Université de Montréal

Overexpression of Notch1 Ectodomain in Macrophages Induces Vascular Defects and Promotes Tumor Progression

par

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Faculty of Medicine

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Overexpression of Notch1 Ectodomain in Macrophages Induces Vascular Defects and Promotes Tumor Progression

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To my parents,

and Zhongwei

To Paul,

Résumé

Le récepteur Notch est conservé dans un grand nombre d'organismes allant des oursins jusqu'aux êtres humains. Il contient le domaine extracellulaire (N^{EC}) qui renferme principalement les répétitions de motifs de type EGF ainsi que le domaine intracellulaire (N^{IC}). Le signal de Notch est surtout amorcée par une interaction récepteur-ligand. Les signaux transmis par le biais de Notch contrôlent le destin des cellules, allant de l'hématopoïèse à l'angiogenèse, et ils conduisent au cancer et aux maladies vasculaires lors de conditions pathologiques. L'angiogenèse est un des mécanismes rendant compte de la formation de nouveaux vaisseaux sanguins. Elle est régulée de près par l'équilibre entre les signaux pro- et anti-angiogéniques et reste latent à l'état adulte. Lorsque l'équilibre est rompu, l'angiogenèse devient pathologique et soutient alors plusieurs maladies. Les macrophages agissent en tant qu'importants stimuli dans le micro-environnement tissulaire pour réguler l'angiogenèse car, après leur activation, ils peuvent sécréter un grand nombre de facteurs pro- et anti-angiogéniques.

C'est en étudiant la leucémie des cellules T induite par rétrovirus, que l'équipe du Dr Jolicoeur a découvert que Notch1 était tronqué par insertion d'ADN viral, créant ainsi une surexpression des domaines $N1^{EC}$ et $N1^{IC}$. Nous avons formulé l'hypothèse que $N1^{EC}$, tout comme $N1^{IC}$, était impliqué dans la formation des tumeurs. Par la suite, nous avons généré des souris transgéniques (Tg) -CD4C/$N1^{EC}$- exprimant $N1^{EC}$ dans les cellules T et dans les cellules de la lignée dendritique/macrophage (Mø). Contre toute attente, ce fut une maladie vasculaire qui s'est développée chez ces souris Tg, et non une leucémie T-cellulaire. On a d'abord observé la maladie vasculaire surtout dans les foies Tg. Suite à une série d'expériences *in vivo* et *in vitro*, on a trouvé que la maladie est liée à une angiogenèse aberrante. Nos résultats essentiels indiquent que la surexpression de $N1^{EC}$ chez les

macrophages induit des malformations vasculaires par le biais d'un mécanisme paracrine. On a ensuite observé la maladie vasculaire dans les utérus des femelles Tg stériles ou moins fertiles. Semblable au cas du foie, les cellules hématopoïétiques (macrophages) jouent un rôle clé dans ce phénotype. Finalement, les souris Tg (CD4C/$N1^{EC}$) furent mêlées à la progression, aux métastases et à l'angiogenèse des tumeurs, toutes détectées avec des modèles de tumeurs différents.

En conclusion, la surexpression de $N1^{EC}$ dans les macrophages induit des maladies vasculaires dans le foie, l'infertilité chez la souris femelle, et encourage tant la croissance des tumeurs que l'angiogenèse tumorale elle-même. Une étude plus poussée des molécules présentes dans les macrophages ciblés par $N1^{EC}$ (requis pour l'angiogenèse et pour la progression des tumeurs) pourrait nous éclairer sur les mécanismes des maladies vasculaires et de la croissance tumorale chez l'être humain, soit somme toute, sur le potentiel thérapeutique de l'anti-angiogenèse et de l'anti-cancer.

Mot-clés: CD4C/$N1^{EC}$, $N1^{EC}$, macrophage, vaisseaux, foie, femelle, et tumeur

Summary

Notch receptor is conserved in many organisms, ranging from sea urchins to humans. It contains the extracellular (N^{EC}) domain—mainly including EGF-like repeats—and the intracellular (N^{IC}) domain. The Notch signalling is predominantly initiated by a receptor-ligand interaction. The signals transmitted through Notch control cell fate, ranging from hematopoiesis to angiogenesis, and lead to cancer and vascular diseases in pathological conditions. Angiogenesis is one of the mechanisms that accounts for new blood vessel formation. It is tightly regulated by the balance of pro- and anti-angiogenic signals and is quiescent during adulthood. When the balance is disrupted, angiogenesis becomes pathologic and sustains many diseases. Macrophages act as important stimuli in the tissue microenvironment to regulate angiogenesis, since activated macrophages can secrete a large number of pro- and anti-angiogenic factors.

While studying retrovirus-induced T-cell leukemia, Dr. Jolicoeur's team found that Notch1 was truncated by viral DNA insertion, thus generating overexpression of $N1^{EC}$ and $N1^{IC}$ domains. We hypothesized that $N1^{EC}$, as $N1^{IC}$, was involved in tumor formation, so we further generated Tg mice (CD4C/$N1^{EC}$) expressing $N1^{EC}$ in T-cells and in cells of the macrophage ($M_{\varnothing}$)/dendritic lineage. Unexpectedly, vascular disease, not T-cell leukemia, developed in these Tg animals. First, the vascular disease was predominately observed in the Tg liver. By a series of *in vivo* and *in vitro* experiments, the disease was found to be involved in an aberrant angiogenesis. Our results indicate that overexpression of $N1^{EC}$ in macrophages induces vascular malformations by a paracrine mechanism. Then, the vascular disease was observed in the uterus of sterile or less fertile Tg females. Similar to the liver, hematopoietic cells (macrophages) play a key role in this phenotype. Finally, the

CD4C/N1^{EC} Tg mice implicated in tumor progression, metastasis, and angiogenesis detected with distinct tumor models.

In conclusion, the overexpression of N1^{EC} in macrophages induces vascular liver disease and female infertility, and promotes tumor growth and angiogenesis. Further study of molecules in the macrophages targeted by N1^{EC} required for angiogenesis and tumor progression may provide new insights into the mechanism of human vascular disease and tumor growth, as well as into the therapeutic potential of anti-angiogenesis and anti-cancer.

Keywords: CD4C/N1^{EC} Tg mice, N1^{EC}, macrophages, vessel, liver, female, and tumor

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List of Abbreviations

AAH: atypical adenomatous hyperplasia
Ang: angiopoietin
ANGPTL3: Angiopoietin-like protein 3
AGM: aorta-gonad-mesonephros
AGS: Alagille syndrome

bFGF: basic fibroblast growth factor
bHLH: basic helix-loop-helix
BM: bone marrow
BMEs: bovine microendothelial cells

CAM: chicken chorioallantoic membrane
CADASIL: cerebral autosomal-dominant arteropathy with subcortical infracts and leukoencephalopathy
CLPs: common lymphoid progenitors
CFU-GM: colonyforming unit, granulocyte-macrophage
COX: cyclooxygenase
CSL: CBF1(RBPjk)/Suppressor of hairless/Lag-1

Dl: Delta
Dll1: Delta-like 1
DMH: 1,2-Dimethylhydrazine dihydrochloride
DN3: double negative stage 3
Dsh: Dishevelled
DSL: Delta/Serrate/Lag-2

ECs: endothelial cells
ECM: extracellular matrix
EGF: epidermal growth factor
EGFR: epidermal growth factor receptor
ER: estrogen receptor
E[*spl*]: Enhancer of split

FC: follicular
FGF: fibroblast growth factor
FL: fetal liver
Flk: fetal liver kinase
Flt: *fms*-like tyrosine kinase

GLUT-1: glucose transporter-1
GM-CSF: granulocyte/macrophage-colony stimulating factor
GOM: granular osmophilic materials

HA: hyaluronic acid

HAF: human angiogenic factor
HBV: hepatitis B virus
HBx: hepatitis B virus X-protein
HCC: hepatocellular carcinoma
HCV: hepatitis C virus
HDAC: histone deacetylase
HES: Hairy/Enhancer of split
HGF/SF: hepatocyte growth factor/scatter factor
HHT: hereditary hemorrhagic telangiectasia
HIF: hypoxia-inducible factor
HME: human macrophage metalloelastase
HMEC-1: human dermal microvascular endothelial cells
HNF: hepatocyte nuclear factor
HPV16: human papilloma virus-16
HRE: hypoxia response element
HSC: hematopoietic stem cells
HUVECs: human umbilical vascular endothelial cells
HRMECs: human renal microvascular endothelial cells

ICAM: intercellular adhesion molecule
IGF-1: Insulin-like growth factor-1
IFN: interferon
IL: interleukin
INOS: inducible nitric oxide synthase

JAG: Jagged

KC: Kupffer cell
KDR: kinase insert domain-containing receptor

LFA-1: lymphocyte function-associated molecule
LNR:
LSECs: sinusoidal endothelial cells of the liver
LYVE-1: lymphatic vascular endothelial receptor

MACS: magnetic cell sorting
MAML1: Mastermind-like-1
MAPK: mitogen activated protein kinase
MASH1: mammalian achaete-scute-homologue 1
MCP: monocyte chemotactic protein
MCP-1: monocyte chemoattactant protein
M-CSF: macrophage-colony stimulating factor
MDF-ECI: macrophage-derived endothelial cell inhibitor
MECIF: monocyte-derived endothelial cell inhibitory factor
MIP: macrophage inflammatory protein
MHC: major histo-compatibility complex
MIF: macrophage migration inhibition factor
MMPs: matrix metaloproteases

MMTV: mouse mammary tumor virus
MøI: macrophage index
MSR: macrophage scavenger receptor
MZ: marginal zone
MZB: splenic marginal-zone-B cells

N^{EC}: Notch extracellular domain
N^{IC}: Notch intracellular domain
NK: natural kill
NLS: nuclear localization sequences
NO: nitric oxide
NOS: Nitric oxide synthase
Nrarp: Notch-regulated ankyrin-repeat protein

OAH: Ordinary adenomatous hyperplasia
OC: oral contraceptives
OFUT1: O-fucosyltransferase 1

PAI: plasminogen activator inhibitor
PD-ECGF: platelet-derived endothelial cell growth factor
PDGF: platelet derived growth factor
PDGFR-b: platelet derived growth factor b
PECAM-1: platelet/endothelial cell adhesion molecule-1
PEST: praline, glutamate, serine, threonine-rich
PGE2: prostaglandin E2.
PH: Partial hepatectomy
PlGF: Placenta growth factor
PLNs: peropherial lymphnodes
PNS: peripherial neural system
P-Sp: para-aortic splanchnopleura
PS: presenilins
PTB: phosphotyrosine-binding

Ser: Serrate
SD: spondylocostal dysostosis
SHH: Sonic hedgehog
SKIP: Ski-related Protein
SMRT: Silencing Mediator of Retinoid and Thyroid hormone receptor
SOPs: sensory organ precursors
SPARC: secreted protein, acidic, and rich in cysteine.
Su(Dx): Supressor of Deltex
Su(H): Supressor of Haireless

TAD: transcriptional activator domain
T-ALLs: human T-lymphoblast leukaemia
TAMs: tumor-associated macrophages
TAN-1: translocation-associated Notch homologue
TGF: transforming growth factor

TIMP: tissue inhibitor of metaloprotease
TNF: tumor necrosis factor
TSC: tuberous sclerosis
TSP-1: Thrombospondin-1

u-PA: urokinase plasminogen activator
u-PAR: urokinase plasminogen activator receptor

VEGF: vascular endothelial growth factor
VEGFR: vascular endothelial growth factor receptor
VHL: Von Hippel-Lindau protein
VM: venous malformation
VPF: vascular permeability factor
vSMCs/PCs: vascular smooth muscle cells/pericytes

YS: yolk sac

Preface

The present thesis, consisting of six chapters, has as a theme: the overexpression of Notch1 ectodomain in macrophages induces vascular defects and promotes tumor progression.

The first chapter is a general introduction and literature review of all the relevant works of the research project. It is divided into eight parts. The Notch fundamental role is mentioned in the first part. Then the function of Notch ectodomain follows in the second part, from which the rationales of the research arise. The third and fourth parts describe the physiological and pathological role of Notch, centered on the hematopoietic development and tumors and end with an emerging roles of Notch in vascular development and disease. Knowledge of vasculogenesis and angiogenesis is reviewed in the fifth section, in which tumor angiogenesis is introduced. Since macrophages are targeted by our Tg $N1^{EC}$ and since our Tg mice developed vascular disease, it is reasonable to understand the relationship between macrophages and angiogenesis. The explanation is to be found in part six. Why are the liver and uterus the main organs affected by aberrant angiogenesis in our Tg mice? The seventh and the eighth part will help you to learn more.

Chapter 2 exposes the rationale, hypothesis, objective, and an overview of the project. Chapter 3-5 are the experimental results planned to be published in three papers. Chapter 6 presents general conclusions and perspectives. Each chapter has its own reference list for reader's convenience. The papers included are the following:

1. Overexpression of Notch1 ectodomain in macrophages induces vascular malformations in Tg mice.

   Li, X., Calvo, E.L., Kay, D.G., Chrobak, P., Cool, M., Hanna, Z. and **P. Jolicoeur.**

2. Expression of the mouse Notch1 extracellular domain in macrophages leads to sterility in female Tg mice

   Li, X., Chrobak, P., and **P. Jolicoeur.**

3. Notch1 ectodomain expressed in macrophages promotes tumor progression, metastasis, and angiogenesis.

   Li, X. and **P. Jolicoeur.**

I provided a major contribution earning the first authorship in these papers. The contributions of the coauthors to these publications are acknowledged: Dr. Calvo has collaborated for the first paper at the beginning and has performed hepatectomy. Dr. Kay for his assistance in some pathological works. Dr. Chrobak for participating in bone marrow and fetal liver cell transplantation. Dr. Cool performed the chimeric experiments. Dr. Hanna for designing the CD4C promoter. Other collaborators, but not coauthors, also contributed to the papers and are gratefully acknowledged. Benoît Laganière for preparing the CD4C/N1^{EC} construction and generating the Tg mice. Ginette Massé for manipulating C3L5 tumor cells and for helping to isolate LSECs and to measure subcutaneous C3L5 tumors. Isabelle Corbin, Jean-René Sylvestre and Paule for taking care the mice. Isabelle Labrosse for helping to design some primers for VEGF, angiopoietin, and Ehprin B4. Chunyan Hu and Lin Jia for *in situ* hybridyzation. Viorica for PECAM-1 and α-actin immunohistochemistry. Dr. Paul Jolicoeur, acted as my thesis supervisor, thanks to his scientific insight and advice throughout the course of all these studies.

Chapter 1.

INTRODUCTION

Multicellular organisms arise by a process of progressive change that we call development. The development of a multicellular organism begins with a single cell. Sidney Brenner (quoted in Wilkins, 1993) has remarked that the animal development proceeds in either of two ways. Most invertebrates are specified predominantly by the "European style" that is to say the developmental fate of each cell is determined by its ancestoral lineage. Conversely, most vertebrates are specified predominantly by the "American style" in which there is a great deal of mixing between cells, and cell fate is determined by its neighbors. Each cell starts off with similar potentials and develops according to the cell type it interacts with. This type of cell fate determination is called conditional specification, because the fate of a cell depends upon the condition of cell-cell interaction.

The role of cell-cell interactions in determining the cell fate is to distinguish two cell types with amplified signals from a cell population. The multiplicity of outcomes arising from the repeated use of such pleiotropic signals depends on the context in which the signals are received. These signals can be caused by some chance factors on the cell membrane. Among these chance factors, the most prevalent ones are members of Notch family (Artavanis-Tsakonas et al., 1999; Mumm and Kopan, 2000). Signals transmitted through Notch control cell fate in a wide array of developmental processes, ranging from neurogenesis to oogenesis (Kimble and Simpson, 1997). Gain- or loss-of-function of Notch gene typically results in an increased abundance of cells adopting one fate at the expense of an alternate fate. In specific contexts, Notch also influences apoptosis, cellular

proliferation, and the organization of tissue boundaries, activities that further contribute to its broad role in morphogenesis, as well as vasculogenesis and angiogenesis (Artavanis-Tsakonas et al., 1999; Selkoe and Kopan, 2003; Iso et al., 2003). The cell-cell interaction controlled by Notch does not stop at birth and persists throughout adulthood.

1.1 Notch family and fundamental role

In 1917, Thomas Hunt Morgan and colleagues described a strain of *Drosophila* with notches, which are absent in the wild type but clearly visible at the border of their wing blades (Morgan, 1917) (Fig. 1a). This curious trait was attributed to a partial loss of function (haploinsufficiency) of a gene, which was later named Notch gene. Notch gene, which was first cloned from *Drosophila* in the mid-1980s by the teams of Artavanis-Tasakonas (Wharton et al., 1985) and Young (Kidd et al., 1986), encodes a receptor with a single transmembrane domain. Notch receptor genes are conserved in many organisms, ranging from sea urchins to humans (Radtke and Raj, 2003).

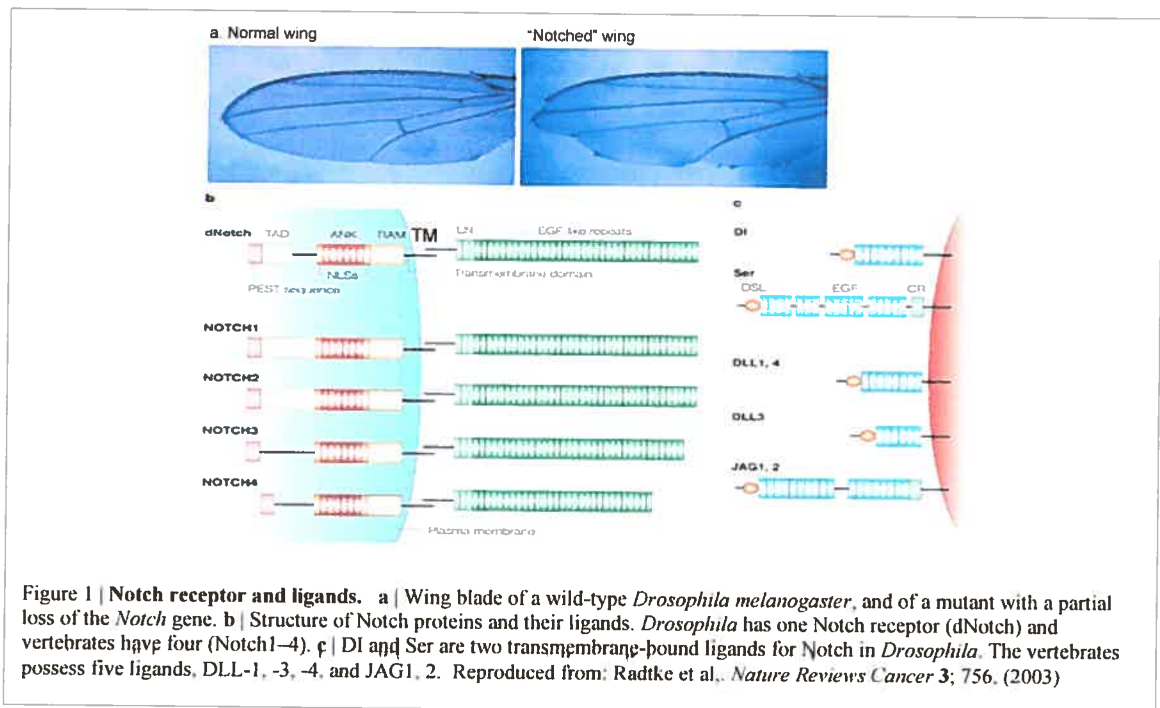

Figure 1 | **Notch receptor and ligands.** **a** | Wing blade of a wild-type *Drosophila melanogaster*, and of a mutant with a partial loss of the *Notch* gene. **b** | Structure of Notch proteins and their ligands. *Drosophila* has one Notch receptor (dNotch) and vertebrates have four (Notch1–4). **c** | Dl and Ser are two transmembrane-bound ligands for Notch in *Drosophila*. The vertebrates possess five ligands, DLL-1, -3, -4, and JAG1, 2. Reproduced from: Radtke et al., *Nature Reviews Cancer* **3**; 756, (2003)

1.1.1. Notch family and structure

Evolutionary divergence between invertebrates and vertebrates has been accompanied by at least two round gene duplications: flies posses a single Notch gene, worms two (glp-1 and lin-12), and mammals four (Notch1, 2, 3, and 4).

At the heart of Notch signaling is the Notch receptor. Although synthesized as a single precursor protein, Notch is cleaved into two parts during its transport to the cell surface and, as a consequence, exists as the heterodimeric receptor (Radtke and Raj, 2003) (Fig. 1b). The extracellular domain includes many repeats of a protein module ressembling epidermal growth factor (EGF)-like domain and three membrane-proximal Notch-specific repeats (LNR) (Fleming, 1998). The EGF-like repeats mainly participate in ligand binding on an adjacent cell, whereas the LNR prevent signaling in the absence of ligand (Rebay et al., 1991). Four functionally distinct, important regions have been identified within the Notch intracellular domain. In N- to C-terminal order, they are the RAM domain and the six ankyrin (also known as CDC10) repeats interacting with downstream effectors of Notch pathway, a transcriptional activator domain (TAD), and the proline, glutamate, serine, threonine-rich (PEST) sequence regulating the stability of proteins. Two nuclear localization sequences (NLS) are present prior to, and following, the ankyrin repeats (Fig. 1b) (Fortini et al., 1993; Struhl et al., 1993; Stifani et al., 1992; Lieber et al., 1993).

1.1.2. Core Notch signaling pathway

The core elements of the Notch signaling system include Notch receptors, DSL ligands (Delta and Serrate in *Drosophila* and vertebrates, Lag-2 in *C.elegans*), CSL DNA-binding proteins (CBF1/RBPjk in vertebrates, suppressor of hairless [Su(H)] in *Drosophila*, Lag-1 in *C.elegans*), and target genes, such as the HES and HERP families of

basic helix-loophelix transcriptional factors (Selkoe and Kopan, 2003; Nakagawa et al., 2000; Fischer et al., 2004) (Fig. 2).

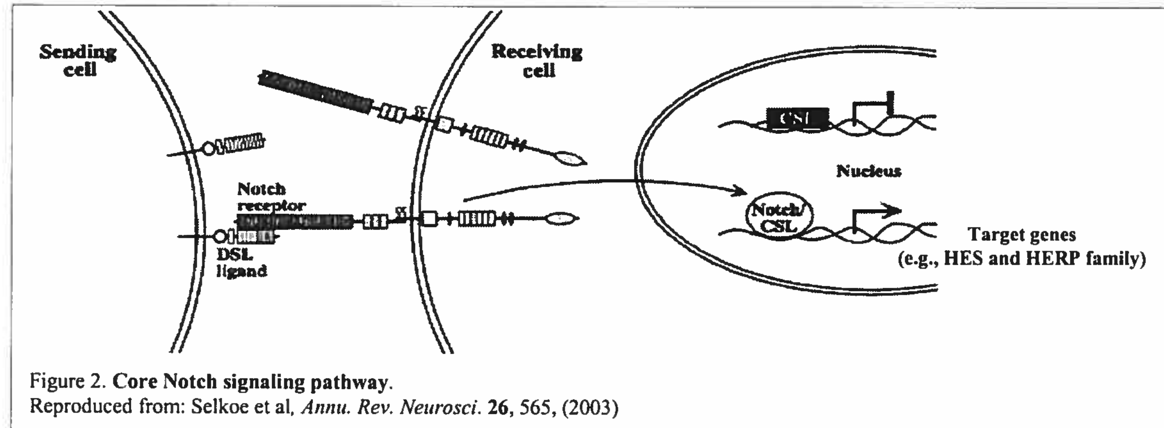

Figure 2. **Core Notch signaling pathway.**
Reproduced from: Selkoe et al, *Annu. Rev. Neurosci.* **26**, 565, (2003)

1.1.2.1 Notch receptors and ligands

In *Drosophila*, a single Notch receptor has 36 EGF-like repeats and one LNR repeat in the extracellular domain, as well as four distinct regions, RAM, CDC10, TAD, and PEST domain, in the intracellular part (Radtke and Raj, 2003). Mammals, such as mice and humans, have four Notch receptors encoded by four different Notch genes: Notch1, Notch2, Notch3, and Notch4 (Del Amo et al., 1993; Weinmaster et al., 1992; Lardelli et al., 1994; Uyttendaele et al., 1996). Although the structures of the four Notch receptors are overall very similar, they do show differences in the extracellular and the intracellular parts. The Notch1 and Notch2 receptors contain 36 EGF-like repeats in their ectodomain, whereas Notch3 harbors 34 and Notch4 only 29. Additional differences are found within the intracellular domain; specifically, Notch1 contains a strong TAD, while Notch2 contains a weak TAD, and no TAD is present in Notch3 and Notch4 (Radtke and Raj, 2003) (Fig. 1b).

While two Notch ligands, Delta (Dl) and Serrate (Ser), are present in *Drosophila*, mammals possess five ligands named Delta-like-1, -3, and -4 (DLL1, 3, and 4) (Bettenhausen et al., 1995; Dunwoodie et al., 1997; Shutter et al., 2000) and Jagged1 and

Jagged2 (JAG1 and JAG2), which are Ser-like ligands (Lindsell et al., 1995; Shawber et al., 1996). Each ligand is also a single transmembrane protein that contains EGF-like repeats in the extracellular domain. Different from the Notch receptor, it contains a conserved DSL (Delta/Serate/Lag-2) domain that can bind to specific EGF-like repeats of the Notch ectodomain on the adjacent cells (Radtke and Raj, 2003). The intracellular domain of the Notch ligands is less known. The main structural differences between the members of the ligand family are the number and spacing of the EGF-like repeats and the presence of a cysteine-rich domain, which is located downstream of the EGF-like repeats in Ser, JAG1, and JAG2 (Fig. 1c).

1.1.2.2. Initiation of Notch signaling

Notch signaling is initiated by a receptor-ligand interaction between two neighbouring cells, which leads to a couple of successive proteolytic cleavages at three sites—S1, S2, and S3—that in turn liberate the cytoplasmic portion of Notch (N^{IC}) from the membrane (Fig. 3) (Baron et al., 2002; Kopan, 2002; Selkoe and Kopan, 2003).

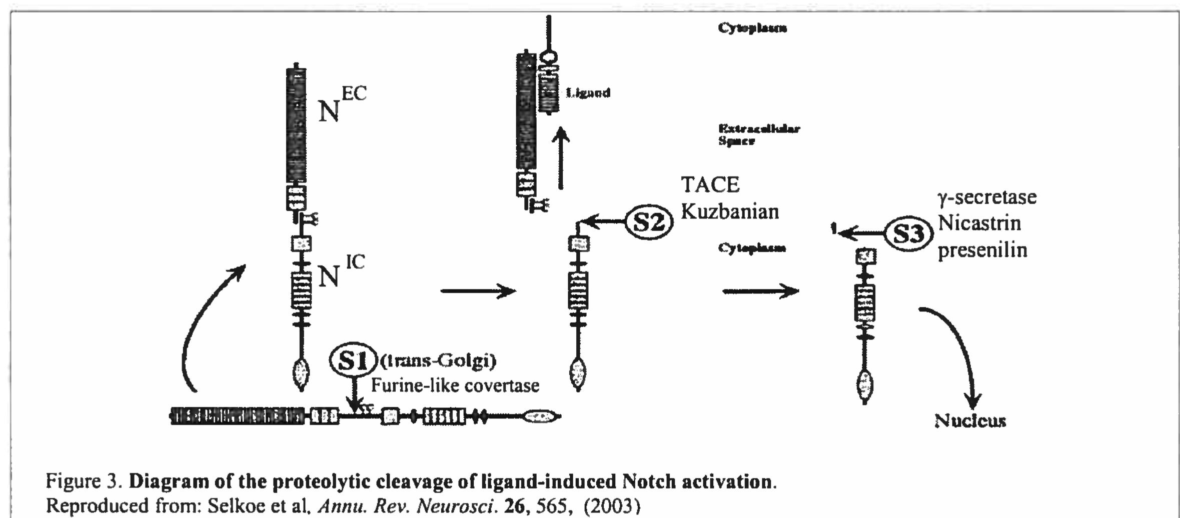

Figure 3. **Diagram of the proteolytic cleavage of ligand-induced Notch activation.**
Reproduced from: Selkoe et al, *Annu. Rev. Neurosci.* **26**, 565, (2003)

S1 cleavage, in the extracellular domain of Notch, occurs constitutively in the trans-Golgi network and is mediated by a furin-like convertase, followed by reassembly of

the fragments to form the heterodimeric Notch receptor at the cell surface. This event has been most closely characterized with respect to mammalian Notch, but there is evidence that the fly Notch is similarly processed (Selkoe and Kopan, 2003). S2 cleavage, by a disintegrin/metalloprotease, occurs in response to ligand binding and releases the majority of the extracellular domain. This cleavage is believed to be mediated by TACE (Henrique et al., 1995) in vertebrates and might be mediated by a related (but distinct) protein Kuzbanian in *Drosophila* (Pan and Rubin, 1997), although the precise role of the latter protein is still controversial. S3 cleavage is caused by the resulting membrane-anchored fragment referred to as Notch extracellular truncation and mediated by γ-secretase. A wealth of studies has demonstrated that both Presenilin and Nicastrin are involved in S3 cleavage (Kopan, 2002; Lai, 2002a). This cleavage finally releases N^{IC}. The mechanism of this particular cleavage is of considerable interest as it is a key event that regulates the nuclear translocation of N^{IC} and its second life as a transcriptional co-activator. Within the nucleus, the released N^{IC} binds to the transcription factor CSL and finally activates targets of Notch signaling (Zhou et al., 2000a; Oswald et al., 2001; Fryer et al., 2002).

1.1.2.3. The mechanism of Notch signaling

A well-established mechanism relies on a ligand-induced release of the N^{IC} and the interaction of this fragment with members of CSL family of transcription factors within the nucleus (Lai, 2002b); however, there is increasing evidence that Notch can signal in CSL-independent modes (Martinez et al., 2002).

CSL-dependent Notch signaling: The resulting soluble N^{IC} is translocated to the nucleus where it binds to and activates transcription factors: the CSL. CSL proteins bind specific DNA sequences to regulate gene expression. In mammals, HES (Hairy/Enhancer of Split) and HERP (HES-related genes with endothelial specificity) are the direct targets

of Notch/CSL-dependent signaling (Sasai et al., 1992; Bicknell and Harris, 2004; Iso et al., 2001). The HES proteins inhibit activity of other bHLH proteins, such as the *Mash-1* and *NeuroD* (de la Pompa et al., 1997), thereby suppressing transcription of the lineage genes; however, the interactions between Notch and CSL appear to be more complex and the effects more diverse than originally described. In the absence of the Notch signal, CSL can mediate a repression of gene transcription through the recruitment of the co-repressor protein SMRT (Silencing Mediator of Retinoid and Thyroid hormone receptor), SKIP (Ski-related Protein), and a HDAC (histone deacetylase) (Kao et al., 1998). The binding of N^{IC} displaces the SMRT co-repressor and its associated HDAC enzyme to relieve transcriptional repression (Morel and Schweisguth, 2000; Tani et al., 2001). Furthermore, the Notch ankyrin repeats and TAD-containing regions are involved in recruiting the histone acetylase protein PCAF and GCN5, which may act catalytically to produce an open chromatin conformation (Kurooka and Honjo, 2000). Finally, the activator complex recruits Mastermind (Mam), which contains a transcription activator domain (Wu et al., 2000) (Fig. 4). The two-state model CSL function allows for variety in the requirements for *in vivo* transcriptional activation of target genes. For example, while some genes respond simply to N^{IC}-dependent removal of the repressor function of CSL, other genes require N^{IC} to drive the CSL into an activator complex (Morel and Schweisguth, 2000; Klein et al., 2000; Li and Baker, 2001).

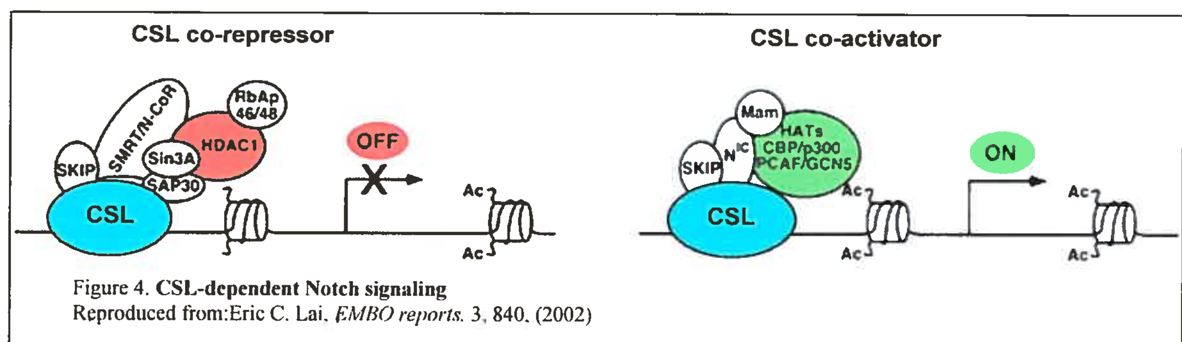

Figure 4. **CSL-dependent Notch signaling**
Reproduced from:Eric C. Lai. *EMBO reports.* 3, 840. (2002)

CSL-independent Notch signaling: In *Drosophila*, the minute analysis of Notch and Su(H) mutant phenotypes has shown that the Notch phenotype is slightly stronger than that of Su(H) mutant embryos. This suggests that the CSL-dependent signaling pathway does not mediate all the functions of Notch (Rusconi and Corbin, 1999; Zecchini et al., 1999). Further support has come from the analysis of a gain-of-function of Notch alleles that alter adult peripheral nervous system (PNS) development. In this mutant, represented by Ax^{59d}, Ax^{M1}, and *Mcd* alleles, the sensory bristles do not develop, thereby suggesting that sensory organ precursors (SOPs) fail to differentiate. As a result, PNS development is not correctly initiated. In addition, this phenotype cannot be rescued by removing the Su(H) function (Brennan et al., 1999c; Ramain et al., 2001). This, in turn, suggests that the increased Notch signaling is occurring via a Su(H)-independent signaling pathway. In vertebrates, it is less clear whether Notch can signal independently of CSL. Unlike *Drosophila*, it is currently impossible to compare the phenotypes of mice that completely lack the Notch functions with mice lacking CBF1 functions since this requires a generation of mice that lack all four Notch genes. Nonetheless, several cell culture experiments, in particular on the differentiation of the myogenic cell line C2C12 into myotube (Nofziger et al., 1999; Kuroda et al., 1999), have provided some evidence for CSL-independent signaling (Martinez et al., 2002). Weinmaster and co-workers have demonstrated that expressing truncated forms of N^{IC}, which cannot activate a CBF1-dependent promoter, can prevent the C2C12 differentiation even in the presence of a dominant, negative CSL protein (Nofziger et al., 1999), therefore suggesting a CSL-independent Notch activity. A second line of evidence in support of a CSL-independent signal is that the co-culture of C2C12 cells (expressing co-liner forms of Notch) with Jagged1-expressing cells failed to activate a

CBF1-dependent reporter gene but still prevented their differentiation into myotubes (Bush et al., 2001).

Some molecules are not integral components of the Notch pathway but modulate Notch signal transduction (Egan et al., 1998). These regulators include Wingless, Deltex, and Numb, acting as Notch/CSL-independent signaling.

Wingless is a member of the Wnt family of proteins that regulate many developmental processes through the activation of the Frizzled class of cell surface receptors (Cadigan and Nusse, 1997). Wingless through Fizzled activates the β-catenin-dependent signaling via the activation of the cytoplasmic protein Dishevelled (Dsh). Evidence of the genetic interactions between Wingless and Notch can be seen from the fact that a repressive activity of Wingless can be regulated by Notch (Couso and Martinez, 1994; Rulifson and Blair, 1995; Lawrence et al., 2001), and the Notch activity can be blocked by Dsh (Axelrod et al., 1996). These results provide a molecular mechanism for the inhibitory cross-talk between two pathways. Wingless has also been shown to inhibit an early Su(H)-independent function of Notch in the selection of muscle founder-cell clusters (Brennan et al., 1999a). Wnt acts to block Notch either directly through its reported binding to N^{EC} (describe later) or, more likely, indirectly by stimulating Dsh to block GSK3 and/or to recruit Deltex/Notch interaction (Foltz et al., 2002; Wesley and Saez, 2000) (Fig. 5).

The N-terminal region of Deltex mediates binding to the Notch ankyrin repeats (Diederich et al., 1994). Based on its mutant phenotypes and its genetic interaction with Notch alleles (Xu and Artavanis-Tsakonas, 1990; Gorman and Girton, 1992), Deltex has been identified as a positive regulator of the Notch pathway. Mutations of *Drosophila* Deltex cause several adult phenotypes similar to Notch loss-of-function, including wing

notches and thickened veins (Xu and Artavanis-Tsakonas, 1990; Gorman and Girton, 1992). As both N- and C-terminal regions of Deltex contain the domains related to ubiquitination (Jackson et al., 2000), Deltex can also act as a repressor of Notch signaling by mediating its degradation (Yun and Bevan, 2003). Other work in mammalian cell culture has suggested that mammalian Deltex may function on Notch through down-regulation of Ras and JNK-dependent signals (Ordentlich et al., 1998) (Fig. 5).

Numb is a phosphotyrosine-binding (PTB) domain protein that binds to two regions of Notch: the RAM domain and the C-terminus via its PTB. Numb plays a role in the process of asymmetric cell division. During neurogenesis in flies, mice, and in avian, the asymmetric distribution of Numb ensures that only one daughter cell inherits a Numb protein that down-regulates the Notch activity via endocytic pathway, thus biasing the Notch-mediated signaling (Lu et al., 1998; Spana and Doe, 1996; Wakamatsu et al., 1999, Baron et al., 2002) (Fig. 5).

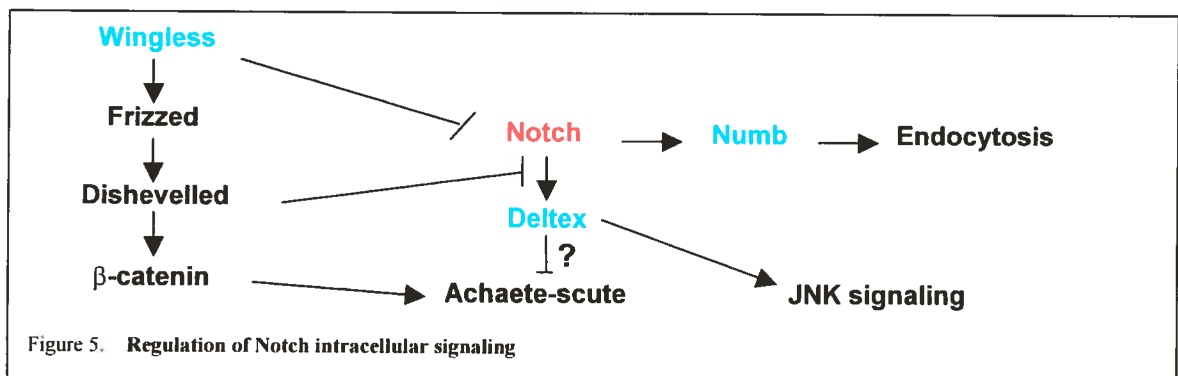

Figure 5. **Regulation of Notch intracellular signaling**

1.1.2.4. Termination of Notch signaling pathway

After N^{IC} acts in the nucleus of mitotic cells, it must be removed, as the daughters will often again rely on Notch signaling to determine their fate (Kopan, 1999). It has been recently demonstrated that the regulation of ubiquitin pathway performed by Sel-10 (Hubbard et al., 1997), Notch-regulated ankyrin-repeat protein (Nrarp) (Lamar et al.,

2001), and Neutralized (Lieber et al., 1993) might play a role in the termination of Notch signaling (Baron et al., 2002), as does the E3 ubiquitin ligase Supressor of Deltex [Su(Dx)] (Cornell et al., 1999).

Ubiquitination of proteins takes place via a multistep mechanism, with ubiquitin first being covalently linked to an E1 enzyme before being passed to another covalently linked intermediate, the E2 enzyme, and finally onto the target protein via an E3-ubiquitin ligase-dependent step (Hershko and Ciechanover, 1998). The E3 proteins have been linked to Notch pathway regulation (Baron et al., 2002). The Notch receptor, therefore, is subject to being regulated by ubiquitination at different levels in the signaling pathway.

Sel-10 was originally identified in *C.elegans* as a negative regulator of Notch signaling and found to bind to the N^{IC} (Hubbard et al., 1997). Recent reports demonstrate that mammalian homologues of the Sel-10 protein can stimulate ubiquitination of N^{IC} and trigger its proteasome-dependent degradation (Gupta-Rossi et al., 2001; Oberg et al., 2001). The association of mammalian Sel-10 with nuclear N^{IC} is dependent on a phosphorylation event and also requires the Notch PEST sequence (Rogers et al., 1986). In *Drosophila*, whether Sel-10 is involved in a proteasome-dependent degradation is not yet known (Schweisguth, 1999).

Nrarp is a small protein encoding two ankyrin repeats (Baron et al., 2002). Nrarp binds to N^{IC} only in presence of *Su*(H), thereby forming a ternary complex. The expression of Nrarp is itself dependent on Notch signaling, suggesting that Nrarp may work in a feedback loop to limit the extent and duration of the Notch signaling. Although not yet directly implicated in ubiquitination, Nrarp stimulates degradation of the Xenopus N^{IC} (Lamar et al., 2001). So far, the degradation mechanism is not known.

Neutralized is localized at the cell membrane and is thought to act upstream of the release of N^{IC}, because the expression of the latter rescues the Neutralized phenotype (Lieber et al., 1993). In *Drosophila*, the Neutralized has been biologically shown to be a ubiquitin ligase and to contain a recognizable Ring finger domain (Yeh et al., 2001). Mutation of Neutralized results in a disruption of Notch signaling in a certain number of tissues, including lateral inhibition during neurogenesis, but not all of the functions of Notch (Lai and Rubin, 2001a; Lai and Rubin, 2001b).

1.1.3. The fundamental role of Notch signaling

1.1.3.1. Participation in cell-fate decision

Notch pathway is already implicated in several processes of development in a certain number of species. In *Drosophila*, the Notch protein is indispensable for the determination of numerous types of cells, for example, organogenesis, neurogenesis, and myeogenesis, as well as the development of the wing and eye (Artavanis-Tsakonas et al., 1999; Egan et al., 1998). In mammals, four homologuous proteins are expressed from three-layer of embryos, namely endoderm, mesoderm, and ectoderm. They play a critical role in the differentiation of numerous cell types, including neurogenic, haematopoietic, and endothelial cells (ECs) (Morrison et al., 2000; Kumano et al., 2003; D'Amore and Ng, 2002; Lawson et al., 2002). The role of Notch pathway in the development of mammals has been proved in the mice deficient for Notch1, Notch2, and their ligands (Swiatek et al., 1994; Conlon et al., 1995; Hrabe et al., 1997). All the mice have severe problems during their development that cause embryonic and perinatal lethality. For example, in mice lacking Notch1 the homozygote embryos develop normally until the 9th embryonic day but die before the 11.5^{th} day. The histological analysis indicated that the majority of the dead

cells were particularly concentrated in the neuroepithelium of the central nervous system (Swiatek et al., 1994). Consistent with these results, the RNA expression of Notch1 was detected in the pre-somite mesoderm at the 7th day of the embryonic development. At the 9th day, the major site of expression was neuroepithelium, as well as tissues of the neural crest (Del Amo et al., 1992; Reaume et al., 1992). Notch1 also plays a role in the developmental pattern of chickens (Caprioli et al., 2002) and in the differentiation of the oligodendrocytes of rats (Wang et al., 1998b). This indicates that the Notch signaling pathway influences a diversity of tissues in various species.

Even though Notch and its ligands are often expressed in the same cell, the receptor is critically activated by the interaction with the ligand localized on the adjacent cells. The cells may be lied to by the homotypic interaction (equipoentential cells) or by the heterotypic interaction (non-equipotential cells) to process different roles.

Lateral inhibition between equivalent cells:

"Lateral inhibition" was first termed by V. B. Wigglesworth to describe a process for the constant density of bristles in the developing insect (Wigglesworth, 1940). From the observation of the effective separation between the existing and newly formed bristles, he postulated that an existing bristle inhibits surrounding cells, such that a new bristle-forming cell could only arise outside the range of its inhibitory influence.

The best characterized components of the inhibitory machinery are the members of the Notch-Delta signaling pathway in the insect neuroblast development. In 1990, Simpson proposed a cell-contact-inhibition model for Notch-Delta-mediate lateral inhibition, according to which the epithelial cells are inhibited directly by the contacting neural cell (Simpson, 1990). Genetic mosaics show that whereas Notch is needed in the cells that are to become neuroblasts, the Delta gene is needed in the adjacent cells that induce the

epidermal phenotype. Later on, a similar model was proposed by Greenwald and Rubin with Lin-12 in *C.elegans* (Greenwald and Rubin, 1992) in order to explain the spacing patterns of neuroblasts in the proneural clusters of epidermal and neural precursors. Initially, all the cells have equal potentials and signaling; however, when, by chance, there is an event causing one of the cells to produce more signals (say, Delta product), this activates the receptors (Notch) on the adjacent cells and, consequently, reduces their signaling level. Since the signaling levels (Notch) on the adjacent cells are low, the neighbors of those low-signaling cells will tend to be, themselves, high-level signalers (Delta). In this way, a spacing of neuroblasts is produced. Inactivating Notch or Delta causes a failure of lateral inhibition resulting in an excess of neural cells at the expense of the epidermal tissues in both embryonic and adult nervous systems. This demonstrates that Notch and Delta are required for the inhibition of the formation of extra neural precursors from a proneural equivalence group (Heitzler and Simpson, 1991; Parks and Muskavitch, 1993). Among other members of the Notch pathway, Su(H) and E(spl) behave, respectively, as a transducer and downstream nuclear effector of the Notch-mediated inhibition in genetic and biochemical experiments (Schweisguth and Posakony, 1994; Delidakis and Artavanis-Tsakonas, 1992).

Notch-mediated lateral signaling is not only restricted to neural tissues. It also controls a number of other cell-fate decisions between equivalent cells in *C.elegans* (Wilkinson et al., 1994). In mouse models, Notch is implicated in a series of processes of lateral inhibition in the generation of somite precursors (Conlon et al., 1995), hematopoietic cells (Washburn et al., 1997), and others (Lewis, 1998).

Determination of non-equipotent cells:

Notch signaling can also occur between two developmentally-distinct cells referred to as inductive cell-fate decision (Artavanis-Tsakonas et al., 1995). In this case, Notch and its ligands are expressed exclusively on two different cell types. The cell expressing the receptor, and therefore the recipient of the Notch signal, is induced to differentiate into a particular cell lineage. For example, a bipotential mouse neural-crest stem cell can be induced by Notch to adopt a glial-cell fate, as opposed to a neural one by Notch ligands expressed on neuroblasts (Morrison et al., 2000). Mouse thymic epithelial cells expressing ligands for Notch1 induce early lymphocyte precursors to adopt the T-cell fate as soon as they enter the thymus, whereas in the absence of Notch1 signaling these precursors take on the B-cell fate as the default pathway (Pear and Radtke, 2003).

1.1.3.2. Induction of terminal differentiation

Instead of influencing the choice between two possible cell fates, Notch signaling between developmentally-related cell types can induce or enhance a terminal differentiation. In the adult mouse-skin keratinocytes, where Dll is not expressed, Jagged-mediated Notch signaling triggers a terminal-differentiation program by inducing early differentiation markers and cell-cycle arrest (Rangarajan et al., 2001). In the human skin, this program is initiated by Dll (Lowell et al., 2000).

1.1.3.3. Maintenance of an undifferentiated state

In addition to participating in the binary cell-fate decision and in the induction of terminal-differentiation, Notch signaling also preserves some cells, such as stem cells, in their native state. Notch signaling in the vertebrate nervous system is usually thought to influence the balance between the progenitor cell pool and its progenitor-differentiating

progeny (Lewis, 1996). Gain-of-function studies on chickens and frogs using a dominant active N^{IC} show that forced Notch signaling prevents progenitors from undergoing neurogenesis, whereas blocking this pathway leads to excessive neurogenesis and depletion of the progenitor pool (Chitnis et al., 1995; Henrique et al., 1997). Similarly, the exposure of hematopoietic stem cells to JAG1 increases the proportion of stem cells, as opposed to differentiating cells; therefore, Notch signaling induces these cells to retain a stem-cell-like character (Varnum-Finney et al., 1998).

Taken together, the fundamental role of Notch signaling is mainly to control the cell-fate decision during development and, meanwhile, to take part in the differentiation and the maintenance of stem cells (Fig. 6).

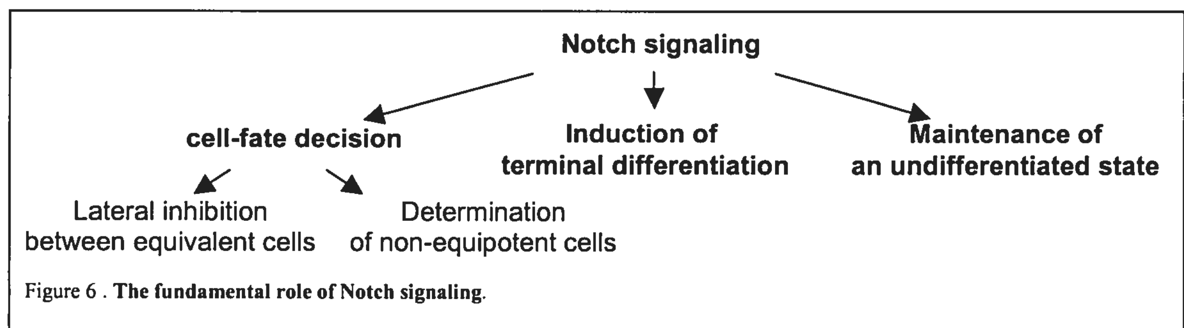

Figure 6 . **The fundamental role of Notch signaling.**

1.2. Function of Notch ectodomain

Since the Notch gene was first cloned (Wharton et al., 1985), one important goal has been to understand the meaning of the repeated modular structure of the ectodomain. Does each EGF-like module have a specific function? Do some modules act simply as a spacer to correct the position of different binding sites? Interspecies sequence conservation within EGF repeats 11–13, 23–27, and 31–34 of *Drosophila* Notch suggests that these regions may form functional subdivisions of the Notch extracellular domain (Wesley, 1999; Wesley and Saez, 2000). Genetic data from *Drosophila* further suggests that there is a functional diversity within the Notch extracellular domain (Kelley et al., 1987).

Mutations in different EGF-like repeats show selective effects in different tissues or have positive or negative consequences on the Notch signaling pathway. For instance, a single amino acid substitution in the EGF-like repeat 14 (the split allele) affects Notch activation specifically in the eye and in the sensory bristle development, while Abuptex alleles of Notch, clustered within the EGF-like repeats 24–29, produce dominant gain-of-function phenotypes in the sensory bristle development and in the wing veins (Kelley et al., 1987; de Celis and Garcia-Bellido, 1994). It is possible, therefore, that extracellular interactions of Notch may confer an additional level of regulation, perhaps in a tissue-specific manner. From analysis of the extracellular interactions of Notch, a picture of the functional specification of the regions within the extracellular domain begins to emerge.

1.2.1. Cross-talk with Notch ligands

The first region of the Notch extracellular domain to be defined as having a functionally distinct role was the binding site of *Drosophila* Notch for its two ligands: Delta and Serrate. Using a co-culture of the *Drosophila*-S2 cells (expressing ligand or receptor) (Rebay et al., 1991), the EGF-like repeats 11 and 12 were both necessary and sufficient for binding of Notch to both Delta and Serrate on adjacent cells; this is called a trans-interaction. Furthermore, through a point mutation within the EGF-like repeat 12 in *Drosophila*, the importance of this region has been established *in vivo* due to an abolition of the ligand-dependent Notch signal (de Celis et al., 1993).

Apart from the trans-interacting complex, other forms of interactions of Notch with Delta and Serrate ligands may also regulate Notch activity. Cis-interaction between Notch and its ligands within the same cell has been identified (Fehon et al., 1990; Jacobsen et al., 1998) but does not appear to activate the receptor in an autocrine manner. Instead, it has been proposed that the cis-interaction mediates a dominant, negative activity of the ligand

on the receptor by reducing the capability to receive a signal from the adjacent cells when the ligand is in excess. Another level of ligand-receptor interaction may come from the proteolytic processing of the ligands. Proteolytically cleaved and secreted forms of ligand have been identified, raising the question as to whether or not this might allow action of the ligand at a distance from where it is expressed (Klueg et al., 1998; Qi et al., 1999).

1.2.2. Cross-talk with other factors

Scabrous

In *Drosophila*, Scabrous has been shown to bind to the Notch receptor through the EGF-like repeats 19–26 (Powell et al., 2001). It is a secreted fibrinogen-related protein interacting with the Notch extracellular domain. Its mutation causes a tissue-specific phenotype in the eye and sensory bristles (Mlodzik et al., 1990). Overexpression of Scabrous inhibits Notch signaling in the *Drosophila* eye and wing (Lee et al., 2000; Powell et al., 2001). On the other hand, the ectopic Scabrous expression blocked the ligand-dependent Notch activity but not that of constitutively-active Notch intracellular domain. This fact suggests that Scabrous acts at the level where the ligand binds to the receptor (Lee et al., 2000). Intriguingly enough, in the S2-cell culture, Scabrous stabilizes Notch at the cell surface (Powell et al., 2001). How this might lead to a modulation of the Notch activity remains unrevealed but tethering of Notch to the extracellular matrix (ECM) might offer one possible mechanism for modulating its and/or other's activity.

Wingless

Recent reports confirm the capability of Wingless to directly bind the Notch extracellular domain. Wingless binds to two *in vivo* forms of Notch, i.e., a full-length and a N-terminal truncated form, through a site lying within EGF-like repeats 19–36 (Wesley, 1999). These data are consistent with a region of *Drosophila* Notch, which, when deleted,

alters the observed phenotypic interactions resulting from co-expression of the Notch extracellular domain and the Wingless (Brennan et al., 1999b). In cell cultures, the association of Wingless with Notch invoked a transcriptional response that differed from that elicited by Delta (Wesley and Saez, 2000). While Delta-dependent signaling was associated with the accumulation of soluble Notch intracellular domain, no such accumulation followed an exposure to Wingless, implying a distinct signaling mechanism (Wesley and Saez, 2000).

1.2.3. Regulation by some factors

Fringe

Fringe acts within the Golgi as an N-acetylglucosaminyltransferase and adds GlcNac group O-linked fucose to specific EGF-like repeats in the Notch extracellular domain (Bruckner et al., 2000; Moloney et al., 2000). Several conserved O-linked fucose sites close to EGF-like repeats 24–26 are critical for Serrate- but not for Delta-dependent Notch signaling (Lawrence et al., 2000). This has been shown to play a key role in special regulations of Notch signaling at the compartment boundaries during pattern formation. Expression of Fringe results in inhibition of Serrate-dependent Notch signaling but not Delta-dependent signaling (Fleming et al., 1997; Panin et al., 1997). The differential effect of Fringe on Serrate and Delta may be due to differences in how the ligands interact with the receptor in the productive signaling complex. When Fringe is expressed in the same tissues as Serrate, Serrate will only signal to Notch in the cells lying adjacent to the Fringe-expressing territory. This is a key step in setting up a spatially restricted zone of Notch signaling at the compartment boundaries. It is interesting to note that the EGF-like repeats 24–26 also lie within a segment of the Notch extracellular domain where the Abruptex-

gain function *Drosophila* Notch alleles are clustered (Kelley et al., 1987). The Abruptex mutations are thought to make Notch resistant to the dominant, negative effects of cis-interacting ligands and also to confer resistance to the consequences of the Fringe activity (de Celis and Bray, 2000).

O-fucosyltransferase-1

O-fucosyltransferase-1 (OFUT1 in *Drosophila*) is another protein that regulates glycosylation of the Notch extracellular domain. The transfer of fucose to Notch by this protein is necessary for the Fringe to function (Shi and Stanley, 2003; Panin et al., 2002). Down-regulation of OFUT1 by RNA interference in soluble extracellular domain of Notch-secreting cells inhibits both Delta-Notch and Serrate-Notch binding, demonstrating a requirement for O-linked fucose for efficient binding of Notch to its ligands. Conversely, overexpression of OFUT1 in cultured cells increases Serrate-Notch binding but inhibits Delta-Notch binding (Okajima et al., 2003), opposing the influence of Fringe on Notch-ligand binding. Mouse embryos lacking *O*-fucosyltransferase-1 die at midgestation with severe defects in somitogenesis, vasculogenesis, cardiogenesis, and neurogenesis. *O*-fucosyltransferase-1 is, therefore, an essential core member of Notch signalling pathways in mammals (Shi and Stanley, 2003).

1.2.4. Participation in trans-endocytosis

Membrane receptors are passively recycled or actively eliminated by endocytosis (Robinson, 1994). This process is very important to regulate the cell signal transduction. A link between endocytosis and Notch signaling was proposed based on the phenotype of the *Drosophila shibire* mutant (Seugnet et al., 1997). Using antibodies to the Notch extra- and intra-cellular domains of *Drosophila*, it has been shown that these two domains can traffic

independently *in vivo*. In the pupal eye, for example, the Notch extracellular domain undergoes trans-endocytosis from the pigment cell (where it is expressed) into the adjacent Delta-bearing cone cells. Both the separation of N^{EC} and N^{IC} and the trans-endocytosis of N^{EC} into the cone cells were disrupted by a *shibire* temperature-sensitive mutation. It was also observed that Delta and Notch become co-localized at the cone cell-pigment cell junction. Since *shibire* is required for Notch signaling, and since endocytosis-defective mutations of Delta fail to signal, it was concluded that the trans-endocytosis mechanism plays a part in the generation of the ligand-dependent cleavage site. This might result in a conformational change (Parks et al., 2000). On the other hand, it has been shown that the halves of the Furin-processed Notch receptors are held together non-covalently. The possibility, therefore, arises that, in some circumstances, signaling could be activated by the physical removal of the Notch extracellular domain without further extracellular cleavage (Rand et al., 2000). The trans-endocytosis model of the Notch activation implies that the generation of the Notch signal requires a membrane-tethered ligand and may explain why soluble-secreted forms of Delta and Serrate act antagonistically on the Notch activity (Qi et al., 1999; Hukriede et al., 1997).

1.2.5. Function of the extracellular domain of Notch ligands in the Notch signaling

While most research was focused on the constitutive intracellular domain of Notch signaling, a few groups were involved in the functional characterization of the extracellular domain of the Notch ligands. Sun and Artavanis-Tsakonas first proved that secreted forms of Delta and Serrate act as antagonists of Notch signaling in *Drosophila* (Sun and Artavanis-Tsakonas, 1996; Sun and Artavanis-Tsakonas, 1997). They examined the function of secreted forms of Delta and Serrate, named as DlS and SerS, by expressing

them under different promoters in the *Drosophila* developing eye and wing. The phenotype associated with the expression of both secreted forms mimics loss-of-function mutations in the Notch pathway. Consistent with these results, Fleming's group found that the soluble form of Serrate, called BD^{G}, acts as a general antagonist of Notch activation (Hukriede et al., 1997); however, Artavanis-Tsakonas and workers showed that a soluble extracellular fragment of Delta (Dl^{EC}) has an apparent agonistic function in the Notch signaling pathway (Qi et al., 1999). DlS and Dl^{EC} are structurally not identical, which could explain their opposing functions.

In *Drosophila*, both soluble ectodomains of Delta and Jagged are implicated in the activation of Notch signaling. Since the extracellular fragments of Notch receptors and Notch ligands are constituted of the same EGF-like repeat, it is reasonable to hypothesize that the Notch soluble ectodomain may also play an important role in the Notch (or other) signaling pathway.

1.2.6. Involvement of Notch ectodomain in the Notch signalling

In our laboratory, Girard et al. (1995) showed that the Notch1 extracellular domain ($N1^{EC}$) was highly expressed in almost all the Notch1-rearranged thymomas of $MMTV^{D}$/c-myc Tg mice infected by Mo-MuLV (Moloney Murine Leukemia Virus) (Girard et al., 1996; Girard and Jolicoeur, 1998). Initially, they intended to identify some of the collaborators of c-myc to induce thymomas in the $MMTV^{D}$/c-myc Tg mice using provirus insertional mutagenesis. They found that quite a high proportion (52%) of these tumors contained Notch1 mutations. Furthermore, they revealed that the provirus was mainly inserted upstream of the exon coding for the transmembrane domain of Notch1. These mutations led to a high expression of truncated Notch1 intracellular domain ($N1^{IC}$) and

full-length Notch1 RNAs and proteins. Interestingly enough, in almost all these Notch1-rearranged tumors, high levels of $N1^{EC}$ could be detected by Western blotting.

Later on, following Girard's observations, Hoemann et al. found that two distinct Notch1 insertional mutations were involved in these thymomas (Hoemann et al., 2000) (Fig. 7). The first type of proviral insertion, named "type I," is in genomic regions

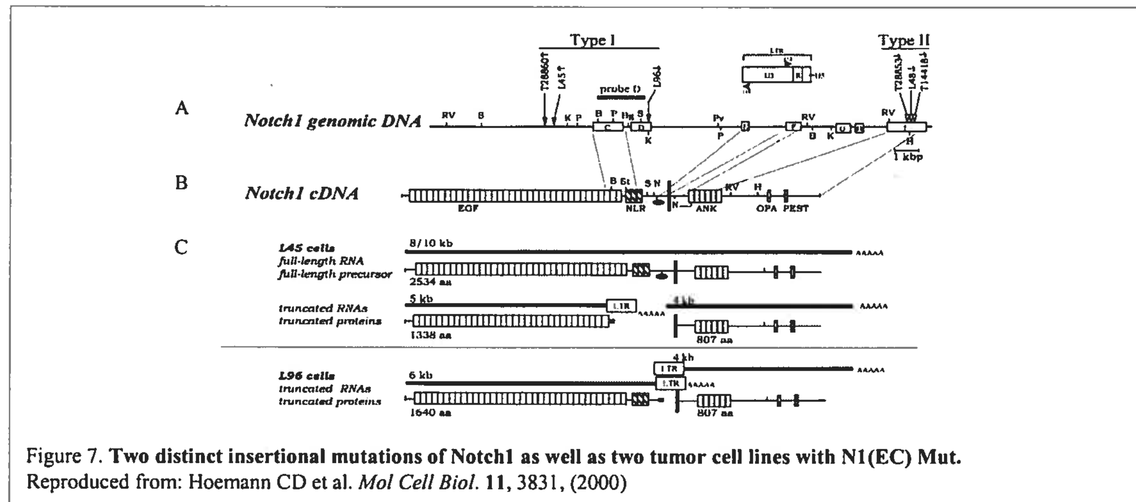

Figure 7. **Two distinct insertional mutations of Notch1 as well as two tumor cell lines with N1(EC) Mut.**
Reproduced from: Hoemann CD et al. *Mol Cell Biol.* **11**, 3831, (2000)

coding for sequences between the 34th EGF-like repeat and the transmembrane domain (Fig. 7A and 7B). The second kind of insertion, termed "type II," is within an 800-nucleotide span at the C-terminal region of Notch1. "Type I" insertion comprises the majority of mutations. The extracellular fragment produced from this specific insertion was called $N1(EC)^{Mut}$ (Fig. 7A and 7B). It was demonstrated that the Notch1 ectodomain could also be generated by normal processing of wild-type Notch1 precursors and was named $N1(EC)^{Wt}$. In order to analyze the putative $N1(EC)^{Mut}$ proteins, two tumor cell lines, L96 and L45 (Fig. 7C), were selected, each harboring distinct type I insertions that produced both intra- and extracellular domains (as detected by Western blotting) (Girard et al., 1996). $N1(EC)^{Mut}$ in the L96 cell line contains 36 EGF-like repeats and a LNR domain, whereas L45 solely contains the 36 EGF-like repeats. It was found that the soluble extracellular fragments of Notch1, $N1(EC)^{Mut}$, were different from the processed

extracellular domain, $N1(EC)^{Wt}$. While $N1(EC)^{Wt}$ is located on the cell surface and is sensitive to trypsin, $N1(EC)^{Mut}$ is secreted from the cell surface but is resistant to trypsin. As a consequence, $N1(EC)^{Mut}$ and $N1(EC)^{Wt}$ are not only structurally, but also functionally, different. $N1(EC)^{Mut}$ resides in the secretory pathway and most probably plays an important role in the tumor formation by interacting with Notch-ligand and/or other signaling pathway.

