Epithelial Expression of Angiogenic Growth Factors Modulate Arterial Vasculogenesis in Human Liver Development

Luca Fabris,[1,2]* Massimiliano Cadamuro,[1,2]* Louis Libbrecht,[3] Peggy Raynaud,[4] Carlo Spirlì,[5] Romina Fiorotto,[2,5] Lajos Okolicsanyi,[2] Frederic Lemaigre,[4] Mario Strazzabosco,[1,5,**] and Tania Roskams[3]**

Intrahepatic bile ducts maintain a close anatomical relationship with hepatic arteries. During liver ontogenesis, the development of the hepatic artery appears to be modulated by unknown signals originating from the bile duct. Given the capability of cholangiocytes to produce angiogenic growth factors and influence peribiliary vascularization, we studied the immunohistochemical expression of vascular endothelial growth factor (VEGF), angiopoietin-1, angiopoietin-2, and their cognate receptors (VEGFR-1, VEGFR-2, Tie-2) in fetal human livers at different gestational ages and in mice characterized by defective biliary morphogenesis (*Hnf6*$^{-/-}$). The results showed that throughout the different developmental stages, VEGF was expressed by developing bile ducts and angiopoietin-1 by hepatoblasts, whereas their cognate receptors were variably expressed by vascular cells according to the different maturational stages. Precursors of endothelial and mural cells expressed VEGFR-2 and Tie-2, respectively. In immature hepatic arteries, endothelial cells expressed VEGFR-1, whereas mural cells expressed both Tie-2 and Angiopoietin-2. In mature hepatic arteries, endothelial cells expressed Tie-2 along with VEGFR-1. In early postnatal *Hnf6*$^{-/-}$ mice, VEGF-expressing ductal plates failed to incorporate into the portal mesenchyma, resulting in severely altered arterial vasculogenesis. *Conclusion:* The reciprocal expression of angiogenic growth factors and receptors during development supports their involvement in the cross talk between liver epithelial cells and the portal vasculature. Cholangiocytes generate a VEGF gradient that is crucial during the migratory stage, when it determines arterial vasculogenesis in their vicinity, whereas angiopoietin-1 signaling from hepatoblasts contributes to the remodeling of the hepatic artery necessary to meet the demands of the developing epithelium. (HEPATOLOGY 2008;47:719-728.)

In the normal hepatic microarchitecture, intrahepatic bile ducts are closely associated with a branch of the portal vein and with 1 or 2 branches of the hepatic artery. These branching anatomical structures run together inside the portal spaces between the hepatic lobules.[1] Furthermore, intrahepatic bile ducts are nourished by a network of capillaries that form a peribiliary plexus (PBP) that appears during the developmental stages[2] and

Abbreviations: Ang, angiopoietin; ASMA, alpha smooth muscle actin; PBP, peribiliary plexus; sDP, single-layer ductal plate; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

From [1]CeLiveR, Center for Liver Research, Ospedali Riuniti di Bergamo, Bergamo, Italy; [2]Department of Surgical and Gastroenterological Sciences, University of Padua, Italy; [3]Department of Pathology, University of Leuven, Belgium; [4]Hormone and Metabolic Research Unit, Université Catholique de Louvain and Institute of Cellular Pathology, Brussels, Belgium; and [5]Department of Internal Medicine, Yale University, New Haven, CT.

Received May 7, 2007; accepted August 31, 2007.

Supported by Fondazione San Martino and the Ministero Istruzione Universita' e Ricerca (Cofinanziamento 2005, research grant 2003060498-001), the Yale Liver Center (DK34989), Telethon (grant E.1253 to L.F.), Progetto di Ricerca di Ateneo (CPDA058897 to L.F.), and FADE. F.L. was supported by grants from the Belgian State Program on Interuniversity Poles of Attraction, the Belgian Fund for Scientific Medical Research, and the D.G. Higher Education and Scientific Research of the French Community of Belgium. P.R. holds a fellowship from the Université Catholique de Louvain. C.S. is a recipient of an AASLD/Liver School Award.

**These authors contributed equally to this work. **These senior authors contributed equally to this work.*

Address reprint requests to: Mario Strazzabosco, M.D., Ph.D., Department of Internal Medicine, Section of Digestive Diseases, Yale University, 333 Cedar Street, LMP 1080, New Haven, CT 06520. E-mail: Mario.Strazzabosco@yale.edu; fax: 203-795-7273.

Published online in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/hep.22015

Potential conflict of interest: Nothing to report.

Supplementary material for this article can be found on the HEPATOLOGY Web site (http://interscience.wiley.com/jpages/0270-9139/suppmat/index.html).

is crucial for maintaining the integrity and function of the biliary epithelium.[3] The close anatomical relationship between intrahepatic bile ducts and hepatic arterial vascularization becomes evident in the early stages of human liver development, when the hepatic artery branches are formed in close proximity with ductal plates,[4] the precursors of the intrahepatic bile ducts. The association between bile ductules and arterial vascularization is also maintained in disease conditions. In ductular reaction, a histological lesion common to many forms of liver injury, the increase in bile ductules at the portal tract interface is paralleled by an increased number of hepatic arterioles and capillaries.[5] Accordingly, in an experimental rat model of selective cholangiocyte proliferation (α-naphthylisothiocyanate treatment), extensive neovascularization of the arterial bed accompanies the increased cholangiocyte mass.[6] Inactivation of the *Hnf6* or *Hnf1*β, transcription factors involved in intrahepatic bile duct epithelium development,[7] resulted in anomalies of the hepatic artery branches in addition to the expected bile duct abnormalities.[8] A similar pattern has been observed also in human liver diseases related to ductal plate malformation (DPM). In these congenital cholangiopathies, the dysmorphic bile ducts are surrounded by an increased number of vascular structures with a pattern resembling a "pollard willow."[9]

The intimate anatomical and functional association between bile ducts and arterial vasculature during both normal and pathological biliary development suggests that the developing bile ducts actually drive arterial development in the liver.[10] However, the nature of the signals originating from the bile ducts is presently unknown. We have found that vascular endothelial growth factor (VEGF) and angiopoieitin-1 (Ang-1) are markedly upregulated in the cystic biliary epithelium of fibropolycystic liver diseases, developmental cholangiopathies associated with DPM. The intensity of VEGF expression in cystic epithelium closely correlated with the microvascular density around the biliary cysts, suggesting that the angiogenic factors produced by cholangiocytes are responsible for the aberrant vascularization described in DPM.[11] Similarly, in several liver diseases, proliferation of VEGF-expressing bile ductules is accompanied by hyperplasia of the peribiliary microvascular bed.[12]

We thus hypothesized that angiogenic growth factors produced by ductal plate cells and cholangiocytes could provide the molecular link between the developing biliary and arterial structures. To this aim, we examined the immunohistochemical expression of VEGF, angiopoietins, and their cognate receptors in developing liver epithelial and vascular cells in human fetuses of different gestational ages and in *Hnf6*$^{-/-}$ mice. Our results were consistent with the hypothesis and suggest that throughout the different stages of liver development, biliary and arterial structures communicate via highly coordinated and stage-specific reciprocal expression of angiogenic factors and receptors. The correct development of the arterial supply to the biliary tree is also dependent on normal incorporation and branching of the biliary tree. In its absence, the angiogenic signals mediated by VEGF and angiopoietin cannot be correctly targeted to generate complex branching structures.

