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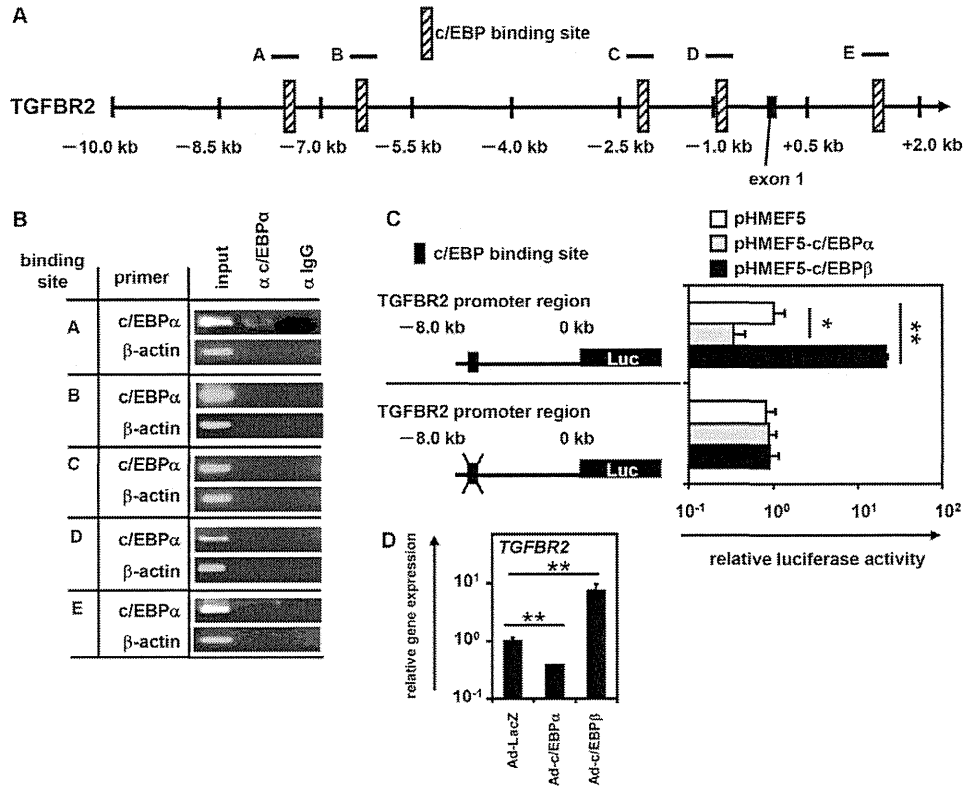

Fig. 4. *TGFBR2* promoter activity and expression are negatively regulated by c/EBPα and positively regulated by c/EBPβ. (A) Candidate c/EBP binding sites (hatched boxes) in the *TGFBR2* promoter region as predicted using rVista 2.0 (see supplementary material Fig. S7). (B) hESCs (H9 cells) were differentiated into hepatoblasts and then a ChIP assay performed. The antibodies and primers employed are summarized in supplementary material Tables S1 and S4. (C) HEK293 cells were transfected with firefly luciferase (Luc) expression plasmids containing the promoter region of *TGFBR2*. In addition, empty plasmid (pHMEF5), c/EBPα expression plasmid (pHMEF5-c/EBPα) or c/EBPβ expression plasmid (pHMEF5-c/EBPβ) was transfected. After 36 hours, a dual luciferase assay was performed. Base pair positions are relative to the translation start site (+1). (D) HepG2 cells (TGFBR2-positive cells) were transduced with 3000 VPs/cell of Ad-LacZ, Ad-c/EBPα or Ad-c/EBPβ for 1.5 hours and cultured for 48 hours. The expression level of *TGFBR2* in HepG2 cells was measured by real-time RT-PCR. On the *y*-axis, the gene expression level in Ad-LacZ-transduced cells was taken as 1.0. $^{*}P<0.05$, $^{**}P<0.01$. Error bars indicate s.d. Statistical analysis was performed using the unpaired two-tailed Student's *t*-test ($n=3$).

development, fetal hepatoblasts were purified from E13.5 mice. The gene expression level of *TGFBR2* in fetal mouse hepatoblasts was negatively or positively regulated by c/EBPα or c/EBPβ, respectively (Fig. 6A,B). The promotion of hepatocyte differentiation by Ad-c/EBPα transduction was inhibited by Ad-TGFBR2 transduction, whereas the inhibition of cholangiocyte differentiation by Ad-c/EBPα transduction was rescued by Ad-TGFBR2 transduction (Fig. 6C). In addition, the promotion of hepatocyte differentiation by si-c/EBPβ transfection was inhibited by Ad-TGFBR2 transduction, whereas the inhibition of cholangiocyte differentiation by si-c/EBPβ transfection was rescued by Ad-TGFBR2 transduction (Fig. 6D). Taken together, these results led us to conclude that c/EBPα and c/EBPβ could determine the cell fate of fetal mouse hepatoblasts by negatively and positively regulating TGFBR2 expression, respectively. Our *in vitro* differentiation system could also prove useful in elucidating the molecular mechanisms of human liver development.

DISCUSSION

The purpose of this study was to better understand the molecular mechanisms of the hepatoblast fate decision in humans. To elucidate the molecular mechanisms of liver development, both conditional knockout mouse models and cell culture systems are useful. For example, DeLaForest et al. demonstrated the role of HNF4α in hepatocyte differentiation using hESC culture systems (DeLaForest et al., 2011). The technology for inducing hepatocyte differentiation from hESCs has recently been dramatically advanced (Takayama et al., 2012a). Because it is possible to generate functional HBCs from hESCs, which can self-replicate and differentiate into both hepatocyte and cholangiocyte lineages (supplementary material Fig. S1 and Fig. 1), the differentiation model of HBCs generated from hESCs should provide a powerful tool for analyzing the molecular mechanisms of human liver development.

In this study, the molecular mechanisms of the hepatoblast fate decision were elucidated using hESC culture systems. HBCs cultured on human LN111 expressed hepatoblast markers (supplementary material Fig. S1) and had the ability to differentiate into both hepatocyte-like cells and cholangiocyte-like cells (Fig. 1). Because a previous study showed that low and high concentrations of TGFβ were required for hepatocyte and cholangiocyte differentiation, respectively (Clotman et al., 2005), we expected that TGFBR2 might contribute to the hepatoblast fate decision. Although TGFβ1, β2 and β3 are all ligands of TGFBR2, TGFβ3 did not promote cholangiocyte differentiation (Fig. 2). This might have been because only TGFβ3 is unable to upregulate the expression of *SOX9*, which is the key factor in bile duct development *in vivo* and cholangiocyte differentiation *in vitro* (Antoniou et al., 2009). We examined the function of TGFBR2 in the hepatoblast fate decision, and found that its overexpression promoted cholangiocyte differentiation, whereas TGFBR2 knockdown promoted hepatocyte differentiation (Fig. 3). Although an exogenous TGFβ ligand was not added to the differentiation medium, the endogenous TGFβ ligand present in Matrigel, which was used in our differentiation protocol, might have bound to TGFBR2. It might also be that the cells committed to the biliary lineage express TGFβ, as a previous study showed that bile duct epithelial cells express TGFβ (Lewindon et al., 2002).

To examine the molecular mechanism regulating TGFBR2 expression, the *TGFBR2* promoter region was analyzed (Fig. 4). *TGFBR2* promoter activity was negatively regulated by c/EBPα and positively regulated by c/EBPβ. c/EBPα overexpression downregulated *TGFBR2* promoter activity in spite of the fact that c/EBPα protein has no repression domain (Yoshida et al., 2006). CTBP1 and CTBP2 (Vernochet et al., 2009) are known to be co-repressors of c/EBPα, and as such constitute candidate co-repressors recruited to the c/EBP binding site in the *TGFBR2* promoter region.

