

morphogenetic protein (BMP), another factor involved in the liver specification (Rossi et al., 2001). By these signals, pre-hepatic cells become proliferative and begin to form the liver bud. Following the formation of primary liver bud from E8.5 to E9.0, the basement membrane surrounding the liver bud is lost by E9.5, and pre-hepatic cells migrate as cords into the surrounding STM (Matsumoto et al., 2001; Sosa-Pineda et al., 2000; Zaret, 1998). These pre-hepatic cells are also designated as hepatoblasts and a number of studies have indicated that hepatoblasts give rise to both hepatocytes and cholangiocytes, biliary epithelial cells (Germain et al., 1988; Rogler, 1997; Shiojiri, 1994; Shiojiri et al., 1991; Spagnoli et al., 1998). As cell sorting using antibodies (Abs) is a powerful means to isolate and characterize a specific cell type, efforts have been made to search for specific cell-surface antigens on hepatoblasts. We previously reported that DLK1, also known as Pref-1, was strongly expressed in the E10.5 mouse liver bud and that DLK1⁺ cells isolated from E14.5 livers expressed albumin and formed colonies composed of hepatocyte and cholangiocyte lineages when cultured in the presence of hepatocyte growth factor (HGF) and epidermal

growth factor (EGF) (Tanimizu et al., 2003). Thus, DLK1⁺ cells contain hepatoblasts and DLK1 is a useful marker to enrich highly proliferative hepatoblasts from fetal liver. Kubota et al. showed that the RT1A1⁻ OX18^{low} ICAM-1⁺ fraction of E13 rat fetal liver contained hepatoblasts (Kubota and Reid, 2000). Suzuki et al. developed a single cell-based assay designated the hepatic colony-forming unit in culture (H-CFU-C) and showed that the CD45⁻ TER119⁻ c-Kit⁻ CD29⁺ CD49f⁺ and CD45⁻ TER119⁻ c-Kit⁻ c-Met⁺ CD49f^{+/low} fraction of E13.5 mouse liver contained hepatic progenitor/stem cells (Suzuki et al., 2000). Thus, the colony-forming capacity is a criterion to evaluate hepatoblasts. They also showed that CD45⁻ TER119⁻ c-Kit⁻ c-Met⁺ CD49f^{+/low} cells of E11.5 mouse liver had high H-CFU-C potential. On the other hand, Minguet et al. reported that CD45⁻ TER119⁻ c-Kit^{low} cells in E11 mouse liver contained the earliest hepatic progenitors, also displaying features of liver-repopulating stem cells (Minguet et al., 2003); however, despite finding these markers, negative selection markers are less informative for the localization of hepatoblasts. Thus, cell-surface antigens expressed on hepatoblasts are valuable for localization and prospective

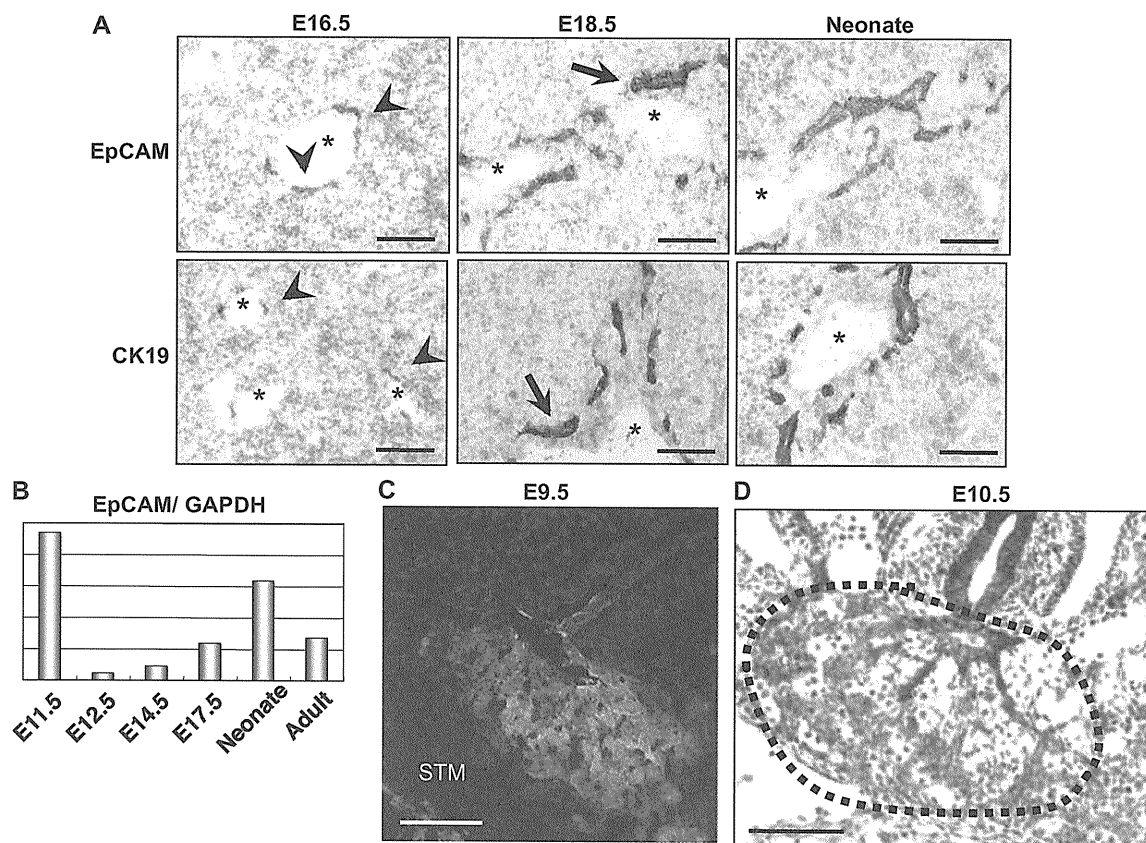


Fig. 1 – Expression profiles of EpCAM during liver development. (A) Immunostaining of E16.5, E18.5 and neonatal liver sections with either anti-EpCAM or anti-CK19 Ab. In E16.5 liver, arrowheads point to mono-layered bile duct progenitor cells. (Arrows) In E18.5 liver, arrows point to bi-layered ductal plates strikingly stained with both Abs. Asterisk: portal veins. (B) Expression of EpCAM in liver is analyzed by real-time RT-PCR during liver development (E11.5, E12.5, E14.5, E17.5, neonate and adult liver). The expression level of EpCAM is normalized by GAPDH (C) IHC of an E9.5 liver section with anti-EpCAM (green) and anti-HNF4 α (magenta) Abs. Both gut endoderm cells and liver precursor cells spreading into STM are stained with anti-EpCAM Ab. (D) IHC of an E10.5 liver section with anti-EpCAM Ab by DAB staining. Dotted line encircles liver primordium. Scale bars: 100 μ m.

isolation of hepatoblasts in the early stage of liver development.

Hepatic stem/progenitor cells are also known to be present in the adult liver and are thought to serve as reservoir cells in the case of massive hepatic necrosis (Dabeva and Shafritz, 1993; Fausto et al., 1993; Sell, 1990; Thorgeirsson et al., 1993). In rodents, oval cells with the ability to differentiate into both hepatocytes and cholangiocytes appear in the periportal region following hepatic injury under a condition that restricts hepatocyte proliferation (Evarts et al., 1990). Although both oval cells in adults and hepatoblasts in embryos exhibit similar characteristics, i.e., proliferation and differentiation into hepatocytes and cholangiocytes (Shiojiri et al., 1991; Tian et al., 1997), their origin and relationship remain unknown. Because of their common characteristics, it is possible that they exhibit common cell-surface molecules. In fact, DLK1 was shown to be expressed in a subpopulation of rat oval cells (Jensen et al., 2004; Tanimizu et al., 2004). EpCAM is a cell-surface glycoprotein expressed on some normal as well as neoplastic epithelial cells (Armstrong and Eck, 2003; Momburg et al.,

1987). In the adult liver, it is known to be expressed on cholangiocytes, but not hepatocytes (de Boer et al., 1999). Recently, we and another group reported that EpCAM is expressed in mouse and rat oval cells (Okabe et al., 2009; Yovchev et al., 2008). In contrast, Dan et al. reported that multipotent progenitor cells derived from human fetal liver expressed EpCAM (Dan et al., 2006). However, the expression profiles of EpCAM, e.g., location and developmental stages in human fetal liver are not known. Schmelzer et al. have reported that pluripotent precursors of hepatoblasts expressed EpCAM and were located in ductal plates in human fetal liver (Schmelzer et al., 2007). However, the expression profile of EpCAM from the onset of liver organogenesis through the formation of ductal plates has not been known. In this report, we investigated the expression of EpCAM at an early stage of mouse liver development and show that EpCAM is the earliest and a transient marker for hepatoblasts during hepatogenesis. Moreover, the expression profile of EpCAM and DLK1 provides evidence for the developmental pathways of liver progenitors for not only hepatoblasts, but also extrahepatic bile ducts and mesothelium.

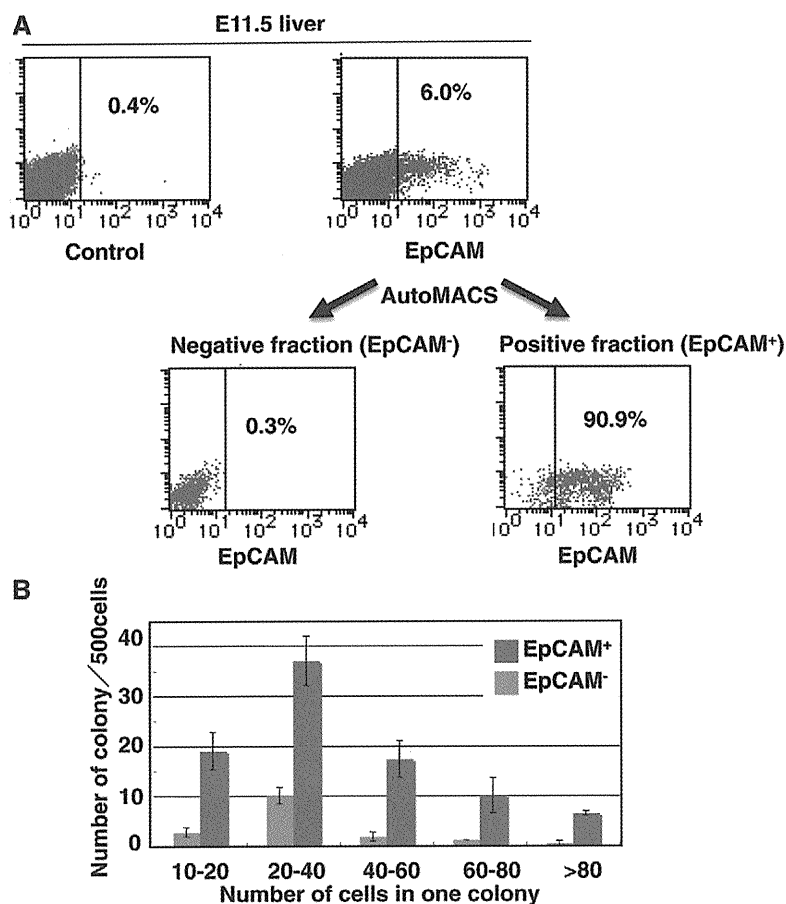


Fig. 2 – Characterization of EpCAM⁺ cells in E11.5 liver. (A) FCM of E11.5 liver cells with anti-EpCAM Ab (upper panels). EpCAM⁺ and EpCAM⁻ cells sorted by AutoMACS were reanalyzed as shown (lower panels). (B) Colony-forming potential of EpCAM⁺ cells in E11.5 liver. EpCAM⁺ cells and EpCAM⁻ cells were isolated from E11.5 liver by AutoMACS. Each 500 cells were cultured to evaluate colony formation.

