

RESEARCH ARTICLE

Axon guidance genes control hepatic artery development

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ABSTRACT

Earlier data on liver development demonstrated that morphogenesis of the bile duct, portal mesenchyme and hepatic artery is interdependent, yet how this interdependency is orchestrated remains unknown. Here, using 2D and 3D imaging, we first describe how portal mesenchymal cells become organised to form hepatic arteries. Next, we examined intercellular signalling active during portal area development and found that axon guidance genes are dynamically expressed in developing bile ducts and portal mesenchyme. Using tissue-specific gene inactivation in mice, we show that the repulsive guidance molecule BMP co-receptor A (RGMA)/neogenin (NEO1) receptor/ligand pair is dispensable for portal area development, but that deficient roundabout 2 (ROBO2)/SLIT2 signalling in the portal mesenchyme causes reduced maturation of the vascular smooth muscle cells that form the tunica media of the hepatic artery. This arterial anomaly does not impact liver function in homeostatic conditions, but is associated with significant tissular damage following partial hepatectomy. In conclusion, our work identifies new players in development of the liver vasculature in health and liver regeneration.

KEY WORDS: Liver development, Slit-Robo signalling, Liver mesenchyme

INTRODUCTION

The liver is composed of lobules consisting of hepatocyte cords and sinusoids that radiate from the portal area to the central vein. Each portal area contains a portal vein surrounded by a dense mesenchyme in which hepatic arteries, bile ducts, nerves and lymphatics are encapsulated (Krishna, 2013). Portal lymphatics collect fluid filtered through the sinusoids and drained via the space of Disse (Tanaka and Iwakiri, 2016). Nerves regulate blood flow in

the liver and their development is controlled through bile duct-mediated secretion of nerve growth factor (Tanimizu et al., 2018). Bile ducts drain bile produced by hepatocytes towards the intestine. Finally, the portal vein provides nutrients and oxygen to the liver; the artery contributes less than half (~25%) of the hepatic blood flow but supplies ~50% of the oxygen.

During development, the portal mesenchyme is derived from the septum transversum (Asahina et al., 2009, 2011). It instructs differentiation of liver progenitors (hepatoblasts) towards biliary cells (cholangiocytes), and subsequently promotes bile duct morphogenesis (reviewed by Gordillo et al., 2015; Lemaigre, 2020; Ober and Lemaigre, 2018; Tanimizu and Mitaka, 2017). Reciprocally, unknown signals from the bile ducts promote perinatal expansion of the portal mesenchyme, as evidenced by hypotrophy of the mesenchyme resulting from cholangiocyte-specific inactivation of the SRY-related HMG box transcription factors (SOX) 4 and SOX9 (Poncy et al., 2015).

Hepatic arteries develop in close proximity to the maturing bile ducts. The arteries' endothelium is likely recruited by developing cholangiocytes through secretion of vascular endothelial growth factor A (Fabris et al., 2008; Gouysse et al., 2002). The vascular smooth muscle cells (VSMCs) forming the artery's tunica media are considered to derive from portal mesenchymal cells (Libbrecht et al., 2002; Villeneuve et al., 2009). Proper arrangement of the arterial VSMCs requires the integrity of the bile ducts, as hepatocyte nuclear factor (HNF) 6 (ONECUT1) or HNF1 β knockout mouse livers have disrupted bile ducts and fail to develop normal arteries (Clotman et al., 2003). Development of the hepatic artery occurs according to a similar pattern in mouse and human, except that artery morphogenesis in mouse occurs postnatally, whereas human hepatic arteries form during the fetal period (Clotman et al., 2003).

Here, our objective is to identify genes and signalling pathways that control morphogenesis of the hepatic artery. Our strategy was to search for ligand/receptor pairs expressed in the developing portal mesenchyme and bile ducts and to test their function using gene inactivation strategies. With this approach, we uncovered that several axon guidance genes are expressed in the developing portal area. Although repulsive guidance molecule BMP co-receptor A (*Rgma*) and neogenin (*Neo1*) genes are dispensable for development of the hepatic artery, we found that roundabout 2 (ROBO2)/SLIT2 signalling promotes development of the VSMCs lining the hepatic artery.

RESULTS

Maturation of the portal mesenchyme and hepatic artery development

At the onset of portal mesenchyme development in human fetal liver, the smooth muscle cell marker α smooth muscle actin (α SMA; ACTA2) is detected in a large proportion of mesenchymal cells. Later, at around 20 weeks of development, it becomes restricted to VSMCs lining the artery and portal vein, whereas the rest of the

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mesenchyme is α SMA negative (Libbrecht et al., 2002; Villeneuve et al., 2009). We found that this process is conserved in mouse: α SMA expression was detected throughout the portal mesenchyme at postnatal day (P) 1, and progressively became confined to the tunica media of the hepatic artery (Fig. 1A). Staining of smooth muscle protein 22 α (SM22 α ; also called transgelin, TAGLN), which is co-expressed with α SMA (Fig. S1), was used to visualise the cells in three dimensions (Fig. 1B, Movie 1). SM22 α -expressing cells irregularly surrounded the portal vein at embryonic day (E) 17.5 and P1. At P6, these cells concentrated to form the hepatic arteries near the hilum, whereas the periphery of the lobes still displayed SM22 α -expressing cells scattered in the mesenchyme around the portal vein. Bile duct morphogenesis undergoes profound changes between E16.5 and E18.5 (Takashima et al., 2015; Tanimizu et al., 2016) and, accordingly, preceded formation of the arteries, as illustrated by staining for the biliary markers SOX9 and osteopontin (OPN; SPP1) (Fig. 1).

Axon guidance signals are expressed in developing bile ducts and portal mesenchyme

To search for potential mesenchyme–bile duct interactions driving hepatic artery development, we separately purified the portal

mesenchyme and cholangiocytes from mouse livers at E16.5 and E18.5. We reasoned that these stages correspond to the initiation of intense bile duct remodelling, and may therefore also represent a potential transition between developmental stages of the portal mesenchyme. Cholangiocytes were purified from *Sox9-GFP* livers, which express GFP specifically in cholangiocytes (Demarez et al., 2018), and mesenchymal cells were isolated from *Sm22 α -Cre; Rosa26^{eYFP}* livers. The latter express eYFP from the *Rosa26* locus when recombined by *Sm22 α -Cre*, which is reportedly active in the developing portal mesenchyme (Hofmann et al., 2010). We confirmed that *Sm22 α -Cre* is active in portal mesenchymal cells: eYFP colocalised in prenatal *Sm22 α -Cre; Rosa26^{eYFP}* livers with α SMA⁺ and α SMA[−] mesenchymal cells, and postnatally with the VSMCs lining the portal vein and hepatic artery (Fig. 2). Labelling of the mesenchyme using *Sm22 α -Cre* was further confirmed by co-staining of eYFP in portal fibroblasts expressing CD90 (THY1) (Fig. 2). We did not detect eYFP in bile ducts, hepatocytes or endothelial cells. We concluded that *Sm22 α -Cre* is appropriate to investigate gene function in developing portal mesenchymal cells.

GFP⁺ cholangiocytes and eYFP⁺ mesenchymal cells were purified at E16.5 and E18.5 and subjected to RNA sequencing (Fig. 3A,B). We postulated that genes involved in hepatic artery

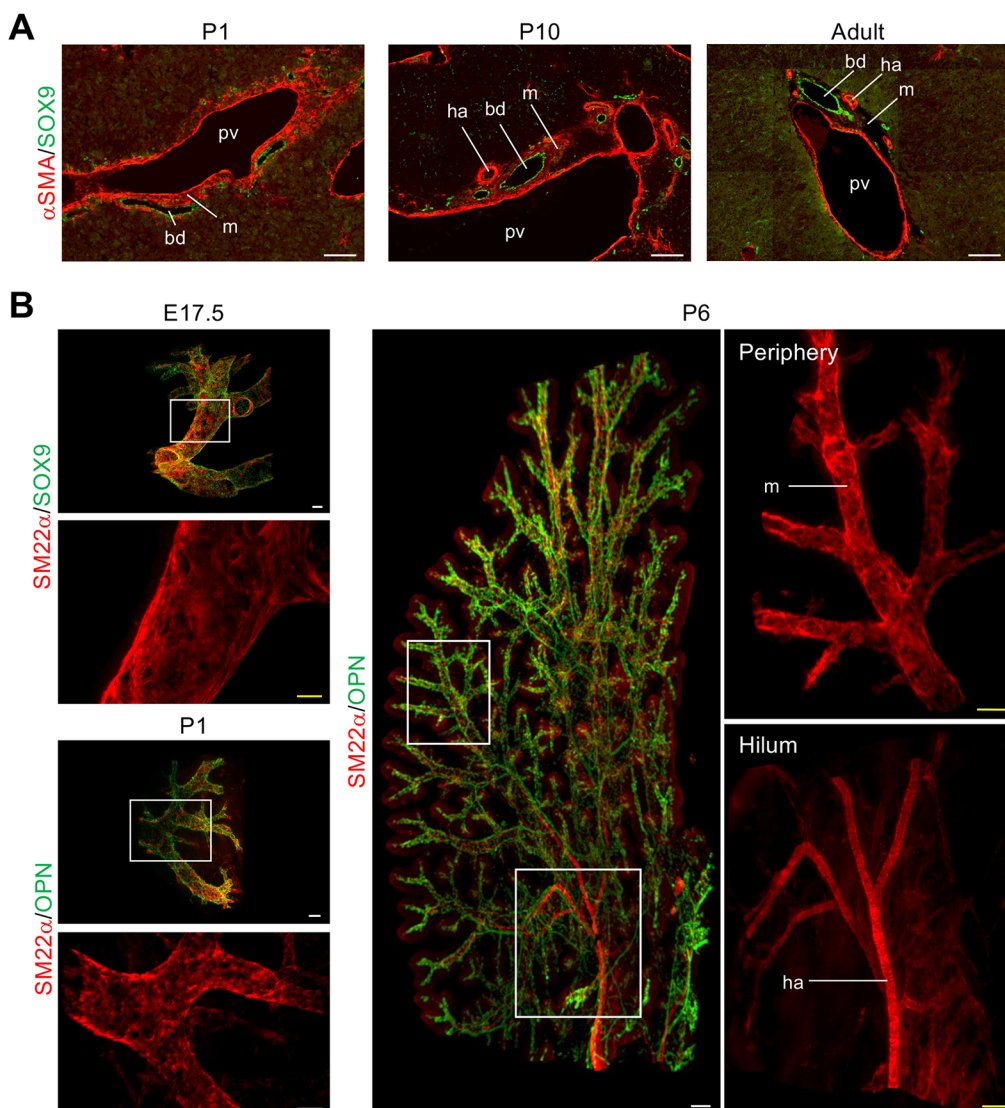


Fig. 1. Expression of the smooth muscle cell marker α SMA in the developing portal area. (A) 2D sections show that α SMA-expressing cells are scattered in the portal mesenchyme of postnatal mouse livers and become restricted to the tunica media of hepatic arteries as artery development proceeds. (B) The same process is illustrated in 3D, using SM22 α staining. Development of the artery is first seen near the hilum of the liver. At the periphery of the lobes, SM22 α -expressing cells are still scattered in the mesenchyme around the portal vein. SOX9 and OPN labelling illustrate bile duct morphogenesis. Boxed areas are shown at higher magnification below and to the right of main panels. bd, bile duct; ha, hepatic artery; m, mesenchyme; pv, portal vein. Scale bars: 50 μ m (white); 100 μ m (yellow).