Materials and Methods

Liver Tissue. Human liver tissue specimens came from 10– to 36–gestational week fetuses (n = 16) obtained from autopsies of spontaneous or medical abortions; routine histopathological examination of the fetuses did not show any liver abnormalities. Control adult liver samples (n = 2, both from men, aged 49 and 18 years) were obtained from liver grafts that could not be transplanted because of iatrogenic lesions. Liver tissue was snap-frozen in liquid nitrogen–cooled isopentane and stored at −80°C. Informed consent and local regional ethical committee approval were obtained before tissue collection.

Liver sections of HNF6-knockout (*Hnf6*$^{-/-}$) mice were provided by Dr. Frederic Lemaigre.[7] Five *Hnf6*$^{-/-}$ mice and 5 controls were studied. The biliary system of the mouse matures only after birth[8]; therefore, mice were studied on postnatal days P1, P3, and P4. For immunohistochemical studies liver tissue was either frozen in liquid nitrogen–cooled isopentane or fixed in formalin and embedded in paraffin. All animals received humane care, and the study protocol was approved by the local institutional committee of the Catholic University of Louvain (Brussels, Belgium).

Histopathological Evaluation. Portal spaces were stratified according to biliary maturation into 3 developmental stages: ductal plate, migratory, and bile duct stages.[13,14] In the ductal plate stage, the single-layer ductal plate (sDP) represents the most immature biliary structure, recognizable as a thin monolayer of biliary-type cuboidal cells localized at the interface between the hepatic parenchyma and the mesenchyme of the nascent portal tract. This structure then originates the double-layer ductal plate (dDP), when a second layer of biliary-type epithelium is formed and a slit-like lumen begins to be observed. As maturation proceeds, tubular segments with a crescent-shaped lumen originating from the ductal plate progressively migrate into the portal tract, forming the incorporating bile duct (migratory stage). In the incorporated bile duct stage, bile duct (BD) maturation is completed, and the epithelium in the portal space appears

Table 1. Expression of Angiogenic Growth Factors by Liver Cell Type during Different Developmental Stages

Developmental stage	Marker/cell type	VEGF	VEGFR-1	VEGFR-2	Ang-1	Ang-2	Tie-2
Ductal plate (single layer)	CK19	+	+	±	−	−	+
	CD34	−	+	±	−	−	−
	ASMA	−	−	−	−	−	−
	Hep	±	−	−	+	−	−
Ductal plate (double layer)	CK19	+	+	±	−	−	±
	CD34	−	+	−	−	−	−
	ASMA	−	−	−	−	±	±
	Hep	±	−	−	+	−	−
Migratory stage	CK19	+	±	−	−	−	−
	CD34	−	+	−	−	−	−
	ASMA	−	−	−	−	+	+
	Hep	±	−	−	+	−	−
Bile duct stage	CK19	±	−	−	−	−	−
	CD34	−	+	−	−	−	+
	ASMA	−	−	−	−	+	+
	Hep	±	−	−	+	−	−

+ Diffuse expression; ± scattered or weak expression; − no expression.

round-shaped in cross section and surrounded by a thick layer of mesenchymal tissue. Overall, 320 portal spaces were analyzed and stratified as ductal plate (sDP = 75; dDP = 136), migratory (n = 79), or bile duct (n = 30) stage.

Immunohistochemistry. Details on the immunohistochemical techniques and antibodies used are provided in the Supplementary Methods.

Results

Human Fetal Liver. Table 1 summarizes the expression of angiogenic growth factors by liver cell type according to the maturational stage of the portal space. Absorption experiments resulted in the complete deletion of the signal of the primary antibody, confirming the genuineness of the immunohistochemical reactions.

During the ductal plate stage, when periportal hepatoblasts in contact with the mesenchyme surrounding the portal vein switch their phenotype, they began to form cytokeratin 19 (CK19)–positive single-layered cords, the single-layer ductal plate (Fig. 1A). We found that the sDPs expressed VEGF (Fig. 1D) along with being strongly positive for the VEGF receptor VEGFR-1 (Fig. 1E) and more faintly positive for VEGFR-2 (not shown); these cells were also positive for Tie-2 but were negative for both Ang-1 and Ang-2. On the other hand, the hepatoblasts expressed Ang-1 (Fig. 2) but not Ang-2 (Fig. 1F) and very little VEGF (Fig. 1D). In contrast with the biliary precursors, the hepatoblasts maintained this pattern of expression throughout the different developmental stages.

During this stage, CD34-positive cells (Fig. 1B) could be observed as scattered single cells or as short linear strings localized along the margins of the nascent portal space; these cells were positive for VEGFR-1 (Fig. 1E) and negative for VEGFR-2, Ang-1, Ang-2, and Tie-2. A distinct subpopulation of cells, arranged as single cells or as small clusters outside the portal space, in the parenchyma, and coexpressing CD34 and VEGFR-2 (Fig. 3A–D) likely represented hemangioblasts, the earliest precursor of endothelial cell.[15,16]

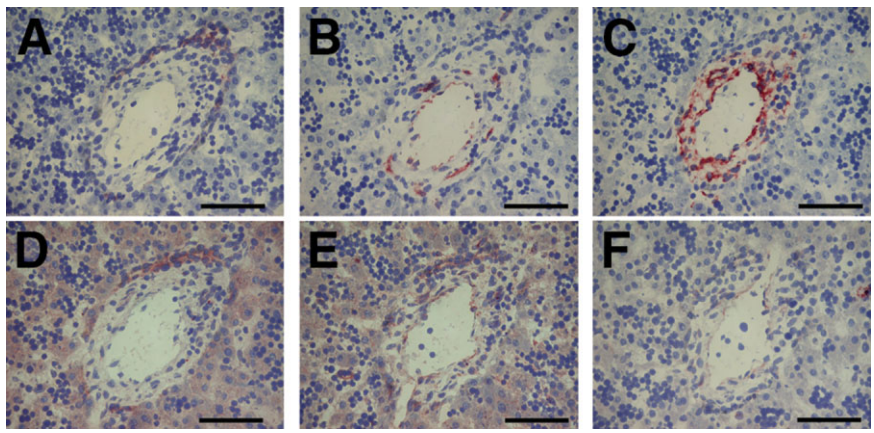

Fig. 1. Comparison of sequential expression of angiogenic growth factors with different cell-type markers in the single-layer ductal plate stage: (A) CK19, (B) CD34, (C) ASMA, (D) VEGF, (E) VEGFR-1, and (F) Ang-2. VEGF (D) was only expressed by ductal plate cells (A), whereas VEGFR-1 immunoreactivity (E) could be found in both ductal plate (A) and endothelial cells (B). Both the developing portal space and hepatoblasts were negative for Ang-2 (F). Scale bar A–F: 100 μm.