Development (2014) doi:10.1242/dev.103168

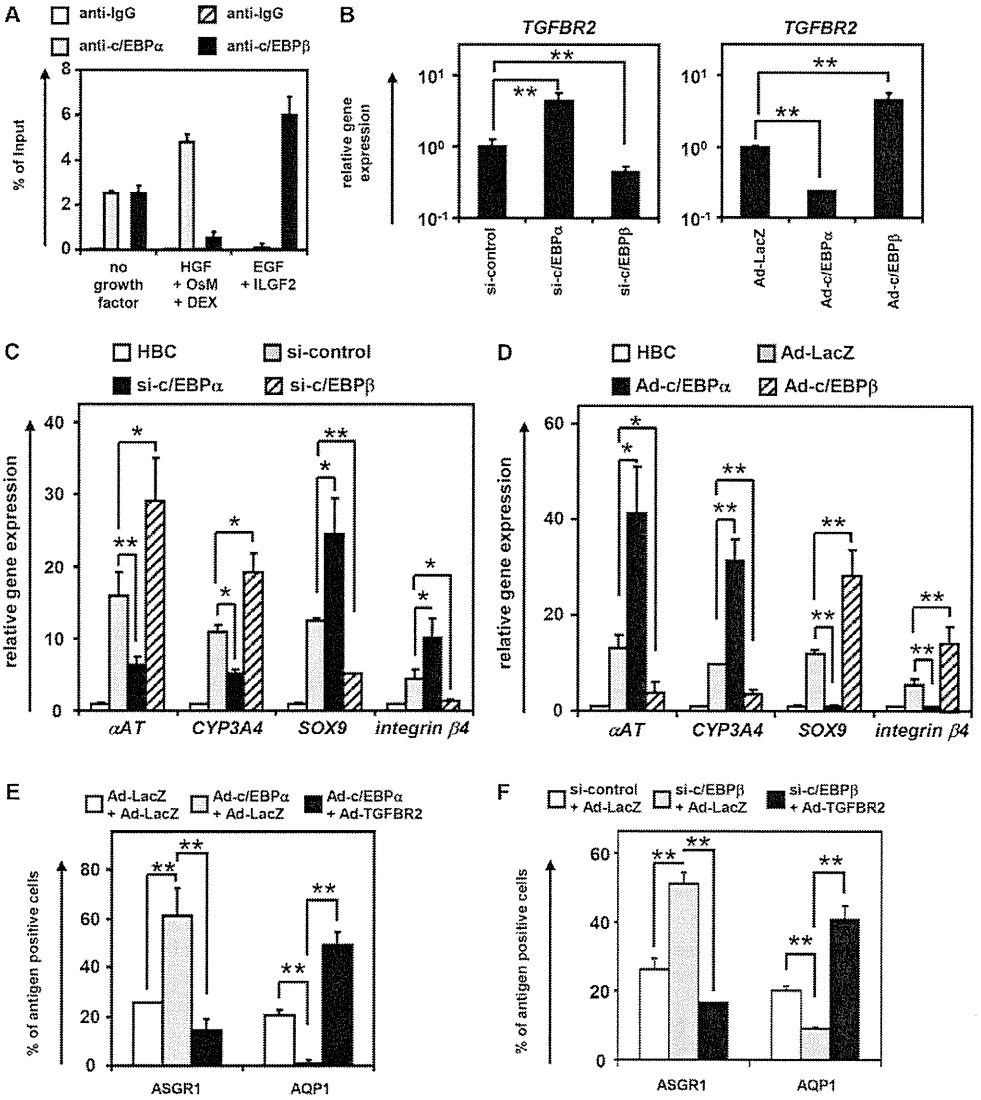

Fig. 5. c/EBPα and c/EBPβ promote hepatocyte and cholangiocyte differentiation by regulating *TGFBR2* expression, respectively. (A) HBCs were differentiated into hepatocyte-like cells or cholangiocyte-like cells according to the scheme outlined in Fig. 1A. On day 10 after hepatocyte or cholangiocyte differentiation, recruitment of c/EBPα or c/EBPβ to the *TGFBR2* promoter region was examined by ChIP assay. (B-D) HBCs were transfected with 50 nM si-control, si-c/EBPα or si-c/EBPβ and cultured in differentiation hESF-DIF medium for 10 days (B left, C). The expression levels of *TGFBR2* and hepatocyte and cholangiocyte markers were then measured by real-time RT-PCR. (B right, D) HBCs were transduced with 3000 VPs/cell of Ad-LacZ, Ad-c/EBPα or Ad-c/EBPβ for 1.5 hours and cultured in differentiation hESF-DIF medium for 10 days. The expression levels of *TGFBR2* and hepatocyte and cholangiocyte markers were then measured by real-time RT-PCR. On the *y*-axis, the gene expression level in the si-control-transfected or Ad-LacZ-transduced cells was taken as 1.0 in B, and levels in HBCs were taken as 1.0 in C and D. (E) HBCs were transduced with 3000 VPs/cell each of Ad-LacZ + Ad-LacZ, Ad-c/EBPα + Ad-LacZ, or Ad-c/EBPα + Ad-TGFBR2 for 1.5 hours and cultured in differentiation hESF-DIF medium for 10 days. The efficiency of hepatocyte or cholangiocyte differentiation was measured by estimating the percentage of ASGR1-positive or AQP1-positive cells, respectively, by FACS analysis. (F) HBCs were transduced with 3000 VPs/cell of Ad-LacZ or Ad-TGFBR2 and then transfected with 50 nM si-control or si-c/EBPβ and cultured in hESF-DIF medium for 10 days. The efficiency of hepatocyte or cholangiocyte differentiation was measured by estimating the percentage of ASGR1-positive or AQP1-positive cells, respectively, by FACS analysis. **P*<0.05, ***P*<0.01. Error bars indicate s.d. Statistical analysis was performed using the unpaired two-tailed Student's *t*-test (*n*=3).

Proteome analysis of c/EBPα would provide an opportunity to identify the co-repressor of c/EBPα. Because large numbers of nearly homogeneous hepatoblasts can be differentiated from hESCs, as compared with the isolation of fetal liver hepatoblasts, hepatocyte differentiation technology from hESCs might prove useful in proteome analysis.

We found that Ad-c/EBPα transduction could promote hepatocyte differentiation by suppressing TGFBR2 expression (Fig. 5). Our findings might thus provide a detailed explanation of the phenotype of *c/EBPα* knockout mice; that is, hepatocyte differentiation is inhibited and cholangiocyte differentiation is promoted in these mice (Yamasaki et al., 2006). We also found that Ad-c/EBPβ transduction could promote cholangiocyte differentiation by enhancing TGFBR2 expression. Because both c/EBPα and c/EBPβ can bind to the same binding site, reciprocal competition for binding is likely to be influenced by regulating c/EBPα or c/EBPβ expression. Therefore, the expression ratio between c/EBPα and c/EBPβ might determine the cell fate of hepatoblasts by regulating the expression level of TGFBR2. We confirmed that our findings could be reproduced in fetal mouse hepatoblasts (Fig. 6). Because a previous study had