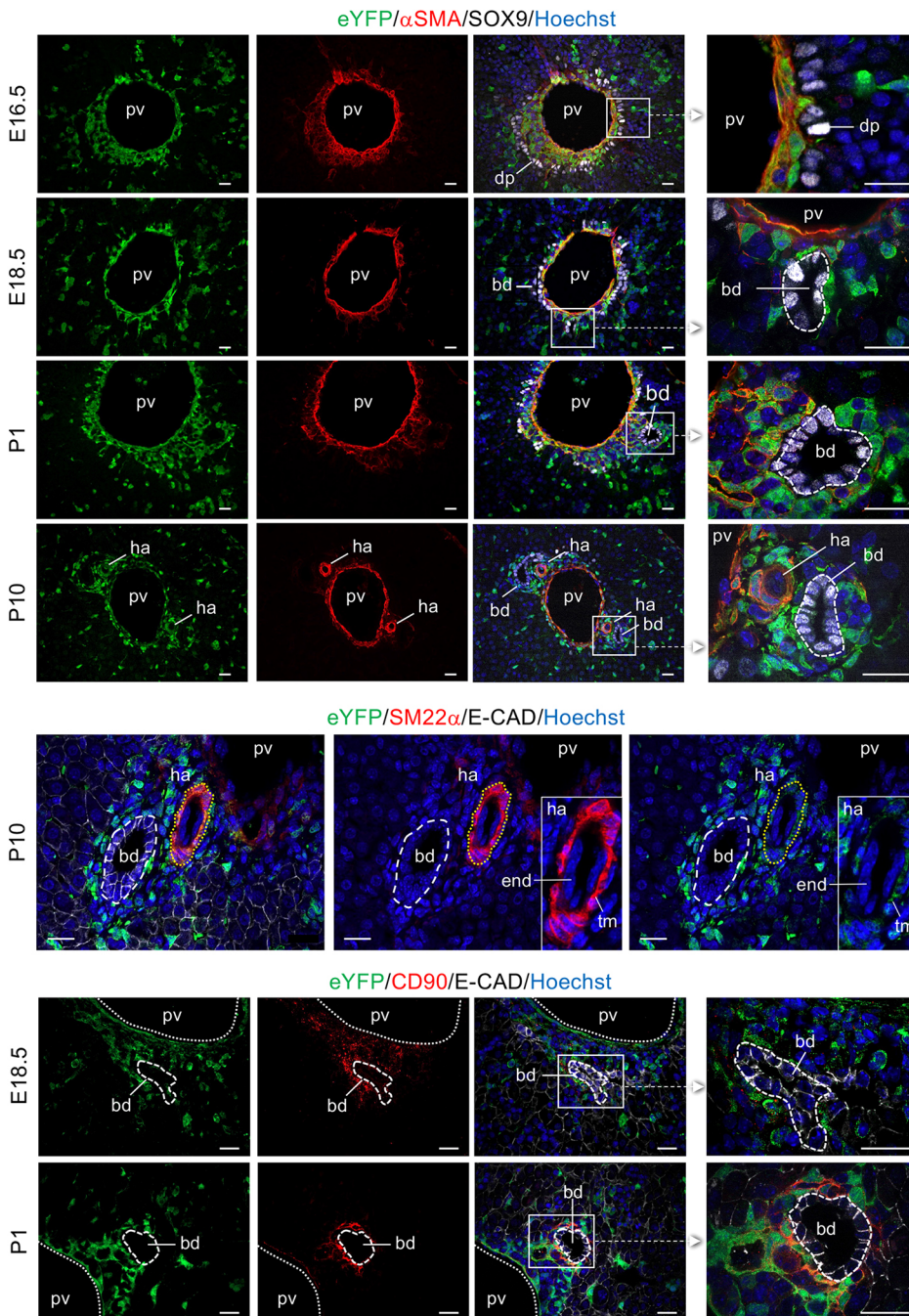


Fig. 2. Activity of *Sm22α-Cre* in developing liver. eYFP staining of livers from *Sm22α-Cre*; *Rosa26^{eYFP}* mice reflects the activity of *Sm22α-Cre* in the portal tract at the indicated developmental stages. eYFP expression is found in the portal tract mesenchyme, including in α SMA-expressing cells and CD90⁺ fibroblasts, but not in SOX9⁺/E-CAD⁺ biliary cells and in arterial endothelial cells. Boxed areas are shown at higher magnification to the right of main panels or as insets. Dotted and dashed lines delineate marked regions. bd, bile duct; dp, ductal plate; end, endothelium; ha, hepatic artery; pv, portal vein; tm, tunica media. Scale bars: 20 μ m.

development should display differential expression between E16.5 and E18.5. We focused on genes coding for ligands produced by cholangiocytes and for their cognate receptors in mesenchymal cells, or vice versa. Fig. 3B provides a pathway enrichment analysis of genes showing a $\log_2$ fold increase ≥ 1 ($P_{\text{adj}} < 0.05$) from E16.5 to E18.5. This analysis revealed that the axon guidance pathway is enriched during development of both the portal mesenchyme and cholangiocytes.

Expression of *Slit/Robo* and *Rgma/Neo1* genes

We then decided to first focus on the receptor *Robo2* identified by earlier single-cell RNA sequencing data as being strongly expressed in mesenchymal cells during liver development (Wang et al., 2020). According to our RNA-sequencing analysis, its expression showed

a significant increase from E16.5 to E18.5 in the portal mesenchyme (Fig. 3C). Spatially, expression of *Robo2* mRNA was detected in the portal mesenchyme: RNAscope *in situ* hybridisation localised *Robo2* mRNA mainly to the embryonic portal mesenchyme, predominantly in cells adjacent to developing ducts (E16.5–E18.5). This observation was confirmed at the protein level by immunostaining (Fig. 4A). After birth, at P1 and P10, the number of *Robo2*-expressing cells in the portal mesenchyme progressively decreased. It was detected at low levels in a limited number of periportal CD90-expressing fibroblasts (white arrowheads in Fig. 4A) and in a subset of α SMA-expressing cells at P1. Later, at P10, it was no longer found in α SMA-expressing VSMCs lining the portal vein or hepatic artery (Fig. 4A), in line with single-cell RNA-sequencing data, which, in adult liver mesenchymal cells, detected