Since 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. We therefore generated Tg mice expressing $N1^{EC}$ in T cells and in cells of the macrophage/dendritic lineage using the regulatory sequences of the human CD4 gene ($CD4C/N1^{EC}$). Unexpectedly, vascular disease, not thymomas, developed at high frequency in these Tg animals. The vascular malformations mainly arose in the liver but also in other organs; for example, uterus and spleen. In addition, a higher progression of tumor was observed in the Tg mice treated with carcinogen (DEN) and injected tumor cells (C3L5). Intensive studies with liver demonstrate that expression of $N1^{EC}$ in macrophages (M_{Φ}) mediates the liver disease through a paracrine loop. This will consist of a key body of this thesis and will be described in detail later.

1.3. Physiological role of Notch signaling

As described before, the fundamental role of the Notch is in the cell-fate decision. Evidently, this function of Notch varies from the hematopoietic to the vascular system.

1.3.1. Notch and hematopoietic development

The best-known function of the Notch in a physiological situation is on T-cell commitment from multipotent progenitor cells; however, recent studies indicate that Notch

also takes part in additional cell-fate decisions, such as the generation of embryonic hematopoietic stem cells (HSC) and splenic marginal-zone-B cells (MZB) (Maillard et al., 2003).

1.3.1.1. Notch and embryonic hematopoietic stem cells

Hematopoietic progenitor cells are derived from the extraembryonic and the intraembryonic regions during embryogenesis. The best characterized hematopoietic sites are the yolk sac (YS), the embryo proper aorta-gonad-mesonephros (AGM), and the para-aortic splanchnopleura (P-Sp). The YS is the site for the primitive hematopoiesis, whereas definitive hematopoiesis takes place in AGM and P-Sp.

Hirai et al. found that both primitive and definitive hemotopoiesis were impaired in Notch1-deficient, but not Notch2-deficient, mice (Kumano et al., 2003). AGM+Sp cultures from the Notch1-deficient mice were unable to normally generate hematopoietic colonies, and cells isolated from the YS of these mice were unable to rescue conditioned newborn recipients. Importantly, this hematopoietic defect is reproduced in presence of γ–secretase inhibitors, but only up to day E10.5 of embryogenesis. This suggests that the Notch activity is not required once the initial HSC pool has been established (Maillard et al., 2003). In summary, Notch1 signaling plays a critical role in generating the earliest hematopoietic cells.

A gain-of-function mutation of Notch can also promote adult HSC self-renewal in both mice and humans (Karanu et al., 2000; Stier et al., 2002). Many of these effects are replicated in HSCs from mice expressing HES-1 transgene (Kunisato et al., 2003). Probably, Notch pathway is involved in hematopoiesis in adults, partially via a HES-1 mediated activity; however, neither Notch- nor CSL-knockout studies have shown a clear role of Notch in the adult HSC so far.

1.3.1.2. Notch and T-cell commitment

HSCs give rise to common lymphoid progenitors (CLPs) mainly in the bone marrow. The CLPs can specify various lineages, including T and B cells, as well as other cells. The CLPs that migrate from bone marrow to thymus develop into T cells, while B cell-differentiation takes place in bone marrow. The first step, however, is the commitment of CLPs to either of the two lineages.

T- versus B-cell development

In mice, Notch plays a critical role in the T- versus B-lineage specification. This was confirmed by both gain- and loss-of-function Notch mutation in mice (Maillard et al., 2003). Gain-of-function mutation leads to a thymic-independent development of the immature T cells and to a loss of B cells, while loss-of-function mutation results in hypotrophied thymus lacking T cells but abounding in B cells. Furthermore, the function of Notch in the T/B decision is Notch1 specific and CLS dependent (Han et al., 2002). Consistent with these results, the constitutive expression of the Notch regulators, such as Fringe (Koch et al., 2001), Deltex (Izon et al., 2002), and Nrarp (Yun and Bevan, 2003), blocks Notch signaling and T-cell development. Interestingly, data from Radtke's group indicated that some extrathymic niches also provide the proper signaling environment for Notch-mediated T-cell commitment (Wilson et al., 2000).

Notch ligands are important for the T versus B lineage specification. Both Jagged and Delta-like family members are shown to be expressed in thymic epithelial cells, thymocytes (Felli et al., 1999; Anderson et al., 2001), and dendritic cells. Also, the *in vitro* and *in vivo* experiments showed that Dll-1 and Dll-4 expression in an appropriate stromal environment may be sufficient to trigger and sustain the T-cell development (Jaleco et al., 2001; Schmitt and Zuniga-Pflucker, 2002). Although Jagged1 is unable to do so *in vitro* in

at least one experimental system (Jaleco et al., 2001), *in vivo* experiments have indirectly indicated that Jagged1 also takes a part in the T-cell commitment (Koch et al., 2001). There are not enough data available from individual knockouts of the Notch ligands to confirm these results because some knockout mice (i.e., Jagged-1, Dll-1, and Dll-3) are early lethal and Dll-4 knockouts have not yet been described.

αβ versus γδ T-cell development

After negotiating the T versus B lineage specification, pre-T cells must negotiate a second cell-fate choice: in favor of αβ or γδ T-cell decision. The role of Notch in the αβ/γδ commitment is controversial. Earlier results from Washburn's study, using mixed bone marrow chimeras reconstituted with Notch1+/+ and Notch1+/- marrow cells, show that Notch1 could favor the αβ over the γδ T-cell commitment (Washburn et al., 1997); however, recent results from Toribio's group show that the overexpression of $N1^{IC}$ in human thymic progenitor cells leads to an increased ratio of γδ over αβ T cells in human-mouse fetal thymic organ cultures (Garcia-Peydro et al., 2003). Finally, using cre-lox technology, conditional deletion of Notch1 in thymocytes at the double negative stage (DN3) of differentiation caused a severely impaired αβ T-cell differentiation, whereas the γδ T-cell development remained normal (Wolfer et al., 2002). These results indicated that the αβ/γδ T-cell commitment had been made before the DN3 (double negative stage 3), and the Notch1 gene was deleted after this commitment. The role of Notch ligand in the αβ/γδ commitment was also observed in Jagged-2 knockout mice, with a reduced number of thymic γδ T cells but with a preserved αβ T-cell differentiation (Jiang et al., 1998).

CD4 versus CD8 T-cell development

Several groups have published results indicating a role of Notch in the CD8/CD4 T-cell decision but that role is still controversial (Singer, 2002). With the aid of gain-of-

function mutations, Rober et al. first reported that the Notch1 activation increases the ratio of CD8+ over CD4+ SP T cell (Robey et al., 1996); however, the studies with conditional Notch1 knockout mice are difficult to reconcile with a role of Notch1 in augmenting the ration of CD8/CD4 T cells (Wolfer et al., 2002). These results suggested that the gain-of-function findings are not physiological or that other members besides Notch1 can influence the CD4 versus CD8 lineage decision. Another possibility is that Notch1 signaling is critical to induce early progenitors to select T/B lineage cell-fate decision rather than to skew the later development of T cells.

1.3.1.3. Notch and marginal B-cell development

Analyses of CLS and Notch2 conditional knockout mice show that Notch2 can act in a nonredundant manner to specify the marginal zone (MZ) versus the follicular (FC) B-cell fate in the spleen (Saito et al., 2003; Tanigaki et al., 2002). These results were supported in mice with a targeted inactivation of MINT, a newly described Notch inhibitor (Kuroda et al., 2003).

1.3.2. Notch and vascular development

The first indication of Notch function in vascular development came from analysis of the Notch1 expression in the embryonic ECs (Del Amo et al., 1993). Soon after, Notch4 was detected, particularly in developing vessels (Uyttendaele et al., 1996; Shirayoshi et al., 1997). Moreover, the ligand Dll4 was recently found to be specific to ECs (Shutter et al., 2000; Mailhos et al., 2001). Until now, all the receptors and ligands have been expressed in at least one vascular compartment, e.g., ECs of arteries, veins, and capillaries, and vascular smooth-muscle cells or pericytes (Iso et al., 2003). The expression varies from several species, including human, rat, mouse, chicken, and zebrafish (Shawber and Kitajewski,

2004; Martinez et al., 2002). Except for Notch4 and Dll4 that are specific to ECs, the other receptors and ligands are widely expressed in many different cells types and tissues.

Loss-of-function studies of Notch signaling in mice demonstrated that Notch function is essential for remodeling of the vascular plexus during development. Mouse embryos deficient in Jagged1, Notch1, Notch1/Notch4, or the presenilins (PS) die between days E9.5–10.5 and display severely disorganized vasculature (Krebs et al., 2000; Xue et al., 1999; Herreman et al., 1999). Although Notch1 deficient mice also display similar vascular defects, it is often less severe than those observed in the Notch1/Notch4 double null mice (Krebs et al., 2000) (Fig. 8. Compared B with C and D, as well as I with J).

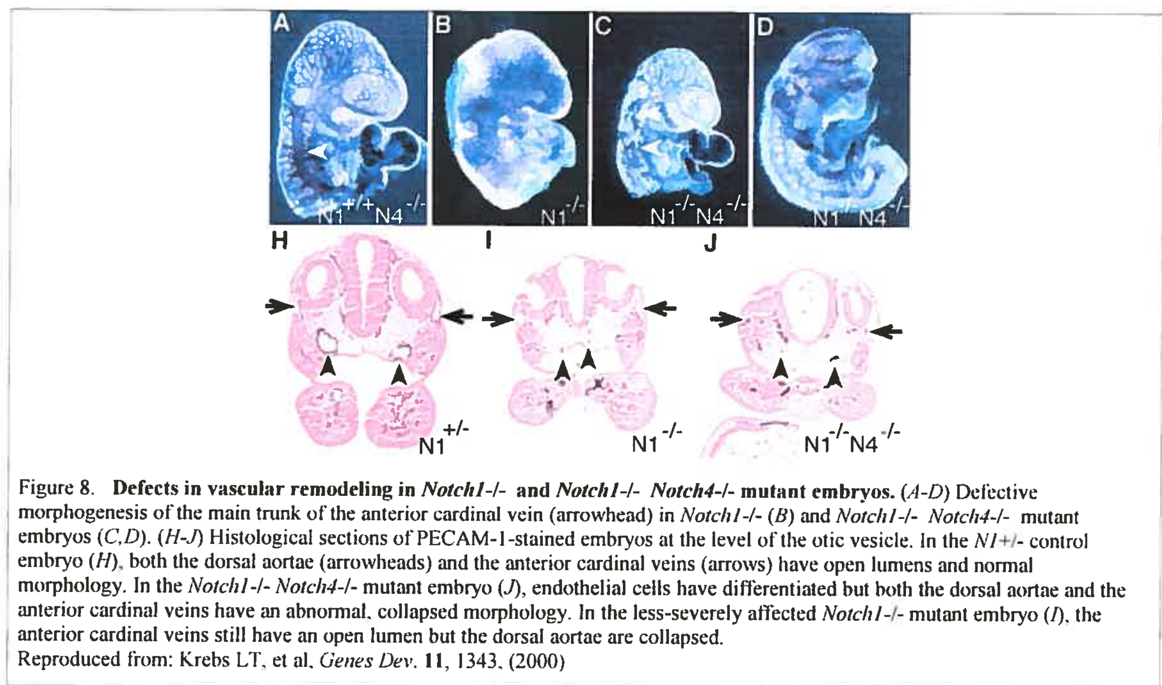

Figure 8. **Defects in vascular remodeling in *Notch1-/-* and *Notch1-/- Notch4-/-* mutant embryos.** (*A-D*) Defective morphogenesis of the main trunk of the anterior cardinal vein (arrowhead) in *Notch1-/-* (*B*) and *Notch1-/- Notch4-/-* mutant embryos (*C,D*). (*H-J*) Histological sections of PECAM-1-stained embryos at the level of the otic vesicle. In the *N1+/-* control embryo (*H*), both the dorsal aortae (arrowheads) and the anterior cardinal veins (arrows) have open lumens and normal morphology. In the *Notch1-/- Notch4-/-* mutant embryo (*J*), endothelial cells have differentiated but both the dorsal aortae and the anterior cardinal veins have an abnormal, collapsed morphology. In the less-severely affected *Notch1-/-* mutant embryo (*I*), the anterior cardinal veins still have an open lumen but the dorsal aortae are collapsed.
Reproduced from: Krebs LT, et al. *Genes Dev.* **11**, 1343, (2000)

Thus, Notch1 and Notch4 are functionally redundant during the vascular development. In addition, with gain-of-function studies of Notch4, transgenic embryonic mice, in which Notch4 is controlled via an endothelium specific promoter, show similar vascular defects and die at E10.5 (Uyttendaele et al., 2001). The same phenotype produced by both decreased and increased Notch indicates that appropriate levels of Notch signaling in ECs are critical for embryonic vascular development.

1.3.2.1. Arterial/venous specification

Notch contributes to arterial/venous specification during the vessel development (Shutter et al., 2000; Lawson et al., 2001; Lawson et al., 2002). For a long time, people have considered the artery and vein as different functional vessels just because of the mechanics of blood flow; however, the molecular mechanisms governing the differentiation and the organization of the rudimentary vessels into arteries and veins are now being uncovered (Wang et al., 1998a; Fuller et al., 2003).

Expression studies of Notch in mammals provided a few hints of a role for Notch in the arterial/venous specification. Notch receptors and ligands are expressed throughout the vasculature quite early in development; however, later on they seem to be restricted to the arteries (Villa et al., 2001; Shawber and Kitajewski, 2004), though more extensive studies showed that they are also expressed in venous vasculature (Iso et al., 2003). This can be seen most clearly in the developing heart outflow and in the male gonads of the mouse. From the developing E9.5 to E13.5 heart outflow tracts, one can find the dynamic localization of Notch4. It is shifted from the anterior cardinal vein to the ECs of aorta (Uyttendaele et al., 1996; Villa et al., 2001). At the embryonic day 11.5 of the male gonad, Notch1, Notch4, and Dll4 are expressed in both the venous and the arterial ECs but, by the next day, become restricted to the arterial vessel only (Brennan et al., 2002). The transitory expression of these genes has suggested a role of Notch in the maintenance of the arterial phenotype.

Studies in zebrafish showed that Notch signaling works in the arterial/venous differentiation, since Notch inhibits the venous state (Lawson et al., 2002). Mutations in Notch or in the Notch-target transcription-factor gridlock caused a disruption in the assembly of the aorta. Meanwhile, a loss in the arterial cell marker ephrin-B2 and an

increase in the venous marker EphB4 were observed (Lawson et al., 2001; Zhong et al., 2001). Consistent with these results, the constitutive activation of Notch suppressed the expression of the other venous marker, *fms*-like tyrosine kinase-4 (flt4) (Lawson et al., 2001); therefore, Notch may promote the development of the artery and inhibit the differentiation of the vein through a gridlock activation. Unfortunately, these results cannot be reproduced in the mouse model with deficient hey2, a mammary comterpart of gridlock (Donovan et al., 2002; Gessler et al., 2002). The reason for this species difference is currently unknown. It may be due to a functional compensation with hey1, which is often co-expressed with hey2 in the arterial ECs (Nakagawa et al., 2000). What seems to be consistent with this hypothesis is that mice lacking both hey1 and hey2 display a constriction of the aortae, though no loss of the major arteries (Chien and Olson, 2002). In cultured human microvascular ECs, a high level of ephrin-B2 was stimulated by an active form of Notch4 (Shawber et al., 2003). Hence, an action of Notch in the arterial/venous specification is quite clear.

1.4. Pathological role of Notch signaling

The Notch activity is important not only in a physiological situation but also in a pathological condition. Mutations of Notch receptors and ligands in mammals lead to abnormalities in many tissues, including hematopoietic disease, solid tumor, neurological disease, and vascular disease (Radtke and Raj, 2003; Gridley, 2003).

1.4.1. Notch and neoplasms

1.4.1.1. T-cell leukemias

The oncogenic role of Notch in a human T-lymphoblast leukaemia (T-ALL) was first identified by Ellisen and his colleagues when they discovered the first human

homologue of the *Drosophila* Notch gene in the T-cell leukemia with a t(7;9) chromosomal translocation (Reynolds et al., 1987; Ellisen et al., 1991). This gene was named TAN-1 for "translocation-associated Notch homologue" and subsequently became the human Notch1. The t(7;9) translocation results in a constitutively expressed truncated form of Notch intracellular domain. It is proposed that the aberrant expression of Notch1 intracellular domain provokes T-cell neoplasm in humans (Ellisen et al., 1991). Studies with mice showed that excessive Notch signaling during the T-cell development leads to T-cell neoplasia (Radtke et al., 1999; Pear et al., 1996). Other results from Jolicoeur's and Overbaugh's groups demonstrated that T-cell neoplasia can also be caused by DNA provirus insertion within Notch1 (Girard et al., 1996; Rohn et al., 1996). In addition, the forced expression of the Notch ligand Dll4 also induces T-cell leukemia (Yan et al., 2001) and induces a lethal T-cell lymphoproliferative disease (Dorsch et al., 2002). Notch activity participates not only in the initiation of T-ALLs but also in their maintenance by recruiting transcription co-activators, such as Mastermind-like-1 (MAML1) (Weng et al., 2003).

1.4.1.2. Epithelial tumors

Before the oncogenic role of Notch1 in human T-ALLs was revealed, Gallahan & Callahan uncovered the tumorigenic role of Notch in the development of epithelial tumors in mouse models (Gallahan and Callahan, 1987). They characterized a frequent insertion site of the mouse mammary tumor virus (MMTV) in these mice and named this region of integration *int-3*. Subsequently, Uyttendaele et al. identified it as the Notch4 locus (Uyttendaele et al., 1996). Similar to the overexpression of $N1^{IC}$ in human T-ALL leukeamia, $N4^{IC}$ is overexpressed in mouse mammary tumors provoked by MMTV insertion. Later on, the mammary adenocarcinorma phenotype discovered in $N4^{IC}$/int-3

transgenic mice further confirmed the oncogenic role of Notch4 in mice (Gallahan et al., 1996; Jhappan et al., 1992). Interestingly, studies from Dr. Jolicoeur's group showed that the mouse Notch1 locus is also a target of MMTV integration. This insertion resulted in $N1^{IC}$ that, in synergy with ERBB2, is thought to be responsible for the development of mammary tumors in these mice (Dievart et al., 1999). Recent results show that Notch1 was also expressed in murine prostate epithelial cells and increased in prostate cancer cells, suggesting that Notch signaling participates in prostatic development and in prostatic tumorigenesis (Shou et al., 2001). Notch signaling pathway is also activated in some human cancers, for example, breast cancer and colon adenocarcinoma (Weijzen et al., 2002; Zagouras et al., 1995). The function of Notch in these diseases is supposed to prevent neoplastic cells from responding to differentiation cues in their immediate environment.

1.4.1.3. Basal-cell carcinomas

Notch signaling in mouse and human skin induces skin differentiation via WAF1, NF-kB, and AP-1 nuclear factors (Rangarajan et al., 2001; Nickoloff et al., 2002; Chu et al., 2002). Mice with Notch1-deficient epithelia develop spontaneous basal-cell-carcinoma-like tumors with down-regulation of WAF1, Shh, and Wnt (Nicolas et al., 2003). Consistent with this result, a reduction of Notch signaling was observed and, as a consequence, caused a down-regulation of Shh signaling in human basal-cell carcinomas. This indicates that Notch signaling is involved in basal-cell carcinomas through Wnt and Shh signaling (Thelu et al., 2002), where Notch acts as a tumor suppressor (Radtke and Raj, 2003).

1.4.1.4. Small-cell lung cancer

As acting on epithelial cell fate in the nervous system (de la Pompa et al., 1997), Notch signaling also determines the epithelial cell fate during mammalian lung development (Borges et al., 1997; Ito et al., 2000). Notch1 and HES1 are highly expressed in airway epithelial cells, whereas mammalian achaete-scute-homologue 1 (MASH1) is restricted to clusters of pulmonary neuro-endocrine cells. It seems that Notch is selectively involved in epithelial cell fate rather than neuro-endocrine cell fate. Interestingly enough, a high-level expression of MASH1 proteins is a characteristic of the primary small-cell lung cancer (Radtke and Raj, 2003). Furthermore, $N1^{IC}$ and $N2^{IC}$ induce a cell-cycle arrest in small-cell lung cancer cells and regulate MASH1 in a negative manner (Sriuranpong et al., 2001; Sriuranpong et al., 2002). Hence, Notch signaling plays a suggestive role in small-cell lung cancer.

1.4.1.5. Cervical cancer

Cervical cancer is caused in part by Human papilloma virus-16 (HPV16) infection. With a complementary role of Notch, the HPV16 oncoproteins E6 and E7 can transform cells (Dyson et al., 1989). In the early stages of cervical cancer, as a tumor suppressor, the Notch activity is reduced, thereby increasing E6 and E7 activation and, conversely, overexpression of Notch1 represses transcription of E6 and E7 (Talora et al., 2002). But in the later stage, the Notch activation can act in synergy with E6 and E7 towards a cellular transformation (detected in both cultured cells derived from cervical cancers and cells within these tumors) (Weijzen et al., 2002; Zagouras et al., 1995; Daniel et al., 1997). This suggests that Notch signaling participates in the cervical tumorigenesis as both an oncogene and a tumor suppressor (Radtke and Raj, 2003).

1.4.2. Notch and inherited disease syndromes

The disruptions of Notch signaling pathway is also involved in human autosomal diseases, for instance, spondylocostal dysostosis (SD). Vertebral segmentation defects associated with rib anomalies are characteristics of SD. These patients exhibit short trunk dwarfism due to multiple hemivertebrae accompanied by rib fusions and deletions. Both autosomal-dominant and -recessive modes of inheritance have been reported (Mortier et al., 1996). One form of autosomal-recessive SD was mapped to chromosome 19q13, where the Dll3 gene is located (Turnpenny et al., 1999). Positional cloning studies have demonstrated that mutations, including protein truncations and missense mutations, in the human Dll3 gene cause this form of autosomal-recessive SD (Bulman et al., 2000). Interestingly enough, this human phenotype has been reproduced and confirmed in Dll3-deficient mice (Dunwoodie et al., 2002). These results suggest that a loss of Dll3 function is related to the human SD. Mice homozygous for a null mutation of the Lfng gene exhibit very similar phenotypes to those observed in the Dll3 mutant mice and in SD patients (Zhang et al., 2002b; Zhang and Gridley, 1998). This indicates that the Dll3 gene is not the sole cause of SD syndromes and that Lfng (another Notch pathway component) is also a candidate gene for autosomal-recessive SD.

Alagille syndrome (AGS) and cerebral autosomal-dominant arteropathy with subcortical infracts and leukoencephalopathy (CADASIL) are other examples of human autosomally inherited diseases that involve Notch signaling (Gridley, 2003) (see below). Both the diseases affect the vascular system and will be discussed next.

1.4.3. Notch and human vascular disease

Notch plays an important role in vessel development in animals. Its importance in the vascular development is highlighted by the genetic defects that arise in humans with

mutations in the Notch pathway, i.e., the AGS and the CADASIL (Bicknell and Harris, 2004; Iso et al., 2003; Shawber and Kitajewski, 2004).

1.4.3.1. AGS

Studies on AGS revealed the role of Notch signaling in organ-specific angiogenesis. AGS is an autosomal dominant disorder characterized by developmental abnormalities of the liver, heart, kidney, eye, skeleton, and, at lower penetrance, several other organs (Krantz et al., 1997; Krantz, 2002; McElhinney et al., 2002; Eldadah et al., 2001). This wide spectrum of defects correlates with the expression of JAG1 in the embryo (Loomes et al., 2002; Loomes et al., 1999; McCright et al., 2002). Genetic studies have demonstrated mutations in the JAG1 gene in 70% of AGS patients and were identified as critical causes for this disease (Li et al., 1997; Spinner et al., 2001). These mutations include 72% premature termination condons, 15% splicing mutations, and 13% missense mutations. The missense mutations are clustered within the extracellular domain of JAG1. Three to seven percent of the AGS patients with deletions encompassing the entire JAG1 gene have phenotypes similar to those involving intragenic mutations (Spinner et al., 2001). Hence, AGS results from JAG1 haploinsufficiency (Spinner et al., 2001; Krantz, 2002). AGS exhibits a variable expression, even within the family members carrying identical JAG1 mutations, but high penetrance (Krantz et al., 1997; Krantz, 2002). One notable feature exhibited by the patients is the vascular defects observed in liver, heart, and kidney (Shawber and Kitajewski, 2004). The relationship between Notch signaling and AGS has been further elucidated by the phenotypes of mouse AGS models (McCright et al., 2001; McCright et al., 2002).

In order to establish a successful mouse AGS model, Gridley's group generated JAG1-deficient mice. Mice homozygous for the JAG1 show vascular defects in the embryo

and in the yolk sac but die early *in utero* because of these defects. Mice heterozygous for the JAG1 mutation exhibit eye defects but do not exhibit some other phenotypes found in humans, providing a disappointing model for this disease (Xue et al., 1999). They also generated mice homozygous for a hypomorphic Notch2 mutation-Notch2^{del1} (McCright et al., 2001). Vascular defects in these mice were mainly observed in the kidney. JAG1 gene was observed to be colocalized to Notch2 in the defective kidney of the Notch2^{del1} mutants. They further generated mice doubly heterozygous for the JAG1 null allele and a Notch2 hypomorphic allele (designated *J1N2+/-*) (McCright et al., 2002). These mice have, surprisingly, reproduced most of the clinically relevant phenotypes, including vascular defects (hepatic, cardiac, and renal) observed in the AGS patients. Thus, the Notch2 gene acts as a genetic modifier to interact with a JAG1 mutation so as to create a more representative model for AGS.

Most AGS patients are diagnosed with liver disease because of a paucity of bile ducts. A similar liver phenotype has been observed in *J1N2+/-* mice (McCright et al., 2002). Bile ducts are usually accompanied by portal veins. During the liver development, the hepatoblasts juxtapose to the portal vein and differentiate into epithelial cells of the bile ducts (Shiojiri et al., 2001). While JAG1 is expressed in the ECs and in the pericytes of the portal vein, Notch2 is expressed in the neighboring epithelial cells that give rise to the bile ducts (McCright et al., 2002). Hence, the liver defects in the bile duct differentiation may result from JAG1-Notch2 signaling between the portal-vein cells (ECs and pericytes) and the bile-duct cells (epithelial cells).

More than 90% of the individuals with JAG1 mutations exhibit cardiovascular abnormalities along with various defects, ranging from the pulmonary vale to the aortae (McElhinney et al., 2002). These diverse defects correlate with the expression of Notch

(Notch1,2,4) and its ligands (JAG1,2) in the developing murine heart. The co-expression of JAG1 with Notch2 was found in the pulmonary artery, in the walls of the atria, and in the ventricular myocardium (Loomes et al., 2002; Villa et al., 2001; Loomes et al., 1999). While Notch2 expression is restricted to the ECs, JAG1 expression extends into the mural cells. Consistent with this expression pattern, half of Notch2^{del1} homozygous die prior to day E16.5 because of severe heart defects (McCright et al., 2001). Multiple heart-related defects were displayed in *J1N2*+/- mice and in hey2-deficient mice (McCright et al., 2002; Donovan et al., 2002; Gessler et al., 2002). Thus, the heart defects might arise from the disruption of JAG1-Notch2 signaling between mural and ECs.

Renal anomalies also occur in 23–74% of the AGS patients. This disease is well characterized in Notch2^{del1} and *J1N2*+/- mice (McCright et al., 2002; McCright et al., 2001). About half of Notch2^{del1} homozygous mice survive until birth, but some still die perinatally from defects in the renal glomerular development. Vascularization of the glomeruli includes the migration of ECs into glomerular cleft, the formation of capillary loops, and the formation of complex capillary tufts. In Notch2^{del1} homozygous, the defects begin from the capillary loop stage (McCright et al., 2002). In these mutant glomeruli, specialized vascular smooth-muscle cells and mesangial cells are entirely absent. In *J1N2*+/- mice, a similar renal phenotype exists but seems less severe (McCright et al., 2001). Interestingly, JAG1 is expressed in ECs and/or in mesangial cells while Notch2 is in surrounding podocyte precursors (McCright et al., 2001). One can speculate that the disruption of JAG1-Notch2 signaling among ECs, mesangial cells, and podocyte precursors leads to a destabilization of the vascular bed of glomeruli.

1.4.3.2. CADASIL

Insights into the role of Notch in vascular homestasis can be drawn from the human disease CADASIL. CADASIL is an autosomal-dominant vascular disorder. Affected patients exhibit migraine, dementia, and a variety of other symptoms. The neuropathological symptoms arise secondary to a slow developing arteropathy. The histo-pathological characteristic for this disease is a degeneration of vascular smooth-muscle cells, accompanied by a reduction of the vessel wall thickness and a loss of ECM (Tournier-Lasserve et al., 1993; Chabriat et al., 1995; Ruchoux et al., 1995). In a majority of patients, mutations in the Notch3 gene are identified as a cause of the syndrome CADASIL (Joutel et al., 1996). Some 66% of these mutations occur in the extracellular domain exon 3 and 4, which encode EGF-repeat 2 through 4 in their entirety. These mutations in CADASIL patients lead to an odd number of cyteines in the affected EGF-repeat (Joutel et al., 1996). Using bioinformatic analysis, it was discovered that the Notch3 mutations are gain-of-function mutations in the CADASIL disease (Donahue and Kosik, 2004). The CADASIL phenotype correlates with the expression of Notch3 in arterial vascular smooth-muscle cells. This expression has been identified in both humans and rodents (Joutel et al., 2000; Prakash et al., 2002). Significantly, in the CADASIL patients, the accumulation of the protein of Notch3 ectodomain was observed at the cytoplasmic membrane of the vascular smooth-muscle cells. It suggests that these mutations impair the clearance of the Notch3 ectodomain from the cell surface (Joutel et al., 2000). Also, there is an abnormal deposition of particles in the ECM, referred to as granular osmophilic materials (GOM) (Joutel et al., 2000). These results indicate that Notch3 functions to maintain cell-cell interactions or communications between the vascular smooth-muscle cells and the ECs.

A recent study by Ruchoux et al. has recreated the CADASIL vessel pathology in a mouse model (Ruchoux et al., 2003). In these transgenic mice, a human Notch3 cDNA containing a common CADASIL mutation (Arg90cys) was expressed in the vascular smooth-muscle cells. As a consequence, the vasculature of these mice exhibit classic CADASIL arteriopathy, including an age-dependent accumulation of Notch3 extracellular domain and GOM deposits. It was found that the accumulation of Notch3 ectodomain and the GOM deposit are caused by smooth-muscle cell viability and adhesion (Ruchoux et al., 1995). Consistent with a role of Notch3 in the cell survival, *in vitro* experiments with smooth-muscle cells expressing either Notch3 or hey1 show that Notch3 inhibits apoptosis via an anti-Fas pathway (Wang et al., 2002; Wang et al., 2003). All together, Notch3 maintains the arterial vessel homeostasis by promoting the smooth-muscle cell survival.

CADASIL syndromes provide the very first evidence that the ectodomain of Notch signaling is implicated in a human disease. Particularly, the mutations in the extracellular domain influence the vascular homeostasis through mediating the communication of smooth-muscle cells to ECs.

1.5. Endothelial cells, vasculogenesis, and angiogenesis

More than 10^{12} ECs are lining inside the blood vessels in humans, covering a vast extension area of more than 1000 m^2 (Jaffe, 1987). They form the endothelium, which is considered a sparse organ system. Not only do they form the structural basis of blood vessels, but they also exert a complex array of specialized functions, including vasculogenesis and angiogenesis (Risau, 1995; Augustin et al., 1994).

1.5.1. Developmental and physiological conditions

1.5.1.1. Endothelial cell development

Hematopoiesis in the mouse is initiated in the embryo approximately 12 hours after the onset of gastrulation (Haar and Ackerman, 1971). During the process of gastrulation, the embryonic epithelium invaginates through the primitive streak. Mesodermal cells are induced during this process, which then migrate widely throughout the extraembryonic membranes (YS) and the embryo proper (P-Sp & AGM). In response to induction of the mesoderm, progenitors of the blood cells and the vascular ECs are the first differentiated cell types to form in the developing vertebrate embryo. Endothelial progenitor cells are called angioblasts, which are defined as a cell type that has the potential to differentiate into an EC but has not yet acquired all the characteristic endothelial markers or formed a lumen (Risau, 1995). The first angioblasts emerge in the so-called blood islands in the YS as early as day 7.5 of gestation. These are cell clusters whose internal members give rise to blood cells, whereas the exterior ones flatten and develop into ECs. The formation of blood vessels from these *in situ* differentiating ECs is called **vasculogenesis**, which forms the embryonic primitive vascular network. The latter will mature through subsequent steps, including sprouting, pruning, and remodeling into small and large vessels, called **angiogenesis** (Risau, 1997; Folkman and D'Amore, 1996). The angioblasts from AGM/P-Sp region (Choi, 1998; Garcia-Porrero et al., 1995; de Bruijn et al., 2000) form the first main embryonic blood vessels, aorta, and cardinal veins, which become connected to the heart and the extraembryonic vessels (Risau, 1995). In addition, the angioblasts also differentiate from the fetal liver (Choi, 1998) and the mesodermal precursors within organ rudiments of lung, pancreas, and spleen (Pardanaud et al., 1989; Sariola et al., 1983).

With a decade of studies, the molecular mechanisms that underlie the angioblast differentiation are becoming more and more clear. While fibroblast growth factors (FGFs) were the first purified proteins demonstrated to induce the mesoderm in animal pole explants (Kimelman and Kirschner, 1987; Slack et al., 1987), the vascular endothelial growth factor receptor-2 (VEGFR-2, also known as fetal liver kinase-1 [flk-1] in the mouse and kinase insert domain-containing receptor [KDR] in the human) was the first protein known to be expressed in a population of mesodermal cells giving rise to angioblasts. Later, during embryonic development, this molecule becomes restricted to EC, consistent with the function of its ligand VEGF as a specific endothelial cell growth and vascular permeability factor (Ferrara et al., 1992). While VEGFR-2 is expressed in the mesoderm of the 7-day mouse, VEGF is expressed in the endoderm of the 7.5-day mouse. Because the endoderm is adjacent to the mesoderm, a paracrine relationship may exist. VEGF secreted by endoderm may support the differentiation of VEGFR-2-expressing mesodermal cells to angiblasts. Some reports show that hematopoietic cells share some identical markers, for example, CD34, SCL, CD31, Runx-1, and VEGFR-2, with angioblasts. Hence, a bipotent population of cells called “hemangioblasts” may exist, which are capable of differentiating into both blood cells and endothelium (Bloor et al., 2002). The different markers expressed in embryonic angioblasts are maintained during embryonic vasculogenesis and angiogenesis and down-regulated in adult vessels; however, they can be reexpressed in pathological angiogenesis (Risau, 1997; Hanahan, 1989; Pepper, 1997).

1.5.1.2. Heterogeneity of endothelial cells

ECs present many common functional and morphological features, however, quiescent and resting ECs in the adult form a highly heterogeneous cell population that

varies in different organs (Turner et al., 1987; Garlanda and Dejana, 1997). The sinusoidal endothelial cells of the liver (LSECs) have a unique phenotype compared with both macrovascular and microvascular ECs from other organs (Limmer and Knolle, 2001). The LSECs express high levels of the molecules necessary for interaction with leukocytes. They also constitutively express all co-stimulatory molecules as well as MHC class I and II molecules necessary for the presentation of antigen to T cell, suggesting they are unique cells where T-cell priming occurs outside lymphatic tissues (Limmer and Knolle, 2001). Interestingly, they express a number of molecules that are typically found in cells of myeloid origin, such as CD4 and CD11c. In addition, they express several patterns of recognition receptors that enable them to act as scavenger cells (Knolle and Limmer, 2003).

Even in different vessel calibers within an organ, the ECs are different. The structural heterogeneity of ECs is a perfect example of their optimized adaptation to microenvironmental needs (Garlanda and Dejana, 1997; Augustin et al., 1994). A good example is the liver, which contains different types of ECs: fenestrated and discontinuous in the sinusoids, and continuous in the large vessels (McCuskey and Reilly, 1993). They heterogeneously express surface antigens associated with different leukocytes (Nagura et al., 1986). While the LSECs express weak PECAM-1 (platelet/endothelial cell adhesion molecule-1), the large vessel ECs express strong PECAM-1 (Couvelard et al., 1996). The sinusoidal cells in the portal and the central part of the liver are also distinct. At the portal part, the endothelial cell fenestrae are larger but comprise less of the endothelial surface area than they do in the pericentral lobule (McCuskey, R.S. 1988). They further show heterogeneity with respect to the recognition of apoptotic cells (Dini and Carla, 1998).

How do ECs take different pathways of differentiation? One of the determinants is the local environment in which ECs differentiate, especially their interaction with surrounding cells. This interaction may occur through the release of soluble mediators, cell-to-cell adhesion, and the synthesis and organization of matrix proteins on which the endothelium adheres and grows (Garlanda and Dejana, 1997; Augustin et al., 1994). For example, contact with parenchymal cells can control the EC phenotype. Hepatocytes have been shown to be involved in determining the phenotype of LSECs (Modis and Martinez-Hernandez, 1991).

1.5.1.3. Blood vessel formation

The formation of new blood vessels is vital during embryogenesis. Two mechanisms account for the formation of blood vessels: vasculogenesis and angiogenesis (Fig. 9). A description of vasculogenic process was first provided as it occurs in frogs, fish, and avians (Clark, 1918; Sabin, 1920; Stockard, 1915), and the concept of de novo blood vessel formation was established at the same time (Sabin, 1920). Risau (1988) later used

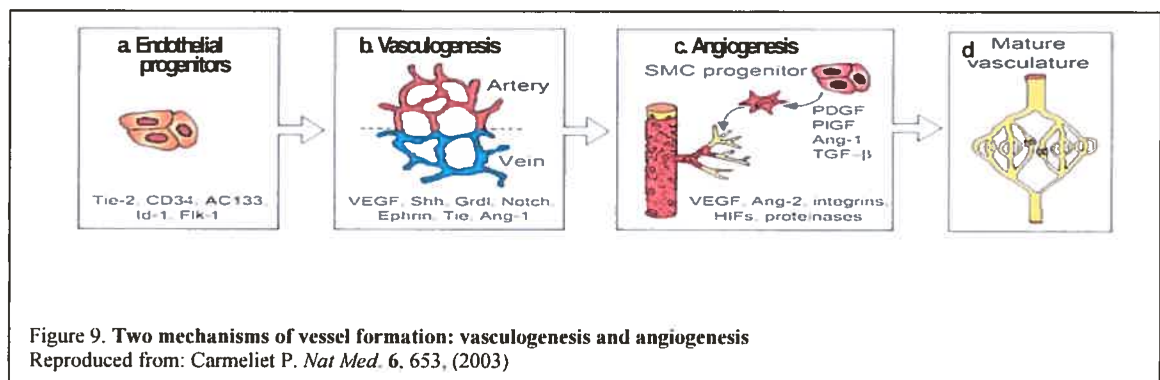

Figure 9. **Two mechanisms of vessel formation: vasculogenesis and angiogenesis**
Reproduced from: Carmeliet P. *Nat Med.* **6**, 653, (2003)

the term "vasculogenesis" to describe de novo blood vessel formation occurring in embryoid bodies (Risau et al., 1988). Some essential steps involved in this process are: 1) the birth of angioblasts; 2) angioblast aggregation (Fig. 9a); 3) elongation of angioblasts into cord-like structures; 4) the organization of isolated vascular segments into capillary-like networks and concomitant with step four; 5) endothelialization and lumenization

(Drake, 2003) (Fig. 9b). Recent studies have shown that circulating progenitor cells (possibly angioblasts) (Rafii, 2000) from embryo and adult bone marrow also contribute to vasculogenesis (Grant et al., 2002; Carmeliet, 2003). These results expanded the scope of vasculogenesis occurrence beyond the embryonic stages of development to include involvement of neovascular processes throughout the development as well as in the adult (Drake, 2003). Hence, two vasculogenic mechanisms can contribute to forming blood vessels: *in situ* vasculogenesis from mesoderm and vasculogenesis from circulating mesodermal or progenitor cells.

"Angiogenesis" describes the formation of new blood vessels from the existing vasculature (Carmeliet, 2000; Bergers and Benjamin, 2003) (Fig. 9c). The classical angiogenesis process, sprouting angiogenesis, is characterized by at least three dynamic steps: 1) a modulation of interactions between ECs with the ECM, which induces the degradation of ECM (Fig. 10b); 2) an initial increase and subsequent decrease in the migration of ECs, which allow the cells to translocate toward the angiogenic stimulus and to stop once they reach their destination (Fig. 10c); 3) an increase of endothelial cell

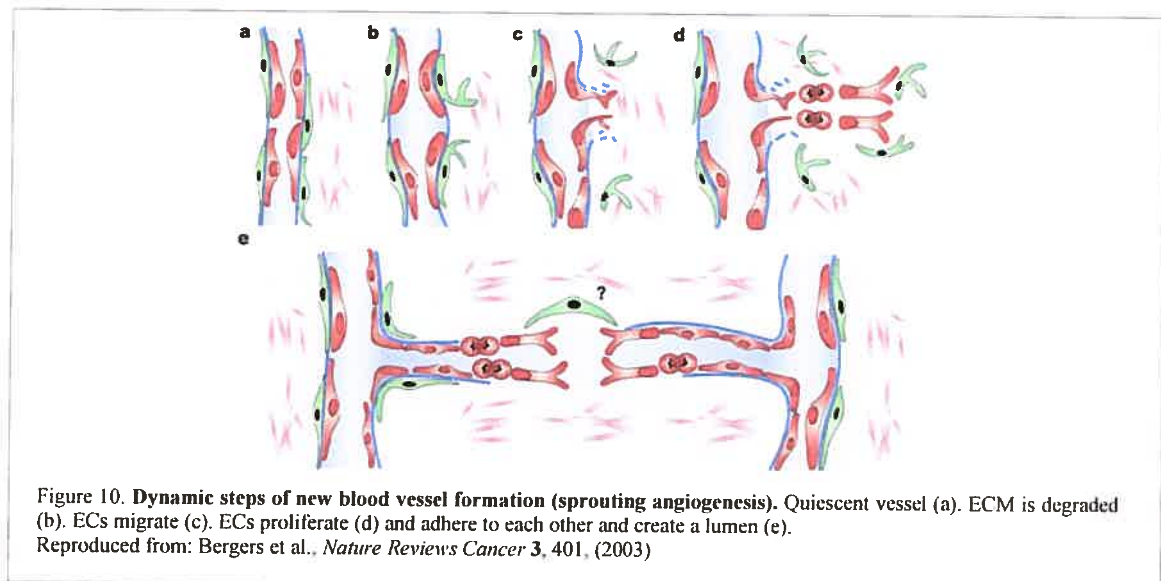

Figure 10. **Dynamic steps of new blood vessel formation (sprouting angiogenesis).** Quiescent vessel (a). ECM is degraded (b). ECs migrate (c). ECs proliferate (d) and adhere to each other and create a lumen (e).
Reproduced from: Bergers et al., *Nature Reviews Cancer* **3**, 401, (2003)

proliferation, which provides new cells for the growing and elongating vessel, and a subsequent return to the quiescent state once the vessel is formed (Bergers and Benjamin, 2003) (Fig. 10d and 10e). During this time, the vessel wall becomes mature, as ECs integrate tightly with supporting cells (smooth-muscle cells and pericytes [SMCs/PCs]) and surrounding matrix (Fig. 9c and 9d). In contrast, vasculogenesis is initially free of smooth-muscle cells, pericytes, and other associated cells (Yancopoulos et al., 2000). Further changes in size and mural structure of vasculature lead to the formation of arteries, veins, and lymphatics, each with its own characteritics. The angiogenesis process is usually quiescent in the adult, occurring only during the female menstrual cycle, in wound healing, and some pathological cases (described later) (Hanahan and Folkman, 1996).

1.5.1.4. Molecular regulation of vessel formation

The genetic and molecular mechanisms that control the development of the vascular system are now beginning to be elucidated. Morphogenic programs that control both the formation of vessels and the maintenance of established vessels are regulated by environmental influences. They are importantly orchestrated by a balance of pro-angiogenic and anti-angiogenic factors (Jain, 2003). In the pro-angiogenic factors, besides growth factors, molecules involved in matrix remodeling and cell adhesion also participate in these programs (Bazzoni et al., 1999).

Angiogenic factors

The FGF family

FGFs were previously thought to be important angiogenic factors but direct *in vivo* evidence is still lacking. They have profound effects in various endothelial cell assays, but they do not play a major role in the morphogenesis of the vascular system in contrast to their crucial function in mesoderm induction. Capillary ECs in embryonic tissues and

organs do not express detectable levels of mRNA-encoding FGF-receptors (Wanaka et al., 1991; Peters et al., 1992). ECs of larger vessels do express FGF-R and respond to FGF *in vivo* (Peters et al., 1992; Lindner et al., 1990). This is probably important for regenerative processes but not for vasculogenesis and angiogenesis (Risau and Flamme, 1995). Hence, FGFs were known to be non-specific factors since they could act on many other cell types (Risau, 1997).

The VEGF family

Presently, only VEGF is specific to the blood vessel formation (Ferrara, 1999; Eriksson and Alitalo, 1999). There are five characterized VEGF relatives in mammals: VEFG-A through VEGF-D, as well as Placenta growth factor (PIGF). The various members of the VEGF family have overlapping abilities to interact with three receptor kinases: VEGFR-1/Flt-1, VEFGR-2/Flk-1, and VEGFR-3/Flt-4 (Eriksson and Alitalo, 1999; Yancopoulos et al., 2000) (Fig. 11a). VEGF-A seems to have the ability to induce

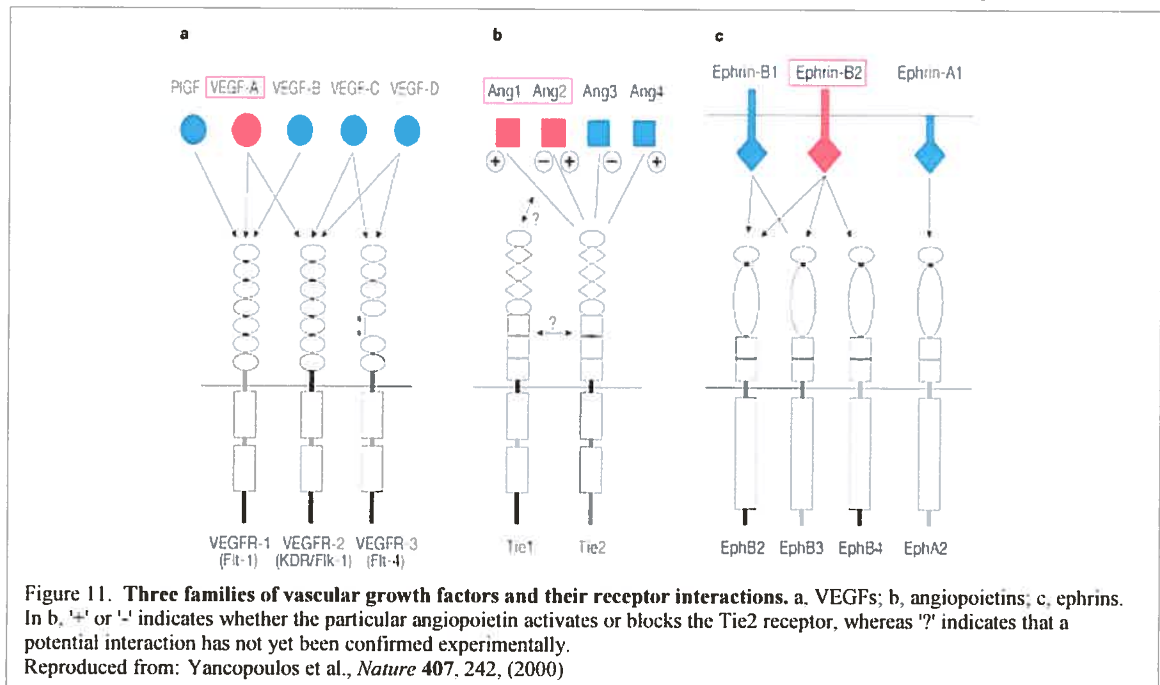

Figure 11. **Three families of vascular growth factors and their receptor interactions.** a, VEGFs; b, angiopoietins; c, ephrins. In b, '+' or '-' indicates whether the particular angiopoietin activates or blocks the Tie2 receptor, whereas '?' indicates that a potential interaction has not yet been confirmed experimentally.
Reproduced from: Yancopoulos et al., *Nature* **407**, 242, (2000)

vascular endothelial cell proliferation but initially was defined as vascular permeability factor (VPF) due to its ability to induce vascular leak (Ferrara, 1999). Mice lacking VEGF-

B have smaller hearts, suggesting that it may play a role in coronary vascularization and growth (Bellomo et al., 2000). Transgenic overexpression of VEGF-C leads to lymphatic hyperplasia, supporting a role of VEGF-C in lymphatic development (Olofsson et al., 1999). Little is known about the normal physiological role of VEGF-D (Eriksson and Alitalo, 1999). PlGF is the first relative of VEGF (Carmeliet, 2000; Persico et al., 1999). It has been suggested to be involved in adult vascular remodeling observed with mice lacking PIGF (Carmeliet, 2000; Carmeliet et al., 2001). VEGFR-2 and VEGF-A are absolutely critical for the earliest stage of vasculogenesis. Mice lacking VEGF-A or VEGFR-2 are embroid lethal since they failed to develop enough ECs and have very few large blood vessels (Shalaby et al., 1995; Carmeliet et al., 1996; Ferrara et al., 1996); however, mice lacking VEGFR-1 have an excess number of ECs and form disorganized vessels in the embryos (Fong et al., 1995). Mice generated with VEGFR-1 lacking tyrosine kinase domain show normal vessel, suggesting that VEGFR-1 acts as a decoy receptor (Hiratsuka et al., 1998). Thus, VEGFR-2 is a major receptor to mediate VEGF-A effect on the vessel formation, and VEGFR-1 acts as a negative regulator of VEGF signaling to ensure a proper number of endothelial cell formations (Yancopoulos et al., 2000). VEGFR-3 seems to play a crucial role in lymphatic vessel development, though it is also involved in the blood vessel development (Taipale et al., 1999).

Recent findings show that lack of VEGF is not only involved in embryonic lethality but also leads to early postnatal death due to vascular deficiencies (Miquerol et al., 2000; Carmeliet et al., 1999). In adult mice, VEGF inactivation is much less traumatic and, seemingly, VEGF-deficient vessels continue to undergo remodeling (Ferrara et al., 1998; Gerber et al., 1999). VEGF does not seem to have a continuous maintenance function for much of adult vasculature; however, VEGF influences normal vascularization and

angiogenesis response in adults when inappropriately overexpressed (Yancopoulos et al., 2000) .

The Angiopoietin family

The angiopoietins (Angs) were discovered as the second group of factors specific to vessels long after the observation of VEGF family (Suri et al., 1996; Maisonpierre et al., 1997; Valenzuela et al., 1999) (Fig. 11b). They seem to be some of VEGF's most important partners (Yancopoulos et al., 2000). The specificity of angiopoietins for vessels results from the restricted distribution of their receptors, Tie-1 and Tie-2, to ECs. Both Tie-1 and Tie-2 are receptor tyrosine kinases. Major attention is focused on Tie-2, since four ligands, Ang-1, Ang-2, Ang-3, and Ang-4, bind primarily to Tie-2. Little is known about Tie-1. Ang-1 and Ang-2 are the most important and studied ligands. While Tie-2 is expressed on EC (except its expression on hematopoietic lineage), Ang-1 and Ang-2 are expressed by the supporting cells and ECs, respectively (Gale and Yancopoulos, 1999).

Compared to VEGF, the angiopoietin family plays a later role in vessel formation. While mice lacking VEGF-A and VEGFR2 failed to form primitive vessels, embryos lacking Ang-1 or Tie-2 develop a rather normal primary vasculature (Suri et al., 1996; Sato et al., 1995); however, remodeling and stabilization of this primitive vasculature in these mice is severely perturbed, leading to embryo lethality (Folkman and D'Amore, 1996; Suri et al., 1996). Ultrastructural examination indicates that ECs failed to interact and adhere properly to underlying supporting cells, which are the cells producing Ang-1 and act in a paracrine fashion on ECs expressing Tie-2. Hence, the angiopoietin family seems not to induce vascular formation but to influence integration of ECs and their supporting cells, allowing them to receive other critical signals from their environment (Suri et al., 1996; Yancopoulos et al., 2000). Notably, recent studies show that in mouse neonates lacking

supporting cells, recombinant Ang-1 restored the deficiencies of the larger vessels, as well as rescued edema and hemorrhage (Uemura et al., 2002). Ang-2 was discovered as the antagonist of Tie-2 to destabilize quiescent vessels leading to the sprouting of new ones (Maisonpierre et al., 1997). Transgenic overexpression of Ang-2 disrupts blood vessel formation in the mouse embryo resembling those of Ang-1 and Tie-2 knockout mice, indicating that Ang-2 acts as a Tie-2 antagonist *in vivo*. In adult mice and humans, whereas Ang-1 is expressed widely in supporting cells of normal tissues, Ang-2 is expressed only at endothelium of vascular remodeling tissues, such as sprouting and regressing vessels in the ovary (Maisonpierre et al., 1997; Goede et al., 1998) or in the tumors (Holash et al., 1999b; Holash et al., 1999a; Zagzag et al., 1999). It was proposed that autocrine induction of Ang-2 in endothelium blocked the constitutive stabilizing influence of paracrine Ang-1, allowing ECs to revert to a more plastic and destabilized state reminiscent of developing vessels (Yancopoulos et al., 2000). The destabilizing role of Ang-2 is regulated by VEGF. In the absence of VEGF, the destabilization is prone to regression. On the other hand, in the presence of VEGF, the destabilized vessels become more sensitive to angiogenic factors leading to new sprouting vessels; however, mice lacking Ang-2 failed to develop an aortic wall, where Ang-2 is highly expressed in transgenic mice. Thus, Ang-2 not only acts as a blocker of Tie-2 but may also play an angiogenic role in vessel formation, at least in some cases (Yancopoulos et al., 2000).

The ephrin family

Other factors specifically related to vessels are the Eph-ephrin family (Gale and Yancopoulos, 1999; Yancopoulos et al., 2000) (Fig. 11c). Ephs are also receptor tyrosine kinases, including at least 14 distinct members with eight ligands—ephrins. These receptors and ligands have been described in both man and mouse (Flanagan and

Vanderhaeghen, 1998). Both Eph receptors and ephrin ligands are rather unique among RTK family. While the Eph receptors are closely related to cytoplasmic tyrosine kinases within their kinase domain (Hanks and Quinn, 1991), the ephrin ligands must be membrane-attached to activate their receptors (Gale and Yancopoulos, 1999; Davis et al., 1994). This membrane attachment promotes ligand clustering, which is necessary to activate their receptors on adjacent cells, whereas monomeric soluble ligands seem to act as antagonist (Davis et al., 1994; Winslow et al., 1995). All of the known ephrins are tethered naturally to the cells in which they are expressed. These ligands are divided into two subgroups: the ephrin-A with three members (A1–A3) and ephrin-B with 5 members (B1–B5). Ephrin-A and -B subgroups bind to the EphA (1–8) and EphB (1–6) receptors, respectively (Gale and Yancopoulos, 1999).

Signaling through the Eph receptors can be a bidirectional event between directly interacting cells (Holland et al., 1996; Bruckner et al., 1997). Initially, Eph-ephrin was mainly found to play roles in the neural system to regulate cell mixing and establish boundaries between distinct cellular compartments aiming to pattern brain and somites (Xu et al., 1996; Durbin et al., 1998). It is now known that some Eph family members also play roles in vascular development (Gale and Yancopoulos, 1999). *In vitro* findings show that both ephrin-A1 and ephrin-B1 are differentially capable of inducing human umbilical ECs (HUVECs) and human renal microvascular ECs (HRMECs) to form tubules (Daniel et al., 1996). *In vivo* studies were performed by Wang et al. (Wang et al., 1998a) using ephrin-B2 knockout mice in which ephrin-B2 gene was replaced by lac-Z gene. Lac-Z expression analysis revealed that ephrin-B2 specifically marked arterial ECs at the earliest stages of vascular development and that Eph-B4 specifically and reciprocally marked only the venous endothelium. This was the first time to propose that molecular differences are, in

part, programmed genetically in arterial versus venous endothelium. Furthermore, they found that EphB4-ephrin-B2 not only simply served as vein-artery makers but also systemically influenced remodeling of vessels in multiple sites, for example, yolk sac, head region, and heart trabeculation, during early embryo development (Wang et al., 1998a). Angiogenesis deficiencies in ephrin-B2 knockout mice were highly reminiscent of the defects observed in the mice lacking Ang-1 or its receptor Tie-2 (Sato et al., 1995; Suri et al., 1996). Adame et al. found other B-class Eph receptors, such as EphB2 and EphB3, to be involved in major vessel formation, especially overlapping with EphB4 in venous endothelium (Adams et al., 1999). Results from EphB4-deficient mice show that EphB4 may be a special case for ephrin-B2 (Gale and Yancopoulos, 1999).

Later studies by Yancopoulos et al. showed that ephrin-B2 continuously marks arteries during later embryonic development, as well as in adults. The expression of ephrin-B2 also extends to supporting cells, suggesting that ephrin-B2 may also regulate formation of arterial muscular walls. In adult setting of angiogenesis, a high reexpression of ephrin-B2 was examined in the new vessels of tumor sites and reproductive organs, indicating that ephrin-B2 is also important for angiogenic settings (Yancopoulos et al., 2000). Recent *in vitro* studies show that EphB4-ephrin-B2 acts as a forward-reverse signaling axis and as a versatile vascular cell-cell interaction and communication system (Fuller et al., 2003). These findings complete the *in vivo* phenotype in which vein-artery is determined by EphB4-ephrin-B2, indicting the general role of the ephrin family in boundary formation in both neural and vascular systems.

The Notch family

Notch is a new member that has appeared in the angiogenic factor's list recently due to its emerging role in vessel formation (Gridley, 2001; Iso et al., 2003).

In vitro studies have provided a mainly negative role of Notch signaling in angiogenesis (Iso et al., 2003). In bovine microendothelial cells (BMEs) experimentally exposed to fibrin, the Jagged-1 transcripts were upregulated. Interestingly, the addition of an antisense oligonucleotide to Jagged-1 to these BMEs enhanced their invasion and tube formation in the underlying gel during FGF-induced angiogenesis (Zimrin et al., 1996), suggesting a negative role of Notch signaling. An extracellular domain of Jagged1 (soluble Jagged1) secreted from NIH 3T3 cells, which could act as a dominant, negative factor, was also found to increase tube formation. These cells further formed tissue masses in nude mice with prominent angiogenesis (Wong et al., 2000). More experiments demonstrated that soluble Jagged-1 inhibited endogenous Jagged-1-mediated Notch signaling to induce angiogenesis (Small et al., 2001). Recently, Leong et al. reported that an activated Notch-4 in human dermal microvascular ECs (HMEC-1) may inhibit angiogenesis, partly by promoting β1 integrin-mediated adhesion to underlying matrix (Leong et al., 2002). All these results support the negative role of Notch signaling in angiogenesis; however, these effects are highly controversial since several other experiments concluded with a positive role of Notch signaling in angiogenesis. Not only did soluble Jagged-1 proteins inhibit cell migration and decrease cell-matrix adhesion (Lindner et al., 2001) but the expression of Jagged-1 or activated Notch-4 in cultured brain ECs also induced microvessel-like structures (Uyttendaele et al., 2000). Hence, one should be careful in interpreting such data, because the function of Notch signaling seems to depend on the cell context.

Jagged-1 and Notch-1/4 embryonic-deficient mice and Notch-4 transgenic mice have already been mentioned earlier to demonstrate the essential role of Notch signaling in the vessel development. In all these mouse models, vasculogenic formation of the head, yolk sac, and intersomitic vessels are unaffected. Initial establishment of the vascular

network does appear to be significantly comprised; however, there is a failure to reorganize these rudimentary vessels into large vessels and branches, suggesting that these Notch receptors and ligands are not required for vasculogenesis but essential for angiogenesis. In the mice lacking Notch-1 and Notch-1/Notch-4, a collapse or discontinuity of aortae and anterior cardinal veins was observed (Krebs et al., 2000). In contrast, in the embryos that expressed the activated Notch-4 specifically in ECs, a dilation of these vessels was revealed and is associated with a failure of the recruitment of smooth-muscle cells to the ECs to organize the vessel wall (Uyttendaele et al., 2001). This phenotype is very similar to those observed in the Tie-2/Ang-1 knockout mice and the Ang-2 transgenic mice (Suri et al., 1996; Yancopoulos et al., 2000). One may speculate that Notch signaling can determine endothelial-mural cell interactions. The failure of the Notch mutant embryos to remodel their vasculature may be due partly to defects in the organization of the pericytes. Consistent with this speculation, while Notch-3 and Jagged-1 are expressed in SMCs, Notch-1, Notch-4, and Jagged-2 are expressed in the ECs of E13.5 embryos (Uyttendaele et al., 1996; Villa et al., 2001). Thus, Notch signaling may provide a signal for mensenchymal-endothelial cell interactions in that it helps the stabilization of the newly formed vessels.

