5*I*). Finally, we can generate chimera animals by injecting the SeVdp-iPS cells into embryos (Fig. 5*J*).

DISCUSSION

Discovery of the methods for achieving cell reprogramming with ectopic expression of defined factors has had a great impact on modern bioscience. However, to establish this technology for practical applications, we still have to overcome several difficult hurdles, such as the dramatic improvement of reprogramming efficiency and reproducible generation of fully differentiation-competent iPS cells without allowing the expression of residual exogenous genes on the chromosomes. Among these issues, chromosomal integration of reprogramming genes is the most critical factor affecting the characteristics of iPS cells (9). Incomplete suppression of exogenous reprogramming genes might affect the pluripotency of iPS cells. Furthermore, even if the integrated gene cassette is suppressed by epigenetic modification, differentiation of mouse iPS cells has frequently reactivated the integrated genes and induced cancers in iPS cell-derived offspring, as some of the reprogramming genes have intrinsic tumorigenic activity (24). Therefore, methodologies for generating fully pluripotent human iPS cells carrying no remnant of exogenous genes have been investigated but with limited success (9). As far as we know, the SeVdp vector described here is the only system that fulfills all the requirements to accomplish this goal with high efficiency and without laborious procedures.

Efficient gene delivery and expression are the primary factors affecting iPS cell generation, and this SeVdp vector has a great advantage over other delivery systems. As a typical recombinant viral vector, it can deliver genes much more efficiently than a nonviral system. In addition, its exceptionally broad host range should be quite beneficial; these viruses can infect almost all cell types from avian to human (42). Among others, the efficiency of the SeVdp vector to induce stable gene expression in human HSCs (~80%) is remarkably higher than that of retroviral vectors under multiple infection cycles with the aid of recombinant fibronectin fragments (~30%) (43). These characteristics have emerged partly because the SeVdp recognizes ubiquitous sialic acid as the primary receptor. Furthermore, the SeVdp has its own RNA-dependent RNA polymerase and requires only ubiquitous cytosolic proteins for transcription/replication (44). Thus, the SeVdp vector induces active transcription just after the nucleocapsid is delivered into the cytoplasm.

The enablement of stable gene expression without chromosomal integration is the most remarkable characteristic of these SeVdp vectors. DNA-based vectors (including retro/lentiviral vectors) can accomplish stable gene expression either by chromosomal integration or by episomal replication, depending on the cellular replication machinery. However, these characteristics make it difficult to remove the stabilized DNA from the cells; this depends either on a complex excision process using DNA recombinase or on passive elimination in the absence of selection. On the other hand, recombinant RNA viral vectors (except for retro/lentiviral vectors) cannot achieve stable gene expression, partly because they trigger cellular defense systems and induce apoptotic death in the host cells. The SeVdp vector is the only RNA-based platform inert enough to allow stable gene expression in sensitive HSCs, thanks to its unique gene mutations/alterations for escaping the host defense system. Furthermore, as the stability of SeVdp genomic RNA depends on the activity of viral RNA polymerase, interference of the polymerase with siRNA can be used to eliminate the genome from infected cells, as shown here.

Coinfection of conventional F-defective SeV vectors installed with *Oct4*, *Sox2*, *Klf4*, and *c-Myc* separately has been reported to generate iPS cells (45, 46). Although these reports demonstrated the potential of SeV vectors in cell reprogramming, our data have clearly demonstrated that this SeVdp vector provides a superior alternative to this approach. Among other factors, the cytopathic nature of the conventional SeV vectors based on wild-type SeV, including simple F-defective SeV vectors (30, 47), limits their utility in cell re-

FIGURE 5. **Reprogramming of MEFs with SeVdp vectors installed with reprogramming genes.** *A*, the genome structure of SeVdp(*c-Myc/Klf4/Oct4/Sox2*) is shown. *B*, shown is genome structure of SeVdp(*Klf4/Oct4/Sox2*) and SeVdp(*Zeo/hKO/c-Myc*), coexisting in a single cell, is shown. *C* and *D*, shown is the efficiency to reprogram MEF/Nanog-GFP. MEF/Nanog-GFP cells (1.25×10^5) were infected with SeVdp vectors, and retroviral vectors were installed with *c-Myc/Klf4/Oct4/Sox2* as described under "Experimental Procedures." Then 1.0×10^3 of infected cells were seeded onto the feeder cells in 6-well plates and cultured for 14 days (*C*) or for the indicated days (*D*). The number of iPS colonies expressing GFP was determined under fluorescent microscopy. Reprogramming efficiency was indicated as the ratio of the number of EGFP-positive colonies to that of MEF/Nanog-GFP seeded in the well (*C*) or to that of infected MEF/Nanog-GFP seeded in the well (*D*). *C*, shown is a comparison of reprogramming efficiency with the SeVdp(*c-Myc/Klf4/Oct4/Sox2*) vector and with retroviral vectors. *SeVdp(M/K/O/S)*, SeVdp(*c-Myc/Klf4/Oct4/Sox2*); *RvMX4*, coinfection of ecotropic retroviral vectors installed with *c-Myc*, *Klf4*, *Oct4*, and *Sox2* separately. *D*, shown is a comparison of reprogramming efficiency by a single infection of SeVdp(*c-Myc/Klf4/Oct4/Sox2*) (*SeVdp(M/K/O/S)*) and by coinfections of SeVdp(*Klf4/Oct4/Sox2*) and SeVdp(*Zeo/hKO/c-Myc*) (*SeVdp(K/O/S)* + *SeVdp(M)*). *E*, characterization of mouse iPS cells generated with SeVdp(*c-Myc/Klf4/Oct4/Sox2*) is shown. The ES-like colonies emerging from MEF/Nanog-GFP cell lines were fixed, incubated with specific primary antibodies against SeV NP antigen (*left*), Sox2 (*middle*), and SSEA-1 (*right*), then stained with secondary antibodies conjugated with Alexa 555. The cells were then counterstained with DAPI and examined by fluorescence microscopy as described under "Experimental Procedures." *Nanog* (*left*), expression of GFP driven by the Nanog promoter. *F*, gene expression analysis with semiquantitative RT-PCR is shown. Aliquots (2 μg) of total RNA prepared from the cells indicated were analyzed as described under "Experimental Procedures." *Lane 1*, MEF; *lane 2*, MEF infected with control vector (SeVdp(*Bsr/ΔF/KO*)); *lane 3*, MEF infected with SeVdp(*M/K/O/S*) on day 5 infection; *lane 4*, SeVdp-iPS cell clone #2-1; *lane 5*, SeVdp-iPS cell clone #9-1; *lane 6*, SeVdp-iPS cell clone #13; *lane 7*, SeVdp-iPS cell clone #16; *lane 8*, SeVdp-iPS cell clone #21; *lane 9*, mouse iPS cell generated with retrovirus vectors (RvMX4); *lane 10*, mouse ES cell (clone D3). *ECAT1*, ES cell-associated transcript 1; *FGF4*, fibroblast growth factor 4. *G*, methylation analysis of Oct4 and Nanog promoters is shown. Methylation profile of CpG in genomic DNA was analyzed by bisulfite sequence analysis as described under "Experimental Procedures." *Open circles*, unmethylated cytosine; *closed circles*, methylated cytosine. The ratio of methylated cytosine is indicated as a percentage of total cytosine residues analyzed. *H*, a telomerase assay is shown. Telomerase activity in total cell extract prepared from 1×10^3 cells was analyzed as described under "Experimental Procedures" and is indicated as the amount of $(dT\text{-}TAGGG)_n$ synthesized. *I*, histology of teratomas derived from SeVdp-iPS cells is shown. Teratoma formation was studied at 6 weeks after the subcutaneous injection of 1×10^6 SeVdp-iPS cells from clone #13 into SCID mice. *a–c*, low magnification; *scale bar* = 100 μm. *d–h*, high magnification observation; *scale bar* = 20 μm. *d*, cartilage; *e*, epidermis; *f*, hair follicle; *g*, sweat gland; *h*, intestinal gland. *J*, adult chimeras derived from SeVdp-iPS cells (clone #13) are shown. *Dark hair* indicates donor contribution.

programming. As revealed in our previous work (12) and in the present article, the cytopathogenicity of SeV depends on multiple factors including escaping from cytokine induction and acute membrane dysfunction, and it is not possible to evade this by simple deletion of all the structural genes (*M*, *F*, and *HN*) (27). Furthermore, the use of a single-gene defective SeV vector raises safety and regulatory concerns, as the potential of the SeV vector to self-replicate was diminished but was not completely abolished by a single gene defect, as shown in Table 1. This issue was neither investigated nor addressed in previous reports describing single gene defective vectors (30, 47) but has been resolved here for the first time.

