Vascular Biology, Atherosclerosis and Endothelium Biology

Overexpression of Notch1 Ectodomain in Myeloid Cells Induces Vascular Malformations through a Paracrine Pathway

Xiujie Li,* Ezequiel Calvo,* Marc Cool,*
Pavel Chrobak,* Denis G. Kay,* and
Paul Jolicoeur*†‡

From the Laboratory of Molecular Biology,* Clinical Research
Institute of Montreal; the Department of Experimental Medicine,†
McGill University; and the Department of Microbiology and
Immunology,‡ Université de Montréal, Montreal, Quebec, Canada

We previously reported that truncation of *Notch1* (N1) by provirus insertion leads to overexpression of both the intracellular (N1^{IC}) and the extracellular (N1^{EC}) domains. We produced transgenic (Tg) mice expressing N1^{EC} in T cells and in cells of the myeloid lineage under the regulation of the CD4 gene. These CD4C/N1^{EC} Tg mice developed vascular disease, predominantly in the liver: superficial distorted vessels, cavernae, lower branching of parenchymal vessels, capillarized sinusoids, and aberrant smooth muscle/endothelial cell topography. The disease developed in lethally irradiated normal mice transplanted with Tg bone marrow or fetal liver cells as well as in *Rag*^{-/-} Tg mice. In nude mice transplanted with fetal liver cells from (ROSA26 × CD4C/N1^{EC}) F₁ Tg mice, abnormal vessels were of recipient origin. Transplantation of Tg peritoneal macrophages into normal recipients also induced abnormal vessels. These Tg macrophages showed impaired functions, and their conditioned medium inhibited the proliferation of liver sinusoid endothelial cells *in vitro*. The *Egr-1* gene and some of its targets (*Jag1*, *FIII*, *FXIII-A*, *MCP-1*, and *MCP-5*), previously implicated in hemangioma or vascular malformations, were overexpressed in Tg macrophages. These results show that myeloid cells can be reprogrammed by N1^{EC} to induce vascular malformations through a paracrine pathway. (*Am J Pathol* 2007, 170:399–415; DOI: 10.2353/ajpath.2007.060351)

main.¹ The N^{EC} domain is largely made of tandem epidermal growth factor-like repeats. On binding of N^{EC} to its ligands, Delta or Serrate/Jagged, the N^{EC} is shed by the action of a disintegrin and metalloprotease protease at a site located within the extracellular juxtamembrane region, whereas intracellular cleavage generates N^{IC}, which enters the nucleus and activates transcription.² The Notch pathway plays a role in normal cell fate decision and has also been found to be involved in vascular disease.^{3,4}

Although the constitutively active truncated form of Notch (N^C) has been extensively studied in different biological systems, studies of N^{EC} have been less numerous. N^{EC} has been shown to bind to its ligands on neighboring cells, thus permitting intercellular signal transmission.⁵ N^{EC} and its ligands can also interact within the same cell, probably within the endoreticulum or the Golgi apparatus.⁶ In addition, N^{EC} interacts with other ligands such as F3/contactin,⁷ CNN3/Nov,⁸ or Wingless.⁹

Studies involving *in vivo* expression of artificially generated ectodomain of Notch ligands in *Drosophila* and in other systems have demonstrated that they can function both as agonist and antagonist.^{10,11} In human, one vascular disease, cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy, is caused by mutations in the ectodomain of Notch3 (N3^{EC}).¹²

While studying retrovirus-induced T-cell leukemia in mice, we previously found that Notch1 was frequently mutated by provirus insertions in these tumors at two distinct genomic sites.¹³ In “type I” mutation, a majority of the integrations occurred in genomic regions coding for sequences between the 34th epidermal growth factor-like repeat and the TM domain. Tumors harboring such

Supported by grants from the National Cancer Institute of Canada and the Canadian Institute of Health Research (to P.J.).

Accepted for publication September 26, 2006.

Supplemental material for this article can be found on <http://ajp.amjpathol.org>.

Address reprint requests to Dr. Paul Jolicoeur, M.D., Ph.D., Clinical Research Institute of Montreal, 110 Pine Ave. West, Montreal, QC, Canada H2W 1R7. E-mail: jolicop@ircm.qc.ca.

Members of the *Notch* (N) gene family encode heterodimeric transmembrane (TM) receptor that contains an extracellular (EC), a TM, and an intracellular (IC) do-

insertions expressed abundant truncated, mutated Notch1 ectodomain proteins [N1^{EC(Mut)}] and high levels of truncated N1^{IC} proteins. Although the N1^{EC(Wt)} proteins processed normally were found at the cell surface, the N1^{EC(Mut)} proteins could not be detected at the cell surface but were secreted in the medium of expressing cells.

Because the role of the N1^{EC(Mut)} in thymoma development has not yet been rigorously analyzed, we hypothesized that N1^{EC}, as N1^{IC}, may be involved in tumor formation, possibly by virtue of its distinct cellular localization. We therefore generated Tg mice expressing N1^{EC} in T cells and in cells of the macrophage/dendritic lineage. Unexpectedly, vascular disease, but not thymomas, developed at high frequency in these Tg animals through a paracrine loop involving expression of N1^{EC} in macrophages.

Materials and Methods

Mice

The C3H/HeN, Rag1^{-/-}, and ROSA26 mice¹⁴ were from Harlan (Indianapolis, IN), the Jackson Laboratory (Bar Harbor, ME), and Dr. David Lohnes (University of Ottawa, Ottawa, ON, Canada), respectively. The L-SIGN/green fluorescent protein Tg mice will be described separately (C. Hu, C. Forestier, Z. Hanna, P. Jolicoeur, unpublished data).

Generation of CD4C/N1^{EC} Tg Mice

The 4.42-kb *HindIII/StuI* N1^{EC} fragment, with a stop codon at amino acid residue 4420, was ligated through *EcoRI* to the 15-kb CD4C sequences. This CD4C promoter represents a chimera between the mouse CD4 enhancer and the human CD4 promoter.¹⁵ The resulting CD4C/N1^{EC} recombinant DNA was excised from the plasmid and microinjected into the pronucleus of (C57BL/6 × C3H) F2 one-cell embryos. Tg founders were bred on the C3H/HeN background.

Chimeric Mice

Chimeric mice were generated by aggregating morulas (2.5 days postcoitum) pairwise, as described previously.¹⁶ These were then transferred into CD1 pseudopregnant female mice.

RNA Purification and Northern Blot Analysis

RNA from different cells and tissues was isolated using Trizol (GibcoBRL, Carlsbad, CA), and 15 to 20 μ g from each sample was electrophoresed on formaldehyde agarose gels and processed for hybridization using a ³²P-labeled 1.9-kb probe M corresponding to the *BamHI-BamHI* fragment of the full-length Notch1 cDNA, essentially as described previously.¹⁷

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis and Real-Time RT-PCR

RNA (1 μ g) was added to RT-PCR reactions containing the relevant primers at a concentration of 0.6 μ mol/L, essentially as described previously.¹³ Primer sets for sense (S) and antisense (AS) amplifications for the following genes were used: for CD4C/N1^{EC} [5'-CCCCACTGGGCTCCTGGTTGCAGC-3' (S) and 5'-GTATGAAGACTCAAAGGGCAG-3' (AS)]; and as control, for hypoxanthine guanine phosphoribosyl transferase [5'-GTTGGATACAGGCCAGACTTTGTTG-3' (S) and 5'-GATTCAACTTGCGCTCATCTTAGGC-3' (AS)]. Quantitative real time PCR amplification was performed in duplicate with primers in combination with SYBR green (SYBR green PCR kit; Qiagen, Valencia, CA) on the Mx4000 apparatus (Stratagene, La Jolla, CA). The following primers were used: for MCP-1 (Ccl2), 5'-CAGCAAGATGATCCCAATGA-3' (S) and 5'-AGTGCTTGAGGTGGTTGTGG-3' (AS); for MCP-5 (Ccl12), 5'-GTCCTCAGGTATTGGCTGGA-3' (S) and 5'-GGGTCAGCACAGATCTCCTT-3' (AS); for Fcrl1, 5'-ACAATTTTGGAGTGGCAACC-3' (S) and 5'-TCACGATCTCGTCTGTGAGG-3' (AS); for FxIII-A, 5'-GAGCTCGGAAACACCAGTC-3' (S) and 5'-CACCGAATCCTTGGTGAGTT-3' (AS); for Jag1, 5'-ATCGCATCGTACTGCCTTTC-3' (S) and 5'-ATTGCCGGCTAGGGTTTATC-3' (AS); for Egr1, 5'-AGCGCCTTCAATCCTCAAG-3' (S) and 5'-GAGTCGTTTGGCTGGGATAA-3'; and for S16, 5'-CTTC-TGGGCAAGGAGCGATTT-3' (S) and 5'-GACTGTCGGA-TGGCATAAATTGG-3' (AS). For each real-time PCR reaction, different volumes of cDNA (depending on the level of expression of each gene), 1× SYBR green PCR master mix, and 10 pmol/ μ l of primers were mixed in a 20- μ l reaction volume in 8× strip tubes (Stratagene) covered with optical cap (Stratagene). All PCR protocols included a 15-minute polymerase activation step followed by 40 cycles consisting of a 94°C denaturation for 30 seconds, annealing at 60°C for 30 seconds, and an elongation step at 72°C for 1 minute. Melt curves (Stratagene), agarose gel electrophoresis, and standard sequencing procedures were used to examine each sample for purity and specificity. Results were normalized according to the average amount of the endogenous (S16) gene. Data were collected and analyzed with the provided application software.

Protein Extraction and Western Blot Analysis

Protein extraction from cells or tissues and Western blot analysis with rabbit polyclonal anti-Notch1^{extra2} (1781-B) antibodies against N1^{EC} were performed as previously described.¹³

Perfusion

Microfil (Flow Tech, Carver, MA) perfusion (0.5 to 2.5 ml) was performed via the apex of the left ventricle, as before,¹⁸ and via the portal vein or the vena cava or through the umbilical vein (for embryos).

