




## ORIGINAL ARTICLE

# Ductular reaction promotes intrahepatic angiogenesis through Slit2–Roundabout 1 signaling

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## Abstract

**Background and Aims:** Ductular reaction (DR) expands in chronic liver diseases and correlates with disease severity. Besides its potential role in liver regeneration, DR plays a role in the wound-healing response of the liver, promoting periductular fibrosis and inflammatory cell recruitment. However, there is no information regarding its role in intrahepatic angiogenesis. In the current study we investigated the potential contribution of DR cells to hepatic vascular remodeling during chronic liver disease.

**Approach and Results:** In mouse models of liver injury, DR cells express genes involved in angiogenesis. Among angiogenesis-related genes, the expression of Slit2 and its receptor Roundabout 1 (Robo1) was localized in DR cells and neoangiogenic vessels, respectively. The angiogenic role of the

**Abbreviations:** AH, alcohol-related hepatitis; ARLD, alcohol-related liver disease; CDE, choline-deficient, ethionine-supplemented; DDC, 3,5-diethoxycarbonyl-1,4-dihydrocollidine; DR, ductular reaction; EpCAM, epithelial cell adhesion molecule; FC, fold change; GO, gene ontology; HUVEC, human umbilical cord endothelial cell; KRT19, keratin 19; MELD, Model for End-Stage Liver Disease; qPCR, quantitative real-time PCR; Robo, Roundabout; SLIT-2, Slit Guidance Ligand 2; Sox9, SRY (sex determining region Y)-box 9; VWF, von Willebrand factor; WT, wild type; YFP, yellow fluorescent protein.

Mar Coll and Silvia Ariño contributed equally to this work.

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[Correction added on December 20, 2021, after first online publication: Affiliation for Mercedes Fernandez was corrected to affiliation 1]

Slit2–Robo1 pathway in chronic liver disease was confirmed in ROBO1/2<sup>-/+</sup> mice treated with 3,5-diethoxycarbonyl-1,4-dihydrocollidine, which displayed reduced intrahepatic neovascular density compared to wild-type mice. However, ROBO1/2 deficiency did not affect angiogenesis in partial hepatectomy. In patients with advanced alcohol-associated disease, angiogenesis was associated with DR, and up-regulation of SLIT2–ROBO1 correlated with DR and disease severity. In vitro, human liver-derived organoids produced SLIT2 and induced tube formation of endothelial cells.

**Conclusions:** Overall, our data indicate that DR expansion promotes angiogenesis through the Slit2–Robo1 pathway and recognize DR cells as key players in the liver wound-healing response.

## INTRODUCTION

Chronic liver injury drives a wound-healing response in order to maintain liver function and hepatic structural properties. The wound-healing response activates well-coordinated repair mechanisms including hepatocyte proliferation, extracellular matrix turnover, and angiogenesis, which induces new blood vessel formation and vascular system remodeling. In this context, the ductular reaction (DR) expands as a regenerative response of the liver, sustaining biliary compartment remodeling. DR cells are a heterogeneous population of biliary cells ranging from reactive cholangiocytes to immature liver progenitor cells. The potential of the biliary compartment to contribute to biliary and parenchymal regeneration has been a focus of intense investigation in the field of liver disease.<sup>[1-5]</sup> However, few studies have assessed the interaction of DR cells with their surrounding environment and their role in the wound-healing response of the liver.

Patients with underlying alcohol-related liver disease (ARLD) and heavy alcohol intake can develop an episode(s) of alcohol-related hepatitis (AH), which is an acute-on-chronic condition associated with poor short-term prognosis. AH is characterized by inflammatory infiltration, fibrosis, hepatocellular damage, and expansion of the DR, which has been associated with disease severity and short-term mortality.<sup>[6,7]</sup> We have recently described that DR cells exhibit a proinflammatory profile in AH, increasing liver inflammation and participating in neutrophil chemotaxis.<sup>[8]</sup>

Slit Guidance Ligand (SLIT) ligands were first described as secreted chemorepellents of growing axons and migrating neurons that act through Roundabout (Robo) receptors. In recent years, it has been described that guidance cues, which are responsible for neuronal development, have a crucial role in vessel formation.<sup>[9-11]</sup> Among the three SLIT proteins, SLIT2 has emerged as a proangiogenic factor by inducing sprouting of new vessel formation in angiogenic tissues. SLIT2 can both

positively and negatively modulate angiogenesis by binding to ROBO1 and ROBO4, respectively.<sup>[12-14]</sup> A study on skin and retina showed that Slit2 binding to Robo4 negatively regulates new vessel formation by counteracting VEGF-mediated angiogenesis.<sup>[15]</sup> The potent proangiogenic role of Slit2 has been described mainly in cancer and ischemic diseases,<sup>[16-19]</sup> in which secreted Slit2 by solid tumors binds to Robo1 and is expressed in vascular endothelial cells, promoting angiogenesis. Conversely, tumor growth can be inhibited by blocking Robo1 activity.<sup>[13,20]</sup> There are few studies assessing the role of the Slit2–Robo1 axis in liver diseases. In this regard, the Slit2–Robo1 pathway was found to be overexpressed in liver cancer, and expression of Slit2 and Robo1 was enhanced in fibrotic liver, promoting liver fibrogenesis through HSC activation.<sup>[21]</sup> However, it remains unknown whether the Slit2–Robo1 pathway exerts a proangiogenic role in liver regeneration and chronic liver injury.

In this study we hypothesized that DR cells participate in liver tissue remodeling in ARLD. In order to identify pathways in which DR cells are involved, we analyzed the transcriptome of DR cells in a mouse model of chronic liver injury. We identified that liver angiogenesis is a significantly enriched biological process related to DR cells. Additionally, we investigated the role of the Slit2–Robo1 pathway in intrahepatic angiogenesis. The results of our study indicate that DR cells promote liver angiogenesis and that the Slit2–Robo1 pathway is a mechanism underlying this fundamental repair process.

## PATIENTS AND METHODS

### Human biopsies and samples

Liver tissue samples were obtained from fragments of nontumoral cirrhotic liver tissue surrounding colon metastasis collected at the time of liver resection or from explants from liver transplantation due to ARLD,

NASH, and HCV. The study was approved by the Ethics Committee of the Hospital Clinic of Barcelona, and all patients included in this study provided written informed consent.

## Patient cohorts

### ARLD cohort

The ARLD cohort consisted of patients with AH and MELD  $\geq 21$  ( $n = 11$ ), patients with AH and Model for End-Stage Liver Disease (MELD) score  $< 21$  ( $n = 18$ ), patients with compensated cirrhosis ( $n = 9$ ), and healthy individuals ( $n = 10$ ).

### Chronic liver disease cohort

The chronic liver disease cohort consisted of patients with AH and MELD  $\geq 21$  ( $n = 11$ ), patients with AH and MELD  $< 21$  ( $n = 18$ ), patients with compensated cirrhosis ( $n = 9$ ), patients with ARLD and criteria of steatohepatitis ( $n = 12$ ), patients with NAFLD ( $n = 10$ ), noncirrhotic HCV ( $n = 10$ ), and healthy individuals ( $n = 10$ ).

The baseline characteristics at the time of liver biopsy of the patients included in the ARLD and chronic liver disease cohorts are described in Argemi et al.<sup>[22]</sup> Moreover, raw sequencing data are available in the Database of Genotypes and Phenotypes of the National Center for Biotechnology Information (phs001807.v1.p1).

### AH cohort

The AH cohort consisted of patients with AH ( $n = 29$ ). Patient characteristics are detailed in Table S1.

### Cirrhotic organoids cohort

The cirrhotic organoids cohort consisted of patients with liver cirrhosis ( $n = 5$ ). The etiologies of cirrhosis are described in Table S2.

Informed consent in writing was obtained from each patient, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the appropriate institutional review committee.

## Animals

Because complete or partial genetic deletion of *Robo1* is embryonically lethal, we explored the role of the Slit2–Robo1 pathway in mice with partial genetic deletion of both *Robo1* and *Robo2* which are viable.

*ROBO1*<sup>-/+</sup>*ROBO2*<sup>-/+</sup> (also referred to as *ROBO1/2*<sup>-/+</sup>) mice with a BALB/c background were kindly supplied by Dr. Jian-Guo Geng from the University of Michigan. Littermates of *Robo1/2*<sup>-/+</sup> mice were used in all studies. Details on chronic liver injury and regeneration mouse models are described in the Supporting Information. All animal experiments were approved by the Ethics Committee of Animal Experimentation of the University of Barcelona and conducted in accordance with the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals*.

## Gene ontology analysis

A microarray analysis of yellow fluorescent protein–positive (YFP<sup>+</sup>) cells isolated from HNF1 $\beta$ CreER<sup>YFP</sup> and wild-type (WT) mice receiving a 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) or a choline-deficient, ethionine-supplemented (CDE) diet for 3 weeks ( $n = 3$  in all groups) was performed previously.<sup>[23]</sup> In order to assess new biological processes related to DR, we performed a functional analysis using the transcriptomic data generated and deposited in the National Center for Biotechnology Information's Gene Expression Omnibus (GSE51389). A description of the Gene Ontology (GO) analysis (Geneontology.org) performed is detailed in the Supporting Information.

## Liver tissue and organoid analysis

Details on immunostaining of liver tissue and organoids as well as gene expression, western blot, and hydroxyproline analysis of liver tissue samples are included in the Supporting Information.