2. Results and discussion

2.1. Expression of EpCAM during mouse liver development

To investigate the expression profiles of EpCAM during mouse liver development, immunohistochemistry (IHC) using anti-EpCAM antibody (Ab) was performed. It is known that EpCAM is constitutively expressed in biliary epithelial cells in the normal adult liver (de Boer et al., 1999; Hreha et al., 1999) Because intrahepatic bile duct cells are known to develop in the periportal area in the late-gestational stage E16.5, we examined the expression of EpCAM as well as cytokeratin (CK)19, a marker for biliary epithelial cells in livers from E16.5 to neonatal stages by immunostaining. Both CK19 and

EpCAM were weakly expressed as a discontinuous single-layered ring in the periportal area of E16.5 liver and their expression became stronger at E18.5 and the neonate stage when ductal plates with a partly bi-layered ring were clearly formed (Fig. 1A). Consistent with the immunostaining result, EpCAM was highly expressed in the late-gestational stage, E17.5, and the neonate stage by real-time PCR (Fig. 1B). Because the formation of ductal plates has not yet occurred in mid-gestational stages (Lemaigre, 2003; Shiojiri, 1997; Tanimizu et al., 2003), it is reasonable that EpCAM expression is low around E14.5. Interestingly, however, EpCAM was expressed highly in E11.5 liver (Fig. 1B). To investigate the expression of EpCAM at the onset of liver organogenesis, we immunostained transverse sections of E9.5 embryos with anti-EpCAM Ab and anti-

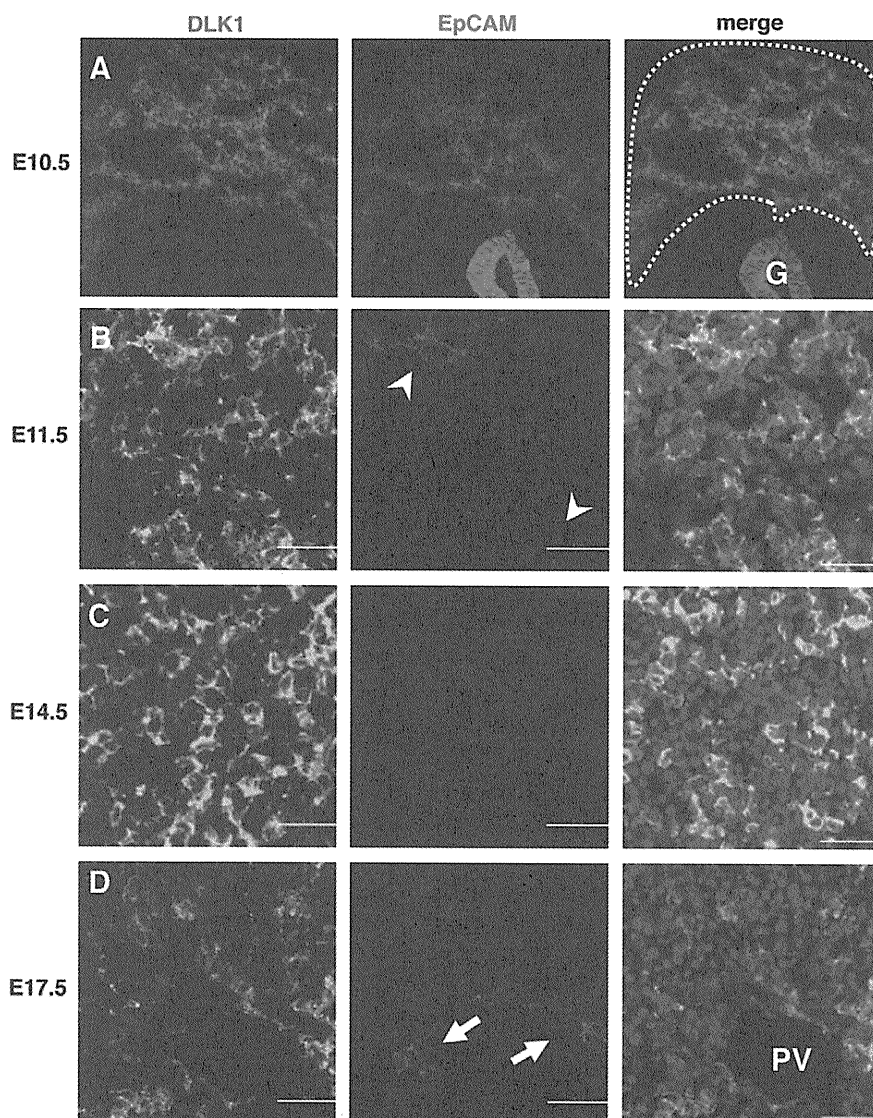


Fig. 3 – Expression profiles of EpCAM and DLK1 during liver development. (A–D) IHC of liver sections with anti-EpCAM (red) and anti-DLK1 (green) Abs at various developmental stages (E10.5, E11.5, E14.5 and E17.5) Whereas cord-like cells in STM were stained with both EpCAM and DLK1 at E10.5 (dotted line), gut endoderm cells were stained with only anti-EpCAM Ab. While faint signals of EpCAM were detected at E11.5 (arrowheads), no significant signal of EpCAM was detected at E14.5. In E17.5 liver, some cells with an intense EpCAM signal but without DLK1 signal were observed around the portal vein (arrows). G, gut; PV, portal vein; Scale bars: 40 μ m.

hepatocyte nuclear factor (HNF4 α) Ab. IHC clearly demonstrated that EpCAM is expressed in HNF4 α ⁺ epithelial hepatoblasts forming liver bud, but not in STM (Fig. 1C). At E10.5, the EpCAM⁺ hepatoblasts spread out from the foregut into the STM region to form hepatic cords (Fig. 1D). These results indicated that the earliest hepatoblasts emerged from EpCAM⁺ foregut endoderm continued to express EpCAM at the onset of hepatogenesis.

2.2. Colony-forming capacity of EpCAM⁺ cells at an early stage of liver development

To characterize EpCAM⁺ cells at an early stage of liver development, we generated anti-EpCAM Ab applicable for flow cytometric analysis (FCM) and investigated its expression profile in fetal liver cells. Because E10.5 liver was too small to dissect precisely and to prepare a sufficient number of cells for FCM, we used fetal liver cells of E11.5 embryos (Fig. 2A). FCM revealed that the percentages of EpCAM⁺ cells in total liver cells were approximately 6%. To further characterize hepatoblasts in EpCAM⁺ cells, we isolated each cell fraction using an automatic magnetic cell sorter (AutoMACS) and evaluated their proliferation potential by in vitro colony formation assay, as previously reported (Tanimizu et al., 2003) (Fig. 2B). Approximately 20% of EpCAM⁺ cells formed colonies, whereas only about 3% of EpCAM⁻ cells did so. In addition, large colonies composed of over 80 cells were formed predominantly in EpCAM⁺ cells, suggesting that

highly proliferative progenitor cells are present in EpCAM⁺ cells at E11.5. This result also indicated that EpCAM⁻ cells include colony-forming cells, although they are less proliferative; therefore, sorting by an anti-EpCAM Ab is effective to purify the highly proliferative population at an early stage of liver development.

2.3. Expression profile of EpCAM in DLK1⁺ cells during liver development

We have previously reported that DLK1⁺ cells in E14.5 liver contain highly proliferative and bipotential hepatic progenitors, indicating that DLK1 is a hepatoblast marker in E14.5 liver (Tanimizu et al., 2003). To investigate the relationship between EpCAM and DLK1, transverse sections of E10.5 embryos were immunostained with anti-DLK1 and anti-EpCAM Abs. As shown in Fig. 3A, EpCAM was expressed in the fetal gut where no DLK1 expression was detected. In the STM region, most EpCAM⁺ cells forming apparent hepatic cords expressed a high level of DLK1. Since hepatoblasts emerge from the foregut endoderm, EpCAM⁺DLK1⁻ endoderm cells in the foregut were considered to give rise to hepatoblasts with DLK1, forming the hepatic bud and primordium. Fluorescent signals of EpCAM in the liver by IHC became weaker along with development. Only faint signals of EpCAM were detected in DLK1⁺ cells at E11.5, but no clear signals of EpCAM were observed in E14.5 liver (Fig. 3B and C). In the late-gestational stage, E17.5, strong EpCAM signals reappeared around portal veins

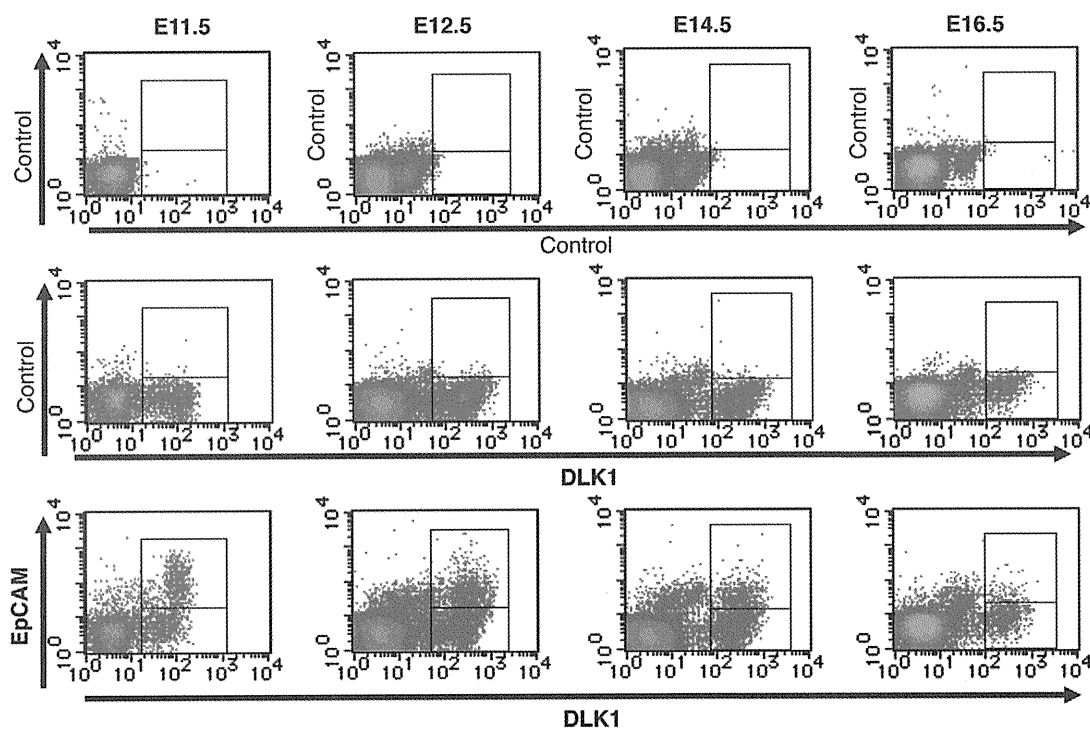


Fig. 4 – FCM of fetal liver cells with anti-EpCAM and anti-DLK1 Abs at various stages. At E11.5, there was an apparent population with high expression of EpCAM and DLK1. The expression level of EpCAM is rapidly downregulated and the population of EpCAM⁺DLK1⁺ cells is markedly reduced after E12.5. As shown in the upper control panel, hepatoblasts show autofluorescence property at E12.5 or later.

Table 1 – Percentage of each cell population in liver during development.

	E11.5	E12.5	E14.5	E16.5
EpCAM ⁺ DLK1 ⁺ /total cells (%)	8.4	3.6	1.9	0.3
DLK1 ⁺ /total cells (%)	18.0	10.4	8.1	3.9
EpCAM ⁺ DLK1 ⁺ cells (%)	46.7	34.4	23.5	6.7

as a ductal plate (Figs. 1A and 3D). These EpCAM⁺ cells were negative for DLK1, as previously reported (Tanimizu et al., 2003).

To reveal the detailed expression profiles of EpCAM and DLK1 during liver development, we performed FCM of fetal liver cells at E11.5, E12.5, E14.5 and E16.5, because it allows more sensitive and quantitative detection than IHC. FCM revealed that approximately 20% of nucleated cells in E11.5 liver were DLK1⁺ cells, about half of which expressed EpCAM (Fig. 4). Interestingly, the percentage of EpCAM⁺ cells in the

DLK1⁺ cell population was rapidly reduced along with liver development (Table 1). Moreover, the expression level of EpCAM after E12.5 was very low compared with that of E11.5 liver. Downregulation of EpCAM in DLK1⁺ cells by FCM was consistent with the results of IHC and RT-PCR in fetal liver from E11.5 to E17.5 (Figs. 1B and 3A–D). These results suggested that EpCAM is highly expressed at the onset of liver organogenesis, dramatically decreased along with hepatic differentiation, and then restricted to intrahepatic bile duct cells at a later stage.

2.4. Colony formation capacity of EpCAM⁺DLK1^{high} cells and EpCAM⁻DLK1^{low} cells in E11.5 liver

FCM showed that E11.5 liver cells contained at least three populations (Fig. 5A). Because the expression level of DLK1 in EpCAM⁺ cells is higher than EpCAM⁻ cells, E11.5 liver could be divided into EpCAM⁺DLK1^{high}, EpCAM⁻DLK1^{low} and EpCAM⁻DLK1⁻ cells. To further examine the feature of

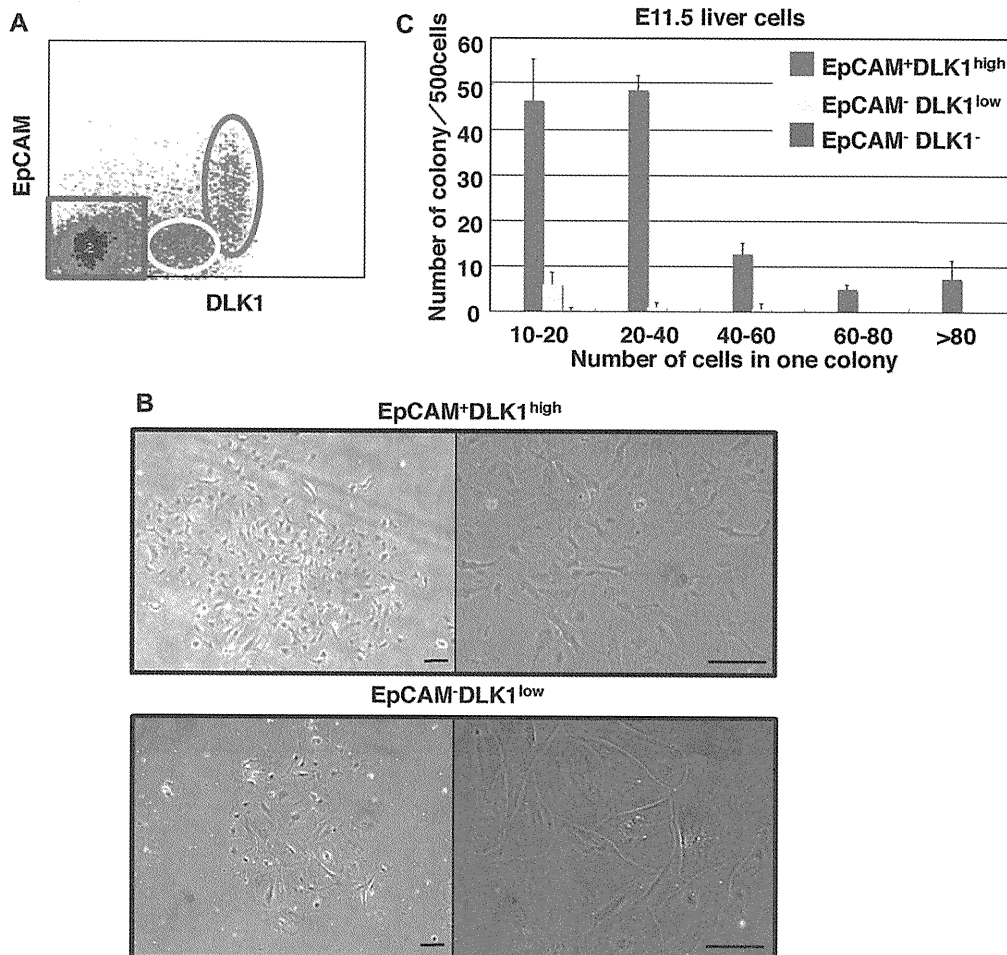


Fig. 5 – Characterization of EpCAM⁺ and EpCAM⁻ cells in E11.5. (A) FCM of E11.5 liver cells with anti-DLK1 and anti-EpCAM Abs. **(B)** Representative morphology of colonies derived from EpCAM⁺DLK1^{high} cells (upper panels) and EpCAM⁻DLK1^{low} cells (lower panel). Scale bars: 100 μ m **(C)** Colony-forming potential of each cell population isolated from E11.5 liver. EpCAM⁺DLK1^{high}, EpCAM⁻DLK1^{low} and EpCAM⁻DLK1⁻ cells were isolated from E11.5 liver by FACSvantage and each 500 cells were cultured to evaluate colony formation. EpCAM⁺DLK1^{high} cells predominantly formed many and large colonies.