As mentioned earlier, Notch signaling plays a role in arterial/venous specification and maintenance of the arterial differentiation. In fact, the phenotype observed in the Notch-1 or Notch-1/Notch-4 deficient mice closely resembles that in the ephrin-B2 knockout mice (Krebs et al., 2000; Wang et al., 1998a; Shawber and Kitajewski, 2004). Using zebrafish genetics, ephrin-B2 was proved to act as a downstream signaling of Notch (Lawson et al., 2002). In this study, sonic hedgehog (SHH) was required for the arterial specification and the induction of VEGF-A that, in turn, induced the expression of Notch,

suggesting that SHH and VEGF-A are upstream of Notch signaling. Interestingly, ectopic expression of Notch rescued ephrin-B2 expression in the absence of VEGF-A, indicating that ephrin-B2 is downstream of Notch signaling (Fig. 12). In mouse models, Notch also

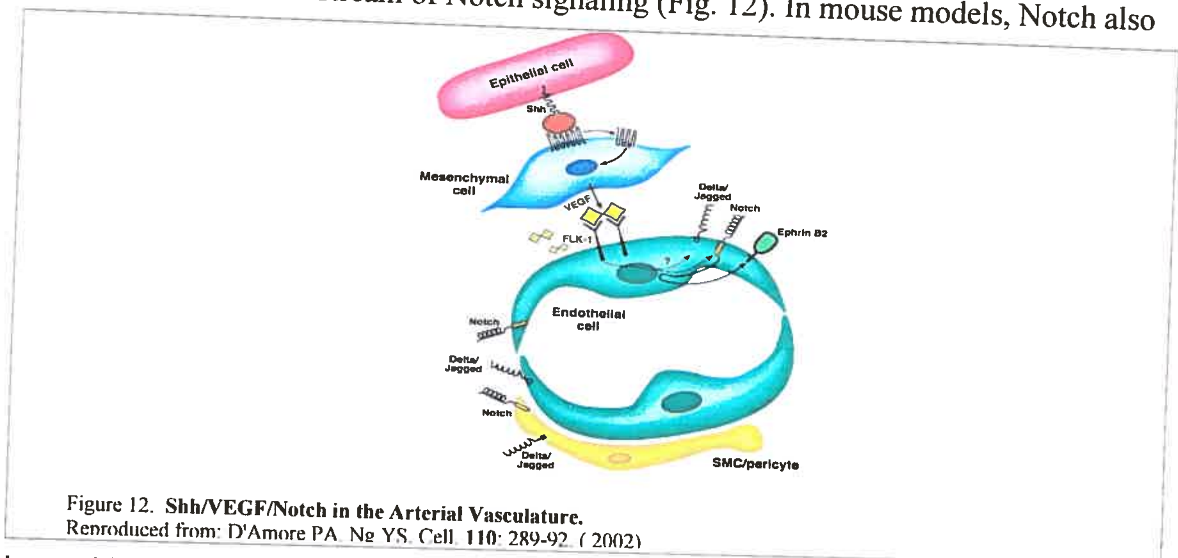

Figure 12. **Shh/VEGF/Notch in the Arterial Vasculature.**
Reproduced from: D'Amore PA, Ng YS. Cell. 110: 289-92. (2002)

is capable of regulating ephrin-B2 release in cultures as VEGF-A does (David J. Anderson, 2004). In human ECs, both Notch-1 and Dll4 are induced by VEGF-A (Liu et al., 2003). Hence, VEGF-Notch-ephrin-B2 cascade seems to exist in several species. These findings place Notch squarely in the midst of other known angiogenic regulators.

Other factors involved in angiogenesis

Platelet-derived growth factor (PDGF) and its receptor-b (PDGFR-b) are expressed by developing vSMCs/PCs and lack of its signaling leads, not only to pericyte loss but also to endothelial changes followed by capillary dilation (mycroaneurysm) and rupture (Lindahl et al., 1997). The EC changes associated with mycroaneurysm formation are likely secondary to the pericyte loss. In the mice lacking PDGF-b and PDGFR-b, vasculogenesis does not rely on the recruitment of vascular accessory cells, but the subsequent remodeling does depend on mesenchymal-endothelial cell interactions (Lindahl et al., 1997; Hellstrom et al., 1999). This phenotype is similar to that observed in the Notch-deficient mice (Krebs et al., 2000). It is, therefore, interesting to explore the

potential collaboration of Notch and PDGF signaling pathways in vascular smooth-muscle cell organization or stabilization.

TGF-β1 promotes vessel formation mainly by stimulating a generation of ECM and inducing differentiation of mesenchymal cells to pericytes. The TGF-β1-ALK5 pathway positively regulates vessel formation (Goumans et al., 2002).

Molecules involved in ECM remodeling and EC migration

While growth factors and their receptors play a key role in angiogenesis, cell-matrix interaction also regulates angiogenesis (Bazzoni et al., 1999; Hood and Cheresh, 2002; Dejana, 2004; DeClerck et al., 2004). The specialized ECM exits as a thin layer called basement membrane that provides supporting structure on which ECs grow. The major macromolecular components of matrix are type IV collagen, laminin, heparin sulfate proteoglycans, fibronectin, and entactin. They serve as a store of various growth factors and proenzymes influencing endothelial cell behavior, such as differentiation, proliferation, and migration, and are involved in vessel development (Brooke et al., 2003; DeClerck et al., 2004).

Dynamic remodeling of the ECM, including deposition, degradation, and re-deposition of new ECM components, implicates an important role during angiogenesis (Egeblad and Werb, 2002). *In vitro*, both laminin and collagen are deposited during endothelial tube formation (Madri, 2001). Consistent with these results, *in vivo* experiments showed that fibronectin particularly regulates vascular formation, as antibody inhibitors of fibronectin block angiogenesis (Kim et al., 2000). Local matrix degradation by matrix metalloproteinases (MMPs) is required for invasion of pre-existing vascular cells through the basement membrane and surrounding stroma, followed by vascular cell migration and proliferation. The proteolysis of collagen can influence EC tube formation

because inhibition of MMPs blocks this process (Madri, 2001). In addition, cleavage of collagen and fibrin has also been implicated as an important step during the invasive stage of angiogenesis (Seandel et al., 2001; Hiraoka et al., 1998). MMPs can directly regulate angiogenesis as MMP-2 was observed to be involved in angiogenesis using *in vitro* CAM (chicken chorioallantoic membrane) assay, and MMP-9 and MMP-14 null mice have impaired angiogenesis during embryonic development (Vu et al., 1998; Zhou et al., 2000b). Furthermore, tissue inhibitors of MMPs (TIMP-1), which regulate the MMP activity on the cell surface, inhibit angiogenesis in *in vivo* anti-tumor assay (Ikenaka et al., 2003).

The proteolytic degradation of collagen may expose cryptic adhesive sites that are crucial for EC migration and proliferation. Some cryptic sites (i.e., RGD) are recognized by members of the integrin family, which participates in EC migration by regulating detachment/attachment to the ECM and maintaining communication between the EC and its neighborhood. The integrin family consists of at least 25 distinct pairs combined by α and β chain (Hood and Cheresh, 2002). Not only do they mediate cellular adhesion to ECM proteins in intercellular spaces and basement membrane, but they also transduce intracellular signals that promote migration of ECs on the surrounding ECM (Aplin et al., 1998; Schwartz and Shattil, 2000). Of the wide spectrum of integrin subunit combinations, studies in experimental angiogenesis models and in mutant mice indicate that several integrins play key roles in regulating angiogenesis. In normal animals, neither $\alpha v\beta 3$ nor $\alpha 5\beta 1$ is expressed by quiescent endothelium but both are significantly upregulated in response to angiogenic growth factors during angiogenesis (Brooks et al., 1994a; Kim et al., 2000). Their expression is controlled by HoxD3 transcription factor that is expressed in ECs to regulate the angiogenic switch (Boudreau N, 1997, Zhong J, 2003). Once $\alpha v\beta 3$ and

$\alpha5\beta1$ are expressed, angiogenesis depends on both integrins, as antagonists of each can block angiogenesis *in vivo* (Brooks et al., 1994a; Kim et al., 2000). In mutant mice, embryonic deletion of $\alpha5\beta1$ induces defects in the organization of the emerging vasculature (Goh et al., 1997) and defects in the vessel formation by ECs *ex vivo* (Francis et al., 2002; Taverna and Hynes, 2001). Similar to $\alpha5\beta1$, embryo lacking $\alpha4\beta1$ leads to placenta and cardiovascular malformations (Yang et al., 1995). Newborns lacking αv die a few hours after birth with significant defects in brain development, including failure of blood vessel formation (Bader et al., 1998). Some integrins are implicated in angiogenesis by regulation of VEGF. While $\alpha v\beta5$ promote VEGF-mediated angigenesis, antagonists of $\alpha2\beta1$ and $\alpha1\beta1$ suppress VEGF-mediated angigenesis to regulate blood vessel formation (Friedlander et al., 1995; Senger et al., 1997; Jin and Varner, 2004).

Anti-angiogenic factors

Compared to the pro-angiogenic factors, less information is available on the role of angiogenesis inhibitors (either termed anti-angiogenic factors) in normal conditions. Thrombospondin-1 (TSP-1) has significant anti-angiogenic effects on EC proliferation, adhesion, and spreading (Iruela-Arispe et al., 1996). The potent anti-angiogenic agents endostatin and angiostatin are able to induce the apoptosis of ECs and to inhibit EC migration and proliferation (Shichiri and Hirata, 2001; Griscelli et al., 1998). Another group of angiogenic inhibitors are TIMPs (Moses, 1997). They can block the role of MMPs to prevent angiogenesis.

1.5.2. Pathological conditions

Neovascularization may, however, also contribute to the pathogenesis of several disorders, including non-neoplastic angiogenic dependent disease and neoplastic disease

(Folkman, 1995). The point at which a "normal" process differs from "pathological" angiogenesis is in the tightly regulated balance of pro- and anti-angiogenic signals (Bergers and Benjamin, 2003) (Fig.13). When the balance is disrupted, angiogenesis

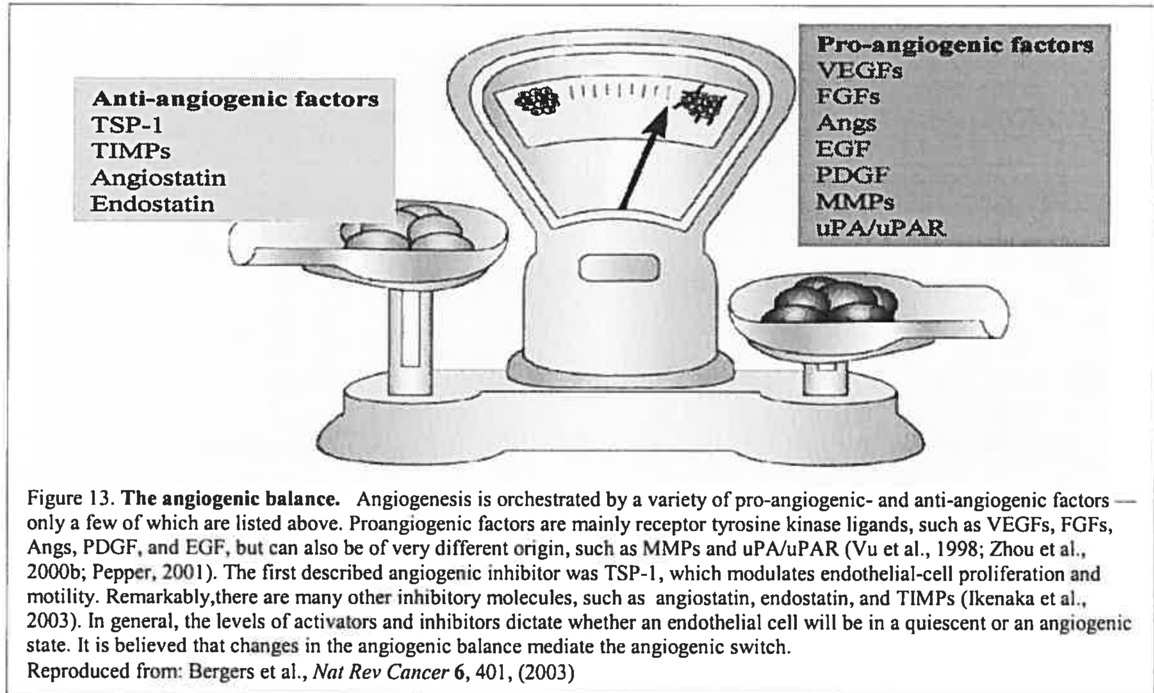

Figure 13. **The angiogenic balance.** Angiogenesis is orchestrated by a variety of pro-angiogenic- and anti-angiogenic factors — only a few of which are listed above. Proangiogenic factors are mainly receptor tyrosine kinase ligands, such as VEGFs, FGFs, Angs, PDGF, and EGF, but can also be of very different origin, such as MMPs and uPA/uPAR (Vu et al., 1998; Zhou et al., 2000b; Pepper, 2001). The first described angiogenic inhibitor was TSP-1, which modulates endothelial-cell proliferation and motility. Remarkably,there are many other inhibitory molecules, such as angiostatin, endostatin, and TIMPs (Ikenaka et al., 2003). In general, the levels of activators and inhibitors dictate whether an endothelial cell will be in a quiescent or an angiogenic state. It is believed that changes in the angiogenic balance mediate the angiogenic switch.
Reproduced from: Bergers et al., *Nat Rev Cancer* **6**, 401, (2003)

becomes pathologic and sustains the progression of many non-neoplastic and neoplastic diseases (Folkman, 1995).

1.5.2.1. Angiogenesis in non-neoplastic disease

In 1995, Folkman termed "angiogenesis disease" as underlying conditions dominated by the abnormal growth of microvessel-angiogenesis that is either excessive or deficient and is usually caused by dysregulation of angiogenic factors (Folkman, 1995). In some human diseases, such as retinal neovascularization, atherosclerotic plaques, and ischemic heart or limb, the neovascular formations are triggered by hypoxia in the diseased area accompanied by high levels of VEGF (Senger et al., 1997; Aiello et al., 1994). In an experimental model of rheumatoid arthritis, excessive production of angiogenic factors from infiltration macrophages ($M_{\varnothing s}$), immune cells, or inflammatory cells may mediate the

ingrowth of a vascular pannus in a joint. TNP-470, one inhibitor of angiogenesis, has been used for inhibiting vascular pannus in these animal models (Peacock et al., 1992). In other human diseases, such as psoriasis and gastric ulcers, the defective microvessels are associated with bFGF and TSP-1, respectively (Nickoloff et al., 1994; Esaki et al., 2002). Recent findings reveal even more angiogenic factors related to neovascularization in human diseases and animal models (Carmeliet, 2003; Sullivan and Bicknell, 2003).

1.5.2.2. Angiogenesis in neoplastic disease

Hemangioma

Some interesting human diseases are the vascular anomalies, which occur mainly in the skin and sometimes in other organs. In 1982, Mulliken and Golwacki classified vascular anomalies into "hemangiomas" (often called "infantile or juvenile hemangiomas") and "malformations" (sometimes called "carvernous hemangiomas") (Folkman, 1995; Vikkula et al., 1998; Mulliken and Glowacki, 1982). Hemangioma is restricted to a rapidly growing vascular tumor in infancy, but regresses during adolescence and adulthood. For clarity, hemangiomas are benign tumors that exhibit an early and rapid proliferation phase during the first year of life, characterized by endothelial and pericytic hyperplasia, followed by a slow but steady involution phase that may last for years (Mulliken and Glowacki, 1982; Vikkula et al., 1998). Vascular malformations are lesions comprised of dysplastic vessels lined by quiescent endothelium. These malformations are further classified into venous malformation, capillary malformation, arteriovenous malformation, lymphatic malformation, and a combined malformation. In contrast to infantile hemangiomas, vascular malformations are usually obvious at birth but never regress and sometimes further expend during adulthood (Vikkula et al., 1998). Later, in 1988, the term

hemangioma was used to describe a variety of vascular tumors or lesions that consist of ECs, pericytes, and as yet uncharacterized stromal cells (Grant et al., 1988).

Hemangioma is represented as a relatively pure form of angiogenesis (Folkman, 1995). So far, some molecules are found to be involved in these diseases, though studies on those are not intensive enough.

Transgenic mice expressing Middle T oncogene developed vascular lesions, and the mouse endothelioma cells expressing this gene induced hemangiomas in various organs and in a variety of species, such as mice, chicks, and others (Williams et al., 1989). Activated mutant Fps/Fes allele-expressing mice display widespread (lymphnodes, uterus, and others) multi-focal hemangiomas (Greer et al., 1994). In humans, the hemangioma basic scheme of histopathological classification has been refined by immunohistochemical characterization in each of the phases (Takahashi et al., 1994). During the proliferation stage, VEGF and bFGF angiogenic factors are increased, indicating that the upregulation of angiogenesis occurs. Collagen IV is present at this stage, suggesting that the collagen breakdown is necessary for the growing capillaries. E-selectin (adhesion molecule) and MCP-1 (monocyte chemoattactant protein) are upregulated, supporting the fact that the adhesion of ECs and the recruitment of monocytes are important for the proliferation stage (Vikkula et al., 1998; Takahashi et al., 1994). In contrast, during the involution stage, TIMP-1 is increased, suggesting regression of the vessels (Takahashi et al., 1994). Recent results showed that hemangiomas display high levels of immunostaining for GLUT-1 (glucose transporter-1) (North et al., 2000), a surface protein that is highly expressed in embryonic and fetal ECs but is lost in most adult tissues, except the placenta and central nervous system (Marchuk, 2001). The similarity in gene expression profile between hemangiomal and embolic placental vessel suggests the "placental origin" of hemangiomas

and explains their exclusively perinatal or congenital occurrence. In addition, the strong gender predilection of hemangioma toward female over male infants (3:1) suggests hormonal effects in hemangiogenesis (Marchuk, 2001).

Of the five types of vascular malformations, venous malformation (VM) is the most common anomaly, accounting for up to 50% of patients (Vikkula et al., 1998). VM has a bluish-purple color, is often raised, and can be emptied by compression. VMs are most often located in the skin and/or mucosal membranes, although visceral lesions may also occur; for example, VM of salivary gland and hepatic cavernous hemangioma. In order to know what VMs are and how they form, immunochemistry with E-slectin and α-actin was performed to identify two major cellular components of the vessel wall: ECs and SMCs (Vikkula et al., 1996; Kraling et al., 1996). This study revealed a deficient recruitment of smooth-muscle cells to the vessel wall, but no EC proliferation defects. Random mapping for two separate families with autosomal dominantly-inherited VMs located the defective gene to chromosome 9. This gene was identified as mutant Tie-2 (Vikkula et al., 1996; Boon et al., 1994), which is normally expressed in ECs and involved in angiogenesis (Suri et al., 1996). More molecules, for example, PDGF-B and TGF-β, may also be involved in this disease, as both molecules were found important for SMC recruitment and differentiation, respectively (Lindahl et al., 1997; Hirschi et al., 1998). In addition, ECM produced by ECs and SMCs may also play a role in VMs. Little is known about the molecular mechanisms of capillary malformation (Vikkula et al., 1998). TGF-b was found to be involved in arteriovenous malformation since two separate genes, HHT1 and HHT2 (hereditary hemorrhagic telangiectasia 1 and 2), involved in this disease are TGF-b binding proteins (McAllister et al., 1994; Vincent et al., 1995; Johnson et al., 1996). There are few molecular clues for human lymphatic malformations available, but LYVE-1 (lymphatic

vascular endothelial receptor), VEGF-C, and Flt-3 have been identified specifically for lymphatic malformation in animal models (Yancopoulos et al., 2000; Jain, 2003). It is possible that both may also be important for human lymphatic vessel malformation.

Most hemangiomas do not need treatment; however, approximately 10% of cases cause serious tissue damage, interfere with vital organ functions, or become life threatening (Folkman, 1995). Clinically, corticosteroid administration results in a dramatic regression of hemangiomas, up to approximately 30% within a week. Interferon alpha-2a has also been used to regress life-threatening tumors (Folkman, 1995).

Tumor angiogenesis

It is now well accepted that tumor growth and metastasis require vascularization to provide both nourishment and a route for tumor cell extravasation. When tumors are less than 1 mm^2, and rarely larger than 2 to 3 mm^2, they have their proper metabolic needs met by diffusion. Most tumors in humans persist *in situ* for months or years without neo-vascularization but then become vascularized when a subgroup of cells in the tumor "switches" to an angiogenic phenotype (Hanahan and Folkman, 1996; Bergers and Benjamin, 2003). Tumor progression is comprised of a series of stages: hyperplasia, dysplasia, adenoma, and carcinoma. The angiogenic switch can occur at different stages depending on the tumor type and the environment. The classical characteristics of tumor angiogenesis have been demonstrated in various animal models. One typical example is the K14-HPV16 mice, which express oncogenes E6 and E7, and develop squamous-cell carcinomas of skin and cervix (Coussens et al., 1999; Bergers et al., 1998; Elson et al., 2000a). In these mice, E6 and E7 alone are not sufficient to cause an "angiogenic switch," but further inactivation of p53 and RB is necessary to induce neovascularization, which occurs in the early stage and is a prerequisite for tumor formation (Bergers and Benjamin,

2003). The tumors finally develop through distinct stages of hyperplasia, angiogenic dysplasia, and, eventually, to highly vascularized invasive tumors that are similar to many human cancers. During tumor progression, tumor cells may, per se, overexpress some pro-angiogenic factors or reduce some anti-angiogenic factors, may mobilize some angiogenic factors from ECM or may recruit Møs, producing their own angiogenic factors. All these changes may disrupt the local equilibrium between pro- and anti-angiogenic factors (Fig. 13) to induce neovascularization (Bergers and Benjamin, 2003).

Some of the pro- and anti-angiogenic factors described earlier are involved in tumor angiogenesis, the most important of those being VEGF. VEGF-A, VEGF-C, and VEGF-D are all produced by human tumors (Ferrara, 2002). PIGF also has a unique role in cancer (Carmeliet et al., 2001). In several different tumor settings, angiopoietins are also increased in collaboration with VEGF (Yancopoulos et al., 2000). Both soluble extracellular domain of Ephrin class A (EphA2-Fc and EphA3-Fc) and class B (ephrinB2 and EphB4) inhibit tumor growth and angiogenesis (Brantley et al., 2002; Martiny-Baron et al., 2004). Very little information is available on the role of Notch signaling in tumor angiogenesis. In MMP-2-deficient mice, tumor angiogenesis and growth is reduced compared to normal mice (Itoh et al., 1998). MMP-9 has also been found to be a part of angiogenic switch in two typical transgenic models of tumor progression in skin and pancreatic islets (Coussens et al., 2000; Bergers et al., 2000). This proteinase makes VEGF available for interaction with its receptors so that the proteinase influences tumor angiogenesis (Bergers et al., 2000). In mouse models, integrins play a diverse role in tumor angiogenesis. Increased levels of integrins are observed to be associated with increased cell invasion and metastasis (Felding-Habermann et al., 2002; Ramos et al., 2002). Many studies have shown that $\alpha v\beta 3$ and $\alpha v\beta 5$ inhibitors block angiogenesis, tumor growth, and

tumor metastasis by inducing apoptosis in proliferating ECs (Brooks et al., 1994b; Cheresh and Stupack, 2002). These results are supported by the recent findings that loss of β3 or β5 does block angiogenesis induced by Del-1 (Zhong et al., 2003), though one study showed that loss of β3 or β5 promotes angiogenesis (Reynolds et al., 2002). Some anti-angiogenic factors are decreased in tumors while angiogenic factors are increased. Mice lacking TSP-1 develop larger and more vascularized breast tumors than the parental strain due to high levels of MMP-9 production (Rodriguez-Manzaneque et al., 2001). In breast cancer patients, overall vascular density correlates to poor cancer prognosis, and TSP-1 expression is inversely correlated with malignant progression of mammary and lung carcinomas, as well as melanomas (Weidner, 1998; Zabrenetzky et al., 1994; Weinstat-Saslow et al., 1994).

Tumor vessels are architecturally often immature, exhibiting tortuosity, blind ends, and increased permeability, resulting in spatial heterogeneity of blood supply both between and within individual tumors because of angiogenic switch (Vaupel et al., 1989; Bergers and Benjamin, 2003). Since the "angiogenic switch" is tightly related to tumor growth and metastasis, treatment of tumor is no longer restricted to merely kill tumor cells. Anti-angiogenic therapies and normalization of tumor vessels are already underway in clinical trial. TNP470, DC101 (VEGF receptor inhibitor), SU5416 (VEGF receptor inhibitor) or BB-94 (broadspectum MMP inhibitor) have been used to treat several tumors with either more realistic animal models or clinical trials (Yoshida et al., 1998; Tong et al., 2004; Heymach et al., 2004; Wielockx et al., 2001). Cyclooxygenase (COX2) inhibitors, that are anti-flammatory agents with anti-angiogenic activities that downregulate the expression of FGF and VEGF factors, have been used to prevent several tumors (Gasparini et al., 2003).

Evidences show that efficiency from two drugs is better than from one (Bergers and Benjamin, 2003). Avestin...

1.6. Macrophages

Angiogenesis in either physiological or pathological conditions needs to induce the formation of its own vasculature and this process is mainly controlled by released factors from local cells in the tissue microenvironment. Recent evidence suggests that Møs act as important stimuli in the microenvironment to regulate angiogenesis in both normal and diseased tissues since activated Møs can secrete a large number of pro- and anti-angiogenic factors (Table 1) (Crowther et al., 2001; Sunderkotter et al., 1994; Bingle et al., 2002; Leek and Harris, 2002).

Proangiogenic factors	Angiogenic inhibitors
VEGF/VP	TGF-β
PDGF	TSP-1
bFGF	IFNγ
HGF/SF	TIMPs
EGF	PAI-1
IGF-1	MECIF
TP(PD-ECGF)	MD-ECI
TNF-α	....
Angiotropin	
HAF	
Substance P	
IL-1β	
IL-4	
IL-6	
IL-8	
PGE2	
SPARC	
MMPs	
uPA/uPAR	
Plasmin	
......	

Table 1. **Macrophage-derived factors in angiogenesis.** IGF-1, produced by activited macrophages, involves in inflammatory angiogenesis; PD-ECGF, involves in both wound and tumor angiogenesis; PGE and SPARC involves in wound angiogenesis; MECIF inhibits thymidine incorporation in cultured HUEC *in vitro*; MDF-ECI, macrophage-derived endothelial cell inhibitor, secreted by adherent human macrophages.
Reproduced from: Sunderkotter et al., *J.Leukoc.Biol.* **55**, 410, (1994)

1.6.1. Macrophage development

The term "macrophage" was invented by Metchnikoff (Metchnikoff E 1893) to describe large mononuclear phagocytic cells able to take up microorganisms. Later,

mononuclear phagocytes were defined as a family including **committed hematopoietic precursors, their immediate progeny blood monocytes,** and **the cells in tissues** derived from transendothelial migration and maturation of monocytes to become professional phagocytes-**macrophages**. Similar to ECs, MØs are also derived from mesenchymal progenitors of the mesoderm (Lichanska and Hume, 2000; Herbomel et al., 1999). In vertebrates, MØs are the only other mature hematopoietic cells (except erythroid linage) present in yolk sac (Keller et al., 1999; Lichanska and Hume, 2000). The first MØs further infiltrate into the fetal liver, and in combination with the microenvironment of the liver initiate the establishment of definitive hematopoiesis. These MØs in the liver resemble the MØs of the hematopoietic islands in the adult bone marrow. With the onset of hematopoiesis in the liver, the number of phagocytes continues to increase to a point where they are one of the most abundant cell types in the embryo, amounting to as much as 10% to 15% of total cells in many organs (Lichanska and Hume, 2000). During these processes, MØs quickly become mature "fetal macrophages" expressing the surface maker F4/80, the macrophage scavenger receptor (MSR) and lysozyme, and the macrophage-specific transcription factor, P.U.1 (Morris et al., 1991). Herbomel et al. found that embryonic MØs are endowed with a proliferation capacity through a rapid differentiation pathway, which bypasses the monocytic series well-documented in the adult hematopoietic organs (Herbomel et al., 1999).

1.6.2. Heterogeneity of macrophages

In adults, MØs are derived from the blood monocytes that originate from bone marrow. These monocytes first enter tissues by adhering to the ECs via an interaction of cell surface molecules, for example, lymphocyte function-associated molecule (LFA-1) on monocytes and intercellular adhesion molecule 1 (ICAM-1) on ECs (Koshikawa et al.,

2000). Adhered monocytes pass through the endothelium of blood vessels into surrounding tissues. Finally, they undergo differentiation into tissue MØs and remain there as resident MØs (Auger, M. J., Roos, J. A. 1992). MØs in different tissues have distinct phenotypes induced by tissue-specific factors. For example, Langerhans cells, dendritic cells, alveolar MØs, peritoneal MØs, and Kupffer cells (KCs) are differently distributed in skin, lymph nodes, lung, peritenium, and liver, respectively. The resident tissue MØs release distinct tissue-specific combinations of 1 or more than 150 proteins in their secretory repertoire (Auger, M. J., Roos, J. A. 1992, Nathan et al., 1980). Further specialization and activation of MØs is prompted by local stimuli produced during pathological processes such as the presence of cytokines, adhesion molecule binding, or interaction with foreign/infectious agents. Recently, Paulnock et al. have demonstrated differential gene expressions during IFN-γ-dependent and -independent activation of MØs, thereby indicating the potential for a diversity of macrophage phenotypes (Paulnock et al., 2000). Even in the same organ, MØs exist in a heterogeneous manner. A rather good example is the liver, which possesses resident macrophages, termed as KCs. The KCs can be subfractionated into classes of different diameters, which show functional differences and different localization within the liver lobule (Hardonk MJ, 1989). It turns out that more KCs are distributed in the portal area than in the central part. Moreover, portal KCs are more active than those located in the central zone.

1.6.3. Macrophage functions

MØs can perform a multitude of functions such as phagocytosis, endocytosis, cytotoxicity, cell-cell interactions, and angiogenesis (Adams, 1992, Nathan, 1987; Crowther et al., 2001).

1.6.3.1. Scavengers

The behavior of Møs acting as scavengers in many conditions is well documented (Aderem and Underhill, 1999). Both embryonic and adult Møs can phagocytize apoptotic cells and bacteria (Herbomel et al., 1999; Willett et al., 1999; Lichanska and Hume, 2000). This phagocytosis function is accomplished by specific receptors. By far the biggest and the most diverse group of these receptors are the scavenger receptors (Franc et al., 1999). Møs are recruited to the sites of infection by either chemotactic molecules emanating from bacteria, or by cytokines released by Møs without the help of lymphocytes (Willett et al., 1999; Herbomel et al., 1999).

1.6.3.2. Cell-cell interactions

Møs are not only scavengers, they also secrete an impressive array of growth factors and cytokines in various situations, as well as various proteins capable of remodeling the ECM (Auger, W.E. 1992). Hence, they might be involved in organogenesis. Herbomel et al. found that, in the zebrafish yolksac, Møs in the circulation valley interact closely with erythroblasts, sometimes seemingly almost engulfing them, but then releasing them back into circulation (Herbomel et al., 1999). This intimate interaction resembles the nursing role of mammalian Møs towards immature erythroid cells (Crocker, P. R. and Milon, G 1992). Maybe this interaction between young Møs and pre-erythroblasts is important for the maturation of both cell types (Herbomel et al., 1999). However, the molecules involved in this interaction are unclear. Another example is that the KCs interact with the ECs in the liver, which will be discussed later.

1.6.3.3. Angiogenesis

Among their functions, the role of MØs in angiogenesis has recently received much more attention by researchers. They influence almost all phases of angiogenesis, particularly the inflammatory and the tumor angiogenesis (Sunderkotter et al., 1994, Crowther et al., 2001). Some studies have also shown their angiogenic role in the embryonic development (Herbomel et al., 1999, Kurz et al., 2004).

1.6.3.3.1. Activation of macrophages

MØs are set into different functional states by a process called activation (Sunderkotter et al., 1994). Certain MØs can be either tumoricidal or bactericidal, but not both at the same time (Rutherford et al., 1993). The activation process is dependent on a complex regulatory mechanism involving different stimuli. Unlike bacterial infection where MØ activation proceeds through sequential steps, there are no clear activation sequences of MØs in angiogenesis (Rutherford et al., 1993, Adams, D.O. 1992). However some studies have confirmed that MØs need first to be activated in order to exert their angiogenic activity (Steinman, 1988). It has been shown that the human and the murine monocyte did not promote neovascularization unless having been treated with some activators (Meyer et al., 1989; Koch et al., 1986). As described earlier, angiogenesis in healthy organisms is under tight control and the vessels in adults are quite stable. On the other hand, persistent neovascularization, is a characteristic feature of malignant tumors and chronic disease, such as rheumatoid arthritis. Cells able to induce neovascularization should be expected to remain inactive unless being activated. Many of the macrophage-derived angiogenic factors are indeed synthesized or released only by activated MØs (Sunderkotter et al., 1994). MØs can be activated by many factors. LPS is a known stimulus activating MØs but it is not a specific signal (Polverini et al., 1977; Koch et al.,

1986). The particular metabolic conditions, low-oxygen tensions (Knighton et al., 1983) or wound-like concentrations (Jensen et al., 1986) found in wounds could be more specific signals for MøS. MøS can also be activated by some activating cytokines, such as IFN-y, GM-CSF, PAF, or MCP-1 (Adams, 1992). These cytokines are secreted by a number of cells, including the activated ECs. Thus, an attractive model is that MøS are recruited and activated by cytokines released from ECs to the required sites, where MøS secrete angiogenic factors to feed back ECs.

1.6.3.3.2. The influence of macrophages on different phases of angiogenesis

The steps leading to the vessel formation have already been mentioned and MøS can influence almost all of these, from a basement membrane disruption to a direct role in the EC migration and proliferation, as well as an inhibitory role in ECs.

MøS are a critical source to secrete several degrading enzymes, particularly, uPA and MMPs, yielding angiogenic fragments of ECM, and to liberate ECM-bound growth factors (Klimetzek and Sorg, 1977; Nathan, 1987, Adams, 1992) (Table 1). These cause changes in the molecular or mechanical structure of the ECM (Sunderkotter et al., 1994). While these enzymes are capable of degrading almost all components of the ECM, MøS themselves also synthesize tissue inhibitors of the metallo- and serine protease (Wohlwend et al., 1987). The concomitant expression of proteases and its inhibitors may promote simultaneously the outgrowth of new vessels and the protection of neovascularized tissues from excessive proteolysis (Bacharach et al., 1992). MøS also secrete cytokines that influence the ECM (Table 1). Some of them play direct role in the modulation of angiogenesis. For example, TGF-β is one of the various cytokines derived from MøS (Ignotz and Massague, 1986) that has been proved to influence ECM *in vitro* and *in vivo* (Vogelmann et al., 2001), and neovascularization *in vivo* (Roberts et al., 1986). PDGF is

another cytokine secreted by Møs (Nathan and Sporn, 1991), that is expressed in pericytes and regulates ECs as well as vessels (Uutela et al., 2004; Nishishita and Lin, 2004). Besides the influence of Mø on ECM, some factors produced by Møs can directly induce the migration and the proliferation of ECs (Sunderkotter et al., 1994). In particular, two factors, human angiogenic factor (HAF) and angiotropin (Sunderkotter et al., 1994; Leek and Harris, 2002), produced by Møs, appear to have predominant chemotactic effects. Their migratory effects are sufficient for initial neovascularization since the migrating ECs can form sprouts without proliferating (Sholley et al., 1984). In addition, many factors such as VEGFs, bFGF, TGF-α, GM-CSF, IL-8, and others secreted by Møs (Table 1), induce mitosis in ECs (Sunderkotter et al., 1994). As already mentioned, VEGFs are the only specific endothelial factors, that critically stimulate the proliferation of ECs (Connolly et al., 1989) and yet modulate vasculogenesis and angiogenesis (Yancopoulos et al., 2000). These factors together with others secreted by Møs can also influence the serine and the metalloproteinases production from ECs, by which they locally modulate the capillary basement membrane (Flaumenhaft et al., 1992). Apart from producing angiogenic factors, Møs also secrete angiogenic inhibitors that modulate the equilibrium of vessel formation (Sunderkotter et al., 1994; Crowther et al., 2001; Bingle et al., 2002; Leek and Harris, 2002) (Table 1). The inhibition of neovascularization is necessary to restrict the extent of the new vascular network and to facilitate the differentiation of the capillary sprouts into functionally mature capillaries. TSP-1 (Kang et al., 2001) and IFN-α, γ (Robinson et al., 1985; Zhang et al., 2002a) are important factors released by Møs to inhibit the migration or the proliferation of ECs. Møs also produce some factors such as TGF-α and-β, IL-1, and IL-6 that have been shown to exert complex actions on ECs (inhibitory and also stimulatory effects) (Sunderkotter et al., 1994; Crowther et al., 2001). The contradictory

actions of these cytokines on angiogenesis are usually due to different experimental designs or to concentration-dependent effects.

1.6.3.3.3. Pathologically angiogenic activity of macrophages

The proangiogenic functions performed by Møs will be presented in three well-characterized and angiogenesis-dependent cases: healing wounds, malignant tumors, and hemangiomas.

Although a large body of literature exists on the cellular events occurring in wound healing, most data have been based on animal models or on cells from human wound fluids rather than on the monitoring of the development of human wound tissue per se. From these studies, wound healing has been described as a complex process involving three main phases: inflammation, proliferation, and remodelling. Møs are implicated in each phase. Already, by day 2 (inflammatory phase), they are the predominant leukocytes infiltrating the wound space. Then, about day 7 (proliferation phase), working together with neutrophils, they remove debris and recruit ECs as well as other cells that participate in the synthesis of a new matrix and in the development of vessels. Finally, persisting in the remodelling phase, they proteolytically modify the collagen matrix to improve the mechanical strength and adhesion in order to keep neovessel regressive (Crowther et al., 2001). Møs are also directly associated with wound angiogenesis. The injection of the Møs isolated from the wound fluid (called wound macrophages) into rat corneas triggers neovessel formation (Thakral et al., 1979; Greenburg and Hunt, 1978; Polverini et al., 1977). Furthermore, some experiments indicate that only the wound-associated Møs, but not the unstimulated monocytes or Møs, are capable of inducing angiogenesis (Koch et al., 1986). The corneal angiogenesis is accelerated only when one employs autologous wound Møs (Clark et al., 1976). Similarly, adding Møs stemming from a 3-week-old wound to a

rabbit-ear chamber-model of wounding produces a significant acceleration of angiogenesis (Thakral et al., 1979). Therefore, these studies suggest that the activation of Møs by wound-specific microenvironmental factors, such as hypoxia and lactate, turns out to be essential for their proangiogenic activity in such tissues (Elson et al., 2000b).

Møs are found in a large number of malignant human and murine tumors, where they are often termed tumor-associated macrophages (TAMs). The TAMs are recruited mainly from the circulating monocyte pool (Eccles and Alexander, 1974; Wood and Gollahon, 1977) rather than being co-opted from the resident tissue Møs (Adams and Hamilton, 1984). There exists a great heterogeneity in the number of Møs in individual tumors of a given type, and they can amount to 80% of the total cell mass of certain tumors such as breast carcinomas (Lewis et al., 1995). TAMs are attracted into and/or immobilized in avascular (Leek et al., 1996) and necrotic hypoxic (Leek et al., 1999) areas of vascularized human tumors by the direct immobilizing effect of hypoxia on Møs and by specific factors produced by hypoxic tumor cells (O'Sullivan and Lewis, 1994), The TAMs in such sites are directly stimulated by hypoxia to cooperate with tumor cells so as to promote revascularization (Lewis et al., 2000). Besides the direct effects, hypoxia and other factors might also exert an indirect effect on the proangiogenic activity of TAMs. In addition, the release of cytokines/factors from tumor cells might be modulated, and they in turn recruit/stimulate the TAMs in diseased areas. Tumor cells are known to produce large amounts of VEGFs and bFGFs (Leek et al., 2000; Stupack et al., 1999), which act as potential chemoattractants for Møs. It seems also possible that the activation of TAMs during the extravasation across the tumor endothelium right into the tumor (Takahashi et al., 1997), and/or during the exposure to cytokines/signals within the tumor milieu itself, might modulate the TAMs susceptibility to some factors. It was originally thought that the

main function of the TAMs was to exert direct cytotoxic effects on tumor cells, to phagocytose apoptotic/necrotic cell debris, and to present tumor-associated antigens (when present) to T cells. However, recent data lead one to believe that TAMs can also promote tumor growth and metastasis, as well as tumor angiogenesis by accordingly regulating angiogenic factors (Bingle et al., 2002; Leek and Harris, 2002). (Fig. 14.) Similar to the

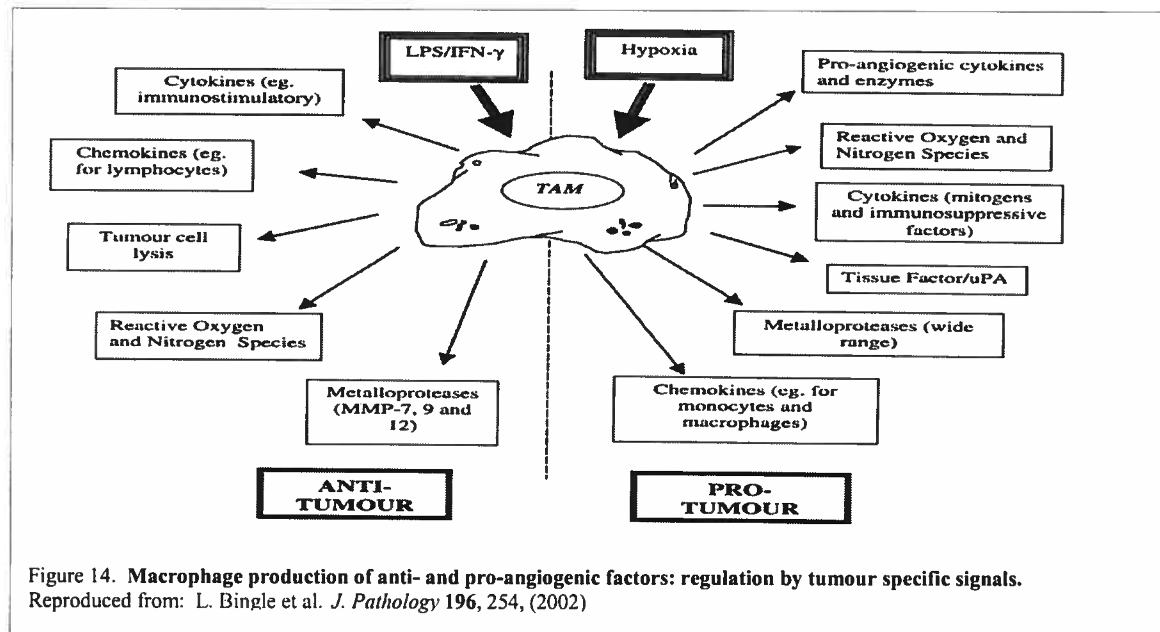

Figure 14. **Macrophage production of anti- and pro-angiogenic factors: regulation by tumour specific signals.** Reproduced from: L. Bingle et al. *J. Pathology* **196**, 254, (2002)

activated Møs described above, the TAMs are the important source of angiogenic factors [such as VEGF (Leek et al., 2000), TNF-α (Pusztai et al., 1994), IL-8 (Fujimoto et al., 2000), and bFGF (Stupack et al., 1999)] and proteases (such as MMPs and uPA). Recent studies show that VEGFs secreted by Møs essentially supports tumor angiogenesis (Barbera-Guillem et al., 2002). Indeed, as early as 1984, Polverini and Leibovich (Polverini and Leibovich, 1984) showed that the TAMs isolated from rodent tumors are capable of stimulating the proliferation of ECs *in vitro* and inducing corneal angiogenesis in a rodent model. So far a direct correlation has been documented between the presence of TAMs and vascularity in breast cancer specimens and mortality (Leek and Harris, 2002; Bingle et al., 2002). This strongly indicates that the presence of TAMs facilitates the

angiogenic process of tumors, thereby promoting metastasis. Recently interesting results have announced that an anti-angiogenic agent (linomide), that selectively inhibited the MØ secretion of TNF-α, blocked stimulatory effects of TAMs on tumor angiogenesis of rat prostate cancer without eliminating their anti-angiogenic effects. This provides a strong evidence that the TAMs play a role in response of the prostate-cancer to anti-angiogenic agents (Joseph and Isaacs, 1998).

The presence of MØs is also linked to the "proliferation hemangiomas" which presents a powerful model to study *in vivo* angiogenesis (Atalay et al., 2003). The CC chemokine MCP-1 is known to be responsible for recruiting the MØs to the sites of infection or inflammation (Graves et al., 1989). As described earlier, MCP-1 is increased in hemangiomas, which attracts MØs (Isik et al., 1996). In this way, MCP-1 could be viewed as a major accessory factor facilitating angiogenesis. A direct role of MCP-1 in angiogenesis has been evidently shown (Salcedo et al., 2000). MCP-1 induces the chemotaxis of human ECs and the formation of blood vessels *in vivo*. The chemotactic response was further inhibited by a monoclonal antibody to the MCP-1. Interestingly, studies with berry powders, potentially suppressing VEGF expression and *in vitro* angiogenesis, show that they significantly inhibit the inducible expression of MCP-1 in ECs. It is noteworthy that endothelioma cells, pre-treated with berry powders, showed a diminished ability to form hemangioma. A decreased infiltration of MØs in the hemangioma of the treated mice was remarkably observed by histological analysis compared to controls (Atalay et al., 2003). These results provide the first evidence that the MØ inhibition plays a role in the anti-angiogenic property of berries in hemangioma.

1.7. Liver and angiogenesis

The liver is the largest gland in the body and it performs an astonishingly large number of tasks that impact all the body systems. While the liver is the prominent site of hematopoiesis during the embryological stage, it shares some fundamental roles during the adult phase, mainly including vascular functions. Present in several pathological situations, angiogenesis occurs in the liver in different contexts such as hemangioma, carcinoma, and chronic diseases.

1.7.1. Liver development and hematopoiesis

The study of embryological liver development which was initiated by von Baer (1828,1833) has been the subject of numerous researches. The results obtained have served as the basic concept for the "classic" of hepatic histogenesis. This concept can be briefly summarized as follows. The liver is formed from two distinct primordia, the hepatic diverticulum of endoderm origin and the vascular network of mesoderm origin. As shown in figure 15, in mouse, at the embryonic day 8.5 to 9.0 (E8.5 to 9.0), the liver endodermal epithelial cells receive stimuli from the mesodermal cells that cause changes in the gene

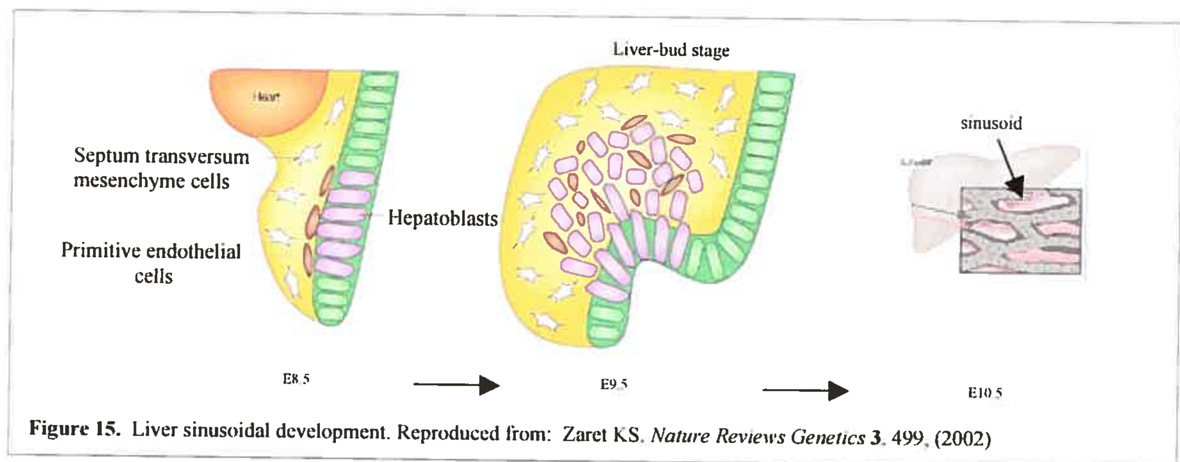

Figure 15. Liver sinusoidal development. Reproduced from: Zaret KS. *Nature Reviews Genetics* **3**, 499, (2002)

expression and the cell division of endodermal cells. Newly specified hepatic cells begin to multiply as a thickening of the ventral endodermal epithelium. At the day E9.5, the

hepatic cells continue to multiply as a bud of cells that proliferate and migrate into the surrounding septum transversum mesenchyme. At the same time, nascent and irregular sinusoids are formed with mostly primitive vascular structures lined by ECs (marked by PECAM-1). At this stage, hematopoietic cells (marked by VEGFR2) could only be occasionally observed to invade these sinusoids. At the day E10, the hematopoietic cells could be clearly detected in the sinusoids. Finally, the hepatic cells form a new domain of condensed tissue mass that becomes vascularized (Zaret, 2002; Matsumoto et al., 2001).

While bone marrow remains the major site of hematopoiesis throughout the entire adult life, during the fetal phase, the liver is the prominent site of hematopoiesis shifted from the YS and SP/AGM that function at pre-liver stages of hematopoiesis (Garcia-Porrero et al., 1995; Dzierzak et al., 1998). Up to 60% of the fetal liver mass consists of blood cells. The hematopoietic activity is determined by the number of islands and their respective volumes. In humans, hematopoiesis attains its maximal activity toward the 6th to 7th month in the embryo and then regresses rapidly. The fetal liver at birth only contains a few disseminated islands, which disappear with the passing first weeks. The question of the origin of liver hematopoietic stem cells has stimulated much research. Jolly and Saragea (1922) admit the possibility of two different stem cell origins: either these cells are the descendants of cells having migrated from the yolk sac or they directly derive from the sinus cells. The majority of authors (Bloom, 1939; Gilmour, 1941; Maximow, 1909,1924) consider the mesenchymal cells of the hepatic parenchyma as the hemocytoblast source. During the prenatal period, a large number of metabolic enzymes are induced within the hepatocytes as the hematopoietic cells migrate elsewhere and the liver prepares to control the metabolite and serum protein levels in the blood, to store glycogen, and finally to detoxify blood with thanks to its special structure.

1.7.2 Liver structure

Understanding the function and the dysfunction of the liver, more than most other organs, depends on understanding its structure. The liver lies in the abdominal cavity, in contact with the diaphragm. Its mass is divided into several lobes, the number and size of which vary among species. The major aspects of the hepatic structure include: 1) the hepatic vascular system and 2) three dimensional arrangements of the liver cells. Of course, the biliary tree is also important, but it will not be considered here. The liver receives approximately 30% of the resting cardiac output and is therefore a very vascularized organ. Unlike in any other organ, blood enters the liver via two separate ways (Fig. 16a). Of great importance is the fact that a majority of the liver's blood supply is venous blood! Roughly speaking, 75% of the blood entering the liver is venous blood coming from the gastrointestinal tract via the portal vein. The remaining 25% of the blood supply to the liver is arterial blood from the systemic circulation via the hepatic artery. The liver is covered with a connective tissue capsule that branches and extends throughout the substance of the liver as septae. This connective tissue divides the parenchyma of the liver into very small units called lobules. The hepatic lobule is the structural unit of the liver. It

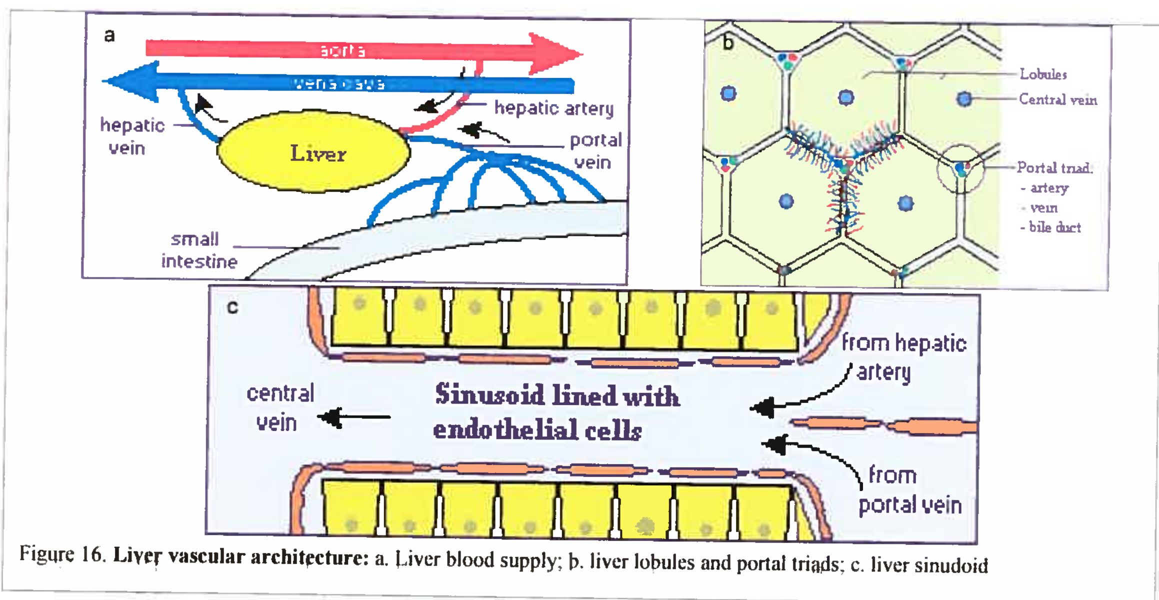

Figure 16. **Liver vascular architecture:** a. Liver blood supply; b. liver lobules and portal triads; c. liver sinudoid

consists of a roughly hexagonal arrangement of plates of hepatocytes radiating outwards from a central vein at the geometrical center. At the vertices of each lobule are regularly distributed portal triads, containing a bile duct and a terminal branch of the hepatic artery and the portal vein (Fig. 16b).

After ramification, terminal branches of the hepatic portal vein and of the hepatic artery empty together as they enter sinusoids, leading to a mixed arterial-venous blood perfusion of the liver (McCuskey, R.S. 1988, Wisse et al., 1985; McCuskey and Reilly, 1993) (Fig. 16c). The sinusoids are distensible vascular channels lined with highly fenestrated ECs as well as other cells (described later), and bounded circumferentially by hepatocytes. As blood flows through the sinusoids, a considerable amount of plasma is filtered into the space between the endothelium and hepatocytes (the "space of Disse"), providing a major fraction of the body's lymph. Finally, blood flows through the sinusoids and empties into the central vein of each lobule that empties to the hepatic vein. The sinusoids are the principle sites for transvascular exchange between blood and hepatocytes. In a normal situation, around 10-15% of the total body's blood volume resides in the liver, with roughly 60% of that in the sinusoids. The structure of the sinusoids is by all means unique. In all mammalian livers, it is composed of 4 different cells: ECs, KCs, fat-storing cells (also termed Ito cells), and pit cells (Balabaud, C. 1988) (Wisse et al., 1996; McCuskey and Reilly, 1993). They form a functional unit together with parenchymal cells. ECs, fat-storing cells, and parenchymal cells are all sessile cells. In contrast, KCs and pit cells seem to be mobile cells, that are likely recruited from extrahepatic sources (bone marrow), and adhering to the endothelial lining. Each cell type takes a distinct position in the liver and has its own specific morphology and functions, and no transitional stages

exist between the cells. Understanding the function of the liver sinusoidal cells and KCs is critical for appreciating the liver gland as a whole.

1.7.3. Functions of liver sinusoidal lining cells (LSECs and KCs)

The LSECs are the most critical cells for the liver sinusoidal lining. They form a fenestrated lining containing open fenestrations of 0.17 μm in diameter that occupy 6-8% of the surface. No intact basal lamina is present under these cells. They filter the fluids exchanged between the sinusoids and the space of Disse through fenestrae (Wisse et al., 1996; Wisse et al., 1985). The LSECs are endowed with a high capacity to phagocytose large particles up to 200 nm in diameter (Steffan et al., 1986). Receptor-mediated endocytosis of the LSECs occurs with a very high efficiency. Some receptors such as mannose and scavenger receptors enable the LSECs to clear rapidly specific substances from blood (Wisse et al., 1996; Nedredal et al., 2003). They also influence the function of Ito cells that contain vitamin A and synthesize and secrete ECMs. For instance, the scavenger receptor of ECs takes up N-teminal propeptide of collagen I. This makes a cooperation between Ito cells producing ECM and ECs clearing ECM to balance the ECM components in the liver. They can produce some factors, for example, PGE2, IL-6, and INF-y. They are the only cells sharing antigens with a peripheral blood Mø subset which has been shown to be capable of presenting soluble antigens (Nagura et al., 1986). Recent studies have found that LSECs act as presenting cells that present antigens to CD4+ and/or CD8+ T cells and thereby contribute to the immune tolerance of the liver (Knolle and Limmer, 2001; Limmer and Knolle, 2001; Knolle and Limmer, 2003). More interesting data revealed that liver ECs can influence hepatic parenchymal cells so as to promote the liver organogenesis (Matsumoto et al., 2001). Notably, LSECs are peculiar cells with

important metabolic products, taking part in the local cytokine cross-talk with other cells. However, factors able to influence KCs are still remained unclear.

KCs are the hepatic Møs that reside in the lumen of the hepatic sinusoid, first identified in the liver by von Kupffer (Kupffer 1876, Carr, 1977). Experimental studies have shown that they locate in the sinusoids by a couple of physical positions like floating in the lumen of sinusoids, touching the pad and/or body of the LSECs (MacPhee et al., 1995). They are in close physical contact with the sinusoidal ECs but their exact role remains unclear. They mainly accumulate in periportal areas to phagocytose and eliminate particulate antigens or pathogens entering the liver along with the portal-venous blood (MacPhee et al., 1992; MacPhee et al., 1995; Knolle and Gerken, 2000). In physical situation, KCs migrate along the liver sinusoids acting as a "street sweeper", removing material from the luminal surface of the sinusoids (MacPhee et al., 1992). The migrating KCs are also a major cause of flow intermittence in the liver sinusoids to promote contact between passaging leukocytes and the LSECs (MacPhee et al., 1995; Limmer and Knolle, 2001). So far, three important functions of KCs have been recognized: clearance of endotoxins from the circulation, production and release of soluble mediators such as cytokines, and presentation of antigens. KCs can be activated by some factors, specifically by endotoxins (Wisse et al., 1996). As a result of their activation, KCs secrete important molecules such as TNF-α, IL-6, IL-1, and IL-10 (Knolle et al., 1995; Wisse et al., 1996; Knolle and Gerken, 2000). It has been well documented that these released factors mainly become beneficial for the host defence to kill tumor cells but also, on the contrary, can cause damage of hepatocytes. The latter constitutes a crucial factor in liver transplantation. The IL-10 released from KCs could suppress CD54 and CD106 on the LSECs to decrease the leukocyte adhesion to the LSECs (Knolle and Gerken, 2000). It could also

downregulate the IL-1 and IL-6 production by LSECs to further influence Ito cells (Knolle et al., 1995). These results support a clear molecular interaction between the KCs and LSECs in the sinusoids (Limmer and Knolle, 2001). Since the activated Møs mediate angiogenesis, questions arise: whether and how the activated KCs influence the function of their adhered LSECs? Do the activated KCs mediate the formation of the liver vessels?

1.7.4 Hepatic Angiogenesis

1.7.4.1. Hepatic hemangiomas

In humans, hepatic hemangioma is the most common benign neoplasm of the liver. Its incidence is estimated to be approximately 20% in the general population (Semelka and Sofka, 1997). The lesions seem to be stable and to be fatal only in 2.5% of large hemangiomas (Zafrani, 1989), most later than 50 years old. However, large hepatic hemangiomas have a mortality rate of 30 to 50 percent (Folkman, 1995).

Hepatic hemangioma, also called cavernous hemangioma, belongs to "endothelial tumors of the liver" that also include rare tumors such as infantile hepatic hemangioendothelioma, angiosarcoma, and epithelial hemangioendothelioma (Hobbs, 1990). In general, cavernous hemangiomas are pathologically large, exhibiting well defined areas of blood-filled spaces lined with a single layer of endothelium separated by fibrous septa. Large hepatic hemangiomas can host hemorrhage, thrombus, calcification, or fibrosis (Semelka and Sofka, 1997; Stringer, 2000). In fact, infantile hepatic hemangioendotheliomas and cavernous hemangiomas are similar lesions but they occur in different age group and have the different histology. As its name indicates, infantile hepatic hemangioendothelioma occurs in infancy. It is highly vascular and is either solitary or multicentric. The vascular lesions contain multiple arterio-venous fistulas. Patients with

multicentric lesions present with hepatomegaly and a high-output congestive heart failure (Hobbs, 1990; Burrows et al., 2001). As many as 87% of these patients may also have cutaneous hemangiomas (Hobbs, 1990). Cavernous hemangioma occurs in adults. Small tumors may be observed with the same prevalence in males and females. By contrast, there exists a general agreement that large hemangiomas are more often symptomatic in women and might be enlarge during pregnancy (Zafrani, 1989; Pepper, 2001; Kaido and Imamura, 2003). Occasional reports of large hepatic hemangiomas in patients taking oral contraceptives (OC) or oestrogens have been published (Zafrani, 1989). Hence, one has been led to suppose that adult hepatic hemangiomas are perhaps secondary to an estrogen effect. In addition, adult hemangiomas are associated with hepatic focal nodular hyperplasia of the liver which is one kind of hepatic tumor. Studies found that six out of 26 hepatic hemangioma patients (23%) were affected with the later benign hepatic tumors, and these six patients were women who had previously used OC. These results suggest that the link between hemangiomas and hepatic tumors is probably not fortuitous, and that OCs might facilitate its clinical recognition by an effect on the growth of the two tumors (Zafrani, 1989).

As far as mice are concerned, hemangiomas have been reported to develop spontaneously, but less frequently in the liver than they do tumors in hepatocellular origin (Booth and Sundberg, 1995). However, in some experimental studies of carcinogens involving several different background mice, high hepatic hemangiomas have been observed (Bannasch, 1983; Sato et al., 1984). For example, BALB/c mice treated with 1,2-Dimethylhydrazine dihydrochloride (DMH) produced 100% incidence of hepatic vascular tumors (Sato et al., 1984). Moreover, some genes were recently identified to be involved in liver vascular defects. von Hippel Lindau (VHL) is a tumor suppressor expressed in most

tissues and cell types (Kessler et al., 1995; Richards et al., 1996). The conditional targeted disruption of this gene in the liver led to severe hepatic cavernous hemangiomas of the liver (Haase et al., 2001). TSC2 is another tumor suppressor and its mutations in humans cause tuberous sclerosis (TSC), a disease characterized by the development of hamartomas in various organs. In the mouse model with TSC2 heterozygous mutation (TSC2+/-), hepatic hemangiomas were developed in 80% of the TSC2+/- mice (Kobayashi et al., 1999), suggesting that the TSC2 is involved in hepatic hamangiomas in the mouse.

1.7.4.2. Hepatocellular carcinoma

In human beings, hepatocellular carcinoma (HCC) is one of the most common malignant tumor in the world, accounting for an estimated 1 million deaths annually. Over 80% of HCC cases worldwide is developed through liver cirrhosis. Major risk factors for HCC include chronic infection with hepatitis B virus (HBV) and hepatitis C virus (HCV), alcohol abuse, and a repeated exposure to aflatoxin B1 (Kim et al., 2002). For experimental carcinogenesis studies, HCC can be induced by some carcinogens in animals (Bannasch, 1983; Hara et al., 2000). Sakamoto et al. divided the development stage of HCC into ordinary adenomatous hyperplasia (OAH), atypical adenomatous hyperplasia (AAH), and well-differentiated HCC depending on the cellular morphology in nodule lesions (Sakamoto et al., 1991).

