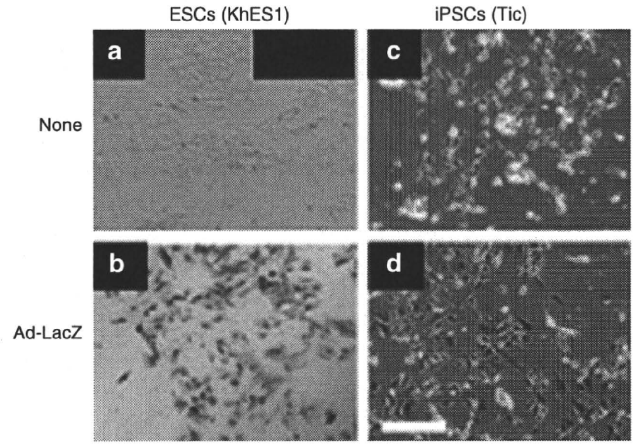

Figure 3 Efficient transgene expression in the human ESC (khES1)- and iPSC (Tic) derived definitive endoderms by using a fiber-modified Ad vector containing the EF-1α promoter. (**a,b**) Human ESC (khES1)-derived and (**c,d**) iPSC (Tic) derived definitive endoderms were transduced with 3,000VP/cell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

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-

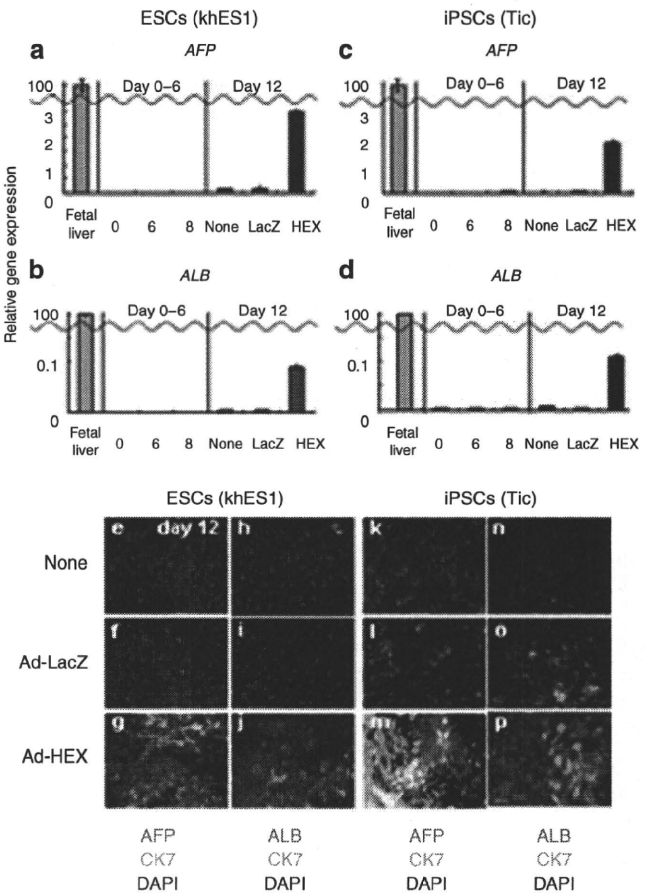

Figure 4 Efficient hepatoblast differentiation from the human ESC (khES1)- and iPSC (Tic) derived definitive endoderms by transduction of the *HEX* gene. (**a–d**) Real-time RT-PCR analysis of the level of (**a,c**) *AFP* and (**b,d**) *ALB* expression in nontransduced cells, Ad-LacZ-transduced cells, 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 = 50μm. Ad, adenovirus; AFP, α-fetoprotein; ALB, albumin; CK7, cytokeratin 7; HEX, Ad-HEX-transduced cells; ESC, embryonic stem cells; iPSC, induced pluripotent stem cell; LacZ, Ad-LacZ-transduced cells; None, nontransduced cells.

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

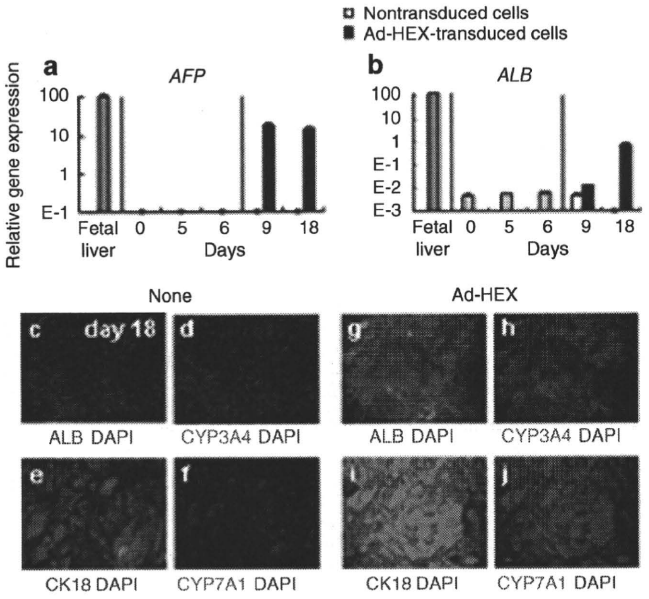

Figure 5 Efficient differentiation of Ad-HEX-transduced hepatoblasts into hepatocytes. (**a**,**b**) Real-time RT-PCR analysis of (**a**) *AFP* and (**b**) *ALB* expression in nontransduced cells and Ad-HEX-transduced cells, both of which were induced from the human iPSC (Tic) derived definitive endoderm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

