UNDERSTANDING THE FUNCTION OF ENDOGLIN THROUGH
THE GENERATION AND CHARACTERIZATION OF ENDOGLIN DEFICIENT MICE
AND THE STUDY OF PATIENTS
WITH HEREDITARY HEMORRHAGIC TELANGIECTASIA

by

Annie Bourdeau

A thesis submitted in conformity with the requirement
For the degree of Doctor of Philosophy
Graduate Department of Immunology
University of Toronto

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0-612-53767-6
ABSTRACT

Understanding the Function of Endoglin
Through the Generation and Characterization of Endoglin Deficient Mice
and the Study of Patients with Hereditary Hemorrhagic Telangiectasia.

Ph.D. 2000, Annie Bourdeau, Department of Immunology, University of Toronto.

Endoglin, (CD105), an integral membrane glycoprotein, is a member of the TGF-β receptor superfamily. It is expressed at high levels on vascular endothelium and also found in several human hematopoietic cells. Hereditary Hemorrhagic Telangiectasia (HHT) is an autosomal dominant disorder associated with mutations in the Endoglin gene, leading to loss of a functional allele (haploinsufficiency). To define the role of endoglin in normal and pathological blood vessel formation, we first investigated its expression in vascular endothelium of HHT patients. We then generated Endoglin deficient mice and analyzed their vascular and immune systems.

The haploinsufficiency model proposed for HHT was first based on in vitro experiments. This thesis further confirms this model. It demonstrates that all blood vessels of HHT1 patients express reduced endoglin in situ, and that severe lesions such as arteriovenous malformations (AVMs) are not attributed to loss of heterozygosity. To generate an animal model for HHT and ascertain the role of endoglin in development, we generated mice lacking one and two copies of the gene.

We show that Endoglin null embryos die at gestational day 10.0-10.5 due to defects in vessel and heart development suggesting that endoglin is critical for both angiogenesis and heart valve formation. Some mice expressing a single allele of Endoglin spontaneously developed clinical signs of HHT. The analysis of disease onset and progression revealed that clinical manifestations were heterogeneous, leading to severe vascular abnormalities and fatal hemorrhages. Our results demonstrate that the HHT mouse model reproduces well the heterogeneous phenotype of the human disease. The data suggest that in addition to reduced expression of endoglin, modifier genes can contribute to heterogeneity and severity of HHT.
As expression of a single allele of Endoglin leads to a vascular disorder, we investigated if it could alter the immune system of Endoglin heterozygous mice. We show that reduced endoglin expression either on vascular endothelium or on subsets of hematopoietic cells leads to alteration of T and B lymphocyte homeostasis.
DEDICATION

To my parents,

Jean Bourdeau

&

Jocelyne Savignac

for their support,

their faith in me, and their love
ACKNOWLEDGMENTS

First and foremost I want to express my deepest gratitude to my supervisor Dr. Michelle Letarte for providing me with an enriching learning environment and teaching me to do science right. I consider Michelle as a friend and will forever be inspired by her selfless dedication to her work. Dr. Dan Dumont also became a good friend and taught me a great deal. His encouragement and support sustained me throughout the good times and the bad times. I am also grateful to the members of my graduate committee Drs Jack Hay and Liliana Attisano for their guidance and support.

How can I express my feelings for Sébastien Trop, first my lab partner, then my husband? Or is it the other way around? I worked with Seb, despaired with Seb, and rejoiced with Seb. He is part of everything that I do, and ever will be. Life in the lab would not have been the same without the technical assistance of Sonia and Urszula, who later became my giggling bodies. Thanks also to Renu and Martin for their friendship and technical support. I owe special thanks to the students I had the good fortune to work with, Jennifer, Melissa, Nelson, Shunji, Sigmund and Merry-Lynn; I only hope that you learned as much from me as I did from you. My sincere appreciation to the staff of the Ontario Cancer Institute and Hospital for Sick Children animal facilities, for taking such good care of my precious little endoglin children. To Kathy, Marie-Eve, Andreas, Nadia and Salma for stimulating discussions and a helping hand when I needed it, thank you. Last but not least, I extend my most sincere thanks to the Medical Council Research of Canada for generous financial support.
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<tr>
<td>aa</td>
<td>Amino acids</td>
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<tr>
<td>ActR-I</td>
<td>Activin receptor type I (also known as ALK-2)</td>
</tr>
<tr>
<td>ActR-IB</td>
<td>Activin receptor type IB (also known as ALK-4)</td>
</tr>
<tr>
<td>ActR-II</td>
<td>Activin receptor type II</td>
</tr>
<tr>
<td>ActR-IIIB</td>
<td>Activin receptor type IIB</td>
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<tr>
<td>AGM</td>
<td>Aorta-gonad-mesonephros</td>
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<tr>
<td>ALK-1</td>
<td>Activin like kinase 1</td>
</tr>
<tr>
<td>ALL</td>
<td>Acute lymphoblastic leukemia</td>
</tr>
<tr>
<td>AML</td>
<td>Acute myeloid leukemia</td>
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<tr>
<td>Ang</td>
<td>Angiopoietins</td>
</tr>
<tr>
<td>AVM</td>
<td>Arteriovenous malformation (Direct connection between a dilated venule and a dilated arteriole without intervening capillaries)</td>
</tr>
<tr>
<td>b-FGF</td>
<td>Basic fibroblast growth factor</td>
</tr>
<tr>
<td>B6n</td>
<td>C57BL/6 backcross</td>
</tr>
<tr>
<td>B6i</td>
<td>C57BL/6 intercross</td>
</tr>
<tr>
<td>BL-CFC</td>
<td>Blast colony-forming cells</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>BMPR-IA</td>
<td>Bone morphogenetic protein receptor IA (also known as ALK-3)</td>
</tr>
<tr>
<td>BMPR-IB</td>
<td>Bone morphogenetic protein receptor IB (also known as ALK-6)</td>
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<tr>
<td>BMPR-II</td>
<td>Bone morphogenetic protein receptor II</td>
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<tr>
<td>CLP</td>
<td>Common lymphoid progenitor</td>
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<td>CMP</td>
<td>Common myeloid progenitor</td>
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<tr>
<td>Co-Smads</td>
<td>Common partner Smads</td>
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<td>DN</td>
<td>Double negative T cells (CD4+CD8−)</td>
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<tr>
<td>DP</td>
<td>Double positive T cells (CD4+CD8+)</td>
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<td>EB</td>
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EC : Endothelial cells
ECM : Extracellular matrix
ED : Embryonic day
End : Endoglin gene
End+/− : Endoglin wild type mice
End+/− : Endoglin heterozygous mice
End−/− : Endoglin homozygous null mice
ES cells : Embryonic stem cells
GMP : Granulocyte macrophage progenitor
HHT : Hereditary Hemorrhagic Telangiectasia
HHT1 : Hereditary Hemorrhagic Telangiectasia type 1
HHT2 : Hereditary Hemorrhagic Telangiectasia type 2
HSC : Hematopoietic stem cells
HUVEC : Human umbilical vein endothelial cells
I-Smads : Inhibitory Smads
IL : Interleukin
Io : Ionomycin
LIF : Leukocyte inhibitory factor
LPS : Lipopolysaccharides
LTR : Long term repopulating
mAb : Monoclonal antibody
MEP : Megakaryocyte/erythrocyte progenitor
MHC : Major histocompatibility complex
Ola− : 129/Ola backcross
P-Sp : Paraortic splanchnopleura
PC : Pericytes
PDGF : Platelet derived growth factor
PMA : Phorbol 12-myristate 13-acetate
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<tr>
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<td>SEB</td>
<td>Staphylococcus enterotoxin B</td>
</tr>
<tr>
<td>SMC</td>
<td>Smooth muscle cells</td>
</tr>
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<td>SP</td>
<td>Single positive T cells (either CD4⁺CD8⁻ or CD4⁺CD8⁺)</td>
</tr>
<tr>
<td>TβR-I</td>
<td>Transforming growth factor beta receptor I (also known as ALK-5)</td>
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<td>TβR-II</td>
<td>Transforming growth factor beta receptor II</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TH</td>
<td>T helper cells</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<tr>
<td>VEGFR-1</td>
<td>Vascular endothelial growth factor receptor type 1 (also known as Flt-1)</td>
</tr>
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<td>VEGFR-2</td>
<td>Vascular endothelial growth factor receptor type 2 (also known as Flk-1)</td>
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CD NOMENCLATURE

CD3: T3 complex; γ, δ, ε, TCRζ subunits, associates with pTα/TCRβ, TCRα/TCRβ and TCRγ/TCRδ receptor subunits to form the pre-TCR, αβ TCR and γδ TCR signaling complex, respectively

CD4: L3T4 antigen, binds MHC class II

CD8: Lyt-2/Lyt-3 (α/β subunits), binds MHC class I

CD10: CALLA, neutral endopeptidase, endopeptidase 3.4.24.11

CD11b: Mac-1, Mo-1, αm integrin chain, CR3, associates with β2 integrin; binds CD54, iC3b, fibrinogen

CD19: B4 antigen, signal transduction molecule that regulates B lymphocyte development, activation, and differentiation

CD24: Heat stable antigen, HSA

CD25: IL-2Rα, component of the high affinity IL-2 receptor complex

CD31: Platelet endothelial cell adhesion molecule, PECAM-1, mediates leukocyte transmigration, homotypic aggregation

CD34: Sialomucin

CD43: Leukosialin

CD45R: B220 antigen, CD45 isoform; tyrosine phosphatase

CD71: Transferrin receptor

CD90: Thy1 antigen

CD105: Endoglin, component of the TGF-β receptor complex

CD117: c-Kit, stem cell factor receptor, SCFR, SCF receptor, tyrosine kinase
CHAPTER ONE

Introduction

1.1 Overview

The regulation of blood vessel assembly and growth is fundamental to embryonic development, organogenesis and the understanding of several of pathologies. The vascular system forms through a combination of vasculogenesis and angiogenesis. These processes are highly regulated by the interaction of several growth factors, such as vascular endothelial growth factor (VEGF), angiopoietins (Ang) and transforming growth factor beta (TGF-β), with their respective receptors (1-4). Endoglin is expressed predominantly on endothelial cells and is present on all types of blood vessels as early as 4 wks of human gestation (5-7). It associates with ligand binding receptors for several members of the TGF-β superfamily (8). Endoglin binds TGF-β1 and TGF-β3 isoforms, through its association with the TGF-β receptor type II (TβR-II). It was shown to modulate several responses to TGF-β1 and is in our view, a regulator of TGF-β1 action in endothelial cells (9, 10). The identification of Endoglin as the gene mutated in Hereditary Hemorrhagic Telangiectasia (HHT), further supports the notion that endoglin plays a critical role in the vascular system (11). This disease is associated with arteriovenous malformations (AVMs) that can arise during development but subsequently grow and lead to hemorrhage and severe multi-organ complications (12).

Endoglin was first discovered 15 years ago as a glycoprotein of pre-B leukemic cells (13). It was subsequently found on several normal human hematopoietic cells. These were erythroid precursors as well as fetal pre-B cells, bone marrow stromal cells and macrophages (14-17). This distribution pattern suggested that endoglin was expressed early in development and could play a regulatory role not only in the vascular system but also in the hematopoietic system. Interestingly TGF-β1 and TβR-II null mice have serious defects in yolk sac hematopoiesis and vasculogenesis which lead to death at embryonic day (ED) 10.5 (18, 19). The characterization of endoglin expression in non-affected vessels and AVMs of HHT patients, along with the generation of Endoglin (End) deficient mice, both presented in this thesis, are
aimed at defining the role of endoglin in vascular and hematopoietic systems. The comparison of early events in the development of these systems and the identification of key molecules will be presented in the introduction to set the stage for defining the role of endoglin. Functional characteristics of members of the TGF-β superfamily and their role in regulating several vascular and immune functions will provide the proper context to discuss endoglin and its possible mode of action in blood vessels of normal and HHT affected men and mice as well as in their immune system.
1.2 Development of the vascular and hematopoietic systems

The vascular and hematopoietic systems are the first to become functional in the developing embryo. Our understanding of the molecular mechanisms that control these biological processes is derived, in part, from the study of genetically modified mice. In search of a role for endoglin during development, it is necessary to understand the current concepts that lead to blood vessel formation and homeostasis as well as to primitive and definitive hematopoiesis.

1.2.1 Key steps in mouse embryonic development

Murine embryonic gestation can be divided into four periods from fertilization to birth (Figure 1.1). Early in development, during cleavage and blastulation period, the mouse embryo undergoes a series of slow cell divisions that will generate the primitive endoderm and trophectoderm, the two layers needed for implantation 4.5 days after fertilization. After implantation, pluripotent cells undergo several rapid divisions and form the primitive epiblast from which the fetus will develop. Partitioning of cells occurs via gastrulation at ED6.5 whereby totipotent cells of the epiblast divide, differentiate and rearrange into three distinct germ layers. The ectoderm will form skin and central nervous system, the mesoderm will form blood, bone and muscle while the endoderm will give rise to the respiratory and digestive tracts (20-22).

The vascular and hematopoietic systems originate from the mesoderm. These cells differentiate further to ventral/posterior and dorsal/anterior mesoderm to give rise to both embryonic and extraembryonic structures (Figure 1.2). In the yolk sac, blood islands are first visible at ED7.5. They are composed of an outer layer of angioblasts and an inner cluster of hematopoietic stem cells (HSC). Half a day later, angioblasts differentiate into endothelial cells and primitive hematopoietic cells can be seen. By ED8.5, a primary capillary plexus is established in the yolk sac by vasculogenesis. A more diverse population of definitive hematopoietic cells can be found in the lumen of these vessels. By ED9.5, the primary capillary network forms branches and remodels into larger vessels via the process of angiogenesis (Figure 1.1).
Figure 1.1 Development of murine vascular and immune systems.

