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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

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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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REFERENCES

1. Thomson, JA, Itskovitz-Eldor, J, Shapiro, SS, Waknitz, MA, Swiergiel, JJ, Marshall, VS *et al.* (1998). Embryonic stem cell lines derived from human blastocysts. *Science* **282**: 1145–1147.
2. Takahashi, K, Tanabe, K, Ohnuki, M, Narita, M, Ichisaka, T, Tomoda, K *et al.* (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **131**: 861–872.
3. Makino, H, Toyoda, M, Matsumoto, K, Saito, H, Nishino, K, Fukawatase, Y *et al.* (2009). Mesenchymal to embryonic incomplete transition of human cells by chimeric OCT4/3 (POUSF1) with physiological co-activator EWS. *Exp Cell Res* **315**: 2727–2740.
4. Nagata, TM, Yamaguchi, S, Hirano, K, Makino, H, Nishino, K, Miyagawa, Y *et al.* (2009). Efficient reprogramming of human and mouse primary extra-embryonic cells to pluripotent stem cells. *Genes Cells* **14**: 1395–1404.
5. Lavon, N and Benvenisty, N (2005). Study of hepatocyte differentiation using embryonic stem cells. *J Cell Biochem* **96**: 1193–1202.
6. Khetani, SR and Bhatia, SN (2008). Microscale culture of human liver cells for drug development. *Nat Biotechnol* **26**: 120–126.
7. Baharvand, H, Hashemi, SM and Shahsavani, M (2008). Differentiation of human embryonic stem cells into functional hepatocyte-like cells in a serum-free adherent culture condition. *Differentiation* **76**: 465–477.
8. Hay, DC, Zhao, D, Fletcher, J, Hewitt, ZA, McLean, D, Urruticoechea-Uriguen, A *et al.* (2008). Efficient differentiation of hepatocytes from human embryonic stem cells exhibiting markers recapitulating liver development in vivo. *Stem Cells* **26**: 894–902.
9. Shiraki, N, Umeda, K, Sakashita, N, Takeya, M, Kume, K and Kume, S (2008). Differentiation of mouse and human embryonic stem cells into hepatic lineages. *Genes Cells* **13**: 731–746.
10. Song, Z, Cai, J, Liu, Y, Zhao, D, Yong, J, Duo, S *et al.* (2009). Efficient generation of hepatocyte-like cells from human induced pluripotent stem cells. *Cell Res* **19**: 1233–1242.
11. Agarwal, S, Holton, KL and Lanza, R (2008). Efficient differentiation of functional hepatocytes from human embryonic stem cells. *Stem Cells* **26**: 1117–1127.
12. Si-Tayeb, K, Noto, FK, Nagaoka, M, Li, J, Battle, MA, Duris, C *et al.* (2010). Highly efficient generation of human hepatocyte-like cells from induced pluripotent stem cells. *Hepatology* **51**: 297–305.
13. Duan, Y, Ma, X, Zou, W, Wang, C, Bahbahan, IS, Ahuja, TP *et al.* (2010). Differentiation and characterization of metabolically functioning hepatocytes from human embryonic stem cells. *Stem Cells* **28**: 674–686.
14. Cai, J, Zhao, Y, Liu, Y, Ye, F, Song, Z, Qin, H *et al.* (2007). Directed differentiation of human embryonic stem cells into functional hepatic cells. *Hepatology* **45**: 1229–1239.
15. McLin, VA and Zorn, AM (2006). Molecular control of liver development. *Clin Liver Dis* **10**: 1–25, v.
16. Shiojiri, N (1981). Enzymo- and immunocytochemical analyses of the differentiation of liver cells in the prenatal mouse. *J Embryol Exp Morphol* **62**: 139–152.
17. Shiojiri, N (1984). The origin of intrahepatic bile duct cells in the mouse. *J Embryol Exp Morphol* **79**: 25–39.
18. Ingelman-Sundberg, M, Oscarson, M and McLellan, RA (1999). Polymorphic human cytochrome P450 enzymes: an opportunity for individualized drug treatment. *Trends Pharmacol Sci* **20**: 342–349.