## In vitro angiogenesis assay

Growth factor–reduced Matrigel (BD Biosciences, San Jose, CA) was completely thawed at 4°C for 16–24 h before conducting the tube formation assay. Liquid Matrigel was added to a previously chilled 24-well plate (300  $\mu$ l/well). The plate was then incubated for 1 h at 37°C to allow Matrigel solidification. Human umbilical cord endothelial cells (HUVECs; 60,000 cells/well) were resuspended in basal medium (DMEM-F12, 1% Glutamax, 1% 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid [Hepes], and 1% penicillin–streptomycin) or basal medium with recombinant SLIT2 protein (2 ng/ml). To further explore the role of organoid-derived SLIT2 in promoting angiogenesis, we preincubated HUVECs for 2 h with or without 10 mg/ml of sheep antihuman ROBO1 antibody (R&D Systems) in basal medium. Following this, HUVECs were stimulated with conditioned medium with and without  $\alpha$ ROBO1 antibody (10 mg/ml).

In all conditions, HUVECs were incubated for 12 h at 37°C, 5% CO<sub>2</sub>. Then, the medium was removed, and the endotubes were rinsed with PBS and fixed with 4% paraformaldehyde for 15 min. Tubules were visualized by phase-contrast microscopy, and six images per well at ×4 were taken. Tube formation was evaluated using the Angiogenesis Analyzer tool (ImageJ software).

### Liver organoids generated from cirrhotic liver tissues

Liver organoids were generated from cirrhotic liver tissue samples and cultured following the protocol previously described with minor modifications.<sup>[8]</sup> Briefly, liver tissue was digested using Collagenase XI (Sigma-Aldrich) and Dispase II (ThermoFisher) for 30 min at 37°C. After washing and erythrocyte lysis steps, pelleted cells were embedded in basement matrix extract (BME; Ambio, Abingdon, UK) and seeded in 24-well plates. Following BME solidification, organoid expansion medium was added. Organoid expansion medium consisted of basal medium (Advanced DMEM/F12 [ThermoFisher], 1% Glutamax [ThermoFisher], 1% Hepes [Life Technologies, Carlsbad, CA], and 1% penicillin–streptomycin [Lonza, Basilea, Switzerland]) supplemented with % N2 and 1% B7 without vitamin A (both from Life Technologies); 1.25 mM *N*-acetylcysteine, 10 mM nicotinamide, and 10 nM gastrin (Sigma-Aldrich); 50 ng/ml EGF, 100 ng/ml FGF-10, 25 ng/ml HGF, 25 ng/ml Noggin, and 500 ng/ml Rspo1 (Peprotech, London, UK); and 5 μM A8301, 0.5 μM CHIR99021, 10 μM forskolin, and 10 μM of Y27632 (Axon Medchem, Groningen, the Netherlands). Y27632 was added only the first 3 days after isolation. The expansion medium was replaced every 2–3 days, and cells were split once a week. At a confluence of 70%–80%, organoid expansion medium was replaced by basal medium for 18 h. Organoid conditioned medium was collected and stored at –80°C until the HUVECs tubulogenic assay and ELISA SLIT2 quantification were performed.

### Serum SLIT2 detection

SLIT2 serum levels from healthy individuals and patients with AH as well as from supernatants of liver organoids generated from patients with cirrhosis were evaluated with a specific ELISA kit for SLIT2 (Cusabio Biotech Co., Wuhan, China) following the manufacturer's protocol.

### Statistical analysis

Values are expressed as mean ± SEM. Statistical differences between groups were analyzed using the

Student *t* test, two-way ANOVA with Bonferroni correction, or the Mann-Whitney *U* test when appropriate with GraphPad Prism 5.0 (San Diego, CA).  $p \leq 0.05$  was considered statistically significant. Correlations between variables were evaluated using Spearman's rho or Pearson's *r*, when appropriate.

## RESULTS

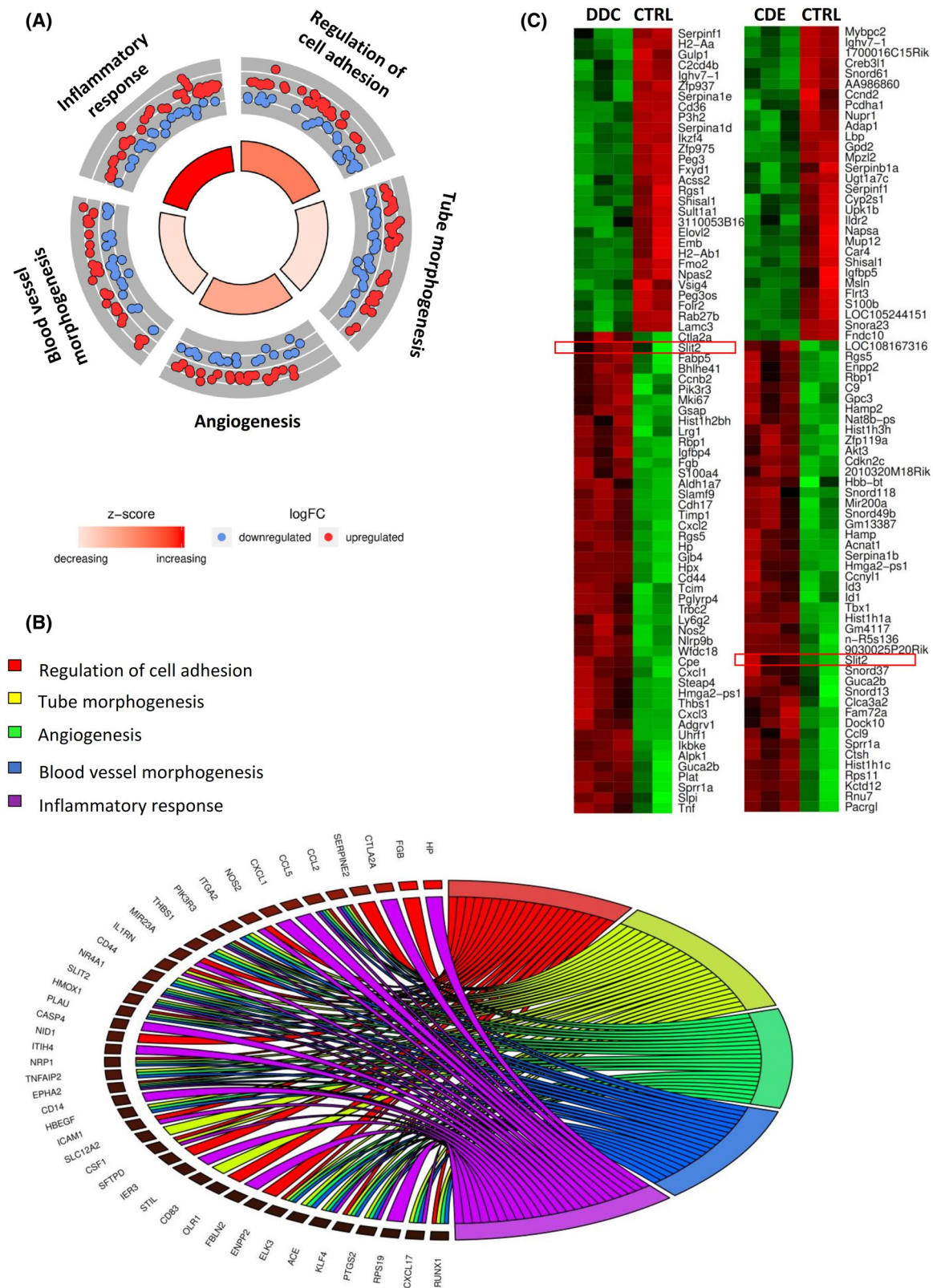
### Mouse DR cells exhibit enriched expression of genes related to angiogenesis

In order to identify biological functions associated with DR cells, we evaluated transcriptomic data obtained from biliary cells, isolated as YFP<sup>+</sup> cells by fluorescence-activated cell sorting, from HNF1βCreER<sup>YFP</sup> mice. DR cells were isolated from mice fed with a diet enriched in DDC, a CDE diet, or a chow diet for 3 weeks and from control mice. GO analysis revealed that the top five enriched biological processes associated with DR cells were inflammatory response (gene ratio 37/315,  $p = 1 \times 10^{-9}$ ), followed by regulation of cell adhesion (gene ratio 31/315,  $p = 8 \times 10^{-9}$ ), angiogenesis (gene ratio 25/315,  $p = 9 \times 10^{-8}$ ), tube morphogenesis (gene ratio 33/315,  $p = 3.7 \times 10^{-7}$ ), and blood vessel morphogenesis (gene ratio 26/315,  $p = 1.18 \times 10^{-6}$ ) (Figure 1A). Because very little is known regarding the capacity of DR cells to participate in angiogenesis, we decided to focus on this key repair mechanism. In order to investigate the mechanism by which DR contributes to liver angiogenesis, we examined the molecules underlying the GO terms related to angiogenesis (Figure 1B). *Slit2*, a well-described proangiogenic factor, was the gene included in all the categories. Moreover, *Slit2* was found to be significantly up-regulated in YFP<sup>+</sup> cells isolated from the DDC and CDE models compared to those sorted from control mice (fold change [FC] = 2.2,  $p = 0.004$  and FC = 7.48,  $p = 0.002$ , respectively) (Figure 1C).