Development (2014) doi:10.1242/dev.103168

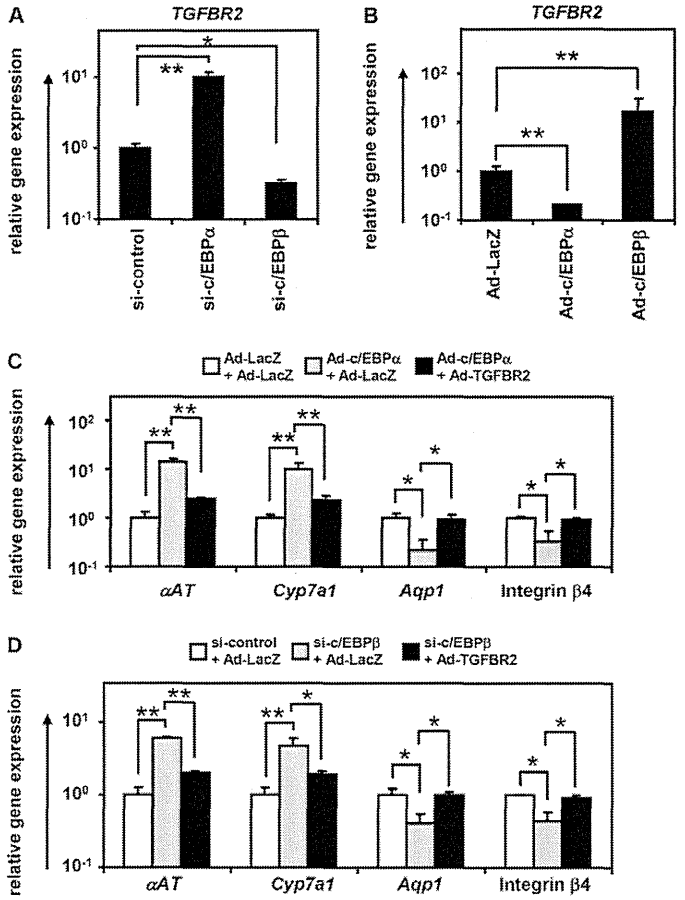

Fig. 6. c/EBPs control the differentiation of fetal mouse hepatoblasts through regulation of TGFBR2 expression. Fetal mouse hepatoblasts (Dlk1-positive cells; the purity was over 98%) were sorted from E13.5 mouse liver. (A) Fetal mouse hepatoblasts were transfected with 50 nM si-control, si-c/EBPα or si-c/EBPβ and cultured for 5 days. The expression of *TGFBR2* was measured by real-time RT-PCR. (B) Fetal mouse hepatoblasts were transduced with 3000 VPs/cell of Ad-LacZ, Ad-c/EBPα or Ad-c/EBPβ for 1.5 hours and cultured for 5 days. The expression of *TGFBR2* was measured by real-time RT-PCR. On the *y*-axis, the gene expression level in the si-control-transfected cells or Ad-LacZ-transduced cells was taken as 1.0. (C) Fetal mouse hepatoblasts were transduced with 3000 VPs/cell each of Ad-LacZ + Ad-LacZ, Ad-c/EBPα + Ad-LacZ, or Ad-c/EBPα + Ad-TGFBR2 for 1.5 hours and cultured for 5 days. On day 5, the expression levels of hepatocyte (*αAT* and *Cyp7a1*) and cholangiocyte (*Aqp1* and integrin β4) markers were measured by real-time RT-PCR. (D) Fetal mouse hepatoblasts were transduced with 3000 VPs/cell of Ad-LacZ or Ad-TGFBR2 and then transfected with 50 nM si-control or si-c/EBPβ and cultured for 5 days. On day 5, the gene levels of hepatocyte (*αAT* and *Cyp7a1*) and cholangiocyte (*Aqp1* and integrin β4) markers were measured by real-time RT-PCR. On the *y*-axis, the gene expression level in the si-control-transfected or Ad-LacZ-transduced cells was taken as 1.0. **P*<0.05, ***P*<0.01. Error bars indicate s.d. Statistical analysis was performed using the unpaired two-tailed Student's *t*-test (*n*=3).

shown that the addition of hepatocyte growth factor (HGF) to hepatoblasts upregulated the expression of c/EBPα and downregulated the expression of c/EBPβ (Suzuki et al., 2003), the ratio between c/EBPα and c/EBPβ might be determined by HGF during hepatocyte differentiation.

In this study, we have identified for the first time that *TGFBR2* is a target of c/EBPs in the hepatoblast fate decision (supplementary material Fig. S9). c/EBPα promotes hepatocyte differentiation by downregulating the expression of TGFBR2, whereas c/EBPβ promotes cholangiocyte differentiation by upregulating TGFBR2 expression. This study might have revealed a molecular mechanism underlying the lineage commitment of human hepatoblasts controlled by a gradient of TGFβ signaling. We believe that similar procedures that adopt the model of human pluripotent stem cell (including human iPS cell) differentiation will be used not only for the elucidation of molecular mechanisms underlying human hepatocyte and biliary differentiation but also for investigating the causes of congenital anomalies of the human liver and biliary tract.

MATERIALS AND METHODS

Ad vectors

Ad vectors were constructed by an improved *in vitro* ligation method (Mizuguchi and Kay, 1998; Mizuguchi and Kay, 1999). The human *c/EBPα* and *c/EBPβ* genes (accession numbers NM_004364 and NM_005194, respectively) were amplified by PCR using the following primers: c/EBPα, Fwd 5′-GCTCTAGATGCCGGGAGAACTCTAACTC-3′ and Rev 5′-GCGGTACCAAACCACTCCCTGGGTCC-3′; c/EBPβ, Fwd 5′-GCATCTAGATTCATGCAACGCCTGGTG-3′ and Rev 5′-ATAGGTACCTAAAATTACCGACGGGCTCC-3′. The human *TGFBR2* gene was purchased from Addgene (plasmid 16622). The human *c/EBPα*, *c/EBPβ* or *TGFBR2* gene was inserted into pBSKII (Invitrogen), resulting in pBSKII-c/EBPα, -c/EBPβ or -TGFBR2. Then, human *c/EBPα*, *c/EBPβ* or *TGFBR2* was inserted into pHMEF5 (Kawabata et al., 2005), which contains the human elongation factor 1α (*EF1α*, also known as *EEF1A1*) promoter, resulting in pHMEF5-c/EBPα, -c/EBPβ or -TGFBR2. pHMEF5-c/EBPα, -c/EBPβ or -TGFBR2 was digested with I-*Ceu*I/PI-*Sce*I and ligated into I-*Ceu*I/PI-*Sce*I-digested pAdHM41-K7 (Koizumi et al., 2003), resulting in pAd-c/EBPα, -c/EBPβ or -TGFBR2. The human *EF1α* promoter-driven *lacZ*- or FOXA2-expressing Ad vectors (Ad-LacZ or Ad-FOXA2, respectively) were constructed previously (Takayama et al., 2012b; Tashiro et al., 2008). All Ad vectors contain a stretch of lysine residues (K7) in the C-terminal region of the fiber knob for more efficient transduction of hESCs, definitive endoderm cells and HBCs, in which transfection efficiency was almost 100%, and the Ad vectors were purified as described previously (Takayama et al., 2012a; Takayama et al., 2011). The vector particle (VP) titer was determined by a spectrophotometric method (Maizel et al., 1968).

hESC culture

The H9 hESC line (WiCell Research Institute) was maintained on a feeder layer of mitomycin C-treated mouse embryonic fibroblasts (Merck Millipore) in ReproStem medium (ReproCELL) supplemented with 5 ng/ml FGF2 (Katayama Kagaku Kogyo). H9 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 and the study was approved by the Independent Ethics Committee.

Generation and maintenance of hESC-derived HBCs

Before the initiation of cellular differentiation, the hESC medium was exchanged for a defined serum-free medium, hESF9, and cultured as previously reported (Furue et al., 2008). The differentiation protocol for the induction of definitive endoderm cells and HBCs was based on our previous reports with some modifications (Takayama et al., 2012a; Takayama et al., 2012b; Takayama et al., 2011). Briefly, in mesendoderm differentiation, hESCs were cultured for 2 days on Matrigel Matrix (BD Biosciences) in differentiation hESF-DIF medium, which contains 100 ng/ml activin A (R&D Systems); hESF-DIF medium was purchased from Cell Science & Technology Institute; differentiation hESF-DIF medium was supplemented with 10 μg/ml human recombinant insulin, 5 μg/ml human apotransferrin, 10 μM 2-mercaptoethanol, 10 μM ethanolamine, 10 μM sodium selenite, 0.5 mg/ml bovine fatty acid-free serum albumin (all from Sigma) and 1×B27 Supplement (without vitamin A; Invitrogen). To generate definitive endoderm cells, the mesendoderm cells were transduced with 3000 VPs/cell of FOXA2-expressing Ad vector (Ad-FOXA2) for 1.5 hours on day 2 and cultured until day 6 on Matrigel in differentiation hESF-DIF medium supplemented with 100 ng/ml activin A. For induction of the HBCs, the

Development (2014) doi:10.1242/dev.103168

definitive endoderm cells were cultured for 3 days on Matrigel in differentiation hESF-DIF medium supplemented with 20 ng/ml BMP4 (R&D Systems) and 20 ng/ml FGF4 (R&D Systems). Transient overexpression of FOXA2 in the mesendoderm cells is not necessary for establishing HBCs, but it is helpful for efficient generation of the HBCs. The HBCs were first purified from the hESC-derived cells (day 9) by selecting attached cells on a human recombinant LN111 (BioLamina)-coated dish 15 minutes after plating (Takayama et al., 2013). The HBCs were cultured on a human LN111-coated dish (2.0×10^4 cells/cm^2) in maintenance DMEM/F12 medium [DMEM/F12 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 1× insulin/transferrin/selenium, 10 mM nicotinamide, 0.1 μM dexamethasone (DEX) (Sigma), 20 mM HEPES, 25 mM $NaHCO_3$, 2 mM L-glutamine, and penicillin/streptomycin] which contained 40 ng/ml HGF (R&D Systems) and 20 ng/ml epidermal growth factors (EGF) (R&D Systems). The medium was refreshed every day. The HBCs were dissociated with Accutase (Millipore) into single cells, and subcultured every 6 or 7 days. The HBCs used in this study were passaged more than three times.

