}}{\text{The volume of the lower medium}} \times 100$$

For comparison, the permeability of a substance without a cell sheet was assumed to be 100% (the control permeability), and its relative permeability through a cell sheet was calculated by the following equation:

$$\text{Relative permeability} = \frac{\text{The permeability of a substance through a cell sheet}}{\text{The control permeability}} \times 100$$

The residual amount of a substance was calculated by the following equation:

Residual amount = [the amount of a substance remaining in the upper medium after incubation for 24 h] + [the amount of a substance in the lower medium after incubation for 24 h].

The residual amount without the cell sheet was estimated at 100% (the control residual amount). Relative residual amounts were then calculated for substances by the following equation:

$$\text{Relative residual amount} = \frac{\text{The residual amount of a substance for 24 h with cell sheet}}{\text{The control residual amount}} \times 100$$

Data are expressed as mean ± SD.

Results

Morphologic analysis of cell sheets

The cross sections of single-layered and multi-layered cell sheets were observed. When the culture temperature was decreased from 37°C to 20°C, C2C12 cells or EMCs on a temperature-responsive culture dish were detached as a contiguous cell sheet. Those cell sheets shrunk horizontally due to the cytoskeletal tensile reorganization. As a result, those cell sheets consisted of two or three cell layers, and the thickness of the cell sheets became approximately 20 μm (Fig. 2F, K). The addition of a layered cell sheet to a tissue increased the total tissue's thickness by the thickness of the added cell sheet (Fig. 2F–J, the C2C12 cell sheet; Fig. 2K–O, the EMC sheet). These results show that cell sheet engineering can control the thickness of 3D tissues.

Permeability of the substances through layered cell sheets

Because nutrients (pyruvic acid and glucose) are vital for living tissues, the permeability through the 3D tissue is a basic factor for tissue engineering. Therefore, our first experiments assessed the permeability of the nutrients through