### *Slit2* is expressed by DR cells and *Robo1* by vessels in DDC mice

*Slit2* acts as a regulator of new vessel formation through interactions with either *Robo1* or *Robo4* receptor in the context of cancer and ischemic diseases.<sup>[16–19]</sup> In order to assess the role of *Slit2* in intrahepatic angiogenesis, we first examined the levels of *Slit2* expression and its receptors in liver tissue from DDC and CDE mouse models. *Slit2* was found to be up-regulated in DDC and CDE compared to control livers (FC = 44.8 ± 4.6 and FC = 8.2 ± 3.3, respectively) at similar levels to the DR markers epithelial cell adhesion molecule (*Epcam*) and keratin 19 (*Krt19*). We also observed a significant up-regulation of *Robo1* in both models (FC = 3.9 ± 0.15





**FIGURE 1** Gene expression and GO analysis of a transcriptomic data set derived from DR cells isolated from HNF1 $\beta$ CreER<sup>YFP</sup> and control mice. (A) GO circle plot indicating the top five enriched biological processes related to DR cells of the DDC and CDE diet models. The outer ring illustrates the expression sign (blue, down-regulated; red, up-regulated) of the genes included in each GO term. The color intensity of the inner ring shows the significance measured by the z score for each category. (B) GOChord plot.<sup>[24]</sup> showing genes linked to their assigned GO terms. (C) Heat map displaying the top 75 significantly deregulated genes in YFP<sup>+</sup> cells isolated from mice receiving the DDC or CDE diet for 3 weeks compared to YFP<sup>+</sup> cells of the control mice. CTRL, control. HNF1 $\beta$ , hepatocyte nuclear factor 1 $\beta$  [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

and  $FC = 2.25 \pm 0.3$ , respectively), whereas *Robo4* was not found to be differentially expressed (Figure 2A). In order to confirm that the expression of *Slit2* was restricted to DR cells, we analyzed the gene expression levels of *Slit2* in both YFP<sup>+</sup> isolated cells and a hepatocyte fraction from DDC mice using quantitative real-time PCR (qPCR). While *Slit2* was found to be significantly enriched in DR cells compared to total liver tissue, no expression was found in the hepatocyte fraction (Figure 2B). As expected, we did not find *Robo1* expression in either of the examined cell fractions. Next, by performing immunohistochemistry in DDC livers, we confirmed that SLIT2 was expressed by the ductular structures (KRT19-positive cells), whereas its receptor, ROBO1, was expressed by the endothelial cells comprising the new vessels located near the periportal areas (Figure 2C). It is important to note that *Robo1* expression was not detected in endothelial cells of the portal vein or hepatic artery, indicating that its expression is specific to the neoangiogenic endothelial cells. These results indicate that, in a DDC mouse model, DR structures express the proangiogenic factor SLIT2, which might interact with ROBO1 receptors expressed by the neo-vessels.

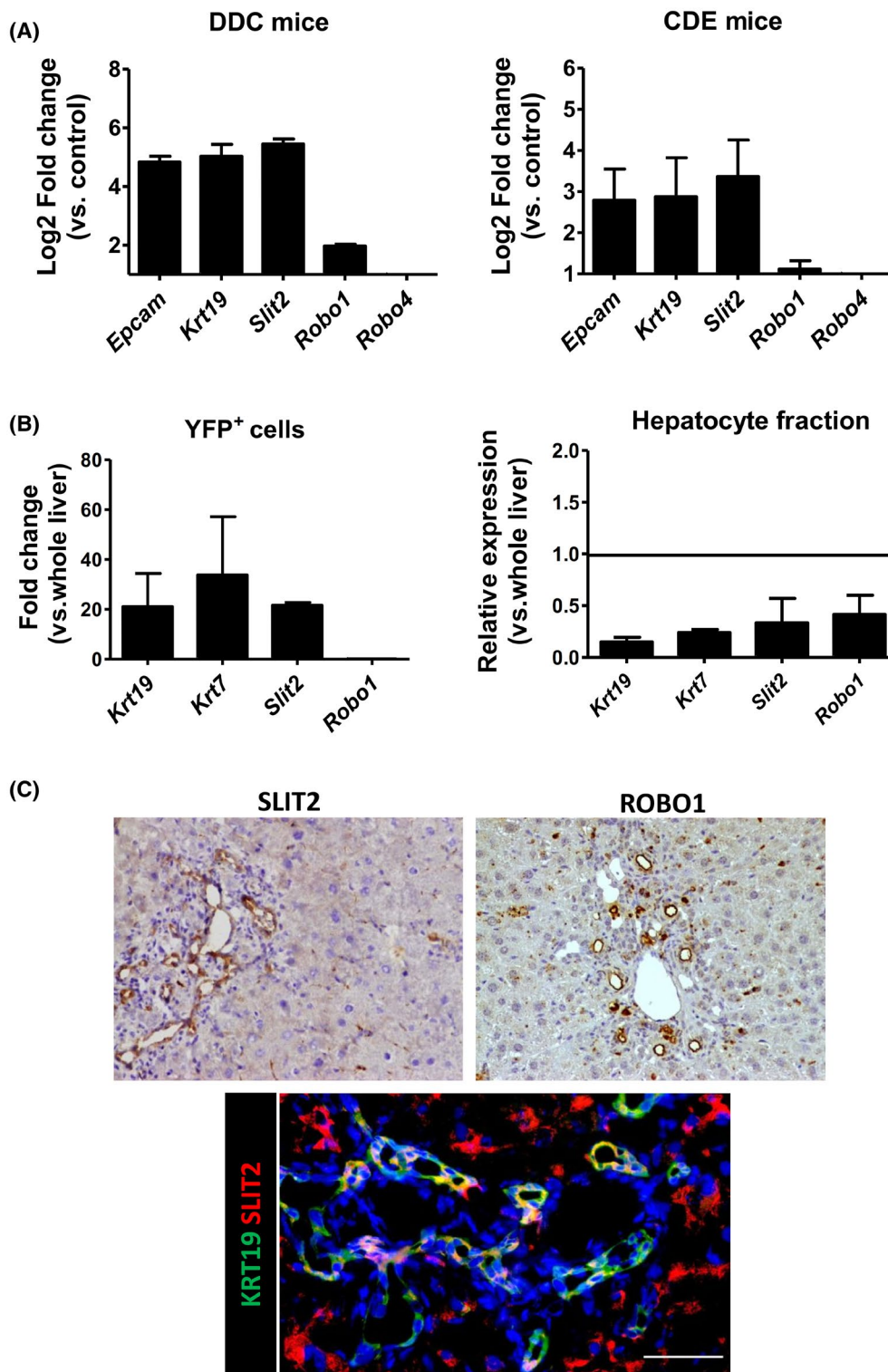
### ROBO1/2<sup>-/+</sup> mice display reduced hepatic angiogenesis compared to WT mice in response to DDC-induced injury

In order to determine the functional role of Slit2–Robo1 signaling in the pathogenesis of chronic liver injury, we used *Robo1/2* partial knockout mice (ROBO1/2<sup>-/+</sup>) and WT littermates with DDC and CDE diets, both involving DR expansion, for 3 and 4 weeks, respectively. Immunohistochemistry of DR key markers such as KRT19 and EpCAM revealed that ROBO1/2<sup>-/+</sup> mice fed the DDC or CDE diet did not display a reduction in DR structures compared to WT mice, indicating that Slit2–Robo1 signaling is not directly involved in ductular cell proliferation (Figure 3A,B). In line with this result, we did not find significant differences in the hepatic expression of genes related to DR such as *Krt19*, *Epcam*, or SRY (sex determining region Y)-box 9 (*Sox9*) (Figures S1A and S2A). In addition, we observed that fibrosis assessed by Sirius red staining; by gene expression of actin alpha 2 smooth muscle, collagen type I alpha 1 chain, matrix metalloproteinase 2, and tissue inhibitor of metalloproteinase 1; and by liver hydroxyproline level determination was not altered in ROBO1/2<sup>-/+</sup> mice treated with DDC or CDE when compared to WT mice (Figures 3A,B, S1B, S2B and S3). Indeed, although a profibrogenic role of Slit2–Robo1 signaling has been previously reported in a mouse model of advanced fibrosis based on chronic CCl<sub>4</sub> administration, it has been attributed to Slit2 expression by HSCs. Interestingly, quantification of

immunohistochemistry staining for CD31 showed a marked reduction of vessels surrounding periportal areas in ROBO1/2<sup>-/+</sup> compared to WT mice in both injury models. However, we did not observe a major gene expression reduction of key markers related to angiogenesis (Figures 3, S1C and S2C). Moreover, we did not observe any differences in liver enzymes in serum of ROBO1/2<sup>-/+</sup> fed the DDC or CDE diet compared to WT (Figure S4A,B). These results demonstrate that Slit2–Robo1 signaling regulates hepatic angiogenesis in both models of chronic liver disease involving DR expansion.

