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Stage-specific regulation of adhesion molecule expression segregates epithelial stem/progenitor cells in fetal and adult human livers

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Abstract
Purpose Regulated expression of cell adhesion molecules could be critical in the proliferation, sequestration, and maintenance of stem/progenitor cells. Therefore, we determined fetal and adult stage-specific roles of cell adhesion in liver cell compartments.
Methods We performed immunostaining for the adhesion molecules, E-cadherin and Ep-CAM, associated proteins, β-catenin and α-actinin, hepatobiliary markers, albumin, α-fetoprotein, and cytokeratin-19, and the proliferation marker, Ki-67. Expression of albumin was verified by in situ mRNA hybridization.
Results In the fetal liver, hepatoblasts showed extensive proliferation with wide expression of E-cadherin, β-catenin, and α-actinin, although Ep-CAM was expressed in these cells less intensely and focally in the cell membrane to indicate weak cell adhesion. Hepatoblasts in ductal plate and bile ducts showed less proliferation and Ep-CAM was intensely expressed in these cells throughout the cell membrane, indicating strong adhesion. In some ductal plate cells, β-catenin was additionally in the cytoplasm and nucleus, suggesting active cell signaling by adhesion molecules. In adult livers, cells were no longer proliferating and E-cadherin, β-catenin, and α-actinin were expressed in hepatocytes throughout, whereas Ep-CAM was expressed in only bile duct cells. Some cells in ductal structures of the adult liver with Ep-CAM coexpressed albumin and cytokeratin-19, indicating persistence of fetal-like stem/progenitor cells.
Conclusions Regulated expression of Ep-CAM supported proliferation in fetal hepatoblasts through weak adhesion and helped in biliary morphogenesis by promoting stronger adhesion in hepatoblasts during this process. Restriction of Ep-CAM expression to bile ducts in the adult liver presumably facilitated sequestration of stem/progenitor cells. This stage-specific and cell compartment-related regulation of adhesion molecules should be relevant for defining how liver stem/progenitor cells enter, exit, and remain in hepatic niches during both health and disease.

Keywords Adhesion molecules · Cell proliferation · Stem cells

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Introduction

Liver development requires regulated controls of cell growth and cell differentiation, through complex interplays between intrinsic cell- and extrinsic membrane-bound and soluble signals [1], and signaling from other cells, for example, embryonic endothelial cells [2]. Tissue homeostasis requires that epithelial cells adhere variably to extracellular matrix components and stroma during cell proliferation, migration, and function [3]. During these processes, cell adhesion molecules, such as cadherins, selectins, integrins, and

immunoglobulin superfamily members, serve critical roles. Adhesion molecules can regulate intracellular signaling, and by regulating their own activities, as exemplified by keratinocytes undergoing terminal differentiation and stratification, where E-cadherin regulated β1-integrin expression [4], may participate in processes relevant to differentiation of stem/progenitor cells. Among adhesion molecules, the epithelial cell adhesion molecule, Ep-CAM, displays unique structure, including epidermal growth factor-like repeats and α-actinin-binding sites [5, 6]. Ep-CAM is expressed in virtually all epithelial cells, with only mature hepatocytes or squamous cells constituting exceptions [7, 8]. Moreover, Ep-CAM expression promotes cell proliferation [8]. Also, adhesion molecules share intracellular signaling partners, for example, α-actinin, which is a constituent of focal adhesion complexes and multiple additional ligands [9], or β-catenin, which is a key member of Wnt signaling pathway [3].

Coordinate regulation of the cell adhesion molecules and their intracellular partners, for example, through interactions between E-cadherin and β-catenin, serves to alter cellular behavior, including persistence of stem cells in niches. For instance, in the small intestine, crypt stem/progenitor cells produce transit-amplifying cells that move upward during replenishment of the villous epithelium or other enterocyte lineages, and downward during the production of Paneth cells [10]. In intestinal crypt areas containing stem cells, β-catenin is expressed in the nucleus at higher levels, reiterating cell adhesion-based regulation of stem/progenitor cell properties.

In this study, we examined fetal stage–specific processes in the liver, where epithelial cells are under development and stem/progenitor cells show proliferation [11–13], and the adult liver, where despite the absence of cell cycling, hepatic progenitor cells may be activated by liver injury or oncogenesis [14–19]. Our consideration was that study of these tissues will offer insight into cell type–specific mechanisms by which expression of adhesion molecules and their relevant intracellular partners was regulated. As adhesion molecules regulate activities of each other, as documented by interactions between Ep-CAM and cadherins [20], we addressed whether differential expression of adhesion molecules will promote proliferation in given liver cell compartments and also help segregate specific cell subpopulations in niches within the liver.

Materials and methods

Materials

Human-specific antibodies were for Ep-CAM (MCA850H, Serotec Ltd., Raleigh, NC), albumin, α-fetoprotein (A6684 and A8452, respectively, Sigma Chemical Co., St. Louis, MO), cytokeratin (CK)-19 (sc-6278, Santa Cruz Biotechnology Inc., Santa Cruz, CA), α-actinin (BP210, Novus Biologicals, Littleton, CO), β-catenin (RB-9035-PO, Neo-Makers, Fremont, CA), E-cadherin (15148-500, Abcam, Cambridge, MA), and Ki-67 (550609, Pharmingen Inc., San Diego, CA). Peroxidase-conjugated mouse-specific goat IgG (A3682), rabbit-specific goat IgG (A1949), and all chemicals were from Sigma. Immunoreagents included Alexa Fluor 488 rabbit-specific goat IgG (A11008, Molecular Probes, Invitrogen Corp., Carlsbad, CA) and Cy3-AffiniPure mouse-specific goat IgG (115-165-205, Jackson ImmunoResearch Labs. Inc., West Grove, PA).

Tissues

The Committee on Clinical Investigations at Albert Einstein College of Medicine approved use of human material. Serial sections were obtained from formalin-fixed, paraffin-embedded surgical pathology specimens of aborted fetal tissues from 7, 12, 15, 16, and 22 weeks of gestation. A total of 10 fetal specimens were studied. Archival serial sections from three formalin-fixed and paraffin-embedded normal adult human livers were available from a previous study [21]. Additional 6 µm thick cryosections were prepared from three other normal adult human livers and two fetal livers of 17 weeks' gestation.

Immunohistochemistry

Sections were deparaffinized by warming to 65°C for 2 h followed by two 10 min washes in xylene and dehydration in graded ethanol. Subsequently, for antigen retrieval, slides were immersed in 10 mM citrate buffer, pH 6.0, heated in a microwave oven for 10 min, and cooled for 20 min at room temperature. For immunostaining, sections were blocked in 3% goat serum in phosphate-buffered saline, pH 7.4 (PBS), for 1 h at room temperature and incubated with primary antibodies in PBS with 3% goat serum (Ep-CAM, undiluted; albumin, 1:100; CK-19, 1:100; α-fetoprotein, 1:100; α-actinin, 1:20; β-catenin, 1:250; E-cadherin, 1:30; and Ki-67, 1:25 dilutions) for 16–18 h at 4°C. The sections were incubated with peroxidase-conjugated mouse- or rabbit-specific goat IgG (1:300) for 1 h at room temperature with color development over 3–10 min in diaminobenzidine. All sections were processed identically after conditions had been optimized.

