# Notch1 Signaling in the Hepatic Microcirculation and Chronic Liver Disease

**Inauguraldissertation**

zur
Erlangung der Würde eines Doktors der Philosophie
vorgelegt der
Philosophisch-Naturwissenschaftlichen Fakultät
der Universität Basel
von

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Basel, 2013

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät
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Basel, den 13.11.2012

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# Table of contents

Page

# 1. Summary

The Notch signaling pathway is an evolutionary conserved pathway that plays essential roles during vascular development and in the regulation of normal and pathological angiogenesis in the adulthood. These roles include angioblast specification, arteriovenous differentiation, regulation of blood vessel sprouting and branching, as well as control of vessel maintenance. In mammals there are four Notch receptors (Notch1 – 4), but only Notch1 and Notch4 are expressed in endothelial cells. Endothelial-specific loss of Notch1 is embryonic lethal, demonstrating its pivotal function in the vascular system. Nevertheless, the role of Notch1 signaling in postnatal vascular physiology is not fully understood. Inducible deletion of Notch1 in mice has been shown to cause nodular regenerative hyperplasia (NRH), which is a histopathological entity of the liver also seen in humans. NRH is thought to appear secondary to microcirculatory disturbances, however the exact pathogenesis is not known. NRH is an important cause of non-cirrhotic portal hypertension. The increased portal pressure can lead to complications including splenomegaly, ascites, or variceal bleeding; the latter is associated with high mortality.
The aim of this thesis is to elucidate the role of Notch1 signaling in the hepatic microcirculation in normal and pathologic conditions. Further, we want to shed some light on the molecular mechanisms implicated in the development of NRH.

In a conditional Notch1 knockout (KO) mouse model we investigated the impact of Notch1 signaling deficiency on the blood vessel homeostasis in the liver. LSEC are normally quiescent. After observing LSEC activation in livers of Notch1 KO mice, resulting in increased cell proliferation, we wanted to explore the ultrastructure of sinusoids in more detail. Scanning electron microscopy analysis of the liver microvasculature revealed phenotypic changes of LSEC, identified by loss of fenestrae. By performing vascular casts, we could discover three-dimensional changes of the hepatic microvasculature. While livers of control mice showed a highly differentiated vascular network, the liver vasculature of Notch1 KO mice was remodeled showing increased branching with larger vessel diameters. In addition, we identified features of intussusceptive angiogenesis in Notch1 KO casts. Time course experiments revealed that vascular changes occur first and that development of portal hypertension and NRH is a secondary phenomenon. To exclude that the observed phenotypic changes are due to loss of Notch1 in cells other than LSEC, a hepatocyte-specific Notch1 KO mouse was generated. This mouse had a completely normal phenotype. Furthermore, different cell populations were isolated from global Notch1 KO mice. Gene expression analysis of the different cell types confirmed

that loss of Notch1 mainly affects the endothelium. Vascular dedifferentiation was found to be mediated by Notch, ephrin, and TEK signaling, all of which are known to regulate LSEC differentiation and quiescence. A very crucial finding, supporting our hypothesis that the NRH phenotype is driven by vascular changes, is the spontaneous development of liver angiosarcoma in Notch1 KO mice. Disruption of Notch1 signaling is sufficient enough to induce malignant transformation of endothelial cells in the liver, reflecting the pivotal role of Notch1 in the hepatic microcirculation.

In a translational study using liver biopsies from NRH patients we assessed the vascular contribution to the development of NRH. Using morphological and molecular approaches, this part of the thesis addressed two main questions: one from a clinical point of view, the other from a basic science perspective.

First, we assessed whether there is an association between the presence of portal hypertension and NRH severity. So far, no one has investigated the relationship between pathologic features and the clinical condition in NRH. Notably, histological assessed nodular transformation correlates well with the presence of portal hypertension. Since most complications occurring in NRH patients are due to increased portal pressure, patients presenting with advanced nodular transformation should be advised to undergo endoscopic screening for varices, since they can cause life-threatening complications.

Based on our findings from the animal study we have hypothesized that NRH is caused by a vascular injury of the sinusoids. In our NRH mouse model we identified dysregulation of a number of genes upon Notch1 deletion, which are involved in endothelial differentiation. Therefore, we wanted to explore if the same set of genes is also regulated in human NRH. To our surprise, the same genes were also found to be dysregulated in the liver of NRH patients, irrespective of the underlying cause of disease. Thus we conclude: Despite different etiologic factors associated with NRH, there is evidence that in all cases the hepatic condition can be traced back to an endotheliopathy mediated by the final common pathway of Notch1/Dll4 and EphrinB2/EphB4 signaling.

Taken together, our study identifies Notch1 as an important player in LSEC differentiation and quiescence. We provide first insights into the molecular mechanism of human NRH, which are in line with our findings from the NRH mouse model. In addition, we showed an oncosuppressive role of Notch1 in the murine liver endothelium, resulting in vascular neoplasms after loss of Notch1.

# 2. Abbreviations

| | |
|---|---|
| Ac-LDL | acetylated low–density lipoprotein |
| ADAM | a distintegrin and metalloprotease |
| AGS | Alagille syndrom |
| AlbCre | albumin Cre |
| alpha-SMA | alpha smooth muscle actin |
| ALT | alanine aminotransferase |
| Ang | angiopoietin |
| AST | aspartate aminotransferase |
| BRDU | 5-bromo-2'-deoxyuridine |
| CADASIL | cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy |
| CSL | CBF-1/Suppressor of Hairless/Lag-1 |
| DAPT | N-[(3,5-Difluorophenyl)acetyl]-L-alanyl-2-phenyl]glycin e-1,1-dimethylethyl ester |
| Dll | delta like |
| DNA | deoxyribonucleic acid |
| EC | endothelial cell |
| ECM | endothelial cell medium |
| Efnb2 | EphrinB2 |
| EGFR | epidermal growth factor receptor |
| eNOS | endothelial nitric oxide synthase |
| EPC | endothelial precursor cell (angiogblast) |
| Ephb4 | Ephrin receptor B4 |
| FGF | fibroblast growth factor |
| Flk | fetal liver kinase |
| Flt | fms related tyrosin kinase |
| FNH | focal nodular hyperplasia |
| GGT | gamma glutamyl transferase |
| GOM | granular osmophilic material |
| GSI | gamma-secretase inhibitor |
| HCC | hepatocellular cancer |
| HCV | hepatitis C virus |
| Hes | hairy-and-enhancer-of-split |
| Hey | hairy-and-enhancer-of-split related |
| HGF | hepatocyte growth factor |
| HIF | Hypoxia inducible factor |
| HPF | high power field |
| HSC | hepatic stellate cells |
| HVPG | hepatic venous pressure gradient |
| INR | international normalized ratio |
| KO | knockout |

| | |
|---|---|
| LDL | low density lipoprotein |
| LSEC | liver sinusoidal endothelial cells |
| MAML | mammalian Mastermind-like |
| MELD | model for end stage liver disease |
| MHC | major histocompatibility complex |
| MMP | matrix metalloproteinase |
| MMTV | mouse mammary tumor virus |
| mRNA | messenger RNA |
| NCPH | non-cirrhotic portal hypertension |
| NICD | notch intracellular domain |
| NO | nitric oxide |
| NRH | nodular regenerative hyperplasia |
| PBS | phosphate-buffered saline |
| PCR | polymerase chain reaction |
| PDGF | platelet-derived growth factor |
| PDGFR | platelet-derived growth factor receptor |
| PHx | partial hepatectomy |
| PlGF | placenta growth factor |
| qPCR | quantitative PCR |
| RBPJ | recombination signal-binding protein-Jκ |
| RT-PCR | reverse-transcription PCR |
| S | site |
| SEM | scanning electron microscopy |
| Shh | sonic hedgehog |
| SMC | smooth muscle cells |
| SOS | sinusoidal obstruction syndrome |
| T-ALL | T-cell acute lymphoblastic leukemia |
| TEK/Tie | tyrosine kinase with immunoglobulin-like and EGF-like domains |
| TGF | transforming growth factor |
| VE-cadherin | vascular endothelial cadherin |
| VEGF | vascular endothelial growth factor |
| VEGFR | vascular endothelial growth factor receptor |

# 3. Introduction

## 3.1 Liver vasculature

### 3.1.1 The hepatic circulation

The liver is the largest internal organ and its complex microcirculation is required for the important liver functions in biosynthesis, metabolism, clearance, and immune response [1].

The liver receives a dual blood supply. Approximately 80% of the blood is received from the portal vein, the remaining 20% are delivered by the hepatic artery. While the portal vein conducts poorly oxygenated, nutrient-rich blood from digestive organs to the liver, the hepatic artery delivers well-oxygenated blood [2]. The portal vein and hepatic artery are further subdivided into the terminal branches, portal venules and hepatic arterioles, which eventually drain into the sinusoids where portal and arterial blood gets mixed. The sinusoids represent the capillary network of the liver and transport the blood from periportal to centrilobular regions in the liver (Figure 1). After perfusion of the liver parenchyma, the blood is collected in terminal hepatic venules, the hepatic vein and finally the inferior vena cava and right atrium [2,3].

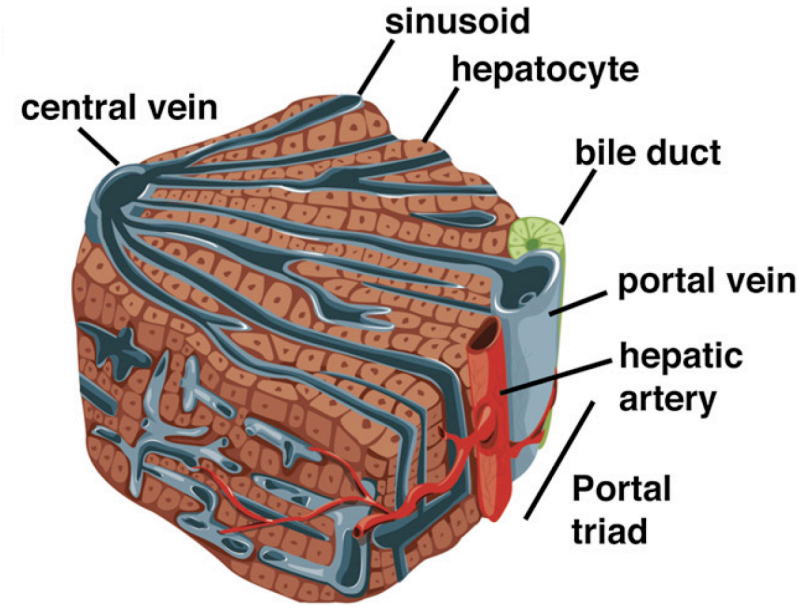


**Figure1. Microanatomy of the hepatic lobule.** The liver receives blood from the portal vein and the hepatic artery, both draining into sinusoids, which represent the capillary network in the liver (from [4]).

### 3.1.2 The hepatic sinusoids and sinusoidal endothelial cells

Hepatocytes (parenchymal cells) comprise 80% of the liver mass, whereas the other 20% are represented by non-parenchymal cells mainly including sinusoidal endothelial cells, Kupffer cells (the resident macrophages of the liver), hepatic stellate cells (the hepatic equivalent to pericytes), and liver-associated lymphocytes [3,5]. The hepatic circulation is predominantly lined by liver sinusoidal endothelial cells (LSEC), which account for 50% of the non-parenchymal cells [3]. To stabilize the vascular structures, extraluminal hepatic stellate cells (HSC) enwrap the sinusoids. They are located within the perisinusoidal space of Disse, which is the separating space between endothelial cells and hepatocytes. LSEC possess specific morphologic features to exert the repertoire of their liver specific functions. Presence of fenestrae, which are pores in the cell supported by cytoskeleton and the lack of a basal lamina characterize this unique LSEC phenotype, distinguishing them from other endothelial cells [3]. Thus, LSEC are a prime example of phenotypic heterogeneity of the endothelium. Non-diaphragmed fenestrae with diameters around 100 – 200 nm are grouped together in so-called “sieve plates” [6,7]. Sieve plates encompass 20 - 50 pores and have a diameter of around 0.1 µm [3]. Distribution and size of fenestrae in the sinusoidal endothelium is not uniform. Depending on the intralobular localization they occupy 6% to 8% of the endothelial surface [2,8]. An actin containing cytoskeleton forms and supports fenestrae and sieve-plates [9,10]. Fenestrae are dynamic structures, which are actively regulated by the fenestrae-associated cytoskeleton. As a result, regulation of endothelial permeability controls the important hepatic function of endothelial filtration. Because of the absence of a basement membrane the endothelium acts as a selective barrier. Thus, the diameter and frequency of sinusoidal fenestrations directly determines the exchange between blood and hepatic parenchyma [8,9]. Chylomicrons, which have a size between 100 - 1000 nm, are not able to leave the sinusoids, while the smaller chylomicron-remnants, 30 - 80 nm in size, are filtered through the sinusoidal fenestrae to enter the space of Disse. It is now well established that the “liver sieve” influences the lipoprotein metabolism and consistent with the fact that chylomicron-remnants are the most artherogenic circulating lipoproteins, fenestrae have an important role in the pathogenesis of artherosclerosis [8,9,11]. Two mechanisms have been described to increase the diameter of fenestrae: disruption of the actin-containing components of the cytoskeleton or application of vascular endothelial growth factor (VEGF) [9,12–14]. Active investigations within the last years brought up an increasing list of agents, which alter the number and diameter of fenestrae. Hormones

like adrenalin or serotonin, drugs such as acetylcholine, toxins like ethanol, but also diseases and aging manipulate the fenestration pattern of LSEC [6].

Another physiological role attributed to LSEC is their scavenger function. The presence of numerous endocytic vesicles in LSEC evinces their high endocytic activity [15]. To clear a multitude of physiological and foreign macromolecules and colloids from the blood, LSEC are specialized in receptor-mediated endocytosis. LSEC display an array of endocytosis receptors at their cell membrane by which they take up a wide range of substances. Among these receptors, the mannose receptor and scavenger receptor are found in LSEC but also in Kupffer cells, demonstrating the complementary function of these two cell types in blood clearance [16,17]. Acetylated low–density lipoprotein (Ac-LDL), whose uptake is mediated through the scavenger receptor, is mainly sequestered by LSEC when compared to Kupffer cells. However, endocytosis of Ac-LDL is not a suitable functional marker for LSEC since extrahepatic endothelial cells also have been reported to endocytose Ac-LDL [16,18,19]. Recently, new members of the scavenger receptor family were found to be expressed exclusively on LSEC. These are stabilin-2 and lymphatic vessel endothelial hyaluronan receptor [19].

The participation of the liver in immunity is well accepted. One aspect that favors a contribution to the immune system is the excellent strategic position in the portal circulation as well as the large surface area of LSEC. The organ is supplied with blood from the gastrointestinal tract via the portal vein and with systemic blood delivered by the hepatic artery. Thus, the liver is constantly exposed to antigens and microbial products and unnecessary activation of the immune system needs to be prevented. LSEC, as the sinusoidal lining cells, are therefore in direct contact with antigens but also with lymphocytes. In the liver, antigen clearance from the blood is mainly performed by LSEC via scavenger-receptor mediated uptake [17]. LSEC interact directly with circulating lymphocytes, such as CD4 T and CD8 T cells. Moreover, LSEC constitutively express molecules for antigen presentation, major histocompatibility complex (MHC) class I and II, and co-stimulatory molecules (CD40, CD80, CD86), which allow LESC to function as antigen-presenting cells [20]. Antigen presentation via MHC class II to naïve CD4 T cells induces a regulatory phenotype ($T_{reg}$) [21]. In addition, LSEC induce CD8 T cell tolerance by cross-presentation of exogenous antigens presented on MHC class I molecules. [22] Usually cross-presentation is restricted to myeloid cells [20]. In contrast to CD8 T cells primed by matured dendritic cells, which acquire a cytotoxic activity, LSEC primed CD8 T cells lack effector functions and lead to tolerance induction rather than enhanced immunity [23]. Interestingly, LSEC induced T cell tolerance is shifted to T cell differentiation into effector cells after viral infection of LSEC [23]. The tolerogenic effect of

LESC is consistent with the phenomenon of liver allograft-induced T cell tolerance, which reduces the event of organ rejection [24]. Another example highlighting the immune function of the liver is the loss of tolerance combined with enhanced generation of antibodies recognizing intestinal bacterial antigens after portosystemic shunting, a situation in which blood from the intestines reaches the systemic circulation without traversing the liver, the main site for metabolism and detoxification [3].

### 3.1.3 Liver sinusoidal endothelial cells in vascular liver disease

While injury of the liver parenchyma can cause chronic liver diseases subsequently leading to vascular remodeling, as it is the case in liver cirrhosis, the opposite scenario is also possible. A number of liver diseases originate in the endothelium with a vascular injury being the cause for hepatic changes. LSEC are an early target of toxins, therefore sinusoidal injury is involved in a number of vascular liver diseases such as sinusoidal obstruction syndrome (SOS), fibrosis, ischemia-reperfusion injury, peliosis hepatis, and nodular regenerative hyperplasia [25]. Vascular damage can occur in the large blood vessels of the liver, as observed in portal vein thrombosis or Budd-Chiari syndrome. Otherwise, endotheliopathy can also be ascribed to the microvasculature with the consequence of SOS or NRH.

#### 3.1.3.1 Sinusoidal obstruction syndrome

Sinusoidal obstruction syndrome used to be named *hepatic veno-occlusive disease*. After experimental evidence proofed that central vein occlusion is not essential for the development of SOS, the name was changed. Because of the finding that this disease is caused by a circulatory damage at the level of the sinusoids, the term SOS is more appropriate than the old name [26]. SOS can be induced by ingestion of pyrrolizidine alkaloids or due to specific drugs (i.e. Gemtuzumab ozogamicin; Mylotarg®) alone or in combination with irradiation. While pyrrolizidine alkaloid-induced SOS is mainly found in non-Western nations, SOS in North America and Western Europe is most commonly caused by myeloablative regimens. These regimens are a combination of high-dose chemotherapy drugs or chemotherapy plus irradiation therapy [26,27]. The primary event in SOS is a toxic insult to the sinusoidal endothelium leading to circulatory obstruction with subsequent liver dysfunction [28]. Studies in the experimental monocrotaline rat model of SOS yielded insights into the pathophysiology of this disease. The cytochrome P450-activated monocrotaline metabolite depolymerizes F-actin, which is the structural

component of the LSEC skeleton [29]. Upon actin depolymerization matrix metalloproteinase-9 (MMP-9) gets activated. MMP-9 is an enzyme that digests extracellular matrix tetherings of LSEC with the consequence that cells start to round up, which in turn leads to gaps in the endothelial barrier [30]. The red blood cells are then able to penetrate into the space of Disse, where they dissect the sinusoidal endothelial lining away from the parenchymal cells. Sinusoidal cells embolize downstream, obstructing the sinusoidal blood flow [28]. Protecting LSEC by the administration of sinusoidal cell glutathation is able to prevent SOS [28,31]. This finding further underlines the primary role of LSEC in the pathogenesis of SOS.

Furthermore, Wang and colleagues showed that conditional knockout of recombination signal-binding protein-Jκ (*RBP-J),* which is the Notch signaling mediating transcription factor, leads to LSEC proliferation and SOS-like pathological changes presented by significant liver congestion, deposition of fibrin-like materials in hepatic sinusoids, and edema in the space of Disse [32].

### 3.1.3.2 Liver Fibrosis

Liver injury results in wound healing with accumulation of extracellular matrix, a process referred to as hepatic fibrosis. Liver fibrosis is reversible if the insult is acute. However, in case of sustained, chronic injury progressive accumulation of scarring tissue replaces normal liver tissue and advanced hepatic fibrosis eventually leads to liver cirrhosis. Deposition of extracellular matrix stimulates hepatocyte regeneration followed by distortion of the hepatic parenchyma and the vascular architecture. These pathological changes are known to activate angiogenic stimuli.

Numerous investigators explored the relationship between angiogenesis and fibrogenesis in chronic liver diseases and it has been shown that the two processes go hand in hand. Two pathways are implicated in the fibrogenic process. First, fibrogenesis and angiogenesis occur in response to chronic wound healing activated by an up-regulation of growth factors, cytokines, metalloproteinases, and molecules involved in extra-cellular matrix remodeling [33,34]. Secondary to the increased tissue hypoxia, which results from progressive sinusoidal capillarization amongst other possible causes, pro-angiogenic pathways are switched on [26,35].

Differentiated LSEC maintain hepatic stellate cells quiescent through VEGF-induced nitric oxide (NO) synthesis and they are even able to promote reversion of activated HSC to quiescence [36]. Stellate cell activation into a proliferative, contractile and fibrogenic cell is a key event in fibrogenesis. Formation of extracellular matrix by activated HSC is the

major mechanism inducing hepatic fibrosis, whereas contractile activity of HSC further increases the intrahepatic vascular tone [37]. Capillarized LSEC, which underwent a dedifferentiation towards a vascular phenotype, lack the property to prevent stellate cell activation because VEGF stimulation of NO production is lost [36]. Interestingly, restoring of LSEC differentiation in capillarized livers induces quiescence of HSC and regression of fibrosis in experimental models. Hence, LSEC differentiation seems to be the central determinant in progression of fibrosis [38].

The two key features of LSEC phenotype are non-diaphragmed fenestrae and absence of a basement membrane. Paracrine VEGF secretion by hepatocytes or HSC, inducing an autocrine production of NO by endothelial nitric oxide synthase (eNOS) in LSEC, is required to maintain LSEC differentiation [39]. Loss of fenestration with development of a continuous basement membrane is termed “capillarization”. In the aging liver a mitigated version called “pseudocapillarization” occurs [40]. These ultrastructural changes of LSEC leading to capillarization impede the exchange between sinusoidal blood and parenchymal cells and cause a mechanical increase of the sinusoidal vascular resistance [41,42]. Capillarization precedes fibrosis in a variety of chronic liver diseases, such as alcoholic liver injury and non-alcoholic fatty liver disease [43–45]. Dedifferentiation of LSEC towards a vascular phenotype, observed in all forms of cirrhosis, impairs hepatic functions and seems to be the initial trigger of this chronic liver disease. Further, capillarization is involved in atherosclerosis and contributes to age-related dyslipidemia [6,11,40]. CD31 (also known as platelet endothelial cell adhesion molecule-1; PECAM-1) has been identified as a marker of capillarization. In normal liver, sinusoidal EC lack expression of CD31 as well as CD34. During the process of sinusoidal capillarization occurring in cirrhosis, the structural changes of LSEC go along with striking changes in the expression pattern of cell-adhesion molecules, including upregulation of CD31 expression on the protein and messenger-RNA level [39,46,47]. In addition, CD31 surface expression correlates inversely with fenestration [39].

#### 3.1.3.3 Non-cirrhotic portal hypertension

In the Western world portal hypertension is the main complication of cirrhosis [48]. However, increased portal pressure is also seen in the absence of hepatic cirrhosis or of chronic liver conditions associated with cirrhosis. This condition is referred to as non-cirrhotic portal hypertension (NCPH) [49]. Portal hypertension is a clinical syndrome defined by an increase in the portal pressure gradient between the portal vein and the inferior vena cava [42]. While normal values range from 1 to 5 mm Hg, increased portal

pressure becomes clinically relevant if values increase to 10 mm Hg or above [42]. NCPH is the collective term for a variety of pathological liver lesions, which were recently classified into four subcategories: idiopathic portal hypertension, diffuse nodular regenerative hyperplasia (see below), partial nodular transformation, and incomplete septal cirrhosis [50]. Differential diagnosis of these lesions is challenging and patients often present more than one of the morphologic alterations. Probably the lesional spectrum is caused by a shared etiology, which is irregular perfusion of the liver. Causes of NCPH can be divided into five categories: immunological disorders, infections, medication or toxins, genetic diseases, and thrombophilia [51]. The pathophysiology of NCPH still remains controversial. Schouten et al. hypothesized that two mechanisms are involved in the development of NCPH. Increased splenic blood flow, caused by elevated NO release in the sinus-lining cells of the spleen, is one mechanism leading to NCPH. The other factor playing a role in NCPH is an increase in intrahepatic resistance resulting from portal venous obliterations. Circulatory lesions due to thrombophilia, immunological disorders, infections, and medication possibly induce portal venous obliteration [51]. The main clinical manifestations are related to complications of portal hypertension, such as variceal bleeding, splenomegaly, and ascites [51]. Liver function generally is preserved and the prognosis depends on the underlying pathologic conditions [49]. Treatment of NCPH patients is focused on complications of portal hypertension as well as on the cure of the initiating hepatic disorder.

