

Fig. 1. HBCs can differentiate into both hepatocyte and cholangiocyte lineages. (A) The strategy for hepatocyte and cholangiocyte differentiation from HBCs. (B,C) The HBC-derived hepatocyte-like cells or cholangiocyte-like cells were subjected to immunostaining with anti-CYP3A4 (red, B) or anti-CK19 (green, C) antibodies, respectively. (D,E) Temporal gene expression levels of hepatocyte markers (α AT and CYP3A4) (D) or cholangiocyte markers (SOX9 and integrin β 4) (E) during hepatocyte or cholangiocyte differentiation as measured by real-time RT-PCR. The temporal gene expression of *TGFBR2* was also examined. The gene expression levels in HBCs were taken as 1.0. (F) 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 and real-time RT-PCR analysis was performed to measure the expression levels of hepatocyte markers (α AT and CYP3A4) and cholangiocyte markers (SOX9 and integrin β 4). * P <0.05, ** P <0.01 (compared with 'before sorting'). Error bars indicate s.d. Statistical analysis was performed using the unpaired two-tailed Student's *t*-test ($n=3$).

TGFBR2 were transplanted into CCl₄-treated immunodeficient mice (Fig. 3E,F). Although some of the si-control-transfected or Ad-LacZ-transduced HBCs remained as HBCs (HNF4 α and CK19 double positive), most of them showed *in vitro* differentiation toward hepatocyte-like cells (HNF4 α single positive) (Fig. 3E, top row). By contrast, Ad-*TGFBR2*-transduced HBCs were predominantly committed to cholangiocyte-like cells (CK19 single positive) and si-*TGFBR2*-transfected HBCs were predominantly committed to hepatocyte-like cells (HNF4 α single positive) (Fig. 3E, bottom row). Ad-*TGFBR2* transduction decreased the percentage of HNF4 α -positive hepatocyte-like cells, whereas it increased the percentage of CK19-positive cholangiocyte-like cells (supplementary material Fig. S5). The hepatocyte functionality of the *in vivo* differentiated HBCs was assessed by measuring secreted human ALB levels in the recipient mice (Fig. 3F). Mice that were transplanted with Ad-*TGFBR2*-transduced HBCs showed lower human ALB serum levels than those transplanted with Ad-LacZ-transduced HBCs, and the mice that were transplanted with si-*TGFBR2*-transfected HBCs showed higher human ALB serum

levels than those transplanted with si-control-transfected HBCs. These data suggest that cholangiocyte or hepatocyte differentiation was promoted by *TGFBR2* overexpression or knockdown, respectively. Thus, based on these data from *in vitro* and *in vivo* experiments, *TGFBR2* plays an important role in deciding the differentiation lineage of HBCs.

***TGFBR2* promoter activity and expression are negatively regulated by c/EBP α and positively regulated by c/EBP β**

A previous study has shown that *TGFBR2* expression is upregulated in *Hnf6* knockout mice (Clotman et al., 2005), although we confirmed by ChIP assay that HNF6 does not bind to the *TGFBR2* promoter region (data not shown). Because c/EBP α is important in the hepatoblast fate decision (Suzuki et al., 2003), we expected that c/EBPs might directly regulate *TGFBR2* expression. The *TGFBR2* promoter region was analyzed to examine whether *TGFBR2* expression is regulated by c/EBPs. Some c/EBP binding sites (supplementary material Fig. S6) were predicted by rVista 2.0 (<http://rvista.dcode.org/>) (Fig. 4A). By performing a ChIP assay, one