hepatoblasts among these cells, we performed in vitro colony formation assays. After 6 days of culture, the number and size of colonies were examined. Approximately 25% of $\text{EpCAM}^+\text{DLK1}^{\text{high}}$ cells formed a colony, whereas $\text{EpCAM}^-\text{DLK1}^{\text{low}}$ cells formed a small number of colonies and no colony was formed from $\text{EpCAM}^-\text{DLK1}^-$ cells (Fig. 5B and C). Moreover, the size of colonies from $\text{EpCAM}^+\text{DLK1}^{\text{high}}$ cells was much larger than those from $\text{EpCAM}^-\text{DLK1}^{\text{low}}$ cells (Fig. 5C). These results indicated that the expression of EpCAM subdivides DLK1^+ cells in E11.5 liver and that it is an excellent marker for early hepatoblasts.

2.5. Colony formation capacity of $\text{EpCAM}^+\text{DLK1}^+$ cells and $\text{EpCAM}^-\text{DLK1}^+$ cells in E14.5 liver

It seemed that E14.5 $\text{DLK1}^{\text{high}}$ hepatoblasts are derived from E11.5 $\text{EpCAM}^+\text{DLK1}^{\text{high}}$ cells (Fig. 4). To address this possibility, we performed FCM of E11.5 and E13.5 liver cells with another 40-1 Ab, which binds to unidentified cell-surface protein expressed on hepatoblasts, but not to DLK1 (data not shown). As shown in Fig. 6A, both E11.5 $\text{DLK1}^{\text{high}}$ cells and E14.5 $\text{DLK1}^{\text{high}}$ cells maintained high expression levels of the 40-1 antigen, strongly suggesting that the majority of

$\text{EpCAM}^+\text{DLK1}^{\text{high}}$ hepatoblasts are losing EpCAM expression to become $\text{EpCAM}^-\text{DLK1}^{\text{high}}$ after E12.5.

While highly proliferative hepatoblasts in E11.5 liver were $\text{EpCAM}^+\text{DLK1}^{\text{high}}$ cells, there were some E14.5 $\text{DLK1}^{\text{high}}$ cells with EpCAM at a low expression level (Fig. 4). To compare colony-forming activity between $\text{EpCAM}^{\text{low}/+}\text{DLK1}^{\text{high}}$ cells and $\text{EpCAM}^{\text{low}/-}\text{DLK1}^{\text{high}}$ cells in E14.5 liver, we sorted each fraction and performed colony formation assay. Both cell populations showed similar colony-forming activity, including large colonies (Fig. 6B). In addition, the colony-forming capacities of both E11.5 $\text{EpCAM}^+\text{DLK1}^{\text{high}}$ and E14.5 $\text{EpCAM}^{\text{low}/-}\text{DLK1}^{\text{high}}$ cells were comparable. These results supported the possibility that E11.5 $\text{EpCAM}^+\text{DLK1}^{\text{high}}$ cells gradually lost EpCAM expression to become $\text{EpCAM}^-\text{DLK1}^{\text{high}}$ cells, while further investigation such as genetic lineage tracing will be required for definitive proof. Importantly, $\text{DLK1}^{\text{high}}$ cells in E14.5 liver include hepatoblasts with high proliferation potential irrespective of EpCAM expression. Thus, EpCAM is the earliest hepatoblast marker but not necessarily a specific marker for hepatoblasts at a later gestational stage in the mouse.

2.6. Characterization of $\text{EpCAM}^+\text{DLK1}^{\text{high}}$ cells and $\text{EpCAM}^-\text{DLK1}^{\text{low}}$ cells in E11.5 liver

We showed that there are at least three major populations, i.e., $\text{EpCAM}^+\text{DLK1}^{\text{high}}$, $\text{EpCAM}^-\text{DLK1}^{\text{low}}$ and $\text{EpCAM}^-\text{DLK1}^-$ cells, in E11.5 liver, which displayed quite distinct size and morphology of their colonies in in vitro assays (Fig. 5). To further characterize these three populations, we sorted each cell fraction and compared their gene expression profiles by RT-PCR (Fig. 7A). $\text{EpCAM}^+\text{DLK1}^{\text{high}}$ cells expressed various genes that are mainly expressed in hepatoblasts or immature hepatocytes, i.e., albumin, alpha-fetoprotein (AFP), transthyretin (TTR), HNF4 α , and c-Met, whereas their expression was weak or hardly detected in $\text{EpCAM}^-\text{DLK1}^{\text{low}}$ cells. By contrast, CK19, was highly expressed in $\text{EpCAM}^-\text{DLK1}^{\text{low}}$ cells, but was very low in $\text{EpCAM}^+\text{DLK1}^{\text{high}}$ cells. $\text{EpCAM}^-\text{DLK1}^-$ cells did not express CK19. Because mouse and rat hepatoblasts do not express CK19, which is a marker for cholangiocytic differentiation (Shiojiri et al., 1991; Tanimizu et al., 2003), there was a possibility that $\text{EpCAM}^+\text{DLK1}^{\text{high}}$ cells contained cells committed to the cholangiocytic lineage. To confirm the results of RT-PCR, we immunostained cytospun cells with anti-albumin and anti-CK19 Abs (Fig. 7B). Consistent with RT-PCR, 86% of $\text{EpCAM}^+\text{DLK1}^{\text{high}}$ cells were positive for albumin, whereas 5% of those cells were positive for CK19. On the other hand, 24% and 18% of $\text{EpCAM}^-\text{DLK1}^{\text{low}}$ cells were albumin⁺ and CK19⁺, respectively. Because intrahepatic bile ducts had not yet been formed at this stage, CK19⁺ cells could be extrahepatic bile duct cells, gallbladder, or another cell type such as mesothelial cells, which also express CK19. Immunostaining of a sagittal section of an E11.5 embryo with anti-CK19 and anti-DLK1 Abs showed that there was an extrahepatic region where DLK1 and CK19 were expressed (Fig. 7C). The CK19⁺ cells in this region were also stained with *Dolichos biflorus* agglutinin (DBA), which selectively binds to extrahepatic bile ducts (Shiojiri, 1997), indicating that sorted DLK1^+ cells included fetal extrahepatic bile duct cells. On the other hand, cells at the mesothelial layer were also positively stained with anti-CK19 (Fig. 7C). These cells were not stained with DBA,

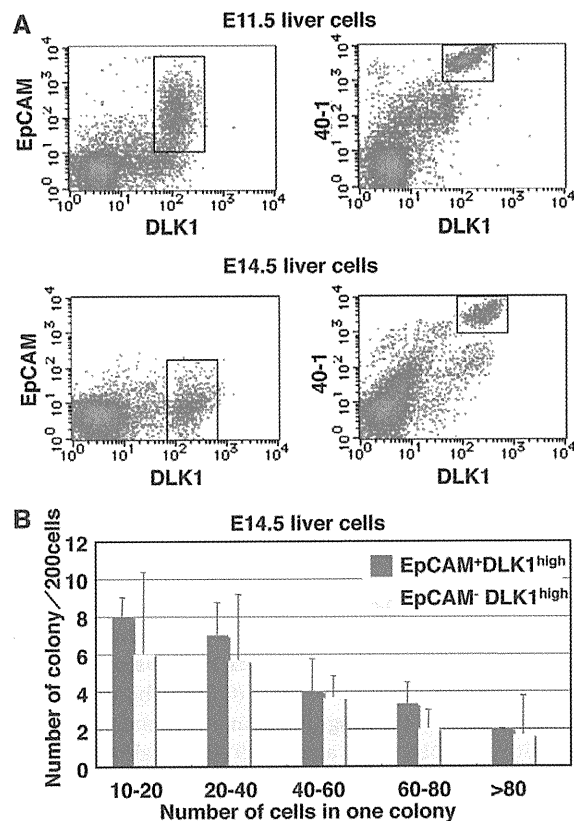


Fig. 6 – Characterization of $\text{EpCAM}^{\text{low}/+}\text{DLK1}^+$ cells and $\text{EpCAM}^{\text{low}/-}\text{DLK1}^+$ cells isolated from E14.5 liver. (A) FCM of E11.5 and E14.5 liver cells with anti-DLK1, anti-EpCAM and 40-1 antibody. (B) Two hundred cells of either $\text{EpCAM}^{\text{low}/+}\text{DLK1}^+$ cells or $\text{EpCAM}^{\text{low}/-}\text{DLK1}^+$ cell were cultured to evaluate colony formation. Both types of cells similarly generated many and large colonies.