Murine gestation can be divided into 4 periods: cleavage and blastulation (ED0-5.0), early organogenesis (ED5.0-10.0), organogenesis (ED10.0-14.5) and fetal growth and development (ED14.5-20.0). Vascular and immune systems arise after gastrulation (ED6.5), and a closer look at the ED7.5-11.5 period is shown. Blood island formation (cerulean blue) occurs in the yolk sac, common to both vascular and hematopoietic systems. Blood islands give rise to angioblasts that will differentiate into endothelial cells (EC) to form vessels by vasculogenesis and angiogenesis (navy blue). Hematopoietic stem cells also develop from the blood islands by primitive and later by definitive hematopoiesis (green). As opposed to yolk sac, the vascular and immune systems do not develop in close proximity in the embryo. Angioblasts are seen at ED7.5 and form endothelial cells that will assemble in embryonic vessels by vasculogenesis and angiogenesis (navy blue). Intra-embryonic hematopoietic progenitors are first seen in the paraaortic splanchnopleura (P-Sp), and then in the aorta-gonad-mesonephros (AGM) region. The (*) indicates hematopoietic cells with long-term repopulating capacities in adult systems. Hematopoiesis switches to fetal liver around ED10.5 (green).
Vascular development in mouse embryos is initiated around ED7.5. Embryonic angioblasts originating from paraxial- and lateral-plate mesoderm migrate and differentiate to form a primitive vessels, which subsequently fuse and remodel through angiogenesis (2, 23, 24). HSC in the embryo do not develop in close contact with angioblasts. Definitive hematopoietic cells have been found primarily in the paraaortic splanchnopleura (P-Sp), and the aorta-gonad-mesonephros (AGM) region of the embryo (Figure 1.1). At ED10.5-11, hematopoiesis shifts to fetal liver and subsequently to bone marrow around the time of birth. The bone marrow remains the major site of hematopoiesis throughout adult life (24-26).

1.2.2 The hemangioblast, a common precursor of endothelial and hematopoietic cells

It has been postulated that HSC and angioblasts, which originate during blood island formation in the yolk sac, were derived from a common precursor, the hemangioblast (Figure 1.2). This is supported mainly by experiments where somites from quail embryos are grafted into chick hosts. In this model, cells expressing the QH1 antigen and thought to represent hemangioblasts, gave rise to progeny belonging either to endothelial or hematopoietic lineages (27). The expression of common surface markers on cells of both lineages also supports a common progenitor (Figure 1.2). Genes that are normally expressed specifically on mature endothelial cells such as vascular endothelial growth factor receptor 2 (VEGFR-2; Flk-1), CD31 (PECAM-1), VE-cadherin, Tie-1 and Tie-2 (Tek) are found on hematopoietic cells enriched for early progenitors (23, 28-30). Furthermore, the phenotype of VEGFR-2 null mice suggests that VEGF receptor is essential for the generation of both hematopoietic and endothelial cells (31, 32). CD34 expressed on hematopoietic precursors and the erythropoietin receptor found primarily on erythroid progenitors, are both expressed in endothelial cells (33, 34) (Figure 1.2).

Evidence for the existence of hemangioblasts was directly demonstrated using an in vitro system of embryonic stem (ES) cell differentiation. Once leukocyte inhibitory factor (LIF) is withdrawn from ES cell cultures, embryoid bodies (EB) develop. After 2.5-3.5 days one cell population called the blast colony-forming cell (BL-CFC) is present, with the potential to form blast colonies when cultured with VEGF. BL-CFC express genes specific for both endothelial
Figure 1.2 Diversity of hematopoietic progenitors in murine yolk sac and embryo.

Mesodermal cells developed during gastrulation, further differentiate giving rise to tissues of yolk sac and embryo. In the yolk sac, hematopoietic stem cells (HSC) and angioblasts which share several surface markers are thought to be derived from a common precursor, the hemangioblast. HSC differentiate into large nucleated erythroid precursors and mature macrophages during primitive hematopoiesis. Later on, yolk sac produces more diverse hematopoietic populations (definitive hematopoiesis) containing anucleated erythroid precursors, myeloid precursors and uncommitted lymphoid precursors. In the embryo, only definitive hematopoiesis (from P-Sp and AGM) occurs, giving rise to several precursors, including committed lymphoid progenitors. Both in extra-embryonic and intra-embryonic tissues, vessels are formed by vasculogenesis and angiogenesis. The connection between the two circulations is established around ED8.5.
and hematopoietic lineages. When cultured with appropriate growth factors, they can give rise to both hematopoietic and endothelial cells (35). These observations support the view that early embryonic hematopoietic and endothelial progenitors develop from a common precursor.

1.2.3 Current models of vasculogenesis and angiogenesis

The earliest blood vessels of an embryo are formed by vasculogenesis, which is defined as differentiation of endothelial cells in situ from angioblasts. These endothelial cells will form the primary vascular plexus (Figure 1.3). Vasculogenesis is responsible for the generation of certain internal organs of endodermal origin (e.g. lung, spleen and pancreas). Once the primary vascular plexus is formed by vasculogenesis, the vascular network expands and matures by angiogenesis (Figure 1.3). This sprouting of new blood vessels from pre-existent ones, is responsible for the vascularization of other developing organs such as brain, kidney and limbs, and for neovascularization in adults (2, 3). Figure 1.3 shows our current understanding of vasculogenesis and angiogenesis that is mostly based on gene targeting experiments (Table 1.1). Both these processes are highly regulated by several growth factors such as VEGF, Ang, TGF-β, basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), ephrins and integrins. The important role of these factors is summarized below except for TGF-β, which will be discussed in section 1.3.

A. Role of the VEGF family

The VEGF family of molecules is comprised of 5 VEGF ligands (VEGF A, B, C, D, and E). They mediate their functions through 3 related receptor tyrosine kinases (VEGFR-1/Flt-1, VEGFR-2/Flk-1, VEGFR-3/Flt-4) reviewed in (36, 37).

The best-studied growth factor is VEGF-A. It is essential for vasculogenesis and modulates its action mainly through the differential use of VEGFR-1 and VEGFR-2. Expression of VEGFR-2 is first observed as mesoderm differentiates and forms the blood islands. It is found in angioblasts, HSC and endothelial cells (38, 39). The germ-line disruption of VEGFR-2 results in the absence of HSC and angioblasts in the embryo and yolk sac, such that neither
(a) Basic fibroblast growth factor (bFGF) induces differentiation of mesodermal cells into angioblasts, the first stage of vasculogenesis. VEGF-A acting through VEGFR-2 induces angioblast differentiation into endothelial cells (EC) whereas its binding to VEGFR-1 triggers formation of a primary vascular plexus. (b) Extracellular matrix (ECM) production which is regulated by TGF-β1, contributes to basement membrane assembly while αvβ3 integrin maintains the integrity of the newly formed vessels. (c) New capillaries are formed by vessel sprouting from pre-existing ones (angiogenesis) or by intussusceptive growth (whereby a large sinusoidal capillary divides into two smaller capillaries) in response to VEGF-A. In adult vasculature, vessels may also grow by sprouting. (d) Quiescent endothelial cells change their shape via αvβ3 integrin, loosen their junctions by reducing VE-Cadherin, degrade ECM with proteinases and loosen pericytes (PC) or smooth muscle cells (SMC) using Ang2, to allow migration and proliferation of new EC in response to VEGF-A. (e) In both embryonic and adult systems, remodeling of vessels to form a mature network is achieved by branching, pruning (regression of excess vessels) and recruitment of mesenchymal cells. PDGF signals the recruitment and proliferation of mesenchymal cells. Their contact with EC activates TGF-β1 that inhibits EC proliferation, induces differentiation of PC and ECM deposition necessary for vessel wall assembly. Sprouting of PC is also mediated by Ang1 interaction with Tie-2. (f) In the final maturation stage, VEGF-A and αvβ3 maintain survival of EC while VE-Cadherin and VCAM-1 support EC fusion. Ang1 and TGF-β1 induce differentiation of PC to SMC and deposition of ECM in response to TGF-β1 provides strength and support for the vessels. The differential expression of ephrinB2 on arterial endothelial cells and EphB4 on the venous ones suggests that these molecules are important in the functional maturation of veins and arteries.
Vasculogenesis

Mesodermal cells → Angioblasts

EGF → Angioblasts

Adult Angiogenesis

Proteinases

VEGF-A

Embryonic Angiogenesis

Intussusception

Sprouting

VEGF-A

Remodeling

VEGF-A

Branching

Pruning

TGF-β1

Ang1

TGF-β1

VEGF-A

Ang1

VE-Cadherin

VCAM

Artery

Capillaries

Vein
hematopoiesis nor vasculogenesis can be initiated. Therefore, VEGFR-2 is required for yolk sac blood island formation and vasculogenesis in the embryo (Table 1.1) (31, 32). The hematopoietic defect is partially rescued when ES cells lacking VEGFR-2 are cultured under specific conditions. This suggests that the environment influences migration and differentiation of early mesodermally derived precursors into hematopoietic cells, a process regulated by VEGFR-2 (40).

Vascular endothelial growth factor receptor 1 (VEGFR-1) is detected on primitive endothelial cells at ED8.0 (38, 53). This receptor is essential for progression of vascular development rather than for endothelial cell differentiation, as demonstrated by the VEGFR-1 null mice (Table 1.1) (43). Mice lacking only the tyrosine kinase domain of VEGFR-1 develop normal vasculature indicating that the extracellular domain of this receptor is needed to support vascular development (44). The expression of VEGF-A correlates well with that of its receptors as it is first detected in both extra-embryonic and embryonic endoderm at ED7.0. Targeted mutations lead to early death of heterozygous mice. This implies that a 50% reduction in VEGF levels delays endothelial cell differentiation and causes impaired vessel assembly leading to death of the embryo at ED11.5 (Table 1.1) (41, 42). Thus, VEGF-A, through differential use of VEGFR-1 and VEGFR-2, is a critical regulator of endothelial cell development. Both in vitro and in vivo experiments have shown that VEGF-A is capable of inducing proliferation, migration and sprouting of endothelial cells that allow formation of tube-like structures (Figure 1.3).

Vascular endothelial growth factor receptor 3 (VEGFR-3) is expressed initially in all embryonic endothelial cells, but as the embryo matures, it decreases in large vessels and becomes specific for lymphatic endothelium in adult tissues (38, 54). VEGFR-3 can bind VEGF C and D (55, 56). Murine embryos with a null mutation in VEGFR-3 show normal formation of the primary capillary plexus and vessel sprouting but defective remodeling of the primary vessel networks into larger blood vessels. Embryonic lethality due to cardiovascular failure is seen at ED12.5 (45) (Table 1.1).
Table 1.1 Important vascular genes and phenotypes of mice following targeting.

<table>
<thead>
<tr>
<th>Targeted gene</th>
<th>Animals</th>
<th>Time of Death</th>
<th>Yolk sac</th>
<th>Embryo</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>VEGF-A</strong></td>
<td>D</td>
<td>ED 11.5</td>
<td>Irregular plexus of small vessels, no large vessel, very few EC</td>
<td>Defective interconnecting vessel network</td>
<td>(41)</td>
</tr>
<tr>
<td>* L D</td>
<td>ED 10.5</td>
<td>Irregular plexus</td>
<td>Disorganized vessel network</td>
<td>(42)</td>
<td></td>
</tr>
<tr>
<td><strong>VEGFR-1</strong> (Flt-1)</td>
<td>L D</td>
<td>ED 8.5</td>
<td>Abnormally large vessels</td>
<td>Disorganized vessel network</td>
<td>(43)</td>
</tr>
<tr>
<td>** L L</td>
<td>Normal</td>
<td>Normal</td>
<td>(44)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VEGFR-2** (Flk-1)</td>
<td>L D</td>
<td>ED 8.5</td>
<td>No vessel</td>
<td>No vessel</td>
<td>(31, 32)</td>
</tr>
<tr>
<td><strong>VEGFR-3</strong> (Flt-4)</td>
<td>L D</td>
<td>ED 12.5</td>
<td>Underdeveloped vessels, no remodeling</td>
<td>Underdeveloped vessels, no remodeling, death by heart failure</td>
<td>(45)</td>
</tr>
<tr>
<td>Angiopoietin-1 (AngI)</td>
<td>L D</td>
<td>ED 12.5</td>
<td>Abnormal vessel morphology</td>
<td>Underdeveloped heart, less complex vascular network</td>
<td>(46)</td>
</tr>
<tr>
<td>Tie-2** (Tek)</td>
<td>L D</td>
<td>ED 9.5</td>
<td>Abnormal vessel morphology, infiltration of RBC, dilated vessels</td>
<td>Vasculature very leaky, no capillary sprout, underdeveloped heart</td>
<td>(48, 49)</td>
</tr>
<tr>
<td><strong>Tie-1</strong> (Tie)</td>
<td>L L P</td>
<td>1.0</td>
<td>Hemorrhage, leaky vessels, small heart</td>
<td>(49)</td>
<td></td>
</tr>
<tr>
<td>*** L D</td>
<td>ED 14.5</td>
<td>Abnormal microvasculature, large vessels</td>
<td>Abnormal microvasculature, hemorrhage</td>
<td>(50)</td>
<td></td>
</tr>
<tr>
<td>EphrinB2</td>
<td>L D</td>
<td>ED 10.5</td>
<td>No vessel fusion, no remodeling</td>
<td>Underdeveloped vessels, no remodeling, underdeveloped heart</td>
<td>(51)</td>
</tr>
<tr>
<td>VE-Cadherin</td>
<td>L D</td>
<td>ED 10.5</td>
<td>No primary vascular plexus</td>
<td>Abnormally large vessels</td>
<td>(52)</td>
</tr>
</tbody>
</table>

Targeted genes and phenotypes observed in +/- heterozygous and -/- homozygous mice. L denotes mice born alive and D mice dying during gestation. Stage of death of the embryos are indicated after birth (P), and during embryogenesis (ED). *Because of the heterozygous lethality, VEGF-/- mice were generated by ES cell aggregation with tetraploid blastocysts. ** Deletion of the kinase domain of VEGFR-1 without affecting the ligand binding region. *** Knockout made by two distinct groups in different strains of mice suggesting influence of genetic background on phenotype.
B. Role of the Angiopoietin family

Other endothelial cell specific receptors are also critical for the development of the vascular and hematopoietic systems. The angiopoietin family is composed of the tyrosine kinase receptors, Tie-1 (Tie) and Tie-2 (Tek). Four angiopoietin ligands (Ang1-4) are known to bind only to the Tie-2 receptor (46, 57, 58). These growth factors, in particular Ang1 and Ang2 are required especially in angiogenic processes such as vascular remodeling and blood vessel maturation (Figure 1.3), as reviewed in (47).