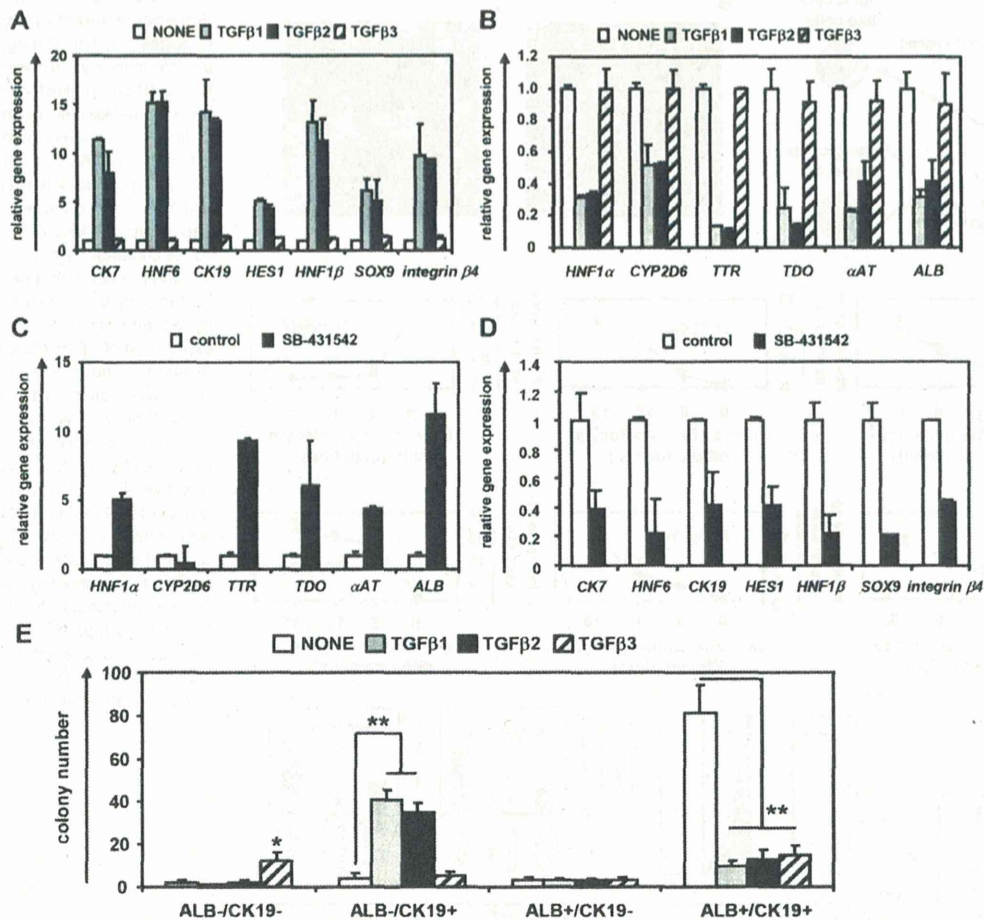


Fig. 2. Hepatocyte and cholangiocyte differentiation from HBCs is regulated by TGF β signaling. (A,B) HBCs were cultured in differentiation hESF-DIF medium containing 10 ng/ml TGF β 1, TGF β 2 or TGF β 3 for 10 days. The expression levels of cholangiocyte (A) and hepatocyte (B) marker genes were measured by real-time RT-PCR. On the y-axis, the gene expression level of cholangiocyte markers in untreated cells (NONE) was taken as 1.0. (C,D) HBCs were cultured in differentiation hESF-DIF medium containing SB-431542 (10 μ M) for 10 days. Control cells were treated with solvent only (0.1% DMSO). Expression levels of hepatocyte (C) and cholangiocyte (D) marker genes were measured by real-time RT-PCR. On the y-axis, the gene expression level of hepatocyte markers in untreated cells (control) was taken as 1.0. (E) HBC colony formation assay in the presence or absence of 10 ng/ml TGF β 1, TGF β 2 or TGF β 3. HBCs were plated at 200 cells/cm² on human LN111-coated dishes. The colonies were separated into four groups based on the expression of ALB and CK19: double-negative, ALB negative and CK19 positive, ALB positive and CK19 negative, and double positive. 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. * P <0.05, ** P <0.01 (compared with NONE). Error bars indicate s.d. Statistical analysis was performed using the unpaired two-tailed Student's t -test (n =3).