Cell reprogramming depends on the simultaneous delivery of multiple genes, on their balanced expression, and on their prompt suppression/removal. These factors affect both the efficiency of iPS cell generation and the quality of the iPS cells (36, 39). Nevertheless, this issue has not been investigated in detail, partly because most of the current viral vectors can accommodate only one or a few extra genes. The capacity of SeVdp vectors to install four reprogramming genes on a single vector was critical both for expressing these genes at a prefixed balance (Fig. 3) and for highly efficient reprogramming (Fig. 5); coinfection of two independent SeVdp vectors failed to accomplish either of these goals (Figs. 3 and 5). This latter phenomenon might be caused by homologous viral interference, which has been observed after coinfections of two independent paramyxoviruses including SeV (48). Otherwise, this phenomenon could reflect that the SeVdp genome is a multicopy replicon with about 40,000 copies per cell (12). In general, it is difficult to manage to produce two (or more) independent multicopy replicons under equal balance when they share the same replication machinery. Active and rapid erasure of SeVdp vectors with specific siRNAs (Fig. 4) is also advantageous to the generation of homogeneous and vector-free iPS cells compared with the passive and unmanageable vector removal that depends on the sequential passages of the cells (45, 46).

Synthetic modified mRNA encoding reprogramming factors has been reported to generate iPS cells highly efficiently (49). This nonviral approach and our SeVdp vector have their own advantages and disadvantages, but the escape from the cellular antiviral defense system is a critical characteristic common to these advanced reprogramming systems. The former has the advantage that the combination of exogenous reprogramming genes and the balance or duration of their expression can be adjusted flexibly. The nonviral approach has another advantage, as the use of recombinant viruses is regulated strictly in general. On the other hand, it is highly dependent on the gene delivery system, as it requires repetitive transfection for 16 days. Therefore, it might not be applicable to cells that are difficult to transfect, such as primary peripheral blood cells. In contrast, adjustment of reprogramming genes and of their expression is not so easy using SeVdp vectors and needs the construction of different vectors for tuning these features. However, once established, the SeVdp vector can always induce steady expression of the installed genes at a fixed balance, and this feature allows highly reproducible and uniform cell reprogramming. Furthermore, prolonged gene expression by a single infection procedure and the wider host range available are advantageous for practical applications, such as reprogramming of blood cells. Combining the results obtained with these two approaches depending on specific research purposes will enable us to create a more advanced system for cell reprogramming.

The field of cell reprogramming is expanding very rapidly, and the tools for safer and efficient reprogramming have become increasingly important both for basic research and for development of medical applications. We are presently investigating the utility of the SeVdp vectors in the genomic reprogramming 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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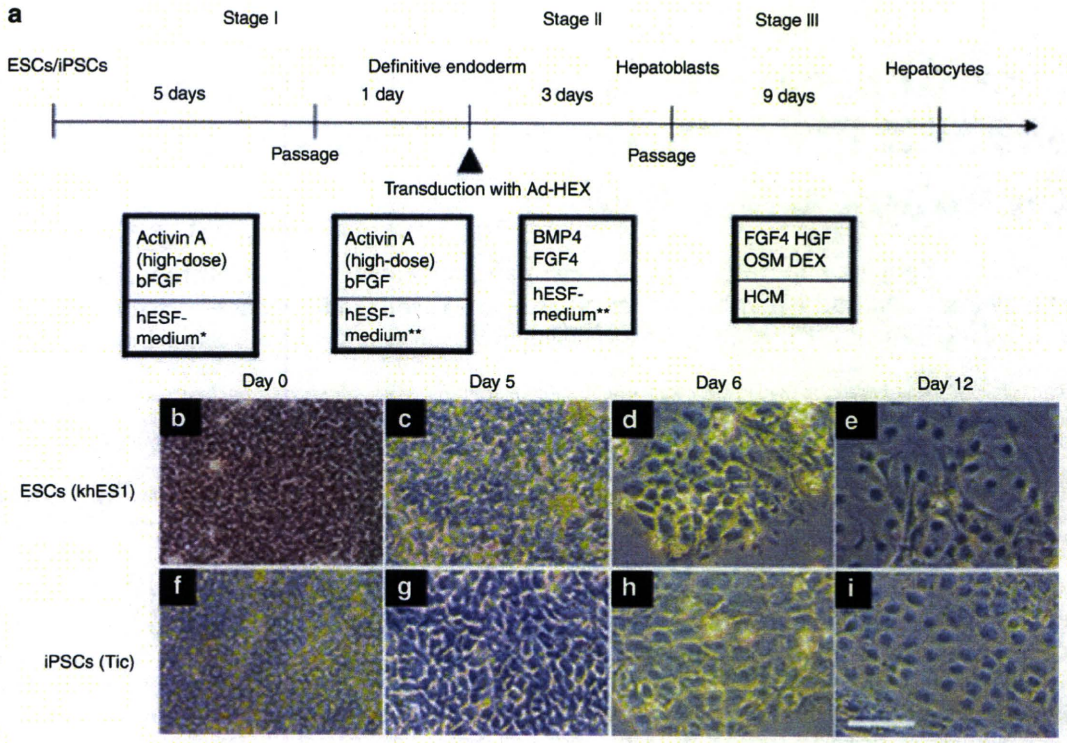

Figure 1 A strategy of differentiation of human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) to hepatoblasts and hepatocytes. **(a)** Schematic representation illustrating the procedure for differentiation of human ESCs (khES1) and iPSCs (Tic) to hepatocytes. **(b–i)** Phase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

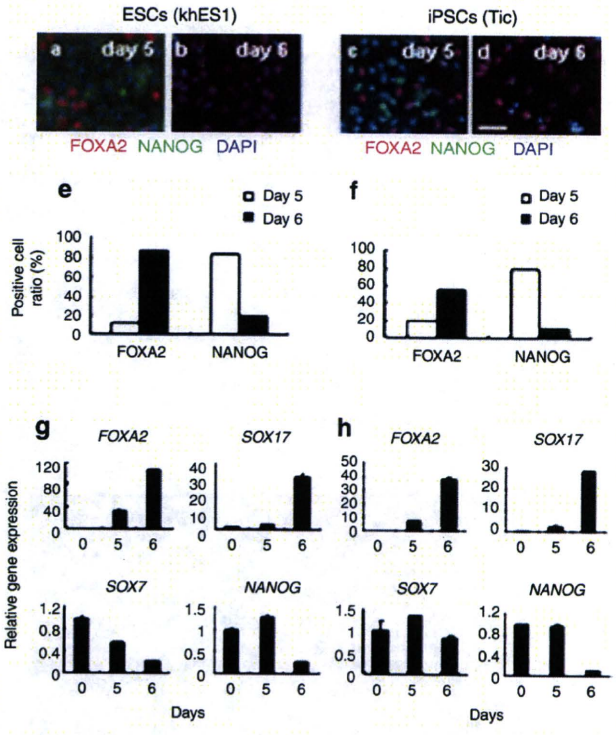

Figure 2 Characterization of the human ESC (khES1)- and iPSC (Tic) derived definitive endoderms. (**a–d**) The immunofluorescent staining of the human ESC (khES1)- and iPSC (Tic) derived differentiated cells before (**a** and **c**; day 5) and after passaging (**b** and **d**; day 6). The cells were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