Tie-2 is first detected at ED7.5 on yolk sac angioblasts and at ED8.5 on angioblasts of the embryo (59) while Ang1 is mainly expressed in mesenchymal or smooth muscle cells surrounding emerging blood vessels (46, 57). As opposed to VEGF-A, Ang1 has been shown to induce chemotactic migration as well as angiogenic sprouting of endothelial cells (60) (Figure 1.3). In both embryonic and adult vasculature, Ang1 reduces microvascular plasma leakage and stimulates vessel growth. This suggested its potential use for controlling vascular permeability in chronic inflammatory disorders and neoplastic diseases (61, 62). Targeted disruption of Ang1 or its receptor Tie-2 and dominant negative expression of Tie-2 lead to lethal developmental cardiac and vascular defects (Table 1.1) (48, 49, 63). Hence this ligand-receptor interaction is implicated in the assembly of vessel wall components. Interestingly, transgenic overexpression of Ang1 yields a striking hyper-vascularization, presumably by promoting vascular pruning (64). An activating mutation in the Tie-2 receptor leads to venous malformations associated with reduction or total absence of smooth muscle cells in the vessel wall (65). Thus, Ang1 and Tie-2 under physiological conditions seem to be involved in the recruitment of smooth muscle cells and regulation of their interaction with endothelial cells (Figure 1.3). It is interesting to note that defects in vascular remodeling can result either from the overexpression of Tie-2 or the absence of this receptor or its ligand. This can be explained if failure to recruit smooth muscle cells leads to proliferation of endothelial cells, as observed in venous malformations (4, 65).
Ang2 also binds to the Tie-2 receptor (57). This ligand, expressed at high levels in regions of vascular remodeling, acts as a natural antagonist of the Ang1/Tie-2 interaction (57, 66). Engineered, overexpression of Ang2 during embryogenesis leads to severe vascular defects similar to those seen in embryos deficient in Ang1 and Tie-2 (57, 67). The lack of Tie-2 receptor not only led to impaired vascular development but also to defective hematopoiesis. An in vitro study demonstrated that Ang1 through its interaction with Tie-2 promotes proliferation and induces adhesion of hematopoietic cells to the extracellular matrix (68).

Our understanding of the contribution of the other tyrosine kinase receptor, Tie-1, to vascular development is limited because no ligand has been defined yet. Tie-1 is first detected on endothelial cells during fusion of blood islands (69). Two groups have generated Tie-1 null mice. These animals have leaky vessels that lead to hemorrhage and death at ED14.5 (50) or within 24h after birth (49) (Table 1.1). Genetic variability between mouse strains could account for the differences in phenotype.

C. Role of other angiogenic factors in embryonic and adult vasculature

Other factors have also been implicated in the formation blood vessels (Figure 1.3). In early development, in vitro findings suggested that bFGF participated in angioblast differentiation via induction of VEGF (2). As the primary plexus is remodeling by angiogenic processes involving branching, and pruning, PDGF induces the recruitment of mesenchymal cells to the vessel wall and stimulates endothelial cell proliferation (70, 71). To complete vessel wall assembly and maturation, the newly described ephrin family of molecules has been implicated during these stages. In particular, ephrin-B2 (membrane bound ligand) and its receptor EphB4 are differentially expressed on arterial and venous endothelial cells (51). Finally, throughout vascular wall assembly and later in mature vessels, the integrins are important molecules for the maintenance and integrity of the vasculature. They are responsible for attachment of cells to extracellular matrix, motility and can even bind metalloproteinases to regulate endothelial cell invasiveness (72-74).
Following embryonic and postnatal development, rapid proliferation of endothelial cells is down-regulated almost to quiescence. However, the capillary endothelial cells maintain their proliferative potential throughout the life of an organism. Angiogenesis does occur under normal physiological situations and is regulated by the same factors as embryonic angiogenesis. Endothelial cell proliferation is dramatically up-regulated during ovarian cycle and pregnancy, for example. Angiogenesis is also seen in wound healing processes and in different pathological conditions. Neovascularization can accelerate disease progression as observed in many neoplastic diseases, or mediate pathogenesis as seen in diabetic retinopathy (3, 75).

1.2.4 Current models of hematopoiesis

A. Embryonic hematopoiesis

During embryonic development, hematopoietic cells develop both in yolk sac and intra-embryonic sites, as described in Figures 1.1 and 1.2 and as reviewed in (76). They are first seen in yolk sac blood islands at ED7.5 (Figure 1.1) where they give rise to embryonic erythrocytes and macrophages (2, 77) (Figure 1.2). The early primitive erythrocytes are large, nucleated cells that produce embryonic globin (ζ, ε, β H1), and constitute the major cell type in primitive hematopoiesis. As the vascular system develops and the heart begins to beat around ED8.5, these primitive erythrocytes circulate in the embryo and persist until mid to late gestation (2). Within 12 to 48h following blood island formation, anucleated erythroid and myeloid precursors are produced by definitive hematopoiesis (78). Lymphoid precursors within the yolk sac have been reported, although these appear to develop from a multipotential uncommitted cell rather than from a lymphoid committed progenitor (79, 80). All yolk sac definitive hematopoietic precursors starting at ED9.0 have the ability to repopulate conditioned newborn mice (donor cells are c-kit+ (CD117) and CD34+ but Sca-1-)(81-83). Later in development, very few totipotent cells found in the yolk sac, were shown to have LTR capacity that probably represent recirculating cells from the embryo (83). The number of definitive hematopoietic cells decreases dramatically after ED10.0 and none were seen after ED12.0.
Prior to establishment of the fetal liver, two regions of the embryo can generate hematopoietic precursors: the P-Sp at ED8.5-9.5 and the AGM at ED10.0-10.5. Both of these sites produce cells of definitive hematopoiesis (79). As within the yolk sac, multipotent progenitors found in P-Sp have the capacity to generate erythroid, myeloid and lymphoid progeny but do not have LTR capacity (79, 80). At ED10.0, the AGM region is colonized with HSC with LTR capacity (83). These cells can repopulate the blood system of the recipient up to 100% in all hematopoietic tissues and lineages, even 8 months post-transplantation. Thus, the AGM region represents the first site of definitive hematopoietic producing cells with a LTR feature (26, 84). All AGM hematopoietic stem cells are positive for c-kit, CD34 and Sca-1 but are negative for mature lineage markers such as CD4, CD8, B220 and Gr-1 (26). Hematopoiesis progresses to the fetal liver, which assumes the predominant hematopoietic role until birth. Late in gestation, hematopoietic precursors seed the bone marrow that becomes the principal site of hematopoietic activity, shortly after birth.

B. Adult hematopoiesis

Over the past several years, multiple models of hematopoiesis have been proposed. Figure 1.4 illustrates a model of HSC differentiation based on data derived from reconstituting activity and clonal succession (85, 86). Adult bone marrow contains HSC, which can give rise to all types of mature hematopoietic cells. Approximately 0.05% of mouse bone marrow cells are Thy-1.1b (CD90.1b) lineage (Lin)-, Sca-1+ and have the capacity to fully reconstitute all blood cell elements and maintain hematopoiesis over the host lifetime (87). Mouse HSC can be further divided into three subpopulations: long-term HSC (LT-HSC), short-term HSC (ST-HSC) and multipotent progenitors. These cells differ from one another by their capacity to self-renew. Long-term HSC give rise to progeny that progressively lose self-renewal capacity and become restricted to one lineage (88, 89).

A clonogenic common lymphoid progenitor (CLP) which can differentiate into T, B, and NK cells was identified in murine bone marrow. This progenitor is IL-7Rα+, Lin-, Sca-1+ and c-kit+ and cannot differentiate into myeloid cells in either in vivo or in vitro systems (90).
Figure 1.4 Hematopoietic differentiation in adult bone marrow.
The bone marrow is the major site of hematopoiesis throughout adult life. Long-term hematopoietic stem cells (LT-HSC) give rise to short-term HSC (ST-HSC) that have limited self-renewal activity. ST-HSC become committed to lymphoid or myeloid lineages by differentiation into common lymphoid (CLP) or myeloid (CMP) progenitors. From there, CLP further differentiate into all the lymphoid lineages including mature T, B, NK cells as well as thymic dendritic cells. CMP differentiate into other oligopotent precursors, the granulocyte/macrophage progenitors (GMP) giving rise to mature monocytes and granulocytic populations and the megakaryocyte/erythrocyte progenitors (MEP) forming platelets and erythroid cells. Adapted from (85, 86).
Similarly, a significant population with CLP activity within the Lin⁻, CD34⁺ and CD10⁺ subset of human bone marrow was found. This population has the capacity to differentiate only into T, B, NK and thymic dendritic cells (91). Evidence of CLP in both murine and human bone marrow confirmed the existence of such oligopotent progenitors. However, it is still unknown whether CLPs are cells exclusively found in the bone marrow or if they can home to the thymus and differentiate into T cells. The microenvironment encountered by CLPs may determine whether they differentiate into B cells by staying in the bone marrow or into T cells if migrating to the thymus (Figure 1.4) (90).

A complementary population to the CLP, the clonogenic common myeloid progenitor (CMP) was identified (92). The CMP gives rise exclusively to myeloid, megakaryocytic and erythroid progenitors and not to lymphoid-derived cells. CMP can further differentiate into either granulocyte/macrophage progenitors (GMP) or megakaryocyte/erythroid progenitors (MEP). In the bone marrow, GMP will generate monocytes, neutrophils, eosinophils and basophils while MEP will yield platelets and erythrocytes. Both CLP and CMP reflect the earliest branch points between the lymphoid and myeloid lineages (92). The differentiation of all progenitors is influenced by the environment. In addition, they appear to be primed for commitment by co-expression of a multiplicity of genes characteristic of various lineages (93). The identification of these genes will further clarify the process of hematopoietic differentiation.
1.3 Role of the TGF-\(\beta\) superfamily of molecules in vascular and hematopoietic systems

The TGF-\(\beta\) superfamily is composed of at least 30 different peptides, which in mammalian cells are divided into 3 major classes: TGF-\(\beta\), activins and bone morphogenetic proteins (BMPs). These factors are synthesized as precursor proteins, which upon cleavage liberate the active growth factor. The mature form of each TGF-\(\beta\) member is a disulfide-linked homodimer of 25–30kDa. They mediate effects through association with heteromeric complexes of serine/threonine kinase receptors of type I and type II which transmit signals to the nucleus through the Smad proteins. Other components of the receptor complexes include betaglycan and endoglin, which modulate responses to ligands. The TGF-\(\beta\) superfamily of molecules act on multiple tissues and have pleiotropic functions: they regulate cell growth and differentiation, and play important roles in early embryonic development and in immune responses (reviewed in (94–98)).

1.3.1 Ligands and their distribution

A. The TGF-\(\beta\) family

The TGF-\(\beta\) family is more divergent from activins and BMPs and consists of 3 mammalian isoforms, TGF-\(\beta1\), \(-\beta2\) and \(-\beta3\). Biologically active TGF-\(\beta\) is a disulfide-linked homodimer of 112 amino acids (aa). All 3 isoforms are structurally similar and exhibit both overlapping and distinct patterns of expression throughout development.

TGF-\(\beta1\) mRNA can be detected as early as in the pre-implantation period, in blastocyst. TGF-\(\beta1\) and \(-\beta3\) are found in the inner cell mass and the trophoderm while TGF-\(\beta2\) is limited to the trophoderm (99, 100). In post gastrulation stage, both extra-embryonic and embryonic vasculature express TGF-\(\beta1\). Starting at ED7.5, TGF-\(\beta1\) can be seen in blood islands and mesodermal cells of the allantois while TGF-\(\beta2\) \(-\beta3\) transcripts are absent (101, 102). Embryos at ED9.5 express TGF-\(\beta1\) in all major blood vessels including dorsal aorta, anterior cardinal veins, caudal arteries and veins and internal carotid artery (101). Within the embryos, variable expression of TGF-\(\beta2\) and \(-\beta3\) is seen. TGF-\(\beta2\) is restricted to the media of major vessels while \(-\beta3\) is observed in media and intima and on mesenchymal cells surrounding umbilical arteries
and veins (103). TGF-β is also implicated in heart development. TGF-β1 mRNA is initially expressed on the heart endocardium and then on endothelial cells of the atrioventricular canal and outflow tract (101). During endocardial cushion tissue formation, TGF-β2 is seen on the outflow tract and the atrioventricular myocardium. After this transformation, TGF-β3 expression is up-regulated in the heart (104, 105).

B. The Activin family

The activin family of ligands is more closely related to the BMPs than to the TGF-βs. Activins are dimeric proteins composed of disulfide linked β-chain subunits βA and βB. Activin-A, is composed of 2 βA subunits and is the most abundant form while Activin-B and Activin-AB are composed of βBβB and βAβB subunits respectively (106). These molecules are primarily known for stimulating the secretion of follicle stimulating hormone (FSH), decreasing the production of ovarian hormones and regulating follicular cell differentiation (reviewed in (107)). However, activins are also involved in many other biological processes outside the pituitary-gonadal axis. In particular, Activin-A was shown to inhibit endothelial cell proliferation (108).

The expression pattern of activin reflects well its potential role in several systems. In pre-implantation murine embryos, activin is present in all cells at morula stage, and then becomes restricted to the inner cell mass of the blastocysts (109). However at implantation, activin is only expressed on the trophectoderm. In subsequent post-implantation stages and until ED10.5 both βA and βB subunits are strictly limited to decidual cells surrounding the embryos. Subsequently, βA subunit is seen in mesenchymal tissues of the heart, developing face and body and in the pre-cartilage condensation in the limbs while βB is expressed in fore and hindbrain and in spinal cord. Both βA and βB are expressed in blood vessels, and in mesenchymal cells of the gonad region (110, 111).

C. The BMP family

The BMP family is the largest and most diverse group of the TGF-β superfamily. It is composed, in particular, of the mammalian members BMP-2 to -8 and BMP-10, which were identified in human and/or mouse. The expression pattern of the BMPs is sometimes overlapping and reflects
their pleiotropic functions. BMP-4 and BMP-7 can be detected in murine embryo as early at ED6.5, at gastrulation (112-114). One day later, BMP-4 is found on the posterior part of the primitive streak (112, 114). At ED 8.5, expression of BMP-2 and BMP-6 is seen along with BMP-4 and -7 on mesodermal cells of the amnion and/or the endodermal component of the yolk sac and the ectoderm (113, 114). All four growth factors are also expressed in areas of the developing neural system. BMP-2 is found in the dorsal surface of the ectoderm, underlying the neural tube. BMP-4 is observed in the posterior mesoderm and the dorsal surface ectoderm and on the presumptive neural crest cells. BMP-6 is detected on the roof plate of the neural tube, while BMP-7 is expressed in the ectoderm surface and the notochord in the neuroepithelium (113, 115, 116).

BMP-2, -4, -6, -7 and the newly described member BMP-10 are implicated in heart development. BMP-2 can be seen on the outer myocardial layer while BMP-4 is in the myocardium. BMP-6 is found on the epithelium of the developing heart. BMP-7 is expressed in atrial and ventricular chambers throughout development (112, 114, 116-119). Later during development, members of the BMP family can be found in mesenchymal condensations. These will give rise to ribs, vertebrae and digits in hypertrophic chondrocytes, to tooth buds, developing eye, and whisker follicles (112, 116, 117, 120-122). The expression of the BMPs in diverse developing systems suggests important roles in embryogenesis at stages of gastrulation, neurogenesis, cardiogenesis, chondrogenesis and limb development.

