Epiplakin1 is expressed in the cholangiocyte lineage cells in normal liver and adult progenitor cells in injured liver.

Akira Matsuo[1, 2], Tetsu Yoshida [1,3], Takanori Yasukawa[1], Rika Miki[1, 2], Kazuhiko Kume[1] and Shoen Kume[1, 2] [1]Department of Stem Cell Biology, Institute of Molecular Embryology and Genetics, Kumamoto University, Honjo 2-2-1, Kumamoto 860-0811, Japan; [2]Global COE "Cell Fate Regulation Research and Education Unit", Kumamoto University, Honjo 2-2-1, Kumamoto 860-0811, Japan; [3]Present address: Department of Ophthalmology Keio University School of Medicine, Tokyo, Japan.

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[§]Corresponding author: Shoen Kume, Ph.D., Fax: +81-96-373-6807
Email: skume@kumamoto-u.ac.jp Division of Stem Cell
Biology Institute of Molecular Embryology and Genetics Kumamoto University
Honjo 2-2-1, Kumamoto 860-0811, Japan

ABSTRACT

We have previously identified Epiplakin1 (Eppk1) as a gene expressed in pancreatic progenitor cells. Here we studied the expression of Eppk1 in developing and regenerating livers in mice. Eppk1 is initially expressed in the early bipotential hepatoblasts and is later confined to the cholangiocytes. After birth, Eppk1 is expressed in the bile duct. In the livers of mice fed with a choline-deficient ethionine-supplemented (CDE) diet, Eppk1-positive cells dramatically increase in number. The Eppk1-positive cells express A6, thereby indicating that they are hepatic progenitor cells. Other cholangiocyte markers, such as Cytokeratins, E-cadherin, Osp and Sox9, are also co-expressed in the hepatic progenitor cells. Some of the Eppk1-positive cells express PCNA, a proliferation marker, thereby suggesting their identities as transient amplifying cells. In conclusion, we have shown that Eppk1 serves as a useful marker for detecting the hepatic progenitor population in the developing and adult liver. The use of Eppk1 as a marker will facilitate studies of mouse hepatic progenitor cells.

INTRODUCTION

The liver contains bipotential progenitor cells, which give rise to hepatocytes and cholangiocytes during development and during regeneration after the injury in the adult liver (Zaret and Grompe, 2008). Liver organogenesis starts in the foregut endoderm at embryonic day 8.5 (E8.5) (Zaret and Grompe, 2008) . At around E13.5, the hepatoblasts differentiate into the hepatocytes or cholangiocytes. Most hepatoblasts in the liver parenchyme differentiate into hepatocytes, while those located close to the portal mesenchyme give rise to the cholangiocytes (Lemaigre and Zaret, 2004; Lemaigre, 2003).

In normal adult liver, most hepatocytes and cholangiocytes are quiescent. However, hepatocytes have a remarkable potential to proliferate. In response to an acute liver injury or a loss of liver mass, proliferation of the hepatocytes takes place to restore homeostasis. However, when this proliferative response of mature hepatocytes is impaired, for example, in severely injured liver, facultative hepatic progenitors become active. The expansion of hepatic progenitors is considered to come from a niche near the terminal bile ducts, called the canal of Hering. These facultative progenitor cells are classically termed “oval cells”. Oval cells function as transit amplifying progenitor cells because their emergence is strongly associated with impaired mature hepatocyte proliferation. This association suggests that they represent a reserve hepatic progenitor population. Repopulation studies in animal models suggest that these progenitors have bipotential differentiation capabilities and differentiate into hepatocytes and cholangiocytes (Duncan et al., 2009; Rountree et al., 2007; Santoni-Rugiu et al., 2005). Mice treated with a choline-deficient ethionine-supplemented (CDE) diet have been widely used as a chronic liver injury model, in which the hepatic progenitor cells are induced (Akhurst et al., 2001).

To understand the nature of hepatic progenitor cells, perspective isolation of hepatic progenitors by cell sorting using several cell surface markers is described. A population of cells in fetal mice was identified to have proliferative capacity and gave rise to the hepatocytes and cholangiocytes (Suzuki et al., 2000; Suzuki et al., 2002). However, cells that express the same markers isolated from the adult liver show limited proliferative capacity (Rountree et al., 2007; Suzuki et al., 2008). Other cell surface molecule, such as TROP2, which is expressed exclusively in oval cells but not in normal cholangiocytes and is therefore useful for the perspective identification of oval cells, is also reported (Okabe et al., 2009).

The plakin family of proteins is known to function in interconnecting cytoskeletal filaments and anchoring them at plasma membrane-associated adhesive junction. Epiplakin1 (Eppk1) is a member of the plakin family of proteins. We previously identified Eppk1 as one of the genes, which was increased during the differentiation of ES cells into Pdx1/GFP++ pancreatic lineage cells (Yoshida et al., 2009). We revealed that Eppk1 is a useful marker for detecting pancreatic progenitor cells in the developing and regenerating pancreas (Yoshida et al., 2008). We also found that Eppk1 is expressed in the gut endoderm during early embryogenesis. In addition, previous reports, using Northern blot analysis and immunostaining, indicate that Eppk1 is expressed in the adult liver (Fujiwara et al., 2001; Spazierer et al., 2003). These results suggest that Eppk1 may serve as a marker for hepatic progenitor cells in addition to pancreatic progenitor cells. Here, we examined the expression of Eppk1 in developing and regenerating mouse livers and demonstrated its usefulness as a novel adult progenitor marker for hepatic lineage cells.