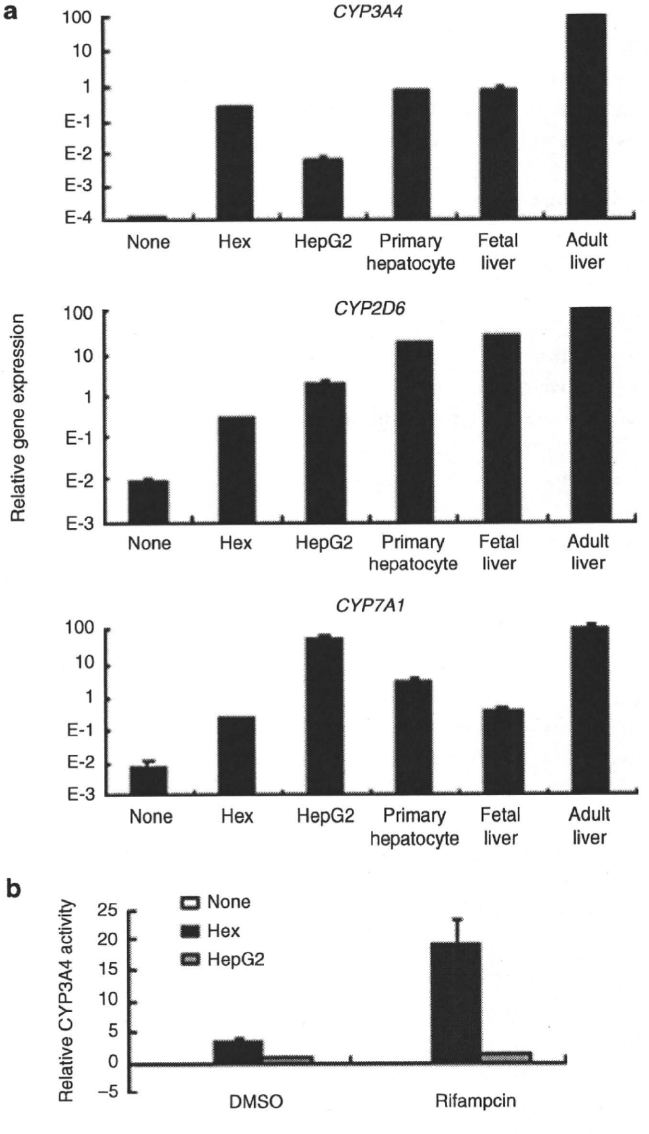

Figure 6 Cytochrome P450 isozymes in human iPSC (Tic) derived hepatocytes. (**a**) Real-time RT-PCR analysis of *CYP3A4*, *CYP7A1*, and *CYP2D6* expression in iPSC (Tic) derived nontransduced cells, Ad-HEX-transduced cells, and fetal and adult liver tissues. (**b**) Induction of CYP3A4 by rifampicin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

ACKNOWLEDGMENTS

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Chemical inhibition of sulfation accelerates neural differentiation of mouse embryonic stem cells and human induced pluripotent stem cells

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ABSTRACT

Pluripotency of embryonic stem cells (ESCs) is maintained by the balancing of several signaling pathways, such as Wnt, BMP, and FGF, and differentiation of ESCs into a specific lineage is induced by the disruption of this balance. Sulfated glycans are considered to play important roles in lineage choice of ESC differentiation by regulating several signalings. We examined whether reduction of sulfation by treatment with the chemical inhibitor chlorate can affect differentiation of ESCs. Chlorate treatment inhibited mesodermal differentiation of mouse ESCs, and then induced ectodermal differentiation and accelerated further neural differentiation. This could be explained by the finding that several signaling pathways involved in the induction of mesodermal differentiation (Wnt, BMP, and FGF) or inhibition of neural differentiation (Wnt and BMP) were inhibited in chlorate-treated embryoid bodies, presumably due to reduced sulfation on heparan sulfate and chondroitin sulfate. Furthermore, neural differentiation of human induced pluripotent stem cells (hiPSCs) was also accelerated by chlorate treatment. We propose that chlorate could be used to induce efficient neural differentiation of hiPSCs instead of specific signaling inhibitors, such as Noggin.

1. Introduction

Embryonic stem cells (ESCs) [1–3] are promising tools for biotechnology and possess key features that should allow their exploitation in the development of cell replacement therapies [4]. Extrinsic signaling pathways are key mechanisms for determining ESC cell fate, and sulfated glycans, such as heparan sulfate (HS), are well known regulators of signal transduction [5]. HS chains are present abundantly on the cell surface of undifferentiated mouse ESCs (mESCs) and functional roles of HS chains have been demonstrated [6,7]. Thus, the modification of sulfated glycans is an attractive approach for developing methods to regulate ESC differentiation.

Sulfated glycans are synthesized in the Golgi as follows. The high energy sulfate donor 3′-phosphoadenosine 5′-phosphosulfate (PAPS), which is required as a substrate for sulfation, is synthesized in the cytosol and nucleus by PAPS synthetase [8], and subsequently is translocated via the PAPS transporter (PAPST) into the Golgi [9–13], where it is used by sulfotransferases to sulfate glycans. Recently, we have demonstrated that the reduction of sulfation by knockdown of *PAPST* using vector-based RNA interference (RNAi) promotes neurogenesis of mESCs [13]. However, the rapid, simple, and safety method for modification of sulfated glycans instead of gene transfer is desired particularly for application of human ESCs (hESCs) to regenerative medicine.

Chlorate inhibits ATP sulfurylase activity of PAPS synthetases by competing with sulfate ions in binding to ATP-sulfurylase resulting in reduction of PAPS [14]. Thus, inhibition of PAPS synthesis by chlorate leads to reduction of sulfation on several sulfated proteins, glycoproteins, glycolipids, and proteoglycans [15,16].

Since induced pluripotent stem cells (iPSCs) were reported [17], the application of human iPSCs (hiPSCs) to regenerative medicine has been expected. In particular, neural induction of hiPSCs is an important research tool for several neural diseases and has been applied to cell replacement therapies. Therefore, methods of efficient and rapid neural induction are required [18]. In this study, we examined whether neural differentiation of hiPSCs in addition to mESCs was enhanced by the chemical down-regulation of sulfation with chlorate.

