tion, inflammatory or anti-inflammatory, of each SIK family kinase action remains to be established. Here, we report that SIK3 is induced by LPS in RAW264.7 macrophages and may repress mRNA expression of secondary genes encoding inflammatory molecules, IL-6, IL-12p40 and iNOS. In contrast, SIK3-KO macrophages showed high expression levels of genes that were down-regulated in SIK3 over-expressed macrophages compared with WT macrophages. Moreover, SIK3-KO mice were highly sensitive to LPS and died after LPS treatment. These phenomena were not observed in KO mice in which other SIK family genes, SIK1 and SIK2, were disrupted.

Despite statistical significance in inflammatory molecule expression between SIK3-KO and WT BMDM, the difference in BMDM was smaller than that in TEPM (see Supporting information, Fig. S2; and Fig. 3a), indicating that observations on the function of SIK3 from TEPM may not be generalizable to other closely related cell types. TEPM are considered to be M1-like macrophages (pro-inflammatory),[32,33] whereas BMDM are to be M2-like macrophages (anti-inflammatory)[34–36] that are characterized by the robust expression of IL-10 following LPS stimulation. The observations of differently originated SIK3-KO macrophages suggest that SIK3 may function in M1-like, rather than in M2-like, macrophages. In addition, other immune compartments that affect macrophage polarization, such as T cells, also have to be considered as a cause of the high mortality of SIK3-KO mice in response to LPS.

Clark *et al.* reported that the TANK-binding kinase 1 inhibitor MRT67307 enhanced IL-10 secretion from bone marrow-derived macrophages after LPS stimulation. MRT67307 also up-regulated the expression of regulatory macrophage (M2b) markers (LIGHT, SPHK1 and Arg1) and down-regulated the production of inflammatory cytokines (IL-6 and TNF-α).[23] The CRTC3–CREB cascade was found to be responsible for MRT67307-mediated enhancement of the *Il10* promoter activity, and an *in vitro* kinase assay suggested AMPK-related kinases as the target of MRT67307. Further screening, modification of compounds, and testing for anti-inflammatory activities helped to identify the potent SIK inhibitors MRT199665, KIN112 and HG-9-91-01 and to determine their inhibitory spectra. Additionally, RNA interference techniques led to the conclusion that all SIK isoforms might play a role in the pro-inflammatory cascades of macrophages via suppression of IL-10 gene expression.[23] The same conclusion was drawn for prostaglandin E_2 or cAMP stimulation in macrophages in a related study.[24] It is not clear

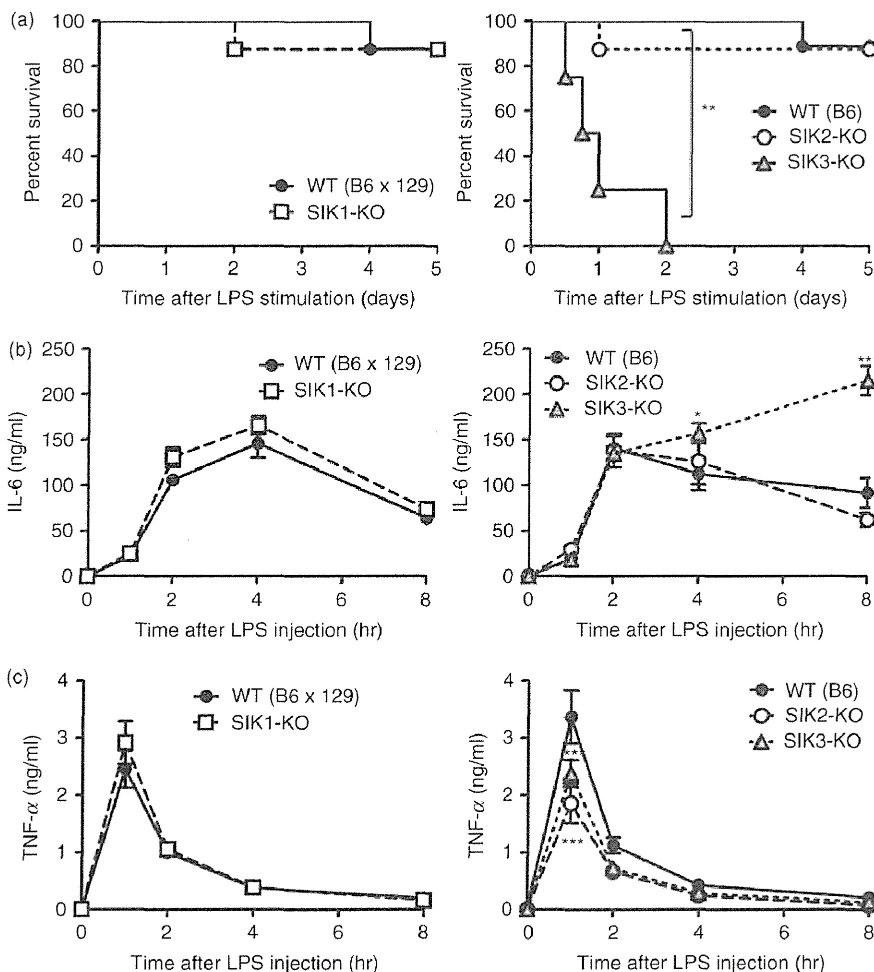

Figure 4. Lipopolysaccharide (LPS) -induced endotoxin shock in each salt-inducible kinase (SIK) -deficient mouse. (a) Left panel: Wild-type (WT) (B6 × 129 background) (n = 8) and SIK1 knockout (-KO) (n = 8); Right panel: WT (C57BL/6 background) (n = 9), SIK2-KO (n = 8), and SIK3-KO (n = 8) mice were injected with LPS (10 mg/kg) and the % survival was plotted. Blood was collected from the tail vein at the indicated time-points, and serum concentrations of interleukin-6 (IL-6) (b) and tumour necrosis factor-α (TNFα) (c) were determined by ELISA. The data are expressed as mean ± SEM. Statistical analyses were performed using the Cox–Mantel test (a) or two-way analysis of variance followed by Bonferroni's post-tests (b, c) for the comparison of the Kaplan–Meier curve for mouse survival and cytokine secretion, respectively. $*P < 0.05$, $**P < 0.01$, $***P < 0.01$.