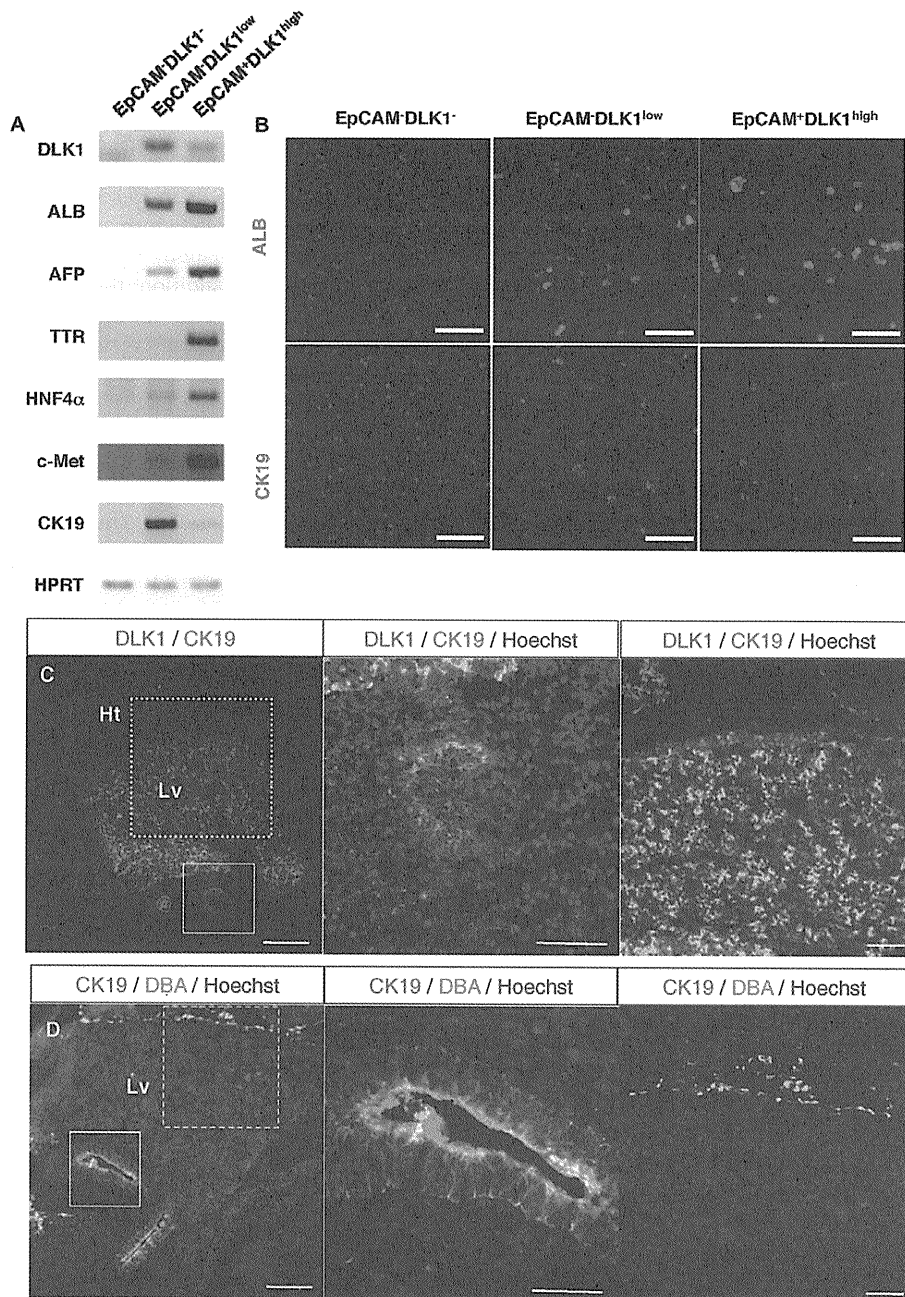


Fig. 7 – Characterization of each population isolated from E11.5 liver by a combination of anti-EpCAM and anti-DLK1 Abs. (A) Gene expression profiles of E11.5 EpCAM⁺DLK1^{high}, EpCAM⁻DLK1^{low} and EpCAM⁻DLK1⁻ cells by RT-PCR. (B) Immunostaining of EpCAM⁺DLK1^{high}, EpCAM⁻DLK1^{low} and EpCAM⁻DLK1⁻ cells with anti-albumin and anti-CK19 Abs by cytospin. (C and D) Immunostaining of a sagittal section of an E11.5 embryo with anti-DLK1 and anti-CK19 Ab or DBA. (C, left) The region of the liver primordium is strongly stained with anti-DLK1 Ab. Scale bars: 100 μ m. (C, middle) Magnification of the enclosed area by a solid square in the left panel shows that the gallbladder is stained with anti-DLK1 and anti-CK19 Abs. Scale bars: 40 μ m. (C, right) Magnification of the enclosed area by a dashed line in the left panel shows that the mesothelium is stained with anti-CK19 Ab. Scale bars: 40 μ m. (D, left) The region close to the liver primordium binds DBA. Scale bars: 100 μ m. (D, middle) Magnification of the enclosed by a solid square area in the left panel shows that the gallbladder is stained with anti-CK19 Ab and DBA. Scale bars: 40 μ m. (D, right) Magnification of the enclosed area by a dotted square area in the left panel shows that the mesothelium is stained with anti-CK19 Ab, but not DBA. Scale bars: 40 μ m. (E and F) Staining of E11.5 liver cells with anti-DLK1 and anti-CK19 Abs (E) or DBA-binding by cytospin (F). (G) Staining of E11.5 liver cells with DBA and anti-EpCAM Ab by cytospin. Scale bars: 40 μ m. Lv, liver; Ht, heart.

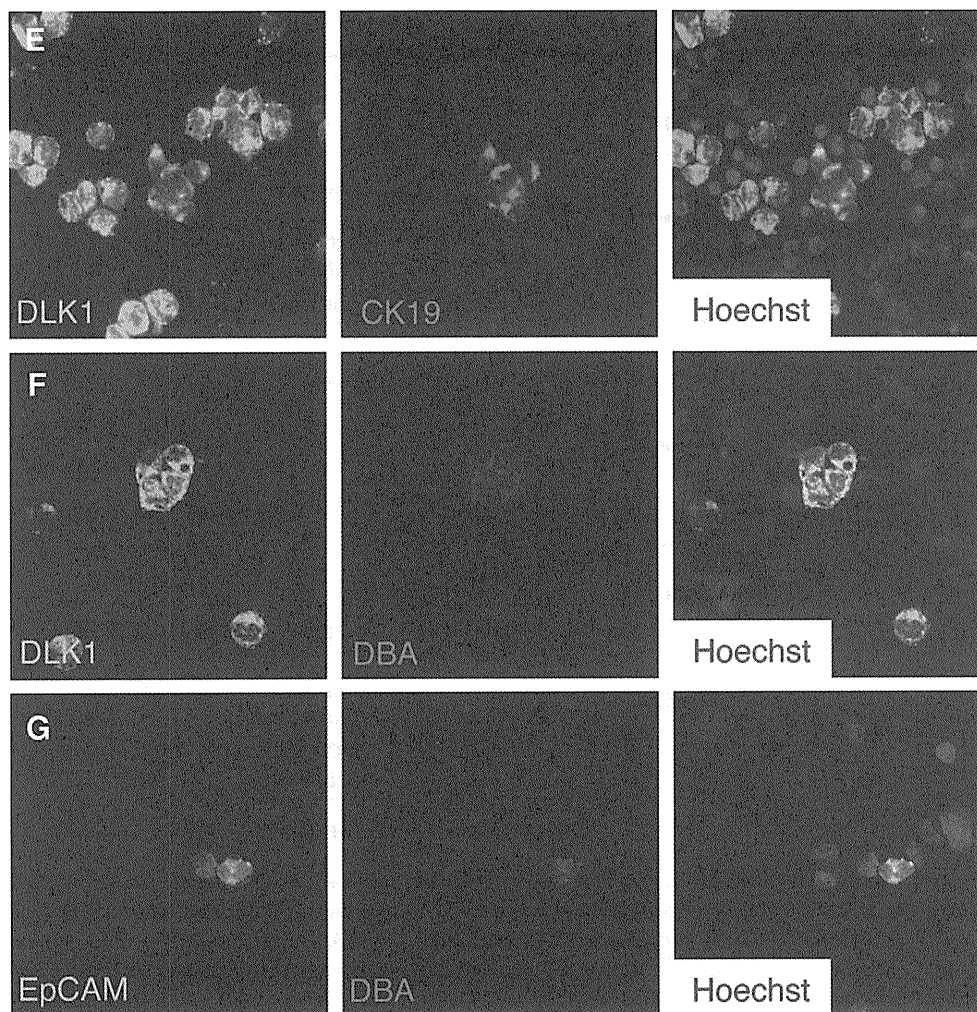


Fig. 7 (continued)

indicating that DLK1⁺ cells also include fetal mesothelial cells (Fig. 7D). The expression of DLK1 in CK19⁺ cells and DBA⁺ cells was confirmed by cytospin (Fig. 7E–G). Almost all DBA⁺ cells were positive for EpCAM, but negative for albumin (data not shown). Therefore, it is likely that low expression of CK19 in EpCAM⁺DLK1^{high} cells is due to DBA⁺ extrahepatic bile duct cells. Thus, EpCAM⁻DLK1^{low} cells in E11.5 liver contain various types of cells, such as low proliferative albumin⁺ hepatocytes and mesothelial cells. The highly extended morphology of small colonies derived from EpCAM⁻DLK1^{low} cells is reminiscent of mesothelium (Fig. 5B). In contrast, EpCAM⁺DLK1^{high} cells contain both highly proliferative hepatoblasts and extrahepatic bile duct cells. Whereas EpCAM⁺DLK1^{high} hepatoblasts lose the expression of EpCAM along with liver development, extrahepatic bile duct cells will maintain its expression.

Although the liver is a central metabolic organ in adults, it mainly functions as a hematopoietic organ in fetuses. Considering the marked change of liver function from the fetal to adult stage, it is not surprising that the characteristics of hepatoblasts and hepatocytes change during liver development. Whereas E-cadherin and Tim2 are constitutively expressed on the hepatocytic lineage throughout liver

development (Nitou et al., 2002; Watanabe et al., 2007), the expression of EpCAM and DLK1 on hepatoblasts is limited at the fetal stage. Therefore, a combination of these markers helps us understand the type of cells and the degree of differentiation. In this report, we show that EpCAM is expressed on hepatoblasts from the onset of liver organogenesis to approximately E14 and also on intrahepatic bile ducts after E16. While DLK1 was previously shown to be expressed on hepatoblasts, our results indicate that DLK1⁺ cells are further subdivided into EpCAM⁺ and EpCAM⁻ cells at an earlier stage. Colony formation assays strongly suggest that EpCAM⁺DLK1^{high} cells are the earliest and highly proliferative hepatoblasts. Moreover, the expression of EpCAM is markedly downregulated within a short period of time (Fig. 3). Fig. 8 summarizes the expression profiles of marker molecules on hepatic cells during mouse development deduced from these results. Recently, we reported that EpCAM⁺ cells include potential hepatic stem cells in adult mouse liver (Okabe et al., 2009). It is interesting to know whether EpCAM⁺ hepatic stem/progenitor cells residing in adult liver are derived from the earliest EpCAM⁺ hepatoblasts or not. In humans, it has been reported that the multipotent stem/progenitor cells de-

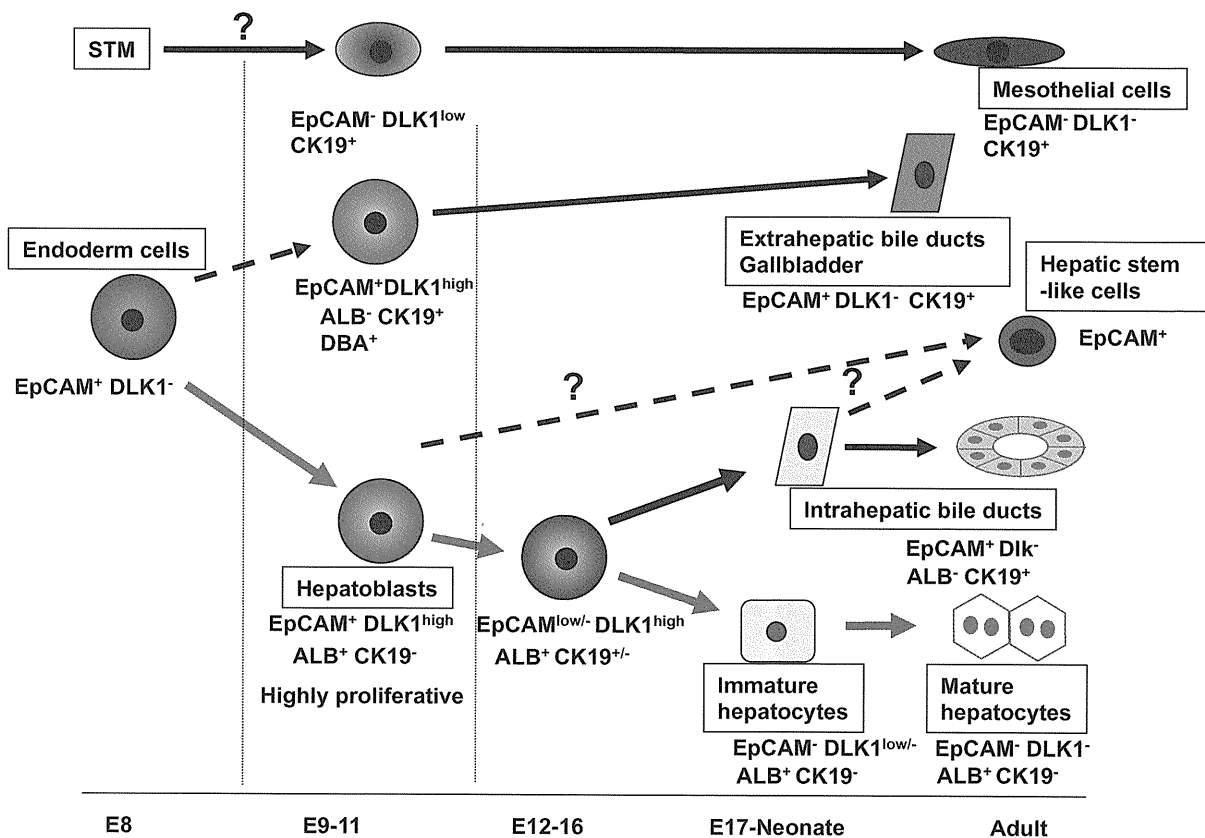


Fig. 8 – Summary of expression profiles of cell-surface markers during mouse liver development. The $\text{EpCAM}^+\text{DLK1}^+$ hepatoblasts emerged from $\text{EpCAM}^+\text{DLK1}^-$ foregut endoderm cells form liver bud and migrate into STM at around E9. In E11.5 liver, $\text{EpCAM}^+\text{DLK1}^{\text{high}}$ cells include highly proliferative hepatoblasts and DBA^+ extrahepatic bile duct cells. By contrast, $\text{EpCAM}^-\text{DLK1}^{\text{low}}$ cells include mesothelial precursor cells and immature hepatocytes with low proliferative potential. Thereafter, $\text{EpCAM}^+\text{DLK1}^{\text{high}}$ hepatoblasts dramatically reduce the expression of EpCAM. At around E16.5, EpCAM is upregulated in ductal plates around a portal vein, while it is absent in hepatoblasts. The origin of EpCAM^+ adult hepatic stem-like cells is not known at present.

rived from fetal liver express EpCAM (Dan et al., 2006; Schmelzer et al., 2006, 2007). Additionally, it is also reported that approximately 95% and 50% of EpCAM^+ cells are hepatoblasts in human fetus and neonate, respectively. Because most of mouse hepatoblasts do not express EpCAM at a later gestational stage as described above, it is likely that the characteristics of hepatoblasts in human are somewhat different from those in mouse. In fact, mouse hepatoblasts do not express CK19, whereas human hepatoblasts are known to express CK19 (Stosiek et al., 1990).

In conclusion, we showed that mouse hepatoblasts exhibit a transient expression of EpCAM during liver development and that the combination of DLK1 and EpCAM expression provided evidence for the developmental pathways of mouse liver precursor cells. Defining cell lineages and differentiation stages is a critical step to understand the mechanism of organogenesis. We showed that the earliest mouse hepatoblasts could be identified and isolated prospectively by the expression of EpCAM as early as E11.5. Further investigation on the profile of cell-surface markers will help us to isolate a specific type of precursor cells and to understand mechanisms of each cell differentiation.