In vitro hepatocyte and cholangiocyte differentiation

To induce hepatocyte differentiation, the HBCs were cultured on a Matrigel-coated dish (7.5×10^4 cells/cm^2) in Hepatocyte Culture Medium (HCM without EGF; Lonza) supplemented with 20 ng/ml HGF, 20 ng/ml Oncostatin M (OsM) (R&D Systems) and 1 μM DEX. To induce cholangiocyte differentiation, the HBCs were cultured in collagen gel. To establish collagen gel plates, 500 μl collagen gel solution [400 μl type I-A collagen (Nitta gelatin), 50 μl 10× DMEM and 50 μl 200 mM HEPES buffer containing 2.2% $NaHCO_3$ and 0.05 M NaOH] was added to each well, and then the plates were incubated at 37°C for 30 minutes. The HBCs (5×10^4 cells) were resuspended in 500 μl differentiation DMEM/F12 medium [DMEM/F12 medium supplemented with 20 mM HEPES, 2 mM L-glutamine, 100 ng/ml EGF and 40 ng/ml ILGF2 (IGF2)], and then mixed with 500 μl of the collagen gel solution and plated onto the basal layer of collagen. After 30 minutes, 2 ml differentiation DMEM/F12 medium was added to the well.

Inhibition of TGFβ signaling

SB-431542 (Santa Cruz Biotechnology), which is a small molecule that acts as a selective inhibitor of activin receptor-like kinase (ALK) receptors [ALK4, ALK5 and ALK7 (also known as ACVR1B, TGFBR1 and ACVR1C)], was used to inhibit TGFβ signaling in HBCs.

Flow cytometry

Single-cell suspensions of hESC-derived cells were fixed with 2% paraformaldehyde (PFA) at 4°C for 20 minutes, and then incubated with primary antibody (supplementary material Table S1) followed by secondary antibody (supplementary material Table S2). Flow cytometry analysis was performed using a FACS LSR Fortessa flow cytometer (BD Biosciences). Cell sorting was performed using a FACS Aria (BD Biosciences).

RNA isolation and reverse transcription (RT)-PCR

Total RNA was isolated from hESCs and their derivatives using ISOGENE (Nippon Gene). cDNA was synthesized using 500 ng total RNA with the 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 system. Relative quantification was performed against a standard curve and the values were normalized against the input determined for the housekeeping gene *GAPDH*. Primers are described in supplementary material Table S3.

Immunohistochemistry

Cells were fixed with 4% PFA. After incubation with 0.1% Triton X-100 (Wako), blocking with Blocking One (Nakalai Tesque) or PBS containing 2% FBS, 2% BSA and 0.1% Triton X-100, the cells were incubated with primary antibody (supplementary material Table S1) at 4°C overnight, followed by secondary antibody (supplementary material Table S2) at room temperature for 1 hour. Immunopositive cells were counted in at least eight randomly chosen fields.

HBC colony formation assay

For the colony formation assay, HBCs were cultured at a low density (200 cells/cm^2) on a human LN111-coated dish in maintenance DMEM/F12 medium supplemented with 25 μM LY-27632 (ROCK inhibitor; Millipore).

Transplantation of clonally derived HBCs

Clonally derived HBCs were dissociated using Accutase and then suspended in maintenance DMEM/F12 medium without serum. The HBCs (1×10^6 cells) were transplanted 24 hours after administration of CCl_4 (2 mg/kg) by intrasplenic injection into 8- to 10-week-old *Rag2/Il2rg* double-knockout mice. Recipient mouse livers and blood were harvested 2 weeks after transplantation. Grafts were fixed with 4% PFA and processed for immunohistochemistry. Serum was extracted and subjected to ELISA. All animal experiments were conducted in accordance with institutional guidelines.

ELISA

Levels of human ALB in mouse serum were examined by ELISA using kits from Bethyl Laboratories according to the manufacturer's instructions.

Culture of mouse Dlk1$^+$ cells

Dlk1$^+$ hepatoblasts were isolated from E13.5 mouse livers using anti-mouse Dlk1 monoclonal antibody (MBL International Corporation, D187-4) as described previously (Tanimizu et al., 2003). Dlk1$^+$ cells were resuspended in DMEM/F12 (Sigma) containing 10% FBS, 1× insulin/transferrin/selenium (ITS), 10 mM nicotinamide (Wako), 0.1 μM DEX and 5 mM L-glutamine. Cells were plated on laminin-coated dishes and cultured in medium containing 20 ng/ml HGF, EGF and 25 μM LY-27632 (ROCK inhibitor).

lacZ assay

Hepatoblasts were transduced with Ad-LacZ at 3000 VPs/cell for 1.5 hours. The day after transduction (day 10), 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) staining was performed as described previously (Kawabata et al., 2005).

Reporter assays

The effects of c/EBPα or c/EBPβ overexpression on *TGFBR2* promoter activity were examined using a reporter assay. An 8 kb fragment of the 5′ flanking region of the *TGFBR2* gene was amplified by PCR using the following primers: Fwd, 5′-CCGAGCTCATGTTTGATGAAGTGTCTAG-CTTCCAAGG-3′; Rev, 5′-GGCTCGAGCCTCGACGTCCAGCCCCT-3′. The fragment was inserted into the *SacI*/*XhoI* sites of pGL3-basic (Promega), resulting in a pGL3-*TGFBR2* promoter region (pGL3-TGFBR2-PR). To generate a *TGFBR2* promoter region containing mutations in the c/EBP binding site, the following primers were used in PCR (mutations are indicated by lowercase letters): Fwd, 5′-CACTAGTATTCAgTG-AtCcgAAAATATGG-3′; Rev, 5′-CACTAGTATTCAgTGAtCcgAAAA-TATGG-3′; this resulted in pGL3-mTGFBR2-PR. HEK293 cells were maintained in DMEM (Wako) supplemented with 10% FBS, penicillin and streptomycin, and 2 mM L-glutamine. In reporter assays, 60 ng pGL3-TGFBR2-PR or pGL3-mTGFBR2-PR was transfected together with 720 ng each expression plasmid (pHMEF5, pHMEF5-c/EBPα and pHMEF5-c/EBPβ) and 60 ng internal control plasmid (pCMV-Renilla luciferase) using Lipofectamine 2000 reagent (Invitrogen). Transfected cells were cultured for 36 hours, and a Dual Luciferase Assay (Promega) was performed according to the manufacturer's instructions.

siRNA-mediated knockdown

Predesigned siRNAs targeting *c/EBPα*, *c/EBPβ* and *TGFBR2* mRNAs were purchased from Thermo Scientific Dharmacon. Cells were transfected with 50 nM siRNA using RNAiMAX (Invitrogen) transfection reagent according to the manufacturer's instructions. As a negative control, we used scrambled siRNA (Qiagen) of a sequence showing no significant similarity to any mammalian gene.

Chromatin immunoprecipitation (ChIP) assay

The ChIP assay kit was purchased from Upstate. Cells were crosslinked using formaldehyde at a final concentration of 1% at 37°C for 10 minutes, and then genomic DNA was fragmented by sonicator. The resulting DNA-protein complexes were immunoprecipitated using the antibodies described in supplementary material Table S1 or control IgG as described in supplementary material Table S2. The precipitated DNA fragments were analyzed by real-time RT-PCR using the primers shown in supplementary material Table S4 to amplify the *TGFBR2* promoter region including the c/EBP binding sites or β-actin locus as a control. The results of quantitative ChIP analysis (Fig. 5A) were expressed as the amount of amplified *TGFBR2* promoter region relative to input DNA taken as 100%.

Statistical analysis

Statistical analysis was performed using an unpaired two-tailed Student's *t*-test. All data are represented as mean ± s.d. (n=3).

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Competing interests

The authors declare no competing financial interests.

Author contributions

K. Takayama, K.K. and H.M. developed the concepts or approach; K. Takayama, Y.N., K.O., H.O. and T.Y. performed experiments; K. Takayama, K.K., M.I., K. Tashiro, F.S., T.H., T.O., M.F.K. and H.M. performed data analysis; K. Takayama, K.K. and H.M. prepared or edited the manuscript prior to submission.