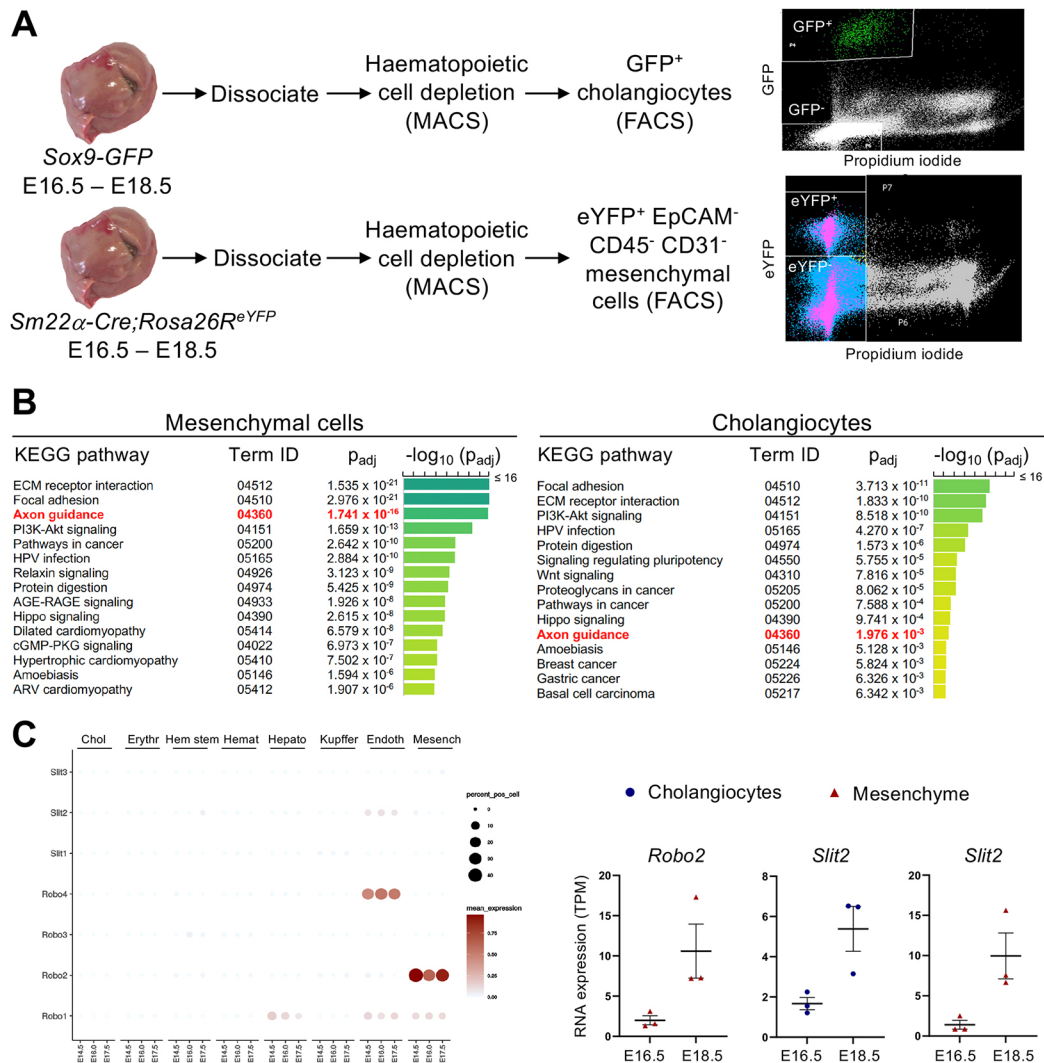


Fig. 3. Purification and gene expression in developing cholangiocytes and portal mesenchymal cells. (A) Flow cytometry purification procedure to isolate GFP⁺ cholangiocytes and eYFP⁺ mesenchymal cells from *Sox9-GFP* and *Sm22α-Cre;Rosa26R^{eYFP}* livers, respectively. FACS, fluorescence-activated cell sorting; MACS, magnetic-activated cell sorting. (B) Pathway enrichment analysis of genes upregulated at E18.5 compared with E16.5 ($\log_2$ fold change ≥ 1 , $P_{\text{adj}} < 0.05$) identifies the axon guidance pathway in both mesenchymal cells and cholangiocytes. (C) Left: Slit/Robo signalling genes in single-cell RNA sequencing analyses from Wang et al. (2020). Right: Transcripts per million (TPM) detected in bulk RNA sequencing from purified developing cholangiocytes and mesenchymal cells. $n=3$ pools of 15 embryonic livers; data are mean $\pm$ s.e.m. Chol, cholangiocytes; Erythr, erythroid progenitors; Hem stem, non erythroid haematopoietic stem cells; Hemato, myeloid/lymphoid/megakaryoid cells; Hepato, hepatoblasts/hepatocytes; Kupffer, Kupffer cells; Endoth, endothelial cells; Mesench, mesenchymal cells.

Robo2 in fibroblasts but not in VSMCs (Fig. S2A) (Lei et al., 2022). Most *Robo2*⁺ cells were located near the duct and artery. We found no evidence for *Robo2* expression in arterial endothelial cells (Fig. 4C). Less relevant to hepatic artery development, but confirming earlier data (Lei et al., 2022), *Robo2* was co-expressed with desmin in parenchymal stellate cells (Fig. S2).

The ROBO ligands are SLIT-1, -2 and -3. Their expression was below detection level in the single-cell RNA sequencing analyses of embryonic liver (Wang et al., 2020). However, using the more sensitive RNAscope *in situ* hybridisation, we detected *Slit3* in portal endothelial cells at a distance from the developing bile ducts and in a small subset of cholangiocytes, whereas *Slit1* was barely detectable in the developing portal area (Fig. S2B). SLIT2 was considered more attractive as a regulator of hepatic artery development. Indeed, *Slit2* expression in liver was strongest in the portal area, where it was detected in cholangiocytes lining developing ducts, in mesenchymal cells and in portal endothelium

(Fig. 4B). This expression was transient in biliary cells (E16.5-P1) and portal endothelium (E16.5-E18.5), but was detected in the mesenchyme at all pre- and postnatal stages tested. In the mesenchyme, *Slit2* was detected in CD90⁺ fibroblasts. It was also detected in α SMA-positive cells near the bile ducts at P1, i.e. prior to artery formation, as well as in a subset of VSMCs lining the artery at P10 (white arrowheads in Fig. 4B). Artery endothelium and parenchymal desmin-expressing stellate cells did not show *Slit2* expression (Fig. 4C, Fig. S2B). Our observations fit with the single-cell RNA-sequencing data of Henderson's and Housset's teams who, later in adult liver mesenchymal cells, essentially detected *Slit2* in fibroblasts and in a subset of VSMCs (Fig. S2A) (Dobie et al., 2019; Lei et al., 2022).

Rgma/Neol was also an interesting ligand/receptor pair because it showed increased expression in the developing ducts and mesenchyme from E16.5 to E18.5 (Fig. S3A). *Rgma* expression was predominantly detected in a subset of developing

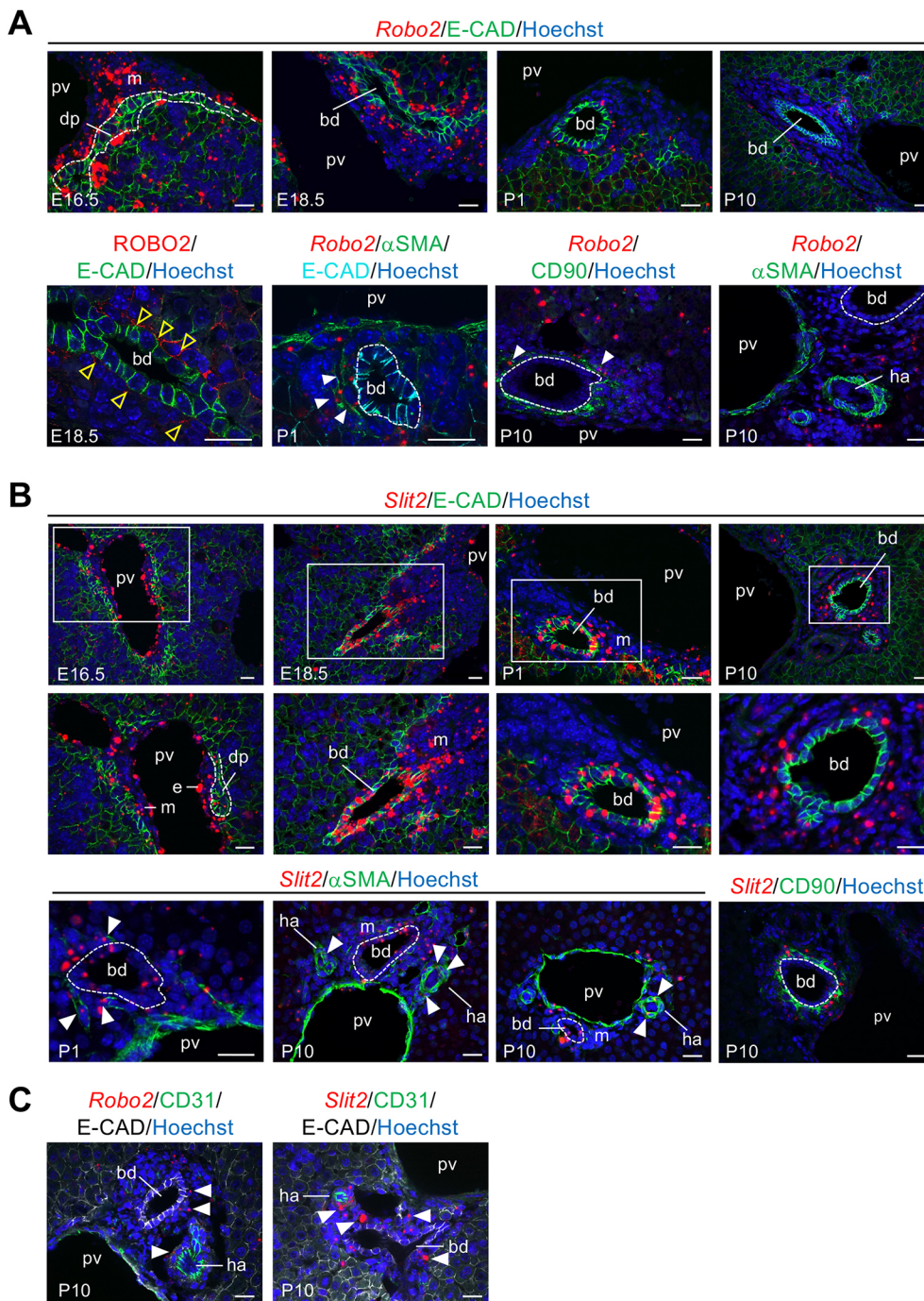


Fig. 4. Expression of *Robo2* and *Slit2* in developing liver. (A) RNAscope *in situ* hybridisation and immunostaining reveals *Robo2* RNA and protein (yellow arrowheads) are expressed in the embryonic and neonatal portal mesenchyme. In the mesenchyme, *Robo2* is detected in a subset of CD90⁺ fibroblasts and at P1 in some α SMA⁺ cells (white arrowheads). At P10, *Robo2* is not detected in the VSMC lining the hepatic artery. (B) *Slit2* is transiently expressed in developing cholangiocytes (high E-CAD expression; E16.5 to P1), as well as in the portal mesenchyme at all stages tested. In the mesenchyme, *Slit2* is detected in CD90⁺ fibroblasts and in a subset of α SMA⁺ cells (white arrowheads) near the bile ducts or lining the artery. (C) There is no evidence for *Robo2* or *Slit2* expression in the hepatic artery endothelium. White arrowheads indicate expression of *Robo2* or *Slit2* in cells distinct from endothelial cells. Boxed areas are shown at higher magnification below. Dashed lines delineate marked regions. bd, bile duct; dp, ductal plate; e, endothelium; ha, hepatic artery; m, mesenchyme; pv, portal vein. Scale bars: 20 μ m.

cholangiocytes and only in very rare mesenchymal cells (Fig. S3B). NEO1 was found in the developing portal mesenchyme, cholangiocytes and hepatic artery (Fig. S3C).

We conclude that SLIT2/ROBO2 and RGMA/NEO1 signalling are candidate regulators of hepatic artery development.

***Rgma*, *Neo1* and biliary expression of *Slit2* are dispensable for hepatic artery and bile duct morphogenesis**

To investigate whether production of SLIT2 by cholangiocytes might impact hepatic artery formation or bile duct morphogenesis, we conditionally inactivated its expression by crossing *Slit2*^{loxP/loxP} mice with *Alfp-Cre* mice. The *Alfp-Cre* transgene induces

recombination of loxP-flanked genes in hepatoblasts, resulting in inactivation of loxP-flanked alleles in the progeny of these cells, namely cholangiocytes and hepatocytes. Hepatic arteries displayed a normal phenotype in P10 *Alfp-Cre;Slit2*^{loxP/loxP} knockout livers, as shown by immunostaining of their tunica media using antibodies against SM22 α and α SMA, or by detection of elastin (Fig. S4A). Moreover, the *Alfp-Cre;Slit2*^{loxP/loxP} mice showed normal biliary ducts (Fig. S4B). The ducts were delineated by well-differentiated and well-polarised cholangiocytes, as evidenced by their transcription factor expression profile (SOX9⁺, HNF4 α ⁺, C/EBP α ⁺), and by the location of β -catenin and E-cadherin (E-CAD; cadherin 1) at their basolateral sides, and mucin 1 at their apical poles. The

muscular layer surrounding the portal vein appeared normal as well, and neural class III tubulin (TUBB3)-expressing nerves were present in the portal space of the mutant livers (Fig. S4C). All animals were healthy at the adult stage. We conclude that expression of *Slit2* in biliary cells is dispensable for normal development of the portal vasculature and bile ducts.