HCC is generally considered as a typical hypervascular tumor (Nakashima et al., 1999; Yamaguchi et al., 1998). With a chronic injury, portal fibroblasts and Ito cells can be transformed into proliferative, fibrogenic, and contractile myofibroblasts by the TGF-β1 and PDGF released from hepatocytes and KCs in injured livers. As a result, the stimulated fibroblasts and Ito cells may produce growth factors, cytokines, and proteinases and finally remodel ECMs (Friedman, 2000). This fundamental change in ECM compositions affects

the functions of all the liver cells such as hepatocytes, LSECs, KCs, and Ito cells. The abnormal functions of these cells might impair the normal liver's blood-supply system including a degeneration of the portal veins and also increasing artery-like vessels (Matsui et al., 1991; Ueda et al., 1992; Chia et al., 1995). The impairment of the blood supply may induce hypoxia (Yamaguchi et al., 1998). Hypoxia can also be induced by an increasing tumor mass per se from OAH, AAH, and early HCC (Kim et al., 2002). Hence, local hypoxia may be a major stimulus to eventually induce angiogenesis in cirrhotic livers. There exist many reports of VEGF expression and hypervascularity that have been detected in the whole progress of all stages of HCC, although the expression level of VEGF varies during the different stages of hepatocarcinogenesis (Kim et al., 2002). Besides VEGF, other factors such as TGF, PDGF, and IGF do play an important role in this angiogenic progress. On the other hand, angiogenic inhibitor-human macrophage metalloelastase (HME), which partakes in angiostatin generation, is now known to be significantly associated with hypervascular tumors (Kaido and Imamura, 2003). Angiogenesis in the HCC probably depends on the balance between HME and VEGF gene expression.

1.7.4.3. Hepatic angiogenesis in liver regeneration and some chronic liver diseases

Partial hepatectomy (PH) is currently being considered as an appropriate model to investigate physiological liver angiogenesis (Michalopoulos and DeFrances, 1997; Wack et al., 2001; Drixler et al., 2002). Following the PH in rodents, the proliferation of hepatocytes starts with a maximum activity in periportal areas (Gerlach et al., 1997) and meanwhile the reconstruction of sinusoids by ECs begins from these areas (Taniguchi et al., 2001). The expression of several proangiogenic growth-factors and of their receptors in

ECs has been recently characterized (Ross et al., 2001). Most of them are expressed at very low level in the resting liver ECs but are significantly increased and are tyrosine-phosphorylated during the regeneration of the liver (Medina et al., 2004). In rat, the VEGF family is upregulated in periportal hepatocytes shortly after PH (Taniguchi et al., 2001). Furthermore, the role of VEGF during liver regeneration was found to be beyond of the simple stimulation of EC cell proliferation: VEGFR1 primes LSECs to produce a series of factors that can protect parenchymal cells from injury and initiate regeneration (LeCouter et al., 2003). Both Ang-1 and Ang-2 are increased later than the VEGF up-regulation (Sato et al., 2001). PDGF and its receptors are also activated during the liver regeneration and this activation plays a potential role in the maturation of the newly formed sinusoids (Ross et al., 2001). Both FGFR and HGF/c-met particularly affect the hepatocyte regeneration rather than the microvascular remodeling (Hioki et al., 1996; Ross et al., 2001). In mouse, some proangiogenic modifiers such as MMPs, TIMPs, and uPA are also required for the EC migration and for the angiogenesis during the later stage of PH (Knittel et al., 2000; Nomura et al., 2002). Hepatic angiogenesis occurs on the top of that in some liver chronic diseases such as chronic inflammatory liver injuries, chronic viral hepatitis, cirrhosis, autoimmune liver diseases, and finally alcoholic liver diseases (Medina et al., 2004).

1.8. Female reproductive system and angiogenesis

The female reproductive organs mainly consist of ovary, uterus, and placenta. These are some of the few adult tissues that exhibit regular intervals of rapid growth. These are also highly vascularized organs and are subjected to high rates of blood flow. Certain phases of reproduction, such as the repair of menstruating uterus and the development of placenta, depend on physiological angiogenesis. The dysfunction of endogenous angiogenic stimulators and inhibitors may underlie several female

reproductive disorders, such as prolonged menstrual bleeding and infertility (Folkman, 1995). To learn more about the mechanisms on bleeding and infertility, understanding the structure of uterus is necessary.

1.8.1 Structure and function of uterus

In all mammals, the uterus develops as a specialization of the paramesonephric or müllerian ducts, which gives rise to two main parts: the corpus (two uterine horns in mouse) and the cervix (Mossman HA, 1987). As the corpus is a pertinent site occurring angiogenesis, it will be mainly described here. The mature uterine corpus wall comprises two functional compartments, the endometrium and the myometrium. The endometrium, which consists of epithelial cells, is the inner layer mucosal lining of the uterus. An important feature of the endometrium is the presence of tubular glands, which dip down from the surface into the underlying lamina propria of the connective tissue. It undergoes important cyclic changes (proliferative and secretion phases) during the menstrual cycle in response to ovarian estrogen and progesterone (Gray et al., 2001). The blood supply in the endometrium is very important in regard to menstruation and pregnancy. The growth of the endometrial vasculature begins during the proliferative phase and then continues throughout the secretory phase of the menstrual cycle in preparation for the implantation. Meanwhile, the endometrium becomes thick and finally sheds to form menstruation, if not implanted (Torry and Rongish, 1992; Zygmunt et al., 2003). Cyclic formation of new blood vessels in the functional endometrium occurs to compensate the lost vessels. This is a monthly cycle in human. The myometrium is a very thick layer of variably oriented smooth muscle cells and blood vessels. It is remarkable for its capacity to undergo a great expansion in order to accommodate the growing fetus during pregnancy.

1.8.2 Uterus angiogenesis

Since endometrium exhibits a rapid cyclical growth and shedding throughout the reproductive life of the female, it provides a valuable model for the study of normal physiological angiogenesis. Three different timing of angiogenesis have been deduced during the menstrual cycle in humans (Torry and Rongish, 1992). The first, which commences during menstruation, is the repair of the vascular bed. The second must occur in concert with the rapid growth of the ECs during the proliferative phase of the cycle. The third phase in which angiogenesis occurs is the arteriole growth during the secretory phase (Rogers and Gargett, 1998). Angiogenesis also manifests itself at the time of embryo implantation and in the placenta after pregnancy. Now the key question is how angiogenesis occurs in the endometrium?

1.8.2.1. Macrophages and uterus

It is interesting to know the relationship between Møs and endometrial angiogenesis because Møs are abundant in the mesenchymal and connective tissue-stroma of the cycling and pregnant uterus. The human endometrium contains a significant proportion of leukocytes (8-35% of all cells). T-cells and Møs are commonly present, although B-cells are absent. Approximately 20% of these are Møs. The absolute numbers and proportions vary during both the menstrual cycle and pregnancy. Throughout pregnancy, the number of Møs increases up to 10-15% of the total cells. In contrast to human, less T-cells exist in the rodent while Møs are the most common resident immune cells widely distributed in the uterus (Mackler et al., 2000). They may account for nearly 10-15% of cells in the virgin uterus and raise up to 22% of cells in the pregnant uterus (Hunt et al., 1985). The increased number of uterine Møs during pregnancy is confirmed by cell counting in the tissue sections. They are distributed throughout the pregnant

endometrium, as well as in stroma and in connective tissues belonging to the myometrium (Mackler et al., 2000; Hunt et al., 2000). In addition, they can traffic between endometrium and myometrium, and these dynamically disseminated Møs contribute to the onset of labor (Mackler et al., 2000). Recent studies show that the uterine Møs can be activated and regulated by hormones. They display an enhanced ability to phagocytose, and to produce a wide number of growth factors, and lastly to synthesize an impressive number of proteases (Hunt et al., 2000). Since Møs are known sources of a number of pro-and anti-angiogenic factors (Sunderkotter et al., 1994), they seem to play a role in controlling endometrial angiogenesis even if detailed studies are still scarce.

1.8.2.2. Factors involved in uterus angiogenesis

As the overall control of endometrial growth and regression is primarily regulated by the circulating levels of estrogen and progestrogen, the role of these steroids in endometrial angiogenesis is worthwhile to be examined. Some studies indicated that estrogen/progestrogen per se do not regulate the endometrial angiogenesis (Goodger and Rogers, 1994) due to highly varying EC proliferation data throughout the cycle. These results are supported by a failure in detection of the expression of estrogen and progestrogen in endometrial ECs (Rogers and Gargett, 1998). However, these studies are contradicted by the following results. Progestrogen is expressed in endometrial ECs from the fifth to ninth week of pregnancy in human (Wang et al., 1992). In mouse models, progestrogen has been shown to directly affect sponge angiogenesis assay as well as endometrial angiogenesis stimulated by an intrauterine levonorgestrel exposure (Hague et al., 2002). In addition, estrogen can dependently induce erythropoietin production and is further implicated in uterine angiogenesis (Yasuda et al., 1998).

Like other tissues, VEGFs and bFGFs appear to be major angiogenic factors in the female reproductive organs. During the menstrual cycle, VEGF and bFGF were shown to be increased three-fold in the secretory phase and to rise toward menstruation as compared with the proliferative phase. Both progesterone and estrogen have been shown to induce VEGF expression in human uterine stromal cells (Zygmunt et al., 2003). In mouse models, VEGF is secreted by uterine natural killer (NK) cells during the mouse pregnancy and may play inducing roles in uterine neovascularization (Wang et al., 2000). FGF was shown to be upregulated by progesterone and prolactin in rat endometrium (Zygmunt et al., 2003); but unlike VEGF, the changes of FGF levels may not necessarily indicate changes in angiogenesis. In other words, along with VEGF, FGF probably participates in maintaining a balance that enables angiogenesis to occur when required (Rogers and Gargett, 1998).

It has been shown earlier that the angiopoietin family is involved in the remodelling of vessels (Suri et al., 1996). Supporting this, Ang-2 mRNA was detected in ovary, uterus, and placenta. These tissues undergo constant vascular remodelling with periods of angiogenesis and vascular regression occurring throughout the reproductive cycle (Maisonpierre et al., 1997). It seems likely that Tie-1, 2 and Ang-1, 2 may play a major role in regulating the growth and regression of endometrial vasculature (Sato et al., 1995; Maisonpierre et al., 1997; Hanahan, 1997; Zygmunt et al., 2003)

Although very little information has been published to date on the role of endometrial angiogenesis inhibitors, anti-angiogenic factors could play a role in this process as they do in general angiogenesis. In the human endometrium, TSP-1 is elevated in the secretory phase as compared to the proliferative phase. In *in vitro* study, TSP-1 is further upregulated by progestrogen (Rogers and Gargett, 1998; Zygmunt et al., 2003). In addition, the endometrium is a rich source of t-PA and u-PA as well as MMP and TIMP,

particularly around the time of menstruation (Koh et al., 1992; Chu Py et al., 2002; Vincent et al., 2002); thus providing these proteins ample opportunity for a role in the regulation of endometrial angiogenesis.

Chapter 2:

Rational, Hypothesis, Objectives, and Overview of the Research

Although many researchers have turned their attention to the study of the roles of Notch signalling in cancer and vascular disease, due to its fundamental function in cell fate decision, most experiments are exhaustively focused on the activation of the Notch intracellular domain; however, a few groups found that the soluble extracellular domain of Notch ligands is implicated in Notch signalling (Qi et al., 1999; Hukriede et al., 1997; Sun and Artavanis-Tsakonas, 1997). Interestingly, while studying retrovirus-induced T-cell leukemia, Dr Jolicoeur's team found that Notch1 was truncated by viral DNA insertion, thus generating overexpression of $N1^{EC}$ and $N1^{IC}$ domains (Girard et al., 1996; Hoemann et al., 2000). We hypothesized that $N1^{EC}$, as $N1^{IC}$, was involved in tumor formation. This hypothesis was recently supported by Aster's group (Weng et al., 2004).

The main objective of this work centers on the generation of Tg mice ($CD4C/N1^{EC}$) expressing $N1^{EC}$ in T-cells and in cells of the macrophage ($M_{\emptyset}$)/dendritic lineage. Most importantly, we wanted to identify the function of Notch ectodomain in thymoma formation. The results obtained from the Tg mice make up the body of this thesis. Unexpectedly, vascular disease, but not thymomas, developed at high frequency in these Tg animals.

In chapter 3, vascular disease was predominately observed in the Tg liver. The liver vascular defects are induced at an early embryonic stage and progress after birth. The phenotype was generated into normal mice after transplantation of bone marrow (BM) or fetal liver (FL) cells from Tg mice and still developed in $Rag1^{-/-}$ Tg mice. These confirm that the disease was induced by hematopoietic cells, particularly myeloid cells. Abnormal vessels were of recipient origin [$lacZ^{-}$ (white)] observed in the nude mice transplanted with $ROSA_{26}$ X $CD4C/N1^{EC}$ Tg FL cells, indicating a paracrine loop acting in this phenotype.

Consistent with these findings, transplantation of Tg peritoneal Møs into normal recipient mice also induced abnormal vessels. *In vitro*, a deficiency of network formation with Tg LSECs was observed by angiogenesis assay. An interaction between LSECs and Møs was shown by co-culture and conditioned media assays. These results show that overexpression of $N1^{EC}$ in myeloid cells induces vascular malformations. Searching for molecules possibly involved in this disease by DNA microarray analysis will lead to the identification of candidates.

In chapter 4, sterile or less fertile Tg females were observed. Histological analyses confirmed that the aberrant vessels presented in the Tg uterus and that enhanced cells with the appearance of Møs were observed in the Tg uterus and placenta. The uterine vascular defects were reproduced in normal females transplanted with Tg FL cells, suggesting that hematopoietic cells induced the uterine vascular defects, which might further affect fertility. Identification of molecules involved in this phenotype will be interesting.

In chapter 5, these mice were observed to facilitate tumor progression and angiogenesis. In the mice treated with DEN carcinogen, more malignant multi-organ tumors are induced in the Tg mice compared to the nTg mice. Severe tumor metastases in Tg mice were shown in the model of transplantation of C3L5 breast tumor cells. Faster primary tumor growth in Tg mice was found in the model transplanted with B78 melanoma cells. In addition, increased vessels and necroses were observed in tumors presented in Tg mice. Hence, overexpression of $N1^{EC}$ in myeloid cells is associated with tumor progression and angiogenesis. The role of Møs in this progression will be further identified.

The ultimate outcome of these studies is to rationally identify the implications of $N1^{EC}$ and macrophages in human diseases and their potential for anti-angiogenesis and anti-cancer.

Chapter 3:

Overexpression of Notch1 Ectodomain in Macrophages Induces Vascular Malformations in Tg Mice.

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In Preparation (Confidential)

Abstract

While studying retrovirus-induced T-cell leukemia, it was found that Notch1 was truncated by viral DNA insertion, thus generating overexpression of Notch1 extracellular (N^{EC}) and intracellular (N^{IC}) domains. We hypothesized that $N1^{EC}$, as N^{IC}, was involved in tumor formation and we further generated Tg mice expressing N^{EC} in T cells and in cells of the macrophage/dendritic lineage (CD4C/$N1^{EC}$). Unexpectedly, no thymomas developed in these Tg animals, but vascular disease did at high frequency.

Vascular disease was predominately observed in liver and found to be involved in an aberrant angiogenesis. The liver vascular defects are induced at an early embryonic stage and progress after birth. The phenotype was generated into normal mice after transplantation of bone marrow (BM) or fetal liver (FL) cells from Tg mice and still developed in $Rag1^{-/-}$ Tg mice. These confirm that the disease was induced by hematopoietic cells, particularly myeloid cells. Abnormal vessels were of recipient origin [$lacZ^-$ (white)] observed in the nude mice transplanted with ROSA26 X CD4C/$N1^{EC}$ Tg FL cells, indicating a paracrine loop acting in this phenotype. Consistent with these findings, transplantation of Tg peritoneal Møs into normal recipient mice also induced abnormal vessels. *In vitro*, a deficiency of tube formation with Tg LSECs was observed by angiogenesis assay. An interaction between LSECs and Møs was shown by co-culture and conditioned media assays. These results show that overexpression of $N1^{EC}$ in myeloid cells induces vascular malformations. Searching for molecules possibly involved in this disease by DNA microarray analysis leads to the identification of candidates.

These results show that overexpression of $N1^{EC}$ in myeloid cells induce severe vascular malformations. This novel pathway may be activated in some human vascular diseases.

Introduction

The Notch gene encodes heterodimeric transmembrane (TM) receptor that contains extracellular domain (EC) and intracellular domain (IC). The N^{EC} has mainly tandem epidermal growth factor (EGF)-like repeats. Mammals have four Notch genes (Notch-1, -2, 3, and -4) and two families of Notch ligands designated "Delta" and "Jagged". Upon binding of the Notch ligands to the N^{EC}, the N^{IC} domain enters the nucleus and activates transcription (Artavanis-Tsakonas et al., 1999; Kopan, 2002). The Notch pathway plays a role in cell fate decision and this signalling pathway is thought to be involved in cancer and vascular disease (Milner and Bigas, 1999; Kumano et al., 2003; Gridley, 2001a; Ellisen et al., 1991b; Joutel and Tournier-Lasserve, 1998).

For a constitutively active N^{IC} can operate as an oncoprotein (Ellisen et al., 1991a; Dievart et al., 1999, Girard and Jolicoeur, 1998; Girard et al., 1996), most studies on Notch activation has been focused on the N^{IC}. Little attention has been given to the N^{EC}, although it serves as an important component of the Notch receptor. The N^{EC} might also play a role in tumor formation since the EGF-like sequences in the N^{EC} play a common role in providing sites for protein-protein interactions (Kelley et al., 1987). The goal of this study was to test this idea with $Notch1^{EC}$ ($N1^{EC}$) directly in a mouse model.

In *Drosophila*, the N^{EC} contains 36 EGF-like repeats, but no two are identical. It can be involved in binding of ligands (de Celis et al., 1993; Rebay et al., 1991) and modified by Fringe (Fleming et al., 1997), where the Abruptex-gain function *Drosophila* Notch alleles are clustered (de Celis and Bray, 2000). Some regions of the N^{EC} may also talk to other signaling pathways such as Wnt and Shh, then influence the intercellular interaction (Glise et al., 2002; Wesley, 1999; Lee et al., 2000; Powell et al., 2001). Studies involving *in vivo* expression of artificially ectodomain of Notch ligands in *Drosophila* and

other systems have demonstrated both agonistic and antagonistic activities (Qi et al., 1999; Hukriede et al., 1997; Sun and Artavanis-Tsakonas, 1997; Sakamoto et al., 2002; Li et al., 1998). In human, two vessel diseases, CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalophathy) (Spinner, 2000; Joutel et al., 2000) and AGS (Alagille Syndrome) (Loomes et al., 1999; Morrissette et al., 2001), are caused by mutations in ectodomain of Notch3 and Jag1, respectively. Given the known implications of the extracellular forms of Notch ligands in experimental systems, as well as information on human diseases involving ectodomain of Notch signaling, a soluble $N1^{EC}$ is likely to play a genetic role between cell-cell interaction via molecular cross-talks.

In mice, while studying retrovirus-induced T-cell leukemia, Jolicoeur and we colleagues revealed the presence of provirus insertional Notch1 mutations in a high proportion (57%) (Girard et al., 1996; Girard and Jolicoeur, 1998). They later showed that these mutations represent two distinct classes, type I and type II (Hoemann et al., 2000). "Type I", a majority of the integration, occurred in genomic regions coding for sequences between the 34th EGF-like repeat and the TM domain. Every tumor bearing this insertion (i.e. L45 tumor cell line) expressed an abundant Notch1 ectodomain named as $N1(EC)^{Mut}$ (Girard et al., 1996). While the $N1(EC)^{wt}$ proteins processed from wild type were shed, the $N1(EC)^{Mut}$ proteins were secreted in the medium of expressing cells (Hoemann et al., 2000). Thus, secreted $N1(EC)^{Mut}$ and processed $N1(EC)^{wt}$ proteins may differently interact with ligands of Notch1 and other molecules and further affect their signaling. However, the role of the $N1(EC)^{Mut}$ in thymomas has not yet to be rigorously analyzed.

We hypothesized that $N1^{EC}$, as N^{IC}, was involved in tumor formation and we further generated Tg mice expressing $N1^{EC}$ in T cells and in cells of the macrophage/dendritic lineage ($CD4C/N1^{EC}$). The design of $N1^{EC}$ was influenced by the

discovery of N1(EC)^{Mut} in the thymomas (i.e. in L45 cell line). Unexpectedly, vascular disease, but not thymomas, developed at high frequency in these Tg animals. The experiments presented below demonstrated that the vascular defects mainly arise from vessel disruptions of Tg liver and show that N1^{EC} causes these defects via its expression in macrophages ($M_{\Phi s}$), thus influencing the defective liver vessel formation through a paracrine loop. Several *in vitro* experiments with isolated LSECs and peritoneal macrophages confirmed this paracrine mechanism.

Our results on these N1^{EC} Tg mice are in agreement with the genetic studies of Notch families in angiogenesis during development, and also provide a model of haemangioma in adulthood, suggesting a new molecular mechanism for the Notch1 pathway in human vascular diseases.

Results

Construction of CD4C/N1^{EC} Tg Mice

To generate the CD4C/N1^{EC} transgene, a truncated N1 cDNA deleted of its TM and cytoplasmic region was ligated downstream of the CD4C regulatory sequences **(Fig. 1A)**. This cDNA has the capacity to code for almost all the N1^{EC} protein, including all the 34 EGF-like repeats of N1^{EC}, but not for its lin-1 motif. Three independent founder lines (F60788, F60787, and F98513) were established and routinely examined for signs of disease.

Expression of the transgene

Tg expression was first assessed by Northern blot analysis on different tissues **(Fig. 1B)**. Three founder lines (F60788, F60787, and F98513) expressed the expected 4.4 kb N1^{EC}-specific Tg RNA at high levels in the thymus and at lower levels in the peripheral lymphoid organs. Expression was very low but detectable in other organs, including in the liver. The expression was also detected in the bone marrow (BM) and in the E14.5 fetus liver by RT-PCR **(Fig. 1C)**. The expected ~180 kD N1^{EC} protein assessed by Western blot analysis **(Fig. 1D)**, was readily detected in the total and sorted CD4^{+}CD8^{+} thymocytes and in peritoneal macrophages. It was also weakly expressed in the liver.

The cell-type specificity of the CD4C regulatory sequences has been extensively documented to be faithful for immature CD4^{+}CD8^{+} and mature CD4^{+} T-cells as well as for cells of the myeloid lineage, including macrophage and dendritic (DC) cells (Hanna et al., 1994; Hanna et al., 2001). *In situ* hybridization (ISH) with Tg-specific hCD4 exon 1 probe confirmed expression in cells morphologically appearing as macrophages **(Supplement 1)**. In the liver, neither hepatocytes nor liver sinusoidal endothelial cells (LSEC) express the transgene at detectable levels by ISH. However, interstitial cells with the morphological

appearance of Kupffer or macrophages express the transgene **(Fig. 1E)**. As expected, the isolated Tg KCs express the CD4C/N1^{EC} but not nTg KCs as detected by RT-PCR **(Fig. 1F)**.

Therefore, these results confirm that the CD4C promoter drives expression of N1^{EC} in the expected cells, i.e. CD4^{+}CD8^{+} thymic T-cells, CD4^{+} T-cells and in cells of the myeloid lineage (macrophages, Kupffer cells) in the CD4C/N1^{EC} Tg mice.

The CD4C/N1^{EC} Tg mice develop severe vascular abnormalities in the liver

Clinically, the CD4C/N1^{EC} Tg mice look healthy and did not develop observable phenotypes after up to 12 months of observation, except for a lower body weight and smaller size (Table 1) as compared to the non-Tg littermates and except that fertility was decreased (F60788) or totally impaired (F60787) in Tg females.

At autopsy, a macroscopic evaluation of the CD4C/N1^{EC} Tg mice revealed an absence of lymphoid organ lymphoproliferation, but revealed a severe liver vascular defect in a high percentage (>97%) of Tg animals (Table 1) from the three founders, easily observable after perfusion with Microfil®. Huge meandered spider-like vessels, crawling on the surface and along the edge of liver and seemingly wrapping the organ, were observed **(Fig. 2A.b)**. Some livers are additionally combined with tumor-like cavities **(Fig. 2A.c and 2A.e)**. Paradoxically, the liver vasculature within parenchyma showed fewer and shorter branches **(Fig. 2B. k and l)**. Vascular malformations were also observed in other organs of Tg mice (brain, heart, spleen, kidney and lungs), but the lesions were much milder and at lower incidence (~10-12%) than in the liver (Table 1). However, in mice of one founder line (F98513) in which the liver phenotype was most prevalent, vascular disease in the lung occurred at high frequency and these were relatively severe. In addition, a significant proportion of Tg mice had an enlarged gallbladder (8/20) and splenomegaly (15/30).

The liver vascular defects related to aberrant angiogenesis with enhanced permeability

The formation of vessels is a two-step process: vasculogenesis and angiogenesis (Carmeliet, 2003). To understand at which step (vasculogenesis or angiogenesis) the N_1^{EC} interfere with vessels of CD4C/N1^{EC} Tg mice, we conducted additional histological experiments. These analyses revealed a variety of vascular malformations. First, the general organization of the lobules appeared abnormal with reverse lobules (that is portal vein in the center of the lobule while central vein in the perilobular area), perilobular sinusoid capillarization and reduced sinusoids in zone 2 **(Fig. 3B).** Occasionally, disrupted arteries were also detected **(Fig. 3B).** Second, the large vessels were not confined within the liver parenchyma, as in non-Tg mice, but rather grew out of the liver, spread along the edges and surfaces of the liver and tend to wrap the whole organ **(Fig. 3A, B)**. Third, these large vessels develop some irregular branchings inward, re-growing into the liver parenchyma from the external surface **(Fig. 2B)**. Very often these sprouting irregular branchings were clustered and dilated **(Fig. 2B)**. Fourth, vascular small vessels-derived from larger vessels inside the parenchyma also gave rise to aberrant and clustered capillaries distributed heterogeneously **(Fig. 3A and B)**. Fifth, vessels were very heterogeneous in their size, some lumen being distended and forming hemangioma-like cavernae **(Fig. 3A)**. Sixth, foci of accumulation of mononuclear cells intermixed with capillary beads and surrounded by morphologically normal appearing liver tissues could be observed **(Fig. 1E and Fig. 3A-B)**.

Immunohistochemistry (IHC) performed with monoclonal antibody Platelet Endothelial Cell Adhesion Molecules-1 (PECAM-1) confirmed the irregular and huge vessels on the surface of the liver, as well as heterogeneous capillaries within the liver

(Fig. 3C.1). IHC using the monoclonal antibody for α-smooth muscle actin (α-SMA) showed an enhanced level in Tg liver tissues **(Fig. 3C.2)**, indicating that the liver might contain higher numbers of SMCs or myoblasts. In addition, the disrupted SMCs were found around the wall of the large vessels, as well as within thrombi **(Fig. 3C.2)**. To confirm the disruption of SMCs and to know whether ECM is also disrupted, Masson's trichrome staining was further performed. With this staining, enhanced positive reaction was showed in Tg liver, indicating both disruption of SMAs and ECM **(Fig. 3C.2)**.

Together, these results suggest that remodeling defects of angiogenesis are occurring in the liver of the CD4C/N1^{EC} Tg mice.

Malformations of vessels often lead to enhanced vascular permeability in a number of different conditions (Dvorak et al., 1999). We assessed leakage of Tg liver vessels using Evans blue (EB) (Thurston et al., 1999). Higher amount of EB was detected in Tg liver than in nTg liver **(Supplement 2A)**. In addition, higher EB fluorescence was observed beneath of ECs of vessel wall of Tg liver. This was absent in nTg liver **(Supplement 2B)**. More importantly, EM analysis showed that a single red cell is entering and passing the feneastrate. No similar phenomenon was found in the nTg liver **(Supplement 2E)**. These data suggest enhanced permeability of liver vessels in Tg mice. Interestingly, high fluorescence was also unexpectedly observed in some MΦs labeled or not by latex beads, that were located in sinusoids **(Supplement 2C and 2D)**. Altogether, both leaky vessels and activated macrophages may lead to the accumulation of EB in liver.

The liver vascular defects in the CD4C/N1^{EC} Tg mice are already apparent during early organogenesis and still develop during adult liver regeneration.

To determine whether this liver phenotype was related to a developmental defect, we examined these Tg mice at earlier points and during the embryonic life. We found that at

E16.5, the liver structure remained relatively normal, but already dilated vessels and intense small vessels extending toward the liver capsule could be observed in Tg mice but not in nTg mice **(Supplement 3A)**. At day 6 postnatal, the phenotype was more apparent and the normal structure of the Tg liver was disrupted and large vessels and capillarized vessels were apparent on the liver surface, but not in nTg liver **(Supplement 3B)**. During adulthood (at day 60) the lobules of Tg liver became progressively more disrupted: huge vessels and tumor-like cavities filled with blood and thrombi were grossly visible on the liver surface. No such abnormalities were ever found in the nTg livers **(Fig. 2A, B and 3A)**. This defect becomes more severe with age. Taken together, these results indicate that this liver vascular phenotype is induced early during embryonic life and progresses in severity as the Tg mice age.

To determine whether the vascular defects of embryonic liver were a pre-requisite for the development of the adult liver disease, we took advantage of the well characterized ability of the liver to regenerate after hepatectomy. Two-month old young adult Tg mice were subjected to partial hepatectomy **(Supplement 4A)** and sacrificed 20 days after this procedure to assess the regenerated lobule. In each mouse assessed (n=5) the regenerated lobule was found to be smaller than the non-Tg one (n=4) (**Supplement 4B**). In addition, gross examination after Microfil® perfusion showed severe vascular defects in Tg regenerated lobules (**Supplement 4C**). These were more severe than those observed in the other non-regenerated lobules of the same mouse. Virtually no normal capillaries were reconstituted during regeneration in hepatectomized Tg liver (**Supplement 4C. g and h**). These Tg regenerated lobes indeed show aberrant vessels emerging at the surface of the lobes, indistinguishable from the phenotype observed in older Tg mice. In contrast, in nTg regenerated liver, normal appearing capillaries were seen (**Supplement 4C. c and d**).

These results suggest that the vascular phenotype of CD4C/N1^{EC} Tg mice can be elicited during neovascularization in adult life. They also show that the vascular defects observed impact on hepatocyte growth.

Bone-Marrow (BM)-derived hematopoietic cells trigger the liver vascular defects.

The CD4C regulatory elements used to express the N1^{EC} cDNA has been found to be quite faithful in allowing expression of various surrogate genes in selected populations of lymphoid (CD4^{+} CD8^{+}, CD4^{+} CD8^{-} T cells) and myeloid (macrophages and DCs) cells (Hanna et al., 1994; Hanna et al., 2001). Their BM progenitors can reconstitute these populations when transfected into lethally-irradiated syngeneic host.

To determine whether hematopoietic cells or cells of other lineages were responsible for inducing this severe liver vascular phenotype, lethally irradiated C3H mice were transplanted with BM cells-derived from CD4C/N1^{EC} Tg and nTg mice. Six months after transplantation, these transplanted mice were sacrificed. As expected, the expression of Tg N1^{EC} RNA was detected in the thymus and lymph nodes of these Tg-bearing recipients by Northern blot **(Fig. 4A.a)**. In addition, Tg RNA expression was detected in the peritoneal MΦs and the liver of these mice using RT-PCR **(Fig. 4A.b)**. These expression data confirmed that these mice were successfully transplanted. Interestingly, all recipient mice reconstituted with Tg BM cells, but none of those reconstituted with non-Tg BM cells, developed a liver vascular phenotype indistinguishable from that described in non-transplanted Tg mice **(Fig. 4B and 4C)**.

These results indicate that BM-derived hematopoietic cells expressing N1^{EC} are responsible for inducing these severe liver vascular defects.

The liver vascular defects still develop in CD4C/N1^{EC} Tg mice bred on Rag1$^{-/-}$ background

To determine 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 Rag-1-deficient background. These Rag-1$^{-/-}$ mice are defective at producing immature CD4^{+} CD8^{+} thymocytes and mature T-cells (CD4^{+} and CD8^{+}) and B-cells (Mombaerts et al., 1992). Initially, mice were typed between 30-40 days and Rag-1$^{-/-}$ Tg mice were found at a much lower frequency (0/80) than expected (15%) from Mendelian segregation suggesting early deaths. By tail typing earlier, it was indeed found that the Rag-1$^{-/-}$ Tg mice were born at the expected Mendelian ratio, but approximately 99% (15/16) of these mice died in the first 5.5 days after birth **(Fig. 5A)**. A moderately severe liver vascular phenotype, indistinguishable from that described in older wild-type Tg mice, could be observed in Rag-1$^{-/-}$ Tg mice **(Fig. 5B)**. Similarly, Rag-1$^{+/-}$ Tg mice, which survived normally, also developed a liver vascular phenotype **(Data not shown)**. Therefore, the liver vascular phenotype which was almost identical morphologically in Rag-1$^{-/-}$ and Rag-1$^{+/-}$ Tg mice is unlikely to explain the very different lifespan of these two groups of mice. The cause of the early death of Rag-1$^{-/-}$ is under investigation. These data strongly suggest that Tg-expressing lymphoid cells (immature CD4^{+}CD8^{+} and mature CD4^{+} T-cells) were dispensable for the development of the liver vascular defects. Thus, myeloid BM-transplantable cells expressing N1^{EC} are involved in the development of the liver vascular disease.

A paracrine loop induces the liver vascular phenotype in CD4C/N1^{EC} Tg mice.

The Tg myeloid cells expressing N1^{EC} could affect liver vascular cells not expressing the transgene and induce them in a paracrine way to show these vascular defects.

Alternatively, Tg expressing cells could themselves differentiate into vascular cells with abnormal functions. To distinguish between these two pathways, three different experiments were set up.

Since both LSECs and KCs are the liver lining cells and interact physically and functionally together (MacPhee et al., 1992; MacPhee et al., 1995; Knolle and Gerken, 2000), it is interesting to know what are the physical relationship between the LSECs and the KCs in our Tg mice. To investigate this possibility, electronic microscopy of liver was performed. Clearly, KCs exist in the sinusoids via at least three different manners: isolated in the sinusoids without interacting with endothelial cells, lying on the foot of the endothelial cells, and directly touching the body of the endothelial cell **(Fig. 6A)**. While one KC was easily seen in the nTg sinusoid, two or three KCs were often found in the Tg sinusoid **(Fig. 6A)**. These data demonstrate a physical interaction of the LSECs and the KCs existing in both nTg and Tg liver sinusoids and more KCs in the Tg liver.

In order to know the vessel defects induced by endothelial cell per se or by transgen expressing myeloid cell, secondly, chimeric mice were generated by fusing ($ROSA_{26}$ X CD4C/$N1^{EC}$) F1 Tg embryos with normal C3H embryos **(Fig. 6B.1)**. $ROSA_{26}$ is a good model to monitor the origin of embryonic stem cells which are β-gal-positive (Zambrowicz et al., 1997; Friedrich and Soriano, 1991). If Tg cells act through a paracrine pathway, abnormal liver vessels would be expected to be derived from both F1 Tg and normal C3H cells, that would be expressing the ROSA marker, β-galactosidase. On the other hand, if $N1^{EC}$ favors the abnormal differentiation of precursors into abnormal vessels, the abnormal liver vessels would be expected to be derived only from the Tg parental cells, that would be not expressing the ROSA marker. As expected, the liver vascular defects were generated in the $ROSA_{26}$ X CD4C/$N1^{EC}$ chimeras, which were large vessels observed on

the surface of the liver **(Fig. 6B.2)**. Importantly, the cytoplasm of the endothelial cells on these defected vessels were blue as detected by β-galactosidase staining and nuclear fast red staining **(Fig. 6B.2)**. These results indicted that these endothelial cells are derived from normal C3H cells. Thus, a paracrine loop probably induces the liver vascular defects.

To confirm the chimera results, finally, fetal liver cells derived from $ROSA_{26}$ X $CD4C/N1^{EC}$ embryo were transplanted to nude mice **(Fig. 6C.1)**. The nude mice were used as hosts mainly to avoid rejection after injection. If Tg cells act through a paracrine pathway, vessels that are originated in the nude mice would be expected to be affected. In this case, the affected vessels should be β-galactosidase staining negative and not show blue color. As expected, the vascular abnormalities were strikingly induced in the recipients transplanting liver cells derived of $ROSA_{26}$ X $CD4C/N1^{EC}$ embryos, with large vessels revealed at the edge of the liver. More importantly, these vessels showed white color that indicated an origin of the host. Meanwhile, they were accompanied by blue color cell populations supposed from the donors **(Fig. 6C.2)**. With the liver section, all the abnormal vessels from the mice assessed stained negative for β-gal activity, while other infiltrating cells, presumably myeloid and lymphoid cells, stained positive **(Fig. 6C.3)**. These results indicated that the defective vessels were derived from the recipient non-Tg mice, thus strongly suggesting that the vascular disease is induced through a paracrine loop.

Taken together, the defective vasculatures in the Tg liver were not caused by endothelial cells per se, but induced by other transgene expressing cells.

Macrophages are sufficient for causing the liver vascular disease.

Following the observation of a paracrine loop, we next search for the identity of the BM-derived expressing cells causing the disease. We hypothesize that the Tg macrophages

act as paracrine cells in order to affect the endothelial cells and further to cause the defective vessels: (1) The macrophages are expressing the CD4C/N1^{EC} transgene in the liver and in other organs, as detected by *in situ* hybridization **(Fig. 1E and supplement 1).** (2) The Kupffer cells purified from the CD4C/N1^{EC} Tg liver strongly expressed the transgene as detected via RT-PCR **(Fig. 1F)**. (3) Higher number of macrophages were observed in the Tg than in the nTg liver, as detected by histological and immunohistochemical analysis **(Fig. 7A)**, as well as by electronic microscopy examination **(Fig. 6A)**. (4) The liver vascular phenotype still persisted in the Tg-bearing mice without T-cells **(Fig. 5B and 6C)**. (5) Macrophages play an important role in the regulation of angiogenesis (Sunderkotter et al., 1994).

In order to confirm this hypothesis, we transplanted macrophages into normal C3H mice. If macrophages act as paracrine cells, the liver vascular phenotype should be reproduced in the mice transplanted with Tg macrophages. Peritoneal macrophages were isolated and then injected into the C3H mice through the tail veins. One to three months after the transplantation, the mice were killed. As expected, the liver phenotype was induced in the C3H mice transplanted with the Tg macrophages but not with the nTg cells as detected by macroscopic analysis (**Data not shown**) and Microfil® **(Fig. 7B)**. As seen in the CD4C/N1^{EC} Tg liver, big vessels were growing out from the liver and then lay on the surface of and at the edge of the liver **(Fig. 7B)**. These abnormalities of the vessels were further confirmed by histological analysis **(Fig. 7C)**.

Therefore, macrophages are sufficient for inducing the liver vascular phenotype in normal C3H mice as found in the Tg mice though the phenotype is much less severe.

Enhanced KCs and decreased LSECs were observed in the Tg liver

With the aim of confirming the paracrine pathway revealed *in vivo*, we planed to purify the LSECs and KCs from the liver via MACS in order to perform some *in vitro* experiments as described in the following. The livers were digested with the collagenase IV and Dnase A. After centrifugation with matrizamide, larger numbers of no parenchymal liver cells (NPLCs) were obtained from the Tg livers than from the nTg livers **(Fig. 8A)**. Interestingly enough, through FACS analysis, a higher percentage (47.2%, n=3) of the Tg Mac-1 positive cells was displayed than for the nTg cells (32.2%, n=3). Conversely, a lower percentage (49.7%) of Tg ICAM-1 positive cells showed up than for the nTg ICAM-1 cells (71.1%) **(Fig. 8B)**. Consistent with these results, more Mac-1 and less ICAM-1 labeling cells were obtained from the Tg NPLCs than from the nTg NPLCs through MACS separation **(Fig. 8C)**, suggesting enhanced macrophages and decreased normal endothelial cells in the Tg livers. These data confirm the *in vivo* results in which more macrophages were observed by EM and less normal branches of the vessels were shown by Microfil® in the Tg livers.

Dysfunctional tube formation with the Tg LSECs and enhanced adhesion of Tg macrophages onto LSECs were observed by *in vitro* Assay.

A fundamental role of endothelial cells is to form vessels *in vivo* (Risau, 1995; Folkman and D'Amore, 1996). In order to investigate the functional difference between the nTg and Tg LSECs *in vitro*, angiogenesis assay was performed with the purified LSECs via MACS. It was observed that less alignments, branches, and tubes were formed by the Tg LSECs on the angiogenesis assay kit at different time (from 1 to 9 hours after the culture) compared to the nTg cells **(Fig. 9A, B, and C)**. This feature of less-number signs is not due to the lesser number of Tg cells **(Fig. 9D)**. These results suggest that the Tg

LSECs are functionally different from the nTg LSECs *in vitro*, matching up the *in vivo* vascular phenotype displayed in the Tg liver.

To confirm the paracrine loop, that is acted through the interaction between the LSECs and KCs, co-culture assay with LSECs and peritoneal macrophages was conducted. After four hours of co-culture, it was found that more clusters of Tg macrophages adhere onto LSECs than those of nTg macrophages **(Fig. 10A)**. Interestingly, the most number of clusters was found in the group with Tg macrophages and Tg LSECs **(Fig. 10)**. These results suggest enhanced adhesion of Tg macrophages onto LSECs. Furthermore, myeloperoxidase activity confirmed high adherence of Tg macrophage and the highest activity detected in Tg macrophages co-cultured with Tg LSECs **(Fig. 10B)**. These results match up the physical interaction between LSECs and KCs, observed *in vivo*, also imply a more important molecular interaction between the activated Tg macrophages and LSECs.

Supernatants from the Tg macrophages inhibit the growth of the nTg LSECs.

To further elucidate the paracrine pathway observed *in vivo*, a conditional media assay was performed with supernatants from the peritoneal macrophages derived from the nTg and Tg mice. Surprisingly, while the normal LSECs treated with a normal media and the nTg macrophage supernatants were confluent after 5 days culture **(Fig. 11a)**, those treated with the Tg macrophage supernatants exhibited a very sparse distribution **(Fig. 11b).** In contrast to a figure of $1X10^6$ LSECs treated with the nTg macrophage supernatants, 5×10^3 cell amounts appeared for the LSECs treated with the Tg macrophage supernatants **(Fig. 11d)**. Therefore, the Tg macrophage supernatants inhibit the LSECs growth *in vitro,* supporting the less tube formation with the Tg LSECs, and providing an evidence on a paracrine loop acting on the defective vessels of the Tg mice.

Molecules possibly required for the vascular phenotype were detected in macrophages and LSECs.

Some effectors of angiogenesis, such as VEGF, angiopoietin, and ephrin, have been reported to induce somewhat similar aberrant vessel growth when overexpressed locally (Yancopoulos et al., 2000; Eriksson and Alitalo, 1999), although never specifically in the liver as observed in CD4C/N1^{EC} Tg mice. We measured levels of some of these factors **(Supplement 6)** in the peritoneal macrophages and LSECs by RT-PCR. Almost all factors, except for Ang-2 and bFGF, were detected in macrophages. Only Ang-1 was lightly increased in the Tg macrophages comparing to the nTg cells, no obvious difference for other factors such as VEGF was detected between the nTg and Tg macrophages. So far, it was found no detectable difference of Flk-1 in the purified LSECs between nTg and Tg mice **(Supplement 6)**. We also measured the levels of VEGF family, angiopoietin family, as well as Eph B4 in liver sections by ISH. No obvious differences were observed by this analysis, with each of these probes between Tg and non-Tg mice (**Data not shown**).

Since N1^{EC} protein has been shown to be released from cells (Hoemann et al., 2000), it could interact with extracellular molecules such as its ligands, especially those already known to be involved in vascular formation such as Delta4 and Jag1. We conducted RT-PCR to measure the levels of some Notch signal pathway **(Supplement 6)** in peritoneal macrophages. No detectable Notch1 intracellular domain was found in both nTg and Tg macrophages, excluding that N1^{EC} protein activates macrophages through Notch1 receptor **(Supplement 6)**. Dll4 expression was shown to be indistinguishable between nTg and Tg macrophages, suggesting that it is not involved in the activation of Tg macrophages. No Dll4 expression was detected in LSECs, supporting that LSECs are not arterial cells (**Supplement 6**). So far, Jag family has not yet been successfully detected by RT-PCR. We

also performed ISH on liver sections with probes derived from Delta4 and Jag1. Delta4 expression was not detected in control non-Tg nor in Tg livers (**Data not shown**). The diseased Tg portal triads gave a seemingly stronger expression of Jag1 than non-Tg mice. **(Data not shown).** Identification of other possible molecules involving angiogenesis is ongoing with help of DNA microarray analysis.

Therefore, neither VEGF nor endogenous Notch1 is activated in the Tg macrophages. Other factors such as Jag1 and MMPs might play a role, at least part, in this disease.

Discussion

CD4C/N1^{EC} mice as an animal model for liver vascular patterning defects similar to human hemangiomas

We demonstrate here that, while thymomas were not observed in the CD4C/N1^{EC} Tg mice, liver vascular patterning defects are highly (97%) present in these mice in contrary to our expectation (Figure 2). The defective vascular patterns in the liver are mainly classified into three classes (Table 2): (1) Huge meandered vessels on the surface/at the edge of the liver. (2) Tumor-like cavities accompanying superficial vessels. (3) Malformations of capillary with the normal appearance of the liver.

The liver vascular defects observed in the CD4C/N1^{EC} are similar to "liver hemangiomas", a rare manifestation of the human disease, because (1) these defects possess some pathological characteristics of general hemangioma: cavity-like lumen filled with thrombi and cavernous vessels formed by single layer of ECs (Semelka and Sofka, 1997; Burrows et al., 2001); (2) perilobular capillarized sinusoids shown in our Tg mice are characteristics of liver haemangioma (Iqbal and Saleem, 1997); (3) the affected CD4C/N1^{EC} Tg mice look healthy after up to 12 months of observation, clinically supporting haemangioma not influencing life-span so much (Iqbal and Saleem, 1997). In fact, the vascular defects observed in CD4C/N1^{EC} Tg mice are reminiscent of the typical mouse liver haemangiomas observed in the mice with a germ-line Tsc2 mutation and the mice with targeted inactivation of VHL (Kobayashi et al., 1999; Haase et al., 2001). However, some differences between these liver vascular defects and typical hemangiomas exist. (1) A focal tumor or multifocal tumors are frequently observed in a typical liver haemangioma in either humans or mouse (Burrows et al., 2001; Greer et al., 1994; Haase et al., 2001). However, the defective vessels are homogenously spread to the entire liver of

CD4C/N1^{EC} Tg mice. (2) While a caveneous haemangioma is the most frequently occurring typical haemangioma (Iqbal and Saleem, 1997), superficial huge vessels are predominantly displayed in the CD4C/N1^{EC} Tg mice.

Thus, the liver vascular disease observed in the CD4C/N1^{EC} Tg mice is beyond of a typical liver haemangioma and displays a complex model.

Angiogenesis process, but not vasculogenesis, is affected in the CD4C/ N1^{EC} Tg mice

As seen in other Notch-ligand deficient and transgenic mice (Krebs et al., 2000; Xue et al., 1999), the vasculogenic formation of the liver vessels are also unaffected in the CD4C/N1^{EC} Tg mice, but there is a failure to reorganize these vessels to normal branches and capillaries. Hence, N1^{EC} is not required for vasculogenesis but essential for angiogenesis. The classical mechanism of angiogenesis is sprouting (Klagsbrun and D'Amore, 1991), meaning that a new branch is formed from an existing vessel by migration and proliferation of ECs and disruption of ECM. However, in addition to sprouting, it is now increasingly recognized that other mechanisms exist for the creation of new blood vessels from the existing vasculature. Such processes is no-sprouting angiogenesis including vessel elongation (Ausprunk et al., 1974) [termed pruning (Risau, 1997)] and intussusception (Burri and Tarek, 1990). Elongation means that vessel is widening but not branching in growing tissues. Intussusception is defined that the lumen of the vessel is divided into two internally by a wall of endothelial cells. As widening and elongating vessels are observed in Tg liver, non-sprouting angiogenesis may be involved in the CD4C/N1^{EC} Tg model. Matrix disruptions around vessels were detectable in the CD4C/N1^{EC} Tg liver, further confirming the aberrant angiogenesis. The proteases released via the activated macrophages may inappropriately degrade the ECM and cause too much ECM deposition. This further influences the angiogenesis process. The defective

endothelial cells (defenestrated) may influence the cooperation between endothelial cells and Ito cells. They cannot clear the ECM produced by Ito cells and result in perisinusoidal fibrosis (Wisse et al., 1996; Saile et al., 2002). In addition, due to some signal disruption, e.g. Ang-1, the smooth muscle cells or pericytes may not be recruited correctly to the endothelial cells. Therefore, the matrix disruptions observed in the CD4C/ $N1^{EC}$ Tg liver may be either a cause or a consequence of defective angiogenesis.

MΦs, but not T-cells, are key mediators to provoke a vascular disease in the liver

Even though both purified DP T-cells and peritoneal macrophages express the $N1^{EC}$ transgene detected by RT-PCR or Western blotting, only the cells with morphological appearance of macrophages express $N1^{EC}$ detected via ISH. This indicates that the expression of the transgene in the macrophages is stronger than that in the lymphocytes.

The outstanding role of MΦs in the liver vascular phenotype has been corroborated by several experiments: (1) BM and FL transplantation has reconstituted the liver vascular disease. (2) This disease was also reproduced in CD4C/$N1^{EC}$ X Rag-$1^{-/-}$ mice. (3) Peritoneal macrophage transplants have yielded similar liver phenotype though much less severely. Therefore, MΦs are key mediators for the liver vascular disease observed in the CD4C/$N1^{EC}$ Tg mice. The following reasons may explain the importance of the macrophages for the liver vascular disease. (1) Although MΦs are not angiogenic per se, they do have the potential to become active in response to some appropriate stimuli (Sunderkotter et al., 1994). The $N1^{EC}$ transgene may be a stimulator to activate the Tg macrophages in the CD4C/$N1^{EC}$ mice. (2) The activated MΦs secrete a myriad of factors and are tightly involved in angiogenesis (Sunderkotter et al., 1994). (3) MΦs are residing in all tissues in greater numbers than other blood-borne cells. Importantly, eighty percent

of the body's MΦs is present in the liver (Knolle and Gerken, 2000), coinciding with the worst disease affecting the liver of CD4C/N1^{EC} Tg mice.

Why does the phenotype mainly occur in the liver?

Endothelial cells are distributed in almost each organ of the body, but their heterogeneity depends on the different microenvironments, allowing them to acquire the organ-specific function, even within different regions of the same organ (Knolle and Limmer, 2001; Gao and Williams, 2001). Consistent with this fact, the vascular defects are observed in several organs of the CD4C/ N1^{EC} Tg mice, but the worst phenotype is being in the liver. The potential differences might arise from the following reasons. First, the liver is a capillary meshwork of the body (Risau, 1997). The specific endothelial cells and the KCs in the liver may discriminate the liver from the other organs. Coinciding with these heterogeneities of the LESCs and the KCs in the liver (MacPhee et al., 1992; Knolle and Gerken, 2000), heterogeneously defective capillarized sinusoids are observed in the different zones of the lobule of the Tg liver. A physical interaction between LSECs and KCs, previously shown by *in vivo* microscopy (MacPhee et al., 1992; MacPhee et al., 1995), is confirmed by our electron-microscopy examinations. Since this interaction is unique to the liver because of the liver-specific microenvironment (Limmer and Knolle, 2001), it may be further beyond a physical contact and cause molecular interactions letting the liver vessels more subject to be affected than the others. In fact, some molecular interactions between them have already occurred, for instance, IL-10 secreted by KCs can act on the LSEC function (Knolle and Gerken, 2000). Since many molecules (such as TNF, IL-1, and IL-6) secreted by the activated macrophages can participate to angiogenesis, the activated Kupffer cells may also release some factors so as to influence the function of the Tg LSECs and further mediate the vessel formation of the Tg liver. Secondly, liver-

specific pro-angiogenic factors such as ANGPTL3 (Goumans et al., 2002) might take part in the severely vascular defects of the liver. Finally, the Ito cells, considered as liver-specific pericytes, might contribute to liver-angiogenesis through additional mechanisms different from those usually attributed to microcapillary pericytes (Medina et al., 2004).

Hence, albeit the expression of the $N1^{EC}$ in the liver seems weak, the liver-specific endothelial cells together with Kupffer cells, the unique interaction of LSECs and KCs, the specific liver-proangiogenic factors, and finally the specific liver-pericytes all in synergy or not may be sufficient to make the worst vascular phenotype occur in the Tg liver.

The Notch1 ectodomain is required for liver vascular patterning defects

The emerging role of the Notch receptors in the vessel development has been well documented during the past few years. As similar phenotypes were observed in both loss- and gain-of-function of Notch (Krebs et al., 2000; Uyttendaele et al., 2001), the conclusion was drawn that the appropriate levels and regulations of the Notch signaling are necessary for the vascular development (Gridley, 2001). Almost all these experiments are focused on the activation of Notch intracellular domain. Up to now, there is only one mouse-model exclusively concerning the function of the ectodomain of Notch pathway, that is to say $Notch3^{EC}$ transgenic mice, in which the vascular phenotype of the human CADASIL is successfully regenerated (Ruchoux et al., 2003). To our knowledge, no other published report demonstrates the role for the ectodomain of other Notch receptors (1, 2, and 4) in vessel development and diseases. For the first time, our results show that the overexpression of the Notch1 ectodomain can be involved in hemangioma-like disease in adult mice. In fact, Notch-deficient mice (Swiatek et al., 1994; Krebs et al., 2000) were generated by mutations sited within the EGF-like repeats, thereby indicating a role for the extracellular domain of Notch in the vessel formation. More over, a couple of *in vitro*

experiments showed that the soluble extracellular domain of JAG1 is implicated in the angiogenesis process (Small et al., 2001; Lindner et al., 2001). In addition, recent experimental studies revealed that soluble fragments of the extracellular domain of some factors such as VEGF, ephrin, and Ang-1 act as dominant negative factors in angiogenesis assays (Kystone meeting-Angiogenesis, 2004). Therefore, as for other soluble factors, $N1^{EC}$ is also required for the maintenance of the vasculature, even in adults.

Although some studies indicate that VEGF is the upstream of Notch-intra signaling pathway, no detectable changes of VEGF were found between the populations of non-Tg and Tg macrophages. Contrary to the function of VEGF which increases the number of vessels (Yancopoulos et al., 2000), the number of normal branches of the vessels in the CD4C/$N1^{EC}$ Tg liver is decreased. That effect is further confirmed by less tube formation of Tg LSECs in *in vitro* angiogenesis assay. These outcomes suggest that VEGF may be implicated in the function of Notch intracellular domain, but not necessarily in the Notch extracellular domain. On the other hand, huge vessels appear in the CD4C/$N1^{EC}$ Tg liver. This is reminiscent of the role of Ang-1 in increasing the size of the vessels. Interestingly enough, elevated levels of Ang-1 were detected in Tg macrophages as compared to non-Tg ones. It is hypothesized that the $N1^{EC}$ expressed in the Tg macrophages enhances the levels of Ang-1, the latter may trans-function on the liver endothelial cells. The detailed mechanism between them will prove to be interesting in the near future.

Following a failure to recruit smooth muscle cells to the vessels and superficial vessels, $N1^{EC}$ may consequently be related to the PDGF family.

The deduced mechanism involved in the liver vascular defects present in the CD4C/$N1^{EC}$ Tg mice

In contrast to the Notch expression in the endothelial cells in other mouse models

(deficient or Tg mice with Notch receptors), the transgene $N1^{EC}$ is mainly expressed in macrophages rather than in endothelial cells. Hence, the vascular disease caused by $N1^{EC}$ does not seem to be induced by the endothelial cells per se. We are presuming that a paracrine pathway between LSECs and macrophages might play a key role in this model. This hypothesis is critically proved by FLT conducted by the $N1^{EC}$ X ROSA as donors and nude mice as hosts, as well as the peritoneal macrophage transplantation. These results indicate that the interaction between LSECs and macrophages is crucial for inducing the liver phenotype. Of great importance, further *in vitro* experiments show that more clusters of Tg macrophages adhere to Tg LESCs observed in co-culture assay. This tends to indicate that besides a physical interaction, a molecular interaction may exist between two types of cells, though the exact molecular mechanisms still remain unclear. The inhibition of the growth of LESCs treated with Tg macrophage supernatants further confirms the paracrine pathway even without a physical contact between the two groups of cells. Thus, a deduced mechanism is: The macrophages are originally activated by the $N1^{EC}$ transgene. The activated macrophages then release some factors, which interact with the liver endothelial cells and then influence the latter's function. At the end, the morphology of the liver vessels is changed by the activated macrophages through a paracrine mechanism.

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Materials and Methods

Mice. The C3H/HeN and Rag1 gene-deficient mice were obtained from Harlan and the Jackson laboratory (Bar Harbor, Maine) respectively. The $Rag1^{-/-}$ mice were bred as heterozygous for at least 6 generations on the C3H/HeN background. The $ROSA_{26}$ mice were kindly obtained from Dr. David Lohnes, formerly from our Institute.

Transgene construction and generation of CD4C/N1^{EC} Tg mice. A 4.42 kbp fragment encoding the Notch1 ectodomain (N1^{EC}) was generated by HindIII/StuI digestion of the Notch1 full-length cDNA and ligated through EcoRI to the 15 kbp CD4C regulatory sequences. This CD4C promoter represents a chimera between the mouse CD4 enhancer and the human CD4 promoter (Hanna et al., 1994). The resulting 20.3 kbp CD4C/N1^{EC} recombinant DNA was excised from the plasmid and microinjected into the pronucleus of (C57BL/6 X C3H) F2 one-cell embryos. Tg founders were identified by tail DNA typing. They were bred on the C3H/HeN background.

Generation of chimeric mice. Chimeric mice were generated by aggregating morulas pairwise, as described (Hogan et al., 1986). Embryos (2.5 days postcoitum, dpc) were treated with an acidic Tyrode solution to remove the zona pellucida of precompacted (4-8 cells) embryos. Pairs of embryos (CD4C/N1^{EC} X CD1) and ($ROSA_{26}$ X CD1) were incubated to aggregate into blastocysts, which 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) and then 15-20 μg from each sample was electrophoresed on formaldehyde agarose gels and processed for hybridization using a ^{32}P-labeled probe, essentially as described (Girard et al., 1996). Probes were the 1.9 kbp probe

M corresponding to the BamHI-BamHI fragment of the full-length Notch1 cDNA (Girard et al., 1996).

RT-PCR analysis. Total RNA was extracted from tissues or cells using Trizol (A2). RNA (1 µg) was added to RT-PCR reactions containing the indicated primers at a concentration of 0.6 µM, essentially as described (Girard et al., 1996). Primer sets for sense (S) and antisense (AS) amplifications for the following genes were used:

Name of gene	Primers
CD4C/N1^{EC}	CCCCACTGGGCTCCTGGTTGCAGC (S) GTATGAAGACTCAAAGGGCAG (AS)
Mouse Notch1	TGTGACAGCCAGTGCAACTC (S) TGGCACTCTGGAAGCACTGC (AS)
Mouse Notch2	ACATCATCACAGACTTGGTC (S) CATTATTGACAGCAGCTGCC (AS)
Mouse Notch3	ACACTGGGAGTTCTCTGT (S) GTCTGCTGGCATGGGATA (AS)
Mouse Notch4	TGCCTGCACAATGGTACCTG (S) TCTGGCTTCAGTGCCTTAAG (AS)
Mouse DLL4	GCAACTGTCCTTATGGCTTTG (S) GCAGGGATTAGGTTGTCCTTC (AS)
MM VEGF	GAGCCTTGTTCAGAGCGGAG (S) CAACGGTGACGATGATGGCA (AS)
MM Flt-1	CTGACCTTCGCATACTGCTCA (S) CTGTCCTCTCTGGGTCTTGG (AS)
MM Flk 1	GTCAGGAAACGCAAAGGCGG (S) CTCCACCCAGCAGAAACCCT (AS)
MM Ang-1	TCCTTTGCATTCTTCGCTGCC (S) GGAAGAGAAATCCGGCTCCAC (AS)
MM Ang2	AGACAGCAGCACAAACTCGG (S) GCTTGGACACCAGCACCTG (AS)
MM Tie-2	CCTGCCAAAAAGCCAGACAGC (S) TTCACTCCACTCCCCCTGCG (AS)
MM ephrin B2	GGATGCATCATCTTCATCGTC (S) GACGCTGTCTGCAGTCCTTAG (AS)
MM Eph B4	ACTCTGCTTTCGGTTCTGTGG CACGGTGGTGAGTCCTTGGA
Mouse bFGF	ATGGCTGCCAGCGGCATCAC (S) GAAGAAACAGTATGGCCTTCTG (AS)
Mouse HPRT	GTTGGATACAGGCCAGACTTTGTTG (S) GATTCAACTTGCGCTCATCTTAGGC (AS)

Protein extraction and Western blot analysis. Protein extraction of cells or tissues and Western blot analysis with polyclonal anti-Notch1^{extra2} (1781-B), antibodies against N1^{EC} were performed as previously described (Hoemann et al., 2000).

Microfil® perfusion. Microfil® (Flow Tech, Carver, MA, USA) is a two-component silicon-rubber curing agent that is used for visualization of the vasculature of other sites in the body, such as the kidney glomeruli (Norlen et al 1978). For postnatal and adult mice, Microfil® perfusion (0.5-2.5 mL) was performed (after thorachotomy under Avertin anaesthesia) via the apex of the left ventricle while the heart was still beating, as described previously (Coral-Vazquez et al., 1999) as well as via the portal vein or the vena cava. Microfil® perfusion (0.2 mL) of E16.5 embryos was performed through the umbilical vein under Avertin anaesthesia. Whole livers were fixed by immersion in 3.7% formaldehyde buffer with PBS, then dehydrated with serial ethanol and cleared in methylsalicylate, as described (Coral-Vazquez et al., 1999).

Tissue sampling and microscopic analysis. For routine histological analysis, mice were killed by CO_2 inhalation or under avertin anesthesia, and organs to be evaluated were dissected and fixed by overnight immersion in 3.7% formaldehyde buffered in PBS. Organs to be assessed were embedded in parraffin, sectioned into 5 μm slices, and stained with hematoxylin and eosin or Masson's trichome, as described previously (Hanna et al., 1998; Kay et al., 2002). Slides were examined by at least two investigators blindly.

Electron microscopy. Mice were perfused with 2% glutaraldehyde in 0.1 M, pH 7.4, cacodylate buffer (10 min). The perfused livers were further fixed in cacodylate buffer (2 h) and therein rinsed with 20% sucrose. The fixed tissue from different areas was cut into 1-mm blocks and subsequently performed series of procedures as described (Breiner et al., 2001).