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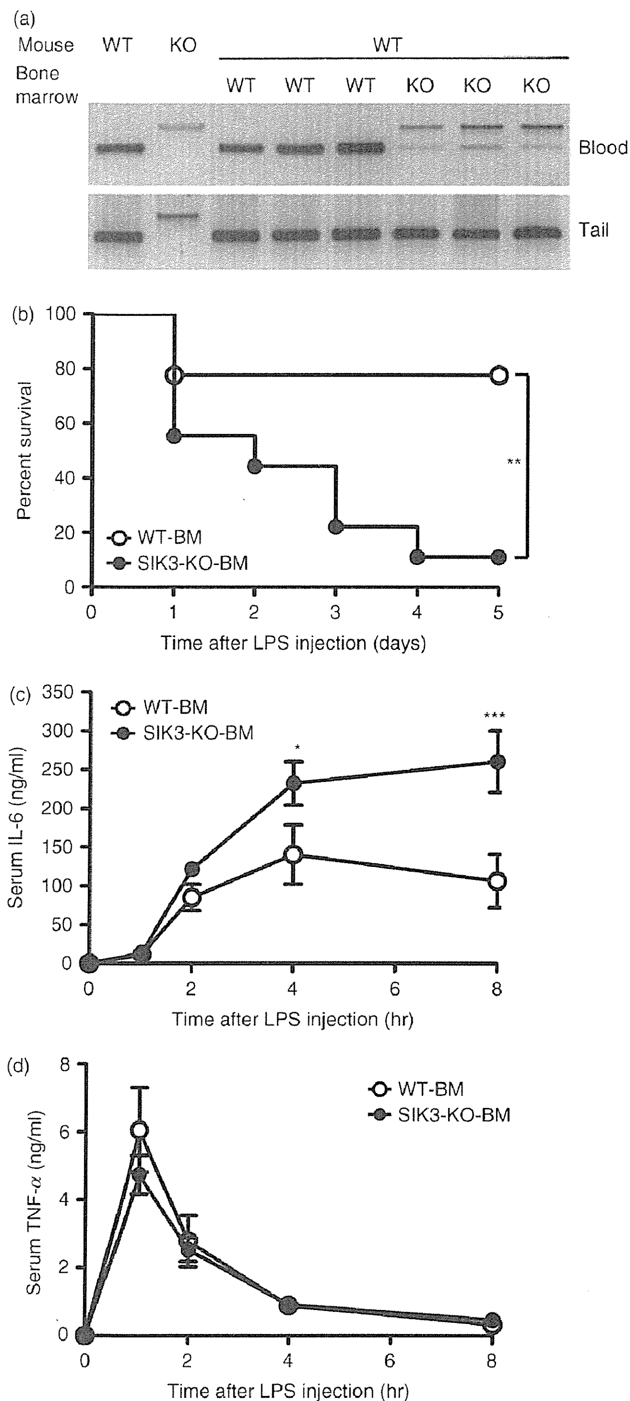

Figure 5. Lipopolysaccharide (LPS) -induced endotoxin shock in wild-type (WT) mice transplanted with salt-inducible kinase 3 knockout (SIK3-KO) bone marrow. (a) DNA was prepared from the blood and tails of mice subjected to bone marrow transplantation and used as the PCR template of SIK3 genotyping. (b) WT mice with SIK3-KO bone marrow (SIK3-KO-BM, $n = 9$) or with WT bone marrow (WT-BM, $n = 9$) were treated with LPS (10 mg/kg) and the % survival was plotted. Blood was collected from the tail vein at the indicated time-points, and serum concentrations of interleukin-6 (IL-6) (c) and tumour necrosis factor-α (TNF-α) (d) were determined by ELISA. The data are expressed as mean ± SEM. Statistical analyses were performed using the Cox–Mantel test (b) or two-way analysis of variance followed by Bonferroni's post-tests (c, d) for the comparison of the Kaplan–Meier curve for mouse survival and for cytokine secretion, respectively. $*P < 0.05$, $**P < 0.01$, $***P < 0.01$.

why the authors chose only SIK2 to demonstrate SIK-mediated repression of IL-10 production in macrophages. The results obtained with our SIK2-KO mice supported the same conclusion. SIK2-KO mice secreted a lower level of TNF-α after LPS treatment than WT mice did. However, serum IL-6 levels did not differ significantly between SIK2-KO and WT mice. In contrast to the results obtained in experiments *in vivo*, enhanced IL-10 mRNA expression accompanied by suppressed iNOS mRNA levels were observed in SIK2-KO TEPM after LPS stimulation, whereas no significant change in TNF-α or IL-6 mRNA level was observed (Fig. S2), suggesting that SIK2 may play a role in pro-inflammatory cascades. These contradictions between observations from KO mice and cultured macrophages may be the result of differences in the experimental conditions and unknown systemic factors that may have offset the pro-inflammatory functions of SIK2 *in vivo*.

In contrast to the SIK2 findings, Yong Kim *et al.*[25] also reported that over-expression of SIK1 and SIK3 resulted in the suppression of TLR4 signalling in RAW264.7 macrophages. They proposed that SIK1 and SIK3 inhibited TLR4 signalling not only through IKK-mediated nuclear factor-κB signalling, but also through p38- and JNK-mediated signalling via an interruption with the TAB2–TRAF6 complex, thereby inhibiting TRAF6 ubiquitination. However, in our study, the SIK1-KO mice or macrophages did not show altered sensitivity to LPS, and, at least in SIK3-KO macrophages, no significant difference was observed in the phosphorylation levels of p38, JNK, IKK or IκBα, the downstream components of TAB2–TRAF6 (Fig. 3f). These results again highlight the different experimental conditions. However, in our experiments using an SIK deficiency animal model, SIK3 was the indispensable kinase among the SIK family with regard to regulation of the inflammatory response in macrophages.

Histone deacetylases are modulators of inflammatory cascades in macrophages and class IIa HDACs (HDAC4, HDAC5, HDAC7, and HDAC9) are the substrates of SIK.[21,22] A recent study indicates that the depletion of the class IIa HDAC4 in macrophages also exacerbated LPS-induced endotoxin shock, and that the inhibition of SIK or LKB1, which is an upstream kinase of SIK, led to the deacetylation of p65 by HDAC4 and the reduction of its recruitment to the *TNFα* and *IL-12β* promoters.[37] In addition, the class IIa HDACs use HDAC3 (a class I HDAC) to repress target gene transcription by forming a repressor complex on promoters.[38,39] Chen *et al.* demonstrated that HDAC3 deficiency in macrophages resulted in the suppression of LPS-induced IL-6 and iNOS expression through the inhibition of the MyD88-independent IRF3–IFN-β–STAT1 signalling pathway.[15] Class I HDACs are also known to deacetylate MAPK phosphatase-1 (MKP-1), which dephosphorylates p38 and inhibits

downstream signalling[40–42] Deacetylation of MKP-1 protein by class I HDACs inhibits its phosphatase activity and stimulates p38 phosphorylation in LPS-stimulated macrophages.[41,42] In addition, the pan-HDAC inhibitor trichostatin A (TsA) inhibits pro-inflammatory cytokine gene expression in macrophages after LPS treatment,[26] which could be due to inhibition of MKP-1.[40,43] Furthermore, Serrat *et al.*[43] indicated that TsA especially affected secondary genes such as those encoding IL-6 and iNOS. These observations suggest the relevance of SIK3 in HDAC-mediated response to LPS. However, we could not show any supportive results that indicate a relationship between HDACs, either class I or IIa, and the enhanced expression of secondary genes in SIK3-deficient macrophages (data not shown).

In conclusion, we found that SIK3 deficiency, but not SIK1 or SIK2 deficiencies, exacerbate endotoxin shock induced by LPS in mice. The increased sensitivity to LPS in SIK3-KO mice was caused by SIK3-deficiency in haematopoietic cells, supposedly in macrophages. Although the involvement of SIK family kinases in the innate immune system has been suggested in cultured macrophages, this study is the first to show the importance of SIKs *in vivo* and indicates that SIK3 plays an important role in anti-inflammatory cascades in LPS-stimulated macrophages. However, it was also true that the strong induction of secondary genes by LPS in SIK3-KO TEPM was not fully reproduced in BMDM despite the presence of statistical significance, suggesting contributions of other cell types to the SIK3-deficient phenotypes. Further studies will be needed to evaluate functions of SIKs during inflammation.