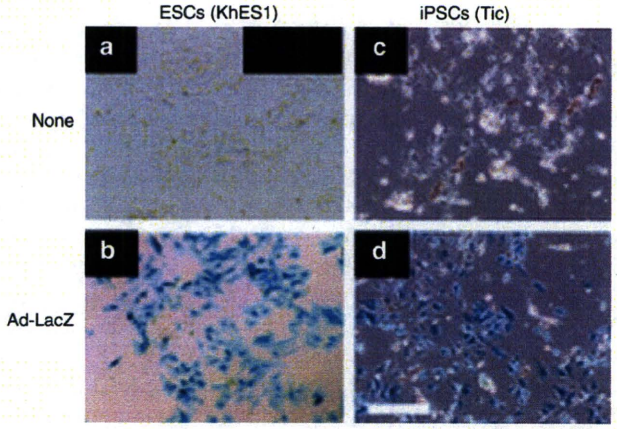

Figure 3 Efficient transgene expression in the human ESC (khES1)- and iPSC (Tic) derived definitive endoderms by using a fiber-modified Ad vector containing the EF-1α promoter. (**a,b**) Human ESC (khES1)-derived and (**c,d**) iPSC (Tic) derived definitive endoderms were transduced with 3,000 VP/cell 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.

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-

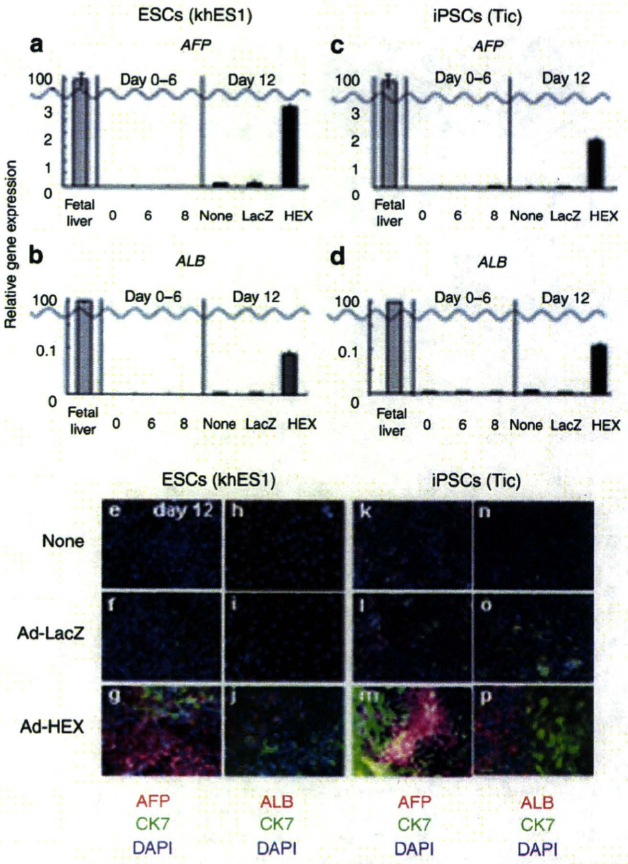

Figure 4 Efficient hepatoblast differentiation from the human ESC (khES1)- and iPSC (Tic) derived definitive endoderms by transduction of the *HEX* gene. (**a–d**) Real-time RT-PCR analysis of the level of (**a,c**) *AFP* and (**b,d**) *ALB* expression in nontransduced cells, Ad-LacZ-transduced cells,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

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

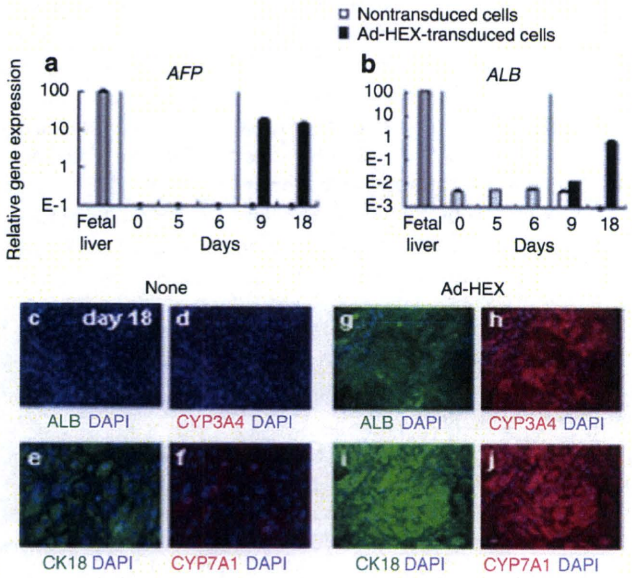

Figure 5 Efficient differentiation of Ad-HEX-transduced hepatoblasts into hepatocytes. (**a**,**b**) Real-time RT-PCR analysis of (**a**) *AFP* and (**b**) *ALB* expression in nontransduced cells and Ad-HEX-transduced cells, both of which were induced from the human iPSC (Tic) derived definitive endoderm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

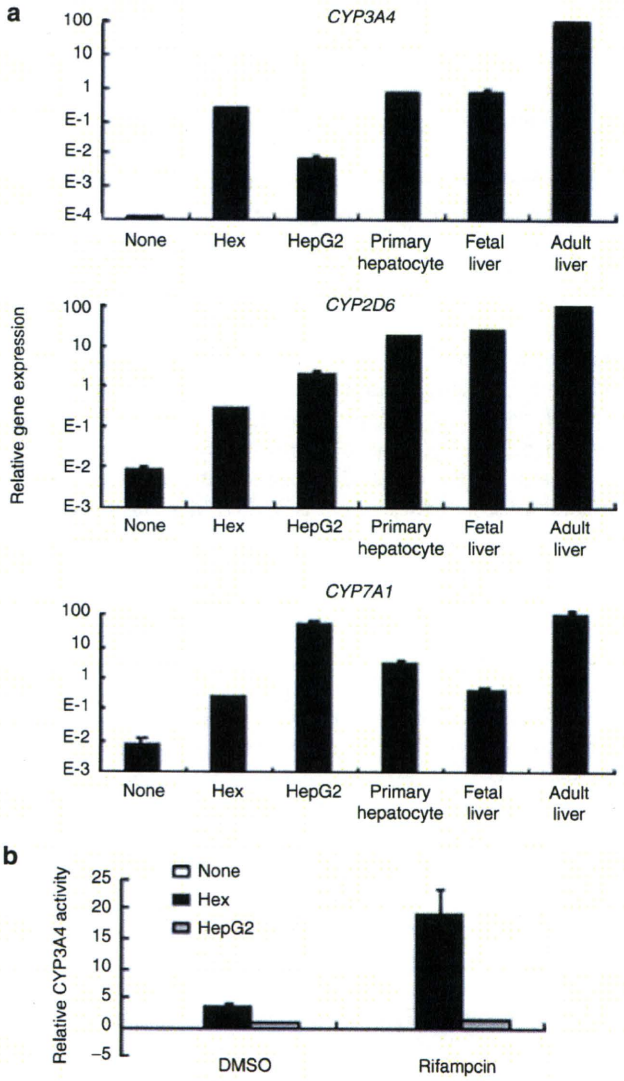

Figure 6 Cytochrome P450 isozymes in human iPSC (Tic) derived hepatocytes. (**a**) Real-time RT-PCR analysis of *CYP3A4*, *CYP7A1*, and *CYP2D6* expression in iPSC (Tic) derived nontransduced cells, Ad-HEX-transduced cells, and fetal and adult liver tissues. (**b**) Induction of CYP3A4 by rifampicin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

iPS 細胞への遺伝子導入を用いた分化誘導の最適化

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Differentiation of Functional Cells from iPS Cells by Efficient Gene Transfer

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Induced pluripotent stem (iPS) cells, which are generated from somatic cells by transducing four genes, are expected to have broad application to regenerative medicine. Although establishment of an efficient gene transfer system for iPS cells is considered to be essential for differentiating them into functional cells, the detailed transduction characteristics of iPS cells have not been examined. By using an adenovirus (Ad) vector containing the cytomegalovirus enhancer/beta-actin (CA) promoters, we have developed an efficient transduction system for mouse mesenchymal stem cells and embryonic stem (ES) cells. Also, we applied our transduction system to mouse iPS cells and investigated whether efficient differentiation could be achieved by Ad vector-mediated transduction of a functional gene. As in the case of ES cells, the Ad vector could efficiently transduce transgenes into mouse iPS cells. We found that the CA promoter had potent transduction ability in iPS cells. Moreover, exogenous expression of a PPARγ gene or a Runx2 gene into mouse iPS cells by an optimized Ad vector enhanced adipocyte or osteoblast differentiation, respectively. These results suggest that Ad vector-mediated transient transduction is sufficient to promote cellular differentiation and that our transduction methods would be useful for therapeutic applications based on iPS cells.