Assessment of Transgene expression by *In situ* Hybridization (ISH). ISH was performed on paraformaldehyde perfusion fixed, paraffin-embedded tissues using a hCD4-exon-1-specific 35S-UTP-labeled antisense probes or control sense RNA probes, as previously described (Hanna et al., 1998a; Hanna et al., 1998b). Tissues from non-Tg control animals hybridized with antisense probes, as well as Tg animal tissues hybridized with sense probes were used as controls.

Immunohistochemistry (IHC). IHC was carried out on freshly frozen 10 μm liver sections. Sections were prepared from liver tissues removed immediately after sacrificing mice. The liver, cut transversely, was embedded in OCT (Miles Chemicals) and immersed into isopentane pre-chilled in liquid nitrogen to the freezing point (viscous liquid). The anti-PECAM-1 (PharMingen clone: 13.3E), the anti-SMA (Sigma clone: 1A4), and the anti-Mac-1 (Cederlane) antibodies were used. IHC with anti-SMA was done on both frozen and formaldehyde perfused 5 μm sections. Incubations with primary antisera were kept 2 hours at room temperature with 1:10 anti-PECAM-1, 1:200 anti-SMA, and 1:200 anti-Mac-1. Following the incubation with the appropriate secondary antibodies conjugated to HRP, the immunoreaction was detected using DAB as a chromogen. Tissues were counterstained with hematoxylin.

β-galactosidase staining. For liver fragments, the liver tissue was perfused with 4% paraformaldehyde for 5 min. The samples were then washed in PBS containing washing buffer solution (2 mM MgCl2, 5mM EDTA, 0.01% sodium deoxycholate and 0.02% NP-40) and stained in fresh X-gal solution at 37 °C overnight as described (Lyden et al., 2001). The X-gal stained liver fragments were directly examined under inverted light microscope. The livers were then embedded in paraffin, sectioned and counter-stained with nuclear fast red to visualize LacZ-negative tissues. For liver sections, the perfused livers were frozen in

OCT and sections (5 μm) cut. Washing and staining were then performed on a slide as described previously (Lyden et al., 2001).

Evans blue. Evans blue (20 μL/10 g) was injected via the tail vein of the mice before 1 to 2 hours killing mice. Protocol to assess the amount of EB was kindly provided by Dr. Jean-Philippe Gratton (IRCM, Canada). Briefly, perfused livers were drying and extracted by formamide. The EB amount in the formamide was measured at 610 nm. The final EB amount per mg 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 Rohdamon fluorescence was finally observed using 10 nm sections by a fluorescent (Zeiss) microscope.

Capture of latex beads. Latex beads (Sigma, L-4530) were injected into mice (2-to 4-month-old) via the tail vein (8.7 X 10^7/200 μl PBS per mouse) one or two hours before killing the mice. Mice were perfused with 4% paraformadehyde under Avertin anaesthesia. The livers were excised and frozen in OCT and liver sections (10 nm) prepared. The latex bead fluorescence (FITC) was observed under a fluorescent (Zeiss) microscope.

Flow cytometry. Single-cell suspensions of thymus, spleen, and liver (NPLCs) were prepared from Tg and non-Tg age-matched littermates. The antibodies used in this study were CD8, CD4, Mac-1 [as previously described (Simard et al., 2002)], and a FITC-coupled anti-mouse ICAM monoclonal antibody (Pharmingen Canada). The experimental procedure was performed as previously described (Simard et al., 2002).

Partial hepatectomy. Partial hepatectomy was achieved under Avertin anaesthesia as described (Wuestefeld et al., 2003; Borowiak et al., 2004). Since Tg mice easily died (3/5) 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.

Bone Marrow transplantation (BMT): After sacrificing the mice (5 nTg and Tg CD4C/N1^{EC} mice per group,10 to 15-week-old.), the bones of the mouse (femur and tibia) were removed and cleaned in RPMI 1640 + penicillin/Streptomycin + L glutamine (bone marrow media) under sterile conditions. The bone marrow was got out by flushing with a 26 G needle full of bone marrow media. Single cell suspension was made using a 26 G needle. Cell suspension was filtered through a Nytex mesh (BSH Thompson, Montreal). After one wash, cells were counted (RBC cell lysis was performed in the counting aliquot). Cells were then resuspended in HBSS solution supplemented with 2% FBS at a concentration of 20 X 10^6 cells per mL and finally injected to hosts, C3H mice.

Fetal liver (FL) cells transplantation (FLT): **Donors-**Fetal livers from 14.5-day-old embryos (E14.5) (CD4C/N1^{EC} X C3H) were harvested. Single cell suspension was made in HBSS supplemented with 10% FBS, under sterile conditions, with a syringe plunger. Cell suspension was filtered through a Nytex mesh (BSH Thompson, Montreal). The remaining fetal tissue was typed for Tg expression by PCR. When typing was known, cells coming from Tg and nTg CD4C/N1^{EC} embryos were pooled, respectively. After one wash, cells were counted (RBC cell lysis was performed in the counting aliquot). Cells were then resuspended in HBSS solution supplemented with 2% FBS at a concentration of 20 X 10^6 cells per mL. **Hosts**-C3H mice or CD1 nude mice (8 to 12 weeks old) were lethally irradiated (950 Rad for C3H mice and 400 Rad for nude mice) using Mark I-68A1 Irradiator (Cs-137, J.L. Shepherds & Associates). Hosts were injected, via the tail vein, with 4 to 15 X 10^6 fetal liver cells (nTg and Tg, respectively) in 0.2 mL of HBSS solution

supplemented with 2% FBS 4-6 hours after irradiation. Mice were analyzed after 2-6 months.

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 medium containing 10% fetal bovine serum (FBSI), β-mercaptoethanol, and P/S. After centrifugation (14 000 rpm) for 5 min at 4 °C, the supernatants were discarded and the residual pellet was washed twice with medium. The pellet was then suspended in a 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, USA) in ice-cold PBS. The purity of macrophages (>95%) was confirmed by FACS analysis with anti-Mac-1 staining.

Macrophage transplantation. Peritoneal macrophages (3×10^6) purified from male mice were intravenously injected into female mice by the tail vein in 200 μL of PBS. One month later, the recipient mice were processed for Microfil® perfusion and histology, in order to observe the liver phenotype.

Isolation of non-parenchymal liver cells (NPLCs). Single cell suspensions were prepared from livers of two or three 10-12-week-old mice according to a published protocol (Do et al., 1999) with minor modifications. Briefly, 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 medium, pH 7.4, at 37 °C for 20-45 min with occasional shaking (once per five min). The resulting cell suspension, diluted in 40 mL of serum-free RPMI, was

centrifuged 3 min 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 min. The interface cells containing LSECs and Kupffer cells were harvested and centrifuged at 1500 rpm for 10 min to pellet the cells. These cells were further processed for FACS analysis and MACS purification.

Purification of liver sinusoid endothelial cells (LSEC) with MACS. LSECs and Kupffer cells were isolated by anti-CD11b-conjugated and by biotinylated ICAM-1/streptavitin-conjugated magnetic bead cells sorting, respectively, according to a published protocol (Do et al., 1999). Approximately 10^6 LSECs were obtained from two livers. Their purity (80%) was confirmed by take up of Dil-Ac-LDL (Molecular probe).

LESCs culture. Freshly isolated LSECs were plated onto 0.1% (w/v) gelatin-coated 48-well NUNC plates with medium containing DMEM/F12 (Invitrogen), 15% FBSI, and 100 μg/mL ECGS (Sigma). Ten hours later, non-adherent cells were washed off and fresh medium was added. Cells grew to confluence in 5 days and were ready for angiogenesis assay, conditioned medium assay, and co-culture assay.

Conditioned medium assay. For conditioned medium assay, peritoneal macrophages were cultured in 2 mL of MF12 medium containing 15% FBSI 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. The monolayers of LSECs were treated with supernatants of macrophages and 3 days later, the LSEC were observed under the microscope and counted.

Co-culture assay. Isolated LSECs were seeded at a density of 1 X 10^5 per well in a 96-well plate in MF12 medium containing 15% FBSI. Once the LSECs have formed

monolayers, the peritoneal macrophages were isolated and cultured in MF12 medium containing 15% FBSI. Twelve hours later, the macrophages were incubated for 3 hours at 37 °C with latex beads (Sigma, 4 X 10^6/mL), then washed and incubated with LESC. The clusters of macrophages were scored after 4 to 72 h of coculture. Then, an assay for myeloperoxidase was performed (Yasuda et al., 2000).

Angiogenesis assay. When LSECs were confluent, the ECMatrix™ gel (ECM625, Chemicon International, Inc. Temecula, CA) was prepared according to the supplier's instructions. Briefly, 100 μL of 10X Diluent Buffer was mixed with 900 μL of ECMatrix™ solution on ice. Then, 50 μL of the solution was transferred to each well of pre-cooled 96-well tissue culture plates and incubated at 37 °C for at least 1 hour in order to allow the matrix solution to solidify. The LSECs were harvested and suspended in the DMEM/F12 medium containing 15% FBSI and 100 μg/mL ECGS. About 104 cells were seeded per well onto the surface of the ECM™ and incubated at 37 °C. Cellular network structures were recorded between 2 and 12 hours under an inverted light microscope (20X).

Figure legends

Figure 1. Structure of the CD4C/N1^{EC} and its expression in the Tg mice

|**A. Diagram of the structure of the transgene**. Black bar, the CD4C regulatory sequences; strippled bar, the N1^{EC} cDNA containing 36-EGF-like repeats; black bar, the polyadenylation sequences from simian virus 40 (SV40). A stop codon was inserted after the 36 EGF-like repeats at the amino acid residue 4420, thus deleting the whole C-terminal half of the protein. C: Cla, E: EcoRI, N: NotI. |**B. Northern blot analysis of RNA from Tg mice**. RNA (10 μg) was extracted from different tissues and hybridized with ^{32}P-labled probe M, specific to the Notch1 EGF-like repeats. The filters were then washed and rehybridized with the 18S ribosomal-specific probe; T: Thymus; L: liver; LN, lymph node; K: kidney. Negative controls (-): HC11 cells, lane 8 or nTg thymus, lanes 16, 17. Positive controls (+): HC11 cells transfected with N1^{EC} (lane 7) or Tg thymus, lanes 15, 18. |**C. RT-PCR analysis of RNA from Tg BM and FL**. Exogenous N1^{EC} was detected by the two primers, which were designed from the exon 1 of hCD4 and the EGF-like repeats of Notch ectodomain, respectively. BM: bone marrow; FL: fetal liver; KCs: Kupffer cells. Negative (-) control: nTg lymphnodes; positive (+) control: Tg lymphnodes. |**D. Western blot of N1^{EC} proteins**. Total protein extracts (100 μg) from whole thymus or liver, sorted $CD4^+CD8^+$ thymocytes and from isolated peritoneal macrophages of the Tg and non-Tg littermates were evaluated with rabbit polyclonal antibody specific to N1^{EC}. The membrane was then stripped and reached with anti-actin antibodies. Negative (-) and positive (+) controls are respectively HC11 and NI^{EC}-expressing HC11 cells. |**E. *In situ* hybridization.** The liver sections were probed with sense and antisense probe for the exon1 of the hCD4 genes. The clustered cells around the disrupted vessels in the Tg mice were probed with the antisense probe (e, g, i, and j), but not with the sense probe (d and h).

No expression was detected in the nTg mice with either the sense or the antisense probes (a and b). Expression in KC-like cells was particularly detected (g, i, and j). Dark field: a,b,d,e,h, and i. Bright field: c,f,g, and j. a-f: 20X; g:5X. h,i, and j: 40X. |**F. RT-PCR analysis of RNA from Tg KCs**: KCs were purified from the liver and further detected the expression of Tg $N1^{EC}$ by RT-PCR as performed in C.

Figure 2. Liver vascular patterning defects phenotype

|**A. Macroscopic analysis of control non-Tg (a and d) and Tg (b, c, and e) liver (2-4 months).** The Tg livers were either smaller or bigger than the non-Tg ones and had irregular shapes. After perfusion with Microfil®, huge and spider-like white vessels were observed at their surface (b). Multiple, dark red tumor-like structures with convex surface were present on the liver surface (c and e). Cross-sections demonstrated that the tumor-like structures are cavities filled with the blood liquids (g), not seen in nTg liver (f). a,d,f: the nTg mice and their livers, b,c,e,g: the Tg mice and their livers, a and b: the mice perfused with Microfil®; d,e,f,g: the mice perfused with 3.7% formaldehyde. c: fresh Tg mouse-liver before perfusion with 3.7% formaldehyde. f and g: The cross-sections of the livers from the mice (d and e). |**B. Microfil® analysis.** Vascular morphology was examined in each lobe of livers in both the nTg and the Tg mice and similar results were obtained with each lobe. Results from left lobe are shown here (a,c,e,g,j, and l). Note the extensive vascular branching and the homogeneous capillary in non-Tg mice (a-d). In Tg mice, fewer, shorter, and straighter branches were observed (e and f). Quantitation of branching (o). In addition, various remodeling defects of the vasculature were observed in the Tg livers (g-l). Large vessels are growing ectopically out of the liver (arrow) and regrow into the liver parenchyma with clustered and dilated capillaries, which are sprouting from the main branch (h,i, and k). In the parenchyma, fewer large vessels were observed, but

sometimes numerous clustered hemangioma-capillaries are abnormally branching from the main branches (l,m, and n). a,c,e,g,j,l: 0.5X1.0; b,d,f,h,k,m: 0.5X5; i and n: 0.5X10.

Figure 3. Liver and vessel remodeling in CD4/N1^{EC} Tg mice

|**A. Histology.** In contrast to the homogeneous distribution of the vessels of non-Tg livers (a and c), Tg livers show heterogeneously huge vessels and capillaries on the liver surface and within the liver (b and d) and huge cavernous vessels (hemangiomas) (d, asterisk) with irregular shape and filled thrombi within their lumen (d, arrow), that have destroyed normal hepatic structure. a and b: The livers were perfused with Microfil®. C and d: The livers were perfused with 4% formaldehyde. 1.25X. |**B. Characterization of vessels in CD4C/N1^{EC} Tg mice.** Liver sections, for electronic microscopy, were processed with hematoxyline & eosine staining. The nTg and Tg livers are shown (a-d and a'-d'). The liver of non-Tg mice (a,b) shows homogenous and 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 shows heterogenous and irregular lobules (a'b'). Reverse lobules in which PV, PA, and BD are located in its center instead of CV (b'). Normally intercalating (d-S1) and paralleled (c-S2) sinusoids are clearly distributed in the non-Tg lobule Zone "1" and Zone "2", respectively (b'). However, in the Tg lobules (b'), sinusoids in the Zone "1" become dilated and capillarized (d'-S1) and filled with blood cells (angiectasis). The parallele sinusoids have disappeared in some regions of the Zone "2", where more than 5 hepatocytes are clamped together without sinusoids (c'-S2). PA: portal artery; PV: portal vein; CV: central vein; S: sinusoids; BD: bile duct (dark arrow); Green arrow: large vessels on the surface of the liver. a, a': 2.5X; b, b': 10X; c, c', d, d': 40X. |**C. Immunochemistry. 1. P-ECAM-1.** In nTg liver, the vessels were homogeneously stained with P-ECAM-1, especially the large vessels (C1a), but heterogeneously distributed vessels were observed

in the Tg liver, particularly with capillarized vessels (C1b,c). Large vessels on the liver surface were also stained (C1d). No staining was detected with the 2nd Ab (C1c',d'). C1-a,b:1X5; c,c',d,d': 1X10. **2. a-SMA immunohitochemistry and Masson's trichrome staining.** Few or no cells around vessel were stained brown with a-SMA in the nTg liver (C2a). Note that the stained cells, supposed to be smooth muscle cells, were heterogeneously distributed around the vessels of the Tg liver (C2b-d). Accumulated cells around disrupted vessels were stained in blue color (supposed to be extracellular matrix) with Masson's trichrom staining in the Tg liver (C2f), but not in the nTg liver (e). (g,h) HE staining. C2-a,e: 5X; b,f: 2.5X; c,d,g,h: 10X.

Figure 4. Bonne Marrow Transplants (BMT) yielded the liver vascular defects

The 10 to14-month-old C3H mice were transplanted with BM cells and were analyzed after 4-month transplantation. |**A. RNA expression of the $N1^{EC}$ in the C3H hosts transplanted with the BM cells from the Tg mice:** The Tg expression in the thymus and in the LNs was detected by Northern blotting (a) and the expression in the peritoneal macrophages and in the liver was detected by RT-PCR (b). 18S rRNA and HPRT serve as control for Northern blot and RT-PCR, respectively. (+): positive control, Tg LNs; (-): negative control, nTg LNs; a: 1.3.5-Thymus; 2.4.6-lymph-nodes. b: 1.3-Tg BM cells to C3H mice; 2.4-nTg BM cells to C3H mice. |**B. The liver phenotype reconstituted by BMT:** All mice transplanted with the nTg BM (a) or Tg BM (b) cells were perfused with Microfil®. Higher magnification of the Tg liver (c) aims at showing defective vessels and cavities on the surface of the liver. |**C. Liver vascular anormalies observed by Microfil®:** The livers perfused with Microfil® from the "Figure B" were observed with microscopy. Huge vessels were apparent on the surface of the liver from mice reconstituted with Tg BM cells (d and e, arrow). A large cavity (d, asterisk) and a cluster of capillaries-

hemangioma vessels (d, circle) were also observed. No such lesions develop in the liver of mice transplanted with non-Tg BM cells (a and c). a,b: 0.5X1.6; c,d,e: 0.5X10.

Figure 5. Reproduced liver phenotype in CD4C/N1^{EC} X Rag$^{-/-}$ mice

|**A. Mice survival was monitored at three time points: P3.5-4.5, P5.5-6.5, and adults.** Few Rag $^{-/-}$ mice expressing N1^{EC} survived after day p5.5 stage compared to Rag $^{+/-}$ mice expressing N1^{EC}. |**B. Genotyping and the liver vascular phenotype: 1. Genotyping.** CD4+CD8+ double positive thymocytes were detected by FACS analysis, in both the postnatal and the adult Rag $^{+/-}$ mice, which was > 93%. This population was absent in the Rag$^{-/-}$ mice (N1^{EC} + and N1^{EC} -). **2. The liver vascular phenotype.** The nTg liver (a and b) and Tg (c and d) liver with or without lymphocytes were compared. At the P3.5, irregular capillaries (arrow-head), large vessel (arrow), and cavities (stars) were displayed in the Tg Rag $^{-/-}$ mice (d,i,j,h, and j'), similar to the phenotype observed in the Tg Rag $^{+/-}$ mice (c and g). These phenotype were not seen in the nTg mice (a,e b, and f). In Tg Rag $^{-/-}$ adults, huge vessel along the edge of the liver with irregular capillaries sprouting from them were observed (d). These phenotypes are similar to those shown in the Tg Rag $^{+/-}$ liver (c). p3.5: a-d,i,j: 3.2X0.5; e-h,j': 10X0.5 in bright field. Adult: a-d 0.5X4.0 in dark field.

Figure 6. A paracrine pathway induces the liver vascular phenotype

|**A. A physical interaction between KCs and LSECs was observed in the sinusoids of both the nTg and Tg liver**. KCs can stay in sinusoid as floating in the sinusoid (b), touching the pad of the endothelial cell (a and d), and interacting with the body of the endothelial cell (c). In the Tg liver, two KCs are often present in the sinusoid (d) compared with the one KC in the nTg sinusoid (a and c). |**B. Chimeras.** The ES cells at the day E2.5 were harvested from $ROSA_{26}$ X CD1 and CD4C/N1^{EC} X CD1 mice. They were fused together and 24 hours later implanted in to pseu-pregnant CD1 mice (1). X-gal staining

was performed on the livers from chimeras (2). In the Tg chimera, large vessel located on the surface of the liver is stained in blue color (b) showing ROSA-origin. No such vessel was observed in the nTg liver (a). |**C. FLT.** Fetus liver cells (at day E14.5 to 15.5) from CD4C/N1^{EC} X ROSA$_{26}$ mice were transplanted into nude mice (1). Two months later, mice were killed. The transgene was detected by RT-PCR. (2). The liver vascular phenotype was only reproduced in the Tg expressing mice. A piece of liver with X-gal staining (3) demonstrated white large vessels on the surface of the liver (arrowhead), accompanied by blue color mass of cells (b, arrow). No such large vessels were found in the liver from the mice transplanted with the nTg cells (a). In the liver section, abnormal vessels (arrow) and increased numbers of hematopoietic cells, including macrophages (asterisk), were observed in the liver from the mice transplanted with the Tg cells (d), but not in mice receiving non-Tg cells (c). The abnormal vessels in d stained negative (white), but were surrounded by blue hematopoietic cells. Mouse 1: transplanted with nTg cells and mouse 2 and 3 with Tg cells. a and b: 5 X; c and d: 20 X

Figure 7. Macrophages are sufficient to induce the liver vascular phenotype

|**A.** Infiltration of macrophages around abnormal Tg vessels. Frozen liver sections were used for IHC using the monoclonal Ab 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 in the Tg liver (b). Higher magnification showed large stained cells (c). a and b: 10X; c: 100X. |**B. Macrophage transplantation.** Peritoneal macrophages (3×10^6) from Tg and non-Tg mice were transplanted by intravenous inoculation into normal mice (8 to 10-week-old) and recipient animals were killed 1 month later and perfused with Microfil® (a-c, and a'-c'). Note the homogeneous vasculature of the liver from a mouse transplanted with the non-Tg macrophages (a, b, and

c), and the abnormal vessels observed in the liver of a mouse transplanted with Tg macrophages (a', b', and c'). Large vessels are growing out of the liver and lying on its surface (b' arrow) and walking along the edge of the liver (c' arrow-head). a and a': 0.5X0.8; b, b', c, and c' 0.5X5.0. (d and e). Histological analysis performed on the Microfil®-perfused livers showed superficially clustered large vessels in the liver from a mouse transplanted with Tg macrophages (e), but not in the liver from a mouse transplanted with non-Tg cells (d). 5X.

Figure 8. Profile of liver lining cells in the CD4C/N1^{EC} Tg mice

All the liver cells used in our experiments were purified from livers of 10 to 14-week-old mice. To get non paranchymal liver cells (NPLCs), a digested liver (Collagenase IV and DNase I) was centrifuged in metrizamide gradient. |**A.** Different number of the NPLCs was obtained from nTg and Tg liver. |**B. NPLCs-FACS.** FACS analysis performed with ICAM-1 FITC and mac-1 PE (identify the LSECs and the KCs respectively) show different cell percentage between nTg and Tg NPLCs. |**C. LSECs and KCs were purified from NPLCs by MACS separation.** Different cell-number was obtained from nTg and Tg NPLCs (2-3 pooled livers) (a and b). The LSECs were confirmed by "uptake" of Dil-Ac-LDL (**Data not shown**) showing 80% purity and confluent cultures formed monolayer cell-sheet with the typical spindle shaped cells (c). The KCs were confirmed by Giemsa-Granules staining, showing 90% purity (d).

Figure 9. Functional abnormalities of the Tg LSECs in the network formation

Purified LSECS were cultured at 37 °C and then used for the angiogenesis assay. |**A. network formation was detected by Angiogenesis assay kit.** Closing networks were formed by nTg LSECs (a). Few or no networks were formed by Tg cells (b). |**B. Distinct signs of network formation were evaluated in both nTg and Tg LSECs.** Alignment:

two distinct cells align together (a); Branch: more than two cells were connected together to form branch (b); network: alignments and branches progressed to form networks (c). Lower amounts of each sign were observed in Tg group than in nTg one (B). |**C. Less signs were demonstrated in Tg LSECs than in nTg LSECs at different time.** The signs of tube formation were not recorded after 9 hours culture, because endothelial cells were going to die on the kit after 8 hours, consisting with the kit instructions. |**D. Quantification of cells:** After counted on the signs, cells in the kit were harvested and quantified by micro-BCA. 0.55X2.5.

Figure 10. Enhanced adhesion of CD4C/N1^{EC} Tg macrophages *in vitro*.

|**A. Increased colony formation of Tg macrophages *in vitro*.** Peritoneal macrophages from Tg or non-Tg mice were co-cultured with Tg and non-Tg LSECs, labeled with latex beads and Dil-Ac-LDL, respectively. The number of clusters formed by macrophages clumped onto the LSECs was different between nTg and Tg macrophages beginning at 3-4 hours after the co-culture (a-d). Each clusters formed by Tg macrophages (b and d) had a more compacted shape than nTg macrophages (a and c). More clusters were observed on Tg LSECs (c and d) than on nTg LSECS (a and b). 5X. |**B. Quantification.** |**C. Myeloperoxidase activity:** Macrophage adherence was confirmed by this assay.

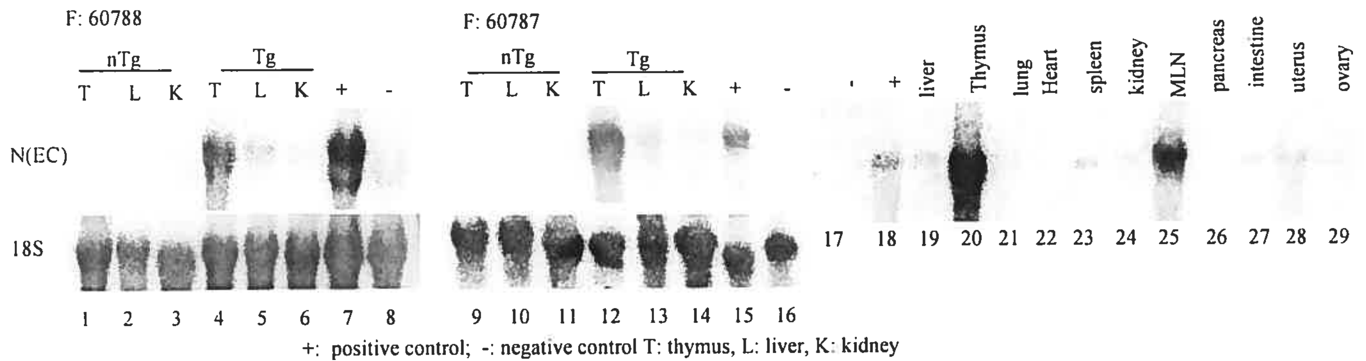
Figure 11. Supernatants of the Tg macrophages inhibit the growth of nTg LSECs

Purified LSECs were incubated with conditioned medium from nTg and Tg peritoneal macrophages in 48-well plates. After 6 days incubation, cell density was observed (a-c, 20X) and the number of cells was counted (d).

Figure 1.

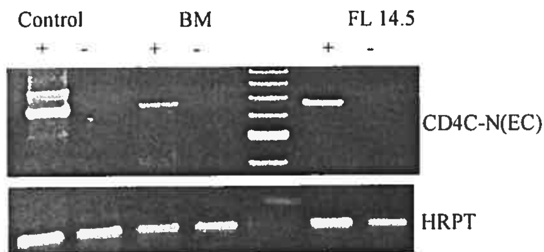
A. CD4C/N1(EC) construct

B. RNA expression of the N1(EC) in theCD4C/N1(EC) Tg mice -Northern Blot



+: positive control; -: negative control T: thymus, L: liver, K: kidney

C. RNA expression of the N1(EC) in the CD4C/N1(EC) Tg mice by RT-PCR



D. Protein expression of the N1(EC) in the CD4C/N1(EC) Tg mice by Western Blot

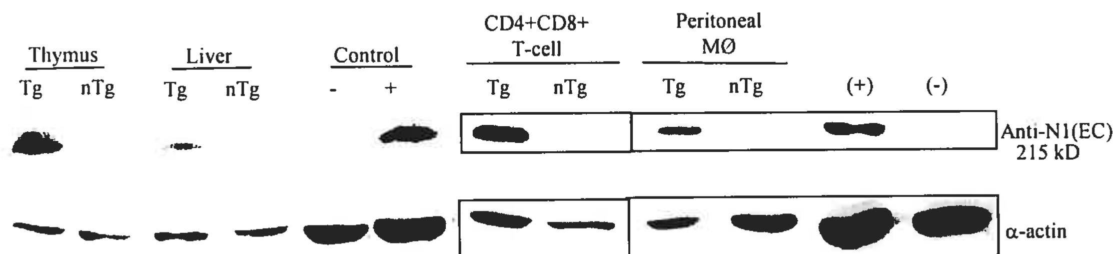
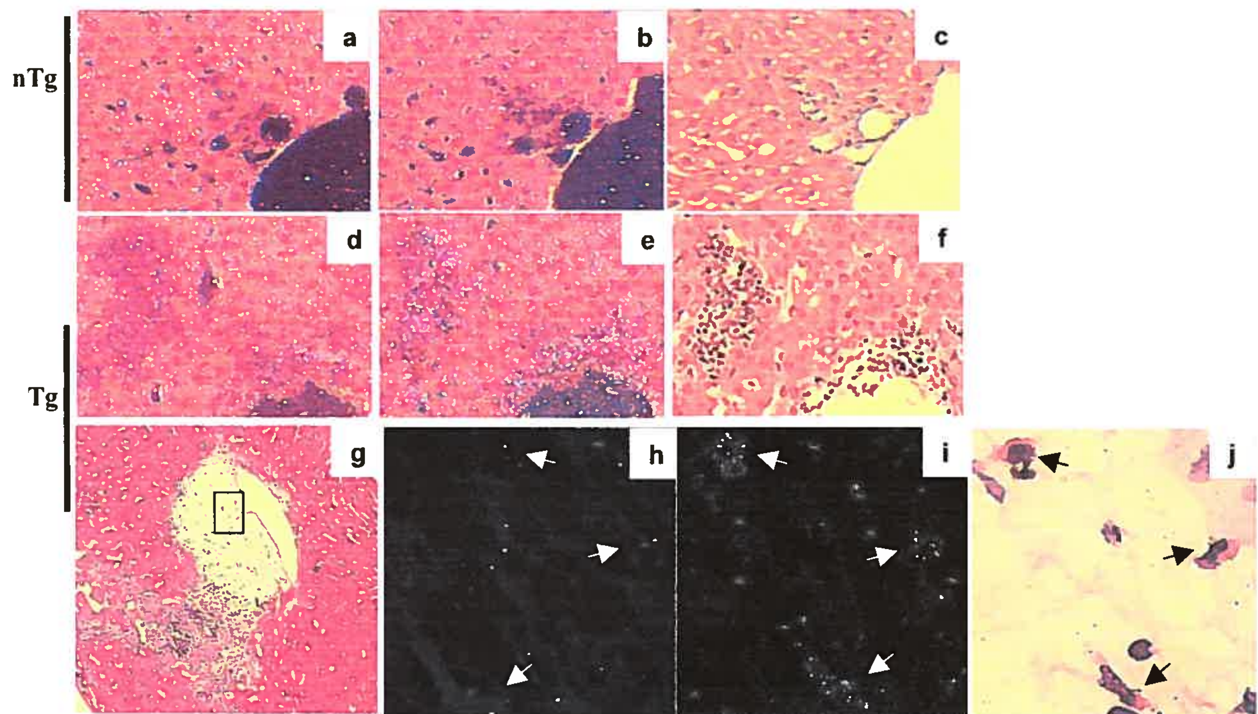


Figure 1.

E. RNA expression of the N1(EC) in the liver by *in situ* hybridization



F. CD4C/N1(EC) expression in the KCs detected by RT-PCR

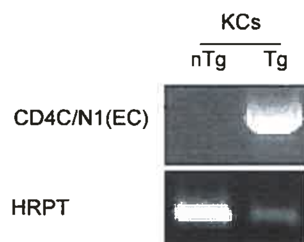
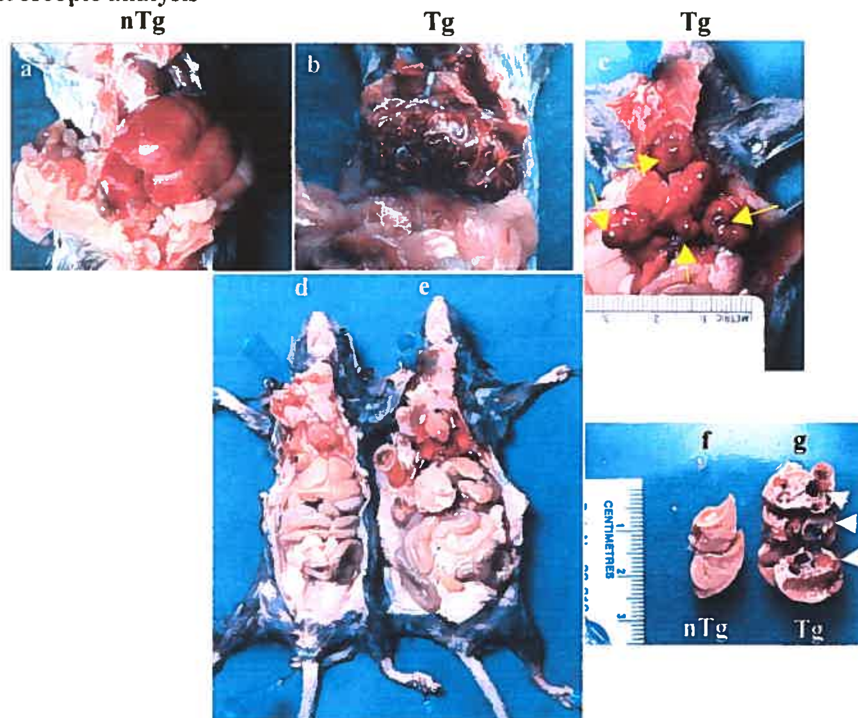


Figure 2.

A: Macroscopic analysis



B. Microfil:

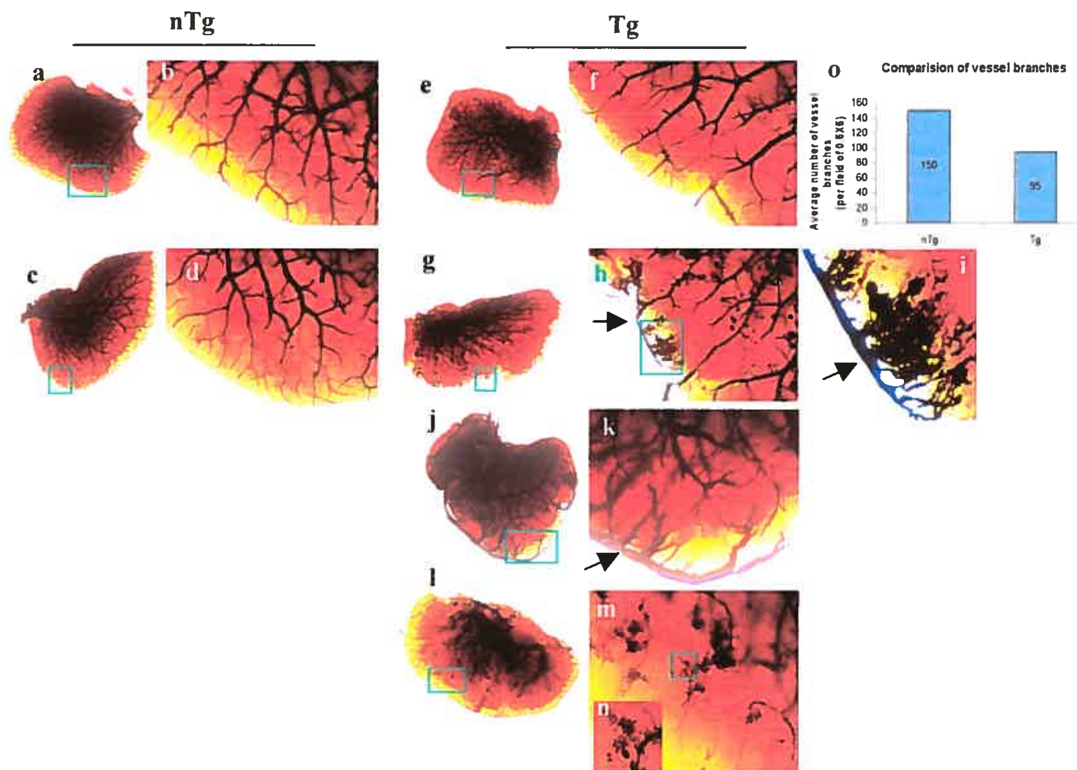
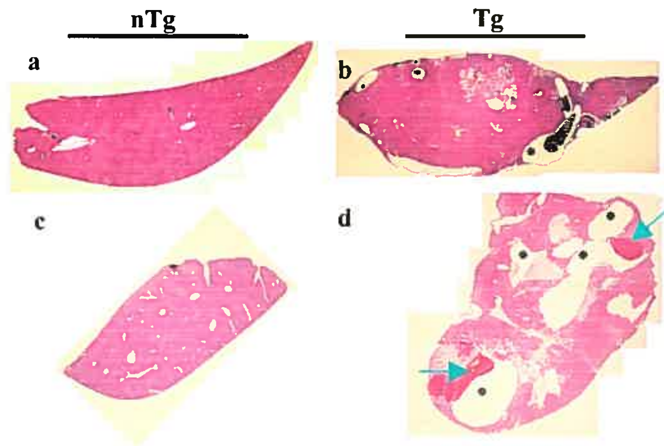


Figure 3.

A. Histology



B. Characterization of vessels

Figure 3.

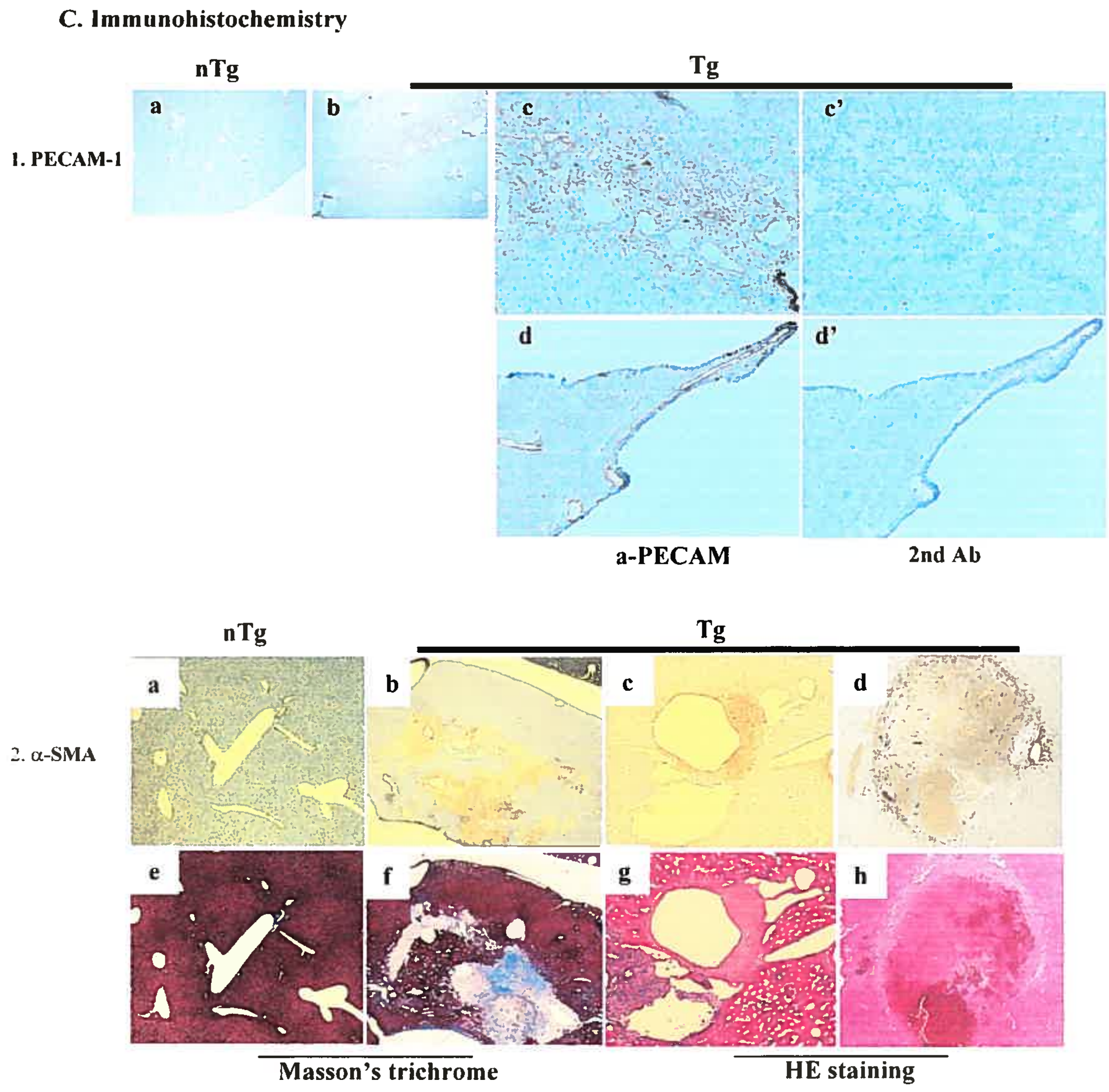
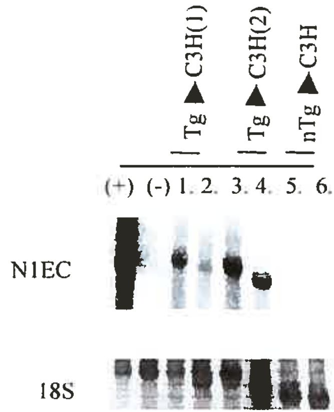


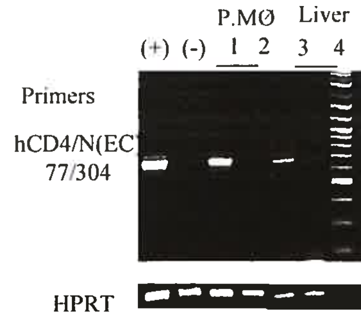
Figure 4.

A. Expression of transgene

a. Northern Blot

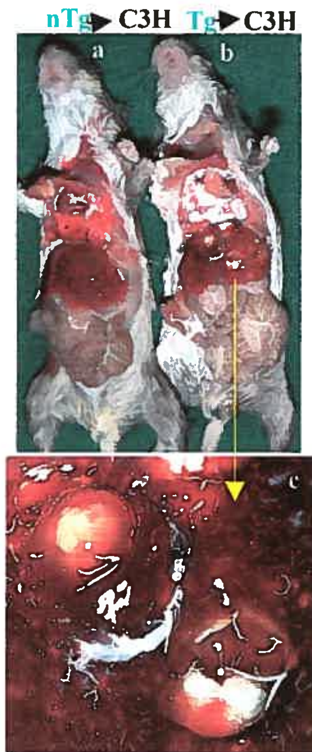


b. RT-PCR



1,3: Tg ▶ C3H, 2,4: nTg ▶ C3H

B. Liver phenotype



C. Reconstitution of vascular abnormalities

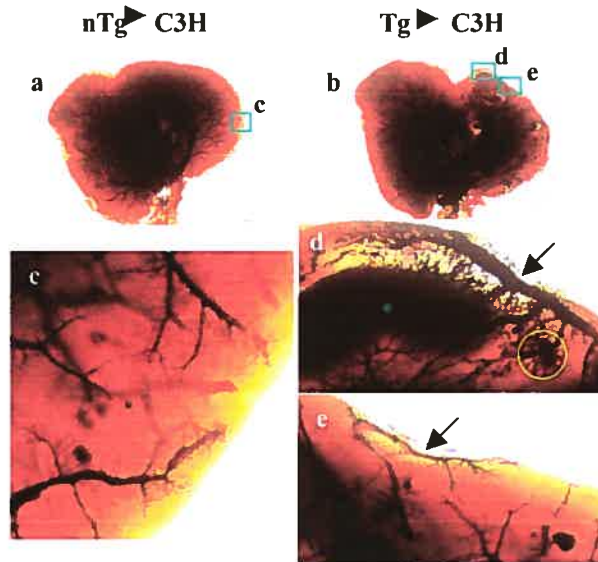
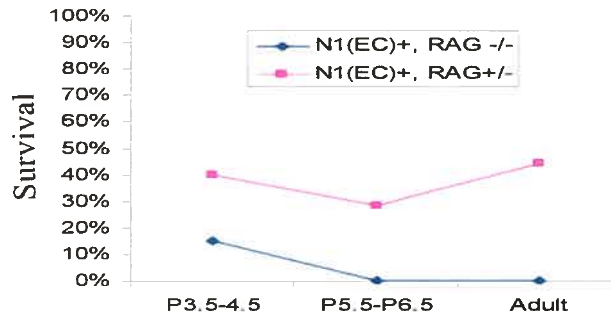


Figure 5.

A. Mendelian Ratio



B. Expression of CD4+CD8+ in the thymus and the vascular phenotype in the liver

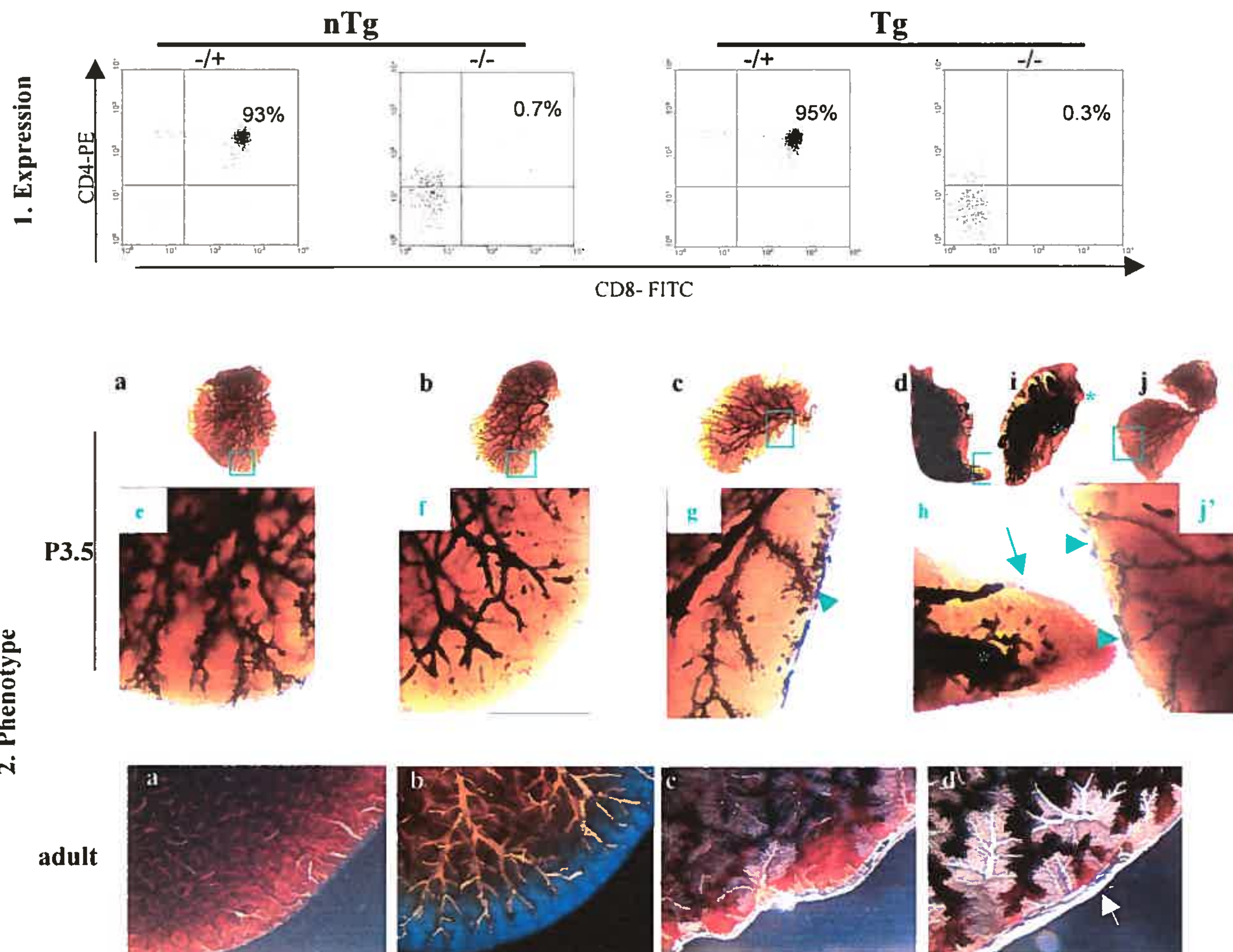
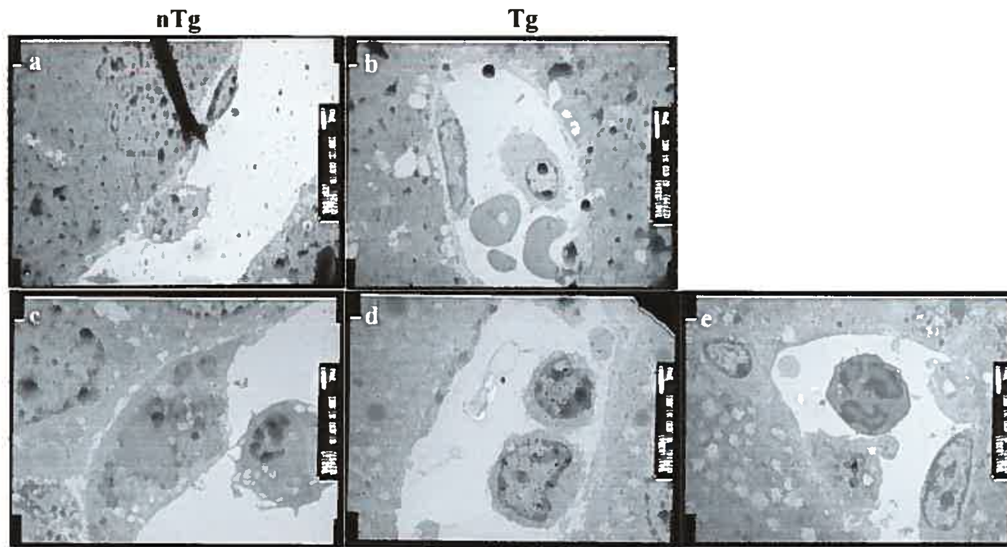
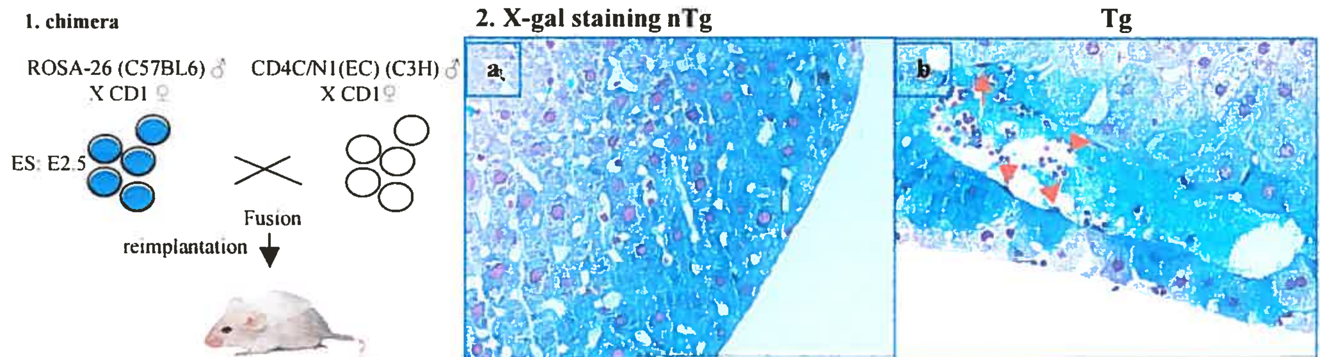


Figure 6.

A: Physical Interaction of Kupffer cells and LSECs observed in the sinusoids of CD4C/N1(EC) Tg mice by EM



B. Chimeras with ROSA and CD4C/N1(EC)



C. FLT

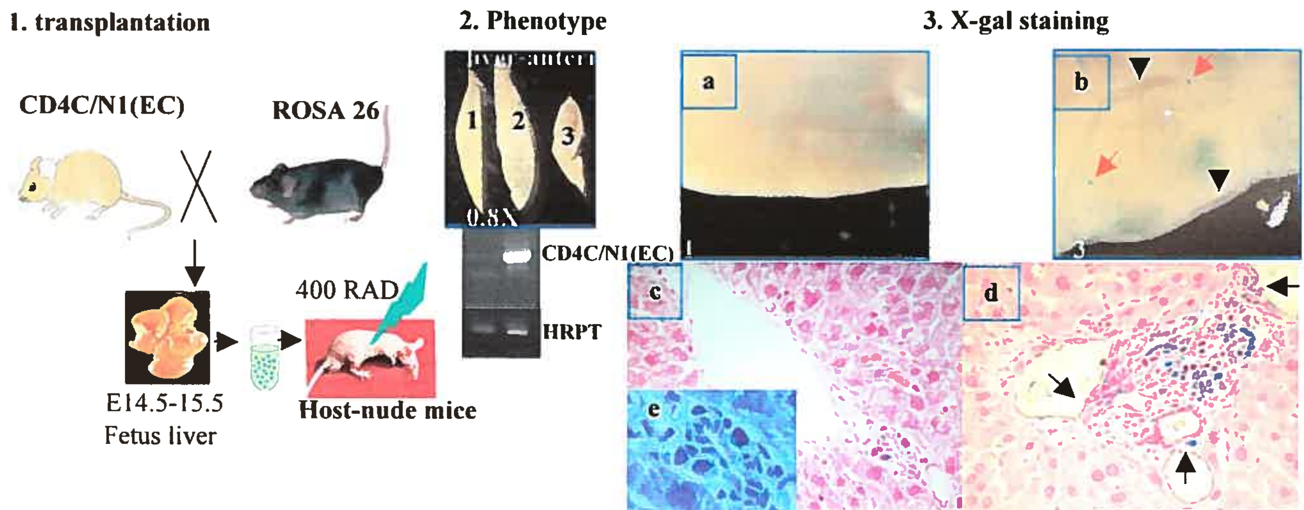
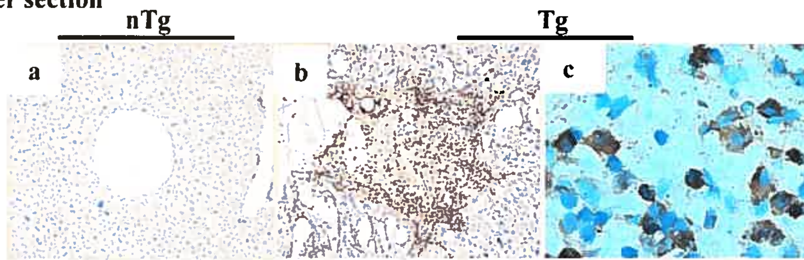


Figure 7.

A. IHC-mac-1 for liver section



B. Macrophage transplantation

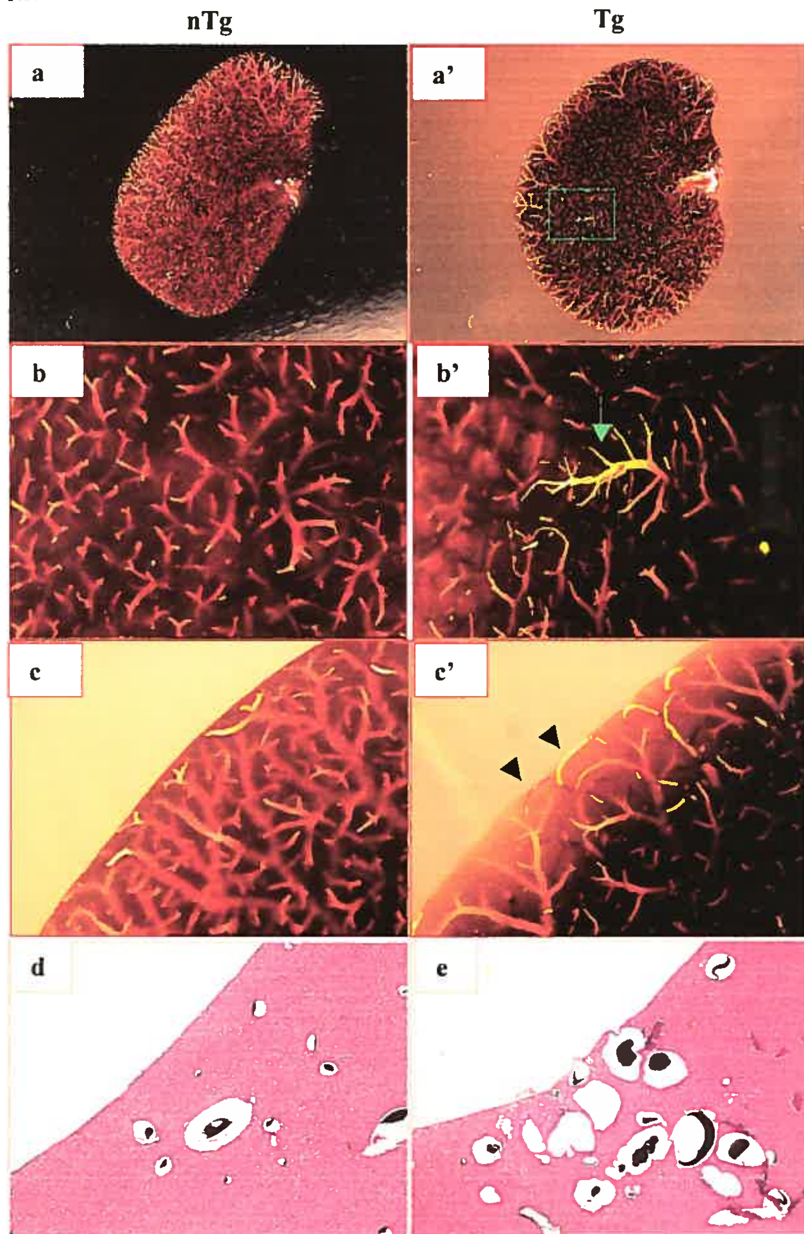
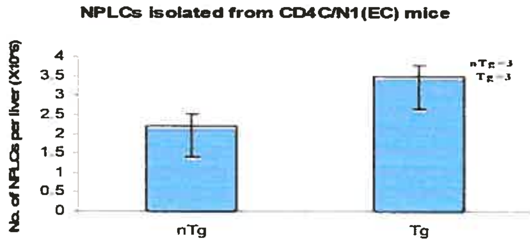


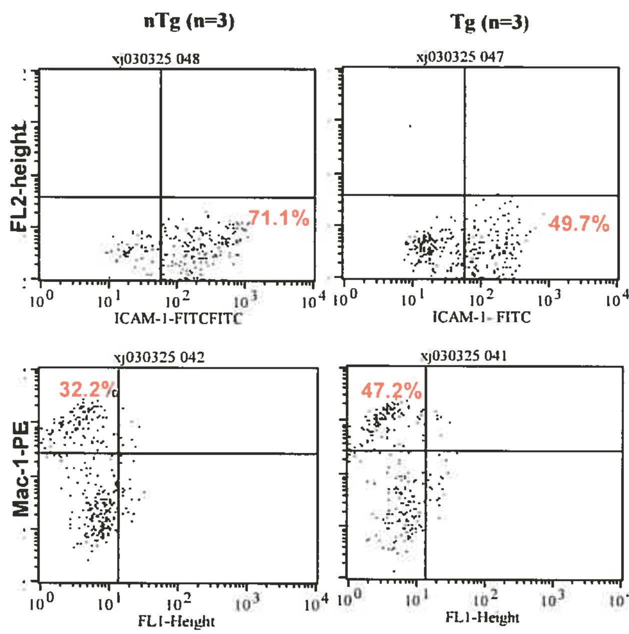
Figure 8.

A. Number of NPLCs



B. NPLCs-FACS

C. LSECs and KCs isolated via MACS



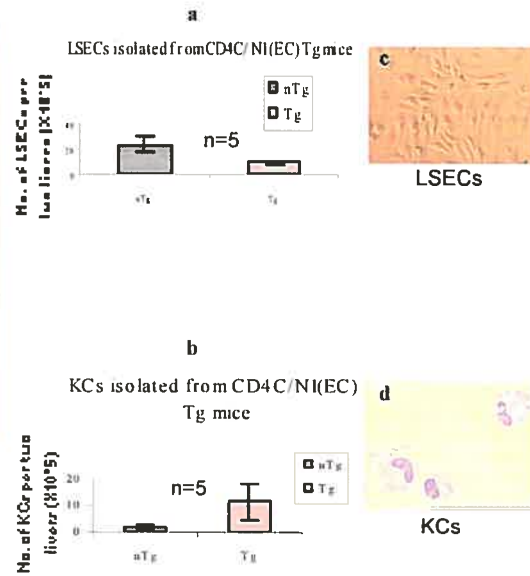
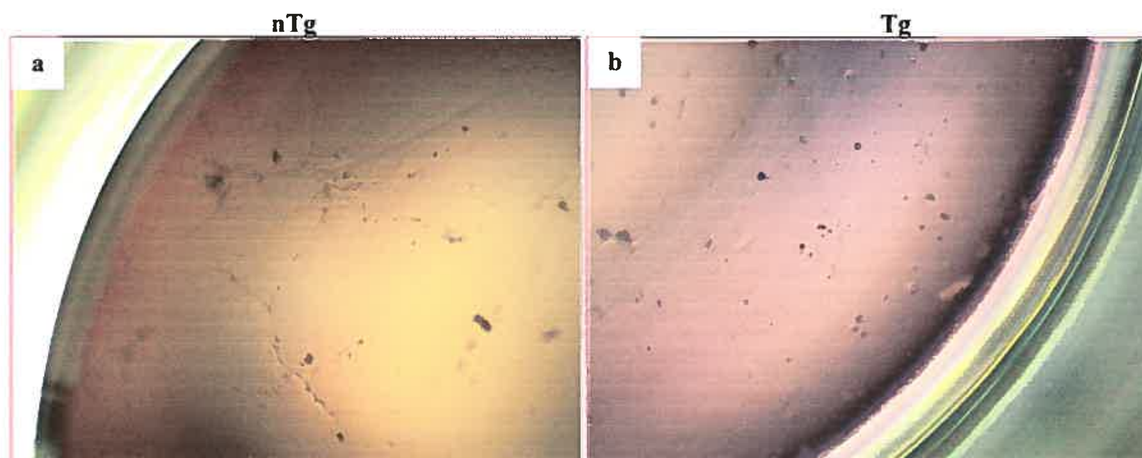
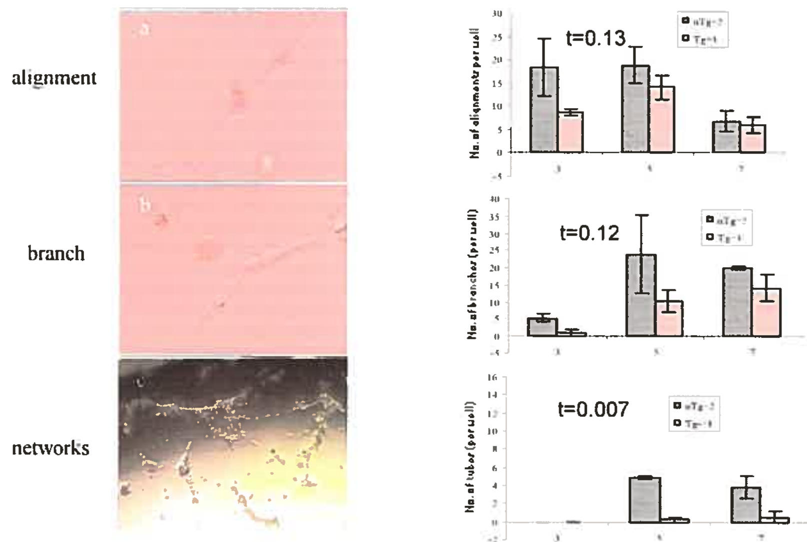


Figure 9.