c/EBP binding site was found in the *TGFBR2* promoter region (Fig. 4B). A reporter assay of the *TGFBR2* promoter region showed that *c/EBP* β activates *TGFBR2* promoter activity, whereas *c/EBP* α inhibits it (Fig. 4C). In addition, *TGFBR2* expression was downregulated by Ad-*c/EBP* α transduction, whereas *TGFBR2* was upregulated by Ad-*c/EBP* β transduction in HepG2 cells (*TGFBR2* positive) (Fig. 4D). We ascertained the expression of *c/EBP* α or *c/EBP* β (*CEBPA* or *CEBPB* – Human Gene Nomenclature Committee) in the Ad-*c/EBP* α - or Ad-*c/EBP* β -transduced cells, respectively (supplementary material Fig. S4). These results demonstrated that the promoter activity and expression of *TGFBR2* were directly regulated by both *c/EBP* α and *c/EBP* β .

***c/EBPs* determine the cell fate decision of HBCs via regulation of *TGFBR2* expression**

To elucidate the relationship between *TGFBR2* and *c/EBPs* (*c/EBP* α and *c/EBP* β) in the hepatoblast fate decision, we first examined the

temporal gene expression patterns of *TGFBR2*, *c/EBP* α and *c/EBP* β in hepatocyte and cholangiocyte differentiation. During hepatocyte differentiation, *TGFBR2* expression was downregulated, whereas *c/EBP* α was upregulated (supplementary material Fig. S7A, top). During cholangiocyte differentiation, *c/EBP* α was downregulated, whereas *TGFBR2* and *c/EBP* β were upregulated (supplementary material Fig. S7A, bottom). In addition, the ratio of *c/EBP* α to *c/EBP* β was significantly increased in hepatocyte differentiation, but significantly reduced in cholangiocyte differentiation (supplementary material Fig. S7B). High-level expression of *c/EBP* α was detected in *TGFBR2*-negative cells, but not in *TGFBR2*-hi cells (supplementary material Fig. S7C). By contrast, high-level expression of *c/EBP* β was detected in *TGFBR2*-hi cells, but not in *TGFBR2*-negative cells. These results suggest that *TGFBR2* is negatively regulated by *c/EBP* α and positively regulated by *c/EBP* β in the differentiation model from HBCs as well as in the HepG2 cell line (Fig. 4).