RESULTS

Eppk1 is expressed in early embryonic hepatic progenitor population

During embryogenesis, Eppk1 is expressed in the foregut lip at the anterior intestinal portal (AIP) and the primitive gut tube at E8.5, and in the whole gut tube including the pancreatic buds at E9.5 (Yoshida et al., 2008). Since Eppk1 is expressed in the progenitor cells in the embryonic pancreas and regenerating pancreas in the adult, we examined its expression in the developing liver. At E10.5, Eppk1 expression is observed in the hepatic primordium, expressing α-fetoprotein (AFP), a hepatoblast marker (Fig. 1A) (Shiojiri et al., 1991). Eppk1 expression rapidly decreases at E11.5 (Fig. 1B) and becomes almost undetectable at E12.5 or E13.5 (Fig. 1C, D). In contrast to the rapid down regulation of Eppk1 expression, AFP expression persisted at these stages. While Eppk1 expression remains decreased at E13.5, hepatocyte nuclear factor 4a (HNF4a), a hepatocyte marker, is expressed in the hepatoblast, and then becomes restricted to the hepatocytes around E13.5 (Fig. 1D). Taken together, Eppk1 marks the earliest hepatoblast population in the hepatic primordium, and then expression decreases rapidly.

Eppk1 is expressed in the cholangiocytes during liver development

At stages E15.5 and on, Eppk1 expression gradually increases again (Fig. 2A, B). We then analyzed the cell type that expressed Eppk1. Smooth muscle actin (SMA) is a marker that indicates smooth muscle cells lining the portal vein (Clotman et al., 2003). Notch signaling is implicated in the formation of biliary duct, and Jagged1 (Jag1) is expressed in the portal vein epithelium (McCright et al., 2002). Eppk1-expressing cells are observed close to the SMA-positive smooth muscle cells and Jag1-positive portal vein epithelium (Fig. 2A, B, C). At E16.5, Eppk1 is expressed in the ductal plate cells near the portal vein (Fig. 2B). Previous studies indicate that cholangiocyte progenitors appear close to the portal vein and form the ductal plate at E13.5-E15.5 (Shiojiri, 1994). The above results suggest that Eppk1 is expressed in the cholangiocyte progenitors.

We then examined the expression of Eppk1 and several differentiation markers. Cytokeratins (CKs) are known to be expressed in the cholangiocytes in developing liver and also in bile ducts after birth (Lemaigre, 2003). Thus, we compared the expression of Eppk1 and CKs, using an anti-pan-CK antibody. The Eppk1 expression much overlaps with that of CKs, during embryonic development and in the adult (Fig. 2D, E, F). During embryonic development, intense Eppk1-positive staining is observed close to the portal vein, enriched in Hnf4a-negative cells (Fig. 2D, E, G, H). Eppk1 staining is observed sometimes in the parenchyme near the portal vein at a much lower level (Fig 2D,E). This Eppk1-positive staining in the parenchyme is not universally observed, but rather confined to a specific population of cells close to the ductal plate, which might represent ductal plate precursor cells.

The bile duct development begins with the appearance of ductal plate precursor cells adjacent to the portal vein at around E14.5 (Lemaigre, 2003). Recent studies show that bile duct formation occurs via a mode of tubulogenesis characterized by a transient asymmetric expression of cholangiocyte and hepatocyte markers (Antoniou et al., 2009). Eppk1 expression is observed in the forming primitive duct, marked by E-cadherin expression (Supplementary Fig. S1).

In normal adult, Eppk1 expression is observed in the bile duct (Fig. 2F), but not in the Hnf4a-positive hepatocytes, although a low level of Eppk1 expression is observed in some hepatocytes that reside close to the portal vein (Fig. 2I).

Taken together, these results indicate a close correlation of Eppk1 expression with the cholangiocyte development and the duct formation temporally and spatially.

Eppk1 marks the adult progenitor population in a regenerating liver after chronic liver injury

We then examined Eppk1 expression in a regenerating liver using a choline deficient, DL-ethionine supplemented (CDE) diet-induced chronic liver injury model. Mice continuously on CDE diets for 2 weeks

developed steatohepatitis. In control mice, Eppk1-expressing cholangiocytes are observed near the portal vein (Fig. 3A). In contrast, in CDE diet-fed mice, Eppk1-positive cells increased not only in regions proximal to the portal vein but also in distal regions throughout the hepatic lobule (Fig 3B). A6 antigen is previously identified as a marker, which is expressed in the cholangiocyte in normal liver and increased in the regenerating liver (Engelhardt et al., 1990). In the CDE diet-fed injured liver, cells double positive for A6 and Eppk1 dramatically increased (Fig 3D). These results indicate that Eppk1 is expressed in the progenitor cells, which increase by injury (Fig.3B, D). Eppk1 expression also increased in the margin of hepatocytes that reside near the portal vein (Fig 3B, D, Supplementary Fig. S2A). Quantification of the Eppk1-positive and A6-positive cells reveals that most A6-positive cells also express Eppk1 (Supplementary Fig. S2B).