3. Materials and methods

3.1. Mice

C57BL/6 mice (Nihon SLC, Hamamatsu, Japan) were used for all experiments. All experiments with animals were performed according to institutional guidelines.

3.2. Antibodies

Anti-DLK1 (24-11) monoclonal antibody (mAb) and 40-1 mAb recognizing hepatoblasts were generated by immunization of a rat with E14.5 mouse fetal liver cells deprived of hematopoietic cells, and screened as previously reported (Suzuki et al., 2008). The anti-DLK1 mAb is commercially available from MBL International (Woburn, MA). The anti-EpCAM mAbs were generated by immunization of a rat with Ba/F3 transfectants expressing mouse EpCAM cDNA (Okabe et al., 2009). Hybridomas expressing mAb were established by fusion of lymphocytes and P3X myeloma cells with polyethylene glycol as described previously (Hara et al., 1999). Specificity of mAb was confirmed by FCM using Ba/F3 trans-

fectants constitutively expressing mouse EpCAM. Each mAb against DLK1, EpCAM and 40-1 was FITC-labeled or biotinylated and used for FCM. The rabbit anti-mouse CK19 polyclonal Ab was raised against the C-terminal peptide, HYNLPTPKAI, and the serum was used for immunostaining, as previously described (Tanimizu et al., 2003). Rhodamine *Dolichos biflorus* agglutinin was purchased from Vector Laboratories (Burlingame, CA). Anti-HNF4 α Ab (H-171) (Santa Cruz Biotechnology) and anti-mouse CD326 (EpCAM) (G8.8) (BD Pharmingen) were used for IHC.

3.3. Immunohistochemistry

Whole embryos or fetal liver were fixed by incubation in a 4% paraformaldehyde solution at 4 °C overnight, followed by dehydration sequentially in 15% sucrose and 30% sucrose solution overnight, and then embedded in OCT. Cryostat sections (7 μ m) were incubated with each Ab, followed by a biotin- or fluorescein-conjugated secondary Ab. The signals were visualized using the Vectastain ABC Elite kit (Vector Laboratories, Burlingame, CA) or fluorescence microscopy. Primary Abs used were: anti-EpCAM 1:200 (BD Pharmingen, San Diego, CA); anti-DLK1 1:200 (MBL); anti-CK19 1:1000. Secondary Abs used were conjugated to FITC or PE (BD Pharmingen).

3.4. Preparation and isolation of liver cells

Fetal liver cells were prepared according to the method of Kamiya et al. (1999). These cells were used for FCM analysis and isolation of EpCAM⁺ cells. EpCAM⁺ cells were isolated using AutoMACS according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, E11.5 liver cells were incubated with anti-FcR Ab for the block of FcR, and then incubated with FITC-conjugated anti-EpCAM mAb. After washing with phosphate-buffered saline (PBS), cells were resuspended in AutoMACS running buffer, and 100 μ l/ml of anti-FITC-labeled microbeads (Miltenyi Biotec) were added. After washing with the running buffer, the cells were loaded onto a magnetic column and EpCAM⁺ cells were eluted from the column after the depletion of EpCAM⁻ cells. Positive and negative selections were performed twice to prepare EpCAM⁺ cells and EpCAM⁻ cells. The purity of each population was over 90%. EpCAM⁺DLK1⁺, EpCAM⁻DLK1⁺ and EpCAM⁻DLK1⁻ cells from E11.5 liver were isolated by FACS-Vantage using FITC-conjugated anti-DLK1 mAb, biotinylated anti-EpCAM mAb and streptavidin-APC. Dead cells were excluded by propidium iodide (1 μ g/ml) staining and FL-3 gating.

3.5. Colony formation assay

Fetal liver cells were cultured as previously described (Tanimizu et al., 2003). Briefly, cells were plated at 50 cells/cm² in DMEM/F12 medium supplemented with 10% FBS, 1 \times ITS, 10 mM nicotinamide (Wako, Tokyo, Japan), 0.1 mM dexamethasone, 5 mM L-glutamine, 20 ng/ml EGF (PeproTech, London, UK), and 20 ng/ml HGF (R&D, Minneapolis, MN) on a type I collagen-coated dish. After 6 days of culture, the number of colonies was determined.

3.6. RNA extraction and reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was extracted from each cell preparation with Trizol reagent (Invitrogen, Carlsbad, CA). Total RNA and random hexamer primers were used to synthesize cDNA using the First-strand cDNA synthesis kit (Amersham Pharmacia Biotech, Piscataway, NJ). The PCR primers and conditions are described below: the samples were denatured at 95 °C for 20 s, followed by 30–40 thermal cycles; denaturation at 95 °C for 10 s, annealing at 56 °C for 20 s, and extension at 72 °C for 90 s, with a final extension at 72 °C for 5 min. PCR primers of albumin, AFP, HNF4 α , CK19 were described previously (Tanimizu et al., 2003). The other primers used are: TTR (5'-atccacaagctcctgacag-3' and 5'-actgctttggcaagatcctgg-3'), c-Met (5'-ggaagagggcattttggctg-3' and 5'-agcacaccgaaggacca cac-3'), HPRT (5'-gactgaaagacttgctcgag-3' and 5'-ccagcaagcttg caaccttaaca-3'). The real-time PCR for EpCAM was performed using LightCycler (Roche) according to manufacturer's protocol. The used primers of EpCAM were 5'-agggcgatccagaac aacg-3' and 5'-atggctgtaggggcttctc-3'.

Acknowledgements

This work was supported in part by a grant for Promotion of Independence for Young Investigators of Japan Science and Technology Agency (JST), and a Grant-in-Aid for Scientific Research and Global COE Program from the Ministry of Education, Sports, Science and Technology, Japan.

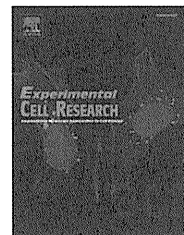
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Research Article

Hedgehog signal activation coordinates proliferation and differentiation of fetal liver progenitor cells[☆]

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ARTICLE INFORMATION

Article Chronology:

Received 25 February 2009

Revised version received 11 May 2009

Accepted 16 June 2009

Available online 24 June 2009

Keywords:

Hedgehog signaling

Fetal liver

Hepatoblast

ABSTRACT

Hedgehog (Hh) signaling plays crucial roles in development and homeostasis of various organs. In the adult liver, it regulates proliferation and/or viability of several types of cells, particularly under injured conditions, and is also implicated in stem/progenitor cell maintenance. However, the role of this signaling pathway during the normal developmental process of the liver remains elusive. Although *Sonic hedgehog* (*Shh*) is expressed in the ventral foregut endoderm from which the liver derives, the expression disappears at the onset of the liver bud formation, and its possible recurrence at the later stages has not been investigated. Here we analyzed the activation and functional relevance of Hh signaling during the mouse fetal liver development. At E11.5, *Shh* and an activation marker gene for Hh signaling, *Gli1*, were expressed in Dlk^+ hepatoblasts, the fetal liver progenitor cells, and the expression was rapidly decreased thereafter as the development proceeded. In the culture of Dlk^+ hepatoblasts isolated from the E11.5 liver, activation of Hh signaling stimulated their proliferation and this effect was cancelled by a chemical Hh signaling inhibitor, cyclopamine. In contrast, hepatocyte differentiation of Dlk^+ hepatoblasts *in vitro* as manifested by the marker gene expression and acquisition of ammonia clearance activity was significantly inhibited by forced activation of Hh signaling. Taken together, these results demonstrate the temporally restricted manner of Hh signal activation and its role in promoting the hepatoblast proliferation, and further suggest that the pathway needs to be shut off for the subsequent hepatic differentiation of hepatoblasts to proceed normally.

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Introduction

The liver is the largest organ in the adult human body and plays a central role in metabolism, serum protein synthesis, detoxification and the maintenance of systemic homeostasis. It is composed of many types of cells including hepatocytes, the parenchymal component executing the majority of the organ's functions, and other non-parenchymal cells such as cholangiocytes, Kupffer cells, hepatic stellate cells (HSCs), and endothelial cells. Among those

cell types, hepatocytes and cholangiocytes, the two epithelial lineages in the organ, derive from a common precursor cell population in the developing liver [1]. Elucidating the nature and the regulatory mechanisms of these hepatic progenitor cells, so-called hepatoblasts, is a fundamental issue to understand how liver architecture and function are properly manufactured.

Liver organogenesis starts at embryonic day (E) 8.5 in the mouse, when liver diverticulum is formed from the ventral foregut endoderm in response to fibroblast growth factor (FGF), secreted

[☆] This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (17014016) and from the CREST program of Japan Science and Technology Agency.

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from the cardiac mesoderm, and bone morphogenetic protein (BMP), secreted from the septum transversum mesenchyme (STM) [1–4]. Hepatoblasts emerging from the ventral endodermal epithelium proliferate by the help of endothelial cells and form the liver bud [5]. Proliferation of hepatoblasts is known to be regulated by various extracellular signals, such as hepatocyte growth factor (HGF) [6], transforming growth factor β (TGF β) [7] and Wnt [8]. Around E10, hematopoietic cells immigrate into the fetal liver from the aorta–gonad–mesonephros region and the placenta, and expand their population tremendously in the microenvironment provided by the fetal liver till birth. During this period of time, hematopoietic cells enhance differentiation of hepatoblasts into hepatocytes by producing cytokines [9]. As hematopoiesis switches from the fetal liver to the bone marrow, liver organogenesis progresses to become a center for metabolism. In this process, hepatocyte differentiation is enhanced by oncostatin M (OSM) [10], HGF [11] and extracellular matrices (ECMs) [12].

The *Hedgehog* (*Hh*) gene, initially identified from a genetic screen in *Drosophila melanogaster*, encodes a secreted factor with multiple biological activities, ranging from regulation of cell proliferation and differentiation to pattern formation as a morphogen [13,14]. In mammals there are three Hh orthologs, namely Sonic hedgehog (Shh), Indian hedgehog (Ihh) and Desert hedgehog (Dhh). Hh is synthesized as a ~45 kDa precursor protein and cleaved to generate the biologically active amino-terminal Hh signaling domain (HhN). In the absence of Hh ligands, the Hh receptor Patched (Ptc) inhibits the activity of another membrane-bound protein, Smoothed (Smo) [15,16]. Binding of Hh to Ptc inactivates its inhibitory effect toward Smo, thereby releasing Smo activity to emanate downstream signals. The Glioblastoma (Gli) family of transcription factors constitutes the key components of the pathway activated by Smo, which then enters the nucleus and stimulates transcription of multiple target genes, including *Gli1* and *Ptc1*, to control multiple cellular functions. Through its range of biological activities, Hh signaling plays crucial roles in development of many fetal tissues and organs, and also in homeostasis of adult tissues by maintaining tissue stem/progenitor cells [17,18]. Notably, aberrant activation of Hh signaling is often associated with formation of multiple types of solid tumors, including hepatocellular carcinoma, cholangiocarcinoma and hepatoblastoma in the liver [19–21].

With regard to the liver, many studies to date have already implicated Hh signaling in adults under several pathological conditions. For instance, HSCs, bile ductular cells and fibroblastic cells express Hh related genes to regulate proliferation and viability of these cells when the liver is damaged [20,22–24]. On the other hand, whether and how the signaling is involved in developmental process of the organ remains little investigated. While *Shh* is initially expressed broadly in the ventral foregut endoderm in mouse embryos around E8.5, its expression becomes lost in the hepatic endoderm at E9.0–9.5, the region which subsequently forms the liver bud [25–28]. Indeed, a study using *Hex* knockout mice has implied that the *Shh* expression needs to be shut off at this stage for the liver bud formation and hepatoblast development to proceed normally [28]. Although expression of Shh as well as other Hh ligands at later stages during liver development has not been directly addressed, it is of interest to note that, in the *Ptc-lacZ* knock-in mice that express lacZ under the control of the Hh-responsive *Ptc* promoter activity, some lacZ signals can be detected in the fetal liver at E11.5 [29], indicating possible activation and functional implication of Hh signaling therein.

In this paper, we address a possible functional involvement of Hh signaling in mouse fetal liver organogenesis. Our results have demonstrated temporally restricted and cell type-specific patterns of ligand expression and pathway activation, and their functional impact on hepatoblast regulation.

Materials and methods

Mice

C57BL/6 mice were purchased from Nihon SLC (Shizuoka, Japan). All procedures were performed according to the guideline set by the institutional animal care and use committee of the University of Tokyo.

Isolation of *Dlk*⁺ fetal liver cells by magnetic cell sorter (MACS)

Mouse *Dlk*⁺ fetal liver cells were obtained according to the methods by Kamiya et al. [10] and Tanimizu et al. [30]. Briefly, mouse fetal livers were dissociated with liver digestion medium (Gibco) followed by hemolysis with a hypotonic buffer. Dissociated cells were incubated with biotinylated anti-*Dlk* antibody [31]. After a wash, cells were incubated with streptavidin-labeled microbeads (Miltenyi Biotec) and isolated by an automatic magnetic cell sorter (AutoMACS; Miltenyi Biotec).

Fluorescence-activated cell sorting (FACS)

E11.5 mouse fetal livers were dissociated according to the method described above. Dissociated cells were incubated with antibodies and sorted by the FACS-Vantage system (Becton Dickinson Biosciences). Anti-p75NTR antibody and anti-mouse *Dlk* antibody were generated by Suzuki et al. [31], and anti-Stab2 antibody by Nonaka et al. [32]. Signals were fluoresced using Alexa Fluor 488-conjugated anti-rat IgG (Invitrogen) and Alexa Fluor 647-conjugated anti-rat IgG (Invitrogen). Phycoerythrin (PE)-conjugated anti-CD45 (553081), fluorescein isothiocyanate (FITC)-conjugated anti-Ter119 (557915) and PE-conjugated anti-Ter119 (553673) were purchased from BD biosciences. Dead cells were stained with propidium iodide (PI).