1.3.2 Receptor activation and signaling mechanisms

A. TGF-β receptor activation

The TGF-β receptor superfamily contains serine/threonine kinases of types I and II, which mediate the effects of the growth factors. Both types of receptors are glycoproteins of about 55–60kDa for type I and 75-85kDa for type II with core polypeptides of 500 and 570 aa respectively. They have a small extracellular domain (~150 aa), a single spanning transmembrane region and a conserved cytoplasmic kinase domain (123-128). In addition, the type I receptors have in their cytoplasmic region a unique short glycine and serine rich motif
(the GS domain, SGSGSG) that is phosphorylated upon receptor activation. They also contain a Leu-Pro motif that serves as binding site for negative regulation of the receptor signaling function (94, 127, 129).

Figure 1.5 demonstrates a current model of the signaling pathways for the TGF-β superfamily (reviewed in (98, 130)). To date, there are three distinct mechanisms of receptor activation. As it was first defined for the prototypic TGF-β1, the dimeric structure of the ligand brings together pairs of type I and II receptors. TGF-β binds the constitutively phosphorylated TβR-II, which then recruits TβR-I in the complex. TβR-II phosphorylates TβR-I in the GS domain and TβR-I propagates downstream signals by phosphorylating Smad proteins (129, 131). Activin mediates its function through this mode of activation by binding to ActR-II or ActR-IIB, recruiting and phosphorylating ActR-IB. The second mode of binding, is characteristic of the BMPs. BMP-2 and BMP-4 can bind BMPR-IA (ALK-3) or BMPR-IB (ALK-6) independently of the type II receptor. To transmit signals, the cooperation of BMPR-II, ActR-II or ActR-IIB is required. The BMP-7 is known to bind very weakly to BMPR-II receptors, so that cooperation with ALK-3, ALK-6 or ActR-I (ALK-2) is required for binding and generating downstream signals (Figure 1.5).

B. The Smads, mediators of TGF-β superfamily signaling

The Smads are a family of highly conserved proteins that are the effectors of various TGF-β signaling pathways. Eight distinct mammalian Smads have now been identified that can be regrouped into 3 subclasses: receptor activated Smads (R-Smads) such as Smads 1, 2, 3, 5 and 8; a common-partner Smad (Co-Smad), Smad4; and the inhibitory Smads (I-Smads), Smads 6 and 7 (Figure 1.5). The R-Smads and Co-Smad both have Mad-homology domains (MH1 and MH2) on N and C termini separated by a proline rich linker region. At basal state, the MH1 domain interacts with MH2 domain to inhibit their mutual transcriptional and biological activities. In addition, the MH2 domain of the R-Smad contains phosphorylation motifs, SSXS, which are absent in the Co-Smad and are involved in several important protein-protein interactions. (reviewed in (97, 98)).
Figure 1.5 Signaling by TGF-β superfamily members.
The activin, TGF-β and BMP families of ligands mediate their functions through association with several combinations of type II and type I receptors, members of the TGF-β superfamily. The type I receptors transduce signals via two distinct pathways by the activation of intracellular receptor Smads (R-Smads): the Smad2/3 pathway in blue and the Smad1/5/8 in green. The R-Smads then form complexes with the common partner Smad4 (Co-Smad; orange) and translocate into the nucleus where they regulate transcription of target genes. Inhibitory Smads (I-Smads; light blue) are induced by ligand stimulation and interfere with receptor association with the R-Smads. Adapted from (98).
The Smad signaling cascade is initiated by the activated type I receptor kinase, which recognizes and phosphorylates the R-Smads on two C-terminal serine residues in their MH2 domain, thus relieving the auto-inhibitory MH1-MH2 interaction. As shown in Figure 1.5, two Smad signaling pathways have been defined. Smads 2 and 3 interact with activated TβR-I (ALK-5) and ActR-IB and signal for TGF-β and activin (131) while Smads 1, 5 and 8 interact with activated BMPR-IA/IB and ActR-I and signal for the BMPs (132-135). Interestingly ALK-1, which was shown recently to bind TGF-β1 in endothelial cells (136) phosphorylates Smads 1 and 5 (134), suggesting cross talk between the pathways. The recruitment of R-Smads to the receptor complex is achieved through the Smad anchor for activation (SARA), a membrane associated protein capable of binding unphosphorylated Smad2 and Smad3. SARA is dissociated upon phosphorylation of the R-Smads (137). Activation of these signaling proteins allows heterodimerization with Smad4, forming preferentially trimers (133, 138). The complex of R–Smad and Co-Smad is then translocated to the nucleus, where it is involved in transcriptional regulation of target genes. Mammalian Smad7 is a general inhibitor of the superfamily while Smad6 is preferentially a BMP inhibitor. These Smads are quickly induced upon stimulation by ligand and provide a mechanism of autoinhibition (139-141) (Figure 1.5). These I-Smads are induced by laminar shear stress on vascular endothelium and may play a regulatory role in vascular remodeling (142, 143).

1.3.3 Effects on vasculogenesis/angiogenesis of targeted disruption of ligands and receptors of the TGF-β superfamily

Many members of the TGF-β superfamily, their receptors or downstream signaling molecules are essential for mouse embryogenesis, as demonstrated by gene targeting experiments. Phenotypes described in mutant mice and summarized in Table 1.2 suggest that they are essential for gastrulation, cardiovascular development and fetal growth and maturation.

Most members of the BMP signaling pathway appear to be needed for egg cylinder formation. BMP-4, BMPR-II, ActR-I, ActR-IB, and BMPR-IA null mice die around gastrulation with little or no mesodermal differentiation (144-148). Also utilized in this pathway are the
ActR-II or ActR-IIIB receptors. While embryos lacking either of these receptors develop to term with no gross defects in mesoderm formation (149), those lacking both ActR-II and ActR-IIIB fail to elongate the primitive streak resulting in defective mesoderm formation (150). BMP-2 also signals through this pathway, but the mutant mice have a less severe phenotype; they do form mesodermal cells but fail to close the pro-amniotic canal leading to death (118). Mice lacking downstream signaling proteins such as the common Smad4, fail to initiate gastrulation and show abnormal visceral endoderm development (151, 152). Although Smad2 has not been shown to signal for BMPs, the null mice also die early in embryogenesis due to defective mesoderm formation (153) (Table 1.2).

Components of the TβR-II mediated signaling pathway have been shown to be important in the differentiation and growth of the vascular system. Targeting mutations in the TGF-βI gene results in primary defects in yolk sac vasculogenesis and hematopoiesis which lead to death at about ED10.5 in 50% of the homozygotes and 25% of the heterozygotes (18). Vessels of the extra-embryonic lesions are small, weak, disorganized, and incomplete contacts between endothelial and mesothelial layers lead to leakage of blood cells into the yolk sac cavity. The defects seen in the TGF-βI deficient mice are not attributed to improper differentiation of the mesoderm into endothelial cells but rather to inadequate differentiation affecting endothelial tube formation (18). Improper endothelial adhesiveness can be explained by the role of TGF-β1 in stimulating synthesis of extracellular matrix (ECM) proteins, blocking degradation of ECM by inhibiting the secretion of proteases and inducing the production of inhibitors of metalloproteinases (154) (Figure 1.3). Furthermore, weak and disorganized blood vessels can be attributed to loss of TGF-β1 effects on vessel wall assembly including endothelial cell proliferation and migration as well as induction of mesenchymal cell differentiation to pericytes and smooth muscle cells (155-157) (Figure 1.3).
Table 1.2 Phenotype of mice with targeted mutation in genes of the TGF-β superfamily.

<table>
<thead>
<tr>
<th>Targeted gene</th>
<th>Animals</th>
<th>Time of Death</th>
<th>Phenotypes</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early development/gastrulation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>BMP-2</strong></td>
<td>L D</td>
<td>7-10.5</td>
<td>Abnormal amnion/chorion formation, cardiac developmental defects</td>
<td>(118)</td>
</tr>
<tr>
<td><strong>BMP-4</strong></td>
<td>L D</td>
<td>6.5-9.5</td>
<td>Gastrulation defects leading to little or no mesoderm differentiation</td>
<td>(144)</td>
</tr>
<tr>
<td><strong>ActR-II</strong></td>
<td>L D</td>
<td>8.5-9.5</td>
<td>Absence of amnion/allantois structure, embryo develops outside yolk sac</td>
<td>85% of -/- with hypoplasia of mandibles, delayed testis maturation or female sterility</td>
</tr>
<tr>
<td><em>ActR-II/ ActR-IIB</em></td>
<td>L D</td>
<td>6.5-8.5</td>
<td>Gastrulation defects leading to no mesoderm formation or primitive streak</td>
<td>(150)</td>
</tr>
<tr>
<td><strong>BMPR-II</strong></td>
<td>L D</td>
<td>6.5-8.5</td>
<td>Gastrulation defects leading to no mesoderm differentiation</td>
<td>(145)</td>
</tr>
<tr>
<td><strong>ActR-I</strong></td>
<td>L D</td>
<td>7.5-8.5</td>
<td>Abnormal visceral endoderm and disorganized mesodermal cells</td>
<td>(146)</td>
</tr>
<tr>
<td><strong>ActR-IB</strong></td>
<td>L D</td>
<td>7.5-8.5</td>
<td>Disorganized epiblast and extra-embryonic ectoderm</td>
<td>(147)</td>
</tr>
<tr>
<td><strong>BMPR-IA</strong></td>
<td>L D</td>
<td>7.5-8.5</td>
<td>Gastrulation defects leading to no mesoderm differentiation</td>
<td>(148)</td>
</tr>
<tr>
<td><strong>Smad2</strong></td>
<td>D</td>
<td>20%</td>
<td>Absence of egg cylinder extra-embryonic portion and no gastrulation</td>
<td>10% of +/- show cranio-facial anomalies and defects in left-right patterning</td>
</tr>
<tr>
<td><strong>Smad4</strong></td>
<td>L D</td>
<td>6.0-7.5</td>
<td>Abnormal visceral endoderm and no gastrulation</td>
<td>+/- mice develop multiple gastric polyps &amp; cancer after 1 year</td>
</tr>
<tr>
<td>Vascular development</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>TGF-B1</strong></td>
<td>D</td>
<td>25%</td>
<td>Abnormal vasculogenesis &amp; hematopoiesis, improper endothelial cell differentiation</td>
<td>50% of +/- inflammatory responses and abnormal modulation of immune response</td>
</tr>
<tr>
<td><strong>TβR-II</strong></td>
<td>L D</td>
<td>10.5</td>
<td>Abnormal vasculogenesis &amp; hematopoiesis,</td>
<td>(19)</td>
</tr>
<tr>
<td><strong>Endoglin</strong></td>
<td>L D</td>
<td>10-11.5</td>
<td>Lack of large yolk sac vessels, impaired angiogenesis, heart defects, hemorrhage</td>
<td>A fraction of +/- show signs of HHT</td>
</tr>
<tr>
<td><strong>ALK-1</strong></td>
<td>L D</td>
<td>10.5</td>
<td>Abnormally large vessels in yolk sac, defect in vascular SMC differentiation</td>
<td>(136)</td>
</tr>
<tr>
<td><strong>Smad5</strong></td>
<td>L D</td>
<td>9.5-11.5</td>
<td>Disorganized yolk sac vessels, gut &amp; heart defects, chorion/allantois fusion</td>
<td>(160)</td>
</tr>
<tr>
<td>Targeted gene</td>
<td>Animals</td>
<td>Time of Death</td>
<td>Phenotypes</td>
<td>References</td>
</tr>
<tr>
<td>--------------</td>
<td>---------</td>
<td>---------------</td>
<td>------------</td>
<td>------------</td>
</tr>
<tr>
<td>+/−</td>
<td>−/−</td>
<td>During gestation</td>
<td>After birth</td>
<td></td>
</tr>
<tr>
<td>late embryonic and perinatal development</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGF-β2</td>
<td>L D</td>
<td>ED19.0-66% P1.0</td>
<td>Cyanotic &amp; dying of respiratory distress; ear, heart, eye, urogenital &amp; cranio-facial defects</td>
<td>(161)</td>
</tr>
<tr>
<td>TGF-β3</td>
<td>L L</td>
<td>P1.0</td>
<td>Cleft palate defect, abnormal and fragile lung vasculature</td>
<td>(162, 163)</td>
</tr>
<tr>
<td>Activin βA</td>
<td>L L</td>
<td>P1.0</td>
<td>Lack whiskers, lower incisors and have cleft palate defect</td>
<td>(149)</td>
</tr>
<tr>
<td>Activin βB</td>
<td>L L</td>
<td>P1.0</td>
<td>Defective eyelid development &amp; female reproductive system</td>
<td>(164)</td>
</tr>
<tr>
<td>BMP-7</td>
<td>L L</td>
<td>P1.4 w</td>
<td>Abnormalities in skeletal, kidney and eye development</td>
<td>(165)</td>
</tr>
<tr>
<td>ActR-IIb</td>
<td>L L</td>
<td>P1.2 w</td>
<td>Severe cardiac defects affecting heart and artery position, septation, &amp; abnormal spleen</td>
<td>(166)</td>
</tr>
<tr>
<td>Smad3</td>
<td>L L</td>
<td>P1.8 mo</td>
<td>Immune dysregulation, abscess formation, accelerated wound healing, cancer</td>
<td>(167, 168)</td>
</tr>
<tr>
<td>Smad6</td>
<td>L L</td>
<td>P&lt;3w 90%</td>
<td>Hyperplasia of cardiac valves &amp; outflow tract, septation defect, aortic ossification</td>
<td>(169)</td>
</tr>
</tbody>
</table>

Targeted genes and phenotypes observed in +/- heterozygous and -/- homozygous mice. L denotes mice born alive and D mice dying during gestation. Stage of death of the embryos is indicated after birth (P), and during embryogenesis (ED). * Indicates knockout of both ActR-II and ActR-IIb.
An almost identical phenotype was observed in the TβR-II knockout mice (19). The TβR-I (ALK-5) knockout has not yet been reported. However, the knockout for ALK-1, a TβR-I present in endothelial cells, has been described (136). ALK-1 null embryos also die at mid-gestation of severe vascular abnormalities characterized by excessive fusion of capillary plexus into cavernous vessels and hyperdilation of large vessels. It was suggested that this phenotype was explained in part by a requirement for ALK-1 in the differentiation and recruitment of vascular smooth muscle cells (136). As ALK-1 signals for TGF-β1 through the Smad1/5 pathway, it is interesting that the Smad5 null mice showed several defects reminiscent of ALK-1 and TGF-β1 deficient mice. A disorganized yolk sac vasculature was observed along with developmental abnormalities in amnion, gut and heart, which lead to death between ED9.5 and 11.5 (160) (Table 1.2). These phenotypes are similar to that of End null mice to be described in Chapter 3.