To further characterize the Eppk1-positive population, we examined the expression patterns of several other molecules that are known to be expressed in the hepatic progenitor cells during regeneration, such as CKs (Jelnes et al., 2007) and E-cadherin (Ueberham et al., 2007). We found that Eppk1-positive cells co-express CKs and E-cadherin in the normal and injured liver (Fig. 4A-D). Osteopontin (Osp) was recently described as being expressed in the cholangiocytes at the apical surface (Antoniou et al., 2009), but its expression has not been reported in the hepatic progenitor cells induced during regeneration yet. Since cholangiocyte markers, CKs and E-cadherin, are expressed in the hepatic progenitor cells (Fig 4 A-D), we tested the expression of Osp in the progenitor cells. Osp expression overlaps with that of Eppk1 (Fig. 4E-F). Sox9 is reported as a novel cholangiocyte marker (Antoniou et al., 2009), which we found to be co-expressed with A6 and E-cadherin not only in bile ducts but also in cells induced in CDE diet-fed injured liver (Supplementary Fig. S3). The above results indicate that the hepatic progenitor cells express different cholangiocyte markers, and further reinforce a molecular similarity between these two cell types. Taken together, our findings indicate that Eppk1 marks the adult hepatic progenitor cells that give rise after chronic injury. Previous reports in injury models in the rat and mouse indicate that the hepatic progenitor cells are transit amplifying cells (Apte et al., 2008; Hu et al., 2007). Wnt/β-catenin signaling is reported as a candidate signaling pathway to activate transit amplifying cells (Apte et al., 2008; Hu et al., 2007). In normal adult liver, Eppk1 and β-catenin are co-expressed in the bile ducts (Fig 4I). In the CDE diet-induced injured liver, the Eppk1- and β-catenin-positive oval cells increased in number (Fig. 4J). Upon injury, a marked increase in PCNA-positive proliferating cells is observed (Fig. 4H. The Eppk1-expressing cells include both PCNA-positive (Fig. 4H', close arrowheads) and -negative populations (Fig. 4H', open arrowheads). These results indicate that Eppk1-expressing cells represent transit amplifying cells that proliferate when stimulated by chronic injury.

DISCUSSION

Previous studies indicate that Eppk1 is expressed in epithelial tissues, including the liver (Fujiwara et al., 2001; Spazierer et al., 2003). In the liver, it was reported to be expressed at the margins of hepatocytes, with additional less pronounced staining of bile canaliculi (Spazierer et al., 2003). To confirm this discrepancy, we tested the antibody reported by Spazierer et al. Their antibody also gave similar results with ours that Eppk1 marks the cholangiocytes in normal liver and the hepatic progenitor cells in regenerating liver, with a much lower expression in the hepatocytes (Supplementary figure S4A, B). Moreover, *in situ* hybridization showed a marked increase in Eppk1-expressing cells in CDE diet–fed injured liver compared to normal liver, using an Eppk1 cDNA fragment as a probe (Supplementary figure S4C,D). Taken together, we conclude that Eppk1 is predominantly expressed at a high level in the cholangiocytes, and at a substantially low level in the hepatocytes. Upon injury Eppk1-expressing cells markedly and transiently increased in the hepatic progenitor cells. Therefore, our findings show that Eppk1 is a novel cholangiocyte and hepatic progenitor cell marker. Eppk1 is one of the plakin family proteins, which is identified as cytolinker proteins, which are associated with cytoskeletal elements and junctional components. It is reported that Eppk1 is one of the candidate molecules, associated with the activation of the EGF pathway (Blagoev et al., 2003), which is also reported as a signaling component that lies down stream of Wnt/β-catenin (Tan et al., 2005). However, in Eppk1 deficient mice, we found that the bile ducts develop normally and that the apical surface and tight junctions are normal. Therefore the lack of Eppk1 protein does not affect the cell adhesion or the formation of the bile duct in the liver (A.M.

unpublished results).

In this study, we show that Eppk1 marks the very early bipotential hepatoblasts in E10.5. Then at E11.5, Eppk1 expression decreases rapidly (Fig. 1). Later, in the developing liver, Eppk1 is re-expressed in cells that reside close to the portal vein (Fig. 2). In contrast, Sox9, a cholangiocyte marker, is detected early at E10.5 in the endodermal cells in the liver primordium and is undetectable in the hepatoblasts. Sox9 is expressed in the cholangiocyte progenitors, which align around the portal vein at E11.5-13.5, prior to the re-expression of Eppk1 (Antoniou et al., 2009). Osp expression is specifically detected in the cholangiocytes at around E15.5 (Antoniou et al., 2009). Cytokeratin19 is expressed at a low level in the hepatoblasts, and then becomes restricted to the cholangiocyte lineage (Lemaigre, 2003). E-cadherin is expressed in the hepatoblasts and hepatocytes throughout the liver, also with higher expression in the cholangiocyte lineage (Doi et al., 2007). In the adult, Eppk1 is expressed in the bile duct in normal liver, and upon chronic injury, it is expressed in the transient amplifying progenitor cells (Fig 3), where its expression overlapped with that of Osp, E-cadherin and CKs (Fig. 4). Moreover, the present paper is the first to show that Osp and Sox9 may be useful as novel hepatic progenitor cell markers (Fig. 4E-F and Supplementary Fig. S3). AFP and Albumin are expressed in the hepatoblasts, but not cholangiocytes. Both AFP and albumin are reported to be re-expressed in the adult progenitor cells in injured liver (Thompson et al., 2010). EpCAM is expressed in the hepatoblast at E11.5, a stage after Eppk1 expression decreased, and later in the cholangiocyte lineage, and also in adult hepatic progenitor cells (Tanaka et al., 2009). Taken together, Eppk1 expression is unique compared to the above mentioned markers and therefore it is useful for the analysis of the origin of cholangiocytes during embryonic development and their contribution during regeneration.