Real-time RT-PCR

Total RNA samples were isolated with TRIzol reagent (Invitrogen). Reverse transcription to cDNA templates was performed using random primers and Superscript III reverse transcriptase (Invitrogen). Real-time RT-PCR experiments were conducted by use of a LightCycler (Roche Diagnostics) and SYBR Premix Ex Taq (Takara-bio). Primers for *Glyceraldehyde-3-phosphate dehydrogenase* (*Gapdh*) were used as the normalization control. The pairs of primers used were as follows: 5'-TGAACGGGAAGTCACTGG-3' (*Gapdh* primer, sense), 5'-TCCACCACCTGTTGCTGTA-3' (*Gapdh* primer, antisense), 5'-CCAAGCCAACITTTATGTCAGGG-3' (*Gli1* primer, sense), 5'-AGCCCGCTTCTTTGTTAATTTGA-3' (*Gli1* primer, antisense), 5'-CCAATTACAACCCGACATC-3' (*Shh* primer, sense), 5'-GCATTTAACTTGCTTTGACCT-3' (*Shh* primer, antisense), 5'-TGCATTGCTCTGTCAGTCTG-3' (*Ihh* primer, sense), 5'-GCTCCCGTCTCTAGGC-3' (*Ihh* primer, antisense), 5'-CATCTGGAGCCATGTACCTT-3' (*Tat* primer, sense),

5'-TCCAGCATCATCACCTCG-3' (*Tat* primer, antisense), 5'-GGCTGGCTACCAAGAGTCTG-3' (*Cps1* primer, sense), 5'-TGTATCCACTCCAT-AAATTGCAG-3' (*Cps1* primer, antisense).

Immunocytochemistry

Dlk⁺ and Dlk⁻ fetal liver cells isolated using MACS were mounted on glass slides by cytospinning and fixed using 4% paraformaldehyde (PFA). Cultured Dlk⁺ cells were fixed using 4% PFA at day 7. Fixed cells were treated with L.A.B. solution (Polysciences) at room temperature for 10 min for antigen retrieval, and then incubated with anti-Dlk antibody, anti-Gli1 antibody (Chemicon) and anti-Ki67 antibody (BD Pharmingen). Signals were visualized using Alexa Fluor 488-conjugated anti-rat IgG (Invitrogen), Alexa Fluor 555-conjugated anti-rabbit IgG (Invitrogen) and Alexa Fluor 488-conjugated anti-mouse IgG (Invitrogen), respectively, and observed under a fluorescence microscope. Nuclei were counterstained with Hoechst33342 (Sigma). In the cultured samples, Ki67⁺ cells and nuclei were manually counted under a fluorescence microscope in three independent fields.

Culture of Dlk⁺ cells

Dlk⁺ fetal liver cells were resuspended in Dulbecco's Modified Eagle Medium (DMEM; Gibco) containing 10% fetal bovine serum (FBS; JRH), 2 mM L-glutamine (Gibco), 1× nonessential amino acid solution (Gibco), 1× insulin/transferrin/selenium (ITS; Gibco), 50 µg/ml of gentamicin, 10⁻⁷ M of dexamethasone (Dex; Sigma), and 10 ng/ml of murine OSM (R&D systems). Cells were plated at 2.1 × 10⁴ cells/cm² on gelatin-coated dishes. Recombinant N-terminus of Shh (ShhN; R&D systems), 2 µM purmorphamine (Wako Pure Chemical Industries), 0.1% of ethanol (EtOH) and 5 µM of cyclophamide (Wako Pure Chemical Industries) were added where indicated. ShhN was used at a final concentration of 0.5 µg/ml except for Supplementary Fig. 2. Culture media were replaced every 2 days.

Quantification of cell proliferation

Cell proliferation was quantified by using the cell proliferation reagent WST-1 (Roche Diagnostics) according to the manufacturer's instructions. Dlk⁺ cells were cultured in 96-well microplates at the same density as above with 100 µl/well of media. Culture media were replaced every 2 days. After 7 days of culture, 10 µl of WST-1 was added to each well and the cells were further incubated for about 1 h. The optical absorbance of the samples was measured at 450 nm.

Colony formation assay

Dlk⁺ cells from E11.5 fetal livers were resuspended in DMEM/F12 (Gibco) containing 10% FBS, 10 mM nicotinamide, 5 mM L-glutamine, 1× ITS, 10 ng/ml of murine epidermal growth factor (Peprotech), 10 ng/ml of human HGF (Peprotech), 50 µg/ml of gentamicin, and 10⁻⁷ M Dex. Five hundred cells per well were plated on collagen-coated 6-well plates. At day 3, equal amount of fresh media was added. After cultivation for 6 days, cells were fixed with 4% PFA and dried up. Cell numbers and colony sizes were manually counted under microscopic observation.

Measurement of ammonia concentrations

Ammonia concentration was measured as previously described [33]. Briefly, culture media were replaced with fresh media containing 2 mM of NH₄Cl at day 10. Cells were further incubated for 48 h, and the culture media were subjected to analyses at various time points by the modified indophenol method using Ammonia-test Wako (Wako Pure Chemical Industries).

Statistics

Data are presented as the mean plus standard error (SE). Statistical significance was assessed by means of a Student's *T* test. A *P* value less than 0.05 was considered statistically significant.

Results

Hh signaling is transiently activated in E11.5 hepatoblasts

In a previous study using the *Ptc-LacZ* mice, where Hh signal activation can be monitored *in situ* by the LacZ reporter expression, some positive signals were observed in the liver of E11.5 mouse embryos [29], indicating that Hh signaling may be operating in the fetal liver at this stage. However, the nature of the Hh-responsive cell(s) as well as activation of the signaling at other developmental stages is not precisely clarified. Thus, we set out to investigate expression of *Hh* genes during the course of fetal liver development by real-time RT-PCR analyses using whole liver samples. As shown in Fig. 1A, expression of *Shh* was evident at E11.5, and decreased gradually as the development proceeded. Accordingly, *Gli1*, a direct transcriptional target of Hh signaling, showed a similar expression pattern. By contrast, expression of *Ihh* increased toward the birth, and then dramatically decreased thereafter. *Dhh* was not detected in these samples (data not shown), consistent with the notion that it is mainly expressed in the testis and Schwann cells [34,35].

As Hh signaling has been implicated in maintenance of hepatic stem/progenitor cells in the adult liver [29], we speculated that it might also play a role in embryonic hepatoblast regulation. Alternatively, the liver serves as one of the hematopoietic organs at fetal stages and Hh signaling has been shown to promote embryonic hematopoiesis and vasculogenesis in pre- or early-gastrulation-stage epiblasts [36], raising a possibility that the signal may target blood cells and/or endothelial cells in the fetal liver. To discriminate these possibilities, hepatoblasts were isolated from the fetal liver by auto-MACS using the antibody against Dlk, a well-established surface marker for hepatoblasts [30], and subjected to RT-PCR analyses. The purities of the isolated Dlk⁺ and Dlk⁻ cell fractions were each more than 95% (data not shown). As shown in Fig. 1B, *Gli1* was more strongly induced in Dlk⁺ hepatoblasts than in Dlk⁻ cells, while *Shh* was produced in both cell fractions of the E11.5 and E12.5 livers. These results indicate the transient nature of Hh signal activation during fetal liver development, which primarily targets hepatoblasts at E11.5.

Expression of the ligand *Shh* was observed in E11.5 Dlk⁻ cells, which is a heterogenous population composed of many types of cells. In order to characterize the Shh-producing cells, we isolated various cell fractions from the E11.5 fetal liver by FACS and analyzed the *Shh* expression (Fig. 1C). We sorted the fetal liver cells

based on the expression of CD45, Ter119, Stab2 and p75NTR, the surface markers for hematopoietic cells, erythroid cells, hepatic sinusoidal endothelial cells [32] and precursors of HSC/portal fibroblasts [31], respectively, in combination with Dlk. As a result, *Shh* was identified to be produced mainly in Dlk⁺ hepatoblasts and the CD45⁻/Dlk⁻ fraction, while little or no expression was observed in hepatic sinusoidal endothelial cells, hematopoietic cells or precursors for HSC. The CD45⁻/Dlk⁻ fraction, though not rigorously characterized at the moment, is supposed to contain mesenchymal cells.

To further examine the character of the Hh-responsive cells, we next carried out immunocytochemistry with E11.5 fetal liver cells (Fig. 2). Freshly isolated Dlk⁺ hepatoblasts were mostly Gli1⁺ (Figs. 2A–D; higher magnification images shown in Supplementary Fig. 1) while Dlk⁻ cells were composed of both Gli1⁺ and Gli1⁻ cells (Figs. 2I–L), consistent with the results obtained by the RT-PCR analyses (Fig. 1B). Since Hh signaling is known to enhance cell proliferation in many types of cells and tissues, we also stained the cells with a marker for cell proliferation activity. In both Dlk⁺ and Dlk⁻ fractions, Ki67⁺ proliferating cells were observed among

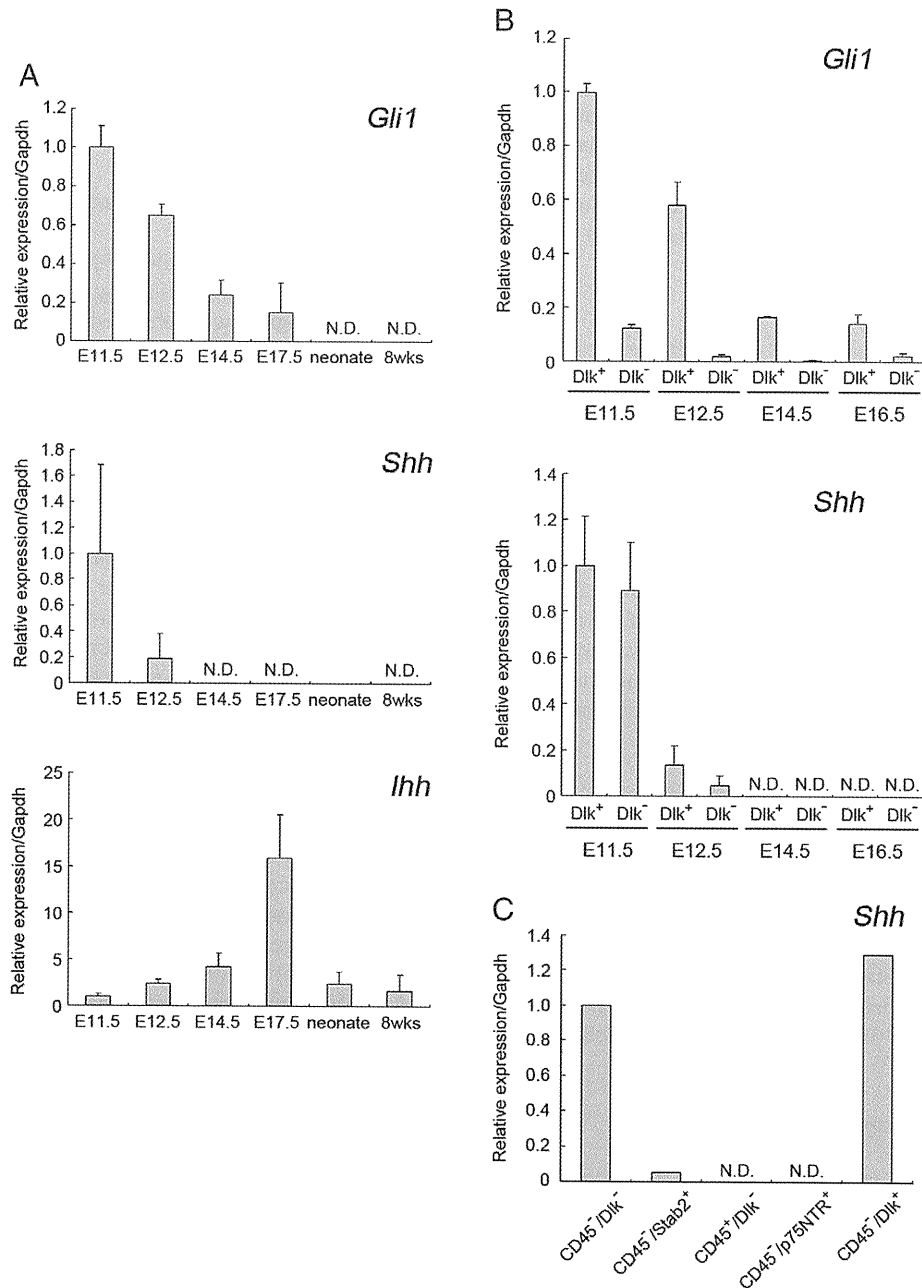


Fig. 1 – Expression of *Gli1*, *Shh* and *Ihh* during the liver development. Relative expression of *Gli1*, *Shh* and *Ihh* in whole liver samples (A; $N = 4$), MACS-sorted samples (B; $N = 4$) and FACS-sorted samples of the E11.5 fetal liver (C) were analyzed by real-time PCR. Expression levels were normalized using that of *Gapdh*. PI-stained dead cells and Ter119⁺ erythroid cells were eliminated from all samples in (C). N.D., not detected.