#### 3.1.3.4 Nodular regenerative hyperplasia

Nodular regenerative hyperplasia is a rare liver disease characterized by the transformation of hepatic parenchyma into small regenerative nodules accompanied by no or only little fibrosis. Steiner was the first one describing this hepatic lesion with alternating areas of regeneration and atrophy lacking fibrous bands, scars, or septa, clearly distinguishing this condition from cirrhosis [52]. A large autopsy study by Wanless found NRH to occur at an incidence of 2.6%. In the same study, Wanless proposed a classification for nodular transformation: micronodular transformation corresponds to parenchymal nodules smaller than 3 mm in diameter in the absence of fibrous tissue. Nodularity was graded 0 to 3+ depending on their distinct appearance. Presence of fibrous septa was also graded ranging from 0 to 3. To meet the diagnosis of NRH, the hepatic lesions had to comply with grade 3 nodular transformation and grade 0 to 1 fibrous septa [53]. The pathogenesis of NRH seems to be related to hemodynamic disturbances in the liver microvasculature. Impaired portal venous blood flow leads to

hepatocyte atrophy, whereas regenerative nodules express the hyperplastic reaction of hepatocytes induced by maintained or increased blood supply [53–55]. The first hypothesis on the pathogenetic mechanism of NRH was proposed by Wanless et al. suggesting that obliterative vascular lesions cause local ischemia, which then induce atrophy and consequently regenerative nodule formation [54]. This etiologic concept has matured within the last decades and it is now accepted that circulatory impairment is the response to a vascular lesion on the sinusoidal level [26,56]. NRH has been described in the setting of several systemic diseases including hematologic disorders, autoimmune diseases, and vascular disorders, as well as in association with certain drugs [57]. Liver function is generally preserved in patients with NRH; accordingly the course of disease is indolent in most patients. The most common manifestation of NRH is non-cirrhotic portal hypertension [49,57,58]. Although NRH usually evolves without symptoms, some patients become symptomatic with a clinical picture that is dominated by portal hypertension and its complications such as gastroesophageal varices, splenomegaly and ascites [57]. Management of NRH patients is aimed to eliminate the underlying causes and to treat the sequelae of the resultant portal hypertension.

## 3.2 Angiogenesis

### 3.2.1 Mechanisms of vascular development

The vascular system is one of the first organs that develops during embryogenesis because oxygen and nutrient supply is mandatory to assure further development of the organism [59]. Blood vessel formation and remodeling involves three distinct mechanisms: vasculogenesis, angiogenesis, and arteriogenesis (Figure 2). De novo blood vessel formation, named vasculogenesis, refers to differentiation of mesoderm-derived angioblasts into endothelial cells, which then coalesce to generate the primary vascular plexus [60]. Angiogenesis, defined as new blood vessel formation from pre-existing vessels, expands the primitive vascular network by sprouting and non-sprouting (intussusceptive) angiogenesis. During arteriogenesis the arterial lumen is increased and mural cells (pericytes in the microvasculature and smooth muscle cells in arteries and veins) are recruited to stabilize the nascent vessels [61–63]. Finally a mature hierarchical circulatory system is established. The formation of such a complex network relies on precisely concerted signaling pathways of which the VEGF pathway is the one studied most extensively.

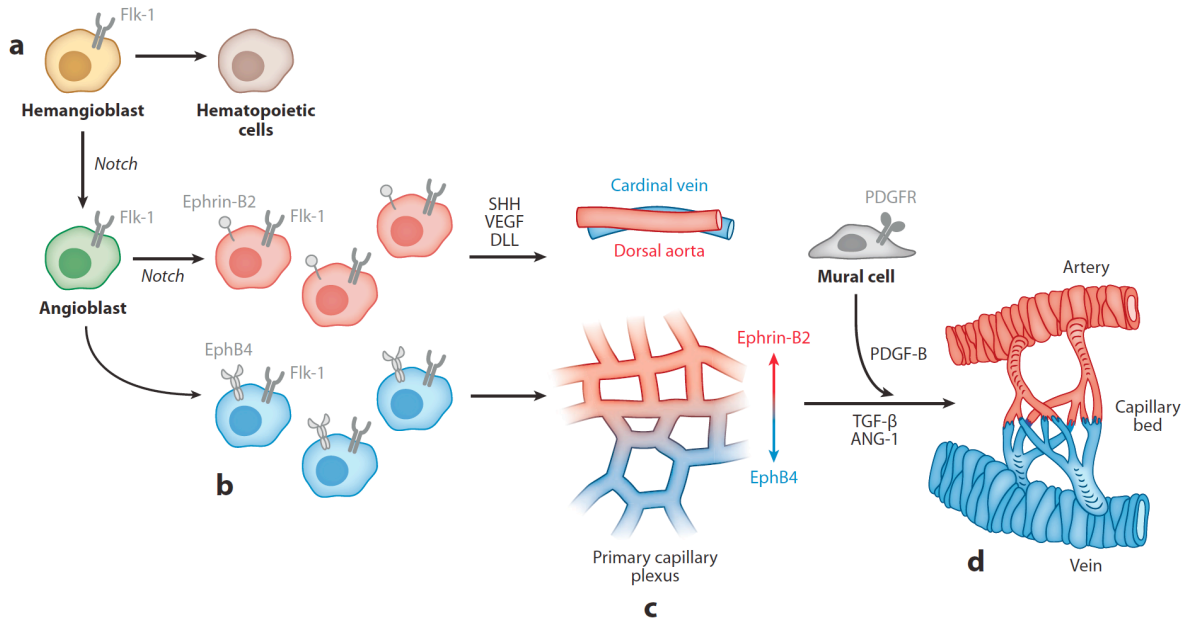


**Figure 2. Vascular development.** Endothelial cells and hematopoietic cells derive both from a common progenitor, the hemangioblast, which is characterized by VEGFR-2 (Flk-1) expression. Stimuli such as Notch signaling drive the angioblast differentiation (a). Notch signaling is involved in determining the arterial and venous fate of endothelial cells by promoting arterial cell fate (b). In the embryo the dorsal aorta and the cardinal vein derive directly from angioblasts promoted by vascular endothelial growth factor (VEGF), Sonic hedgehog (SHH), and Notch signaling. In the yolk sac, upon fusion of angioblasts a primitive capillary plexus arises. The Ephrin system is important for arterial-venous specification with Ephrin-B2 as an arterial marker and Eph-B4 expressed on veins (c). Finally, stabilization and maturation of the new blood vessels is achieved by recruitment of mural cells, a process mediated by platelet-derived growth factor B (PDGF-B), transforming growth factor β(TGF-β), and angiopoietin-1 (ANG-1) signaling (d) (from [64]).

Vascular endothelial growth factors (VEGF) and its receptors are the major players in embryonic and adult angiogenesis [59,65,66]. The mammalian VEGF family consists of five members, VEGF-A (also referred to as VEGF), VEGF-B, VEGF-C, VEGF-D, and placenta growth factor (PlGF). The VEGF ligands interact with three tyrosine kinase receptors, VEGF receptor (VEGFR)-1 (also Flt-1), VEGFR-2 (also known as KDR in humans or Flk-1 in mice), and VEGFR-3 (also Flt-4) [67,68]. VEGF-A binds to VEGFR-1 and VEGFR-2 and mediates the majority of its biological effects through VEGFR-2 signaling [65,66]. VEGF/VEGFR-2 signaling stimulates endothelial cell proliferation, migration, differentiation, sprouting, survival, and vascular permeability [63,66–68].

Vasculogenesis is controlled by a number of signaling molecules like fibroblast growth factors (FGF), the hedgehog family of morphogens, Neuropilins, transforming growth

factor-β (TGF-β) and its receptors, but the most pivotal role is dedicated to VEGF signaling with important roles in both, vasculogenesis and angiogenesis [63]. The indispensable role of VEGF/VEGFR-2 signaling at early developmental stages is reflected by the fact that VEGFR-2 marks hemangioblasts, a multipotent precursor, which can differentiate into cells of the hematopoietic lineage and into angioblasts [69]. Furthermore, numerous knockout studies underscored the crucial role of the VEGF/VEGFR system in the embryonic vascular development [66,68,70,71]. Mice lacking a single VEGF-A allele die at embryonic day 9.5, highlighting the significance of VEGF dosage for efficient embryonic vessel development [71,72].

### 3.2.2 Physiological angiogenesis

The formation of new blood vessels originating from preexisting vessels is a well-characterized process implicated in the development of the cardiovascular system. Following the process of vasculogenesis, angiogenesis gives rise to a mature and complex vascular bed by remodeling of the primitive vascular labyrinth. This example of angiogenesis under physiological conditions is assigned to embryogenesis. Physiological angiogenesis in the adult is a rather infrequent event and occurs in the female reproductive cycle (ovulation, menstruation, implantation, pregnancy), during wound healing, and in the process of tissue regeneration [73–76].

Angiogenesis can take place by distinct mechanisms (Figure 3). Sprouting angiogenesis, which is the most widely studied mode of angiogenesis, refers to the formation of new blood vessels from a preexisting one (Figure 3a). The outgrowth of a novel vessel is initiated by numerous stimulatory angiogenic signals and requires proteolytic activities for the degradation of the endothelial basement membrane [77]. Growth factors activate endothelial cells to sprout, proliferate, mature, and remodel, finally resulting in expansion of the vascular network [78]. Vasculogenesis (Figure 3b) describes a mode of vessel formation by recruitment of endothelial progenitor cells followed by incorporation into vessel walls [79]. Intussusception is a specific form of angiogenesis where new vessels are formed by splitting of pre-existing vessels (Figure 3c).

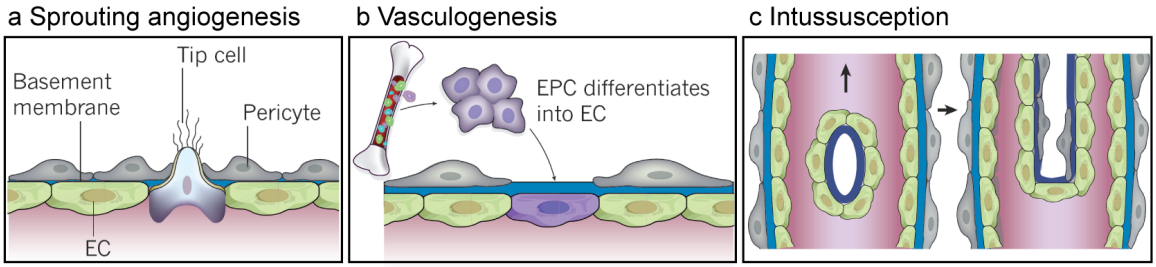


**Figure 3. Mechanisms of blood vessel formation.** Angiogenesis can occur by sprouting angiogenesis (a), by recruitment of endothelial precursor cells (EPC) which undergo differentiation into endothelial cells (EC; b), or by splitting angiogenesis, a process known as intussusception (c). Modified from [79].

Intussusception is a mode of blood vessel formation that is not well understood. This new concept of blood vessel formation was first discovered during postnatal growth in the rat lung where it permits the increase of the capillary network [80]. In the third week of rat life and within the first two years in humans, the volume of the lung increases by more than 20 times with expansion of the capillary volume of more than 30 times. Such an immense increment asks for a fast growth of the vasculature. Studying electron micrographs of rat lung vascular casts revealed that intussusceptive angiogenesis represents such a rapid process because it does not require proliferation of endothelial cells. Instead, remodeling of endothelial cells increases their volume in preparation for the following steps: transluminal contact formation, interstitial pillar generation, pillar growth in diameter, and endothelial cell retraction which eventually leads to separated vessels (Figure 4). In simplified terms, the capillary network extends within itself [81].

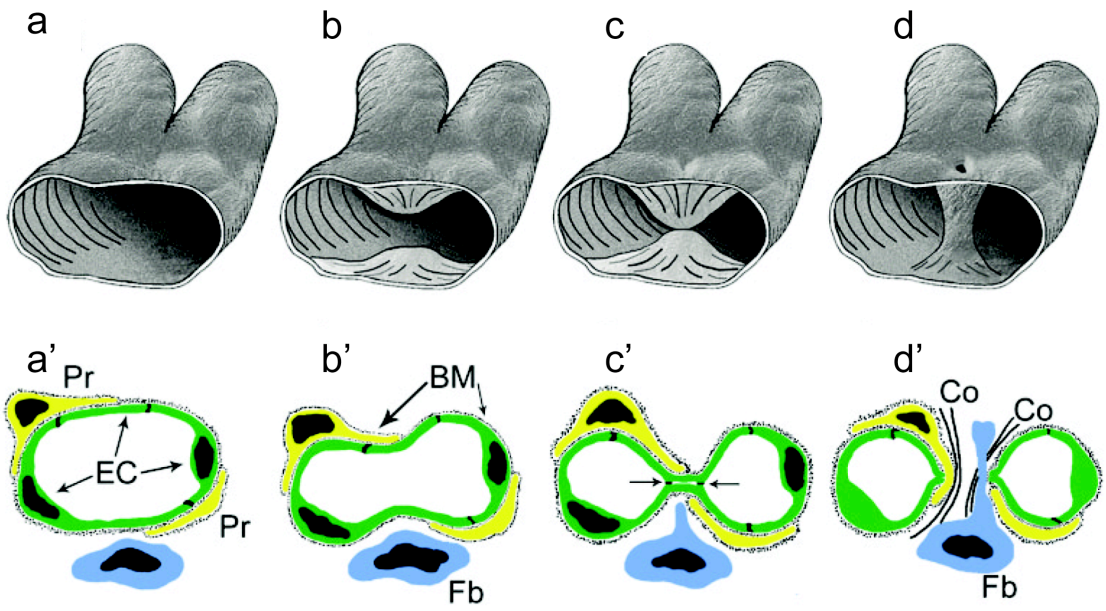


**Figure 4. Intussusceptive angiogenesis.** Schematic illustration of the sequential steps involved in intussusceptive vascular growth. The process is initiated with the protrusion of opposite vessel walls towards the inside of the vessel lumen (a, a', b, b'). When the endothelial cells have established an intercellular contact (c, c'), the endothelial layer and the basal membrane become perforated and a pillar is formed in the center of the vessel lumen (d, d'). Invading pericytes (Pr) and fibroblasts (Fb) produce collagen fibrils to stabilize the pillars (c', d') (from [81]).

Endometrial growth in the menstrual cycle as well as vessel formation in the placenta after pregnancy depends on tightly regulated angiogenic processes, both involving growth and remodeling of the vasculature [73]. Injury of the dermis initiates neovascularaization as part of a successful wound healing process. The initial phase in wound healing is hemostasis resulting in fibrin-rich clot formation. Activated platelets and the fibrin-matrix, which forms the clot, provide a variety of angiogenic factors, including VEGF-A, that stimulate the sprouting of new vessels into the wound [82,83].

Liver regeneration represents another case of angiogenesis in the physiological setting that offers a reproducible model to study hepatic angiogenesis. After loss of hepatic tissue, the liver starts to regenerate and grows back to its normal mass [84,85]. Most of the current knowledge on the mechanisms regulating this highly complex and orchestrated phenomenon is gained from studies in the two-third partial hepatectomy (PHx) model performed in rodents. Higgins and Anderson introduced this experimental setup [86]. In a surgical procedure three of the five liver lobes are removed intact without damage of the residual lobes. In mice and rats, tissue regeneration is completed after five to seven days and the remaining liver enlarged to restore the original organ mass [84]. In

contrast to hepatic toxin (e.g. carbon tetrachloride) induced liver regeneration, PHx provides a regenerative impulse without inducing a response to inflammation or tissue damage. Liver regeneration after PHx involves sequential changes in gene expression, growth factor production, and morphologic structure. It is carried out by participation of all the existing mature liver cell populations, which are normally quiescent [84]. Hepatic angiogenesis is required to support the rapid increase in liver tissue and possibly terminates the regenerative process [87].

Given that there is no tissue damage after PHx to trigger the healing scenario, there must be another stimulus to induce the changes of signaling pathways in the liver. Even though the role of hemodynamic events after PHx are not well understood, it seems that the tremendous changes in the hepatic blood flow pattern induce hepatocyte proliferation through activation of hepatocyte growth factor (HGF) [88]. Hepatocytes are the first cells starting to proliferate, growing to avascular clusters. Cells located within the cluster are outside the oxygen diffusion distance of 100 - 200 µm [87,89]. Thus, they are exposed to hypoxic conditions which activate the transcription factor hypoxia inducible factor-1 (HIF-1) followed by upregulation of downstream targets such as VEGF and VEGFR-1 [90]. VEGF secreted by proliferating hepatocytes peaks shortly after PHx [91,92]. VEGF promotes autocrine proliferation of hepatocytes besides stimulating the proliferative activity of sinusoidal endothelial cells in a paracrine fashion [91,93]. The enhanced expression of VEGF and its receptors coincides with the peak of endothelial proliferation [89,94]. VEGF does not only exert a proliferative stimulus to LSEC, but also permits migration of LSEC by breaking down the surrounding extracellular matrix through upregulation of collagenases, MMP, and urokinase-type plasminogen activator [95,96]. In addition, regulation of endothelial cell proliferation during hepatic regeneration involves other angiogenic factors including platelet-derived growth factor receptor beta (PDGF-R beta), epidermal growth factor receptor (EGF-R), and the angiopoietin/Tie system [89,94]. Angiopoietin-1 (Ang-1) mediates vessel stability through interaction with Tie-2, whereas Ang-2 antagonizes this effect [89]. Despite the close homology of Tie-1 with Tie-2, the former does not bind to angiopoietins and it’s exact role in angiogenesis remains to be elucidated [97]. Angiopoietin/Tie factors peak at 96 h after PHx, except for Ang-2, which shows the highest expression after 168 h. Ang-1 is likely to induce maturation of sinusoids presumably by interaction between endothelial cells and pericytes mediated through Tie-2 expressed on endothelial cells. Ang-2 in the absence of VEGF inhibits angiogenesis and might be associated with the cessation of the regenerative process [92]. These findings indicate a central role of angiogenesis in the course and termination of liver regeneration.

### 3.2.3 Pathological angiogenesis

Angiogenesis underlies a tightly controlled system consisting of pro-angiogenic and anti-angiogenic factors. Disequilibrium in the angiogenic balance in favor of pro-angiogenic molecules turns on the “angiogenic switch” and contributes to the pathogenesis of many disorders such as cancer, psoriasis, rheumatoid arthritis, and diabetic retinopathy [74,76,98]. Pathologic angiogenesis recapitulates plenty of processes seen in physiological angiogenesis. Under both conditions, the same regulators are operating in a highly coordinated fashion. The difference between those two situations is that in pathological angiogenesis blood vessels grow uncontrolled without a termination phase once the vascular perfusion is established. Pathologic conditions drive the angiogenic cascade in a continuous manner eventually leading to development of “angiogenic diseases” [64]. Deregulated vessel growth in terms of insufficient vascular growth also impacts health, as it is associated with several diseases including heart ischemia, hypertension, neurodegeneration, osteoporosis, and many more [76,99].

In the pathologic situation of cancer, tumor angiogenesis is switched on. Tumor growth heavily depends on angiogenesis. As soon as the tumor reaches a size of 1-2 mm, formation of new blood vessels is necessary to supply tumor tissue with oxygen and nutrients by which further tumor growth is allowed. Because of the rapid tumor expansion and the irregular pattern of the tumor vasculature some cells become exposed to a hypoxic environment. Blood vessel recruitment in tumors is powered by tumor released factors and tumor hypoxia [100]. However, the tumor vessels are disorganized and leaky, reflecting their pathological nature. Although tumor vessel growth uses physiological mechanisms of angiogenesis it is obvious that in pathological stages coordination and restriction of the sequential events is impaired [101,102].

In the liver, pathological angiogenesis has been described during inflammatory and fibrotic processes in several chronic liver diseases of viral and autoimmune origin and in hepatocellular carcinoma [103–105]. In addition, excessive angiogenesis has been linked to the development of portal hypertension [106].

Portal hypertension is known to be associated with vascular changes occurring during chronic liver diseases. These vascular changes are not restricted to the liver, but are also present in the splanchnic and systemic circulation. Accordingly, there is an intrahepatic (increased vascular resistance) and an extrahepatic (increased hyperdynamic splanchnic circulation) contribution to the development of portal hypertension (Figure 5). It has long been known that cirrhosis is a vascular disease. In this context it appeared obvious that portal hypertension, which is most commonly caused by cirrhosis, is related to vascular

abnormalities. Indeed, numerous investigators substantiated this assumption by demonstrating that portal hypertension is a consequence of architectural changes of the hepatic circulation [39,107,108]. More recent findings also identified neovascularization implicated in the genesis of portal hypertension [103].

Intrahepatic vascular alterations:

LSEC regulate the hepatic vascular tone by releasing vasoactive substances. However, in liver cirrhosis and portal hypertension the vasomotor property is deranged; production/bioavailability of vasodilators (i.e. nitric oxide) is decreased, whereas vasoconstrictors (i.e. thromboxane A2) are increased [42]. With this imbalance of vasodilators and vasoconstrictors, endothelial dysfunction contributes to an increased intrahepatic resistance.

LSEC constitute the first line of defense, thus they are exposed to diverse types of liver injury. Inflammation, oxidative stress, insulin resistance, and alcohol are thought to be the main triggers of liver injury causing sinusoidal dysfunction [109]. In the liver, LSEC play important roles in the regulation of vasomotor control [109], blood clearance, immunity [110], hepatocyte growth [111], angiogenesis, and sinusoidal remodeling [112]. Therefore, LSEC dysfunction leads to impairment of these physiological effects. In liver cirrhosis, the sinusoidal circulation is characterized by “endothelial dysfunction” which results in a diminished bioavailability of endothelial relaxing factors, such as NO. This in turn causes an increase in the intrahepatic vascular resistance [113–115]. LSEC dysfunction can be seen as the primary event in the development of portal hypertension, as it initiates a cascade of structural and functional changes further contributing to the progression of portal hypertension. One consequence of LSEC dysfunction is the disruption of the crosstalk between LSEC and HSC, which is responsible for maintaining the sinusoidal homeostasis [39,116]. NO produced by LSEC preserves the quiescent state of HSC [107]. Because hypoactive endothelial cells in the cirrhotic liver generate reduced levels of NO, HSC become activated to a myofibroblastic phenotype [107]. Indeed, activated HSC contribute to accumulation of extracellular matrix and promote hepatic fibrosis following acute or chronic liver injury [33,37]. Fibrosis as well as active contraction of HSC gives rise to increased hepatic resistance based on a mechanic process [117]. At the same time the enhanced contractility and the presence of collagen induce capillarization of the sinusoids [39,107,118]. Furthermore, decreased NO production in LSEC disrupts the autocrine regulation of the LESC phenotype with dedifferentiation and capillarization [39]. As a consequence of defenestration and presence of a basement membrane during capillarization, the metabolic exchange with hepatocytes is physically impeded, sinusoids become more rigid, and hence

capillarization further enhances mechanically the sinusoidal vascular tone [42]. Altogether, LSEC dysfunction is a key factor in the pathophysiology of portal hypertension.

Extrahepatic vascular alterations:

Portal pressure is an important factor that triggers endothelial dysfunction in the intestinal microcirculation leading to the hyperdynamic state, characterized by splanchnic and peripheral vasodilatation, increased plasma volume, and increased cardiac output [119]. Dysfunctional endothelial cells, showing NO hyperproduction, are seen in cirrhosis and portal hypertension [42,120–122]. NO produced by eNOS is a vasodilatory agent considered as the major player in regulating arterial vasodilatation in the splanchnic and systemic circulation [120,123,124]. Besides NO, other vasodilator molecules are also found to be elevated in hyperactive endothelial cells, contributing to the arterial vasodilatation in the intestinal and splanchnic system [125–127]. The net effect of endothelial cell hyperactivation in the splanchnic and systemic circulation is vasodilatation in these vascular beds. In response to peripheral arterial vasodilatation, owing to increased vasodilators and decreased vasoconstrictors, a state of hypovolemia is simulated with ensuing activation of vasoconstrictive and volume-retaining neurohumoral mechanisms as well as increased cardiac output [128,129]. Compensatory sodium and water retention aims to perpetuate perfusion pressure by increasing the intravascular volume [130]. The hyperdynamic splanchnic circulation is mediated by arterial vasodilatation and expansion of blood volume [131]. As a result the blood flow in the splanchnic organs is increased with subsequent increase in portal venous inflow. In turn, increased flow to the portal vein aggravates portal hypertension [132,133].

To counterbalance the high pressure in the portal circulation, collateral vessels are formed. Collateral formation related to portal hypertension was traditionally thought to result from the opening of preexisting vessels [42,134]. However, recent studies demonstrated a role of angiogenesis in splanchnic hyperemia and collateral formation [135,136]. Portal pressure is sensed at the intestinal microciruculation, probably communicated by mechanical forces, switching on pro-angiogenic pathways [119]. Indeed, portal pressure has been shown to induce release of VEGF and PlGF providing evidence that angiogenesis plays a role in portal hypertension [137–139]. Such angiogenic growth factors facilitate splanchnic neoangiogenesis. Two effects result from these newly formed vessels. On the one hand, angiogenesis increases the splanchnic vascular bed size and therefore promotes increased blood supply in splanchnic organs. As they eventually drain into the portal vein, collaterals exacerbate portal hypertension [42,137]. On the other hand, formation of new blood vessels contributes to portal-

systemic collaterals, including esophageal and gastric varices, which are responsible for variceal bleeding, related with high mortality [140]. In addition, collaterals are also involved in portal-systemic shunting, thereby causing severe complications such as portal–systemic encephalopathy [140].