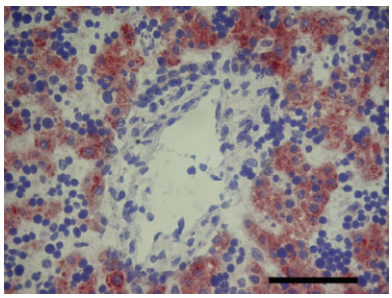

Fig. 2. Expression of Ang-1 by hepatoblasts. Ang-1 was diffusely expressed by hepatoblasts, which continued in the subsequent maturational stages (scale bar: 100 μm).

At this stage, alpha smooth muscle actin (ASMA)–positive cells (Fig. 1C) were also observed as single cells scattered inside the nascent portal space or as small clumps of cells around the central portal vein branch. Their phenotype was clearly different from that of the CD34-positive cells, as they were negative for VEGFR-1 (Fig. 1E) and VEGFR-2 and also for Ang-1 and Ang-2 (Fig. 1F). However, a subpopulation of ASMA-positive cells, characterized by a spindle shape and short cytoplasmic processes, expressed the angiopoietin receptor Tie-2 (Fig. 4A).

As the ductal plate was duplicated by a second layer of CK19-positive cells, it acquired a circular shape with a slit-like lumen over variably long segments of its perimeter. In this structure, the double-layer ductal plate (Fig. 5A), cells retained the angiogenic phenotype of sDP and expressed both VEGF (Fig. 5D) and VEGFR-1 (Fig. 5E).

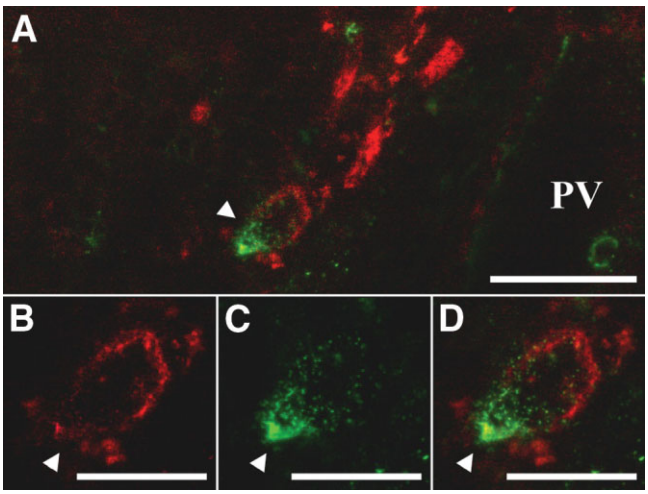

Fig. 3. Expression of VEGFR-2 by a subpopulation of CD34-positive cells. Dual immunofluorescence with VEGFR-2 (FITC) and CD-34 (Texas Red). VEGFR-2 (A–D merged, C unmerged) was expressed by a small subset of CD34-positive cells (A–D merged, B unmerged) characterized by a round shape and a location either at the margin of the nascent portal tract or scattered in the parenchyma. This immunophenotype (CD34/VEGFR-2+) has been shown to be displayed by hemangioblasts (arrowheads, VEGFR-2 expression by the CD-34-positive cells; scale bar for A and B–D: 50 and 25 μm, respectively; PV, portal vein).

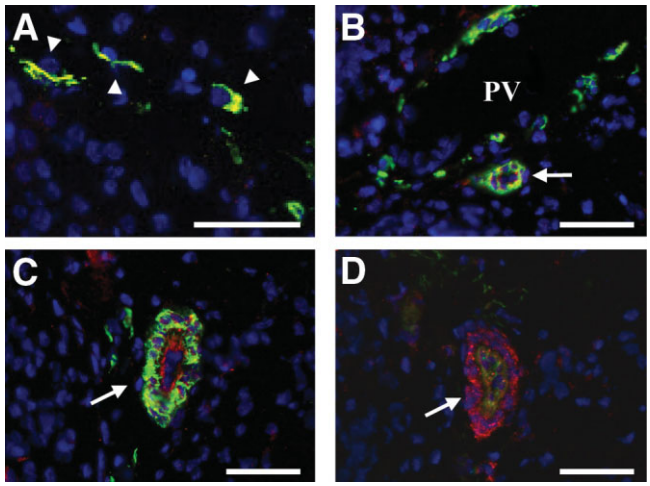

Fig. 4. Expression of Tie-2 by vascular cells during progressive maturation of hepatic artery. Dual immunofluorescence with (A–C) Tie-2 (Texas Red) and ASMA (FITC) and (D) Tie-2 (Texas Red) and CD34 (FITC); (A–D) DAPI nuclear staining. (A) In the ductal plate stage Tie-2 was expressed by ASMA-positive cells scattered inside the portal mesenchyma and characterized by a spindle shape with short cytoplasmic processes (coincident staining yellow, arrowheads), features consistent with portal myofibroblast (scale bar: 50 μm). (B) In immature hepatic artery Tie-2 was homogeneously expressed by ASMA-positive cells arranged in circular structures of increasing size (arrow) but was absent in the inner endothelial cell layer (scale bar: 50 μm). (C) In mature hepatic artery with progressive lumen opening, Tie-2 was expressed not only by mural cells (coincident staining), but also by the inner endothelial cell layer, which did not show coincident staining (arrow; scale bar: 50 μm), as confirmed by (D) coincident staining with the specific endothelial marker CD34 (arrow; scale bar: 50 μm). PV, portal vein.

The CD34-positive cells (Fig. 5B) were now organized in small clumps surrounded by a sheet of ASMA-positive cells (Fig. 5C) forming small circular structures without a recognizable lumen that were localized in the portal mesenchyma between the ductal plate (DP) and the portal vein. In these structures, the CD34-positive cells were constantly positive for VEGFR-1 (Fig. 5E) and negative for VEGFR-2, whereas the ASMA-positive cell layer showed homogeneous expression of Tie-2 and scattered expression of Ang-2 (Fig. 5F). A discontinuous layer of ASMA-positive and Tie-2-positive cells was also detectable around the lumen of the central portal vein.

During the migratory stage some of the CK19-positive ductal plate cells gradually migrated into the portal mesenchyme to form a tubular structure (incorporating bile ducts, iBDs), whereas the remaining cells of the DP were progressively deleted (Fig. 6A). In the iBD cells, expression of VEGF (Fig. 6D) and VEGFR-1 (Fig. 6E) was maintained, Tie-2 was gradually down-regulated, and VEGFR-2 became negative (not shown).