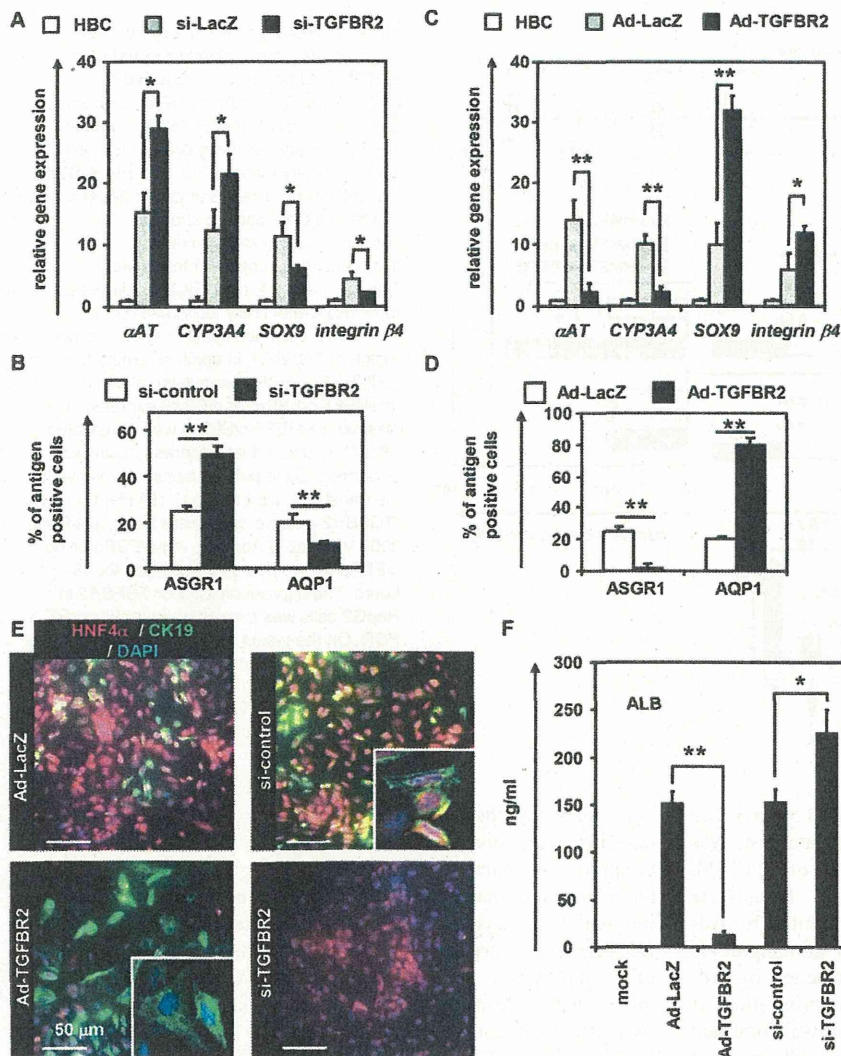


Fig. 3. TGFBR2 regulates bi-directional differentiation of HBCs.

(A) HBCs were transfected with 50 nM control siRNA (si-control) or TGFBR2-suppressing siRNA (si-TGFBR2) and cultured in differentiation hESF-DIF medium for 10 days. The expression levels of hepatocyte (α AT and CYP3A4) or cholangiocyte (SOX9 and integrin β 4) markers were measured by real-time RT-PCR. On the y-axis, the gene expression level in HBCs was taken as 1.0. (B) On day 10 after siRNA transfection, the efficiency of hepatocyte or cholangiocyte differentiation was measured by estimating the percentage of ASGR1-positive or AQP1-positive cells, respectively, by FACS analysis. (C) HBCs were transfected with 3000 VPs/cell of Ad-LacZ or Ad-TGFBR2 for 1.5 hours and cultured in differentiation hESF-DIF medium for 10 days. Expression levels of hepatocyte or cholangiocyte marker genes were measured by real-time RT-PCR. On the y-axis, gene expression levels in the HBCs was taken as 1.0. (D) On day 10 after Ad vector transduction, the efficiency of hepatocyte or cholangiocyte differentiation was measured by estimating the percentage of ASGR1-positive or AQP1-positive cells, respectively, by FACS analysis. (E, F) The si-control, si-TGFBR2, Ad-LacZ- or Ad-TGFBR2-transfected/transduced HBCs (1.0×10^6 cells) were transplanted into CCl₄-treated (2 mg/kg) Rag2/Il2rg double-knockout mice by intrasplenic injection. (E) Expression of human HNF4 α (red) and CK19 (green) was examined by double immunohistochemistry 2 weeks after transplantation. Nuclei were counterstained with DAPI (blue). (F) Levels of human ALB in recipient mouse serum were measured 2 weeks after transplantation. * $P < 0.05$, ** $P < 0.01$ (compared with Ad-LacZ-transduced or si-control-transfected cells). Error bars indicate s.d. Statistical analysis was performed using the unpaired two-tailed Student's *t*-test ($n=3$).