Tissue Sampling and Microscopic Analysis

Routine histological analysis and staining with hematoxylin and eosin or Masson's trichrome was performed as described previously.¹⁸ Slides were examined by at least two blinded investigators.

In Situ Hybridization

In situ hybridization was performed with hCD4-exon-1-specific [³⁵S]UTP-labeled antisense or control sense RNA probes, as previously described.¹⁸

IHC and Immunofluorescence Studies

Immunohistochemistry (IHC) was performed with anti-Mac-1 (Cedarlane) or polyclonal anti-Notch1^{extra2} (1781-B) antibodies, as previously described.¹⁸ This was followed by incubation with the anti-rabbit IgG coupled to Alexa Fluor 680 (Molecular Probes, Eugene, OR). GFP was visualized by fluorescent microscopy (Zeiss).

Confocal Microscopy

Sections were incubated overnight at 4°C with primary antibodies: anti-PECAM-1 (CD31) (1:10) (clone 13.3E; PharMingen) and anti-smooth muscle actin (1:200, clone 1A4; Sigma, St. Louis, MO). This was followed by incubation with appropriate secondary antibodies: biotinylated rabbit anti-rat IgG (E0468; DAKO) and streptavidin Alexa Fluor 488 (Molecular Probes) for anti-PECAM-1 and anti-mouse IgG coupled to Alexa Fluor 680 (Molecular Probes) for anti-smooth muscle actin. Sections were analyzed by confocal microscopy (Zeiss LSM 510), as before.¹⁹

BM and Fetal Liver Cells Transplantation

Recipient C3H or nude mice were lethally or partially irradiated (900 and 400 rads, respectively). Approximately 4 to 15 × 10⁶ bone marrow (BM) or fetal liver cells were injected into the tail veins of the irradiated mice. Mice were analyzed 2 to 6 months later.

Electron Microscopy

Mice were perfused with 2% glutaraldehyde in 0.1 mol/L cacodylate buffer, pH 7.4 (10 minutes). The perfused livers were further fixed in cacodylate buffer (2 hours) and therein rinsed with 20% sucrose. The fixed liver tissues from different areas were cut into 1-mm blocks and subsequently prepared for electron microscopy.

β-Galactosidase Staining

For liver fragments, the livers were perfused with 4% paraformaldehyde for 5 minutes. The samples were then washed in phosphate-buffered saline (PBS) containing washing buffer solution (2 mmol/L MgCl₂, 5 mmol/L ethylenediamine tetraacetic acid, 0.01% sodium deoxycholate,

and 0.02% NP-40) and stained in fresh X-gal solution at 37°C overnight. The X-gal-stained liver fragments were directly examined under inverted light microscope. The livers were then embedded in paraffin, sectioned, and counterstained with nuclear fast red to visualize LacZ-negative tissues. For liver sections, the perfused livers were frozen in OCT, and sections (5 μm) were cut. Washing and staining were then performed on a slide.

Evans Blue

Evans blue (EB; 20 μl/10 g) was injected via the tail vein. Protocol to assess the amount of EB was kindly provided by Dr. Jean-Philippe Gratton from our Institute. In brief, perfused livers were dried and extracted by formamide. The EB amount in the formamide was measured at 610 nm. The final EB amount per milligram of the dry liver was calculated. To observe the EB fluorescence, the mice were perfused with 4% formaldehyde after the EB injection. The liver and spleen were excised and frozen in OCT. The EB Rohdmon fluorescence was finally observed with 10-nm sections under a fluorescence (Zeiss) microscope.

Capture of Latex Beads

Latex beads (L-4530; Sigma) were injected into 2- to 4-month-old mice via the tail vein (8.7 × 10⁷/200 μl of PBS per mouse) 1 or 2 hours before sacrificing the mice. Mice were perfused with 4% paraformaldehyde under Avertin anesthesia. The livers were excised and frozen in OCT, and liver sections (10 μm) were prepared. The latex bead fluorescence (fluorescein isothiocyanate) was observed under a fluorescent (Zeiss) microscope.

Partial Hepatectomy

Partial hepatectomy was achieved under Avertin anesthesia, as described previously.^{20,21} Because Tg mice easily died (three of five) when both their median and left lobes were removed, only the median lobe was removed for both non-Tg and Tg mice, to favor survival. Three weeks after surgery, mice were sacrificed and analyzed.

Purification of Peritoneal Macrophages

Peritoneal macrophages were collected from 8-week-old mice without any stimulation. The resident cells in the abdominal cavity were collected with 15 ml of RPMI containing 10% inactivated fetal bovine serum (FBS), β-mercaptoethanol, and penicillin (100 U/ml)-streptomycin (100 μg/ml). After centrifugation (1400 rpm) for 5 minutes at 4°C, the supernatants were discarded, and the residual pellet was washed twice with medium. The pellet was then suspended in culture medium. For the co-culture assay, the cells were cultured in 10 ml of medium as described above. Twelve hours later, the cells were first washed with warm (37°C) culture medium and PBS. Then, the adherent cells were harvested with a cell scraper (Corning Incorporated, Corning, NY) in ice-cold

PBS. The purity of macrophages (>95%) was confirmed by fluorescence-activated cell sorting (FACS) analysis with anti-Mac-1 staining.

Isolation of Nonparenchymal Liver Cells

The livers were cut with sterile scissors and then homogenized using plunger of syringe (5 ml). Each liver slurry was digested with 0.02% (w/v) collagenase and 0.002% (w/v) DNase in 10 ml of serum-free RPMI 1640, pH 7.4, at 37°C for 20 to 45 minutes with occasional shaking (once per 5 minutes). The resulting cell suspension, diluted in 40 ml of serum-free RPMI, was centrifuged for 3 minutes at 300 rpm, 4°C. The cells from each liver were resuspended in 2.5 ml of ice-cold serum-free RPMI 1640 and gently mixed with 3.5 ml of ice-cold 30% (w/v) metrizamide (Sigma). The cells were then centrifuged at 2500 rpm for 20 minutes. The interface cells containing liver sinusoidal endothelial cells (LSECs) and Kupffer cells were harvested and centrifuged at 1500 rpm for 10 minutes to pellet the cells. These cells were further processed for FACS analysis and magnetic cell sorting purification.

Co-Culture Assay

Isolated LSECs were seeded at a density of 1×10^5 per well in a 96-well plate in MF12 medium containing 15% FBS. Once the LSECs have formed monolayers, the peritoneal macrophages were isolated and cultured in MF12 medium containing 15% FBS. Twelve hours later, the macrophages were incubated for 3 hours at 37°C with latex beads (4×10^6 /ml; Sigma), washed, and incubated with LSECs. The clusters of macrophages were scored after 4 to 72 hours of co-culture.

Macrophage Transplantation

Peritoneal macrophages (3×10^6) purified from male mice, as previously described,¹⁸ were injected into female mice by the tail vein in 200 μ l of PBS. One month later, the recipient mice were processed for Microfil perfusion and histology.

Purification of LSECs with MACS

LSECs and Kupffer cells were isolated by anti-CD11b-conjugated and biotinylated intercellular adhesion molecule-1/streptavidin-conjugated magnetic bead separation, respectively, according to a published protocol.²² Their purity (80%) was confirmed by uptake of Dil-Ac-LDL (Molecular Probes).

LSEC Culture

Freshly isolated LSECs were plated onto 0.1% (w/v) gelatin-coated 48-well NUNC plates with medium containing Dulbecco's modified Eagle's medium/F12 (Invitrogen, Carlsbad, CA), 15% FBS, and 100 μ g/ml endothelial cell growth supplement (Sigma). Ten hours later, nonadher-

Figure 1. Structure of the CD4C/N1^{EC} DNA and its expression in Tg mice. **A:** Structure of the transgene. White bar, the CD4C regulatory sequences, including the mouse CD4 enhancer (enh), the human CD4 promoter (prom) with exon 1 and intron 1; stippled bar, the N1^{EC} cDNA containing 36 epidermal growth factor-like repeats; black bar, the polyadenylation signal from simian virus 40 (SV40). A, *Aat*II; Bs, *Bss*II; E, *Eco*RI; N, *Not*I. **B:** Northern blot analysis of RNA from CD4C/N1^{EC} Tg mice. RNA (10 μ g) was extracted from different tissues and hybridized with ³²P-labeled probe M, specific to the Notch1 epidermal growth factor-like repeats. The filters were then washed and rehybridized with the ribosomal 18S-specific probe (1 to 16). On the other hand, the agarose gel was stained with ethidium bromide before transfer to the membrane and photographed under UV irradiation (lanes 17 through 25). T, thymus; L, liver; LN, lymph nodes; K, kidney. Negative (–) controls (C): HC11 cells, lane 8, or nTg thymus, lanes 16 and 17. Positive (+) controls (C): HC11 cells transfected with N1^{EC}, lane 7, or Tg thymus, lanes 15 and 18. **C:** Western blot of N1^{EC} proteins. Total protein extracts (100 μ g) from whole thymus or liver, sorted CD4⁺CD8⁺ thymocytes, and peritoneal macrophages of the Tg and non-Tg littermates were evaluated with anti-N1^{EC} antibody. The membrane was then stripped and probed with anti-actin antibodies. Negative (–) and positive (+) controls (C) are, respectively, HC11 and N1^{EC}-expressing HC11 cells.

ent cells were washed off, and fresh medium was added. Cells were grown to confluence for 5 to 10 days before being trypsinized and replated for conditioned medium and co-culture assays.

Conditioned Medium Assay

Peritoneal macrophages were cultured in 2 ml of MF12 medium containing 15% FBS at 37°C in 5% CO₂. Two hours later, the medium was gently changed. Seventy-two hours later, cell-free supernatants were harvested and frozen at –20°C until LSECs were ready.

