

Potential hepatic stem cells reside in EpCAM⁺ cells of normal and injured mouse liver

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Hepatic oval cells are considered to be facultative hepatic stem cells (HSCs) that differentiate into hepatocytes and cholangiocytes in severely injured liver. Hepatic oval cells have also been implicated in tumorigenesis. However, their nature and origin remain elusive. To isolate and characterize mouse oval cells, we searched for cell surface molecules expressed on oval cells and analyzed their nature at the single-cell level by flow cytometric analysis and in the *in vitro* colony formation assay. We demonstrate that epithelial cell adhesion molecule (EpCAM) is expressed in both mouse normal cholangiocytes and oval cells, whereas its related protein, TROP2, is expressed exclusively in oval cells, establishing TROP2 as a novel marker to distinguish oval cells from normal cholangiocytes. EpCAM⁺ cells isolated from injured liver proliferate to form colonies *in vitro*, and the clonally expanded cells differentiate into hepatocytes and cholangiocytes, suggesting that the oval cell fraction contains potential HSCs. Interestingly, such cells with HSC characteristics exist among EpCAM⁺ cells of normal liver. Intriguingly, comparison of the colony formation of EpCAM⁺ cells in normal and injured liver reveals little difference in the number of potential HSCs, strongly suggesting that most proliferating mouse oval cells represent transit-amplifying cells rather than HSCs.

KEY WORDS: Hepatic stem cell, Oval cell, EpCAM, TROP2 (TACSTD2), Hepatocyte, Cholangiocyte, Liver injury

INTRODUCTION

Most of the metabolic functions in the liver are carried out by hepatocytes that form hepatic cords, whereas cholangiocytes form bile ducts that drain bile produced by hepatocytes. During development, hepatocytes and cholangiocytes, two types of hepatic epithelial cells, derive from hepatoblasts emerging from the foregut endoderm (Notenboom et al., 2003; Zaret, 2000). Hepatoblasts are highly proliferative and express hepatocytic proteins such as albumin (ALB) and alpha-fetoprotein (AFP), an immature hepatocyte marker. As the liver develops, hepatoblasts propagate and those close to the portal mesenchyme differentiate into cholangiocytes, while the rest become mature hepatocytes (Lemaigre, 2003). Therefore, hepatoblasts are thought to be hepatic stem/progenitor cells in the fetus.

By contrast, it is highly controversial whether adult mammalian liver contains hepatic stem cells (HSCs). Adult liver has a remarkable potential to regenerate from severe parenchymal loss, even though hepatocytes and cholangiocytes are mitotically dormant under normal conditions. Hepatocytes have a remarkable potential to self-replicate (Fausto, 2004; Michalopoulos and DeFrances, 1997) and are capable of at least 80 doublings by serial transplantation (Overturf et al., 1997), allowing the liver to regenerate. However, liver injury that limits this pathway accompanies the proliferation of a potential stem/progenitor cell compartment at the interface of the biliary tree and hepatic cords, which is known as the ductular reaction (Alison et al., 1996; Roskams et al., 2004; Theise et al., 1999). These undifferentiated

epithelial cells are often referred to as ‘oval cells’ because of their ovoid nucleus (Farber, 1956). Upon activation, oval cells expand into liver parenchyma from the portal area. Oval cells express both ALB and cytokeratin 19 (CK19; KRT19 – Mouse Genome Informatics), which are hepatocytic and cholangiocytic markers, respectively, and are believed to differentiate into hepatocytic and biliary lineages, similar to hepatoblasts in embryonic liver. Thus, oval cells are thought to be facultative stem/progenitor cells in adult liver. Although oval cells have been most extensively studied in rodents, similar cells have been found in connection with various human liver diseases and are implicated in tumorigenesis (Fausto, 2004; Lee et al., 2006). Whether oval cells constitute HSCs has been debated in numerous reports involving various rodent injury models using chemical reagents, including carcinogenic agents. The 2-acetylaminofluorene (2-AAF)/partial hepatectomy (PH) model, in which hepatocyte proliferation is blocked by 2-AAF prior to PH, has been extensively used to characterize oval cells in rats (Evarts et al., 1987; Laishes and Rolfe, 1981). However, the same procedure does not generate oval cells in mice and alternatives such as the use of a choline-deficient ethionine-supplemented diet (Akhurst et al., 2001) and a 3,5-diethoxycarbonyl-1,4-dihydro-collidine (DDC)-containing diet (Preisegger et al., 1999; Wang et al., 2003) have been developed. Although the proliferating epithelial cells that are present in the periportal region upon injury caused by various insults are referred to as oval cells, it remains unclear whether oval cells generated via different protocols in different species have the same characteristics. A major problem in characterizing oval cells is the lack of appropriate cell surface markers to identify and isolate the oval cell compartment. Therefore, despite a large number of studies, the exact nature of oval cells – including their origin, stemness and bi-directional differentiation – is still poorly understood. Because of the difficulty in performing clonal analysis of HSCs, it has also been unclear whether HSCs exist in normal liver.

The aim of this study was to identify cell surface molecules on mouse oval cells and to analyze their nature at the clonal level. To this end, we utilized the 2-AAF/PH rat model and the DDC diet

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mouse model to generate oval cells and found that epithelial cell adhesion molecule (EpCAM) and the related molecule, TROP2 (TACSTD2), were upregulated in these livers. EpCAM was expressed in normal cholangiocytes and also in oval cells in the liver of mice fed the DDC diet (DDC liver). By contrast, TROP2 was expressed in almost all EpCAM⁺ cells in DDC liver, but not in normal liver, indicating that TROP2 is a novel marker to distinguish between normal cholangiocytes and oval cells. Furthermore, we isolated EpCAM⁺ cells from DDC liver and demonstrated that clonally expanded cells were able to differentiate into hepatocytes and cholangiocytes. Finally, we provide evidence for the presence of potential HSCs in EpCAM⁺ cells of normal liver and compare their characteristics before and after oval cell activation.

MATERIALS AND METHODS

Animals

C57BL/6 mice (Japan SLC, Hamamatsu, Japan) at 8–12 weeks were used for all experiments. All experiments with animals were performed according to institutional guidelines. The diet containing 0.1% DDC was purchased from CLEA, Japan. Mouse oval cells were activated by feeding with the diet containing 0.1% DDC.

Identification of cDNA encoding a membrane protein

Total RNA was prepared from the non-parenchymal fraction of rat liver treated with 2-AAF/PH at 7 days after PH as described previously (Tanimizu et al., 2004b). Total RNA was amplified using the MessageAmp aRNA Amplification Kit (Ambion), and used to construct a cDNA library in the pMXs-SST vector using the SuperScript Choice System (Invitrogen, Carlsbad, CA, USA). The cDNA library contained 4.3×10^6 independent clones. The signal sequence trap method was performed as described previously (Kojima and Kitamura, 1999).