Key words——regenerative medicine; induced pluripotent stem (iPS) cell; adenovirus vector; mesenchymal stem cell; embryonic stem (ES) cell

1. はじめに

幹細胞（stem cells）は自己複製能と分化多能性という大きく2つの特徴を有する細胞であり，目的の細胞へ分化させることにより創薬や再生医療への応用が期待されている．再生医療への応用が期待されている幹細胞には，造血幹細胞，神経幹細胞，間葉系幹細胞，ES（embryonic stem）細胞，iPS（induced pluripotent stem）細胞などがある．このうち，ES 細胞は受精卵（胚）から樹立され，iPS 細胞は体細胞に4種の遺伝子（Oct-3/4m Sox2, Klf4, c-Myc）を導入することにより作製される．しかしながら，ES 細胞や iPS 細胞を直接生体に移植するには困難な場合も多く，マウス生体に投与するとランダムに分化しテラトーマ（奇形腫）を形成する．したがって，治療目的には幹細胞を *in vitro* で目的の細胞に分化させた後生体に移植することが望ましいと考えられる．幹細胞を骨，心筋，脂肪，血液などの目的の細胞に分化させるには，培養液に特定のサイトカインや増殖因子等の液性因子を加える方法がとられているが分化効率は十分ではない．そこで，われわれは各種幹細胞に機能遺伝子を導入することにより効率よく分化させることができないかと考え研究を進めている．一般に，幹細胞は遺伝子導入が困難であり，リポフェクション法やレトロウイルスベクター系など通常用いられる方法では十分な導入効率が得られない．われわれは高効率かつ一過性に目的遺伝子を発現させることができるアデノウイルス（Ad）ベクターを用いて機能遺伝子を導入

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することにより，幹細胞の分化誘導効率を向上させることを目指した．

2. 間葉系幹細胞への遺伝子導入

間葉系幹細胞は骨髄由来のストローマ細胞であり，骨，軟骨，脂肪，心筋系列などの中胚葉系細胞に分化することができ，未分化状態で細胞を容易に増殖させることができる．[1] また，最近では，間葉系幹細胞は神経細胞，肝細胞，インスリン産生細胞などの外胚葉や内胚葉系の細胞へも分化するという報告もあり，再生医療や組織工学への応用が強く期待されている．間葉系幹細胞の分化を制御する手段の1つとして，細胞分化に関与する遺伝子を導入することが挙げられる．Ad ベクターを用いた間葉系幹細胞への遺伝子導入も試みられてきたが，間葉系幹細胞は Ad 受容体 CAR (coxsackievirus and adenovirus receptor) を発現していないためにその導入効率は極めて低く，遺伝子導入には高タイターのベクターを必要としていた．[2,3] われわれは，独自に開発した種々のファイバー改変型 Ad ベクターを用いて間葉系幹細胞にレポーター遺伝子を導入し，その発現効率を比較検討した．[4,5] 間葉系幹細胞を多く含有する画分であるマウス骨髄ストローマ細胞を用いて遺伝子導入効率を測定した結果，間葉系幹細胞にはファイバーにポリリジン残基を挿入した K7 型ベクターが最も適しており，従来型ベクターの 100 倍以上の遺伝子導入効率を示すことが明らかとなった [Figs. 1 (A) and (B)]．RGD 型ベクターは従来型ベクターに比較し 10 倍程度の導入効率を示した．また，種々のプロモーターを用いて比較検討したところ，CA プロモーターが最適であった [Fig. 1 (C)]．したがって，間葉系幹細胞には CA プロモーターを有する K7 型 Ad ベクターを用いることにより最も高効率に遺伝子導入できることが明らかとなった．間葉系幹細胞は様々な系列の細胞に分化するというだけではなく，担がんマウスに投与された場合には腫瘍に集積する性質を有している．[6] し

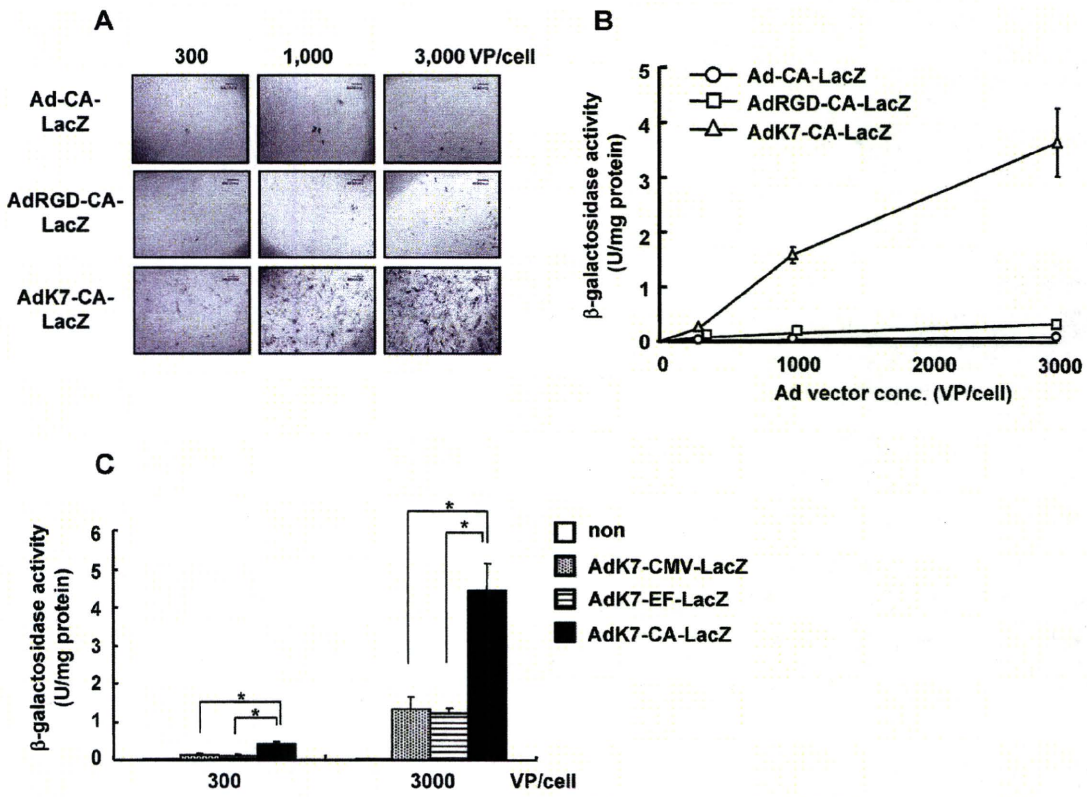

Fig. 1. Gene Transduction Efficiency in Mouse Primary BMSCs by Various Types of Ad Vectors

Mouse primary BMSCs were transduced with the indicated doses of LacZ-expressing Ad vectors for 1.5 h. Two days later, (A) X-gal staining and (B) luminescence assay were carried out. Similar results of X-gal staining were obtained in three independent experiments. Scale bar indicates 200 μm. (C) Optimization of promoter activity in BMSCs using LacZ-expressing AdK7. BMSCs were transduced with each Ad vector at 300 or 3000 VP/cell, and LacZ expression in the cells was measured. The data (B and C) are expressed as mean±S.D. (n=3). *p<0.01.

