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Embryonic Ductal Plate Cells Give Rise to Cholangiocytes, Periportal Hepatocytes and Adult Liver Progenitor Cells

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Abstract

Background & Aims—Embryonic biliary precursor cells form a periportal sheet called the ductal plate, which is progressively remodeled to generate intrahepatic bile ducts. A limited number of ductal plate cells participate in duct formation; those not involved in duct development are believed to involute by apoptosis. Moreover, cells that express the SRY-related HMG box transcription factor 9 (SOX9), which include the embryonic ductal plate cells, were proposed to continuously supply the liver with hepatic cells. We investigated the role of the ductal plate in hepatic morphogenesis.

Methods—Apoptosis and proliferation were investigated by immunostaining of mouse and human fetal liver tissue. The post-natal progeny of SOX9-expressing ductal plate cells was analysed after genetic labeling, at the ductal plate stage, by Cre-mediated recombination of a *ROSA26R^{YFP}* reporter allele. Inducible Cre expression was induced by *SOX9* regulatory regions, inserted in a bacterial artificial chromosome. Livers were studied from mice under normal conditions and during diet-induced regeneration.

Results—Ductal plate cells did not undergo apoptosis and showed limited proliferation. They generated cholangiocytes lining interlobular bile ducts, bile ductules and canals of Hering, as well as periportal hepatocytes. Oval cells that appeared during regeneration also derived from the ductal plate. We did not find that liver homeostasis required a continuous supply of cells from SOX9-expressing progenitors.

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Conclusions—The ductal plate gives rise to cholangiocytes lining the intrahepatic bile ducts, including its most proximal segments. It also generates periportal hepatocytes and adult hepatic progenitor cells.

Keywords

hepatic cell differentiation; liver development; liver regeneration

Introduction

Hepatocytes and cholangiocytes derive from common embryonic liver precursor cells, which are referred to as hepatoblasts. In developing liver, the segregation of the hepatocyte and cholangiocyte lineages is detectable by the expression of the transcription factor SRY-related HMG box transcription factor 9 (SOX9) in a subset of hepatoblasts which encompass the biliary precursor cells¹. Hepatoblasts that remain SOX9-negative are located throughout the liver parenchyma and differentiate toward hepatocytes by gradual acquisition of hepatocyte-specific functions^{2, 3}. After birth, periportal and pericentral hepatocytes progressively display distinct properties, a phenomenon called liver zonation⁴. In contrast, embryonic SOX9-positive biliary precursor cells line up around the periportal mesenchyme to constitute a near-complete and single-layered sleeve of cells which is termed “ductal plate” (reviewed in references⁵⁻⁷). Each ductal plate gives on average rise to two bile ducts per portal tract⁸, and only a minority of ductal plate cells participate in bile duct morphogenesis. It has been proposed that the ductal plate cells not involved in bile duct formation regress, and that such regression occurs by apoptosis⁹. In parallel, there is evidence that ductal plate cells have stem cell properties. Indeed, when they are isolated by immunoselection and maintained in culture, ductal plate cells display self-renewal capacity and an ability to differentiate toward hepatoblast-like cells. When transplanted *in vivo*, these cells engraft in liver and give rise to cells that have characteristics of mature epithelial liver cells^{10, 11}. Recently, it was also proposed that SOX9-expressing cells in fetal and adult liver continuously generate hepatic cells under normal physiological conditions¹².

These observations prompted us to revisit the fate of the ductal plate cells *in vivo*. Here we address this issue by evaluating apoptosis and proliferation of the ductal plate cells at several stages of development, and by using a genetic lineage tracing approach. Our data show that ductal plate cells give rise to periportal hepatocytes and to cholangiocytes lining the interlobular bile ducts, the intralobular ductules and the canals of Hering. Oval cells, which appear during diet-induced liver regeneration and are believed to derive from the intralobular ductules or canals of Hering harboring facultative stem cells (reviewed in reference¹³), also originate from the ductal plate.

Materials and Methods

Animals

Experiments were performed with approval of the University Animal Welfare Committee. *SOX9-CreER^{T2}* mice express the T2 variant of tamoxifen-inducible cyclization recombinase-estrogen receptor ligand binding domain (CreER^{T2}) and were obtained by injection into fertilized oocytes of a bacterial artificial chromosome containing the cDNA of CreER^{T2} cloned in-frame into the SOX9-coding region¹⁴. *ROSA26R^{YFP}* mice were as described¹⁵. Tamoxifen (Sigma, Bornem, Belgium) was dissolved in corn oil at a concentration of 30 mg/ml and injected intraperitoneally in pregnant mice at embryonic day (E) 15.5 at 100 mg/kg of body weight. For diet-induced liver regeneration, mice were fed a choline-deficient (MP Biomedicals, Irvine, CA, USA) ethionine supplemented (Sigma, Bornem, Belgium; 0.15% in water) diet (CDE), or a 3,5-diethoxycarbonyl-1,4-dihydro-

collidine (DDC) diet (Sigma, Bornem, Belgium; 0.1% DDC in standard diet (Altronim, Lage, Germany)).