Disruption of other members of the TGF-β superfamily lead to phenotypes in late developmental and perinatal stages (Table 1.2). For example, disruption of TGF-β3 leads to very fragile vessels, particularly in lungs but also gives cleft palate defects (162, 163) Both TGF-β3 and -β2 were found important for epithelial-mesenchymal transformation processes (161, 163). TGF-β2 null mice die during gestation of multiple developmental defects affecting a wide range of organs such as heart, lung, cranium, limb, spinal cord, eye, inner ear and urogenital system (161).

1.3.4 Role of TGF-β family members in hematopoiesis and immune system

Members of the TGF-β superfamily, particularly TGF-β1, have been extensively studied because of their important regulatory effects on the developing and mature immune system. TGF-β1 is produced by most immune cell types and controls their differentiation, proliferation and activation status by autocrine and paracrine regulation of its secretion. TGF-β1 is the most potent immunosuppressive cytokine and is essential for the maintenance of normal immune function, as reviewed by Letterio & Roberts (170).
A. Role of TGF-β determined by transgenic mice

More than 50% of the *TGF-β1* null mice die during gestation (18) while live born null mice exhibit severe immune dysregulation leading to death at 3 to 4 wks. These mice develop multifocal perivascular infiltration of inflammatory cells in many tissues and show increased numbers of proliferating cells in lymphoid organs. Lymphocytes isolated from these mice are defective in their proliferative response to mitogens. This is probably due to impaired IL-2 synthesis or secretion (171-173). In addition, no epidermal dendritic Langerhans cells are found in the *TGF-β1* deficient mice thereby blocking immune activation (174). Uncontrolled B cell responses are seen which include elevated IgE levels, lack of anti-inflammatory IgA, and an excess of complement-binding IgG and IgM antibodies. These features contribute significantly to the inflammation seen in these animals (175). *TGF-β1* null mice also develop circulatory anti-ds-DNA and anti-ss-DNA as well as glomerular immune complex deposits and show features similar to human systemic lupus erythematosus (SLE), Sjogren’s syndrome, graft versus host disease (GVHD), and polyomyositis. This autoimmune phenotype has been mainly ascribed to the presence of activated CD4⁺ T cells (176-179).

The onset of inflammation in *TGF-β* deficient animals coincides with enhanced expression of major histocompatibility complex (MHC) class I and II molecules (171, 180). Mice homozygous for both *TGF-β1* and *MHC class II* lack inflammatory infiltrates, circulating autoantibodies and glomerular immune complexes. Mice deficient in both β2-microglobulin and *TGF-β1* show reduced tissue inflammation and decreased severity of the autoimmune phenotype. This suggests a role for both MHC class I and II mediated responses in these animals (181, 182). Crossing *TGF-β1* mice with SCID (severe combined immunodeficient mice) results in progeny with no inflammatory lesions and no signs of autoimmunity, implying a role for B and T cells (177).

Recently, the role of TGF-β in lymphocytes was studied by two groups using restricted expression of a dominant negative *TβR-II* in T cells. No TGF-β signaling was possible in the targeted T cells of these transgenic mice. Both groups demonstrated that TGF-β is required for T cell homeostasis (183, 184). In one case, the major finding was a CD8⁺ T cell
lymphoproliferative disorder that resulted in massive expansion of the lymphoid organs (183). In the second case, circulating autoimmune antibodies and inflammatory infiltrates were demonstrated and T cells differentiated spontaneously into T helper cells (TH), TH1 and TH2 cytokine secreting cells (184). Smad3 transduces signals for the activated TβR-I (ALK-5). Mice with a null mutation die between 1 and 8 months of leukocytosis with massive inflammation and abscess formation. T cells isolated from these animals were completely resistant to inhibition by TGF-β1 demonstrating that Smad3 is required for mediating the regulatory effects of TGF-β1 in T cells (168).

B. Role of TGF-β in growth, differentiation and maturation of the immune system

In the thymus, during development of T cells, TGF-β1 inhibits the progression and differentiation of immature single positive CD4+CD8low precursor thymocytes into CD4+CD8+ double positive (DP) thymocytes, influencing T cell receptor (TCR) selection process (185). Furthermore in absence of TGF-β1, DP thymocytes are not efficiently deleted in the thymus. The additive effect of TGF-β1 and TNF-α (tumor necrosis factor alpha) on DP thymocytes favors the expansion of CD8 T cells (186). Maturation of precursors of TH cells into TH1 and TH2 subpopulations is driven by cytokines. TH1 cells produce interferon gamma (IFNγ) and Interleukin 12 (IL-12) while TH2 cells secrete IL-4, IL-5, IL-6 and IL-10. TGF-β1 can inhibit the production of and response to cytokines associated with each subset. T cells producing TGF-β are classified as a distinct TH3 subset and also produce IL-4 and IL-10 (187, 188).

TGF-β1 also affects B cell development from pre-B cell stage to immunoglobulin-secreting plasma cells (189). It is a potent inhibitor of antigen or mitogen driven B cell proliferation and can induce apoptosis. In general, apoptosis is regulated by p53, down-regulation of bcl2 and up-regulation of BAX. TGF-β1 down-regulates bcl2, thus inducing cellular apoptosis (190). In mature B cells, TGF-β1 inhibits expression of IgM, IgD, IgA κ and λ chains, but stimulates isotype switching recombination of IgG2b in mouse and of isotype IgA in both mouse and human (191).
During early response to injury and inflammation, TGF-β1 released from platelets acts as a pro-inflammatory agent by recruiting and activating resting monocytes, and inducing the synthesis of cytokines such as IL-1. As monocytes differentiate into macrophages, they secrete TGF-β1 to facilitate tissue remodeling and wound closure (192). At this stage, TGF-β1 participates in resolution of the inflammatory response by down-regulation of TNF-α, peroxide (H₂O₂) and nitric oxide (NO) production by macrophages and enhances production of IL-10, a potent immunosuppressive factor (193). These mechanisms will reduce matrix deposition and limit the extent of tissue fibrosis (194).
1.4 The role of endoglin in vascular and hematopoietic systems

Endoglin was identified, cloned and sequenced in our laboratory. It was characterized as a glycoprotein that binds TGF-β1 and -β3 (195). More recently it was also shown to bind to other members of the TGF-β superfamily by association with their respective ligand binding receptors (8). Endoglin is also the gene mutated in Hereditary Hemorrhagic Telangiectasia type 1 (HHT1), a disease associated with arteriovenous malformations (11). Endoglin was first isolated in human where its expression, structure and function were characterized. The study of the role of murine endoglin has more recently been undertaken with the generation of End deficient mice by us (Chapters 3, 4 and 5) and independently by two other groups (158, 159).

1.4.1 Tissue distribution

A. Marker of endothelial cells

Endoglin was discovered in a study aimed at defining surface proteins of pre-B acute lymphoblastic leukemia (ALL) cells. Monoclonal antibody (mAb) 44G4 produced against pre-B ALL cells showed an intense reactivity to vascular endothelial cells in thymus and tonsils (13). Endoglin was next found to be expressed in human kidney, on glomerular mesangium, interstitium and vascular endothelium (196). The distribution of endoglin was further investigated by immunostaining various tissues including lymph nodes, spleen, lungs, liver and umbilical cord. In all tissues, mAb 44G4 reacted strongly with endothelial cells of capillaries, arterioles, small arteries, venules, high endothelial venules as well as with the arteries and vein of the umbilical cord (5). Endoglin was recognized as an endothelial marker and assigned the CD105 cluster at the 5th Leukocyte International Workshop (6). To date, approximately 40 mAb recognizing different regions of the extracellular domain of human endoglin have been characterized and are used in diverse applications (6, 197, 198).

Endoglin is constitutively expressed at high levels on endothelial cells. However its expression can be modulated by the activation status of the endothelium and is up-regulated during angiogenic processes. In vitro, endoglin was found to be invariably high on cultured human umbilical vein endothelial cells (HUVEC) and low on fibroblasts. In addition, we have
demonstrated that endoglin is expressed on mesenchymal cells of the adventitial layer of normal brain arteries, leptomeneningial vessels and intra-cerebral arterioles (199). Stimulation with IFN-γ or irradiation increases expression of endoglin (200, 201). In in vivo situations where endothelial cell proliferation occurs such as inflammation and skin lesions (cutaneous melanoma, granulation tissue and psoriasis), endoglin expression was shown to be considerably up-regulated (200). Several other studies demonstrated increased endoglin levels under pathological or inflammatory situations. It was enhanced on perifollicular endothelial cells of patients with autoimmune thyroiditis and on newly formed vessels in hepatic inflammation associated with chronic hepatitis C. The vascular endothelium of neoplasms such as angiosarcoma, various carcinoma, Hodgkin’s disease, lymphoma, osteosarcoma and solid tumors also show up-regulated endoglin expression (202-204).

B. Endoglin in human gestation

Endoglin is present early in human development. It is predominantly expressed on endothelial cells of many embryonic tissues as early as 4 wks of gestation. Tissues originating from ectoderm and endoderm, as well as mesoderm-derived structures such as cartilage and muscles, are negative for endoglin (7). Its pattern of expression in the developing heart is most striking. It is found on the endocardium throughout gestation but is transiently up-regulated on cushion tissue mesenchyme of both atrioventricular canal and outflow tract at 5-8 wks of gestation during heart septation and valve formation (7). Endoglin is also expressed throughout pregnancy on the syncytiotrophoblast of placenta, the fetal cell layer which interfaces with maternal blood (205). It is also seen transiently on cytotrophoblasts differentiating along the invasive pathway from 6-12 wks of gestation (206).

C. Endoglin in hematopoietic cells

Although endoglin is predominantly expressed on endothelial cells, its identification on human pre-B leukemic cell lines suggests a possible role in hematopoiesis. Endoglin distribution is restricted, but includes several lineages of hematopoietic cells. Pro-B and pre-B leukemic cell lines express endoglin, while T and B leukemic cell lines do not (5, 13, 207). Fresh leukemic
cells obtained from children with pre-B ALL and acute myeloid leukemia (AML) bear low levels of endoglin (207).

Endoglin is also found on some normal hematopoietic cells. Endoglin expression was demonstrated in fetal bone marrow, on early B-lineage precursor cells (CD19⁺ and CD34⁺) and pro-erythroblasts (CD71⁺, glycophorin A⁺) (14, 15). In adult bone marrow, only pro-erythroblasts were positive for endoglin (15) while B, T, NK, and myeloid cells were negative (14). Endoglin was also found on stromal cells derived from fetal and adult bone marrow. These cells responded with increased proliferation after stimulation with TGF-β1 or with an antibody to endoglin (15, 208). In peripheral blood, endoglin is absent from B and T lymphocytes and monocytes. However, upon activation of monocytes in culture, endoglin is up-regulated; it is also present on circulating and tissue macrophages (16, 209). A more recent study has analyzed the expression of endoglin in circulating CD34⁺ progenitors (210). These cells are capable of restoring hematopoiesis in autologous and allogeneic recipients after myeloablation (211, 212). Fractionation of the circulating CD34⁺ cells on the basis of endoglin expression yielded two distinct populations of hematopoietic progenitors. The CD34⁺ endoglin⁺ cells had biological properties characteristic of primitive hematopoietic precursors. They were a proliferating population, defined as cytokine low-responding primitive progenitors, with high cloning efficiency and high LTR potential (210, 213). This suggests that endoglin is expressed in early hematopoietic development.

D. Endoglin expression in the mouse

Endoglin is also expressed strongly on blood vessels and capillaries in all murine tissues examined (214). It is present on lymph node high endothelial venules in situ and on derived cell lines (215). In addition, mesenchymal-like stromal cells found in connective tissue of intestine, stomach, heart, muscle, uterus, ovary and testes were found to express endoglin (214). It is also expressed on the murine fibroblast cell line NCTC-2071 derived from normal subcutaneous arteriolar and adipose tissue and at low levels on 3T3 embryonic fibroblasts (214).
1.4.2 Endoglin structure

Endoglin was immunoprecipitated and subsequently purified by affinity to mAb 44G4 and shown to be a homodimeric glycoprotein composed of 90 kDa monomers (5, 13, 216). The cDNA was cloned from a human placental expression library and shown to code for a type I integral membrane protein (217). Endoglin has an extracellular domain of 586 aa, a single transmembrane region of 25 aa and a 47-residue with 19 constitutively phosphorylated serine/threonine in the cytoplasmic tail (217). Human endoglin is heavily glycosylated with four potential N-linked glycosylation sites. After N-glucosidase or endoglycosidase F treatment, the molecular mass of the glycoprotein is reduced by 20 kDa (216). One potential O-linked region was also found proximal to the transmembrane domain and upon O-glycanase and neuraminidase treatment of HOON cells, the molecular mass was further reduced by 15 kDa (216, 217).

Murine endoglin is 74% identical to human endoglin at the nucleotide level and 76% at the protein level (214, 218). Murine endoglin is also heavily glycosylated with five potential N–linked glycosylation sites, and a potential O-linked region proximal to the transmembrane domain (214).

Human endoglin was mapped to chromosome 9q33-34 (219). It was subsequently characterized at the DNA level and shown to contain 15 exons (11). Murine endoglin maps to a region of chromosome 2 that is syntenic with human chromosome 9q (220). The cDNA sequence has 2902 bp and contains 363 bp of 5'-untranslated region, a full open reading frame of 1959 bp and 580 bp of 3'-untranslated regions including polyadenylation signal AATAAA located 9 bp upstream of the poly(A)+ tail (214).

The promoter region of human endoglin has also been studied. Transcriptional start sites were found to be located 350 bp upstream from the translation initiation codon (Figure 1.6). The majority of the promoter activity is located within the proximal promoter region –81/+350 bp. The 5'-flanking region of the endoglin gene lacks consensus TATA and CAAT boxes, but contains two GC-rich regions and consensus motifs for Sp1, ets, GATA, AP-2, NFκB, and
Figure 1.6 Alignment of nucleotide sequence of the 5’-flanking region of human and murine endoglin genes.