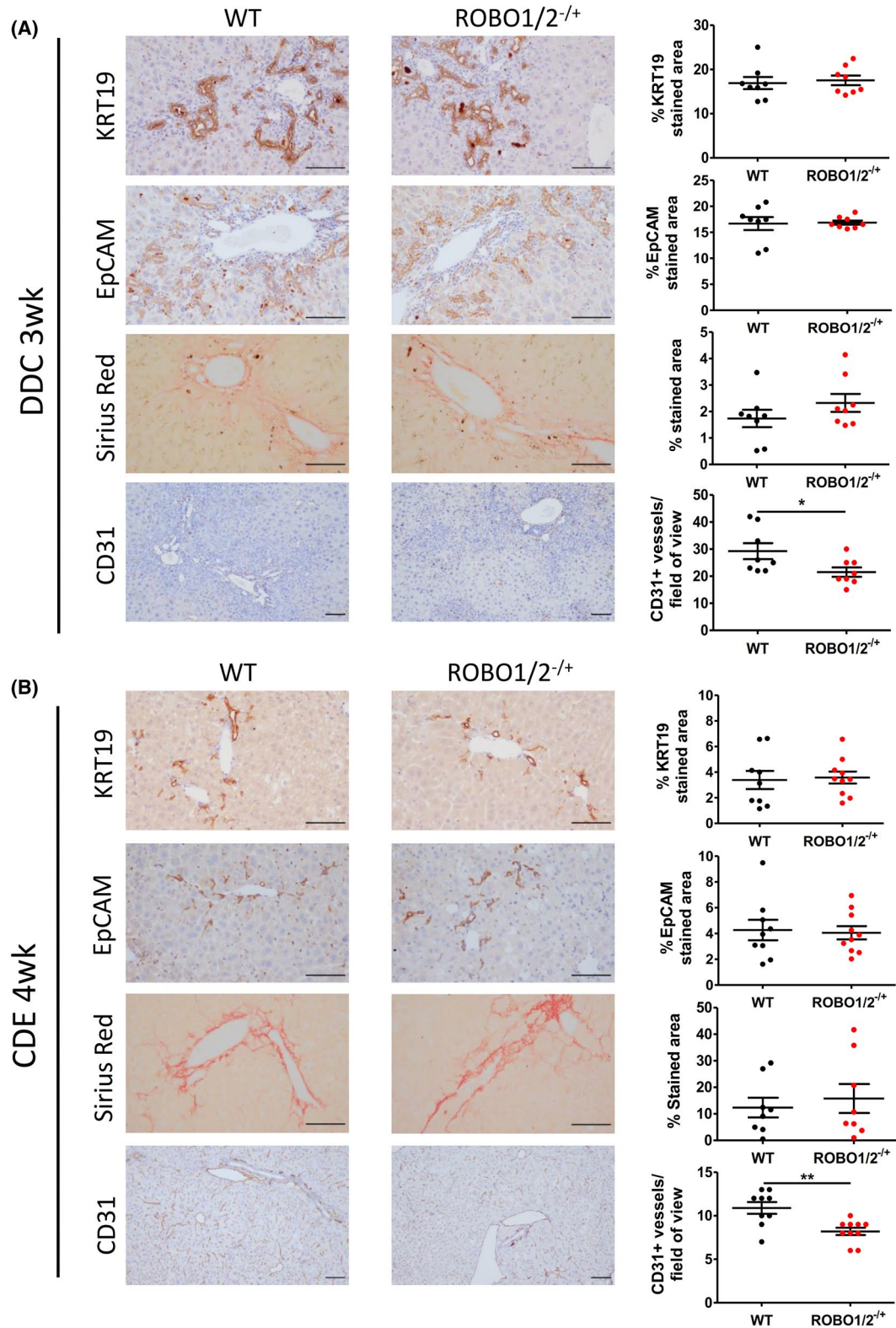
### Slit2–Robo1 signaling does not participate in modulating physiological angiogenesis in response to partial hepatectomy

After partial hepatectomy, angiogenesis and angiocrine growth factors play a key role in the correct regeneration of the liver.<sup>[25]</sup> Because Slit2–Robo1 has a role in regulating hepatic angiogenesis in chronic liver damage, we assessed whether this signaling pathway participates in the regeneration mechanisms of a healthy liver. With this objective, we first performed two-thirds partial hepatectomy in C57BL/6J mice. We assessed the hepatic gene expression of *Slit2* and *Robo1* together with DR markers (*Epcam*, *Krt19*, *Sox9*) at different stages of liver regeneration after partial hepatectomy, considering early (0, 24, 48, and 72 h) and late (Days 7 and 28) time points after surgery. Interestingly, we found increased *Slit2* expression at 48 h, 72 h, and Day 7 after partial hepatectomy compared to intact liver at 0 h. Besides, liver expression of *Robo1* was significantly up-regulated at 72 h after the surgery (Figure 4A). Next, to assess the potential role of Slit2–Robo1 signaling in physiologic angiogenesis, we performed partial hepatectomy in ROBO1/2<sup>-/+</sup> mice and WT littermates. We evaluated liver regeneration at 48 h and Day 7 after partial hepatectomy. We did not find significant differences in the number of proliferating cells within the parenchyma, assessed by immunohistochemistry of KI67 between ROBO1/2<sup>-/+</sup> and WT mice at early (48 h) and late (7 days) time points after surgery (Figures 4B and S6B). Likewise, liver regeneration index, calculated as liver weight/body weight ratio, did not change either (Figures S5A and S6A). Regarding liver progenitor cell expansion, we found a down-regulation of *Epcam* and *Krt19* gene expression in ROBO1/2<sup>-/+</sup> mice at early, but not at late, time points after partial hepatectomy when compared to WT mice. However, we did not see any differences in DR expansion as assessed by immunohistochemistry of KRT19 at 48 and 7 days after surgery (Figures 4B,C, S5B and S6C), suggesting that liver regeneration is not affected by *Robo1* deficiency in partial hepatectomy. To specifically investigate whether angiogenesis was impaired



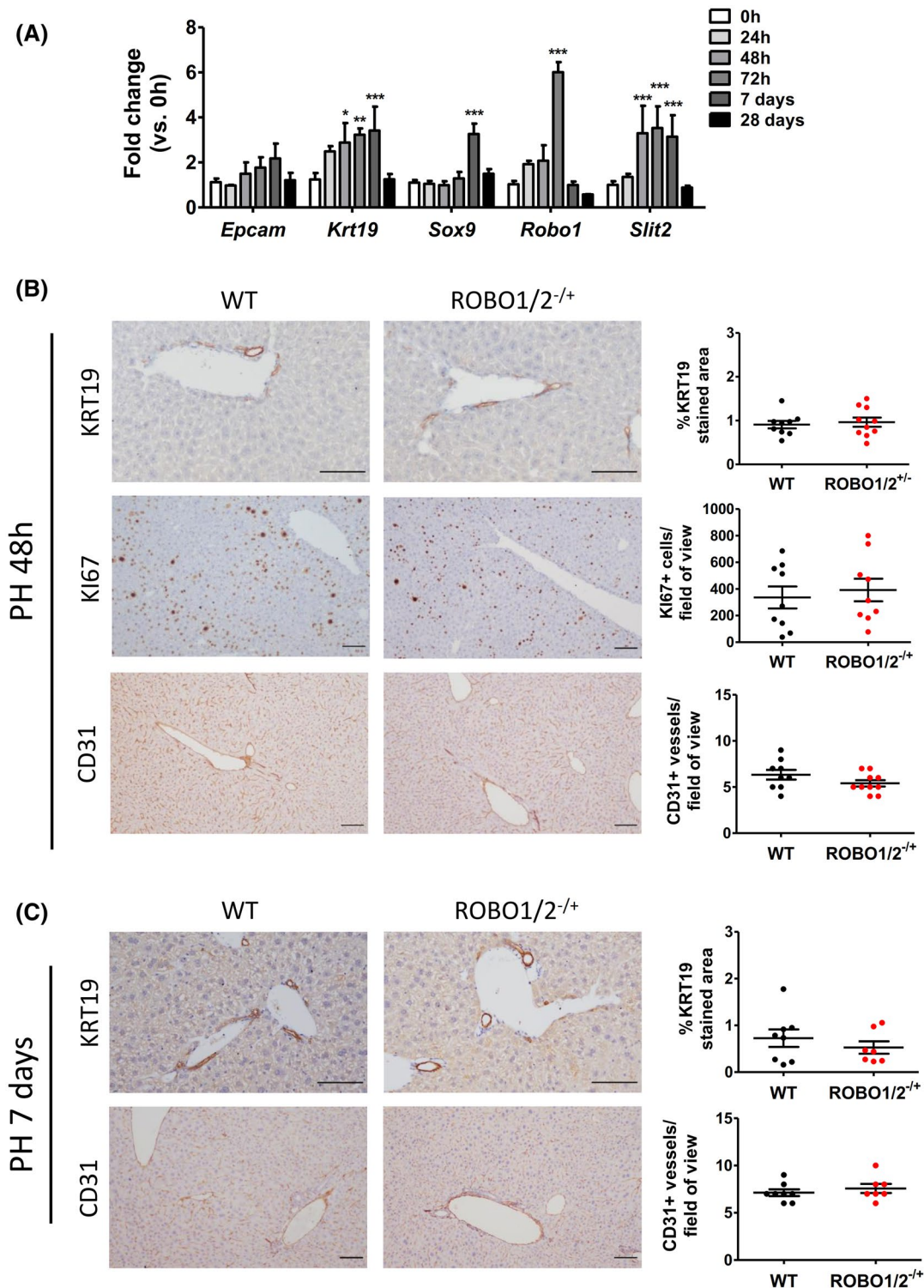
**FIGURE 2** Gene and protein expression of Slit2–Robo1 signaling in mouse models involving DR proliferation. (A) Gene expression analysis by qPCR of *Slit2*, *Robo1*, *Robo4*, and DR markers (*Krt19* and *Epcam*) in whole liver tissue isolated from mice receiving the DDC ( $n = 5$ ) or the CDE diet for 3 weeks ( $n = 5$ ). Expression values are represented as log<sub>2</sub> FC versus control mice. (B) Levels of gene expression measured by qPCR of key DR markers (*Epcam* and *Krt19*) and *Slit2* and its receptor *Robo1* in YFP<sup>+</sup> cells and hepatocyte fraction from livers of mice receiving the DDC diet for 3 weeks. Expression values are represented as FC versus the expression in whole liver tissue. (C) Representative images at  $\times 10$  of SLIT2 and ROBO1 immunohistochemistry in liver tissues of a mouse receiving the DDC diet for 3 weeks show the expression of SLIT2 by DR cells and ROBO1 expression restricted to new vessels. Immunofluorescence of KRT19 (green) and SLIT2 (red) confirms colocalization of SLIT2 within the DR. Nuclear counterstaining was performed with DAPI (blue). Scale bar, 100  $\mu$ m. Data are presented as mean  $\pm$  SEM [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]