2. Materials and methods

2.1. Cell culture

R1 [19] and E14TG2a [20] mESC lines were maintained on mouse embryonic fibroblasts (MEFs) inactivated with 10 μg/ml

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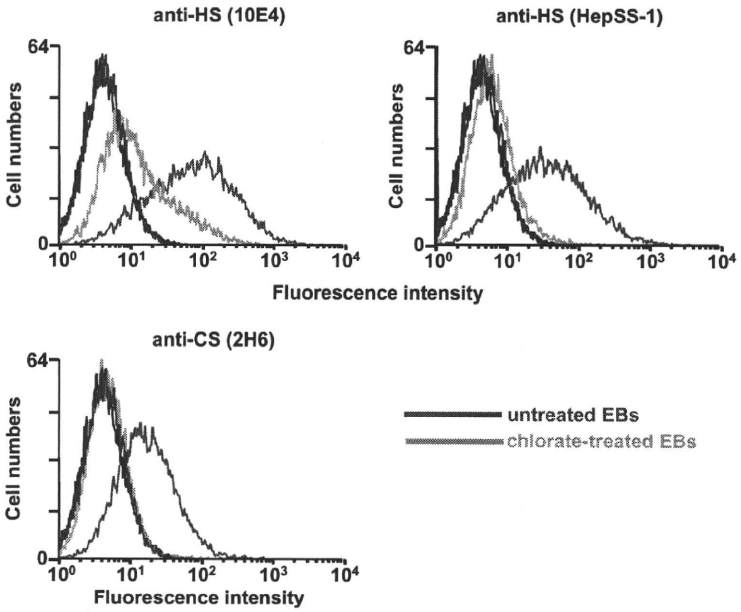

Fig. 1. Chlorate treatment reduces sulfation of HS and CS in EBs. FACS analysis of cells 3 days after EB formation using an anti-HS antibody (10E4 or HeppSS-1) or an anti-CS antibody (2H6) (black and blue lines represent the IgM isotype control for untreated and chlorate-treated EBs, respectively). Chlorate treatment was performed for 24 h from 2 days after EB formation. Three independent experiments were performed and representative results are shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

mitomycin C (Sigma) in ESC medium (DMEM supplemented with 15% FBS {Hyclone}, 1% penicillin/streptomycin {Gibco}, 0.1 mM 2-mercaptoethanol {Gibco}, and 0.1 mM non-essential amino acids {Gibco}) with 1000 U/ml LIF (Chemicon). hiPSC clones (MRC-hiPS_Fetch {NIHS0604} and MRC-hiPS_Tic {JCRB1331}) [21] were maintained on inactivated MEFs in iPSellon (Cell-Sight) with 10 ng/ml bFGF (Wako). All mESC experiments were performed using the R1 line and most results were confirmed using the E14TG2a line.

For embryoid body (EB) formation, mESCs or hiPSCs were transferred to low cell binding 60 mm dishes (Nunc) and cultured in ESC medium without LIF or iPSellon without bFGF, respectively. Before EB formation, hiPSCs were preplated on gelatin-coated dishes to remove feeder cells. For neuronal differentiation, 1 μM all-trans retinoic acid (RA) (Sigma) was added 4 days after EB formation. Then, 5 days after EB formation, EBs were plated onto PDL/laminin-coated 60 mm dishes (Becton Dickinson) in DMEM-F12 containing N2 supplement (Gibco).

To down-regulate sulfation, 50 mM chlorate (Sigma) was added from 2 days after EB formation throughout EB culture.

2.2. FACS analysis

FACS analysis was performed 3 days after EB formation. After EDTA treatment, the cell suspension was incubated with primary antibodies diluted in FACS buffer (0.5% bovine serum albumin and 0.1% sodium azide in PBS). After washing, the cell suspension was incubated with FITC-conjugated secondary antibody (Sigma) diluted in FACS buffer. Cell sorting and analysis were performed using a FACSAria Cell Sorter (Becton Dickinson). Primary antibodies were as follows: mouse IgM isotype control (Chemicon), the anti-HS antibody 10E4 (Seikagaku Corp.), the anti-HS antibody HepSS-1 (Seikagaku Corp.), the anti-chondroitin sulfate (CS) antibody 2H6 (Seikagaku Corp.).

2.3. Immunoblotting

Cells were lysed with lysis buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM Na_3VO_4, 10 mM NaF, protease inhibitors). Isolation of nuclear extracts was performed as described previously [6]. Samples containing 5 μg of cell lysate or nuclear extract were separated by 10% SDS–PAGE and transferred onto PVDF membranes (Millipore). After blocking, the membranes were incubated with primary antibodies as follows: anti-ERK1/2 (Cell Signaling Technology), anti-phosphorylated ERK1/2 (Thr183/185; Cell Signaling Technology), anti-phosphorylated Smad1 (Ser463/465; Cell Signaling Technology), anti-β-actin (Sigma), anti-β-catenin (Cell Signaling Technology), anti-lamin B_1 (Zymed), and anti-βIII-tubulin (Chemicon). The membranes were then incubated with the appropriate peroxidase-conjugated secondary antibodies (Cell Signaling Technology), washed and developed with ECL Plus reagents (GE Healthcare).

2.4. Immunostaining

After neural differentiation on PLL/laminin-coated glass chamber slides (Iwaki), cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% saponin. After washing and subsequent blocking, cells were stained with an anti-βIII-Tubulin antibody. After washing, cells were stained with an FITC-conjugated secondary antibody and counterstained with propidium iodide (PI). Immunofluorescence images were obtained using an LSM5Pascal confocal laser scanning microscope (Carl Zeiss).

2.5. Real-time PCR

Real-time PCR was performed as described previously [6]. For hiPSCs, FastStart Universal SYBR Green Master (Roche) was used. Primer sets and probes are listed in Supplementary Table 1.