Conditional inactivation of *Rgma* in developing cholangiocytes using *Sox9-Cre^{ER};Rgma^{loxP/loxP}* mice, and cholangiocyte- and mesenchyme-specific inactivation of *Neol* using *Sox9-Cre^{ER};Neol^{loxP/loxP}* and *Sm22α-Cre;Neol^{loxP/loxP}* mice, did not reveal biliary, mesenchymal or hepatic artery defects either (Figs S5–S7). This was concluded from our immunostainings for markers of cholangiocyte differentiation and polarity (SOX9, MUC1, E-CAD), hepatic artery [SM22α, αSMA, CD31 (PECAM1)], hepatocytes (HNF4α, CEACAM1), mesenchyme (vimentin) and lymphatics (LYVE1), which did not differ between control and knockout livers. The mice were healthy.

***Slit2* expression in mesenchymal cells is dispensable for bile duct development, but is required for normal morphogenesis of hepatic arteries**

We next addressed the role of *Slit2* expression in the portal mesenchyme by crossing *Sm22α-Cre* and *Slit2^{loxP/loxP}* mice. Here again we found no biliary anomaly: at P10, *Sm22α-Cre;Slit2^{loxP/loxP}* mice developed bile ducts with normal morphology and surrounded by well-polarised and well-differentiated cholangiocytes (Fig. S8A). There was no evidence of cholestasis, as shown by normal plasma bilirubin levels (Fig. S8B). Moreover, retrograde injection of ink in the common bile duct followed by tissue clearing did not reveal anomalies of the intrahepatic biliary tree (Fig. S8C). TUBB3-expressing nerves were detected in the portal space of the control and mutant livers, as well as LYVE1⁺ lymphatics (Fig. 5A, Fig. S8A).

However, immunostaining for αSMA, SM22α and smoothelin (SMTN) revealed arterial anomalies in the *Sm22α-Cre;Slit2^{loxP/loxP}* mice at P10. The arterial phenotype was variable, with approximately 50% of animals (6/11) displaying no detectable phenotype, whereas the others (5/11) presented with a reduction in the thickness of the tunica media ranging from nearly no VSMCs surrounding the arterial endothelium to a limited decrease in the arterial wall's thickness. Fig. 5A illustrates three phenotypes observed in *Sm22α-Cre;Slit2^{loxP/loxP}* mice, namely the presence of normal arteries, the lack of detectable VSMCs around the arteries, and an intermediate phenotype with reduced thickness of the artery's muscular layer. This phenotype was confirmed quantitatively: measurement of the ratio of the surface of the muscle layer to the total artery area revealed a significant reduction in the knockouts (Fig. 5B). Accordingly, the ratio of the arterial luminal area to the surface of the total artery area was increased in the mutant livers (Fig. 5B).

To avoid a potential bias related to arterial size, these measurements were performed on hepatic arteries with a cross-sectional area inferior or superior to 2000 μm². The number of animals and hepatic arteries measured in these experiments is shown in Table S1. The phenotypic heterogeneity did not reflect variable efficiency of *Sm22α-Cre*-mediated inactivation of the *Slit2* gene. *Sm22α-Cre* is indeed widely active in the portal mesenchyme of all animals as determined by eYFP induction in *Sm22α-Cre;Rosa26R^{eYFP}* livers (Fig. 2). Moreover, *in situ* RNA hybridisation using a BaseScope probe targeting the floxed exon 8 of *Slit2* showed efficient inactivation of the gene, in all portal areas (Fig. S9). Therefore, the heterogeneous phenotype resulted from biological rather than technical variability.

We next hypothesised that the reduced tunica media impacts the overall morphology of the hepatic artery and visualised the artery using anterograde injection of ink in the gastroduodenal artery. Control livers displayed straight artery branches, whereas localised tortuosity was observed in a subset of artery branches of *Sm22α-Cre;Slit2^{loxP/loxP}* livers (red arrows in Fig. 6). Portal vein morphology was normal, as evaluated by ink injections (Fig. S10). Consistent with the phenotypic variability of the mice, only a subset of mice displayed these morphological anomalies.

We conclude that the expression of *Slit2* in the portal mesenchyme is dispensable for bile duct formation, but is necessary for normal development of the VSMCs that constitute the tunica media of the hepatic artery.

***Robo2* expression in mesenchymal cells is dispensable for bile duct development, but is required for normal morphogenesis of hepatic arteries**

Because SLIT2 is a ligand of ROBO2, we hypothesised that the latter is involved in hepatic artery development and generated *Sm22α-Cre;Robo2^{loxP/loxP}* mice in which *Robo2* is inactivated in the mesenchyme. Similar to *Slit2* inactivation in the mesenchyme, no evidence was found for abnormal differentiation and polarity of cholangiocytes, or for abnormal morphogenesis of bile ducts in *Sm22α-Cre;Robo2^{loxP/loxP}* mice (Fig. S8). Portal nerves (TUBB3⁺) and lymphatics (LYVE1⁺) also appeared normal, and liver function, assessed by plasma levels of the hepatic enzymes albumin and bilirubin, was unaffected (Fig. 5A, Fig. S8A,B). The efficiency of *Sm22α-Cre*-mediated inactivation of *Robo2* was demonstrated using BaseScope *in situ* hybridisation targeting the floxed exon 5 (Fig. S9).

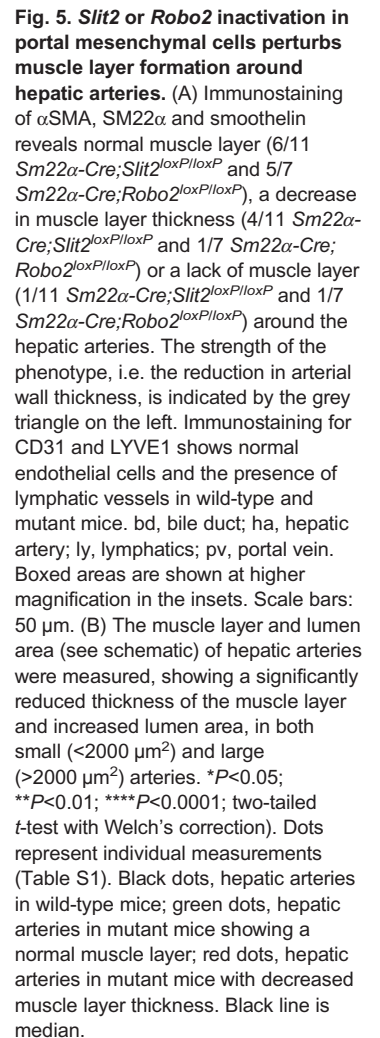
However, again similar to *Sm22α-Cre;Slit2^{loxP/loxP}* mice, a subset of *Sm22α-Cre;Robo2^{loxP/loxP}* mice displayed hepatic artery anomalies. Two mice out of seven showed reduced or lack of detectable muscular layer, as evidenced by αSMA, SM22α and smoothelin immunostaining (Fig. 5A). This phenotype was confirmed quantitatively using the same measurements as those performed in *Sm22α-Cre;Slit2^{loxP/loxP}* livers (Fig. 5B). Ink injection into the hepatic artery further revealed perturbed morphology, with artery branches showing localised tortuosity, again like in SLIT2-deficient livers (Fig. 6). Portal vein morphology was normal (Fig. S10).

The arterial phenotype in the absence of *Robo2* was mild. Given that *Robo1* is expressed in the mesenchyme (Fig. S2), we envisaged that *Robo1* could at least in part compensate for the absence of *Robo2* and generated *Sm22α-Cre;Robo2^{loxP/loxP};Robo1^{-/-}* mice. The newborns died shortly after birth (in line with observations by others who have investigated *Robo1^{-/-}* mice; Li et al., 2015). The early death of the *Sm22α-Cre;Robo2^{loxP/loxP};Robo1^{-/-}* newborns precluded the analysis of the hepatic artery at P10 in the liver lobes. However, hepatic arteries at the onset of their development were detected near the hilum at P0 (Fig. S11). Together, these data do not provide evidence for a compensation of *Robo2* deficiency by *Robo1*.

Because the phenotype resulting from *Robo2* inactivation in the portal mesenchyme is similar to that of *Slit2* inactivation, we suggest that ROBO2 is the receptor of SLIT2 during development of the hepatic artery's tunica media.

SLIT2/ROBO2 signalling controls maturation of vascular smooth muscle cells lining the hepatic artery

During vessel formation, VSMCs undergo profound changes, including their maturation from an immature synthetic state,



expression of phenotypic markers. Because this transition is expected to occur perinatally, i.e. when the VSMCs aggregate around the hepatic artery endothelium (Fig. 1), we purified the portal mesenchyme at P10 and measured the expression of synthetic and contractile markers. Considering the inter- and intra-organ heterogeneous arterial phenotype (Fig. 5) and the need for sufficient amounts of liver tissue for purification of portal mesenchyme, it was impossible to keep a part of the liver tissue for histological analysis to verify whether or not it displayed a morphological arterial defect.