The CD34-positive (Fig. 6B) and ASMA-positive cells (Fig. 6C) were now organized in well-formed circular

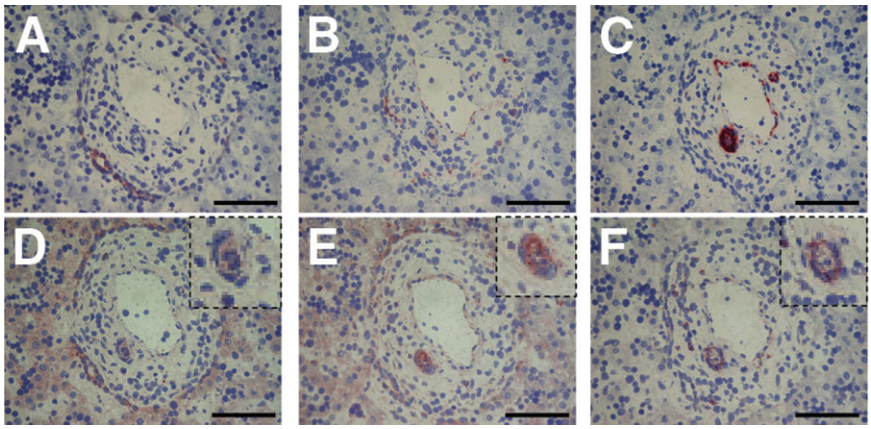

Fig. 5. Comparison of sequential expression of angiogenic growth factors with different cell-type markers in the double-layer ductal plate stage: (A) CK19, (B) CD34, (C) ASMA, (D) VEGF, (E) VEGFR-1, and (F) Ang-2. As in the previous stage, VEGF (D) was still expressed by ductal plate cells acquiring a double-layered structure (A), whereas there was VEGFR-1 immunoreactivity (E) in both ductal plate and endothelial cells, which started to form circular buds (B) surrounded by a sheath of ASMA-positive cells (C), where some Ang-2 immunoreactivity could be observed (F). Figure insets provide major details; scale bar A–F: 100 μm.

structures of increasing size containing a small, slit-like lumen, in which the ASMA-positive cells assumed an onion skin–like appearance. In this structure (immature hepatic artery), the CD34- and ASMA-positive cells showed different angiogenic phenotypes. The inner CD34-positive cells maintained homogeneous and strong expression of VEGFR-1 (Fig. 6E), whereas the outer ASMA-positive cells expressed Tie-2 (Fig. 4B) and showed increased immunoreactivity for Ang-2 (Fig. 6F). Some additional CD34-positive cells expressing VEGFR-1 but not surrounded by ASMA-positive cells closely aggregated around the migrating portion of the ductal plate. They assumed the shape of small capillaries rather than vessels, and likely represented the nascent peribiliary plexus (Fig. 6B, arrowhead). Additional ASMA-positive cells coexpressing Tie-2 and Ang-2 formed a very recognizable although thin regular wall around the central portal vein.

Once migrated into the portal space, the bile ducts were surrounded by the mesenchyme and assumed a tubular morphology (incorporated bile ducts, BD), whereas the ductal plate cells, at the margin of the portal mesenchyme, disappeared (Fig. 7A). This was the incorporated bile duct stage. In these structures, VEGF expression became fainter (Fig. 7D), and VEGFR-1 expression ceased (Fig. 7E).

At this stage, the hepatic artery branches increased in number in the portal space and showed a very recognizable open lumen in which CD34-positive cells (Fig. 7B) were surrounded by a thicker wall of ASMA-positive cells (Fig. 7C), the mature hepatic artery. Unlike in the immature artery, in the mature hepatic artery, Tie-2 was also expressed by the inner endothelial cell layer (Fig. 4C-D), along with VEGFR-1 (Fig. 7E), whereas the ASMA-positive cells still strongly coexpressed Ang-2 (Fig. 5F) and Tie-2 (Fig. 4C). The PBP was extended by the increased number of CD34-positive cells, which were immunoreactive for VEGFR-1, in parallel with the progressive enlargement of the BD (Fig. 7B,E, insets).

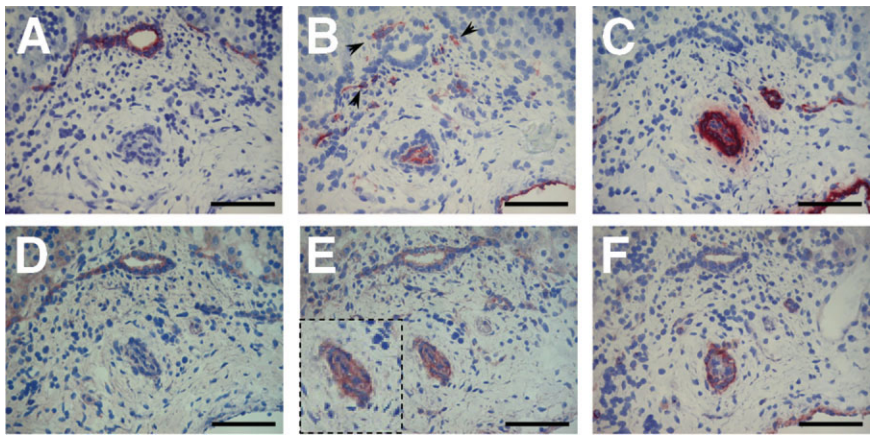

Fig. 6. Comparison of sequential expression of angiogenic growth factors with different cell-type markers in the migratory stage: (A) CK19, (B) CD34, (C) ASMA, (D) VEGF, (E) VEGFR-1, and (F) Ang-2. VEGF (D) was still expressed by incorporating bile duct cells (A), on which VEGFR-1 expression was getting fainter (E). However, strong VEGFR-1 expression was maintained in endothelial cells (B) assuming a circular shape but not yet lining a discernible lumen (E, inset, for further details) and covered by an outer layer of ASMA-positive cells (C), the immature hepatic artery. In the immature hepatic artery ASMA-positive cells expressed Ang-2 (F). Some additional CD34-positive cells aggregated in the vicinity of the incorporating bile duct to form the nascent PBP (arrowheads in B). Scale bar A–F: 100 μm.

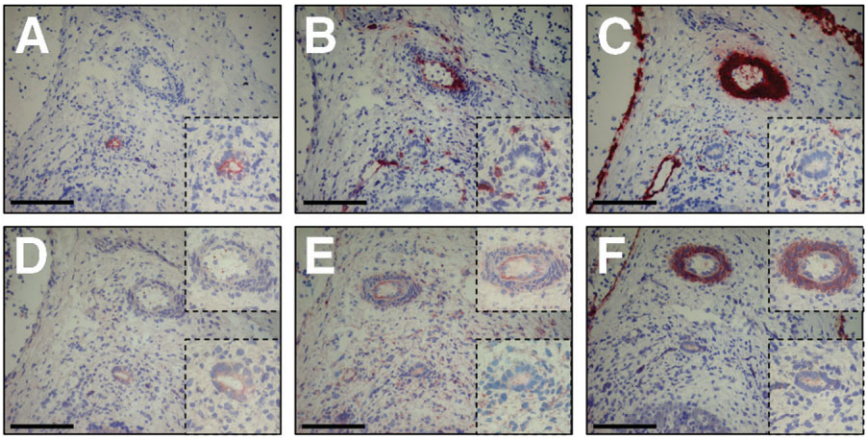

Fig. 7. Comparison of sequential expression of angiogenic growth factors with different cell-type markers in the bile duct stage: (A) CK19, (B) CD34, (C) ASMA, (D) VEGF, (E) VEGFR-1, and (F) Ang-2 (F). VEGF expression (D) faded on the incorporated bile duct (A); this biliary structure was negative for VEGFR-1 (E), which was still expressed by endothelial cells (B) lining a very discernible lumen and surrounded by a thicker wall of ASMA-positive cells (C), the mature hepatic artery. Homogeneous immunoreactivity for Ang-2 (F) was present in mural cells (C) of the mature hepatic artery (scale bar A–F: 100 μm). In PBP, endothelial cells expressing VEGFR-1 aggregated around the incorporated bile duct and increase in number parallel to bile ducts (B, E, insets).