Results

Tg Expression of N1^{EC} Induces Severe Liver Vascular Abnormalities

The truncated N1^{EC} mutant fragment was ligated downstream of the CD4C regulatory sequences to generate CD4C/N1^{EC} Tg mice (Figure 1A). Three independent Tg founder lines (F60787, F60788, and F98513) were established. The CD4C sequences have previously been shown to drive expression of surrogate genes in imma-

ture double positive (DP) CD4⁺CD8⁺ and mature CD4⁺ T cells, as well as in cells of the myeloid lineage.¹⁵ Northern (Figure 1B) and Western (Figure 1C) blot analyses confirmed Tg expression at high levels in the thymus, in DP thymocytes, and in peritoneal macrophages but at lower levels in peripheral lymphoid organs, as expected. Expression was much lower in other organs, including in the liver.

Clinically, the CD4C/N1^{EC} Tg mice look healthy for up to 12 months of the observation period, although they were smaller than non-Tg littermates, as assessed at 3 months of age (F98513: non-Tg, 26.7 ± 2.9 g, versus Tg, 20.3 ± 0.5 g, $P < 0.005$). In addition, fertility was decreased (F60788) or totally impaired (F60787) in Tg females. Necropsy revealed a severe liver vascular disease in a high percentage of Tg animals from the three founders [F60787: 62 of 63 (98.4%); F60788: 35 of 39 (89.7%); and F98513: 39 of 40 (97.5%)], easily observable after perfusion with Microfil. Huge meandering spider-like vessels, crawling on the surface and along the edge of liver and seemingly wrapping the organ, were observed (Figure 2, B, F, and G). These external vessels projected branches into the parenchyma, but only superficially, often forming tufts and dilatations (Figure 2, F and G). Paradoxically, the liver vasculature within the parenchyma showed fewer and shorter branches (Figure 2, D, E, and H). In some cases, section of the Tg livers showed huge vascular cavernae (Figure 2J). Vascular malformations also developed in other organs of Tg mice [spleen (Figure 2L), brain, heart, and kidney; data not shown], but the lesions were milder and occurred at lower frequency (~10 to 15%). Interestingly, the thymus, in which transgene expression was the highest, did not develop obvious vascular abnormalities (data not shown).

The absence of major vascular disease in the thymus suggested that the cell population producing N1^{EC} may be important to elicit this phenotype. This was confirmed by constructing Tg mice expressing N1^{EC} in another cell population, namely in the mammary epithelial cells under the regulation of the mouse mammary tumor virus long terminal repeat (MMTV/N1^{EC}). Despite high levels of N1^{EC} protein produced in these Tg mammary glands, vascular abnormalities did not develop (data not shown).

Reverse Lobule Organization, Inverse Smooth Muscle-Endothelial Cell Topography, and LSEC Capillarization in CD4C/N1^{EC} Tg Mice

Further histological evaluation of CD4C/N1^{EC} Tg livers confirmed the presence of cavernous vascular lesions (Figure 3E) and revealed a variety of anomalies. On the liver surface, large veins could be observed (Figure 3B). In the parenchyma, the lesions were multifocal, usually with area of parenchyma appearing normal (Figure 3B). In affected areas, Masson's trichrome staining showed enhanced positive reaction (data not shown). Occasionally, disrupted arteries were also detected (Figure 3D; data not shown). Small vessels inside the parenchyma gave rise to aberrant and clustered capillaries distributed heterogeneously (Figure 3B). The large vessels within the parenchyma were very

Figure 2. Vascular disease of the liver and spleen in CD4C/N1^{EC} Tg mice. **A** and **B**: Macroscopic analysis of control non-Tg (**A**) or Tg (**B**) liver. The Tg livers had irregular shapes. After perfusion with Microfil, huge and spider-like white vessels were observed at their surface. **C–H**: Microscopic analysis. Note the extensive vascular branching and the homogeneous capillary networks in non-Tg mouse (**C**). **D**: In Tg mice, fewer and shorter branches were observed. **E**: Quantitation of branching. In addition, various remodeling defects of the vasculature were observed in the Tg livers. **F**, **G**, and **H**: Large vessels growing ectopically out of the liver (arrow), clustered and dilated capillaries, fewer large parenchymal vessels, and hemangioma capillaries (**H**, inset). **C**, **D**, **G**, and **H**: 0.5 × 5; **F** and **I**: 0.5 × 10. **I** and **J**: Low power view of sectioned livers. Tg (**J**) but not non-Tg (**I**) livers show hemangioma-like cavernae (asterisks) with thrombi (arrow) within their lumen. **K** and **L**: Large and superficial vascular malformations are observed in Tg spleen (**L**) but not in non-Tg spleen (**K**).

heterogeneous in size, some lumen being distended (Figure 3E). Foci of accumulation of lymphocytes and mononuclear cells intermixed with abnormal vessels and surrounded by morphologically normal-appearing hepatocytes could be observed (see below).

In severely affected areas, especially in older Tg mice, the liver structure itself was abnormal. The portal vein was frequently found to be located in the center of the lobule where the central vein is normally found, thus apparently generating a reverse lobule organization. In addition, interruption of sinusoids was manifested as capillarization of perilobular sinusoids in zone 1 (S1) and reduction of center sinusoids in zone 2 (S2) (Figure 3, B and D).

Confocal microscopic analysis of vessels was next performed to detect endothelial cells and smooth muscle cells (SMCs) by double staining with anti-CD31 and anti-smooth

muscle actin antibodies, respectively. In the large vessels at the surface of Tg livers, this analysis showed that endothelial cells form a discontinuous layer and are abnormally distributed around vessels, being located in a reverse position relative to that of the SMCs, which are arranged in what appears to be a monolayer (Figure 3G). This cell organization is quite distinct from what is observed in large vessels from non-Tg livers where endothelial cells and SMCs are intermixed in a single monolayer (Figure 3F). In the Tg intrahepatic large vessels, the SMCs do not form a uniform multilayer as they do in non-Tg vessels but are rather scattered around, loosely attached, and seem to migrate away from the endothelial cells (Figure 3, H and I). Finally, these experiments revealed an enhanced labeling of Tg sinusoids with anti-CD31 (Figure 3J) relative to that of non-Tg ones (Figure 3F), suggesting the capillarization of LSECs.²³

To further confirm the apparent anomalies of the LSECs, we visualized the LSECs with the GFP marker, by breeding the CD4C/N1^{EC} Tg mice with Tg mice expressing GFP specifically in LSECs among liver cells. In the liver of non-Tg mice, GFP-positive sinusoids were homogeneously distributed, radiating in regular arrows (Figure 3K). In contrast, in Tg livers, the GFP-positive sinusoids were dilated, distributed in patches, and less numerous (Figure 3, L and M). The lower number of GFP⁺ cells was confirmed by FACS analysis (non-Tg: 42.8% versus Tg: 19.5%, $P < 0.05$), (data not shown). Finally, electron microscopy confirmed the abnormalities of Tg sinusoids (Supplemental Figure 1).

Because malformations of vessels such as those documented here often lead to enhanced vascular permeability in a number of different conditions, we also assessed leakage of Tg liver vessels using EB.²⁴ Although the total amount of EB retained in Tg liver was higher than in non-Tg livers [Tg, 0.25 mg/mg tissue ($n = 5$), versus non-Tg, 0.15 mg/mg tissue ($n = 4$), $P < 0.05$], histological evaluation showed that EB fluorescence beneath of ECs of vessel walls of Tg was rare and spotty (data not shown). Interestingly, the higher fluorescence of Tg liver also appeared to reflect accumulation of EB in Tg macrophages located within sinusoids (see below).

The Liver Vascular Malformations Arise Early During Organogenesis and Develops During Adult Liver Regeneration

At embryonic day (E) 16.5, dilated and numerous small vessels extending toward the liver capsule could already be observed in Tg mice, although the liver structure and branching remained relatively normal (Figure 4, A–D). In

6-day-old Tg mice, the phenotype was more apparent: the liver structure was disrupted, and large as well as capillarized vessels were apparent on its surface (Figure 4, E–H). To determine whether the vascular malformations of embryonic liver were a prerequisite for the development of the adult liver disease, we took advantage of the ability of the liver to regenerate after hepatectomy as a model of liver angiogenesis.²⁵ Two-month-old adult mice were subjected to partial hepatectomy and sacrificed 20 days later. In each Tg mouse assessed ($n = 5$), the regenerated lobule was found to be smaller than the non-Tg one ($n = 4$) (Figure 4I) and to show severe vascular malformations (including large vessels crawling at its surface, decreased branching, and clustered capillaries sprouting from them) (Figure 4, I [d] and M), even more severe than those observed in the nonhepatectomized lobules of the same Tg mouse (Figure 4L). Virtually no normal capillaries were reconstituted during regeneration of Tg livers (Figure 4M). Therefore, the vascular phenotype of CD4C/N1^{EC} Tg mice can be elicited in adult life during neovascularization. In these conditions, growth of hepatocytes is significantly impaired.

BM-Derived Tg Hematopoietic Cells Are Responsible for the Development of the Liver Vascular Disease

To determine whether hematopoietic cells or cells of other lineages were responsible for inducing this liver vascular phenotype, lethally irradiated C3H mice were transplanted with BM cells derived from CD4C/N1^{EC} Tg mice and assessed 3 to 4 months later. All recipient mice ($n = 10/10$) reconstituted with Tg BM cells (Figure 5B), but none of those ($n = 0/6$) reconstituted with non-Tg BM cells (Figure 5A) developed a liver vascular disease indistinguishable from that described in nontransplanted Tg mice.