Overall, angiogenic processes constitute a pathological hallmark of portal hypertension and inhibition of angiogenesis may be a novel approach to prevent disease progression and development of portal hypertension-associated complications [136,141].

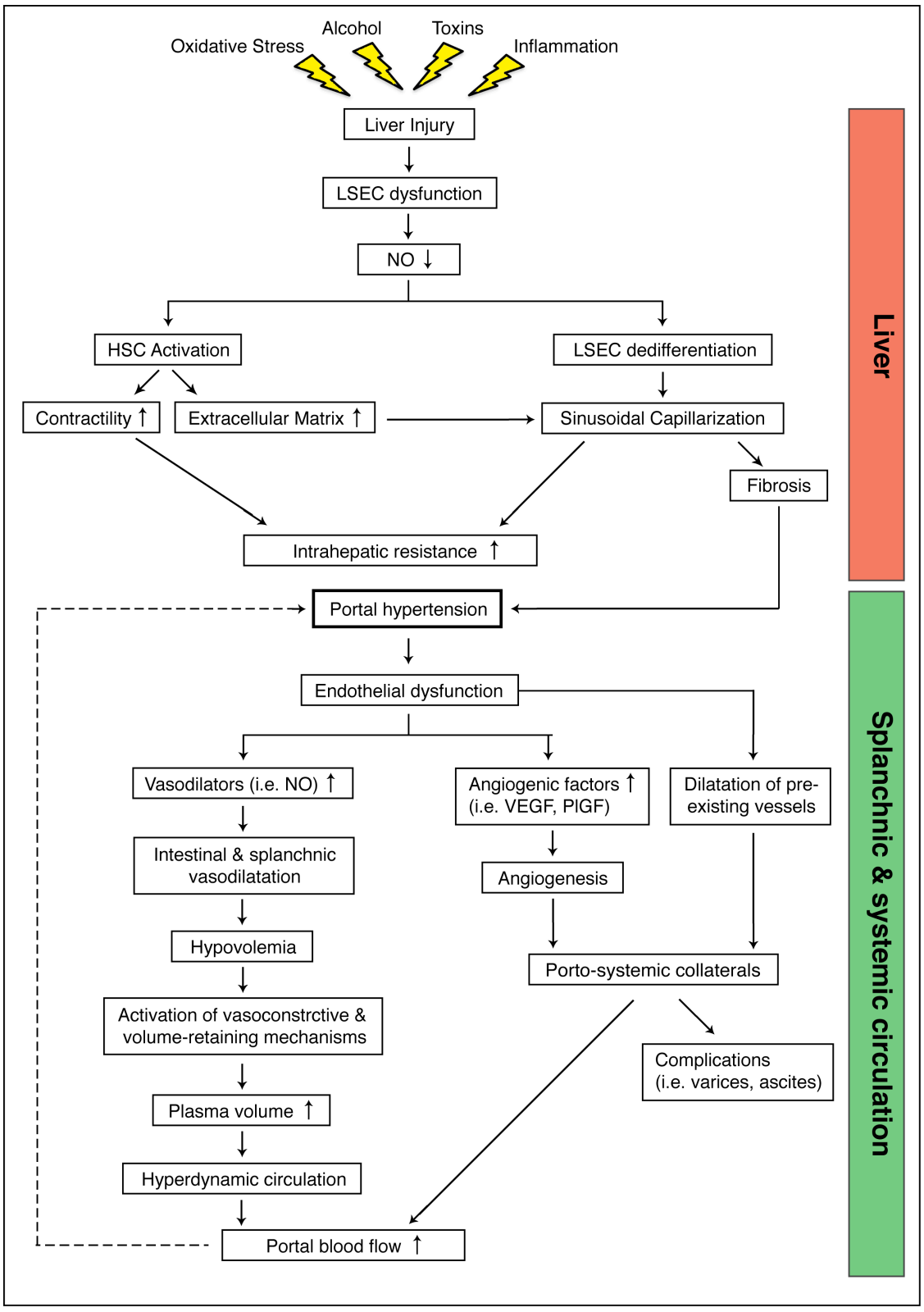


**Figure 5. Pathophysiology of portal hypertension.** Overview of the factors implicated in development of portal hypertension. LSEC dysfunction and endothelial dysfunction in the splanchnic and systemic circulation play a central role in development of portal hypertension and disease progression.

## 3.3 Notch signaling

### 3.3.1 The Notch signaling pathway

Notch signaling is a highly conserved pathway involved in developmental and physiological cellular processes including cell fate decisions, differentiation, boundary formation, cell proliferation, and cell apoptosis [142,143]. Notch receptors and Notch ligands are both single-pass type I transmembrane proteins mediating intercellular communication to control pattern formation [144]. In mammals there are four Notch receptors (Notch1-4) and five Notch ligands belonging to the Delta and Jagged evolutionary conserved classes: Delta-like 1 (Dll1), Dll3, Dll4, Jagged1, and Jagged2 [144,145]. The Notch receptor undergoes three proteolytical cleavages, named site 1 (S1), site 2 (S2), and site 3 (S3). The first cleavage is essential for receptor maturation and occurs in the Golgi complex by a furin convertase, allowing the receptor to present as a heterodimer at the cell surface [143,145]. The latter Notch cleavages at S2 and S3 depend on receptor-ligand interaction (Figure 6) [145].

Ligand binding to the Notch receptor activates Notch signaling by inducing two proteolytical cleavages. Upon ligand-receptor interaction a conformational change in the juxtamembrane receptor region is induced leading to S2 cleavage by ADAM metalloproteases ADAM10 (also known as Kuzbanian) and ADAM17 (also known as tumor necrosis factor $\alpha$ converting enzyme). The second cleavage triggers the following cleavage at the site S3 within the Notch transmembrane domain and is mediated by the multi-protein complex $\gamma$-secretase. The final cleavage releases the Notch intracellular domain (NICD), which can then translocate to the nucleus where it interacts with the DNA-binding protein CSL [144–146]. The CSL (named CBF1/RBPj$\kappa$ in mammals) transcription factor is a negative regulator of Notch target genes. In the absence of NICD, CSL associates with transcriptional co-repressors. Binding of NICD to CSL displaces co-repressors and recruits co-activators, in particular mammalian Mastermind-like 1 (MAML1). The trimeric complex recruits histone acetylases and chromatin remodeling factors, which promotes transcription activation of target genes such as genes of the *Hairy* and *Enhancer-of-split* locus in Drosophila and the related *Hes* and *Hey* genes (i.e. *Hes1* or *Hey1*) in mammals [144,146,147]. Given that Notch signaling is activated upon cell-to-cell contact, the pathway is important for directing cell fate specification, morphogenesis and organogenesis during development [143,145]. Consequently, dysregulation of Notch signaling has been implicated in a multitude of developmental defects, but is also involved in several human diseases, including many tumors [148,149].

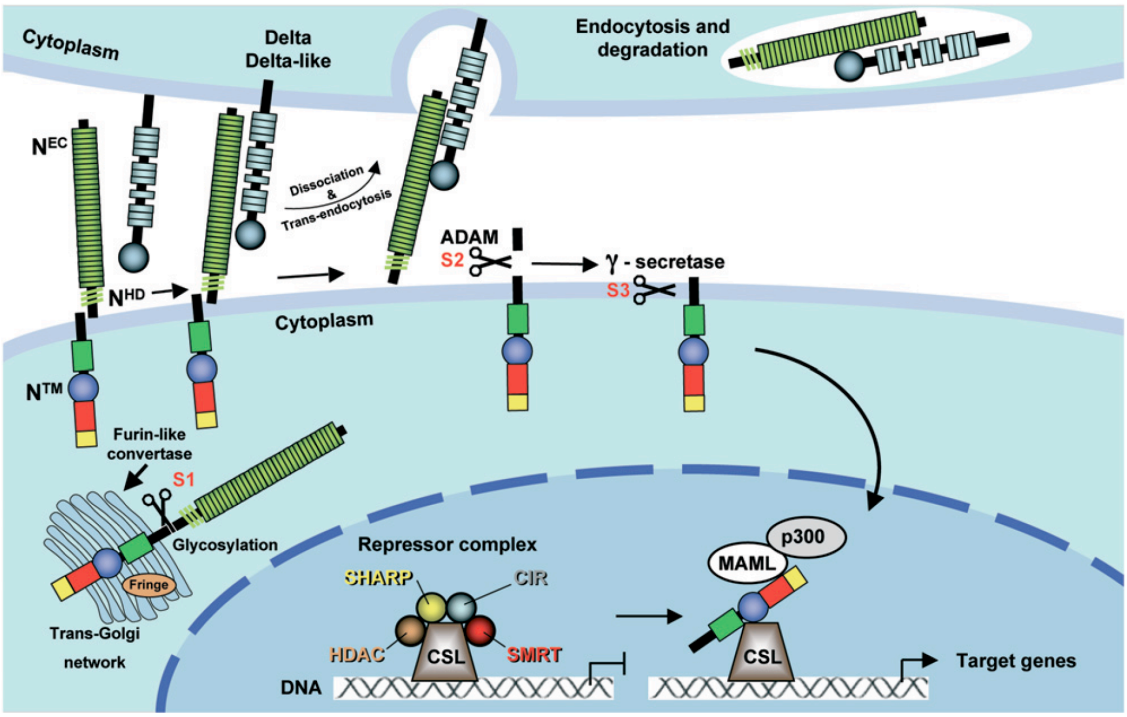


**Figure 6. The Notch signaling pathway.** The Notch receptor is cleaved by a Furin-like convertase at site S1 within the secretory pathway. This cleavage event converts the Notch receptor into a heterodimer consisting of the Notch extracellular domain ($N^{EC}$) and the Notch transmembrane and intracellular domain ($N^{TM}$). The Notch receptor is activated upon binding to a ligand presented by a neighboring cell. This interaction induces a conformational change by which the site S2 is exposed for cleavage by ADAM metalloproteases. The generated membrane-anchored truncated $N^{TM}$ becomes a substrate for the γ-secretase complex. The γ-secretase cleaves the intermediate NTM at site S3, which releases NICD. NICD then enters the nucleus, where it binds to the transcription fator CSL. NICD-CSL interaction facilitates displacement of transcriptional repressors (HDAC, SHARP, CIR and SMRT) and recruits coactivators such as MAML and CBP/p300. The active transcription complex switches on transcription followed by upregulation of target genes. $N^{HD}$, Notch heterodimerization domain; HDAC, histone-deacetylase; CIR, CBF1-interacting corepressor; SMRT, silencing mediator of retinoid and thyroid receptors; SHARP, SMRT/HDAC-1-associated repressor protein; CBP, CREB binding protein; CSL, CBF1/RBPj in mammals, Suppressor of Hairless in Drosophila, LAG in C. elegans (from [150]).

### 3.3.2 Notch signaling in vascular development

The Notch signaling pathway plays a crucial role during vascular development, evidenced by the expression of Notch receptors and ligands in endothelial cells [151,152]. Gene inactivation strategies in mice have demonstrated that Notch signaling is crucial for the vascular-plexus remodeling of the primitive blood vessels. Global knockout of Notch1 and

Jagged1, Notch1/Notch4 double mutation, and Dll4 haploinsufficiency are embryonic lethal due to vascular defects [153–155]. The phenotype of these lethal Notch pathway mutants is characterized by the absence of angiogenic vascular remodeling of the primitive plexus. Notably, specific deletion of Notch1 in the endothelium recapitulates the vascular phenotype of Notch1 null embryos indicating that the observed growth arrest results from Notch1 loss in vessels [156]. Although Notch4 homozygous mutant mice are viable and show no obvious phenotype, Notch1/Notch4 double null embryos often display a more severe vascular phenotype than Notch1 homozygous mutant embryos [152]. Interestingly, generation of a transgenic mouse expressing activated Notch4 specifically in the endothelium exhibited a similar phenotype to that seen in Notch1 deficient mice with embryonic death [153,155]. The observation of angiogenic defects not only in knockout (loss-of-function) mice but also in transgenic (gain-of-function) mice suggests that embryonic vascular remodeling and morphogenesis depends on precisely regulated levels of Notch activity. Taken together, the severe phenotypes as a consequence of genetic ablation targeting Notch pathway molecules reveals the importance of Notch signaling during vessel pattern and network remodeling.

Further, Notch signaling has been shown to regulate a number of events: artery-vein differentiation in endothelial and vascular smooth muscle cells, sprouting and branching of blood vessels during both normal development and tumor angiogenesis, as well as vascular smooth muscle cell differentiation [154].

The role of Notch signaling in cell-fate determination has been well established in multiple cell-types over the last years. Considering the artery-specific expression of several Notch receptors and ligands, it is obvious that Notch signaling is involved in the regulation of arteriovenous cell differentiation [157–159]. For many decades it had been believed that hemodynamic parameters trigger differentiation of arteries and veins before the concept of a genetically predetermined expression pattern was established. Whether an endothelial cell acquires an artery or vein identity, is defined by a genetic program already before the onset of blood flow. Notch signaling plays an important role during this arteriovenous differentiation as confirmed by genetic ablation studies [154,155,160]. Lawson et al. showed in zebrafish that disruption of Notch signaling leads to arteriovenous malformations. Notch mutant embryos displayed loss of artery-specific markers such as ephrin B2, at the same time venous markers like EphB4 and VEGFR3 were ectopically expressed in the dorsal aorta [160]. The ectopic expression of activated Notch results in repression of a venous phenotype with a decrease of venous marker. However, Notch signaling activation was not able to induce arterial differentiation ectopically [160]. These findings suggest that the Notch signaling pathway regulates

arteriovenous differentiation by repressing the venous phenotype rather than inducing arterial differentiation during embryonic vascular development [160]. Further investigations revealed that arterial specification of endothelial cells in zebrafish embryos arises from a complex signaling cascade linking the Notch pathway to VEGF signaling. VEGF, whose expression is induced by sonic hedgehog (Shh) acts upstream of Notch signaling, which mediates arterial differentiation by enhancing the expression of the artery-specific marker ephrinB2, which is a direct target of the Notch pathway [161,162].

Angiogenesis is the process of new blood vessel formation from an existing vascular network. VEGF is known to induce angiogenic sprouting by activating quiescent endothelial cells, which subsequently adopt different phenotypes depending on their role in the growing sprout [163]. The cell in the front of the sprout is the tip cell. Its very motile but non-proliferative features allow the tip cell to explore the environment for growth factor signals and to lead the outgrowth of vessel sprouts. The cells following behind the tip cell become stalk cells, which are highly proliferative cells that form the vascular lumen [163]. To guarantee an organized vascular extension of the primary capillary plexi a mechanism is required that limits the number of outgrowing vessel sprouts and restricts the nomination of tip cells to a small subset of endothelial cells. Several studies in the mouse retina, zebrafish embryo, tumor xenograft models, and in vitro cell culture assays have shown that the Notch pathway regulates which cell turns into a tip cell and which cells differentiate into stalk cells in response to VEGF signaling (Figure 7) [164–168]. In endothelial tip cells Dll4 and VEGFR-2 are highly expressed compared to neighboring stalk cells, where Notch activity is prominent. Dll4 expressing endothelial cells activate Notch signaling and negatively regulate VEGFR-2 expression in adjacent cells. Thereby Dll4/Notch signaling suppresses the tip-cell fate and thus prevents sprouting of these cells. Disruption of Dll4/Notch signaling goes along with increased tip cell formation, sprouting and branching of the vasculature [164–167]. On the molecular level, tip cell markers like *Pdgfb* and *Unc5b* are upregulated at the vascular front in Dll4 mutant retinas [164,165]. In summary, Dll4/Notch signaling, stimulated by VEGF, restricts the tip cell phenotype to a confined number of cells, thereby preventing excessive vessel sprouting and promoting normal vascular morphogenesis.

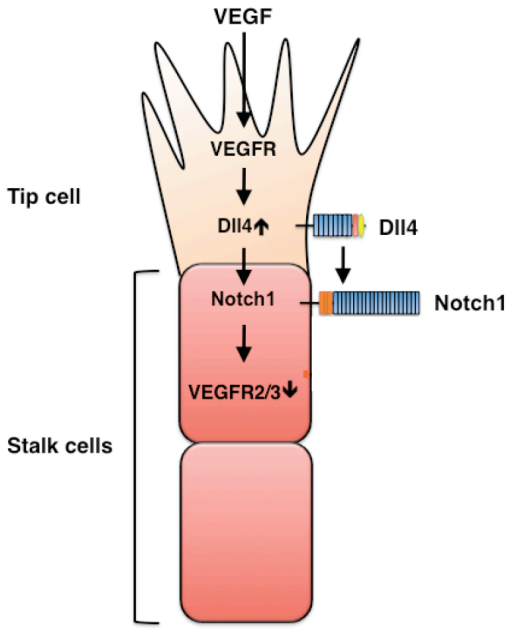


**Figure7. Tip-stalk cell differentiation.** The tip cell becomes selected by sensing a higher levels of VEGF. VEGF/VEGFR signaling induces expression of Dll4 in tip cells. Dll4, in turn, activates Notch signaling in adjacent cells to reduce expression of VEGFR2 and VEGFR3. Dll4-mediated Notch signaling is required for stalk cell specification by efficiently suppressing the tip-cell fate in these cells. Thereby, Notch signaling limits the number of tip-cells and vascular sprouts. Adapted from [169].

### 3.3.3 Notch signaling in vascular disease

Most of the Notch pathway roles in blood vessels were studied in models for developmental angiogenesis. Embryonic lethality of null mutant mice for several members of the Notch pathway made analysis of the Notch signaling in the adult vascular physiology difficult. Thus, Notch functions in the postnatal vasculature are not well understood. The identification of mutations in Notch pathway components associated with human vascular diseases improved our understanding of Notch signaling in vascular homeostasis. Notch signaling impairment has been found to be involved in two hereditary vascular pathologies: the Alagille syndrome (AGS) and the cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL).

The inherited degenerative vascular disease CADASIL is due to Notch3 mutations [170]. This adult-onset disease manifests with stroke and dementia [171,172]. The pathology underlying the condition is a progressive degeneration of the vascular smooth muscle cells (SMC) and the accumulation of granular osmophilic material (GOM) [170]. The specific Notch3 expression pattern, highly restricted to the smooth muscle layer, implicates abnormalities in vascular smooth muscle cells eventually leading to their degeneration [153]. CADASIL is considered to be a systemic vascular disorder since destruction of the smooth muscle layer and GOMs has also been found in muscle and skin biopsies [173]. However, vascular perturbations are restricted to the brain [174]. The late-onset of CADASIL underlines the importance of Notch signaling in adult stages by

regulating vascular homeostasis and maintaining differentiation of the mature blood vessels.

AGS is caused by a mutation in the *Jagged1* gene. In a small group of AGS patients, mutations in the Notch2 receptor were identified [175]. The clinical presentation of this autosomal dominant disorder is defined by developmental abnormalities of the liver, heart, eye, and skeleton [176,177]. Initially, AGS has been described as a syndrome characterized by bile duct paucity in conjunction with cardiac defects, skeletal abnormalities, ocular abnormalities, and characteristic facial features. Vascular abnormalities, already appreciated in the alternative name “arteriohepatic dysplasia”, have been described in multiple AGS patients supporting the idea of a vascular involvement in this disorder as well as the significant role of Notch signaling in vascular homeostasis [178].

Very little is known about the role of Notch signaling in the adult liver vasculature and the molecular mechanisms regulating hepatic vascular maintenance are not well understood. RBP-J is the key mediator of Notch signaling for all four Notch receptors. Deletion of RBP-J in mice leads to defects in LSEC with SOS-like disease and impaired liver regeneration, suggesting a fundamental role of Notch signaling in the liver vasculature. [32] In mice, rats, and monkeys chronic Dll4 blockade induces histopathological changes of the liver, affecting endothelial cells as well as hepatocytes [179]. Furthermore, Notch1 heterozygosity induces vascular tumors in mice, predominantly occurring in the liver reflecting that Notch1 expression is essential in the normal liver [180].

Although Notch signaling has been shown to play a role in the human adult liver in physiological and disease state, further studies are needed to elucidate how the Notch pathway coordinates homeostasis of the adult hepatic vasculature [181].

## 3.4 Aim of the thesis

The Notch1 signaling pathway is crucial for angiogenic vascular remodeling during embryogenesis, but its role in postnatal angiogenesis remains elusive. Since the knowledge on the function of Notch1 signaling in the liver vasculature is very limited together with the fact that our laboratory has a conditional Notch1 KO mouse model available, which shows a liver phenotype, we made it to our goal to characterize the role of the Notch1 pathway in the adult hepatic microcirculation and in pathologic conditions.

Global loss of Notch1 results in embryonic lethality due to vascular defects [152]. Generation of endothelial-specific deletion of Notch1 was found to recapitulate the vascular defects observed after global Notch1 KO and proves an endothelial defect to be responsible for the angiogenic failure during embryogenesis rather than failure of extrinsic signals from nonvascular tissue [156]. However, the role of Notch1 signaling in adult angiogenesis remains to be clarified. Therefore, we wanted to assess the role of Notch1 in the liver vasculature and LSEC with specific regard to vascular homeostasis, differentiation, and quiescence.

In our laboratory it has been previously shown, that deletion of Notch1 induces nodular regenerative hyperplasia in mice [182]. This finding has made the MxCre Notch1 KO mouse an excellent experimental model to study the pathomechanisms of a liver pathology that is also present in humans. Although it has long been established that human NRH results from hemodynamic disturbances, the exact pathogenesis is still not known. Furthermore, data on molecular mechanisms of the disease are not available. Thus, the aim of our animal study was to dissect the mechanisms involved in the development of NRH and to assess how Notch1 deficiency is associated to the development of this liver condition. In support of the hypothesis that NRH is linked to a vascular injury we wanted to identify angiogenic processes contributing to the pathological changes and to study endothelial markers associated with this disease.

NRH is a rare liver condition that in some patients can lead to development of portal hypertension. NRH has been shown to be associated with autoimmune, hematological, infectious, neoplastic, or drug-related causes. Unlike the large post-mortem autopsy study by Wanless, all reported conditions associated with NRH are based on single case reports or case series and systemic population studies on NRH are missing. In a translational study using patient liver biopsies we aimed to establish a correlation

between NRH severity and the presence of portal hypertension. In analogy to our mouse model, we analyzed if the same set of genes are also regulated in patients with NRH favoring a vascular injury at the sinusoidal level as the underlying cause of this disease. Thus, our study provides first insights into the molecular pathogenesis of NRH.

# 4. Results

## 4.1 Disruption of Notch1 induces vascular remodeling, intussusceptive angiogenesis, and angiosarcomas in livers of mice

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Gastroenterology 2012, Volume 142, Issue 4

# Disruption of Notch1 Induces Vascular Remodeling, Intussusceptive Angiogenesis, and Angiosarcomas in Livers of Mice

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**BACKGROUND & AIMS:** Notch signaling mediates embryonic vascular development and normal vascular remodeling; Notch1 knockout mice develop nodular regenerative hyperplasia (NRH). The pathogenesis of NRH is unclear, but has been associated with vascular injury in the liver sinusoids in clinical studies. We investigated the role of Notch1 signaling in liver sinusoidal endothelial cells (LSECs). **METHODS:** We studied *MxCre Notch1*$^{lox/lox}$ mice (conditional knockout mice without tissue-specific disruption of *Notch1*); mice with hepatocyte-specific knockout were created by crossing *Notch1*$^{lox/lox}$ with *Alb-Cre*$^{+/-}$ mice. Portal vein pressure was measured; morphology of the hepatic vasculature was assessed by histologic and scanning electron microscopy analyses. We performed functional and expression analyses of isolated liver cells. **RESULTS:** *MxCre*-induced knockout of *Notch1* led to NRH, in the absence of fibrosis, with a persistent increase in proliferation of LSECs. *Notch1* deletion led to de-differentiation, vascular remodeling of the hepatic sinusoidal microvasculature, intussusceptive angiogenesis, and dysregulation of ephrinB2/EphB4 and endothelial tyrosine kinase. Time-course experiments revealed that vascular changes preceded node transformation. *MxCre Notch1*$^{lox/lox}$ mice had reduced endothelial fenestrae and developed portal hypertension and hepatic angiosarcoma over time. In contrast, mice with hepatocyte-specific disruption of *Notch1* had a normal phenotype. **CONCLUSIONS: Notch1 signaling is required for vascular homeostasis of hepatic sinusoids; it maintains quiescence and differentiation of LSECs in adult mice. Disruption of Notch1 signaling in LSECs leads to spontaneous formation of angiosarcoma, indicating its role as a tumor suppressor in the liver endothelium.**

*Keywords:* Vascular Tumor; Liver Cancer; Ephrin Signaling; Sinusoidal Capillarization.

