VEGF is crucial for the hepatic vascular development required for lipoprotein uptake

Brian Carpenter[1,*,†], Yuankai Lin[1,*], Stephanie Stoll[1], Robert L. Raffai[1,2], Robert McCuskey[3] and Rong Wang[1,‡]

[1]Pacific Vascular Research Laboratory, Division of Vascular Surgery, Department of Surgery, University of California, San Francisco, CA 94143-0507, USA
[2]Veterans Administration Medical Research Center, Surgical Services (112G), San Francisco, CA 94121, USA
[3]Department of Cell Biology and Anatomy, University of Arizona College of Medicine, Tucson, AZ 85724, USA
*These authors contributed equally to this work
†Present address: Auvation Limited, Aberdeen Science and Technology Park, Balgownie Drive, Bridge of Don, Aberdeen AB22 8GU, UK
‡Author for correspondence (e-mail: rongw@itsa.ucsf.edu)

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Summary

Hepatic lipid catabolism begins with the transport of lipoprotein remnants from the sinusoidal vasculature into hepatocytes by endocytosis via microvilli. To test the hypothesis that fenestrated sinusoidal endothelial cells (SECs) are crucial for this process, we selectively disrupted SECs by downregulating vascular endothelial growth factor (VEGF) signaling, using hepatocyte-specific, tetracycline-regulatable expression of a VEGF receptor that can sequester VEGF but cannot relay its signal. Newborn mutant livers appeared grossly normal, but displayed a dark-red color that was distinguishable from normal physiological lipid-rich pink livers. Mutant sinusoidal networks were reduced and their SECs lacked fenestrae. Hepatocellular lipid levels were profoundly reduced, as determined by Oil Red O staining and transmission electron microscopy, and fewer hepatocytic microvilli were evident, indicating impaired lipoprotein endocytosis. Levels of apolipoprotein (APO) E bound to mutant sinusoidal networks were significantly reduced, and fluorescently-labeled murine remnant lipoproteins injected into the blood stream failed to accumulate in the space of Disse and diffuse into hepatocytes, providing evidence that reduced hepatocellular lipid levels in mutant livers are due to impaired lipoprotein uptake. Temporal downregulation of VEGF signaling revealed that it is crucial at all developmental stages of hepatic vascular morphogenesis, and repression of the dominant-negative effect can rescue the phenotype. These findings provide the first genetic evidence that VEGF dynamically regulates SEC fenestration during liver organogenesis, a process that is required for lipoprotein uptake by the liver.

Key words: Angiogenesis, Space of Disse, Sinusoids, Vascular development

Introduction

The adult liver is a highly vascularized tissue, a feature that is crucial for its function. Each hepatocyte is flanked by sinusoidal vascular channels, which are lined by sinusoidal endothelial cells (SEC), with the space of Disse separating hepatocytes and sinusoidal cells (McCuskey, 1994). The SECs are fenestrated and lack a basement membrane, which allows the direct exchange of blood plasma on hepatocyte surfaces (Wisse et al., 1985). The fenestrae also allow efficient 'sieving' of macromolecules, including lipoproteins and chylomicron remnants, from the blood to the hepatocytes for processing (Braet and Wisse, 2002). Efficient removal of cholesterol-rich lipoproteins from the blood by the liver occurs by receptor-mediated endocytosis on hepatic microvilli within the space of Disse (Cooper, 1997; Havel and Hamilton, 2004; Mahley and Ji, 1999). This process is important in preventing hypercholesterolemia and diseases such as atherosclerosis.

Proper formation of the intricate sinusoidal vascular network provides all hepatocytes with direct access to the blood plasma and efficient lipoprotein metabolism. A cooperative effort between hepatocytes and endothelial cells (ECs) during liver organogenesis may be required for the proper development of SECs and the space of Disse. In support of this, Matsumoto et al. recently showed that ECs are required for liver bud emergence into the septum transversum mesenchyme at embryonic day 9.5 (E9.5), and that ECs delimit the extent of hepatic cell migration (Matsumoto et al., 2001). These interactions suggest that there are specific signals that are communicated between ECs and endoderm that stimulate liver organogenesis. Morphological studies have highlighted the importance of the space of Disse, and SEC and hepatocyte structural features, such as fenestrations and microvilli, respectively, for effective lipoprotein-remnant passage from the blood to hepatocytes (Fraser et al., 1995). It is therefore likely that there are communication signals between these two cell lineages that are responsible for establishing the structural characteristics of these cell types. We have chosen to undertake a genetic approach to test the hypothesis that disruption of the liver vasculature would affect the ability of the liver to properly function in lipoprotein homeostasis.

Development

Vascular endothelial growth factor (VEGF) is a potent angiogenic regulator required for embryonic development. Mice heterozygous for the VEGF allele ($VEGF^{+/-}$) are embryonic lethal by E12 (Carmeliet et al., 1996; Ferrara et al., 1996). $VEGF^{+/-}$ and $VEGF^{-/-}$ embryos display severely abnormal vasculature and organ development. In addition to being required for embryonic development, VEGF is also required for continued development in neonatal mice (Gerber et al., 1999). In the liver, hepatocytes of $VEGF^{-/-}$ mice have an abnormal morphology and an underdeveloped sinusoidal network. Additionally, VEGF inactivation leads to a significant increase in EC apoptosis in neonatal mice, suggesting a role for VEGF in EC survival (Gerber et al., 1999).

Communication between hepatocytes and ECs through VEGF signaling has been suggested by the expression patterns of VEGF by hepatocytes (Mochida et al., 1998; Yamane et al., 1994), and of its tyrosine kinase receptors VEGFR1 (FLT1) and VEGFR2 (FLK1/KDR) by ECs (de Vries et al., 1992; Quinn et al., 1993). Differential signaling of VEGF through its receptors highlights the intricacies of this communication system in the liver. Signaling through VEGFR1 induces SECs to release various cytokines that stimulate hepatocyte proliferation; signaling through VEGFR2 stimulates SEC proliferation (LeCouter et al., 2003). During regeneration of the adult liver following partial hepatectomy, expression of both VEGF and its receptors is upregulated (Mochida et al., 1998; Ross et al., 2001; Sato et al., 2001; Shimizu et al., 2001). Adult rats undergoing partial hepatectomy concomitant with VEGF administration showed a significant increase in both SEC and hepatocyte proliferation (Taniguchi et al., 2001). Conversely, rats treated with an anti-VEGF antibody following partial hepatectomy displayed significantly reduced proliferation of SECs and hepatocytes (Taniguchi et al., 2001).

In this study, we hypothesized that VEGF signaling plays an important role in the development of the space of Disse and SEC structure during liver organogenesis. We chose to downregulate VEGF activity by using a liver-specific, conditional VEGF-knockdown system. We show here that downregulation of VEGF signaling does not cause detectable gross abnormalities in liver development, but that it selectively abrogates hepatic sinusoidal structure, resulting in incomplete lining of the sinusoidal lumen, decreased network complexity, lack of fenestrae, impaired lipoprotein uptake and decreased hepatocellular lipid content. These results demonstrate that VEGF signaling is both important for SEC development and required for lipoprotein uptake in the liver.