Preparation of liver cells and flow cytometry (FCM)

A single-cell suspension from DDC and normal livers was obtained by a modified two-step collagenase perfusion method as described previously (Seglen, 1976). In short, livers were perfused with liver perfusion medium (Invitrogen) at a flow rate of 3 ml/minute for 5 minutes. Then, the liver was perfused with basic perfusion solution (136 mM NaCl, 5.4 mM KCl, 5 mM CaCl₂, 0.5 mM NaH₂PO₄ 2H₂O, 0.42 mM Na₂HPO₄ 12H₂O, 10 mM HEPES pH 7.5, 5 mM glucose and 4.2 mM NaHCO₃) containing 0.5 g/l collagenase type IV (Sigma, St Louis, MO, USA) at a flow rate of 3 ml/minute for 8 minutes. The digested liver was transferred to a glass dish and chopped into small pieces using a surgical knife in D-PBS (Dulbecco's phosphate-buffered saline). Cells dispersed by pipetting were passed through a 70-μm cell strainer and the flow-through fraction was used for the next step as the first cell suspension. The undigested clot on the strainer

was recovered and redigested with basic perfusion solution containing 0.5 g/l collagenase type IV, 0.5 g/l pronase (Roche Diagnostics) and 50 mg/l DNase I (Sigma) by stirring for 20 minutes at 37°C. This digested liver was also passed through a 70-μm cell strainer and the flow-through fraction was combined with the first cell suspension. After centrifugation at 700 rpm (100 g) for 2 minutes, the supernatant was transferred to a new tube and the centrifugation repeated until no cell pellet was visible. The final supernatant was centrifuged at 1200 rpm (300 g) for 5 minutes and the precipitated cells were used as non-parenchymal cells (NPCs) for FCM analysis. Aliquots of cells were blocked with anti-FcR antibody, co-stained with fluorescein- and biotin-conjugated antibodies, washed, incubated with allophycocyanin-conjugated streptavidin (Invitrogen), and analyzed by FACSCalibur (Becton Dickinson). Dead cells were excluded by propidium iodide staining.

Antibodies and immunohistochemistry (IHC)

The anti-EpCAM monoclonal antibody was generated by immunization of a rat with a Ba/F3 cell transfectant overexpressing EpCAM cDNA. The establishment of hybridoma clones was performed as described previously (Hara et al., 1999). The anti-EpCAM monoclonal antibody was biotinylated using Amersham ECL Protein Biotinylation Module (GE Healthcare, UK) or fluorescein-conjugated using the Fluorescein Labeling Kit-NH₂ (Dojindo Molecular Technologies), and then used for all FCM analyses. The rabbit anti-mouse CK19 polyclonal antibody was raised against the C-terminal peptide, HYNLPTPKAI, and the serum was used for IHC as previously described (Tanimizu et al., 2003). The biotin-conjugated anti-mouse TROP2 antibody was purchased from R&D Systems. The anti-human Ki67 antibody was purchased from Becton Dickinson. Frozen sections (8 μm) of livers were prepared using a HM505E cryostat (Microm International) after fixation with 4% paraformaldehyde, and incubated with each antibody, followed by a biotin- or fluorescein-conjugated secondary antibody. The signals were visualized by fluorescence microscopy.

RNA extraction and reverse transcription PCR (RT-PCR)

Total RNA was extracted from each cell preparation using Trizol reagent (Invitrogen). Total RNA (1 μg) and random hexamer primers were used to synthesize cDNA using the First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech). The samples were denatured at 94°C for 5 minutes, then subjected to 25–40 cycles of denaturation at 94°C for 30 seconds, annealing at 52–57°C for 30 seconds, and extension at 72°C for 1 minute, with the final extension at 72°C for 5 minutes. PCR primers for mouse genes are shown in Table 1. The quantitative real-time RT-PCR was performed using a LightCycler ST300 (Roche) and the following primers (5' to 3'): rat *Epcam*, TCTACAAGGAAGAGATCAGCAAAA and TGTGTATC-TACCCATCTCCTTT; rat *Trop2*, GACCAAATGTGTTGGCCTGT and GTCACAGCTGGGAGGAAAAT; rat *Hprt*, GACCGGTTCTGT-CATGTCG and ACCTGGTTCATCATCACTAATCAC.

Table 1. Oligonucleotides used in RT-PCR

Gene	Forward (5' to 3')	Reverse (5' to 3')
<i>Epcam</i>	CGGCTCAGAGAGACTGTGC	GATCCAGTAGGTCCTCACGC
<i>Ck19</i>	GTCCTACAGATTGACAATG	CACGCTCTGGATCTGTGACAG
<i>Ck7</i>	GGGATGACCTCCGCAACACC	CTCCAGCAGCTTGCGGTAGG
<i>Alb</i>	CATGACACCATGCTGCTGAT	GCCTTTCCACCAGGGATCCAC
<i>Afp</i>	CTGGAGTGTCTGCAGGATGG	CCACAGCCGGACCATTTCTC
<i>Gapdh</i>	GGAGCGAGACCCCACTAA	GTGTAGCCCAAGATGCC
<i>Hprt</i>	GACTGAAAGACTTGCTCGAG	CCAGCAAGCTTGCAACCTTAACAA
<i>G6Pase</i>	AACCCATTGTGAGGCCAGAGG	TACTCATTACACTAGTTGGTC
<i>Tat</i>	TGCATCTCTGAAGACATG	CTTCTCTCTGGTGTAGCTCT
<i>Cps</i>	ACTGAGAGATGCTGACCTA	CCTGGAAATTGGTGAGGAGA
integrin β4	GACCTATGAAGAAGGTGCTC	GGCTCAGATTGCGTGCCATAG
<i>Ggt1</i>	GCTGAGCTGATTGAGCATCCG	GGTTGATGAAGTTGGGCGAGC
mucin 1	GAGCGCCAGCCTTGAGTTTG	GGAGGCACTACTGTGGACTG
<i>Trop2</i>	CTGACCTAGACTCCGAGCTG	CCAACCCATCTGGTCTGAGG
claudin 4	GACTTTGACCCCTGCAGAGG	GGCCACAGGCTGTTATGAGC
<i>Cd44</i>	CAGAGGCGACTAGATCCCTC	GAGTCACAGTGCGGGAAGCTC
<i>Slc10a1</i>	AGATCAAGGCTCACTTCTGG	AGAAGTCCTTCTGCAAGCTG
<i>Tdo2</i>	ACAATGAAGAAGACAGAGC	TGTAGTCTCTCCAAAGTTA

Culture of EpCAM⁺ cells and colony formation assay

NPCs were prepared as described above. EpCAM⁺ cells were sorted by FACS Vantage SE (Becton Dickinson). The cells were suspended in the standard medium (Williams' medium E containing 10% FBS, 10 mM nicotinamide, 2 mM L-glutamine, 0.2 mM ascorbic acid, 20 mM HEPES pH 7.5, 1 mM sodium pyruvate, 17.6 mM NaHCO₃, 14 mM glucose, 100 nM dexamethasone, 1 × ITS (insulin, transferrin, selenium X) and 50 mg/ml gentamicin) and seeded on a type-I collagen-coated dish. human EGF, human recombinant HGF and mouse IL6 were added to the culture to a final concentration of 10 ng/ml each. After the establishment of cell lines, IL6 was excluded from the culture medium because it was confirmed to have no apparent effect. For colony formation assays, EpCAM⁺ cells were sorted by a two-step selection (see Fig. S1 in the supplementary material) and plated at 1 × 10⁴ cells per 35-mm dish. The isolated cells were cultured for 9 days and then the number and size of colonies were counted.

Differentiation into the hepatocytic lineage in vitro

Clonally expanded cells (3 × 10⁵ per well) were cultured in the standard culture medium in a 6-well plate. After 2 days, 20 ng/ml Oncostatin M (OSM) and 1% DMSO were added into the confluent culture. After 5 days, the medium was changed to standard culture medium containing 20 ng/ml OSM, 1% DMSO and 17% Matrigel (growth factor reduced). After 3 and 5 days, the cultured cells were used for RNA preparation and periodic acid-Schiff (PAS) staining as described (Kamiya et al., 1999).