A lineage tracing experiment is a powerful tool to follow the fate of progenitor cells. However, lineage tracing studies on the cholangiocyte development and the regeneration of adult injured liver are still very limited. In previous studies, the cholangiocytes and progenitor populations were identified by immunohistochemical analyses. There are reports that Foxl1-is a marker of bipotential hepatic progenitor cells (Sackett et al., 2009). However, the origin of the Foxl1-positive population after injury remains elusive, since Cre expression in the Foxl1Cre;R26RlacZ mouse line was not inducible in the above study. Many cholangiocyte markers are also expressed in the adult hepatic progenitor cells, thereby supporting the idea that the origin of adult hepatic progenitor population is the duct or Canal of Hering. Future Eppk1 lineage tracing experiments would address this issue.

Previously, we described that Eppk1 is expressed in the progenitor population of the pancreas and the retina, both in the developing embryo and in the adult (Yoshida et al., 2010; Yoshida et al., 2008). Moreover, in the liver, our findings show that Eppk1 marks the progenitor population of early hepatoblasts (embryonic progenitors) in the earliest liver primodium, the cholangiocyte progenitors and oval cells (adult progenitors). There are several genes that resemble Eppk1 in that they are expressed in different progenitor/stem cell systems. For example, Osp resembles Eppk1 in that it is also expressed in the pancreas in both the progenitor cells and in the ducts (Kilic et al., 2006) (Fig. 4). Similarly, Sox9 is expressed in the progenitor and ducts in the pancreas (Lioubinski et al., 2003; Seymour et al., 2007), as well as in retinal progenitor cells and Muller glial cells in the retina (Poche et al., 2008). Likewise, Lgr5 characterizes the intestine and hair follicle stem cell (Barker et al., 2007; Jaks et al., 2008). In contrast, the molecular mechanisms underlying the stem/progenitor cell system remain to be revealed.

In sum, our data reveal Eppk1 as a novel marker to detect the population of cholangiocyte progenitors in the developing liver, and the oval cells in injured liver (Fig. 5). Future lineage tracing experiments, using Eppk1 would aid in understanding the molecular mechanisms and the role of hepatic progenitor cells in the maintenance of liver homeostasis.

MATERIALS AND METHODS

Animals

Wild type mice were obtained from Japan SLC. For timed pregnancies, it was assumed that morning of plug detection corresponds to E0.5. Embryo ages were rounded to the nearest half-day. All animal procedures were performed

in accordance with the guidelines for the care and use of animal at the Kumamoto University.

Liver injury model using CDE diet

Wild type C57BL/6 (Japan SLC) mice were used. Mice were fed with a CD (Choline Deficient, MP Biomedicals, Cat#. 9602214) diet with drinking water supplemented with 0.15% or 0.165% DL-ethionine (wt/vol) (Sigma, Cat#. E5139) for 2 weeks, as described (Akhurst et al., 2001; Jelnes et al., 2007).

Tissue preparation and processing

Mouse embryonic and adult tissues were fixed in 4% paraformaldehyde at 4^{o}C overnight. For frozen sections, paraformaldehyde was replaced with 30% sucrose (W/V) in PBS overnight and embedded in OCT compound (Sakura Fine technical Co., Japan). Sections were cut at 10 μm. For paraffin embedding, after fixation in 4% paraformaldehyde at 4 ^{o}C overnight, tissues were embedded in paraffin. Sections were cut at 5 μm. For hemaotoxylin eosin (HE) staining, paraffin sections were dewaxed, rehydrated and stained with hematoxylin and eosin.

Immunohistochemistry

Sections were boiled in Target Retrieval Solution (Dako Cytomation, Cat# s1699) for 5 minutes at 105˚C for antigen retrieval. Sections were permeabilized with 0.1% Triton X-100 in PBS, blocked with Blocking One (Nacalai tesque) or M.O.M. Immunodetection Kit (Vector). Primary antibodies used were: rabbit anti-Eppk1 (Yoshida et al., 2010), mouse anti-AFP (1:500, MONOSAN, Cat# MON4035), goat anti-Hnf4a (1:500, Santa Cruze, Cat#sc-6556), mouse anti-smooth muscle actin (1:500, SMA Sigma, Cat#sc-6556 A5228), goat anti-Jag1 (1:500, R&D, Cat#AF599), mouse anti-pan-Cytokeratin (1:500, Abcam, Cat# ab11213), mouse anti-E-cadherin (1:100, BD Transduction Laboratories, Cat# 610182), rat anti-A6 (1:500, a kind gift from Dr. VM Factor (Engelhardt et al., 1990), goat anti-Osteopontin (1:500, R&D, Cat#AF808), mouse anti-β-catenin (1:500, BD Transduction Laboratories,Cat#610153), mouse anti-PCNA (1:500, Oncogene, NA03-200UG), rabbit anti-Sox9 (1:250, Millipore, Cat#AB5535) antibodies. Secondary antibodies used were: Alexa488-conjugated, Alexa568-conjugated or Alexa594-conjugated antibodies (1:1000, Molecular Probes). Nuclei were counterstained with DAPI (Roche). Slides were mounted with mountant, Perma Fluor (LAB VISION Co.). Optical sections were viewed using a scanning laser confocal imaging system (TCSSP2 AOBS, Leica Microsystems). Nonconfocal images were acquired using an Olympus IX-71 microscope (Olympus Optical) equipped with a Nikon digital sight DS-5M.