Materials and methods

Mouse strains

Generation of the *TRE-VEGFR2T* mouse strain was performed as previously described (Wang et al., 2001). Briefly, a truncated murine *VEGFR2*, encoding amino acids 1-828, fused with a hemaglutin (HA) tag at the C terminus, was isolated as a 2.48 kb *Xho*I and *Hin*dIII fragment from pBSK-Flk1-HA [gift from T. Quinn, University of California San Francisco (UCSF) (Quinn et al., 1993)]. This fragment was inserted by blunt-end ligation into the *Xba*I site of pUHG 10-3 (gift from H. Bujard, European Molecular Biology Laboratory). A 4.48 kb *Xho*I and *Ase*I DNA fragment containing the *tetracycline* (*tet*) *response element* (*TRE*), the human CMV minimal promoter with heptamerized upstream tet-operators, the murine *VEGFR2T*-coding sequence, and the rabbit β-globin intron and poly(A) sequence was isolated and used for microinjection to derive mice from the FVB/N background. Founder lines were established and were screened using primers from the *TRE* (5′-GTCGAGTAGGCGTGTACG-3′) and from the murine *VEGFR2T* gene (5′-GAATCACGCTGAGCATTGGGC-3′). *LAP-tTA* mice (Kistner et al., 1996) and *TRE-lacZ* mice (Redfern et al., 1999) were maintained predominantly in a FVB/N background. $VEGFR2^{lacZ}$ mice (Shalaby et al., 1995) were maintained in a mixed background. Doxycycline (Dox) was administered in the chow diet (Bio-Serv). Tet (Sigma), at a final concentration of 500 μg/ml, was administered in 5% sucrose drinking water. All animals were treated in accordance with the guidelines of the UCSF Institutional Animal Care and Use Committee.

Western blot

Western-blot analyses were performed with a mouse anti-HA antibody (clone 12CA5, Exalpha Biologicals) or anti-albumin antibody (Accurate Chemical and Scientific Corporation) at 20 μg/ml in blocking solution.

VEGF ELISA assay

VEGF protein concentration was determined using the Quantikine Mouse VEGF Immunoassay (R&D Systems) according to the manufacturer's recommended protocol.

Histology and immunohistochemistry

Tissues were fixed in 4% paraformadehyde (PFA)/phosphate-buffered saline (PBS) for 24 to 48 hours and processed according to standard histology procedures. Paraffin sections were analyzed for glycogen storage using the Periodic Acid-Schiff (PAS) Staining System (Sigma). Proliferating cell nuclear antigen (PCNA) staining was performed using the Zymed Kit (Zymed Laboratories). Apoptosis was analyzed using a terminal deoxynucleotidyl transferase biotin-dUTP nick-end labeling (TUNEL)-staining kit (Roche).

Immunohistochemistry was performed as previously described (Wang et al., 2001). Rabbit anti-VEGFR2 antibody (TO14) (Brekken et al., 1998) was used at a final concentration of 4 μg/ml. Rat anti-CD31 antibody (clone MEC 13.3, PharMingen) was used at 5 μg/ml. Rabbit anti-murine apolipoprotein (APO) E and anti-murine APOB antibodies (Raffai and Weisgraber, 2002) were used at 1:1000 dilution. Signals were amplified with Vectastain Elite ABC kits (Vector Laboratories). Signals were visualized using the DAB peroxidase substrate kit (Vector Laboratories); sections were counterstained with Hematoxylin.

Vascular perfusion

Using a 26-gauge needle, anesthetized newborn pups were perfused by injection of 10 ml of PBS, followed by 5 ml of 1% PFA into the left ventricle of their hearts.

Preparation of DiI-labeled murine lipoproteins and lipoprotein-uptake experiment

Murine remnant lipoproteins were prepared from plasma of $Apoe^{h/h}Ldlr^{-/-}$ mice deficient in the low-density lipoprotein receptor as previously described (Raffai, 2003; Raffai and Weisgraber, 2002). Remnant lipoproteins in this mouse model are composed of APOB48 and APOB100-containing lipoproteins, both enriched with APOE (R.L.R., unpublished). Purified remnants were dialyzed against PBS (pH 7.2) and labeled with the fluorescent probe 3,3′-dioctadecylindocarbocyanine (DiI) (Molecular Probes), as described (Pitas, 1981), and were adjusted to a concentration of 0.5 mg/ml protein. DiI-labeled remnant lipoproteins (30 μl) were injected into the hearts of anesthetized newborn pups. After 10 minutes of circulation, the livers were harvested and fixed in 4% PFA/PBS at 4°C overnight, equilibrated in 30% sucrose overnight, and embedded in optimal cutting temperature (OCT). Frozen liver sections (10 μm) were mounted with VECTASHIELD mounting medium (Vector Laboratories).

β-Galactosidase staining

Mouse embryos were fixed in PBS with 0.2% glutaraldehyde, 5 mM EGTA, 1 mM $MgCl_2$ for 1-3 hours at 4°C, then rinsed in PBS. For whole-mount staining, embryos were incubated overnight at room temperature in PBS containing 1 mg/ml X-gal, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM $MgCl_2$ and 0.02% NP40. After staining, samples were washed in PBS, fixed in 4% PFA and stored in 2% PFA. For thick sections (100-200 μm), fixed tissues were embedded in 4% low-melting agarose/PBS, sectioned using a vibratome (VT1000S, Leica) and stained as described above.

Oil Red O staining

Tissues were fixed in 4% PFA/PBS at 4°C overnight, equilibrated in 30% sucrose overnight, and embedded in OCT. Frozen liver sections were stained with Oil Red O (Sigma) for 10 minutes and counterstained with Hematoxylin.

Electron microscopy

For transmission electron microscopy (TEM), anesthetized newborn pups were perfused with 0.1 M sodium cacodylate (pH 7.4) and then fixative solution (2% glutaraldehyde, 1% PFA, 0.1 M sodium cacodylate pH 7.4). The liver was harvested and fixed at 4°C for 1-2 hours, dissected into 1-2 mm sections and fixed for an additional hour at 4°C. Standard procedures for TEM were then performed.

Routine methods were used to prepare liver specimens for scanning electron microscopy (SEM) (McCuskey, 1986). Livers were fixed by perfusion with 0.1 M cacodylate buffer, followed by 1.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). Pieces of fixed livers were dehydrated in a graded ethanol series, critical-point dried, fractured, sputter-coated with 10 nm gold and examined using the XL35 scanning electron microscope (Philips Electronic Instruments).

Results

Downregulation of VEGF signaling specifically in the liver

To ascertain the role of SECs in hepatic lipoprotein uptake, we knocked down VEGF signaling in the developing mouse liver. We constructed a truncated VEGF receptor 2 (VEGFR2T), containing the native extracellular and transmembrane domains but lacking the cytoplasmic tyrosine kinase domain, under the control of a *TRE*. Expression from the *TRE* promoter requires an active tet-transactivator (tTA); Tet, or its more stable analog Dox, binds to and inactivates tTA, thereby repressing *TRE*-driven expression. For easy detection, an *HA* tag was fused to the C terminus of the *VEGFR2T* cDNA (*TRE-VEGFR2T*; Fig. 1A). To sequester VEGF specifically in the liver, we restricted the expression of VEGFR2T to hepatocyte membranes by employing a second mouse strain *LAP-tTA*, the tTA driver line with tTA expression under the control of the *LAP* (C/EBPβ) promoter (Kistner et al., 1996). As determined by western blot using an anti-HA antibody (Fig. 1B), VEGFR2T expression was restricted to the livers of double-transgenic pups (*LAP-tTA/TRE-VEGFR2T*). Very little difference in VEGFR2T expression was observed between the four founder lines generated. Furthermore, VEGFR2T expression was suppressed in the liver of double-transgenic newborns born to mothers who received a Dox diet during pregnancy (Fig. 1B).