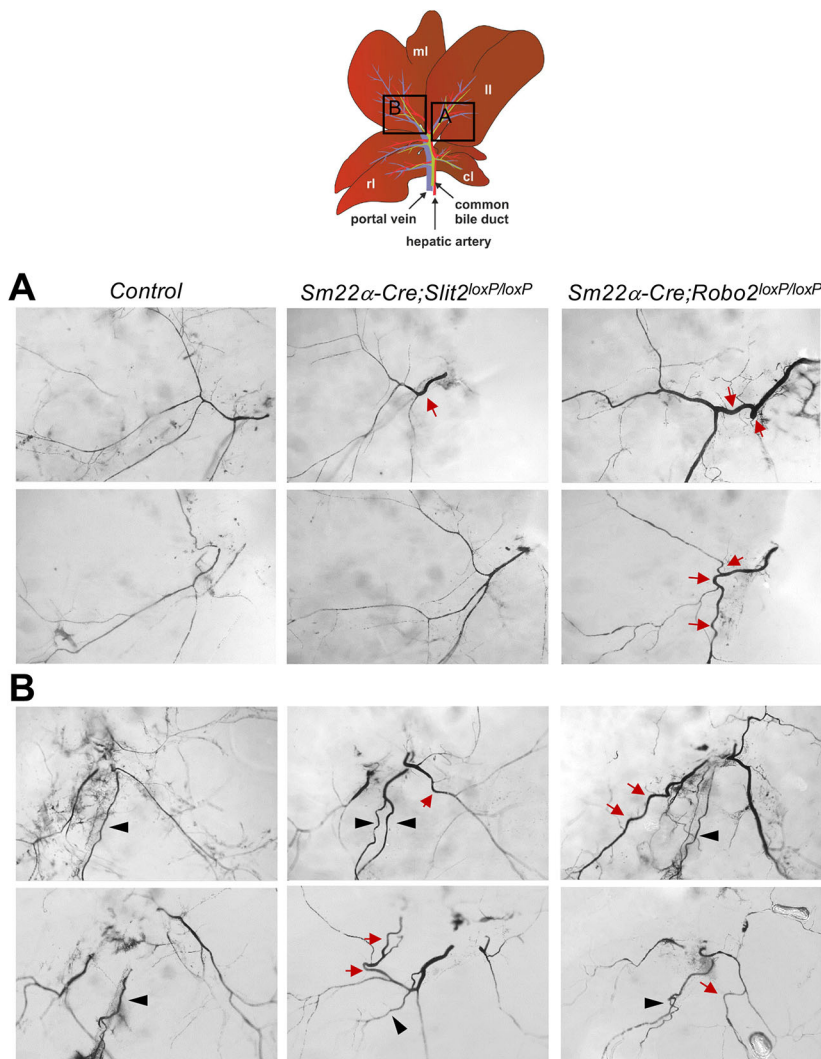


Fig. 6. *Slit2* or *Robo2* inactivation in portal mesenchymal cells modifies the shape of hepatic arteries. (A,B) Ink injection in the gastroduodenal artery followed by clearing of the median lobe (A) and the left lobe (B) shows irregular morphology (red arrows) of the arteries in 2-month-old *Sm22α-Cre;Slit2^{loxP/loxP}* (2/4) and *Sm22α-Cre;Robo2^{loxP/loxP}* (2/4) mice, compared with wild-type mice ($n=5$). Black arrowheads indicate the gallbladder artery. Schematic above depicts the regions shown in the image panels. cl, caudate lobe; ll, left lobe; ml, median lobe; rl, right lobe.

To circumvent this problem, we decided to evaluate the state of the mesenchyme by RT-qPCR analysis of a phenotypic index that corresponds to the ratio of expression of synthetic (*Coll15a1*, *Fbln2*, *Dnmt1*) versus contractile (*Cnn1*, *Eln*, *Smtn*) markers (Owens et al., 2004; Zhuang et al., 2017). Expression of these markers was normalised to that of the pan-mesenchyme marker vimentin. This approach was justified by our observation that expression of the mature contractile markers smoothelin and elastin is reduced in *Sm22α-Cre;Slit2^{loxP/loxP}* and *Sm22α-Cre;Robo2^{loxP/loxP}* mice at P10 (Figs 5A and 7A). The phenotypic index, calculated as indicated in Fig. 7B, shows that the synthetic state is increased in purified portal mesenchymal cells from mice that lack *Slit2* or *Robo2* expression, reflecting a reduction in the number of cells reaching VSMC maturity. Proliferation of α SMA⁺ cells at P10, analysed by phospho-histone H3 labelling, was low or undetectable at P10 in control liver as well as in mutant livers (Fig. 7C). Therefore, we concluded that the increased ratio of synthetic versus contractile gene expression reflects a failure of normal maturation of VSMCs in the mutant livers.

Tissular damage following partial hepatectomy

Sm22α-Cre;Slit2^{loxP/loxP} and *Sm22α-Cre;Robo2^{loxP/loxP}* mice grew well and were healthy in homeostatic conditions. It is known that,

following partial hepatectomy, the portal blood flow is increased in the remaining lobes. To mitigate vascular shear stress, the hepatic artery contracts to restrict blood flow. This is the so-called 'hepatic artery buffer response' (Lautt, 2007). Given that the *Slit2* and *Robo2* knockout mice had reduced tunica media, we tested whether this might impact liver morphology after partial hepatectomy. We performed 70% partial hepatectomy and analysed the phenotype 24 h later (Fig. 8). In contrast to control animals, *Sm22α-Cre;Robo2^{loxP/loxP}* mice displayed haemorrhagic and necrotic areas near the portal tract. Their hepatic arteries displayed a reduced layer of smooth muscle cells, as described above in younger animals. This was associated with cholestasis as evidenced by hyperbilirubinemia. Hepatocyte damage was further illustrated by elevated plasma levels of aspartate aminotransferase. A subset of partially hepatectomised *Sm22α-Cre;Slit2^{loxP/loxP}* livers also displayed haemorrhagic and necrotic areas; these were less frequent than in *Sm22α-Cre;Robo2^{loxP/loxP}* mice and were not associated with hyperbilirubinemia; only a slight increase in plasma aspartate aminotransferase was noticed in a subset of *Slit2*-deficient animals (Fig. 8). We concluded that the tissular damage observed after partial hepatectomy might at least in part be caused by inappropriate hepatic artery contraction leading to insufficient protection against excessive blood pressure and haemodynamic stress.

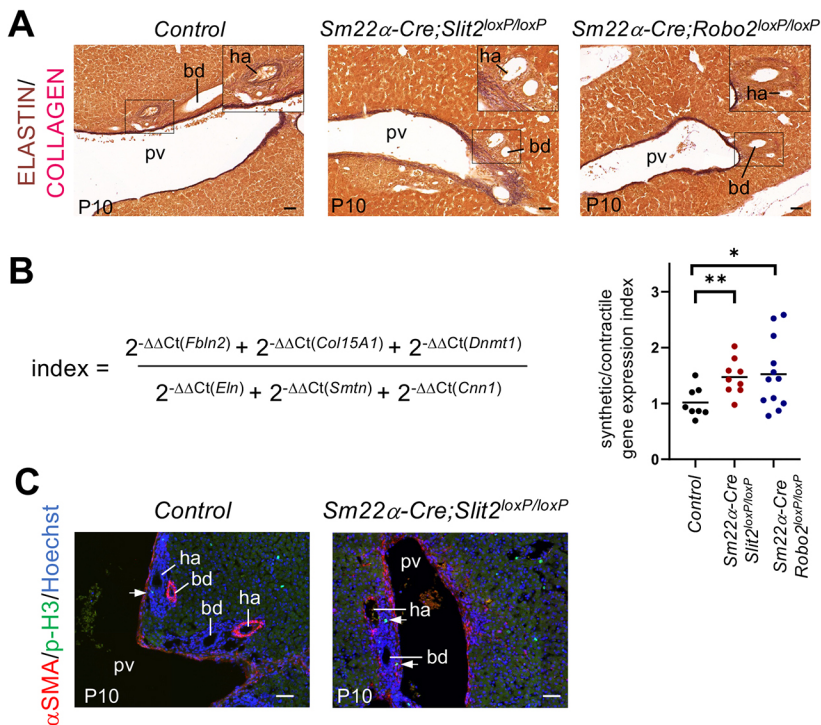


Fig. 7. *Slit2* or *Robo2* inactivation in portal mesenchymal cells affects the maturation of VSMCs.

(A) Reduced elastin staining around hepatic arteries in *Sm22α-Cre;Slit2^{loxP/loxP}* and *Sm22α-Cre;Robo2^{loxP/loxP}* mice. Boxed areas are shown at higher magnification in insets. (B) Left: The maturation of VSMCs was evaluated using an index calculated by the sum of expression of markers of the synthetic state divided by the sum of expression of markers of the contractile state. Expression levels are relative to the pan-mesenchymal marker vimentin and were calculated by RT-qPCR using the $\Delta\Delta\text{Ct}$ formula. Right: The maturation index of VSMCs in *Sm22α-Cre;Slit2^{loxP/loxP}* and *Sm22α-Cre;Robo2^{loxP/loxP}* mice at P10 suggests that the lack of *Slit2* or *Robo2* expression favours maintenance of a synthetic state. *Cnn1*, calponin 1; *Col15A1*, collagen 15a1; *Dnmt1*, DNA methyltransferase 1, *Eln*, elastin; *Fbln2*, fibulene 2; *Smtn*, smoothelin. Dots in graph represent individual animals (control, $n=8$; *Sm22α-Cre;Slit2^{loxP/loxP}*, $n=8$; *Sm22α-Cre;Robo2^{loxP/loxP}*, $n=12$); black lines represent means; * $P<0.05$; ** $P<0.01$ (paired t -test). (C) Proliferation visualised using phospho-histone H3 (p-H3) labelling does not uncover a difference in the proliferation of αSMA^+ cells in control and *Slit2*-deficient portal mesenchyme. Only rare proliferating cells were detected in the periportal area (white arrows). bd, bile duct; ha, hepatic artery; pv, portal vein. Scale bars: 50 μm .

DISCUSSION

Available knowledge on the role of axon guidance genes in the liver is limited to disease conditions. More specifically, the SLIT/ROBO pathway promotes fibrosis and neoangiogenesis during fibrosis and development of ductular reactions (Chang et al., 2015; Coll et al., 2022; Lei et al., 2022; Pi et al., 2022; Zeng et al., 2018). In hepatocellular carcinoma, expression levels of ROBO and SLIT genes are predictive of tumour stage (Avci et al., 2008). Here, we illustrate how portal mesenchymal cells become organised to form the hepatic artery and propose that SLIT2/ROBO2 signalling regulates the formation of the artery's tunica media by promoting maturation of the VSMCs.

Expression of *Robo2* and *Slit2* was observed at P1, i.e. prior to artery formation, in CD90^+ portal fibroblasts and in αSMA^+ cells located near the bile ducts. When the artery wall had developed at P10, *Slit2* but not *Robo2* was detected in the VSMCs forming the tunica media. *Slit2* or *Robo2* was absent from the hepatic artery's endothelium. This profile raises the possibility that during hepatic artery development SLIT2/ROBO2 signalling occurs between fibroblasts and αSMA^+ cells that are VSMC precursors. In this scenario, fibroblasts would promote VSMC maturation. Alternatively, SLIT2/ROBO2-mediated signalling between αSMA^+ VSMC precursors may be required for VSMC maturation. Yet another alternative is that SLIT2/ROBO2-mediated interactions between fibroblasts would secondarily impact on VSMC development. How intercellular signalling is mediated to control VSMC development remains unknown.