The published sequence of the human endoglin promoter (221) was aligned with the murine promoter sequence (unpublished data). Identical residues are highlighted in orange and deleted regions are indicated by a dash line. Numbering in the left margin is by reference to the transcription start site (+1) determined by primer extension analysis in human and deduced by homology in the murine sequence. The cDNA sequence of exon 1, starting with ATG codon, is shown in bold.
Mad, as well as TGF-β-, glucocorticoid-, vitamin D-, and estrogen-responsive elements (221). A strong promoter activity was demonstrated specifically in endothelial cells but not in epithelial cells and fibroblasts (222). These findings suggested that the endoglin promoter could be used for the transcriptional targeting of endothelial cells by gene therapy. In the process of elaborating a strategy to inactivate the endoglin gene in mice, we characterized a genomic clone containing 2443 bp of the promoter region and compared its sequence to the reported human promoter (Figure 1.6). The highest similarity is in the proximal promoter region suggesting that several of the regulatory elements might be conserved between human and mouse (Bourdeau et al., unpublished data).

1.4.3 Endoglin function

Cloning of betaglycan, known as a receptor type III for TGF-β, revealed homology to endoglin (223). This was in fact the first suggestion that endoglin might functionally be related to TGF-β receptors. Subsequent analysis of the binding capacity of iodinated TGF-β to HUVEC demonstrated that endoglin binds with high affinity to TGF-β1 and TGF-β3 isoforms (KD~50 pM) and does not associate with TGF-β2 (195). Endoglin was also shown to bind TGF-β1 in pre-B leukemic cells (224) and stromal cells (208). Endoglin was subsequently shown to bind TGF-β only in association with TβR-II (8). It is also an accessory protein for activin-A, BMP-7 and BMP-2 via association with their respective ligand binding receptor (8) (Figure 1.7). No functional role for endoglin has been found to date with members of the TGF-β superfamily other than TGF-β1 and TGF-β3. Although the exact role of endoglin in the TGF-β receptor complex has yet to be determined, it can modulate several responses to TGF-β1. In the U-937 monocytic cell line, TGF-β1 inhibits proliferation leading to a down-regulation of c-myc mRNA. These two effects were abolished when U-937 cells were transfected with endoglin (10). In addition, fibronectin synthesis and adhesion were no longer stimulated by TGF-β1 (10). When endoglin was overexpressed in rat myoblasts, the ability of TGF-β1 to inhibit cellular proliferation and stimulation of plasminogen activator inhibitor 1 (PAI-1) synthesis was substantially decreased (225).
Figure 1.7 Endoglin and its association with multiple receptors of the TGF-β superfamily. The type II (RII) and type I (RI) receptors are related Ser/Thr kinases, where RII is constitutively phosphorylated and transphosphorylates RI in the GS domain, upon ligand binding. For TGF-β1, TGF-β3, activin A and BMP-7, the RII receptors bind ligand whereas for BMP-2, the RI receptors ALK-3 and ALK-6 bind ligand. Endoglin, which is constitutively phosphorylated, interacts with these ligand binding receptors and is thus a component of the heteromeric receptor complex. Diagram adapted from (8).
Endoglin is present in endothelial cells and in several subsets of immune cells and has been demonstrated to regulate TGF-β1 responses. We propose that it plays a major role in mediating effects of this growth factor in vascular and immune systems. A recent study suggested a role for endoglin in the modulation of TGF-β1 effects on neovascularization processes (226). Using anti-sense oligodeoxynucleotides (AS ODN) to endoglin, these authors observe a 50% inhibition of cDNA and protein expression, but no inhibition of TGF-β1 binding to HUVEC and no interference with DNA synthesis. However, reduced endoglin expression partially antagonized TGF-β1 effects on proliferation and migration of HUVEC and on vessel formation in vitro (226).

The expression of endoglin on mesenchymal fibroblast-like or stromal cells suggests a functional role in these cells. When endoglin was overexpressed in mouse NCTC929 fibroblasts, decreased migration in chemotactic and wound healing assays was noticed. Dramatic changes in cellular morphology were observed in these endoglin transfectants due primarily to reduced production of extracellular matrix components (227). During heart development, endoglin is expressed at high levels on the endocardium. It is also transiently up-regulated on cushion tissue mesenchyme in human embryos at 5-8 wks of gestation, during heart septation and valve formation (7).

Endoglin is also transiently up-regulated on placental cytotrophoblasts invading the spiral arteries of human uterus (206). To explore the role of endoglin in trophoblast differentiation, AS ODN or antibodies to endoglin were used to treat explants from first trimester chorionic villi. Blocking endoglin expression on cytotrophoblasts stimulated their differentiation along the invasive pathway and the production of fibronectin associated with trophoblast growth and migration. These effects were reversible by addition of exogenous TGF-β2 but not TGF-β1 and TGF-β3 suggesting that endoglin is necessary for the mediation of their inhibitory effects on extravillous trophoblast differentiation (228).
1.5 Hereditary Hemorrhagic Telangiectasia

1.5.1 Description of clinical signs

HHT, also known as Rendu-Osler-Weber syndrome, is a vascular disorder inherited in an autosomal dominant fashion and with an estimated prevalence of 1:8000. HHT is primarily associated with recurrent nose bleeds (epistaxis), telangiectases, visceral lesions and with a family history of bleeding (229). In many patients, the first sign of HHT is epistaxis, caused by telangiectases in the nasal mucosa (12, 230). A telangiectasia is a dilated blood vessel, believed to originate by focal dilation of a post-capillary venule that eventually fuses directly with a dilated arteriole, bypassing the capillary network (231). Telangiectases can also be found on the intestinal mucosa. Particularly in elderly patients, gut telangiectases can cause intestinal bleeds and lead to severe anemia and transfusion (232). A recent study have shown that ~25% of HHT patient ≥60 years old develop such complications (233). Dilation of larger vessels can produce AVMs known to occur in the majority of patients in the lung, and brain (15-20%) but also in the liver (12, 234). The shunting of blood through these lesions can lead to serious complications such as hypoxemia, stroke, brain abscess, heart failure and fatal hemorrhage (230).

Only 10 to 15% of cerebral AVMs are HHT related and observed predominantly in newborns and children (235, 236). These lesions present as multiple cortical or micro AVM with a single feeding artery and single draining vein (237). They cause high cardiac output due to increased blood pressure often leading to heart failure and death (235, 236, 238-240). Such lesions are thought to originate from a disordered mesodermal differentiation between 3 and 8 wks of gestation (241).

70% of pulmonary AVMs are associated with HHT and they present as either diffuse telangiectases or large complex structures consisting of a bulbous aneurysmal sac between dilated feeding arteries and draining veins (230, 234). Pulmonary AVMs are frequently seen in young adults and their prevalence is increased during pregnancy (242, 243). This has been ascribed to increases in the concentration of estrogens and progesterone in the circulation, and to increase in vascular volume (up to 40% at term) (244).
It has been reported that liver involvement occurs in 8-16% of HHT patients, based on the detection of large hepatic AVMs by liver imaging studies. These vascular abnormalities are often associated with fibrosis, telangiectases, and cirrhosis (245). All these clinical manifestations demonstrate the heterogeneity in the phenotype.

1.5.1 Genetics of HHT

HHT is a heterogenous disorder in terms of its clinical manifestations. This has been explained in part at the molecular level by linkage analysis and mapping of two different loci and possibly a third or fourth locus.

Endoglin had been mapped to chromosome 9q33-34 (219) prior to mapping of HHT to the same chromosomal region (11, 246). It was then demonstrated that endoglin was the gene mutated in HHT1 (11). Forty-one distinct mutations in Endoglin have now been reported in HHT1 patients (11, 209, 247-254). Deletions and insertions of nucleotides, and nonsense and missense mutations can be found that are distributed throughout the End gene (Figure 1.8). Our laboratory has demonstrated by metabolic labeling that mutated forms of endoglin, including missense mutations, are transient intracellular species that do not reach the cell surface (250, 255). This suggests that in HHT1 patients a reduction in the level of functional endoglin (haploinsufficiency) rather than a dominant negative effect of the mutant protein is responsible for HHT1. Although every family appears to have a distinct mutation, it is unlikely that the type of mutation or its position will affect the clinical outcome, as haploinsufficiency is the underlying mechanism of HHT1. It has been demonstrated in eight families with HHT1, including some with null alleles, that disease severity was not correlated with the type of mutation (255). Current data suggest that HHT1 families have a much higher incidence of pulmonary AVM and cerebral AVM than other HHT families (256).

HHT type 2 (HHT2) was mapped to a second locus on chromosome 12q (257, 258). The target gene was identified as ALK-1, an activin-like kinase receptor type I of the TGF-β receptor superfamily (259). ALK-1 is expressed at high levels in endothelial cells and placenta. When overexpressed in COS-1 cells, ALK-1 can associate with either TβR-II or ActR-II upon binding
Figure 1.8 Genomic map of the human endoglin gene illustrating the mutations reported for HHT1 families.

The genomic map of endoglin is shown with exons and introns relatively to scale. Exon 1 starts with the ATG methionine codon. Interrupted lines refer to introns incompletely sequenced. The 41 mutations published to date are illustrated. The types of mutation: Deletion (D, dark blue), Insertion (I, red), Splice site (S, light blue), Nonsense (N, orange) and Missense (M, green) are shown below for exonic mutations or above for intronic mutations. Adapted from (230).
of their respective ligands (260). Recently ALK-1 was shown to bind TGF-β1 in endogenous endothelial cells in association with TβR-II (136). Twenty-one distinct mutations were shown in HHT2 patients with each family carrying a distinct mutation (261-264). Haploinsufficiency in ALK-1 is also the suggested predominant mechanism in HHT2 patients (264). A later onset of disease and a lower frequency of pulmonary AVMs and cerebral AVMs are observed in ALK-1 families (256).

At least two families with HHT, one with liver complications and one with pulmonary involvement have been shown not to be linked to 9q 33-34 or 12q (265, 266). This suggests that a third or even fourth locus may exist that would account for HHT in these families. Since both endoglin and ALK-1 can be found in the same TGF-β1 receptor complex (Figures 1.5 and 1.7), we propose that the downstream signaling pathway is critical to vascular development and homeostasis. Such a pathway must be defective in HHT. We can speculate that the third and possibly fourth gene responsible for HHT are involved in this pathway or its regulation.
1.6 Thesis objectives

1.6.1 Rationale

Endoglin is expressed predominantly on endothelial cells of all types of blood vessels and as early as 4 wks into human gestation. It is also found on some subsets of hematopoietic cells including human erythroid precursors, fetal pre-B cells bone marrow stromal cells and macrophages. This distribution suggests that endoglin could have effects on cells of both lineages very early in development. The fact that endoglin was first identified on pre-B leukemic cells, and its association with the dominant vascular disorder, Hereditary Hemorrhagic Telangiectasia type 1, further support the notion that endoglin plays a critical role in these systems. The haploinsufficiency model is now very well documented for HHT and implies that having a single functional allele of endoglin leads to this disorder. Endoglin is present in the TGF-β receptor complex and can modulate responses to TGF-β. This pleiotropic growth factor is known to have very potent effects on both endothelial and hematopoietic cells by regulating their proliferation, differentiation and the maintenance of homeostasis. These important functions have been confirmed with the TGF-β1 null mice, which show serious defects in their vascular and immune systems.

1.6.2 Hypothesis

Our hypothesis is that critical levels of endoglin are essential for normal blood vessel development and for maintenance of homeostasis in both vascular and hematopoietic systems.

1.6.3 Specific objectives

The main objectives of this thesis are to define the contribution of endoglin to normal and pathological events affecting blood vessel formation as well as its impact on hematopoietic lineages and the immune response. We started our investigations with the study of normal and pathological samples from HHT patients and pursued with the generation and characterization of mice lacking one or both alleles of the End gene. The results obtained from each of the following specific objectives are reported in chapters 2 to 5.
Chapter 2. Endoglin Expression is reduced in Normal Vessels but still Detectable in Arteriovenous Malformations of patients with Hereditary Hemorrhagic Telangiectasia

The haploinsufficiency model proposed for HHT was first based on *in vitro* experiments using HUVEC or activated monocytes. To investigate whether all blood vessels of HHT patients express reduced levels of endoglin *in situ*, we established a method to quantify endoglin on sections of tissues derived from HHT patients. To determine if the development of vascular lesions is triggered by further reduction or focal loss of endoglin, we also examined sections of arteriovenous malformations. Our studies demonstrate that all vessels present in normal vascular beds express reduced levels of endoglin and that AVMs which represent profound changes in the vessel wall structure are not caused by focal loss of endoglin in these lesions.

Since a reduction in endoglin can lead to the vascular abnormalities associated with HHT, and since AVMs are presumed associated with defects in early vascular development, we inferred that endoglin must play a crucial role in vascular development. To address this question, we targeted the murine *End* gene by homologous recombination. We hypothesized that the *End* null mice would die at mid-gestation of abnormal vascular development, similarly to the *TGF-β1* and *TβR-II* null mice. We were also hoping that the heterozygous mice would develop HHT, like their human counterparts.

Chapter 3. Characterization of Mice Lacking Endoglin and Development of a Murine Model of Hereditary Hemorrhagic Telangiectasia

To study the role of endoglin in development, we derived *End* null mice. As no live HHT1 homozygous human has been documented and because of multiple lethal phenotypes associated with key molecules in vascular development, we postulated that the null phenotype would be embryonic lethal. The expression of endoglin on the endocardium throughout human heart development and its transient up-regulation on cushion tissue mesenchyme during valve formation and heart septation prompted us to investigate not only vessel abnormalities but also possible heart defects in these mice. We demonstrated that *End* null mice die at mid-gestation of vascular and cardiovascular defects similar to those of *TGF-β1* and *TβR-II* null mice. Because of
the embryonic lethality, we did not assess endoglin function in early hematopoiesis and in later stages of gestation or post-natally.

The haploinsufficiency associated with HHT1, as confirmed in Chapter 2, implies that mutation at a single allele leads to a loss of function. As HHT is an autosomal dominant disease with varied penetrance, we postulate that End heterozygous (End") mice might show a variable vascular phenotype, particularly on different genetic backgrounds. In this chapter we report the first few cases of HHT mice, identified by telangiectases and external bleeds and suggest that the genetic background influences disease onset.

Chapter 4. Heterogeneity of Vascular Defects in Endoglin Heterozygous Mice with Hereditary Hemorrhagic Telangiectasia suggests Involvement of Modifier Genes

Mice with one functional allele of the End gene were found to spontaneously develop clinical signs of HHT. To characterize onset, progression and mechanisms of disease, we followed over a period of one year, End" mice generated on two background strains, C57BL/6 and 129/Ola. An earlier onset and higher prevalence of HHT was associated with the 129/Ola strain. Human HHT is associated with telangiectases, epistaxis and some patients can develop gastrointestinal, hepatic, pulmonary and/or cerebral complications. Therefore, we characterized the extent of external and visceral HHT manifestations in these mice. We report multiple vascular abnormalities and their sequelae, which are similar to those found in human. Our data suggest that endoglin haploinsufficiency and effects of modifier genes are responsible for heterogeneity and severity of HHT. Our mouse model is unique and provides an excellent tool for studying HHT pathogenesis and novel therapeutic approaches.