To determine the onset and tissue specificity of VEGFR2T expression, the *LAP-tTA* mice were bred with a *lacZ* reporter line, *TRE-lacZ* (Redfern et al., 1999). β-Galactosidase activity was observed specifically in the livers of double-transgenic (*LAP-tTA/TRE-lacZ*) embryos as early as E10.5 (Fig. 1C). At

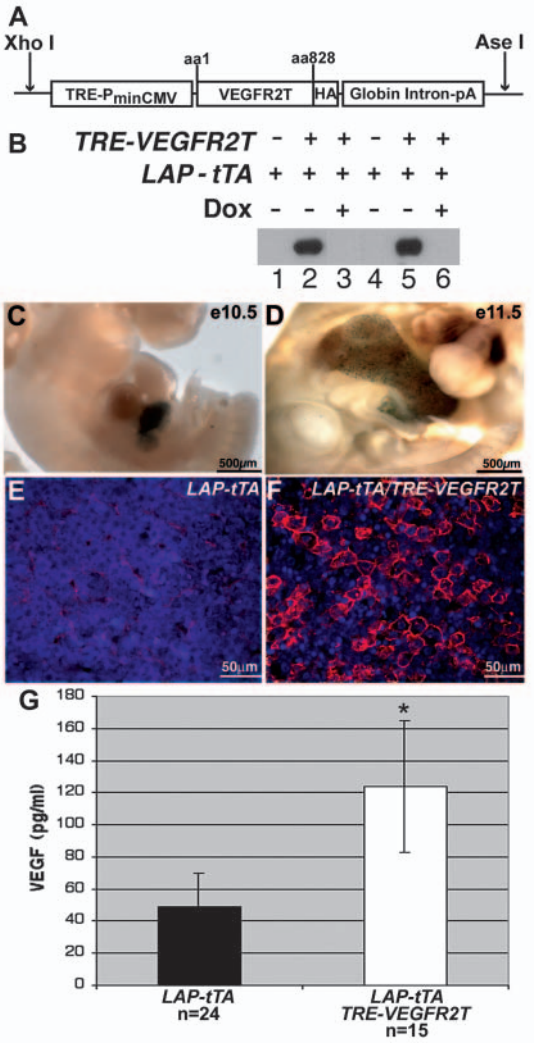

Fig. 1. Dox-controlled VEGFR2T expression in hepatocytes. (A) Schematic diagram of the transgene *TRE-VEGFR2T*. (B) Western blotting of newborn liver using an anti-HA antibody. Lanes 1-3, *TRE-VEGFR2T* sub-line 4377.5; lanes 4-6, sub-line 3647.5. (C,D) Expression of tTA by *lacZ* reporter assay in (C) E10.5 and (D) E11.5 embryos. (E,F) Immunostaining for the extracellular domain of VEGFR2 on *LAP-tTA* control liver (E) and *LAP-tTA/TRE-VEGFR2T* mutant liver (F). Nuclei are stained blue with DAPI. (G) VEGF levels in liver by ELISA. Error bars represent s.d. *P<0.01.

E11.5, a liver-specific, speckled *lacZ* expression pattern was observed (Fig. 1D). These results indicate that tTA is expressed in a subset of hepatocytes early after the initial onset of liver organogenesis (E9.5) (Matsumoto et al., 2001).

To determine the membrane expression pattern of VEGFR2T, liver sections were examined by immunofluorescent staining with an antibody against the extracellular domain of VEGFR2. In *LAP-tTA* control livers, a normal sinusoidal pattern of VEGFR2 expression was observed, while expression of VEGFR2T in double-transgenic pups was primarily localized to hepatocyte plasma membranes (Fig. 1E,F). Importantly, the level of VEGFR2T expression in hepatocytes was far greater than VEGFR2 expression by ECs. The VEGF levels in the mutant newborn livers were two and a half times those in control livers (Fig. 1G). An increase in

Table 1. Observed phenotypes in newborn VEGFR2T transgenic pups

Genotype (subline)	Total newborns (12 litters)	% Born dead	% Liver dark red	% Jaundiced
LAP-tTA TRE-VEGFR2T (3579.5)	19	26.3	100	63.8
LAP-tTA TRE-VEGFR2T (3647.5)	13	38.5	100	30.7
LAP-tTA	39	0	0	0

hepatic VEGF levels would be expected to result in increased liver vascularization (Dor et al., 2002). However, morphological analyses of livers from *LAP-tTA/TRE-VEGFR2T* mice did not show an increase in blood vessels, but rather a decrease (Fig. 3E,F, Fig. 6), suggesting that VEGFR2T expression impaired vascular network formation. Similar elevations of VEGF levels accompanying decreased vasculature have been observed with other VEGF-sequestering molecules (Kim et al., 2002). Our VEGFR2T-expressing mice, however, represent a novel and effective model system for studying tissue-specific effects of VEGF knockdown.

VEGFR2T-expressing newborn mice exhibit a dark-red liver phenotype

We next focused on the phenotypic analysis of newborn mice that expressed VEGFR2T throughout embryonic development. Analysis of the progeny of two *TRE-VEGFR2T* transgenic lines revealed that 26-38% of double-transgenic newborns were stillborn, and 30-63% were jaundiced (Table 1). By comparison, control *LAP-tTA* littermates never exhibited signs of jaundice and all were born alive. While the body weight and liver:body weight ratios of both *LAP-tTA* and *LAP-tTA/TRE-VEGFR2T* newborns were comparable (Fig. 2B,C), 100% of double-transgenic livers were dark-red in appearance, in contrast to the pink color of normal control livers (Fig. 2A). No detectable abnormalities were observed in all other organs examined. Given the 100% penetrance of the dark-red liver phenotype, this was the most striking gross phenotype of the double-transgenic mice.

To confirm that hepatic VEGFR2T expression was responsible for the observed phenotypes, VEGFR2T expression was suppressed throughout embryogenesis by feeding pregnant mothers Dox. As expected, suppression of VEGFR2T expression resulted in no double-transgenic pups having a jaundice phenotype (data not shown) or a dark-red liver (Fig. 2A).

Because our transgene was expressed in hepatocytes, we investigated whether it had induced any direct effects in hepatocytes that may have been responsible for the observed phenotype. Both liver albumin (Fig. 2D) and glycogen levels (data not shown) were similar in double-transgenic and control livers. Serum bilirubin levels were comparable in mutants and controls (data not shown). Furthermore, no visual differences were observed between mutant and control hepatocytes that were isolated from adult livers and cultured in vitro (data not shown). Recent studies have shown that VEGF signaling through EC-specific receptors can regulate hepatocyte proliferation during adult liver regeneration (LeCouter et al., 2003). We therefore evaluated hepatocyte proliferation in VEGFR2T-expressing mice. Newborn liver sections stained with PCNA showed no significant change in hepatocyte proliferation in double-transgenic livers (Fig. 2E), demonstrating that hepatocyte growth is normal in these mice.