Although the heterogeneity of the liver mesenchyme has been well characterised in adults (Andrews et al., 2022; Dobie et al., 2019; Lei et al., 2022), investigation of the cell–cell and molecular mechanisms driving VSMC maturation requires better knowledge of the portal mesenchymal cell populations in the pre- and perinatal periods. Lei et al. identified five subpopulations of VSMCs characterised by specific markers (Lei et al., 2022). However, these markers were expressed at levels too low to allow accurate

investigation in mesenchyme purified at P10 (data not shown), likely indicating that VSMC maturation is not yet terminated at that stage. Furthermore, increased knowledge of the spatial functions of the mesenchymal cells in the portal space is needed. Indeed, we note that *Robo2* expression is predominantly localised in the vicinity of bile ducts, and that inactivating *Slit2* or *Robo2* did not impact development of the narrow muscular layer surrounding the portal vein.

Several guidance molecules play a role in artery patterning, but the role of SLIT-ROBO signalling in this context remains obscure (Finney and Orr, 2018). SLIT2 produced by endothelial cells and VSMCs is a known repellent of arterial VSMCs (Liu et al., 2006). As this function was investigated *in vitro*, its physiological relevance remains unclear. Portal VSMCs fated to form the hepatic artery may exhibit migrational defects in the absence of SLIT2/ROBO2 signalling. Such defects could be associated with a lack of VSMC maturation. Migrational anomalies cannot easily be investigated *in vivo* in developing mouse liver. We currently lack information about VSMC precursors that would enable their purification and allow migrational behaviour in response to SLIT-ROBO stimuli to be tracked *in vitro*. Also, *in vitro* data on VSMCs contradicted *in vivo* findings (Owens et al., 2004), thereby prompting the need to design assays that recapitulate the hepatic artery specificities *in vitro*. Endothelial cells can recruit VSMCs during arteriogenesis. The lack of *Slit2* or *Robo2* detection in the hepatic artery's endothelium prompts us to rule out that the arterial defect seen in the mutant mice results from an endothelial cell defect.

We noted a significant inter-individual phenotypic variability of the arterial phenotype. Although the reason for such variability was not investigated, we note that the mice were in a mixed genetic background. The arterial phenotype caused by the inactivation of *Slit2* and *Robo2* in the mesenchyme was mild in homeostatic conditions. Our analyses of double *Robo1/Robo2* knockouts did not support that ROBO1 might compensate for the

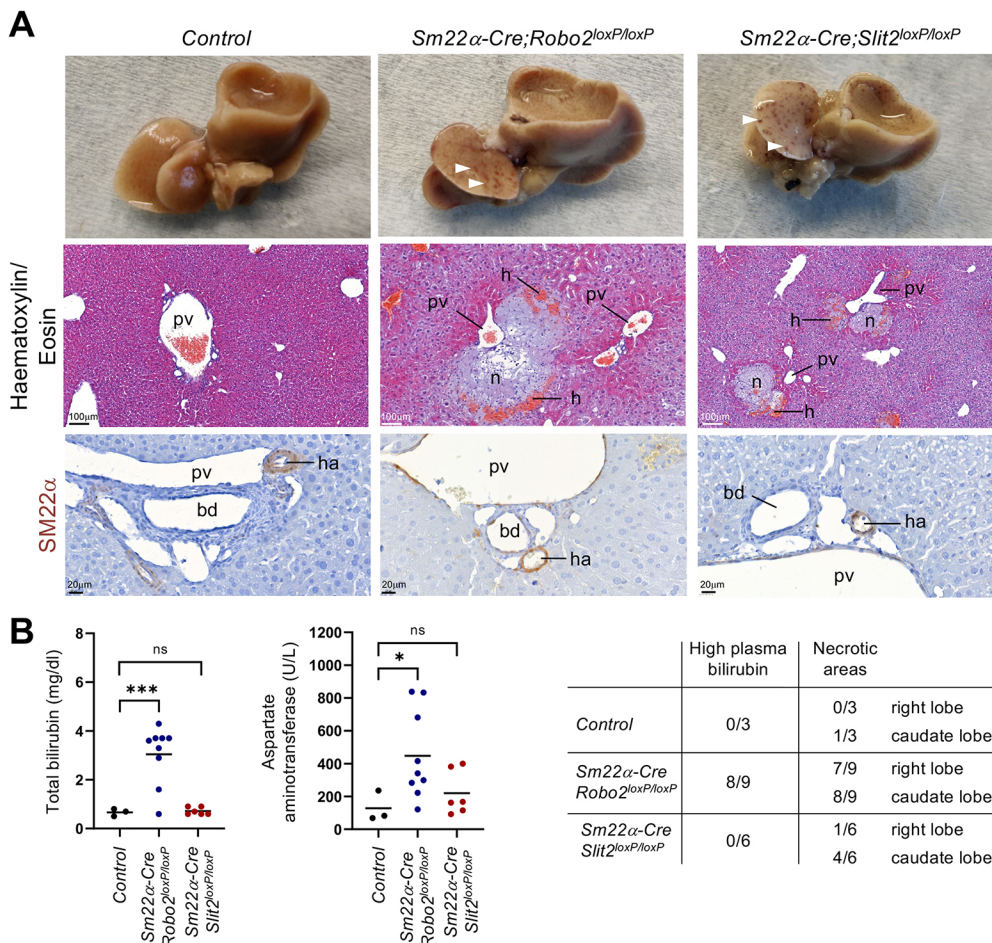


Fig. 8. Tissue damage following partial hepatectomy of mice with reduced muscle layer around the hepatic artery. (A) Adult control, *Sm22α-Cre;Slit2^{loxP/loxP}* and *Sm22α-Cre;Robo2^{loxP/loxP}* mice were subjected to 70% partial hepatectomy and analysed 24 h later. Haemorrhages were detected at the lobe surface (white arrowheads), and haemorrhagic and necrotic areas were detected near the portal tract on tissue sections of the mutant animals. SM22α immunostaining showed hepatic arteries with reduced muscular layer, as observed in younger animals (see Fig. 5). (B) Graphs illustrate plasma levels of total bilirubin and aspartate aminotransferase in mice 24 h after partial hepatectomy. Black lines represent means. * $P<0.05$; *** $P<0.001$ (paired t -test). ns, not significant. The table shows the number of partially hepatectomised mice displaying hyperbilirubinemia and tissular damage. bd, bile duct; h, haemorrhage; ha, hepatic artery; n, necrosis; pv, portal vein.

lack of ROBO2. Developing bile ducts, periportal mesenchyme and portal endothelium express *Slit3* (Fig. S2B), which may potentially compensate for the absence of *Slit2*. However, SLIT3 preferably binds to ROBO1 and ROBO4 (Zhang et al., 2009) and our RT-qPCR data did not show changes in *Slit3* mRNA levels in *Sm22α-Cre;Slit2^{loxP/loxP}* and *Alfp-Cre;Slit2^{loxP/loxP}* livers at P10 (not shown). In humans and primates, unmyelinated nerves are localised along the hepatic artery (Bioulac-Sage et al., 1990; Chapman and Eagles, 2008). These nerves might produce angiogenic growth factors, which may contribute to hepatic artery morphogenesis and potentially compensate in part for the absence of *Slit2* and *Robo2* expression in the portal mesenchyme.

Because formation of bile ducts precedes that of the arteries, we did not anticipate the presence of prenatal or early postnatal anomalies of the bile duct as a result of arterial defects. However, our data suggest that following partial hepatectomy, the reduced thickness of the tunica media in mice with deficient *Slit2* or *Robo2* results in the hepatic artery being unable to contract properly and protect the tissue from vascular stress, leading to tissular damage. The necrotic and haemorrhagic areas were devoid of bile plugs, eliminating the possibility that they correspond to biliary infarcts. The tissular damage is also unlikely to result from hypoxia caused by insufficient arterial blood supply. Indeed, this does not fit well with our observation that arteries are dilated. Also, livers from adult *Sm22α-Cre;Robo2^{loxP/loxP}* mice did not show overexpression of carbonic anhydrase 9, a target of HIF-1 and marker of hypoxia (data not shown).

Future investigation of hepatic mesenchyme development requires *Cre*-driver mice with higher cell specificity than *Sm22α-Cre*. Greenhalgh et al. listed mouse lines with *Cre* activity in non-parenchymal cells of the liver (Greenhalgh et al., 2015). Although *Pdgfrb-Cre* and *Lrat-Cre* are active in adult portal mesenchyme, no information is available on prenatal activity (Henderson et al., 2013; Mederacke et al., 2013). Here, we selected the *Sm22α-Cre* line described by Holtwick et al. (2002). The alternative *Sm22α-CreKI* is reportedly not functional in the embryo (Zhang et al., 2006). *Vimentin-Cre^{ER}* is not active in the portal mesenchyme of homeostatic liver (Troeger et al., 2012). *Gata4-G2-Cre* marks the septum transversum cells, but also a subset of CD45 (PTPRC)⁺ leucocytes, which may interfere with our objective to investigate portal mesenchymal cells, because CD45⁺ cells are present in the periportal space (Delgado et al., 2014).

In conclusion, our work raises novel questions and identifies future needs to tackle the functional understanding of the portal mesenchyme in development. It also reveals that SLIT/ROBO signalling is a regulator of liver vasculature development in health and regeneration.

MATERIALS AND METHODS

Animals

Mice (*Mus musculus*) received humane care and the research protocol was approved by the Animal Welfare Committee of the Université Catholique de Louvain with numbers 2018/UCL/MD/014, 2022/UCL/MD/16, and 2020/UCL/MD/020. All mice were housed under a 12 h light/dark cycle in individually ventilated cages supplied with RM3 chow (801700, Tecnilab,

Someren, Netherlands), acidified water, and polyvinyl chloride play tunnels. *Alfp-Cre*, *Sma22-Cre*, *Sox9-Cre^{ER}*, *Robo1^{-/-}*; *Robo2^{loxP/loxP}*, *Robo2^{loxP/loxP}*, *Rgma^{loxP/loxP}* and *Slit2^{loxP/loxP}* mice have been described (Domyan et al., 2013; Holtwick et al., 2002; Kellendonk et al., 2000; Kopp et al., 2011; Long et al., 2004; Lu et al., 2007; Rama et al., 2015) and were kindly provided by F. Tronche, W. Martinet, M. Sander, F. Spagnoli, V. Mirakaj and A. Chédotal. *Rosa26^{CreYFP}* and *Sox9-GFP* mice have been described (Gong et al., 2003; Srinivas et al., 2001). Transgenic mice were in mixed background, except for *Sox9-GFP*, which are in CD1 background. Sex of embryos and newborns was not determined. Age is indicated in the figure legends.