For fluorescence microscopy, 5 µm cryosections were fixed in 4% paraformaldehyde in PBS for 5 min at 4°C, washed twice for 5 min each in PBS and blocked in 3% goat serum for 30 min at room temperature. Primary

antibodies (*β*-catenin, 1:250; Ep-CAM, undiluted) were applied either separately or together for 1 h at room temperature. Tissue sections were washed four times in PBS for 5 min each and incubated with secondary antibodies (Alexa Fluor 488 goat IgG, 1:500; Cy3-AffiniPure goat IgG, 1:500) for 30 min in the dark. Sections were washed four times for 5 min each in PBS, counterstained with 4,6-diamidino-2-phenylindole (DAPI, D3571, Molecular Probes) and examined under epifluorescence.

In situ hybridization

A 330-base-pair fragment was excised from a full-length human albumin cDNA plasmid with *Xba* I and *Nco* I enzymes, which cleave nucleotides 850 and 1,179, according to the NM 000477 human albumin locus. This corresponded to the albumin sequence between exons 7 and 9 (positions 9,541 and 12,572; NCBI human albumin Gene ID, 213). Purified albumin fragment was subcloned into pGEM-3Z plasmid (Promega Corp., Madison, WI) with verification of insert orientation by DNA sequencing. The pGEM-3Z-Alb plasmid was linearized with either *EcoR*I or *Hind*III for 6 h, and linearized DNA was extracted with phenol/chloroform and recovered by ethanol precipitation. Digoxigenin-labeled sense- and antisense-strand RNA probes with SP6 and T7 RNA polymerases, respectively, were synthesized (DIG RNA Labeling Kit; Roche Applied Science, Indianapolis, IN) according to the manufacturer. Briefly, 20 μl of master mix for each probe was prepared to 1 mM ATP, CTP, GTP, 0.65 mM UTP, 1 unit/μl RNase inhibitor, and 2 unit/μl SP6 or T7 RNA polymerases, and samples were incubated for 2 h at 37°C. Two microliters of 10 unit/μl RNase-free DNase I and 10 μl of total yeast RNA (10 mg/ml) were added to each sample for 30 min at 37°C to destroy DNA templates. Probes were precipitated with 3 volumes 95% ethanol and 0.1 volume 5 M LiCl for 3 h at −80°C, recovered under 13,000*g* for 20 min at 4°C, and suspended in 90 μl water, 5 μl total yeast RNA, and 5 μl 5 M LiCl. This was followed by two precipitations using 5 μl (first time) and 2.5 μl (second time) of total yeast RNA and LiCl. Probes were suspended in 100 μl water with 1 μl RNAse inhibitor. Probe yield was estimated in ethidium bromide gels, and labeled probes were stored under −80°C until use.

Tissue sections of 5 μm thickness were subjected to in situ hybridization, essentially as described previously [22], with minor modifications. After fixation in ethanol, washing, and dehydration, endogenous peroxidase activity was blocked by treating sections for 30 min at room temperature with 3% hydrogen peroxide in methanol. Fifty nanograms of digoxigenin-labeled riboprobes were used per slide with hybridization for 18 h at 45°C. Sections were incubated for 1 h in 2% blocking solution (Roche) and incubated for 2 h with peroxidase-conjugated digoxigenin antibody (1:100, Roche) in 1% blocking solution followed by color development over 20 min with diaminobenzidine (DAB).

Tissue analysis

For morphometry, multiple areas of tissue sections were randomly analyzed using portal areas for centering under 400× magnification. To determine the proliferation index, the fraction of Ki-67-stained cells per 1,000 cells was obtained. Three independent observers graded tissues in a blinded fashion and interobserver differences were reconciled by consensus. The intensity of gene expression was graded semiquantitatively from 0 (negative) to 4 (maximally positive staining in any tissue).

Statistical methods

Where applicable, significance of differences was analyzed by *t*-tests, χ^2 tests, or analysis of variance (ANOVA). P values < 0.05 were considered significant.

Results

The general liver organization after 7 weeks of gestation differed from later gestational stages, where the acinar structure was better defined and discrete portal and perivenous areas became apparent. Bile ducts and ductal plates were not well formed in fetal livers between 7 and 12 weeks, and mature-appearing bile ducts were observed only after 15 weeks. At all stages, fetal livers contained a large number of hepatoblasts and hematopoietic cells (Fig. 1). The architecture of adult livers was normal without hepatic injury, steatosis, mitotic activity, or bile duct proliferation.

E-cadherin expression was not different in subsets of parenchymal liver cells

E-cadherin was well expressed in the membrane of parenchymal and ductal cells in fetal and adult livers (Figs. 2a–d). Similarly, α-actinin was expressed in both fetal and adult liver cells. As α-actinin is a component of focal adhesion complexes, this likely indicated that cell signaling mediated by focal adhesion complexes was active in various cell types. However, cell-to-cell variability in the intensity of α-actinin expression in the fetal liver may have

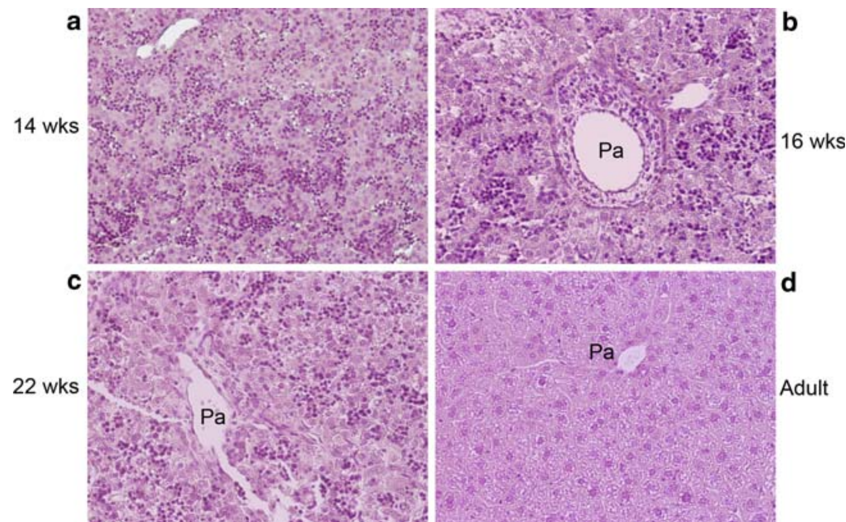

Fig. 1 General tissue organization showing representative examples of fetal livers (14-, 16-, and 22-week gestations) and adult liver to indicate the integrity of tissues studied. Note that fetal tissues contain large numbers of hematopoietic cells. Since acinar organization and biliary morphogenesis requires time, the liver after 14 weeks of gestation is shown to indicate mostly vascular spaces (**a**), which is particularly the appearance of fetal livers between 7 and 10 weeks of gestation, while the ductal plate surrounding the portal area is more readily seen in the liver after 16 weeks of gestation (**b**) and bile duct development is more advanced in the liver after 22 weeks of gestation (**c**). By contrast, the adult liver shows absence of hematopoietic cells and ductal plate structure (**d**). Pa = portal area. Original magnification, 200×; hematoxylin and eosin staining

been consistent with some differences (Fig. 2a). On the other hand, in ductal plate cells in the fetal liver, the closely associated partner of E-cadherin, β-catenin, was often more abundant in the cell membrane and/or cytoplasm. In some ductal plate cells, we observed β-catenin in the nucleus as well (Fig. 2a and see below). In the adult liver, β-catenin was expressed in bile duct cells at relatively lower levels (Fig. 2d), whereas fetal bile duct cells expressed β-catenin more intensely (Fig. 2b), suggesting differences in cell responses to adhesion-mediated signals.

