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Tbx3 controls the fate of hepatic progenitor cells in liver development by suppressing *p19^{ARF}* expression

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Although the T-box family of transcription factors function in many different tissues, their role in liver development is unknown. Here we show that *Tbx3*, the T-box gene that is mutated in human ulnar-mammary syndrome, is specifically expressed in multipotent hepatic progenitor cells, 'hepatoblasts', isolated from the developing mouse liver. *Tbx3*-deficient hepatoblasts presented severe defects in proliferation as well as uncontrollable hepatobiliary lineage segregation, including the promotion of cholangiocyte (biliary epithelial cell) differentiation, which thereby caused abnormal liver development. Deletion of *Tbx3* resulted in the increased expression of the tumor suppressor *p19^{ARF}* (*Cdkn2a*), which in turn induced a growth arrest in hepatoblasts and activated a program of cholangiocyte differentiation. Thus, Tbx3 plays a crucial role in controlling hepatoblast proliferation and cell-fate determination by suppressing *p19^{ARF}* expression and thereby promoting liver organogenesis.

KEY WORDS: Tbx3, Liver, Hepatoblast, p19^{ARF} (Cdkn2a), Differentiation, Mouse

INTRODUCTION

The identification of transcription factors that regulate proliferation and differentiation of organ progenitor cells is crucial for understanding the fundamental mechanisms of development, regeneration, and disorders of any given organ. In the vertebrate developing liver, multipotent hepatic progenitor cells (also known as hepatoblasts) proliferate and give rise to both hepatocytes and cholangiocytes as descendants (Lemaigre and Zaret, 2004). This essential event in liver organogenesis requires transcription factors that act either as central inducers or suppressors for the proliferation and differentiation of these cells. For example, hepatic nuclear factor 4α (Hnf4α), a transcription factor in the nuclear receptor family, activates many downstream genes responsible for hepatocyte differentiation, including the homeodomain transcription factor Hnf1α (Tian and Schibler, 1991; Li et al., 2000). The homeodomain transcription factor Hnf6 (Onecut1) is believed to attenuate early biliary commitment of hepatoblasts, but later, Hnf6 also positively regulates cholangiocyte differentiation and bile duct morphogenesis (Clotman et al., 2002; Suzuki et al., 2003a). The cell-lineage restriction into hepatocytes or cholangiocytes is additionally controlled by the basic leucine-zipper transcription factor C/EBPα (CCAAT/enhancer binding protein α; Cebpα), and deletion or suppression of C/EBPα blocks hepatocyte differentiation and concomitantly induces biliary development (Tomizawa et al., 1998; Suzuki et al., 2003a; Yamasaki et al., 2006).

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Our previous studies enabled the prospective isolation of hepatoblasts from the developing mouse liver by combining flow cytometry and fluorescence-conjugated antibodies (Suzuki et al., 2000; Suzuki et al., 2002). In particular, cells marked by the hepatocyte growth factor (Hgf) receptor c-Met displayed distinctive activities in response to Hgf stimulus, including self-renewing cell divisions and differentiation into both hepatocytes and cholangiocytes (Suzuki et al., 2002; Suzuki et al., 2003a). Isolating c-Met$^+$ c-Kit$^-$ CD45$^-$ Ter119$^-$ (also known as Met, Kit, Ptprc and Ly76, respectively – Mouse Genome Informatics) cells achieved a much higher enrichment of hepatoblasts, and thus this method could facilitate the identification of a discrete set of transcription factors that are activated in this specific cell population. Using this strategy, we examine here the developmental role of the T-box family of transcription factors in the proliferation and differentiation of hepatoblasts.

MATERIALS AND METHODS

Immunostaining

Hepatic tissue sections and cultured cells were fixed and incubated with primary antibodies against Hnf4α (Santa Cruz, Santa Cruz, CA), Tbx3 (Santa Cruz), BrdU (Amersham, Little Chalfont, UK), E-cadherin (BD Biosciences, San Jose, CA), N-cadherin (BD Biosciences), albumin (Bethyl, Montgomery, TX, for tissue sections; Biogenesis, Poole, UK, for cultured cells), CK7 (Chemicon, Temecula, CA), cleaved caspase 3 (Cell Signaling, Danvers, MA), PCNA (Santa Cruz), p19^{ARF} (Abcam, Cambridge, UK) and Myc-Tag (Cell Signaling). Detailed information on the antibodies is available upon request. After washing, the sections and the cells were incubated with Alexa 488- and/or Alexa 555-conjugated secondary antibodies specific to the appropriate species (1:200; Molecular Probes, Eugene, OR), followed by incubation with DAPI.

Gene expression analysis

Reverse transcriptase (RT)-PCR and quantitative (q)PCR were conducted as described (Suzuki et al., 2003a; Suzuki et al., 2003b). PCR primers and probes are described in our previous papers (Suzuki et al., 2000; Suzuki et al., 2002; Suzuki et al., 2003a) and are available upon request. For qPCR analysis of *p19^{ARF}* expression, we used SYBR Premix Ex Taq II (Takara, Japan) according to the manufacturer's instructions.