The hepatic sinusoidal microvasculature is composed of a highly specialized and differentiated endothelium characterized by a discontinuous, fenestrated endothelial lining without a basement membrane.[1] The liver sinusoidal endothelial cells (LSECs) comprise half of the nonparenchymal cells of the liver and play a central role in many physiological functions, including liver organogenesis, liver regeneration, control of the vasomotor tone, scavenger functions, blood cell trafficking, prevention of hepatic stellate cell (HSC) activation, and production of paracrine factors such as hepatocyte growth factor and interleukin-6.[1–5] Chronic liver disease can lead to endothelial dysfunction and dedifferentiation of LSECs with loss of fenestrations, deposition of a basement membrane, and surface expression of CD31, a process that has been termed *sinusoidal capillarization* and that precedes liver fibrosis.[6,7] The determinants regulating the normal, differentiated LSEC phenotype are only incompletely understood. Paracrine secretion of vascular endothelial growth factor (VEGF) by hepatocytes and HSCs as well as autocrine production of nitric oxide by endothelial NO synthase have been shown to be essential to maintain the phenotype of LSECs.[6] However, other signaling pathways, the absence of shear stress, and interendothelial and heterotopic contact with HSCs might be additional important determinants of LSEC differentiation.

The Notch signaling pathway is an intercellular signaling mechanism that controls endothelial cell differentiation, arteriovenous specification, and vascular development.[8,9] Notch signaling mediates intercellular communication through membrane-bound receptors (Notch1–4) and ligands (Jagged-1, -2, and Delta-like-ligand [Dll]1, 3, and 4).[8,10] The Notch signaling pathway involves ligand-induced activation of the receptor, proteolytic cleavage, and subsequent translocation of its intracellular domain (NICD) to the nucleus, where it acts as a transcriptional regulator. Notch1 is expressed in normal liver and has been detected in hepatocytes, biliary epithelial cells, and LSECs.[11] Jagged-1 and Notch2 mutations have been shown to cause Alagille syndrome, a condition leading to bile duct paucity and cardiovascular defects at birth.[12,13] Notch1, 3, and 4 and their ligands Jagged-1, Jagged-2, and Dll4 are expressed in blood vessels.[14] Mutations in

*Abbreviations used in this paper:* BrdU, 5-bromo-2'-deoxyuridine; DAPT, N-[(3,5-difluorophenyl)acetyl]-L-alanyl-2-phenyl]glycine-1,1-dimethylethyl ester; Dll4, Delta-like-ligand-4; ECM, endothelial cell medium; HSC, hepatic stellate cell; KO, knockout; LSEC, liver sinusoidal endothelial cell; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NRH, nodular regenerative hyperplasia; pIpC, polyiosinic-polycytidylic acid; SEM, scanning electron microscopy; VEGF, vascular endothelial growth factor; WT, wild-type.


0016-5085/$36.00
doi:10.1053/j.gastro.2011.12.052

Notch3 cause inherited vascular diseases, such as the degenerative vascular disorder cerebral autosomal-dominant arteriopathy with subcortical infarcts and leukoencephalopathy.[8] However, the function of Notch1 in the liver is unknown.

We previously reported that knockout of the receptor Notch1 in mice leads to development of nodular regenerative hyperplasia (NRH) in the liver.[15] NRH is a specific histopathologic entity in human beings that can be complicated by portal hypertension; however, the etiology and pathogenesis of NRH remain unclear. Several reports have linked NRH to vascular injury of the sinusoidal microcirculation (ie, by toxins, drugs, arteritis, or thromboembolic events).[16–19] The observation that the final common pathway of NRH might be an endotheliopathy and that Notch1 signaling is crucial for vascular differentiation led us to explore the role of Notch1 as a regulator of LSEC phenotype in the sinusoidal microcirculation.

In the present study, we show that Notch1 signaling is required for vascular homeostasis of hepatic sinusoids by inducing quiescence and differentiation of LSECs in adult mice. Disruption of the Notch1 pathway leads to LSEC proliferation, vascular remodeling, capillarization with loss of fenestrae, and to the development of portal hypertension and, eventually, angiosarcoma.

## Materials and Methods

### *Animals, Induction of Notch1 Deletion, Genotyping, and Southern Blotting*

MxCre Notch1$^{lox/lox}$ mice on a C57Bl/6 background carry the Cre-recombinase under the murine Mx1 promoter. To induce recombination, 300 $\mu$g of polyiosinic-polycytidylic acid (pIpC) (InvivoGen, San Diego, CA) was injected intraperitoneally in 4-week-old mice at days 0, 3, and 6, resulting in efficient deletion of *Notch1* in the liver already after 1 day.

Hepatocyte-specific Notch1 knockout (KO) mice were generated by crossing MxCre- Notch1$^{lox/lox}$ mice with AlbCre$^{+/-}$ mice on a C57Bl/6 background (Jackson Laboratory, Bar Harbor, ME), expressing Cre-recombinase under the hepatocyte-specific albumin promoter. Single transgenic Notch1$^{lox/lox}$ or Cre+ littermates of each strain were used as controls. Newborn mice were genotyped as previously described.[20] The efficiency of *Notch1* deletion was checked regularly by DNA extraction from livers and subsequent Southern blot analysis with a Notch1-specific probe as previously described.[20] *Notch1* deletion was consistent in LSECs and hepatocytes during the whole observation period.

Animals were maintained in the animal facility of the Department of Biomedicine at the University Hospital Basel, in a specific-pathogen-free environment on a 12-hour light and 12-hour dark schedule. Food and drinking water were provided ad libitum. All experiments were approved by the veterinary office of Basel.

### *Macroscopic and Microscopic Assessment of Livers After Notch1 Deletion*

Mice were harvested at different time points after recombination as indicated in the text. After measuring the body weight, the livers were removed, assessed macroscopically, and weighed. From all livers, parts were immediately shock frozen for further analysis, the rest was fixed in 4% formaldehyde and processed for standard histologic assessment with H&E staining.

For 5-bromo-2'-deoxyuridine (BrdU) labeling of cells in the S phase of the cell cycle, mice were given 0.8 mg/mL of BrdU (Sigma, Buchs, Switzerland) by drinking water for 1 week. BrdU incorporation was detected by immunohistochemistry with an anti-BrdU antibody (Roche Diagnostics, Rotkreuz, Switzerland) and avidin biotin complex (ABC-Eite; Vectra Laboratories, Geneva, Switzerland). The average amount of positive hepatocytes and LSECs was assessed in 10 high-power fields by 2 independent observers (M.T.D., D.S.) in a blinded fashion.

For immunohistochemistry, formalin-fixed and paraffin-embedded 4-$\mu$m–thick mouse liver sections were dewaxed, rehydrated, pretreated for 5 minutes in an autoclave with Tris/EDTA/citrate (TEC) buffer, pH 8, incubated with the respective primary antibody (Supplementary Table 1) and counterstained with hematoxylin. Standard indirect immunoperoxidase procedures were used (ABC-Elite).

### *Measurement of Portal Vein Pressure*

Wild-type (WT) and KO mice 2, 8, and 16 weeks after pIpC injection and *Notch1* deletion, were anesthetized with an intraperitoneal injection of 2 $\mu$g/g body weight of a narcotics mix (medetomidine 1 mg/mL, climazolam 10 mg/mL, fentanyl 50 $\mu$g/mL). A midline laparotomy was performed and the portal vein was cannulated through the superior mesenteric vein with a 30-gauge needle connected to a highly sensitive pressure transducer (MLT1050 Pressure Transducer; AD Instruments, Spechbach, Germany). The external zero reference point was placed at the midportion of the animal. The measurements were recorded on a multichannel computer-based recorder.

### *Fluorescein Isothiocyanate–Formaldehyde–Treated Serum Albumin Monomer Application*

Eight weeks after pIpC injection, WT and KO mice were anesthetized as described. A midline laparotomy was performed and 1.12 $\mu$g/g body weight of fluorescein isothiocyanate–formaldehyde–treated serum albumin monomer (a gift from Dr Bard Smedsrød, University of Tromsø, Norway)[21] was injected into the portal vein. Thirty minutes later the mice were exsanguinated and the livers were harvested. Sections were obtained as described earlier and analyzed by fluorescence microscopy.

### *Scanning Electron Microscopy*

Methylmethacrylate corrosion casts were performed as followed: the vasculature of the murine liver was perfused in situ via the portal vein with a freshly prepared solution of Methylmethacrylate (Mercox, Vilene Company, Tokyo, Japan) containing 0.1 mL of accelerator per 5 mL of resin. One hour after perfusion, the livers were excised and transferred to a 15% KOH solution for the dissolution of the tissue, which was achieved within 3–4 weeks. After washing, casts were dehydrated in ethanol and dried in a vacuum desiccator.

For normal scanning electron microscopy (SEM) analysis, the liver was perfused with fixative (2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer [pH 7.4, 350 mOsm]). Then the liver was excised, cut into small pieces, and immersed in the same fixative as used for perfusion. Samples then were processed as the casts. All samples were mounted on aluminium stabs, sputter-coated with gold, and viewed under a Philips XL 30 Super FEG scanning electron microscope (Philips, Zurich, Switzerland). Sinusoidal diameters were evaluated by measuring the narrowest site between two branching points using CellP soft-

ware (Olympus Soft Imaging System, Muenster, Germany). Branching was assessed by counting branching points and normalizing to the total tube length of the capillary network in calibrated TIFF files.

To evaluate fenestrae and sieve plates, planar endothelial areas were photographed and saved as calibrated TIFF files; fenestrae and sieve plates were quantified and morphometrically analyzed in representative areas using CellP software.

### *Cell Culture*

Isolated primary human LSECs (ScienCell, Carlsbad, CA) were used between passages 2 and 6. Their phenotypic characterization has been described previously.[22] LSECs were cultured in endothelial cell medium (ECM; ScienCell), and supplemented with 10% fetal bovine serum (FBS), L-glutamine (1 mmol/L), penicillin (100 IU/mL), and streptomycin (100 $\mu$g/mL). In experiments investigating supernatant from murine hepatocytes, transformed murine LSECs (a gift from Dr Vijay Shah, Mayo Clinic) were used as previously described.[23]

### *LSECs, HSCs, and Hepatocyte Isolation and Conditioned Medium*

LSECs, HSCs, and hepatocytes were isolated from Notch1 KO and WT mice and purified as previously described.[24] Briefly, livers were perfusion-digested with collagenase (Liberase TM Research Grade; Roche, Basel, Switzerland) followed by separation of hepatocytes and nonparenchymal cells by centrifugation. LSECs and HSCs were isolated further by centrifuging over a Percoll gradient and purified by selective adhesion. Purity of isolated cells was verified by fluorescein isothiocyanate–FSA uptake in LSECs, by retinoid autofluorescence in HSCs, and by hepatocyte nuclear factor 4 alpha (Supplementary Table 1) immunostaining in hepatocytes, confirming greater than 90% purity. Isolated cells were used immediately for RNA extraction. Isolated hepatocytes and HSCs also were cultured in plastic culture dishes containing Dulbecco's modified Eagle medium overnight. Hepatocytes subsequently were incubated with serum-free ECM. After 24 hours supernatant was harvested, filtered through a 0.2-$\mu$m filter, and aliquots were stored at $-20$°C. HSCs were used further for co-culture tube formation assay.

### *3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Assay*

Cell viability and proliferation of murine LSEC was assessed by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. LSECs were seeded in 96-well plates in complete ECM. After 4 hours cells were washed twice and incubated overnight at 37°C with conditioned medium derived from Notch1 KO or WT hepatocytes. FBS–free ECM was used as a negative control. Cells then were incubated for 24 hours at 37°C with 333 $\mu$g/mL MTT solution. Absorbance was measured at 490 nm.

### *Vascular Tube Formation Assay*

Cultured LSECs were seeded on Matrigel-coated, 4-well chamber slides (Lab-Tek, Fisher Scientific, Wohlen, Switzerland) with a total of $2 \times 10^4$ cells per well.[22] The complete wells were photographed after 16 hours of incubation at 37°C in FBS–free ECM. Total vascular tube length per well was analyzed digitally using the software CellP. In some experiments, LSECs were incubated with the Notch inhibitor N-[(3,5-difluorophenyl) acetyl]-L-alanyl-2-phenylglycine-1,1-dimethylethyl-ester at 1 $\mu$mol/L (DAPT, Sigma) versus dimethyl sulfoxide or co-cultured with isolated HSCs in a ratio of 3:1.

### *RNA Extraction, Quantitative Reverse-Transcription Polymerase Chain Reaction*

Total RNA was extracted from isolated cells by column separation and DNase treatment (Nucleospin kit; Machery-Nagel, Oensingen, Switzerland) according to the manufacturer's instructions. A total of 1 $\mu$g of RNA was reverse-transcribed and real-time polymerase chain reaction was performed using the SYBR green polymerase chain reaction master mix (Applied Biosystems, Foster City, CA). Intron-spanning primers are listed in Supplementary Table 2. All reactions were run in duplicate using a 7500 Fast Real-Time Polymerase Chain Reaction System (Applied Biosystems). Messenger RNA (mRNA) expression levels of the transcripts were normalized to RPL19 using the $\Delta$Ct method.

### *Statistical Analysis*

Comparisons were performed by the Student *t* test (unless otherwise specified) using Graph Pad Prism 4.0 software (in the figures *designates statistical significance when $P < .05$, **$P < .01$, and ***$P < .001$, respectively).

## Results

### *Deletion of Notch1 Leads to LSEC Activation and Portal Hypertension*

We previously reported that deletion of *Notch1* in MxCre Notch1$^{lox/lox}$ mice, further described as KO mice, leads to increased liver weight, hepatocyte proliferation, nodular transformation, and phenotypic changes identical to NRH described in patients[15] (Figure 1*A–C*). To explore the concept, that a cause of NRH might be a dysfunctional endothelium in the liver, we examined the sinusoidal compartment in these KO mice. KO mice showed areas with widened sinusoidal spaces located in the periphery of the nodules (Figure 1*B*). LSECs were found to have increased proliferation rates 14 days after pIpC injection, suggesting persistent activation (Figure 1*C* and *E*). Increased hepatocyte proliferation occurred already after 6 days and was localized mainly in the center of the nodules (Figure 1*C* and *D*). Because portal hypertension is a known complication of NRH, we assessed portal pressure in KO mice with fully developed NRH by cannulation of the portal vein 16 weeks after pIpC injection. In comparison with controls, KO mice developed a significant increase in portal pressure (Figure 1*F*). Unexpectedly, portal pressure already was increased significantly before the onset of NRH (ie, 2 weeks after pIpC injection; Figure 1*F*), suggesting the presence of increased intrahepatic vascular resistance before nodular transformation of the liver parenchyma.

### *Loss of Notch1 Signaling Induces Vascular Remodeling Through Intussusceptive Angiogenesis*

Next, to explore the 3-dimensional morphology of the hepatic microcirculation, we studied vascular casts by SEM. Surprisingly, KO mice showed extensive remodeling of the sinusoids with a plexus-like appearance visible as early as 14 days after pIpC injection

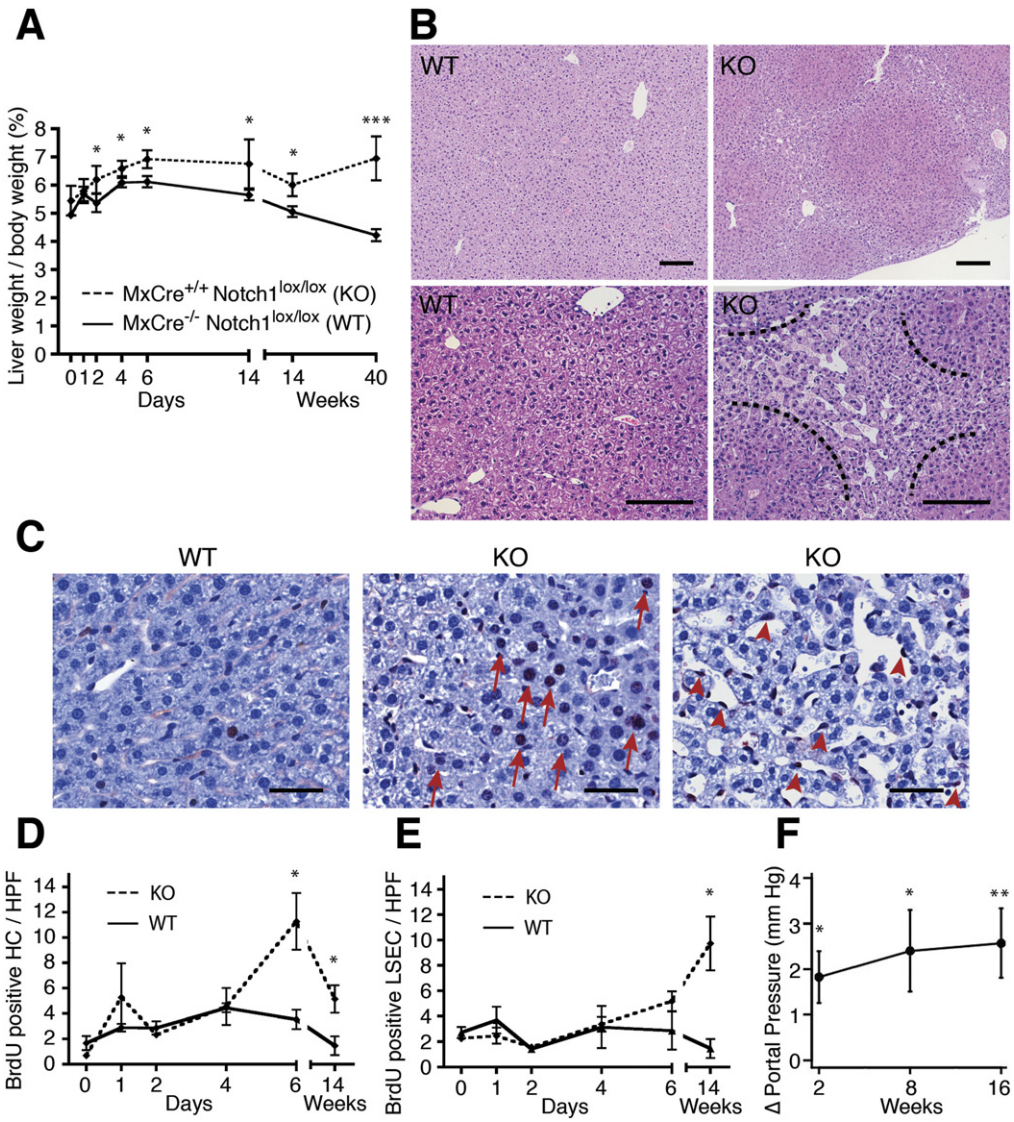



**Figure 1.** Notch1 knockout in MxCre mice leads to hepatocyte and LSEC proliferation and development of increased portal vein pressure. (*A*) Development of liver weight vs body weight ratio in MxCre Notch1$^{lox/lox}$ (KO) and MxCre- Notch1$^{lox/lox}$ (WT) mice over time after the first intraperitoneal pIpC injection (n ≥ 7 animals per time point, mean with standard deviation). (*B*) H&E-stained liver sections of KO mice 14 weeks after recombination. *Black dotted lines* outline regenerative nodules. At the periphery of nodules, dilated sinusoids can be identified *(bar = 250 μm)*. (*C*) BrdU (0.8 mg/mL) was given by drinking water 7 days before the harvest. Liver sections were stained for nuclear BrdU incorporation *(bar = 100 μm)*. KO mice had BrdU-positive hepatocytes localized in the regenerative nodules (*arrows*), whereas positive LSECs were found mainly in the dilated sinusoids at the periphery of the nodules (*arrowheads*). Shown are representative pictures 14 weeks after pIpC injection. (*D*) The average amount of BrdU-positive hepatocytes in 10 random high-power fields per animal was assessed and showed increased hepatocyte proliferation rate (n ≥ 6 animals per time point, mean with standard error of the mean). (*E*) BrdU-positive LSECs were quantified as described in *panel D* and showed, compared with hepatocytes, a delayed but significant increase in proliferation rate. (*F*) KO mice developed increased portal vein pressure. The pressure was measured by canulation of the portal vein at the indicated time points after pIpC injection (n ≥ 4 animals per time point, mean difference in comparison with controls with standard deviation).



(Figure 2*A* and *B*). Further, casts already revealed abundant formation of pores 2 days after pIpC injection (Figure 2*A* and *B*, *arrowheads*), which are the hallmark of intussusceptive angiogenesis. This is in contrast to sprouting angiogenesis, which is the primary mode of blood vessel formation (by endothelial cell proliferation, migration, sprout formation with recanalization). Intussusceptive angiogenesis on the other hand defines the angiogenic process in which transluminal tissue pillars develop within capillaries, small arteries, and veins (Figure 2*C*), and subsequently fuse, thus delineating new vascular entities or resulting in vessel remodeling (Supplementary Figure 1). The process of angiogenesis and vascular remodeling led to an increased variation of the sinusoidal diameter (Figure 2*D*) and increased branching within the microcirculation (Figure 2*E*). Accordingly, complementary experiments with isolated human LSECs showed increased vascular tube formation after pharmacologic Notch inhibition by the γ-secretase inhibitor DAPT in vitro (Figure 2*F*).

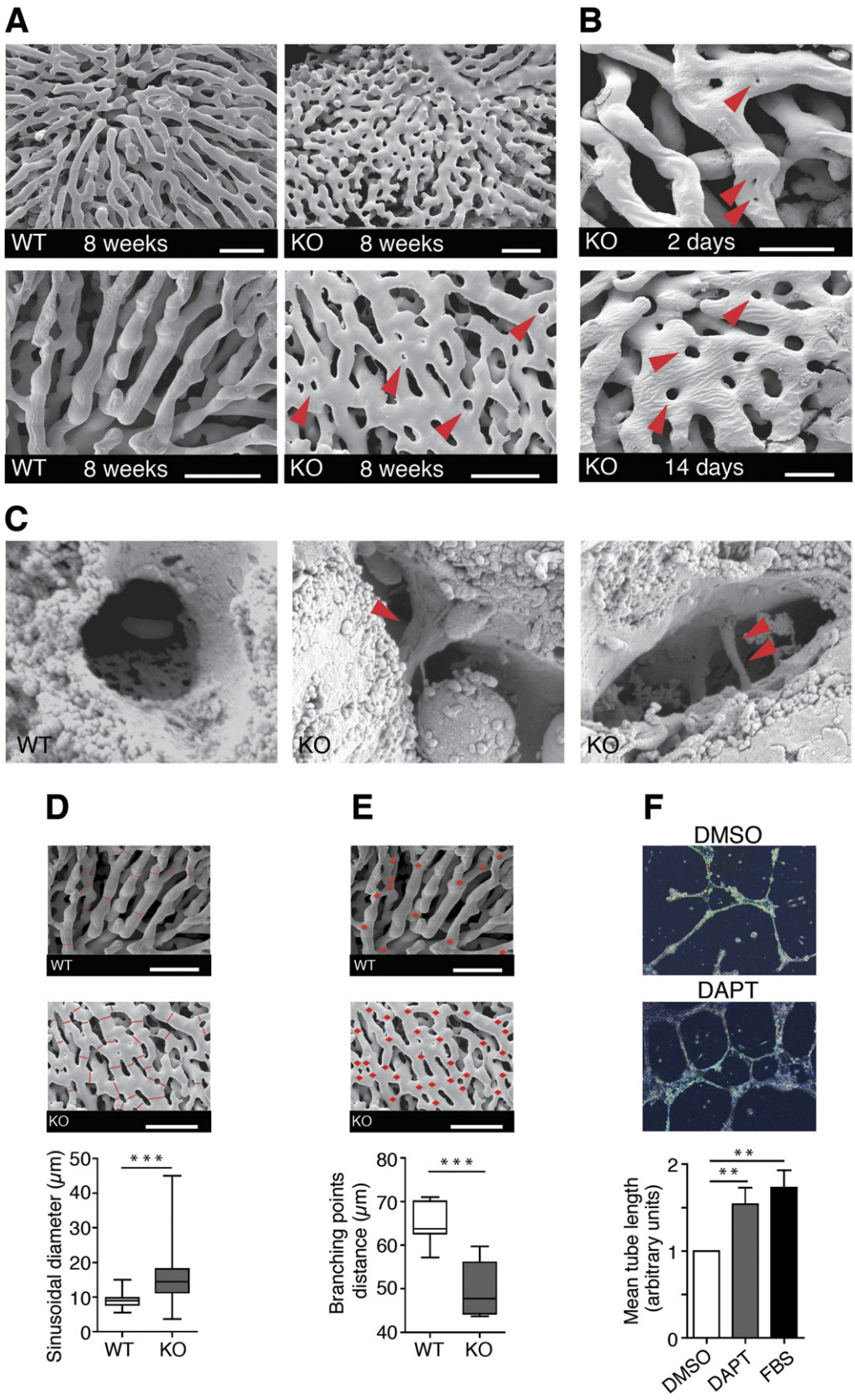



**Figure 2.** Loss of Notch1 signaling leads to vascular remodeling through intussusceptive angiogenesis. (*A*) SEM analysis of vascular casts in WT and KO mice 8 weeks after pIpC injection showed architectural changes of the hepatic microcirculation in KO mice (low magnification at top, higher magnification at bottom; *bar = 50 μm*). At higher magnification, casts revealed pores (*red arrowheads*), the hallmark of intussusceptive angiogenesis. (*B*) Time-course SEM analysis in KO revealed pore formation (*red arrowheads*) 2 days after the first pIpC injection and partial remodeling of the sinusoids after 2 weeks (bar = 20μm). (*C*) Endovascular pillars, the corresponding intraluminal structures to the pores of intussusception, were found in KO SEM samples but not in WT 2 weeks after pIpC injection (*middle picture* with adjacent erythrocyte of ~7.5 μm next to the pillar). (*D*) Sinusoidal diameters were analyzed in mice 8 weeks after pIpC injection by measuring the diameter of the sinusoids between 2 branching points at the narrowest site. Deletion of *Notch1* led to a strong variation in diameters with a significantly increased average diameter (n = 3 animals per group with > 250 data points assessed per group; $P < .0001$, Mann–Whitney test). (*E*) Branching points of the sinusoidal vascular network were quantified in mice 8 weeks after pIpC injection (*red diamonds*) by dividing the total tube length of the vessels by the quantified branching points within the network. *Notch1* deletion led to increased branching with shorter vascular intersections. (*F*) Pharmacologic inhibition of Notch signaling by DAPT (1 μmol/L) led to increased angiogenesis of primary human LSECs in vitro. Total vascular tube formation of human LSECs on Matrigel in 4-well chamber slides was quantified digitally after 16 hours of incubation (3 independent experiments in duplicates).