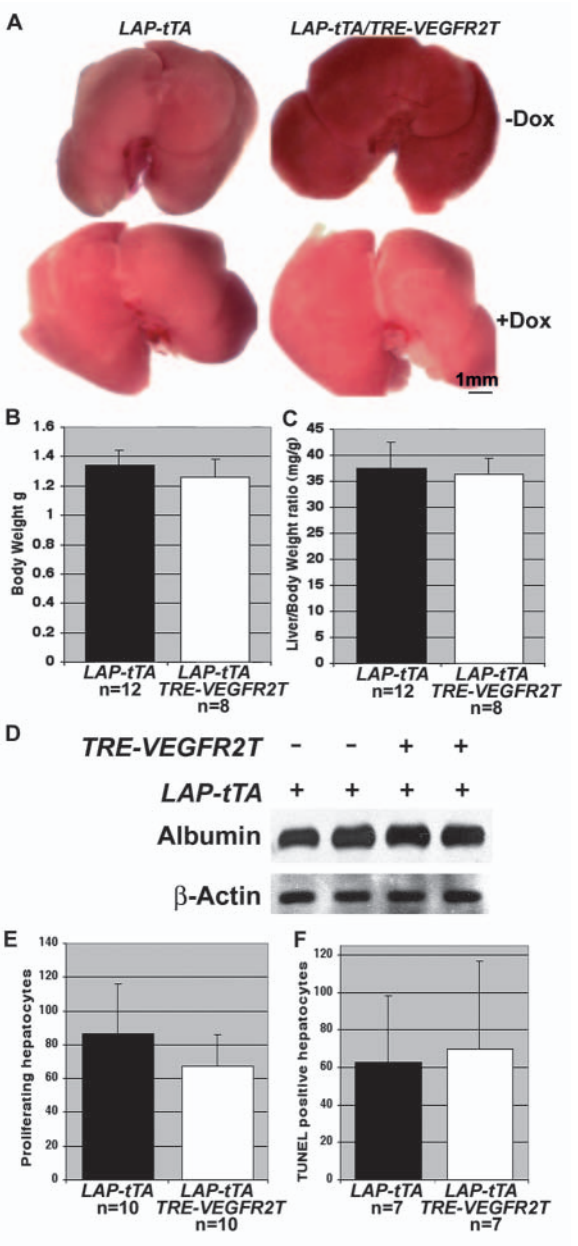

Fig. 2. Gross morphological and histological phenotype of *LAP-tTA/TRE-VEGFR2T* newborn mice. (A) A dark-red appearance distinguishes the *LAP-tTA/TRE-VEGFR2T* mutant livers from controls. Dox suppresses this phenotype. (B,C) Body weight and liver/body weight ratios of *LAP-tTA/TRE-VEGFR2T* newborns are comparable to *LAP-tTA* controls. (D) Western blotting with an anti-albumin antibody. (E) Insignificant reduction in hepatocyte proliferation in mutant livers by PCNA staining. (F) No significant difference in apoptosis. The number of proliferating cells (E) or apoptotic cells (F) is per 40× microscopic field. Error bars represent the s.d.

Hepatocyte cell death, as determined by TUNEL assay, was also not significantly different in livers of control and mutant mice (Fig. 2F). Thus, downregulation of VEGF activity did not result in any detectable alteration of the function of hepatocytes.

VEGF depletion in the embryonic liver results in vascular defects

By design, we expected that the endothelial component of the liver would be disrupted by VEGFR2T. Hematoxylin and Eosin (H&E) staining of liver sections revealed that there was a significant increase in red blood cells in the mutant livers (Fig. 3A,B), suggesting a defective circulation. The vasculature in the livers of newborn mice was further examined by immunohistochemistry using an EC-specific anti-CD31 antibody. In the mutant livers, ECs were present, but were less organized and more discontinuous than in control livers, resulting in fewer or collapsed-looking sinusoidal channels (Fig. 3E,F). It is likely that the red blood cells observed in H&E stains are trapped in these collapsed sinusoids. In fact, analysis of perfused newborn mutant livers showed that, although some of the red blood cells could be flushed out, others remained trapped (Fig. 3D). As expected, fewer red blood cells were observed in control livers (Fig. 3A,C). Given that VEGFC and VEGFD, critical for lymphatic ECs, can also bind to VEGFR2 (Tammela, 2005), we stained for a lymphatic-specific EC marker, LYVE1. Immunostaining using an anti-LYVE1 antibody did not reveal any apparent difference in the expression patterns or levels of LYVE1 between control and mutant newborn livers (Fig. 3G,H), suggesting that the lymphatic development in *LAP-tTA/TRE-VEGFR2T* was not affected.

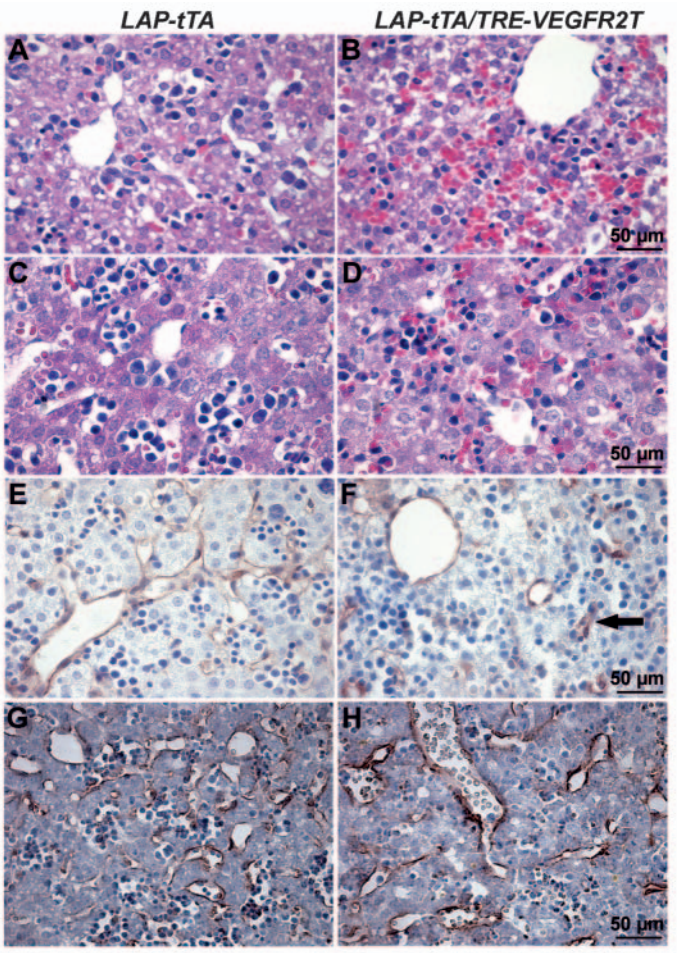

Fig. 3. The vascular sinusoidal network is disorganized in mutant livers. (A,C,E,G) Control livers; (B,D,F,H) mutant livers. (A-D) H&E staining of unperfused (A,B) and perfused (C,D) newborn livers shows that red blood cells accumulate in the livers of *TRE-VEGFR2T/LAP-tTA* transgenic newborns and cannot be flushed out (D). (E,F) Anti-CD31 staining (brown) shows that the microvascular networks in *TRE-VEGFR2T/LAP-tTA* livers fail to fully develop when compared with controls, with some sinusoids appearing to be collapsed (arrow in F). (G,H) Anti-LYVE1 staining (brown) shows a similar expression pattern and level.