ChIP experiments showed that c/EBP α or c/EBP β is recruited to the TGFBR2 promoter region containing the c/EBP binding site in hepatocyte-like cells or cholangiocyte-like cells, respectively (Fig. 5A), suggesting that c/EBP α and c/EBP β oppositely regulate TGFBR2 promoter activity in the differentiation from HBCs. We confirmed that c/EBP α or c/EBP β was mainly recruited to the TGFBR2 promoter region containing the c/EBP binding site in TGFBR2-negative or TGFBR2-positive cells, respectively (supplementary material Fig. S7D). Taken together, we concluded that c/EBP α and c/EBP β are able to regulate the cell fate decision of HBCs via regulation of TGFBR2 expression. During differentiation from HBCs, TGFBR2 expression was negatively regulated by c/EBP α and positively regulated by c/EBP β (Fig. 5B). To examine whether c/EBP α or c/EBP β could regulate the differentiation from HBCs, *in vitro* gain- and loss-of-function analyses were performed. si-c/EBP α transfection downregulated hepatocyte marker gene expression, whereas it upregulated cholangiocyte marker genes (Fig. 5C). By contrast, si-c/EBP β transfection upregulated hepatocyte marker and downregulated cholangiocyte marker gene expression (Fig. 5C). In accordance, Ad-c/EBP α transduction upregulated hepatocyte marker genes and downregulated cholangiocyte markers (Fig. 5D), whereas Ad-

c/EBP β transduction downregulated hepatocyte markers and upregulated cholangiocyte marker genes. Promotion of hepatocyte differentiation by Ad-c/EBP α transduction was inhibited by Ad-TGFBR2 transduction, whereas inhibition of cholangiocyte differentiation by Ad-c/EBP α transduction was rescued by Ad-TGFBR2 transduction (Fig. 5E). In addition, promotion of hepatocyte differentiation by si-c/EBP β transfection was inhibited by Ad-TGFBR2 transduction, whereas inhibition of cholangiocyte differentiation by si-c/EBP β transfection was rescued by Ad-TGFBR2 transduction (Fig. 5F). We further confirmed that inhibition of hepatocyte differentiation by si-c/EBP α -transfection was rescued by si-TGFBR2 transfection (supplementary material Fig. S8). Taken together, these results led us to conclude that c/EBP α and c/EBP β could determine the cell fate of HBCs by negatively and positively regulating TGFBR2 expression, respectively (supplementary material Fig. S9).

c/EBPs organize the differentiation of fetal mouse hepatoblasts through regulation of TGFBR2 expression

We have demonstrated that c/EBPs may determine the HBC fate decision via regulation of the expression level of TGFBR2. To examine whether our findings could be replicated in native liver

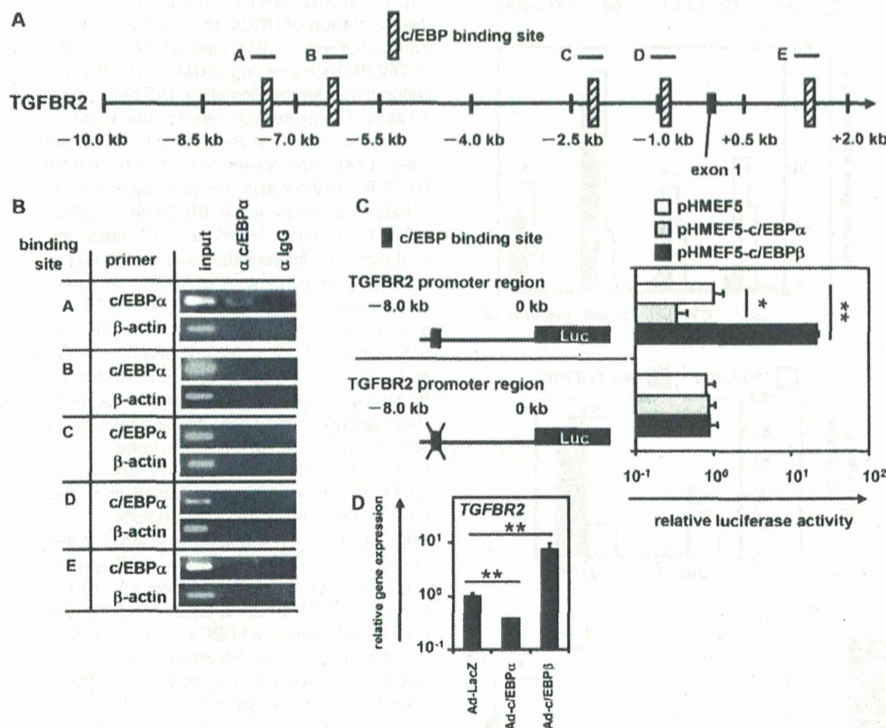


Fig. 4. *TGFBR2* promoter activity and expression are negatively regulated by *c/EBPα* and positively regulated by *c/EBPβ*. (A) Candidate *c/EBP* binding sites (hatched boxes) in the *TGFBR2* promoter region as predicted using rVista 2.0 (see supplementary material Fig. S7). (B) hESCs (H9 cells) were 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 transfected 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.