Flow cytometry and cell culture

Single-cell suspensions were prepared from the livers of wild-type, *Tbx3$^{+/-}$* or *Tbx3$^{-/-}$* mouse embryos and incubated with fluorescence-conjugated antibodies as described (Suzuki et al., 2002). We used phycoerythrin (PE)-

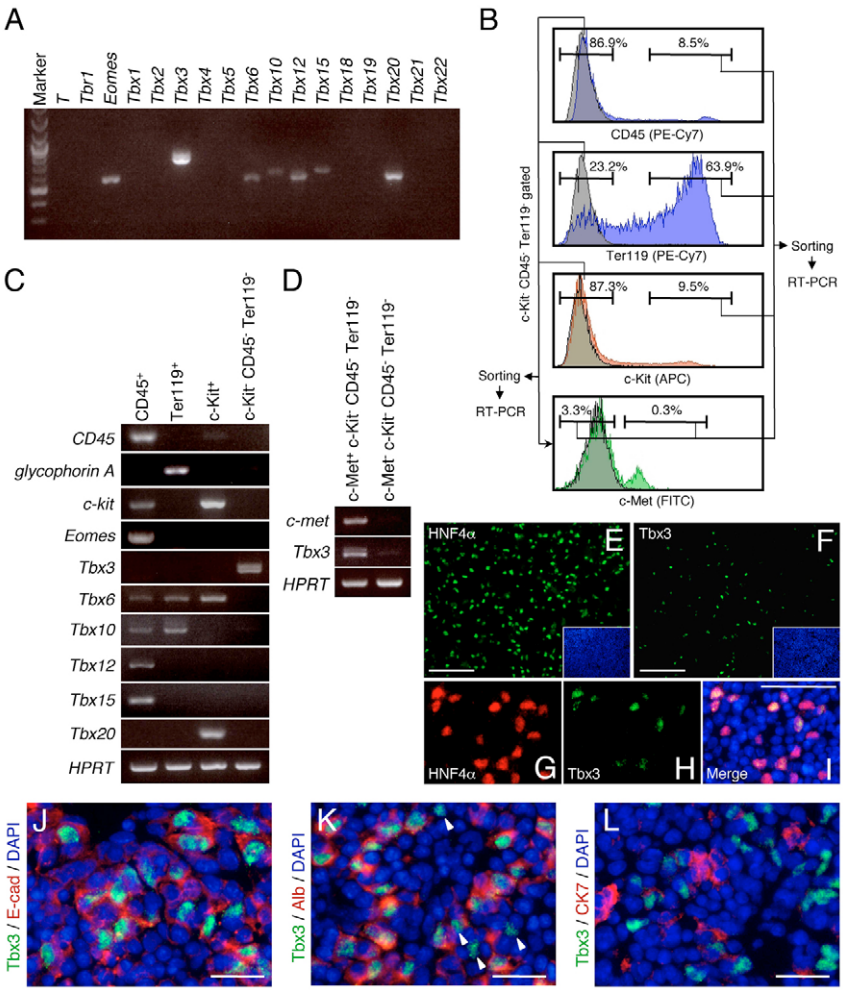

Fig. 1. Specific expression of Tbx3 in hepatoblasts isolated from the developing mouse liver. (**A**) Of the 17 mouse T-box genes, *Eomes*, *Tbx3*, *Tbx6*, *Tbx10*, *Tbx12*, *Tbx15* and *Tbx20* were expressed in the E13.5 mouse liver, as assessed by RT-PCR. The upper band in the *Tbx3* lane represents a transcript variant of this gene (Bamshad et al., 1999). (**B**) Flow-cytometric fractionation of E13.5 liver cells into CD45$^+$, Ter119$^+$, c-Kit$^+$, c-Kit$^-$ CD45$^-$ Ter119$^-$, c-Met$^-$ c-Kit$^-$ CD45$^-$ Ter119$^-$, and c-Met$^+$ c-Kit$^-$ CD45$^-$ Ter119$^-$ cell populations. Cells in each fraction were sorted and analyzed. (**C**,**D**) RT-PCR analyses of T-box gene expression in cells sorted from each fraction shown in B. Erythroid cell-specific glycophorin A was recognized by anti-Ter119 antibody. (**E**,**F**) Hnf4α (E) and Tbx3 (F) immunofluorescent images of E13.5 liver sections. Insets denote individual cells as labeled with DAPI (blue). (**G-I**) Co-immunofluorescence staining of Hnf4α and Tbx3 showed that a subpopulation of Hnf4α$^+$ cells was marked by either strong or weak staining of Tbx3. (**J**) All Tbx3$^+$ cells expressed E-cadherin. (**K**,**L**) Tbx3 was expressed in Alb$^{-/low}$ primitive hepatic cells (arrowheads) and in a subpopulation of Alb$^+$ differentiating hepatocytes (K), and in a few CK7$^+$ cholangiocytes (L). Scale bars: 100 μm in E,F; 50 μm in I; 25 μm in J-L.