19. Hunter, MP, Wilson, CM, Jiang, X, Cong, R, Vasavada, H, Kaestner, KH *et al.* (2007). The homeobox gene Hhex is essential for proper hepatoblast differentiation and bile duct morphogenesis. *Dev Biol* **308**: 355–367.
20. Bogue, CW, Ganea, GR, Sturm, E, Ianucci, R and Jacobs, HC (2000). Hex expression suggests a role in the development and function of organs derived from foregut endoderm. *Dev Dyn* **219**: 84–89.
21. Martinez Barbera, JP, Clements, M, Thomas, P, Rodriguez, T, Meloy, D, Kioussis, D *et al.* (2000). The homeobox gene Hex is required in definitive endodermal tissues for normal forebrain, liver and thyroid formation. *Development* **127**: 2433–2445.
22. Keng, VW, Yagi, H, Ikawa, M, Nagano, T, Myint, Z, Yamada, K *et al.* (2000). Homeobox gene Hex is essential for onset of mouse embryonic liver development and differentiation of the monocyte lineage. *Biochem Biophys Res Commun* **276**: 1155–1161.
23. Bort, R, Signore, M, Tremblay, K, Martinez Barbera, JP and Zaret, KS (2006). Hex homeobox gene controls the transition of the endoderm to a pseudostratified, cell emergent epithelium for liver bud development. *Dev Biol* **290**: 44–56.
24. Xu, ZL, Mizuguchi, H, Sakurai, F, Koizumi, N, Hosono, T, Kawabata, K *et al.* (2005). Approaches to improving the kinetics of adenovirus-delivered genes and gene products. *Adv Drug Deliv Rev* **57**: 781–802.
25. Tashiro, K, Inamura, M, Kawabata, K, Sakurai, F, Yamanishi, K, Hayakawa, T *et al.* (2009). Efficient adipocyte and osteoblast differentiation from mouse induced pluripotent stem cells by adenoviral transduction. *Stem Cells* **27**: 1802–1811.
26. Tashiro, K, Kawabata, K, Sakurai, H, Kurachi, S, Sakurai, F, Yamanishi, K *et al.* (2008). Efficient adenovirus vector-mediated PPAR gene transfer into mouse embryoid bodies promotes adipocyte differentiation. *J Gene Med* **10**: 498–507.
27. Kubo, A, Chen, V, Kennedy, M, Zahradka, E, Daley, GQ and Keller, G (2005). The homeobox gene HEX regulates proliferation and differentiation of hemangioblasts and endothelial cells during ES cell differentiation. *Blood* **105**: 4590–4597.
28. Kovesdi, I, Brough, DE, Bruder, JT and Wickham, TJ (1997). Adenoviral vectors for gene transfer. *Curr Opin Biotechnol* **8**: 583–589.
29. Kawabata, K, Sakurai, F, Yamaguchi, T, Hayakawa, T and Mizuguchi, H (2005). Efficient gene transfer into mouse embryonic stem cells with adenovirus vectors. *Mol Ther* **12**: 547–554.
30. Koizumi, N, Mizuguchi, H, Utoguchi, N, Watanabe, Y and Hayakawa, T (2003). Generation of fiber-modified adenovirus vectors containing heterologous peptides in both the HI loop and C terminus of the fiber knob. *J Gene Med* **5**: 267–276.
31. Asahina, K, Fujimori, H, Shimizu-Saito, K, Kumashiro, Y, Okamura, K, Tanaka, Y *et al.* (2004). Expression of the liver-specific gene Cyp7a1 reveals hepatic differentiation in embryoid bodies derived from mouse embryonic stem cells. *Genes Cells* **9**: 1297–1308.
32. Moll, R, Franke, WW, Schiller, DL, Geiger, B and Krepler, R (1982). The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. *Cell* **31**: 11–24.
33. D'Amour, KA, Agulnick, AD, Eliazer, S, Kelly, OG, Kroon, E and Baetge, EE (2005). Efficient differentiation of human embryonic stem cells to definitive endoderm. *Nat Biotechnol* **23**: 1534–1541.
34. Touboul, T, Hannan, NR, Corbineau, S, Martinez, A, Martinet, C, Branchereau, S *et al.* (2010). Generation of functional hepatocytes from human embryonic stem cells under chemically defined conditions that recapitulate liver development. *Hepatology* **51**: 1754–1765.