Human fetal liver specimens

Tissue samples were obtained from spontaneous or therapeutic abortion, in compliance with the French legislation, the 1975 Declaration of Helsinki, and the European Guidelines for the use of human tissues.

Immunofluorescence

Mouse liver preparation and immunofluorescence analysis were as described¹⁶ (Supplementary Table). Immunodetection of Cre was carried out with Tyramide Signal Amplification kit (Molecular Probes, Invitrogen, Merelbeke, Belgium). For multiple immunostaining with primary antibodies raised in the same species, staining-elution cycles were performed as described¹⁷. Pictures were taken with a Zeiss Cell Observer Spinning Disk confocal microscope or an Axiovert200 fluorescence microscope (Carl Zeiss, Zaventem, Belgium).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)

TUNEL assay was performed with *In Situ* Cell Death detection kit, Fluoresceine (Roche Applied Science, Mannheim, Germany) according to manufacturer's instructions. Liver samples were dewaxed, rehydrated and treated by microwave irradiation in 0.1M citrate buffer, pH 6.0. TUNEL reaction with fluoresceine-coupled dUTP was performed prior to immunofluorescence detection of E-cadherin. DNaseI pretreatment served as positive control.

Results

Lack of apoptosis and low proliferation of ductal plate cells

To investigate the fate of ductal plate cells we first looked at apoptosis in developing mouse liver. The ductal plate was identified by staining for SOX9 or E-Cadherin. Co-stainings for activated Caspase 3 or TUNEL were performed from E15.5 to E18.5, namely from the initiation of ductal plate formation to the stage at which it is actively regressing. No apoptosis was detected in ductal plate cells at any stage, indicating that apoptosis is not the main mechanism of remodeling (Figure 1A-B). Similarly, analysis of a human fetal liver at 11 weeks of gestation did not reveal apoptosis in developing ducts. Human embryonic liver showed biliary structures at several stages of maturation, namely ductal plate cells with small lumina and adjacent to the parenchyma, as well as more mature ductal structures becoming incorporated in the mesenchyme (Figure 1A-B). Apoptosis was detected by activated caspase 3 and TUNEL stainings outside the ductal plate on the same sections of mouse and human liver, thereby providing positive controls (data not shown).

Proliferation of SOX9-expressing ductal plate cells was also assessed in mouse liver (E15.5 to E18.5) using co-immunodetection of Ki67, which labels cells in the G1, S, G2 and M phases of proliferation. The data show that only a very low number of ductal plate cells was proliferating (arrowheads, Figure 1C), while proliferation was intense in the parenchyma.

A mouse model for genetic lineage tracing of ductal plate cells

Since the ductal plate cells were not undergoing apoptosis, we determined their fate using a genetic lineage tracing approach. To this end we first validated a mouse line that expresses inducible CreER^{T2} in SOX9-expressing cells¹⁴ (*SOX9-CreER^{T2}* mice). The cell-type specificity of CreER^{T2} expression was analyzed from E14.5 to E16.5 by immunofluorescent

co-detection of CreER^{T2} and SOX9, and of CreER^{T2} and the hepatoblast marker HNF4. As shown in Figure 2, 18 h after tamoxifen injection, CreER^{T2} was exclusively found in nuclei of SOX9⁺ ductal plate cells, and never in HNF4⁺ hepatoblasts; 87.47% of SOX9-expressing cells showed nuclear CreER^{T2} (Supplementary Figure 1A). We concluded that CreER^{T2} expression in developing liver is specific to SOX9-expressing ductal plate cells.

The ductal plate gives rise to cholangiocytes and to periportal hepatocytes

To trace the fate of SOX9⁺ ductal plate cells we administered a single dose of tamoxifen to pregnant females from intercrosses between *SOX9-CreER^{T2}* mice and *ROSA26R^{YFP}* mice. Tamoxifen transiently induces CreER^{T2} nuclear translocation in SOX9⁺ cells, leading to recombination of the *ROSA26R^{YFP}* locus and to permanent expression of YFP in the cells' progeny. We also verified that CreER^{T2} remained inactive in the absence of tamoxifen (Supplementary Figure 1B). Pregnant mice were injected at E15.5, and the livers of *SOX9-CreER^{T2};ROSA26R^{YFP}* offspring were collected after birth. Interlobular bile ducts expressed YFP in the postnatal period, confirming the presumed ductal plate origin of the bile ducts. These YFP⁺ bile ducts were identified by co-staining for YFP and SOX9, osteopontin (OPN) or cytokeratin 19 (CK19)¹ (Figure 3A).

A number of hepatocytes also expressed YFP in the postnatal period. They displayed the typical polygonal shape of hepatocytes and expressed the hepatocyte marker HNF4, but not the biliary markers SOX9, OPN and CK19 (Figure 3A). These hepatocytes were located in the periportal area. Given that the hepatic lobule is zoned along its porto-central axis, we verified that the YFP-expressing hepatocytes displayed the expected enzymatic profile of periportal hepatocytes. This was the case, since they expressed carbamoyl-phosphate-synthetase1 (CPS1), but not glutamine synthetase (GS) (Figure 3B). These hepatocytes remained periportal and maintained a periportal enzymatic profile; also, they did not display differential proliferation as compared to YFP⁻ hepatocytes (Supplementary Figure 2A).