In vitro differentiation into the cholangiocytic lineage

Cellmatrix Type I-A (Nitta Gelatin) was used for three-dimensional culture to induce cholangiocytic differentiation according to the manufacturer's instructions. In short, 0.3% Cellmatrix Type I-A, a 5 × DMEM containing 250 mg/ml gentamicin, and the reconstitution buffer (0.05 M NaOH, 200 mM HEPES pH 7.5, 262 mM NaHCO₃) were mixed at a ratio of 7:2:1. This mixture was mixed with an equal volume of 5 × 10⁴ cells suspended in standard culture medium without dexamethasone and nicotinamide but with human recombinant HGF (20 ng/ml). The cell suspension was poured into a 6-well plate and left at 37°C to form a gel. Then, the culture medium was gently laid onto the gel.

RESULTS

Screening for cell surface markers of oval cells

To identify the cell surface molecules expressed on hepatic oval cells, we utilized the signal sequence trap (SST) method, which can efficiently isolate genes encoding a protein with a signal sequence (Kojima and Kitamura, 1999; Watanabe et al., 2007). As there are several protocols for generating oval cells in rats and mice, the characteristics of oval cells might not be the uniform. We therefore searched for cell surface molecules expressed on oval cells in the two species using different protocols (see Fig. S2 in the supplementary material). To this end, we first constructed an SST cDNA library from non-parenchymal cells (NPCs) of rat liver subjected to 2-AAF/PH treatment and identified 54 membrane proteins (see Table S1 in the supplementary material). First, we compared the expression of their counterpart genes for mouse between normal and DDC liver by RT-PCR, and found that *Epcam*, *Trop2*, mucin 1, claudin 4, *Cd44*, integrin β3, *Lyve1*, *gp130* (*Il6st*) and *Fxyd5* were significantly upregulated in DDC liver relative to normal liver. Chronic liver injury caused by DDC diet induces oval cells in mice, whereas PH and carbon tetrachloride-induced acute hepatitis do not. Next, the expression of the candidate genes was compared by northern blotting among normal liver, DDC liver and models of acute hepatitis, resulting in the identification of six of the nine genes that were specifically upregulated in the DDC liver, but not in the other livers (Fig. 1A). The remaining three genes, *Lyve1*, *gp130* and *Fxyd5*, were upregulated in an acute hepatitis model, suggesting that they might be involved in inflammation (data not shown). Because the expression of EpCAM, mucin 1, CD44 and

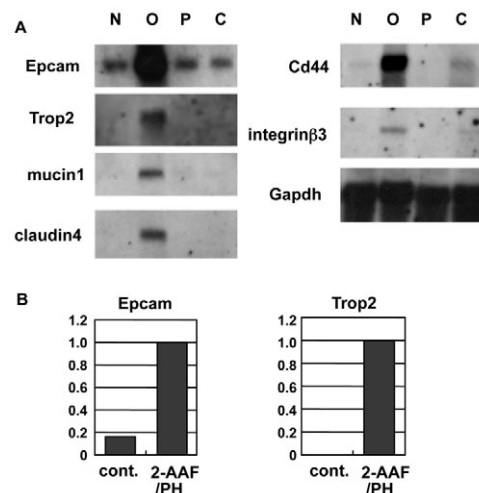


Fig. 1. Expression profiles of candidate genes in normal and injured mouse and rat liver. (A) Northern blot analysis of candidate genes in mouse liver. The expression of these genes was selectively upregulated in DDC liver, but not in injured liver without oval cell activation. **(B)** Quantitative RT-PCR of *Epcam* and *Trop2* in rat liver. Whereas *Epcam* was expressed in normal rat liver (cont.) and upregulated in 2-AAF/partial hepatectomy (PH)-treated liver, *Trop2* was not expressed in normal liver but was expressed in 2-AAF/PH-treated liver. N, adult mouse normal liver; O, DDC liver (6 weeks); P, liver 48 hours after 70% PH; C, liver 24 hours after carbon tetrachloride administration.

claudin 4 has been reported to be upregulated in the rat oval cell fraction (Yovchev et al., 2007), the characteristics of mouse oval cells in the DDC model appear similar to those of rat oval cells in the 2-AAF/PH model. In this study, we have focused on two structurally related type-I membrane proteins, EpCAM and TROP2. EpCAM is known to be expressed in many types of normal epithelial cell as well as in tumor cells (Armstrong and Eck, 2003; Went et al., 2004). In the liver, EpCAM is expressed on cholangiocytes but not on hepatocytes (de Boer et al., 1999; Momburg et al., 1987). Consistent with previous studies (Yovchev et al., 2007; Yovchev et al., 2008), real-time PCR showed that *Epcam* was expressed in normal rat liver and its expression was upregulated by 2-AAF/PH (Fig. 1B). As for mouse oval cells, Gleiberman et al. reported that EpCAM is expressed in oval cells upon carbon tetrachloride-induced liver injury (Gleiberman et al., 2005). However, the expression status of EpCAM in DDC liver and the nature of isolated EpCAM⁺ cells have remained unknown. By contrast, *Trop2* was expressed in both rat and mouse injured liver, but not in normal liver (Fig. 1). TROP2 is a member of the EpCAM family and exhibits nearly 50% homology with EpCAM. TROP2 has been shown to be expressed in various tumors, whereas its expression in the liver was not known. We further examined the expression of EpCAM and TROP2 on mouse oval cells in DDC liver and investigated the nature of isolated EpCAM⁺ cells.

Expression of EpCAM in mouse hepatic oval cells

As shown in Fig. 2A, after 4 weeks of DDC diet feeding, the mouse liver turned black because of hepatic porphyria resulting from the inhibition of the heme biosynthetic pathway (Fonia et al., 1996). Hematoxylin and Eosin (H&E) staining was performed in adult normal liver and DDC liver. The DDC liver clearly showed numerous small cells with a large nucleus around the portal veins (Fig. 2B). Immunohistochemistry (IHC) for CK19, a marker for oval cells and