### *Notch1 Signaling Is Essential for LSEC Differentiation*

The hepatic microcirculation is a highly differentiated endothelium characterized by the presence of fenestrae, sieve plates, and scavenger receptor activity. We therefore analyzed whether these LSEC-specific features are influenced through Notch1 signaling. The deletion of *Notch1* induced an almost complete loss of fenestrae and

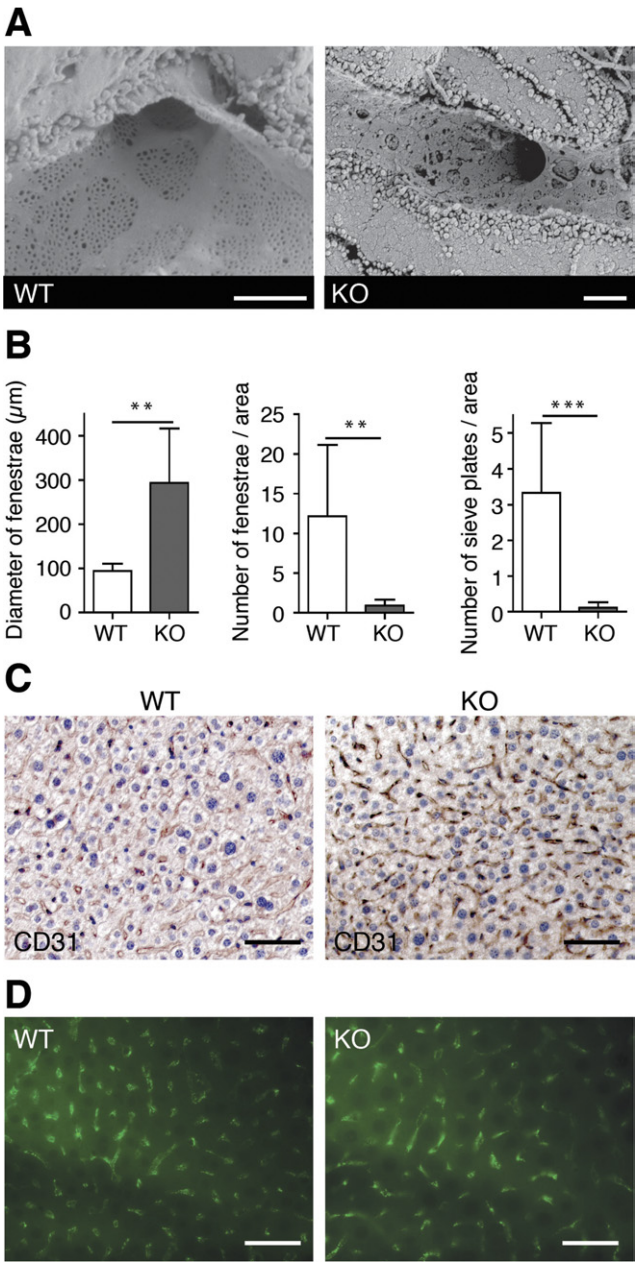



**Figure 3.** Notch1 signaling is essential for LSEC differentiation. (*A* and *B*) SEM of hepatic sinusoids revealed a significant reduction of fenestrae and sieve plates after loss of *Notch1* by morphometric analysis *(bar = 2 μm)*. The remaining fenestrae in KO mice had a significantly increased diameter (n = 4 animals per group, 2 weeks after pIpC injection). (*C*) Immunohistochemistry for the endothelial surface marker CD31 showed up-regulation in the membrane of LSECs of KO mice in comparison with WT *(bar = 100 μm*; 12 weeks after pIpC injection), indicating dedifferentiation and capillarization of the sinusoids. (*D*) LSEC-specific uptake of fluorescein isothiocyanate–serum albumin was not influenced after deletion of *Notch1*, indicating normal scavenger function *(bar = 100 μm*; 12 weeks after pIpC injection).

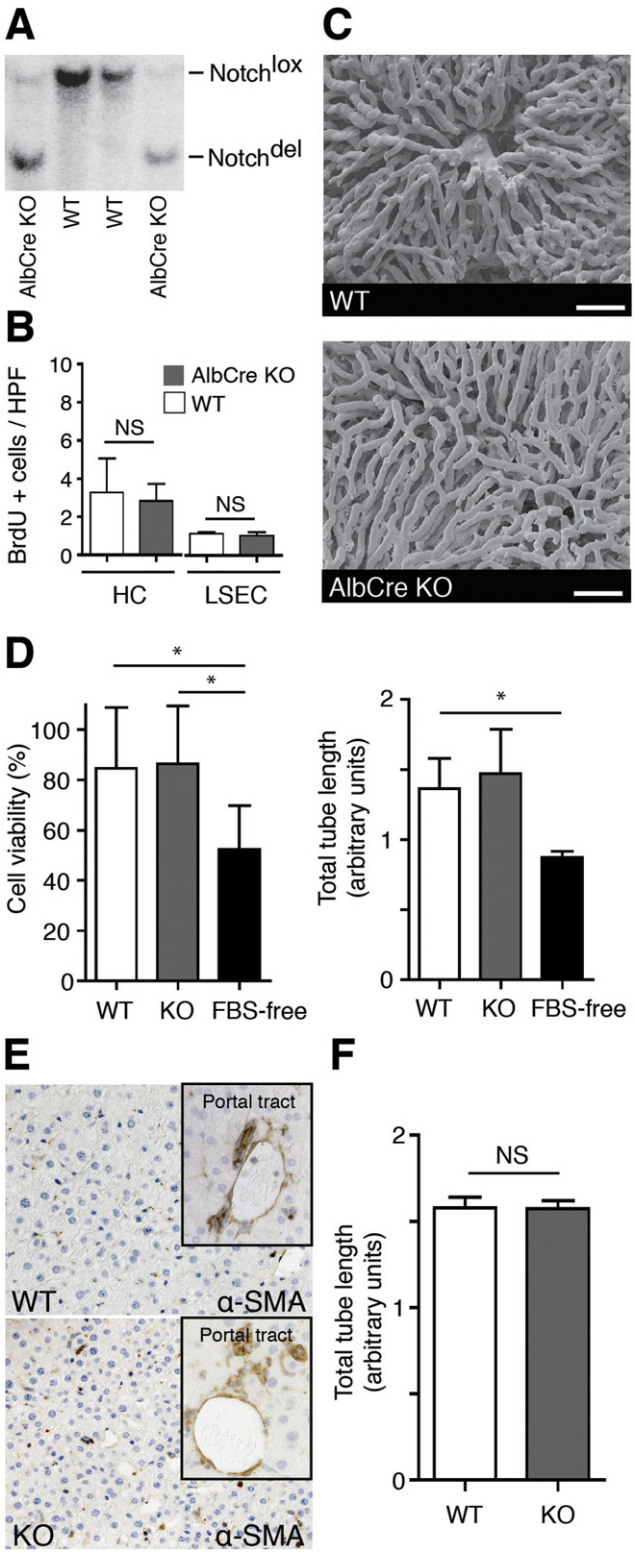
sieve plates within 2 weeks (Figure 3*A* and *B*). The remaining fenestrae were characterized by a significant increase in diameter (Figure 3*B*). This dedifferentiation was evidenced further by up-regulation of CD31, which is a marker of capillarization in diseased LSECs in disorders such as fibrosis and hepatocellular carcinoma and have been shown to be correlated inversely with LSEC fenestration[6] (Figure 3*C*). To assess another specific function of LSECs, namely the clearance of certain macromolecules by scavenger receptors,[21] we studied the uptake of fluorescein isothiocyanate–albumin after portal vein injection by fluorescence microscopy (Figure 3*D*). This specific function remained unchanged after *Notch1* KO, highlighting that LSECs after loss of *Notch1* retain a certain degree of differentiation and functionality.

To exclude the possibility that the observed changes of the hepatic microvasculature in MxCre Notch1$^{lox/lox}$ mice are only secondary to effects on hepatocytes, we generated a hepatocyte-specific knockout by crossing Notch1$^{lox/lox}$ with AlbCre-positive mice (AlbCre KO mice). The successful recombination was confirmed by Southern blot (Figure 4*A*). These hepatocyte-specific *Notch1* KO mice were phenotypically normal (liver parenchyma without nodularity microscopically and macroscopically, normal liver weight, normal vascular morphology) and showed no increased proliferation rates of hepatocytes or LSECs at the same time points as in MxCre KO mice (Figure 4*B* and *C*, and data not shown). Isolated LSECs incubated with conditioned media from normal versus MxCre *Notch1* KO primary hepatocyte cultures showed no difference in the MTT proliferation assay or in vitro capillary tube formation (Figure 4*D*). Expression of mitogens such as VEGF, fibroblast growth factor, angiopoietins, and transforming growth factor $\beta$1 also were unchanged in isolated hepatocytes and HSCs, making a paracrine effect in vivo in our model unlikely (Supplementary Figure 2). Finally, the absence of HSC activation by $\alpha$-smooth muscle actin immunohistochemistry (Figure 4*E*), the complete lack of fibrosis on histology, and the missing effect of co-cultured *Notch1*-deficient HSCs on in vitro angiogenesis (Figure 4*F*) make HSC as drivers of the vascular remodeling in our model unlikely.

### *Interruption of Notch1 Signaling Leads to Regulation of the LSEC Receptors EphrinB2/EphB4*

Prior studies have delineated the role of Notch signaling in functional vessel patterning and differentiation in developmental angiogenesis[8]; however, Notch signaling in LSECs of the adult liver is less well defined. Expression analysis of Notch members in isolated LSECs and hepatocytes from WT and KO animals showed 7-fold increased *Notch1* mRNA levels in WT LSECs versus hepatocytes (Figure 5*A* and Supplementary Figure 3*B*). Deletion was almost complete as early as 3 days after pIpC injection (Figure 5*A* and Supplementary Figure 3*B*). To assess how *Notch1* deletion induces vascular remodeling, we analyzed genes known to promote vascular growth and maturation. We found a significant and persistent down-regulation of *Dll4*, as well as the receptor tyrosine kinase *EphrinB2* in LSECs, whereas angiopoietins, fibroblast growth factors, VEGF, and VEGF-receptor 2 were not regulated after Notch1 deletion (Figure 5*B* and Supplementary Figures 2 and 3). Endothelial tyrosine kinase

(Tek) was down-regulated significantly only after 12 weeks, which might be a secondary effect after vascular remodeling and development of NRH. A decrease of EphrinB2 after Notch1 KO could not be detected by immunohistochemistry; however, EphB4 was strongly up-regulated in the endothelial lining of the sinusoids in comparison with controls (Figure 5C).




### *Notch1 KO Mice Develop Hepatic Angiosarcomas*

Interestingly, long-term experiments revealed spontaneous development of hepatic tumors in Notch1 KO mice 50 weeks after pIpC injection with a penetrance of 86%, whereas none of the Cre- mice showed any phenotypical changes (Figure 6A). Histologic assessment further characterized these tumors as angiosarcomas with highly dysplastic endothelial cells as confirmed by CD34 staining (Figure 6B and C).

## Discussion

Mammalian endothelial cells display remarkable heterogeneity in structure and function in different organs.[1] The molecular determinants of the organ-specific phenotype of endothelial cells are poorly understood. We investigated the role of Notch1 receptor, which is known to regulate arteriovenous differentiation during embryogenesis, in the hepatic endothelium of adult mice. Our findings provide loss-of-function evidence that Notch1 is required for vascular homeostasis of hepatic sinusoids by inducing quiescence and differentiation of LSECs. Disruption of the Notch1 pathway leads to intussusceptive angiogenesis, LSEC proliferation, loss of fenestrae, sinusoidal capillarization with portal hypertension, and, eventually, malignant transformation (Figure 7).

The hepatic microcirculation is composed of highly differentiated endothelial cells with distinct gene and protein expression profiles as well as a distinct phenotype characterized by fenestrations, sieve plates, and lack of a basement membrane.[1,25] In the adult, LSECs acquire a quiescent, nonangiogenic state. Nevertheless, LSECs retain considerable proliferation and growth potential, which is activated during physiological processes such as liver regeneration as well as in pathologic conditions such as tumor angiogenesis in hepatocellular carcinoma. Signaling pathways that activate LSECs (ie, VEGF, placental

**Figure 4.** Phenotypic changes in the liver are not hepatocyte- or HSC-driven. (*A*) Notch1$^{lox/lox}$ mice were crossed with mice carrying Cre under control of the albumin promoter (AlbCre KO). Southern blot analysis of liver homogenates in 8-week-old mice proved successful recombination (Notch$^{del}$). The remaining Notch$^{lox}$ band reflects mainly the nonhepatocyte cell population in the liver homogenate. (*B*) The average amount of BrdU-positive hepatocytes and LSECs in 10 high-power fields in liver sections of AlbCre KO mice (16 weeks) was assessed and showed no difference in proliferation rate (n = 5 animals per genotype). (*C*) Morphology of vascular casts from 12-week-old AlbCre KO mice was normal *(bar = 50 μm)*. (*D*) Cell viability of transformed murine LSECs assessed by MTT assay and tube length formation assessed by tube formation assay showed no significant difference after being treated for 24 hours with conditioned fetal bovine serum (FBS)-free media derived from isolated primary hepatocytes from either WT or MxCre Notch1$^{lox/lox}$ (KO) mice (3 independent experiments in duplicates). (*E*) Quantification of activated HSC by α-smooth muscle actin (α-SMA) immunohistochemistry showed no difference between WT and MxCre Notch1$^{lox/lox}$ (KO) mice (12 weeks after pIpC injection). (*F*) Tube formation of murine LSECs co-cultured with isolated HSCs (3 days after pIpC injection) was not different (3 independent experiments in triplicates).

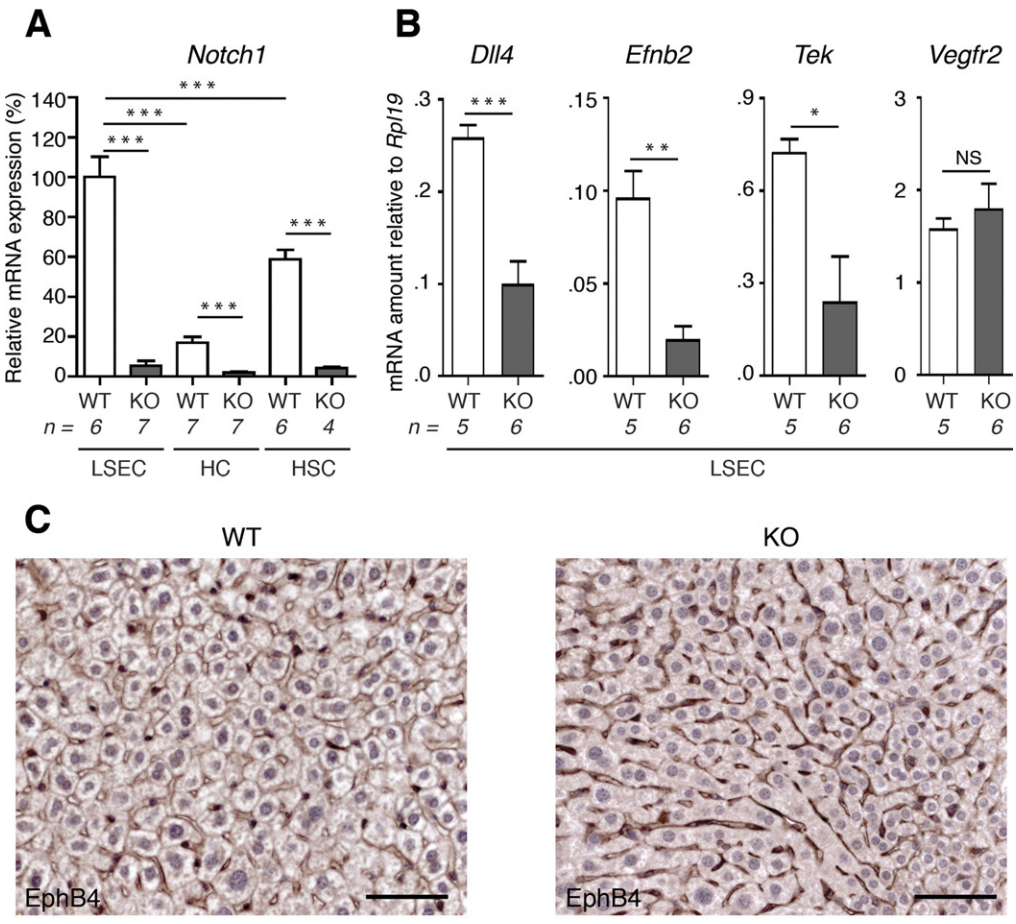



**Figure 5.** Interruption of Notch1 signaling leads to LSEC activation through regulation of EphrinB2/EphB4. (*A*) Quantitative reverse-transcription polymerase chain reaction analysis of *Notch1* mRNA in isolated LSECs, hepatocytes, and HSCs of WT and KO mice relative to the amount in WT LSECs after normalization to *Rpl19*. (*B*) mRNA expression of *Dll4*, *Efnb2*, *Vegfr2*, and Tek from isolated LSECs (n indicated below the plots). Values are normalized to *Rpl19* expression. *Bars* show mean with standard error of the mean. (*C*) EphB4 immunohistochemistry showed increased staining in the LSECs of KO mice *(bar = 100 μm)*. All analyses were performed with mice 12 weeks after pIpC injection.

growth factor) have been studied extensively.[7] However, signals that induce and maintain quiescence and differentiation of LSECs are poorly understood. An important signaling pathway for differentiation in endothelial and vascular smooth muscle cells during vascular development is Notch, which controls endothelial cell migration, arteriovenous specification, and regulation of blood vessel sprouting and branching during normal and pathologic angiogenesis.[8,26] KO mice that lack components of the Notch pathway have severe vascular defects and die in utero.[26] There are only limited studies on the function of Notch in the adult and differentiated vasculature. Carlson et al[27] reported that endothelial expression of constitutively active Notch4 elicits reversible arteriovenous malformations in the liver, uterus, and skin of adult mice. The precise molecular function of Notch signaling in the vascular specification remains unknown. Several developmental studies have shown that Notch modulates the VEGF-VEGF-receptor signaling and Ephrin signaling in endothelial cells, which are crucial for proliferation, migration, differentiation, and endothelial cell survival.[26] In isolated LSECs of our *Notch1* KO mice, we have found a down-regulation of *Dll4*, Tek, and *EphrinB2* (Figure 5). Tek is an important receptor tyrosine kinase in differentiated endothelial cells and has been shown to regulate vessel maturation and quiescence.[28] The role of the tyrosine kinase receptor EphB4 and its ligand ephrinB2 in the hepatic microcirculation is not well defined. Up-regulation of EphB4 expression was detected in hepatic sinusoidal vessels during vascular remodeling in rats after carbon tetrachloride–induced liver injury or bile duct ligation.[22,29] Das et al[29] showed that ephrinB2/EphB4 signaling promotes chemotaxis of LSECs through a pathway that involves extracellular regulated kinase (Erk), Kruppel-like factor 2, and VEGF. In gain- and loss-of-function experiments, Notch, through the ephrinB2/EphB4 pathway, recently was found to be the molecular regulator in the balanced growth of arteries and veins during early development.[30,31] Notch signaling controls this equilibrium by regulating vascular size and by promoting arterial differentiation, thereby dictating the ratio of arterial to venous ECs. EphrinB2/EphB4 signaling regulates this balance by sorting differential ECs into the respective vessels.[30,31] Hence, our study in the liver microcirculation of adult mice supports this concept, that Notch through ephrinB2/EphB4 signaling regulates vascular differentiation and size.

Interestingly, SEM analysis in our study revealed morphologic characteristics of an alternative form of angiogenesis after *Notch1* KO, termed *intussusceptive angiogenesis* (Figure 2). Generation of new vessels by intussusception is a unique mechanism of vascular growth that allows rapid expansion and remodeling of microvessels. This mode of angiogenesis was first described in the vasculature of

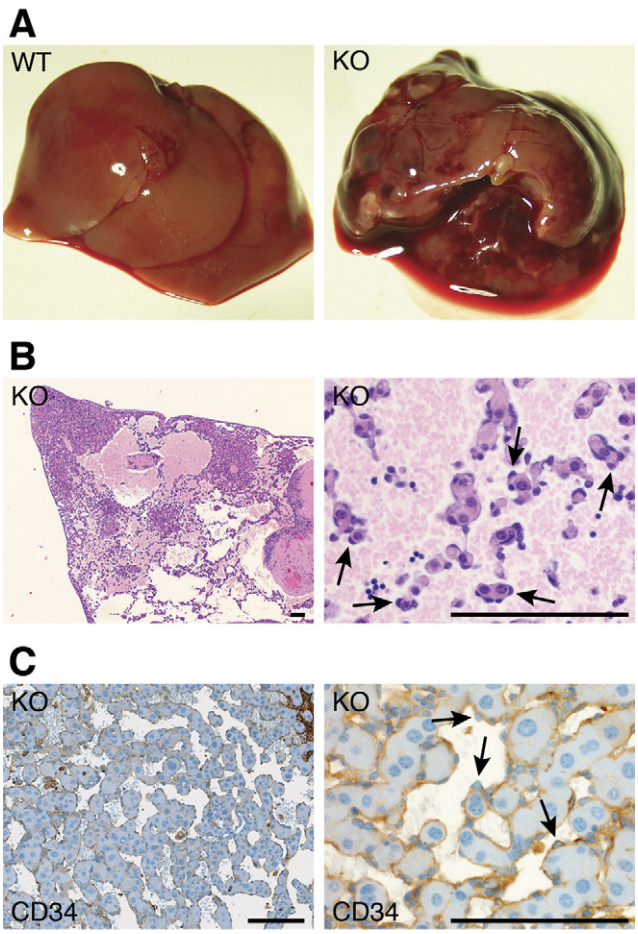



**Figure 6.** Notch1 KO mice develop hepatic angiosarcomas. (*A*) Macroscopic images of livers 50 weeks after plpC injection, showing cavernous, blood-filled tumors in the KO. (*B*) H&E and (*C*) CD34 immunohistochemical staining of hepatic angiosarcomas in KO mice 50 weeks after plpC injection *(bar = 200 μm)*. *Black arrows* indicate endothelial cells with highly dysplastic nuclei.

developing lungs,[32] but also has been found to contribute in physiological angiogenesis during liver regeneration as well as in pathologic angiogenesis in chronic liver disease and hepatocellular carcinoma.[7,33,34] The molecular signaling regulating intussusceptive angiogenesis remains unclear and might involve several angiogenic pathways such as the VEGF and mammalian target of rapamycin pathways.[34,35] Our study suggests that Notch signaling is another, albeit negative, regulator and that absence of the Notch1 receptor in LSECs induces this specific form of angiogenesis and vascular remodeling.