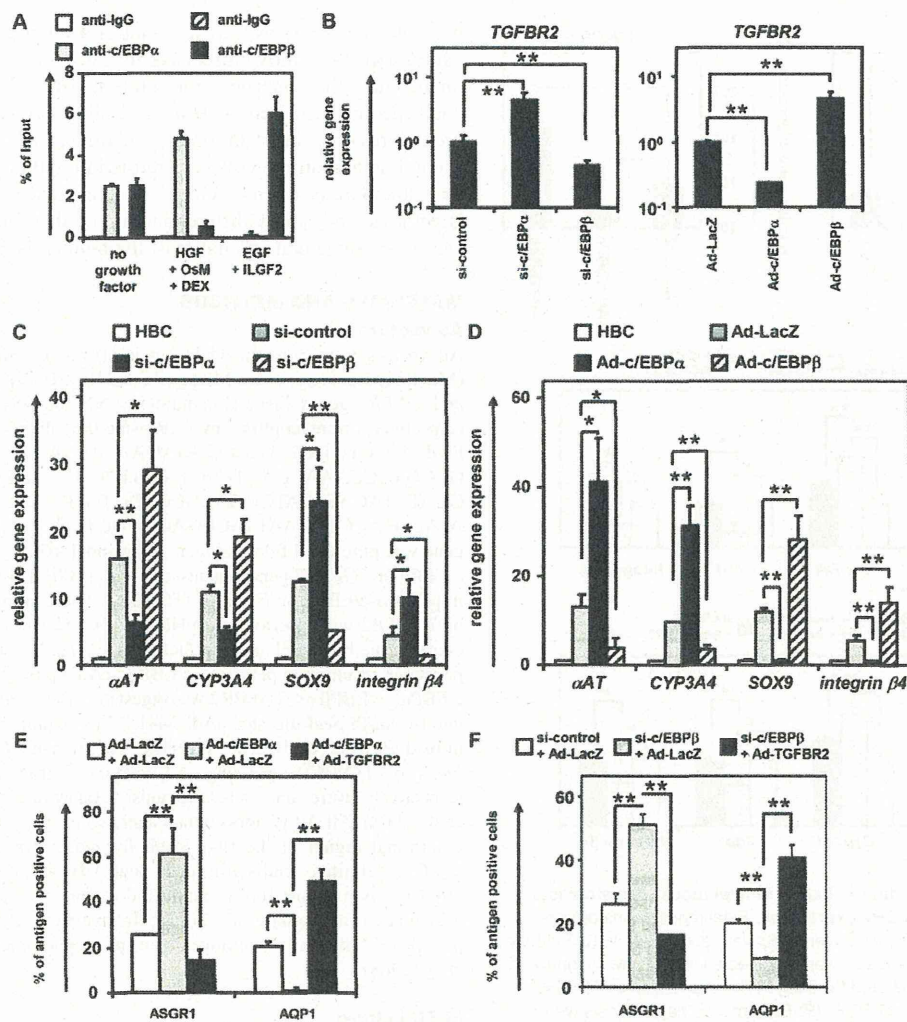


Fig. 5. *c/EBPα* and *c/EBPβ* promote hepatocyte and cholangiocyte differentiation by regulating *TGFBR2* expression, respectively. (A) HBCs were differentiated into hepatocyte-like cells or cholangiocyte-like cells according to the scheme outlined in Fig. 1A. On day 10 after hepatocyte or cholangiocyte 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 transfected 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 transfected 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 transfected 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