たがって，間葉系幹細胞は分化させた細胞自身を治療に利用するだけでなく，抗腫瘍性サイトカイン等を発現する間葉系幹細胞をがんに対する細胞治療薬として利用できる可能性があり，現在検討している．

3. 間葉系幹細胞の分化誘導

間葉系幹細胞への遺伝子導入にはCAプロモーターを有したK7型Adベクターが最適であることが明らかとなったので，次に本ベクターを用いて機能遺伝子を導入し分化誘導効率を向上させることを試みた．Runx2はRuntファミリーに属する転写因子であり，Runx2遺伝子欠損マウスは骨芽細胞分化が初期で停止し骨形成が欠損することが知られている．[7] そこで，Runx2遺伝子をマウス骨髄ストローマ細胞に導入した結果，従来の分化誘導法と比較しアルカリホスファターゼ活性の上昇及びカルシウムの沈着がみとめられ，著明に骨芽細胞への分化誘導効率が促進されることが明らかとなった［Figs. 2(A) and (B)］．また，それに伴い，タイプIコラーゲン，オステリクス，オステオカルシン等の骨芽細胞マーカー遺伝子の発現も上昇した［Fig. 2(C)］．さらに，このようにして分化誘導した骨芽細胞をマウスに移植したところ，Fig. 3に示すように異所性の骨形成がみとめられ，*in vivo*においても機能を有していることが示された．以上より，本ベクターは*in vitro*及び*in vivo*において間葉系細胞を効率よく骨芽細胞へ分化誘導するのに適したベクターであることが示された．

4. マウスES細胞及びマウスiPS細胞への遺伝子導入

ES細胞は胚盤胞内部細胞塊由来の細胞であり，無限に増殖するとともにすべての機能細胞に分化する性質を有する．一方，iPS細胞は体細胞に4種の遺伝子（Oct-3/4, c-myc, Sox2, Klf4）を同時に導入することにより得られる人工多能性幹細胞であり，倫理的な問題を回避できることから再生医療への応用が大きく期待されている．[8] しかしながら，これら幹細胞の分化を自由に制御する技術はいまだ確立されておらず，その原因の1つとして効率よい遺伝子導入法が確立されていないことが挙げられる．これまで，ES細胞に対しては，プラスミドDNAを用いたエレクトポレーション法（プラスミドDNAを電気的刺激により細胞内に導入し，染色体にわずかに目的遺伝子と薬剤耐性遺伝子が組み込まれた細胞を薬剤で選択する方法），[9] レトロウイルスベクター，[10] レンチウイルスベクター，[11] ポリオーマウイルスの複製機構を利用したスーパートランスフェクション法（ポリオーマウイルスの複製起点を含んだプラスミドDNAがマウスES細胞ではエピゾーマルに増幅できる性質を利用した方法）[12] などが外来遺伝子導入法として用いられてきた．しかしながら，これらは半永久的に導入遺伝子を発現し続ける方法であり，ES細胞やiPS細胞の分化制御，特に医療目的などの細胞分化後には発現を停止させたい場合には好ましくない．Adベクターは導入遺伝子が宿主染色体へ組込まれることなく，染色体外にエピゾームとして存在することから（増幅しない），遺伝子発現が一過性であり，ES細胞やiPS細胞を目的の機能細胞に分化させた後は導入遺伝子の発現が消失するものと期待される．そこで，筆者らは，マウスES細胞及びiPS細胞に最も適したAdベクターによる遺伝子導入法の確立を試みた．その結果，マウスES細胞やiPS細胞はAd受容体CARを高発現しており，従来型アデノウイルスベクターが最適であることが明らかとなった．[13,14] また，RSV, CMV, CA（β-actin promoter/CMV enhancer），EF-1αの4種のプロモーターを用いて検討した結果，ES細胞及びiPS細胞ではCA及びEF-1αプロモーターを用いた場合にのみ遺伝子発現がみられ，RSVやCMVプロモーターはほとんど機能しなかった［Fig. 4(A)］．これまでAdベクターはES細胞への遺伝子導入には不適と考えられてきたが，これは多くの場合，最も一般的に用いられているCMVプロモーターを用いて検討されてきたためであり，ウイルスの細胞へのエントリー自体には問題がないことが示された．ただし，CAプロモーターを用いた場合にはES細胞のみならずその支持細胞（フィーダー細胞）である胚繊維芽細胞にも遺伝子発現がみられたのに対し，EF-1αプロモーターを用いた場合にはほぼES細胞特異的に遺伝子発現可能であった．これは，EF-1αプロモーターの活性が胚繊維芽細胞に比べES細胞において相対的に高いことが原因と考えられる．したがって，目的により両プロモーターを使い分けることによって，再生医療への幅広い応用が期待できる．