Several YFP⁺ cells were located in the periportal mesenchyme and were distinct from hepatocytes and from cholangiocytes lining interlobular ducts. These cells had a flattened morphology and lined the biliary ductules and the canals of Hering (Figure 4), as defined according to the current nomenclature¹⁸. The ductules were delineated by small CK⁺ cholangiocytes and surrounded by laminin (Figure 4A). The canals of Hering (Figure 4B) were delineated on one side by hepatocytes and on the other side by CK⁺ cells; the latter had a basal pole lined by laminin, as shown earlier in rodents¹⁹. Expression of CEACAM, which stains the bile canaliculi at the apical pole of hepatocytes, further confirmed the identification of the canals of Hering.

Finally, although the SOX9-expressing cell populations (cholangiocytes lining interlobular ducts, ductules and canals of Hering) contained YFP-labeled cells, we found no evidence that the liver becomes colonized by cells that express YFP and that derive from SOX9⁺YFP⁺ progenitors (Figure 3A). This was in contrast with the observations of Furuyama and coworkers¹².

Thus, we concluded that SOX9⁺ ductal plate cells give rise to cholangiocytes lining the bile ducts including the most proximal biliary structures, as well as to periportal hepatocytes.

Ductal plate cells are the embryonic precursors of oval cells

When the liver is injured under conditions that prevent hepatocyte proliferation, the organ regenerates by proliferation and differentiation of oval cells²⁰. These cells are transit-amplifying cells, considered to derive from facultative progenitor cells located most likely in the ductules or canals of Hering (reviewed in references^{13, 21, 22}). Since biliary cells, including those lining the ductules and canals of Hering, originate from the ductal plate, we

determined if the ductal plate is the embryonic origin of oval cells. To this end, tamoxifen was administered at E15.5 to *SOX9-CreER^{T2};ROSA26R^{YFP}* embryos, and 4 weeks after birth the mice were fed a CDE diet until the livers were collected 21 days after the onset of the diet. Such diet is known to induce oval cell-dependent liver regeneration. Oval cells expressed the oval/biliary cell markers CK19, SOX9 and OPN²³ as well as the oval cell-specific marker TROP2²⁴. A number of oval cells expressed YFP, indicating that their embryonic origin is the SOX9⁺ ductal plate (Figure 5). Not all oval cells expressed YFP, which is consistent with the fact that CreER^{T2} is not detectable in the nucleus of 100% of the ductal plate cells at E15.5, and with the efficiency of CreER^{T2} activity (Figure 2; Supplementary Figures 1A and 3). Therefore, we quantified the percentage of YFP⁺SOX9⁺ oval cells (ratio of YFP⁺SOX9⁺ oval cells over SOX9⁺ oval cells). The results showed that some periportal areas had close to 100% of SOX9⁺ oval cells that co-expressed YFP, suggesting that at least in these periportal areas nearly all oval cells derived from the ductal plate (Figure 5, Supplementary Figure 3).

A regenerative response similar to an oval cell response is also induced in mice fed a DDC diet²⁵. To further test if oval cells derive from the ductal plate, tamoxifen was administered at E15.5 to pregnant females, and their 4 week-old *SOX9-CreER^{T2};ROSA26R^{YFP}* progeny was fed a DDC diet for 15 days, before analysis of the liver. Proliferation of atypical ductular structures was observed, as expected (Figure 6). The ductular structures expressed the oval/biliary cell markers SOX9, OPN and CK, and most ductular cells co-expressed YFP, indicating that they derive from the ductal plate. We concluded that the SOX9-expressing ductal plate cells give rise to at least a fraction of adult hepatic progenitor cells.

Discussion

During liver development the biliary precursor cells constitute the ductal plate, of which only a fraction of the cells is considered to participate in the formation of the biliary tree. It was proposed that, at least in humans, ductal plate remodeling occurs by proliferation and apoptosis⁹. Our analysis of mouse and human fetal livers did not reach the same conclusion, since no apoptosis and nearly no proliferation was found in the ductal plate. In addition, we show that the ductal plate differentiates toward cholangiocytes and periportal hepatocytes. The latter finding is in line with recent data¹², which we extend here by demonstrating that the ductal plate gives rise to cholangiocytes lining the interlobular bile ducts, the ductules and the canals of Hering, as well as to periportal, but not pericentral, hepatocytes.

Several metabolic functions are exerted according to a porto-central gradient, but the steepness of each gradient differs, making it difficult to make a clear-cut distinction between hepatocyte sub-types. An exception is GS expression, which is strictly pericentral and non overlapping with CPS1. No YFP⁺ cell expressed GS, eliminating the possibility that ductal plate-derived hepatocytes contribute to the pericentral hepatocytes. CPS1 expression extends from the periportal zone to the midlobular zone. Co-labeling of YFP and CPS1 was always detected within the six to eight hepatocytes around the portal space, which corresponds to the periportal area called zone 1⁴. Based on this anatomical location we propose that ductal plate-derived hepatocytes are periportal. However, considering the limitations of such anatomical interpretation, and considering that some section areas show a low number of cells between the portal space and the central vein, we cannot rule out that some mid-lobular hepatocytes derive from the ductal plate.