Portal hypertension is a known complication in patients with NRH. In fact, NRH is the most frequent cause of intrahepatic, noncirrhotic portal hypertension in the Western world.[17] In our murine model, we interpret the observed increase in portal pressure even before the onset of NRH as a result of increased intrahepatic resistance induced by frictional forces of the remodeled microcirculation and increased branching points on the blood, which from a fluid dynamics perspective is a visco-elastic fluid. Such a nonideal fluid is subject to a complex rheology with frictional forces caused by shear stress through vessel convergence, divergence, varying diameters, turns, and surface irregularities. Two recent independent computational fluid dynamic studies have confirmed increased shear force in vascular beds with intussusceptive angiogenesis in vivo and in silico.[36,37] However, we cannot exclude that increased splanchnic inflow and the nodular transformation of the liver parenchyma also might contribute to the increased portal pressure in Notch1 KO mice.

Notch1 has been shown to be an oncogene in many solid tumors and in leukemia. Depending on the tissue type, Notch1 also rarely can function as a tumor-suppressor gene

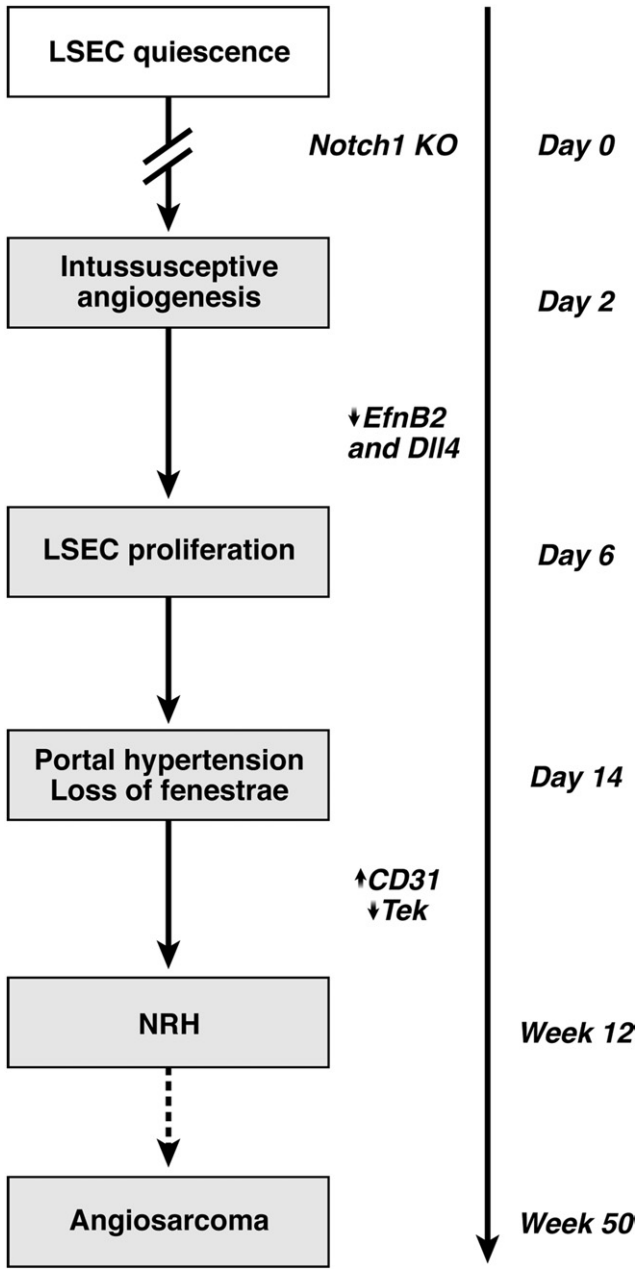



**Figure 7.** Time line of phenotypic changes after Notch1 KO. In adult liver Notch1 is required to maintain endothelial quiescence and differentiation. Disruption of Notch1 signaling induces intussusceptive angiogenesis, proliferation, and portal hypertension, eventually leading to sinusoidal capillarization, NRH, and angiosarcomas.

(ie, in squamous cell carcinoma of skin and lung). Recently, blockade of Dll4 as well as loss of heterozygosity of Notch1 led to vascular tumors in animals.[38,39] In our model, we observed persistent and cell autonomous LSEC proliferation, dedifferentiation, and eventually malignant transformation (Figure 7). Therefore, our findings of spontaneous development of hepatic angiosarcoma establish Notch1 also as a tumor-suppressor gene in LSECs.

Thus, the present study identifies Notch1 as an important signaling pathway in LSECs that governs vascular structure and function in the liver, regulates intussusceptive angiogenesis, and acts as an endothelial tumor-suppressor gene.

## Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2011.12.052.

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Received January 7, 2011. Accepted December 29, 2011.

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*Acknowledgments*

The authors thank Sylvia Ketterer and Ursula Dürmüller for excellent technical assistance and are indebted to Bard Smedsrød and Dmitri Svistounov for providing the fluorescein isothiocyanate–formaldehyde-treated serum albumin monomer and teaching the liver cell isolation method. The authors also thank Vijay Shah and Robert Huebert for providing transformed murine liver sinusoidal endothelial cells.

Michael Dill and Sonja Rothweiler contributed equally to this work.

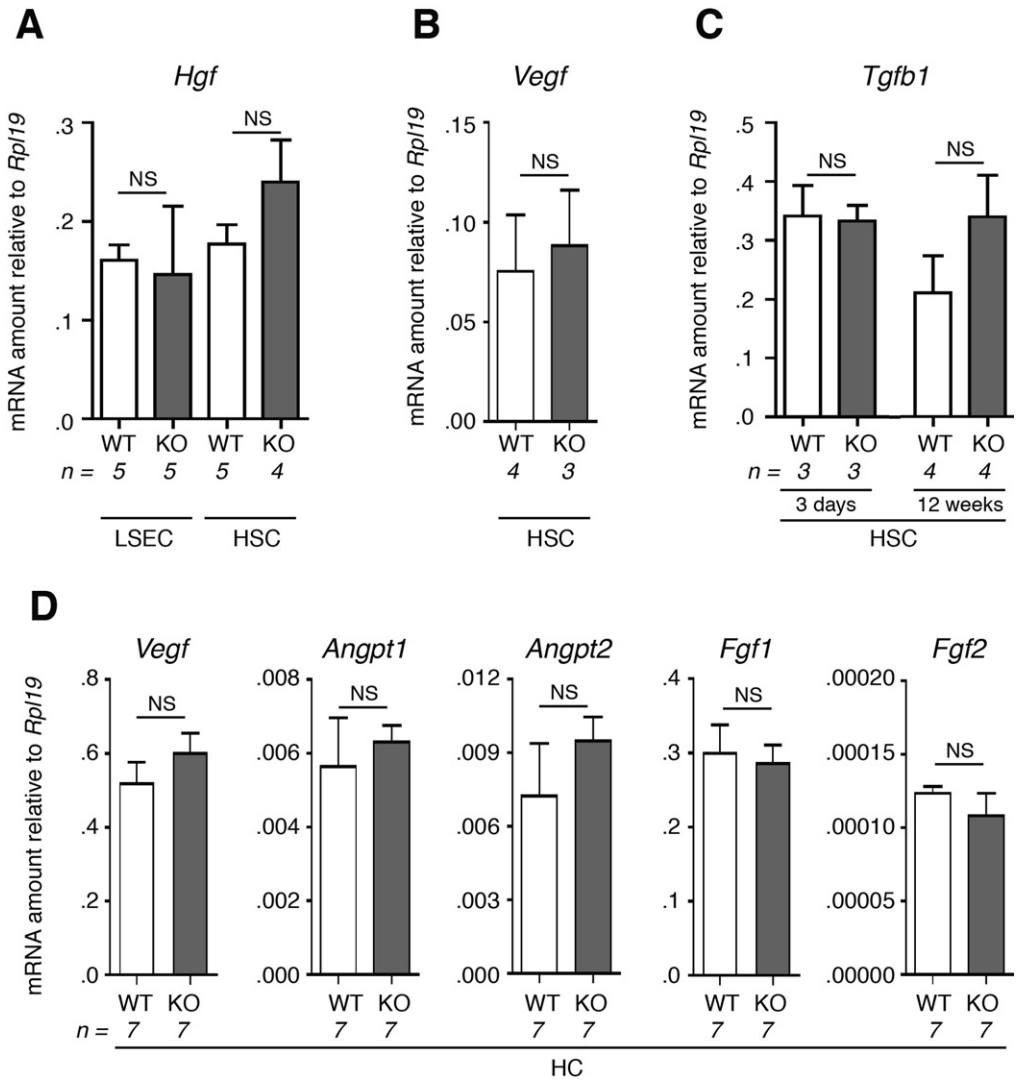
*Conflicts of interest*

The authors disclose no conflicts.

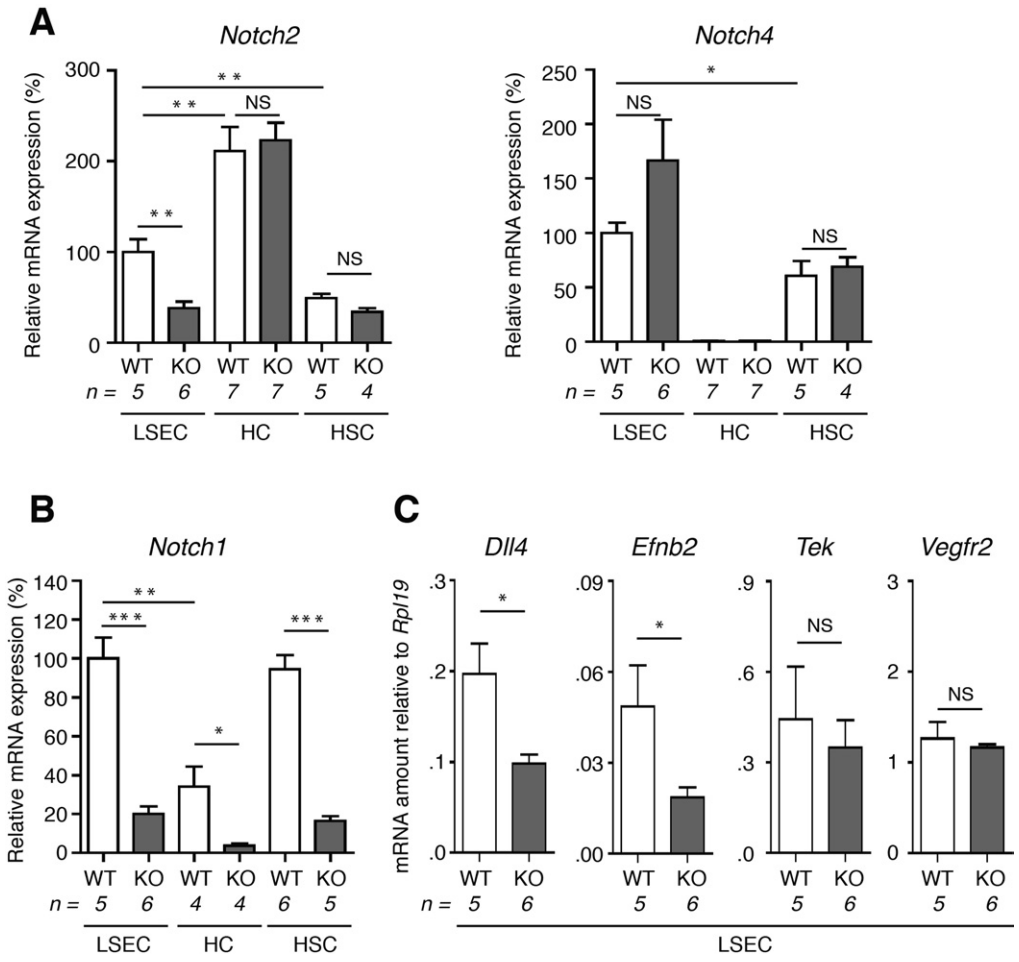
*Funding*

Supported by a Swiss National Science Foundation (SNF) grant 323500-123714 (M.T.D.), and an SNF Score grant 32323B_123815 (D.S.).

**Supplementary Figure 1.** Schematic representation of intussusceptive angiogenesis. Graphic diagram showing the 3-dimensional process generating new vessels by intussusceptive angiogenesis. Protrusions of opposing endothelial cells into the vascular lumen establish interendothelial contacts. The newly formed pillar increases in girth and leads to consecutive splitting of the vessel into 2 daughter vessels. Modified from Burri PH, Hlushchuk R, Djonov V. Intussusceptive angiogenesis: its emergence, its characteristics, and its significance. Dev Dyn 2004; 231:474–488 and Makanya AN, Hlushchuk R, Djonov VG. Intussusceptive angiogenesis and its role in vascular morphogenesis, patterning, and remodeling. Angiogenesis 2009;12:113–123.




**Supplementary Figure 2.** mRNA expression of growth factors from different hepatic cell compartments. (*A*) mRNA amount of *Hgf* relative to *Rpl19* is not increased in isolated LSECs and HSCs from KO mice 12 weeks after pIpC injection. (*B*) mRNA amount of *Vegf* is not increased in isolated HSCs from KO mice 12 weeks after pIpC injection. (*C*) mRNA amount of transforming growth factor $\beta$ 1 (*Tgfb1*) is not increased in isolated HSCs from KO mice early and late after pIpC injection. (*D*) mRNA amount of the growth factors *Vegf*, *Angpt1*, *Angpt2*, *Fgf1*, and *Fgf2* are not increased in isolated hepatocytes from KO mice 12 weeks after pIpC injection. All *bars* show mean with standard error of the mean (n is indicated below the plots).




**Supplementary Figure 3.** mRNA expression from isolated LSECs, hepatocytes, and HSCs of WT and KO mice. (*A*) Relative expression of mRNA of *Notch2* and *Notch4* from LSECs, hepatocytes (HCs), and HSCs isolated 12 weeks after pIpC injection from WT and KO mice. Shown is the amount relative to LSEC WT expression after normalization to *Rpl19*. *Notch3* had a very low expression both in LSECs and HCs of WT and KO animals and is not shown. (*B*) Relative expression of mRNA of *Notch1* from LSECs, HCs, and HSCs isolated 3 days after the first pIpC injection from WT and KO mice. Shown is the amount relative to LSEC WT expression after normalization to *Rpl19*. (*C*) mRNA expression of *Dll4*, *Efnb2*, Tek, and *Vegfr2* from isolated LSECs 3 days after pIpC injection. Values are normalized to *Rpl19* expression. All *bars* show mean with standard error of the mean (n is indicated below the plots).

## 4.2 Notch1 and EphrinB2 are Downregulated in Liver Sinusoidal Endothelium of Patients with Nodular Regenerative Hyperplasia

Sonja Rothweiler, Luigi Terracciano, Luigi Tornillo, Michael T. Dill, Markus H. Heim and David Semela.

Liver International, accepted 12 June 2013, Epub ahead of print.

Liver INTERNATIONAL
OFFICIAL JOURNAL OF THE INTERNATIONAL ASSOCIATION FOR THE STUDY OF THE LIVER

Liver International ISSN 1478-3223

ORIGINAL ARTICLE

# Downregulation of the Endothelial Genes Notch1 and EphrinB2 in Patients with Nodular Regenerative Hyperplasia

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**Keywords**
Capillarization – endotheliopathy – portal hypertension

**Abbreviations**
ALP, Alkaline phosphatase; alpha-SMA, alpha smooth muscle actin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; DAPT, N-[(3,5-Difluorophenyl)acetyl]-L-alanyl-2-phenyl] glycin e-1,1-dimethylethyl ester; Dll4, Delta-like 4; EfnB2, ephrinB2; FNH, focal nodular hyperplasia; GGT, gamma glutamyl transferase; GSI, gamma-secretase inhibitor; HSC, hepatic stellate cells; HVPG, hepatic venous pressure gradient; INR, international normalized ratio; KO, knockout; LSEC, liver sinusoidal endothelial cells; MELD, model for end-stage liver disease; MMP, matrix metalloproteinase; NRH, nodular regenerative hyperplasia; RT-PCR, reverse-transcription PCR; SEM, scanning electron microscopy; Tek, TEK tyrosine kinase, endothelial; VEGF, vascular endothelial growth factor.

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Received 7 February 2013
Accepted 12 June 2013

DOI:10.1111/liv.12261

## Abstract

*Background & Aims:* Nodular regenerative hyperplasia (NRH) is a rare liver disease characterized by small regenerative nodules without fibrosis and can cause portal hypertension. Aetiology and pathogenesis of NRH remain unclear. We have recently shown that Notch1 knockout induces NRH with portal hypertension through vascular remodelling in mice. The aim of this study was to analyse histological and clinical data of NRH patients and to explore if the endothelial pathways identified in our NRH mouse model are also regulated in human NRH. *Methods:* Patients were identified retrospectively from the pathology database. Clinical and laboratory patient data were retrieved. mRNA expression was measured in liver biopsies from a subset of NRH patients. *Results:* Diagnosis of NRH was confirmed in needle biopsies of 51 patients, including 31 patients with grade 1, 12 patients with grade 2 and 8 patients with grade 3 NRH. Grade 3 nodularity significantly correlated with the presence of portal hypertension: 50% of the patients with grade 3 NRH vs. 6.5% with grade 1 ($P = 0.0105$). mRNA expression analysis in liver biopsies from 14 NRH patients and in primary human sinusoidal endothelial cells revealed downregulation of identical genes as in the murine NRH model, which are implicated in vascular differentiation: Notch1, delta-like 4 (Dll4) and ephrinB2. *Conclusions:* In this large NRH needle biopsy cohort, we demonstrated that advanced nodularity correlates with presence of portal hypertension. Downregulation of the endothelial signalling pathways Dll4/Notch1 and ephrinB2/EphB4 supports the hypothesis that human NRH is caused by a sinusoidal injury providing first insights into the molecular pathogenesis of this liver condition.

Nodular regenerative hyperplasia (NRH) is a rare liver disease characterized by diffuse nodular transformation of the hepatic parenchyma into small regenerative nodules. Typically mild or no fibrosis is observed, distinguishing these lesions from cirrhosis (1, 2). Dilated sinusoids are found in internodular regions, whereas in the nodules they are usually narrow (3). Only histology showing these features allows the diagnosis of NRH.

Clinical presentation varies from patient to patient. The course of disease is often asymptomatic and liver function is usually preserved (2, 4). In case of a symptomatic presentation, the primary clinical manifestation

is portal hypertension complicated by variceal bleeding, splenomegaly and ascites (5–8).

NRH has been reported to occur in association with rheumatological disorders, haematological malignancies and other conditions (2, 9). Several drugs have also been associated with the development of NRH, among which azathioprine is the most frequent reported drug (2, 8–10). Originally, it has been suggested that NRH is caused by obliterative changes such as thromboembolic events leading to compromised hepatic blood flow with subsequent atrophy in ischaemic regions and reactive hyperplasia in well-perfused regions (2). Many of the reported associations can be explained by this causal concept as they all can be traced back on the common feature of heterogeneous hepatic blood supply. However, circulatory impairment in the hepatic microcirculation in the absence of thromboembolic events has also been reported (6, 7, 11, 12). In this context, we have previously shown that knockout of the receptor Notch1 in liver sinusoidal endothelial cells induces NRH with portal hypertension through vascular remodelling and capillarization of the liver microvasculature (13, 14). Molecular analysis in these NRH mice showed that the vascular changes are mediated through the endothelial ephrinB2/EphB4 pathway supporting the hypothesis that the primary event in NRH is a sinusoidal endotheliopathy preceding hepatocyte proliferation (2).

The aim of this study was to analyse histological, clinical and biochemical features of NRH patients as well as to determine if the endothelial genes found in our mouse model are also regulated in human NRH.

## Material and methods

### Patient selection

In this retrospective study, we searched the liver biopsy database of the Pathology Institute of the University Hospital Basel for entries between 1996 and 2011 containing the words nodular regenerative hyperplasia. The database comprises all liver biopsies that were performed at the University Hospital Basel or biopsies sent to the Pathology Department for second opinion. Patients who underwent a liver biopsy within the study time were included if their liver biopsy showed histological features exclusively of NRH without further features of additional chronic liver disease. The reason for liver biopsy in the NRH patients was unclear elevation of liver function tests after negative hepatopathy screening. Clinical records were retrieved for the identified NRH patients. To complete the medical history for patients, general practitioners or the attending doctor was also contacted. Patients with concomitant liver disease were excluded.

### Histological analysis

The diagnosis of NRH was based on histological assessment of needle biopsies. Liver biopsies were stained with haematoxylin-eosin, reticulin (Novotny) and Sirius red. All biopsies were reassessed for nodularity, fibrosis and sinusoidal dilatation by LTe, an expert liver pathologist at the University Hospital Basel. NRH was diagnosed on haematoxilyn-eosin, Sirius red and reticulin staining, according to well-established criteria in liver biopsies, including liver cell nodularity as well as the presence of regenerating liver cell plates, surrounded by compressed, atrophic liver cell plates in the absence of/or with minimal fibrosis (15, 16). Nodular hyperplasia was classified into grade 0–3. Briefly, absent nodularity was rated as grade 0; mild, moderate and prominent nodularity as appearing on H&E and reticulin staining were graded with 1, 2, and 3 respectively. The nodularity grading also takes into account the extent of nodular transformation in the biopsy. Sinusoidal dilation was assessed as present or absent. Several weeks after the first histological examination, livers were examined a second time to confirm the NRH diagnosis and nodularity grading as well as to assess the intraobserver variability.

### Laboratory tests

Data for the following laboratory tests were retrieved: alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamyl transferase (GGT), alkaline phosphatase (ALP), platelet counts, total bilirubin, albumin and the international normalized ratio (INR). Tests were usually performed on the day of the biopsy, otherwise test results within a period of one month prior or after the biopsy were used.

### Assessment of portal hypertension

Presence of portal hypertension was assigned if any of these conditions were observed: hepatic venous pressure gradient (HVPG) >10 mmHg; ascites, splenomegaly or dilated portal vein (>13 mm in transverse diameter); oesophageal or gastric varices or portal-hypertensive gastropathy assessed by endoscopy. Isolated splenomegaly in patients with an underlying primary disease such as haematological malignancies was not included as a criterion of portal hypertension.

### Immunohistochemical studies

For CD34 immunohistochemistry, 3–5 μm paraffin-embedded liver sections were stained using the automated Benchmark XT system (Ventana Medical Systems, Tucson, AZ) with the commercially available prediluted monoclonal antibody (790-2927, Ventana) and the Enhanced DAB Detection Kit (Ventana).

### RNA extraction and quantitative RT-PCR

Upon written informed consent, 14 patients agreed to donate a second liver biopsy for research purposes taken immediately through the same coaxial needle as the

diagnostic liver biopsy. The protocol was approved by the ethical commission of Basel. Supplementary Table 1 summarizes detailed information on these 14 patients. As controls ($n = 10$), hepatitis C patients showing a Metavir activity/fibrosis score A1F0 or A1F1 were used. Additional controls included biopsies from patients with cirrhosis ($n = 7$) and focal nodular hyperplasia (FNH, $n = 8$). RNA was extracted from human liver tissue and from liver sinusoidal endothelial cells (LSEC) using TRIzol reagent (Invitrogen, Basel, Switzerland) according to the manufacturer's instruction. RNA was reverse transcribed with Moloney Murine Leukaemia Virus Reverse Transcriptase (Promega Biosciences, Wallisellen, Switzerland) in the presence of random primers (Promega) and deoxynucleoside triphosphates. SYBR green-based real-time polymerase chain reaction (SYBR green PCR master mix; Applied Biosystems, Foster City, CA) was performed. Intron-spanning primers were designed for Rpl19, Notch1, Hes1, EfnB2, EphB4, Dll4 and TEK tyrosine kinase, endothelial (Tek) (Supplementary Table 2). All reactions were carried out in duplicate on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems). Messenger RNA (mRNA) expression levels of the transcripts were normalized to RPL19 using the ΔCt method.

### Cell culture

Isolated primary human LSEC (ScienCell Research Laboratories, Carlsbad, CA, USA) were used between passages 2 and 6. LSEC were cultured in endothelial cell medium (ScienCell) supplemented with 5% foetal bovine serum, 1% endothelial growth supplement (ScienCell) and 1% penicillin/streptomycin (ScienCell). For mRNA analysis, cells were serum-starved overnight and were then treated with the γ-secretase inhibitor *N*-[*N*-(3,5-difluorophenacetyl)-l-alanyl]-*S*-phenylglycine *t*-butyl ester (DAPT, Sigma, St. Louis, MO) for 24 h, with azathioprine (Pro Concepta Zug AG, Zug, Switzerland) 150 μM for 24 h or with methotrexate (Pfizer AG, Zürich, Switzerland) 1 or 10 μg/mL for 24 h respectively.