Adベクターを導入することによりES細胞やiPS細胞が有する本来の性質が失われると再生医療

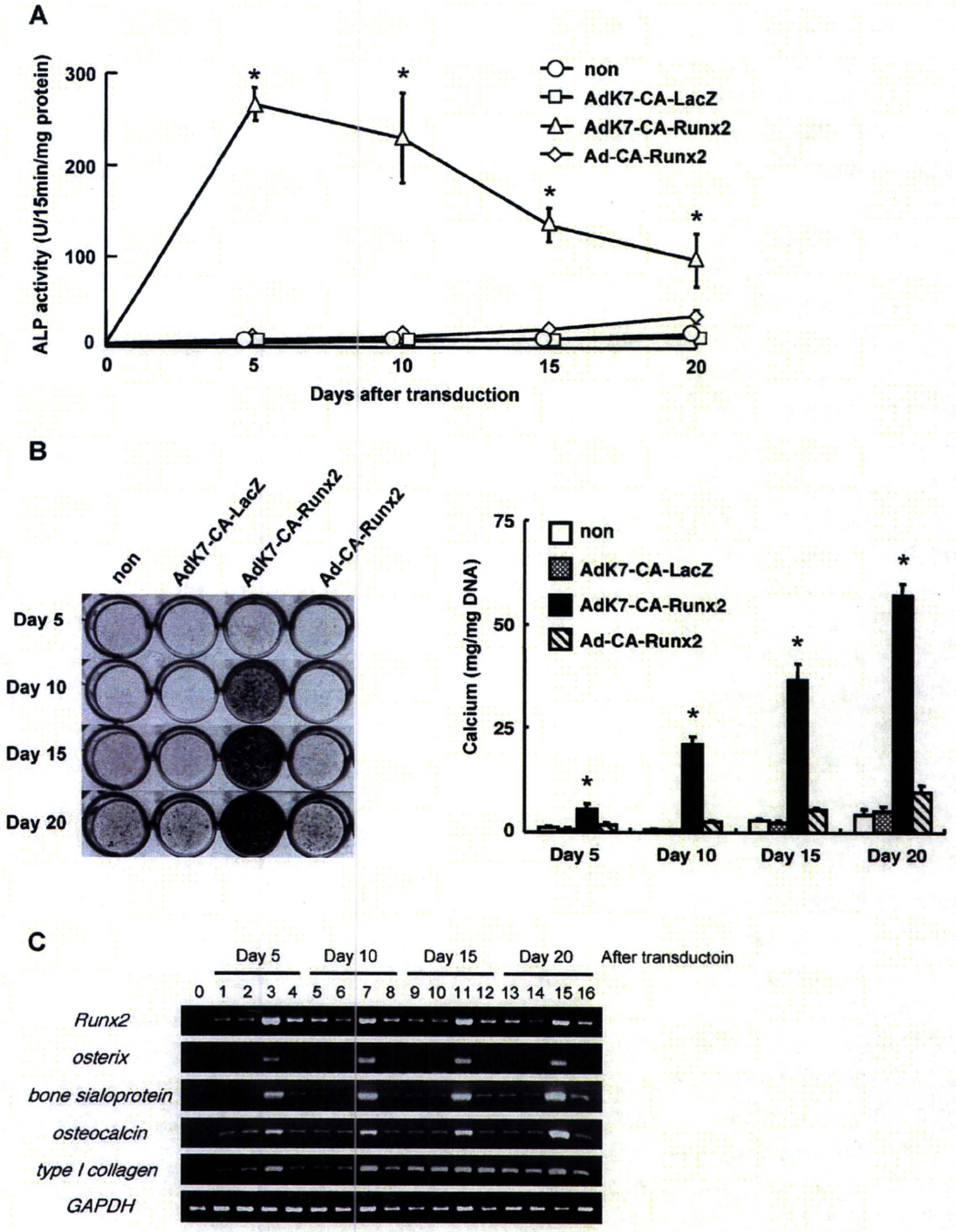

Fig. 2. Promotion of *in Vitro* Osteoblastic Differentiation in BMSC Transduced with AdK7-CA-Runx2

BMSCs were transduced with each Ad vector at 3000 VP/cell for 1.5 h, and were cultured for the indicated number of days. (A) Alkaline phosphatase activity, (B, left) matrix minerarization, and (B, right) calcium deposition in the cells was determined as described in Materials and Methods. The data are expressed as mean ± S.D. (n=3). *p<0.01 as compared with non-, AdK7-CA-LacZ-, or Ad-CA-Runx2-transduced cells. (C) RT-PCR was carried out using primers for Runx2, osterix, bone sialoprotein, osteocalcin, collagen type I, and GAPDH. Lane 0: non-treated BMSCs; lanes 1, 5, 9, and 13: BMSCs with osteogenic supplements (OS); lanes 2, 6, 10, and 14: BMSCs with OS plus AdK7-CA-LacZ; lanes 3, 7, 11, and 15: BMSCs with OS plus AdK7-CA-Runx2; lanes 4, 8, 12, and 16: BMSCs with OS plus Ad-CA-Runx2.

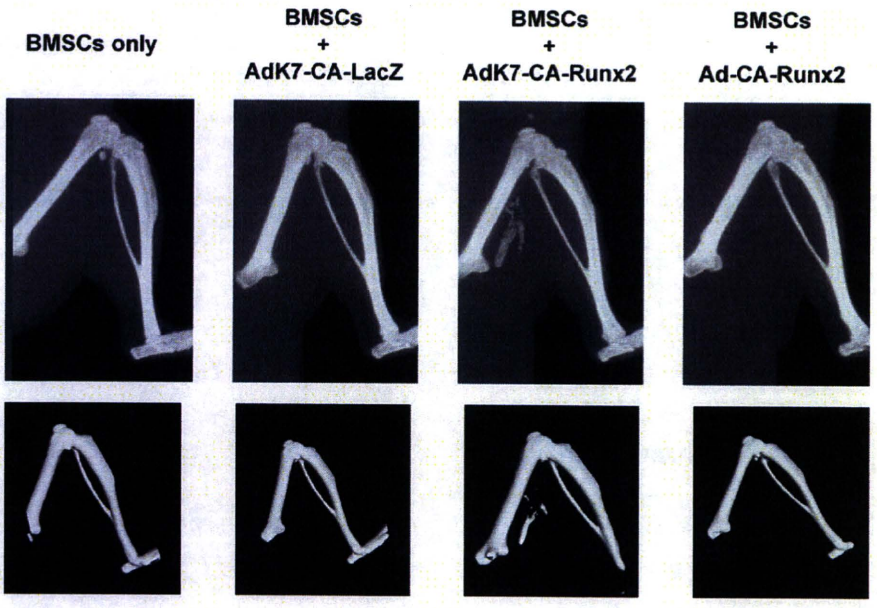

Fig. 3. *In vivo* Ectopic Bone Formation of Mouse BMSCs by AdK7-mediated Runx2 Gene Transduction

BMSCs were transduced with indicated Ad vectors at 3000 VP/cell for 1.5 h. On the following day, cells (2×10^6 cells) were suspended in PBS and injected into the hind limb biceps muscle of nude mice. Four weeks later, bone formation was analyzed by the microCT system. Similar results were obtained in two independent experiments. Upper, X-ray images; lower, 3D reconstitution images.

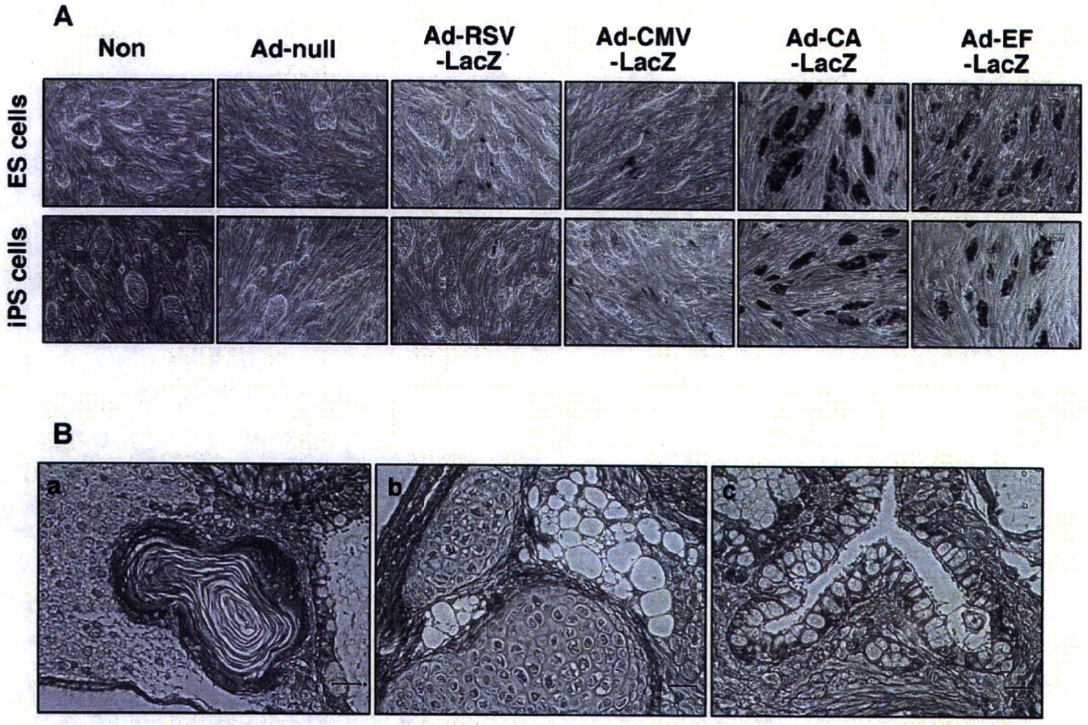

Fig. 4. Efficient Transgene Expression in Mouse iPS Cells by Using an Ad Vector Containing the CA and the EF-1α Promoter

(A) Mouse ES cells or iPS cells were transduced with LacZ-expressing Ad vector at 3000 VP/cell. On the following day, X-gal staining was carried out. Similar results of X-gal staining were obtained in three independent experiments. (B) Paraffin sections of the teratomas derived from Ad-CA-mCherry-transduced iPS cells were prepared, and sections were stained with hematoxylin and eosin. a, ectoderm (epidermis); b, mesoderm (cartilage and adipocyte); c, endoderm (gut epithelium).