**FIGURE 3** Slit2–Robo1 signaling mediates angiogenesis in both DDC and CDE mouse models. Representative immunohistochemical staining of KRT19, EpCAM, Sirius red, and CD31 staining in ROBO1/2<sup>-/+</sup> and WT mice receiving (A) the DDC diet for 3 weeks ( $n = 8$  in both groups) or (B) the CDE diet for 4 weeks ( $n = 9$  in WT group,  $n = 10$  in ROBO1/2<sup>-/+</sup> group). In both injury models, DR/progenitor cell expansion and fibrogenesis were evaluated by quantification of positive staining areas for KRT19/EpCAM and Sirius red, respectively, measured by ImageJ Software. Neovascularization was quantified by counting the number of CD31-positive vessels surrounding the periportal areas/field of view. Scale bars, 100  $\mu$ m. Data are presented as mean  $\pm$  SEM. Groups were compared by  $t$  test analysis. \* $p < 0.05$  and \*\* $p < 0.01$  [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]





**FIGURE 4** Slit2–Robo1 signaling does not participate in partial hepatectomy regeneration. (A) Gene expression analysis of liver progenitor cell markers (*Krt19*, *Epcam*, and *Sox9*), *Slit2*, and *Robo1* in mouse livers at early (24, 48, and 72 h after surgery) and late (Days 7 and 28) time points after two-thirds partial hepatectomy in WT mice ( $n = 3–5$  mice/group). FC is represented as expression at each time point versus expression at Day 0. Grouped analysis was compared by two-way ANOVA with Bonferroni's posttest correction. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ . (B) Two-thirds partial hepatectomy was evaluated in WT and ROBO1/2<sup>-/+</sup> mice ( $n = 9$  in WT group and  $n = 10$  in ROBO1/2<sup>-/+</sup> group), being sacrificed at 48 h after surgery. (C) Two-thirds partial hepatectomy was performed in WT and ROBO1/2<sup>-/+</sup> mice ( $n = 8$  in WT group and  $n = 7$  in ROBO1/2<sup>-/+</sup> group), and animals were sacrificed at Day 7 after surgery. (B,C) Representative images of cell proliferation at 48 h evaluated by KI67 staining. (B,C) Representative immunohistochemical staining for CD31 and KRT19 in livers at 48 h and 7 days after partial hepatectomy. DR expansion and cell proliferation were measured by the quantification of positive staining for KRT19 and KI67, respectively, by ImageJ software. New vessel formation was evaluated by counting CD31<sup>+</sup> vessels/field of view. Scale bars, 100  $\mu$ m. Data are presented as mean  $\pm$  SEM. Groups were compared by *t* test. PH, partial hepatectomy [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

in *ROBO1/2<sup>-/+</sup>* mice, we quantified CD31-stained vessels within the liver tissue. We did not observe differences in the formation of new vessels at early or late time points after partial hepatectomy, indicating that Slit2–Robo1 signaling does not significantly contribute to physiological angiogenesis (Figure 4B,C). These results suggest that, although there is an activation of the Slit2–Robo1 pathway after partial hepatectomy, other mechanisms, possibly mediated by VEGF, are more relevant in driving angiogenesis during hepatic regeneration of healthy liver.

### DR and the Slit2–Robo1 pathway are associated with ARLD progression

DR is a histological key feature of AH and increases along ARLD progression. Given the proangiogenic role of SLIT2 in mouse models involving DR expansion, we hypothesized that DR contributes to tissue repair along ARLD progression by promoting angiogenesis through Slit2–Robo1 signaling. In order to assess this hypothesis, we first showed that neoangiogenesis was taking place near the DR structures in liver tissues from patients with cirrhosis and AH by performing a double immunohistochemistry of von Willebrand factor (VWF) and KRT7 (Figure 5A). Next, we examined the levels of *SLIT2* and *ROBO1* expression as well as the gene expression profile of gene sets related to angiogenesis and DR in RNA sequencing data from total liver tissue from a cohort of patients encompassing the whole spectrum of ARLD.<sup>[22]</sup> The patients included in this cohort were grouped according to ARLD stage: severe AH, patients with AH and MELD  $\geq 21$  ( $n = 11$ ); nonsevere AH, patients with AH and MELD  $< 21$  ( $n = 18$ ); patients with compensated cirrhosis ( $n = 9$ ); and healthy individuals ( $n = 10$ ). Interestingly, the expression profile of the angiogenesis gene set markedly increased with ARLD progression, suggesting that intrahepatic angiogenesis is a relevant regeneration mechanism underlying ARLD. As expected, the DR gene signature increased in ARLD progression, and we found that it followed a similar gene expression pattern as angiogenesis. In addition, *SLIT2*

and *ROBO1* expression displayed a similar trend to DR and angiogenesis sets, showing significant up-regulation in severe compared to nonsevere AH (Figure 5B). Next, we sought to determine if DR and angiogenesis were linked biological processes in patients with chronic liver diseases of different etiologies. For this analysis we included a group of patients with nonadvanced chronic liver disease with the following etiologies: ARLD with histologic criteria of steatohepatitis ( $n = 12$ ), NAFLD ( $n = 10$ ), and HCV-infected patients without cirrhosis ( $n = 10$ ). The hepatic expression of DR markers (*KRT7* and *KRT19*) strongly correlated with the expression of angiogenesis markers such as platelet/endothelial cell adhesion molecule and *VWF* (Figure 5C) as well as with *SLIT2* and its receptor *ROBO1* (Figure 5D). This result suggests that DR expansion and angiogenesis are activated not only along ARLD progression but also in chronic liver diseases of other etiologies. Moreover, our findings indicate that the Slit2–Robo1 pathway is associated with fundamental repair processes in chronic liver disease such as DR and angiogenesis. In addition, the expression of the angiogenic signaling factor *VEGFA* and the kinase insert domain receptor and Fms-related receptor tyrosine kinase 1 did not correlate with the hepatic expression of DR markers (Figure S7), suggesting that in chronic liver disease DR-mediated angiogenesis may not be related to *VEGFA* signaling.

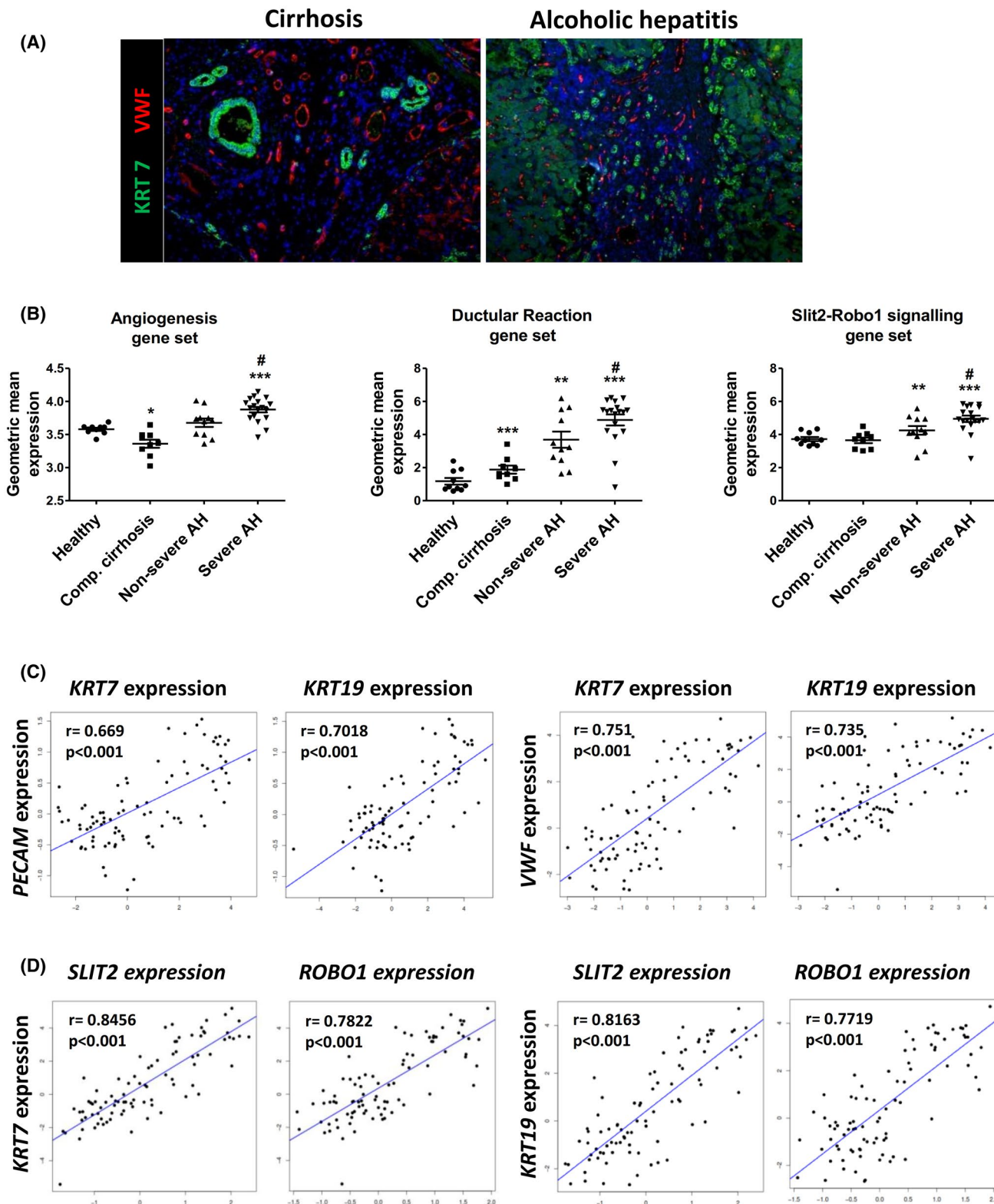
### The Slit2–Robo1 pathway is enhanced in patients with AH