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FIGURE LEGEND

Fig. 1

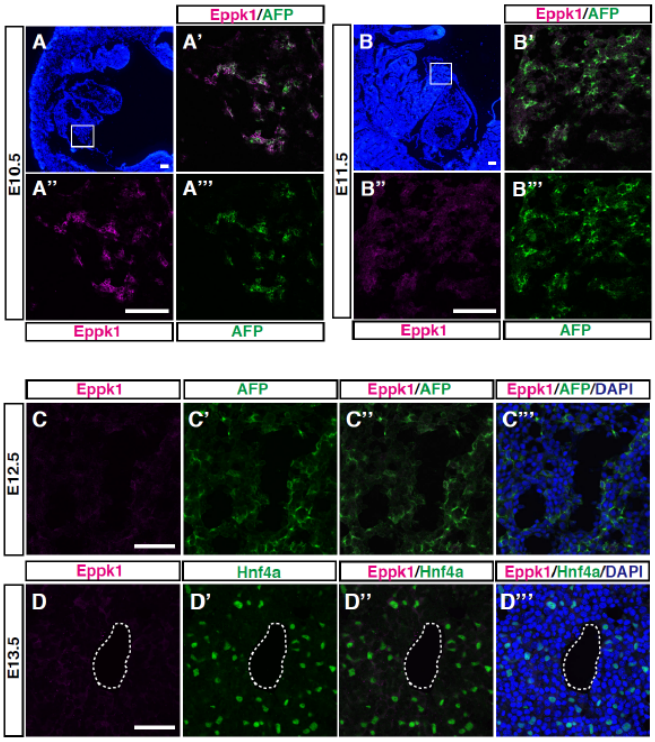


Fig.1. Eppk1 is expressed in early liver development.

Eppk1 is expressed in the liver primodium and the hepatoblasts, but not the hepatocytes.
(A-D) Immunostaining of Eppk1 (magenta) and (A-C) AFP (green) or (D) Hnf4a (green) at (A) E10.5, (B) E11.5, (C) E12.5 and (D) E13.5. Nuclei (blue) are counter stained with DAPI. Eppk1 expression is observed in (A) E10.5 liver primordium, overlapping with AFP staining, (B-D) but decreases at later stages. (D) Hnf4a is detected in the hepatocytes at E13.5, where Eppk1 is not expressed. (A'-D''') High magnification views of the area enclosed by rectangles in A-D. Portal vein, PV. Scale bars: 50 μm.

Fig. 2

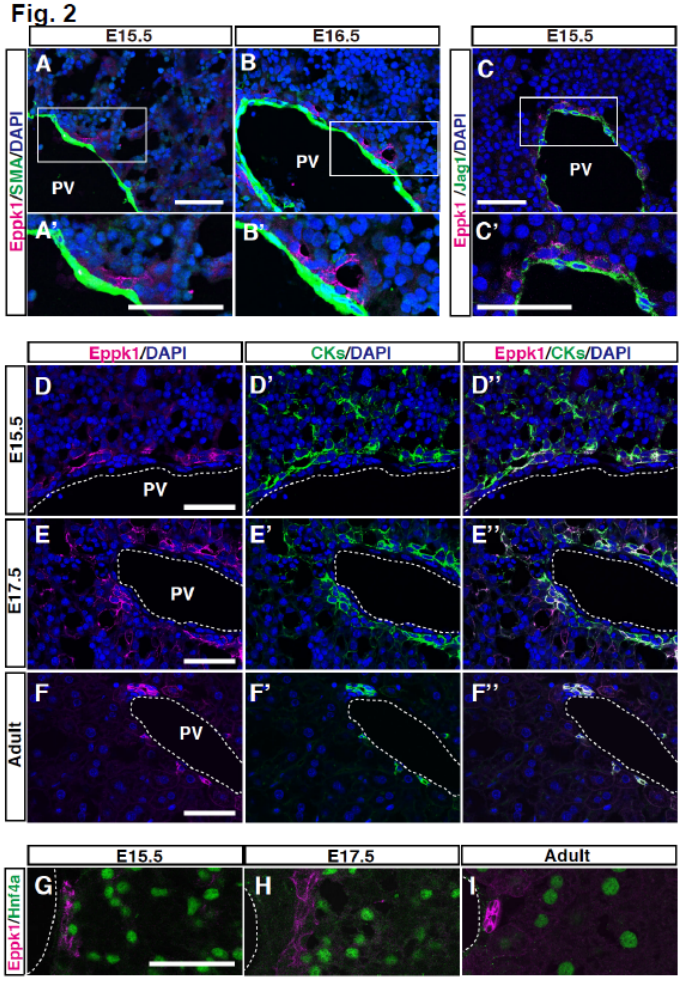


Fig.2. Eppk1 is expressed in developing liver

Eppk1 is expressed in the progenitor cells of the cholangiocyte during liver organogenesis.
(A-C) Eppk1 (magenta)-expressing cells locate (A, B) close to SMA-positive smooth muscle cells lining the portal vein (green) and (C) Jag1-positive portal vein endothelium (green). (D-F) Eppk1 (magenta)-positive progenitor cells also express CKs (green) at (D) E15.5, (E) E17.5 and in (F) the adult. (G-I) Eppk1 expression is observed in the primary duct but not in the HNf4a-positive hepatocytes at (G) E15.5, (H) E17.5 and in (I) the adult. Nuclei are counter stained with DAPI (blue). Scale bars: 50 μm.