The Liver Vascular Disease Still Develops in CD4C/N1^{EC} Tg Mice Bred on Rag1-Deficient Background

To distinguish whether lymphoid or myeloid cells expressing the Tg N1^{EC} protein were responsible for inducing the liver vascular phenotype, the CD4C/N1^{EC} Tg mice were bred on *Rag-1*-deficient (*Rag-1*^{-/-}) background which is defective at producing immature CD4⁺ CD8⁺ thymocytes, mature T cells (CD4⁺ and CD8⁺), and B cells.²⁶ Unexpectedly, most (>95%) of the *Rag1*^{-/-} Tg mice died before birth, and only a few survived beyond 5 days after birth, whereas only 1 mouse (instead of 40

Figure 3. Liver and vessel remodeling in CD4/N1^{EC} Tg mice. **A–E:** H&E staining. Non-Tg liver (**A** and **C**) shows regular lobules, where the central vein (CV) is located in the center surrounded by portal vein (PV) and biliary duct (BD). In contrast, the Tg liver (**B**) shows irregular and heterogeneous lobules with reverse lobule (**B** and **D**). **C:** Intercalating and paralleled sinusoids are distributed in the non-Tg lobule zone “1” (S1) and zone “2” (S2), respectively. **D:** In the Tg lobules, sinusoids in S1 become dilated and capillarized and the parallel sinusoids have disappeared in some regions of S2, where more than five hepatocytes are clamped together. **E:** Hemangioma-like caverna in Tg liver. PA, portal artery; S, sinusoids; **dark arrows**, BD; **green arrows**, large vessels on the surface of the liver. Magnification: $\times 2.5$ (**A** and **B**); $\times 10$ (**C** and **D**); and $\times 5$ (**E**). **F–J:** Vessel remodeling. Confocal microscopy performed with anti-CD31 (fluorescein isothiocyanate) and anti-smooth muscle actin (red Texas) antibodies. A large vessel at the surface of the Tg liver (**G**) was compared with that of a normal liver (**F**). Notice the discontinuity of the Tg endothelial cells and their aberrant location relative to the SMC layer. Also note heterogeneously distributed CD31 positivity in Tg sinusoids (**J**) but not in non-Tg (**F**) sinusoids. **H** and **I:** Intrahepatic large vessel of non-Tg (**H**) or Tg (**I**) liver. Notice the scattering of the SMC away from the endothelial cells, in Tg liver. **K–M:** Disruption of Tg sinusoids in double (CD4C/N1^{EC} $\times$ L-SIGN/GFP) Tg mice close to large vessels (*). Note the decreased and patched staining in the Tg liver (**L** and **M**).

Figure 4. The liver vascular disease of CD4C/N1^{EC} Tg mice begins during embryogenesis and develops after partial hepatectomy (PH) in adult CD4C/N1^{EC} Tg mice. **A–H:** Both embryonic (E16.5 days) (**A–D**) and postnatal (P6) (**E–H**) stages were examined. Livers were perfused with Microfil (**A**, **C**, **E**, and **G**) to show the vascular morphology and those fixed with 4% formaldehyde used for H&E staining (**B**, **D**, **F**, and **H**). Homogenous vessels were observed in the non-Tg livers (**A** and **E**). In the Tg livers at E16.5, heterogeneous and denser distribution of capillaries (especially extending the liver capsule) is evident, although the liver structure and vessel branching remained relatively normal (**C**). At P6, the phenotype was more apparent. Large clustered vessels and dilated capillaries (arrowhead) close to the surface of the Tg liver and decreased branching are observed. **G** and **H:** In addition, the liver structure was disrupted. **E** and **F:** No such abnormalities were ever found in the non-Tg livers. Magnification: $\times 5$ (**A**, **C**, **E**, and **G**); $\times 20$ (**B**, **D**, **F**, and **H**). **I–M:** PH involves ligation of two-third quarter lobes. **I:** Macroscopic analysis. Nonhepatectomized (**a** and **c**) and hepatectomized (**b** and **d**) livers from the CD4C/N1^{EC} Tg mice (**c** and **d**) and their non-Tg (**a** and **b**) littermates were compared after perfusion with Microfil. In non-Tg mice, note the large lobe regenerating after PH (**b**, arrowhead) relative to the nonhepatectomized liver (**a**, arrowhead). Such hypertrophy is not observed in Tg mice (**d**, arrowhead). **J–M:** Microscopic analysis. The vasculature of both nonhepatectomized (**J**) and regenerated hepatectomized (**K**) livers of the non-Tg mice is homogeneous. Abnormal vessels are present in both nonhepatectomized (**L**) and regenerated hepatectomized (**M**) Tg livers (Magnification, $\times 5$).

mice expected from Mendelian segregation) reached adulthood. The few born *Rag1*^{−/−} Tg mice exhibited a moderately severe liver vascular phenotype, indistinguishable from that observed in *Rag-1*^{+/-} or in older wild-type Tg mice (Figure 5, C and D). The cause of the early death of *Rag-1*^{−/−} Tg mice is under investigation. These data strongly suggest that myeloid BM-transplantable cells expressing N1^{EC} are involved in the development of the liver vascular disease.

Macrophages of CD4C/N1^{EC} Tg Mice Accumulate in Areas of Malformed Vasculature and Show Functional Abnormalities

The critical role of the nonlymphoid hematopoietic cells in the development of liver vascular disease led us to assess the interaction of these cells with the liver vascula-

Figure 5. Liver vascular disease develops in mice transplanted with BM cells from CD4C/N1^{EC} Tg mice or in Rag1^{-/-}CD4C/N1^{EC} Tg mice. **A and B:** Transplantation. Recipient C3H mice (3 to 4 months old) transplanted with non-Tg and Tg BM cells were perfused with Microfil and analyzed 3 to 4 months after transplantation. Huge vessels were apparent on the surface of the liver from mice reconstituted with Tg BM cells (**B**, arrow). A large cavity (**B**, asterisk) and a cluster of capillaries-hemangioma-like vessels (**B**, circle) were also observed. No such lesions develop in the liver of mice transplanted with non-Tg BM cells (**A**) 0.5 × 10. **C and D:** Analysis of Rag1^{-/-}CD4C/N1^{EC} Tg mice. The mice were observed at 3 days postnatally or in adulthood, although very few Rag1^{-/-} Tg mice survived later than 5.5 days postnatally. Irregular capillaries (arrowheads), large vessels (arrows) along the edge of the liver, and a cavity (asterisk) were observed in Rag1^{-/-} or Rag1^{+/-} Tg mice (**D**), but were absent in non-Tg mice (**C**) P3.5, 0.5 × 10 in bright field. Adult, 0.5 × 4.0 in dark field.

ture. *In situ* hybridization with Tg-specific hCD4 exon 1 probe revealed no detectable expression in hepatocytes nor in LSECs but showed expression in cells with the morphological appearance of Kupffer cells (KCs) or macrophages (Figure 6, A and B). Further evaluation of Tg RNA expression in purified KCs and LSECs by RT-PCR confirmed the strong expression in KCs and revealed a weak expression in LSECs (Figure 6B), consistent with the CD4 expression reported in human LSECs.²⁷ Immunofluorescence analysis with anti-N1^{EC} antiserum also

confirmed expression of N1^{EC} protein in Tg macrophages (Figure 6C). Interestingly, N1^{EC} localization in cells seemed heterogeneous, with a high proportion being in vesicular structures (Figure 6C, b–e), suggesting active secretion. Secretion of soluble N1^{EC} was confirmed by Western blot analysis of supernatants (but not in the pellets) from plated peritoneal macrophages (Figure 6D).

Histological evaluation showed an increased number of macrophage-like cells surrounding the defective ves-

sels of Tg liver (Figure 6A). This was confirmed by IHC with CD11b staining (Figure 6E) and by EM, where it was easy to document a higher number of macrophages (KCs) in the Tg than in the non-Tg sinusoids (Figure 6F). These were often clustered, forming groups of two or more cells (data not shown). FACS analysis of nonparenchymal liver cells also showed a higher percentage of CD11b⁺ cells in Tg (47.2%, $n = 3$) than in non-Tg (32.2%, $n = 3$) mice (data not shown). In addition, purified Tg KCs were larger than non-Tg ones. This was also the case for peritoneal macrophages (Figure 6C). These results suggest an activated state for these cells.

Tg macrophages also exhibited functional defects *in vivo*. They captured latex beads (Figure 6G) and accumulated Evans blue (Figure 6H) at a higher rate than non-Tg ones. Together, these results strongly suggest that Tg macrophages are activated and reprogrammed by the expression of N1^{EC}.

The Liver Vascular Phenotype Develops in CD4C/N1^{EC} Tg Mice by a Paracrine Pathway

The Tg myeloid cells expressing N1^{EC} could affect liver endothelial cells and induce them, in a paracrine way, to develop these vascular defects. On the other hand, Tg-expressing cells could themselves differentiate into endothelial cells to form defective vessels. To distinguish between these two pathways, chimeric mice were first generated by fusing CD4C/N1^{EC} Tg embryos with ROSA26 embryos. The chimeric ROSA26-CD4C/N1^{EC} mice developed typical liver vascular defects, although milder than CD4C/N1^{EC} themselves (Figure 7B). Importantly, some endothelial cells on some defective vessels were found to stain positive for β -galactosidase (β -gal; Figure 7B), indicating their origin from the ROSA26 parent not overexpressing N1^{EC}. Thus, a paracrine loop probably induces the liver vascular disease.

To confirm these results, fetal liver cells derived from Tg (ROSA26 $\times$ CD4C/N1^{EC}) F₁ or from control (ROSA26 $\times$ non-Tg) F₁ embryos were transplanted into nude mice. The transplanted recipient mice receiving Tg cells ($n = 8$ of 8), but not those receiving non-Tg cells, developed liver vascular abnormalities (Figure 7D). All abnormal vessels from the mice assessed ($n = 2$ of 2) stained negative for β -gal activity, whereas other infiltrating cells, presumably myeloid and lymphoid cells, stained positive (Figure 7D). These results indicated that the defective vessels were derived from the recipient non-Tg nude mice, thus strongly suggesting that the liver vascular disease is induced through a paracrine loop.

Transplanted Tg Macrophages Are Sufficient to Induce the Liver Vascular Disease in Normal Recipient Mice

Next, we transplanted Tg or non-Tg peritoneal macrophages into normal C3H mice through the tail vein. Mice were sacrificed 1 to 3 months after transplantation and analyzed. Transgene expression could be detected by RT-PCR in the liver of recipient mice injected with Tg macrophages but not in those receiving non-Tg macrophages, as expected (data not shown). Interestingly, malformations of vessels developed in liver of recipient mice injected with Tg macrophages ($n = 3$ of 5) (Figure 7, F and H) but not in that of mice transplanted with non-Tg macrophages (Figure 7, E and G). The phenotype was very similar to that documented in CD4C/N1^{EC} Tg mice, although less severe. It shows large vessels growing out from the liver and crawling at the surface and at the edge of the liver, as well as reduced normal branching (Figure 7, F and H).