To confirm the association of DR with the Slit2–Robo1 pathway, we evaluated the hepatic expression of *SLIT2* and its receptor *ROBO1* in a cohort of patients with AH ( $n = 29$ ) and healthy individuals ( $n = 5$ ). The characteristics of the patients are described in Table S1. *SLIT2* and *ROBO1* expression was increased in patients with AH compared to controls (FC =  $3.4 \pm 0.4$ , and FC =  $2.35 \pm 0.19$ , respectively) (Figure 6). As expected, the hepatic expression of *SLIT2* significantly correlated with the expression of its receptor, *ROBO1*. Interestingly, in the same cohort of patients with AH,

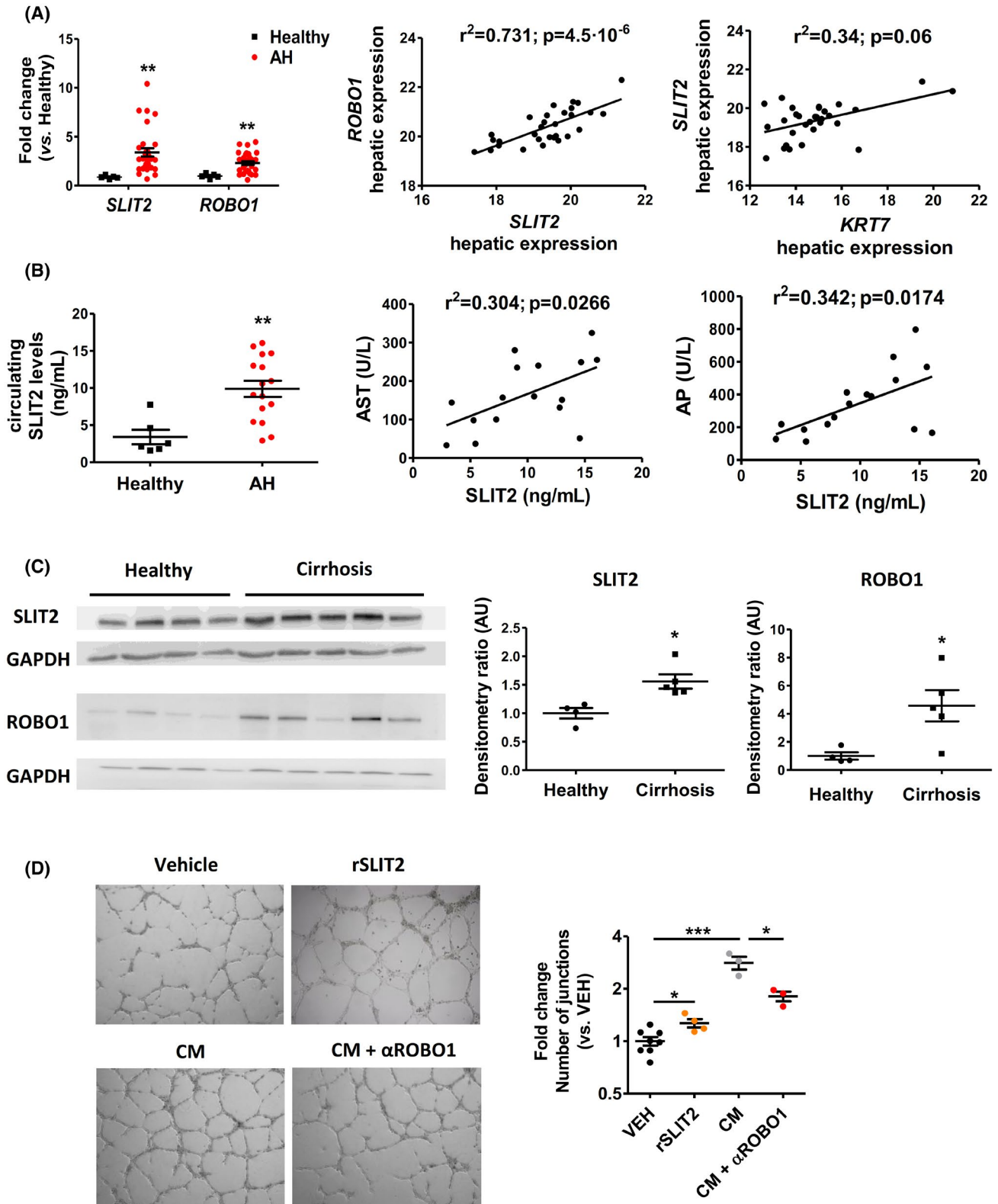
**FIGURE 5** Expression profile of Slit2–Robo1 signaling, angiogenesis, and DR gene sets along ARLD progression. (A) Representative double immunostaining for KRT7 and VWF in paraffin-embedded sections of liver tissues from patients with cirrhosis and patients with AH showing neoangiogenesis surrounding the periportal areas where DR expands. Nuclear counterstaining was performed with DAPI (blue). (B) Hepatic gene expression levels of angiogenesis, DR gene sets, and Slit2–Robo1 signaling in patients with compensated cirrhosis, patients with nonsevere AH (MELD  $< 21$ ), severe AH (MELD  $\geq 21$ ), and healthy individuals. (C) Correlations of hepatic gene expression of DR markers (*KRT7* and *KRT19*) with angiogenesis markers (*PECAM* and *VWF*) and with *SLIT2* and *ROBO1* expression (D). The liver transcriptomic data were obtained from a cohort of patients encompassing the ARLD spectrum: patients with early alcohol-associated steatohepatitis were nonobese individuals with high alcohol intake, mild elevation of transaminases, and histologic criteria of steatohepatitis ( $n = 12$ ), patients with histologically confirmed AH by biopsy (nonsevere,  $n = 18$ ) and liver explants from patients with AH who underwent early transplantation (severe,  $n = 11$ ) were compared to nondiseased human livers (healthy,  $n = 10$ ), patients with noncirrhotic HCV infection (compensated cirrhosis,  $n = 10$ ), patients with NAFLD according to Keiner's criteria and without alcohol abuse ( $n = 9$ ), and patients with compensated HCV-related cirrhosis ( $n = 9$ ). Data are presented as mean  $\pm$  SEM. Gene expression was analyzed versus healthy ( $*p < 0.05$ ,  $**p < 0.01$ , and  $***p < 0.001$ ) and versus nonsevere AH ( $\#p < 0.05$ ). PECAM, platelet/endothelial cell adhesion molecule [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

SLIT2 expression strongly correlated with the hepatic expression of KRT7, suggesting that DR cells are a cell source of SLIT2 in patients with AH (Figure 6A). Next, we investigated the serum levels of SLIT2 in a subset of patients with AH ( $n = 16$ ) and healthy individuals ( $n = 6$ ). We observed that circulating SLIT2 levels were

significantly increased in patients with AH compared to healthy individuals ( $9.8 \pm 0.9$  versus  $3.4 \pm 1.1$  ng/ml) (Figure 6B). Additionally, circulating levels of SLIT2 positively correlated with serum levels of aspartate aminotransferase and alkaline phosphatase, suggesting that SLIT2 increases together with liver injury in AH.







### DR cells from cirrhotic livers secrete SLIT2 and induce tubulogenesis

We recently reported that liver organoids derived from cirrhotic liver tissue mimic DR cells and represent a relevant *in vitro* model to study DR.<sup>[8]</sup> In order to confirm

that human DR cells secrete SLIT2, we generated liver biliary organoids from patients with cirrhosis of different etiologies (NASH,  $n = 1$ ; ARLD,  $n = 2$ ; and HCV,  $n = 2$ ). The cirrhotic tissues used to generate organoids expressed higher levels of SLIT2 and ROBO1 proteins compared to control livers (Figure 6C). We also