Fig. 3

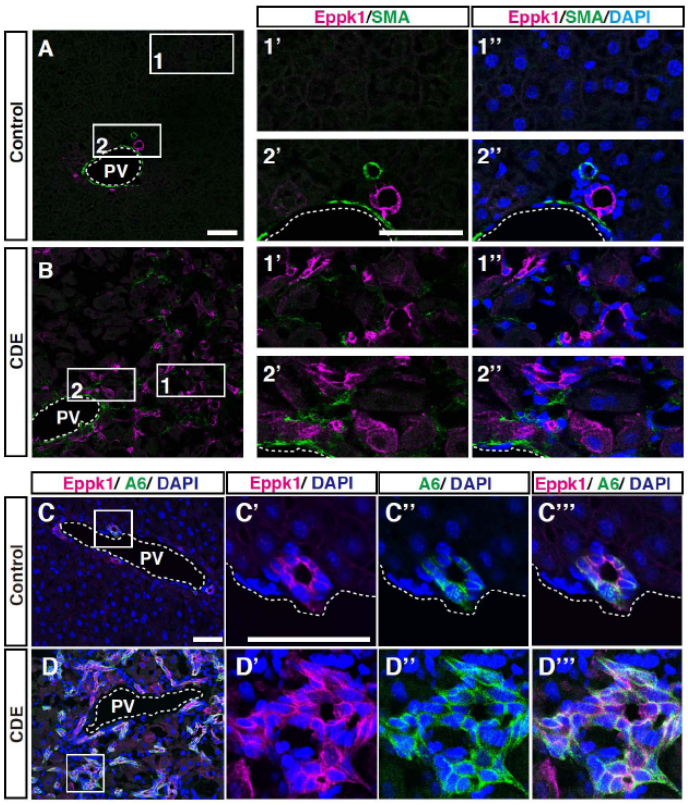


Fig.3. Eppk1 is expressed in the adult hepatic progenitors.

Eppk1 is co-expressed with A6 in the adult hepatic progenitor cells.
(A-B) In the liver of mice fed with CDE diet, Eppk1-positive cells increased not only (B-2) near the portal vein, but also (B-1) throughout the hepatic lobule. In contrast, Eppk1-positive cells are observed (A-2) near the portal vein but not in the (A-1) lobule in normal liver. (C-D) Eppk1 (magenta) and A6 (green) are co-expressed in (C) the cholangiocytes in normal adult liver, and (D) the injured liver of CDE diet fed mice.
(C'-D''') High magnification views of the area enclosed by rectangles in C-D. Nuclei are counter stained with DAPI (blue). Scale bars: 50 μm.

Fig.4.

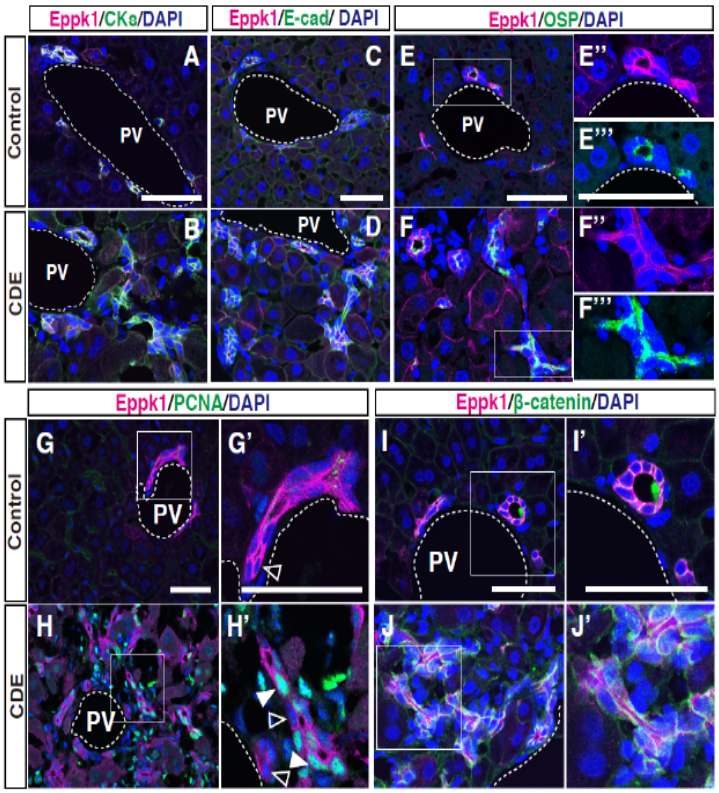


Fig.4. Eppk1-expressing cells co-expressed with CKs, E-cadherin and OSP in the adult progenitors and are transit amplifying cells that proliferate upon chronic injury.