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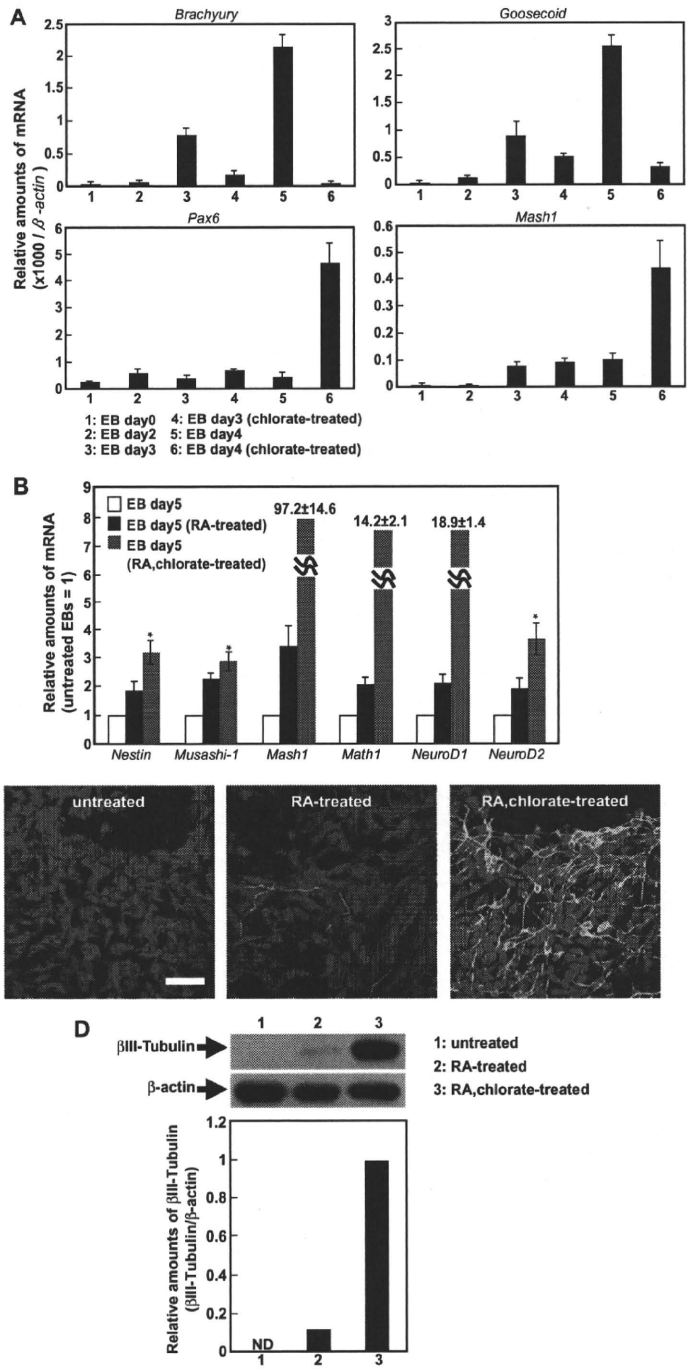

Fig. 2. Reduction of sulfation accelerates neural differentiation of mESCs. (A) Real-time PCR analysis of markers of mesoderm (*Brachyury*, *Goosecoid*) and ectoderm (*Mash1*, *Pax6*) in EBs harvested on each day shown. Relative amounts of each mRNA were calculated after normalization to *β-actin* mRNA. The values shown are the means ± SD of three independent experiments. (B) Real-time PCR analysis of neural markers, such as neural stem/progenitor cell markers (*Nestin*, *Musashi-1*) and proneural markers (*Mash1*, *Math1*, *NeuroD1* and *NeuroD2*), 5 days after EB formation. The results are shown after normalization against the values obtained with untreated EBs (value = 1). The values shown are the means ± SD of duplicate measurements from two independent experiments and significant values are indicated; $^{*}P < 0.05$, compared with the RA-treated EBs. (C) Immunocytochemical staining 2 days after replating of EBs. Representative confocal images from two independent experiments are shown (βIII-tubulin, green; PI, red). Scale bar, 20 μm. (D) Western blotting 2 days after replating of EBs. Representative immunoblots from two independent experiments are shown. The histogram shows mean densitometric readings of βIII-tubulin/loading controls. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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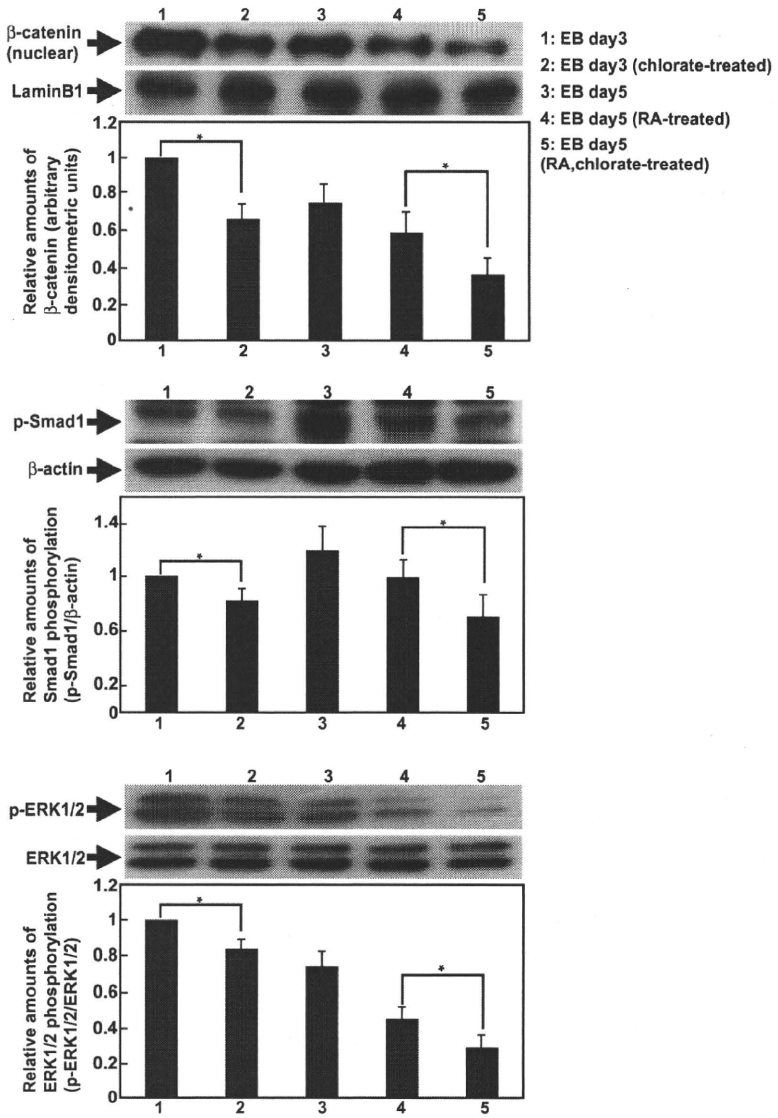