Supernatants from the Tg Macrophages Inhibit the Growth of Non-Tg LSECs

The interaction of Tg macrophages with LSECs (the most abundant endothelial cells of the liver) was next investigated *in vitro*. Co-culture of Tg and non-Tg macrophages with normal non-Tg LSECs showed that Tg macrophages tend to cluster (>4 cells) and form a higher number of colonies than non-Tg ones (Fig. 7I, a–c). This result is consistent with the higher number of macrophages observed *in vivo* in sinusoids of Tg livers (Figure 6F).

We next investigated whether factors released from macrophages could affect the morphology and growth of LSECs *in vitro*. Normal non-Tg LSECs were enriched and cultured *in vitro* in the presence of conditioned medium from Tg and non-Tg peritoneal macrophages. Supernatants from Tg macrophages were found to inhibit the growth of LSECs in this assay compared with supernatants from the non-Tg peritoneal macrophages (Figure 7I, d). This inhibitory effect was still apparent when Tg macrophage supernatants were depleted of N1^{EC} with anti-N1^{EC} antibodies but could not be observed after incubation of LSECs with supernatants from HC11 mammary epithelial cells transfected with N1^{EC} and containing equivalent levels of N1^{EC} as Tg macrophages (data not shown). These results suggest that factors other than N1^{EC} itself are inhibiting LSECs growth *in vitro*. Incubation of endothelial cells from

Figure 6. CD4C/N1^{EC} Tg macrophages express N1^{EC} and show impaired functions. **A–D:** Tg expression in macrophages. Tg RNA expression was detected by *in situ* hybridization in KC-like cells in the Tg liver section (**A**) and in purified Tg KCs (**B**) and further confirmed N1^{EC} in Tg KCs by RT-PCR (**B**). Tg N1^{EC} protein expression was detected in macrophages by IHC (**C**) ($\times 40$) or in macrophage supernatant by Western blot (**D**) (1: 400 $\times$ g supernatant; 2: 100,000 $\times$ g (45 minutes) supernatant; 3: 100,000 $\times$ g pellet). PR, Ponceau Red. **E:** Infiltration of macrophages around abnormal Tg vessels. Frozen liver sections were used for IHC using the monoclonal antibody for Mac-1. The non-Tg (**a**) and Tg (**b** and **c**) livers were compared. Note that, in contrast to the weak staining detected in the non-Tg liver section (**a**), a strong staining was observed around vessels in the Tg liver (**b**). Higher magnification showed large stained cells (**c**). Magnification: $\times 10$ (**a** and **b**); $\times 100$ (**c**). **F:** EM. Quantitation of KCs present in the sinusoids of Tg (28 fields) or non-Tg (11 fields) liver. **G:** Increased phagocytosis of Tg macrophages. Two hours before sacrifice, latex beads (8.7×10^7 /mouse) were injected into 4- to 6-month-old non-Tg ($n = 3$) or Tg ($n = 4$) mice by tail vein. Frozen liver sections were used to count the beads captured by KCs. Note that Tg KCs capture more beads than non-Tg cells. **H:** Increased accumulation of EB in Tg macrophages. EB was injected via the tail vein, and mice were sacrificed 1 to 2 hours after injection. Frozen liver sections were prepared and examined by fluorescence microscopy. Strong fluorescence was observed in cells having macrophage/monocyte morphology in Tg sinusoids. Magnification, $\times 20$.

Figure 7. The liver vascular disease develops in CD4C/N1^{EC} Tg mice through a paracrine pathway that seems to involve macrophages. **A** and **B**: Liver vascular disease in chimeric mice. Chimeric mice were generated by fusing E2.5-day embryos from ROSA26 with those of CD4C/N1^{EC} Tg or non-Tg mice. Chimeric mice were analyzed at 5 to 6 months of age by X-gal staining of their livers. In ROSA26 Tg chimera (**B**), large vessels located on the surface of the liver developed, and some of them stained blue, indicating their ROSA26 parental origin. No such vessels were observed in the ROSA26 non-Tg chimeric livers (**A**). **C** and **D**: Fetal liver cell transplantation. Fetal liver cells from control (non-Tg × ROSA26) F₁ and from Tg (CD4C/N1^{EC} Tg × ROSA26) F₁ E14.5-day embryos were transplanted into 2- to 3-month-old nude mice. Two months later, mice were sacrificed and analyzed by X-gal staining ($n = 2$ for each group). Abnormal β -gal-negative vessels (arrows) and infiltration of β -gal-positive hematopoietic cells, including macrophages (asterisk), were observed in the liver from the mice transplanted with Tg cells (**D**), but not in mice receiving non-Tg cells (**C**). Magnification, $\times 20$. **E-H**: Macrophage transplantation. Peritoneal macrophages (3×10^6) from 3- to 4-month-old Tg and non-Tg mice were transplanted by intravenous inoculation into 2- to 3-month-old normal mice, and recipient animals were sacrificed 1 month later and perfused with Microfil (**E** and **F**). Note the abnormal vessels (arrows) in the liver of a mouse transplanted with Tg macrophages (**F** and inset). Magnification, 0.5×5.0 . **G** and **H**: Histological analysis performed on the Microfil-perfused livers showed superficially clustered large vessels in the liver from a mouse transplanted with Tg macrophages (**H**), but not in the liver from a mouse transplanted with non-Tg ones (**G**). Magnification, $\times 5$. **I**: Interaction of CD4C/N1^{EC} Tg macrophages with LSECs. **a-c**: Peritoneal macrophages from Tg or non-Tg mice were co-cultured with normal LSECs, labeled with latex beads or Dil-Ac-LDL, respectively. The number of clusters ($5\times$) formed by non-Tg (**a**) or Tg (**b**) macrophages clumped onto the LSECs was counted after 3 to 4 hours of incubation and quantitated (**c**). **d**: Supernatants of Tg macrophages inhibit the growth of non-Tg LSECs. Purified LSECs were incubated with conditioned medium (CM) from non-Tg or Tg peritoneal macrophages in 48-well plates. After 5 days of incubation, cells were counted.

another tissue (rat lungs) with Tg supernatants had no inhibitory effect (data not shown), suggesting that LSECs were more susceptible than other endothelial cells to Tg macrophage factors. Together, these data are consistent with the lower branching of liver vessels *in vivo* and suggest that factors released from macrophages may be involved in the development of some aspects of the liver disease in CD4C/N1^{EC} Tg mice.

Molecular Alterations in Macrophages of CD4C/N1^{EC} Tg Mice

Levels of expression of candidate genes that may be dysregulated in Tg macrophages were next measured in purified peritoneal macrophages by RT-PCR analysis. Levels of molecules known to bind to N1^{EC} (Delta 1, Delta 3, Delta 4, Jag1, Jag2, and *cnn3*) were first measured.

Figure 8. Expression analysis of candidate genes in macrophages of CD4C/N1^{EC} Tg mice. **A:** RNA was extracted from non-Tg and Tg peritoneal macrophages. Expressions of the indicated genes were measured by quantitative PCR. Values for each gene are expressed relative to S16 expression. These results were reproduced with either pooled cells (three mice/non-Tg or Tg group, $n = 2$, pooled experiments) four times or cells from individual mice ($n = 3$, non-Tg or Tg) twice. All statistical differences were obtained by the Student's *t*-test, except for MCP-5 and Egr-1 where the F-test was used. **B:** Egr-1 binding sites in the indicated genes, as searched with the MatInspector/Genomatrix software⁷⁵ or by sequence comparison for MCP-1. This latter site is identical to the Egr-1 site of the mouse luteinizing hormone- β gene.⁷⁶

Among these, only Jag1 was differentially expressed (data not shown). This was confirmed by quantitative PCR, which showed ~3-fold increased expression in Tg macrophages (Figure 8). We next measured expression of well-documented angiogenic molecules: vascular endothelial growth factor, Flt-1, Flk-1, angiopoietin 1 and 2, Tie-2, EphB4, EphrinB2, basic fibroblast growth factor, coagulators [tissue factor (FIII) and FXIII)], MCP-1, and MCP-5. The latter two candidates were measured because mouse MCP-5 is most homologous to human MCP-1,²⁸ which has been shown to be elevated in human hemangiomas.²⁹ Comparable levels of expression for most of these genes were found in Tg and non-Tg macrophages (data not shown). However, levels of MCP-5 (*Ccl12*), MCP-1 (*Ccl2*), FIII, and FXIII-A were higher in Tg than in non-Tg macrophages (Figure 8). In particular, MCP-5 was increased ~40-fold (range, 10 to 150) (Figure 8). Because MCP-1³⁰ and FIII^{31–33} genes have been described as targets of the transcription factor Egr-1, expression of the latter was measured and found to be increased ~4-fold in Tg macrophages relative to non-Tg ones (Figure 8). Together, these results indicate that N1^{EC}-expressing macrophages produce enhanced levels of pro-angiogenic molecules.

Discussion

Macrophages Are Key Mediators of Vascular Lesions Induced by N1^{EC} in CD4C/N1^{EC} Tg Mice

We demonstrate here that CD4C/N1^{EC} Tg mice apparently develop three distinct vessel anomalies: enhanced growth of large meandering ectopic vessels at the surface of the liver, impaired vascular branching within the

liver parenchyma, and formation of cavernous vascular lesions.