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Supplementary material

Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.103168/-/DC1

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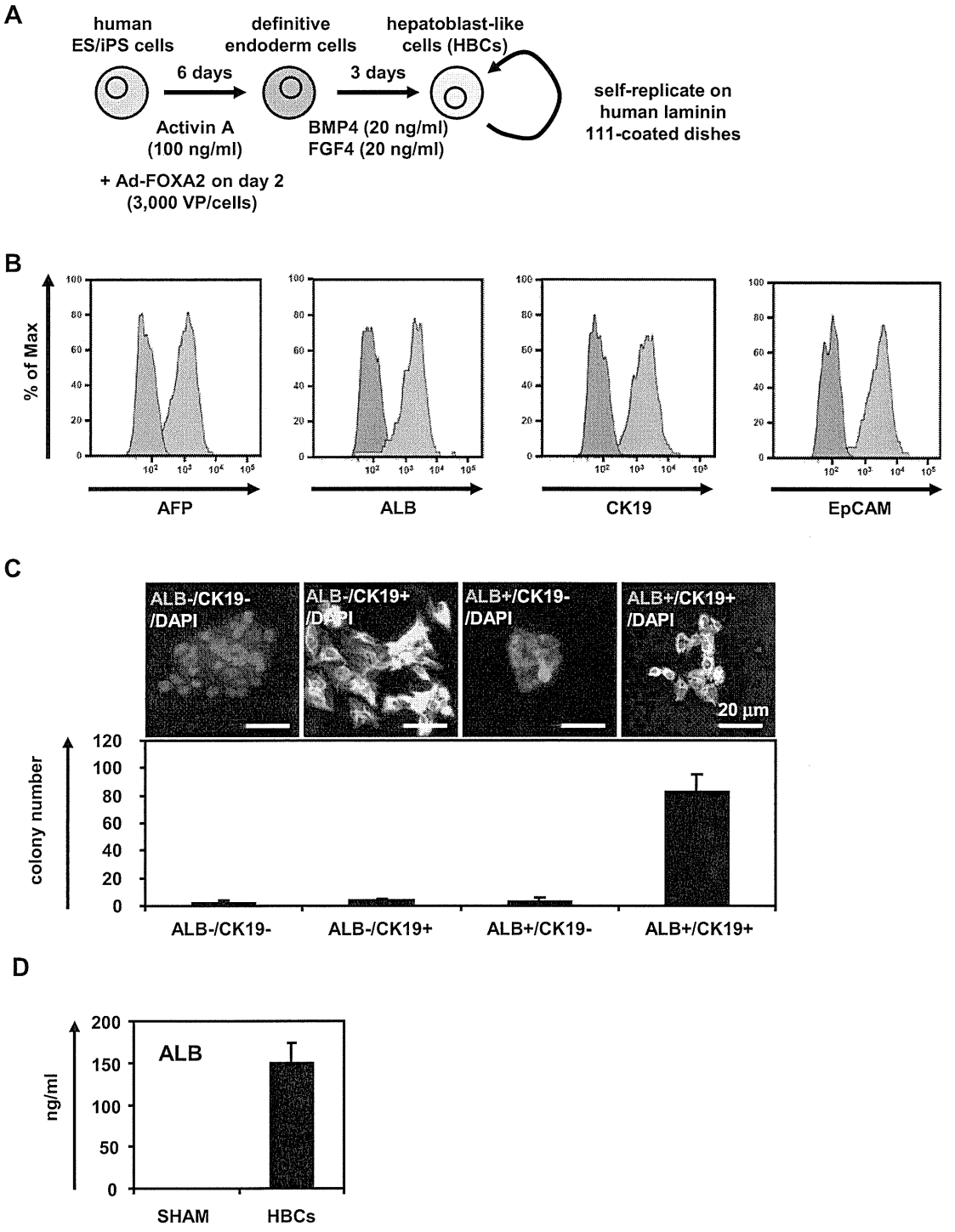

Fig. S1 The hepatoblast-like cells (HBCs) generated from hESCs were characterized.

(**A**) hESCs were differentiated into the HBCs via definitive endoderm cells. The HBCs were maintained on human LN111. (**B**) The expression levels of hepatoblast markers (AFP, ALB, CK19, and EpCAM) in the HBCs were examined by FACS

analysis. (**C**) Clonal assay of the HBC was performed. The HBCs were plated at a density of 200 cells/cm^2 on human LN111-coated 96-well plates. The colonies were separated into four groups based on the expression of ALB and CK19 (ALB and CK19 double-negative, ALB negative and CK19 positive, ALB positive and CK19 negative, and ALB and CK19 double-positive groups). The numbers represent wells in which the colony was observed in three 96-well plates (total 288 wells). Five days after plating, the cells were fixed with 4% PFA and used for double immunostaining. Nuclei were counterstained with DAPI (blue). (**D**) The HBCs were transplanted into CCl_4 (2 mL/kg)-treated Rag2/IL2 receptor gamma double-knockout mice. The human ALB level in recipient mouse serum was measured at 2 weeks after transplantation.

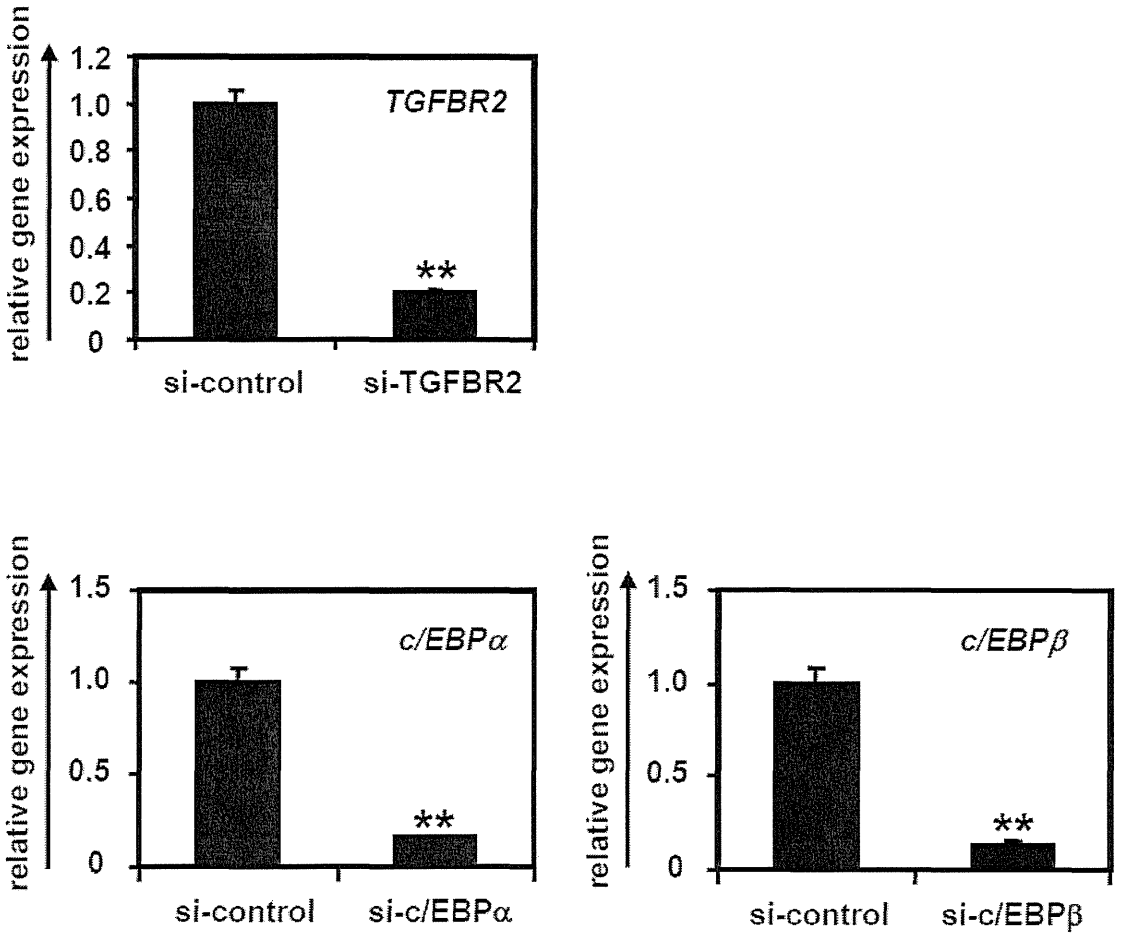

Fig. S2 c/EBPα, c/EBPβ, or TGFBR2 were knocked-down in the HBCs by si-c/EBPα, si-c/EBPβ, or si-TGFBR2 transfection, respectively.

The HBCs were transfected with 50 nM of si-control, si-c/EBPα, si-c/EBPβ, or si-TGFBR2. Two days after transfection, the gene expression levels of *c/EBPα*, *c/EBPβ*, or *TGFBR2* were examined by real-time RT-PCR in si-c/EBPα-, si-c/EBPβ-, or si-TGFBR2-transfected cells, respectively. On the y axis, the gene expression levels of *c/EBPα*, *c/EBPβ*, or *TGFBR2* in si-control-transfected cells were taken as 1.0. $^{**}P<0.01$ (compared with the si-control-transfected cells).