VEGF depletion in the embryonic liver impairs hepatic lipoprotein uptake

Given the 100% penetrance of the dark-red liver appearance, we further investigated this most striking phenotype. Because some mammals accumulate hepatic lipids during the late stages of gestation (Bohmer and Havel, 1975), the dark-red liver is the first indication that compromised VEGF signaling may affect lipoprotein uptake. To test this hypothesis, we analyzed

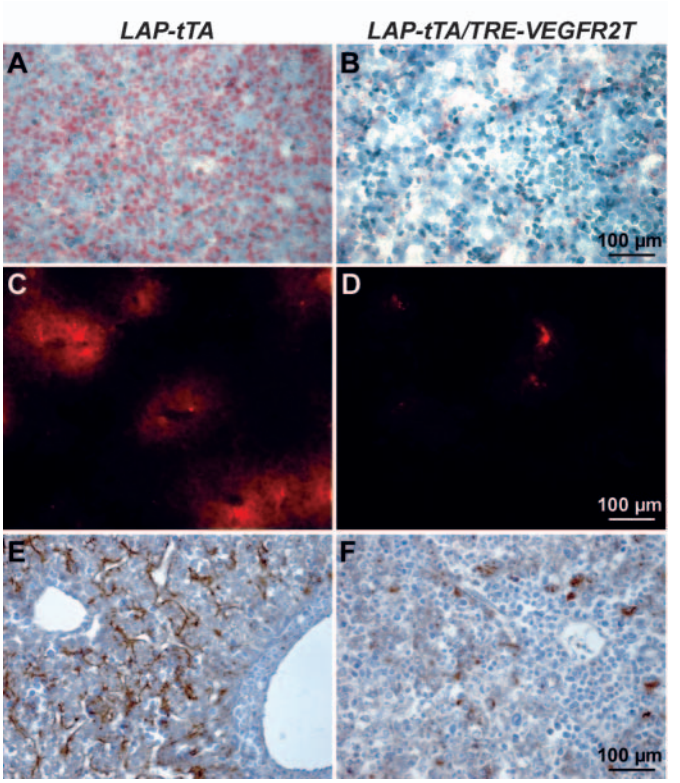

Fig. 4. Lipid accumulation in hepatocytes is impaired in *LAP-tTA/TRE-VEGFR2T* mice. (A,B) Oil Red O staining of liver sections reveals decreased hepatocellular lipids in mutant livers (B) when compared with littermate control livers (A). Samples are counterstained with Hematoxylin. (C,D) Infusion experiment with DiI-labeled remnant lipoproteins demonstrates the quick and efficient uptake and transportation of serum lipid by hepatocytes in control livers (C) but not in mutant livers (D). (E,F) Anti-APOE staining shows continuous APOE accumulation in the space of Disse of control livers (E), whereas, in the mutant livers, an area with little or disrupted APOE distribution is evident (F).

newborn liver sections with Oil Red O, which specifically stains neutral lipids. Mutant livers displayed a profound decrease in cytoplasmic lipid levels (Fig. 4B). However, both mutant and control littermates possessed healthy milk sacs, suggesting that the difference in hepatocellular lipid levels was not a consequence of malnourishment, but rather of lipid uptake from the blood. Some mutant livers exhibited patchy Oil Red O staining, reflecting the non-uniform expression pattern of tTA, and thus VEGFR2T, in the liver (Fig. 1D), further suggesting that VEGFR2T expression, not nursing, was the primary cause for the defect in lipid accumulation.

To confirm that the absence of lipid droplets in mutant livers was due to defective lipoprotein uptake, we infused fluorescently labeled, murine remnant lipoproteins into the bloodstream of control and mutant newborn pups. Ten minutes after the injection of DiI-labeled remnant lipoproteins into the heart, an intense and diffuse fluorescence pattern was observed in hepatocytes of control livers, indicating a robust uptake of labeled lipoproteins (Fig. 4C), as has been previously described in other mouse models of lipoprotein clearance (Ji et al., 1995). By contrast, the mutant livers showed a much weaker and more constricted pattern of fluorescence, indicating a significant reduction in the sequestration and uptake of fluorescently labeled remnant lipoproteins (Fig. 4D). Thus, VEGFR2T expression compromised hepatic lipid uptake.

A possible explanation for the deficient lipid uptake is that the interface between SECs and parenchymal cells is disrupted in the mutant livers, thereby reducing the ability of plasma lipoproteins to exit the sinusoidal blood vessels and be taken up by hepatocytes. To test this hypothesis, we employed electron microscopy to visualize the structure and interactions of SECs and hepatocytes, and to determine the integrity of the space of Disse. Multiple differences were immediately evident between littermate control livers and the VEGFR2T mutant livers. Mutant livers had a sparse and poorly developed sinusoidal network (arrows in Fig. 5A,B), with numerous blood cells present in the disrupted sinusoidal spaces (Fig. 5C,D). Additionally, hepatocytes in the mutant livers appeared to be more scattered than those in control livers, and were often oddly shaped (Fig. 5C,D) and exhibited fewer microvilli projections into the space of Disse (Fig. 5E,F). Similar to observations made by Oil Red O staining, electron microscopy analysis showed numerous lipid droplets in the parenchymal cells of control livers, whereas the parenchymal cells of the mutant livers contained few or no lipid droplets (Fig. 5A-D). The space of Disse had an abnormal morphology in mutant livers, often resulting in large gaps between SECs