への応用は困難となる．そこで，三胚葉への分化能が保持されているかどうかを検討するために，Adベクター導入後のiPS細胞を用いてテラトーマ形成実験を行った［Fig. 4(B)］．その結果，遺伝子導入されたiPS細胞はもとのiPS細胞と同様に外胚葉・中胚葉・内胚葉いずれにも分化可能であることが明らかとなり，Adベクターは ES細胞やiPS細胞の分化能を妨げることなく，効率よく遺伝子導入ができるベクターであることが明らかとなった．

5. 遺伝子導入によるES細胞の分化制御

最適化されたAdベクターを用いてES細胞に機能遺伝子を導入し，実際にES細胞の分化を制御できるかどうかについて検討した．マウスES細胞はフィーダー細胞由来のサイトカインLIF（leukemia inhibitory factor）がその未分化維持に必須であることが知られている．LIFは受容体に結合後，下流のSTAT3（signal transducer and activator of transcription 3）を介してシグナルを伝達する．そこで，EF-1αプロモーターを有した従来型Adベクターを用いてSTAT3のdominant-negative変異体(STAT3F)のcDNAをマウスES細胞に導入することにより，LIFの下流シグナルを阻害させたところ，LIF存在下でもES細胞は三胚葉すべての細胞に分化することが明らかとなった［Fig. 5(A)］．ES細胞の未分化維持にはLIF以外にもNanogなどの転写因子が必須であることが明らかとなっている．そこで，先述のベクターを用いてSTAT3FとNanogを同時に発現させたところ，STAT3Fによる細胞分化シグナルがNanog発現により阻害され，ES細胞は未分化状態を維持し続けた［Fig. 5(B)］．したがって，Adベクターを用いることでES細胞の分化を自由に制御できる可能性が示された．

6. ES細胞及びiPS細胞の高効率分化誘導

Adベクターを用いた遺伝子導入技術が分化誘導系に応用できるかどうかを検討するために，マウスES細胞及びiPS細胞へ機能遺伝子を導入することを試みた．ES細胞及びiPS細胞からの分化モデルとして脂肪細胞への分化誘導を行うとともに，脂肪細胞分化に必須の遺伝子であるPPARγ（peroxisome proliferator-activated receptor gamma）遺伝子[15]をES細胞及びiPS細胞へ導入することにより，脂肪細胞への分化効率が上昇するかどうかを検討した．両細胞へLacZ遺伝子（コントロール）又

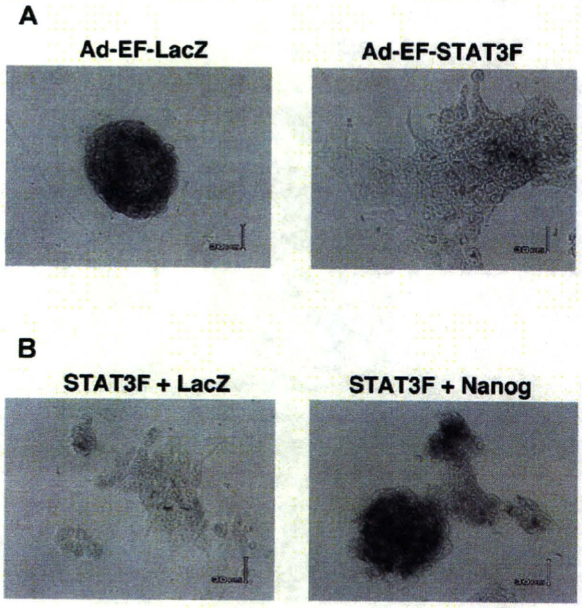

Fig. 5. Introduction of Functional Genes into Mouse ES Cells by Ad Vectors Containing EF-1α Promoter

Mouse ES cells (1×10^4 cells) were seeded, and on the following day, the cells were transduced with 3000 VP/cell of Ad-EF-LacZ or Ad-EF-STAT3F for 1.5 h (A). Mouse ES cells were also co-infected with 3000 VP/cell of Ad-EF-STAT3F and 3000 VP/cell of Ad-EF-lacZ or Ad-EF-Nanog for 1.5 h (B). On day 3, each cell was infected again by the same vectors. On day 5, alkaline phosphatase staining was performed. Alkaline phosphatase-positive cells indicate undifferentiated ES cells. Similar results were obtained in three independent experiments.

はPPARγ遺伝子を導入し，脂肪細胞分化用の液性因子（インスリン，デキサメタゾンなど）を含む培地中で15日間接着培養した．オイルレッドO染色により脂肪細胞への分化効率を評価した結果，iPS細胞の脂肪細胞への分化効率はES細胞の分化効率よりも低いものの，液性因子を作用させて培養することにより両細胞とも脂肪滴が観察された［Fig. 6(A)］．さらに，液性因子のみを用いた分化誘導法と比較し，液性因子を加えさらにAdベクターによるPPARγ遺伝子を導入したES細胞及びiPS細胞は，極めて効率よく脂肪細胞へ分化していることも示された．すなわち，従来の方法ではES細胞において約50%の細胞が，iPS細胞において20–30%の細胞がそれぞれオイルレッドOで染色されたのに対し，PPARγ遺伝子を導入したES細胞及びiPS細胞においては80–90%の細胞がオイルレッドOに染色された．また，Adベクターを用いたPPARγ遺伝子導入による脂肪細胞への分化効率の上昇は，脂肪細胞特異的なマーカー遺伝子の発現上昇によっても確認された．なお，LacZ遺伝子を導

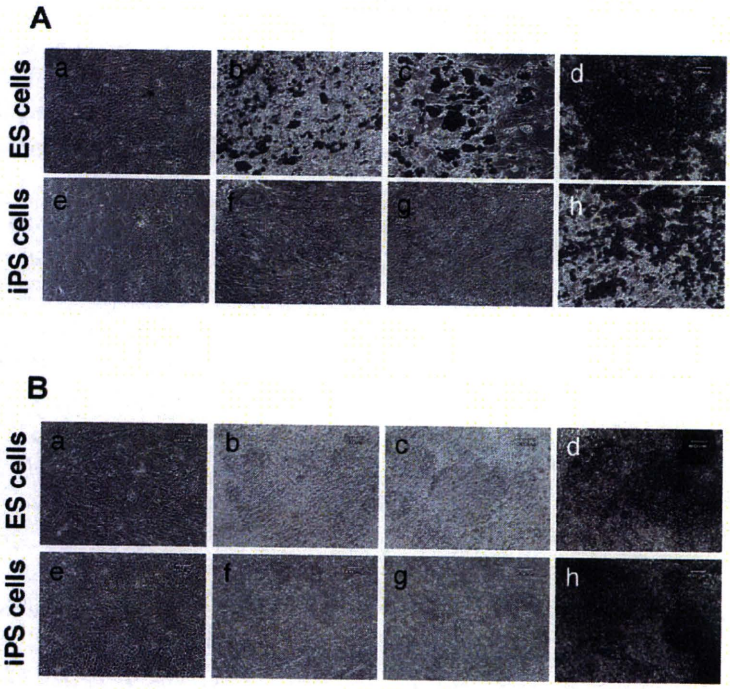

Fig. 6. Efficient Adipocytic or Osteoblastic Differentiation from Mouse ES Cells and iPS Cells by the Transduction of the PPARγ or Runx2 Gene

(A) ES-EBs or iPS-EBs were transduced in triplicate with 10000 VP/cell of Ad-CA-LacZ or Ad-CA-PPARγ. After plating onto a gelatin-coated dish on day 7, ES-EBs and iPS-EBs were cultured for 15 days in the presence or absence of adipogenic supplements (AS). After cultivation, lipid accumulation was detected by oil red O staining. a, non-treated ES-EBs; b, ES-EBs with AS; c, ES-EBs with AS plus Ad-CA-LacZ; d, ES-EBs with AS plus Ad-CA-PPARγ; e, non-treated iPS-EBs; f, iPS-EBs with AS; g, iPS-EBs with AS plus Ad-CA-LacZ; h, iPS-EBs with AS plus Ad-CA-PPARγ. The scale bar indicates 60 μm. (B) ES-EBs or iPS-EBs were transduced in triplicate with 10000 VP/cell of Ad-CA-LacZ or Ad-CA-Runx2. After culturing for 15 days with or without osteogenic supplements (OS), matrix mineralization in the cells was detected by von Kossa staining. a, non-treated ES-EBs; b, ES-EBs with OS; c, ES-EBs with OS plus Ad-CA-LacZ; d, ES-EBs with OS plus Ad-CA-Runx2; e, non-treated iPS-EBs; f, iPS-EBs with OS; g, iPS-EBs with OS plus Ad-CA-LacZ; h, iPS-EBs with OS plus Ad-CA-Runx2. The scale bar indicates 60 μm.