The discrepancy between earlier data⁹ and our observations on apoptosis and proliferation most likely results from technical reasons. Our data suggest that neither apoptosis nor proliferation are the most significant determinants of the fate of ductal plate cells. We propose that most remodeling of the ductal plate occurs by differentiation towards

cholangiocytes and hepatocytes. However, we cannot determine if subpopulations of SOX9-expressing cells contribute to one and/or the other cell type.

Importantly, our data do not support a recent model of hepatic homeostasis. SOX9-expressing cells in adult liver line interlobular ducts, intralobular ductules and canals of Hering. Furuyama and coworkers¹² proposed that SOX9-expressing cells are progenitors which continuously supply the liver with hepatic cells. In these experiments 8 week-old *SOX9-IRES-CreERT2;ROSA26R^{YFP}* mice were injected with tamoxifen, and the progeny of SOX9-expressing cells were proposed to colonize the liver. In our experimental setting, SOX9-expressing cells in adult liver were YFP-labeled as a result of CreERT²-mediated recombination in the embryo; no ectopic expression of CreERT² was detected after tamoxifen injection. We found no evidence that the progeny of YFP-labeled SOX9⁺ cells colonize the liver. Instead, YFP⁺ cells persisted as biliary cells or periportal hepatocytes. This was verified until 7 months of age (data not shown). A definitive reason explaining the discrepancy between our work and that of Furuyama cannot be provided. Still, a number of points need attention. The *SOX9-CreERT²* mouse line used in our study expresses Cre recombinase from a bacterial artificial chromosome, thereby leaving the genomic *SOX9* loci intact. In the work of Furuyama and coworkers, IRES-CreERT² recombinase coding sequences were inserted in the 3' untranslated region of the *SOX9* locus. This raises the possibility that SOX9 expression levels may not be maintained at physiological levels, leading to a potential impact on the physiology of SOX9-expressing cells. We cannot eliminate the possibility that our work and that of Furuyama and coworkers trace the fate of distinct subpopulations of SOX9⁺ cells. Still, Furuyama and coworkers proposed that proliferation of SOX9⁺ cells contributes to the supply of hepatic cells. Our quantifications of proliferating SOX9⁺ cells showed progressive decrease of proliferation with age (Supplementary Figure 2B). The decreasing and low proliferation levels from birth to week 8 does not provide support for a mode of liver homeostasis that depends on SOX9⁺ cell proliferation. Finally, in our hands, injection of tamoxifen in 8 week-old mice (a single dose of 10 mg/kg body weight of tamoxifen) induces ectopic expression of SOX9 in hepatocytes (Supplementary Figure 2C). This raises the possibility that, in adult mice, tamoxifen induces ectopic CreERT²-mediated recombination of a ROSA reporter locus in hepatocytes. In that case, lineage tracing would not distinguish between the progeny of normal SOX9⁺ cells or of hepatocytes expressing tamoxifen-induced SOX9.

During CDE and DDC diet-induced liver regeneration, oval cells proliferate. There is mounting evidence that they derive from the ductules or canals of Hering (reviewed in references^{21, 22}). Our data indicate that oval cells emerge from the progeny of the ductal plate. Oval cells are unlikely to derive from the ductal plate-derived hepatocytes, since the latter are injured during the diet (data not shown). The oval cells are also unlikely to derive from interlobular ducts, since there is no correlation between the periportal distribution of YFP⁺ oval cells and YFP⁺ interlobular duct cells (Supplementary Figure 3). Finally, there is controversial evidence that bone marrow cells may be a source of hepatic progenitor cells (reviewed in reference²²), raising the possibility that YFP⁺ oval cells in our experiments derive from bone marrow cells which would express *SOX9-CreERT²* at E15.5. This is unlikely: first, bone marrow precursor cells are mainly located in the parenchyma of fetal liver, and *SOX9-CreERT²* expression in E15.5 fetal liver was exclusively found in the ductal plate, not in cells colonizing the parenchyma; second, bone marrow precursors are also found in the circulation from E12.5 to E17.5, but these cells are not distinct from the fetal liver hematopoietic cells, since they display the same stem cell surface markers; third, hematopoietic stem cells are not detectable in the limb bone until E17.5²⁶.