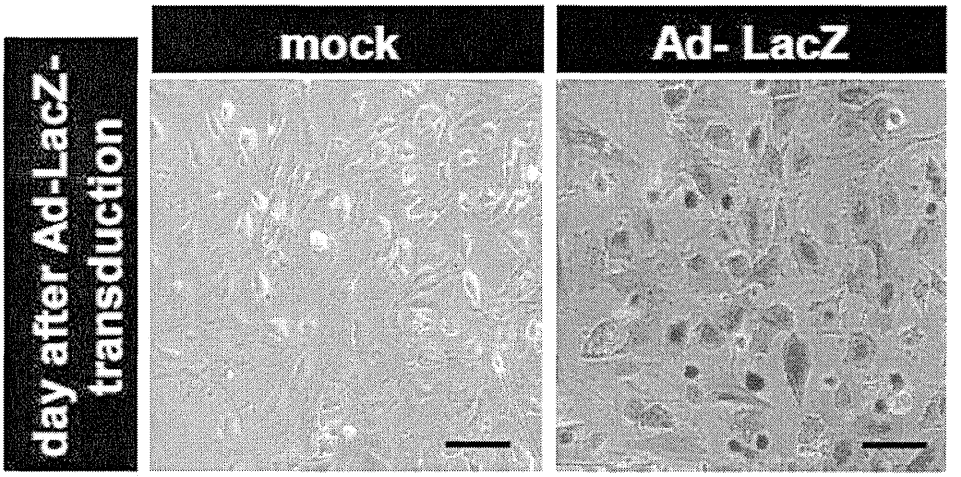

Fig. S3 Ad vectors efficiently transduced the HBCs.

The HBCs were transduced with 3,000 VP/cell of Ad-LacZ for 1.5 hr. The day after transduction, X-gal staining was performed. The scale bars represent 50 μm.

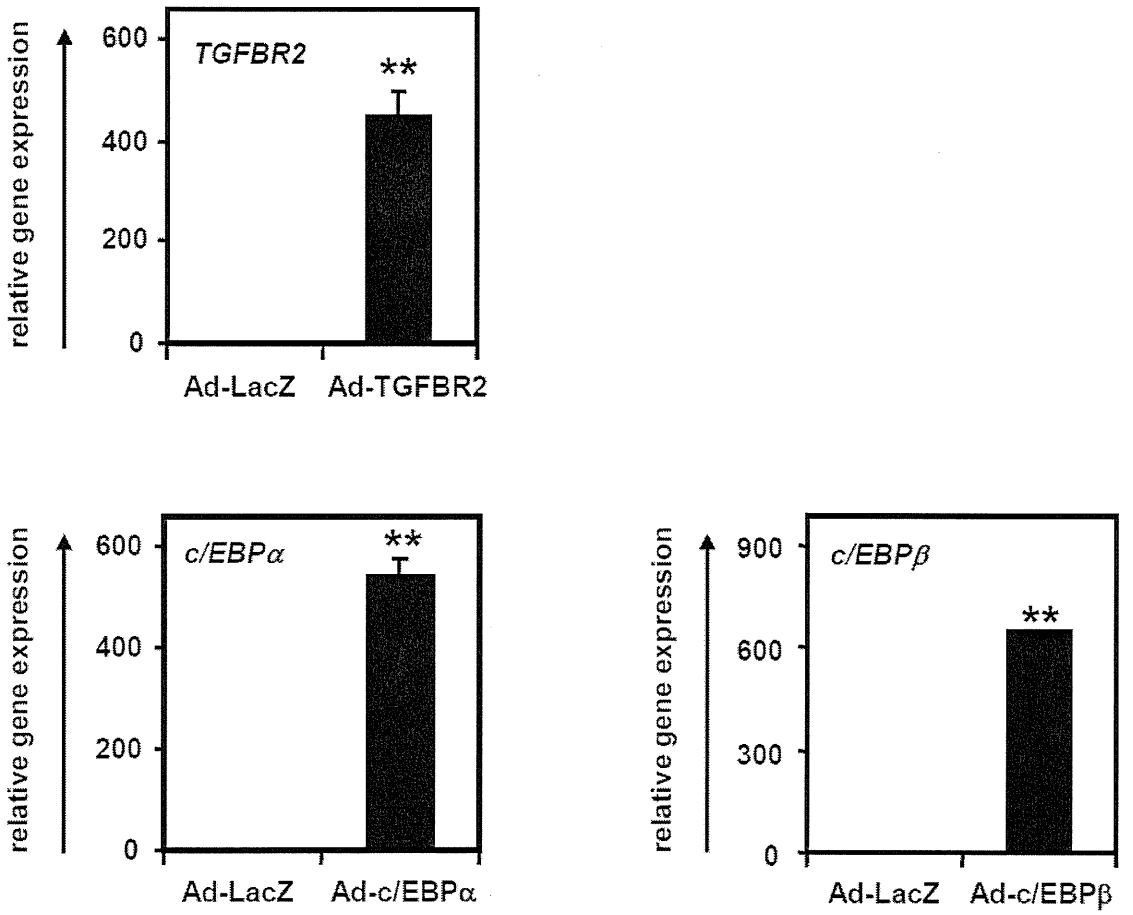

Fig. S4 c/EBPα, c/EBPβ, or TGFBR2 were overexpressed in the HBCs by Ad-c/EBPα, Ad-c/EBPβ, or Ad-TGFBR2 transduction, respectively.

The HBCs were transduced with 3,000 VP/cells of Ad-c/EBPα, Ad-c/EBPβ, or Ad-TGFBR2 for 1.5 hr. Two days after Ad vectors transduction, the gene expression levels of *c/EBPα*, *c/EBPβ*, or *TGFBR2* were examined by real-time RT-PCR in Ad-c/EBPα-, Ad-c/EBPβ-, or Ad-TGFBR2-transduced cells, respectively. On the y axis, the gene expression levels of *c/EBPα*, *c/EBPβ*, or *TGFBR2* in Ad-LacZ-transduced cells were taken as 1.0. $^{**}P<0.01$ (compared with the Ad-LacZ-transfected cells).

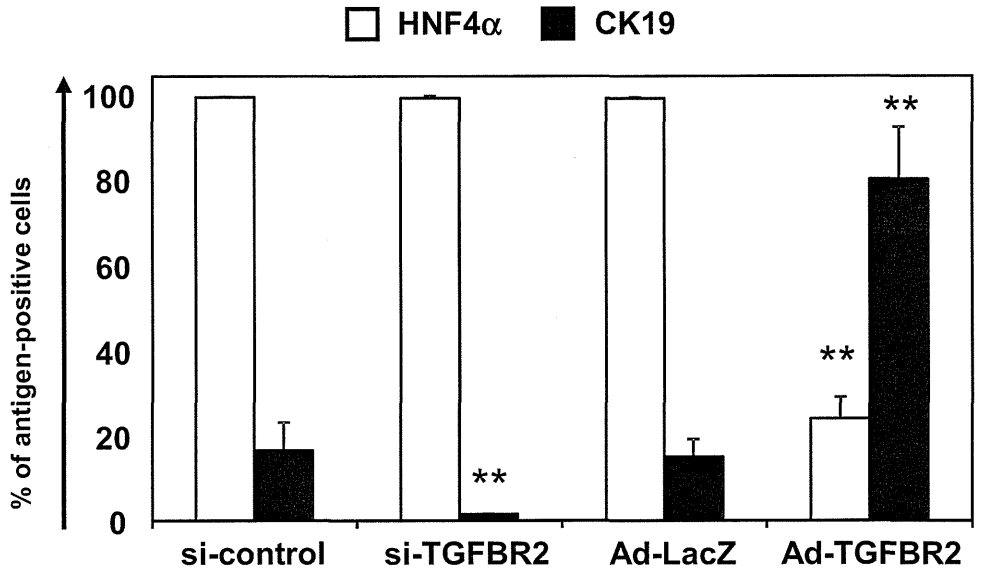

Fig. S5 TGFBR2 overexpression or knockdown in the HBCs promotes cholangiocyte or hepatocyte differentiation, respectively.

The si-control-, si-TGFBR2-, Ad-LacZ- or Ad-TGFBR2-transduced HBCs (total of 1.0×10^6 cells) were transplanted into CCl_4 (2 mL/kg)-treated Rag2/IL2 receptor gamma double knockout mice by intrasplenical injection. Expressions of HNF4α and CK19 were examined by immunohistochemistry at 2 weeks after transplantation. Semiquantitative analysis of the immunofluorescent staining was performed in the human cell clusters. $^*P<0.05$; $^{**}P<0.01$.