Cy7-conjugated anti-CD45, Ter119 monoclonal antibodies (mAbs) (Pharmingen, San Jose, CA), allophycocyanin (APC)-conjugated anti-c-Kit mAb (Pharmingen), and fluorescein isothiocyanate (FITC)-conjugated anti-c-Met mAb. The c-Met mAb was produced in cultures of a hybridoma cell line raised by fusing mouse myeloma cells to rat lymphocytes obtained by inoculating rats with 293T cells expressing the entire coding sequence of mouse *c-Met* fused to a C-terminal Flag-Tag (MBL, Nagoya, Japan). BrdU-incorporating cells were stained using the APC BrdU Flow Kit (BD Biosciences, San Jose, CA). The fluorescence-labeled cells were analyzed and separated with FACS Aria (BD Biosciences). For single-cell culture analysis, cells identified on clone sorting by FACS Aria were cultured in individual wells of type-IV-collagen-coated 96-well plates, and clonal colonies formed from each cell were analyzed as described (Suzuki et al., 2002).

Expression constructs

The entire coding sequences of mouse *Tbx3* and *p19^{ARF}* were obtained by RT-PCR using embryonic liver-derived total RNA and then inserted into pCMV-Tag3B (Stratagene, La Jolla, CA) and pIRES2-eGFP (Clontech, Palo Alto, CA), respectively. Tbx3-shRNA (target sequence AAAGTCGTCACTT-TCCACAAA), driven by a mouse U6 promoter, was amplified by PCR and inserted into a vector containing a puromycin-resistance gene. p19^{ARF}-siRNA (Stealth RNAi; target sequence GCTCTGGCTTTCGTGAACATGTTGT) was designed and synthesized (Invitrogen, Carlsbad, CA). Transfection of hepatoblast cultures was carried out with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. To check the effect of Tbx3-shRNA, cells cotransfected with Tbx3-shRNA and Tbx3/pCMV-Tag3B (Myc-Tbx3) were analyzed by western blotting using antibodies against Myc-Tag (1:1000) and β-actin (1:2000; Abcam).

RESULTS AND DISCUSSION

The T-box genes, defined by a common DNA-binding T-box domain, are involved in many aspects of embryonic and extraembryonic tissue development (Naiche et al., 2005). Until now, however, there has been no report regarding the contribution of T-box genes in the developing or adult liver. As an initial approach, we examined the hepatic expression of multiple T-box genes in E13.5 mouse embryos and identified the specific expression of *Eomes*, *Tbx3*, *Tbx6*, *Tbx10*, *Tbx12*, *Tbx15* and *Tbx20* (Fig. 1A). To determine which cell type(s) expressed these genes, cells that were first fractionated into either CD45$^+$, Ter119$^+$, c-Kit$^+$, or c-Kit$^-$ CD45$^-$ Ter119$^-$ cell populations were isolated separately and analyzed (Fig. 1B). Significantly, only *Tbx3*, the T-box gene that is mutated in human ulnar-mammary syndrome (Bamshad et al., 1997), was expressed in c-Kit$^-$ CD45$^-$ Ter119$^-$ hepatic epithelial cells, and *Tbx3* expression was restricted to this cell population (Fig. 1C). Further fractionation of c-Kit$^-$ CD45$^-$ Ter119$^-$ cells into c-Met$^+$ or c-Met$^-$ cells revealed that *Tbx3* expression was much higher in the c-Met$^+$ c-Kit$^-$ CD45$^-$ Ter119$^-$ hepatoblast population (Fig. 1B,D). Immunofluorescence staining revealed that although Hnf4α$^+$ primitive hepatic cells coexpressed Tbx3 in E9.5 and E10.5 hepatic primordia (see Fig. S1A-L in the supplementary material), by E13.5 Tbx3 was detected only in a portion of Hnf4α$^+$ cells, including primitive hepatic cells and differentiating hepatocytes (Fig. 1E-I). The Tbx3$^+$ Hnf4α$^+$ cells in E13.5 liver were also marked by the