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Disclosures

The authors have no conflict of interests.

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PROTOCOL EXCHANGE | COMMUNITY CONTRIBUTED

A simple improvement of the conventional cryopreservation for human ES and iPS cells

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Abstract

In this study, a simple method for the cryopreservation of human embryonic stem (ES) and induced pluripotent stem (iPS) cells is proposed. It is based on the conventional slow-freezing method with 10% DMSO and modified mainly in a thawing protocol without specific equipment or reagents. Recovery rate of the cells cryopreserved by this method was equally high, which is comparable to that of the cells frozen by the vitrification method. In the case of vitrification method, it requires practiced hand because cells can be terribly damaged upon failure of rapid-warming process and strict maintenance of low temperature is required throughout the cryopreservation. On the contrary, our method is available for novices and cryopreserved cells can maintain cell recovery rate for one week after transfer from -150 °C to -80 °C condition. This simple modified method would gain widespread acceptance.

Subject terms: Cell culture Developmental biology

Keywords: Human pluripotent stem cells Cryopreservation Slow-freezing method

Introduction

Human embryonic stem (hES) cells1 and human induced pluripotent (hiPS) cells2, 3 have great potential as a possible source of cells for the regenerative medical transplantation of various tissues and also for other research applications, such as developmental biology, toxicology, and drug discovery. Today, a number of hES and hiPS cell lines have been established around the world. To promote these great potential researches using hES and hiPS cell lines, the prerequisite is the foundation of cell banks of well-characterized and safety-tested cells for research which serve as seed stocks for therapeutic applications. Currently, several cell banks are distributing such cell lines all over the world. The International Stem Cell Banking Initiative has developed a consensus on the principles of best practice for the

banking, testing, and distribution of hES cells4, 5, 6, 7, 8. During cell banking, although most of the procedures share many of the principles of routine cell culture, a number of significant steps exist in the case of hES and hiPS cells. One of them is cryopreservation9.

Both hES and hiPS cells have been well-known to be sensitive to the cryopreservation9, 10, 11. Two cryopreservation methods are commonly used for hES and hiPS cells which are vitrification12, 13, 14, 15 and slow freezing16, 17, 18, 19. At this moment, vitrification method is considered to be preferable for the local storage because of the high survival rate12, 15, 16, 20. However, vitrified cells can be terribly damaged upon failure of rapid-warming process without practiced hand compared to slow-frozen cells. Furthermore, if the low temperature is not strictly maintained, cell viability decreases rapidly. Therefore, when vitrified cells are transported, it needs liquid nitrogen dry-shippers with careful documentation for the regulatory requirements and transports companies with technical expertise to undertake such shipments, resulting in the high costs involved. As for slow-freezing method with dimethyl sulfoxide (DMSO), although it has been long trusted as a stable cryopreservation for a wide variety of cells including mouse ES cells, it produces low levels of recovery or spontaneously differentiation for hES and hiPS cells.

Until now, several approaches have been investigated for the improvement of cryopreservation for hES and hiPS cells such as the technique based on stabilizing hES colonies adherent to or embedded in a Matrigel matrix17, and the protocol using of Rho-associated kinase (ROCK) inhibitor21, 22. Considering clinical application of hES and hiPS cells, xeno-free defined freezing medium should be required. An effective serum- and xeno-free chemically defined freezing procedure was reported by Hovatta's group23. Another group has proposed in situ cryopreservation using gas permeable culture casettes24. However, there is not any evidence that the cells can stably preserve their phenotypes for more than 10 years, excluding the conventional cryopreservation of the cells with DMSO. Thus, it is suggested that although these new methods would be worth to be challenged, the conventional cryopreservation should be backed up as a precaution.

We here show that a simple modification of the conventional slow freezing cryopreservation with 10% DMSO increased cell viability after thawing. This method requires neither specific equipment nor reagents and only the thawing way is changed, but resulting cells have comparable growth ability to those of vitrified cells. Even when using defined medium, such as our growth factor defined medium hESF925, 26, 27, 28, or Thomson's mTeSR medium29, this method could work well. Because the conventional slow freezing cryopreservation is quite familiar, this simple method would gain widespread acceptance.

Reagents

Critical: All reagents and materials used must be sterile.

- Gelatin (0.1%, Millipore, cat. no. ES-006-B)
- ES qualified fatal bovine serum (GIBCO, cat. no.10439)
- DMEM-F12 (GIBCO, cat. no. 12660-012)
- DMEM high glucose (GIBCO, cat. no. 11965-092)

•Knockout serum replacement (KSR, Invtirogen, cat. no. 10828-028)
•L-Glutamine (200 mM, Invitrogen, cat. no. 25030-081)•
•β-Mercaptoethanol (55 mM, Invitrogen, cat. no. 21985-023)
CAUTION: β-Mercaptoethanol is toxic. Avoid inhalation, ingestion and skin contact during use.
•Non-essential amino acid solution (0.1 mM, Invitrogen, cat. no. 11140-035)
•Basic fibroblast growth factor (bFGF, sigma-Aldrich, cat. no. F0291)
CRITICAL: The activity of bFGF is variable across companies
•Dispase (Roche, cat. no. 04942078001)
•2.5% trypsin (Invitrogen, cat. no. 15090-046)
•Collagenase IV (Invitrogen, cat. no. 17104-019)
•Dimethyl sulfoxide (DMSO, Sigma-Aldrich, cat. no. D2650)
CAUTION: Keep this reagent away from sources of ignition. Take precautions to prevent the buildup of electrostatic charge. Personal protection: protective gloves and safety glasses..
CRITICAL: High purification of DMSO should be used. Store in an airtight container after opening.
•Feeder cells, Mitomycin C treated primary mouse embryonic fibroblasts (PMEF) (PMEF-CF; DS Pharma Biomedical Co.,Ltd, cat. no. BBASF1214) and (PMEF-H; Chemicon, cat. no. PMEF-H)
CRITICAL: PMEF-CF and PMEF-H are used for hES and hiPS cells respectively.
•WA09 hES cells (WiCell, Madison, WI, USA) and JCRB1331 hiPS cells (JCRB1331, Osaka, Japan)

Reagent setup
β-Mercaptoethanol For a 1,000× stock (100 mM), dissolve 70 µl in 10 ml PBS (the final concentration is 0.1 mM). Store at 4 °C in 36 months from date of manufacture.

CAUTION: β-Mercaptoethanol is toxic. Avoid inhalation, ingestion and skin contact during use. Personal protection: gloves, safety glasses and good ventilation.

bFGF Dissolve 25 µg bFGF in 1-ml sterile Tris-base (5 mM). Store at -20 °C in 250–500 µl aliquots in three months. The final concentration should be 100 ng ml−1.
CRITICAL: Avoid repeated freeze-thaw cycles. Thaw and add bFGF to the medium just prior to the use.