At this stage the central portal vein was not modified further, and the CD34-positive cells remained positive for VEGFR-1 but negative for Tie-2.

In adult liver, expression of angiogenic growth factors differs from that observed in the last fetal maturational stage. Unlike in the BD stage, VEGF immunoreactivity was negative in cholangiocytes and faintly expressed by hepatocytes with a cell membrane aspect (as already reported[11]), whereas VEGFR-1 was expressed only by endothelial cells (ECs) and VEGFR-2 was not expressed at all. However, whereas the hepatocytes still expressed Ang-1, Tie-2 immunostaining was restricted to ECs and became negative in mural cells, where Ang-2 expression also disappeared (data not shown).

Hnf6 $^{-/-}$ ***Mice.*** Timing of artery formation differs between humans and mice, only occurring in the latter after birth, as previously observed by Clotman.[8] At P1 small clumps of ASMA-positive cells were in the vicinity of single- and double-layered ductal plates. These primordial structures then acquired a clear artery profile with an ASMA-positive cell layer around an endothelium from P4 onward, closely paralleling the migration of incorporating bile ducts into the portal mesenchyma. In the wild-type (WT) mice, VEGF was expressed by both hepatocytes and cholangiocytes, where it decorated ductal plates (Fig. 8A) as well as incorporating bile ducts (Fig. 8B). Only when the VEGF-expressing cholangiocytes organized as an incorporating bile duct did ASMA-positive cells begin to form a well-structured arterial profile (Fig. 8C). As shown in Fig. 8D, VEGFR-1 immunoreactivity was localized to endothelial cells, which in controls were surrounded by a layer of ASMA-positive cells. In the *Hnf6*$^{-/-}$ mice (Fig. 8F,G) on day P4, biliary cells retained a ductal plate configuration without progressing toward a migratory stage, and ASMA-positive mural cells did not assemble with endothelial cells (Fig. 8H,I). Similar to in humans, in mice, Ang-1 was diffusely expressed by hepatocytes but was absent from cholangiocytes (not shown). On the other hand, its receptor Tie-2 was expressed by ASMA-positive cells, which were organized as a regular wall around the endothelium in the controls (Fig. 8E) but remain irregularly scattered in the portal mesenchyma in the *Hnf6*$^{-/-}$ mice (Fig. 8L). Overall, these data indicate that in *Hnf6*$^{-/-}$ mice, defects of incorporating ducts expressing VEGF are associated with severely perturbed development of hepatic arteries, which may be a result of hampered EC recruitment during the migratory stage.

Discussion

The intimate anatomical relationship between intrahepatic bile ducts, hepatic artery branches, and the peribiliary vascular plexus is a common morphological feature, observed not only during liver development but also in response to liver injury. The fine-branching patterns of both epithelial and vascular structures seem to develop in a coordinated fashion. Data from experimental animals[6,8] and congenital cholangiopathies[11,17,18] suggested that a signal originating from the bile duct cells directs arterial vasculogenesis during development and arterial neoangiogenesis in response to biliary damage. We recently showed that in cystic cholangiopathies related to DPM, such as autosomal dominant polycystic kidney disease and Caroli's disease, the biliary epithelium retains an immature phenotype characterized by up-regulation of VEGF and angiopoietins. We speculated that angiogenic factor production by cholangiocytes promotes the aberrant vascularization around the cyst in order to provide its vascular supply.[11]

In the present study we investigated the expression of angiogenic growth factors (VEGF, Ang-1, Ang-2) and their cognate receptors (VEGFR-1, VEGFR-2, and Tie-2) during biliary and arterial development in humans. Our findings showed strong expression of VEGF

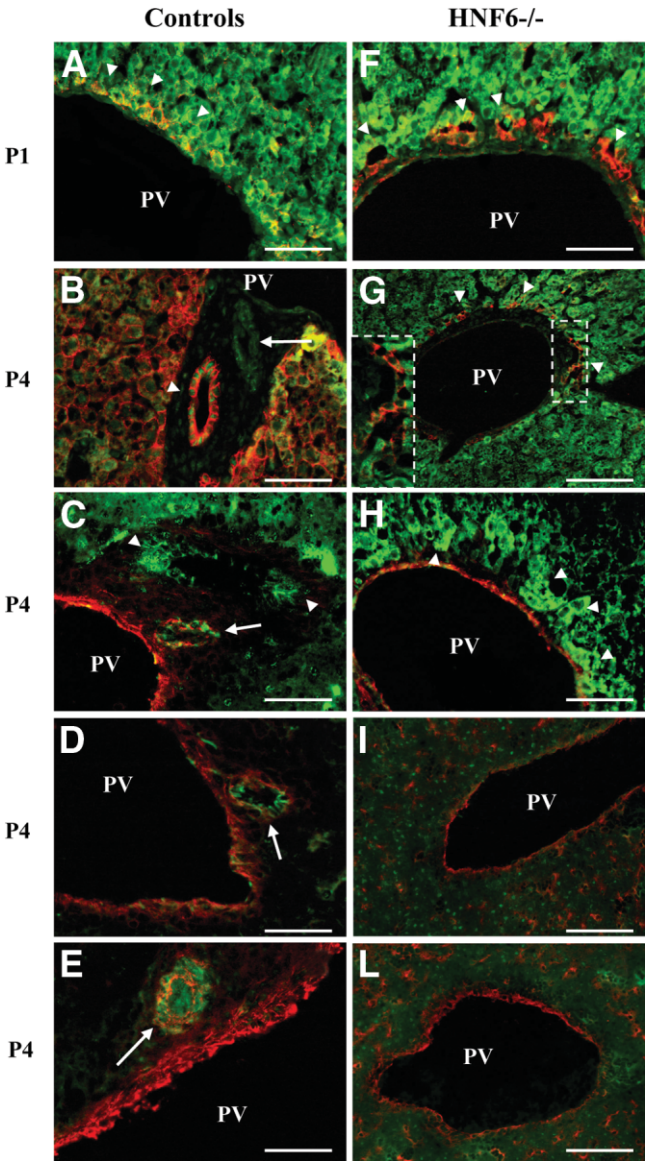

Fig. 8. Expression of angiogenic growth factors in *Hnf6*$^{-/-}$; and control mice on P1–P4. Dual immunofluorescence with: (A–B, F–G) pan-CKs (Alexa Fluor 594) and VEGF (Alexa Fluor 488); (C, H) ASMA (Alexa Fluor 594) and VEGF (Alexa Fluor 488); (D, I) ASMA (Alexa Fluor 594) and VEGFR-1 (Alexa Fluor 488); and (E, L) ASMA (Alexa Fluor 594) and Tie-2 (Alexa Fluor 488). VEGF was expressed by both hepatocytes and cholangiocytes organized as either (A, F) ductal plate (P1; scale bar: 50 μm) or (B, G) incorporating BDs, which normally develop in controls but not in *Hnf6*$^{-/-}$ mice (P4; scale bar for B and G: 50 and 100 μm, respectively). (C, H) Only when VEGF was expressed by incorporating BDs did ASMA-positive cells form a structured arterial profile, which did not develop when VEGF was expressed by ductal plate cells in *Hnf6*$^{-/-}$ mice (P4; scale bar: 50 μm). (D, I) VEGFR-1 immunoreactivity was restricted to endothelial cells, which were surrounded by a layer of ASMA-positive cells in controls but not in *Hnf6*$^{-/-}$ mice (P4; scale bar for D and I: 50 and 100 μm, respectively). (E, L) Tie-2 was expressed by ASMA-positive cells, forming a regular wall around the endothelium in controls and remaining irregularly scattered in the portal mesenchyma in *Hnf6*$^{-/-}$ mice (P4; scale bar for E and L: 50 and 100 μm, respectively). PV, portal vein; arrowheads, ductal plate/BD; arrows, hepatic artery.