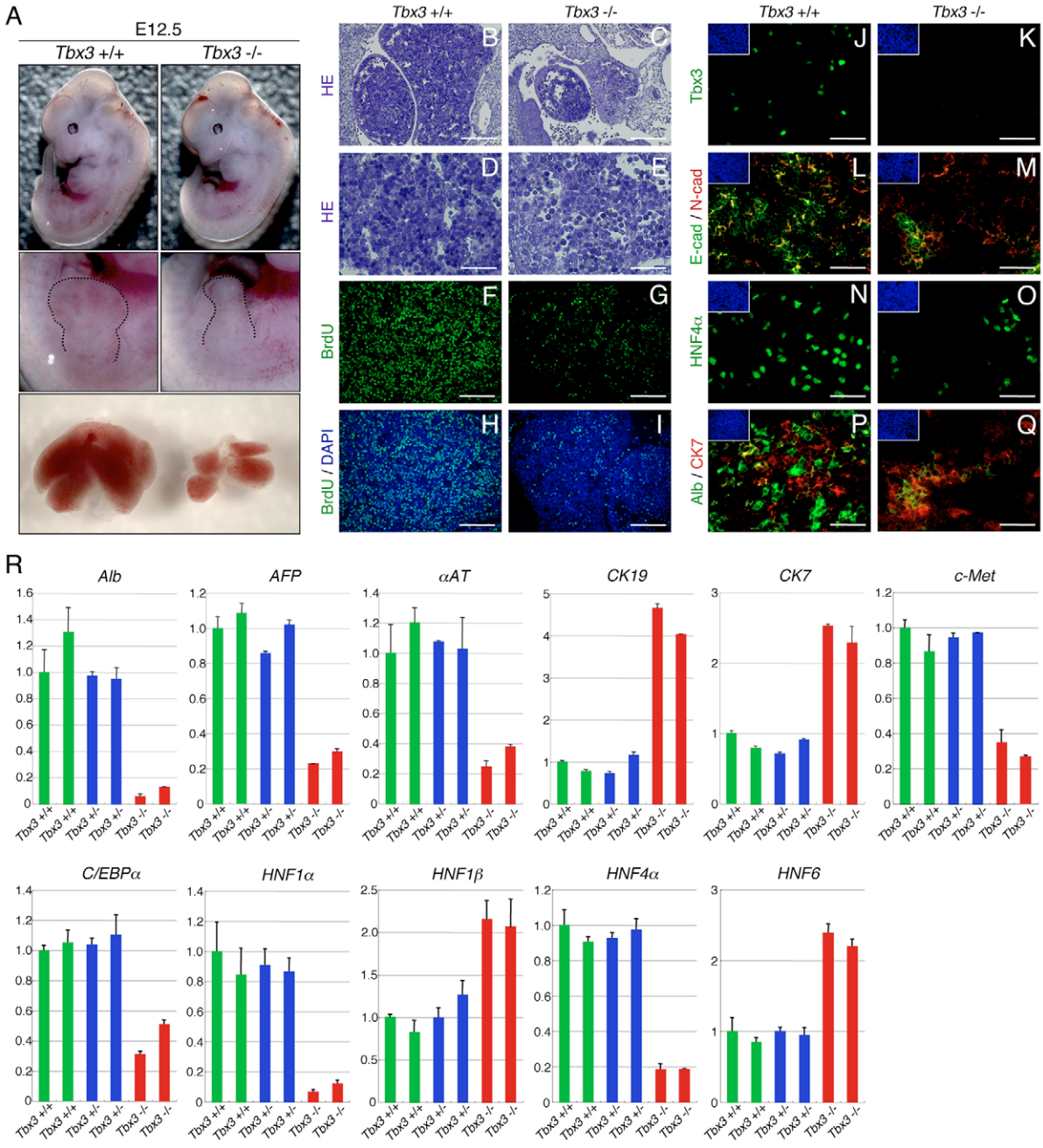

Fig. 2. Defects in proliferation and differentiation of hepatic epithelial cells in *Tbx3*$^{-/-}$ embryos. (**A**) Morphology of Tbx3$^{+/+}$ wild-type and Tbx3$^{-/-}$ mouse embryos (E12.5). Besides the abnormal development of Tbx3$^{-/-}$ hind limbs (upper and middle panels), the *Tbx3*$^{-/-}$ liver was much smaller than that of wild type (bottom panel). (**B-E**) Hematoxylin and Eosin (HE) staining of liver from wild-type (B,D) and Tbx3$^{-/-}$ (C,E) embryos. (**F-I**) BrdU immunofluorescent images of wild-type (F,H) and *Tbx3*$^{-/-}$ (G,I) liver cells. (**J-Q**) When compared with the wild-type liver (J,L,N,P), the number of E-cadherin^{+} epithelial cells, but not of N-cadherin^{+} (E-cadherin^{-}) mesenchymal cells, was significantly smaller in the absence of Tbx3 (M). Within *Tbx3*-deficient liver epithelial cells, primitive hepatic cells and differentiating hepatocytes that expressed Hnf4α and Alb constituted only a small population (O,Q), whereas the number of CK7^{+} cholangiocytes was relatively large (Q). Insets denote individual cells as labeled with DAPI. (**R**) Gene expression analysis by qPCR for E12.5 liver from wild-type, *Tbx3*$^{+/-}$ and *Tbx3*$^{-/-}$ embryos. Two mice of each genotype were analyzed separately. All data were normalized to the values of a wild-type liver and fold differences are shown. Bar represents mean ±s.d. (n=3). Scale bars: 200 μm in B,C; 100 μm in F-I; 50 μm in D,E,J-Q.

epithelial cell marker E-cadherin and were categorized into albumin$^{-/low}$ (Alb$^{-/low}$) primitive hepatic cells and Alb^{+} differentiating hepatocytes, but scarcely into cytokeratin 7^{+} (CK7; also known as Krt7 – Mouse Genome Informatics) cholangiocytes (Fig. 1J-L). In the later stages of liver development, however, Tbx3 expression decreased and became faint in Hnf4α^{+} cells during the advancement of hepatocyte differentiation (see Fig. S1M-V in the supplementary material). These results suggest that Tbx3 plays a role in an early phase of hepatogenesis, especially in the regulation of hepatoblast activities.