KSR Thaw at 4 °C overnight. Store aliquots at -20 °C up to12 months. CRITICAL: We recommend that KSR is ideally used in a week after thawing.

L-Glutamine Prepare 1:100 dilution, store aliquots at -20 °C in three months.

Non-essential amino acid solution Prepare 1:100 dilution, store aliquots at -20 °C in 12 months from date of manufacture.

Human pluripotent cell medium For WA09 hES cells, add 200 ml KSR, 5 ml L-Glutamine, 1.85 ml β-Mercaptoethanol and 8 ml non-essential amino acid solution into 800 ml DMEM-F12 medium. For JCRB1331 hiPS cells, add 200 ml KSR, 10 ml L-Glutamine, 1.85 ml β-Mercaptoethanol and 8 ml non-essential amino acid solution into 800 ml DMEM-F12 medium. Store at 4 °C and should be used in one week.

Dispase Add 20 mg dispase to 10 ml DMEM medium (high glucose). This gives a final concentration of 2 mg/ml. Sterilize through a 0.22 µm filter. It is possible to dispense enzymes in small aliquots and store at -20 °C for 6 months. Defrost dispase should be ideally used in three days after thawing. Avoid freezing and thawing.

Collagenase IV Dissolve 10 mg in 10 ml distilled water (the final concentration is 1 mg/ml). Sterilize through a 0.22 µm filter. It is possible to dispense enzymes in small aliquots and store at -20 °C for 6 months. Defrost collagenenase should be ideally used in three days after thawing.

CaCl2 Dissolve 0.11 mg in 10 ml distilled water (the final concentration is 0.1 M). Sterilize through a 0.22 µm filter. It is possible to dispense enzymes in small aliquots and store at 4 °C.

Collagenase-Trypsin-KSR solution (CTK) Add 5 ml of 2.5% trypsin, 5 ml of 1 mg/ml collagenase IV, 0.5 ml of 0.1 M CaCl2, and 10 ml of KSR into 30 ml of PBS. It is possible to dispense enzymes in small aliquots and store at -20 °C for 3 months. CTK should be ideally thawed when used. Avoid freezing and thawing.

Slow freezing medium Mix DMSO and KOSR in the ratio 1:9.
CRITICAL: The flow freezing medium should be prepared freshly and stored on ice before use.

Vitrification medium (DAP213) Dissolve 0.59 g of acetamide in 6 ml of Human pluripotent cell medium, subsequently add 1.42 ml of DMSO and 2.2 ml of propylene glycol. Fill up to 10 ml with the medium. Store aliquots at −80 °C for three months.

Fixation buffer (pH9.5) for Alkaline phosphatase staining Refer Table 1 for the recipes.

Equipment

- 15 and 50 ml Conical tube (BD, cat. no. 352096, cat. no. 352070)
- 25 cm2 vent bulb culture flask (Corning, cat. no. 430639)
- 5, 10, and 25 ml Plastic disposable pipette (VWR, cat. no. 89130-896, 89130-898, 89130-900)
- transfer pipette (Scientific, cat. no. SC-233-J).
- 0.22 µm Pore size filter (Millipore, cat. no. SLGP033RS)
- 0.22 µm Vacuum filtration (500 ml, Thermo Scientific, cat. no. 291-4520)
- Disposable syringes, 10 and 20 ml (Terumo, cat. no. ss-10ESz, ss-20ESz)

- Glass Pasteur pipettes, 9 inches. Sterilize by autoclave (Fisher Scientific, cat. no. 13-678-6B)
- Cryovial (Thermo Scientific, cat. no. 5000-0020)
- Centrifuge (KOKUSAN, H-60R)
- Inverted phase-contrast microscope (4, 10, 20 and 40× objectives) (OLYMPUS, CKX41)
- Purifier class II biosafety cabinet (LABCONCO, cat. no. 302310030)
- Micropipette (0.5–10, 10–100 and 100–1,000 μl) (Eppendorf, cat. no. 4910000018, 4910000042, 4910000069)
- Pipette aid (Drummond Scientific Company, PA-400)
- Tissue culture incubator, with humidity and gas control to maintain 37 °C and 95% humidity in an atmosphere of 5% CO2 in air (YAMATO, IP 400)
- Water bath (TAITEC, SDminiN)
- Hemocytometer (Onecell, OC-C-SO2)
- Cryovial storage rack (Thermo Scientific Nalgene, cat. no. 238-024)
- Liquid nitrogen tank (Thermo Scientific, TY509X4)

Procedure

Passaging of hES/iPS cells:
Maintain WA09 and JCRB1331 cells on feeder layers of mitomycin C-treated primary mouse embryonic fibroblasts (PMEF) in gelatin-coated 25 cm2 tissue flasks. Passage colonies every 5-7 days depending on colony growth. Exchange culture medium every day except the day following the passaging.

Freeze stock of hES/iPS Cells:
1. Remove the differentiated area completely by using the Pasteur pipette in advance.
2. Examine all the plates containing hES/iPS Cells by inverted phase-contrast microscopy. Cultures used for cryopreservation should show no signs of microbial contamination and should be subconfluent (the distance between the colonies is < 10-20% of the average colony diameter). Confluent cultures may be less amenable to freezing.
3. Label cryogenic vials with the name of the cell line, passage number, lot number and the date.
4. Remove the culture medium from flasks.
5. Add 1.5 ml dispase (1U/ml) per the T25 flask and incubate for 2-5 min until the edges of the colonies start to curl.
CRITICAL: Treatment time of dispase varies and depends on the cell lines and quality of colonies used.6. Gently scrape with scraper and pipetting with 10 ml pipette so as not to be small clumps.
CRITICAL: Cell colonies should not be disrupted in small size.
7. Transfer to a 15 ml centrifuge tube and spin at 20 X g, 2 min, 4 °C.
CRITICAL: It is recommended to centrifuge at low g forces (20 x g) to prevent feeder cells collecting at the bottom of the tube together.
8. Carefully aspirate supernatant without disturbing the cell pellet.

CRITICAL: Finger tap the tube gently to disperse the pellet.

9. Resuspend the cells in freshly prepared ice-cold freezing medium (10% DMSO/culture medium).

CRITICAL: The freezing medium should be prepared freshly and kept on ice before use.

10. Transfer 200 µl aliquots into each labeled cryogenic vial.

CRITICAL: Small volume may result in higher cell survival rates. It is recommended the volume of fluid during cryopreservation is 200 ul rather than 500 ul.

11. Place the vials on ice for a few minute.

CRITICAL: Vials are allowed to sit on ice for 15 min. A prolonged storage on ice is likely to cause a further decline of cell viability.

12. Store them in the freezing container (BICELL or Mr.Frosty®) and keep it in a -80 °C deep freezer overnight.

CRITICAL: It is recommended to finish suspension within 30 min. Prolonged immersion cause decline in cell viability.

13. Next day transfer the vials to the liquid nitrogen tank (in the vapor phase, -150 °C).

CRITICAL: Immersion of vials into the liquid phase of liquid nitrogen may lead to viral or cross contamination.

CRITICAL: It is recommended to abort long term storage in a -80 °C deep freezer. An extended storage at -80 °C causes the decline in cell recovery. Storage in a -80 °C deep freezer within 7 days after transferring from liquid nitrogen tank is permissible.