and Ang-1 in developing bile duct cells and hepatoblasts, respectively. We also showed that their cognate receptors were expressed in endothelial and mural cells with a stage-specific pattern. In immunohistochemical studies, only fixed times could be analyzed as a snapshot of what is actually a dynamic process, as is the case in arterial vasculogenesis. However, a working model can be proposed. Our findings concur with the hypothesis that angiogenic growth factors generated by liver epithelial cells mediate the cross-talk between the developing biliary tree, the hepatic arterial branches, and the PBP during human liver ontogenesis.

Unlike vascular cells, which were persistently negative for VEGF throughout the different developmental stages, biliary epithelial cells were strongly positive for VEGF from the earliest maturational stages (sDP). VEGFR-1 and VEGFR-2 were also expressed in cholangiocyte precursors, but the expression was lost in the incorporated and incorporating ducts, respectively. On the other hand, VEGFR-1 was strongly expressed by cells positive for the endothelial marker CD34 throughout development. VEGFR-1 expression could be detected in CD34-positive cells, even in the most immature vascular structures that, rather than being arranged in a circular configuration, formed small clumps or short strings of cells adjacent to the ductal plate. We also observed a population of cells positive for both CD34 and VEGFR-2 but negative for VEGFR-1; these cells were localized at the margin of the portal tract or inside the parenchyma (Fig. 3A–D). This immunophenotype has been associated with hemangioblasts, that is, residual embryonic stem cells with wide-spectrum differentiation potential toward hematopoietic and endothelial lineages.[15,16]

VEGF is the most important promoter of vasculogenesis, the process by which endothelial cells differentiate and proliferate within a previously avascular tissue and then coalesce to form a primitive tubular network. The ability of cholangiocytes to secrete VEGF and express VEGF receptors is quite interesting. We previously showed that in ductal plate malformations and other human liver diseases, reactive cholangiocytes express VEGFR-1 and VEGFR-2.[11] We also showed evidence that cultured cholangiocytes secrete VEGF and that VEGF can stimulate cholangiocyte growth *in vitro* and activate ERK1/2, a common pathway for cholangiocyte mitogens (Spirlì et al., unpublished data), thus proving that VEGF and VEGF receptor immunoreactivity actually indicates the production and functional activity of these proteins in cholangiocytes from humans as well as in rodent models. This suggests the existence of an autocrine mechanism during development and in reactive cholangiocytes after liver damage. In the present study we focused on the paracrine role of angiogenic growth factors released during liver development by epithelial cells to influence arterial vasculogenesis.

In the early steps of vasculogenesis, the effects of VEGF are mediated by the VEGFR-2 receptor. In contrast, VEGFR-1 is involved in a later phase of vasculogenesis, when hemangioblast commitment must be suppressed to allow the correct assembly of endothelial cells into functional and branched vascular channels.[19] Therefore, by secreting VEGF, ductal plate cells are capable of directing the patterning of EC formation and branching via a paracrine mechanism, acting first on VEGFR-2 expressed by endothelial progenitors and then on VEGFR-1 expressed by endothelial cells in the process of assembling the vessels.

With the progressive incorporation of the tubular part of the ductal plate into the portal mesenchyma (migratory stage), CD34-positive/VEGFR-1 positive cells follow 2 patterns: on one side they begin to form a thin network of capillaries strictly adjacent to the incorporating bile duct; on the other side they assemble in larger and more complex vascular structures, alongside but not in contact with the migrating ducts. The smaller capillary-like structures in intimate contact with the iBD likely represent the precursor of the PBP. In fact, as previously described by Nakanuma,[13] in the following stage they grow and mature in parallel to the development of bile ducts. The reciprocal expression of VEGF in ductal structures and of VEGFR-1 in capillary and ductal structures suggests that VEGF, released by the developing bile ducts and acting on VEGFR-1 expressed in endothelial cells and cholangiocytes, modulates synchronous and parallel maturation of both the PBP and the intrahepatic bile ducts.

VEGF release by cholangiocytes acts as a signal for mural and endothelial cells to assemble in close vicinity to the maturing bile ducts. The correct development of ductular structures during the migratory stage seems to be crucial to driving proper assembly of the nascent artery profiles. This was clearly demonstrated in the *Hnf6*$^{-/-}$ mice, where ductal plate–like structures were present but did not progress beyond the stage of partial duplication. In this animal model, on P4, ductal plates failed to incorporate into the portal mesenchyma as bile ducts, and no nascent hepatic artery could be observed in these portal spaces. In contrast, at the same point, the WT animals showed hepatic arteries developing in close proximity to the incorporated bile ducts. This indicates that in addition to the vasculogenic signals, the correct development of the arterial supply to the biliary tree is dependent on the normal incorporation and branching of the biliary tree. In its absence, the cross-talk mediated by VEGF cannot be correctly targeted, and therefore, the proper recruitment of ECs alongside the migrating ducts is perturbed.

When the artery is properly assembled, CD34-positive cells are surrounded by a thin layer of ASMA-positive cells, as observed in the immature hepatic artery. These ASMA-positive cells express Tie-2, the receptor for both Ang-1 and Ang-2, and therefore can be activated by the Ang-1 produced by hepatoblasts. Ang-1 plays a relevant role in the early stages of arterial vasculogenesis. Tie-2 is known to be expressed by mural precursor cells, which respond to angiopoietins by migrating and secreting proteolytic enzymes such as the matrix metalloproteinase-2.[20] Therefore, Ang-1 can modulate the recruitment of supporting cells to the developing vasculature, a step necessary to stabilize vessels during arteriogenesis.[21] Mural cells are thought to derive from a mesenchymal precursor different from that of endothelial cells and activated by angiopoietins.[20] In keeping with this hypothesis, we observed that during the ductal plate stage, when the ASMA-positive cells were not yet organized around the cluster of CD34-positive cells, a subpopulation of ASMA-positive cells expressed Tie-2. These cells, whose mesenchymal origin was indicated by their ASMA immunoreactivity, were characterized by a spindle-shaped morphology and were scattered in the portal space (Fig. 4A). These cells were actually similar to the myofibroblast-like cells we previously described in the developing portal space as the cell source of the smooth muscle layer of the hepatic artery branch.[4]

After the recruitment of mural cell precursors, a stabilizing layer of smooth muscle cells and pericytes was assembled around the endothelium, a process known as angiogenic remodeling. We found that at this stage some ASMA-positive cells expressed Ang-2 in addition to Tie-2. In contrast with adult cells, in mural cell precursors Ang-2 does not counteract the stimulatory effect of Ang-1 but rather functions as a Tie-2 agonist.[20] In particular, angiopoietins are capable of inducing a close integration of ECs with mural cells by affecting junctional molecules or by acting as adhesive protein.[22] Our data suggest that the process of angiogenic remodeling of the immature hepatic arteries is regulated both by the paracrine effect of Ang-1 produced by hepatoblasts and by the autocrine effect of Ang-2 on the same mural cells.