Fig. 3. Signaling via several pathways is decreased in chlorate-treated EBs. Western blotting of downstream signaling components 3 or 5 days after EB formation. Representative immunoblots from two independent experiments are shown. The histograms show mean densitometric readings ± SD of β-catenin or the phosphorylated proteins/loading controls after normalization against the values obtained with untreated EBs cultured for 3 days (value = 1). Values were obtained from duplicate measurements of two independent experiments and significant values are indicated; $^*P < 0.05$.

3. Results and discussion

Firstly, we examined whether sulfation of glycans in EBs was reduced by chlorate treatment. HS and CS are functionally important sulfated glycans that are expressed until at least 8 days after EB formation in mESCs [13]. Thus, we focused on HS and CS in this study. Three days after EB formation, FACS analysis of chlorate-treated EBs revealed poor staining by the anti-HS and the anti-CS antibodies, indicating that sulfation on HS and CS was reduced (Fig. 1).

Signaling by both Wnt and BMP is essential for the choice of lineage between mesoderm and ectoderm: the reduction of both Wnt and BMP signaling inhibits mesodermal differentiation and enhances ectodermal differentiation [22,23]. Thus, we assumed that a reduction in Wnt and BMP signaling at the point when mesoderm starts to differentiate (2–4 days after EB formation) would accelerate neural differentiation. Therefore, we examined EB differentiation in response to chlorate treatment 2 days after EB formation. Real-time PCR analysis at 0–4 days after EB formation showed that the expression of early mesoderm markers (*Brachyury*, *Goosecoid*) did not increase in a time-dependent manner in chlorate-treated EBs, although it did in untreated EBs (Fig. 2A). In contrast, expression of ectoderm markers (*Mash1*, *Pax6*) was higher in chlorate-treated EBs than in untreated EBs at 4 days after EB formation (Fig. 2A). These results indicate that chlorate treatment from 2 days after EB formation affected lineage choice between mesoderm and ectoderm: it inhibited mesodermal differentiation but induced ectodermal differentiation. As shown in

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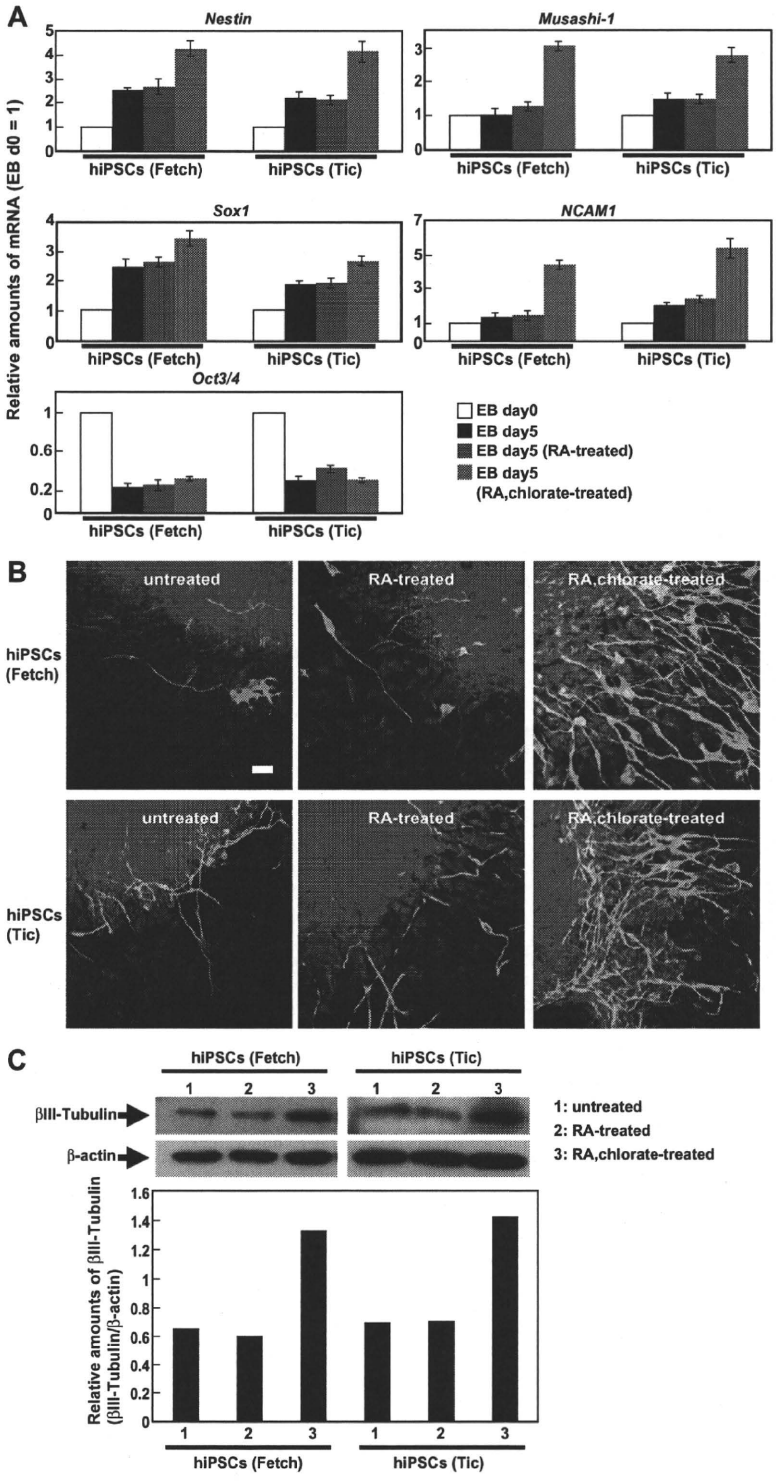