Neo1^{loxP/loxP} mice were generated using the cloning-free CRISPR-Cas9 system (Aida et al., 2015). Guide RNAs were designed using CRISPRdirect (Naito et al., 2015). Two guide RNAs targeting sequences 5' and 3' of exon 4 were designed. Each guide RNA consisted of a crRNA (20 nucleotides), corresponding to the target sequence with a tail (16 nucleotides), and a tracrRNA (67 nucleotides; 1072534, Integrated DNA Technologies), containing the hairpin recognised by Cas9 with an extremity complementary to the crRNA tail. Two long, single-strand oligonucleotides containing homology arms and loxP sequence were synthesised (Integrated DNA Technologies) as Ultramer DNA and used as repair template for homologous direct repair. crRNAs and single-stranded DNA oligonucleotides were as follows: crRNA-1 (5'), 5'-CGGCTGTTGTACCCTCGTG-3'; crRNA-2 (3'), 5'-ACATCAACCCCTCTACTGAC-3'; Ultramer 1 (5'),

5'-AGTCAGTAGGAAGGGACACAGGTATTGAGTTTGTAGTCTCGGCTGTTGTACCCTCGTAGCataactctgtataATGTATGctatacgaagtattGTGTGGTATTGTATAGATTTCATGTTGTGTCTTCCTCATGTGAC-CAGAAAGCACCATTGTAA-3'; Ultramer 2 (3'),

5'-CTTGAAAGGCATTTTATGTTGTGTCTATGGTGTAAAGAAAAGCATGCTTCCAGTCGAATTCataactctgtataATGTATGctatacgaagtattAGTAGAGGGGTTGATGTTGTCTGCTGCTGTTAACTTCA-TTATAGCTAGCTTGCAAGG-3' (lower case letters represent loxP sites).

Annealed crRNA/tracrRNA (2.4 pmol/μl), recombinant Cas9 nuclease V3 (200 ng/μl; Integrated DNA Technologies) and both single-strand DNA donors (5 ng/μl each) were mixed in IDTE buffer (11-01-02-02, Integrated DNA Technologies) and injected into the pronucleus of B6D2F2 mouse zygotes. Injected zygotes were incubated in KSOM medium at 37°C for at least 2 h before being transferred into oviducts of CD1 pseudo-pregnant female mice. Identification of the positive founders with insertion of both loxP sites was done by PCR genotyping and confirmed by Sanger sequencing. Cre-mediated recombination induces the deletion of loxP-flanked exon 4, creating a frame shift and premature stop codon, leading to a putative small protein of 255 aa instead of full-length neogenin 1.

Partial hepatectomy

In order to take into account circadian variations in liver regeneration, all procedures were performed between 07:00 and 12:00. During the operative procedures, animals were allowed to breathe spontaneously in a glass cylinder filled with a mixture of oxygen (2 l/min) and isoflurane (2.5%) (IsoFlo, Zoetis-Belgium SA). Animals had a median laparotomy after subcutaneous injection of 5 ml of warm physiological sterile solution to increase the circulatory blood volume. Liver ligaments were gently freed and the left lateral lobe and median lobe were lifted with cotton-tipped sticks out of the abdomen. Seventy per cent hepatectomy was performed by a non-resorbable suture at the basis of the median and left lateral lobe and removal of the above lobes as described (Higgins and Anderson, 1931; Nevzorova et al., 2015). Care was taken to avoid constriction of the lumen of the inferior intrahepatic vena cava and of the hepatic hilum.

Purification of mesenchymal cells and cholangiocytes

For RNA-sequencing analysis of embryonic cells, pools of 15 livers at E16.5 or E18.5 were dissected and mechanically dissociated using scalpel blades as described (Van Liedekerke et al., 2022). Briefly, *Sox9-GFP* or *Sm22α-Cre;Rosa26^{CreYFP}* livers were used for purification of cholangiocytes or mesenchymal cells, respectively. Subsequent chemical dissociation was performed in DMEM-F12 (31870-025, Gibco, Life Technologies) containing 1 mg/ml collagenase IV (LS00418, Worthington Biochemical Corporation), 1 mg/ml dispase (17105-041,

Gibco, Life Technologies) and 0.1 mg/ml of DNase I (11284932001, Roche) for 30 min at 37°C. An equal volume of 10% fetal bovine serum in PBS was added to stop the digestion, and cells were resuspended in 2 mM EDTA, 0.5% bovine serum albumin (BSA) in PBS and filtered through a 40 μm cell strainer (087711, Fisher Scientific). Haematopoietic cells were eliminated by magnetic cell sorting using the Lineage Cell Depletion kit (130090858, Miltenyi Biotec), which contains antibodies against CD5, CD45R (B220), CD11b (ITGAM), Gr-1 (Ly-6G/C; GSR), TRBV7-4 and Ter-119 (LY76). The cells were subjected to flow cytometry (BD FACSAria III, sample and collection tubes maintained at 4°C) and GFP⁺ cholangiocytes or eYFP⁺ CD45⁻ CD31⁻ EpCAM⁻ mesenchymal cells were purified. Antibodies used in flow cytometry are listed in Table S2. Total RNA was isolated using RNAqueous[®] Micro kit (AM1931, Invitrogen) according to the manufacturer's protocol. RNA quality was evaluated using the Agilent RNA 6000 Pico Kit (50671513, Agilent Technologies) and Bioanalyzer[™] (Agilent Technologies) for measuring concentration and calculation of RNA integrity number (RIN).

For RT-qPCR analysis of portal mesenchymal cells at P10, control and transgenic livers at P10 were dissected and mechanically dissociated using scalpel blades as described above. Trypsin-EDTA 0.05% (25300054, Gibco, Life Technologies) was used for 3 min at 37°C to help dissociation. Cells were resuspended in 2 mM EDTA, 5% fetal bovine serum in PBS, filtered through a 40 μm cell strainer and centrifuged at 50 g with a minimum deceleration. Supernatant was collected and centrifuged for 5 min at 290 g and the pellet retained. Total RNA was isolated using the RNeasy Mini Kit (74104, QIAGEN) according to the manufacturer's protocol, and was processed for reverse transcription using the First Strand cDNA Synthesis kit (K1612, Thermo Fisher Scientific). cDNA was used to perform qPCR using KAPA SYBR[®] FAST Universal 2X qPCR Master Mix (KK4602, Sopachem Life Sciences) and primers designed to amplify target genes (Table S4) on a CFX96 Real Time System (Bio-Rad) thermal cycler. Gene expression levels were determined by the 2^{-ΔΔCT} method following normalisation to vimentin as a reference gene. Technical triplicates were run for all samples and, as a control, a reaction without cDNA was performed.

Sequencing and bioinformatics workflow

For bulk RNA sequencing, read quality control was performed using FastQC software v0.11.7 (Babraham Institute, Cambridge, UK). Low-quality reads were trimmed and adapters were removed using Trimmomatic software v0.35 (Bolger et al., 2014). Reads were aligned using HISAT2 software v2.1.0 (Kim et al., 2015) on GRCm38 mouse genome. Gene counts were generated using HTSeq-count (v0.5.4p3) software (Anders et al., 2015) and gencode.vM15.annotation.gtf annotation file. Differential expression analyses were performed using DESeq2 (v1.24.0) (Love et al., 2014). Pathway analysis was performed using g:Profiler (Raudvere et al., 2019). The RNA-seq data have been deposited in the Gene Expression Omnibus database under accession numbers GSE163062 and GSE224219.

The count tables for the single-cell RNA sequencing re-analysis of the data of Wang et al. (2020) were kindly provided by the authors. The tables were combined, normalised using the cell-pooling approach as described (Lun et al., 2016) and log-transformed before visualisation.

Dual RNAscope/immunofluorescence and BaseScope

RNAscope RNA *in situ* hybridisation was performed on 6 μm sections of formalin-fixed, paraffin-embedded tissues (FFPE), according to the manufacturer's protocol for manual RNAscope[®] 2.5 HD Assay-RED (322350, Advanced Cell Diagnostics Inc.). The tissue sections were incubated at 60°C for 1 h, deparaffinised in xylene (twice, 5 min each) and dehydrated in 99% ethanol (twice, 2 min). Endogenous peroxidase was blocked with hydrogen peroxide for 10 min at room temperature (RT) followed by two short washes with deionised water. Slides were heated for 10 s at 100°C in deionised water, and antigen retrieval was performed for 15 min at 100°C using RNAscope[®] Target retrieval. Tissue sections were washed in deionised water and 99% ethanol. Slides were dried for 5 min at RT and tissues were delineated using an ImmEdge Hydrophobic Barrier Pen (310018, Advanced Cell Diagnostics Inc.). Slides were incubated for 15 min with RNAscope[®] Protease plus (diluted at 1/5 in deionised water) at 40°C, washed with deionised water and incubated with the probe for 2 h at 40°C.

The tissue sections were washed with RNAscope® Wash buffer and six amplifications were performed (using six reagents AMP1-AMP6). The signal detection followed using RNAscope® Fast A and B reagents for 10 min at RT. The slides were kept in PBS overnight before immunostaining was performed. Sections were blocked for 45 min at RT in 3% milk, 10% BSA, 0.3% Tween 20 in PBS. Primary and secondary antibodies were diluted in 10% BSA, 0.3% Tween 20 in PBS, and incubated, respectively, for 90 min at 37°C and 90 min at RT. Images were taken with Cell Observer Spinning Disk (Carl Zeiss) and Zen Blue software. Primary and secondary antibodies are described in Tables S1 and S2.

BaseScope RNA *in situ* hybridisation was performed on 6 µm sections of FFPE tissues, according to the manufacturer's protocol for BaseScope RED v2 kit, as described (Martens et al., 2021). The custom probes were designed to bind specifically to exon 8 of the *Slit2* gene, or to exon 5 of the *Robo2* gene.