Chapter 5. Endoglin Heterozygous Mice show B and T cell defects

Both stromal and endothelial components of the microenvironment are required to support survival and differentiation of hematopoietic precursors. Endoglin is expressed not only on vascular endothelium but also on several hematopoietic subsets in humans and modulates TGF-β responses in monocytes. Since we demonstrated that a single copy of endoglin can lead to human and mouse HHT, we hypothesize that it may also dysregulate development and function of
hematopoietic cells and alter immune responses. We first determined the expression pattern of endoglin in lymphoid tissues. Using healthy End<sup>−/−</sup> mice of C57BL/6 and 129/Ola backgrounds, we analyzed cellularity and lymphocyte subset distribution in thymus and spleen of mice ranging in age from 6 to 24 wks. We also measured proliferative responses, cell cycle characteristics and ability to undergo apoptosis. Histological examination of sections from thymus and spleen was performed to visualize the architecture and cellular components of these organs. We also studied the distribution of myeloid and lymphoid cells in bone marrow. We demonstrated that End<sup>−/−</sup> mice show defects in both thymus and peripheral lymphoid organs. Our data suggest a role for endoglin in the regulation of T and B lymphocyte homeostasis.

**Chapter 6. Discussion**

The characterization of endoglin expression in the vasculature of HHT patients, along with the analysis of End deficient mice presented in this thesis, were aimed at defining endoglin function in vascular and hematopoietic systems. In early development we established that endoglin is essential for angiogenesis. We propose an integrated model of vessel development whereby endoglin regulates TGF-β1 effects on vessel wall assembly and stabilization of endothelial tubes and possibly alters responses of other angiogenic factors. The establishment of a murine model of HHT has revealed great similarities with the human disorder. A comparison of external signs and visceral involvement associated with human and mouse HHT will be discussed. We propose a new model for the generation of vascular lesions in HHT, where the co-inheritance of a mutated endoglin and modifier genes, contribute to the heterogeneity and severity of clinical manifestations. Vascular changes in vessels seen in arteriovenous malformations may also be due to alterations in the signaling pathways in which endoglin and ALK-1 are implicated. In lymphoid organs, vascular abnormalities could influence the development and maturation of the immune system. We will discuss the possibility that the immune phenotype, observed in End heterozygous mice, is cell-autonomous or dictated by the microvascular environment.
CHAPTER TWO

Endoglin Expression is reduced on Normal Vessels but still Detectable in Arteriovenous Malformations of Patients with Hereditary Hemorrhagic Telangiectasia Type I

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In this chapter, I developed a new quantitative immunohistochemistry method and elaborated a computerized densitometry analysis that allowed the quantitation of endoglin in situ on normal vessels and AVMs from HHT patients. To ascertain the HHT1 status of the patients, Ms Paquet characterized endoglin at the protein level, while Ms Cymerman found the mutation in the endoglin gene. Pedigrees and clinical information were provided by Dr. Meschino, Ms McKinnon and Dr. Guttmacher. I also characterized the vessel wall components within AVM. This analysis was done in collaboration with Dr. Becker, pathologist, who commented and confirmed our observations. This project was supervised by Dr. Letarte.

By copyright permission of the American Society for Investigative Pathology, the contents of this chapter was first published essentially in this form in \textit{The American Journal of Pathology} : Bourdeau A., Cymerman U., Paquet M-E., Meschino W., McKinnon W.C., Guttmacher A.E., Becker L., Letarte M. 2000. Endoglin expression is reduced in normal vessels but still detectable in arteriovenous malformations of patients with Hereditary Hemorrhagic Telangiectasia type1. \textit{Am. J. Pathol.} 156:3, 911-923.
2.1 Abstract

Endoglin is predominantly expressed on endothelium and is mutated in Hereditary Hemorrhagic Telangiectasia type 1. We report the analysis of endoglin in tissues of a newborn (family 2), who died of a cerebral arteriovenous malformation and in a lung specimen surgically resected from a 78-year-old patient (family 5) with a pulmonary AVM. The clinically affected father of the newborn revealed a novel mutation that was absent in his parents, and identified as a duplication of exons 3 to 8 by quantitative multiplex polymerase chain reaction. The corresponding mutant protein (116kDa monomer), and the missense mutant protein (80kDa monomer) present in family 5 were detected only as transient intracellular species and were unreactive by Western blot and immunostaining. Normal endoglin (90kDa monomer) was reduced by 50% on peripheral blood-activated monocytes of these HHT1 patients. When analyzed by immunostaining and densitometry, presumed normal blood vessels of the newborn lung and brain, and vessels adjacent to the adult pulmonary AVM, showed a 50% reduction in the endoglin/PECAM-1 ratio. A similar ratio was observed in the cerebral and pulmonary AVM, suggesting that all blood vessels of HHT1 patients express reduced endoglin in situ, and that AVMs are not attributed to a focal loss of endoglin.
2.2 Introduction

Hereditary Hemorrhagic Telangiectasia, also known as Rendu-Osler-Weber syndrome, is an autosomal dominant vascular dysplasia that affects 1:10,000 individuals. This disorder is associated with epistaxis and telangiectases in the majority of patients and pulmonary and cerebral arteriovenous malformations in 15-20% of cases (12, 234). These abnormalities are caused primarily by the dilatation of post capillary venules which eventually connect directly to arterioles without intervening capillaries (231). Direct shunting of blood through cerebral AVMs may lead to ischemic and/or hemorrhagic infarctions (267).

Only a small proportion of cerebral AVMs observed in the general population is associated with HHT. Cerebral AVMs have been observed predominantly in newborns and children and may present with either high-output congestive heart failure, migraine-like headaches or rupture, which may lead to death (235, 236, 238-240). Such lesions are thought to originate from a disordered mesodermal differentiation between 3 and 8 wks of gestation (241). The expression of factors such as VEGF and the vascular tyrosine kinase receptor Tie-1 in cerebral AVMs suggest active angiogenesis in these malformations (268, 269).

Of pulmonary AVMs, at least 70% of cases are associated with HHT (230, 234, 270). These range from diffuse telangiectases to large complex structures consisting of a bulbous aneurysmal sac between dilated feeding arteries and draining veins (230). Current data suggest that HHT1 families have a much higher incidence of pulmonary AVM than HHT2 families (11, 256, 271, 272). Cerebral AVMs often cluster in families with a higher prevalence of pulmonary AVM and are thus likely also more frequent in HHT1 families.

HHT is a heterogeneous disorder in terms of its clinical manifestations. This is explained in part at the molecular level by the involvement of at least two different loci (11, 246). The candidate gene for HHT1 was mapped to chromosome 9q33-34 (246, 273) and was identified as End (11), which codes for a homodimeric integral membrane glycoprotein expressed at high levels on vascular endothelial cells (217) and previously mapped to 9q33-34 (219). Endoglin was first shown to be a component of the TGF-β1 and TGF-β3 receptor complexes (195). More recently, it was demonstrated to interact with the ligand-binding receptor for several members of
the TGF-β superfamily, including activin and BMPs (8). Thirty-nine distinct mutations in the End gene have now been reported in HHT1 patients (11, 230, 247-252), and most families carry a distinct mutation.

The ALK-1 gene, coding for an activin-like kinase receptor type I of the TGF-β receptor superfamily, maps to chromosome 12q and is mutated in HHT2 (258, 261, 274). The clinical HHT2 phenotype is characterized by a later onset of disease and lesser penetrance; eighteen distinct mutations in the ALK-1 gene have been described (262, 263).

With HUVEC and activated monocytes in culture obtained from HHT1 patients, we have demonstrated that mutated forms of endoglin are transient intracellular species that do not reach the cell surface (209, 252). This suggests that a reduction in the level of functional endoglin (haploinsufficiency) rather than a dominant negative effect of the mutant protein is responsible for HHT1. In the current study, we were able to examine tissues of a newborn, who died subsequent to the rupture of a cerebral AVM, and a lung specimen resected from an elderly patient with a pulmonary AVM. This permitted us to establish that reduced endoglin levels can also be observed in situ in seemingly normal vessels. We also studied vessels of the vascular lesions, cerebral and pulmonary AVM respectively, and determined that they still express endoglin.
2.3 Materials and Methods

2.3.1 Clinical evaluation and patient samples

Informed consent was obtained from all participants for blood samples and surgical specimens. All procedures were reviewed and approved by the Research Ethics Board of the Research Institute at the Hospital for Sick Children. All members of families with HHT are given a number, which is referred to with the prefix H (for HHT).

In family #2 (Figure 2.1a), patient H9 died at seven days of a cerebral hemorrhage due to the rupture of one of the two cerebral AVMs present, followed by heart failure; autopsy was performed 8h after death. Paraffin embedded sections (5-7 μm) of cerebral AVM and unaffected vascular beds were obtained from the Pathology Department, Hospital for Sick Children, Toronto. Controls samples were prepared similarly from newborns who died of unrelated causes. Blood samples were received from patients H300, H262, H283, H299, H3, H4 and from placenta and umbilical cord from newborn H11.

Family #5 has four generations of clinically affected individuals as illustrated in Figure 2.1b. A lung AVM was surgically resected from patient H12, a 78 year old, non-smoking woman. This specimen was dissected into the lesion itself and adjacent areas. Fresh tissues were used for preparation of DNA, protein extracts and formalin-fixed, paraffin-embedded tissue blocs. Sections (5-7 μm) of pulmonary AVM and unaffected lung adjacent area were obtained by Dr. J. Tessitore, Pathology Department, University of Vermont College of Medicine, Burlington. Blood samples were obtained from patients H150, H170. Normal lung samples from 68-years-old, non-smoking woman were obtained by Dr. B. Mullen, Pathology department, Mount Sinai Hospital, Toronto.

2.3.2 Cell culture, metabolic labeling and immunoprecipitation

Activated monocytes were prepared from peripheral blood of patients and control, by adherence to plastic and culture for 16h at 37°C, as previously described (198, 250). Cells were washed with serum-free media and incubated for 30 min in methionine-free Dulbecco’s Modified Eagle Medium (DMEM) prior to labeling with 100μCi/ml 35S-methionine (trans-label, ICN
Figure 2.1 Pedigrees of two families with HHT1.

(a) Pedigree of HHT family 2. Patient H283 and H262 were both unaffected and are the biological parents of patient H3 who presented with telangiectases, nosebleeds and pulmonary AVM. His wife, patient H4 was unaffected and had two miscarriages at 7 wks of gestation. Their first child, patient H9 died of a cerebral hemorrhage followed by heart failure caused by rupture of one of the two cerebral AVM present. Their second child, patient H11, was born normally and was confirmed unaffected. (b) Pedigree of family 5 with a history of HHT1 for four generations. Patient H12 has nosebleeds, telangiectases and a pulmonary AVM. Patient H150 presented with a pulmonary AVM and telangiectases whereas patient H170 had nosebleeds and telangiectases. Black symbols = HHT1- affected individuals; Gray symbols = HHT1 suspected individuals; White symbols = unaffected individuals.
Pharmaceuticals, Montreal, Quebec, Canada) for 3.5h. Cells were then solubilized in 0.01M Tris, pH 7.5, 0.128M NaCl, 1mM EDTA, 1% Triton X-100 plus protease inhibitors (lysis solution) and immunoprecipitated with saturating amounts of mAb P3D1 or P4A4. mAb P3D1 (and mAb Sn6h used in Western blot and immunostaining) recognize an epitope in the first 204 aa residue of the extracellular domain of endoglin (corresponding to exons 1-5). mAb P4A4 reacts with an epitope encoded by exon 7 and located between aa 277 and 331 (6). To quantify endoglin expression and correct for differences in yield between samples, aliquots of total lysates were precipitated with 10% TCA, collected on glass fiber filters and total incorporation into proteins was determined. Equivalent amounts of labeled proteins (in cpm) were fractionated by SDS-PAGE (4-12%, Novex Experimental Technology, San Diego, CA, USA) under reducing (0.05M dithiothreitol, DTT) and non-reducing conditions. Gels were exposed on X-OMAT AR film (Eastman Kodak Company, Rochester, NY, USA) and radioactivity in each band was quantified by Phosphorimager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA).

2.3.3 Western Blot

Peripheral blood activated-monocytes were solubilized in lysis solution. Protein concentration was estimated with a Bio-Rad assay (BioRad Laboratories Ltd, Mississauga, ON, Canada) and known amounts were fractionated on SDS-PAGE (8%) under non-reducing conditions. Proteins were transferred electrophoretically onto nitrocellulose membranes which were then blocked for 1h in Tris-buffered saline-tween TBS-T (0.02M Tris pH 7.5, 0.137M NaCl, 0.1% Tween 20) containing 5% skim milk. Membranes were incubated for 1h with mAb Sn6h (ascites, diluted 30000 fold), followed by 1h with horseradish peroxidase-conjugated rabbit anti-mouse IgG (H&L, 10,000 fold dilution; Jackson Immunolabs, Bio-Can, Mississauga, ON, Canada). Endoglin was detected by enhanced chemiluminescence (ECL detection kit, Amersham Life Sciences, Oakville, ON, Canada).
2.3.4 Mutation Analysis by QMPCR and sequencing

Genomic DNA was extracted from blood lymphocytes, placenta and lung specimens using DNAZOL (Life Technologies Inc.). Purity and quality of the DNA and accurate estimation of the concentration are critical for fragment analysis by Quantitative Multiplex polymerase chain reaction (QMPCR). All 15 exons of endoglin were amplified in five PCR reactions using one selected Cy 5.5 fluorescent conjugated primer for each exon as previously described (252). Quantitative amplification was achieved with 18 to 22 cycles, annealing T° ranging from 51 to 55°C, optimized primer concentrations ranging from 80 to 800 nM and 150 ng of genomic DNA. The amplification of a c4 fragment (329 or 282bp) derived from a gene on chromosome 15 was included as internal standard in each of the reactions. The Multiplex PCR pools and fragment sizes (in bp) were as follows: reaction 1, exon 9b (149), exon 4 (283), C4 standard (329), exon 2 (363), exon 6 (389) and exon 11 (426); reaction 2, exon 12 (154), C4 standard (282), exon 10 (304), exon 1 (314), and exon 8 (373); reaction 3, exon 9a (222), exon 5 (238), exon 13 (255) and C4 standard (282); reaction 4, exon 14 (269), exon 7 (289) and c4 standard (329); reaction 5, exon 3 (251) and c4 standard (329).