The large vessels at the surface of the liver are reminiscent of normal large vessels surrounding some organs and often large tumors. Such vessels seem to be programmed to “wrap” or cover an organ, suggesting that their fate may be controlled by hematopoietic myeloid cells through the Notch1 pathway. These superficial Tg vessels seem to be unable to grow within the liver parenchyma and only superficially penetrate the parenchyma. The impaired vascular branching paradoxically observed within the liver parenchyma of Tg mice is possibly related to this inhibition, suggesting the presence of anti-angiogenic factors within the parenchyma. These putative inhibitory factors seem to be uniformly distributed, even in regions showing minimal other vascular abnormalities. The *in vitro* inhibitory growth of LSECs by conditioned medium from Tg macrophages may reflect this *in vivo* phenotype. Finally, the malformed vessels, including the large vascular cavities in Tg liver are similar to some human liver vascular lesions.^{34,35} These are frequent and are often associated with cutaneous hemangiomas, the liver representing the most common extracutaneous site of hemangiomas.^{36,37} The human liver vascular lesions are also known to be quite heterogeneous,^{34,36–39} being classified either as vascular malformations, which typically never regress, or as hemangiomas, which frequently involute.^{37,40} The lesions of CD4C/N1^{EC} Tg mice seem to be similar to nonregressing human vascular malformations, although the large Tg cavernous lesions, often solitary, may share some features of human cavernous hemangiomas.⁴¹

Several independent experiments point toward the role of N1^{EC}-expressing hematopoietic myeloid cells, in particular of macrophages, in the development of the complex vascular phenotype of CD4C/N1^{EC} Tg mice, although other myeloid cell populations, such as dendritic cells in which the CD4C promoter is active,¹⁵ may contribute to the phenotypes. Interestingly, the strong effect of N1^{EC} seems to be cell-specific: in CD4C/N1^{EC} or MMTV/N1^{EC} Tg mice, expression of high levels of N1^{EC} in thymic T cells and in mammary epithelial cells, respectively, do not induce a vascular disease in the thymus or in the mammary glands. A role of macrophages in angiogenesis has been well documented.^{42,43} Macrophages, under specific conditions, have the capability of secreting a myriad of proangiogenic and some anti-angiogenic factors.^{42,43} They seem to be critical for angiogenesis in wound healing and in malignant tumors.⁴⁴ Interestingly, accumulation of myeloid cells (monocytes, macrophages, or dendritic cells) has been observed in infantile human hemangiomas^{45,46} and in experimental hemangiomas.⁴⁷ In this latter model, inhibition of MCP-1 led to decreased infiltration of macrophages in hemangiomas and to diminished ability to form hemangiomas.⁴⁷ Our results confirm and extend these data by showing that N1^{EC}-expressing macrophages provide both pro- and anti-angiogenic signals.

Vascular abnormalities develop in several organs of the CD4C/N1^{EC} Tg mice but are more severe and frequent in the liver. This high organ selectivity may arise for

the following reasons. First, the liver contains specific endothelial cells (LSECs) that may be specifically sensitive to the factors produced by the N1^{EC}-expressing Kupffer cells. Second, in no other organ of the body is the interaction of the endothelial cells (LSECs) with macrophages (Kupffer cells) closer than in the liver.^{48,49} Third, most (80%) of the body macrophages reside in the liver, where they are designated Kupffer cells. This very high number of Kupffer cells may itself be a determinant to reach a critical local concentration of factors required to induce the vascular phenotypes. The weak expression of the N1^{EC} transgene detected in LSECs is unlikely to have a major impact on the development of the vascular disease in the liver, because the N1^{EC}-mediated vascular phenotype is fully inducible by transplantation of Tg BM or fetal liver cells, whereas mouse LSECs have been shown not to be transplantable.⁵⁰

It has already been well documented, through loss- or gain-of-function mutants, that Notch signaling is involved in vascular development.³ However, these findings differ significantly from those reported here. Most of the vascular defects observed in these mutant mice develop during early embryogenesis. In addition, most studies performed with gain-of-function mutants represent work with the truncated intracellular form of Notch family members. In contrast, the phenotypes of the CD4C/N1^{EC} Tg mice can develop in adulthood and are induced by the expression of N1^{EC}, implicating for the first time this molecular form in a pathological process. Interestingly, expression of an N3^{EC} mutant in smooth muscle cells of Tg mice was shown to recapitulate the vascular phenotype of human cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy disease.⁵¹ A truncated N1^{EC} and N4^{EC} may also contribute to the vascular phenotype described in double N1+N4 gene-deficient mice, because the mutation generated truncated N1^{EC} and N4^{EC} proteins of a significant size.⁵²

The Pathogenesis of the Liver Vascular Disease of CD4C/N1^{EC} Tg Mice

Because transplanted BM precursors or peritoneal N1^{EC}-expressing macrophages can reproduce the major features of vascular disease, a paracrine pathway involving the interaction of N1^{EC}-expressing macrophages or factor(s) derived from them with resident endothelial cells is postulated. Consistent with this hypothesis, we have documented significant changes of small vessels or LSECs in these Tg mice, both *in vivo* (capillarization, enhanced CD31 positivity, and decreased sprouting) and *in vitro* (decreased proliferation). Transdifferentiation of macrophages into endothelial cells^{53,54} is less likely, as shown with Tg-ROSA26-transplanted mice.

In CD4C/N1^{EC} Tg mice, it seems that Tg macrophages are reprogrammed by the expression of N1^{EC}. We have documented that the Tg macrophages show signs of activation and exhibit functional defects. This activation is also supported by the increased expression of the Egr-1 transcription factor in Tg macrophages. *Egr-1* seems to be a stress-responsive gene,⁵⁵ and its activation

regulates the expression of several proangiogenic genes,^{56–58} including *MCP-1*³⁰ and *Fli1*^{31–33,59} (see below). The reprogramming of macrophages by N1^{EC} must be unique, because no other signaling molecule has yet been described, to our knowledge, as being able to confer to macrophages the angiogenic properties observed in these Tg mice.

Because N1^{EC} normally binds to Notch1 ligands¹ or to other ligands (F3/contactin, CNN3, and Wingless),^{7–9} such N1^{EC}-ligand interaction could occur within intracellular compartments (possibly in the Golgi apparatus or the intrareticulum) or on the plasma membrane of macrophages. Therefore, the putative binding of N1^{EC} to a ligand might initiate an autocrine loop. It may function as agonist or antagonist on the receptor(s) to which it binds, similar to the action of Notch ligand ectodomain in *Drosophila*.^{10,11} We have excluded that Tg N1^{EC} could activate signaling of the endogenous wild-type N1 itself within macrophages. Tg mice (CD4C/N1^{intra}) expressing the activated intracellular domain of Notch1 (N1^{intra}) in the same target cells, including macrophages, through the same regulatory sequences (CD4C) as the CD4C/N1^{EC} Tg mice presented here do not develop a vascular phenotype.⁶⁰ However, the overexpressed Jag1 documented in N1^{EC}-expressing macrophages may represent the ligand(s) of N1^{EC} in these cells.

Reprogrammed N1^{EC}-expressing macrophages produce factors affecting neighboring endothelial cells in a paracrine manner. Both positive angiogenic factors (inducing growth of the ectopic superficial vessels and of large cavernous vascular lesions) and negative angiogenic factors (blocking parenchymal vessel branching) seem to be produced simultaneously. N1^{EC} itself is unlikely to be among these paracrine pro- or anti-angiogenic factors, because its overexpression by other cells in other organs (thymus of CD4C/N1^{EC} Tg mice and mammary glands of MMTV/N1^{EC} Tg mice) was not found to induce the development of a vascular phenotype. Rather, other factor(s) whose expression is (are) modulated in N1^{EC}-mediated reprogramming of macrophages might be implicated.

Our initial screen to identify genes that may be transmitting such paracrine signals showed a notable lack of detectable changes of vascular endothelial growth factor levels in Tg macrophages. This is consistent with the decreased vascular branching in CD4C/N1^{EC} Tg liver, in contrast with the enhanced angiogenesis that would be expected if vascular endothelial growth factor levels were elevated.⁶¹ Moreover, Tg rabbits overexpressing vascular endothelial growth factor in the liver under the regulation of the human α -antitrypsin promoter develop vascular malformations⁶² but no superficial large vessels, as observed in CD4C/N1^{EC} Tg mice. Our search, however, revealed interesting candidates (*MCP-1*, *MCP-5*, *Fli1*, *FXIII-A*, *Jag1*, and *Egr-1*) already known to be implicated in angiogenesis. Overexpression of Jag1 by Tg macrophages could deliver signal to nearby endothelial cells through the Notch receptor(s) and induce neovascularization. Such signaling by Jag1 expressed in carcinoma cells was recently found to enhance significantly tumor angiogenesis.⁶³ This could occur through Notch4, be-

cause expression of constitutively active Notch4 in endothelial cells was shown to induce vascular malformations.^{64,65} The enhanced expression of coagulation factors FIII and FXIII-A in Tg macrophages is of interest. Tissue factor (FIII) regulates angiogenesis,⁶⁶ and its pharmacological inhibition can block basic fibroblast growth factor-induced angiogenesis *in vivo*.⁶⁷ In contrast, FXIII-A may be pro- or anti-angiogenic, through its association with $\alpha_v\beta_3$ integrin present on vascular endothelial cells.^{68,69} Finally, the very high levels of MCP-5 and the high levels of *MCP-1* in Tg macrophages are likely to be involved in the observed vascular phenotype and in particular may contribute to the development of cavernous lesions. *MCP-1* has been shown to be involved in many angiogenic events.^{70,71} In human hemangiomas, the levels of *MCP-1* (most homologous to murine MCP-5²⁸) are elevated, and its enhanced expression has been suggested to be involved in the development of these lesions.²⁹ Recently, an *in vivo* murine model of hemangioendothelioma was found to be totally dependent on *MCP-1*.^{47,72} It is worth noticing that two (*MCP-1* and *FIII*) of these genes represent known targets of the Egr-1 transcription factor,^{30,59} whereas the other three (*MCP-5*, *Jag1*, and *FXIII-A*) harbor Egr-1 DNA-binding sequences (Figure 8). This strongly suggests that the *Egr-1* represents a key intermediate in the N1^{EC}-mediated vascular disease of these Tg mice. This would be consistent with its known role in various biological processes involving neovascularization and/or angiogenesis.^{56,57,73,74}

In conclusion, our data show that expression of N1^{EC} can reprogram macrophages in such a unique way that they acquire the ability to significantly affect neighboring endothelial cells, most likely by producing several pro- or anti-angiogenic factors, thus leading to the development of vascular cavernous lesions and additional vascular patterning defects. Our data suggest a new molecular mechanism for the involvement of the Notch1 pathway in vascular diseases. They implicate the soluble ectodomain of Notch1 in a myeloid cell-specific process involving a paracrine loop. In particular, these N1^{EC} Tg mice provide a novel model of human vascular disease, the development of which is dependent on myeloid cells. In some pathological processes, cleavage and production of N1^{EC} may occur aberrantly within macrophages, thus leading to their reprogramming, in particular through the overexpression of Egr-1, as documented here. Thus, a similar N1^{EC}-regulated macrophage-mediated process may be involved in some human diseases, such as hemangioma, tumor growth, and metastasis and may represent a target for therapeutic benefit.