Ep-CAM was differentially expressed in liver cells

Ep-CAM was expressed throughout the first and second trimesters, although with qualitative and quantitative changes during that period. At 7 weeks, 50–70% of parenchymal cells expressed Ep-CAM, although expression was low-grade (1.3 ± 0.1). Only occasional periportal cells expressed Ep-CAM at higher intensity (grade 2.7 ± 0.1) (Fig. 3a). Midgestation between 12 and 16 weeks, 60–90% parenchymal cells expressed Ep-CAM, with higher expression intensity (grade 2.1 ± 0.1), compared to that at 7 weeks (Fig. 3b and c). At 12–16 weeks, Ep-CAM was most intensely expressed in ductal plate cells, grade 3.3 ± 0.1, $P < 0.05$, χ^2 test. Ep-CAM expression in biliary cells was lower intensity, grade 2.4 ± 0.1, compared to

ductal plate cells, $P < 0.05$, χ^2 test. At 22 weeks, intensity of Ep-CAM expression in parenchymal liver cells and bile duct cells was similar to midgestational stages (Fig. 3d). By contrast, in the adult liver, Ep-CAM was expressed in only bile duct cells (Fig. 3e). The intensity of Ep-CAM expression in the adult liver was much less: grade 1.1 ± 0.0. These observations indicated developmental stage—and cell type—specific regulation of this adhesion molecule.

Intracellular distribution of Ep-CAM was also different

To verify the distribution pattern of Ep-CAM in liver cells, we analyzed additional tissues with fluorescence microscopy (Fig. 4). These studies verified that Ep-CAM expression was different in fetal liver cell subsets. For instance, in ductal plate and bile duct cells, Ep-CAM was distributed throughout the cell membrane (Fig. 4a). By contrast, Ep-CAM was distributed focally in the cell membrane of hepatoblasts in the liver parenchyma (Fig. 4b). As before, in the adult liver, Ep-CAM was present in only bile duct cells (Fig. 4c and d).

Relationship between Ep-CAM and β-catenin expression

In further studies, to colocalize β-catenin and Ep-CAM in cells, we costained Ep-CAM and β-catenin along with DAPI counterstaining to visualize cell nuclei (Fig. 5). These studies demonstrated interspersed cells in ductal plates with high-level expression of Ep-CAM in the cell membrane with cytoplasmic and nuclear localization of

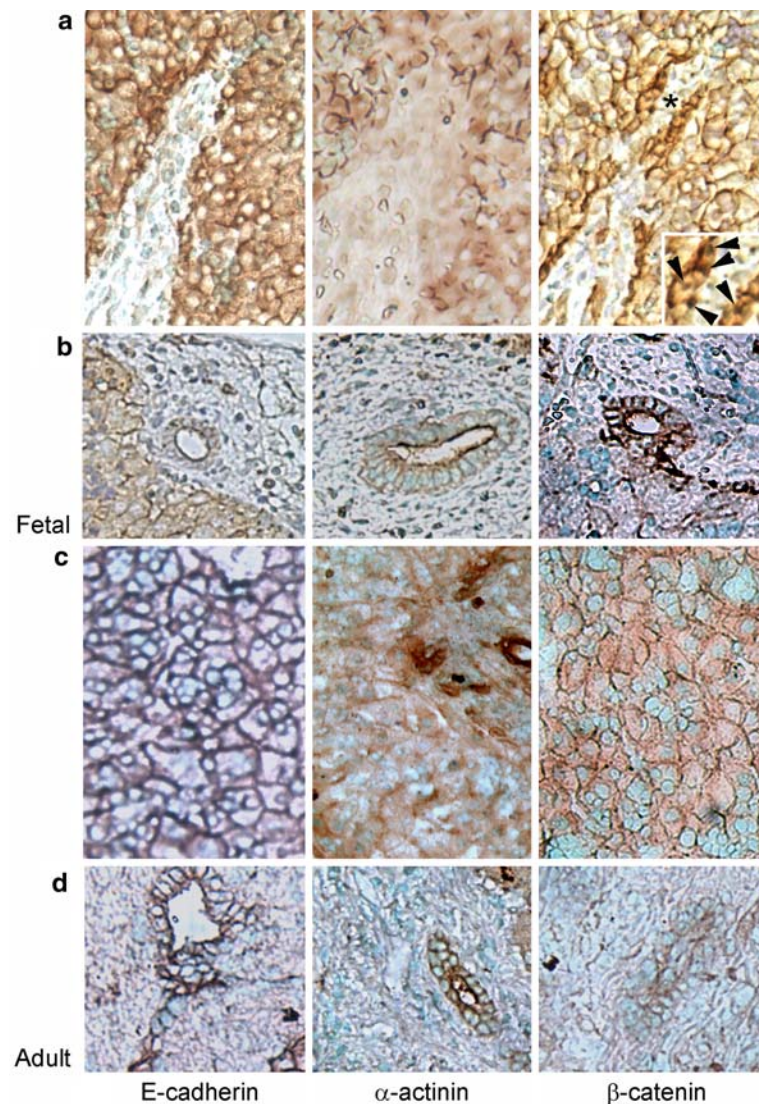

Fig. 2 Regulated expression of E-cadherin, α-actinin, and β-catenin. Panels in **a** and **b** show immunostainings using 17-week fetal liver sections with midlobular areas (**a**) and portal areas containing bile ducts (**b**). Remaining panels are from adult livers. E-cadherin and α-actinin were localized in the cell membrane, and cytoplasm in the fetal liver, although these were localized only in the cell membrane in the adult liver; α-actinin was better expressed in bile duct cells in the adult liver. β-catenin was largely restricted to the cell membrane in both parenchymal fetal and adult liver cells. However, in some ductal plate cells in the fetal liver, β-catenin was distributed in the cytoplasm and nucleus (arrows in inset in panel **a** showing magnified view of area marked by asterisk). These cells simultaneously expressed α-actinin and E-cadherin. In adult hepatocytes and bile duct cells, β-catenin was abundantly expressed and was localized only in the cell membrane. Original magnification, 400×, methyl green counterstain

β-catenin (Fig. 5a). By contrast, in the parenchyma of fetal livers, we did not observe such cells, where β-catenin had translocated to the nucleus of Ep-CAM-expressing cells (Fig. 5b). These studies indicated that altered β-catenin distribution likely contributed to Ep-CAM-associated cell signaling and suggested that cell adhesion was a playing role in altering cellular properties in various parts of the liver lobule. Therefore, we examined the prevalence of stem/progenitor cells in the parenchyma, ductal plate, and bile duct structures, where differences were observed in the pattern of Ep-CAM expression.