**Table 1.** Patient characteristics and conditions associated with nodular regenerative hyperplasia

| Characteristics | Values |
|---|---|
| Age at diagnosis (years) | 49.2 (9–75) |
| Sex | |
| Male | 28 (55%) |
| Female | 23 (45%) |
| Ethnicity | |
| Caucasian | 46 (90%) |
| Hispanic | 3 (6%) |
| African | 1 (2%) |
| Asian | 1 (2%) |
| Rheumatological disorders | |
| Vasculitis | 4 (8%) |
| Systemic lupus erythematosus | 2 (4%) |
| Rheumatoid arthritis | 1 (2%) |
| Haematological disorders | |
| Hodgkin's lymphoma | 1 (2%) |
| Osteomyelofibrosis | 1 (2%) |
| Acute lymphoblastic leukaemia | 1 (2%) |
| Chronic myelogenous leukaemia | 1 (2%) |
| Factor V Leyden thrombophilia | 1 (2%) |
| Drugs | |
| Azathioprine | 4 (8%) |
| Miscellaneous | |
| Sarcoidosis | 1 (2%) |
| Antimitochondrial antibody seropositivity | 1 (2%) |

### Statistical analysis

Diagrams, unpaired Student's *t*-tests and Fisher's exact test were performed using GraphPad Prism version 4.00 for Macintosh (GraphPad Software, San Diego, CA, www.graphpad.com). Kappa statistic was calculated to determine the intraobserver variability in the evaluation of histological NRH nodularity grading.

## Results

### Patient demographics

Fifty-one patients with a confirmed histological diagnosis of NRH were identified. The computer search of the pathology report database for NRH resulted in 221 patients. However, 127 patients had to be excluded as the biopsy was performed externally and sent to the Pathology Department of the University Hospital Basel for a second opinion. Liver biopsies with less than 5 portal tracts or liver biopsies too much fragmented for a reliable assessment of the lobular architecture were excluded, which was the case in 6 patients. A further 25 patients were eliminated for not meeting the criteria for NRH. Another 12 cases were excluded because of other or concomitant causes of liver disease (i.e. primary biliary cirrhosis, chronic viral hepatitis, Budd-Chiari syndrome), leaving 51 patients with a confirmed diagnosis of NRH in the study. These 51 patients had percutaneous biopsies except one patient with a transjugular biopsy only (total of three passes allowing reliable diagnosis of NRH).

This study group included 28 men and 23 women with a mean age at diagnosis of 49 years (range 9–75 years) (Table 1). Among the 51 NRH patients, 24 were not reported to have any disease, condition or drug related to NRH. The associations found in the remaining 27 patients were classified into four groups: rheumatological disorders, haematological malignancies, drugs and miscellaneous (Table 1).

### Liver function tests and presence of portal hypertension

For 46 patients, liver biochemical tests were available and retrieved (Fig. 1). The majority of patients showed only minor abnormalities. However, increased levels

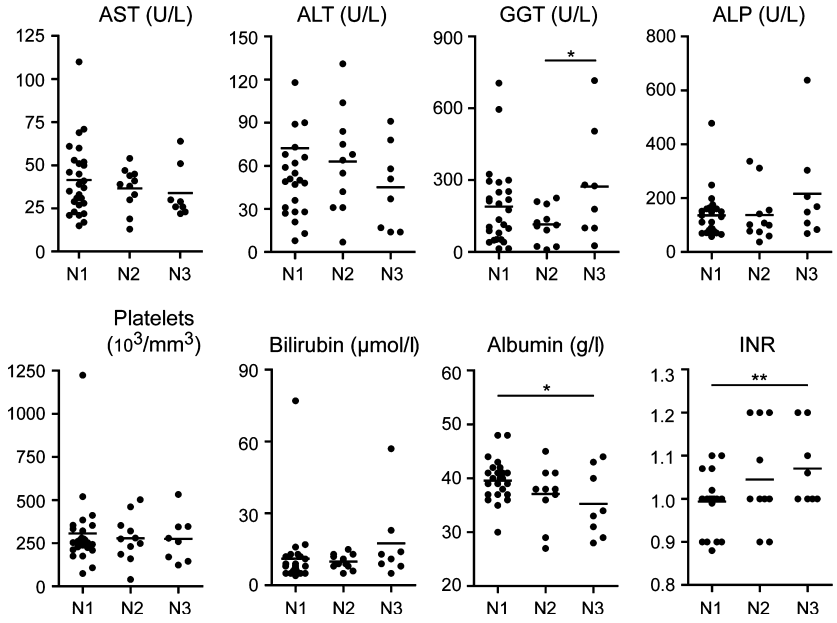


**Fig. 1.** Liver function tests at the time of biopsy in NRH patients. Liver function tests, platelet counts, total bilirubin, albumin and INR in NRH patients according to NRH grading (N1-3). Each circle represents an individual patient, bars represent mean. *$P < 0.05$, **$P < 0.01$.

(1.5 times the upper limit of normal) were found for alanine aminotransferase in 21 patients (41.2%), for gamma glutamyl transferase in 30 patients (58.8%) and for alkaline phosphatase in 8 patients (15.7%). Serum albumin was decreased in patients with grade 3 NRH compared to grade 1 NRH ($P = 0.0231$).

Imaging studies were available for 37 patients, of which 4 patients additionally underwent assessment of hepatic venous pressure. Nine patients (17.6%) presented with clear signs of portal hypertension: oesophageal varices, ascites, splenomegaly, portal-hypertensive gastropathy and/or widened portal vein (Table 2). Varices were observed in six patients identified with portal hypertension. Of 4 patients in whom HVPG measurement was performed, 3 had a high pressure gradient (>10 mmHg). The fourth patient showed normal HVPG (<6 mmHg) most probably explained by collateral circulation observed on angiography during HVPG measurement in wedged position, but had otherwise clear signs of portal hypertension (varices, splenomegaly, portal-hypertensive gastropathy, ascites). In total, 15 patients had an upper gastrointestinal endoscopy of whom 7 patients showed signs of portal hypertension (Table 2).

**Table 2.** Assessment of portal hypertension

| Nodularity | Patient # | Signs of portal hypertension |
|---|---|---|
| 3 | a | HVPG 22 mmHg, ascites, splenomegaly, portal-hypertensive gastropathy |
| | b | Ascites, dilated portal vein |
| | c | Ascites, splenomegaly |
| | d | HVPG 4 mmHg, varices, ascites, splenomegaly, portal-hypertensive gastropathy |
| 2 | e | Varices |
| | f | Varices, ascites, splenomegaly, dilated portal vein |
| | g | HVPG 13 mmHg, varices, splenomegaly |
| 1 | h | Varices, splenomegaly, portal-hypertensive gastropathy, dilated portal vein |
| | i | HVPG 14 mmHg, varices, splenomegaly |

### Association between histological grading and presence of portal hypertension

Nodularity of the liver parenchyma was classified into grade 1, grade 2 and grade 3 depending on the degree of nodular transformation. Among the 51 patients diagnosed with NRH, grade 1, grade 2 and grade 3 were observed in 31 (60.8%), 12 (23.5%) and 8 (15.7%) patients respectively. In the biopsies showing nodular regeneration grade 1 (Fig. 2A), nodular transformation could be identified focally in the liver biopsy, whereas in a biopsy of grade 2 (Fig. 2B), nodularity was found to a larger extent. Grade 3 (Fig. 2C) was assigned to biopsies with a very distinct nodular transformation found throughout the biopsy specimen. Sinusoidal dilatation was found in all patients with grade 3 NRH, in 75.0% of those with grade 2 NRH and in 58.1% of patients having grade 1 nodular transformation. A second blinded microscopic evaluation of the biopsies showed an excellent agreement for the evaluation of NRH nodularity staging with a kappa coefficient of 0.931. The mean percentage agreement between the first and second evaluation was 96.2%. Presence of portal hypertension was associated with advanced degree of nodularity, namely

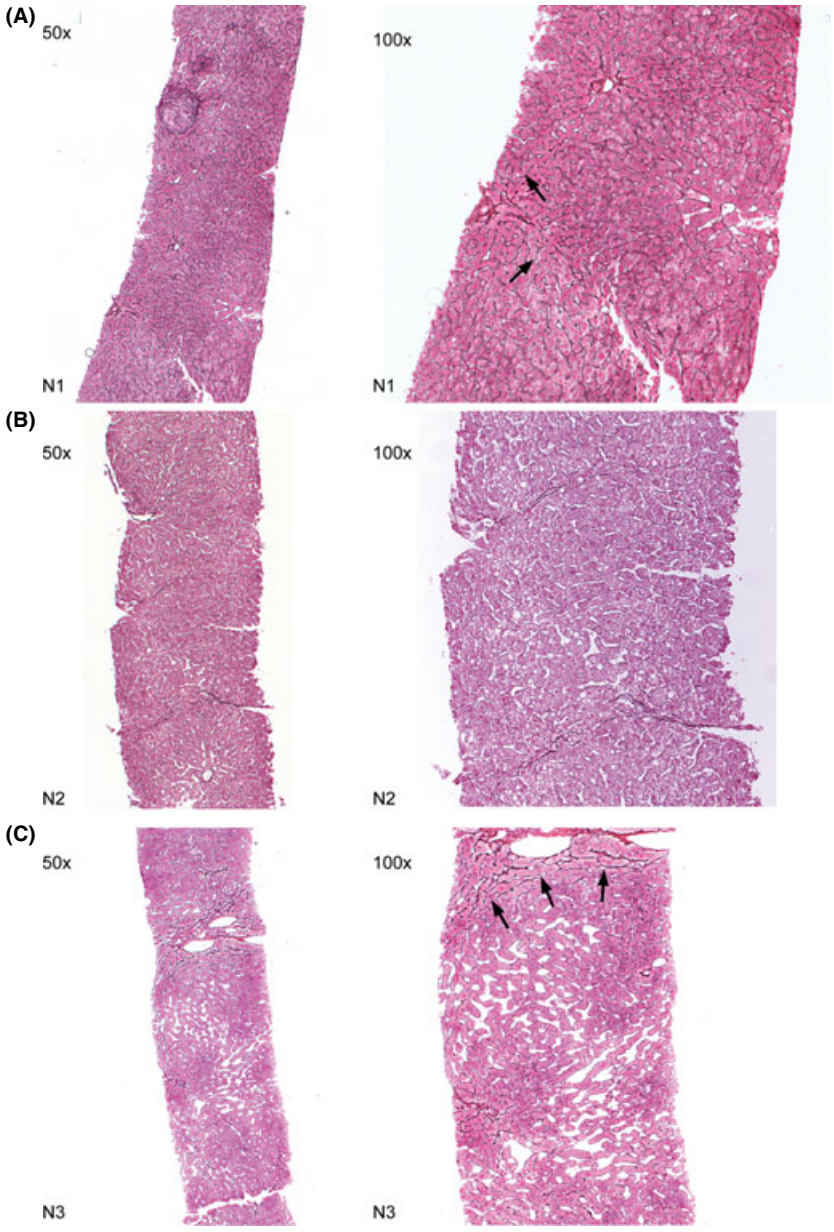


**Fig. 2.** Histological assessment of liver biopsies from NRH patients by Novotny staining showing grade 1 (A, mild nodularity), grade 2 (B, moderate nodularity) and grade 3 (C, severe nodularity) nodularity. Arrows indicate compressed regions at the periphery of nodules. Note dilated sinusoids in grade 3 NRH biopsy. Original magnification: left panel 50×, right panel 100×.

**Table 3.** Presence of portal hypertension

| Nodularity | Portal hypertension, *n* (%) |
|---|---|
| 3 | 4/8 (50%) |
| 2 | 3/12 (25%) |
| 1 | 2/31 (6.5%) |

in the group of patients diagnosed with grade 3 NRH, portal hypertension was reported in 50% ($P = 0.0105$, grade 3 vs. grade 1, Fisher's exact test). In contrast, portal hypertension was identified in only 25% of the cases with grade 2 NRH and in 6.5% of patients with grade 1 NRH (Table 3).

### CD34 expression and histological features of NRH

In normal liver, CD34 is absent in hepatic sinusoids except for periportal LSEC (Fig. 3A) (17). However, CD34 has been shown to be a marker of sinusoidal capillarization and is upregulated in chronic liver

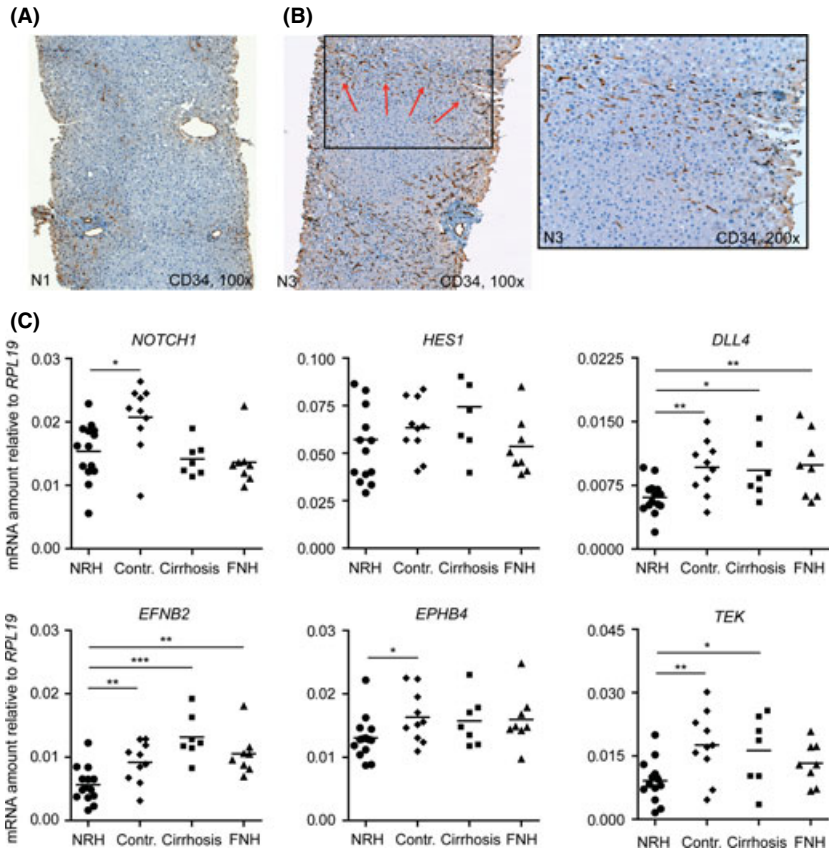


**Fig. 3.** Changes in endothelial gene expression in liver biopsies from NRH patients. Expression of CD34 in NRH livers with grade 1 nodularity (A) and with advanced grade 3 nodularity (B). CD34 immunohistochemistry with positive endothelial cells at the periphery of nodules (arrows). Original magnification: 100× (A, B left panel) and 50× (B right panel). (C) Gene expression analysis for Notch1, Hes1, Dll4, ephrinB2, EphB4 and Tek in 14 NRH patients in comparison to controls ($n$ = 8), to cirrhotic patients ($n$ = 7) and to patients with focal nodular hyperplasia (FNH, $n$ = 8).

disease (18). Analysis of CD34 expression in 47/51 (92.2%) patients showed that in NRH biopsies with advanced nodularity, CD34 positivity was more pronounced in selected patients: CD34 was expressed to a greater extent in compressed regions which are noticed at the periphery of nodules, where sinusoids and hepatic plates are compressed by the regenerative, hypertrophic hepatocytes (Fig. 3B). We identified enhanced sinusoidal CD34 expression in four patients with grade 3 nodularity and in one patient with grade 2 nodularity. Interestingly, increased CD34 expression was observed predominantly in patients presenting with signs of portal hypertension (4 out of the 5 cases had portal hypertension). Notably, the patient with NRH grade 2 and upregulated CD34 expression was also found to have portal hypertension. NRH patients with grade 1 nodularity showed no increased CD34 expression in comparison to controls.

### Gene expression analysis in NRH patients

We have previously shown that endothelial dysfunction is the primary event leading to development of NRH in mice (13, 14). In this NRH mouse model, we have identified dysregulation of the endothelium-specific signalling pathways Dll4/Notch1 and ephrinB2/EphB4, which are known to regulate endothelial differentiation and vascular remodelling. To explore if these endothelial pathways are also dysregulated in patients diagnosed with NRH, we have performed expression analysis in human tissue from liver biopsies. For 14 of our NRH patients, a second liver biopsy specimen for research purposes was available (detailed information see Supplementary Table 1).

As control group, we have chosen patients with minimally active chronic hepatitis C (A1F0 and A1F1) as liver biopsies from healthy subjects were not available. Low fibrosis scores were selected to match NRH biopsies in terms of no to minimal fibrosis. Liver biopsies from patients with FNH and cirrhosis were used as additional control groups to assess specific expression of Dll4/Notch1 and ephrinB2/EphB4 in liver diseases associated with regenerative processes, vascular abnormalities and/or portal hypertension. Notably, the control group with cirrhosis patients showed a similar amount of portal hypertension compared to the NRH patients (2/7

cirrhotics vs. 2/14 NRH patients with portal hypertension). In NRH patients, mRNA expression of the Notch1 receptor, its ligand Dll4, the EphB4 receptor and its ligand ephrinB2 as well as Tek were significantly reduced compared to control patients (Fig. 3C). Notch1 was also found to be downregulated in other conditions with vascular remodelling and sinusoidal capillarization such as cirrhosis and FNH. However, downregulation of Dll4 and ephrinB2 was specific for NRH and was not regulated in cirrhosis and FNH (Fig. 3C).

One limitation of human studies is the impracticality to isolate pure LSEC from NRH liver biopsies. To overcome this drawback, we performed complementary experiments using isolated primary human LSEC from resected livers. LSEC incubated with the gamma-secretase inhibitor DAPT, to inhibit Notch signalling, confirmed regulation of Dll4 and ephrinB2 (Fig. 4A). The same gene signature was found in primary human LSEC treated with azathioprine, which is known to induce NRH in patients (Fig. 4B). In contrast, methotrexate, which is an antimetabolite like azathioprine but not associated with NRH, did not downregulate the expression of Dll4/Notch1, Tek or ephrins (data not shown).

## Discussion

In this large liver biopsy-based NRH cohort, we investigated the relationship between histological grading, clinical presentation and molecular changes with specific attention to endothelial involvement in this disease. This study established a correlation between portal hypertension and degree of liver nodularity in NRH patients. Downregulation of the endothelial specific genes Notch1, Dll4, ephrinB2 and Tek supports the hypothesis that human NRH is caused by an endotheliopathy of the hepatic microcirculation (Fig. 5).

The exact pathogenesis of NRH remains unclear. One of the pathological concepts describes NRH as a result of a reactive hyperplastic response of hepatocytes induced by impaired hepatic blood flow possibly initiated by vascular obliteration. In 1856, the pathologist Rudolf Virchow postulated that thrombus formation is causally related to abnormalities in blood flow (stasis), hypercoagulability of the blood and endothelial injury (19). In keeping with Virchow's concept, all three categories can be involved in the pathogenesis of NRH by causing impaired hepatic blood flow. Vascular obstruction can occur at the sinusoidal level or at the level of portal vein branches. In addition, in the literature there are many cases describing NRH in association with disorders affecting the vasculature such as Felty's syndrome (20), polyarteritis nodosa (21), rheumatoid vasculitis (22) or hereditary haemorrhagic teleangiectasia (23), all supporting the concept that vascular lesions are the trigger towards the common pathological mechanism of impaired hepatic blood flow. However, only few studies addressed the role of sinusoidal endothelial cells in NRH. In a murine NRH model, we have recently shown that deletion of the receptor Notch1 leads to disruption of LSEC homoeostasis leading to persistent LSEC proliferation, intussusceptive angiogenesis, loss of fenestrae, capillarization of the hepatic microcirculation and eventually portal hypertension (14). Hepatocyte proliferation and nodular transformation of the liver

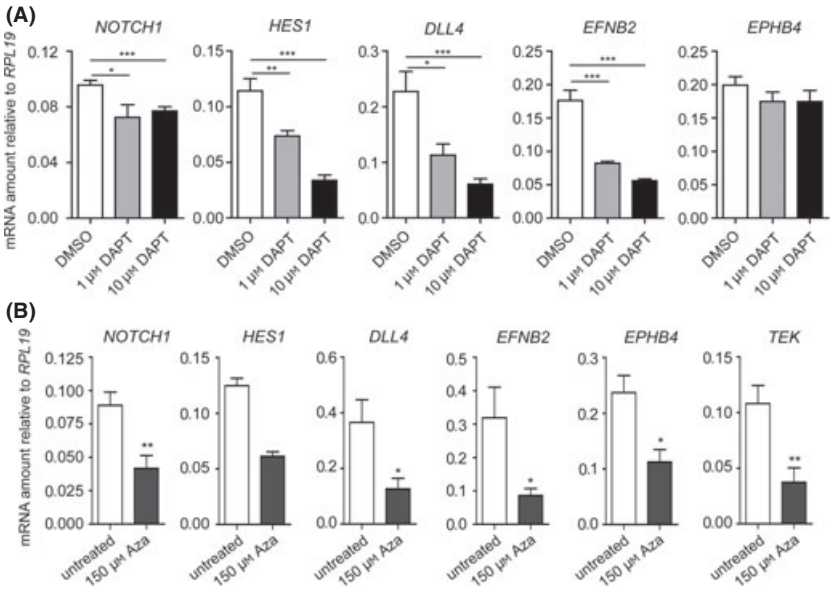


**Fig. 4.** Gene expression analysis in isolated human liver sinusoidal endothelial cells (LSEC). (A) Gene expression analysis after incubating LSEC for 24 h with the Notch inhibitor *N*-[*N*-(3,5-difluorophenacetyl)-l-alanyl]-*S*-phenylglycine *t*-butyl ester (DAPT). (B) Endothelial genes are downregulated in LSEC treated with azathioprine for 24 h. Data represent three individual experiments performed in duplicates. Mean+SEM. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

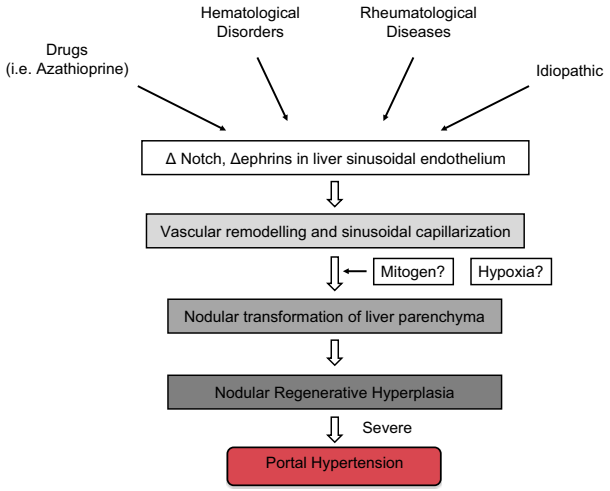


**Fig. 5.** Schematic summary of findings in NRH based on current analysis in humans and in an established murine model: Different aetiologies can lead to dysregulation of genes in the liver sinusoidal endothelium (i.e. Notch, ephrins). Changes in endothelial gene expression induce vascular remodelling and capillarization of the sinusoidal circulation. In response to vascular remodelling, hepatocytes proliferate, which leads to nodular transformation of the liver parenchyma mediated by an unknown mitogenic stimulus and/or hypoxia. A subgroup of NRH patients can develop portal hypertension during the course of disease.

parenchyma were secondary events only following vascular remodelling and led to development of pronounced NRH in these mice. These experimental findings suggest that NRH is an adaptive response to impaired vascular function in the liver. The process of vascular dedifferentiation in this model was found to be mediated by Notch, ephrin and Tek signalling, all of which are key players in LSEC differentiation and quiescence (14). In our murine NRH model, we have shown that these endothelial signalling pathways are required for vascular homoeostasis and normal function of the hepatic sinusoids by inducing quiescence and differentiation of LSEC and that disruption of Notch1 leads to NRH and portal hypertension. Here, we confirm for the first time specific regulation of these endothelial signalling pathways in human liver biopsies from NRH patients and *in vitro* in isolated primary human LSEC. Notch1 signalling has been extensively studied in vascular development and has been shown to mediate cell-autonomous regulation of vessel sprouting, branching and vascular diameter in endothelial cells (24, 25). Similar to our NRH mouse model, deregulated Notch signalling has been shown to promote pulmonary arterial hypertension in humans and mice through vascular remodelling and increased vascular resistance (26). Notch1 has emerged as a critical mediator in multiple processes of the angiogenic response such as orchestration of tip/stalk cell specification during angiogenesis, spatial restriction of the VEGF response and determination of the arterial or venous cell fate specification (27, 28). Furthermore, ephrinB2 has been shown to be a direct Notch target (29). EphrinB2 and its receptor EphB4 control segregation and sorting of arterial and venous endothelial cells allowing remodelling and maturation of the primitive capillary networks into arteries and veins (30, 31). In the liver vasculature, ephrinB2/EphB4 signalling has been reported to regulate microvascular structure during remodelling in chronic liver disease (32). Moreover, ephrinB2/EphB4 and Notch loss-of-function mutants show arteriovenous malformations (33, 34). Considering the multifactorial aetiology of NRH, other pathways could also be involved in the pathogenetic process of this disease. Such a candidate could be Angiopietin-1 (Ang-1), the major agonist for Tek. Tek is involved in vessel maturation and quiescence and has very recently been shown to potentiate the Dll4/Notch signalling leading to vascular quiescence (35). In a transgenic mouse model, it has been shown that deletion of Ang-1 is embryonically lethal because of vascular abnormalities. In contrast, deletion of Ang-1 after E13.5 did not result in an overt phenotype suggesting a dispensable role for Ang-1 in adult, quiescent vasculature (36). Of note, in our previous murine NRH study, no significant differences in Ang-1 and Ang-2 expression were observed in hepatocytes isolated from NRH mice. On the other hand, the endothelial receptor Tek was significantly lower expressed in NRH mice compared to wildtype mice, which is in line with the findings in the human NRH biopsies (14). In summary, these preclinical studies highlight the pivotal role of the Notch pathway and the ephrin signalling in angiogenesis, endothelial differentiation and vascular homoeostasis. Analysing Notch1 expression in our liver biopsies from NRH patients, FNH patients and patients with cirrhosis showed an overall downregulation when compared to controls. Strikingly, Dll4 and ephrinB2 were found to be regulated in NRH patients only but not in patients with liver cirrhosis or FNH. The discovery of such a restricted gene expression pattern was unexpected as the analysed NRH patients present different aetiologic factors. The specific downregulation of Dll4 and ephrinB2 in our patient cohort (comprising different aetiologies of NRH) indicates that the underlying mechanism in these patients can be attributed to an endotheliopathy mediated through the common final pathway of Dll4/Notch1 and ephrinB2/EphB4 signalling. These molecular findings are in line with a recently published murine NRH model (14). However, other mechanisms independent of Notch and ephrin signalling might be involved in the pathogenesis of NRH because of other causes (i.e. thrombosis).