Acknowledgments

We thank Benoît Laganière, Viorica Lascau, Isabelle Corbin, Isabelle Labrosse, Ginette Massé, Jean-René Sylvestre, and Lin Jia for excellent technical assistance and Chunyan Hu, Louis Lamarre, and Zaher Hanna for helpful discussions.

References

1. Artavanis-Tsakonas S, Rand MD, Lake RJ: Notch signaling: cell fate control and signal integration in development. *Science* 1999, 284:770–776
2. Kopan R: Notch: a membrane-bound transcription factor. *J Cell Sci* 2002, 115:1095–1097
3. Gridley T: Notch signaling during vascular development. *Proc Natl Acad Sci USA* 2001, 98:5377–5378
4. Shawber CJ, Kitajewski J: Notch function in the vasculature: insights from zebrafish, mouse and man. *Bioessays* 2004, 26:225–234
5. Rebay I, Fleming RJ, Fehon RG, Cherbas L, Cherbas P, Artavanis-Tsakonas S: Specific EGF repeats of Notch mediate interactions with Delta and Serrate: implications for Notch as a multifunctional receptor. *Cell* 1991, 67:687–699
6. Sakamoto K, Ohara O, Takagi M, Takeda S, Katsube K: Intracellular cell-autonomous association of Notch and its ligands: a novel mechanism of Notch signal modification. *Dev Biol* 2002, 241:313–326
7. Hu QD, Ang BT, Karsak M, Hu WP, Cui XY, Duka T, Takeda Y, Chia W, Sankar N, Ng YK, Ling EA, Maciag T, Small D, Trifonova R, Kopan R, Okano H, Nakafuku M, Chiba S, Hirai H, Aster JC, Schachner M, Pallen CJ, Watanabe K, Xiao ZC: F3/contactin acts as a functional ligand for Notch during oligodendrocyte maturation. *Cell* 2003, 115:163–175
8. Sakamoto K, Yamaguchi S, Ando R, Miyawaki A, Kabasawa Y, Takagi M, Li CL, Perbal B, Katsube K: The nephroblastoma overexpressed gene (NOV/ccn3) protein associates with Notch1 extracellular domain and inhibits myoblast differentiation via Notch signaling pathway. *J Biol Chem* 2002, 277:29399–29405
9. Wesley CS: Notch and wingless regulate expression of cuticle patterning genes. *Mol Cell Biol* 1999, 19:5743–5758
10. Sun X, Artavanis-Tsakonas S: Secreted forms of DELTA and SERRATE define antagonists of Notch signaling in *Drosophila*. *Development* 1997, 124:3439–3448
11. Qi H, Rand MD, Wu X, Sestan N, Wang W, Rakic P, Xu T, Artavanis-Tsakonas S: Processing of the Notch ligand delta by the metalloprotease kuzbanian. *Science* 1999, 283:91–94
12. Joutel A, Corpechot C, Ducros A, Vahedi K, Chabriet H, Mouton P, Alamowitch S, Domenga V, Cecillion M, Marechal E, Maciazek J, Vayssiere C, Cruaud C, Cabanis EA, Ruchoux MM, Weissenbach J, Bach JF, Bousser MG, Tournier-Lasserre E: Notch3 mutations in CADASIL, a hereditary adult-onset condition causing stroke and dementia. *Nature* 1996, 383:707–710
13. Hoemann CD, Beaulieu N, Girard L, Rebai N, Jolicoeur P: Two distinct Notch1 mutant alleles are involved in the induction of T-cell leukemia in c-myc transgenic mice. *Mol Cell Biol* 2000, 20:3831–3842
14. Zambrowicz BP, Imamoto A, Fiering S, Herzenberg LA, Kerr WG, Soriano P: Disruption of overlapping transcripts in the ROSA beta geo 26 gene trap strain leads to widespread expression of beta-galactosidase in mouse embryos and hematopoietic cells. *Proc Natl Acad Sci USA* 1997, 94:3789–3794
15. Hanna Z, Rebai N, Poudrier J, Jolicoeur P: Distinct regulatory elements are required for faithful expression of human CD4 in T cells, macrophages and dendritic cells of transgenic mice. *Blood* 2001, 98:2275–2278
16. Hogan B, Beddington R, Costantini F, Lacy E: *Manipulating the Mouse Embryo: A Laboratory Manual*. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press, 1994
17. Girard L, Hanna Z, Beaulieu N, Hoemann CD, Simard C, Kozak CA, Jolicoeur P: Frequent provirus insertional mutagenesis of Notch1 in thymomas of MMTVD/myc transgenic mice suggests a collaboration of c-myc and Notch1 for oncogenesis. *Genes Dev* 1996, 10:1930–1944
18. Kay DG, Yue P, Hanna Z, Jothy S, Tremblay E, Jolicoeur P: Cardiac disease in transgenic mice expressing human immunodeficiency virus-1 nef in cells of the immune system. *Am J Pathol* 2002, 161:321–335
19. Poudrier J, Weng X, Kay DG, Paré G, Calvo EL, Hanna Z, Kosco-Vilbois MH, Jolicoeur P: The AIDS disease of CD4C/HIV transgenic mice shows impaired germinal centers and autoantibodies and develops in the absence of IFN-g and IL-6. *Immunity* 2001, 15:173–185
20. Borowiak M, Garratt AN, Wustefeld T, Strehle M, Trautwein C, Birchmeier C: Met provides essential signals for liver regeneration. *Proc Natl Acad Sci USA* 2004, 101:10608–10613