In conclusion, our work shows that the ductal plate in embryonic liver gives rise to cholangiocytes lining all segments of the intrahepatic bile ducts, as well as to periportal

hepatocytes and hepatic progenitor cells. Our data bear significance to understand the mechanisms of biliary malformations and of liver regeneration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

SOX9	SRY-related HMG box transcription factor 9
Cre	cyclization recombinase
CreER	cyclization recombinase-estrogen receptor ligand binding domain
E	embryonic day
CDE	choline-deficient ethionine supplemented
DDC	3,5-diethoxycarbonyl-1,4-dihydro-collidine
YFP	yellow fluorescent protein
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
OPN	osteopontin
CK19	cytokeratin 19
P	postnatal day
CPS1	carbamoyl-phosphate-synthetase1
GS	glutamine synthetase
CEACAM	carcinoembryonic antigen-related cell adhesion molecule

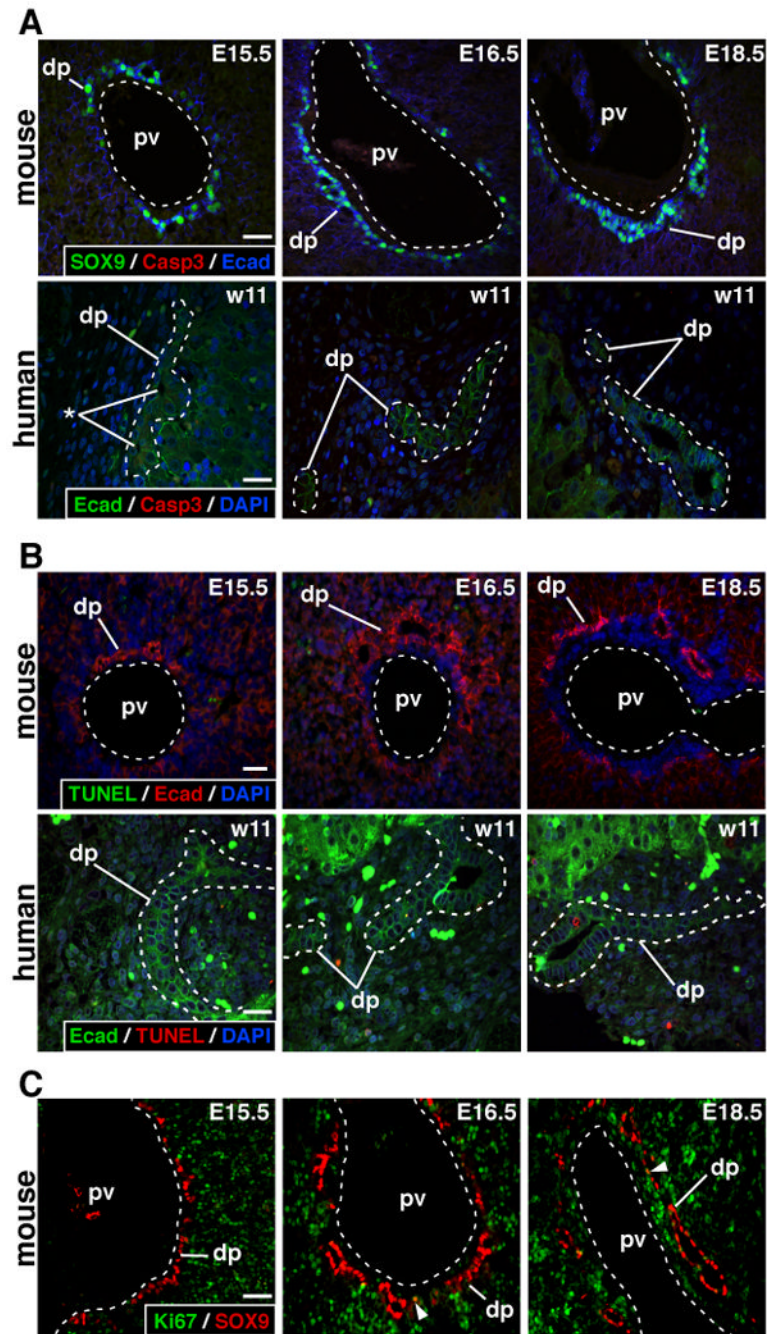


Figure 1.

Lack of apoptosis and very low proliferation in ductal plate. (A-B) Mouse and human ductal plates were analyzed at the stages indicated. No evidence for apoptosis (activated caspase 3 and TUNEL) was found. (C) Only a few SOX9⁺ ductal plate cells were proliferating (Ki67; arrowhead). dp, ductal plate; pv, portal vein; *, lumen of developing duct; size bar, 20 μm.