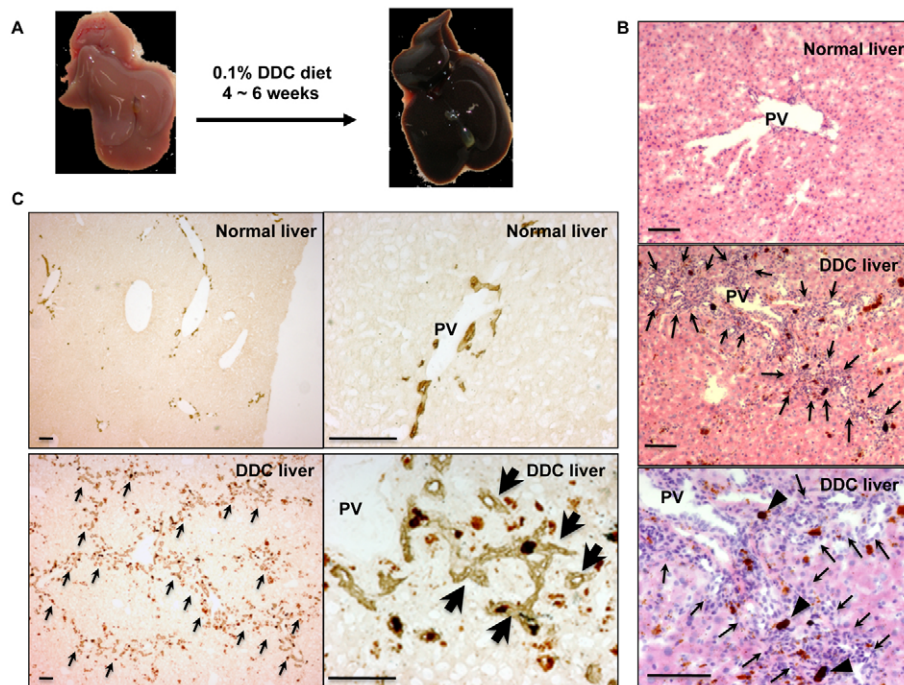


Fig. 2. DDC diet causes hepatic injury and oval cell activation. (A) The liver turned black after mice were fed a DDC diet. (B) H&E staining of a frozen section of mouse normal liver (top) and 4 weeks after DDC feeding (middle and bottom). Numerous small cells appeared around the portal veins in the DDC liver (arrows). The brown clots represent the deposition of iron hemes (arrowheads). (C) Immunohistochemistry (IHC) with anti-CK19 antibody showed that these numerous small cells included CK19-expressing oval cells (arrows) in DDC liver. PV, portal vein. Scale bars: 100 μ m

cholangiocytes, demonstrated that these cells included oval cells as well as other CK19-negative cells, such as inflammatory cells and fibroblasts (Fig. 2C). Since EpCAM expression was upregulated in DDC liver, it was examined by IHC using sections of normal liver and of liver from mice fed DDC for 1 or 4 weeks. EpCAM⁺ bile ducts were located adjacent to the portal vein in normal liver as reported previously (de Boer et al., 1999; Hreha et al., 1999; Joplin et al., 1990), whereas there were many EpCAM⁺ cells forming ductular structures away from the portal vein in DDC liver (Fig. 3A). IHC with both anti-EpCAM and anti-CK19 antibodies demonstrated that all CK19⁺ cells expressed EpCAM in DDC liver (Fig. 3B). Thus, all oval cells expressing CK19 also expressed EpCAM. To further investigate the EpCAM⁺ cells, we generated rat monoclonal antibodies against mouse EpCAM that were applicable for flow cytometry (FCM). FCM of NPCs prepared from DDC liver showed that neither CD45 (PTPRC) nor PECAM was expressed on EpCAM⁺ cells, indicating that EpCAM⁺ cells are not hematopoietic or endothelial cells (Fig. 3C). Furthermore, the isolated EpCAM⁺ cells were individually examined by immunostaining after cytopspin. Almost all the sorted cells were immunostained with anti-CK19 antibody as well as A6 antibody, a mouse oval cell marker (Engelhardt et al., 1993) (Fig. 3D). Hepatic oval cells are known to be highly proliferative. To investigate their proliferation in vivo, the isolated EpCAM⁺ cells were stained with anti-Ki67 antibody (Fig. 3E). Whereas the percentage of Ki67⁺ cells in the isolated EpCAM⁺ cells was ~1% in normal liver, it was 12.2% after 1 week on the DDC diet and increased to 17.4% after 4 weeks (Fig. 3F). These results strongly suggested that EpCAM is expressed in proliferating oval cells and that anti-EpCAM antibody is useful for isolating oval cells from mice fed with DDC.

TROP2 is a novel marker for mouse oval cells

Because TROP2 expression was specifically upregulated in both the mouse and rat injury models with oval cell activation (Fig. 1), TROP2 was anticipated to be expressed in oval cells. To reveal TROP2-expressing cells in normal and injured liver, we performed IHC with anti-EpCAM and anti-TROP2 antibodies. In contrast to

EpCAM, TROP2 was not expressed in normal mouse liver (Fig. 4A). However, numerous TROP2⁺ cells appeared around the portal area in DDC liver (Fig. 4B). Double immunostaining of TROP2 and EpCAM clearly showed that most of the EpCAM⁺ cells co-expressed TROP2 in DDC liver, suggesting that TROP2 is a novel marker for oval cells (Fig. 4B). Although we could not distinguish the original bile duct in DDC liver, almost all EpCAM⁺ cells expressed TROP2. FCM of the NPCs prepared from DDC liver also showed that the expression level of TROP2 and the population of TROP2⁺ cells among EpCAM⁺ cells gradually increased upon ingestion of the DDC diet (Fig. 4C). Consistent with the result of IHC, whereas TROP2 was not present in cholangiocytes expressing EpCAM at day 0, almost all EpCAM⁺ cells became TROP2⁺ in the DDC-fed mice and EpCAM⁺ TROP2⁻ cells were hardly detected after 4 weeks, suggesting that cholangiocytes themselves might also begin to express TROP2 by oval cell activation in the DDC model. Alternatively, it is also possible that TROP2⁺ oval cells differentiate into mature cholangiocytes and replace the pre-existing cholangiocytes damaged by DDC administration.

Characterization of mouse oval cells

To reveal the characteristics of mouse oval cells, the gene expression profile of freshly isolated EpCAM⁺ cells from DDC liver was examined by RT-PCR. As previously reported in rat oval cells, mouse EpCAM⁺ cells also expressed both cholangiocyte markers [*Ck19*, *Ck7* (*Krt7*) and *Ggt1*] and a hepatocyte marker (*Alb*), whereas the other NPCs did not (Fig. 5A). Consistent with the previous report that AFP expression was rarely detected in mouse oval cells (Jelnes et al., 2007), AFP was not detected in mouse EpCAM⁺ cells (data not shown). Rat oval cells were reported to express c-KIT, CD34 and THY1 (Petersen et al., 1998). It was also reported that CD133 (PROM1) is expressed in both mouse and rat oval cells (Rountree et al., 2007; Suzuki et al., 2008; Yovchev et al., 2008). Taking advantage of FCM using the anti-EpCAM antibody, we investigated the expression of these oval cell markers in EpCAM⁺ cells before and after DDC feeding (Fig. 5B) and found