21. Wuestefeld T, Klein C, Streetz KL, Betz U, Lauber J, Buer J, Manns MP, Muller W, Trautwein C: Interleukin-6/glycoprotein 130-dependent pathways are protective during liver regeneration. *J Biol Chem* 2003, 278:11281–11288
22. Do H, Healey JF, Waller EK, Lollar P: Expression of factor VIII by murine liver sinusoidal endothelial cells. *J Biol Chem* 1999, 274:19587–19592
23. Couvelard A, Scoazec JY, Dauge MC, Bringuier AF, Potet F, Feldmann G: Structural and functional differentiation of sinusoidal endothelial cells during liver organogenesis in humans. *Blood* 1996, 87:4568–4580
24. Thurston G, Suri C, Smith K, McClain J, Sato TN, Yancopoulos GD, McDonald DM: Leakage-resistant blood vessels in mice transgenically overexpressing angiopoietin-1. *Science* 1999, 286:2511–2514
25. Medina J, Arroyo AG, Sanchez-Madrid F, Moreno-Otero R: Angiogenesis in chronic inflammatory liver disease. *Hepatology* 2004, 39:1185–1195
26. Mombaerts P, Iacomini J, Johnson RS, Herrup K, Tonegawa S, Papaioannou VE: RAG-1-deficient mice have no mature B and T lymphocytes. *Cell* 1992, 68:869–877
27. Scoazec JY, Feldmann G: Both macrophages and endothelial cells of the human hepatic sinusoid express the CD4 molecule, a receptor for the human immunodeficiency virus. *Hepatology* 1990, 12:505–510
28. Sarafi MN, Garcia-Zepeda EA, MacLean JA, Charo IF, Luster AD: Murine monocyte chemoattractant protein (MCP)-5: a novel CC chemokine that is a structural and functional homologue of human MCP-1. *J Exp Med* 1997, 185:99–109
29. Isik FF, Rand RP, Gruss JS, Benjamin D, Alpers CE: Monocyte chemoattractant protein-1 mRNA expression in hemangiomas and vascular malformations. *J Surg Res* 1996, 61:71–76
30. Giri RK, Selvaraj SK, Kalra VK: Amyloid peptide-induced cytokine and chemokine expression in THP-1 monocytes is blocked by small inhibitory RNA duplexes for early growth response-1 messenger RNA. *J Immunol* 2003, 170:5281–5294
31. Bea F, Puolakkainen MH, McMillen T, Hudson FN, Mackman N, Kuo CC, Campbell LA, Rosenfeld ME: Chlamydia pneumoniae induces tissue factor expression in mouse macrophages via activation of Egr-1 and the MEK-ERK1/2 pathway. *Circ Res* 2003, 92:394–401
32. Pawlinski R, Pedersen B, Kehrle B, Aird WC, Frank RD, Guha M, Mackman N: Regulation of tissue factor and inflammatory mediators by Egr-1 in a mouse endotoxemia model. *Blood* 2003, 101:3940–3947
33. Group ER, Donovan-Peluso M: Lipopolysaccharide induction of THP-1 cells activates binding of c-Jun, Ets, and Egr-1 to the tissue factor promoter. *J Biol Chem* 1996, 271:12423–12430
34. Burrows PE, Dubois J, Kassarian A: Pediatric hepatic vascular anomalies. *Pediatr Radiol* 2001, 31:533–545
35. Semelka RC, Sofka CM: Hepatic hemangiomas. *Magn Reson Imaging Clin N Am* 1997, 5:241–253
36. Nord KM, Kandel J, Lefkowitz JH, Lobritto SJ, Morel KD, North PE, Garzon MC: Multiple cutaneous infantile hemangiomas associated with hepatic angiosarcoma: case report and review of the literature. *Pediatrics* 2006, 118:e907–e913
37. Drolet BA, Esterly NB, Frieden IJ: Hemangiomas in children. *N Engl J Med* 1999, 341:173–181
38. Miyayama S, Matsui O, Zen Y, Yamashiro M, Ryu Y, Minami T, Notsumata K, Tanaka N: Focal hepatic lesions mimicking cavernous hemangioma supplied by the portal vein. *Hepatol Res* 2006, 36:70–73
39. Iqbal N, Saleem A: Hepatic hemangioma: a review. *Tex Med* 1997, 93:48–50
40. Vikkula M, Boon LM, Mulliken JB, Olsen BR: Molecular basis of vascular anomalies. *Trends Cardiovasc Med* 1998, 8:281–292
41. Kim GE, Thung SN, Tsui WM, Ferrell LD: Hepatic cavernous hemangioma: underrecognized associated histologic features. *Liver Int* 2006, 26:334–338
42. Sünderkotter C, Steinbrink K, Goebeler M, Bhardwaj R, Sorg C: Macrophages and angiogenesis. *J Leukoc Biol* 1994, 55:410–422
43. Moldovan L, Moldovan NI: Role of monocytes and macrophages in angiogenesis. *EXS* 2005, 94:127–146
44. Crowther M, Brown NJ, Bishop ET, Lewis CE: Microenvironmental influence on macrophage regulation of angiogenesis in wounds and malignant tumors. *J Leukoc Biol* 2001, 70:478–490
45. Nguyen VA, Furhapter C, Romani N, Weber F, Sepp N: Infantile hemangioma is a proliferation of beta 4-negative endothelial cells adjacent to HLA-DR-positive cells with dendritic cell morphology. *Hum Pathol* 2004, 35:739–744
46. Ritter MR, Reinisch J, Friedlander SF, Friedlander M: Myeloid cells in infantile hemangioma. *Am J Pathol* 2006, 168:621–628
47. Atalay M, Gordillo G, Roy S, Rovin B, Bagchi D, Bagchi M, Sen CK: Anti-angiogenic property of edible berry in a model of hemangioma. *FEBS Lett* 2003, 544:252–257
48. MacPhee PJ, Schmidt EE, Groom AC: Evidence for Kupffer cell migration along liver sinusoids, from high-resolution in vivo microscopy. *Am J Physiol* 1992, 263:G17–G23
49. Limmer A, Knolle PA: Liver sinusoidal endothelial cells: a new type of organ-resident antigen-presenting cell. *Arch Immunol Ther Exp (Warsz)* 2001, 49(Suppl 1):S7–S11
50. Gao ZH, Williams GM: Vascular endothelial-cell turnover: a new factor in the vascular microenvironment of the liver. *Trends Immunol* 2001, 22:421–422
51. Ruchoux MM, Domenga V, Brulin P, Maciazek J, Limol S, Tournier-Lasserre E, Joutel A: Transgenic mice expressing mutant Notch3 develop vascular alterations characteristic of cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy. *Am J Pathol* 2003, 162:329–342
52. Krebs LT, Xue Y, Norton CR, Shutter JR, Maguire M, Sundberg JP, Gallahan D, Closson V, Kitajewski J, Callahan R, Smith GH, Stark KL, Gridley T: Notch signaling is essential for vascular morphogenesis in mice. *Genes Dev* 2000, 14:1343–1352
53. Moldovan NI, Goldschmidt-Clermont PJ, Parker-Thornburg J, Shapiro SD, Kolattukudy PE: Contribution of monocytes/macrophages to compensatory neovascularization: the drilling of metalloelastase-positive tunnels in ischemic myocardium. *Circ Res* 2000, 87:378–384
54. Fernandez Pujol B, Lucibello FC, Gehling UM, Lindemann K, Weidner N, Zuzarte ML, Adamkiewicz J, Elsasser HP, Muller R, Havemann K: Endothelial-like cells derived from human CD14 positive monocytes. *Differentiation* 2000, 65:287–300
55. Gashler A, Sukhatme VP: Early growth response protein 1 (Egr-1): prototype of a zinc-finger family of transcription factors. *Prog Nucleic Acid Res Mol Biol* 1995, 50:191–224
56. Worden B, Yang XP, Lee TL, Bagain L, Yeh NT, Cohen JG, Van Waes C, Chen Z: Hepatocyte growth factor/scatter factor differentially regulates expression of proangiogenic factors through Egr-1 in head and neck squamous cell carcinoma. *Cancer Res* 2005, 65:7071–7080
57. Fahmy RG, Dass CR, Sun LQ, Chesterman CN, Khachigian LM: Transcription factor Egr-1 supports FGF-dependent angiogenesis during neovascularization and tumor growth. *Nat Med* 2003, 9:1026–1032
58. Khachigian LM, Collins T: Inducible expression of Egr-1-dependent genes: a paradigm of transcriptional activation in vascular endothelium. *Circ Res* 1997, 81:457–461
59. Cui MZ, Parry GC, Oeth P, Larson H, Smith M, Huang RP, Adamson ED, Mackman N: Transcriptional regulation of the tissue factor gene in human epithelial cells is mediated by Sp1 and EGR-1. *J Biol Chem* 1996, 271:2731–2739
60. Priceputo E, Bouallaga I, Zhang Y, Li X, Chrobak P, Hanna ZS, Poudrier J, Kay DG, Jolicoeur P: structurally distinct ligand-binding or ligand-independent Notch1 mutants are leukemogenic but affect thymocyte development, apoptosis, and metastasis differently. *J Immunol* 2006, 177:2153–2166
61. Yancopoulos GD, Davis S, Gale NW, Rudge JS, Wiegand SJ, Holash J: Vascular-specific growth factors and blood vessel formation. *Nature* 2000, 407:242–248
62. Kitajima S, Liu E, Morimoto M, Koike T, Yu Y, Watanabe T, Imagawa S, Fan J: Transgenic rabbits with increased VEGF expression develop hemangiomas in the liver: a new model for Kasabach-Merritt syndrome. *Lab Invest* 2005, 85:1517–1527
63. Zeng Q, Li S, Chepeha DB, Giordano TJ, Li J, Zhang H, Polverini PJ, Nor J, Kitajewski J, Wang CY: Crosstalk between tumor and endothelial cells promotes tumor angiogenesis by MAPK activation of Notch signaling. *Cancer Cell* 2005, 8:13–23
64. Carlson TR, Yan Y, Wu X, Lam MT, Tang GL, Beverly LJ, Messina LM, Capobianco AJ, Werb Z, Wang R: Endothelial expression of constitutively active Notch4 elicits reversible arteriovenous malformations in adult mice. *Proc Natl Acad Sci USA* 2005, 102:9884–9889
65. Uyttendaele H, Ho J, Rossant J, Kitajewski J: Vascular patterning

- defects associated with expression of activated Notch4 in embryonic endothelium. *Proc Natl Acad Sci USA* 2001, 98:5643–5648
66. Belting M, Dorrell MI, Sandgren S, Aguilar E, Ahamed J, Dorfleutner A, Carmeliet P, Mueller BM, Friedlander M, Ruf W: Regulation of angiogenesis by tissue factor cytoplasmic domain signaling. *Nat Med* 2004, 10:502–509
 67. Hembrough TA, Swartz GM, Papathanassiou A, Vlasuk GP, Rote WE, Green SJ, Pribluda VS: Tissue factor/factor VIIa inhibitors block angiogenesis and tumor growth through a nonhemostatic mechanism. *Cancer Res* 2003, 63:2997–3000
 68. Dallabrida SM, Falls LA, Farrell DH: Factor XIIIa supports microvascular endothelial cell adhesion and inhibits capillary tube formation in fibrin. *Blood* 2000, 95:2586–2592
 69. Dardik R, Loscalzo J, Inbal A: Factor XIII (FXIII) and angiogenesis. *J Thromb Haemost* 2006, 4:19–25
 70. Kim MY, Byeon CW, Hong KH, Han KH, Jeong S: Inhibition of the angiogenesis by the MCP-1 (monocyte chemoattractant protein-1) binding peptide. *FEBS Lett* 2005, 579:1597–1601
 71. Salcedo R, Ponce ML, Young HA, Wasserman K, Ward JM, Kleinman HK, Oppenheim JJ, Murphy WJ: Human endothelial cells express CCR2 and respond to MCP-1: direct role of MCP-1 in angiogenesis and tumor progression. *Blood* 2000, 96:34–40
 72. Gordillo GM, Onat D, Stockinger M, Roy S, Atalay M, Beck FM, Sen CK: A key angiogenic role of monocyte chemoattractant protein-1 in hemangioendothelioma proliferation. *Am J Physiol Cell Physiol* 2004, 287:C866–C873
 73. Lee YS, Jang HS, Kim JM, Lee JS, Lee JY, Li KK, Shin IS, Suh W, Choi JH, Jeon ES, Byun J, Kim DK: Adenoviral-mediated delivery of early growth response factor-1 gene increases tissue perfusion in a murine model of hindlimb ischemia. *Mol Ther* 2005, 12:328–336
 74. McCaffrey TA, Fu C, Du B, Eksinar S, Kent KC, Bush H Jr, Kreiger K, Rosengart T, Cybulsky MI, Silverman ES, Collins T: High-level expression of Egr-1 and Egr-1-inducible genes in mouse and human atherosclerosis. *J Clin Invest* 2000, 105:653–662
 75. Quandt K, Frech K, Karas H, Wingender E, Werner T: MatInd and MatInspector: new fast and versatile tools for detection of consensus matches in nucleotide sequence data. *Nucleic Acids Res* 1995, 23:4878–4884
 76. Lee SL, Sadovsky Y, Swirloff AH, Polish JA, Goda P, Gavrilina G, Milbrandt J: Luteinizing hormone deficiency and female infertility in mice lacking the transcription factor NGFI-A (Egr-1). *Science* 1996, 273:1219–1221