35. Vallier, L, Touboul, T, Brown, S, Cho, C, Bilican, B, Alexander, M *et al.* (2009). Signaling pathways controlling pluripotency and early cell fate decisions of human induced pluripotent stem cells. *Stem Cells* **27**: 2655–2666.
36. Shiraki, N, Yoshida, T, Araki, K, Umezawa, A, Higuchi, Y, Goto, H *et al.* (2008). Guided differentiation of embryonic stem cells into Pdx1-expressing regional-specific definitive endoderm. *Stem Cells* **26**: 874–885.
37. Morrison, GM, Oikonomopoulou, I, Migueles, RP, Soneji, S, Livigni, A, Enver, T *et al.* (2008). Anterior definitive endoderm from ESCs reveals a role for FGF signaling. *Cell Stem Cell* **3**: 402–415.
38. Sumi, T, Tsuneyoshi, N, Nakatsuji, N and Suemori, H (2008). Defining early lineage specification of human embryonic stem cells by the orchestrated balance of canonical Wnt/beta-catenin, Activin/Nodal and BMP signaling. *Development* **135**: 2969–2979.
39. Xu, RH, Peck, RM, Li, DS, Feng, X, Ludwig, T and Thomson, JA (2005). Basic FGF and suppression of BMP signaling sustain undifferentiated proliferation of human ES cells. *Nat Methods* **2**: 185–190.
40. Keller, G (2005). Embryonic stem cell differentiation: emergence of a new era in biology and medicine. *Genes Dev* **19**: 1129–1155.
41. Selden, C, Shariat, A, McCloskey, P, Ryder, T, Roberts, E and Hodgson, H (1999). Three-dimensional *in vitro* cell culture leads to a marked upregulation of cell function in human hepatocyte cell lines–an important tool for the development of a bioartificial liver machine. *Ann N Y Acad Sci* **875**: 353–363.
42. Soto-Gutiérrez, A, Navarro-Alvarez, N, Zhao, D, Rivas-Carrillo, JD, Lebkowski, J, Tanaka, N *et al.* (2007). Differentiation of mouse embryonic stem cells to hepatocyte-like cells by co-culture with human liver nonparenchymal cell lines. *Nat Protoc* **2**: 347–356.
43. Kubo, A, Kim, YH, Irion, S, Kasuda, S, Takeuchi, M, Ohashi, K *et al.* (2010). The homeobox gene Hex regulates hepatocyte differentiation from embryonic stem cell-derived endoderm. *Hepatology* **51**: 633–641.
44. Hacein-Bey-Abina, S, Von Kalle, C, Schmidt, M, McCormack, MP, Wulffraat, N, Leboulch, P *et al.* (2003). LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science* **302**: 415–419.
45. Sakamoto, N, Tsuji, K, Muul, LM, Lawler, AM, Petricoin, EF, Candotti, F *et al.* (2007). Bovine apolipoprotein B-100 is a dominant immunogen in therapeutic cell populations cultured in fetal calf serum in mice and humans. *Blood* **110**: 501–508.
46. Furue, MK, Na, J, Jackson, JP, Okamoto, T, Jones, M, Baker, D *et al.* (2008). Heparin promotes the growth of human embryonic stem cells in a defined serum-free medium. *Proc Natl Acad Sci USA* **105**: 13409–13414.
47. Mizuguchi, H and Kay, MA (1998). Efficient construction of a recombinant adenovirus vector by an improved *in vitro* ligation method. *Hum Gene Ther* **9**: 2577–2583.
48. Mizuguchi, H and Kay, MA (1999). A simple method for constructing E1- and E1/E4-deleted recombinant adenoviral vectors. *Hum Gene Ther* **10**: 2013–2017.
49. Maizel, JV Jr, White, DO and Scharff, MD (1968). The polypeptides of adenovirus. I. Evidence for multiple protein components in the virion and a comparison of types 2, 7A, and 12. *Virology* **36**: 115–125.
50. Suemori, H, Yasuchika, K, Hasegawa, K, Fujioka, T, Tsuneyoshi, N and Nakatsuji, N (2006). Efficient establishment of human embryonic stem cell lines and long-term maintenance with stable karyotype by enzymatic bulk passage. *Biochem Biophys Res Commun* **345**: 926–932.

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