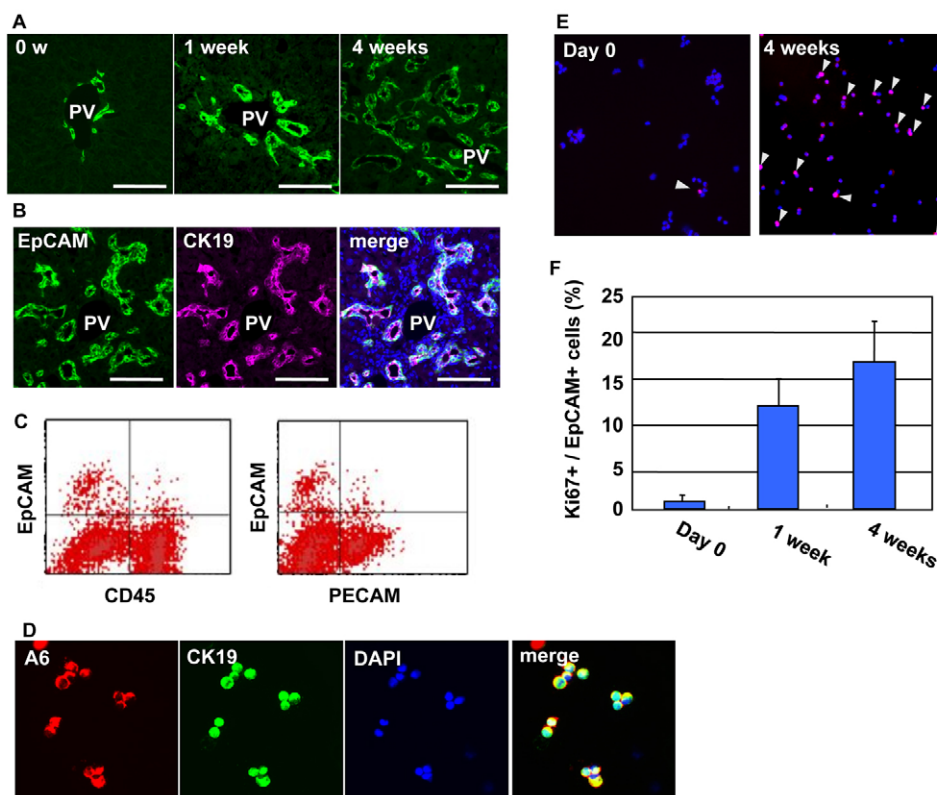


Fig. 3. EpCAM is a cell surface marker for mouse oval cells. (A) IHC of frozen liver sections with anti-EpCAM antibody after DDC feeding. EpCAM was expressed in cholangiocytes around the portal vein of normal mouse liver (0 w). Feeding DDC caused the proliferation of EpCAM⁺ cells (1 and 4 weeks). (B) IHC of frozen liver sections with anti-EpCAM and anti-CK19 antibodies after 4 weeks of DDC feeding. (C) Flow cytometry (FCM) of non-parenchymal cells (NPCs) prepared from the liver of mice fed DDC for 4 weeks with anti-EpCAM antibody and either CD45 or PECAM antibody. EpCAM⁺ cells were negative for CD45 (hematopoietic marker) and PECAM (endothelial marker). (D) Immunostaining of EpCAM⁺ cells with anti-A6 and anti-CK19 antibodies by cytospin. EpCAM⁺ cells expressed both molecules. (E) Immunostaining of EpCAM⁺ cells sorted from normal and DDC liver with anti-Ki67 antibody by cytospin. Many EpCAM⁺ cells from DDC liver were stained with Ki67 (arrowheads). (F) The percentage of Ki67⁺ cells among EpCAM⁺ cells after DDC treatment. The data are derived from five different fields of view. Error bars, s.d. PV, portal vein. Scale bars: 100 μ m.

that c-KIT, CD34 and THY1 were not expressed in EpCAM⁺ cells regardless of injury. The expression pattern of CD133 was similar to that of EpCAM, indicating that both molecules are originally expressed in normal cholangiocytes. By contrast, TROP2 was exclusively expressed in oval cells of DDC liver. These results indicate that TROP2 is induced by oval cell activation and that it is a novel marker for oval cells in mice.

Primary culture of EpCAM⁺ cells from DDC liver

It has been thought that oval cells are proliferative *in vivo* and possess the potential to differentiate into hepatocytic and cholangiocytic lineages. Therefore, oval cells are considered

facultative stem/progenitor cells. To assess such characteristics, we cultured the EpCAM⁺ cells isolated from DDC liver *in vitro*. The EpCAM⁺ cells purified with a cell sorter were seeded onto type-I collagen in the presence of EGF, HGF and IL6. After plating, adherent cells began to proliferate (Fig. 6A) and covered the entire dish after 1 month. The *in vitro* proliferating EpCAM⁺ cells were apparently homogeneous and exhibited epithelial cell morphology even after serial passages (Fig. 6B). They could grow and survive for more than 6 months, maintaining a homogenous morphology. These results suggested that EpCAM⁺ cells derived from DDC liver include cells with high proliferative potential.

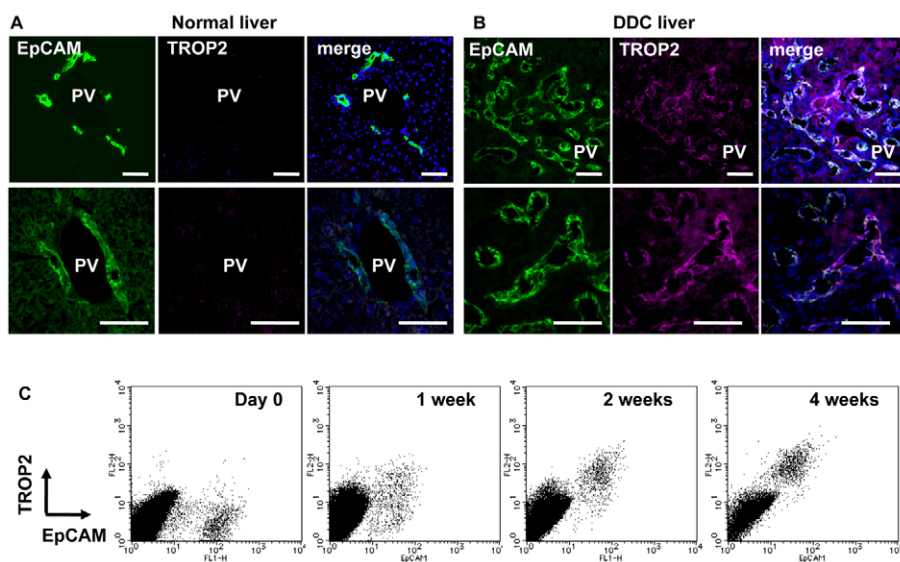


Fig. 4. Expression of EpCAM and TROP2 in normal and injured mouse liver.

(A, B) IHC of frozen sections of normal liver (A) and the liver of mice fed DDC for 5 weeks (B) with anti-EpCAM and anti-TROP2 antibodies. TROP2 was expressed in oval cells but not in normal cholangiocytes. (C) FCM of NPCs with anti-EpCAM and anti-TROP2 antibodies after DDC feeding. TROP2 begins to be expressed in EpCAM⁺ cells as DDC feeding proceeds. PV, portal vein. Scale bars: 100 μ m.

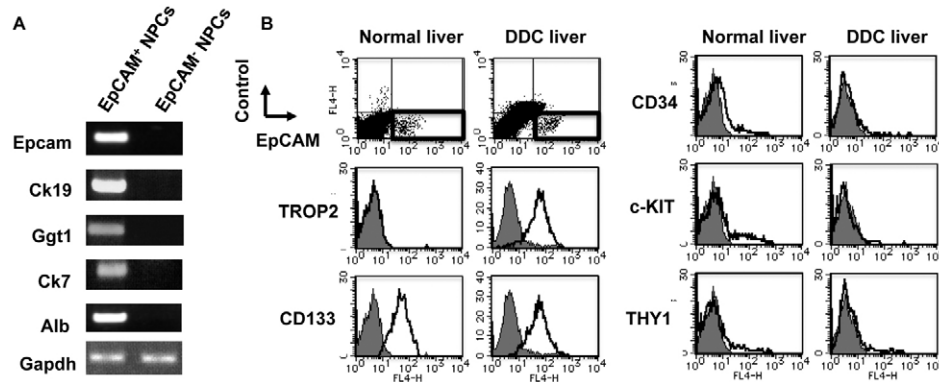


Fig. 5. Characterization of freshly isolated EpCAM⁺ cells. (A) RT-PCR of freshly isolated EpCAM⁺ and EpCAM⁻ cells from the liver of mice fed DDC for 4 weeks. NPCs from DDC liver were divided into EpCAM⁺ and EpCAM⁻ cells by FACS Vantage, then RT-PCR was performed. (B) FCM of EpCAM⁺ cells from normal and DDC livers with known oval cell markers. EpCAM⁺ cells surrounded by bold lines were reanalyzed with other antibodies (TROP2, CD133, CD34, c-KIT, THY1) as shown.