(A, C, E) In normal liver, Eppk1-positive (magenta) cells co-express (A) CKs (green), (C) E-cadherin (green) and (E) Osp (green). (B, D, F) In the liver of mice fed with CDE diet, Eppk1-positive adult progenitor cells co-express (B) CK, (D) E-cadherin and (F) OSP. In most cells, Eppk1, CK, E-cadherin and Osp co-localized.
(G) Eppk1-positive cells are quiescent in normal liver. (H) Upon injury, many Eppk1-positive (magenta) progenitor cells express a proliferation marker, PCNA (green), although some cells remain PCNA-negative (open arrowhead). (I) Wnt/β–catenin is co-expressed with Eppk1 in the bile duct in normal liver. (J) In CDE treated liver, Eppk1-, β–catenin-double positive cells increase in number. Nuclei are counter stained with DAPI (blue). Scale bars: 50 μm.

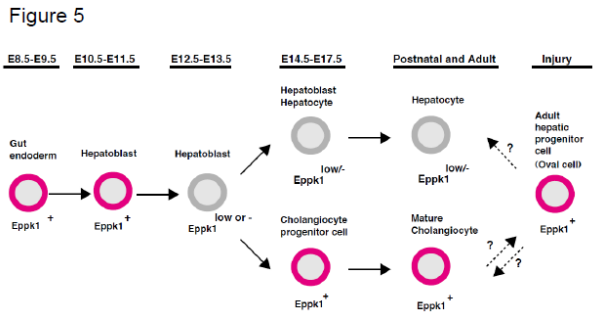
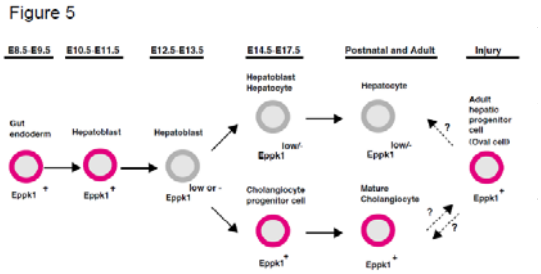
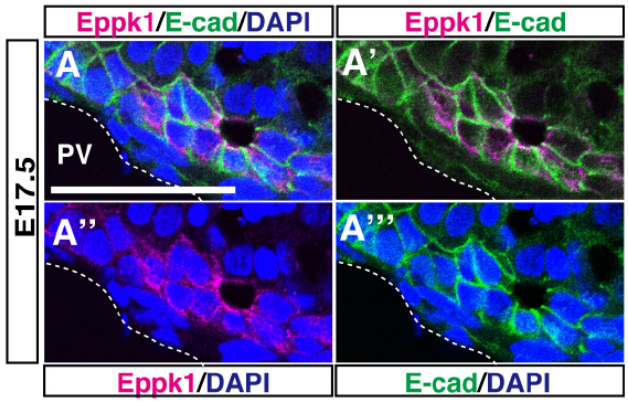


Fig.5. A schematic representation of Eppk1 expression pattern.

Eppk1 is expressed in the gut endoderm during early embryogenesis at E8.5 and E9.5 (Yoshida et al, 2008). In the developing liver, Eppk1 is expressed in the earliest bipotential hepatoblasts, before differentiation occurs. Then, Eppk1 expression decreases in differentiating cells and again increases in progenitor cells, which have adopted the cholangiocyte fate. Upon injury, transit amplifying cells so called 'oval cells' increase in number. The oval cells are hepatic progenitor



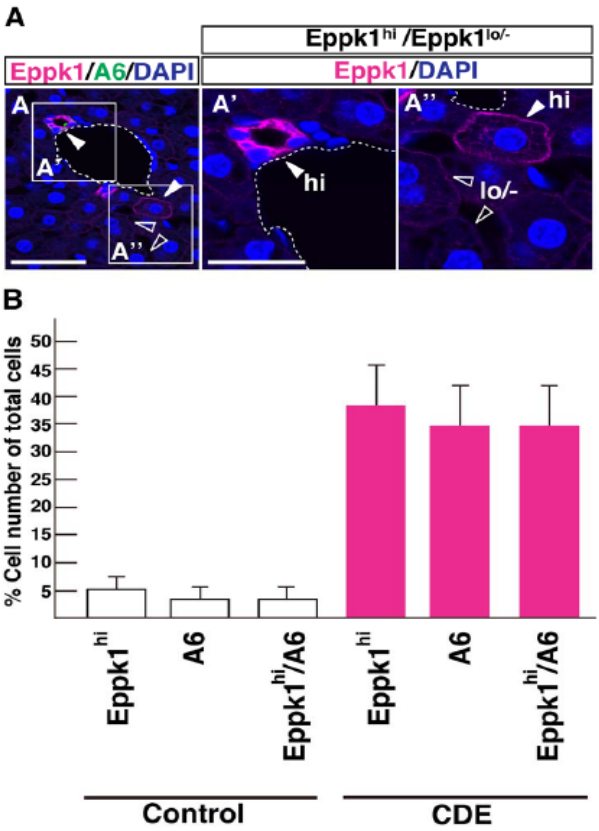
Supplementary Fig.S1.



Supplementary Fig. S1.

Eppk1 is expressed in the forming primitive duct, marked by E-cadherin expression.
(A) Eppk1-positive cells are cholangiocytes progenitors. Eppk1 expression (magenta) is observed in the forming primitive duct, similarly with that of the E-cadherin (green) expression. Nuclei are counter stained with DAPI (blue). Scale bars: 50 µm

Supplementary Fig.S2.