Regulation of liver gene expression and proliferation in specific cell compartments

To identify cells with CK-19, albumin, and α-fetoprotein markers associated with hepatobiliary development, we performed further analysis. Hepatoblasts in fetal liver parenchyma expressed CK-19 with changes in cell distributions during gestation. At 7 weeks, some but not all parenchymal cells adjacent to portal-like areas stained for CK-19, whereas up to 70% parenchymal and 30% ductal plate cells showed albumin (Fig. 6a). The pattern of α-fetoprotein expression was similar to albumin. Subsequently, during midgestational stages between 12 and 16 weeks, CK-19-positive cells were mostly in ductal plates or bile ducts. The intensity of CK-19 expression in these cells was greater, reaching up to grade 4 (Fig. 6b). At 22 weeks, when CK-19 was less well expressed in ductal plate cells, hepatoblasts in periportal areas expressed CK-19 only weakly at grade 1 intensity. With further gestation, albumin expression was changed, such that by 15 weeks, albumin was expressed at grades 3–4 (Fig. 6b). Similarly, α-fetoprotein was expressed most in midlobular-area cells (Fig. 6b). In adult livers, CK-19 was in only bile duct cells

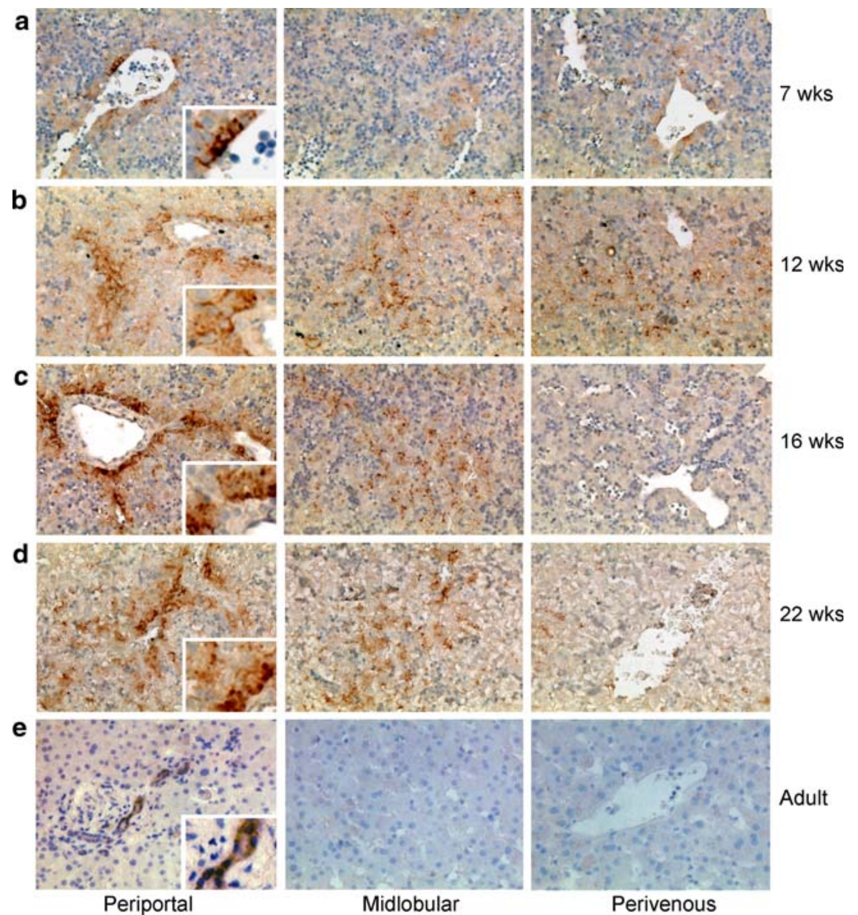

Fig. 3 Ep-CAM expression in fetal and adult human livers. The left, middle, and right rows demonstrate, respectively, periportal, midlobular, and perivenous areas of the liver lobule. Ep-CAM staining is seen as brown peroxidase product. Note that Ep-CAM was expressed in periportal cells (insets show higher magnification views), and in hepatoblasts at 7 weeks, where bile ducts have not yet appeared (**a**). Ep-CAM was expressed more intensely at 12, 16, and 22 weeks of gestation, especially in ductal plate cells in periportal areas (**b**–**d**). In the adult liver, Ep-CAM was expressed only in the bile ducts, panel **e**, and not in hepatocytes. Original magnification, 200×, toluidine blue counterstain

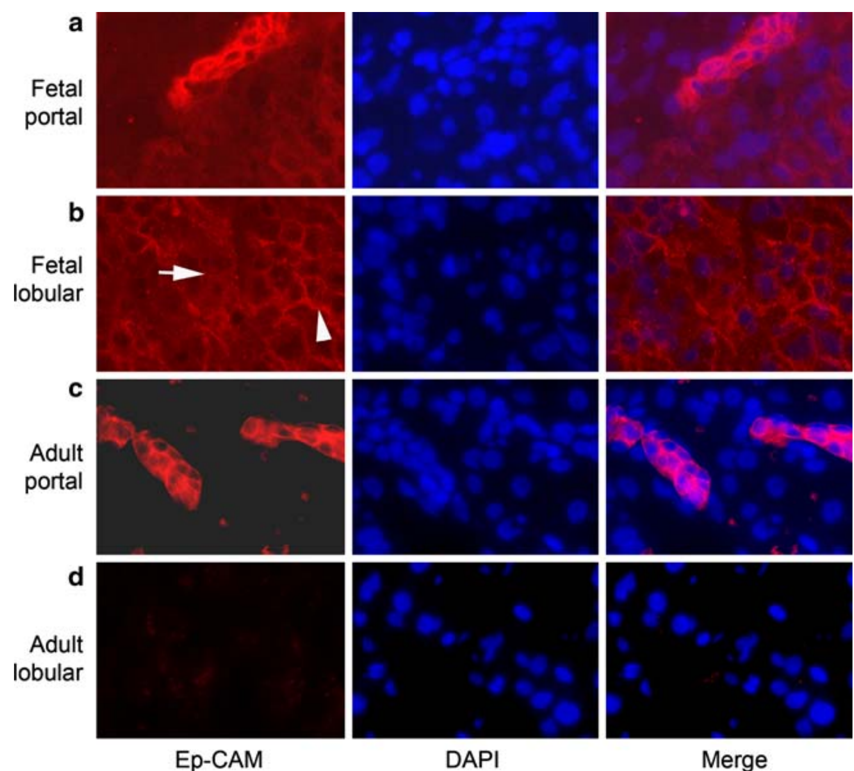

Fig. 4 Immunofluorescence localization of hepatic Ep-CAM. Sequential images of tissues stained for Ep-CAM (red color) and DAPI (blue) with merged images in panels on extreme right are shown. Panels in **a** and **b** show 16-week-old fetal liver and panels in **c** and **d** show adult liver. Note expression of Ep-CAM at high levels throughout cell membranes in ductal plate cells in fetal liver (**a**) and bile duct cells in adult liver (**b**). In hepatoblasts, Ep-CAM expression was less intense, and Ep-CAM was distributed in a focal manner (arrow, **b**) in cell membrane (arrowhead, **b**). Adult hepatocytes did not express Ep-CAM. Original magnification, 400×