**FIGURE 6** Patients with AH display increased hepatic expression of Slit2–Robo1 signaling and enhanced SLIT2 serum levels. DR cells from cirrhotic liver secrete SLIT2 and mediate angiogenesis. (A) *SLIT2* and *ROBO1* expression levels measured by qPCR in liver biopsies of patients with AH ( $n = 29$ ) and healthy individuals ( $n = 5$ ). Correlation of hepatic gene expression assessed by qPCR of *SLIT2* with *ROBO1* and *KRT7* in patients with AH ( $n = 29$ ). The regression coefficient ( $r^2$ ) and  $p$  value are indicated. (B) Serum levels of SLIT2 measured in healthy controls ( $n = 6$ ) and patients with AH ( $n = 16$ ) by ELISA. Data are presented as mean  $\pm$  SEM. \* $p < 0.05$ , \*\* $p < 0.01$ . (C) Protein expression analysis by western blot of SLIT2 and ROBO1 in livers from patients with cirrhosis ( $n = 4$ ) and healthy subjects ( $n = 4$ ). Quantification of protein levels was performed by densitometric analysis. As a loading control, an antibody against glyceraldehyde 3-phosphate dehydrogenase was used. (D) Tubulogenic assay performed by exposing HUVECs to organoid basal medium ( $n = 8$  technical replicates), human recombinant Slit2 (2 ng/ml;  $n = 4$  technical replicates), conditioned medium of three cirrhotic organoids ( $n = 3$  organoids generated from 3 different cirrhotic liver tissue explants), and conditioned medium plus  $\alpha$ ROBO1 antibody for 12 h. Angiogenic capacity was evaluated by counting the junctions formed using the Angiogenesis Analyzer tool of ImageJ software. Data are representative of three independent experiments, represented as the mean FC  $\pm$  SEM versus organoid basal medium group average. Groups were compared by  $t$  test. \* $p < 0.05$  and \*\*\* $p < 0.001$ . AP, alkaline phosphatase; AST, aspartate aminotransferase; AU, arbitrary units; CM, conditioned medium; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; VEH, vehicle [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

confirmed the presence of DR in all the cirrhotic tissue samples examined by KRT7 immunohistochemistry (Figure S8A). As expected, we detected the SLIT2 protein in all the supernatants of the organoids generated ( $17 \pm 2.8$  ng/ml) (Table S2 and Figure S8B), and we observed SLIT2 expression by immunofluorescence in the liver organoid (Figure S8C). Next, with the purpose of demonstrating the proangiogenic potential of DR cells, we performed a tubulogenic assay by exposing HUVECs to conditioned medium derived from cirrhotic liver organoids ( $n = 3$ ). As a positive control, we incubated HUVECs with recombinant SLIT2 protein (2 ng/ml). Compared to the basal medium, liver organoid conditioned medium significantly increased angiogenesis, as assessed by the quantification of the number of junctions formed by HUVECs. To further demonstrate that Slit2 secreted by DR cells mediates angiogenesis, we treated HUVECs with conditioned medium containing  $\alpha$ ROBO1 blocking antibody. Interestingly, we observed that the proangiogenic ability of DR cells was significantly attenuated after ROBO1 blockade (Figures 6D and S8D). Altogether, these results suggest that DR cells release the proangiogenic factor SLIT2, thus contributing to angiogenesis.

## DISCUSSION

In this study we show that DR may promote intrahepatic angiogenesis in chronic liver diseases. We show that DR structures express SLIT2, whereas its receptor ROBO1 is expressed by endothelial cells of small vessels. We describe that the Slit2–Robo1 pathway regulates intrahepatic angiogenesis in chronic liver injury but not in physiological liver regeneration induced by loss of liver cell mass. Moreover, we describe that intrahepatic angiogenesis and DR expansion are concomitant mechanisms taking place along ARLD progression. With the use of a human in vitro model of DR cells, we demonstrate the capacity of DR cells to produce SLIT2 and induce angiogenesis. Collectively, these data indicate that DR cells trigger intrahepatic liver angiogenesis through the Slit2–Robo1 pathway.

Under chronic liver damage, the intrahepatic vascular bed undergoes a critical structural modification by enhancing the number of sinusoidal vessels and producing shunts connecting central and portal venules to reduce sinusoidal vascular resistance.<sup>[26]</sup> In the present study, we show that intrahepatic angiogenesis increases with disease progression, indicating that liver angiogenesis behaves as a dynamic process following the same pattern as other repair mechanisms such as fibrogenesis. In this regard, previous studies have evaluated the therapeutic potential of preventing hepatic angiogenesis as a strategy to reduce disease progression. However, the beneficial effects of the administration of antiangiogenic drugs in animal models of chronic liver injury is still controversial.<sup>[27,28]</sup> In fact, angiogenesis is a fundamental mechanism underlying liver injury repair and regeneration, and therefore, it may play a beneficial role in the wound-healing response to injury. To understand to what extent the intrahepatic angiogenesis contributes to disease progression and tissue healing in chronic liver diseases is a very important aspect with clinical implications that should be directly investigated.

DR cells are known to play a role in liver fibrosis and inflammation.<sup>[8,29,30]</sup> However, there is a lack of information regarding the potential of DR in promoting angiogenesis. In order to elucidate whether DR participates in angiogenesis, we performed a GO analysis with the transcriptomic data generated from DR cells isolated from mice subjected to chronic injury involving DR expansion (3 weeks of DDC and CDE diets). Interestingly, angiogenesis was found to be one of the most enriched biological processes associated with mouse DR. In humans, in a large cohort encompassing the whole spectrum of ARLD, we confirmed that DR expansion correlates with intrahepatic expression of angiogenic factors and markers and that both mechanisms paralleled disease progression.

Intrahepatic angiogenesis takes place under the physiological context of liver regeneration and under pathological conditions.<sup>[31,32]</sup> To investigate whether the Slit2–Robo1 pathway promotes angiogenesis in these contexts, we used chronic injury (3 and 4 weeks

of DDC and CDE diets, respectively) and liver regeneration (partial hepatectomy) models in mice with a partial deletion of *Robo1* and *Robo2* genes because complete deletion of *Robo1* is embryonically lethal. Interestingly, we show that the Slit2–Robo1 pathway promotes liver angiogenesis in response to chronic injury but does not contribute to remodeling the vascular bed during liver regeneration after partial hepatectomy. These data indicate that angiogenesis driven by the Slit2–Robo1 pathway may be taking place only in the context of chronic liver injury where DR expansion is present but not in a regenerative response in a healthy liver, where there is no involvement of the DR. These results suggest that the Slit2–Robo1 pathway may be a potential target to enhance liver wound healing in chronic liver disease. In fact, it is known that Slit2–Robo1 signaling participates in promoting angiogenesis in several pathological contexts such as retinopathy,<sup>[33]</sup> ischemia,<sup>[18]</sup> or endometriosis.<sup>[34]</sup> In addition, Slit2 expression in solid tumors promotes tumor-induced angiogenesis by acting through Robo1 expressed in vascular endothelial cells.<sup>[13]</sup> Further studies are needed to elucidate whether intrahepatic Slit2 contributes to angiogenesis in other pathological liver settings such as tumor angiogenesis.

Little is known regarding the mechanisms that trigger pathological intrahepatic angiogenesis. To date, the main cell type inducing liver angiogenesis in a pathological setting is liver endothelial sinusoidal cells, although HSCs also directly contribute to new vessel formation by producing proangiogenic factors such as VEGF, platelet-derived growth factor, and angiopoietin-1 or in a paracrine manner by activating liver sinusoidal endothelial cells.<sup>[35,36]</sup> Our study focused on intrahepatic angiogenesis underlying chronic liver diseases with DR expansion such as ARLD. In this context, we showed that the neoangiogenesis, which is histologically visualized as small vessels with varying diameter and positive staining for VWF or CD31, takes place near the proliferative ductular structures. To confirm the angiogenic role of DR cells, we generated biliary three-dimensional organoids derived from cirrhotic liver tissue, which have been shown to retain the phenotypic and transcriptomic features of DR cells mimicking in vitro DR.<sup>[8]</sup> In this regard, we demonstrate that all DR organoids generated from different etiologies (ARLD and NASH), secrete SLIT2 and induce HUVEC tube formation.

Interestingly, serum SLIT2 levels are increased and correlate with the severity of liver injury in patients with AH, the most severe form of ARLD that is characterized by extensive DR. These results are in agreement with previous data showing increased circulating levels of SLIT2 in patients with fibrosis.<sup>[21]</sup> In addition, activated HSCs produce and are also targets of SLIT2, contributing to fibrogenesis. These results indicate that DR cells

are not the only cell source of Slit2 in chronic liver disease and suggest that Slit2 secreted by DR cells could contribute to wound healing not only by inducing angiogenesis but also by targeting HSCs and promoting fibrogenesis.

Although we have shown that DR cells promote angiogenesis through the Slit2–Robo1 pathway, DR may also produce other proangiogenic factors. Histologically, it has been shown that liver progenitor cells derived from patients with primary biliary cholangitis express VEGFA and VEGFC at the protein level.<sup>[37]</sup> However, in the ARLD cohort, we did not find a correlation between the hepatic expression of VEGFA and the expression pattern of genes related to DR, suggesting that intrahepatic expression of VEGFA does not occur in parallel with DR expansion.

DR cells have the potential to contribute to liver regeneration by giving rise to new hepatocytes when their replication capacity is hampered. In this study, we provide evidence to support that DR cells contribute to tissue remodeling by promoting angiogenesis. In addition, we have recently reported that in chronic liver damage DR cells produce chemoattractant agents to induce neutrophil recruitment that might also participate in liver tissue repair.<sup>[8]</sup> Taken together, these results suggest that DR has a major role in liver wound healing, driving fundamental repair mechanisms.

Overall, this report provides evidence that DR cells mediate intrahepatic angiogenesis through the Slit2–Robo1 pathway and recognizes DR cells as important contributors to wound-healing processes in chronic liver disease.

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## CONFLICT OF INTEREST

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## AUTHOR CONTRIBUTIONS

Mar Coll participated in the design of the study, performed experiments, and drafted the manuscript. Silvia Ariño participated in all the experiments and critically reviewed the manuscript. Ester Garcia-Pras and Javier Gallego performed angiogenesis in vitro assays. Celia Martínez-Sánchez performed data analysis. Beatriz



Aguilar-Bravo participated in liver organoid generation. Delia Blaya, Julia Vallverdú, and Teresa Rubio-Tomás helped with the maintenance and genotyping of the ROBO1/2<sup>+/-</sup> mouse colony. Juan Jose Lozano performed bioinformatics analysis. Elisa Pose and Isabel Graupera recruited the patients and critically reviewed the manuscript. Andrea Fernández-Vidal performed the hepatic hepatectomy surgeries. Albert Pol contribute to the critical review of the manuscript. Ramón Bataller provided the ARLD sequencing data set and critically reviewed the manuscript. Jian-Guo Geng provided the ROBO1/2<sup>+/-</sup> mice and critically reviewed the manuscript. Mercedes Fernandez and Pere Ginès interpreted data and contributed to the critical review of the manuscript. Pau Sancho-Bru conceived and designed the study, critically reviewed the manuscript, and supervised the study.