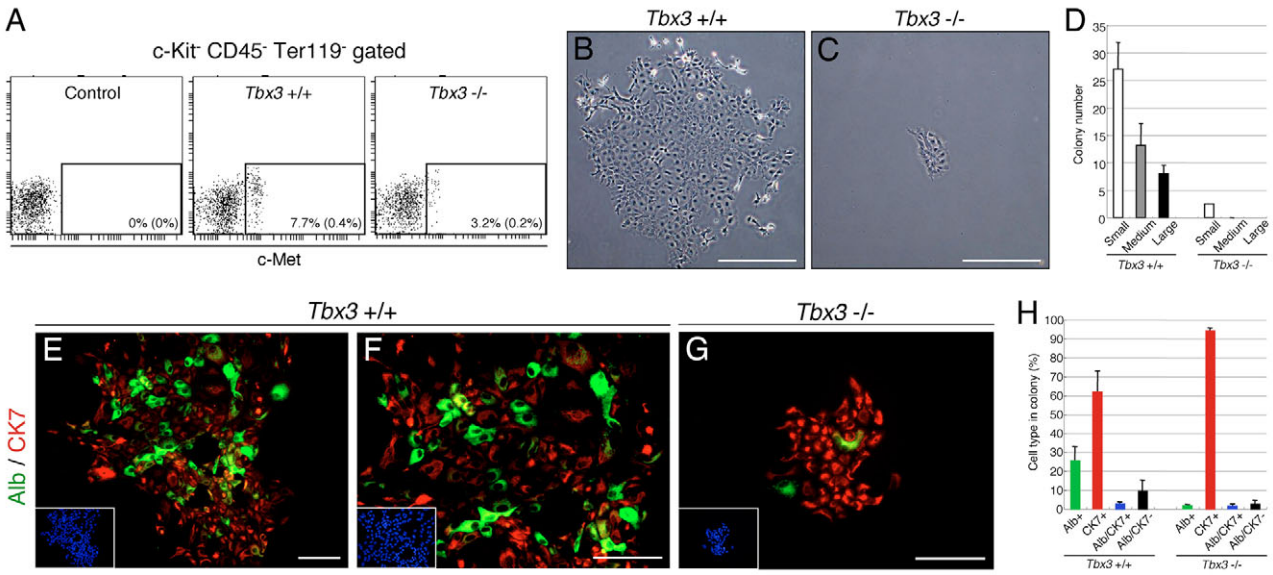

Fig. 3. Tbx3 regulates the proliferation and the cell-lineage determination of hepatoblasts. (**A**) CD45$^+$, Ter119$^+$, and c-Kit$^+$ cells were gated and removed from the initial wild-type and *Tbx3*$^{-/-}$ mouse liver tissue specimens (E12.5). The c-Kit$^-$ CD45$^-$ Ter119$^-$ hepatic epithelial cells were then fractionated based on c-Met expression. For the in vitro colony assay, the sorting gate was set for the c-Met$^+$ c-Kit$^-$ CD45$^-$ Ter119$^-$ cell population. The ratios of c-Met$^+$ cells in c-Kit$^-$ CD45$^-$ Ter119$^-$ cells and in unfractionated total cells are shown by the percentage values outside and within the parenthesis, respectively. Wild-type cells stained with an isotype control antibody were used as a control. (**B**,**C**) Clonal colony formation upon 5-days single-cell culture of c-Met$^+$ c-Kit$^-$ CD45$^-$ Ter119$^-$ cells isolated from wild-type (B) or *Tbx3*$^{-/-}$ (C) livers. (**D**) Numbers of large, medium and small colonies per 150 wells, as formed by wild-type or *Tbx3*$^{-/-}$ liver-derived c-Met$^+$ c-Kit$^-$ CD45$^-$ Ter119$^-$ cells after 5 days of single-cell culture. The average of three independent experiments (mean ±s.d.). (**E-G**) Co-immunofluorescence staining of Alb and CK7 was conducted for clonal colonies formed by c-Met$^+$ c-Kit$^-$ CD45$^-$ Ter119$^-$ cells isolated from wild-type (E,F) and *Tbx3*$^{-/-}$ (G) livers after 18 days of culture. Insets denote individual cells labeled with DAPI. (**H**) Following co-immunofluorescence staining of Alb and CK7, the percentage of Alb$^+$, CK7$^+$, Alb/CK7$^+$, and Alb/CK7$^-$ cells in each colony was determined. The average of 24 colonies (mean ±s.d.). Scale bars: 500 μm in B,C; 100 μm in E-G.