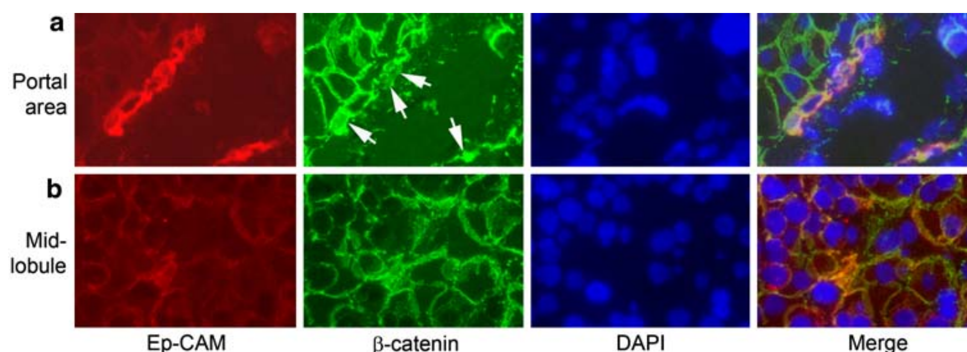

Fig. 5 Colocalization of Ep-CAM and β-catenin expression in the fetal liver. Sequential images of 16-week fetal liver to localize Ep-CAM and β-catenin along with nuclear staining using DAPI are shown. Merged images are on the extreme right. Panel **a** shows ductal plate cells containing Ep-CAM with β-catenin in cytoplasm and nucleus in some cells (arrows; merged image showing yellow cell membranes with coexpression of Ep-CAM and β-catenin indicating colocalization of these proterins). Panel **b** shows liver parenchyma with β-catenin in only the cell membrane, and weaker expression of Ep-CAM compared with bile duct cells and ductal plate cells. Original magnification, 650×

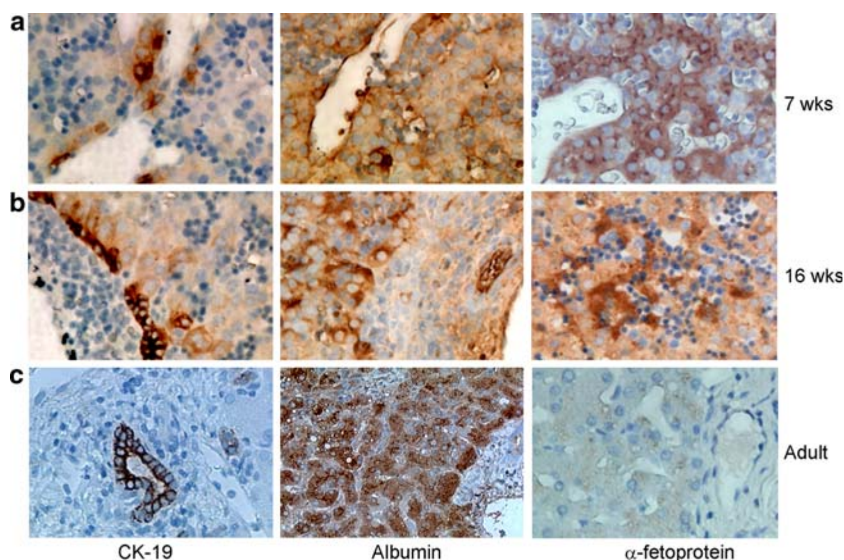

Fig. 6 Comparison of gene expression profiles in fetal and adult human livers. In fetal livers, CK-19 was expressed in ductal plate cells and parenchymal hepatoblasts, whereas CK-19 expression was restricted to bile ducts in the adult liver (panels on left). Albumin was expressed in fetal hepatoblasts and ductal plate cells (center panels), whereas α-fetoprotein was expressed in only parenchymal hepatoblasts and ductal plate cells (panels on right). Adult hepatocytes showed extensive albumin, but not α-fetoprotein staining. Original magnification, 400×, toluidine blue counterstain

(Fig. 6c). Albumin was expressed at high levels in adult hepatocytes (Fig. 6c), while, as expected, α-fetoprotein was not expressed in the adult liver.

Fetal liver contained rapidly cycling hepatoblasts

Ki-67 staining showed extensive proliferation in hepatoblasts throughout the fetal liver, particularly at 7 weeks, in $52 \pm 6\%$ cells, and also during subsequent gestation (Fig. 7a and b). In adult livers, cells rarely expressed Ki-67 (Fig. 7c). Cell proliferation was significantly less in ductal plate cells, and bile duct cells showed least proliferation (Fig. 7e). At 22 weeks, proliferation indices were 31 ± 10, 20 ± 5, and $10 \pm 1\%$ in parenchymal cells, ductal plate cells, and bile duct cells, respectively, $P < 0.001$, ANOVA.

Ep-CAM expression and proliferation activity in specific cell compartments

To address whether cell subsets with Ep-CAM expression were undergoing proliferation and differentiation, we used albumin and CK-19 expression for defining cell commitment along hepatic or biliary lineages, respectively. Representative areas from fetal liver at 15 weeks showed that the most prominent cell proliferation was in the mid-zonal areas of the liver lobule (Fig. 8a), whereas cells in ductal plates expressing Ep-CAM and CK-19 more abundantly were not actively proliferating (Fig. 8b and c). These proliferating parenchymal cells in the fetal liver contained albumin along with CK-19 in many cells (Fig. 7d). In these areas, fetal liver cells also expressed α-fetoprotein (see Fig. 5). Ep-CAM was expressed in proliferating albumin/α-fetoprotein/CK-19-positive cells in a