Characterization of the cell lines established from EpCAM⁺ cells

As in freshly isolated EpCAM⁺ cells, CK19 and ALB were expressed in the established cell lines after 30 days of culture, as evidenced by RT-PCR and immunostaining (Fig. 6C,D). *Afp*, a marker of fetal liver progenitor cells, was expressed in the cultured cells (Fig. 6C), whereas it was not expressed in freshly isolated mouse oval cells, suggesting that more immature cells might be selected from EpCAM⁺ cells of NPCs in DDC liver. Alternatively, these proliferating cells might acquire a more hepatoblast-like character when cultured in vitro as previously reported (Schmelzer et al., 2007). In accordance with the latter idea, *Epcam* expression gradually decreased in culture (Fig. 6C). At the onset of mouse liver development, EpCAM is highly expressed in delta-like 1 homolog (DLK1)-positive hepatoblasts; however, its expression in hepatoblasts is dramatically reduced as liver development proceeds (our unpublished data). The reduction of EpCAM expression in cultured cells observed in this study is in line with the possibility that EpCAM⁺ cells might shift from an HSC-character to a more hepatoblast-like character.

To investigate the bipotency of cultured hepatic stem-like cells derived from EpCAM⁺ cells (HSCEs) at the clonal level, we randomly picked several clones and finally established 12 independent cell lines (HSCE1-12). All the expanded clones expressed both hepatocytic (*Alb*) and cholangiocytic (*Ck7*, *Ck19*) genes (see Table S2 in the supplementary material). The expression of *c-Met*, which is known to be expressed in oval cells, was also detected in all clones. Whereas tyrosine aminotransferase (*Tat*), a marker of perinatal hepatocytes, was weakly expressed in some of the clones, glucose-6-phosphatase (*G6Pase*; *G6pc*), another marker of perinatal hepatocytes, and carbamoyl phosphate synthetase (*Cps*), an adult hepatocyte marker, were rarely expressed (2/12 and 0/12, respectively). *Afp*, an immature hepatocyte marker, was expressed in most of the clones (10/12). These results indicated that HSCEs maintain immature characteristics.

Differentiation of clonally expanded EpCAM⁺ cells into the hepatocytic lineage in vitro

To address whether the established clones can differentiate into hepatocytic and cholangiocytic lineages, we used two clones, HSCE1 and HSCE2, and examined their differentiation potential. To induce hepatocytic differentiation and maturation, we utilized an in vitro culture system reported previously (Kamiya et al., 1999; Kamiya et al., 2002). Oncostatin M (OSM) is a powerful inducer of the differentiation of fetal hepatocytes and the addition of

Engelbreth-Holm-Swarm (EHS) gel enhances hepatocyte maturation. Dimethyl sulfoxide (DMSO) is also known to maintain the differentiation of hepatocytes in culture (Isom et al., 1985; Sakai et al., 2002). By combining these methods, the expression of metabolic enzymes and transporters was monitored in two clones (Fig. 7A). Because both clones exhibited a similar profile, only the results of HSCE1 are shown. The expression of *G6Pase*, *Cps*, *Tat*, tryptophan-2,3-dioxygenase (*Tdo2*) and solute carrier family 10 (sodium/bile acid cotransporter family), member 1 (*Slc10a1*) was markedly induced by the addition of OSM, DMSO and EHS gel (Fig. 7B). By contrast, the expression of *Ck19* and of *Afp*, a marker of immature fetal hepatocytes, was downregulated. In addition, the PAS reaction showed that there were many clusters of hepatocytic

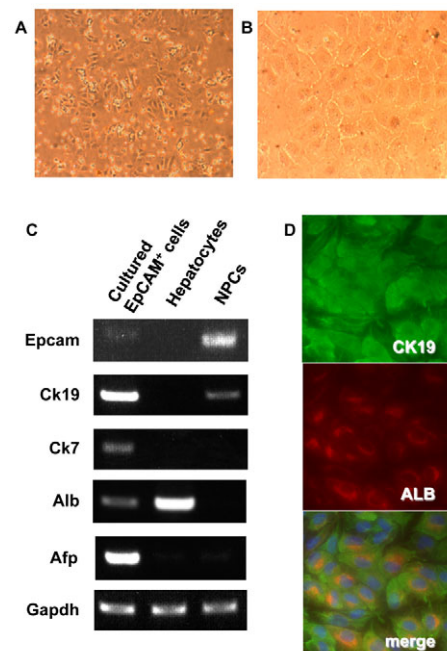


Fig. 6. EpCAM⁺ cells derived from DDC liver have high proliferative potential. (A,B) In vitro culture of EpCAM⁺ cells. Freshly isolated EpCAM⁺ cells from DDC liver were seeded on type-I collagen-coated dishes in the presence of HGF, EGF and IL6. The morphology of the cells after 5 days of culture (A) and after several passages (B) is shown. (C) RT-PCR of EpCAM⁺ cells after 30 days of culture. *Afp* was strongly expressed in the cultured cells. (D) Immunostaining of the cultured cells with anti-CK19 and anti-ALB antibodies.

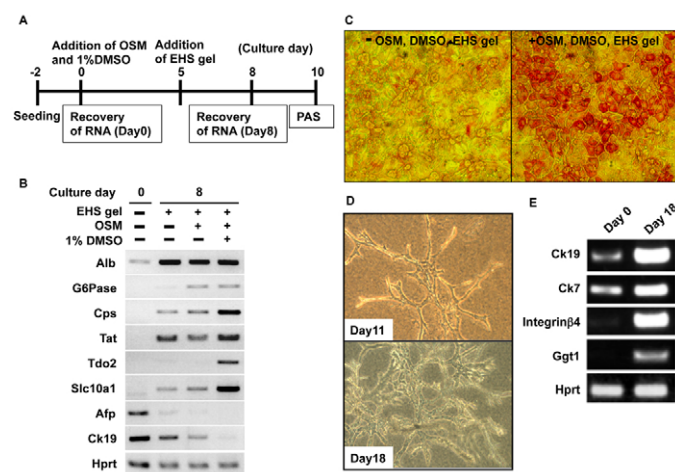


Fig. 7. Clonally expanded HSCEs can differentiate into both hepatocytes and cholangiocytes. (A) Experimental design for the differentiation of hepatic stem-like cells derived from EpCAM⁺ cells (HSCEs) into hepatocytic cells. (B) RT-PCR of clone HSCE1 after hepatocytic differentiation. The addition of OSM, DMSO and EHS gel strongly induced the expression of hepatocytic genes and downregulated that of hepatoblastic and cholangiocyte genes. (C) PAS staining of HSCE1. The addition of OSM, DMSO and EHS gel strongly induced the accumulation of glycogen. (D) Morphological changes of HSCE1 after cholangiocyte differentiation. Tubules and branching morphology were clearly observed after 11 days of culture. (E) RT-PCR of HSCE1 after cholangiocyte differentiation. The expression of cholangiocyte marker genes was markedly upregulated in HSCE1.

cells accumulating glycogen (Fig. 7C). These results strongly suggested that clonally expanded HSCEs differentiate into the hepatocytic lineage.