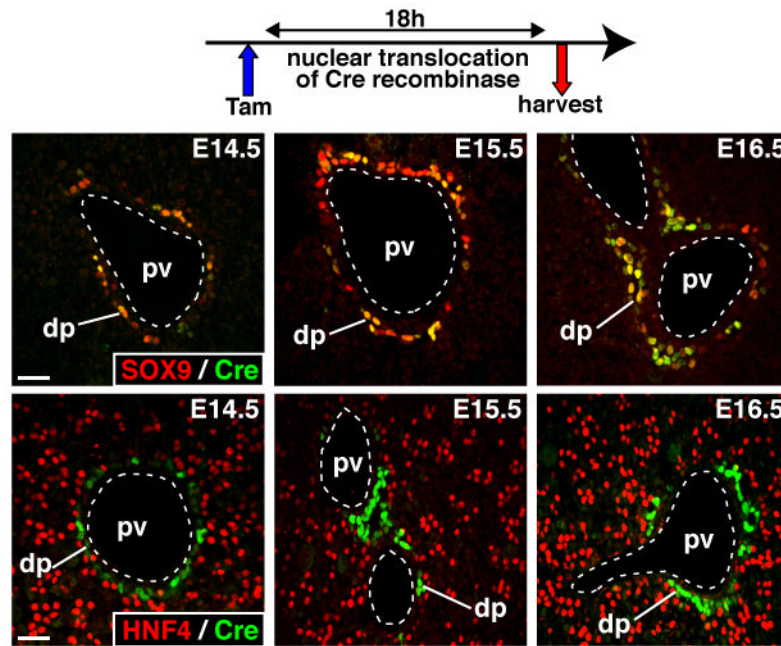


Figure 2. Expression of CreER^{T2} in developing liver is restricted to SOX9-expressing ductal plate cells. Pregnant females were injected with tamoxifen 18h prior to collection of the liver at the stages indicated. The livers were stained to detect CreER^{T2} and biliary (SOX9) or hepatoblast (HNF4) markers. dp, ductal plate; pv, portal vein; size bar, 20 μ m.

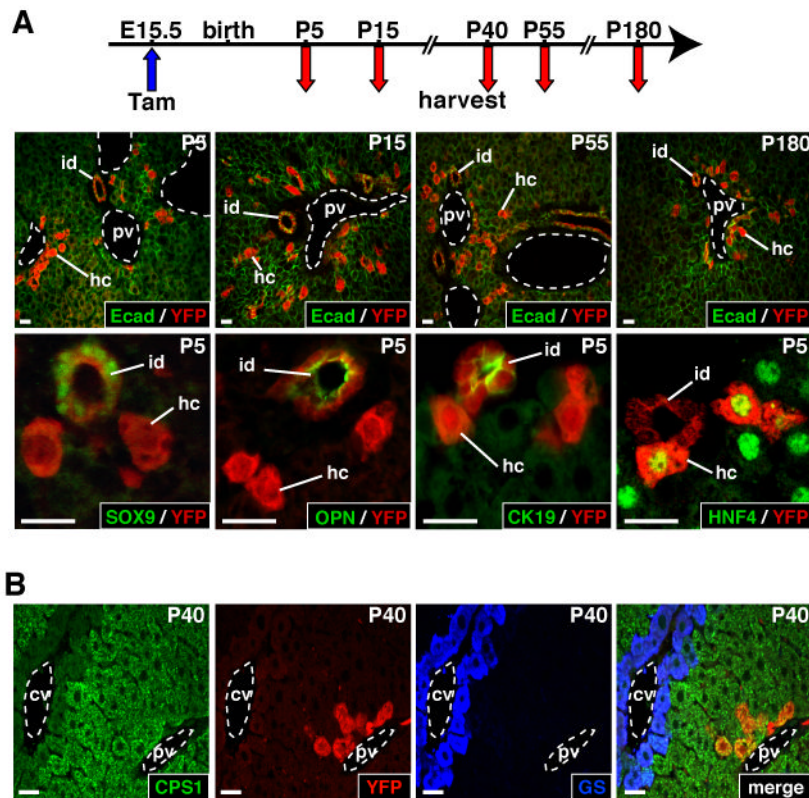


Figure 3. The ductal plate gives rise to cholangiocytes lining interlobular bile ducts and periportal hepatocytes. Pregnant females were injected with tamoxifen at E15.5, and the *SOX9-CreER^{T2};ROSA26R^{YFP}* offspring were analyzed at the time points indicated. (A) The YFP⁺ progeny of the ductal plate consisted of interlobular ducts and of hepatocytes which persisted at least until 7 months of age. The biliary markers (SOX9, OPN, CK19) were expressed in the YFP⁺ ducts but not in YFP⁺ hepatocytes; the latter expressed the hepatocyte marker HNF4. (B) The YFP⁺ hepatocytes were restricted to the periportal zone and expressed periportal (CPS1) but not perivenous (GS) markers. cv, central vein; hc, hepatocyte; id, interlobular bile duct; pv, portal vein; size bar, 20 μ m.