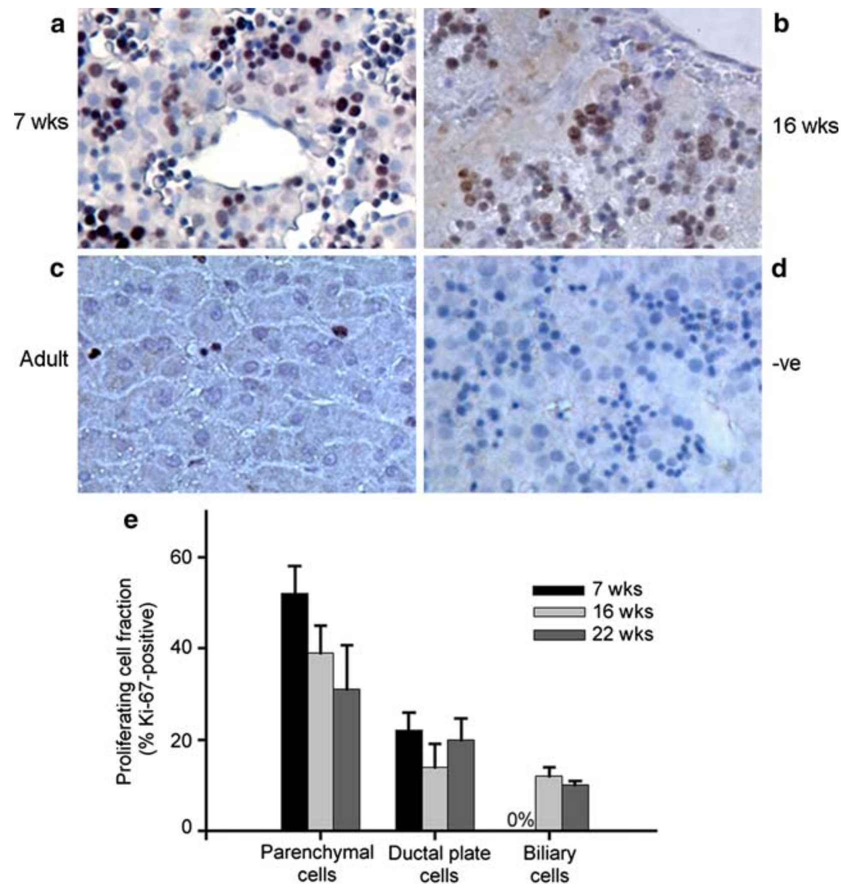

Fig. 7 Analysis of liver cell proliferation. Ki-67 staining showing extensive proliferation in fetal hepatoblasts at 7 and 16 weeks (**a**, **b**). Ductal plate and bile duct cells showed less proliferation. Panel **c** shows an adult liver, where Ki-67 staining was restricted to blood cells (arrows). Panel **d** shows negative control without the primary antibody. Chart in **e** shows cumulative analysis of cell proliferation. Proliferation within each cell compartment was not significantly different, although differences in hepatoblasts and ductal plate cells or biliary cells were significant. Original magnification, 400×, toluidine blue counterstain

focal membranous pattern and at relatively low intensity levels (grade 1). By contrast, Ep-CAM was expressed more extensively in ductal plate cells, where highly CK-19-positive cells were most prevalent. Also, CK-19 was prominent in ductal areas, which contained occasional cells with albumin expression, albeit rarely with Ki-67 staining, suggesting diminished cell cycling.

In adult livers, where cell proliferation was lacking, we did observe cells with Ep-CAM, albumin, and CK-19 (Fig. 8a–d). These adult cells were not in the liver parenchyma and were instead in ductal structures. We identified such cells in approximately 2% of bile ducts in 21 portal areas, where 80 ductal structures contained Ep-CAM/albumin/CK-19-expressing cells. These cells showed rare evidence for proliferation, basolateral membranous staining for Ep-CAM and CK-19, and albumin staining in occasional cells (Fig. 8).

To further verify presence of stem/progenitor cells defined by hepatobiliary gene expression in duct structures, we additionally examined fetal and adult livers for albumin mRNA expression with in situ hybridization (Fig. 9). These studies established that albumin mRNA was abundantly expressed in adult hepatocytes, and in fetal hepatoblasts (Fig. 9a). Moreover, bile ducts contained cells with albumin mRNA expression in both fetal and adult livers (Fig. 9b).

Discussion

The major findings of this study were as follows: (1) E-cadherin was well expressed in fetal hepatoblasts throughout the liver, irrespective of their proliferation state. (2) Expression of Ep-CAM was related to cell proliferation in the fetal liver—lower level or focal expression of Ep-CAM was observed in rapidly proliferating hepatoblasts in the parenchyma, whereas hepatoblasts in ductal plates and bile ducts showed less proliferation, but more intense Ep-CAM expression throughout the cell membrane. (3) Fetal hepatoblasts showed variable α-actinin expression, while β-catenin was well expressed in a membranous pattern in hepatoblasts; however, some ductal plate cells showed β-catenin in the cytoplasm and nucleus. (4) And, in adult livers, Ep-CAM was expressed in only bile ducts. In some adult cells in bile duct structures, the pattern of Ep-CAM and hepatobiliary gene expression was reminiscent of fetal hepatoblasts.

This localization in the liver of E-cadherin, Ep-CAM, and their partners α-actinin and β-catenin, indicated that adhesion molecules contributed to the fate of epithelial cell subsets during rapid liver development, helped in acinar and ductal morphogenesis, and participated in the establishment of stem cell niches. These considerations are