CRITICAL: It is reported that vitrification protocols may be superior to slow freezing. We showed our slow freezing method was equally successful as vitrification method.

PAUSE POINT: The cells can be stored indefinitely in the vapor phase of liquid nitrogen.

14. Document the details of vials and storage location in the appropriate inventory.

Preparation of feeder seeded culture flasks before thawing of hES/hiPS cells

15. Coat the flasks with 0.1% gelatin (3 ml/25 cm2 flask).

16. Incubate the flasks for 1 hour at 37 °C or overnight at room temperature.

17. Rinse the flasks with PBS.

18. Inoculate mitomycin c-treated primary mouse embryonic fibroblasts (PMEF) at 5-7×103 cells/cm2 in DMEM (high glucose) containing 10% heat inactivated FBS.

CRITICAL: Prepare the feeder seeded culture flasks in a few days in advance of the thawing or passaging of hES/hiPS cells, and use them within 5 days.

CRITICAL: Density of feeder cells should be determined by colony morphology of hES/hiPS cells in advance.

Thawing of hES/hiPs cells

19. Prior to using of feeder seeded flasks, replace the medium with the human pluripotent cell medium twice.

20. Pre-warm 10 ml of the human pluripotent cell medium in a 15 ml tube in a 37 °C water bath.

21. Rapidly thaw out frozen cells by adding the pre-warmed medium directly into a vial with the transfer pipette.
CRITICAL: In a case of thawing the frozen cells in a 37 °C water bath or in a 37°C incubator, the cells may be seriously damaged.
22. Gently collect the cells in the 15ml tube and wash vial with the pre-warmed medium.
23. Centrifuge the tube at 90x g, 2 min, room temperature.
24. Aspirate the medium and finger tap the tube gently to disperse the pellet.
25. Resuspend the cells in 5-7 ml of pre-warmed culture medium and transfer the suspension into a 25 cm2 flask.
26. Gently move the flask several times along horizontal and vertical directions to distribute the cells evenly.
27. Incubate the flasks in an incubator at 37 °C in a humidified atmosphere of 5% CO_2.
28. Culture medium should be exchanged every other day until hES/hiPS cells are ready for passaging.
CRITICAL: Do not move the flask on the day following the passaging.

Alkaline phosphatase staining to determine the growth of hiPS/hES cells
29. Remove the culture medium and rinse once with PBS for 1 min at room temperature.
30. Fix the colonies with the fixation buffer for 1 min at room temperature.
31. Wash twice with DDW.
32. Start the color reaction by incubating in the reaction buffer blocking off the light.
33. Stop the reaction after 15 min by washing twice with DDW.
34. Count the stained colonies.

Timing

Steps 1–14, Freeze stock of hES/iPS: 1hour
Steps 15–18, Preparation of feeder seeded culture flasks before thawing of hES/hiPS cells: 5-10 days
Steps 19–29, Thawing of hES/hiPS: 10–15 minutes
Steps 30–35, Alkaline phosphatase staining: 20–30 minutes

Anticipated Results

Simple modified method of conventional slow freezing cryopreservation for hES and hiPS cells described here have equally successful recovery rate and ability to maintain undifferentiated state as those of vitrification method (Fig. 1, Fig. 2). Thawing method by addition of pre-warmed medium directly into the frozen vial also leads to high cell viability after recovery from cryopreservation (Fig. 3). Additionally, shortening keeping time on ice before freezing (Suppl. Fig. 1), use of dispase (Suppl. Fig. 2), shortening cell suspension time after the dissociation process (Suppl. Fig. 3), reducing fluid volume from 500 to 200 ul (Suppl. Fig. 4), implementation of freezing process in vapor phase of liquid nitrogen (-150 °C) within 7 days after slow freezing process in a -80 °C deep freezer (Suppl. Fig. 5) increase viability of the

cryopreserved cells by the slow freezing method. Moreover, cryopreserved cells are maintained high recovery rate and undifferentiated state within 7 days if the vials were transferred from a liquid nitrogen gas tank to a -80 deep freezer (Suppl. Fig. 6).

Our method may be available for novices, because it is simple and easy method that requires neither specific equipment nor reagents (Fig. 1). Moreover, our method allows for keeping in a -80 °C deep freezer within one week after transferring from a liquid nitrogen tank, which make favorable to transportation as compared to vitrification method. Also, this cryopreservation is available for serum-free culture such as culture with hESF9 medium and hESF9a2i medium25, 26, 27, 28, 29.

We believe that our method can be employed by stem cell banking and researchers and will most likely lead to the expected outcome.

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Figures

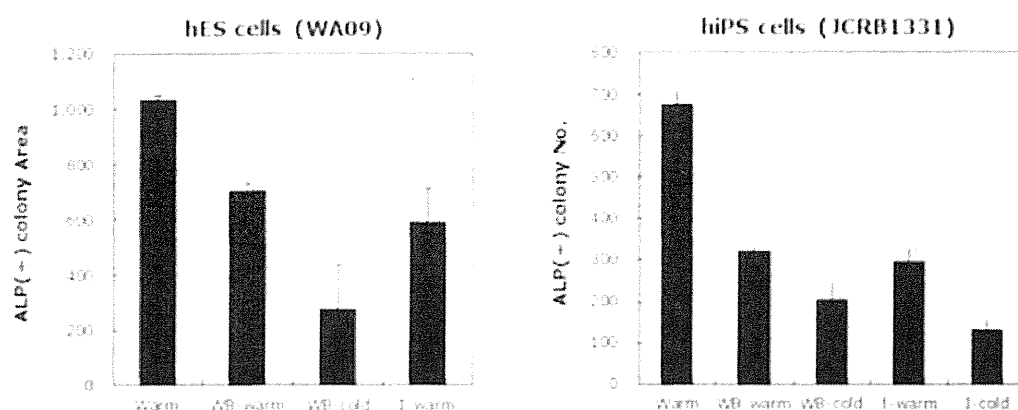
Figure 1.: Schematic diagram illustrating the process of our cryopreservation method

Our cryopreservation protocol is based on the conventional slow-freezing method with 10% DMSO and modified simply without specific equipment or reagents. Recovery rate of the cells cryopreserved by this method was equally high in comparison with the vitrification method. Even when using defined medium, such as our growth factor defined medium hESF925, 26, 27, 28, or mTeSR medium29, this cryopreservation method could work well.

Figure 2. : Comparison between slow-freezing and vitrification method

The cells were frozen by each cryopreservation method. The colonies with ALP positive were measured at 6 days (H9) or 7 days (JCRB1331) after thawing. Vitrification method was performed according to Fujioka, T. and Suemori, H16. Briefly, cells were resuspended with 200 μl of DAP213 and rapidly placed into cryovials and then transferred to the vapor phase of the liquid nitrogen (within 15 sec). Bars indicate the means ± S.D. (n=3).