Immunofluorescence and immunohistochemistry

Immunofluorescence and immunohistochemistry were performed on 6 µm sections of FFPE or sucrose-embedded frozen tissues. FFPE tissue sections were deparaffinised three times (3 min each) in xylene, then incubated for 3 min each in 99%, 95%, 70% and 30% ethanol and deionised water. Antigen retrieval was performed by microwave heating for 10 min or using the Lab Vision PT Module (Thermo Fisher Scientific), in 10 mM citrate pH 6 or Tris-EDTA pH 9, depending on the antibody. Sections were permeabilised for 5 min in 0.3% Triton X-100 in PBS before blocking for 45 min in 3% milk, 10% BSA, 0.3% Triton X-100 in PBS. For preparation of frozen sections, livers were collected, briefly washed in PBS, and fixed overnight in 4% paraformaldehyde (PFA). Tissues were incubated in sucrose 15% in PBS for 6–12 h and overnight in sucrose 30% in PBS, before embedding in OCT and freezing in liquid nitrogen-cooled isopentane. Frozen sections were thawed at RT for 20 min and rehydrated in PBS for 10 min. Antigen retrieval was performed by 5 min incubation in 10 mM citrate pH 6 preheated at 95°C, and sections were rinsed in deionised water and PBS, before blocking as above.

Primary antibodies were diluted in blocking solution at 4°C overnight and secondary antibodies were diluted in 10% BSA, 0.3% Triton X-100, 10 µg/ml bisBenzimide Hoechst H33258 (Sigma-Aldrich, B2883) in PBS at 37°C for 1 h. For immunohistochemistry, antibody binding was visualised using 3,3'-diaminobenzidine tetrachloride (ab64238, Abcam), and 30% Haematoxylin (109249, Merck Millipore) was used to counterstain the tissue. Pictures were taken with Cell Observer Spinning Disk (Carl Zeiss) and Zen Blue software, or with CaseViewer on scanned tissues. Primary and secondary antibodies are described in Tables S2 and S3.

Haematoxylin-Eosin and elastin-collagen staining

Stainings were performed on 6 µm sections of FFPE tissues. For Haematoxylin-Eosin staining, tissue sections were deparaffinised three times (3 min each) in xylene, then incubated for 3 min each in 99%, 95%, 70% and 30% ethanol and deionised water. Tissue sections were stained for 7 s in 100% Haematoxylin solution (109249, Merck Millipore), washed for 2 min in tap water, stained for 14 s in Eosin solution (3137.2, Carl Roth) and washed with tap water until the stain faded. Dehydration was performed in 30%, 70%, 95%, 99% and 100% ethanol and slides were washed in xylene until slides were mounted with DPX (1005790500, VWR).

For elastin-collagen staining, the Elastica Van Gieson Staining Kit (8275.1, Carl Roth) was used, and the manufacturer's protocol was adapted to our tissues. Tissue sections were deparaffinised three times (3 min each) in xylene, then rehydrated for 3 min in 99% and 80% ethanol, and stained 30 min in Esorcinol Fuchsin solution. Slides were washed with tap water until the stain faded, washed with deionised water, differentiated for a few seconds with ethanol 80%, and washed with deionised water to interrupt the differentiation. Finally, tissue sections were stained for 3 min with the Van Gieson solution and washed briefly in 70%, 95% and 100% ethanol, followed by xylene before mounting slides with DPX.

Blood collection and plasma analysis

Six- to eight-week-old mice were anaesthetised and blood was collected by intracardiac puncture. Plasma samples were analysed for total bilirubin,

albumin, alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase and lactate dehydrogenase (Fuji Dri-Chem NX500 analyzer).

Ink injection and tissue clearing

Six- to eight-week-old mice were anaesthetised and transcardially perfused with PBS for 3 min (perfusion rate 5 ml/min). Protocols for bile duct and portal vein injections were as described (Hankeova et al., 2021) and were adapted for ink injection. Briefly, black ink (Higgins Drawing Ink) was injected in the common bile duct using PE10 tubing (427401, BD Bioscience) attached to a 30 g needle/2 ml syringe. White ink (Higgins Drawing Ink) was injected into the portal vein using a catheter (Peripheral IV cannulae, BDAM381312, VWR) attached to a 5 ml syringe. To visualise the hepatic arteries, we injected ink in the gastroduodenal artery according to a protocol adapted from that described in rats (Sheu et al., 2013; Shinozaki et al., 2004; Tada et al., 2006). The gastroduodenal artery was cleaned from surrounded tissue, and surgical suture (Surgicryl 910, 15150217, SMI AG) was loosely wrapped around the artery. An incision was made in the gastroduodenal artery using spring scissors (2 mm, 15000-03, Fine Science Tools). PE10 tubing was attached to a 30 g needle/5 ml syringe, and the end of the tubing was stretched and cut at an angle to create a bevelled edge. The common hepatic artery was clamped, the tubing was inserted in the gastroduodenal artery, and the suture was tightened on the tubing, before injecting ink. Following ink injection, liver lobes were incubated on a rocking platform in 50% methanol for 4 h at RT, in 100% methanol overnight at RT and in benzyl alcohol (BA), benzyl benzoate (BB) solution (1BA: 2 BB) for a few hours at RT. Images were taken with a Stereo Microscope Stemi SV6 (Zeiss) and a Moticam X3 camera.

Tissue labelling and three-dimensional microscopy

Embryonic and newborn livers were fixed overnight in 4% PFA at 4°C. The liver lobes were dissected after fixation and post-fixed in 4% PFA at 4°C overnight. Tissue bleaching was carried out as described (Renier et al., 2014). The samples were dehydrated for 2 h at RT in ascending concentrations of methanol in PBS (50%, 80%, 100%), and then incubated overnight at 4°C with a 6% hydrogen peroxide solution in 100% methanol. Samples were rehydrated for 2 h at RT in descending concentrations of methanol (100% twice, 80%, 50%) and subjected to two 1-h washes in PBS. Samples were kept at 4°C for further processing.

For immunostaining, the samples were permeabilised and blocked in PBS containing 0.2% gelatin (Merck) and 0.5% Triton X-100 (Roth) (PBSGT) at RT (Belle et al., 2014). They were transferred to a solution containing primary antibodies (Table S2), 0.1% saponin (10 µg/ml) and heparin (10 µg/ml) in PBSGT for 10 days at 37°C with agitation. Samples were then washed six times (30 min each) in PBSGT at RT. Secondary antibodies (Table S3) were diluted in a solution containing 0.1% saponin (10 µg/ml) and heparin (10 µg/ml) in PBSGT and filtered through a 0.22 µm filter. Samples were incubated overnight at 37°C in the secondary antibody solution. After six washes (30 min each) in PBSGT at RT, samples were stored in the dark at 4°C until tissue clearing.

For tissue clearing, a modified 3DISCO clearing protocol was used. All incubation steps were performed in the dark at RT in a fume hood, on an agitator, using a 15 ml centrifuge tube. Samples were first dehydrated in ascending concentrations (50%, 80% and 100%) of tetrahydrofuran (THF; 186562, Sigma-Aldrich) diluted in H₂O. An initial overnight incubation in 50% THF was followed by 2 h in 80% THF and 2 h in 100% THF. Samples next underwent delipidation for 30 min in dichloromethane (DCM; Sigma-Aldrich) followed by overnight clearing in dibenzyl ether (DBE; Sigma-Aldrich). Samples were then stored in individual light-absorbing glass vials (Carl Roth) at RT. In these conditions, samples could be stored for up to 9 months and imaged without significant loss of fluorescence.

Three-dimensional image acquisitions were performed using an ultramicroscope I (LaVision BioTec-Miltenyi Biotec) with InspectorPro software (LaVision BioTec-Miltenyi Biotec). The light sheet was generated by a laser (wavelength 560 or 640 nm, Coherent Sapphire Laser, LaVision BioTec-Miltenyi Biotec) and focused using two cylindrical lenses with 150 ms time exposure. Two adjustable protective lenses were applied for small and large working distances. A binocular stereomicroscope (MXV10,

Olympus) with a 2× objective (MVPLAPO, Olympus) was used at different magnifications (0.63×, 0.8×, 1×, 1.25×, 1.6×, 2×, 2.5×, 3.2×, 4×, 5× and 6.3×). The corresponding zoom factors and numerical apertures are available on manufacturer website (<https://www.miltenyibiotec.com/BE-en/products/objective-lenses-for-ultramicroscopes.html#150-000-493>). Samples were placed in an imaging reservoir made of 100% quartz (LaVision BioTec-Miltenyi Biotec) filled with DBE and illuminated from the side by the laser light. A Zyla SC CMOS CCD camera (2048×2048 pixel size, Andor Oxford Instruments) was used to acquire images. The step size between each image was fixed at 1 and 2 µm. All tiff images were generated in 16-bit.

For image processing, images, 3D volume, and movies were generated using Imaris software (version 9.9.0, Bitplane-Oxford Instruments). Stack images were first converted to imaris file (.ims) using ImarisFileConverter and 3D reconstruction was performed using the volume rendering function. 3D pictures and movies were generated using the ‘snapshot’ and ‘animation’ tools.

Statistical analyses

Single comparisons of plasma levels in two experimental groups were performed using a paired Student's *t*-test. For morphometric analyses of hepatic arteries, significance was assessed by a Student's *t*-test with Welch's correction. For calculation of adjusted *P*-value in gene expression analyses, a Benjamini–Hochberg correction was applied. *P* < 0.05 was considered significant. No animals were excluded from the statistical analyses, and the operator was aware of experimental groups. Statistical analyses were performed using GraphPad Prism 9.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: L. Gannoun, C.D.S., I.A.L.; Methodology: L. Gannoun, C.D.S., M.B., N.D., Y.A., A.D., Y.H., I.R.; Formal analysis: L. Gannoun, C.D.S., M.B., A.L., C.V.; Investigation: L. Gannoun, C.D.S., S.C., A.D., Y.H.; Writing - original draft: L. Gannoun, C.D.S., Y.A., A.L., C.V., F.P.L.; Writing - review & editing: L. Gannoun, C.D.S., M.B., N.D., A.D., Y.H., I.R., I.A.L., P.J., L. Gatto, F.P.L.; Supervision: P.J., L. Gatto, F.P.L.; Project administration: F.P.L.; Funding acquisition: F.P.L.

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Data availability

RNA-seq data have been deposited in the Gene Expression Omnibus database under accession numbers GSE163062 and GSE224219.

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