To address this issue, *Tbx3*-null (*Tbx3*$^{-/-}$) mice were employed (Ribeiro et al., 2007). The E12.5 *Tbx3*$^{-/-}$ embryonic liver was much smaller than those from *Tbx3*$^{+/+}$ wild-type or *Tbx3*$^{+/-}$ heterozygous embryos (Fig. 2A-C, and data not shown). Adhesion of epithelial cells in the *Tbx3*$^{-/-}$ liver appeared to be normal, but there were many cavities in which epithelial cells were largely replaced by hematopoietic cells, suggesting a decrease in and an abnormality of epithelial cells (Fig. 2B-E). Indeed, when compared with cells from the wild-type liver, the number of BrdU-incorporating or PCNA$^+$ proliferating cells decreased substantially in the absence of *Tbx3* (Fig. 2F-I and see Fig. S2 in the supplementary material) without any increase in the number of apoptotic cells (see Fig. S3 in the supplementary material). Additionally, in the *Tbx3*$^{-/-}$ embryonic liver, the number of E-cadherin$^+$ epithelial cells was also significantly reduced, whereas the number of N-cadherin$^+$ (E-cadherin$^-$) mesenchymal cells was not affected (Fig. 2J-M). These data demonstrated an essential role of Tbx3 in activating the proliferation of immature hepatic epithelial cells. Those *Tbx3*-deficient epithelial cells were composed of a few primitive hepatic cells and differentiating hepatocytes that were marked by the expression of Hnf4α and Alb, as well as a relatively large number of CK7$^+$ cholangiocytes (Fig. 2N-Q). The percentages of PCNA$^+$ cells in Hnf4α$^+$ cells were 68.8% and 27.3% in the livers of wild-type and *Tbx3*$^{-/-}$ embryos, respectively. Quantitative PCR (qPCR) analysis also demonstrated that the expression levels of genes encoding hepatocyte differentiation markers [Alb and α-1-antitrypsin (αAT; Serpina1 – Mouse Genome Informatics)], primitive hepatic cell markers [α-fetoprotein (Afp) and c-Met], and transcription factors involved in the early stage of hepatocyte differentiation (C/EBPα, Hnf1α and Hnf4α) were all markedly diminished in the *Tbx3*$^{-/-}$ embryonic liver, although the expression of genes encoding cholangiocyte markers [CK19 (Krt19) and CK7] and transcription factors that control cholangiocyte differentiation (Hnf1β and Hnf6) (Clotman et al., 2002; Coffinier et al., 2002) were all upregulated (Fig. 2R). Therefore, in the developing liver, Tbx3 acts not only as an activator for proliferation, but also as a regulator for hepatobiliary lineage segregation.

Next, to examine the role of Tbx3 in hepatoblasts directly, liver cells obtained from wild-type and *Tbx3*$^{-/-}$ embryos were fractionated, and cells defined as c-Met$^+$ c-Kit$^-$ CD45$^-$ Ter119$^-$ were sorted out and clonally cultured (1 cell per well). In the liver of the *Tbx3*$^{-/-}$ embryo, the percentage of c-Met$^+$ cells within the c-Kit$^-$ CD45$^-$ Ter119$^-$ hepatic epithelial cell population was less than half of that from wild-type embryos, suggesting a defect in in vivo hepatoblast proliferation (Fig. 3A). After 5 days of culture, wild-type cells isolated from the liver formed large (more than 100 cells), medium (50 to 100 cells) and small (fewer than 50 cells) colonies (Fig. 3B,D). We previously identified cells capable of forming larger colonies as a more primitive hepatic cell type (Suzuki et al., 2000; Suzuki et al., 2002). As a result of deleting the *Tbx3* gene, colony formation by c-Met$^+$ c-Kit$^-$ CD45$^-$ Ter119$^-$ cells was significantly impaired, and only small colonies formed, despite cell attachment onto the bottom of wells being normal (Fig. 3C,D). At day 18 of culture, differentiating Alb$^+$ hepatocytes (~25%) and CK7$^+$ cholangiocytes (~60%) appeared in colonies formed by wild-type cells (Fig. 3E,F,H). In the case of *Tbx3*$^{-/-}$ colonies, however, 95% of the cells became CK7$^+$ cholangiocytes, and only a few Alb$^+$ hepatocytes (~2%) emerged (Fig. 3G,H). Thus, *Tbx3*-deficient