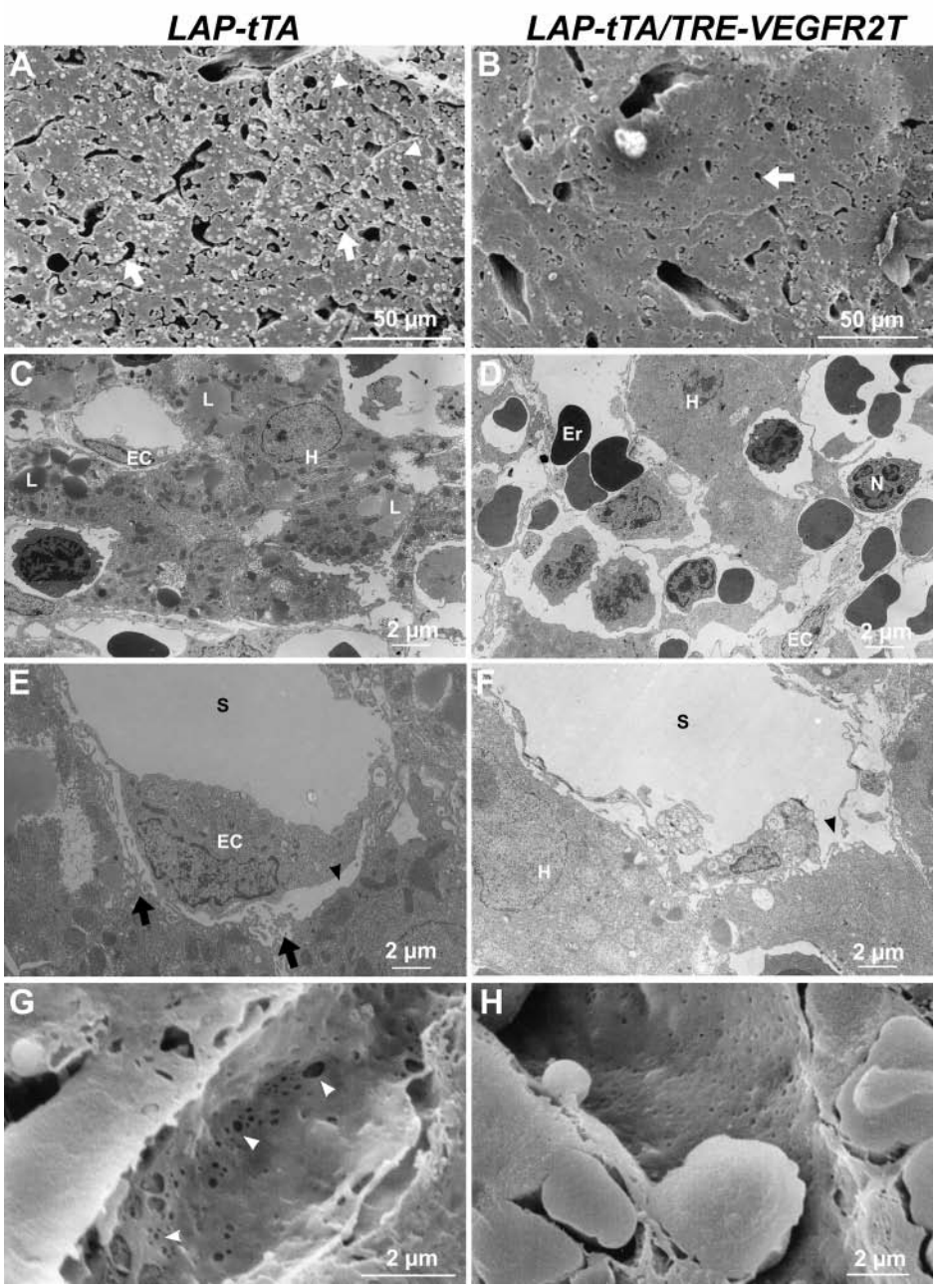

Fig. 5. Electron microscopy of liver sections shows defects in SEC and hepatocyte morphology. There is an abundance of lipid droplets (white arrowheads) in the control (A), but not in mutant (B), livers. SEM of livers shows fewer sinusoidal channels (white arrows) in the mutant (B). TEM of control (C,E) and mutant (D,F) livers reveals abnormal hepatocyte morphology, reduced hepatocyte microvilli projections (black arrows), and disrupted space of Disse (black arrowheads) in the mutant livers. SEM shows that mutant SECs lack fenestrae (H), whereas SECs in control livers are highly fenestrated (white arrowheads in G). EC, endothelial cell; L, lipid; H, hepatocyte; N, neutrophil; Er, erythrocyte; S, sinusoid.

and hepatocytes (Fig. 5E,F). Furthermore, SEM showed that the sinusoidal endothelium of the mutant livers (Fig. 5H) lacked the fenestrations that are characteristic of normal sinusoids (arrowheads in Fig. 5G).

The space of Disse is the major anatomical location for the clearance of intestinally derived, plasma remnant lipoproteins, which is mediated primarily by APOE and cell surface receptors (Mahley and Ji, 1999). Hepatocytes secrete APOE into the space of Disse, enriching sinusoidal surfaces and lipoproteins with APOE. This allows remnant lipoproteins to be rapidly sequestered and internalized into hepatocytes by receptor-mediated processes. The disruption of the space of Disse and the paucity of lipid accumulation in livers observed in our *LAP-tTA/TRE-VEGFR2T* model prompted us to examine whether the distribution pattern of APOE on sinusoidal surfaces had been altered in the mutant livers, thus impairing the capability of hepatocytes to clear plasma remnant lipoproteins derived from the intestine. Immunostaining liver sections with antiserum specific for murine APOE (Raffai and Weisgraber, 2002) revealed that, unlike control livers that displayed abundant amounts of APOE in the space of Disse continuously along with the sinusoidal surfaces, mutant livers displayed many areas with little or disrupted APOE distribution (Fig. 4E,F). A similar staining pattern was seen using antiserum specific for APOB100 and APOB48, the major protein components of remnant lipoproteins (data not shown). In summary, our findings provide the first genetic evidence that VEGF can modulate the integrity of the fenestrated endothelium and the space of Disse that is essential for lipid uptake by the liver.

VEGF is required for vascular development throughout liver organogenesis

Because a disrupted vasculature and impaired lipid uptake are both evident in newborn transgenic mice, we wanted to identify the developmental stage at which these abnormalities manifested themselves. To visualize the liver vasculature throughout embryonic development, we used a *VEGFR2^{lacZ}* reporter allele (Shalaby et al., 1995) to observe blood vessel ECs. At E11.5, the vasculature in the mutant and control livers was comparable, having formed the primary plexus from which the mature vasculature would arise (Fig. 6A,B). In control livers at E12.5, a blood vessel network was apparent (Fig. 6C). However, in the E12.5 mutant livers, the vasculature appeared to be less organized and the vessels were dilated (Fig. 6D). At E13.5, major blood vessels had formed in both the control and mutant livers, but the microvascular network in the mutant livers appeared to be more disorganized, with fewer branches than the control livers (Fig. 6E,F). At the newborn stage, very little microvascular network was evident in the mutant livers (Fig. 6H), supporting the immunohistochemical observations (Fig. 3F).

To further assess the temporal requirement for VEGF in this process, we used the Tet-regulatable feature of our transgenic system. Pregnant mothers were fed with Tet-supplemented drinking water during the initial stages of pregnancy, and Tet was removed at specific time points during gestation (Table 2). Owing to the time required for the complete metabolism of Tet, there is a 3-4 day lag between the time when it is removed from the diet and the time when the transgene (VEGFR2T) is expressed (data not shown). When VEGFR2T expression was

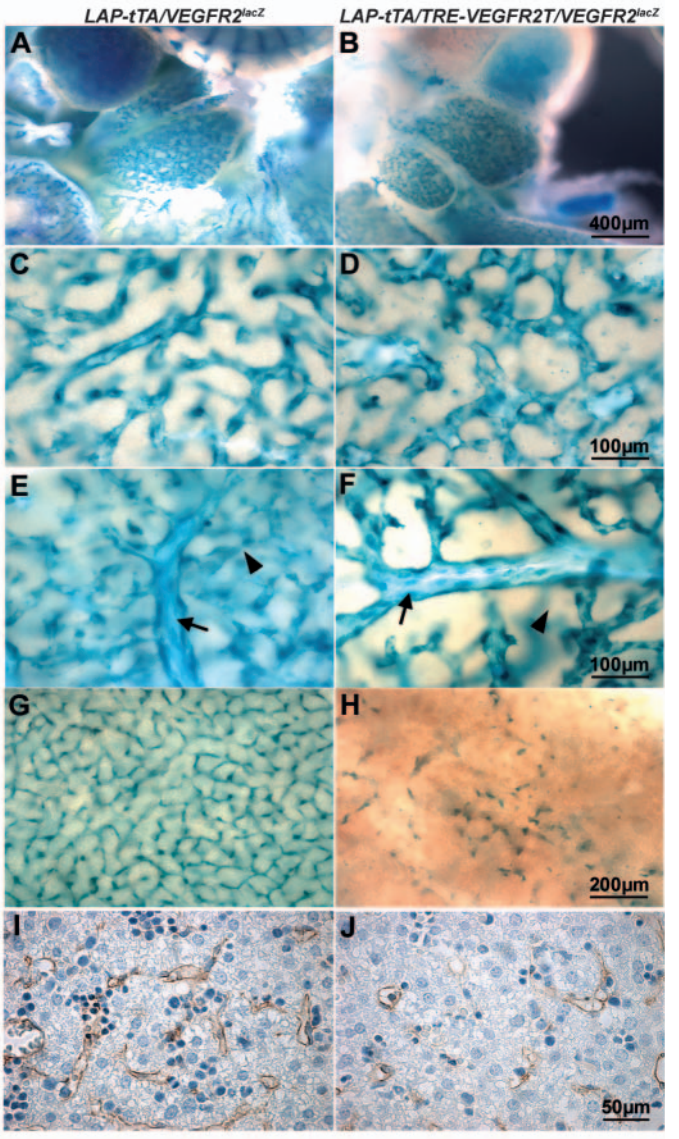

Fig. 6. Vascular development is defective in livers that express VEGFR2T during organogenesis. The developing liver vasculature was observed by β-galactosidase staining of ECs with the *VEGFR2^{lacZ}* allele (blue). The vasculature of control (A,C,E,G) and mutant (B,D,F,H) livers was visualized at E11.5 (A,B), E12.5 (C,D) and E13.5 (E,F), and in newborn pups (G,H). Arrows denote major blood vessels; arrowheads highlight the microvasculature (E,F). (I,J) The sinusoidal network is equally disrupted in newborn mutant livers (J) when Tet was removed at E12.5.