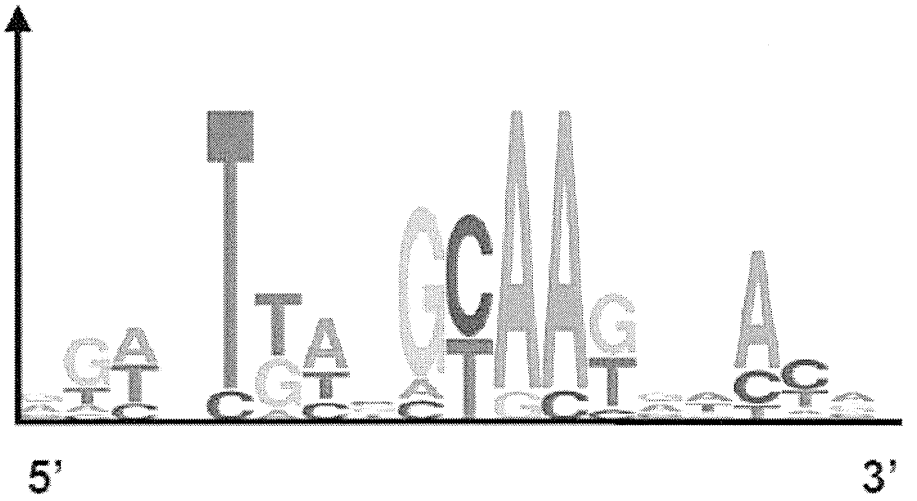

Fig. S6 c/EBP-binding site on the TGFBR2 promoter region

The consensus sequence of the c/EBP-binding site is described. (http://www.cbil.upenn.edu/cgi-bin/tess/tess).

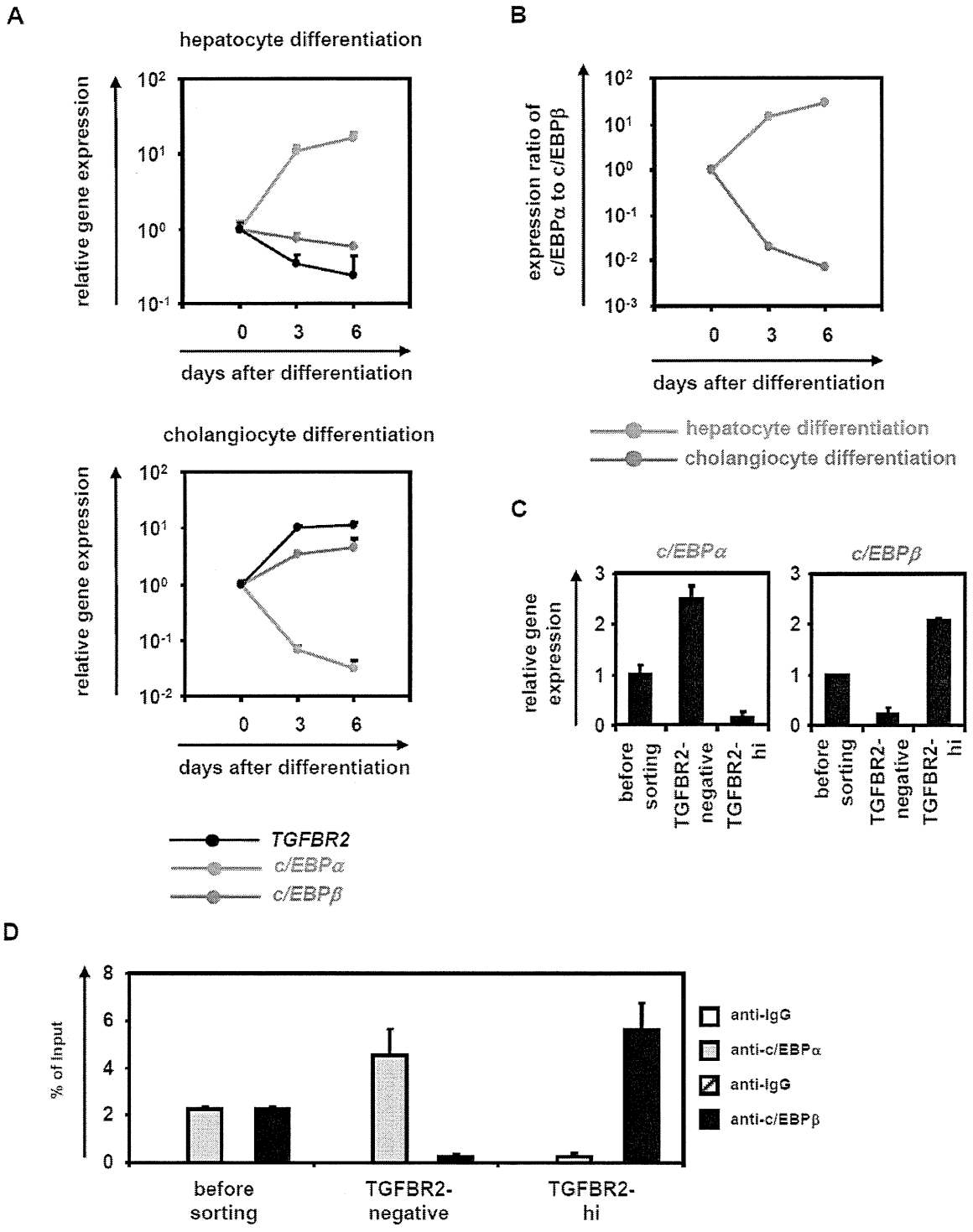

Fig. S7 Temporal gene expression levels of *TGFBR2*, *c/EBPα*, and *c/EBPβ* in hepatocyte and cholangiocyte differentiation.

The HBCs were differentiated into hepatocyte-like cells or cholangiocyte-like cells as shown in figure 1A. (**A**) Temporal gene expression levels of *TGFBR2*,

c/EBPα, and *c/EBPβ* in hepatocyte differentiation and cholangiocyte differentiation of the HBCs were examined by real-time RT-PCR. On the y axis, the gene expression levels in the HBCs were taken as 1.0. (**B**) The temporal ratio of c/EBPα to c/EBPβ was demonstrated in hepatocyte and cholangiocyte differentiation. The ratio of c/EBPα to c/EBPβ in the HBCs was taken as 1.0. (C) The HBCs were cultured on Matrigel for 5 days, and then the expression level of TGFBR2 was examined by FACS analysis. TGFBR2-negative, -lo, and –hi populations were collected as described in figure 1F. Real-time RT-PCR analysis was performed in three populations (before sorting, TGFBR-negative, and TGFBR2-hi) to measure the expression levels of *c/EBPα* and *c/EBPβ*. (**D**) The recruitment of c/EBPα or c/EBPβ to the TGFBR2 promoter region in three populations (before sorting, TGFBR-negative, and TGFBR2-hi) was examined by ChIP assay.

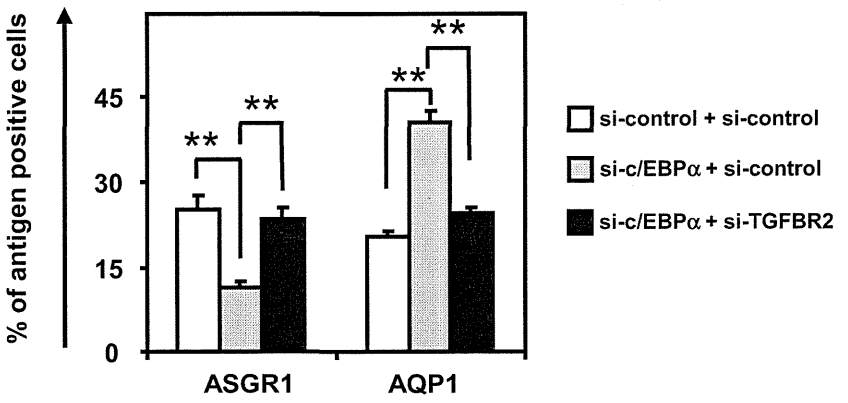

Fig. S8 Inhibition of hepatocyte differentiation by si-c/EBPα transfection was rescued by si-TGFBR2 transfection.

The HBCs were transfected with 50 nM of each of si-control + si-control, si-c/EBPα + si-control, or si-c/EBPα + si-TGFBR2 and cultured with the differentiation hESF-DIF medium for 10 days. The efficiency of hepatocyte or cholangiocyte differentiation was measured by estimating the percentage of ASGR1- or AQP1-positive cells, respectively, using FACS analysis. $*P<0.05$; $**P<0.01$.