Fig. 4. Reduction of sulfation accelerates neural differentiation of hiPSCs. (A) Real-time PCR analysis of neural markers (*Nestin*, *Musashi-1*, *NCAM1* and *Sox1*) and an undifferentiated cell marker (*Oct3/4*) 5 days after EB formation. The relative amounts of each mRNA were normalized by *GAPDH* mRNA. The results are shown after normalization against the values obtained with undifferentiated hiPSCs (value = 1). The values shown are the means ± SD of duplicate measurements from representative experiments. Two independent experiments were performed. (B) Immunocytochemical staining 2 days after replating of EBs. Representative confocal images from two independent experiments are shown (βIII-tubulin, green; PI, red). Scale bar, 20 μm. (C) Western blotting 2 days after replating of EBs. Representative immunoblots from two independent experiments are shown. The histogram shows mean densitometric readings ± SD of βIII-tubulin/loading controls. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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Fig. 3, Wnt/β-catenin, BMP/Smad, and FGF/ERK signaling, which are involved in the mesodermal differentiation of mESCs [24], were reduced in chlorate-treated EBs. Therefore, the inhibition of these signaling pathways by chlorate treatment seemed to cause defects in mesodermal differentiation and induced ectodermal differentiation. Chlorate treatment from the start of EB formation inhibited initial differentiation, as reported previously (data not shown, [25]). Thus, chlorate treatment from the appropriate time induces differentiation into a specific lineage, in particular neural differentiation.

Next, we investigated further the neural differentiation of chlorate-treated EBs. Firstly, we examined the expression of neural differentiation markers (Fig. 2B). Even at 5 days after EB formation, the expression levels of these markers were significantly higher in chlorate-treated EBs than in EBs only treated with RA, which indicated that differentiation into neural stem/neural progenitor cells and neural precursor cells was accelerated by chlorate treatment. These results were thought to be due to the reduction of Wnt and BMP signaling as shown in Fig. 3. Because, signaling by BMP and Wnt inhibits the neurogenesis of mESCs via EB formation [26,27]. Secondly, we examined the ability of chlorate-treated EBs to differentiate into mature neurons. Immunocytochemical staining for the mature neuron marker βIII-tubulin showed that only 2 days after plating, chlorate-treated EBs generated extremely dense networks of neurite outgrowths, which were not seen with EBs only treated with RA (Fig. 2C). We confirmed by western blotting that the level of βIII-tubulin in chlorate-treated cells was significantly higher than that in cells only treated with RA (Fig. 2D). Taken together, these results demonstrated that the reduction of sulfation by chlorate treatment accelerated the neurogenesis of mESCs.

As described above, the reduction of sulfation on HS and CS by chlorate treatment (Fig. 1) caused defects in Wnt/β-catenin, BMP/Smad, and FGF/ERK signaling during EB formation (Fig. 3), which was followed by the acceleration of neural differentiation (Fig. 2). These results are consistent with previous report [13], in which reduction of PAPST-dependent sulfation promotes neurogenesis of mESCs due to the decreased sulfation on HS and CS.

To date, several signaling pathways have been shown to contribute to the neural differentiation of hESCs and hiPSCs [18,28]. The BMP inhibitor Noggin and the inhibitor of the Lefty/Activin/TGFβ pathways SB431542 enhance neural differentiation [18]. Thus, we examined whether the inhibition of signaling pathways by chlorate treatment also enhanced the neural differentiation of hiPSCs. Real-time PCR analysis 5 days after EB formation showed that the expression of several neural stem/progenitor cell markers was higher in chlorate-treated EBs than in EBs only treated with RA, whereas expression of the marker of undifferentiated cells, *Oct3/4*, was reduced equally in both types of EBs (Fig. 4A). These results indicate that chlorate treatment accelerated the neural differentiation of hiPSCs. Furthermore, as shown in Fig. 4B and C, differentiation into mature neurons was induced markedly in chlorate-treated EBs.

In conclusion, we have demonstrated that the down-regulation of sulfation by chlorate treatment could accelerate neural differentiation of hiPSCs as well as mESCs. This acceleration was induced by the addition of chlorate at the correct time, and involved the inhibition of signaling pathways involved in the induction of mesodermal differentiation (Wnt, BMP, and FGF) and inhibition of neural differentiation (Wnt and BMP). This study as well as our previous report [13] have demonstrated that reduction of sulfation is effective to induce neural differentiation compared with the standard method using RA. In our previous report [13], we used RNAi method using retrovirus vector for long-term neural differentiation. This method requires great care and may give damages to the transfected cells. Moreover, the reduction of sulfation is more drastic in chlorate-treated cells than in *PAPST*-knockdown cells. This reflects more rapid induction of neural differentiation in chlorate-treated cells as shown in this report than in *PAPST*-knockdown cells. Furthermore, in hiPSCs including hESCs, efficiency of gene transfer is very low and for clinical application gene transfer should be avoided. Therefore, chlorate is useful for rapid, simple, and safety reduction of sulfation for rapid neural differentiation of hiPSCs, possibly including hESCs. Taken together, we propose that chlorate, which is available at low cost, could be used to achieve efficient and rapid neural induction of hiPSCs as well as mESCs in place of expensive signaling inhibitors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.09.085.