turned on as late as E15.5-E16.5, 100% of newborn mutant mice exhibited a dark-red liver color, suggesting that hepatic lipid uptake was impaired in these mice. Anti-CD31 immunohistochemical staining showed a disrupted sinusoidal network in these mutant mice (Fig. 6J; Table 2), when compared with control mice (Fig. 6I), similar to the vascular defects observed in mice that expressed VEGFR2T throughout development (Fig. 3F). This result suggests that as little as 3-4 days of expression of VEGFR2T can sufficiently deplete VEGF in the liver, and the phenotypic observations are consistent with a role for VEGF in modulating the sinusoidal

Table 2. VEGF signaling is required during all stages of embryonic organogenesis for normal liver vascular development

Off Tet* (dpc)	VEGFR2T on† (dpc)	Mutant liver phenotype	Vasculature‡ phenotype
–	10.5	Dark red	Disrupted
12.5	15.5-16.5	Dark red	Disrupted
13.5	16.5-17.5	Light red/normal	Disrupted
14.5	17.5-18.5	Light red/normal	ND
15.5	18.5-19.5	Normal	Normal

*Pregnant mothers were fed tetracycline (Tet) in their diet until this timepoint.
†When Tet is removed from the diet, there is a 3-4 day delay in transgene expression, due to the time required for Tet in the system to be metabolized.
‡See Fig. 6 and the Discussion in the text.
ND, not determined; dpc, days post-coitum.

network development and SEC fenestrations necessary for proper lipid homeostasis. When VEGFR2T expression is turned on at E16.5-E17.5, newborn mutant mice exhibited a light-red colored liver and a disrupted vasculature. This intermediary phenotype suggests that only 2-3 days may not be sufficient to turn on VEGFR2T expression and to cause full-capacity depletion of VEGF, suggesting either a dose-dependent role of VEGF in SEC development or that the vasculature is fully developed by this stage and is no longer responsive to VEGF signaling.

Collectively, our results demonstrate an important role for VEGF signaling in the development of the liver vasculature during organogenesis. Furthermore, this defective vasculature causes an abnormal space of Disse that does not allow efficient hepatic remnant lipoprotein uptake, which is probably the primary reason for the lack of lipid accumulation in the livers of these mice.

Discussion

Although much is known about the molecular mechanisms of hepatocyte-mediated lipoprotein uptake and catabolism (Havel and Hamilton, 2004; Mahley and Ji, 1999), the regulation of vascular structure in these processes is not understood. We report that VEGF signaling is required for proper sinusoidal development in the liver, and that defects in this signaling pathway result in defective SEC structure and a non-functional space of Disse, causing impaired plasma remnant-lipoprotein uptake in the liver.

Liver-specific VEGF depletion results in defective lipid uptake in hepatocytes

A defect in lipid homeostasis in VEGFR2T-expressing mice was first proposed based on the gross phenotypical observations that their livers displayed a dark-red color (Fig. 2A). In the normal fetal liver, one function of the hepatocyte is to store and process lipids. These vast fat stores give the liver a lighter-colored appearance; reduced lipid stores may, therefore, result in a darker-colored liver phenotype. Several lines of evidence supported this hypothesis. First, histological analysis of the liver with Oil Red O staining revealed markedly decreased intracellular lipid droplets in mutant livers (Fig. 4). Second, TEM confirmed a decrease in hepatocellular lipid droplets in mutant livers (Fig. 5B,D). Third, the absence of robust APOE localization in the sinusoids in mutant livers (Fig. 4E,F) demonstrated an aberrant space of Disse, which is normally enriched with APOE (Mahley and Ji, 1999) and functions to allow efficient remnant-lipoprotein clearance (Raffai and Weisgraber, 2002).

The most direct line of evidence demonstrating that aberrant hepatic-lipid homeostasis in mutant mice was due to defective hepatic-lipoprotein uptake came from a remnant lipoprotein-clearance study using fluorescently labeled, murine remnant lipoproteins. In this experiment, control livers rapidly (in 10 minutes) took up the injected lipoproteins, which diffused broadly into the hepatic layers; whereas, in the mutant livers, the labeled lipoproteins remained mostly restricted to the sinusoids (Fig. 4C,D). This pattern of hepatic lipoprotein uptake is strikingly similar to what Ji et al. reported in their classic experiments using intravenous heparinase to inhibit remnant lipoprotein uptake in liver (Ji et al., 1995).

Defects in hepatocyte morphology, consisting of abnormal cell shape and reduced numbers of microvilli (Fig. 5C-F) also contributed to reduced lipid homeostasis. Hepatocyte microvilli normally extend into the space of Disse, where they are bathed in extra-sinusoidal serum. The microvilli act to increase the surface area of the hepatocyte, allowing a more efficient passage of lipoproteins from the space of Disse. The observed reduction in hepatocyte microvilli in the livers of VEGFR2T-expressing mice may also explain the reduced uptake of lipoprotein remnants observed in mutant mice. It is unclear, however, why the number of microvilli is reduced. It could simply be a consequence of having a disrupted space of Disse in the mutant livers, or it might suggest that further communication between SECs and hepatocytes is necessary to stimulate microvilli projections. A caveat to our experimental design is that the overexpression of VEGFR2T on hepatocyte surface membranes may have a direct effect on hepatocytes, either on their ability to form microvilli or on the assembly of lipoprotein receptors within these villi. However, we have not detected any other abnormalities in the hepatocytes by other methods. Furthermore, in a similar transgenic system, in which the Met receptor was overexpressed in mouse hepatocytes to induce tumor formation, *LAP-tTA/TRE-hMET* mice did not exhibit the same defect (R.W., unpublished). This suggests that the observed defects in lipid homeostasis in our mice are specific to the VEGFR2T transgene and its depletion of VEGF signaling, and are not a consequence of non-specific receptor overexpression on hepatocyte membranes.

VEGF is required for the fenestrations in SECs that are required for lipoprotein passage

SEM revealed a lack of fenestrations in the SECs of the mutant livers, suggesting that VEGF signaling is crucial in the morphogenesis of SECs. SEC fenestrations function as a sieve to allow small lipoprotein remnants to pass out of the sinusoids and into the space of Disse, where they can be endocytosed by receptor-mediated mechanisms on the surface of hepatocytes. A lack of fenestrae would inhibit lipoprotein remnant exit from the sinusoids, thus reducing uptake by the liver and resulting in reduced lipid content in hepatocytes.

VEGF has previously been suggested to play a role in maintaining the integrity of endothelial fenestrations, on the basis of observations that VEGF is continuously expressed by epithelial cells associated with fenestrated endothelium, in

Development

organs such as the kidney and choroid plexus (Breier et al., 1992; Esser et al., 1998). Additionally, ectopic expression of VEGF has been shown to induce fenestrations in normally unfenestrated ECs (Roberts and Palade, 1995). Gerber and colleagues observed a loss of endothelial fenestrations in the glomeruli of the kidney in mice treated with a dominant-negative soluble VEGF-sequestering receptor, further supporting a role for VEGF in endothelial fenestrae development (Gerber et al., 1999). It has also recently been shown that VEGF signaling during development is necessary for establishing endothelial fenestrae in pancreatic islets (Lammert et al., 2003). Our results represent the first demonstration of an essential role for VEGF signaling in establishing and maintaining the fenestrations in the SECs of the developing liver necessary for a functional space of Disse.