There are several reports of NRH developing in response to various drugs such as azathioprine, 6-thioguanine, busulfan or oxaliplatin (10, 37, 38). In a study by Rubbia-Brandt and colleagues, NRH was found to be induced by injury to the liver endothelium through oxaliplatin in patients with colorectal carcinoma. Interestingly, in the same study, they were

able to reveal a protective effect of bevacizumab, an antibody against vascular endothelial growth factor (VEGF). NRH was present in 67 out of 274 patients treated with oxaliplatin. If oxaliplatin was combined with bevacizumab, the incidence of grade 3 NRH was reduced from 28.9% to 11.4%. Azathioprine is the prevalently documented drug associated with NRH (9). In previous *in vitro* experiments, azathioprine was found to be more toxic to sinusoidal endothelial cells than to hepatocytes, further supporting that NRH arises from endothelial damage (39). Using isolated primary human LSEC incubated with azathioprine, we have reproduced our *in vivo* findings and showed that azathioprine induces the same set of endothelial genes found in liver biopsies from NRH patients and in the murine NRH model. These results and the above mentioned studies strongly favour the concept of NRH being caused by a circulatory impairment at the sinusoidal level (12).

In the western world, NRH is the main cause of non-cirrhotic portal hypertension (5, 6). By analysing a needle biopsy cohort of 51 NRH patients, we have identified a correlation between presence of portal hypertension and disease severity. So far, the relationship between grade of nodularity and increased portal pressure has not been assessed. Nonetheless, one must keep in mind that because of the retrospective nature of this study imaging and/or endoscopy reports were not available for 14 patients (27.5%), leading to a potential underestimation of portal hypertension in this analysis. In conclusion, this finding suggests that patients with severe nodularity should be assessed for the presence of portal hypertension.

Interestingly, we observed capillarization demonstrated by immunohistochemistry in some cases of advanced grading and/or presence of portal hypertension, whereas patients with grade 1 nodularity never showed increased CD34 positivity. However, these findings have limitations. Although NRH is a diffuse liver disease, nodular transformation can be distributed in an irregular fashion leading to sampling error. Therefore, specimen size is crucial for accurate diagnosis and grading as lesions may be missed if the sample size is too small (40, 41).

In summary, the present findings establish a correlation between histological grading and the presence of portal hypertension and provide new insights into the molecular biology of NRH. This is the first human study linking LSEC signalling to the pathogenesis of NRH. Although different aetiologic factors have been associated with NRH, our results indicate that the underlying mechanism in these conditions seems to be an endotheliopathy mediated through the common final pathway of Dll4/Notch1 and ephrinB2/EphB4 signalling. Further studies are needed to understand how the multiple conditions associated with NRH affect the signalling in liver sinusoidal endothelial cells.

## Acknowledgements

The authors thank Ursula Dürmüller for excellent technical assistance.

*Financial support:* SNF Score Grant 32323B_123815 to D.S.

*Conflict of interest:* None.

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## Supporting information

Additional Supporting Information may be found in the online version of this article:

**Table S1.** Detailed characteristics of NRH patients used for gene expression analysis.

**Table S2.** Primer sequences used for real-time RT-PCR analysis.

**Supplementary Table 1. Detailed characteristics of NRH patients used for gene expression analysis**

| Nodularity | NRH risk factor | Sex | Ethnicity | Age at Biopsy | Presence of portal hypertension (signs) |
|---|---|---|---|---|---|
| 3 | no | m | Caucasian | 62 | yes (varices, splenomegaly, gastropathy) |
| 2 | MGUS | m | Caucasian | 62 | no |
| 2 | CML leukemia | m | Caucasian | 33 | no |
| 2 | AMA antibody | f | Caucasian | 35 | no |
| 2 | no | m | Caucasian | 42 | yes (HVPG: 13 mmHg, varices, splenomegaly) |
| 1 | no | m | Caucasian | 56 | no |
| 1 | no | f | Caucasian | 25 | no |
| 1 | no | m | Caucasian | 41 | no |
| 1 | no | m | Asian | 49 | no |
| 1 | no | f | Caucasian | 68 | no |
| 1 | no | m | Caucasian | 55 | no |
| 1 | no | f | Caucasian | 63 | no |
| 1 | no | m | Caucasian | 39 | no |
| 1 | no | m | Caucasian | 35 | no |

AMA, anti-mitochondrial antibody; CML, Chronic myelogenous leukemia; MGUS, monoclonal gammopathy of undetermined significance

**Supplementary Table 2. Primer sequences used for real-time RT-PCR analysis**

| Gene | Forward primer sequence | Reverse primer sequence |
|---|---|---|
| *Rpl19* | 5’-GATGCCGGAAAAACACCTTG-3’ | 5’-TGGCTGTACCCTTCCGCTT-3’ |
| *Notch1* | 5'-CTGCATGCGGCTGTGTCT-3' | 5'-GGCTCGGTTCCGGATCA-3' |
| *Hes1* | 5'-AAAGATAGCTCGCGGCATTC-3' | 5'-AGGTGCTTCACTGTCATTTCCA-3' |
| *Efnb2* | 5’-TGCCAGACAAGAGCCATGAA-3’ | 5’-TGATCCAGCAGAACTTGCATCT-3’ |
| *EphB4* | 5’-TTGGAAACTGCTGATCTGAAGTG-3’ | 5’-CCAGGCCGCTCAGTTCCT-3’ |
| *Dll4* | 5'-CCAACTGCCCTTCAATTTCAC-3' | 5'-TGCCAAGCTTCGATGATGAG-3' |
| *TEK* | 5'-CCAAACGTGATTGACACTGG-3' | 5'-TGTGAAGCGTCTCACAGGTC-3' |

# 5. Discussion

## 5.1 The role of Notch1 signaling in liver sinusoidal endothelial cells

Endothelial cells display remarkable heterogeneity in cell morphology, function, gene expression, and antigen constitution [3,183]. Depending on space and time, the endothelium is equipped with appropriate features to meet the demands in different organs. In case of the liver, the endothelial cells lining the sinusoids have acquired a highly specialized phenotype defined by non-diaphragmed fenestrae, clathrin-coated pits and absence of an underlying basement membrane. It has been shown that VEGF plays an important role in the generation and maintenance of LSEC fenestration [184]. Deeper insights in mechanisms regulating the LSEC phenotype were provided by DeLeve and colleagues who identified that VEGF mediated autocrine NO production by sinusoidal endothelial cells is required to maintain the sinusoidal endothelium differentiated [39]. In this study we analyzed the ultrastructure of the liver microcirculation in conditional Notch1 KO mice by scanning electron microscopy. We found dramatic changes of the hepatic microcirculation affecting the sinusoidal architecture and the LSEC fine structure. On the one hand we could show that loss of Notch1 induces vascular remodeling through intussusceptive angiogenesis with increased branching and enlarged vessel diameters. On the other hand, disruption of Notch1 signaling led to modification of the LSEC porosity pattern. The number of fenestrae and sieve plates was drastically decreased, while the few remaining fenestrae displayed an increased diameter. Overall, the sinusoidal porosity was reduced and some segments of the sinusoids were entirely lined by non-fenestrated endothelium resembling a continuous capillary appearance. As it is known that the Notch pathway acts downstream of VEGF, it could be speculated that Notch is part of the signaling axis responsible for fenestrae regulation in LSEC as a molecular mediator conveying the VEGF signal to the actin cytoskeleton.

The effects of Notch signaling are context-dependent: Notch pathway components display distinct distribution patterns in the different organs, tissues, and cell types by which cell fate decisions can be regulated. To define cell fate specifications, Notch intertwines with other signaling pathways. One of them is the Eph/ephrin pathway. The arterial marker EphrinB2 is a known downstream target of Notch signaling [168]. EphrinB2 and its ligand EphB4 are involved in the embryonic arterial and venous endothelial cell specification and their specific expression extends into adulthood to

control tissue homeostasis [185]. In isolated LSEC of our Notch1 KO mice, we showed a reduced expression of EphrinB2. Finding EphrinB2 expression in LSEC challenges the idea that capillaries have neither an arterial nor a venous identity. However, not much is known about molecular mechanisms regulating the phenotype of the hepatic sinusoidal vasculature. According to our findings, EphrinB2 seems to play a role in the sinusoidal circulation. But still, the expression pattern of endothelial markers in hepatic capillaries distinguishing them from capillaries in other vascular beds is not completely elucidated yet. What becomes clear by finding this interaction of the Notch pathway with other signaling pathways is that the liver capillary bed depends not only on one signaling axis, rather on a complex vertical and horizontal interplay of several signal transduction pathways possibly involving cross-talk between LSEC and neighboring cells.

The vasculature in the adult is normally quiescent and endothelial cells have a low proliferation rate. The average turnover time of normal endothelium is more than 1 year [183,186]. By looking at our data we found increased LSEC proliferation in livers of Notch1 KO mice, which evidences that disruption of Notch1 signaling promotes LSEC activation. To ultimately conclude on the role of Notch1 signaling in liver sinusoidal endothelial cells the key experiment would be to perform an inducible deletion of Notch1 specifically in the LSEC population. In our study we performed a global, tissue-unspecific Notch1 deletion as well as a specific deletion in the liver epithelial compartment as a control. Mice with a hepatocyte-specific Notch1 KO did not have an endothelial response or resemble features of nodular regenerative hyperplasia. However, this approach is not sufficient to cleanly rule out the possibility that deletion of Notch1 in other non-hepatocyte or non-endothelial cell lineages contributes to the development of the observed phenotype in MxCre mediated Notch1 deletion. It would be very interesting to see whether specific loss of Notch1 in the endothelium only is sufficient to reproduce the phenotype observed after global knockout. We tried this approach by crossing the floxed Notch1 mice with the VE-cadherin-Cre-ER$^{T2}$ (provided by L. Iruela-Arispe) mice, but we did not achieve a sufficient recombination in the liver endothelium. It has been reported that in adults the expression of VE-cadherin is weak in the vasculature of the liver, which might be the explanation why we did not observe the NRH phenotype after conditional deletion of Notch1 in endothelial cells [187,188]. To circumvent these limitations we isolated the three major liver cell populations; hepatocytes, liver sinusoidal endothelial cells, and hepatic stellate cells. By assessing the gene expression of several mitogens and growth factor receptors upon Notch1 deletion in the different cell types, we could

show that there are no major changes in non-endothelial cells, but expression changes of endothelial molecules in liver sinusoidal endothelial cells. Thus, we conclude that disruption of Notch1 signaling in liver sinusoidal endothelial cells is responsible for the observed phenotypic changes in the MxCre Notch1 KO mouse model. With this conclusion our study provides a novel hint in unveiling the significance of the Notch1 signaling pathway in the maintenance of LSEC quiescence and differentiation.

## 5.2 Endotheliopathy - common feature in nodular regenerative hyperplasia and contributor to portal hypertension

Besides loss of fenestrae we also observed upregulation of CD31, which is absent in differentiated LSEC [189]. Both processes demonstrate capillarization of LSEC induced by Notch1 deletion. Capillarization has been described in other pathologic states of the liver such as fibrosis, hepatitis, alcoholic liver injury, and in the physiological process of aging [40,43,45,190–193]. Therefore it becomes clear that injury of an intact endothelium is involved in the development of numerous diseases in the liver as well as in other organs. Truly, in our experimental approach we observed an increase in the portal pressure, which is later on followed by hepatic parenchyma transformation leading to NRH. Most commonly, portal hypertension is caused by liver cirrhosis and hepatic fibrosis [194]. However, in our animal model we could exclude both of these conditions. We hypothesize that the increased portal pressure is due to an increased intrahepatic resistance, as the consequence of vascular remodeling at the level of the hepatic microcirculation. Determinants of resistance to blood flow are vessel diameter, organization of the vascular network, viscosity of the blood, and mechanical forces acting on the vessels [195]. Based on physical arguments, the structural alterations (increased vessel diameter and increased branching) in our system result in elevation of the blood flow resistance in turn leading to portal hypertension.

Nodular transformation of the liver parenchyma occurred after the increased portal pressure was established. To explain the increased hepatocyte proliferation we investigated several hepatocyte growth factors (i.e. HGF, TGF-beta, FGF, VEGF), but none of them showed an altered expression that would account for the augmented hepatoctye proliferation. It remains an open question which stimulus drives the regenerative hepatic nodule formation. However, based on our findings we believe that hepatocyte proliferation is a secondary adaption in consequence of hemodynamic changes induced by vascular remodeling.

## 5.3 Translational study: confirming findings from the animal model in patients with nodular regenerative hyperplasia

In the western world, NRH is the main cause of non-cirrhotic portal hypertension [58,196]. By analyzing the largest needle biopsy cohort of NRH patients we have identified a correlation between presence of portal hypertension and disease severity. So far the relationship between grade of nodularity and increased portal pressure has not been assessed. According to this correlation we suggest that patients presenting with severe nodularity should be assessed for presence of portal hypertension.

A large body of evidence based on multiple case reports indicates that NRH is the result of a heterogeneous hepatic blood supply possibly initiated by vascular obliteration. In 1856, the pathologist Rudolf Virchow postulated that thrombus formation is causally related to abnormalities in blood flow (stasis), hypercoagulability of the blood, and endothelial injury [197]. In keeping with Virchow's concept all three categories can be involved in the pathogenesis of NRH by causing impaired hepatic blood flow. Vascular obstruction can occur at the level of the microvasculature or the portal vein. In the literature, there are many cases describing NRH in association with vascular disorders such as Felty's syndrome [198], polyarteritis nodosa [199], rheumatoid vasculitis [200], or hereditary hemorrhagic telangiectasia (also known as Osler-Weber-Rendu disease) [201], all supporting the idea that vessel lesions are a trigger to commence impaired hepatic blood flow. We support the idea that NRH is the expression of a vascular pathology at the level of the hepatic microvasculature founded on our results obtained from the Notch1 KO animal study. One aim of this translational study was to confirm these recent findings in human patients. And indeed, we could show the relevance of the same set of genes, identified in mice, in patients diagnosed with NRH.

Literature on the molecular mechanism underlying the pathogenesis of NRH is sparse. In our NRH mouse model we could identify Notch1, Dll4, EphrinB2, and Tek as key players driving the vascular changes that eventually lead to development of NRH. Gene expression analysis in 14 NRH patients confirmed a downregulation of the same set of genes. To ensure that expression differences are exclusive for NRH, adequate control groups were diligently chosen: HCV patients with no inflammation and no fibrosis referred to as "control" group; patients with cirrhosis and focal nodular hyperplasia (FNH) patients were selected as two additional control groups showing liver conditions associated with

vascular changes. Notch1 showed an overall downregulation in patients with NRH, FNH and cirrhosis when compared to controls. Strikingly, expression of Dll4 and EphrinB2 was specifically reduced in NRH patients only. The discovery of such a restricted gene expression pattern was very surprising to us since the analyzed NRH patients present different etiologic factors but somehow the underlying cause in all the patients can be attributed to an endotheliopathy mediated through the common final pathway of Notch1/Dll4 and EphrinB2/EphB4 signaling.

Not only different diseases were reported to be associated with NRH, but also a number of drugs are known to generate NRH. In this context it is worth to mention the recent study by Rubbia-Brandt and colleagues, where NRH was found to be induced by injury to the liver endothelium through oxaliplatin in patients with colorectal carcinoma. Among 274 patients treated with Oxaliplatin, NRH was observed in 24.5%. Interestingly, when Oxaliplatin was combined with Bevacizumab (an anti-VEGF antibody) less patients developed NRH indicating a protective effect of bevacizumab. A possible explanation for the positive effect of bevacizumab on oxaliplatin-associated liver injury is the attenuation of VEGF-induced upregulation of MMP in sinusoidal endothelial cells. It has been shown previously that increased MMP activity permits dehiscence of LSEC from the space of Disse, ultimately leading to sinusoidal obstruction [30]. This report therefore corroborates the theory of NRH being caused by a circulatory impairment at the level of sinusoids.
Al-Hamoudi and colleagues, who reported a case of NRH in association with a florid carcinoid syndrome, provide further evidence for this hypothesis. Signs of a vasculopathy were not present in this case. The authors speculated that NRH is induced by microcirculatory disturbances, which are the consequence of vasoactive hormones including Serotonin secreted by the tumor [202].

## 5.4 Notch1 functions as a tumor-suppressor in the liver vasculature

Given the pleiotropic functions of the highly conserved Notch signaling pathway during embryonic development and in adult tissues, it is not surprising that Notch signaling is deregulated in many cancers. In view of the necessity of extensive fine-tuning to ensure the adequate Notch signal in the appropriate cellular context, aberrant Notch signaling is predestined to be implicated in cancer. Similar to its diverse physiologic functions, the Notch pathway has been described as a double-edged sword in tumorigenesis with oncogenic as well as oncosuppressive activity.

The first evidence for a putative role of Notch in cancer derived from the analysis of T-cell acute lymphoblastic leukemia (T-ALL) that identified a chromosomal translocation within the Notch1 locus [203]. Later, the oncogenic potential of aberrant Notch signaling was described in mouse mammary tumor virus (MMTV)-induced breast cancer [204]. As a retrovirus, MMTV is able to insert its viral genome in the host genome, thereby deregulating the expression of adjacent genes. The Notch4 locus was identified as a common integration site of MMTV resulting in the expression of a truncated constitutively active Notch4 [157,205]. Over the last years, an oncogenic function of Notch has been established in various human malignancies, such as leukemia, breast cancer, colorectal cancer, pancreatic cancer, and melanoma [150]. However, there is growing evidence that Notch is associated with oncosuppressive roles as observed in hematopoietic cells, skin, pancreatic epithelium, and in hepatocytes [206]. These data indicate that Notch signaling in cancer has two faces: depending on the cellular context Notch functions either as an oncoprotein or as a tumor suppressor.

The data derived from our Notch1 KO mouse model reveal a tumor suppressive role for Notch1 signaling in the liver. In our model, we observed activation of liver sinusoidal endothelial cells with persistent proliferation, dedifferentiation, and eventually malignant transformation. Fifty weeks after Notch1 deletion we found spontaneous development of hepatic angiosarcoma with a penetrance of 86%. Therefore, our findings establish Notch1 as a tumor suppressor gene in the liver endothelium. This conclusion is in line with our hypothesis that Notch1 signaling controls tissue homeostasis and vascular quiescence by growth suppression. Other groups have also identified Notch pathway components implicated in the development of vascular tumors. Treatment with a Dll4-specific neutralizing antibody in mice, rats, and cynomolgus monkeys resulted in liver histopathology, including centrilobular hepatocyte atrophy, sinusoidal dilation, and bile duct proliferation. But only in rats blocking of Dll4-mediated Notch signaling induced vascular neoplasms [207]. In a genetic mouse model endothelial specific deletion of Dll4 caused hepatic vascular lesions, which recapitulated the observed vascular alterations evoked by pharmacological inhibition of Dll4/Notch signaling [208]. Continuing the investigations of Notch signaling in cancer, Liu et al. had the objective to determine whether Notch1 has a tumor suppressor activity. Using an elegant genetic approach, where Notch1 loss of heterozygosity is coupled to the level of Notch1 activation, they could demonstrate that loss of Notch1 promotes development of vascular tumors, which were most prevalent in the liver [180]. These findings reflect a tumor suppressor function of Notch1 in the vascular system, further highlighting the importance of Notch1 signaling

in liver endothelial cells [180]. In summary, these results suggest an essential role of Dll4/Notch1 signaling for maintaining integrity and quiescence of liver sinusoidal endothelium and define a tumor suppressor function of the Dll4/Notch1 signaling axis in the liver endothelium.

Angiosarcoma is a rare cancer deriving from malignant endothelial cells. It represents a highly aggressive tumor with metastasis and dismal prognosis [209]. The molecular events implicated in the transformation of differentiated endothelial cells into vascular malignancies are poorly understood. These gaps in knowledge impede the development of novel treatment strategies, which are urgently needed since treatment options for this type of cancer are limited and of transient efficiency [210]. In light of this unfavorable situation, in ongoing experiments we aim to dissect the molecular mechanisms driving angiosarcoma growth as well as to test anti-angiogenic drugs for the treatment of angiosarcoma. In support of an oncosuppressive role of Notch1 in angiosarcoma the Notch pathway might provide a novel therapeutic target in angiosarcomas.

## 5.5 Concluding remarks

The importance of the Notch pathway in vascular development and arterial-fate specification has drawn great attention to this signaling network in vascular cells. For this reason, the understanding of the Notch gene regulatory network in the vascular growth, function, and maintenance has greatly evolved. Nevertheless, the function of Notch1 in the liver remains poorly understood. In this study, we show that Notch1 signaling has the capacity to promote homeostasis of the hepatic microvasculature by maintaining LSEC in a quiescent and differentiated stage. Loss of Notch1 signaling leads to activation of LSEC followed by vascular remodeling, which subsequently causes development of nodular regenerative hyperplasia and portal hypertension with the final result of endothelial cell tumor. Analyzing liver biopsy samples we provide first insights into the molecular pathomechanisms of human NRH by linking the deregulation of endothelial pathways to the pathogenesis of NRH. Our research contributes to a better understanding of Notch1 signaling in the hepatic microcirculation and adds another piece to the evolving picture of the Notch signaling pathway.

# 6. Acknowledgements

I would like to thank David Semela for being a fantastic supervisor during my whole studies as a PhD student. He allowed me to grow into field of research and he was a patient teacher of the intriguing biology of the liver. I really appreciate that I was able to attend many international conferences and that he put a lot of trust in me, i.e. letting me present at the International Liver Congress™ already within my first year of being a PhD student. In this regard, I also want to mention his great support with preparing me well for all the talks I was giving and for teaching me how to make a good presentation. Furthermore, I always enjoyed his enthusiasm about angiogenesis and the uniqueness of the liver vasculature. And this is why we now not only share our passion for sweets, but also for the sinusoidal endothelial cells.

I would like to express my special thanks to Markus Heim for giving me the opportunity to perform my PhD thesis in his laboratory. He enabled me to be integrated in the Hepatology group. Thereby, I experienced an excellent scientific environment as well as brilliant technical and scientific knowledge.

Further, I am very grateful for the valuable collaboration with the Institute of Pathology. I highly appreciated to work with Luigi Terraciano, who familiarized myself with the liver pathology. During my PhD studies I could learn a lot from his longstanding expertise as a pathologist and his enthusiasm about the entire spectrum of histology in normal and diseased liver. I also would like to thank Ursula Dürmüller for introducing me to the immunohistochemistry and being very helpful with all her experience to overcome problems.

I want to thank my colleagues and friends from the Hepatology group. They enriched my everyday working life with valuable advice, great discussions, as well as cheerful conversations during lunchtime and coffee breaks.

Finally, I want to express deep gratitude to my parents, Margarete and Gerd, who always stand behind me, encouraged me to go my way that I have chosen, respecting my decisions and supporting me along the way.

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