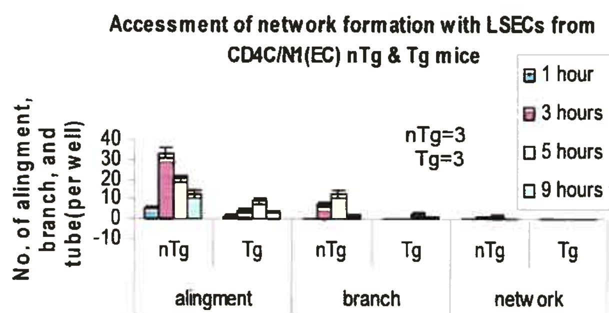
A. Network formation by LSECs on the angiogenesis kit



B. Assessment of network formation between nTg & Tg



C. Dynamic network formation



D. Quantification of cells

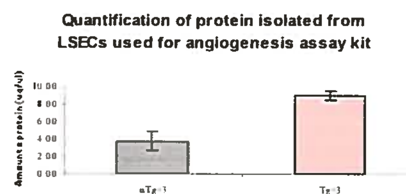


Figure 10.

A. Enhanced cluster formation by Tg MØs cocultured with LSECs

Mф-
Mф+
a
b
c
d
LSEC -
LSEC +

MO-latex beads
LSEC-Ac-DiI-LDL
merge
phase

Colonies formed by macrophages onto LSECs

No. of colonies
25
20
15
10
5
0
*t=0.02
*t=0.005
*t=0.03
MΦ-
MΦ+
MΦ-
MΦ+
lsec-
lsec+
lsec- MΦ-
lsec- MΦ+
lsec+ MΦ-
lsec+ MΦ+

B. Strong adherence of Tg macrophages to LSECs

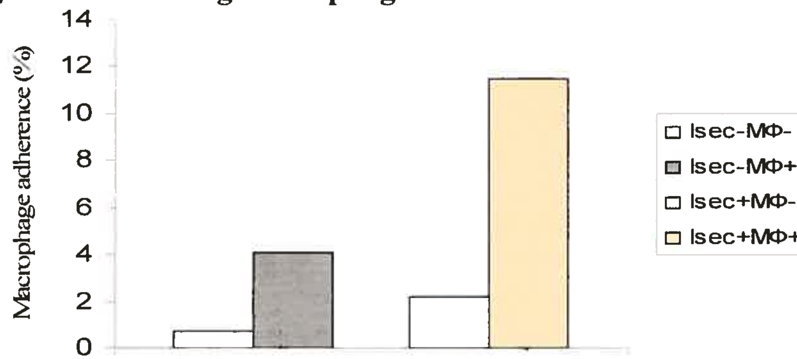


Figure 11.

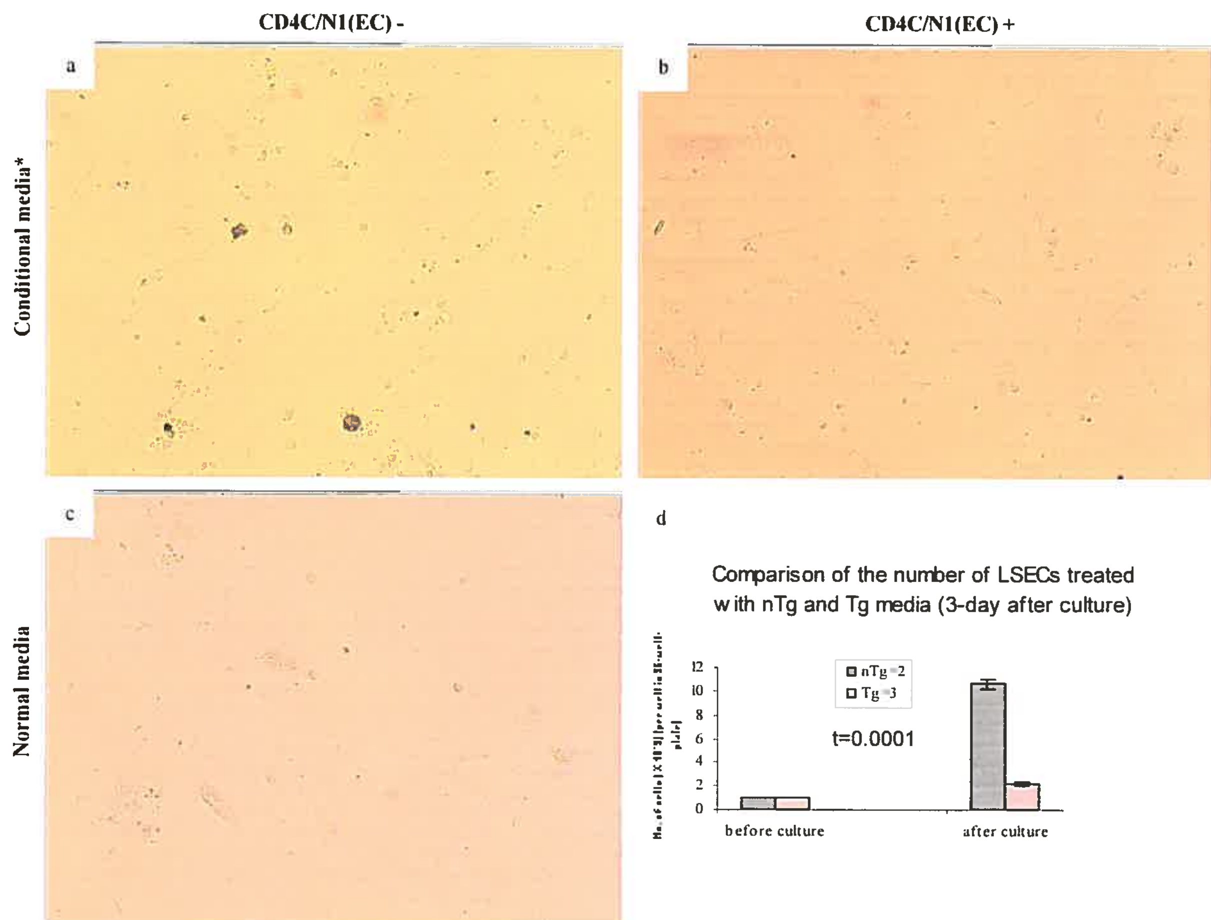


Table 1. Incidence of disease in CD4C/N(EC) Tg mice

Gross Analysis	Founder: 60787		Founder: 60788		Founder: 98513		Total	
Histology analysis	Tg	nTg	Tg	nTg	Tg	nTg	Tg	nTg
Vascular defects in liver	20/21	0/15	23/25	0/25	10/10	0/10	53/56 (97%)	0/50
Vasculaer defects in heart	2/10	0/10	0/10	0/10	N	N	2/20 (10%)	0/20
Vascular defects in brain	2/10	0/10	1/10	0/10	N	N	3/20 (15%)	0/20
Vascular defects in spleen	1/10	0/10	1/10	0/10	N	N	2/20(10%)	0/20
Vascular defects in lung	1/10	0/10	0/10	0/10	10/10	0/10	11/30(37%)	0/30
Vascular defects in kidney	0/10	0/10	1/10	0/10	N	N	1/20 (5%)	0/20
Enlarge spleen	4/10	0/10	4/10	0/10	7/10	0/10	15/30	0/30
Enlarge Gallbladder	5/10	0/10	3/10	0/10	N	N	8/20	0/20
Small body size	10/23	0/19	9/12	0/35	N	N	19/35	0/54
Lung tumor(epithelial cells-filtration)	1/10	0/10	0/10	0/10	N	N	1/20	0/20
Kidney tumor(Macrophages-filtration)	1/10	0/10	0/10	0/10	N	N	1/20	0/20
Female sterile phenotype	9/9	0/10	2/9	0/20	N	N	11/18	0/20

Table 2. Classification of liver vessel defects in CD4C/N1(EC) Tg mice

	Founder line		
Classification	60787	60788	**Total**
Superficial vessel	13/15	10/16	**23/31**
Tumor-like cavity	9/15	3/16	**12/31**
Malformations observed in liver with normal appearance	0/15	3/16	**3/31**

Note: Some Tg mice (8/31) are affected defects with both superficial vessels and tumor-like cavities.

Supplement legends

Figure 1. RNA expression of the hCD4 detected by *in situ* hybridization

All the sections, including LNs (A), thymus (B), and intestine (C), were probed with sense and antisense probe for the exon1 of the hCD4 genes. The expression of hCD4 was always observed in the Tg mice with the antisense probe (Ae, Af, Be, and Ce), but not with the sense probe (Ab; Bb; and Cb). No expression was detected in the nTg mice with either the sense or the antisense probes (Aa, Ad, Ba, Bd, Ca, and Cd). Large cells with the morphological appearance of macrophages were apparently probed (Ag, A1, AI, Bg, and Cg), Dark field: A: a,b,d,e,g; B: a,b,d,e; C: a,b,d,e,g. Bright field: A: c,f,h; B: c,f,g; C: c,f; A: a-f: 5X; g and h: 20X, i: 40X. B and C (a-f): 20X, g: 100X.

Figure 2. Permeability of the Tg liver vessels

One or two hours after injected EB via tail vein, the mice (4 to 6-month-old) were killed. The nTg and Tg livers were compared..|**A. Amount of the Evans Blue (EB).** Perfused livers were drying and extracted by formamide. The amount of the EB extracted from the Tg group is statistically higher than that from the nTg group (a, $P<0.05$). The amount of EB in the spleen was measured as control (b). |**B. Permeability of vessel:** Perfused liver was sectioned and extravazation was observed by fluorescent microscopy. The leaked EB was observed under the flat endothelial cell of the vessel in the Tg liver (a' and b', star signs) but not in the nTg liver (a and b). a,a': 0.55X10; b,b': 0.55X100. |**C. Capture of the EB by liver macrophages:** Macrophages monitored by latex beads (green) show red fluorescence in both the nTg (a-b) and Tg livers (a'-b'). 0.55X100.|**D. Capture of the EB by monocytes in the sinusoids of the Tg liver:** Paired sections were prepared for fluorescence microscopy (a-c) and HE staining (d and e). In the section of the Tg liver,

cells in the sinusoids gave strong fluorescence (b and c). These cells look like monocytes according to HE staining (e). a-c: 20 X; d and e: 10 X.|**E. EM:** Floating red cells were normally observed within the sinusoids of the nTg liver (a). But in the Tg sinusoids, the red cell entered the fenestrate (b arrowhead) as well as the parenchymal tissues (c arrow) beyond vessels.

Figure 3. The liver defects appeared during organogenesis.

Both embryonic (A, E16.5) and postnatal stage (B, P6) were examined. Livers were perfused with Microfil® (a,b and a', b' in A1 and B1) and used for HE staining (c,e and c',e' in A1 and B1), and IHC with P-ECAM-1 (a,b and a', b', c in A2 and B2). Homogenous vessels were observed in the nTg livers (a'-d' in A1, B1, and A2 B2, arrowhead), but heterogeneous in the Tg liver (a'-c' in A1 and B1, arrow). In addition, clustered vessels (A2 and B2: b', red arrowhead), dilated channel (B1d', green arrowhead), and dilated capillaries (B2b', red arrowheads) were in the Tg liver. Tissues stained with only 2nd Ab serve as control (c in A2 and B2). A1 and B1: a, a': 0.5X3.2; b, b': 0.5X10; c, c': 1.25x1.6; e, e': 40X1.6. A2 and B2: a, a', c: 5 X; b, b': 20 X.

Figure 4. Persisted vessel disease after partial hepatectomy in adult mice

|**A. Diagrammatic representation of the partial hepatectomy (PH):** Quarter lobes were ligated during PH as the cross-signs shown in the figure. |**B. Macroscopic analysis:** Non-hepatectomized livers (a and c) and hepatectomized livers (b and d) from the CD4C/N1^{EC} Tg mice (c and d) and their nTg (a and b) littermates were compared, after perfusion with Microfil®. In non-Tg mice, note that lobe generating after PH (b), relative to the non-hepatectomized liver (a). Such hypertrophy is not observed in Tg mice (d, black arrowhead) |**C. Microscopic analysis:** The vascular morphology of both non-

hepatectomized (a and b) and the hepatectomized (c and d) livers of the nTg mice is homogeneous. Abnormal vessels are present in present in both non-hepatectomized (e and f, arrowhead) and hepatectomized Tg livers (g and h, arrowhead). a,e,c,g: 1 X; b,d,f,g: 5 X.

Figure 5. Morphological difference between the nTg LSCEs and Tg LSCEs

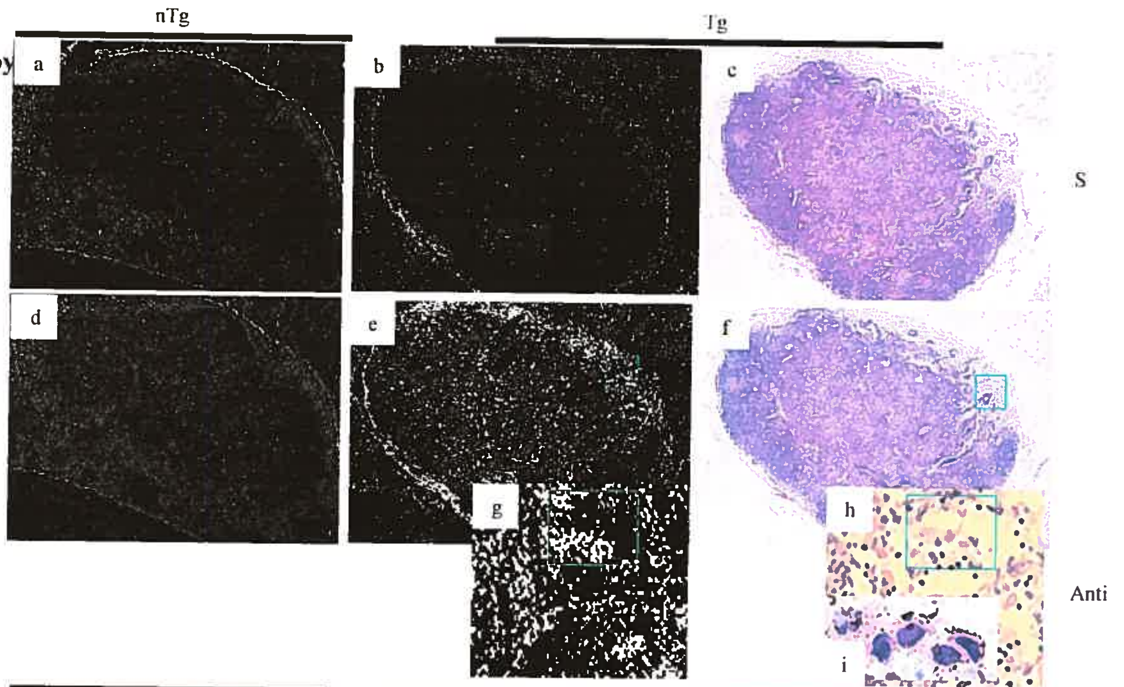
Morphology of the LSECs was first detected by Giemsa-Granules staining (a-b). Endothelial cell-feature was determined by the "uptake" of Dil-Ac-LDL (c). This morphology was further confirmed by the fluorescence observation of the LSECs purified from CD4C/N1^{EC} X l-sign Tg mice (d and e). 20X. When LSECs were harvested from dishes, a different time for detachment between the nTg and the Tg cells was observed (f).

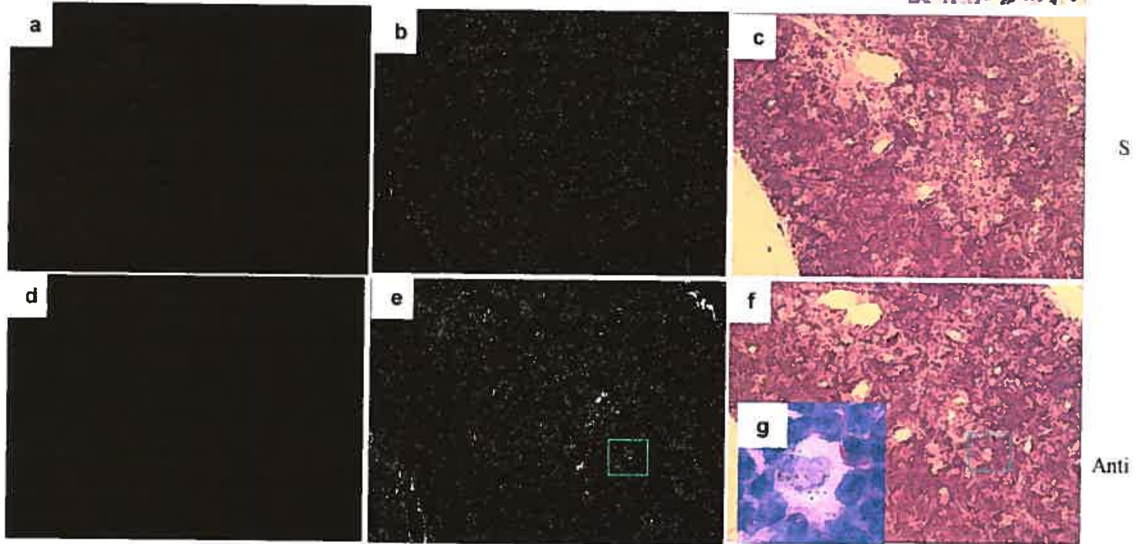
Figure 6. Possible molecules required for the vascular disease

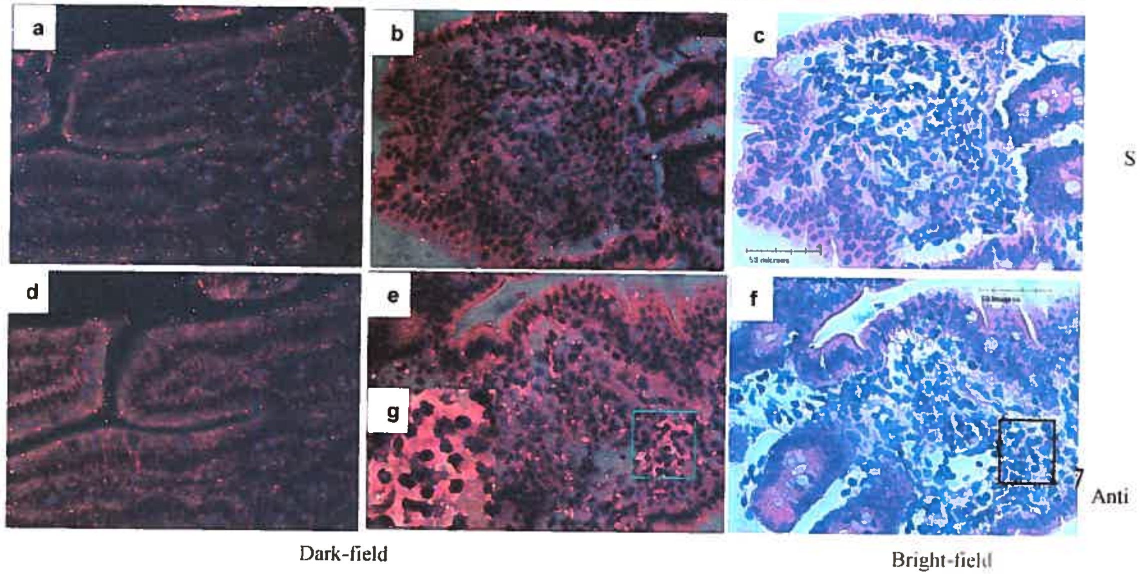
cDNA from macrophages and LSECs were performed RT-PCR and nTg and Tg cells were compared. Macrophage cDNA was diluted in 1:10 and 1:100. Series of primers (Some tyrosine kinase and Notch pathway) were detected in macrophages. Flk-1 and Dll4 were detected in LSECs.

Supplement 1.

E. RNA expression by *in situ* hybridization

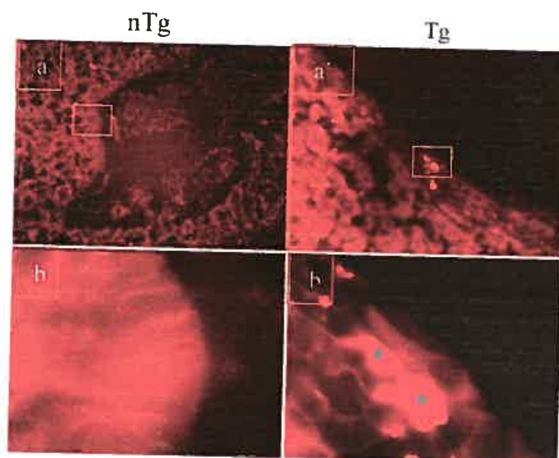




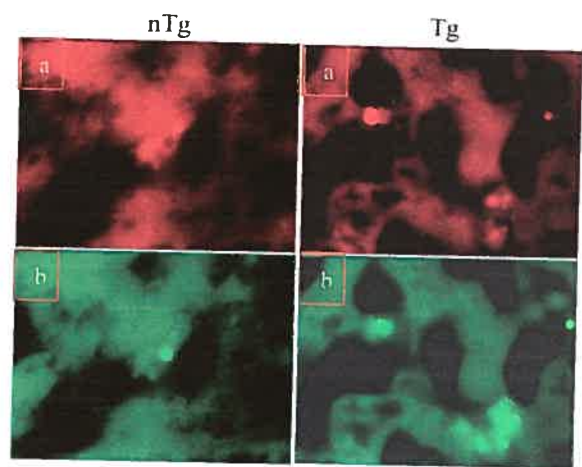


Supplement 2.

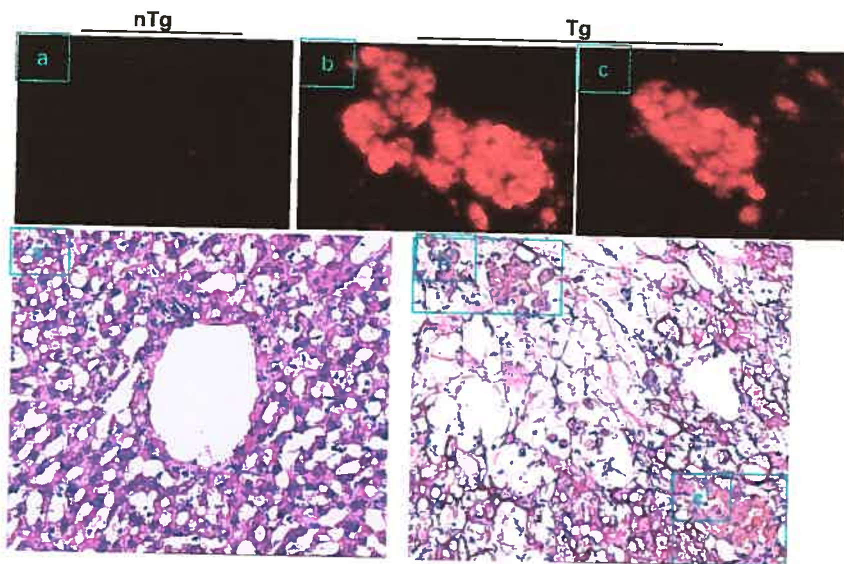
B: Permeability



C: Macrophages

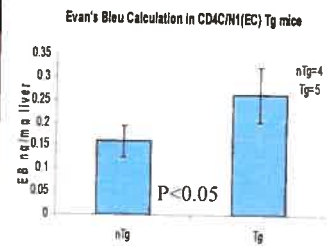


D: Monocytes-Macrophages

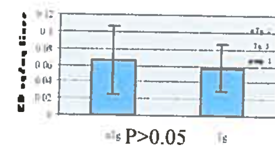


A: Quantification of EB

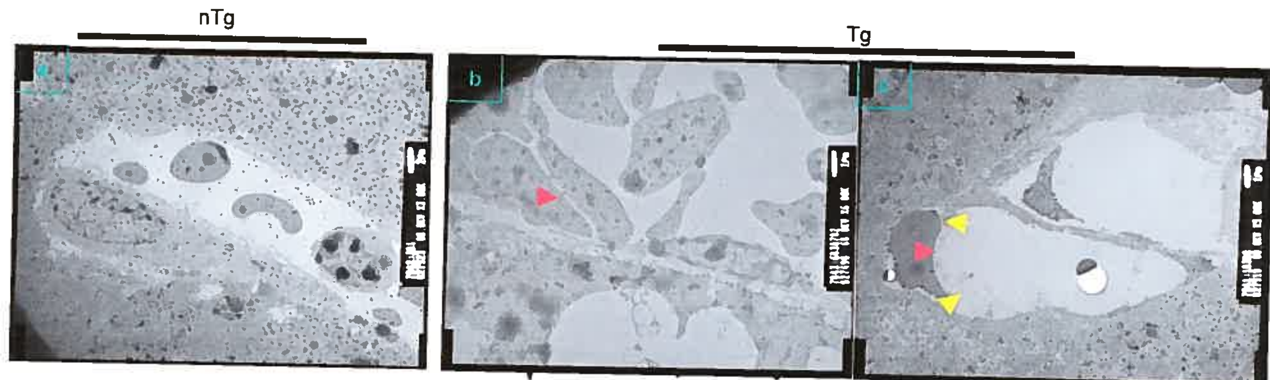
a. Liver



b. Spleen



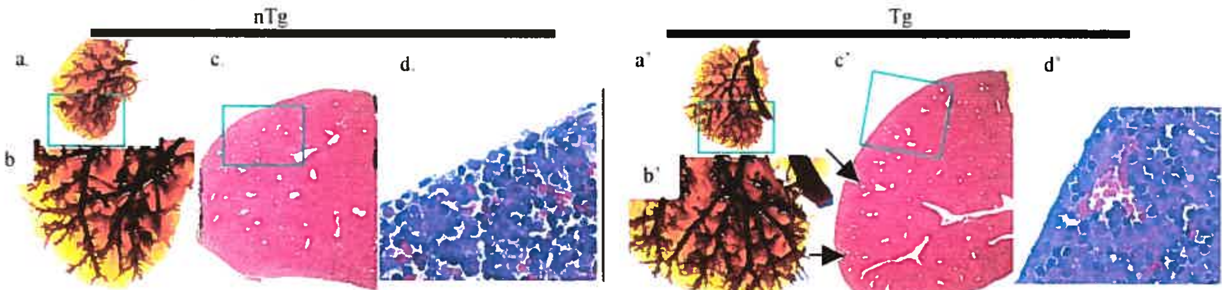
E: EM



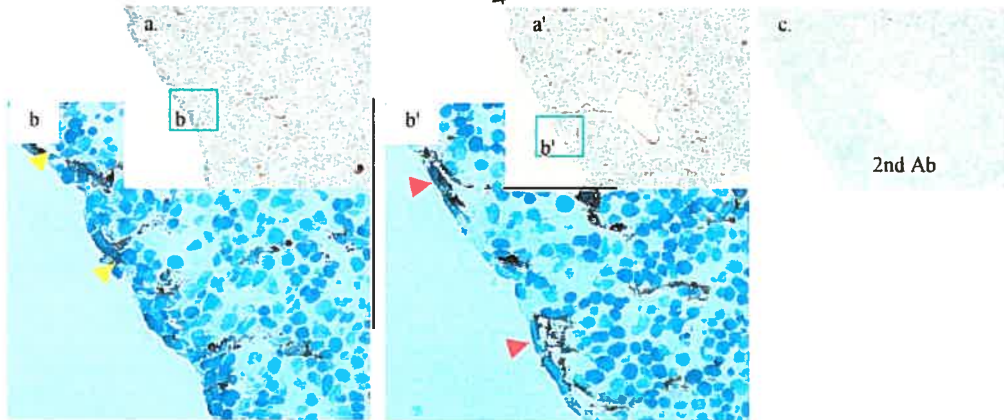
Supplement 3.

A. Embronic stage

1. Microfil & histology



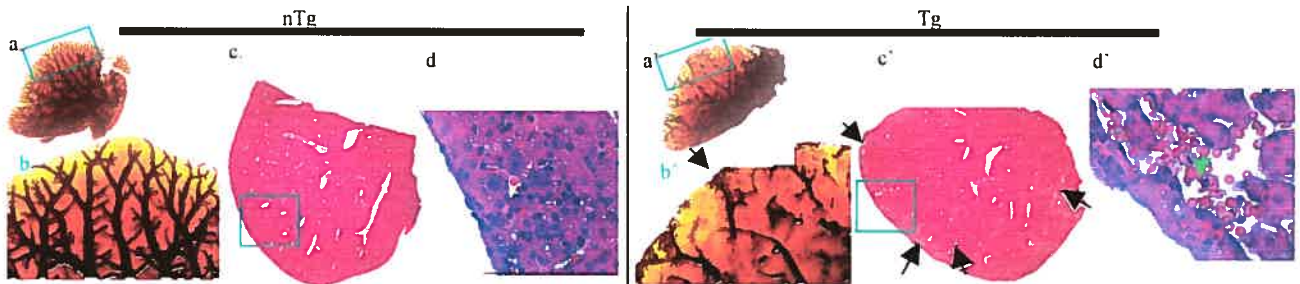
2. IHC: PECAM-1



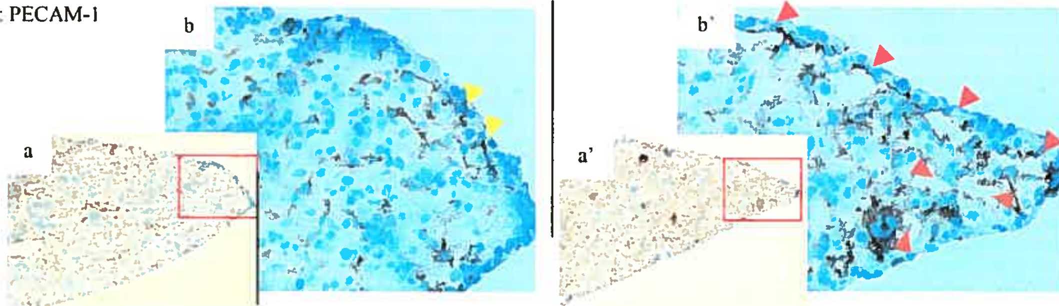
B. Postnatal stage

d

1. Microfil & histology

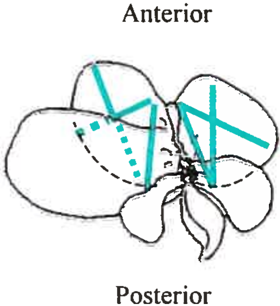


2. IHC: PECAM-1

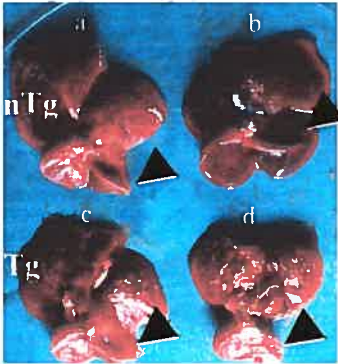


Supplement 4.

A. Digrammatic representation of PH



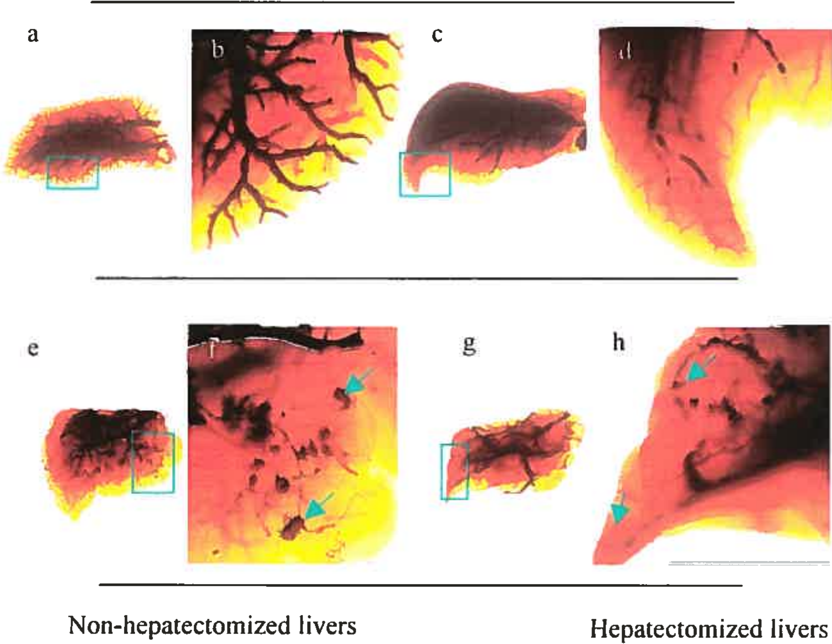
B. Gross analysis



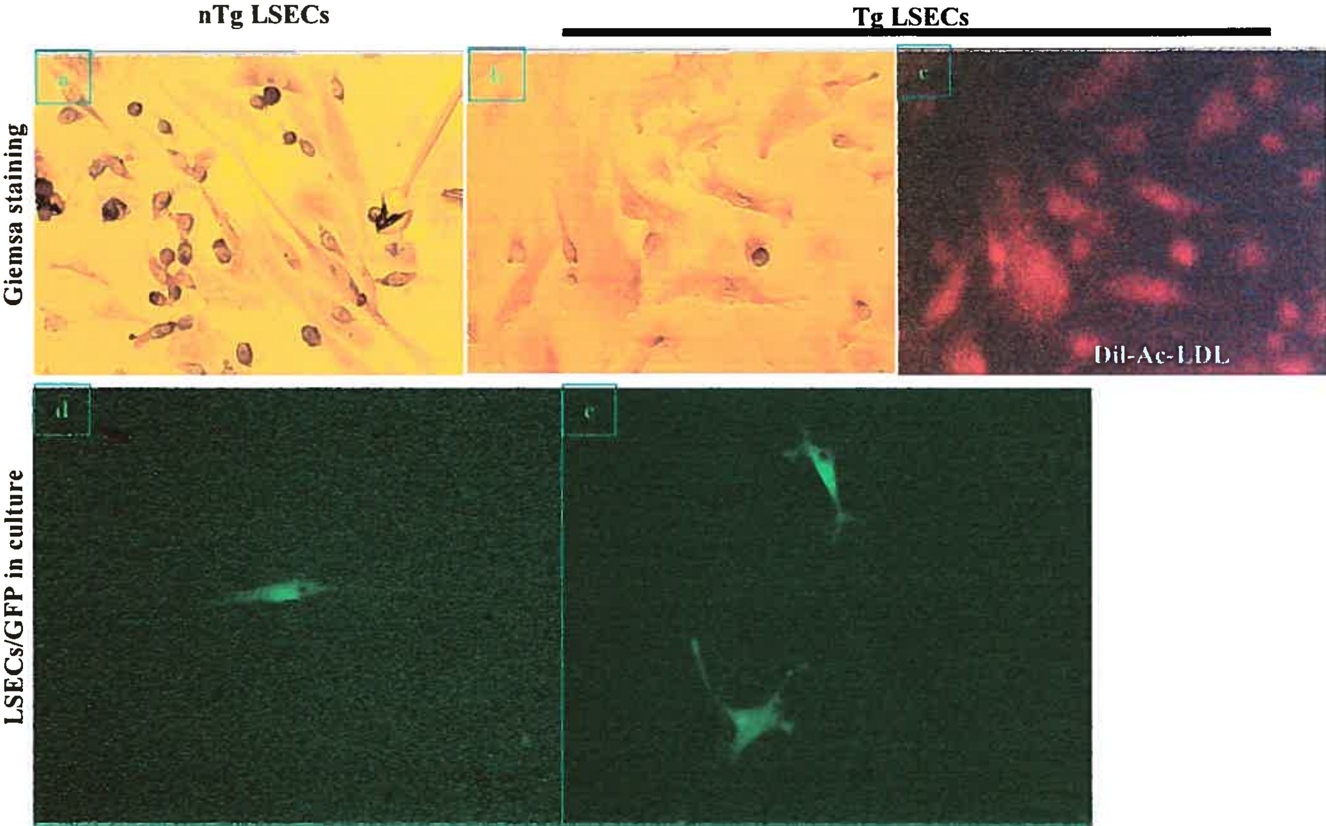
Non-hepatectomized livers

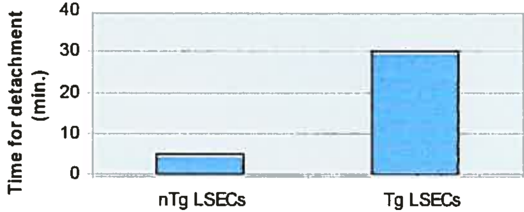
Hepatectomized livers(3-week later)

C. Microfil

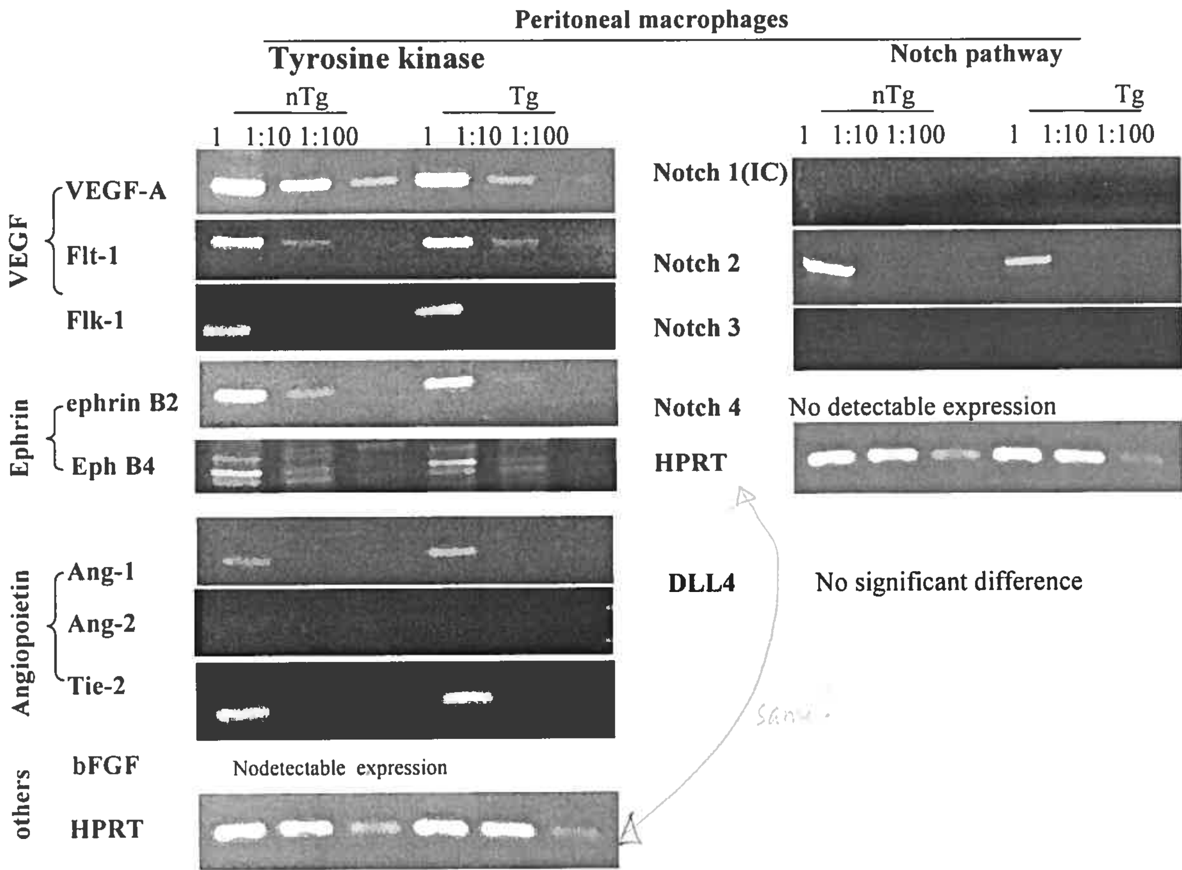


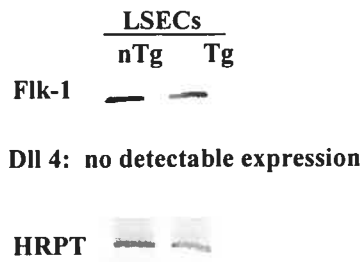
Supplement 5.





Supplement 6.





Chapter 4:

Expression of the Mouse Notch1 Extracellular Domain in Macrophages Leads to Sterility in Female Tg Mice

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Key Words: $N1^{EC}$, CD4C/$N1^{EC}$ Tg mice, vessels, macrophages, and female sterility.

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In Preparation (Confidential)

Abstract

Angiogenesis is usually quiescent in the adult. One exception, however, is the angiogenesis that occurs during endometrial growth, and in the placenta after pregnancy. The dysfunction of angiogenesis in the uterus may underlie several female reproductive disorders, such as infertility. In our previous results, we demonstrated that macrophages are reprogrammed by $N1^{EC}$ Tg and induce liver vascular disease in the Tg mice ($CD4C/N1^{EC}$). Here, we characterized another phenotype: female sterility. Two founder lines have been analyzed. All females of the higher expressor line and 50% of the lower expressor line were sterile. Tg expression was detected in both uterus and ovaries by Northern and Western analysis. The uteri of Tg mice were smaller and exhibited thinning of both myometrium and endometrium as compared to nTg littermate controls. Interestingly, defective vessels were displayed in Tg uteri, especially on the surface, as seen in the Tg livers. Developmental studies demonstrated that while no or few embryos were observed in Tg mice at E6.5, 9.5, and 16.5 stage, many defective vessels were found in each layer of uterus. In addition, hemorrhage was identified in endometrium of exclusively pregnant Tg mice. Moreover, macrophage-like cells were increased in Tg cycling and pregnant uteri, as well as placentas. Finally, the uterine vascular defects were reproduced in nTg recipients which had been transplanted with Tg fetal liver cells. So far, enhanced Notch4 was detected in the Tg-uteri compared to nTg ones. The results suggest that female infertility is directly caused by uterine vascular defects consequent to $N1^{EC}$ Tg expression in hematopoietic cells and possibly macrophages. Further experimentation should be done to elucidate the molecular alterations required for defective uterine vessels and reprogrammed macrophages.

Introduction

Two mechanisms account for the formation of blood vessels: vasculogenesis and angiogenesis. "Vasculogenesis" is mainly involved in the de novo development of blood vessel and has recently been shown to contribute to adult vessel formation (Risau et al., 1988; Grant et al., 2002). "Angiogenesis" describes the formation of new blood vessels from existing vasculature, which occurs during later development of the organization, as well as sporadically throughout adult life (Carmeliet, 2000). Angiogenesis is usually quiescent in the adult. Notable exceptions, however, are the angiogenesis that occurs during the growth of endometrium, and in the placenta after pregnancy (Torry and Rongish, 1992); Indeed, the term angiogenesis was first used to describe vessel growth in the placenta (Folkman and Klagsbrun, 1987). The dysfunction of angiogenesis in the uterus may underlie several female reproductive disorders, such as infertility (Folkman, 1995).

Many factors have been shown to be capable of promoting or inhibiting uterus angiogenesis *in vivo* and *in vitro*. Steroids (estrogen and progesterone) are not only expressed in the endometrial endothelial cells of the pregnant uterus in human (Wang et al., 1992), but have also been proven to affect uterine angiogenesis in animal models (Hague et al., 2002; Yasuda et al., 1998). Both FGF and VEGF are upregulated in endometrium by steroids (Zygmunt et al., 2003). VEGF can be secreted by uterine NK cells during the mouse pregnancy and may participate in uterine neovascularization in mouse models (Wang et al., 2000). Ang-2 mRNA was detected in the endothelia of ovary, uterus, and placenta (Maisonpierre et al., 1997; Goede et al., 1998). It seems likely that Tie-1, 2 and Ang-1, 2 may play a major role in regulating the growth and regression of endometrial vasculature (Sato et al., 1995; Maisonpierre et al., 1997; Hanahan, 1997). In

the human endometrium, TSP-1 is elevated in the secretory phase as compared to the proliferative phase. In *in vitro* study, TSP-1 is upregulated by progesterone (Rogers and Gargett, 1998).

Macrophages are abundant in the uterus and placenta (Hunt and Pollard, 1992; Hunt et al., 2000). They are the most common resident immune cells in the uterus of the rodents (Mackler et al., 2000; Hunt et al., 2000). They may account for nearly 10-15% of cells in the cycling uterus and increase to 22% of cells in the pregnant uterus (Hunt et al., 1985). They are distributed throughout the pregnant endometrium, as well as in stromal and connective tissues belonging to the myometrium (Mackler et al., 2000; Hunt et al., 2000). They can traffic between endometrium and myometrium (Mackler et al., 2000). Recent studies show that the uterine macrophages can be activated and regulated by hormones. Activated macrophages display an enhanced ability to phagocytose, produce a wide number of growth factors, and to synthesize an impressive number of proteases currently known to affect angiogenesis (Hunt et al., 2000; Sunderkotter et al., 1994). Hence, they seem to play a role in controlling uterus angiogenesis, even if detailed studies are still scarce.

Previous results have demonstrated that macrophages are reprogrammed by $N1^{EC}$ Tg and induce liver vascular disease in the Tg mice. These data were used to characterize another phenotype, namely female sterility. This phenotype may be related to uterine vascular defects that can be induced by hematopoietic cells (probably macrophages), as demonstrated through fetal liver transplantation. A paracrine mechanism, similar to that which was discovered in the liver phenotype, might be involved in the female sterility phenotype.

Results

CD4C/N1^{EC} female Tg mice show sterility

In the characterized CD4C/N1^{EC} Tg mice as described in the previous paper (Xiujie et al.), it was found that no or few live offsprings were born from breedings with Tg females, in contrast to seven to nine viable offspring from breedings with Tg males (**Fig. 1b**). Two-month old females were then spontaneously selected from two founder lines (60787 and 60788) to breed with C3H males. In founder line 60787, where the liver vascular disease is severe, no pups was born from 100% Tg mice (5/5), in contrast to an average number of eight babies from C3H mice (4/4). In another founder line, 60788, where the liver vascular disease is less severe, approximately 50% of females were unable to produce babies, while those that could had smaller littler size (on average, four babies from Tg females compared to eight babies from control mice) (**Fig. 1c**). These results suggest that Tg females cannot conceive, or alternatively, that embryos die during gestation.

To determine whether and when the embryos died, we observed embryos at different stages of development (**Fig. 2**). As opposed to the eight or nine embryos obtained from C3H females (**Fig. 2. A, G, and I**), no embryos are observed from Tg females in the line established from founder 60787 (**Fig. 2. B, H, and J**) at E9.5 (one day after placenta formation) or at E16.5 stages, but cavities (**Fig. 2. B and J**) are observed with dead embryonic cells (**Fig. 2. D and F**), suggesting a natural abortion. Even at E6.5 (one day after implantation), no normal embryonic masses are seen in Tg-uterus (**Fig. 2L**). The pregnant Tg uteri are highly vascularized and soft, indicating that response of uterus to hormones might be normal. With another founder line 60788, a few embryos are observed but less than those from normal C3H females (**data not shown**). Northern blot (Xiujie et

al.) and Western blot show that the N1^{EC} transgene is strongly expressed in non-pregnant Tg-uteri and weakly in Tg-ovaries, but not in nTg-tissues (**Fig. 1a**). These results suggest that the Tg may affect reproductive organ function, thereby leading to defective implantation and/or placenta formation, rather than by having an effect on the embryo per se.

Uterine vascular defects in Tg mice

To investigate reproductive organ defects in Tg mice, gross and histological analyses were performed. First we examined non-pregnant mice. Ovaries were indistinguishable between nTg and Tg mice through macroscopic examinations (**Fig. 3A**), and by histological analysis with HE staining, all stages of follicles could be observed in Tg ovaries, a similarity to nTg ovaries (**Fig. 3B. a and b**). Tg-uterus, however, was thinner than that from nTg littermate, especially in the fact that large superficial vessels were visible in the Tg- but not in the nTg-uterus, as seen under macroscopic examination (**Fig 3A**). HE staining further confirmed the large, superficial vessels in Tg-uteri. Also, thinner endometrium and myometrium are observed in Tg-uteri as compared to nTg-uteri (**Fig 3B. c-f**). Next, pregnant mice were examined. Many large vessels were observed within myometrium surrounding the cavities containing the dead embryonic cells (**Fig. 2C and D**), and hemorrhages were clearly found in the endometrium (just beneath epithelial cells) (**Fig. 2E**). Most likely, the vascular defects lead to a disruption of Tg uterine structure and further influence embryo implantation as well as placenta formation.

Increased macrophage-like cells in Tg uteri

To elucidate which cells induce the uterus defects, the following experiments were conducted. With HE staining, increased cells with appearance of macrophages were observed in Tg cycling uteri, as compared to nTg-uteri (**Fig. 4**). These increased cells are

distributed within different layers of uterus; around the defective vessels of connective tissues (between meso- and myometria), as well as among the glands within endometrium (**Fig. 4A-E and G-K**). Sometimes, clustered inflammatory cells were observed in endometrium but not seen in non-Tg uteri (**data not shown**). In pregnant mice, clustered macrophage-like cells were often observed in Tg uteri, as opposed to their homogeneous distribution in non-Tg uteri (**data not shown**). In addition, preliminary result showed enhanced and accumulated macrophage-like cells in Tg placenta compared to non-Tg placenta (**Fig. 4. F, L, and M**). Previous results have shown that the peritoneal macrophages expressed Tg $N1^{EC}$, as detected by RT-PCR and Western blot (Xiujie et al.). Since reprogrammed macrophages induce liver vascular defects via a paracrine loop (Xiujie et al.), it was supposed that the same mechanism might be involved in the uterus vascular defects.

Hematopoietic cells (macrophages?) play a key role in the uterus vascular defects

To prove the above hypothesis, fetal liver transplantation was first performed. The $N1^{EC}$ Tg was successfully detected in macrophages, pLNs, and uteri from mice transplanted with Tg-cells but not with nTg-cells (**Fig. 5A**). Surprisingly, not only uterus vascular defects were reproduced in the Tg-expressing mice, but also hemangiomas were observed, as seen in the liver (**Fig. 5B and 5C**). Histological analysis showed defective vasculature under the surface of the uterus, as well as within the myometrium of Tg-bearing mice, especially growing out of uterus to form hemangiomas (some of them accompanied by endometrium) (**Fig. 6B and 6F**). Interestingly, clustered hematopoietic cells, probably macrophages, were observed around the defective uterus vessels (**Fig. 6H**). Moreover, the thinner and vascularized uteri were reproduced in the nude mice transplanted with the Tg fetal liver cells (**data not shown**).

Increased Notch4 expression in the diseased uteri of Tg mice

To identify molecules required for the uterus defects, RT-PCR was performed in order to test some candidate genes that are expressed in endothelial cells within uterine tissues. It was found that Notch 4 was remarkably increased in the Tg-uterus compared to the nTg-uterus **(Fig. 7)**, supporting the vascular defect theory. Other factors are currently being identified, and detailed mechanisms will be characterized later.

Discussion

Notch has been appeared in the angiogenic factor's list recently due to its emerging role in vessel formation (Iso et al., 2003). For instance, both Notch1/4 embryonic deficient mice and Notch4 transgenic mice have demonstrated the essential role of Notch signaling in vessel development (Krebs et al., 2000; Uyttendaele et al., 2001). Most studies, however, are focused on the activation of the Notch intracellular domain. Subsequently, no reports mention the role of Notch ectodomain in the adult uterine vascular development.

In the present study, it was first found that the CD4/N1^{EC} Tg females display sterility or low fertility. Then it was observed that these sterile mice show thin uteri accompanied by uterus defected vessels. An increased number of macrophage-like cells are detected in the Tg cycling uterus and placenta. Importantly, the uterine vascular defects are reproduced by the transplantation of Tg-FL cells. Similar to the liver vascular phenotype, hematopoietic cells (probably macrophages) might play a key role in the uterine vascular defects.

Since the uterus and its contents demand an increased supply of blood during pregnancy, angiogenesis plays a key role in the pregnancy-associated changes in the reproductive tract. Uterus' vasculature undergoes three main adaptation changes during pregnancy: vasodilation; increased permeability; and growth of new vessels (Reynolds et al., 1992; Torry and Rongish, 1992). These changes interfere with all vessels of each layer (endo-, myo-, and mesometrium) of uterus (Zygmunt et al., 2003). The neovascularization takes place very early in pregnancy and is initiated in extraembryonic areas. It has been shown that there is a close relationship between the state of neovascularization and embryonic development. The normal chronic villous vascularization is essential for the successful development of pregnancy (te Velde et al., 1997). Either poor or increased

vascularization can lead to early pregnancy losses (Vuorela et al., 2000; Qiao et al., 1997; Vailhe et al., 1999). In the Tg mice, with the exception of disordered vessels observed in cycling uteri via macro- and microscopic analyses, hemorrhage was clearly detected in the pregnant Tg-uteri. Also Nothc4, specifically expressed in the endothelial cells, was enhanced in the Tg uteri compared to nTg uteri (Reynolds et al., 1987; Krebs et al., 2000). These data indicate that these defective vessels may abnormally progress during pregnancy and are abnormally initiated in the extraembryonic areas of the Tg mice. This will further cause impaired implantation, trophoblast invasion, and placenta formation, and finally lead to abortion.

Studies have shown that leukocytes have major pregnancy-associated functions including facilitation of implantation, invasion of trophoblast, development of placenta, and, especially, modulation of maternal vasculature (Hunt et al., 2000). The role of uNK (uterine NK) cells has been well documented, probably because they are the most abundant cells in the human uterus. Although macrophages have drawn less attention in experimental studies, they are the most numerous leukocytes in the uterus of the rodents (Mackler et al., 2000; Hunt et al., 2000). An *in vitro* experiment has shown that human macrophages within female reproductive tract can produce the hCG-induced angiogenic factor VEGF. Also, VEGF expression has been described in fetal macrophages within villous stroma (Zygmunt et al., 2003). This indicates a potential angiogenic role for macrophages in the uterus. Previous results demonstrated that reprogrammed Tg-macrophages inhibit the growth of liver sinusoidal endothelial cells, as detected by *in vitro* co-culture assays (Xiujie et al.). It was presumed that increased Tg macrophages might influence the neovascularization not only in the cycling uterus, but especially in

implantation, trophoblast invasion, and placentation during early pregnancy. These will finally be enough to cause disorders of pregnancy.

In summary, $N1^{EC}$ expressed in macrophages not only induces vascular defects in liver but also does so in other organs, particularly the uterus. The uterine vascular defects, as well as reprogrammed macrophages might effect implantation, placenta formation, and fetus formation, leading to female sterility or low fertility (**Fig. 8**). The exact molecular mechanism remains unclear. Future works will identify molecules altered in Tg macrophages, as well as the relationship between macrophages and the endothelial cells of uterus.

Acknowledgements

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Methods and Materials

CD4C/N1^{EC} Transgene construction and generation of CD4C/N1^{EC} Tg mice

As described in the previous paper (Xiujie et al).

Infertility testing

First, eight 10-week-old CD4C/N1^{EC} Tg female and male mice were bred with 16 age-matched C3H males and females (respectively) over a 4-month time period to evaluate infertility. The number of live offspring was compared between the two groups. Then, developmental studies were performed. Embryonic day 6.5, 9.5, and 16.5 (E6.5, E9.5, and E16.5) embryos were isolated and counted from the pregnant nTg and Tg females crossed with C3H mice. The pregnant uteri with embryo were examined by histological analysis.

Protein Extraction and Western Blot Analysis

Protein extraction was accomplished by lysing uteri and ovaries, as performed in the previous paper (Xiujie et al.)

Tissue Sampling and Microscopic Analysis

For routine histological analysis, mice were killed by CO_2 inhalation or under avertin anesthesia, and organs to be evaluated were dissected and fixed by overnight immersion in 3.7% formaldehyde buffered in PBS. Organs to be assessed were embedded in parraffin, sectioned into 5 μm slices, and stained with hematoxylin and eosin, as described previously (Hanna, 1998). Slides were finally chosen randomly. Tissues were examined by at least two investigators.

Microfil® perfusion :

Microfil is a two-component silicon-rubber curing agent that has been used extensively for visualization of the vasculature of other sites in the body, such as the rate kidney glomeruli. For postnatal and adult, Microfil® (Flow Tech, Carver, MA, USA)

perfusion (0.5-2.5 mL) was performed (after thorachotomy under Avertin anaesthesia) via the apex of the left ventricle while the heart still beating, as described previously under Avertin anaesthesia. Whole uteri and livers were fixed by immersion in 3.7% formaldehyde buffer with PBS, then dehydrated with serial ethanol and cleared in methylsalicylate, as described in (Coral-Vazquez et al., 1999).

Fetal liver (FL) cell transplantation

Donors (embryos at day E15.5 from CD4C/N1^{EC} Tg mice) were genotyped by fast PCR of DNA isolated from a couple of organs (spleen, kidney, and gut). Normal C3H mice were lethally irradiated (900 rads). Approximately 4-15 X 10^6 FL cells were injected into the tail veins of the irradiated mice. Mice were analyzed after 2-6 month transplantation

RT-PCR

Total RNA was extracted from liver and peritoneal macrophages using Trizol Reagent (Invitrogen). The cDNA was synthesized from 1ug total RNA by the previous protocol (Xiujie et al.). Primer sets for the following genes were used: CD4C/N1^{EC}: sense CCCCACTGGGCTCCTGGTTGCAGC and antisense GTATGAAGACTCAAAGGGCA-G. Notch4: Sense TGCCTGCACAATGGTACCTG and antisense TCTGGCTTCAGTG-CCTTAAG.

Figure legends

Figure 1. Expression of N1^{EC} mRNA and protein in Tg reproductive tracts and an infertility phenotype in Tg females

A| Total protein extracts (100 ug) from whole uteri and ovaries of Tg and nTg littermates were separated by SDS-PAGE and analyzed by Western blotting with a polyclonal antibody specific for N1^{EC} {362}. B| Offsprings obtained by intercrossing Tg males or females with C3H mice. C| Percentage of sterile females in different founder lines.

Figure 2. Developmental studies on the line established from founder 60787

Macropscopy (G, H, and L) and histological analysis with HE staining (A-F, G, and H) were performed on the pregnant uteri at different developmental stages, E16.5, 9.5, and 6.5. Normal C3H and Tg females were compared. In contrast to normal embryos observed in normal C3H uteri (A, G and I), cavities (B, C and I, black arrow) containing dead embryonic cells (D and F) were detected in Tg pregnant uteri at both E16.5 (A) and E9.5 (F) stages. Note that the large vessels in meso-, myo-, and endometrium (C and D) as well as hemorrhage in the endometrium (E, arrow) of Tg pregnant uterus. Higher magnifications for B are shown in C (5X), D (20X), and E-F (40X). Tg uterus was smaller than the control at E6.5 (L). Embryo masses were indicated (white arrow).

Figure 3. Macro- and micro-scopic analysis of Tg reproductive organs

A| External appearance of reproductive tract. Note that Tg uterus is thinner and has more superficial vessels (b, white arrow) than its littermate. B| Microscopic analysis (HE staning). Note more vessels (d, black arrow) as well as thinner endometrium (f, astericks) and myometrium (f, arrowhead) in the Tg uterus compared with nTg littermate (c and e).

Figure 4. Increased macrophages observed in Tg uteri.

H & E stained sections of uterus and placenta from nTg and Tg mice. Note that more macrophages are distributed in Tg uterus (G-M) compared to non-Tg one (A-F). Uteri are grouped in A-E and G-K and placentas are grouped in F, L, and M. B, C a, H, and I: showing macrophages around vessels located within myometrium. D, E, J, and K: showing macrophages among glands located within endometrium. Macrophages are cells with morphology indicated by arrows. A.G: 5X; B, D, H, J, F, L, and M: 40X; C, E, I, and K: 100X.

Figure 5. Tg expression and vascular phenotype are reproduced in the uterus of the mice transplanted with Tg FL cells

Four months after transplantation with FL cells, the recipients were observed. A| Tg $N1^{EC}$ expression was detected in uterus, LNs, and peritoneal macrophages by RT-PCR. B| Macroscopic phenotype. Coincident with the hemangiomas (filled with Microfil® products) observed in the Tg → C3H chimeric liver (green arrows), some tumors observed in the Tg → C3H chimeric uterus (blue arrows, especially, on the surface of the uterus). C| Microfil perfusion shows clear vascular tumors on the surface of Tg → C3H chimeric uterus (b and d) but not in nTg → C3H chimeras (a and d).

Figure 6. Histological analysis of uterus from FL chimeras

Same organs as seen in Figure 5. were subsequently performed H & E staining. Big vessels (red asterisk) perfused with Microfil® are shown in endometrium (B) and myometrium (F) of Tg → C3H uterus but not in nTg → C3H uterus (A and E). Note that these vessels grow out from the uterus and form the tumor on the uterine surface (D, black arrow) and that the big vessels are usually accompanied by hematopoietic cells (H, blue arrow).

Figure 7. Notch4 is increased in the Tg uteri

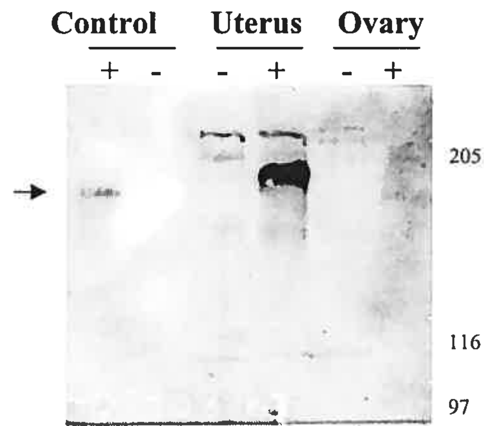
Uterine cDNA and its dilutions (1:10 and 1:100) were performed RT-PCR. Notch4 was detected and nTg and Tg uteri were compared.

Figure 8. Postulated mechanism leading female fertility.

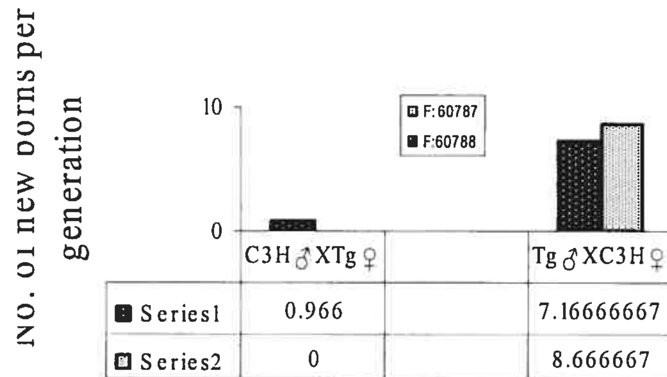
Macrophages activated by $N1^{EC}$ Tg induce uterine vascular defects. Both activated Macrophages and defective vessels could impair implantation, placentation, and fetus formation, leading to infertility.

Figure 1.