VEGF depletion impairs microvascular network formation throughout embryogenesis

In addition to structural defects in SEC, the vascular network is also disrupted, even collapsed, in VEGFR2T-expressing liver, as shown by both immunostaining and electron miscroscopy analyses. A disrupted vasculature would also reduce access of hepatocytes to the sinusoids, which would impair the process of lipoprotein passage from the blood, as we observed. A defective vasculature resulting in impaired blood flow through the liver was also evident by the accumulation of red blood cells. It is possible that these accumulated red blood cells contribute to the color phenotype of the mutant liver, in addition to the decreased hepatic lipid stores. In contrast to the vascular system, the lymphatic system appears to be unaffected. Although VEGFR2 (and VEGFR2T as well) can bind to the lymphatic growth factors VEGFC and VEGFD, lymphatic endothelium-specific anti-LYVE1 staining did not reveal any apparent differences between controls and mutants, suggesting that the impairment of microvascular networks may not be secondary to the depletion, if any, of VEGFC and VEGFD. Thus, VEGFR2T could specifically target VEGF signaling in the liver.

Further analysis of the vasculature during embryonic development, using a *VEGFR2*^{lacZ} reporter allele, showed normal capillary plexus formation at E11.5 (Fig. 6A,B). Expression of the tTA was observed as early as E10.5 (Fig. 1C). It is possible that VEGFR2T expression may not have been induced sufficiently early to affect initial capillary plexus formation. Alternatively, but less likely, VEGF signaling in the liver may not be required during this initial phase of vascular development. However, remodeling of the vasculature after this stage was significantly impaired in VEGFR2T-expressing mice. Although major blood vessels formed in the mutant livers at E13.5 (Fig. 6F), the microvascular network was generally characterized by fewer branches and thicker and more disorganized vessels at E12.5 and E13.5 (Fig. 6D,F) than in control livers. Very little sinusoidal vasculature remained at the newborn stage (Fig. 6J). A similarly compromised sinusoidal vasculature was observed when VEGFR2T-expression was suppressed during development until E15.5-E16.5 (Fig. 6H, Table 2), supporting a role for VEGF signaling in the developing sinusoidal vasculature, and that this signaling is required throughout organogenesis, beginning as early as E12.5 or as late as E16.5. The partial phenotypes we observed in mice that expressed VEGFR2T at E16.5-E17.5 indicate that sinusoidal vascular development is regulated by VEGF in a dose- and/or time-dependent manner. While the embryonic requirement for VEGF in the context of the whole organism has long been known (Carmeliet et al., 1996; Ferrara et al., 1996), our findings represent the first demonstration of a requirement for VEGF signaling for the vascular development in a single organ, the liver.

VEGFR2T represents the most potent system for functionally depleting VEGF in a tissue-specific manner

In this study, we chose to use a membrane-bound VEGF-sequestering model system to functionally deplete VEGF in the developing liver. VEGFR2T is membrane bound, and therefore does not circulate to other organs systems, making it specific to the liver when expressed under the control of the LAP promoter. Overexpression of this allele allows for the sufficient sequestration of secreted VEGF, even if VEGFR2T is not expressed by all hepatocytes within the liver. The Tet-regulation feature of our VEGFR2T system is essential, not only because it allows for temporal control of transgene expression, but also because it allows for transgene suppression in breeding stocks and control animals, which may be necessary when studying otherwise lethal phenotypes. Combination of this regulation system with a liver-specific promoter further strengthens the effectiveness of this model system in dissecting the role of VEGF signaling and angiogenesis in liver development, hepatic regeneration, and liver tumor progression. Additionally, this unique and powerful system can be adapted for studying the effects of numerous other transgenes in many other organ systems.

It should be noted, however, that other systems exist which may prove useful for such analysis. One possible approach to deplete VEGF signaling would be to use a soluble VEGF receptor system, such as VEGF-Trap (Holash et al., 2002), which would effectively sequester secreted VEGF and could eliminate any potential non-specific defects caused by overexpressing a membrane-bound receptor. However, such a soluble receptor system cannot be restricted specifically to the liver, and therefore would be likely to cause significant defects in other organ systems, as has been observed with other soluble VEGF receptors in newborn mice (Gerber et al., 1999).

Cre/*lox*-mediated VEGF knockout is advantageous in that it can also eliminate potential artifacts due to overexpression of membrane-bound receptors, and it can be tissue specific, thereby reducing the occurrence of potentially confounding whole-embryo defects that a soluble receptor may cause. We performed such an experiment in our *LAP-tTA/TRE-Cre/VEGF*$^{floxed/floxed}$ mice, in which Cre expression (and thus VEGF deletion) was hepatocyte specific. However, we observed only a 40% reduction in liver VEGF levels (data not shown). Although these mice did have a slightly underdeveloped sinusoidal vasculature, this defect was not as severe as that observed in VEGFR2T-expressing mice, and there was no observable reduction in hepatocellular lipid stores, as determined by Oil Red O analysis and liver color observations (data not shown). The incomplete knockout of VEGF in this system is likely to be a result of two phenomena. First, *LAP-tTA* (and hence Cre) expression is limited to only a subset of hepatocytes (Fig. 1D and data not shown). Therefore, only a fraction of VEGF alleles are deleted. Second, although

Development

hepatocytes are the primary VEGF-expressing cells in the liver, VEGF is also expressed by other cell types in the liver and can arrive via the circulatory system from other organs. Thus, even VEGF gene deletion in all hepatocytes will not eliminate all sources of circulating VEGF in the liver and would not achieve the sequestration effects of VEGFR2T.

Significance

Morphological evidence has long suggested a strong link between ECs and hepatocytes in the process of lipid uptake by the liver. Our results present the first genetic evidence for the existence of a signaling mechanism derived from ECs responsible for lipid uptake by the liver. We have shown that VEGF signaling in the liver is essential for the development of the functional sinusoidal vasculature required for efficient plasma lipoprotein uptake. Depletion of VEGF in the liver results in a disrupted vascular network, a lack of SEC fenestrations and a non-functional space of Disse.

The role of VEGF in microvascular network and SEC structural development has important pathological implications. Impaired remnant lipoprotein uptake and clearance from plasma by the liver results in premature atherosclerosis and cardiovascular disease (Fraser et al., 1995; Mahley and Ji, 1999). In addition to the defects associated with lipoprotein uptake, defenestration itself is linked to other pathologies, including cirrhosis and cancer (Mori, 1993). Although not necessarily implicated as a cause of these diseases, defenestration of the endothelium occurs in both of these situations. If decreased SEC porosity impairs the transport and/or metabolism of metabolites or other nutrients necessary for normal liver function and development, defenestration could be a factor in the development of these, and other, pathologies.

The demonstration of a crucial role for VEGF signaling in the regulation of SEC fenestration and lipid uptake by the liver is of particular significance given the tremendous progress in the development of anti-VEGF therapies, including the recently approved cancer therapeutic drug Avastin (Genentech) (Muhsin et al., 2004). It is conceivable that continued research and drug development based on VEGF function may clarify the cellular mechanisms of plasma lipid metabolism, and provide the treatment of cardiovascular disease caused by abnormal lipoprotein metabolism and lipid uptake in the liver.

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