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## REFERENCES

- Huch M, Dorrell C, Boj SF, van Es JH, Li VSW, van de Wetering M, et al. In vitro expansion of single Lgr5<sup>+</sup> liver stem cells induced by Wnt-driven regeneration. *Nature*. 2013;494(7436):247–50.
- Tarlow BD, Finegold MJ, Grompe M. Clonal tracing of Sox9<sup>+</sup> liver progenitors in mouse oval cell injury. *Hepatology*. 2014;60(1):278–89.
- Deng X, Zhang X, Li W, Feng R-X, Li LU, Yi G-R, et al. Chronic liver injury induces conversion of biliary epithelial cells into hepatocytes. *Cell Stem Cell*. 2018;23(1):114–22.e3.
- Malato Y, Naqvi S, Schürmann N, Ng R, Wang B, Zape J, et al. Fate tracing of mature hepatocytes in mouse liver homeostasis and regeneration. *J Clin Invest*. 2011;121(12):4850–60.
- Raven A, Lu W-Y, Man TY, Ferreira-Gonzalez S, O'Duibhir E, Dwyer BJ, et al. Cholangiocytes act as facultative liver stem cells during impaired hepatocyte regeneration. *Nature*. 2017;547(7663):350–4.
- Gao B, Bataller R. Alcoholic liver disease: pathogenesis and new therapeutic targets. *Gastroenterology*. 2011;141(5):1572–85.
- Sancho-Bru P, Altamirano J, Rodrigo-Torres D, Coll M, Millán C, José Lozano J, et al. Liver progenitor cell markers correlate with liver damage and predict short-term mortality in patients with alcoholic hepatitis. *Hepatology*. 2012;55(6):1931–41.
- Aguilar-Bravo B, Rodrigo-Torres D, Ariño S, Coll M, Pose E, Blaya D, et al. Ductular reaction cells display an inflammatory profile and recruit neutrophils in alcoholic hepatitis. *Hepatology*. 2019;69(5):2180–95.
- Andrews W, Barber M, Hernandez-Miranda LR, Xian J, Rakic S, Sundaresan V, et al. The role of Slit–Robo signaling in the generation, migration and morphological differentiation of cortical interneurons. *Dev Biol*. 2008;313(2):648–58.
- Andrews WD, Barber M, Parnavelas JG. Slit–Robo interactions during cortical development. *J Anat*. 2007;211(2):188–98.
- Ba-Charvet KTN, Brose K, Marillat V, Kidd T, Goodman CS, Tessier-Lavigne M, et al. Slit2-mediated chemorepulsion and collapse of developing forebrain axons. *Neuron*. 1999;22(3):463–73.
- Suchting S, Heal P, Tahtis K, Stewart LM, Bicknell R. Soluble Robo4 receptor inhibits in vivo angiogenesis and endothelial cell migration. *FASEB J*. 2005;19(1):121–3.
- Wang B, Xiao Y, Ding B-B, Zhang NA, Yuan X-B, Gui L, et al. Induction of tumor angiogenesis by Slit–Robo signaling and inhibition of cancer growth by blocking Robo activity. *Cancer Cell*. 2003;4(1):19–29.
- Park KW, Morrison CM, Sorensen LK, Jones CA, Rao YI, Chien C-B, et al. Robo4 is a vascular-specific receptor that inhibits endothelial migration. *Dev Biol*. 2003;261(1):251–67.
- Jones CA, London NR, Chen H, Park KW, Sauvaget D, Stockton RA, et al. Robo4 stabilizes the vascular network by inhibiting pathologic angiogenesis and endothelial hyperpermeability. *Nat Med*. 2008;14(4):448–53.
- Alajez NM, Lenarduzzi M, Ito E, Hui ABY, Shi W, Bruce J, et al. miR-218 suppresses nasopharyngeal cancer progression through downregulation of survivin and the SLIT2–ROBO1 pathway. *Cancer Res*. 2011;71(6):2381–91.
- Chang P-H, Hwang-Verslues WW, Chang Y-C, Chen C-C, Hsiao M, Jeng Y-M, et al. Activation of Robo1 signaling of breast cancer cells by Slit2 from stromal fibroblast restrains tumorigenesis via blocking PI3K/Akt/β-catenin pathway. *Cancer Res*. 2012;72(18):4652–61.
- Li X, Zheng S, Tan W, Chen H, Li X, Wu J, et al. Slit2 protects hearts against ischemia–reperfusion injury by inhibiting inflammatory responses and maintaining myofilament contractile properties. *Front Physiol*. 2020;27(11):228.
- Chaturvedi S, Yuen DA, Bajwa A, Huang Y-W, Sokollik C, Huang L, et al. Slit2 prevents neutrophil recruitment and renal ischemia–reperfusion injury. *J Am Soc Nephrol*. 2013;24(8):1274–87.
- Wang L-J, Zhao Y, Han B, Ma Y-G, Zhang J, Yang D-M, et al. Targeting Slit–Roundabout signaling inhibits tumor angiogenesis in chemical-induced squamous cell carcinogenesis. *Cancer Sci*. 2008;99(3):510–7.
- Chang J, Lan T, Li C, Ji X, Zheng L, Gou H, et al. Activation of Slit2–Robo1 signaling promotes liver fibrosis. *J Hepatol*. 2015;63(6):1413–20.
- Argemi J, Latasa MU, Atkinson SR, Blokhin IO, Massey V, Gue JP, et al. Defective HNF4α-dependent gene expression as a driver of hepatocellular failure in alcoholic hepatitis. *Nat Commun*. 2019;10(1):1–19.
- Rodrigo-Torres D, Affò S, Coll M, Morales-Ibanez O, Millán C, Blaya D, et al. The biliary epithelium gives rise to liver progenitor cells. *Hepatology*. 2014;60(4):1367–77.
- Walter W, Sánchez-Cabo F, Ricote M. GOplot: an R package for visually combining expression data with functional analysis. *Bioinformatics*. 2015;31(17):2912–4.
- Ding B-S, Nolan DJ, Butler JM, James D, Babazadeh AO, Rosenwaks Z, et al. Inductive angiocrine signals from sinusoidal endothelium are required for liver regeneration. *Nature*. 2010;468(7321):310–5.
- Iwakiri Y, Shah V, Rockey DC. Vascular pathobiology in chronic liver disease and cirrhosis—current status and future directions. *J Hepatol*. 2014;61:912–24.
- Patsenker E, Popov Y, Stickel F, Schneider V, Ledermann M, Sägesser H, et al. Pharmacological inhibition of integrin αvβ3 aggravates experimental liver fibrosis and suppresses hepatic angiogenesis. *Hepatology*. 2009;50(5):1501–11.
- Mejias M, Garci-Pras E, Tian C, Miquel R, Bosch J, Fernandez M. Beneficial effects of sorafenib on splanchnic, intrahepatic, and portocollateral circulations in portal hypertensive and cirrhotic rats. *Hepatology*. 2009;49(4):1245–56.
- Gadd VL, Skoien R, Powell EE, Fagan KJ, Winterford C, Horsfall L, et al. The portal inflammatory infiltrate and ductular reaction in human nonalcoholic fatty liver disease. *Hepatology*. 2014;59(4):1393–405.
- Williams MJ, Clouston AD, Forbes SJ. Links between hepatic fibrosis, ductular reaction, and progenitor cell expansion. *Gastroenterology*. 2014;146(2):349–56.

31. Thabut D, Shah V. Intrahepatic angiogenesis and sinusoidal remodeling in chronic liver disease: new targets for the treatment of portal hypertension? *J Hepatol*. 2010;53:976–80.
32. Fernández M, Semela D, Bruix J, Colle I, Pinzani M, Bosch J. Angiogenesis in liver disease. *J Hepatol*. 2009;50:604–20.
33. Rama N, Dubrac A, Mathivet T, Ni Chárthaigh R-A, Genet G, Cristofaro B, et al. Slit2 signaling through Robo1 and Robo2 is required for retinal neovascularization. *Nat Med*. 2015;21(5):483–91.
34. Guo SW, Zheng Y, Lu Y, Liu X, Geng JG. Slit2 overexpression results in increased microvessel density and lesion size in mice with induced endometriosis. *Reprod Sci*. 2013;20(3):285–98.
35. Taura K, De Minicis S, Seki E, Hatano E, Iwaisako K, Osterreicher CH, et al. Hepatic stellate cells secrete angiopoietin 1 that induces angiogenesis in liver fibrosis. *Gastroenterology*. 2008;135(5):1729–38.
36. Lee JS, Kim JH. The role of activated hepatic stellate cells in liver fibrosis, portal hypertension and cancer angiogenesis. *Korean J Hepatol*. 2007;13:309–19.
37. Franchitto A, Onori P, Renzi A, Carpino G, Mancinelli R, Alvaro D, et al. Expression of vascular endothelial growth factors and their receptors by hepatic progenitor cells in human liver diseases. *Hepatobiliary Surg Nutr*. 2013;2(2):68–77.

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