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V. 参考資料

astellas

日本標準商品分類番号
87799、876349

2010年4月（改訂第6版）

医薬品インタビューフォーム

日本病院薬剤師会のIF記載要領（1998年9月）に準拠して作成

血漿分画製剤

特定生物由来製品、処方せん医薬品

ボルヒール®組織接着用 献血

生体組織接着剤

BOLHEAL®

剤形	外用剤
規格・含量	0.5mL製剤：人フィブリノゲン40mg　人血液凝固第XIII因子37.5単位 アプロチニン液500KIE　日本薬局方　トロンビン125単位 日本薬局方　塩化カルシウム水和物2.95mg 1mL製剤：人フィブリノゲン80mg　人血液凝固第XIII因子75単位 アプロチニン液1,000KIE　日本薬局方　トロンビン250単位 日本薬局方　塩化カルシウム水和物5.9mg 2mL製剤：人フィブリノゲン160mg　人血液凝固第XIII因子150単位 アプロチニン液2,000KIE　日本薬局方　トロンビン500単位 日本薬局方　塩化カルシウム水和物11.8mg 3mL製剤：人フィブリノゲン240mg　人血液凝固第XIII因子225単位 アプロチニン液3,000KIE　日本薬局方　トロンビン750単位 日本薬局方　塩化カルシウム水和物17.7mg 5mL製剤：人フィブリノゲン400mg　人血液凝固第XIII因子375単位 アプロチニン液5,000KIE　日本薬局方　トロンビン1,250単位 日本薬局方　塩化カルシウム水和物29.5mg
一般名	和　名：人フィブリノゲン、人血液凝固第XIII因子、アプロチニン液、トロンビン、塩化カルシウム水和物 洋　名：Human fibrinogen, Human antihemophilic factor XIII fraction, Aprotinin Solution, Thrombin, Calcium Chloride Hydrate
製造販売承認年月日 薬価基準収載年月日 発売年月日	製造販売承認年月日：2009年 6月23日 薬価基準収載年月日：2009年 9月25日 発売年月日：1991年11月28日
開発・製造販売・発売・提携・販売会社名	製造販売：一般財団法人　化学及血清療法研究所 販　売：アステラス製薬株式会社
医薬情報担当者の連絡先・電話番号・FAX番号	

本IFは2010年4月改訂の添付文書の記載に基づき改訂した．

IF利用の手引きの概要

― 日本病院薬剤師会 ―

1. 医薬品インタビューフォーム作成の経緯

当該医薬品について製薬企業の医薬情報担当者(以下，MRと略す)等にインタビューし，当該医薬品の評価を行うのに必要な医薬品情報源として使われていたインタビューフォームを，昭和63年日本病院薬剤師会(以下，日病薬と略す)学術第2小委員会が「医薬品インタビューフォーム」(以下，IFと略す)として位置付けを明確化し，その記載様式を策定した．そして，平成10年日病薬学術第3小委員会によって新たな位置付けとIF記載要領が策定された．

2. IFとは

IFは「医療用医薬品添付文書等の情報を補完し，薬剤師等の医療従事者にとって日常業務に必要な医薬品の適正使用や評価のための情報あるいは薬剤情報提供の裏付けとなる情報等が集約された総合的な医薬品解説書として，日病薬が記載要領を策定し，薬剤師等のために当該医薬品の製薬企業に作成及び提供を依頼している学術資料」と位置付けられる．
しかし，薬事法の規制や製薬企業の機密等に関わる情報，製薬企業の製剤意図に反した情報及び薬剤師自らが評価・判断・提供すべき事項等はIFの記載事項とはならない．

3. IFの様式・作成・発行

規格はA4判，横書きとし，原則として9ポイント以上の字体で記載し，印刷は一色刷りとする．表紙の記載項目は統一し，原則として製剤の投与経路別に作成する．IFは日病薬が策定した「IF記載要領」に従って記載するが，本IF記載要領は，平成11年1月以降に承認された新医薬品から適用となり，既発売品については「IF記載要領」による作成・提供が強制されるものではない．また，再審査及び再評価(臨床試験実施による)がなされた時点ならびに適応症の拡大等がなされ，記載内容が大きく異なる場合にはIFが改訂・発行される．

4. IFの利用にあたって

IF策定の原点を踏まえ，MRへのインタビュー，自己調査のデータを加えてIFの内容を充実させ，IFの利用性を高めておく必要がある．
MRへのインタビューで調査・補足する項目として，開発の経緯，製剤的特徴，薬理作用，臨床成績，非臨床試験等の項目が挙げられる．また，随時改訂される使用上の注意等に関する事項に関しては，当該医薬品の製薬企業の協力のもと，医療用医薬品添付文書，お知らせ文書，緊急安全性情報，Drug Safety Update(医薬品安全対策情報)等により薬剤師等自らが加筆，整備する．そのための参考として，表紙の下段にIF作成の基となった添付文書の作成又は改訂年月を記載している．なお適正使用や安全確保の点から記載されている「臨床成績」や「主な外国での発売状況」に関する項目等には承認外の用法・用量，効能・効果が記載されている場合があり，その取扱いには慎重を要する．