A . WB: anti-Notch1 extra-2



B. Natality of mice from CD4C/N1(EC) females



C. Percentage of sterile mice

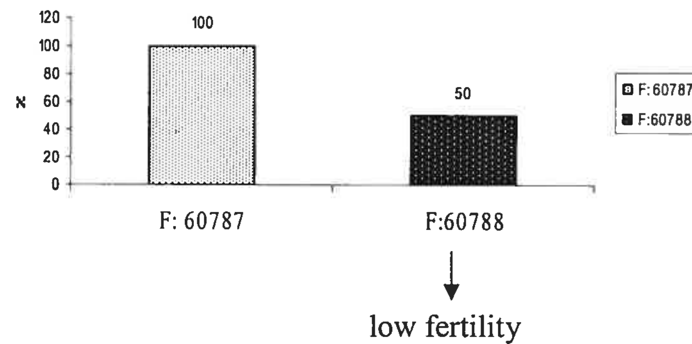
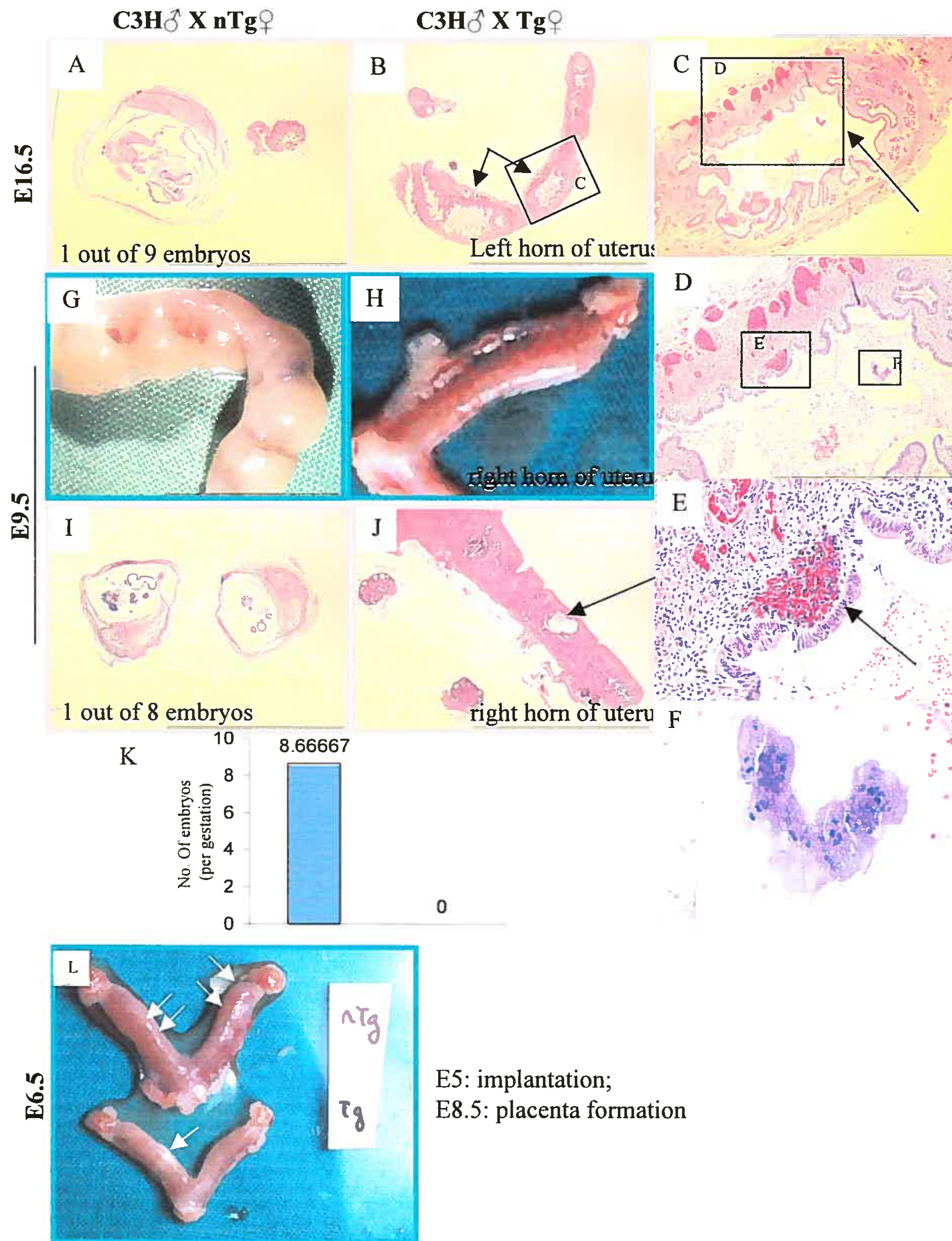


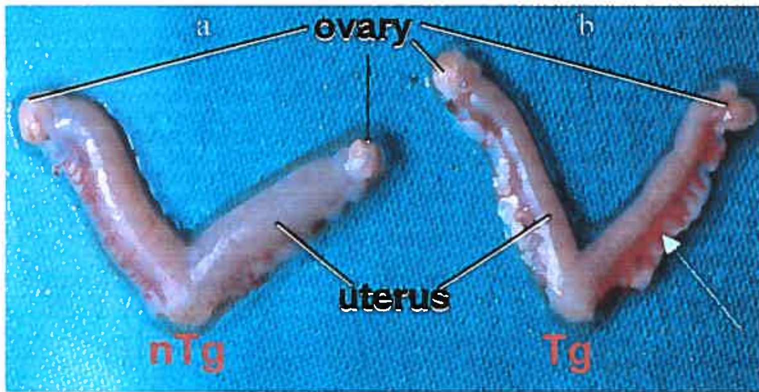
Figure 2.



E5: implantation;
E8.5: placenta formation

Figure 3.

A. Macroscopic examination



B. Microscopic analysis

nTg Tg

Ovary: a, b — 5X

Uterus: c, d — 2.5X

e f

Figure 4.

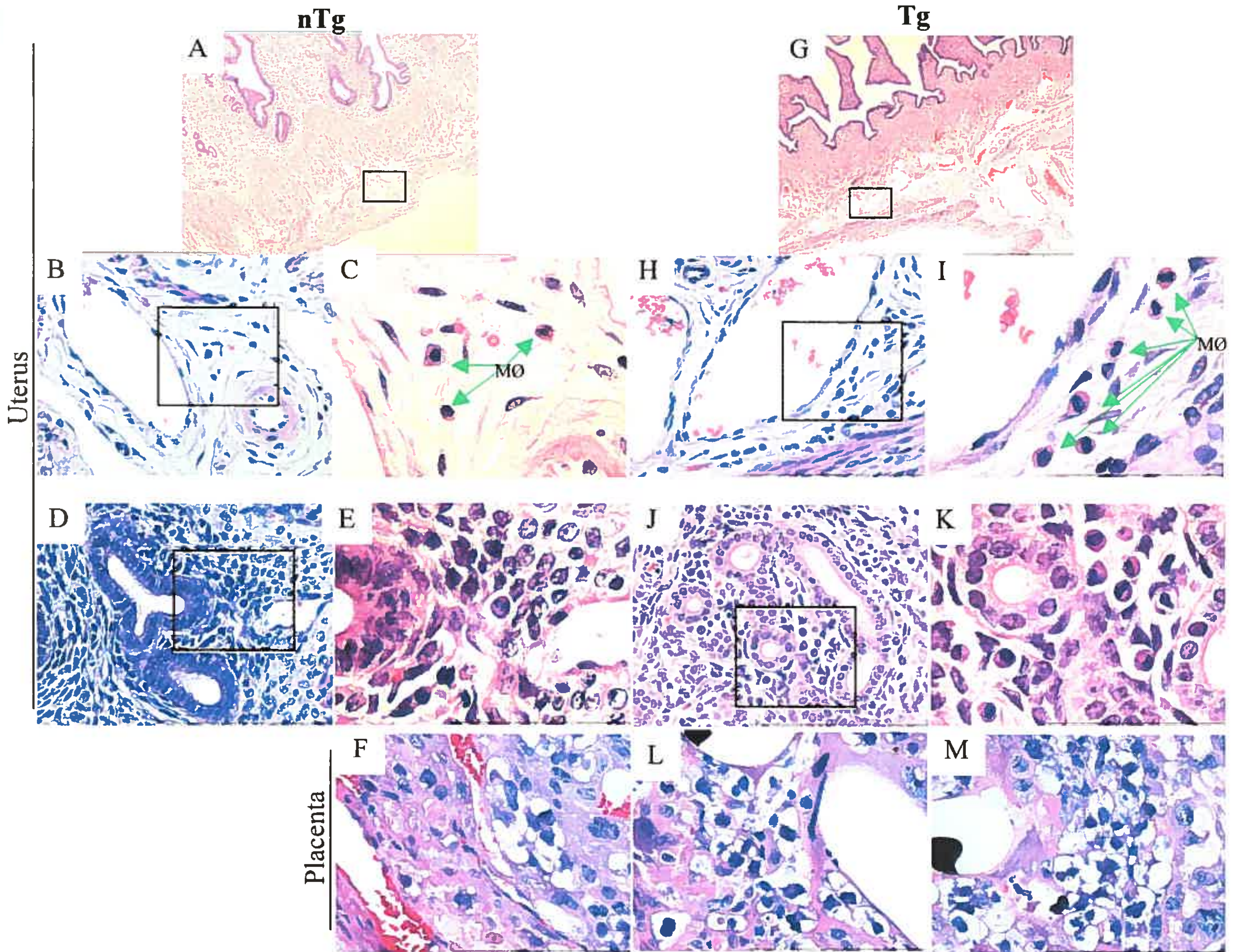
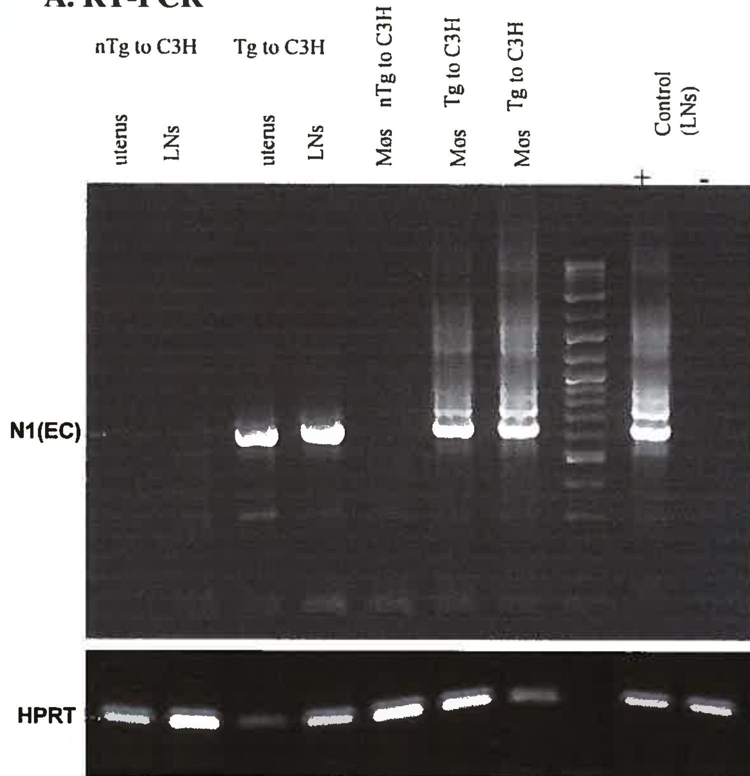
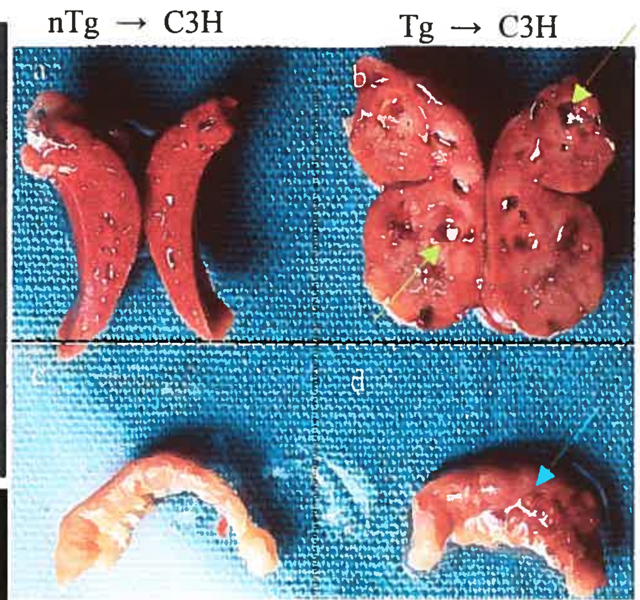


Figure 5.

A. RT-PCR



B. Macroscopic phenotype



C. Microfil

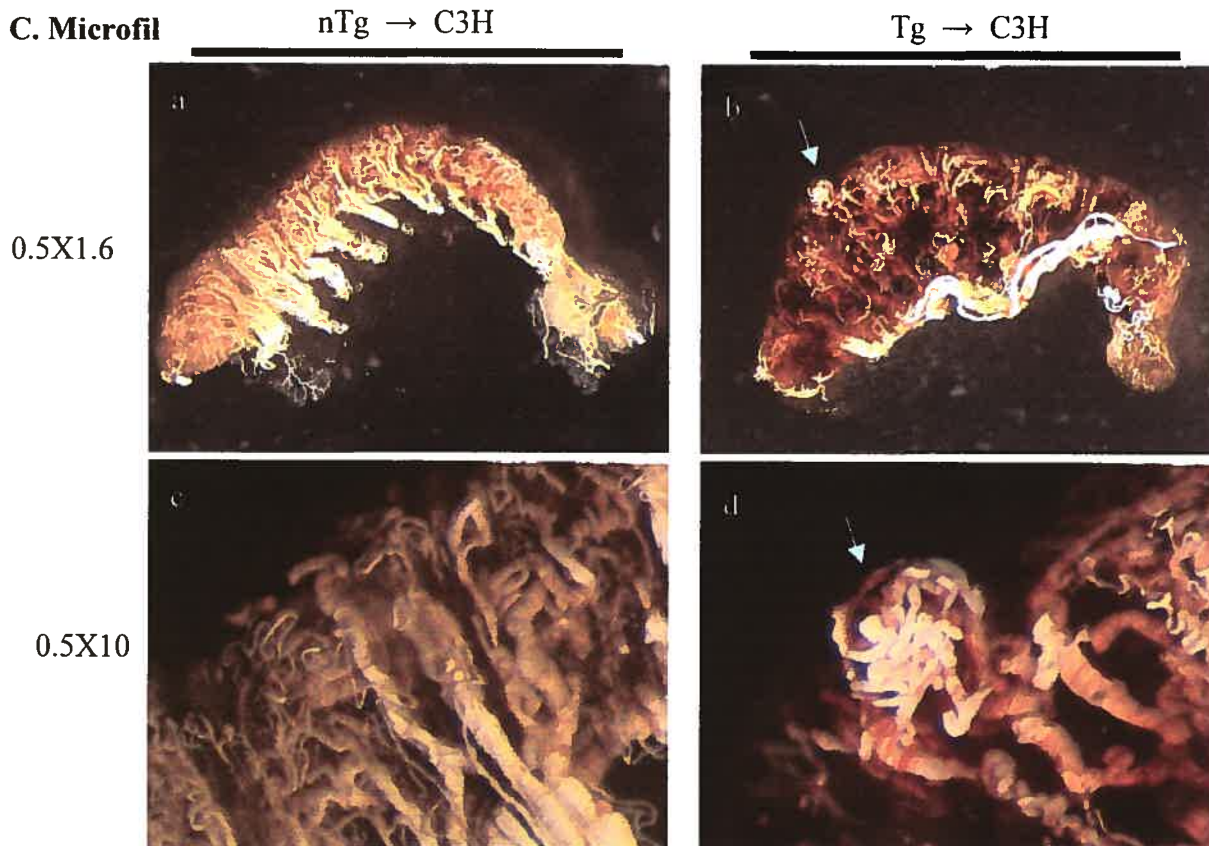


Figure 6.

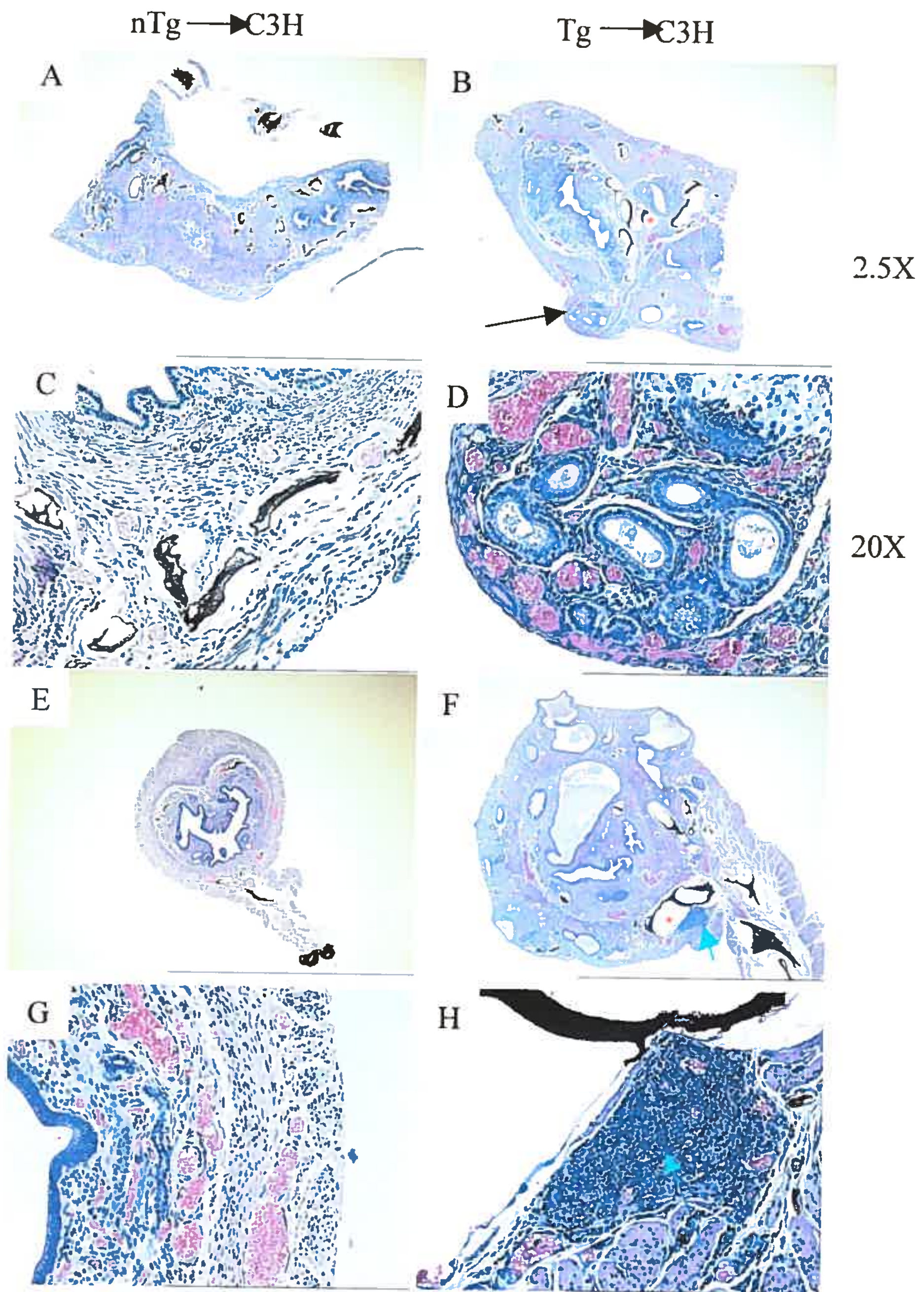


Figure 7.

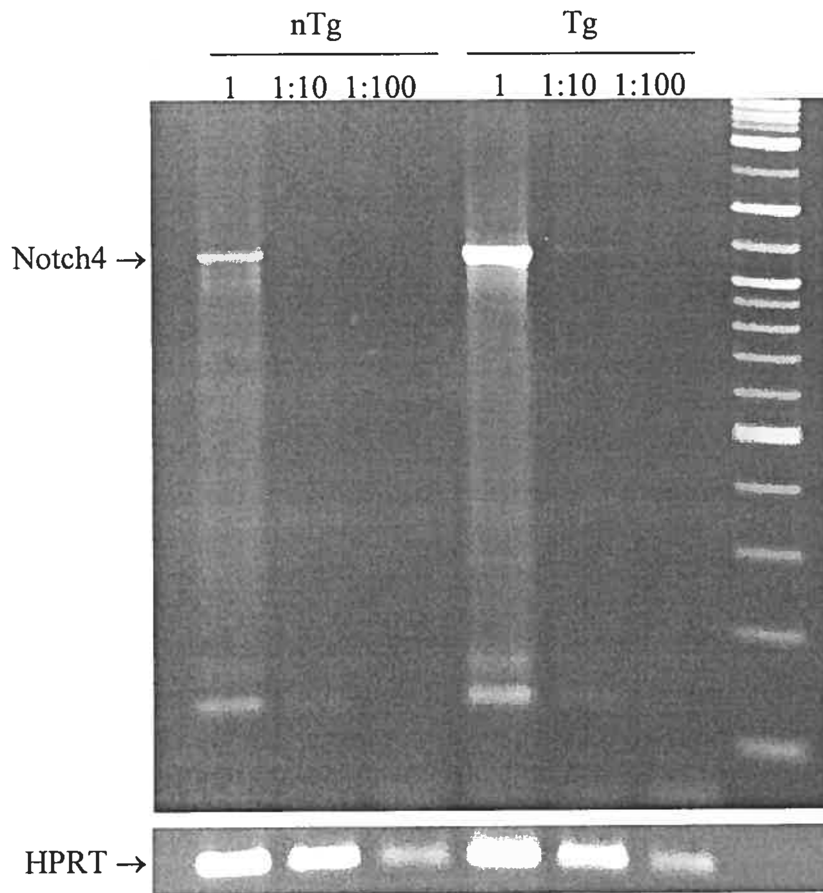
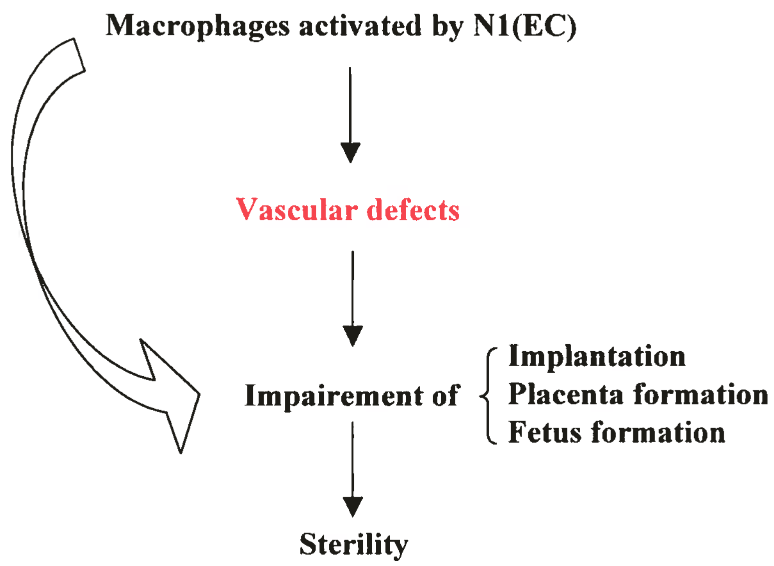


Figure 8.



Chapter 5:

Notch1 Ectodomain Expressed in Macrophages Promotes Tumor Progression, Metastasis, and Angiogenesis

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Key Words: N1^{EC}, macrophages, DEN, C3L5, B78, tumor, angiogenesis.

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In Preparation (Confidential)

Abstract

Previous results have demonstrated that severe vascular patterning defects were discovered in the CD4C/N1^{EC} Tg mice. This is particularly true in the liver, in which macrophages targeted by N1^{EC} Tg play a key role. Since tumors are angiogenesis-dependent and take advantage of pre-existing vasculature, we hypothesized that tumor may grow better in these mice. We first chose diethylnitrosamine (DEN) as a known effective carcinogen to induce tumours. High incidence (100%) of liver tumours was found in the DEN-treated mice. An enhanced percentage of liver carcinomas, accompanied by increased vasculature, was observed in Tg mice (66.7%), as compared to non-Tg mice (26.7%). Lung metastases of hepatic origin were only apparent in Tg mice. In addition, Tg mice exhibit a high percentage of lung carcinomas (50%) and kidney tumors (42.9%). Subsequently, we established other distinct models to evaluate the tumor growth and angiogenesis. Isolated hepatocyte tumors induced by DEN were first injected into the mice, resulting severe metastases (i.e. chest cavity) observed in the Tg mice. We then used another model: subcutaneous transplantation with C3L5 breast tumor cells. Metastases to heart (3/5), kidney (2/4), and pLN (1/4) were only observed in the Tg mice, and both higher number and larger size of lung tumors were displayed in the Tg mice. Finally, B78 melanoma cells were subcutaneously injected into the nude mice transplanted with fetal liver (FL) cells. Faster primary tumor growth in Tg-bearing mice was found compared to that in nTg-bearing ones. In both C3L5 and B78 models, a high degree of vascularization and necrosis were observed in the primary tumors of Tg mice. Hence, N1^{EC} expressed in the macrophages is associated with tumor growth, metastasis, and angiogenesis. Macrophages might play a critical role in these processes, providing good targets for antiangiogenesis and anticancer in the future.

Introduction

It has been known for a long time that solid tumors do not grow in isolation. They instead need a proper microenvironment in order to perform a multistage progressive process. The growth of tumors can not only be directly facilitated by genetic changes (Fidler and Radinsky, 1990) but also be indirectly facilitated by tumor angiogenesis (Folkman, 1971; Tang and Conti, 2004). The progression of primary tumors, as well as metastatic tumors, may be regulated by a combination of autocrine and paracrine signals from tumor cells and stromal cells (Nicolson, 1993; Lin et al., 2002; De Wever and Mareel, 2003).

Molecules such as CSF-1 produced by tumor cells can autonomously regulate tumorigenity and tumor invasiveness (Sapi et al., 1996; Sapi et al., 1998; Filderman et al., 1992). They can also stimulate the proliferation of endothelial cells and the recruitment of macrophages into the tumor-site, which can affect tumor growth by paracrine regulation (Nicolson, 1993; Lin et al., 2002). For tumor metastasis, at early stages, tumor cells should be more dependent on the growth's response to their new secondary microenvironment (Nicolson, 1993). Enhanced adhesion of metastatic cells to the endothelial cells of the organ's microvasculature is important for colonization of tumor cells in the secondary site. Increased responsiveness of cancer cells to paracrine growth factors (i.e.EGF) and inhibitors (i.e.TGF-β1), differently expressed at distinct organ-sites, can cause an organ-specificity of the metastatic colonization. At the later stages of progression, where a widespread dissemination of cancer occurs, the autocrine mechanisms will dominate the growth of metastatic cells (Nicolson, 1993).

Our previous results show that severe vascular patterning defects were occuered in the CD4C/N1^{EC} C3H Tg mice, particularly in the liver (Xiujie et al). Since tumors are

angiogenesis-dependent and take advantage of pre-existing vasculature (Verheul et al., 2004; Tang and Conti, 2004), we hypothesized that tumors may grow better in these mice.

The C3H mouse has been used in the National Toxicology Program (NTP) and National Cancer Institute (NCI) carcinogenesis studies since 1971. Moreover, the liver of this mouse is a sensitive model for evaluating positive tumor responses to the carcinogens (Reynolds et al., 1987). One carcinogen which is effective in liver tumor induction is diethylnitrosamine (DEN), an activator of Ras mutations in the mice (Reynolds et al., 1987; Klaunig et al., 1987; Frey et al., 2000). Other experimental tumor models are approached by transplantations of tumor cells such as C3L5, a highly metastatic cell line that was clonally derived from a spontaneous mammary tumor of C3H mouse (Lala and Orucevic, 1998). A weakly metastatic cell line known as B78, was cloned from a melanoma of C57 mouse (La Porta and Comolli, 1997). Both tumor cells have been used to evaluate the tumor progression, metastasis, and angiogenesis (Lala and Orucevic, 1998; Jadeski and Lala, 1999; Switaj et al., 2004)

In the present study, we have assessed the CD4C/N1^{EC} Tg mice in tumor progression, metastasis, and angiogenesis. In order to achieve this, we devised distinct mouse models based on CD4C/N1^{EC} Tg-bearing mice: hepatocarcinomas induced by DEN; metastasis caused by a transplantation of C3L5 tumor cells; and tumor growth and angiogenesis induced by B78 melanoma cells.

Results

While CD4C/N1^{EC} Tg mice show severe liver vascular defects, obvious hepatocarcinomas were induced in the mice treated with DEN

Previous results have demonstrated that while a thymoma phenotype was expected to be induced, liver vascular pattering defects were unexpectedly discovered in CD4C/N1^{EC} Tg mice: parenchymal tumors are quite rare in these mice (Xiujie et al.). In order to bring to light a possible association between N1^{EC}, or defective vessels, and hepatocyte tumors, an administration of DEN was performed in these mice (**Fig. 1. and Table 1**). As expected, with a DEN treatment, obvious liver parenchymal tumors were successfully induced in both nTg and Tg groups. Following a gross analysis (**Fig. 2**), tumors in male nTg livers can be clearly identified as white nodules of various sizes. Tumor nodules, however, are not easy to identify in Tg livers. Many flat nodules are fused together with big vessels and display a green-red or a blue-red color. The whole liver itself is affected with a decaying appearance (**Fig. 2**). In females, a higher number and larger size of liver tumors were obtained in Tg mice as compared to non-Tg littermates. They are frequently observed at the edge of the liver, accompanied by the defective vessels (**Fig. 2**).

Since tumor growth will increase the weight of the tissue, we weighed the whole body, as well as the liver alone, and subsequently calculated their ratio. There is no significant difference between nTg and Tg for all signs (**Table 2 and Fig. 3A**). However, the ratio in the male group treated with DEN is higher than in the female group treated with DEN (**Fig. 3Ac**). Although the statistic is not significant, it is consistent with a higher number of liver tumors observed in males as described below.

Higher number of large liver tumors were developed in Tg mice

With a careful gross examination, almost 100% of mice in non-Tg and Tg (males and females) were found to have developed hepatocellular tumors (**Table 3A and Fig. 3B**). Although there is no significant statistical difference, more tumors were observed in males than in females (**Fig. 3Ca**), a reflection of the results obtained by other teams (De Maria et al., 2002; Bugni et al., 2001). Hence, the following results comparing the tumors between nTg and Tg will be separated into distinct gender groups. Since the size of a tumor is related to its malignancy, and since carcinoma is usually larger than 5mm in diameter, the size of the liver tumors in our experiments was measured and defined as either "small" (< 5mm in diameter) or "large" (> 5 mm in diameter). In both male and female groups, a higher number of large tumors in Tg mice was found than in non-Tg ones (this difference is significant in female group), though no difference appeared in small tumors (**Fig. 2C. b and a**). Interestingly, large tumors were preferentially present in males than in females (**Table 2. and Fig. 2Cc**), which is consistent with the higher numbers in males overall.

CD4C/N1^{EC} Tg mice developed more hepatocellular carcinomas with enhanced vessels and a higher rate of pulmonary metastases than non-Tg ones, especially in males

In order to assess grades of the liver lesions, a histological analysis was carefully performed with HE staining sections. For both male and female groups of non-Tg mice, the tumor nodules were easily discriminated from compressed adjacent normal parenchymal hepatocytes (**Fig. 4. A-E**), although it was not possible to distinguish nodules with a clear demarcation in Tg mice (**Fig. 4. B-F**). However, more carcinomas (85.7% and 40%) developed in Tg groups compared to nTg mice (50% and 0%) (**Table 3 and Fig. 4I**).

These malignant cells are polymorphous with big nuclei, and form distinct glandular patterns (**Fig. 4. D and H**). They are often found around the huge defective vessels (**Fig. 4. B and F**), and are frequently swimming within vascular channels (**Fig. 4. D and H**). Necrosis, an important sign of angiogenesis (Leek et al., 1999), was found in Tg mice (4/7 and 1/5) but not in non-Tg mice. Interestingly, in Tg males, a high rate of pulmonary metastases (57.1%) were observed (**Fig. 4B.i**), as compared to none detected in other mice. Tg mice developed progressive liver tumors and promoted lung metastasis, especially in males, suggesting that $N1^{EC}$ Tg expressed in macrophages is associated with development and metastasis of the liver tumors initiated by DEN.

CD4C/$N1^{EC}$ Tg mice developed more highly-progressed tumors of pulmonary origin than nTg mice

To investigate metastasis from the liver, other organs were examined. We found that lung was the second organ bearing some tumors. Higher incidence, higher number, and large size of lung tumors were observed in Tg mice as compared to nTg mice (**Table 3 and Fig. 5A**). A significant difference was particularly found in the female group.

To identify the origin of these lung tumors, histological analyses were performed. Surprisingly, almost all tumors dissected from gross analysis samples were critically formed by lung-derived cells, and, with the exception of two cases, they were accompanied by some hepatocytes in Tg males (**Fig. 5B. h and i**). Contrary to the lack of carcinoma found in non-Tg mice, Tg mice from both male and female groups have displayed a high number of carcinomas (42.9% and 60%, respectively) (**Fig 5B. a-g and Table 3**). Additionally, the tumors were larger in males than in females (**Fig. 5B. a-b and e-f; Fig. 3. A-C and D-E**). These results indicate that DEN can also induce lung tumors (Shukla and Taneja, 2002; Kim et al., 1997). The important observation in this case, is that the Tg mice

are more inclined to develop DEN-induced lung tumors than the nTg mice. Hence, the CD4C/N1^{EC} Tg mice, especially males, display accelerated development of lung tumors as well as liver tumors.

CD4C/N1^{EC} Tg mice developed kidney tumors, particularly in males

While many demarcated tumors were easily found in the livers and in lungs (especially in Tg males), kidneys with tumor-like masses were additionally revealed by gross examination. These diseased kidneys were particularly identified in Tg males, compared with almost no disease recorded in the other mice (**Table 3. and Fig. 6A**). The gross characters of these kidneys include a big size, an irregular shape, a rough surface, and a solid tissue.

To further confirm the features of these tumors, histological analysis was performed with the kidney HE staining sections. Very strikingly, a high incidence of glomerular tumors (42.9%) and tubular epithelial carcinomas (57.1%) was revealed in Tg males treated with DEN (**Table 3. and Fig. 6B. b, c. and g**). In contrast, no tumor was histologically observed in non-Tg mice; only two out of four non-Tg males were proven to sustain as hyperplasia (**Fig. 6B. a-g**). Despite the fact that no similar disease was detected in females, one Tg mouse showed tubular epithelial hyperplasia (**Fig. 6B. d and e**). These results indicate that CD4C/N1^{EC} Tg mice promote the development of renal tumors induced by DEN (Ansar et al., 1999).

In summary, multiple-organ tumors (liver, lung, and kidney) and lung metastases were induced in the Tg mice, indicating a role of either Tg N1^{EC} (or cells expressing Tg) or defective vessels caused by Tg in tumor formation and metastases.

Severe metastases were observed in Tg mice transplanted with hepatocyte tumor cells and C3L5 tumor cell line

To further figure out the involvement of CD4C/N1^{EC} in tumor metastasis in Tg mice, hepatocarcinoma cells isolated from liver tumor induced by DEN were first transplanted into the liver of non-Tg and Tg mice via intra-hepatic injections. Most Tg mice, but not nTg mice, died during the injection (**Table 4**). One month after the transplantation, liver-localized tumors were detected in nTg livers, but not in the Tg ones. However, severe metastases were observed in the Tg mice in contrast to mild ones found in the nTg mice (**Table 5 and Fig. 7**). Solely intestinal metastases were noted in the nTg mice, whereas in Tg mice, larger intestinal tumors, as well as additional metastatic tumors were identified: thymus, chest cavity, and pancreas (**Table 5 and Fig. 7**). This outcome hints that CD4C/N1^{EC} Tg mice are associated with tumor metastasis.

Subsequently, another model of tumor metastasis was designed using C3L5, a cell line with a propensity for pulmonary implantation (Lala and Orucevic, 1998). These cells were subcutaneously transplanted into both CD4C/N1^{EC} nTg and Tg mice. Four weeks after the transplantation, the mice were observed. Contrary to expectations, preliminary results showed that the primary tumors in the Tg mice were not larger, but rather smaller than those in the nTg mice (**Fig. 8A and 8B**). However, as expected, the tumors in the Tg mice were more highly-vascularized and hemorrhagic than those in the nTg mice (**Fig. 8B).** In addition, more necroses were found in the Tg primary tumors than in nTg mice (**Fig. 8C**). These facts suggest that the CD4C/N1^{EC} Tg mice are relevant to tumor angiogenesis.

Interestingly, severe metastases were detected in Tg mice compared to nTg mice. With gross examination, large tumors (> 5mm) were clearly spotted in the Tg heart, but not

in the nTg heart (**Fig 9A. a-b and Table 6**). Moreover, a larger size and higher number of tumors was observed in the Tg lung than in the nTg lung (**Fig. 9A. c-d and Table 6**). With histological analyses, the tumors observed in heart and lung were confirmed to be metastatic tumors (**Fig. 9B**). Malignant tumor cells are observed to be invading the myocardium and endocardium of the Tg heart but not the nTg heart (**Fig. 9B. a and b**). While metastatic cells are forming small tumors with well-differentiated pseudoglandular architectures in the nTg lung, tumors in the Tg lung increased in number and exhibited an enlarged size with poorly-differentiated pseudoglandular architectures (**Fig. 9B. c and d**). Interestingly, intra-glomerular metastatic tumors were found in the Tg mice, not in nTg mice (**Fig. 9B. e and f**). In addition, metastatic cells were also seen in the peripheral lymph-node of Tg mice but not in nTg mice (**Fig. 9B. g and h**). These data confirm that the CD4C/N1^{EC} Tg mice are associated with tumor metastasis.

Primary tumor growth in the Tg-bearing nude mice is both faster and displays a higher degree of valcularization

To further confirm that CD4C/N1^{EC} Tg mice facilitate in tumor angiogenesis, another tumor growth model was established using B78, a weakly metastatic cell line (La Porta and Comolli, 1997). The cells were subcutaneously injected into the nude mice, which had been transplanted with nTg or Tg FL cells one month earlier. Tumors in the Tg-bearing mice grew faster than those in the nTg-bearing mice and the large size of tumors could be clearly identified by macroscopic examination (**Fig. 10. A and B**). Neovascularized and hemorrhagic histology was once again observed in the tumors from Tg-bearing mice (**Fig. 10. B and C**). To distinguish the features of tumor vessels between nTg- and Tg-bearing mice, Microfil® perfusion was performed. Of note is the fact that Tg tumors are always difficult to perfuse, and it was necessary to administer even more

Microfil® products (because of aberrant angiogenesis or hemorrhage?). Afterwards, more tortuous and blind-end vessels were observed microscopically in the tumors coming from the Tg-bearing mice, as compared to those from nTg-bearing mice (**Fig. 10D**). Histological examination reveals that more necroses are present in the tumors from the Tg-brearing mice than those from nTg bearing-mice (**Fig. 10D**). These results, in turn, suggest that CD4C/N1^{EC} Tg mice are involved in tumor growth and tumor angiogenesis.

Discussion

Previous results showed that $N1^{EC}$ induces multi-organ vascular defects in Tg mice, especially in the liver, in which macrophages targeted by $N1^{EC}$ Tg play a key role (Xiujie et al). Here, we demonstrated that the CD4C/$N1^{EC}$ Tg mice promote multi-organ carcinogenesis including the liver, lung, and kidney when initiated with DEN. We have also demonstrated that the Tg mice are associated with tumor metastases, as detected in multiple organs in the models transplanted with hepatocarcinoma and C3L5 tumor cells. Moreover, we observed that the Tg is involved in tumor growth with the model of B78 melanoma transplantation. Finally, our data indicated that the CD4C/$N1^{EC}$ Tg mice are associated with tumor angiogenesis: enhanced angiogenesis was observed in hepatocarcinomas induced by DEN and in primary tumors injected by C3L5 and B78 tumor cells in Tg mice.

The tumor must have a vascular supply in order to maintain its respiratory and nutritional requirements (Folkman, 1990), indicating that angiogenesis can be treated as a paracrine factor for tumor progression. Enhanced vascularization was observed in Tg-bearing tumors with the primary features of more malignancy in DEN treatment and faster growth in B78 tumor cell transplantation. These results suggest that the involvement of $N1^{EC}$ in angiogenesis is required for the tumor progression. Some tumor development also takes advantage of pre-existing vasculature (Verheul et al., 2004). In the model treated with DEN, liver malignant cells in Tg mice are often adjacent to large, defective vessels. Malignant tumors are also observed in other Tg organs (lung and kidney) where defective vessels already existed before the DEN treatment (Xiujie et al.). These results suggest that the pre-existing defective vessels might promote with the carcinoma progression by providing more DEN carcinogen to epithelial cells via their permeability (Xiujie et al.). On

the other hand, activated signals expressed in endothelial cells of the pre-existing defected vessels might accelerate malignancy of the epithelial cells. These malignant cells in turn stimulate new aberrant angiogenesis by positive feedback regulation.

The newly formed vessels are easily penetrated by metastatic tumor cells, which thereby enter the systemic circulation (Weidner et al., 1991), indicating that angiogenesis can be of a paracrine nature with regards to tumor metastasis. This might partially explain the phenomenon observed in the model transplanted with C3L5 tumor cells. A high degree of neovascularization is observed in the primary tumors of the Tg mice. However, in contrast to B78 tumor cells, severe metastases to other organs, but not fast tumor growth, are particularly identified in the Tg mice. This suggests that the role of these new vessels might favor metastasis of primary tumor cells to other organs, rather than growing in the injection site. Pre-existing vasculatures might be also advantageous to tumor metastases. Consistent with other reports (Jadeski and Lala, 1999; Lala and Orucevic, 1998), lung metastases are observed in both nTg and Tg mice. However, more severe metastases were found in Tg lungs compared to nTg mice. Also, large metastases to normally rare targets, such as heart and glomeruli, are discovered in the Tg mice. Probably, the pre-existing defective endothelial cells in the Tg mice provide positive paracrine signals for recruiting tumor cells to colonize in the second organs, resulting in multi-organ metastases. Similarly, metastatic hepatocytes to the lung, as observed in DEN-treated Tg mice, might also be related to the newly formed and pre-existing defective vessels.

Another required factor for paracrine regulation of tumor progression and metastasis might be the presence of macrophages. It has been established that the majority of malignant solid tumors contain numerous leukocytes, of which, macrophages make up the major components (Bingle et al., 2002; Elgert et al., 1998). These macrophages are

referred to as tumor-associated macrophages (TAMs), which correlate with prognosis of many human cancers (Bingle et al., 2002). Decreased progression and metastases of breast cancers were observed in the GSF-1$^{-/-}$ mice without macrophages, proving that the latter play a role in tumor progression, metastasis, and angiogenesis (Lin et al., 2002; Lin and Pollard, 2004). TAMs can be first recruited to tumor sites by a range of factors from neoplastic tumor cells, including MCP-1, CSF, and VEGF (Ohno et al., 2003). They then produce cytokines or growth factors such as PDGF, TGF, and VEGF that can directly be involved in tumor progression by paracrine fashion (Leek and Harris, 2002; Sunderkotter et al., 1994). As described in the previous paper, macrophages are reprogrammed by N1^{EC} Tg and exert an angiogenic role through a paracrine loop. Probably, this mechanism also functions in the tumor progression and metastasis. The reprogrammed Tg macrophages could be first recruited by the primary tumors (induced by DEN carcinogen as well as by C3L5 and B78 tumor cells). They then abnormally cluster in the tumors where they can stimulate tumor cell mitogenesis and angiogenesis. In this way, the tumor can either grow fast in the primary site (B78 melanoma) or easily metastasize from the primary site (C3L5 breast tumor). Tumor cells might also be easily colonized in the secondary organs, causing multi-organ metastases due to the reprogrammed macrophages. Hence, the next interesting experiment will be to characterize a role of macrophages in progressed tumors (DEN and B78) as well as in metastatic tumors (C3L5).

It is notable that the most highly necrotic breast tumors are also the most highly angiogenic (Leek et al., 1999). In our models, a high degree of necrosis was observed, accompanied by a hypervascularization in the primary tumors (C3L5 and B78) of the Tg-bearing mice (Table 7). They were also observed in the hepatocarcinomas of the Tg mice induced by DEN, who had the most severe carcinomas and aberrant angiogenesis. These

data suggest that those tumors with the highest levels of necrosis are the most angiogenic and might possess the highest focal macrophages.

Among those tumors induced by DEN, severe tumors are found in males, while a significantly higher number of large tumors was identified in Tg females. This implies that tumors in the male group may have developed too late in both nTg and Tg groups to be compared together. In the metastatic tumors induced by C3L5, repeated experiments with more animals are currently proceeding in order to get ideal statistics.

In conclusion, $CD4C/N1^{EC}$ Tg mice are associated with tumor growth, progression, metastasis, and with angiogenesis by some paracrine mechanisms (defective ECs and reprogrammed macrophages). The exact molecular mechanisms still remain unknown. Further work should be done to identify key molecules required for these paracrine loops, which may facilitate anti-tumor treatments in the future.

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Methods and materials

Animals

CD4C/N1^{EC} Tg mice were generated as previously described (Xiujie et al.). Two groups of CD4C/N1^{EC} transgene littermate mice (nTg and Tg) of both sexes were prepared for the experiments of carcinogen-induced tumor (Table 1.). Genotyping was conducted immediately after DEN injection.

Treatment of animals

On day 14 after birth, a total of thirty mice received a single peritoneal injection of DEN (Sigma; 14ug/g body weight), diluted with phosphate buffered saline (PBS) (Hara et al., 2000). DEN-treated CD4C/N1^{EC} nTg and Tg mice were weighed and sacrificed between 52 to 54-week-old (**Fig. 1**). Major organs, including liver, lung, kidney, prostate, and others were examined for macroscopically visible tumors and the livers were weighed. Then whole mice, including organs, were perfused and fixed with 4% paraformaldehyde. The number of tumors in the liver and in the lung was counted. The size of the tumors in the liver was measured and recorder in two catalogues: < 5 mm in diameter and > 5 mm in diameter.

Tumor cell line and media

C3L5 is a highly metastatic cell line derived by five cycles of repeated *in vivo* selections for spontaneous lung metastases, following a subcutaneous transplantation of C3 cells into the C3H/Hej mice (Jadeski and Lala, 1999). The C3L5 cells used in the present study were kindly provided by Dr. Peeyush Lala (Ontario, CANADA) and cultured according to the provided protocol. Briefly, C3L5 cells were grown from frozen stock and maintained in RPMI 1640 medium (GIBCO. Cat. 31800-089) supplemented with 10% FBSI and 1% penicillin-streptomycin in a humidified incubator, 5% CO_2. On the other

hand, B78, a weakly metastatic cell line, is derived from a C57BL/6J murine melanoma (La Porta and Comolli, 1997). B78 melanoma cells were maintained in DME medium (GIBCO. Cat. 12100-061) supplemented with 10% FCSI in a humidified incubator, 5% CO_2.

Tumorigenicity Assay

Hepatocarcinomas (induced by DEN) were isolated from the liver and were subcutaneously injected into each side of the back of 4-month-old CD1 nude mice. Tumors were harvested when they reached a size of 1.5-2.0 cm in diameter. Then the tumor cells from the nude mice were syngenic into C3H mice for two cycles. These cells were finally transplanted into CD4C/N1^{EC} Tg mice. Briefly, the fresh cells were meshed until reaching almost single-cell suspensions. These suspensions were intra-hepatically injected into the liver (quarter and lateral lobes) of CD4C/N1^{EC} nTg and Tg mice (four-month-old and six mice per group). One month later, the mice were killed and their organs were macroscopically and microscopically examined.

To induce metastases, groups of mice (n=4) were received s.c. injections of $2X10^6$ C3L5 breast cancer cells. All mice were sacrificed at 4 weeks after injection. Metastatic tumors were removed to perform HE staining. To induce skin tumors, $1X\ 10^6$ B78 melanomas were intradermally injected into the nude mice (n=4 per group) transplanted with FL cells, as described below. Skin tumor surface area was measured once per 2 or 3 days until 4 weeks after the tumor cell transplantation. Then the nude mice were sacrificed. Skin tumors were removed and divided into half; therefore paraffin and frozen sections were both obtained from the same sample. Then the sections were performed HE and IHC.

FL cells transplantation (FLT)

For FLT, donors (CD4C/N1^{EC}) were genotyped by fast PCR of liver and other organs. Recipients (nude mice) were lethally irradiated (400 rads). Approximately, 4 X 10^6 FL cells were injected into the tail veins of the irradiated mice. For tumor assay, 8 weeks after FLT, the nude mice were examined via a tumorigenicity assay, as described above.

Histological examination

The fixed organs as described above were embedded in paraffin for routine processing and examination of HE staining sections as previously described (Xiujie et al.).

Figure legends

Figure 1. Experimental design of DEN treatment.

↓ DEN (14μg/g body weight i.p.) S; Sacrifice.

Figure 2. Macrographs of liver tumors of mice treated with DEN.

Gross liver tumors shown in whole nTg (A and D) and Tg mice (B, C, and E) are compared in different male (A-C) and female (D-E) groups. In males, in contrast to nTg liver, exhibit clear tumor nodules (pink arrow) which vary from small (B) to big (A), the appearance of the whole liver looks decaying and tumor nodules are not easy to identify (C). In females, when compared to nTg liver, harbour no or few small tumors (D), however, clear tumor nodules situated at the edge of the liver (black and white arrow) are visible in Tg mice (E). Big-edged vessel is indicated (arrowhead).

Figure 3. Liver and body weight as well as incidence and number of liver tumors.

A| Liver weight, body weight, and the ratio of liver/body weight were compared between nTg and Tg mice. B| Incidence of liver tumors in nTg and Tg mice (male and female group). C| Number of liver tumors classified into two groups "small" (< 5 mm in diameter) and "large" (> 5 mm in diameter) were compared in nTg and Tg mice induced with DEN.

Figure 4. Histological analysis of hepatocyte tumors of mice induced with DEN

Liver sections including tumors from mice treated with DEN were stained with H & E staining. Comparison of the border between tumor and adjacent tissue, vessel, and malignancy of hepatocytes from nTg (A, C, E, and G) and Tg mice (B, D, F, and H). Note that, in nTg livers, tumors (Tu) are well demarcated (A and E, green arrowhead), but no visible remnant of adenoma (Tu) is subsisted in the Tg livers (B and F). Note also big superficial vessels surrounding malignant cells (B and F: blue arrow) and vascular channels among the malignant hepatocytes (D and H: blue arrows indicate ECs and black

arrow indicate red cells) in Tg livers, compared to the vessels in nTg livers (A, C and E, G). Note that hepatocytes with big nucleus like eyes (D) have formed isolated glands in Tg mice (D and H), indicating more malignancy (C and G). Percentage of classified tumors and necrosis is distinctly shown in nTg and Tg mice (I). Tu: tumor, Li: liver.

Figure 5. Incidence, numbers, and histological analyses of lung tumors of mice treated with DEN

A| Comparison of the incidence (a), number (b), and size (c) of lung tumors between nTg and Tg mice. B| Comparison of lung tumors between nTg (a,c, and e) and Tg (b,d, and f) from male and female groups via histological analyses with HE staining. Note that tumors are larger in Tg lungs (b and f) than those in nTg mice (a and e). Also, note the mixed differentiated carcinomas in Tg lung-well differentiated carcinoma with glandular (d, green arrow) and poorly differentiated carcinoma (b, black arrow), compared to the uniform adenomas in nTg lung (c and g). Hepatic metastatic cells are shown in Tg lung (i), not in nTg one (c). Classification of distinct grade of lung tumors between nTg and Tg mice is shown (g).

Figure 6. Incidence and histological analysis of kidney tumors of mice induced with DEN.

A| A high incidence of tumors manifested itself in Tg kidneys, compared to nTg mice. B| Comparison of kidney tumors between nTg and Tg mice via histological analysis with HE staining. Note the tubular epithelial tumors (b and f) and the glomerular tumors (c and g) present in male Tg kidneys but neither in male nTg mice nor in any females (a,f and g).

Figure 7. Tumor formation in CD4C/N1^{EC} Tg mice by transplanting hepatocarcinomas induced by DEN

Tissues fixed with formaldehyde were sectioned and stained with H & E staining. A| Liver localized tumor observed in the nTg mice (n=5, a and c) but not in Tg ones (n=6, b and d). B| Severe metastases of hepatocarciniomas were shown in Tg thymus, intestine, chest cavity, and pancreas compared to only intestine metastasis in nTg mice.

Figure 8. Primary tumor growth observed in CD4C/N1^{EC} Tg mice transplanted with C3L5 breast tumor cells.

A| Measurement of primary tumor growth. B| Comparison of primary tumors in nTg (a, n=3) and Tg (b, n=5) mice by macroscopic examination. Note smaller size of tumor, however, more peripherial neovessels (arrow) and hotspots (asterisk) in Tg mouse (b) compared to nTg mouse (a). C| Necroses are shown in Tg tumor (b) than in nTg tumor (a) by HE stain (1.25X). N: necrosis.

Figure 9. Metastatic tumors observed in CD4C/N1^{EC} Tg mice transplanted with C3L5 breast tumor cells.

A| Comparison of macroscopic tumors in heart and lung in nTg (n=3) and Tg mice (n=5). Heart and lung tumors are indicated in (b. blue arrow) and (c and d. green arrow). B| Comparison of metastatic tumors with histological analyses via H & E staining in heart (a and b), lung (c and d), kidney (e and f), and pLN (g and h) in nTg and Tg mice. All are shown in 20X, except for kidney in 40X.

Figure 10. Faster primary tumor growth observed in Tg-bearing nude mice transplanted with B78 melanoma cells.

A| Tumors surface of nTg-(n=4) and Tg-(n=4) bearing mice are dynamically measured during 3-28 days after tumor transplantation. B| Note that larger, hemorrhagic (blue

arrow), and necrosis (red arrow) are displayed in Tg-bearing mice, but not in nTg-bearing ones. C| More neovascularization is shown in Tg-bearing mice than in nTg-bearing mice. D| Vessel morphology is detected by Microfil® perfusion in nTg- (a and c) and Tg-bearing mice (b and d). Note that more tortuous and blind-end vessels in the Tg-bearing mice (b and d) compared to nTg-bearing ones (a and c). HE staining shows more necroses in Tg-bearing tumor (f) compared to nTg-bearing tumor (e).

Figure 1.

Figure 2.

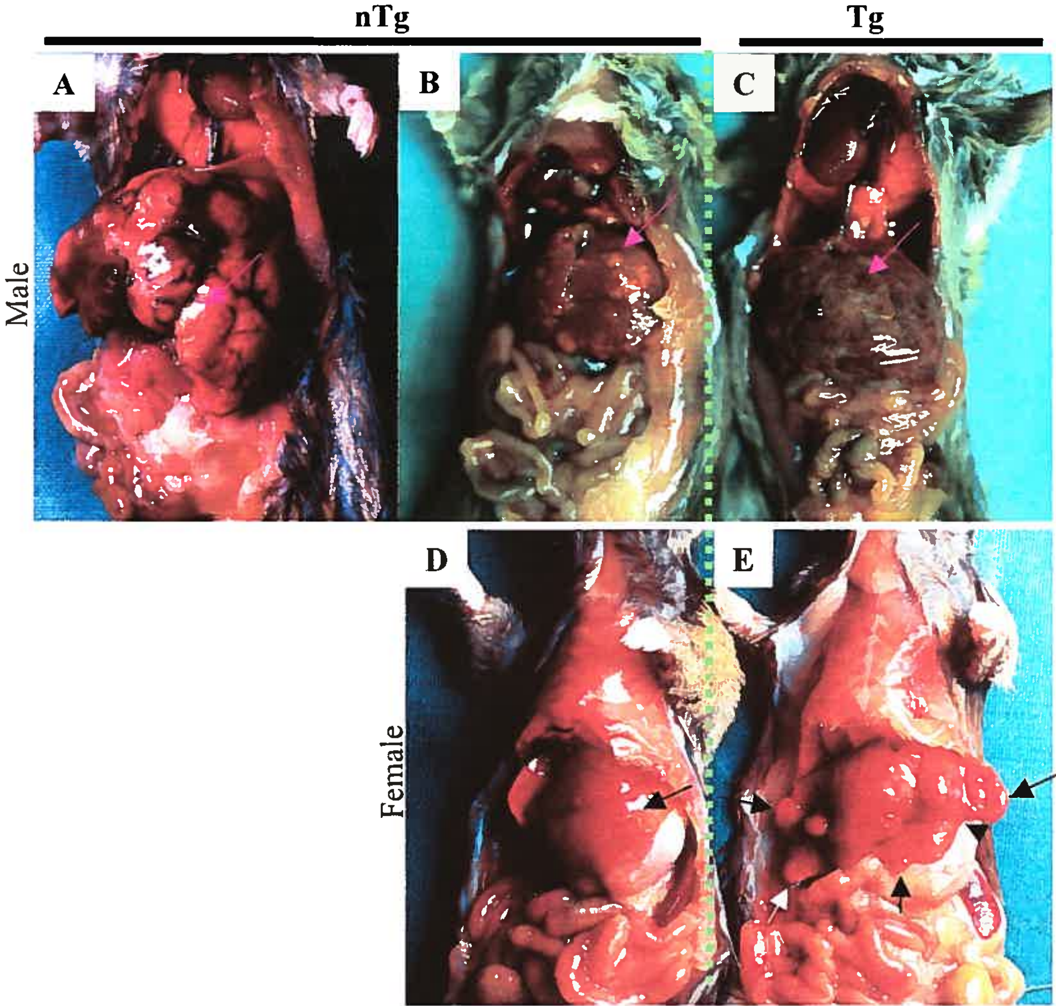


Figure 3.

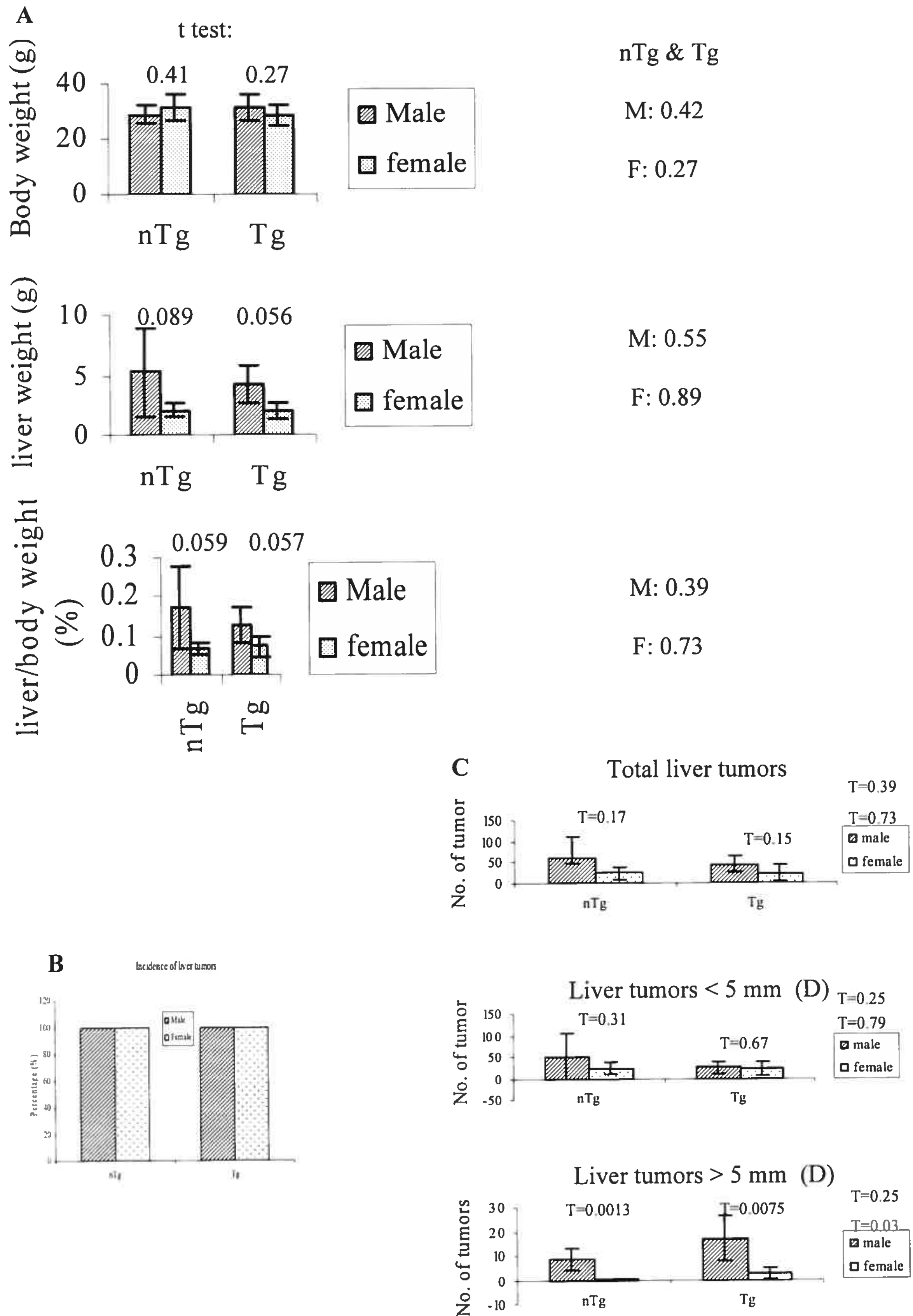


Figure 4.