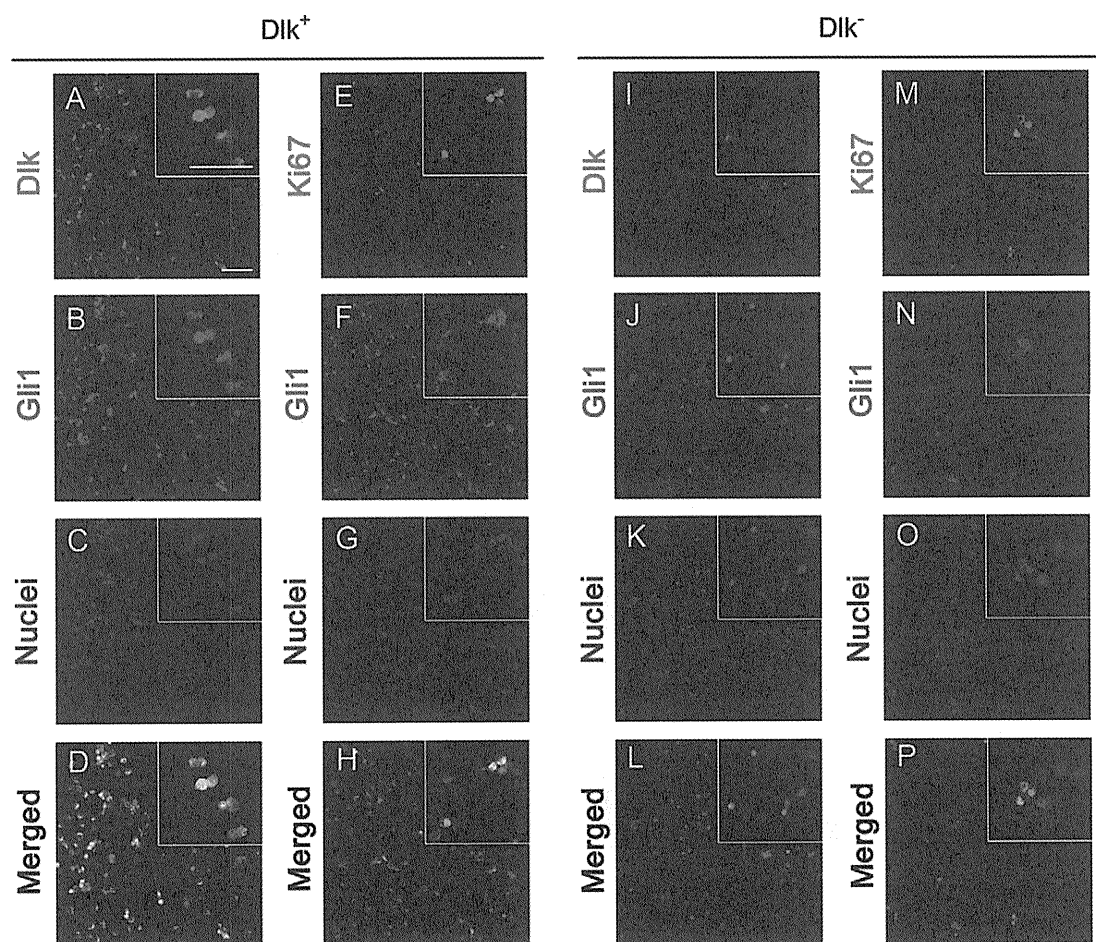


Fig. 2 – Immunocytochemistry of E11.5 Dlk⁺ and Dlk⁻ cells. Dlk⁺ cells (A–H) and Dlk⁻ cells (I–P) of the E11.5 fetal liver were sorted using MACS and mounted on glass slides by cytospinning. (A–D and I–L) Cells were co-stained with anti-Dlk antibody (A and I; visualized by green) and anti-Gli1 antibody (B and J; visualized by red), together with Hoechst33342 to detect the nuclei (C and K; visualized by blue). Merged images for A–C and I–K are shown in panels D and L, respectively. (E–H and M–P) Cells were co-stained with anti-Ki67 antibody (E and M; visualized by green) and anti-Gli1 antibody (F and N; visualized by red), together with Hoechst33342 to detect the nuclei (G and O; visualized by blue). Merged images for E–G and M–O are shown in panels H and P, respectively. A magnified view is indicated as the inset in each panel. All the panels and insets are each shown at the same magnifications, and the white scale bars in panel A represent 100 μ m.

the Gli1⁺ cells (Figs. 2E–H, M–P). Notably, all the Ki67⁺ cells were also positive for Gli1. These results suggest a possible relationship between the Hh signal activation and proliferation in fetal liver cells.

Hh signaling stimulates proliferation of hepatoblasts

In order to investigate the direct effect of Hh signal activation on hepatoblast proliferation, we cultured Dlk⁺ hepatoblasts from the E11.5 fetal liver in the presence of the biologically active form of recombinant Shh (N-terminal fragment of Shh; ShhN). Since Hh signaling was found to be activated at E11.5 but weakened or disappeared at E14.5, we also cultured the hepatoblasts from the latter stage for comparison. In the bulk culture of E11.5 Dlk⁺ cells, proliferation was significantly promoted by the addition of ShhN in a dose-dependent manner, as well as by a small molecule agonist of Hh signaling, purmorphamine [37] (Fig. 3A and Supplementary

Fig. 2). These effects were cancelled in the presence of cyclopamine, a well-established chemical inhibitor of Hh signaling [38]. Proliferation was not affected by cyclopamine alone or EtOH, which was used as the solvent of cyclopamine. On the other hand, neither activation nor inhibition of Hh signaling had any effect on proliferation of E14.5 Dlk⁺ cells (Fig. 3A). Immunocytochemistry of the cultured E11.5 Dlk⁺ cells demonstrated that activation of Hh signaling increased the proportion of Ki67⁺ cells, which were all co-stained with Gli1 (Fig. 3B; higher magnification images shown in Supplementary Fig. 3). This effect was also cancelled by cyclopamine, but not changed by EtOH (data not shown).

To analyze the effect of Hh signaling on the character of hepatoblasts as immature stem/progenitor cells, we next carried out colony formation assays at low cell density, where the cells with stem/progenitor cell activities are considered to be capable of forming relatively large colonies [39]. When Dlk⁺ cells from the E11.5 fetal liver were cultured under this condition, total cell

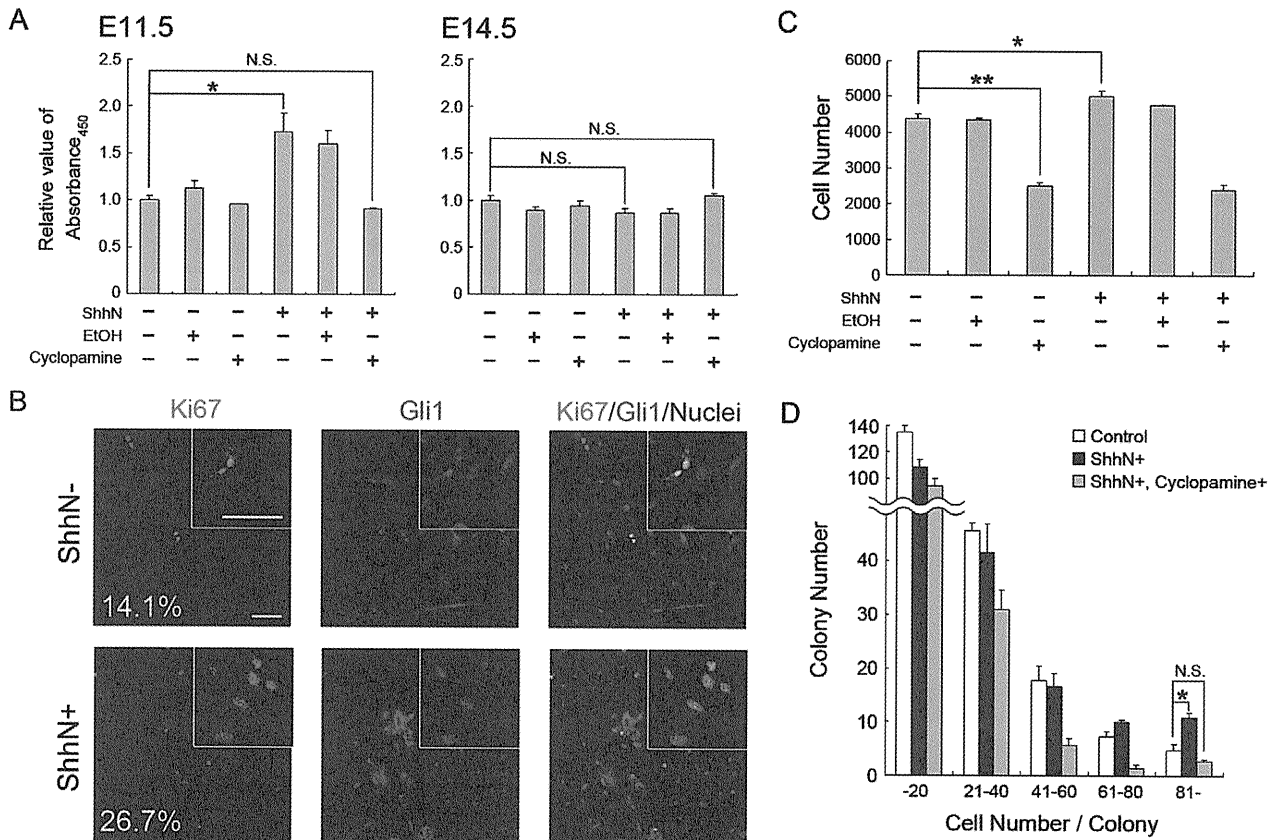


Fig. 3 – Effect of Hh signaling on proliferation of Dlk^+ cells. (A) E11.5 and E14.5 Dlk^+ cells were cultured for 7 days, and cell proliferation was measured by WST-1 assay. $N = 3$. (B) E11.5 Dlk^+ cells were cultured for 7 days and stained with anti-Ki67 antibody (green) and anti-Gli1 antibody (red). Merged images are shown in the right column, where nuclear staining by Hoechst33342 is also included (blue). White numbers show percentages of Ki67⁺ nuclei/total nuclei. White scale bars represent 100 μm . (C) Total cell numbers of the cultured Dlk^+ cells after 6 days under the low cell density culture condition. $N = 3$. (D) The colony size and frequency in (C) are depicted. The results for the cultures in the absence (control) or presence of ShhN, and with cyclopamine in addition to ShhN are represented. $N = 3$. N.S., not significant ($P > 0.1$); * $P < 0.05$; ** $P < 0.01$.

numbers were increased by ShhN application while decreased by cyclopamine (Fig. 3C). Intriguingly, the number of the large colonies (defined as those comprising more than 80 cells in this study) was significantly increased by activation of Hh signaling (Fig. 3D). Taken together, these results indicate that Hh signaling can enhance proliferation of hepatoblasts.

Hh signaling has an inhibitory effect on hepatic differentiation of hepatoblasts

We previously demonstrated that differentiation of hepatoblasts into hepatocytes can be faithfully recapitulated in *in vitro* culture of E14.5 Dlk^+ cells [30], and have noticed that the same differentiation-inducing protocol can also be applicable to E11.5 Dlk^+ cells, as judged by induction of various marker genes for differentiated hepatocytes as well as by several functional assessments (see below, and data not shown). Using this *in vitro* culture system, we aimed to analyze the effect of Hh signaling on hepatocyte differentiation of hepatoblasts. After 10 days of culture, E11.5 Dlk^+ cells underwent differentiation into hepatocytes and expressed marker genes for differentiated hepatocytes, *tyrosine*

aminotransferase (Tat) and *carbamoyl-phosphate synthetase 1 (Cps1)* (Fig. 4A). Hh signaling activity in the cultured cells as monitored by *Gli1* expression declined after induction of the differentiation, consistently with its transition *in vivo* (Fig. 1A). We then administrated ShhN to these cultures to forcibly activate Hh signaling during the course of differentiation, which resulted in dramatic decrease in the expression of *Tat* and *Cps1* (Fig. 4A). A similar effect of ShhN was also observed in the culture of E14.5 Dlk^+ cells (Fig. 4B). Addition of cyclopamine diminished the ectopic Hh signal activation and its inhibitory effect on hepatocyte differentiation. Notably, this resulted in not only restored but rather augmented levels of *Tat* and *Cps1* expression (Fig. 4C). Cyclopamine treatment in the absence of the exogenous ShhN also gave a similar enhancing effect, suggesting that inhibition of the endogenous, autocrine Hh signaling *in vitro* may potentiate hepatic differentiation of hepatoblasts (Fig. 4C).