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Ⅰ．概要に関する項目

1．開発の経緯

ボルヒールは、財団法人 化学及血清療法研究所(現 一般財団法人 化学及血清療法研究所)において開発された生体組織接着剤である。本剤は、血液凝固の最終段階で形成されるフィブリンの作用を利用して組織の接着・閉鎖を行うものであり、フィブリノゲン、血液凝固第XIII因子、トロンビン、塩化カルシウム水和物およびアプロチニンの5つの成分から構成されている。本剤の構成成分であるフィブリノゲン、血液凝固第XIII因子及びトロンビンは、HBs抗原、抗HCV抗体、抗HIV-1抗体、抗HIV-2抗体及び抗HTLV-Ⅰ抗体陰性でかつALT(GPT)値でスクリーニングし、さらにHIV、HBV、HCV、HAV及びヒトパルボウイルスB19についての核酸増幅検査(NAT)を行った国内の健康な献血者の血漿から分離精製されている。さらに、独自の乾燥加熱処理により混入の可能性のあるHIV、サイトメガロウイルス等は不活化される事が確認されている。しかし、ウイルス等の感染性を完全には否定できない。
また本剤は、臨床試験により縫合あるいは接合の困難な場合や血液、体液または体内ガスの漏出をきたす場合に有用であることが確認され、1991年3月に承認を得て販売に至った。なお、本剤は使用成績調査を実施し、1996年3月再審査結果が通知され、「効能・効果」ともに現行どおりの内容で承認された。
その後、平成12年9月19日付医薬発第935号「医療事故を防止するための医薬品の表示事項及び販売名の取扱いについて」に基づく販売名の変更承認を2009年6月に取得し、新販売名を「ボルヒール組織接着用」とした。

2．製品の特徴及び有用性

(1) 国内献血由来の血漿を原材料としている。
(2) ヒトの生理的血液凝固機序に基づいているため、適用部位での自然な創傷治癒過程を助ける。
(3) ヒト由来の純度のよいフィブリノゲン、トロンビン、血液凝固第XIII因子を用いている。
(4) 創傷治癒に関与する血液凝固第XIII因子の含量を高く設定している。
(5) 調製器がセット化されているため調製操作が簡便である。
(6) 溶解性に優れ、溶解後の泡もスムーズに消失するので、無駄なく、有効に使える。
(7) ウイルス不活化を目的として独自の条件による乾燥加熱処理を施している。
(8) 総症例1,452例中、副作用は5例(0.34%)に5件みられ、その内訳は肝機能障害3件(0.21%)、ALT(GPT)上昇1件(0.07%)、発熱1件(0.07%)であった。[再審査終了時]
重大な副作用としてショックが報告されている。

Ⅱ．名称に関する項目

1．販売名	
(1) 和名	ボルヒール組織接着用
(2) 洋名	BOLHEAL
(3) 名称の由来	特になし
2．一般名	
(1) 和名(命名法)	人フィブリノゲン、人血液凝固第XIII因子、アプロチニン液(JAN)、トロンビン(JAN)、塩化カルシウム水和物(JAN)
(2) 洋名(命名法)	Human fibrinogen、Human antihemophilic factor XIII fraction、Aprotinin Solution(JAN)、Thrombin(JAN)、Calcium Chloride Hydrate(JAN)
3．構造式又は示性式	該当しない
4．分子式及び分子量	分子式：フィブリノゲン、血液凝固第XIII因子、アプロチニン、トロンビン：該当資料なし 塩化カルシウム水和物：$CaCl_2 \cdot 2H_2O$ 分子量：フィブリノゲン(340,000) 血液凝固第XIII因子(320,000) アプロチニン(6,512) トロンビン(36,800) 塩化カルシウム水和物(147)
5．化学名(命名法)	該当しない
6．慣用名，別名，略号，記号番号	治験番号：HG-4
7．CAS登録番号	該当しない

Ⅲ．有効成分に関する項目

1．有効成分の規制区分	特定生物由来製品、処方せん医薬品
2．物理化学的性質 (1) 外観・性状	フィブリノゲン(血液凝固第Ⅻ因子を含む)：白色～淡黄色の凍結乾燥粉末であり、溶液を加えるときほどんど無色でわずかに混濁した液となる。 アプロチニン液：無色澄明の液 トロンビン：白色～淡黄色の無晶形の凍結乾燥粉末であり、溶液を加えるとき無色澄明の液となる。 塩化カルシウム水和物：本体は白色の粒又は塊であるが、溶液は無色澄明である。
(2) 溶解性	該当しない
(3) 吸湿性	該当しない
(4) 融点(分解点)，沸点，凝固点	該当しない
(5) 酸塩基解離定数	該当しない
(6) 分配係数	該当しない
(7) その他の主な示性値	該当しない
3．有効成分の各種条件下における安定性	該当資料なし
4．有効成分の確認試験法	(1) フィブリノゲン：免疫電気泳動による免疫化学試験 (2) 血液凝固第Ⅻ因子：モノダンシルカダベリン取り込みゲルろ過法を用いる。 (3) アプロチニン液：日本薬局方外医薬品成分規格「アプロチニン液」の確認試験(2)を準用する。 (4) トロンビン：日本薬局方「トロンビン」の定量法を準用する。 (5) 塩化カルシウム水和物：日本薬局方一般試験法の定性反応のカルシウム塩(2)及び塩化物(2)を準用する。
5．有効成分の定量法	(1) フィブリノゲン：生物学的製剤基準一般試験法のたん白窒素定量法を準用して検体1mL中の総たん白質量及び凝固性たん白質量を測定する。ただし、凝固性たん白質量の測定においては、あらかじめ検体を10分の1の濃度に生理食塩液で希釈し、pH6.6～7.4、20～30℃でトロンビンとカルシウム塩を十分量加えて、生じた凝塊を適当な溶液でよく洗ったものを試料とする。また、たん白質量の計算においては、6.25を6.0とする。 (2) 血液凝固第Ⅻ因子：モノダンシルカダベリン取り込みゲルろ過法を用いる。 (3) アプロチニン液：日本薬局方外医薬品成分規格「アプロチニン液」の確認試験(2)を準用する。 (4) トロンビン：日本薬局方「トロンビン」の定量法を準用する。 (5) 塩化カルシウム水和物：日本薬局方一般試験法の定性反応のカルシウム塩(2)及び塩化物(2)を準用する。