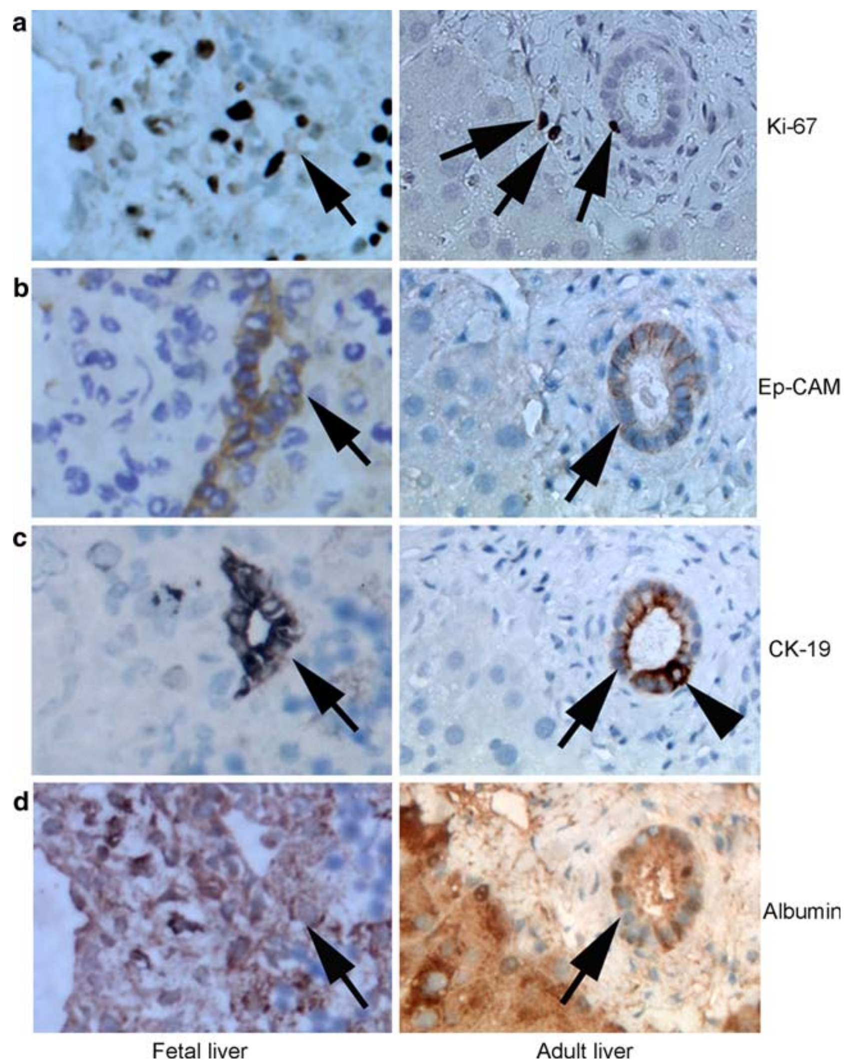

Fig. 8 Cell proliferation and persistence of Ep-CAM-expressing cells. Showing serial sections from 15-week-old fetal liver (left) and adult liver (right), except panel **d** on left. In the fetal liver, hepatoblasts were proliferating (arrow) and ductal plate cells expressing Ep-CAM and CK-19 showed less proliferation (**b**, **c**). In the adult liver, proliferative activity was restricted to an occasional ductular cell or mesenchymal cells in portal areas (arrows, **a**). Adult ductal cells showed weak basolateral Ep-CAM staining (arrow, **b**) and heterogeneous expression of CK-19 (arrows, **c**). Occasional ductal cells in adult and fetal livers expressed albumin (arrows, **d**). Original magnification, 400×, toluidine blue counterstain

schematically summarized in Fig. 10 and further discussed below. Undoubtedly, the processes of organ development, morphogenesis, and homeostasis are complex and assignment of specific mechanisms from the vantage of cell adhesion will require more work.

Cell adhesion and proliferation

Our data here indicate that cell adhesion regulates proliferation of fetal hepatoblasts—a population that constitutes a so-called transit-amplifying compartment. Of note, low-level and focal expression of Ep-CAM in parenchymal fetal hepatoblasts should have supported cell proliferation through weaker anchorage to stroma and superior cell migration capacity. Moreover, Ep-CAM was shown to induce cell proliferation by directly upregulating c-Myc and other cell cycle regulated genes [23]. Alternatively, high levels of Ep-CAM expression in ductal plate and bile duct cells should have decreased proliferation through stronger anchorage of cells to stroma, and other intracellular changes, for example, those involving β-catenin. The absence of Ep-CAM expression in adult hepatocytes, which do not normally proliferate, indicated that molecules imparting stronger adhesion than Ep-CAM contributed, again in agreement with an association between weaker cell adhesion mediated by Ep-CAM and proliferation. In the adult liver, stem/progenitor cells are needed only when mature cells are lost due to liver injury; expression of Ep-CAM was documented in liver cells in focal nodular hyperplasia, cirrhosis, or oncogenesis [24], which represent such situations. Similarly, Ep-CAM was prominently expressed in progenitor cells activated by liver injury in rats [25].

The relationship between cell adhesion and proliferation should be mediated by interactions among cadherins, catenins, and focal adhesion complex subunits. For instance, cytoplasmic E-cadherin and other type I cadherins bind cytoskeletal actin via catenins, often in cadherin–β-catenin–α-catenin–actin sequences [26]. E-cadherin may

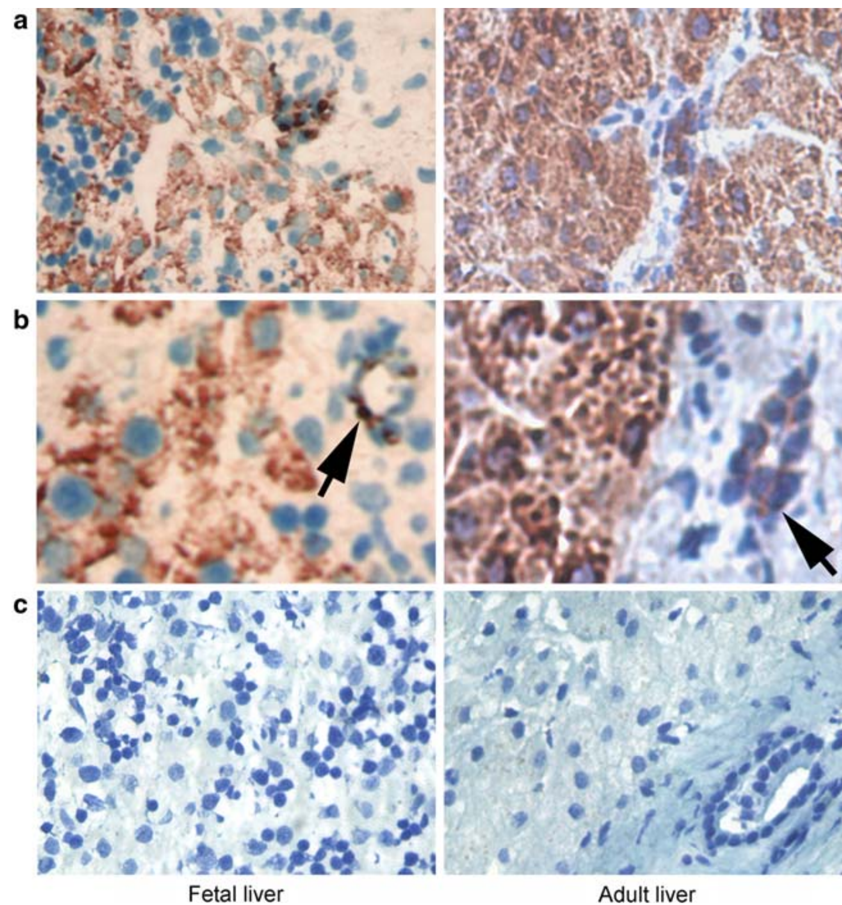

Fig. 9 In situ hybridization to identify albumin mRNA expression. An in situ hybridization using nonradioactive digoxigenin probe is shown. Panels **a** and **b** with hybridization using antisense albumin probes show extensive albumin mRNA in parenchymal cells, and cells in bile ducts (arrows in **b**). In Panel **c**, sense albumin probe shows no hybridization signals. Original magnification, 400×; hematoxylin counterstain

bind cellular integrins [27], which mediate mesenchyme interactions during organogenesis [28]. In a previous study, fetal ductal plate cells were shown to express E-cadherin [29], which was similar to our findings. Cell adhesion is mediated in adult hepatocytes by E-cadherin and N-cadherin, and in adult bile duct cells by E-cadherin, biliary glycoprotein related to the carcinoembryonic antigen, and Ep-CAM [30–32]. Therefore, perturbation of cellular catenin–actin association by Ep-CAM may represent interactions between Ep-CAM and E-cadherin [6, 20]. An interaction between Ep-CAM and E-cadherin shifts cell adhesion from strong to weak [33], which should have been beneficial during rapid cell proliferation in the fetal liver. Other interactions between E-cadherin, β-catenin, and integrins may result in the engagement of protein kinases regulating cell signaling during proliferation. Here, nonphosphorylated β-catenin is stabilized to translocate and associate with the lymphoid enhancer–binding factor/T cell-specific factor, transcription factor, adenomatous polyposis coli tumor suppressor product, scaffolding protein–axin/conductin, and growth factor receptors to promote cell proliferation [34–37]. Also, transforming growth factor-β–activated Smad proteins or integrin signaling can induce kinases linked to Wnt-1, insulin-like growth factor-II, Ras and integrin, to enhance β-catenin activity [38–40]. Identification of cytoplasmic and nucleus-translocated β-catenin in some fetal ductal plate cells, as shown here, suggests the possibility of Wnt activity in these cells, similar to the developmental regulation of β-catenin in mice [41], offering additional mechanistic clues.