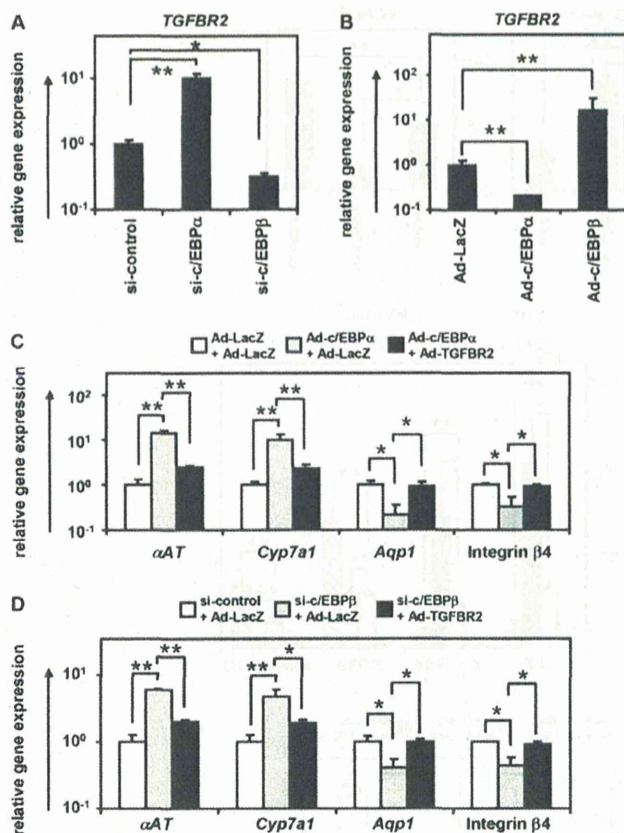


Fig. 6. c/EBPs control the differentiation of fetal mouse hepatoblasts through regulation of TGFBR2 expression. Fetal mouse hepatoblasts (DIK1-positive cells; the purity was over 98%) were sorted from E13.5 mouse liver. (A) Fetal mouse hepatoblasts were transfected with 50 nM si-control, si-c/EBP α or si-c/EBP β and cultured for 5 days. The expression of *TGFBR2* was measured by real-time RT-PCR. (B) Fetal mouse hepatoblasts were transfected with 3000 VPs/cell of Ad-LacZ, Ad-c/EBP α or Ad-c/EBP β 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 transfected with 3000 VPs/cell each of Ad-LacZ + Ad-LacZ, Ad-c/EBP α + Ad-LacZ, or Ad-c/EBP β + Ad-TGFBR2 for 1.5 hours and cultured for 5 days. On day 5, the expression levels of hepatocyte (*α AT* and *Cyp7a1*) and cholangiocyte (*Aqp1* and integrin β 4) markers were measured by real-time RT-PCR. (D) Fetal mouse hepatoblasts were transfected with 3000 VPs/cell of Ad-LacZ or Ad-TGFBR2 and then transfected with 50 nM si-control or si-c/EBP β and cultured for 5 days. On day 5, the gene levels of hepatocyte (*α AT* and *Cyp7a1*) and cholangiocyte (*Aqp1* and integrin β 4) 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/EBP α and downregulated the expression of c/EBP β (Suzuki et al., 2003), the ratio between c/EBP α and c/EBP β 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/EBP α promotes hepatocyte differentiation by downregulating the expression of *TGFBR2*, whereas c/EBP β