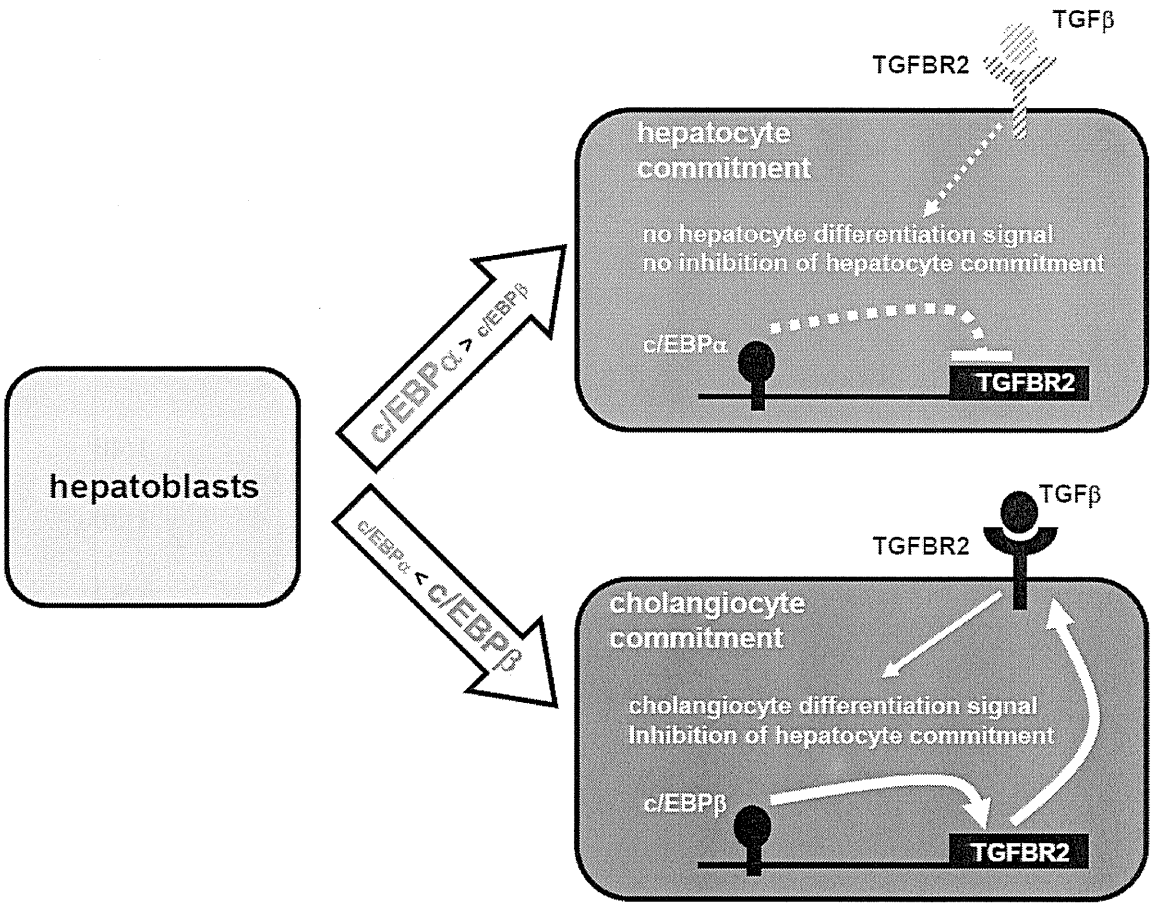

Fig. S9 The lineage segregation of hepatoblasts might be explained by c/EBP-mediated control of TGFBR2 expression.

In hepatocyte differentiation from hepatoblasts, c/EBPα promotes hepatocyte differentiation via negative regulation of TGFBR2 expression. On the other hand, c/EBPβ promotes cholangiocyte differentiation via positive regulation of TGFBR2 expression in cholangiocyte differentiation.

Supplemental Table 1 The primary antibodies used in this study

Antigen	Species	Company (catalog number)	Dilution
CK19	rabbit	Abcam (ab52625)	1/250
AFP	mouse	Cell Signaling (#3903)	1/100
c/EBPβ	rabbit	Santa Cruz Biotechnology (sc-150AC)	1/50
ALB (ELISA)	goat	Bethyl Laboratories (E80-129)	
ALB (FCM)	rabbit	Abcam (ab135575)	1/40
ALB (IHC)	goat	Santa Cruz Biotechnology (sc-46293)	1/200
c/EBPα	rabbit	Abcam (ab40764)	1/50
HNF4α	abcm	Abcam (ab36175)	1/100
TGFBR2	mouse	Santa Cruz Biotechnology (sc-17799)	1/50
ASGR1	goat	Santa Cruz Biotechnology (sc-13467)	1/50
CYP3A4	goat	Santa Cruz Biotechnology (sc-27639)	1/200
AQP1	mouse	Abcam (ab9566)	1/40
EpCAM	mouse	Militenyi Biotec (130-091-254)	1/50

Supplemental Table 2 The secondary antibodies used in this study

Antigen	label	Company	Species	Dilution
rabbit IgG	alexa fluor 488	Molecular Probes	goat	1/1000
rabbit IgG	alexa fluor 488	Molecular Probes	chicken	1/1000
mouse IgG	alexa fluor 488	Molecular Probes	rabbit	1/1000
goat IgG	alexa fluor 488	Molecular Probes	rabbit	1/1000
rabbit IgG	alexa fluor 594	Molecular Probes	mouse	1/1000
goat IgG	alexa fluor 594	Molecular Probes	mouse	1/1000
goat IgG	alexa fluor 594	Molecular Probes	chicken	1/1000
goat IgG	alexa fluor 594	Molecular Probes	donkey	1/1000
mouse IgG	alexa fluor 594	Molecular Probes	chicken	1/1000

Supplemental Table 3 The primers used for real-time RT-PCR in this study

Genes	Primers (forward/reverse; 5' to 3')
CK7	AGACGGAGTTGACAGAGCTG/GGATGGCCCGGTTCATCTC
CK19	CTCCCGCGACTACAGCCACT/TCAGCTCATCCAGCACCCTG
HES1	ATGGAGAAAAATTCCTCGTCCC/TTCAGAGCATCCAAAATCAGTGT
SOX9	TTTCCAAGACACAAACATGA/AAAGTCCAGTTTCTCGTTGA
integrin β4	GCAGCTTCCAAATCACAGAGG/CCAGATCATCGGACATGGAGTT
TO	GGCAGCGAAGAAGACAAATC/TCGAACAGAATCCAACTCCC
αAT	ACTGTCAACTTCGGGGACAC/CATGCCTAAACGCTTCATCA
ALB	GCACAGAATCCTTGGTGAACAG/ATGGAAGGTGAATGTTTTCAGCA
TGFBR2	GGAAACTTGACTGCACCGTT/CTGCACATCGTCCTGTGG
c/EBPα	TTCACATTGCACAAGGCACT/GAGGGACCGGAGTTATGACA
c/EBPβ	CGTGTACACACGCGTTCAG/CTCTCTGCTTCTCCCTCTGC
HNF6	CAAACCCTGGAGCAAACTCAA/TGTGTTGCCTCTATCCTTCCC
HNF1β	ACCAAGCCGGTCTTCCATACT/GGTGTGTCATAGTCGTCGCC
CYP2D6	CTTTCGCCCCAACGGTCTC/TTTTGGAAGCGTAGGACCTTG
TTR	TCATCGTCTGCTCCTCCTCT/AGGTGTCATCAGCAGCCTTT
HNF1α	AACACCTCAACAAGGGCACTC/CCCCACTTGAAACGGTTCCT
CYP3A4	AAGTCGCCTCGAAGATACACA/AAGGAGAGAACACTGCTCGTG
mouse αAT	TTGCTCGACACAACATGGAAT/ACGTCCCAGTTTGACATCTCT
mouse CYP7A1	GCTGTGGTAGTGAGCTGTTG/GTTGTCCAAAGGAGGTTCACC
mouse AQP1	AGGCTTCAATTACCCACTGGA/CTTTGGGCCAGAGTAGCGAT
mouse integrin β4	AGAGCTGTACCGAGTGCATC/TGGTGTCGATCTGGGTGTTCT

Supplemental Table 4 The primers used for ChIP assay in this study

	Primers (forward/reverse; 5' to 3')
c/EBP binding site A	TCACAACTTTCTAAGTCCCAATTTT/ACTGAGGCAGGGACTGTGTC
c/EBP binding site B	AACTGAAATGTCTTCCTTTTTCAA/CAGGAGGAGTAGAGCCAGCA
c/EBP binding site C	GCCACATTGTGTTTTCAGGA/ TTAGCCGAGAATGATGTCACC
c/EBP binding site D	CCAGAGGGCTGTACAGAATCA/ CCAGATTTGCCCAAGACATT
c/EBP binding site E	TGCCTACTGGGTGCTAGAGG/AACCTTCAGAGACAGCGATCA
β-actin	CCGGCGGGGTCTTTGTCTGAGC/GGGCCGGCCGCGTTATTACCA