Figure 3.: Comparison among several thawing method after cryopreservation



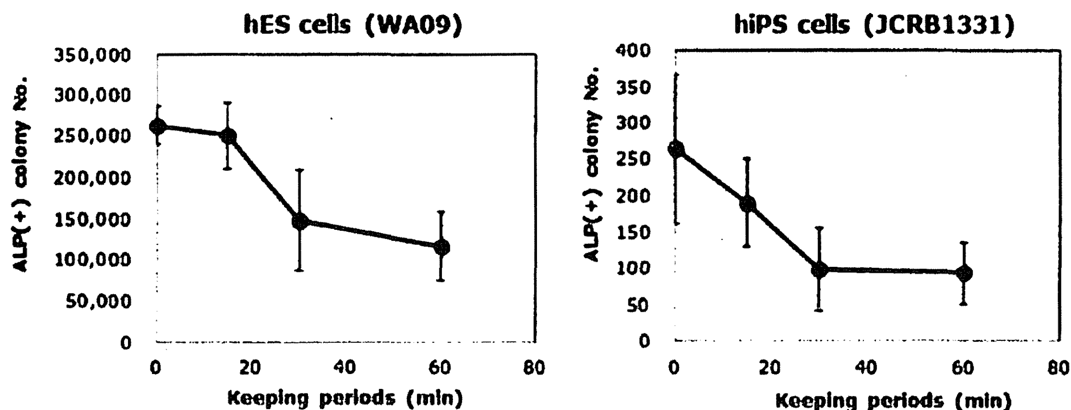
The cells were frozen by slow-freezing method and thawed out by each thawing method. Then, the colonies with ALP positive were measured at 6 days (H9) or 7 days (JCRB1331) after thawing. Warm, directly add pre-warmed medium into frozen cells; WB-warm, thaw with water bath and add pre-warm medium into vial; WB-cold, thaw with water bath and add cold medium into vial; I-warm, thaw with incubator and add pre-warm medium into vial. Bars indicate the means ± S.D. (n=3).

Table 1.: Fixation buffer (pH9.5) for Alkaline phosphatase staining

component	Working concentration	Volume
Citric acid	4.5 mM	0.864 g
Sodium citrate	2.25 mM	0.581 g
Sodium chloride	3.0 mM	0.174 g
Methanol	65 %	650 ml
formaldehyde	3 %	30 ml

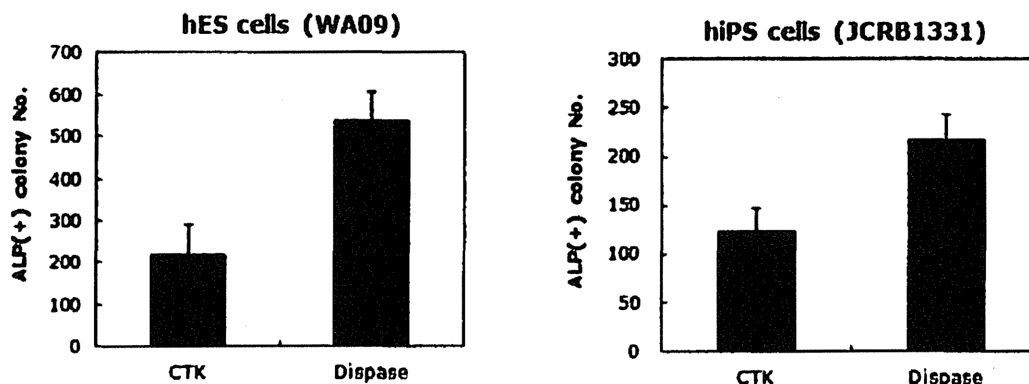
Adjust pH to 9.5. Fill up to 1,000 ml in distilled water

Supplementary Figure 1.: Keeping time on ice before freezing



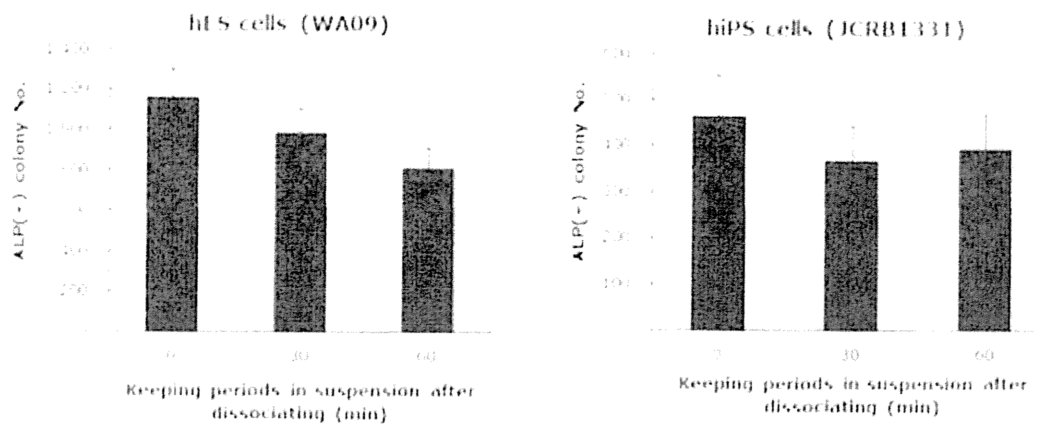
The cells were harvested and kept on ice for each period and then slowly frozen. The colonies with ALP positive were measured at 6 days (H9) or 7 days (JCRB1331) after thawing. Bars indicate the means ± S.D. (n=4).

Supplementary Figure 2.: Effect of dissociation material before cryopreservation



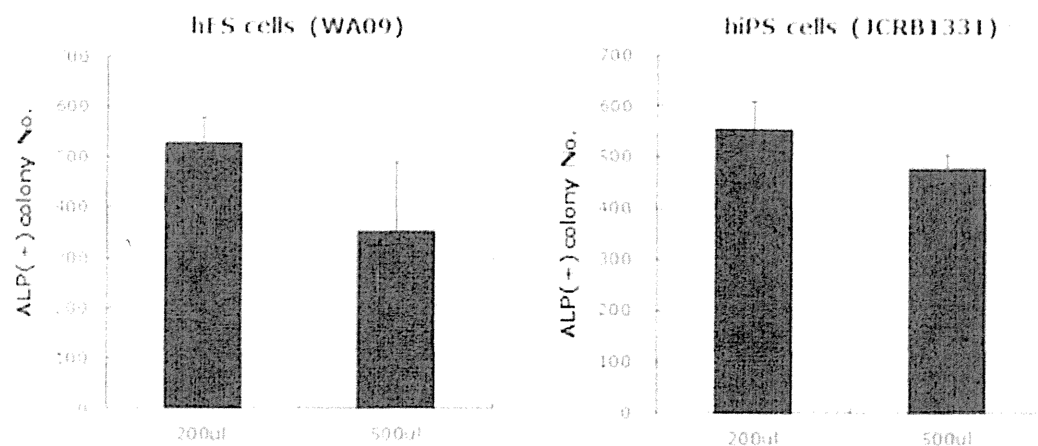
Cells were exposed to 1 ml CTK or Dispase until the edge of colonies began to curling up from the plate in a 37 °C incubator and then harvested. The cells were frozen by modified cryopreservation method. The colonies with ALP positive were measured at 6 days (H9) or 7 days (JCRB1331) after thawing. Bars indicate the means ± S.D. (n=3).