Differentiation of clonally expanded EpCAM⁺ cells into the cholangiocyte lineage in vitro

To evaluate the potential for differentiation into cholangiocytes, we utilized a three-dimensional collagen gel culture that is effective for the formation of tubules (Nishikawa et al., 1996; Tanimizu et al., 2004a). A cell suspension in collagen type-I gel was plated onto a basal layer of a collagen type-I gel and then culture medium containing HGF, an inducer of tubulogenesis, was loaded on top. After 11 days of culture, the formation of tubules was observed, and after 18 days of culture branching structures were clearly evident (Fig. 7D). In addition, RT-PCR revealed that the expression of cholangiocyte marker genes was upregulated in the cultured cells (Fig. 7E), indicating that HSCEs differentiated into the cholangiocyte lineage. Thus, EpCAM was expressed on oval cells in DDC liver and the clonally expanded cells from sorted EpCAM⁺ cells exhibited characteristics of HSCs, i.e. unlimited proliferation and bi-directional differentiation. However, it remained unclear whether the emergence of such potential HSCs was an event restricted to liver injury and whether they were derived from oval cells.

EpCAM⁺ cells in normal liver include potential HSCs

Because EpCAM⁺ cells are present in normal mouse liver, we investigated whether potential HSCs are present among EpCAM⁺ cells of normal liver. Interestingly, the colony formation assay revealed that sorted EpCAM⁺ cells from normal liver formed both

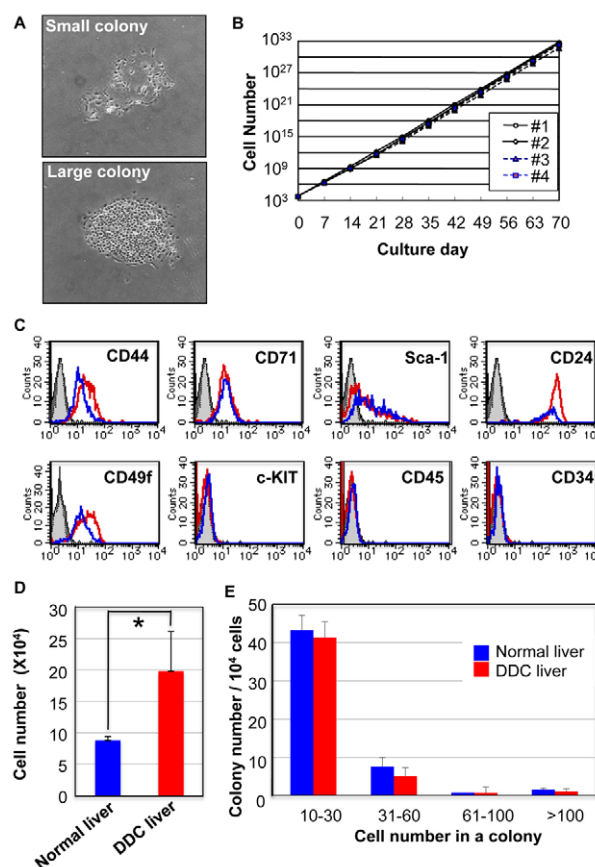


Fig. 8. Comparison between EpCAM⁺ cells isolated from normal and injured liver. (A) Colony formation assay of EpCAM⁺ cells from normal liver. Representative morphology of a small colony (top) and a large colony (bottom). Large colonies composed of more than 100 cells after 9 days of culture proliferate exponentially. (B) Unlimited cell proliferation of the established clones (#1-#4) in in vitro culture. (C) Comparison of cell surface markers between HSCEs from normal (blue line) and injured (red line) liver by FCM. Control IgG is in gray. The expression profiles of cell surface markers were similar in both HSCEs. (D) The number of EpCAM⁺ cells per normal (n=4) or injured (n=6) liver. The number was estimated from the percentage of EpCAM⁺ cells after immunomagnetic bead selection (see Fig. S2 in the supplementary material). There was a significant increase in EpCAM⁺ cells in DDC liver (*P<0.01). (E) Colony formation assay of EpCAM⁺ cells from normal and injured liver of mice fed DDC for 4 weeks. The data are derived from four independent experiments. Error bars, s.d.

small and large colonies (Fig. 8A). These large colonies, which were composed of more than 100 cells, continued to proliferate, and these clones propagated continuously and exhibited bipotency similar to the HSCE clones derived from DDC liver (Fig. 8B and data not shown). In addition, HSCEs derived from normal and DDC liver exhibited a similar expression pattern of cell surface markers, strongly suggesting that they are closely related (Fig. 8C). By contrast, EpCAM⁻ cells from normal or DDC liver did not give rise to any colonies, even when ten times more cells were plated than for EpCAM⁺ (data not shown). If oval cells themselves have characteristics of potential HSCs then oval cell activation by the DDC diet should increase the number of potential HSCs. To test this possibility, we compared the number of EpCAM⁺ cells and potential HSCs between normal and DDC liver (see Fig. S1 in the supplementary material). As shown in Fig. 8D, the number of

EpCAM⁺ cells recovered from a DDC liver was approximately twice that from a normal liver. Interestingly, the numbers of large colonies corresponding to potential HSCs were very similar for normal and injured liver (Fig. 8E). In addition, adult potential HSCs are a very small population of EpCAM⁺ cells in normal and injured liver. Although we cannot exclude the possibility that potential HSCs might increase slightly (not more than 2-fold), it is unlikely that the activation of oval cells by liver injury significantly increases the number of potential HSCs.

DISCUSSION

Oval cells have been considered adult liver stem/progenitor cells (Fausto, 2004; Oertel and Shafritz, 2008). However, previous studies were mostly histochemical, or involved biochemical and molecular biological characterization using cell fractions prepared by density gradient techniques. FCM is a powerful means of characterizing a particular type of cell, as successfully demonstrated by the identification of a very rare population of hematopoietic stem cells using a combination of cell surface markers (Osawa et al., 1996). In fetal liver, we and others reported the isolation and characterization of hepatoblasts by FCM using several markers (Kubota and Reid, 2000; Suzuki et al., 2002; Tanimizu et al., 2003). Although definitive proof of stemness requires a clonal analysis of freshly isolated oval cells, a lack of specific markers has hampered the precise identification and prospective isolation of oval cells from NPCs by FCM. To address this issue, we first searched for cell surface molecules expressed on oval cells and showed that anti-EpCAM antibody is useful for isolating oval cells from the liver of DDC-fed mice by cell sorting. Clonal analyses provide strong evidence that the EpCAM⁺ cells from DDC liver contain adult potential HSCs that possess the capacity for unlimited proliferation and bi-directional differentiation.

Hepatic stem/progenitor cells have been considered to contribute to the regeneration of damaged liver when the proliferation of hepatocytes is restricted, and cell lines with HSC characteristics have been established from chemically damaged and/or genetically dysfunctional liver (Braun et al., 1987; Dumble et al., 2002; Sugiyama et al., 1997; Suzuki et al., 2008; Tirmitz-Parker et al., 2007; Yin et al., 1999). However, it remained unclear whether such cells are present in normal adult liver. Cell lines with HSC characteristics have been established from normal mouse (Fougere-Deschatrette et al., 2006), human (Herrera et al., 2006) and rat (Sahin et al., 2008) liver. However, these cell lines were derived from a hepatocyte-enriched cell population by centrifugation using a Percoll gradient or from unfractionated cells, leaving their origin ambiguous. Recently, Schmelzer et al. reported that EpCAM⁺ cells from postnatal human donors include HSCs (Schmelzer et al., 2007). In this report, we demonstrate that EpCAM⁺ cells isolated from normal or DDC mouse liver by FCM include potential HSCs. Interestingly, only a limited population of EpCAM⁺ cells formed large colonies and the number of potential HSCs was not significantly increased in DDC liver compared with normal liver. Thus, our results strongly suggest that most mouse oval cells do not possess self-renewal potential and are likely to represent transit-amplifying cells that differentiate into mature hepatic cells.