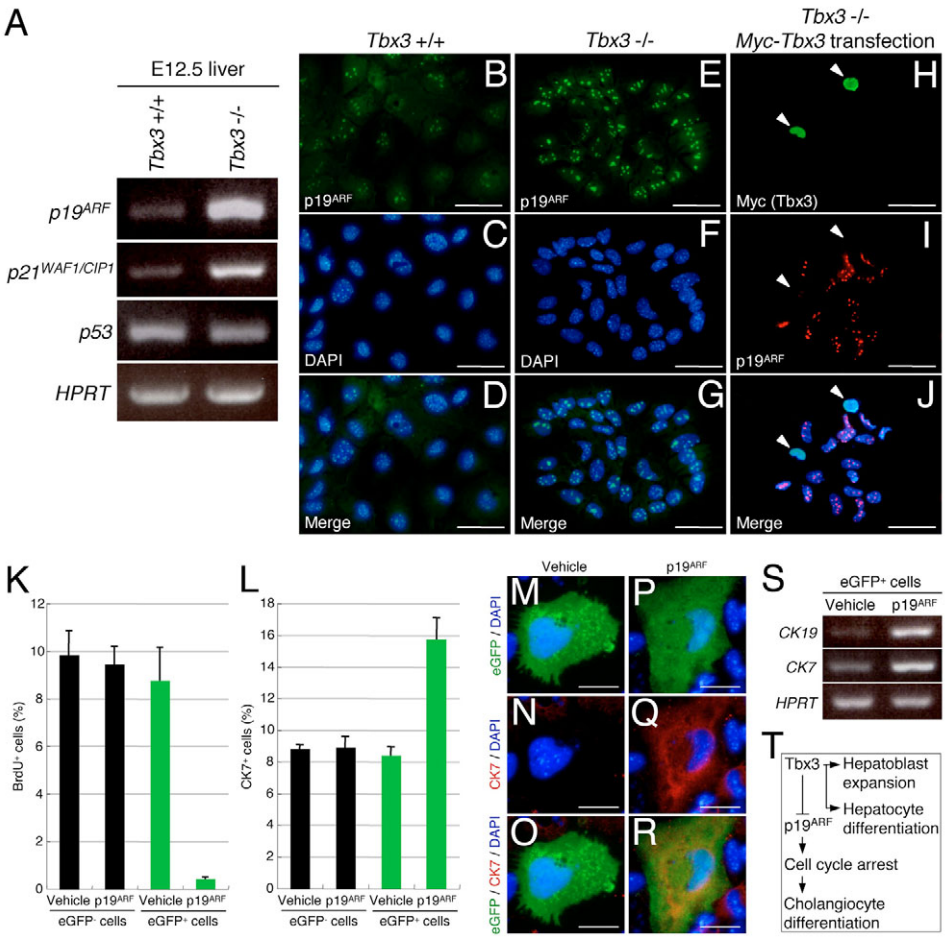

Fig. 4. Negative regulation of p19^{ARF} by Tbx3 is required for controlling the proliferation and the hepatobiliary lineage segregation of hepatoblasts. (**A**) RT-PCR analysis revealed that in *Tbx3*$^{-/-}$ mouse livers, expression of *p19*^{ARF} and *p21*$^{WAF1/CIP1}$ was upregulated, but *p53* expression was unaffected. (**B-G**) Immunofluorescence staining of p19^{ARF} was conducted for clonal colonies formed by c-Met$^+$ c-Kit$^-$ CD45$^-$ Ter119$^-$ cells isolated from wild-type (B-D) and *Tbx3*$^{-/-}$ (E-G) livers after 5 days of culture. Representative data from 18 colonies are shown. (**H-J**) Cells in clonal colonies formed by *Tbx3*$^{-/-}$ liver-derived c-Met$^+$ c-Kit$^-$ CD45$^-$ Ter119$^-$ cells were transiently transfected with Myc-Tbx3 and subsequently stained using antibodies against Myc-Tag (H) and p19^{ARF} (I). Arrowheads indicate Myc-Tbx3-transfected cells. Representative data from 11 colonies are shown. (**K-S**) Cells in cultures of wild-type liver-derived c-Met$^+$ c-Kit$^-$ CD45$^-$ Ter119$^-$ cells were transiently transfected with p19^{ARF}-IRES-eGFP. Then, the percentage of cells immunoreactive for BrdU (K) or CK7 (L) was analyzed by flow cytometry. Vehicle-transfected cells and non-transfected (eGFP$^-$) cells were used as controls. Bar charts represent the average of three independent experiments (mean ±s.d.). Immunofluorescence staining of CK7 for vehicle- or p19^{ARF}-IRES-eGFP-transfected cells confirmed intense CK7 expression in p19^{ARF} (eGFP)-expressing cells (M-R). Also, *CK19* and *CK7* transcripts were increased in cells expressing p19^{ARF} (eGFP), as assessed by RT-PCR following flow-cytometric isolation of eGFP$^+$ cells (S). (**T**) A proposed mechanism underlying the regulation of proliferation and differentiation of hepatoblasts by Tbx3. Scale bars: 50 μm in B-J; 25 μm in M-R.

hepatoblasts suffered severe defects in proliferation and differentiated more efficiently into cholangiocytes. To verify these findings further, we introduced Tbx3 short hairpin RNA (Tbx3-shRNA) into in vitro propagating progeny from a single hepatoblast derived from the c-Met$^+$ c-Kit$^-$ CD45$^-$ Ter119$^-$ cell population (see Fig. S4A in the supplementary material). In the culture of these cells, many cells (~80%) expressed Tbx3 (see Fig. S4B in the supplementary material). Consistent with the results from the *Tbx3*$^{-/-}$ liver study, efficient suppression of endogenous Tbx3 expression inhibited hepatoblast proliferation, repressed the expression of several genes activated during hepatocyte differentiation, and enhanced cholangiocyte marker expression (see Fig. S4C,E-H in the supplementary material). Taken together, we conclude that Tbx3 plays an essential role in hepatogenesis by controlling the proliferation and the cell-lineage decision of hepatoblasts.

In light of the above findings, we next sought to unveil the molecular mechanisms underlying the Tbx3-dependent regulation of hepatoblasts. Analogous to the results shown above, evidence from loss-of-function experiments demonstrated that Tbx3, which was also highly expressed in hepatic carcinoma cells, controlled a hyperproliferative feature of these cells (Renard et al., 2007). In the intestine, the molecular mechanisms governing the homeostatic self-renewal of stem cells and the aberrant proliferation of colorectal cancer cells are similar (Radtke and Clevers, 2005). Thus, in hepatoblasts, Tbx3 might function as it does in malignant cancer cells. Tbx3 is known as a transcriptional repressor for *p19*^{ARF} (*Cdkn2a* – Mouse Genome Informatics) and *p14*^{ARF} (the human ortholog of *p19*^{ARF}), the tumor suppressor genes that stabilize p53 (Trp53) via inactivation of Mdm2 in response to a variety of oncogenic stresses (Honda and Yasuda, 1999; Brummelkamp et al., 2002; Lingbeek et al., 2002; Kim and Sharpless, 2006). This evidence suggests that in