Supplementary Figure 3.: Suspension time after dissociation



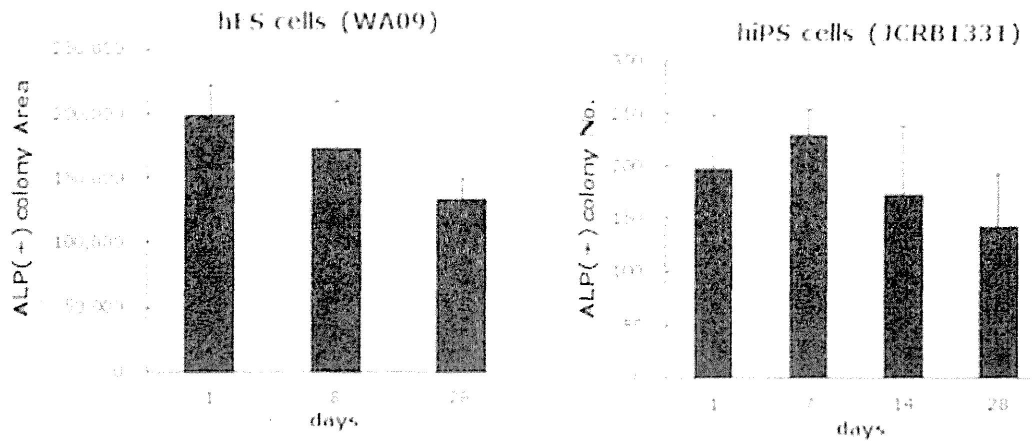
The cells were detached with dispase and kept each period in suspension and then slowly frozen. The colonies with ALP positive were measured at 6 days (H9) or 7 days (JCRB1331) after thawing. Bars indicate the means ± S.D. (n=4).

Supplementary Figure 4.: The volume of fluid during cryopreservation



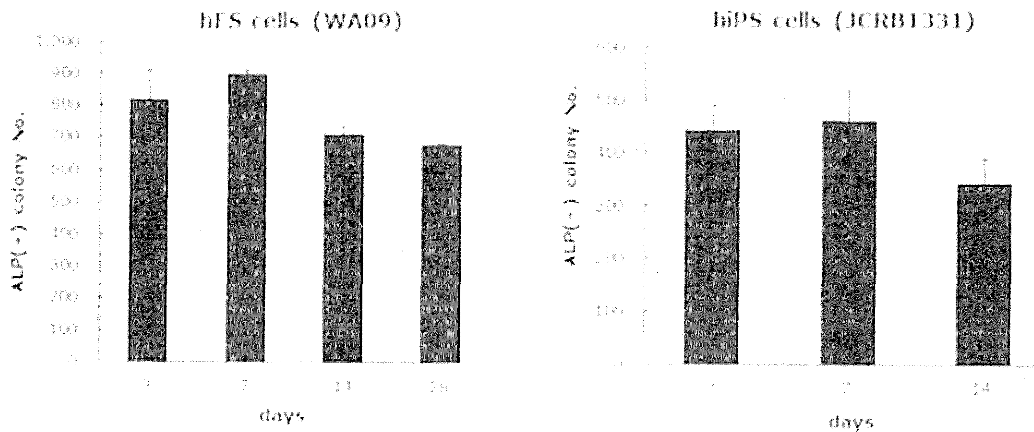
Cells were harvested and then suspended with each volume of PS medium containing 10% DMSO. The cells were frozen by modified cryopreservation method. The colonies with ALP positive were measured at 6 days (H9) or 7 days (JCRB1331) after thawing. Bars indicate the means ± S.D. (n=3).

Supplementary Figure 5.: Preservation period in a -80°C deep freezer before transferring into a liquid nitrogen tank



Cells were harvested and then suspended with PS medium containing 10% DMSO. The aliquot was left in a -80 °C deep freezer for each time and then transferred into a liquid nitrogen tank. The colonies with ALP positive were measured at 6 days (H9) or 7 days (JCRB1331) after thawing. Bars indicate the means ± S.D. (n=3).

Supplementary Figure 6.: Keeping period in a -80 °C deep freezer after transferring from a liquid nitrogen tank



Cells were harvested and then suspended with PS medium containing 10% DMSO. The cells were frozen by modified cryopreservation method. After transferred from a liquid nitrogen tank to a -80 °C deep freezer, the vials were kept each time at -80 °C deep freezer. The colonies with ALP positive were measured at 5 days (H9) or 6 days (JCRB1331) after thawing. Bars indicate the means ± S.D. (n=3).

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Competing financial interests

The authors declare no competing financial interests

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Readers' Comments

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HHEX Promotes Hepatic-Lineage Specification through the Negative Regulation of Eomesodermin

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Abstract

Human embryonic stem cells (hESCs) could provide a major window into human developmental biology, because the differentiation methods from hESCs mimic human embryogenesis. We previously reported that the overexpression of hematopoietically expressed homeobox (HHEX) in the hESC-derived definitive endoderm (DE) cells markedly promotes hepatic specification. However, it remains unclear how HHEX functions in this process. To reveal the molecular mechanisms of hepatic specification by HHEX, we tried to identify the genes directly targeted by HHEX. We found that HHEX knockdown considerably enhanced the expression level of eomesodermin (EOMES). In addition, HHEX bound to the HHEX response element located in the first intron of EOMES. Loss-of-function assays of EOMES showed that the gene expression levels of hepatoblast markers were significantly upregulated, suggesting that EOMES has a negative role in hepatic specification from the DE cells. Furthermore, EOMES exerts its effects downstream of HHEX in hepatic specification from the DE cells. In conclusion, the present results suggest that HHEX promotes hepatic specification by repressing EOMES expression.

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Introduction

The molecular mechanisms of liver development have been clarified by using model organisms such as chicks, *Xenopus*, *zebrafish*, and mice [1–2]. Although these models have many advantages, the molecular mechanisms of human liver development might be different from those of model organisms. The use of differentiation models from human embryonic stem cells (hESCs) for studying human development might resolve these problems, because these differentiation methods mimic human embryogenesis [3]. Previous reports have demonstrated that the definitive endoderm (DE) cells could be efficiently generated from hESCs in the presence of Activin A [4], and that the hESC-derived DE cells have the potential to differentiate into various DE-derived lineages, such as hepatocytes, pancreatic beta-cells, and small intestinal enterocytes[5–7]. In hepatic differentiation, Agarwal et al. reported that the typical gene expression profiles observed in the differentiation model from hESCs are similar to those observed in fetal liver development [8]. In addition, we previously reported that CCAAT/enhancer binding protein-mediated regulation of TGF beta receptor 2 expression determines the hepatoblast fate decision by using a differentiation model from hESCs [9]. The use of differentiation models from hESCs, rather than the usual model organisms, would provide great opportunities to expand our understanding of the molecular mechanisms.