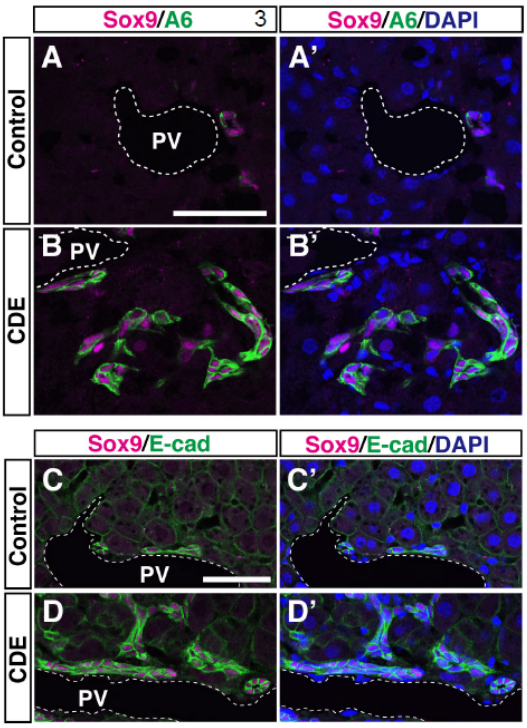


Supplementary Fig. S2.

Eppk1-expressing cells are subdivided into Eppk1 high (Eppk1^{hi}) and Eppk1 low (Eppk1$^{lo/neg}$) populations based on immunohistochemical analysis. (A, A') In normal adult liver, cholangiocytes expressed Eppk1 at a high level (close arrowhead). These cells are defined as Eppk1^{hi} (hi) population. (A, A") A minor hepatocyte population lies close to the portal vein are Eppk1^{hi} (hi). However, most hepatocytes expressed Eppkl at a low (lo) or undetectable level (Eppk1$^{lo/-}$, lo/- open arrowhead). (A', A") High magnification views of the area enclosed by rectangles in A. Nuclei are counter stained with DAPI (blue). Scale bars: 50 µm (A) and 25µm (A', A").
(B) The proportion of Eppk1^{hi}-, A6- and

Eppk1^{hi}/A6-double positive cells in control or CDE-fed liver. The numbers of positive cells were counted in three represented regions, the proportions are shown as average ± standard errors are shown.

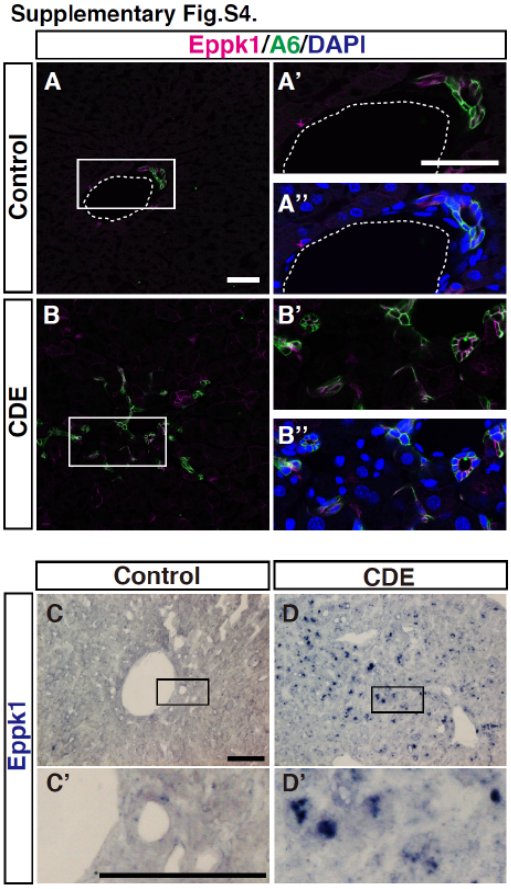
Supplementary Fig. S3



Supplementary Fig. S3

Sox9 is a cholangiocyte marker, and also a candidate adult progenitor cell marker.
(A) Sox9 (magenta) is expressed in the bile duct in normal liver. (B) Sox9 expression increased in the liver of CDE diet fed mice. (C-D) Sox9 expression overlaps with that of E-cadherin (green) in (C) control liver and (D) the injured liver of CDE diet fed mice.
Nuclei are counter stained with DAPI (blue). Scale bars: 50 μm.

Supplementary Fig.S4.



Supplementary Fig. S4

(A, B) Eppk1 expression detected by the antibodies by Spazier et al. (2003) reveals the localization of Eppk1 in the cholangiocytes and adult progenitor cells. The staining in the hepatocytes is low. (C, D) *In situ* hybridization using an Eppk1 antisense probe reveals Eppk1 transcript in (C) normal liver and (D) CDE diet-fed injured liver. Nuclei are counter stained with DAPI (blue) in A, B. Scale bars: 50 μm (A-B") and 100μm (C-D').
Section *in situ* hybridization was performed as described previously (Katsumoto et al., 2009; Matsuura et al., 2009). Mouse Eppk1 consisted of 16 plakin repeat domains (PRDs) (Spazierer et al., 2003). A cDNA fragment encodes the 10th PRD domain is used as a probe. Signal is detected by NBT/BCIP (Roche). The staining in the cytoplasm is non-specific signals, since the sense probe gave similar signals (A.M. unpublished results).