Cell adhesion and organ morphogenesis

The process of liver development includes acinar organization and ductal morphogenesis. Although mechanisms underlying these events are incompletely understood, biliary specification and/or morphogenesis are under genetic controls. For instance, defects in notch signaling pathways, hepatic transcription factors, and homoebox regulators, have been incriminated as primary genetic abnormalities in biliary specification and morphogenesis [42, 43]. It should be noteworthy that our findings do not imply that cell adhesion molecules are causally significant in biliary development. Nonetheless, Ep-CAM likely facilitated biliary morphogenesis by segregating biliary-specified hepatoblasts in the ductal plate and primitive ducts. This putative role of Ep-CAM is strengthened by the

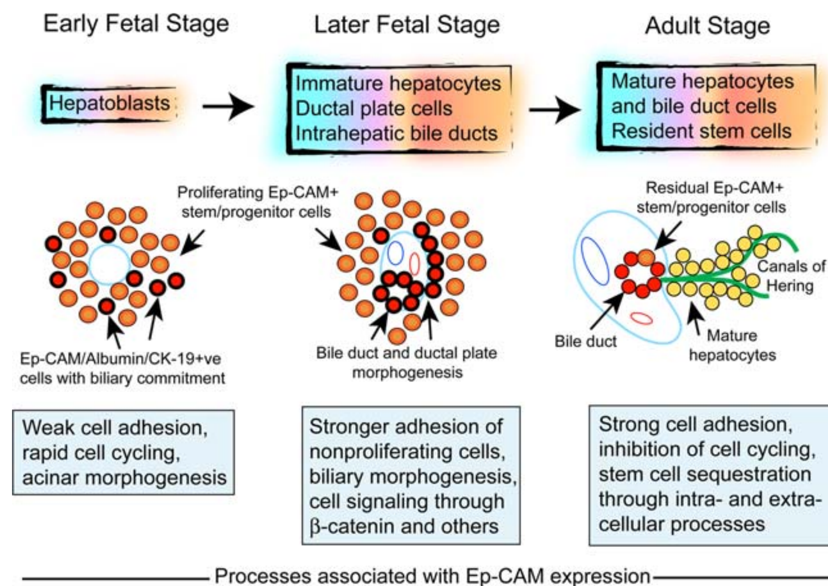

Fig. 10 Schematic representation of changes during liver development. In this scheme, hepatoblasts in the fetal liver parenchyma originate from primitive endoderm and give rise to immature hepatocytes, ductal plate cells, and intrahepatic ducts, which in turn generate mature hepatocytes and biliary cells. The cartoons depict morphogenetic changes during early fetal stage (left), for example, 7 weeks, where the liver contains loosely arranged Ep-CAM+ stem/progenitor cells (cells in pink with black and red borders indicating coexpression of E-cadherin and Ep-CAM, respectively), while at later fetal stages (middle), for example, after 12 weeks, biliary morphogenesis leads to ductal plate cells and onset of biliary morphogenesis with high-level expression of Ep-CAM and CK-19 along with E-cadherin (cells shown in red with black borders). The adult stage (right) is characterized by the familiar hepatic and biliary compartments, although residual fetal-type stem/progenitor cells remain in ductular structures with likely access to the parenchyma through canals of Hering. The boxes indicate that during the early fetal stage, weak cell adhesion through focally expressed Ep-CAM would promote cell proliferation and acinar organization. During later fetal stages (middle), extensive Ep-CAM expression and altered intracellular β-catenin activity, along with other regulatory factors, would impede cell proliferation, facilitate biliary morphogenesis and help sequester stem/progenitor cells in the ductal plate, and primitive bile ducts. In the adult liver (right), strong E-cadherin-mediated adhesion in the absence of Ep-CAM would inhibit proliferation-initiating events in mature hepatocytes, while Ep-CAM will help sequester fetal-type stem/progenitor cells in ductal niches, largely in a quiescent stage, until these cells must replenish lost hepatocytes

observation of ductal organization in Ep-CAM-expressing hepatic progenitor cells emerging from periportal areas [25]. The roles of Ep-CAM in biliary specification and morphogenesis can potentially be examined in models, especially with conditional Ep-CAM knockdown or knockout strategies to inactivate gene expression. Although Ep-CAM has been transgenically expressed to study its role in tumorigenesis [44], Ep-CAM knockout mice are yet to be generated.

Cell adhesion and sequestration of stem/progenitor cells

Our results are compatible with stem-like cells in ductal plate areas of the fetal liver with hepatic (α-fetoprotein, albumin, α-1 antitrypsin) and biliary markers (CK-19, Das-1, γ-glutamyl transpeptidase) [11, 16, 45, 46]. On the other hand, we found that Ep-CAM/CK-19/albumin-positive hepatoblasts in the parenchyma of the fetal liver were the ones proliferating most, as early as after 7 weeks, when ductal plates and bile ducts had not even appeared. Therefore, whether ductal plate and bile duct structures represent niches for stem/progenitor cells rather than sites of stem cell origin are two distinct possibilities. We consider it most likely that parenchymal hepatoblasts are the original stem/progenitor cells in the fetal liver, whereas by contributing in biliary morphogenesis and as forerunners of the Canal of Hering [18], ductal plate structures leave behind fetal-like stem/progenitor cells in the adult liver. This possibility is borne out by the multipotent capacity of Ep-CAM-expressing cells after isolation from the fetal human liver [47]. Similarly, presence of occasional Ep-CAM/CK-19/albumin-positive cells in ductal structures of the adult liver will be in agreement with the persistence of such stem/progenitor cells in this niche [18, 48]. In these structures, Ep-CAM, coupled with coordinate regulation of cell cycling by β-catenin and cellular targets, for example, epidermal growth factor [49], or other mediators, should protect cells from premature activation or differentiation. Through regulated expression of Ep-CAM, β-catenin, and other molecules, hepatic stem cells should thus be maintained in niches, possibly similar to intestinal crypt stem cells, which produce transit-amplifying cells to generate villous epithelium and other lineages [10]. It should be

noted that higher levels of β-catenin are found in crypt areas containing intestinal stem cells. Similarly, sequestered stem/progenitor cells in the adult liver can be activated under suitable circumstances [14–19, 24, 25, 50].

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