A transcription factor, *hematopoietically expressed homeobox* (HHEX), is initially expressed in DE, and then its expression is restricted to the future hepatoblasts, which could segregate into both hepatocytes and cholangiocytes [10]. In the *HHEX*-null embryo, some hepatic gene expression levels are reduced and further hepatic development is prevented [11–12]. These studies indicate that the transcription factor HHEX plays an essential role in hepatic specification from DE. Recently, we reported that overexpression of HHEX by using adenovirus (Ad) vectors in the hESC-derived DE cells markedly promotes the hepatic specification [13]. Moreover, Kubo et al. demonstrated that HHEX promotes this process by synergistically working with bone morphogenetic protein 4 (BMP4), and they expected that HHEX might function with *HNF1 homeobox A* (HNF1α) [14], which is known to be its co-activator [15]. However, the functions of HHEX in this process

are not well understood, and the target genes of HHEX have not been investigated in detail. Therefore, we attempted to identify the target genes of HHEX in the hepatic specification by using a differentiation model from hESCs.

In the present study, to elucidate the functions of HHEX in hepatic specification from DE, we attempted to identify the target genes of HHEX by using the hepatic differentiation model from hESCs. To this end, the candidate target gene of HHEX were verified by performing ChIP-qPCR and luciferase reporter assays, and then loss-of-function assays were performed to clarify the functions of the candidate target gene in the hepatic specification. These results confirmed that *eomesodermin* (EOMES), which is known to regulate DE differentiation, is one of the crucial target genes of HHEX in human hepatic specification from the DE. Our report thus shows for the first time that HHEX promotes hepatic specification through the repression of EOMES expression.

Materials and Methods

hESCs Culture

A hESC line, H9 (WA09, WISC Bank, WiCell Research Institute), was maintained on a feeder layer of mitomycin C-treated mouse embryonic fibroblasts (MEF) (Millipore) with ReproStem medium (ReproCELL) supplemented with 5 ng/ml fibroblast growth factor 2 (FGF2) (KATAYAMA CHEMICAL INDUSTRIES). hESCs were dissociated with 0.1 mg/ml dispase (Roche) into small clumps and then were subcultured every 4 or 5 days. H9 was used following the Guidelines for Utilization of Human Embryonic Stem Cells of the Ministry of Education, Culture, Sports, Science and Technology of Japan after approval by the institutional ethical review board at National Institute of Biomedical Innovation.

In vitro Differentiation

The differentiation protocol for the induction of DE cells and hepatoblasts was based on our previous report with some modifications [13–16–21]. Briefly, hESCs were dissociated by using dispase and suspended in MEF-conditioned ReproStem medium supplemented with 10 ng/ml FGF2, and then plated onto a growth factor reduced Matrigel (BD Biosciences)-coated dish. When hESCs reached approximately 80% confluence, the MEF-conditioned ReproStem medium was replaced with the differentiation RPMI-1640 medium (Sigma) containing 100 ng/ml Activin A (R&D systems) (the differentiation RPMI-1640 medium is consisted with RPMI-1640 medium (Sigma) supplemented with B27 supplement (Invitrogen) and 4 mM L-glutamine), and then cultured for 4 days. For induction of the hepatoblasts, the DE cells were cultured for 5 days in the differentiation RPMI-1640 medium supplemented with 20 ng/ml BMP4 (R&D Systems) and 20 ng/ml FGF4 (R&D Systems).

RNA Isolation and Reverse Transcription-PCR

Total RNA was isolated from hESCs and their derivatives using ISOGENE (Nippon Gene). cDNA was synthesized using 500 ng of total RNA with a SuperScript VILO cDNA Synthesis Kit (Invitrogen). Real-time RT-PCR was performed with SYBR Green PCR Master Mix (Applied Biosystems) using an Applied Biosystems StemOnePlus real-time PCR systems. Relative quantification was performed against a standard curve and the values were normalized against the input determined for the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primer sequences used in this study are described in **Table S1 in File S2**.

Flow Cytometry

Single-cell suspensions of the hESC derivatives were fixed with 2% paraformaldehyde (PFA) at 4°C for 20 minutes and then incubated with the primary antibody, followed by the secondary antibody. Flow cytometry analysis was performed using a FACS LSR Fortessa flow cytometer (BD Biosciences). All the antibodies are listed in **Table S2 in File S2**.

ChIP-qPCR

ChIP assays were performed by using a Chromatin Immunoprecipitation Assay Kit (Millipore) according to the manufacturer's instructions. The hESC-derived cells (approximately 1.0×10^6 cells) were cross-linked with 1% formaldehyde at room temperature for 10 minutes. The cells were washed once with PBS containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mg/ml aprotinin and 1 mg/ml pepstatin A) and then harvested using a cell scraper. The cross-linked cells were centrifuged and resuspended with sodium dodecyl sulfate (SDS) lysis buffer with the protease inhibitors described above, and then incubated on ice for 10 minutes. The cells were sonicated to solubilize and shear cross-linked DNA. The resulting whole cells were centrifuged, and the supernatants were diluted in ChIP Dilution Buffer containing the protease inhibitors described above, then added to Protein A magnetic beads and rotated at 4°C for 30 minutes. Next, the supernatants of these cells were immunoprecipitated with anti-human HHEX antibody (Santa Cruz Biotechnology, sc-15129) or anti-goat IgG antibody at 4°C overnight with rotation. On the following day, the resulting supernatants were added to Protein A magnetic beads and rotated at 4°C for 60 minutes, then washed five times with Low Salt Immune Complex Wash Buffer (one time), High Salt Immune Complex Wash Buffer (one time), LiCl Immune Complex Wash Buffer (one time), and TE Buffer (two times) for 5 minutes per wash with rotation. Bound complexes were added to elution buffer (1% SDS, 0.1 M $NaHCO_3$) at room temperature for 15 minutes with rotation, and then the supernatants were added to 5 M NaCl and were eluted at 65°C for 4 hours. Immunoprecipitated DNA was purified by treatment with 0.5 M EDTA, 1 M Tris-HCl, and 10 mg/ml proteinase K at 45°C for 60 minutes and recovered by phenol/chloroform alcohol extraction and ethanol precipitation. Purified DNA was used as a template for qPCR according to the protocol described in the *RNA isolation and reverse transcription-PCR* section above. All the antibodies are listed in **Table S2 in File S2**. The primer sequences used in this study are described in **Table S1 in File S2**.

Plasmid Constructions

The promoter region of EOMES was cloned. To generate the 5′ untranslated region (UTR) of the EOMES-firefly luciferase reporter construct (pGL3-EOM-5UTR1000), a 1,000 bp 5′ UTR of the human EOMES was amplified by using the following primers: 5′-AGCGGTACCTTCCTCTCTACAAACCTTTCCCACTGGG-3′ and 5′-TAACCATGGGCTTTGCAAAGCGCAGACGGCAGCTGGCTGC-3′ (−1,000/−1 5′ UTR of EOMES; KpnI and NcoI restriction sites incorporated into sense and antisense primers, respectively, are underlined) and to generate the long 5′ UTR of the EOMES-firefly luciferase reporter construct (pGL3-EOM-5UTR4000), a 4,000 bp 5′ UTR of the human EOMES was amplified by using the following primers: 5′-CAGGGTACCGATAACACGTTTTTAGTGGGGGTG-3′ and 5′-TAACCATGGGCTTTGCAAAGCGCAGACGGCAGCTGGCTGC-3′ (−4,000/−1−5′ UTR of EOMES; KpnI and NcoI restriction sites incorporated into sense and antisense primers, respectively, are underlined).