As biliary development proceeds (bile duct stage), the immature vasculature undergoes further remodeling, characterized by sprouting, vessel enlargement, and progressive opening of a lumen. Through this process, the original vascular bed is modified to form a complex network of large vessels that ramifies into smaller branches. At this maturational stage the phenotype of the hepatic arteries is characterized by the additional expression of Tie-2 in VEGFR-1-positive and CD34-positive endothelial cells and by the coexpression of Ang-2 and Tie-2 in ASMA-positive supporting cells, whereas incorporated bile duct cells and hepatoblasts continue to express VEGF and Ang-1, respectively. At this stage, the interplay between VEGF and angiopoietins is crucial to ensuring that

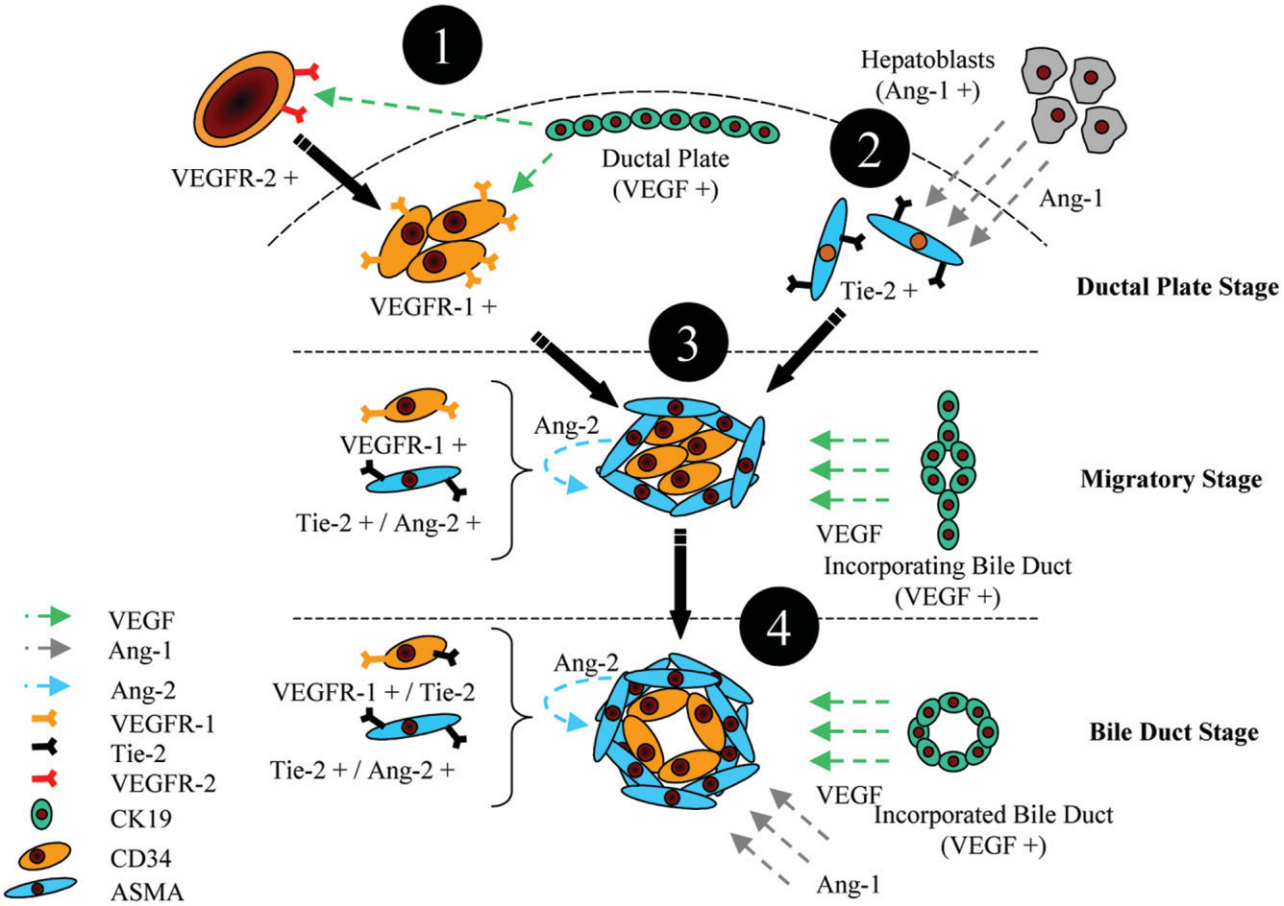

Fig. 9. The working hypothesis was that there is functional cooperation between ductal plate cells and hepatoblasts in driving arterial and PBP vasculogenesis in the developing human liver This interaction is based on the capability of ductal plate cells and hepatoblasts to produce VEGF and Ang-1, respectively, throughout the different maturational stages. During the ductal plate stage, VEGF induces activation of VEGFR-2-positive endothelial cell precursors, which reach the portal mesenchyme in proximity to the developing ductal plates where they form small cell clumps expressing VEGFR-1 (1). On the other hand, Ang-1 recruits Tie-2-positive portal myofibroblasts scattered in the portal space to behave as mural cell precursors (2). In the migratory stage, VEGFR-1-positive endothelial cells and Tie-2-positive mural cells assemble into nascent vascular structures without a recognizable lumen (immature hepatic artery), where mural cells take part autocrinally in arterial remodeling by releasing Ang-2 (3). In addition, some of the CD34-positive cells are drawn by the migrating portions of the ductal plate to form the nascent PBP. In the bile duct stage, endothelial cells acquire Tie-2 positivity along with VEGFR-1, thereby becoming prone to the synergic action of VEGF and Ang-1, which induces lumen formation and progressive vascular enlargement, the mature hepatic artery (4).

different vascular cell types proliferate and migrate in a complementary and coordinated fashion.[23,24] In particular, VEGF, acting in concert with angiopoietin-1, exerts potent mitogenic and motogenic effects on endothelial cells[25,26] and promotes the increased diameter of the lumen. Our finding of additional Tie-2 expression by ECs in mature hepatic artery characterized by progressive lumen opening is thus consistent with this mechanism. Tie-2 expression by ECs is also a feature of the adult arterial vasculature, where mural cells no longer express both Tie-2 and Ang-2, consistent with a reduced need for ongoing arterial remodeling processes in the adult liver.