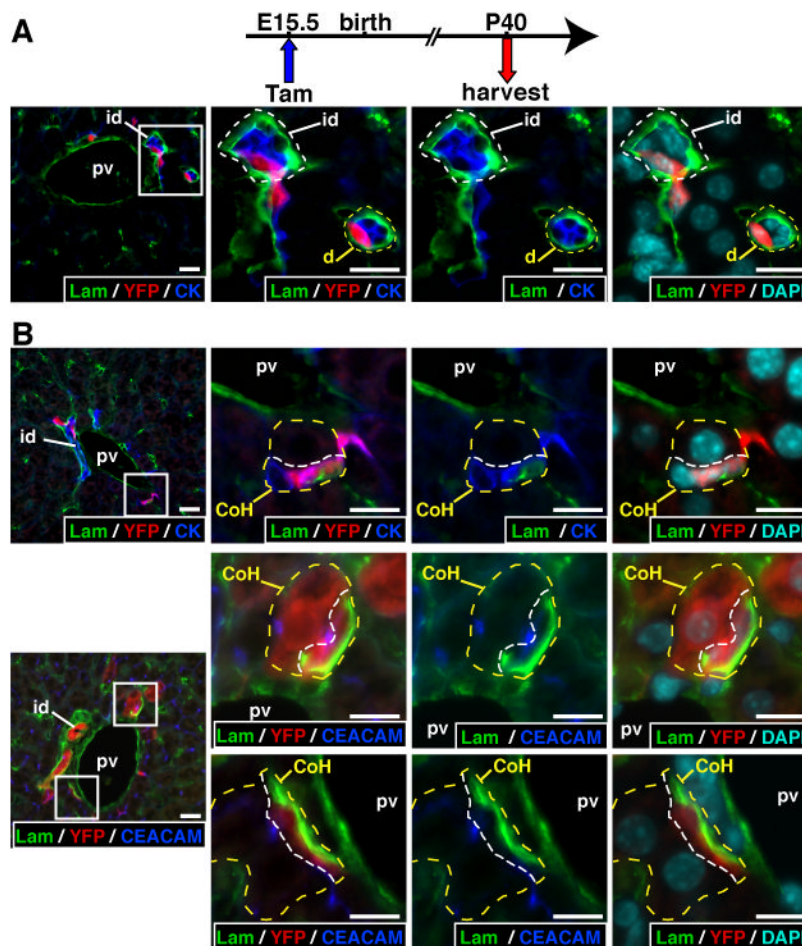


Figure 4.

The YFP⁺ progeny of the ductal plate includes cholangiocytes lining the ductules and the canals of Hering. (A) Laminin (lam) fully surrounds ductules (yellow dotted line) and interlobular ducts (white dotted line). (B) Upper panels: Canals of Hering (yellow dotted line) were delineated by hepatocytes with a large nucleus and cholangiocytes expressing cytokeratin (pan-cytokeratin antibody); the white dotted line separates the cholangiocyte from the hepatocyte. Laminin is only found along the basal pole of the cholangiocytes. Middle and lower panels: canals of Hering are also identified by CEACAM staining which marks the bile canaliculi at the apical pole of hepatocytes. The middle pictures show a canal of Hering lined by a YFP⁺ hepatocyte and a YFP⁺ cholangiocyte. In A-B, the right three pictures correspond to magnified areas delineated in the left picture by a white box. CoH, canal of Hering; d, ductule; id, interlobular bile duct; pv, portal vein; size bar, 10 μm.

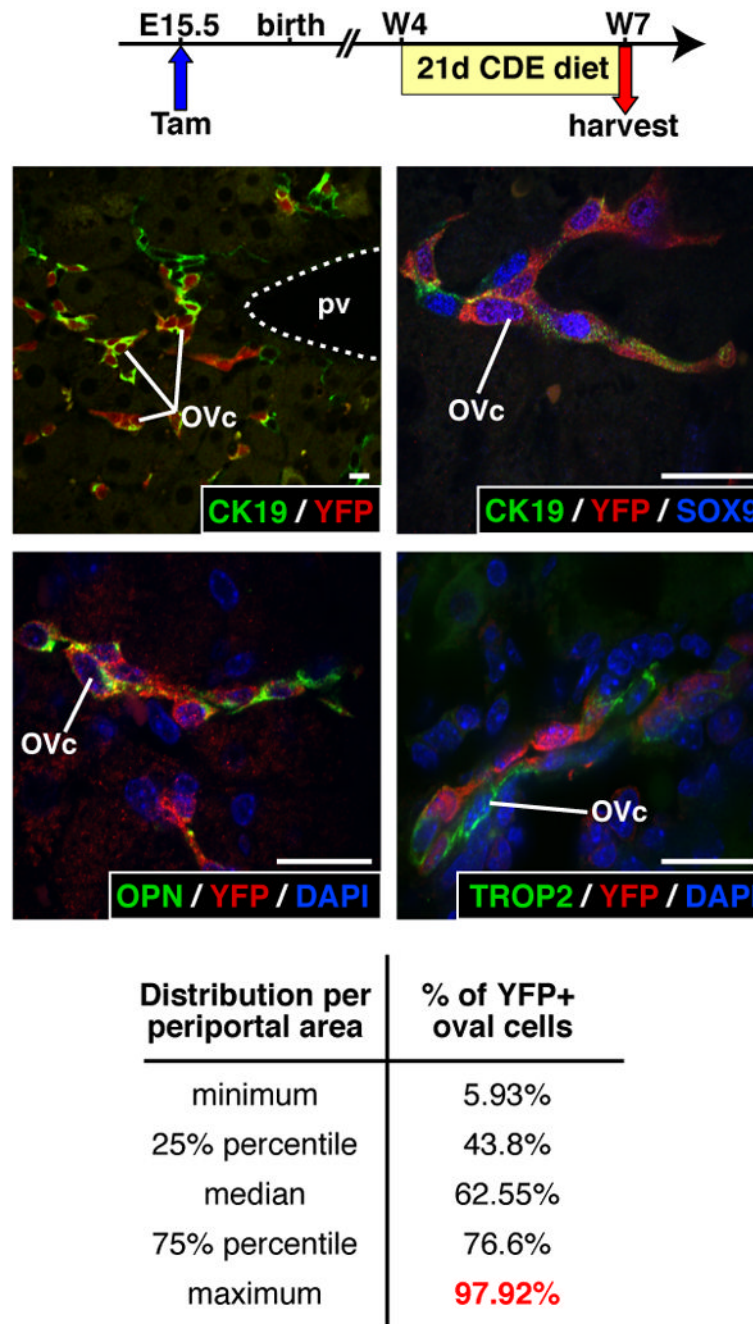


Figure 5.