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 TGF β 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/EBP α* and *c/EBP β* genes (accession numbers NM_004364 and NM_005194, respectively) were amplified by PCR using the following primers: *c/EBP α* , Fwd 5'-GCTCTAGATGCCGGGAGAACTCTAACTC-3' and Rev 5'-GCGGTACCAAAACCACTCCCTGGTCC-3'; *c/EBP β* , Fwd 5'-GCATCTAGATTTCATGCAACGCCTGGTG-3' and Rev 5'-ATAGGTACCTAAAATTACCGACGGGCTCC-3'. The human *TGFBR2* gene was purchased from Addgene (plasmid 16622). The human *c/EBP α* , *c/EBP β* or *TGFBR2* gene was inserted into pBSKII (Invitrogen), resulting in pBSKII-c/EBP α , -c/EBP β or -TGFBR2. Then, human *c/EBP α* , *c/EBP β* or *TGFBR2* was inserted into pHMEF5 (Kawabata et al., 2005), which contains the human elongation factor 1 α (*EF1 α* , also known as *EEF1A1*) promoter, resulting in pHMEF5-c/EBP α , -c/EBP β or -TGFBR2. pHMEF5-c/EBP α , -c/EBP β or -TGFBR2 was digested with *I-CeuI/PI-SceI* and ligated into *I-CeuI/PI-SceI*-digested pAdHM41-K7 (Koizumi et al., 2003), resulting in pAd-c/EBP α , -c/EBP β or -TGFBR2. The human *EF1 α* 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 μ g/ml human recombinant insulin, 5 μ g/ml human apotransferrin, 10 μ M 2-mercaptoethanol, 10 μ M ethanolamine, 10 μ M sodium selenite, 0.5 mg/ml bovine fatty acid-free serum albumin (all from Sigma) and 1 \times B27 Supplement (without vitamin A; Invitrogen). To generate definitive endoderm cells, the mesendoderm cells were transfected 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

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/cm²) in maintenance DMEM/F12 medium [DMEM/F12 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS), $1 \times$ insulin/transferrin/selenium, 10 mM nicotinamide, 0.1 μ M dexamethasone (DEX) (Sigma), 20 mM HEPES, 25 mM NaHCO₃, 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/cm²) 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 μ M DEX. To induce cholangiocyte differentiation, the HBCs were cultured in collagen gel. To establish collagen gel plates, 500 μ l collagen gel solution [400 μ l type I-A collagen (Nitta gelatin), 50 μ l $10 \times$ DMEM and 50 μ l 200 mM HEPES buffer containing 2.2% NaHCO₃ 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 μ l 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 μ l 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 TGF β 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 TGF β 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/cm²) on a human LN111-coated dish in maintenance DMEM/F12 medium supplemented with 25 μ M 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 CCl₄ (2 mg/kg) by intrasplenic injection into 8- to 10-week-old *Rag2^{fl/fl}2rg* 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, $1 \times$ insulin/transferrin/selenium (ITS), 10 mM nicotinamide (Wako), 0.1 μ M 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 μ M 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/EBP α* or *c/EBP β* 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'-CCGAGCTCATGTTTGTATGAAGTGTCTAGCTTCCAAGG-3'; Rev, 5'-GGCTCGAGCCTCGACGTCCAGCCCCCT-3'. The fragment was inserted into the *SacI/XhoI* sites of pGL3-basic (Promega), resulting in a pGL3-*TGFBR2* promoter region (pGL3-TGFB2-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'-CACTAGTATTCAgTGAtCcgAAAATATGG-3'; this resulted in pGL3-mTGFB2-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-TGFB2-PR or pGL3-mTGFB2-PR was transfected together with 720 ng each expression plasmid (pHMEF5, pHMEF5-*c/EBP α* and pHMEF5-*c/EBP β*) 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

Pre-designed siRNAs targeting *c/EBP α* , *c/EBP β* 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 \pm s.d. ($n=3$).

Acknowledgements

We thank Natsumi Mimura, Yasuko Hagihara and Hiroko Matsumura for excellent technical support.

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.

Funding

H.M., K.K., M.K.F. and T.H. were supported by grants from the Ministry of Health, Labor, and Welfare of Japan (MEXT). H.M. was also supported by Japan Research Foundation for Clinical Pharmacology, The Uehara Memorial Foundation. K.O. was supported by Special Coordination Funds for Promoting Science and Technology from MEXT. F.S. was supported by Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO). K. Takayama and Y.N. are Research Fellows of the Japan Society for the Promotion of Science.

Supplementary material

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

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