To further confirm the inhibitory effect of Hh signaling on hepatocyte differentiation, we also tested a metabolic function of the cultured Dlk^+ cells. As *Cps1* is a key enzyme of the urea cycle and synthesizes carbamoyl phosphate from ammonia [40], we chose to measure ammonia clearance activity of the differentiated

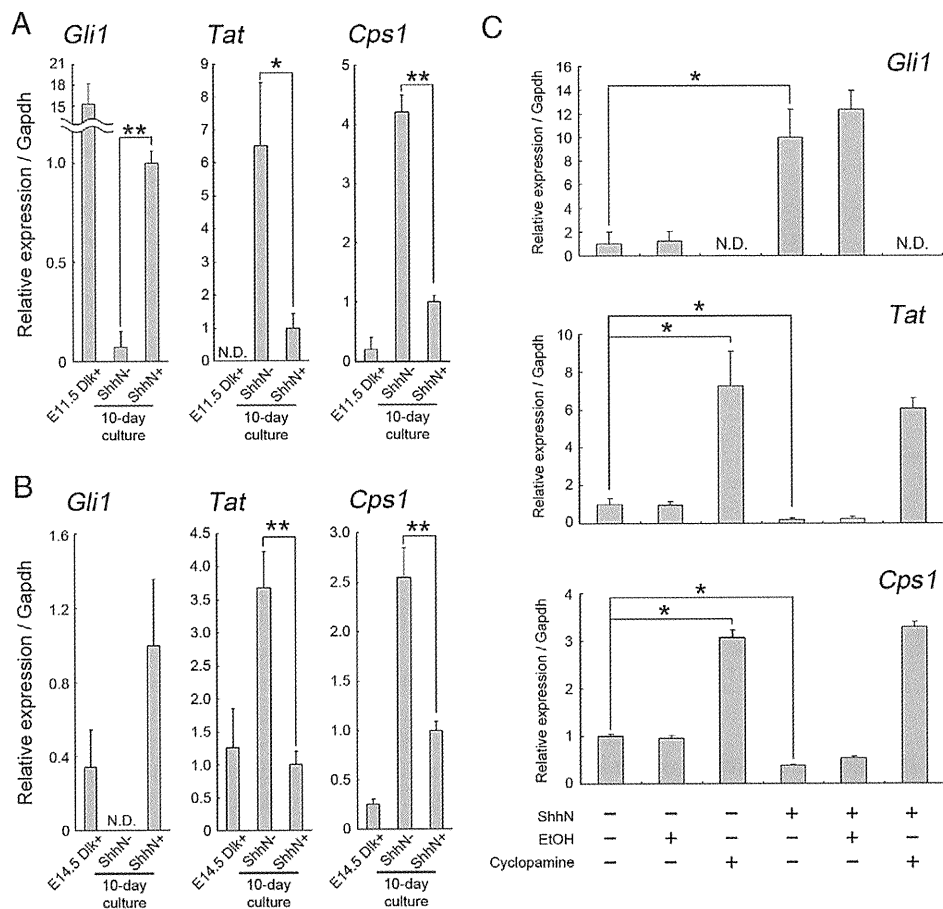


Fig. 4 – Effect of Hh signaling on expression of hepatocyte marker genes. E11.5 *Dlk*⁺ cells (A and C) and E14.5 *Dlk*⁺ cells (B) were cultured for 10 days, and relative expression of *Gli1*, *Tat* and *Cps1* was measured using real-time RT-PCR. Expression levels were normalized using that of *Gapdh*. *N* = 4. N.D. not detected; **P* < 0.05; *P* < 0.01.**

hepatocytes. In both E11.5 and E14.5 hepatoblast cultures, ammonia concentration in the medium was declined by the differentiated cells, and the clearance kinetics was delayed in those cells that were differentiated in the presence of ShhN (Fig. 5, and data not shown). This inhibitory effect of ShhN was cancelled by addition of cyclopamine during the differentiation process. In contrast to the case with the marker gene expression, however, cyclopamine treatment did not further enhance the clearance activity beyond the basal level in this assay. Nonetheless, these results together indicate that Hh signaling inhibits functional differentiation to hepatocytes of embryonic hepatoblasts.

Discussion

The progression of organogenesis relies on multiple modes of interaction between tissues and/or cells and thus is tightly and precisely regulated by coordinated actions of various extracellular signals, such as those mediated by cell adhesion molecules, ECMs, as well as growth factors. With respect to the liver development, critical roles of FGF, BMP, Wnt, and several other growth factors or cytokines have already been well appreciated, yet the entire picture of the regulatory signals throughout the whole process still remains to be elucidated. In

this study, we have added Shh to the list of those signals, which plays an important role in modulating proliferation and differentiation of hepatoblasts.

By using real-time RT-PCR and immunostaining analyses, we showed that Hh signaling was transiently activated in the fetal liver at E11.5, according to the appearance and proliferation of hepatoblasts (Figs. 1 and 2). Indeed, the signal activation was observed primarily in *Dlk*⁺ hepatoblasts, while expression of the corresponding ligand Shh was achieved by both *Dlk*⁺ hepatoblasts and *Dlk*⁻ cells (Figs. 1B and C). Thus, hepatoblasts at this stage are targeted by Hh signaling in both autocrine and paracrine fashions, similar to the situation observed with adult colon cells [41].

In the E11.5 fetal liver, a weaker yet significant level of *Gli1* expression was also detected in *Dlk*⁻ cells, indicating activation and a possible function of Hh signaling therein. Our preliminary experiments employing the FACS-sorted cell fractions suggest that the signal is activated in the p75NTR⁺ precursors of HSC/portal fibroblast (Y. Hirose, T. Itoh, and A. Miyama, unpublished observation). In the adult liver, Shh is known to function as an autocrine activation and viability factor for HSC [22,24]. Since *Shh* expression is absent in fetal p75NTR⁺ cells (Fig. 1C), it is not likely an autocrine but rather a paracrine factor for HSCs in the fetal liver and may play a role in regulating their development, which needs to be addressed in future studies.

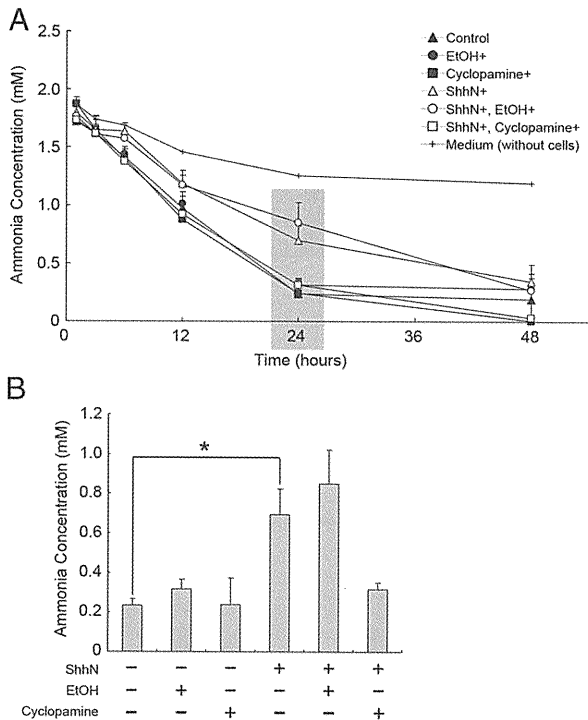


Fig. 5 – Effect of Hh signaling on acquisition of the ammonia clearance ability. After the culture of E11.5 Dlk⁺ cells for 10 days in the presence or absence of ShhN, cyclopamine, and EtOH (vehicle for cyclopamine), the culture media were replaced with the fresh medium containing 2 mM of NH₃. Ammonia concentration in the media was measured at the indicated time points. The values for ammonia concentration at 24 h were extracted in (B). *N* = 3. **P* < 0.05.

In addition to the *Shh* expression at early to mid stages of liver organogenesis, we also observed increasing expression of *Ihh* in the fetal liver, peaking at around the perinatal stage. In the case of this ligand, however, concomitant elevation of *Gli1* expression was not observed (Fig. 1A). Notably, a previous study using C3H10T1/2 cells and some embryonic tissues has reported that the effect of *Ihh* is weaker than that of *Shh* [42]. In fact, we also applied the N-terminal fragment of *Ihh* to the *in vitro* culture of E14.5 Dlk⁺ hepatoblasts to activate Hh signaling, but the effect was weaker than that of *ShhN* (data not shown). Interestingly, *Ihh* has been shown to be expressed in around ductal plates, the precursor for bile ducts, in fetal human livers [29] as well as in bile ductular cells in injured adult livers [20,23]. It is thus of considerable possibility that *Ihh* signaling may be involved in bile duct formation and, as such, local activation of the downstream signaling in/around those limited structures is not strong enough to be detected in our analysis using the whole liver samples.

By adapting our previously established culture protocol of E14.5 Dlk⁺ hepatoblasts, where the cells can proliferate and then undergo hepatocyte differentiation with marker gene expression and functional maturation [30,43], we have approached *in vitro* the functional relevance of the *Shh* expression and signal activation in E11.5 Dlk⁺ hepatoblasts (Figs. 3–5). In the culture of E11.5 Dlk⁺ cells, stimulation with *ShhN* promoted their proliferation (Figs. 3A and B). In accord with this *in vitro* result, some *Gli1*⁺ Hh-

responsive cells were also positive for Ki67 in the freshly isolated Dlk⁺ fraction (Figs. 2A–F). Together with the observation in the colony formation assays at lower cell density, where total cell numbers were increased by *ShhN* stimulation while decreased by cyclopamine (Fig. 3C), our results indicate that Hh signaling activates hepatoblast proliferation in the E11.5 fetal liver. Noticeably, cyclopamine treatment alone (i.e., in the absence of exogenous *Shh* stimulation) significantly affected proliferation of E11.5 Dlk⁺ cells in the colony formation assay (Fig. 3C), but failed to do so under the differentiation-inducing culture condition (Fig. 3A). The latter condition is considered to recapitulate, at least in part, the developmental process *in vivo*, where Dlk⁺ hepatoblasts are prone to lose their *Shh* expression as the differentiation progresses (Figs. 1A and B). Under this condition, the autocrine Hh signaling in hepatoblasts are supposed to be attenuated and hence the inhibitory effect of cyclopamine could not be detected. In contrast, the cells are not actively induced to differentiate in the colony formation assay and are thus considered to better maintain their original character with autocrine Hh signaling, leading to the observed inhibitory effect of cyclopamine on their proliferation.

It should be noted that, in contrast to E11.5 hepatoblasts where *ShhN* showed a growth-stimulatory effect, in the E14.5 cells neither activation nor inhibition of Hh signaling had any effect on proliferation even though application of *ShhN* did seem to activate the signaling therein to a considerable extent (Figs. 3A and 4B). Thus, intrinsic cellular program to respond to Hh signal activation is likely to be changed in hepatoblasts during this period of development, although the underlying molecular basis remains to be elucidated. Considering the fact that the ligand expression in the fetal liver rapidly declines during the same period, this may constitute another level of regulatory mechanism that ensures the progression of the hepatoblast development from proliferating to differentiating stages. It is also interesting to note that, during the culture of E11.5 Dlk⁺ cells under the differentiation-inducing condition, the cells showed a tendency to gradually lose their *Gli1* expression even in the continuous presence of exogenous *ShhN*, resulting after 10 days of culture in a 15-fold lower level of the expression compared to the initial level when the culture was started (Fig. 4A, and data not shown). As continuous Hh signal activation was found to be deleterious for differentiation of hepatoblasts to hepatocytes, it is tempting to speculate that hepatoblasts may also be programmed so that they lose their ability to receive and/or transduce Hh signaling during the course of differentiation, which may help further establish a robust system to warrant proper development of hepatocytes.

As mentioned above, *Ihh* expression has been detected in around ductal plates and bile ducts in fetal and adult livers, respectively. The periportal region containing these structures have been considered as putative hepatic stem/progenitor cell compartments, and a previous study has implicated the role of *Ihh* signal activation there in maintaining resident hepatic stem/progenitor cells [29]. As the formation of ductal plates starts at around E15.5 in mouse embryos [144], it remained unclear whether and how *Ihh* or any other signals were involved in stem/progenitor cell regulation in the fetal liver at earlier stages. In our colony formation assays using E11.5 hepatoblasts, the numbers of relatively large colonies, which are assumed to derive from a more immature stem/progenitor cell population with higher proliferative potentials, were significantly augmented by Hh signal

activation (Fig. 3D). These findings together illuminate the unified activity, as well as the diversified modes of action, of Hh signaling in regulating hepatic stem/progenitor cells. That is, at E11.5 Shh is likely involved in transient expansion of hepatoblasts with their immature phenotype being maintained, while at the later stages Ihh signaling around the ductal structures in turn mediates local maintenance of resident hepatic stem/progenitor cells.

During the course of fetal liver development, Shh expression is initially observed at the very early stage, around E8.5, in the ventral foregut endoderm, and then lost at E9.0–9.5 in the liver bud primordium [25–27]. Based on the results obtained using the *Hex* mutant mice, it is assumed that ectopic expression of *Shh* at the latter stage is deleterious to the proper development of the liver bud, due presumably to its inhibitory activity toward the epithelial morphological transition of the hepatic endodermal cells [28]. Together with these previous findings, our present study has established a hitherto unidentified, highly dynamic feature of the Shh signal activation, as well as its multiple effects on hepatoblast regulation, during the liver organogenesis. Notably, it has been shown that Wnt signaling is initially inhibitory to liver induction at early stages, but rather enhances liver bud growth and differentiation later in development [45]. In a similar way to Wnt signaling, Hh signaling has several distinct and potentially contradictory roles at different stages of embryonic livers, so that expression of the ligand and activation of the signaling pathway need to be strictly regulated for the liver development to proceed normally. It has been reported that the formation of the liver bud occurs in both *Shh*^{-/-} and *Gli2*^{-/-}*Gli3*^{-/-} mouse embryos at E9.5, although the size is smaller than normal in the latter case [46,47]. Future studies using these kinds of *in vivo* models, with a particular focus on the hepatoblast behavior and hepatic differentiation, should pave the way to fully understand the role that Hh signaling plays during the liver organogenesis.

Acknowledgments

We thank Drs. M. Tanaka and N. Tanimizu for valuable suggestions and technical assistance, and the members of the Miyajima laboratory for helpful discussions.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.yexcr.2009.06.018.

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