In conclusion, our study has shown that VEGF produced by the developing bile ducts is likely the signal linking ductal and arterial development in the liver. By secreting VEGF, which acts on endothelial cells and their precursors, bile duct cells promote arterial and PBP vasculogenesis. At the same time, Ang-1, produced by hepatoblasts, induces maturation of the vessel. A similar phenomenon was recently described in peripheral nerves,[27] which were shown to promote arterial differentiation via VEGF. The interdependence of arteries with other branched structures during ontogenesis is thus a general phenomenon. In the liver, however, artery differentiation is a more complex process, requiring the functional interaction of the developing cholangiocytes with hepatoblasts. Therefore, as summarized in Fig. 9, we suggest that cholangiocytes generate a VEGF gradient that is crucial in the migratory stage, when it determines arterial and PBP vasculogenesis in their vicinity, whereas Ang-1 signaling from hepatoblasts contributes to the remodeling of hepatic arteries to meet the demands of the developing epithelium. Interestingly, in several forms of liver disease, cholangiocytes

retain the ability to produce VEGF, consistent with the idea that liver repair recapitulates ontogenesis.[28,29]

Acknowledgment: We thank Dr. Dhanpat Jain (Department of Pathology, Yale University) and Dr. Jordan Pober (Department of Pathology, Yale University) for critically reading the manuscript.

References

1. Crawford AR, Lin XZ, Crawford JM. The normal adult human liver biopsy: a quantitative reference standard. HEPATOLOGY 1998;28:323-331.
2. Terada T, Nakanuma Y. Development of human peribiliary capillary plexus: a lectin-histochemical and immunohistochemical study. HEPATOLOGY 1993;18:529-536.
3. Kono N, Nakanuma Y. Ultrastructural and immunohistochemical studies of the intrahepatic peribiliary capillary plexus in normal livers and extrahepatic biliary obstruction in human beings. HEPATOLOGY 1992;15:411-418.
4. Libbrecht L, Cassiman D, Desmet V, Roskams T. The correlation between portal myofibroblasts and development of intrahepatic bile ducts and arterial branches in human liver. Liver 2002;22:252-258.
5. Desmet V, Roskams T, Van Eyken P. Ductular reaction in the liver. Pathol Res Pract 1995;191:513-524.
6. Masyuk TV, Ritman EL, LaRusso NF. Hepatic artery and portal vein remodeling in rat liver: vascular response to selective cholangiocyte proliferation. Am J Pathol 2003;162:1175-1182.
7. Clotman F, Lannoy VJ, Reber M, Cereghini S, Cassiman D, Jacquemin P et al. The onecut transcription factor HNF6 is required for normal development of the biliary tract. Development 2002;129:1819-1828.
8. Clotman F, Libbrecht L, Gresh L, Yaniv M, Roskams T, et al. Hepatic artery malformations associated with a primary defect in intrahepatic bile duct development. J Hepatol 2003;39:686-692.
9. Desmet VJ. Congenital diseases of intrahepatic bile ducts: variations on the theme "ductal plate malformation." HEPATOLOGY 1992;16:1069-1083.
10. Lemaigre F, Zaret KS. Liver development update: new embryo models, cell lineage control, and morphogenesis. Curr Opin Genet Dev 2004;14:582-590.
11. Fabris L, Cadamuro M, Fiorotto R, Roskams T, Spirli C, Melero S, et al. Effects of angiogenic factor overexpression by human and rodent cholangiocytes in polycystic liver diseases. HEPATOLOGY 2006;43:1001-1012.
12. Van den Heuvel MC, Gouw AS, Boot M, Slooff MJ, Poppema S, de Jong KP. The finest branches of the biliary tree might induce biliary vascularization necessary for biliary regeneration. J Hepatol 2006;44:947-955.
13. Nakanuma Y, Hoso M, Sanzen T, Sasaki M. Microstructure and development of the normal and pathologic biliary tract in humans, including blood supply. Microsc Res Tech 1997;38:552-570.
14. Libbrecht L, Cassiman D, Desmet V, Roskams T. Expression of neural cell adhesion molecule in human liver development and in congenital and acquired liver diseases. Histochem Cell Biol 2001;116:233-239.
15. Ziegler BL, Valtieri M, Porada GA, De Maria R, Muller R, Masella B, et al. KDR receptor: a key marker defining hematopoietic stem cells. Science 1999;285:1553-1558.
16. Pelosi E, Valtieri M, Coppola S, Botta R, Gabbianelli M, Lulli V, et al. Identification of the hemangioblast in postnatal life. Blood 2002;100:3203-3208.
17. Gaudio E, Onori P, Pannarale L, Alvaro D. Hepatic microcirculation and peribiliary plexus in experimental biliary cirrhosis: a morphological study. Gastroenterology 1996;111:1118-1124.
18. Gaudio E, Barbaro B, Alvaro D, Glaser S, Francis H, Ueno, Y et al. Vascular endothelial growth factor stimulates rat cholangiocyte proliferation via an autocrine mechanism. Gastroenterology 2006;130:1270-1282.
19. Fong GH, Zhang L, Bryce DM, Peng J. Increased hemangioblast commitment, not vascular disorganization, is the primary defect in flt-1 knock-out mice. Development 1999;126:3015-3025.
20. Iurlaro M, Scatena M, Zhu WH, Fogel E, Wieting SL, Nicosia RF. Rat aorta-derived mural precursor cells express the Tie2 receptor and respond directly to stimulation by angiopoietins. J Cell Sci 2003;116:3635-3643.
21. Suri C, Jones PF, Patan S, Bartunkova S, Maisonpierre PC, Davis S, et al. Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. Cell 1996;87:1171-1180.
22. Carlson TR, Feng Y, Maisonpierre PC, Mrksich M, Morla AO. Direct cell adhesion to the angiopoietins mediated by integrins. J Biol Chem 2001;276:26516-26525
23. Chae JK, Kim I, Lim ST, Chung MJ, Kim WH, Kim HG, et al. Coadministration of angiopoietin-1 and vascular endothelial growth factor enhances collateral vascularization. Arterioscler Thromb Vasc Biol 2000;20:2573-2578.
24. Kim I, Kim HG, Moon SO, Chae SW, So JN, Koh KN, et al. Angiopoietin-1 induces endothelial cell sprouting through the activation of focal adhesion kinase and plasmin secretion. Circ Res 2000;86:952-959.
25. Suri C, McClain J, Thurston G, McDonald DM, Zhou H, Oldmixon EH, et al. Increased vascularization in mice overexpressing angiopoietin-1. Science 1998;282:468-471.
26. Asahara T, Chen D, Takahashi T, Fujikawa K, Kearney M, Magner M, et al. Tie2 receptor ligands, angiopoietin-1 and angiopoietin-2, modulate VEGF-induced postnatal neovascularization. Circ Res 1998;83:233-240.
27. Mukouyama YS, Shin D, Britsch S, Taniguchi M, Anderson DJ. Sensory nerves determine the pattern of arterial differentiation and blood vessel branching in the skin. Cell 2002;109:693-705.
28. Golding M, Sarraf C, Lalani EN, Alison MR. Reactive biliary epithelium: the product of a pluripotential stem cell compartment? Hum Pathol 1996;27:872-884.
29. Fabris L, Strazzabosco M, Crosby HA, Ballardini G, Hubscher SG, Kelly DA, et al. Characterization and isolation of ductular cells coexpressing neural cell adhesion molecule and Bcl-2 from primary cholangiopathies and ductal plate malformations. Am J Pathol 2000;156:1599-612.