入した細胞ではこのような分化効率の上昇はみとめられなかった．これらの結果から，マウス ES 細胞及び iPS 細胞の脂肪細胞への分化効率は Ad ベクターを用いた PPARγ 遺伝子の導入により改善できることが示された．

次に，Ad ベクターによる遺伝子導入がその他の細胞種への分化誘導系にも有用であるかどうかを検討するため，マウス ES 細胞及び iPS 細胞から骨芽細胞への分化誘導を試みた．今回の分化誘導系においては前述した Runx2 遺伝子を Ad ベクターにより発現させた．Ad-CA-Runx2 を EB（embryoid body）へ 3 回作用させ，骨芽細胞分化用の液性因子（β グリセロリン酸，アスコルビン酸など）中で接着培養し，マウス ES 細胞及び iPS 細胞由来の細胞が石灰化を生ずる骨芽細胞へ分化しているかどうかを von Kossa 染色により解析した．その結果，液性因子を作用させることにより石灰化が検出されたものの，その分化効率は低く，20%以下の細胞しか石灰化を起こしていなかった[Fig. 6(B)]．一方，Runx2 遺伝子を導入した ES 細胞及び iPS 細胞は，液性因子のみで培養した細胞及び LacZ 遺伝子を導入した細胞と比較し，石灰化した細胞が著明に増加していること明らかとなった［Fig. 6(B)］．また，沈着した石灰化を定量化したところ，LacZ 遺伝子導入群においては液性因子のみの誘導法と著差はみとめられなかったものの，Runx2 遺伝子導入細胞においては約 8 倍，石灰化が上昇していた．これらの結果から，Ad ベクターによる Runx2 遺伝子の導入によりマウス ES 細胞及び iPS 細胞から骨芽細胞へ効率よく分化誘導可能であることが示された．このように最適化した Ad ベクターを用いたマウス ES 細胞及び iPS 細胞への分化関連遺伝子の導入により，脂肪細胞及び骨芽細胞への分化効率を飛躍的に改善できることが示され，本遺伝子導入技術はマウス ES 細胞及び iPS 細胞を用いた細胞分化研究に有用であると考えられた．

7. おわりに

これまでにもレトロウイルスベクターなどの恒常的遺伝子発現系を用いてES細胞から標的の細胞への分化誘導は行われてきた．しかしながら，これらの手法では機能遺伝子が染色体に挿入されるため治療用に適しておらず，一過性の遺伝子導入系の分化誘導が望まれていた．今回，われわれはAdベクターを用いたマウスES細胞及びiPS細胞への高効率遺伝子導入法を確立し，さらにその遺伝子導入技術を利用して分化関連遺伝子をマウスES細胞及びiPS細胞へ導入することにより特定の細胞へ効率よく分化誘導することに成功した．なお，異なるiPS細胞株についても今回と同様の結果が得られており，[14] 幅広いES細胞及びiPS細胞株に適用可能であることが示唆されている．また，今回は示していないが，分化が完了した細胞ではAdベクター由来の遺伝子発現はほとんどみとめられないことも確認している．[16] したがって，Adベクターは幹細胞を用いた分化誘導研究において，効率面だけでなく安全面においても非常に有用であると考えられる．現在，筆者らのグループではAdベクターを用いた遺伝子導入技術がその他の細胞種への分化誘導系へ応用可能かどうかに関して，マウス及びヒトのES細胞及びiPS細胞を用いて検討中である．一過性発現を示すAdベクターを用いた遺伝子導入技術は，幹細胞研究・再生医療研究において重要なツールになるものと考えられ，今後のますますの応用が期待される．

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再生医療・細胞治療の規制等に関する欧米の動向
― 臨床応用に関する規制当局の支援の比較 ―

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1―はじめに：細胞・組織加工製品

ヒトの臓器や組織の確保が難しいわが国の状況下において、重篤で生命を脅かす疾患や身体の機能を著しく損なう病態などのうち、治療法に乏しいものに対しての再生医療や細胞治療の実用化を望む声が高まっている。これらの医療に用いることを目的として加工（培養・活性化・足場との複合化等）を施された細胞や組織、あるいは加工された細胞・組織を含む製品は「細胞・組織加工製品」（細胞・組織加工医薬品ないし細胞・組織加工医療機器）と呼ばれ、その開発では世界的にも熾烈な競争が展開されている。ただし細胞・組織加工製品は、細胞という動的で複雑な成分を含むと同時に、製品の態様や特性、臨床上の適用法は多種多様であり、また、その臨床応用に関して限られた経験と知識しか存在しないため、科学的根拠に基づいた品質や安全性等の確保のあり方や開発の合理的な進め方が課題となっている。比較的進んでいると言われる欧米においても、当局は細胞・組織加工製品の実用化を促進するための試行を繰り返しながら規制の枠組みの整備を進めている。

2―リスクベースアプローチ

細胞・組織加工製品は品目ごとの多様性が高いため、欧米では規制や開発の方針・内容を定めるために、それぞれの製品の性質に固有、かつその品質・安全性・有効性に関連するリスクの分析をベースにし、その影響の度合いを科学的に評価するアプローチ方法が採られている[1,2]。これを「リスクベースアプローチ」と呼ぶ。日本では、細胞・組織加工製品を医薬品・医療機器として開発することを目的として薬事法に則って実施される「治験」と、細胞・組織加工製品を用いた治療法の開発を目的として医療法・医師法のもとで行われる「臨床研究」という異なる規制の枠組みが存在する。一方、欧米ではリスクベースアプローチの原則に基づき、商業目的か非商業目的かに拘わらず、原則的には同一の規制がかかる。即ち、大学病院等による非商業目的の「臨床研究」においても国への臨床試験申請並びにICHのGCPへの準拠が要求される点で日本よりも厳しい制度となっている。ただ、日本と比較した場合には、より多くの研究費・開発資金が確保できること、臨床試験のコストが低いこと、臨床試験の公的ネットワークによる臨床試験支援体制およびコンサルタント・CROなどの支援企業が充実していることのほか、規制当局が開発早期から開発者と情報を共有し、製品の目的に沿った柔軟な対応が可能となっていることなど、様々な利点がある。

3―米国の規制

米国ではヒト細胞・組織を利用した製品は遺伝子治療薬と併せてHCT/P（human cell, tissue, or cellular/tissue-based product）と総称される。中でも細胞に一定以上の加工を施したものや遺伝子治療薬などは、公衆衛生サービス法351条により食品医薬品局（FDA）の販売承認が必要とされ、351HCT/Pと呼ばれる（表1）。

1）351HCT/Pの区分

351HCT/Pはその主な作用様式が細胞・組織の生化学的・免疫学的又は代謝的機能に基づく場合には生物製剤、細胞・組織の物理的又は構造的機能の場合には医療機器としての規制を受ける。これを主作用様式の原則と言う[4]。これまでに2品目が生物製剤、5品目が医療機器として販売承認を受けている。

2）相談制度

近年の351HCT/Pの臨床試験申請の過半数は大学などによる非商業目的のものであるが、FDAは臨床試験の申請前に非公式な相談を無料で行うほか、各種の開発段階において相談制度を設けて351HCT/Pの開発を支援している。

生物製剤の開発者とFDAとの相談は大きくタイプA、B、Cの3種に分けられる。タイプAは見解の相違や試験中断等に関する緊急時の相談、タイプBは製品