cancer cells, Tbx3 negatively regulates *p19^ARF^* expression to induce escape from cellular senescence and to activate mitosis. To determine whether this regulatory mechanism is also active in hepatoblasts, we first analyzed hepatic *p19^ARF^* expression in wild-type and *Tbx3*$^{-/-}$ embryos. Intriguingly, liver cells lacking Tbx3 upregulated the expression of *p19^ARF^* and, to a lesser extent, the cyclin-dependent kinase inhibitor *p21^WAF1/CIP1^* (*Cdkn1a* – Mouse Genome Informatics) (Fig. 4A). This *p21^WAF1/CIP1^* expression might be activated either by p19^{ARF}-dependent p53 stabilization (el-Deiry et al., 1993), or as a direct result of *Tbx3* deficiency (Prince et al., 2004). Cells in colonies formed by c-Met$^+$ c-Kit$^-$ CD45$^-$ Ter119$^-$ cells isolated from either wild-type or *Tbx3*$^{-/-}$ liver expressed p19^{ARF} at day 5 of culture, but its expression level was much higher in the cultures of *Tbx3*$^{-/-}$ liver cells (Fig. 4B-G). When Tbx3 expression was restored by transgenes, a significant decrease of p19^{ARF} expression was exhibited in *Tbx3*$^{-/-}$ colony cells (Fig. 4H-J). In addition, the defect in proliferation of Tbx3-shRNA-expressing hepatoblast progeny in culture was partially rescued by introducing p19^{ARF}-small interfering RNA (p19^{ARF}-siRNA) (see Fig. S4D,E in the supplementary material). These results associate Tbx3 with the negative regulation of p19^{ARF} in proliferating hepatoblasts. Moreover, to characterize the relationship between p19^{ARF} activation and the promotion of cholangiocyte differentiation in the *Tbx3*$^{-/-}$ liver, *p19^ARF^* was introduced into cultures of wild-type c-Met$^+$ c-Kit$^-$ CD45$^-$ Ter119$^-$ cells. Consistent with the data from the *Tbx3*$^{-/-}$ liver, the overexpression of *p19^ARF^* resulted in a significant reduction in the number of proliferating cells (Fig. 4K). Surprisingly, *p19^ARF^* overexpression was also effective in upregulating the expression of *CK19* and *CK7* and increased the number of CK7$^+$ cholangiocytes (Fig. 4L-S). Thus, the growth arrest induced by active p19^{ARF} in the absence or suppression of Tbx3 function is sufficient to promote cholangiocyte differentiation from hepatoblasts.

These findings uncovered unique and unexpected roles for Tbx3 in controlling the proliferation and the differentiation of hepatoblasts during liver development (Fig. 4T). The phenotypic features of *Tbx3*$^{-/-}$ embryos, including a diminished liver size, have also been observed in another *Tbx3* mutant mouse line, but such liver defects were only discussed as an effect of deficiencies in the yolk sac or in hematovascular development (Davenport et al., 2003). In our study, although there was no previous evidence to implicate a role for Tbx3 in hepatogenesis, searching for T-box genes that are expressed specifically in hepatoblasts led to the identification of Tbx3 as an essential regulator for the proliferation and differentation of hepatoblasts. Therefore, the phenotypic alterations in the *Tbx3*$^{-/-}$ embryonic liver arose as a direct consequence of the developmental defects of hepatoblasts, although the subsequent misinteractions of hepatoblasts with other hepatic components might also be relevant to that phenotype.

The mechanisms controlling the segregation of hepatobiliary lineages have been a challenging area to understand, as many important genes, including those encoding Hnf4α, Hnf1α, Hnf6, Hnf1β and C/EBPα, have been found to be involved (Tian and Schibler, 1991; Tomizawa et al., 1998; Li et al., 2000; Clotman et al., 2002; Coffinier et al., 2002; Suzuki et al., 2003a; Yamasaki et al., 2006). Our present data indicate that under the loss of Tbx3 function, the growth arrest induced by p19^{ARF} is important for activating a program of cholangiocyte differentiation in hepatoblasts, including the upregulation or downregulation of many hepatic transcription factors. Conversely, the repression of *p19^ARF^* expression by Tbx3 allows hepatoblasts to proliferate and provides these cells with an alternative fate, such as the differentiation into hepatocytes. These facts are further supported by evidence that in the developing liver, both Alb$^-$ and Alb$^+$ cells, but few CK7$^+$ cells, are found in Tbx3$^+$ cells (Fig. 1K,L). Therefore, although a transcriptional hierarchy involved in the lineage determination of hepatoblasts should be elucidated in future analyses, Tbx3 might act as a central regulator for maintaining cells in an undifferentiated state and for activating their proliferation to create the basis of liver organogenesis, until such a time that its expression ceases or is superseded by the subsequent activation of other transcription factors required for differentiation. Because organ progenitor cells consist of a rare population in each environment, the identification of crucial genes that regulate their distinctive potentials is difficult. Our method for prospectively isolating hepatoblasts could be used efficiently to identify new genes, such as Tbx3, that are fundamentally required for their activities, and to improve our understanding of the molecular nature of liver development, regeneration and carcinogenesis.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/135/9/1589/DC1

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