Cholangiocytes proliferate under various pathological conditions. Cholangiocytes proliferate from pre-existing ducts in portal areas after PH or bile duct ligation in rats. Oval cell proliferation or ductular hyperplasia is induced by a number of chemicals, including 2-AAF, DDC (Preisegger et al., 1999) and by a choline-deficient ethionine-supplemented diet (Tian et al., 1997), and the proliferating

cells form disorganized tubular structures that sprout into liver lobules. Selective damage of the periportal zone reduces such proliferation, supporting the notion that oval cells derive from the periportal region, in particular from the canals of Hering that connect the bile canaliculus and the biliary tree (Paku et al., 2001). However, the origin of oval cells is controversial and bone marrow stem cells were suggested to be a source (Petersen et al., 1999; Sell, 2001), although several reports refute this possibility (Menthena et al., 2004; Wang et al., 2003). Oval cells and cholangiocytes are not clearly distinguishable at the molecular level. In fact, oval cells and normal cholangiocytes are known to express many intracellular and membrane proteins in common, including EpCAM and CD133. However, it is also reported that some genes are predominantly expressed in rat or mouse oval cells. The expression of THY1, c-KIT and CD34 in oval cells has been controversial and these are also found in hematopoietic and mesenchymal cells (Dezso et al., 2007; Yovchev et al., 2008). By contrast, TROP2 is not expressed at all in normal liver and only in the oval cells of injured liver, indicating that TROP2 is a useful marker for oval cells.

Intriguingly, after oval cell activation, most of the EpCAM⁺ cells express TROP2, and the original normal cholangiocytes, i.e. the EpCAM⁺ TROP2⁻ cells, are hardly detected by FCM and immunostaining. These results raise the possibility that not only oval cells but also activated cholangiocytes might begin to express TROP2 after liver injury and partly contribute to oval cell proliferation in this mouse model (Fig. 9). Alternatively, oval cells that are activated to proliferate during DDC administration and that express both EpCAM and TROP2 might continue to express both of these markers at least transiently after they differentiate into mature cholangiocytes. Further investigation into the expression profile of TROP2 in cholangiocytes would provide a clue as to the origin of oval cells. Moreover, the function of EpCAM and TROP2 in oval cell activation is an interesting issue that remains to be addressed.

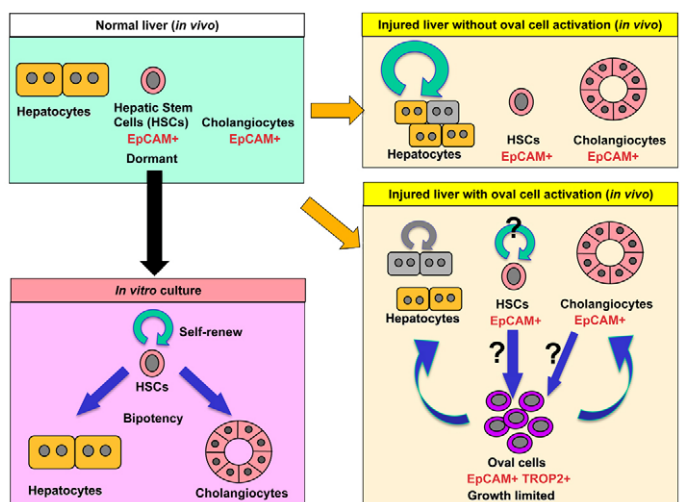


Fig. 9. Model of the hepatic stem/progenitor cell system in vivo and in vitro. Potential HSCs exist in normal liver as EpCAM⁺ cells. They can proliferate unlimitedly and differentiate into both hepatocytes and cholangiocytes in vitro. Upon liver injury without oval cell activation, hepatocytes proliferate and contribute to liver regeneration. Upon liver injury with oval cell activation, EpCAM⁺ TROP2⁺ cells appear around portal veins to regenerate the liver. The oval cells might be partly derived from EpCAM⁺ cholangiocytes. Most oval cells lacked the potential to self-renew in the in vitro colony formation assay.

Oval cells are believed to be involved in the regeneration of liver following injury. In addition, they are also considered as a cellular precursor for hepatocellular carcinoma (HCC) (Knight et al., 2005). Most of the experimental procedures used to induce oval cell proliferation in the liver ultimately lead to tumorigenesis. However, the connection between oval cells and tumorigenesis remains unclear. EpCAM is expressed on many normal as well as neoplastic epithelial cells and tumor-initiating cells (Al-Hajj et al., 2003; Armstrong and Eck, 2003; Momburg et al., 1987). Most recently, Maetzel et al. reported that EpCAM is a potent signal transducer and that its cleaved intracellular domain utilizes components of the Wnt pathway to induce cell proliferation (Maetzel et al., 2009). Such signaling via EpCAM might be involved in oval cell proliferation. Conversely, *Epcam* is itself a Wnt/ β -catenin signaling target gene in HCC cell lines (Yamashita et al., 2007). Consistently, the involvement of the Wnt/ β -catenin pathway in the oval cell response has been reported (Apte et al., 2008; Hu et al., 2007; Yang et al., 2008). We recently reported that *Wnt7a*, *Wnt7b* and *Wnt10a* are upregulated in DDC liver and that the Wnt/ β -catenin pathway is likely to be involved in oval cell activation in vivo (Itoh et al., 2009). Therefore, the correlation between tumorigenesis and the expression of EpCAM in oval cells is plausible. Because EpCAM is widely expressed in normal epithelial cells, including biliary epithelial cells, the expression and signaling of EpCAM should be tightly regulated at the steady state. However, the regulation of EpCAM signaling in normal and malignant stem-like cells remains unknown.

It is also reported that TROP2 is expressed on cells of some normal tissues as well as on cancer cells (El Sewedy et al., 1998; Huang et al., 2005; Ohmachi et al., 2006). Our finding that TROP2, a member of the EpCAM family, is upregulated in oval cells raises the possibility that TROP2 might modulate and/or enhance the intracellular signaling of EpCAM to promote proliferation and migration into liver parenchyma. Alternatively, TROP2 itself might transduce intracellular signaling in a manner similar to EpCAM. In fact, ectopic expression of TROP2 in NIH3T3 cells is sufficient to promote both anchorage-independent growth and tumorigenesis (Wang et al., 2008). Most recently, Goldstein et al. reported that TROP2 identifies a subpopulation of murine and human prostate basal cells with stem cell characteristics (Goldstein et al., 2008), suggesting an association between TROP2 and stem/progenitor cells.

Although chronic liver injury induces the proliferation of oval cells in some rodent models and in human disease, the underlying relationship between oval cells and HSCs and their developmental mechanisms require further investigation. We showed the presence of potential HSCs in normal liver, as well as in injured liver, by isolating EpCAM⁺ cells and demonstrated the expression of TROP2 in oval cells. Further investigation into the interaction between EpCAM and TROP2 in oval cells will help us to understand the mechanisms of growth and differentiation in cancer-initiating cells as well as in normal stem/progenitor cells.

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Supplementary material

Supplementary material available online at
<http://dev.biologists.org/cgi/content/full/136/11/1951/DC1>

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