CDE diet-induced oval cells derive from the ductal plate. Pregnant females were injected with tamoxifen at E15.5, and 4 week-old *SOX9-CreER^{T2};ROSA26R^{YFP}* offspring were fed a CDE diet. YFP⁺ oval cells express typical markers (CK19, SOX9, OPN, TROP2). The percentage of YFP⁺ oval cells $[(YFP^+SOX9^+/SOX9^+) \times 100]$ per periportal area can reach close to 100%. 13,419 SOX9⁺ oval cells were counted over 146 periportal areas. OVC, oval cell; pv, portal vein; w, week after birth; size bar, 10 μ m.

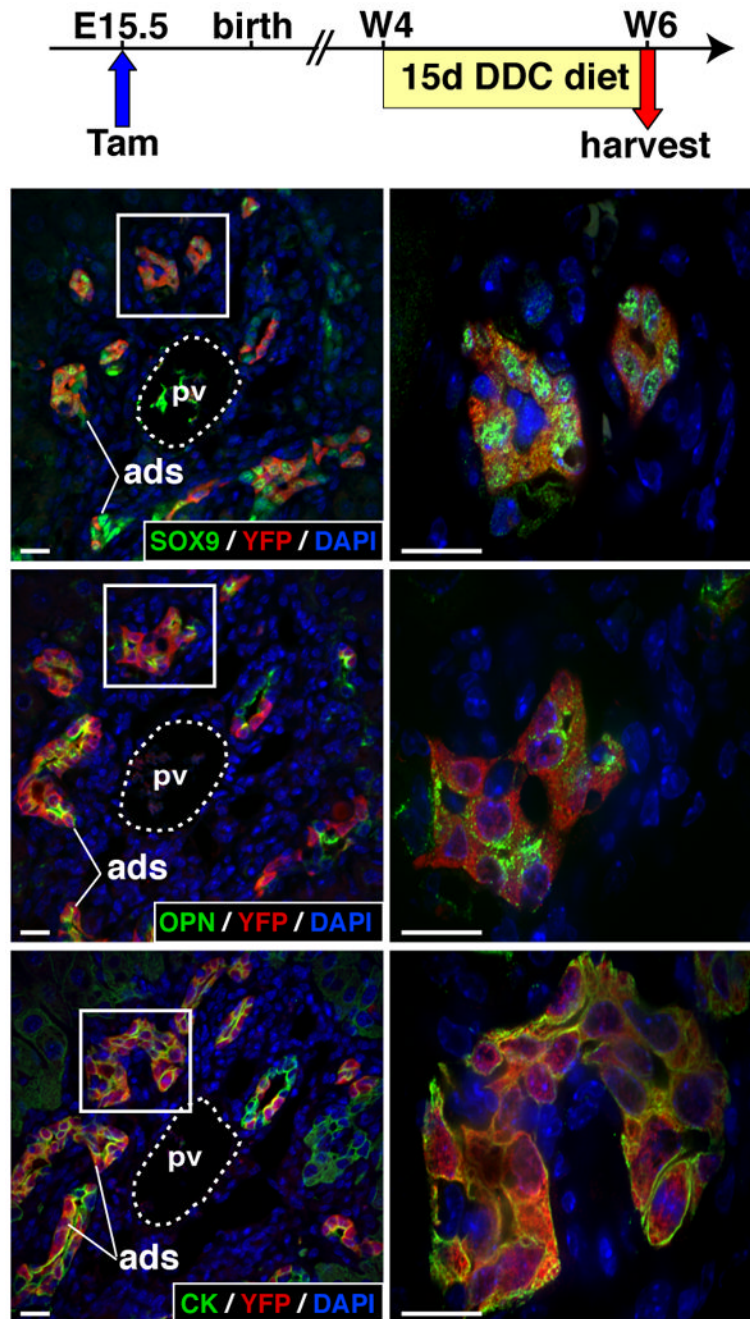


Figure 6.

DDC diet-induced oval cells derive from the ductal plate. Pregnant females were injected with tamoxifen at E15.5, and 4 week-old *SOX9-CreER^{T2};ROSA26R^{YFP}* offspring were fed a DDC diet for two weeks. Atypical ductular structures were induced. They co-express YFP and biliary/oval cells markers SOX9, OPN and CK (pan-cytokeratin antibody). The right pictures correspond to magnified areas delineated by a white box in the left panels. ads, atypical ductular structure; pv, portal vein; w, week after birth; size bar, 10 μ m.