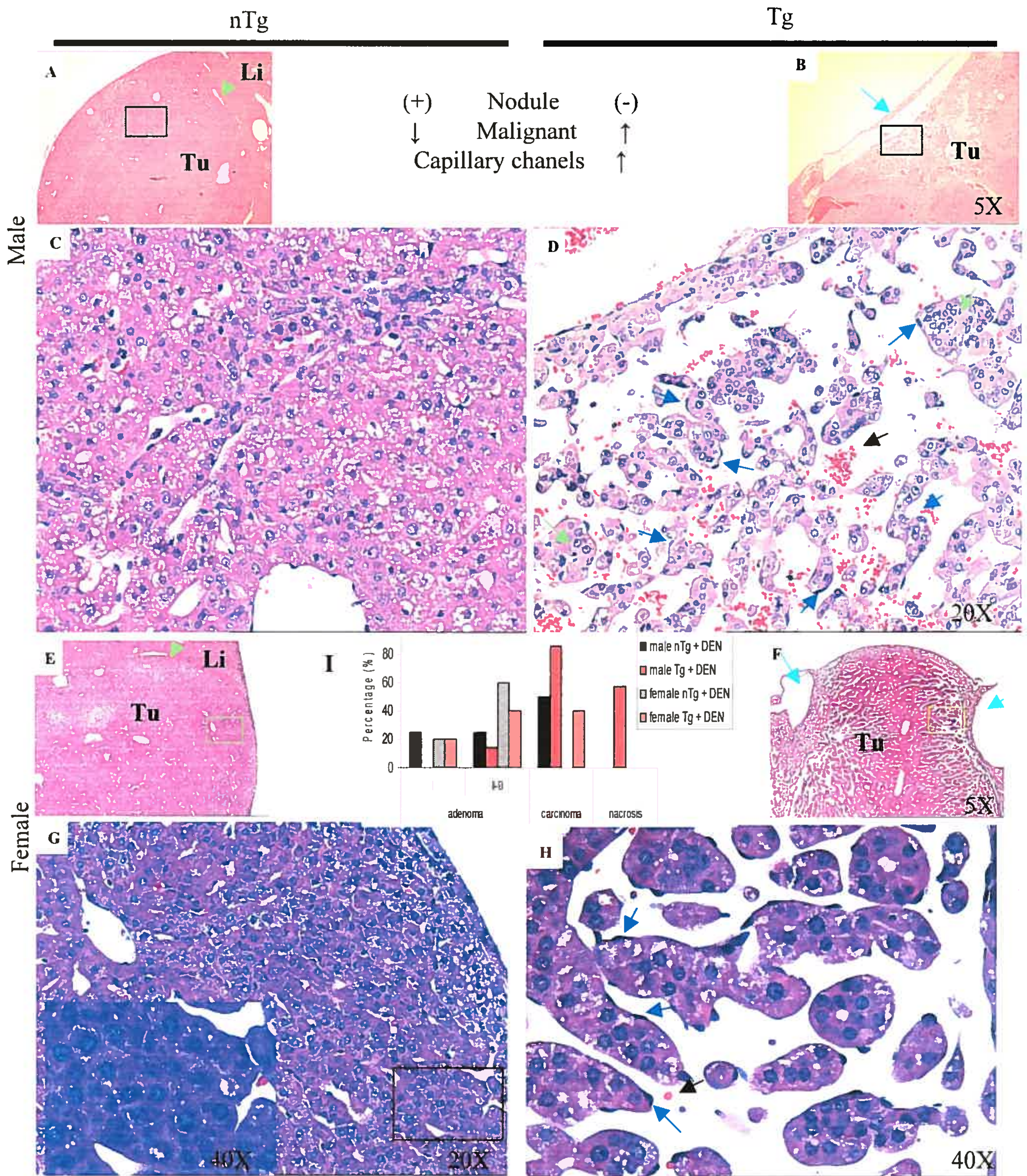
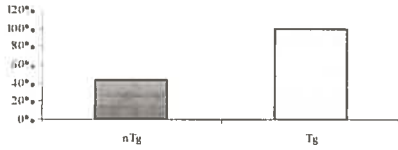


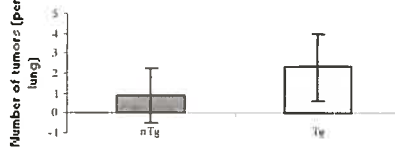
Figure 5.

A

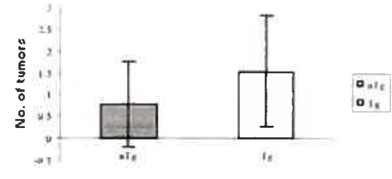
a Incidence of lung tumor in total mice



b Number of lung tumor in total mice treated with DEN



c Size of lung tumors



B

nTg

Tg

Male

a

b

h

2.5X

c

20X

d

20X

i

40X

Female

e

f

5X

Hepatocyte Metastasis only in Tg males

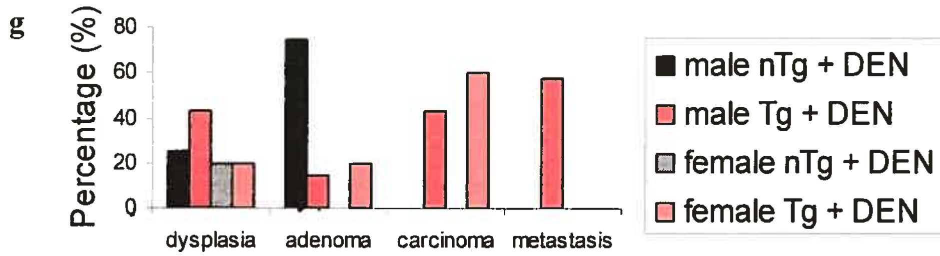
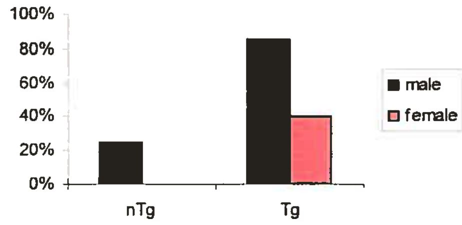
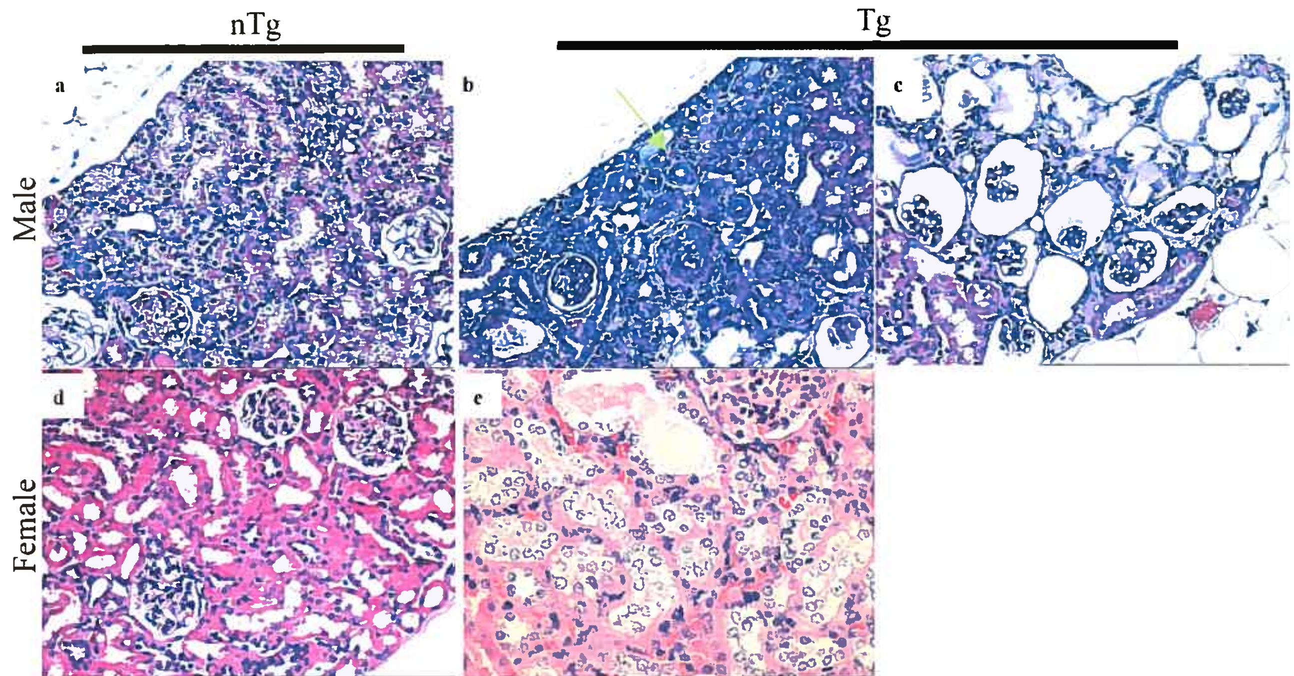


Figure 6.



B. Histology



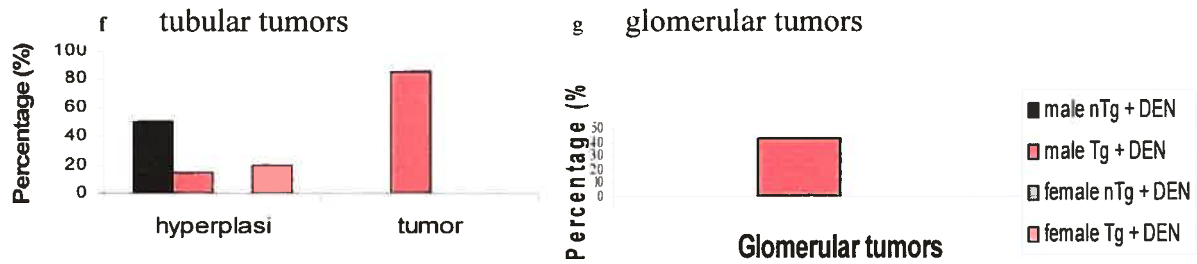
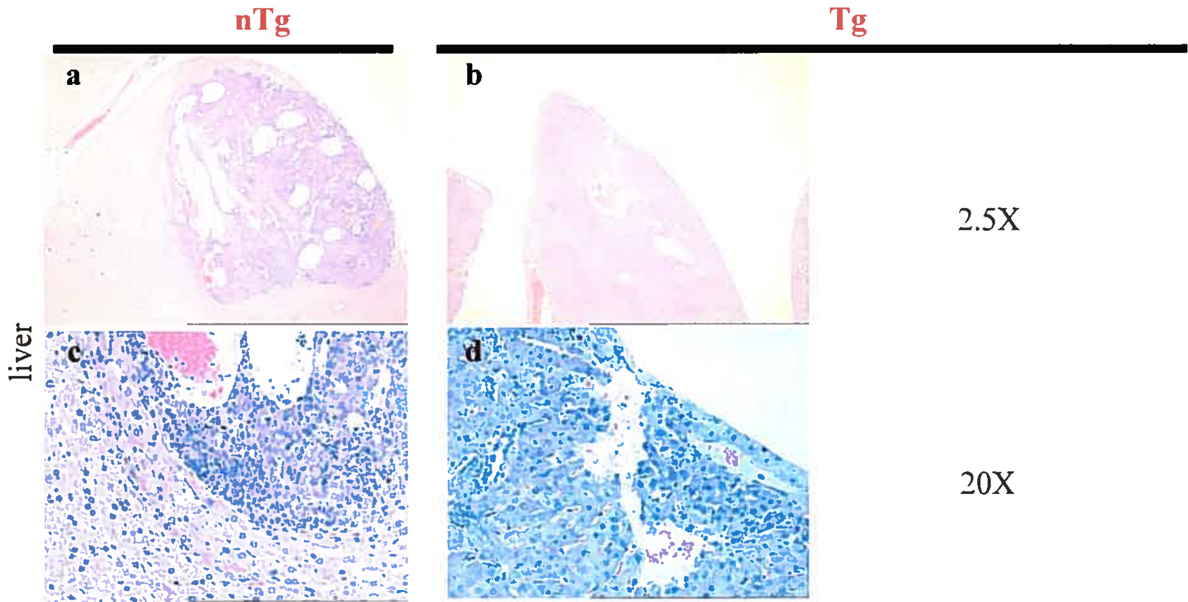


Figure 7.

A: Liver localized tumor



B: Metastatic tumors

thymus

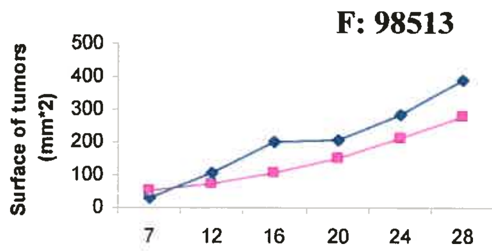
intestine

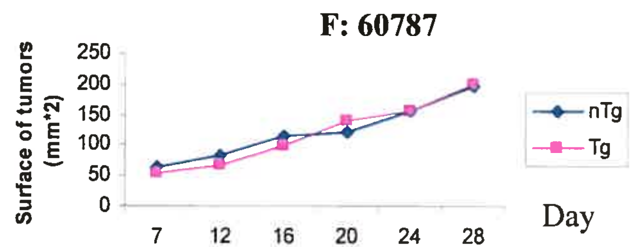
Chest cavity

pancreas

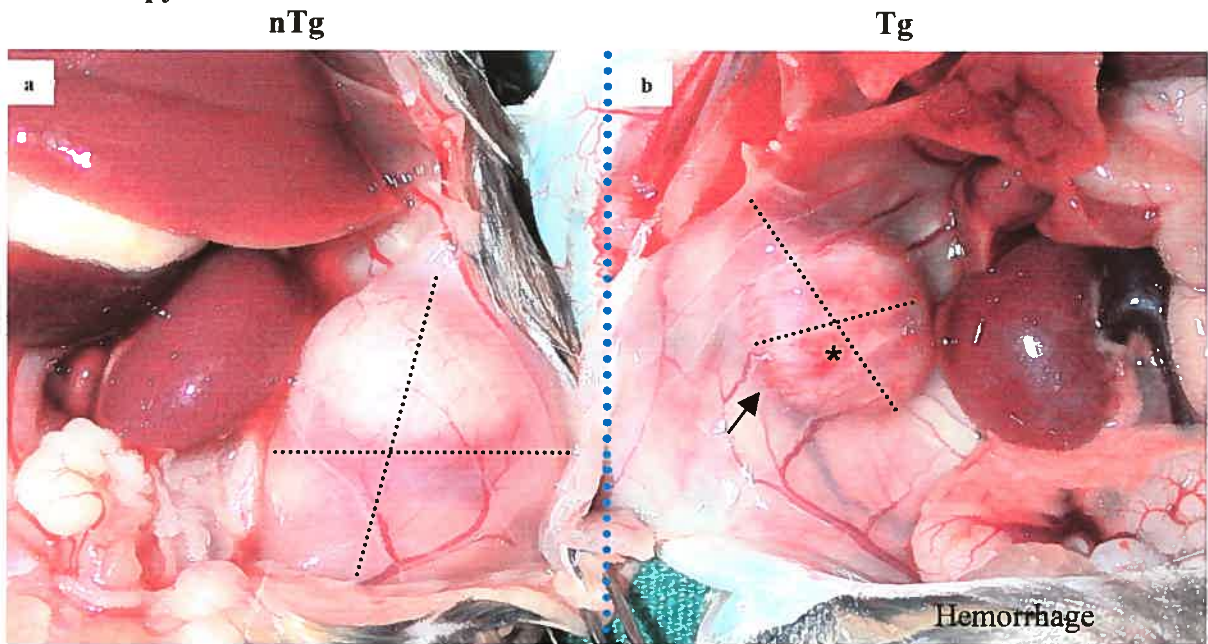
Figure 8.

A. Rate of tumor growth





B. Macroscopy



C. Microscopy

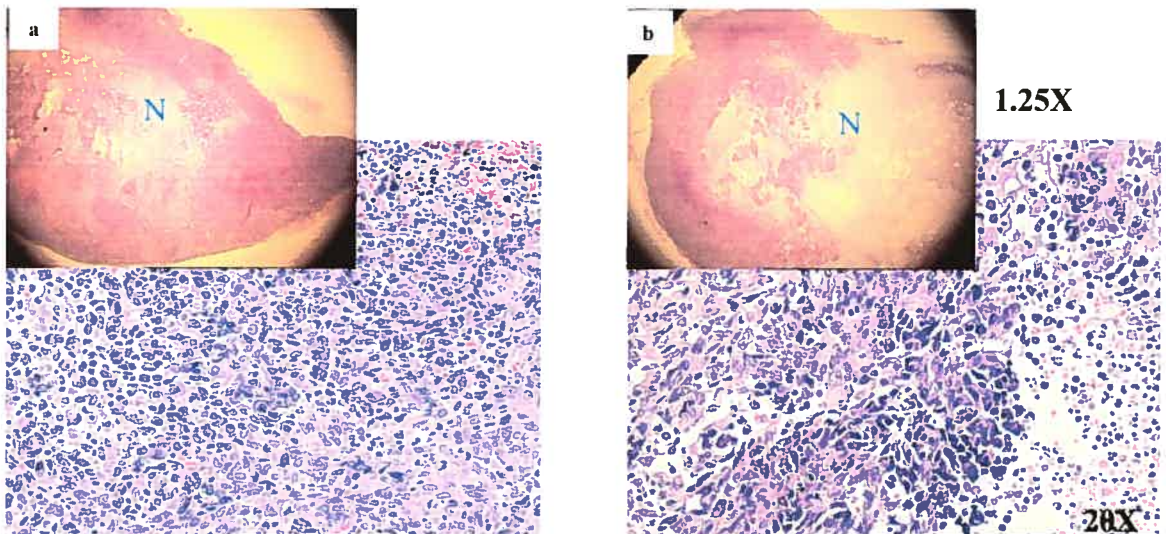


Figure 9.

A. Macroscopy

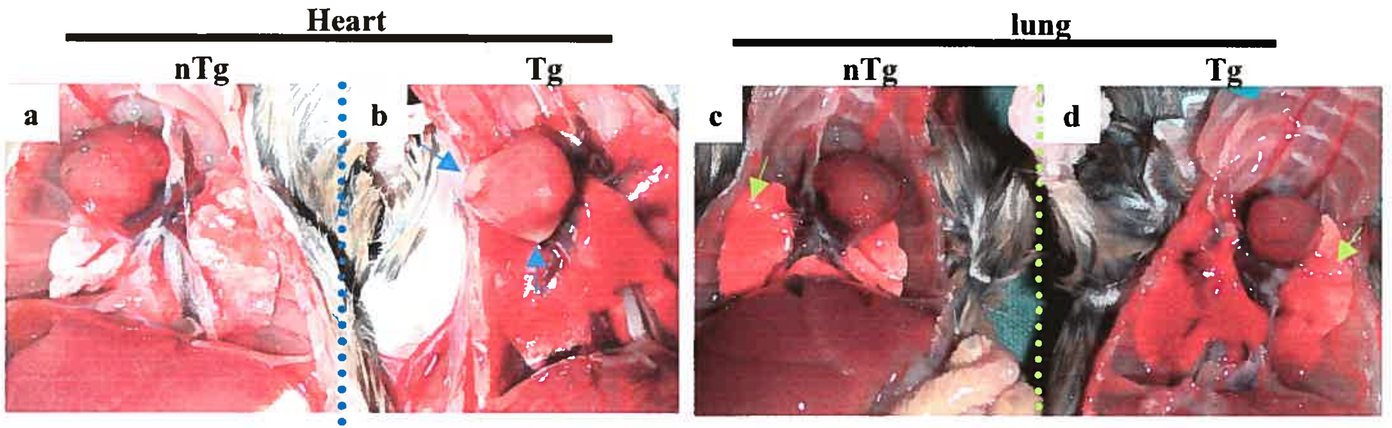


Figure 9.

B. Histology

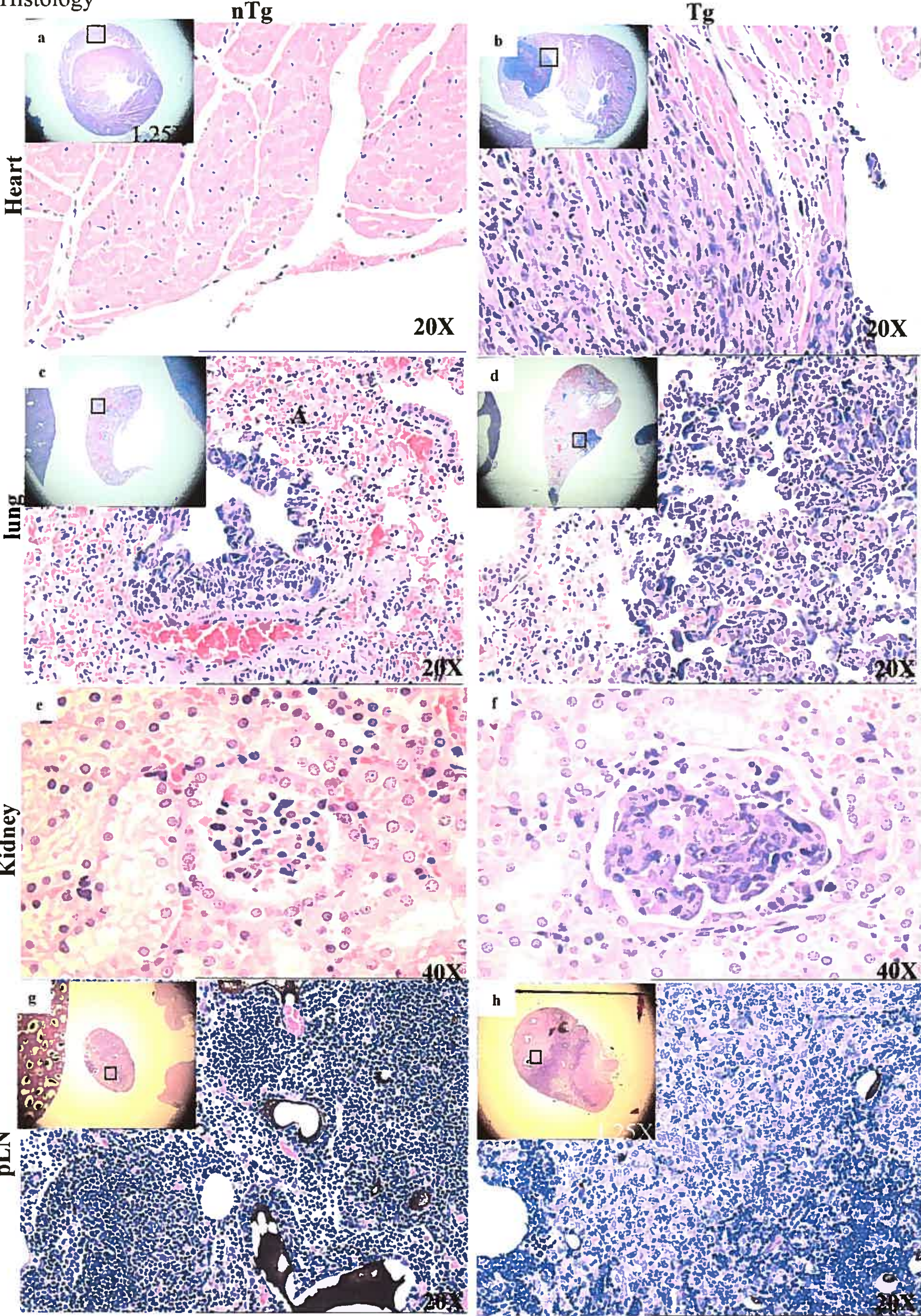
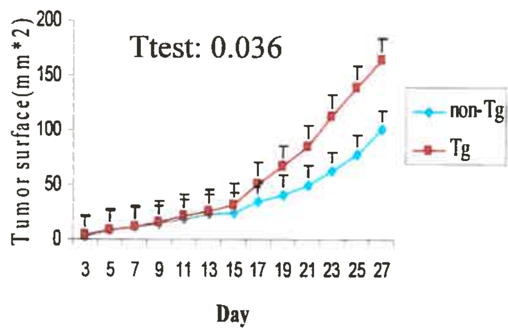
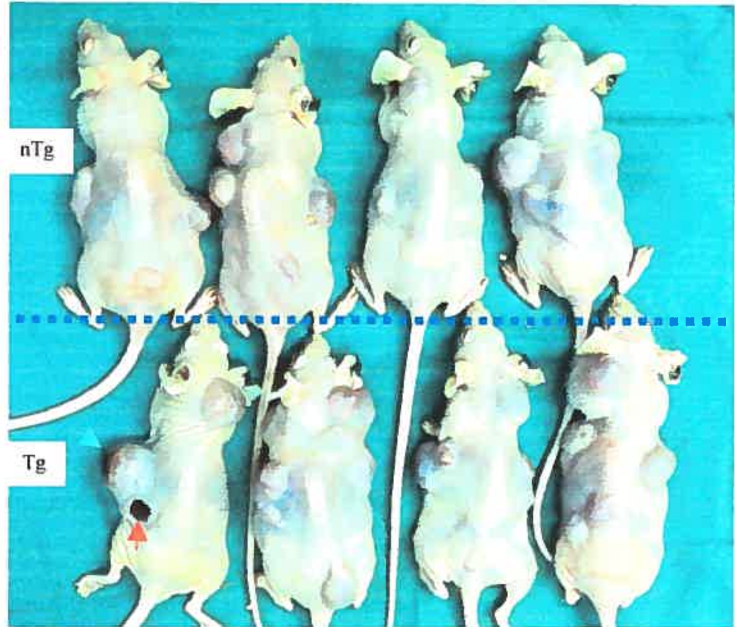


Figure 10.

A: Tumor growth



B: Tumor size



C: Tumor neovascularization

D: Tumor angiogenesis

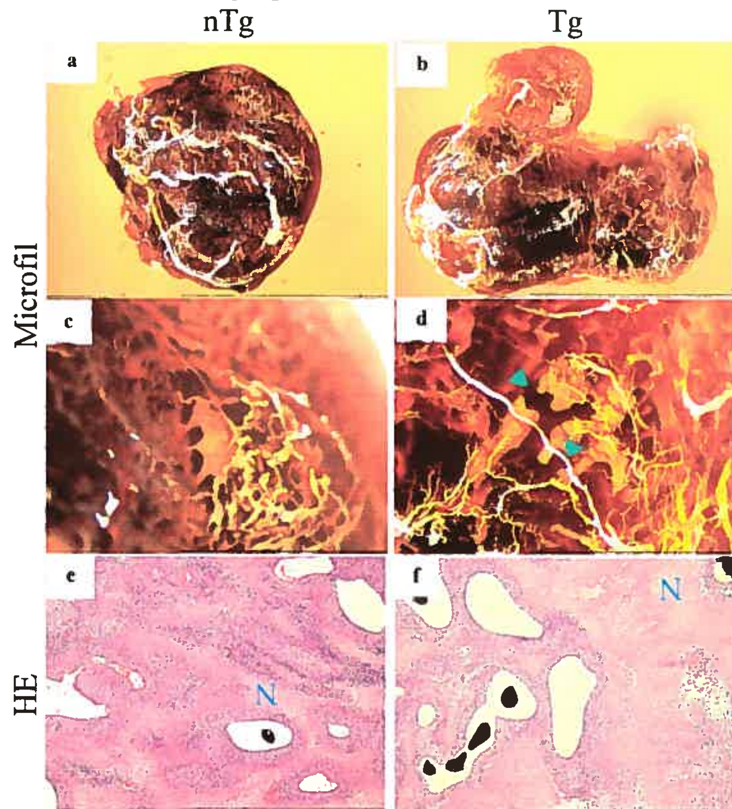


Table 1: Groups studied with DEN

	No. of treated mice			No. of analyzed mice					
				8-month		12-month		total	
	M	F	total	M	F	M	F	M	F
nTg	4	7	11	0	2	4	5	4	7(11)
Tg	9	6	15	* 1	1	7	5	8	6(14)

Table 2. General information of the liver and body weight

Treated group		No.of mice treated with DEN	No.of mice analyzed	body weight	liver wieght	liver/body weight
Male	nTg	4	4	29±3.7	5.2±3.6	0.17±0.106
	Tg	7	7	31.4±4.4	4.2±1.5	0.13±0.045
female	nTg	5	5	31.4±4.4	2.1±0.5	0.07±0.016
	Tg	5	5	28.4±3.6	2.0±0.7	0.07±0.025

Table 3: Summary for liver, lung, and kidney tumors

Summary of tumors in liver, lung, and kidney

A. Liver

Group		Gross	Histology					
			(-)	adenoma		carcinoma	nacrosis	metastasis
				I	II-III			
male	nTg + DEN	4/4 (100%)	0/4 (0%)	1/4 (25%)	1/4 (25%)	2/4 (50%)	0/4 (0%)	0/4 (0%)
	Tg + DEN	7/7 (100%)	0/7 (0%)	0/7 (0%)	1/7 (14.3%)	6/7 (85.7%)	4/7 (57.1%)	4/7 (57.1%)
female	nTg + DEN	5/5 (100%)	1/5 (20%)	1/5 (20%)	3/5 (60%)	0/5 (0%)	0/5 (0%)	0/5 (0%)
	Tg + DEN	5/5 (100%)	0/5 (0%)	1/5 (20%)	2/5 (40%)	2/5 (40%)	0/5 (0%)	0/5 (0%)

B. Lung

Group		Gross	Histology				
			(-)	dysplasia	adenoma	carcinoma	metastasis
male	nTg + DEN	3/4 (75%)	0/4 (0%)	1/4 (25%)	3/4 (75%)	0/4 (0%)	0/4 (0%)
	Tg + DEN	6/7 (85.7%)	0/7(0%)	3/7 (42.9%)	1/7 (14.3%)	3/7 (42.9%)	4/7 (57.1%)
female	nTg + DEN	1/5 (20%)	4/5 (80%)	1/5 (20%)	0/5 (0%)	0/5 (0%)	0/5 (0%)
	Tg + DEN	4/5 (80%)	0/5 (0%)	1/5 (20%)	1/5 (20%)	3/5 (60%)	0/5 (0%)

C. Kidney

Group		Gross	Histology					
			(-)	tubular			glomurula	
				dysplasia	adenoma	carcinoma	N	Y
male	nTg + DEN	1/4 (25%)	2/4 (50%)	2/4 (50%)	0/4 (0%)	0/4 (0%)	0/4 (0%)	0/4 (0%)
	Tg + DEN	6/7 (85.7%)	0/7 (0%)	1/7 (14.3%)	2/7 (28.6%)	4/7 (57.1%)	3/7 (42.9%)	3/7 (42.9%)
female	nTg + DEN	0/5 (0%)	5/5 (100%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)
	Tg + DEN	2/5 (40%)	4/5 (80%)	1/5 (20%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)

Table 4. General information on transplantation of hepatocacinoma

	nTg	Tg
Injected mice	5	6
Survival mice	5	2
Mice with tumor formation	2(F&M)	1(M)
Mice with metastasis	1(M)	1(M)

(remark: 4 Tg mice died soon after injection but not nTg mice)

Table 5. Tumors observed in the different organs of mice transplanted with hepatocarcinoma

	nTg	Tg
liver	Y(2/6)	N
thymus	N	Y
chest cavity	N	Y (two tumors, 5mm & 8mm in diameter)
lung	N	N
pancreas	N	Y
intestine	Y (one tumor, 2mm)	Y (two tumors, 5mm)
other organs	N	N

Table 6: CD4C/N1(EC) Tg mice are associated with metastasis of C3L5 tumor cells

Organs	Tg status	
	nTg	**Tg**
liver	0/3	0/5
* heart	0/3	3/5
* lung	2/3	5/5 (80 tumors/mouse)
	25/25 tumors ≤5mm	1/80 > 5mm
		79/80 < 5mm
mLN	0/3	0/5
* pLN	0/3	1/4
* kidney	0/3	2/4

Chapter 6: Conclusions and Perspectives

5.1. Involvement of Notch1 ectodomain expression driven by CD4C promoter in vascular defects of CD4C/N1^{EC} Tg mice

For a long time, researchers have turned their attention to the study of the roles of Notch signaling in development and cancer due to its fundamental function in cell fate decision (Artavanis-Tsakonas et al., 1999; Milner and Bigas, 1999; Radtke and Raj, 2003). Now, more and more studies show that Notch signaling is also required for the vascular development and for human vascular diseases (i.e. Algille syndrome and cerebal autosomal-dominant arteriopathy with subcortical infracts and leukoencephalopathy) (Sullivan and Bicknell, 2003; Gridley, 2001; Iso et al., 2003; Gridley, 2003). Appropriate expression and function of Notch is necessary for the vascular development (Gridley, 2001), as similar phenotypes were observed in both loss- and gain-of-function of Notch (Krebs et al., 2000; Uyttendaele et al., 2001). However, almost all these experiments are focused on the activation of the Notch intracellular domain except one mouse model of Notch3^{EC} destined to mimic human CADASIL (Ruchoux et al., 2003). For the first time, our results directly show that the overexpression of the Notch1 ectodomain result in hemangioma-like diseases in adults regulated by a paracrine loop (see chapter 2). In our Tg mice, the transgene consist of 1-36 EGF like-repeats of the extracellular domain of Notch1 and lack LNR, TM, and intracellular domains. The aim of this design was to imitate the "type II mutation" of Notch1 discovered in the thymomas (i.e. L45 tumor cell line) from the mice infected by Mo-MuLVs (Girard et al., 1996; Hoemann et al., 2000). This fragment has been shown to be secreted from the cells (Hoemann et al., 2000). The transgene expression is under the control of CD4C promoter, which consist of mouse CD4

enhancer, human CD4 promoter, and human CD4 Exon1. This promoter drives gene expression in CD4+ T-cells, macrophages, and dendritic cells (Hanna et al., 1994; Hanna et al., 2001), therefore, CD4C/N1^{EC} Tg mice were expected to produce thymoma. Contrary to our initial expectation, these mice develop a vascular disease regulated mainly by macrophages, thereby opening a new window for understanding the function of Notch1 ectodomain. In fact, Notch1, 4-deficient mice showing a disruption of vascular development (see below) were generated by mutations situated within the EGF-like repeats, thereby also hinting at a function of the extracellular domain of Notch1, 4. Although we did not succeed to induce thymomas in this model, the role of N1^{EC} in the tumor formation cannot be underestimated. This is strongly supported by the activated mutations in Notch1 ectodomain found in the human T-ALL latently (Weng et al., 2004). Alternative models to study the role of N1^{EC} in tumor formations will be needed.

5.2. A remolding of liver vasculatures is induced and transformed into tumor vessels

As shown in chapter 2, the vasculatures in the Tg livers are aberrantly remolded compared to nTg livers. Three apparent and distinct vessel anomalies could be observed: large meandered ectopic vessels growing at the surface or at the edge of the liver; large vascular cavities and impaired vascular branching within the liver parenchyma. These anomalies are different from those observed in Notch1, 4-deficient (N1$^{-/-}$N4$^{-/-}$) mice (Table 1.), though both phenotypes involve vascular defects caused by abnormal compartments of Notch1. The most severe phenotype of N1$^{-/-}$N4$^{-/-}$ occurs before E9.5, while that of N1^{EC} appears during adulthood although the vascular defects are induced during embryonic stage (E16.5). There are severe vascular defects found in the yolk sac and placenta of N1$^{-/-}$N4$^{-/-}$ mice, but not that severe in N1^{EC} Tg mice since all the babies from CD4C/N1^{EC} father were born normally; however, severe uterus vascular defects were observed in N1^{EC} Tg

mice (Chapter 4). The vascular defects mainly affect the anterior of the embryo of $N1^{-/-}N4^{-/-}$, while they particularly present in the liver of $CD4C/N1^{EC}$ Tg adult mice. In $N1^{-/-}N4^{-/-}$ mice, original large vessels (aortea and anterior cardinal veins) were shown defects with collapsed morphology, while in our Tg mice, normal branches within the liver are decreased but enlarged vessels are increased with superficial grow-favorite. Probably the endothelial cells select alternative cell fate between intra- and superficial-vessels as well as small and big vessels due to an imbalance of impulsive and repulsive signals as they do for artery-vein fate decision (Fuller et al., 2003). This hypothesis needs to be further investigated.

	$N1^{-/-}$ $N4^{-/-}$	$N1^{EC}$
Embryo arrest	yes (E9.5)	no
Timepoint appearing the vascular phenotype	E9.5, severe	E16.5, mild
Timepoint obtained the most severe vascular phenotype	Embryo (< E9.5)	adulthood
Defective organs/tissues	anterior of the embryo yolk sac placenta	liver others organs uterus
Type of defective vessels	large vessels (dorsal aortea, anterior cartinal veins)	large vessels (superficial veins) sinusoids
Features of defective vessels	malformation of large vessels: disorganized collapsed morphology	malformation of large vessels: superficial enlarged size, tortuous, dilation decreased intrahepatic branches cavities, cavenous heamangiomas sinusoidal capillarization

Table 1. Comparison of the phenotype (vascular defects) of Notch1,4 deficient mice and $CD4C/N1^{EC}$ Tg mice

No tumor vessels were demonstrated in $N1^{-/-}N4^{-/-}$ mice, but heamangioma and sinusoidal capillarization, that hint activated endothelial cells, were observed in our Tg mice. Especially, some characteristics of tumor vessel were displayed in our Tg mice, which are tortuous, clustered, dilated, blind-end, and permeable vessels, as well as decreased intra-

hepatic and increased superficial-hepatic vessels (Bergers and Benjamin, 2003; Torry and Rongish, 1992; Holash et al., 1999; Larcher et al., 1998; Thurston et al., 1999). The differences of vascular defects between two kinds of mice may be explained by several aspects: (1) The targets of the $N1^{-/-}N4^{-/-}$ mice are endothelial cells while the targeted cells of $N1^{EC}$ are macrophages; (2) The Notch ectodomains are directly mutated in EGF-like repeats and they are still anchored to the expressing cells in the $N1^{-/-}N4^{-/-}$ mice; however, the $N1^{EC}$ is a soluble molecule EGF-like repeats in the CD4C/$N1^{EC}$ Tg mice. Hence, $N1^{EC}$ may talk more molecules in macrophages and/or in endothelial cells compared to the EGF-like repeats in $N1^{-/-}N4^{-/-}$ mice; (3) The Notch signaling is directly disrupted in the $N1^{-/-}N4^{-/-}$ mice, while the $N1^{EC}$ does not interfere in Notch1 intracellular domain (Isabelle et al, submitted) but provokes other molecular pathway beyond Notch signaling and makes macrophages release altered factors (see bellow). Therefore, the role of $N1^{EC}$ in our model is more complex than that of mutated EGF-like repeats in the $N1^{-/-}N4^{-/-}$ mice. The $N1^{EC}$ expressed in macrophages is not only involved in vessel development but also in transformation of tumor vessel.

Our mouse model of CD4C/$N1^{EC}$ is not only suitable for the genetic studies of Notch functions in angiogenesis during development of animal models, but it also provides a vascular defect-model like hemangioma in human adulthood.

5.3. Macrophages, but not T-cells, are reprogrammed by $N1^{EC}$ to induce a severe liver vascular disease.

Although both CD4+ T-cells and macrophages (peritoneal macrophages and KCs) express $N1^{EC}$ as detected by RT-PCR or Western blotting, only the cells with morphological appearance of macrophages were detected in Tg liver by ISH. This suggests that the Tg expression in the macrophages may be stronger than that in the thymocytes.

This could explain the absence of expected thymoma. The Tg liver phenotype is consistent with the cell distribution. Macrophages are residing in all tissues in greater number than other blood-born cells. Eighty percent of the body's macrophages are present in the liver (Knolle and Gerken, 2000). In our model, clustered macrophages are often detected in the Tg liver but not in the nTg liver. Increased numbers of KCs were also detected by FACS analysis of isolated NPLCs. These results hint at an involvement of macrophages in vascular diseases. Macrophages have the potential to be reprogrammed and secrete a myriad of angiogenic factors in response to some appropriate stimuli (Sunderkotter et al., 1994). The $N1^{EC}$ transgene may provide one such stimulus. In addition to the secretion of the angiogenic factors, Moldovan and his colleagues recently show that in a mouse model with targeted expression of MCP-1 in the myocardium, macrophages implicate in the drilling of tunnels in ischemic myocardium, hinted transdifferentiation of macrophages into endothelial cells (Moldovan, 2000). This transdifferential mechanism was excluded in $CD4C/N1^{EC}$ Tg mice by fetal liver transplantation helped with ROSA marker. Except for roles of macrophages, per se, interactions between macrophages and endothelial cells are also key events in the pathogenesis of vascular disease. These interactions can be mediated by several types of receptor-ligand cross-talk, including integrin-mediated binding and CD40 with its ligands (Lessner, 2004). In our model, not only a strong physical interaction between Tg macrophages and LSECs was observed by EM and co-culture assay, but also their molecular interaction was proved by the transplantation of macrophages into C3H and co-culture assay *in vitro*. The exact nature of the interacting molecules remains to be determined.

The possible raisons why the vascular phenotype predominantly occurs in the liver may include among others: the higher numbers of macrophages residing in the liver (80%

of body's macrophages), a suitable hematopoietic and blood system, and the unique interaction between LSECs and KCs in the liver (see chapter 2).

5.4. Tg macrophages induce the liver vascular defects through a paracrine loop

It is clear that the Tg macrophages express the $N1^{EC}$ Tg and are reprogrammed by its expression. Hence, the vascular disease caused by $N1^{EC}$ does not seem to be mediated by endothelial cells per se. Also, it is not caused by transition macrophages to endothelial cells. This conclusion strongly stems from chimeras and the FLT using the $N1^{EC}$ X ROSA as donors and nude mice as hosts. In this experiment, white colored (host-origin) defective vessels were accompanied by blue color (donor-origin) hematopoietic cells (probably macrophages). Such a combination indicates that a paracrine mechanism (influence of hematopoietic cells on LSECs) is crucial for inducing the liver vascular patterning defects. The transplantation of macrophages into C3H mice also gives rise to the same phenotype, thereby confirming the paracrine mechanism provoked by the Tg macrophages. Furthermore, this paracrine loop is corroborated by two *in vitro* experiments: a co-culture assay showing more clusters of Tg macrophages adhered onto LSECs, and a conditional media assay demonstrating the inhibition of LSECs treated with Tg macrophage culture supernatants. The speculated factors released from macrophages might be growth factors such as TNF, chemokines such as MCP-1, and adhesion molecules such as coagulators.

5.5. Mechanisms on the vascular defects observed in the liver-$N1^{EC}$ activated macrophages via an autocrine loop beyond Notch intracellular domain

Since $N1^{EC}$ is a soluble molecule, it might be either an angonist or antagonist for Notch1 signaling as the ectodomain of Notch ligands did (Sun and Artavanis-Tsakonas, 1996; Sun and Artavanis-Tsakonas, 1997; Qi et al., 1999) (it was our initial hypothesis).

However, the preliminary results showed that endogene Notch1 in macrophages was indistinguishable between nTg and Tg. In order to know whether exogeneous Notch1 intracellular domain ($N1^{IC}$) is influenced by $N1^{EC}$ or not, transgenic mice CD4C/$N1^{EC}$ were bred with CD4C/$N1^{IC}$ Tg mice. The CD4C/$N1^{IC}$ Tg mice were recently established in our laboratory and give rise to thymoma (Isabelle et al, submitted). Neither $N1^{EC}$ nor $N1^{IC}$ was influenced by the other in these double Tg mice. Hence we conclude that $N1^{EC}$ is neither angonist nor antagonist for $N1^{IC}$ and it may involve vascular disease by another pathway.

The interaction between receptor (EGF motifs) and ligand (DSL domain) of neighboring cells is a major event in the initiation of Notch signaling. While much attention has been concentrated at the activation of the Notch expressing cells induced by this interaction, few studies were done on the Notch ligand expressing cells. It is possible that the $N1^{EC}$ directly talks to Notch1 ligands on endothelial cells and make the endothelial cells change (Fig. 1A). However, this possibility was not proved by our preliminary *in vitro* results, in which anti-$N1^{EC}$ could not block increased colonies formed with Tg macrophages onto LSECs, as well as inhibition of the growth of LSECs treated by Tg macrophage media. To completely exclude this possibility, further experiments should be done. On the other hand, another scenario (Fig. 1B) is more attractive compare to Figure A. Macrophages are targeted by $N1^{EC}$ Tg which further promotes vascular disease, probably, through activation of macrophages. The EGF-like repeats contained in the $N1^{EC}$ Tg may bind to the DSL domain of classic Notch ligands such as Jag1, that has been involved in vessel formation and AGS human disease (Xue et al., 1999; Spinner et al., 2001). They may also interact with novel ligands such as F3/contactin and CNN3/Nov

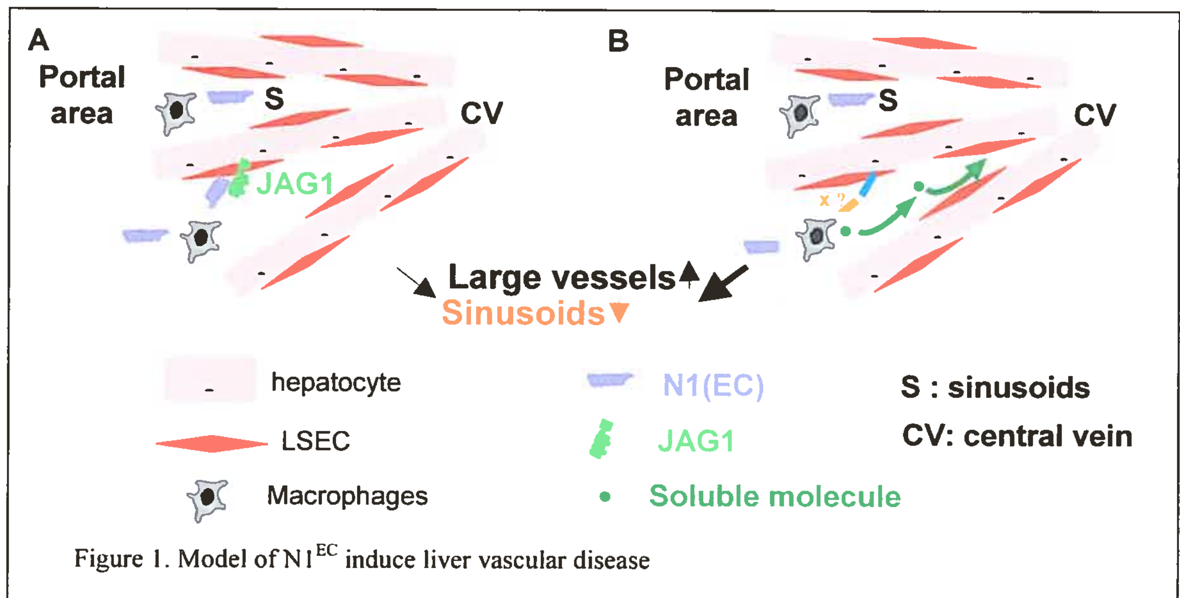


Figure 1. Model of $N1^{EC}$ induce liver vascular disease

(Hu et al., 2003; Sakamoto et al., 2002), that have been recently found to involve in cell adhesion (Revest et al., 1999; Perbal et al., 1999) and angiogenesis (Christian et al., 2003; Lin et al., 2003). In addition, they may interact with other signals such as Shh to perform angiogenic activity (D'Amore and Ng, 2002; Lawson et al., 2002). Through the autocrine interaction of $N1^{EC}$ Tg and Notch ligands, as well as other signals (Shh) expressed in macrophages, the macrophages might be activated (also called reprogrammed). Some transcription factors beyond Notch pathway might be altered. Subsequently, targeted genes of these transcription factors would be produced from macrophages. These genes may be involved in vessel formation varied from growth factors such as PDGF, chemokines such as MCP-1, adhesion molecules such as cadherin, and enzymes such as MMPs. All these molecules may, in turn, influence endothelial cells, finally leading to vessel defects by a paracrine fashion (Fig. 1B). Speculated factors will be confirmed by microarray analysis for peritoneal macrophages. By the same manner, the activated macrophages can affect other organs such as uterus and promote tumor progression (see later).

5.6. Uterus vascular defects observed in sterile CD4C/N1^{EC} Tg females or less fertile are induced by hematopoietic cells (macrophages?)

Besides the liver, that predominantly exhibits the vascular defects as, other organs also show vascular defects in Tg mice, particularly, uterus. The defective Tg uteri show a thinner uterine body and superficial vessels compared to nTg ones. The reason why the vascular defects also occur in the uterus may be that uterus is one of the few adult tissues exhibiting regular intervals of rapid growth and physiological angiogenesis (Folkman, 1995). Interestingly, these mice are sterile or of low fertility as examined in two founder lines (60787 and 60788) and confirmed by developmental studies at E6.5, E9.5, and E16.5 stages. Many defective vessels are observed in different layers (endometrium, myometrium, and mesometrium) of the Tg-pregnant uteri accompanied by hemohrrage in endometrium of the Tg-pregnant uteri but not in non-Tg-pregnant ones. Therefore, the sterile phenotype might be caused by the vascular defects since angiogenesis plays a key role in the pregnancy-associated changes in the reproductive tract. The dysfunction of endogenous angiogenic stimulators and inhibitors underlie several female reproductive disorders, such as infertility (Folkman, 1995).

Similar to the liver vascular phenotype, increased macrophages are found in the cycling Tg uteri compared to the nTg ones. These cells are distributed in each layer of uterus and located around defective vessels and among the glands. Their numbers are also increased and they appear clustered in the Tg pregnant uteri and Tg placenta. This uterus phenotype is reproduced, and even enhanced, by the transplantation of Tg FL cells but not by nTg ones, suggesting that hematopoietic cells play a key role in the uterus defects. Macrophages are the most frequent leukocytes in the uterus of the rodents (Hunt and Pollard, 1992) where they can produce angiogenic factors such as VEGF. It is possible that

macrophages induce this phenotype through a paracrine loop that remains to be determined.

5.7. Involvement of N1^{EC} expressed in the macrophages in the tumor progression and metastases

Although no thymomas were detected in the CD4C/N1^{EC} Tg mice, we obtained hemangiomas, particularly in the liver (chapter 2) and also in the uterus of mice transplanted with Tg-FL cells (chapter 4). These results not only reveal some functions of N1^{EC} expressed in the macrophages on vascular formation but also hint to its role in the formation of vascular tumor. With the aid of distinct mouse models, we have been able to further elucidate its function on the parenchymal tumor progression (chapter 3). In the mice treated with DEN carcinogen, more malignant multi-organ tumors are induced in the Tg mice compared to the nTg mice. Lung metastases originating from the liver are only detected in the Tg mice. The increased metastases are also supported by the transplantation of hepatocarcinomas (induced by DEN) into CD4C/N1^{EC} Tg mice, which develop multi-organ metastases. The best evidence of increased metastases in the Tg mice is obtained with subcutaneous transplantation of C3L5 breast tumor cells (a highly metastatic cell line) into the CD4C/N1^{EC} Tg mice. In that experiment, severe metastases occur in several organs of the Tg mice, in contrast to only one organ in the nTg mice. Interestingly, we have obtained a rare heart metastasis. Glomerular metastasis is also revealed in Tg kidney as an intraglomerular metastasis originated from liver, shown in Figure 2. This is utterly amazing, because kidney is a very rare organ accepting a metastasis due to the presence of inhibitors secreted by the kidney. For example, one of tumor inhibitors, TGF-β1, can be secreted from certain organs, especially, from kidney to fail to metastasis to kidney (Nicolson and Dulski, 1986; Nicolson, 1987; Tucker et al., 1984). These broad metastases,

including heart and kidney, suggest that some factors from the Tg mice might be involved in these processes (described later).

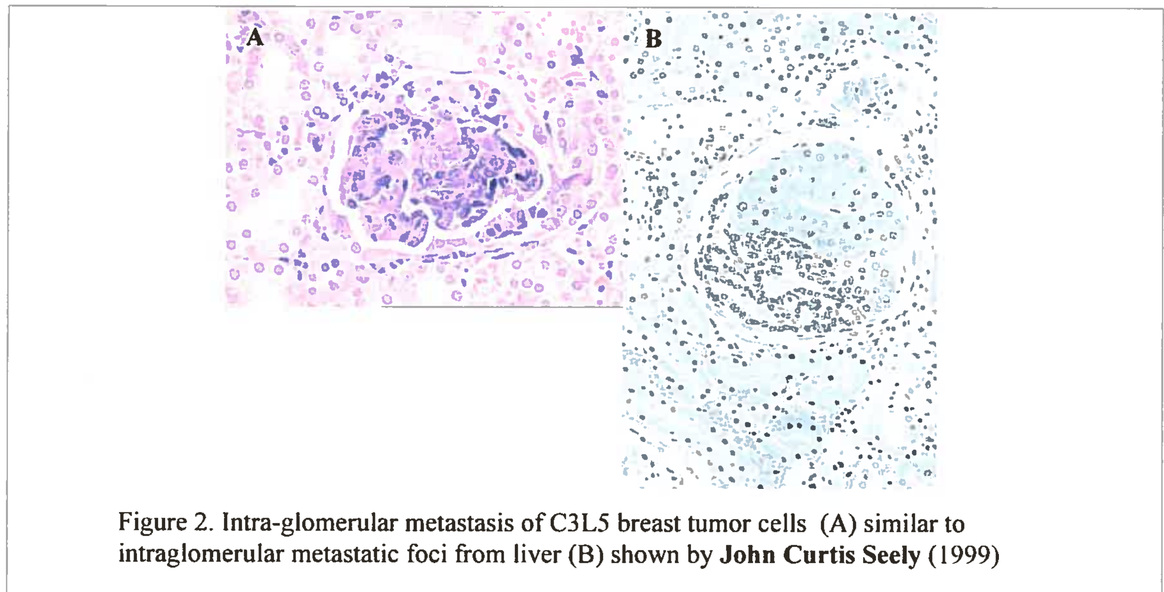


Figure 2. Intra-glomerular metastasis of C3L5 breast tumor cells (A) similar to intraglomerular metastatic foci from liver (B) shown by **John Curtis Seely** (1999)

5.8. Involvement of $N1^{EC}$ Tg expressed in macrophages in tumor angiogenesis and growth

In the model of hepatocarcinomas induced by DEN, increased vascularization and necroses are observed in the Tg livers. High vascularization, haemorrhage, and necrosis also appear in the primary tumors of Tg-bearing mice as compared to those of nTg-bearing mice transplanted subcutaneously with C3L5 breast tumors and B78 melanomas. Necrosis is the visible consequence of tumor ischemia and the most necrotic breast tumors are also the most angiogenic (Leek et al., 1999). Hence, we may conclude that $N1^{EC}$ expressed in the macrophages is associated with tumor angiogenesis. In addition, in the tumor model with the transplantation of B78 melanomas, a faster tumor growth is observed in the nude mice transplanted with Tg fetal liver cells, therefore suggesting involvement of $N1^{EC}$ in tumor growth.

5.9. Factors might be related to the tumor progression and metastasis in the 4C/N1^{EC} Tg mice.

The tumor cells must have a blood supply in order to maintain their respiratory and nutritional requirements. They release factors that stimulate nearby endothelial cells, to form new vessels and secrete molecules that will allow tumoral cells to grow and/or to metastaze (Folkman, 1995). Hence, the vascular nutrition might act as another paracrine effects in the tumor progression. The defective vessels including pre-existing vasculatures and newly formed vessels present in the CD4C/N1^{EC} Tg mice might also behave as paracrine enhancers to accelerate the tumor cell progression and metastasis. Probably, the hepatic tumor progression induced by DEN, the fast tumor growth of the primary B78 tumors, the severe metastases from the transplantation of C3L5 breast tumors might all be related, at least partially, to the defective vessels. For example, increased permeability of Tg vessels would result in leakage of plasma proteins that lead to extravascular fibrin clotting. The fibrin clotting will help stimulate the new ingrowth of blood vessels and macrophages, preparing for tumor metastasis (Wang, 2005). Certainly, this hypothesis should be confirmed in the future.

The growth and progression of primary as well as metastatic tumors are regulated not only by autocrine signals from tumor cells per se, but also by paracrine signals from stromal cells (Nicolson, 1993; De Wever and Mareel, 2003; Marchetti et al., 2003; Lin et al., 2002).

Macrophages are one of the first hematopoietic cells to arrive at the sites of inflammation and act as signaling centers to recruit other stromal cells (Lingen, 2001). They are the major components of majority of malignant solid tumors, termed as tumor associated macrophages (TAMs), and exert paracrine effects (Bingle et al., 2002; Elgert et

al., 1998). The TAMs are first recruited to the tumor sites by factors released from tumor cells and subsequently exert their pro-tumor activities mainly through two ways: angiogenesis and neoplastic cell mitogenesis (Leek and Harris, 2002). In many solid tumor types, the abundance of TAM correlates with tumor progression, metastasis, and poor prognosis (Lin and Pollard, 2004; Nishihira et al., 2003; Knowles et al., 2004). As described in chapter 2, macrophages are reprogrammed by $N1^{EC}$ in our Tg mice and influence LSECs so as to cause defective vessels in the liver. Probably, these reprogrammed macrophages also exert their angiogenic task in tumor growth and metastasis as well as other organs. They are recruited into the primary tumors induced by DEN carcinogens as well as C3L5 and B78 tumor cells. Macrophages abnormally cluster or form hotspot in the tumors and subsequently produce a range of signals. These signals from TAMs stimulate neoplastic cell mitogenesis and aberrant angiogenesis, making tumors either progress/grow faster (as seen in DEN and B78 models) or allow them to metastasize easily (as seen in C3L5 model) from their primary site. Due to the same reasons, tumor cells might also easily reside in a second organ and form multi-organ metastases as seen in the model of transplantation with C3L5 breast tumors. In addition, TAMs can secrete proteases such as plasmin (regulated by u- and t-PA) and MMPs which degrade ECMs surrounding the tumor, enabling the tumor cells to break free from the tumor mass and invade locally, or enter the systemic circulation to form distant metastases (Figure 3).

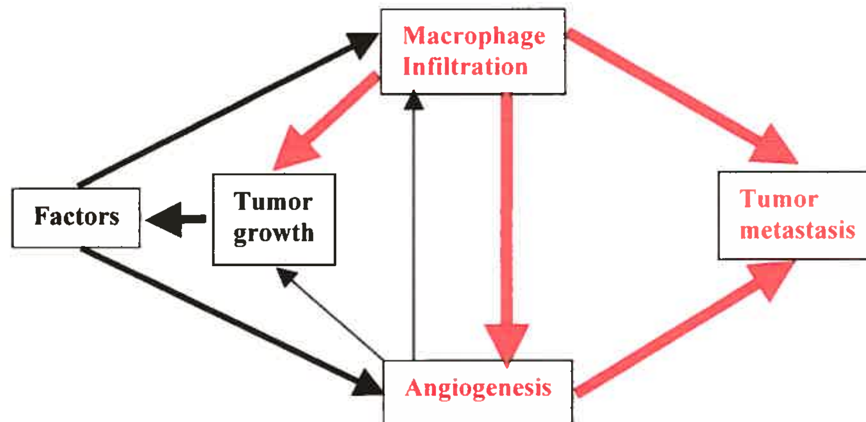


Figure 3. Macrophages and tumor progression and metastasis

5.10. Role of macrophages in anti-angiogenesis and anti-mitogenesis

Except for tumor cells themselves, the tumor microenvironments (blood vessels, inflammatory cells including macrophages, and connective tissues) also play very important roles during tumor pregression (Bissell et al, 2005). High permeability of tumor vessels results in extravascular coagulation, helping tumor growth and metastasis. It made Dvorak think: "tumors: wounds that do not heal" (Dvorak, 1986). Furthermore, macrophages are intricately involved in tumor progression as they are in wound healing. This has inspired researchers to think of tumor as "the wound that will never heal". However, since macrophages could be used as targets for anti-angiogenic and anticancer therapy, one could hope that tumors will heal. There are few studies to achieve this goal. They mainly involve suppression of the involvement of TAMs and use of genes delivered by macrophage vehicles. A significant decreased number and secreting function of TAMs were found in the rats treated with linomide (a quinoline-3-carboxamide) (Vukanovic and Isaacs, 1995), an inhibitor for tumor growth of the prostatic cancer via an anti-angiogenic response (Vukanovic et al., 1993). Macrophages transduced with a gene encoding the pro-drug activating enzyme cytochrom P450 2B6 (under the transcriptional control of a trimer of an HRE), resulted in a decreased viable tumor cells *in vitro* (Griffiths et al., 2000). Kupffer cells present in the liver can also be inactivated *in vivo* and *in vitro* by an

adenoviral gene transfer (Wheeler et al., 2001). The discovery of a paracrine loop through which the reprogrammed macrophages function in our Tg mice further attests the importance of macrophages as being gene delivery vehicles. It opens up the possibility that defective vessels and tumor progression could both be modified by blocking the $N1^{EC}$ pathway involved in the macrophage-mediated angiogenesis and tumor mitogenesis. Of course, such a study will be more complex in an *in vivo* environment and hard to achieve with a high efficiency, macrophage-specific transfection technique.

In summary, for the first time, we discover the functions of the Notch1 ectodomain in the liver vascular defects, female infertility, and tumor progression and angiogenesis. However, the $N1^{EC}$ does not work alone. It needs a special environment-macrophage! All three diseases are associated with the macrophages reprogrammed by $N1^{EC}$. These macrophages subsequently participate in angiogenesis and mitogenesis roles via paracrine mechanisms. Therefore, both $N1^{EC}$ and macrophages would be hoped as targets for anti-angiogenesis and anti-cancer!

In the future, we will attempt to accomplish some experiments to deepen the projects: (1) For the vascular disease observed in the liver, the first important hypothesis is that some transcription factors beyond Notch signaling might be activated in the Tg macrophages, and their targeted genes involved in angiogenesis would be altered. To reach this hypothesis, DNA microarray analysis with peritoneal macrophages will be performed and confirmed by real time PCR analysis. Then an exact molecular pathway for the activation—for example, the cellular compartment of $N1^{EC}$ in macrophages and the relationship of $N1^{EC}$ with its ligands (Jag1), as well as with supposed activated transcription factors on the macrophages—will be identified through *in vitro* experiments with peritoneal macrophages (i.e., Confocal analysis, immunofluorescence, western and

immunoprecipitation). The second hypothesis is that interactions between macrophages and endothelial cells are mediated by some receptor-ligands cross-talk. Co-culture assay with KCs and LSECs using immunofluorescence to identify this molecular cross-talk is interesting. Then, blockage of related molecules (RNAi or knockout mice) will give us a clear answer. To further exclude that the soluble $N1^{EC}$ gives rise to the vascular defects via direct interaction with LSECs, RT-PCR analysis of Notch ligands on the LSECs will be performed first. (2) For the vascular disease observed in the females, it is hypothesized that the macrophages induce the vascular defects in the uteri by a paracrine loop as they do in the liver. The increased macrophages in the uterus and placenta will be confirmed with specific markers (Mac-1). Then molecules screened by microarray analysis from peritoneal macrophages will be detected in uterus and placenta with the hope of altered expression of these molecules in Tg mice compared to nTg mice. Finally, the vascular phenotype of the uterus might be inhibited by blocking these molecules (RNAi or knockout mice). The vascular development of the maternal side of the placenta in the Tg females will be analyzed using HE staining and immunohistochemistry (PECAM-1 and/or vWF). (3) For tumor progression, we will take advantage of the C3L5 model, in which the C3L5 cell line is easy to manipulate and a severe metastasis is clear to find in the Tg mice. A high number of mice (15 mice per group) will be used to get an ideal statistic analysis. We hypothesize that macrophages accelerate the progression of the metastasis. To test this hypothesis, CD4C/$N1^{EC}$ Tg mice will be crossed with CSF-$1^{-/-}$ mice in which macrophages are absent and then C3L5 tumor injection will be performed in CD4C/$N1^{EC}$ X CSF-$1^{-/-}$ mice. Transplantation of chimeric cells (nTg or Tg macrophages with C3L5 tumor cells) into C3H mice is another good experiment to prove the role of macrophages in the tumor metastasis. In order to know molecules expressed in macrophages involved in the tumor

progression, the candidates screened in peritoneal macrophages by DNA microarray will be tested in the C3L5 tumor model. The tumor progression may be blocked by inhibition (RNAi or Knockout) of increased factors expressed in the macrophages. Besides Tg macrophages, we also hypothesis that the defective vessels may accelerate the tumor progression. This is difficult to do because we cannot completely block the defective vessels. But one way can be tried, and that is to block the leakage of the vessels in the C3L5 tumor model. Decreased metastasis would be resulted from blocking leakage of vessels since ingrowth of fibrin and influx of macrophages into tissues is diminished.

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