Thermocycling was performed using DNAEngine (MJ Research) and QMPCR products were run on a MicroGene Blaster Sequencer (Visible Genetics Inc, VGI; Toronto, ON, Canada) for 30 to 40 min. The data was analyzed using GeneObjects DNA analysis software (VGI). The ratio of the peak area for each endoglin exon was calculated relative to that of the c4 internal standard for each patient sample and compared to the normal two-copy control DNA samples. The exons to be sequenced were first amplified with nonlabeled primers identical to those used in QMPCR and then were sequenced as described above (252).

2.3.5 Immunohistochemical staining

Paraffin-embedded sections of lung, spinal cord and cerebral AVM from patient H9 and age-matched controls and from lung specimens from patient H12 and age-matched control were dewaxed by standard procedures, blocked with 5% normal goat serum (Dako, Mississauga, ON, Canada) in TBS-T (0.01M Tris pH 7.4, 0.16M NaCl, 0.2% Tween 20) for 20 min and incubated
at 4°C for 2h with optimal concentrations of primary antibody. These were mAb JC70A to PECAM-1(CD31; hybridoma supernatant diluted 8 fold; Dr. D. Mason, Oxford, U.K.), mAb 1A4 to α-smooth muscle cell actin (ascites diluted 2000 fold; Sigma, Oakville, ON, Canada), non-immune murine IgG1 (10μg/ml; Coulter, Burlington, ON, Canada) and mAb Sn6h to endoglin (ascites diluted 8000-fold; obtained from Dr B. Seon, through the VI Leukocyte International Workshop). mAb Sn6h is the best of about 40 mAb tested at detecting human endoglin in paraffin-embedded tissue sections (197, 275, 276). Slides were washed and incubated for 1h at 4°C with an alkaline phosphatase goat anti-mouse IgG (H+L) Fab’2 (diluted 400 fold; Jackson Immunolabs, Bio-Can). In some experiments, the LSAB+ Dako amplification system was used according to the manufacturer’s instructions (Dako, Mississauga, ON, Canada). The enzymatic reaction (1h at 23°C) was initiated by adding 0.35mM BCIP, 0.45mM NBT (Boehringer Mannheim, Montreal, QC, Canada) and 0.2mM levamisole (Sigma, St-Louis, MI, USA) to block endogenous alkaline phosphatase activity. To facilitate image analysis, sections were not counterstained. Tissue morphology was assessed by hematoxylin and eosin (H&E) and the elastin histochemical stain was performed by the manufacturer’s instructions (Accustain, Sigma, Oakville, Ontario, Canada).

2.3.6 Image Analysis

Images were acquired in black and white directly from the stained tissue sections using an Olympus BX50 microscope linked to a charged-coupled devise (CCD) video camera, using Image Pro Software Analysis (Carsen Medical Scientific Co Ltd, Markham, ON, Canada) and digitization on a Power Macintosh G3 computer. Staining intensity on endothelial cells of arteries, veins and capillaries was quantified as follows using the software NIH Image 1.61/fat for Power Macintosh (http://rsb.info.nih.gov/nih-image/Default.html) and Image Pro-Plus. For each large vessel or a group of capillaries, images were enlarged and 300 measurements were taken on the endothelial layer with the cross hair tool counting the average gray value of the selected pixel (scale of 0-256 shades of gray; 0 = white and 256 = black). Mean density values and standard error of the mean (SEM) were calculated for PECAM-1, endoglin and IgG1 of both
control and patient tissue sections stained in the same experiment. Note that the background mean intensity values, determined with IgG1 control, were less than 15 in all sections. The ratio of the mean density values of endoglin and PECAM-1 was calculated for each vessel. The endoglin/PECAM-1 ratio of the HHT patient was then compared to that of the control and expressed as relative endoglin levels (%).
2.4 Results

2.4.1 Description of a new endoglin mutant protein in family 2

The pedigree of family 2 is illustrated in Figure 2.1a. The clinical diagnosis of HHT was made on examination of patient H3, who presented with daily nosebleeds since childhood and also had telangiectases. Further investigations revealed two small pulmonary AVMs but no cerebral AVMs. His son H9 died at 7 days of a cerebral hemorrhage due to the rupture of one of two cerebral AVMs present, followed by heart failure, rendering the diagnosis of HHT in this child likely. A healthy daughter H11, was born two years later. No definite diagnosis of HHT was made for other family members, although patient H262, mother of H3, and her sister (H300), had frequent nose bleeds in childhood and adolescence, which subsequently subsided.

We first examined the level of endoglin and the presence of potential mutant proteins in peripheral blood activated monocytes from members of this family. Figure 2.2a demonstrates that fully glycosylated endoglin monomer (E; 90kDa) and a partially glycosylated precursor (P; 80 kDa) were immunoprecipitated from control lysates (lanes 1 & 3) with both mAb P3D1 and P4A4 which recognize distinct regions of endoglin. In patient H3 samples, E and P were expressed at reduced levels and a mutant protein M (116 kDa) was detected (lanes 2 & 4). In absence of DTT, the normal dimers of endoglin (E; 160 kDa) and precursor (P; 140 kDa) were observed in control lanes 9 & 11. With patient H3, these normal homodimers were present at lower levels (lanes 10 & 12); additional dimers were seen at 200 and 240 kDa as well as traces of monomers (lanes 10 & 12). The level of surface endoglin (E) was estimated by Phosphorimager and the ImageQuant software, as 50 ± 4% in H3 activated monocytes relative to control (mean of 4 values; Table 2.1). The mutant form (M), in Figure 2.2 represented 45% of the total endoglin (E + P + M) seen in patient H3.

Protein analysis was performed on several other family members, including parents of patient H3, the presumed affected mother (H262) and control father (H283). To our surprise, normal levels of endoglin were found in H262, and no mutant protein was present (Figure 2.2a).
Figure 2.2 Analysis of peripheral blood activated monocytes reveals reduced levels of normal endoglin and presence of a mutant protein in patient H3 but not in his parents.

(a) Cells from the clinically affected patient H3 (family 2) and a normal control (C) were labeled with $^{35}$S-methionine, solubilized in Triton X-100 and immunoprecipitated with mAb P3D1 or P4A4 to endoglin. Equivalent amounts of labeled proteins were fractionated on 4-12% gradient SDS-PAGE, under reducing (lanes 1-4) and non-reducing conditions (lanes 9-12). Fully processed endoglin (E) and partially glycosylated precursor (P) were observed in patient H3 at reduced levels relative to control and a mutant protein (M) is noted. Cells from individuals H262 and H283, mother and father of patient H3, were also analyzed in the same manner (lanes 5-8 and 13-16). Normal E and P forms of endoglin were seen and at normal levels; no mutant protein was observed. (b) Activated monocytes from patient H3 were lysed in Triton X-100 and varying amounts of proteins were fractionated on 8% SDS-PAGE, under non-reducing conditions (−DTT). Gels were transferred and analyzed by Western Blot using mAb Sn6h to endoglin. The control (C) was an extract from HUVEC. Only the normal dimers of endoglin (E) are detected by Western Blot analysis. Molecular mass markers in kilodaltons (kDa) are indicated.
Table 2.1 Clinical and Molecular Data on HHT families 2 and 5.

<table>
<thead>
<tr>
<th>Family #</th>
<th>Patient #</th>
<th>Clinical Manifestations</th>
<th>Endoglin levels on monocytes Mean ± SD</th>
<th>Mutation identified at DNA level</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>3</td>
<td>Nosebleeds, Telangiectases, pulmonary AVM</td>
<td>50 ± 4</td>
<td>Yes (Duplication of exons 3-8)</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>–</td>
<td>nd&lt;sup&gt;1&lt;/sup&gt;</td>
<td>No</td>
</tr>
<tr>
<td>2</td>
<td>9</td>
<td>cerebral AVMs</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>2</td>
<td>11</td>
<td>–</td>
<td>85 ± 13&lt;sup&gt;2&lt;/sup&gt;</td>
<td>No</td>
</tr>
<tr>
<td>2</td>
<td>262</td>
<td>Nosebleeds before puberty&lt;sup&gt;3&lt;/sup&gt;</td>
<td>121 ± 17</td>
<td>No</td>
</tr>
<tr>
<td>2</td>
<td>283</td>
<td>–</td>
<td>83 ± 9</td>
<td>No</td>
</tr>
<tr>
<td>2</td>
<td>299</td>
<td>Nosebleeds before puberty&lt;sup&gt;3&lt;/sup&gt;</td>
<td>97 ± 11</td>
<td>No</td>
</tr>
<tr>
<td>2</td>
<td>300</td>
<td>–</td>
<td>126 ± 20</td>
<td>No</td>
</tr>
<tr>
<td>5</td>
<td>12</td>
<td>Telangiectases, Nosebleeds, pulmonary AVM</td>
<td>nd</td>
<td>Yes (Missense G447C, exon 4)</td>
</tr>
<tr>
<td>5</td>
<td>150</td>
<td>Telangiectases, pulmonary AVM</td>
<td>34 ± 8</td>
<td>Yes (Missense G447C, exon 4)</td>
</tr>
<tr>
<td>5</td>
<td>170</td>
<td>Telangiectases, Nosebleeds</td>
<td>nd</td>
<td>Yes (Missense G447C, exon 4)</td>
</tr>
</tbody>
</table>

<sup>1</sup> nd: not done
<sup>2</sup> Levels of endoglin measured on HUVEC from newborn H11 rather than on activated monocytes as for adults.
<sup>3</sup> Although patients 262 and 299 had nosebleeds in childhood, they have no other signs of HHT and were confirmed not to have the mutation.
Additional family members tested had normal levels of endoglin, and no detectable mutant including patient H11, sister of the proband for whom HUVEC analysis was performed (Table 2.1 and Figure 2.1a).

Because only pathological specimens were available from newborn H9, we needed to establish that the mutant protein, if present, would not be detected by immunostaining which reflects steady-state levels of protein expression. The relative stability of mutant endoglin was thus analyzed by Western blot using mAb Sn6h, which gives optimal detection of endoglin on paraffin-embedded sections (6, 197). Only normal endoglin dimers were reactive with Sn6h in H3 samples; no trace of mutant was observed (Figure 2.2b). Furthermore, the mutant protein was also not reactive with mAb P4A4 by Western blot whereas it was detected as newly synthesized mutant protein by metabolic labeling (Figure 2.2a). We also demonstrated by cell surface biotinylation of activated monocytes from patient H3 that no mutant protein was detected at the cell surface (data not shown). Therefore, this novel mutant form of endoglin, despite its larger molecular mass, is only a transient intracellular species not expressed at the cell surface and not detected at steady-state level, either by Western blot or immunostaining.

2.4.2 Identification of a novel endoglin mutation arising in family 2

DNA samples were subjected to QMPCR to screen for an End mutation that could account for the generation a larger-than-normal protein, as well as resolve the question of a new mutation arising in patient H3 and absent from his parents. Figure 2.3 demonstrates that DNA from patient H3, fractionated in five different reactions so that each exon could be analyzed, contained an additional copy of exons 3 to 8. The area under each peak is proportional to the allele copy number when QMPCR is truly quantitative, as optimized in preliminary studies (252). The ratio of the peak area for exons 3 to 8 from H3 DNA ranged from 1.3 to 1.6 relative to the mean values derived from 12 controls that had been run in the same analysis. This implies that three copies of these exons was present relative to the two-copy control, presumably due to an intronic mutation that included the duplication of exons 3 to 8. Each exon of the End gene was amplified individually from the DNA of patient H3 and sequenced, but no mutation was
Figure 2.3 QMPCR fragment analysis reveals a duplication of exons 3 to 8 in patient H3 of family 2.

DNA samples were analyzed by QMPCR using five different reactions (R1 to R5), optimized so that peak area is proportional to copy number for each exon. Illustrated here are DNA from patient H3 and control for each of the five reactions. The exon number is indicated under the peak and "st" refers to internal size standards. The ratio of each exon is expressed relative to c4 fragment, an internal copy number standard. In each experiment, the peak area was estimated for H3 relative to the mean ratio observed for 12 control samples. The ratios of H3 relative to these controls were 1.0 for exons 1 and 2, ranged from 1.3 to 1.6 for exons 3 to 8, and from 0.84 to 1.0 for exons 9a to 14. Accordingly, peak surface area is greater for exons 3 to 8 in patient H3.
found at splice junctions or in the exons, further supporting an intronic mutation. This mutation was not seen in the parents (H283, H262) of patient H3, confirming that they were not affected and that the End mutation had arisen in patient H3. DNA was also analyzed from H4, H11, H299 and H300 individuals (see Figure 2.1a) and no mutation was found, confirming that they were not affected.

DNA fingerprinting using nine different markers confirmed that individual H3 and his parents H262 and H283 shared the expected alleles, and established that this mutation arose in patient H3. DNA isolated from shavings of the paraffin-embedded cerebral AVM and adjacent brain tissue of newborn H9 was of sufficient quality to confirm five markers shared with his parents H3 and H4, but not pure enough to demonstrate the mutation for QMPCR analysis.

2.4.2 Analysis of endoglin DNA and protein in family 5

Family 5 shows four generations of individuals affected with HHT (Figure 2.1b). Patient H12, age 78, underwent resection of the right lung middle lobe because she was unfit to undergo transcatheter embolization of her pulmonary AVM. Patient H150, also with a pulmonary AVM, was shown to express reduced levels of endoglin in peripheral-blood activated monocytes (34 ± 8%; Table 2.1).

The mutation in this family was reported previously, as a missense mutation in exon 4, a G to C substitution at bp 447 of the endoglin cDNA (251). In the current study, DNA was isolated from the pulmonary AVM lesion itself of patient H12 and, when analyzed it revealed the expected mutation (Figure 2.4). Sequencing also revealed that the normal End allele was still present in the lesion, ruling out a loss of heterozygosity (Figure 2.4).

This mutation leads to a tryptophan-to-cysteine conversion at aa 149. The mutant protein was detectable only by metabolic labeling and as a transient intracellular precursor of 80 kDa, as described previously (198). This transient protein was not seen by Western blot analysis of protein extracts from pulmonary AVM and adjacent area in samples from lung from patient H12. However, normal endoglin was readily detectable in both the highly vascularized pulmonary AVM and the adjacent tissues (data not shown).
Figure 2.4 Normal and mutant alleles of End DNA are present in the pulmonary AVM of patient H12.

DNA extracted from the pulmonary AVM of patient H12 (family 5) was sequenced for exon 4. At position 447, G of the normal allele and C of the mutant allele are both present. This missense mutation was absent in the control DNA sample.
2.4.3 Immunostaining of the vasculature of newborn with cerebral AVM (family 2)

A. Normal Vasculature

Lung and spinal cord resected from newborn H9 at the time of autopsy, and from age-matched controls were analyzed by immunostaining. We first demonstrated that endoglin and PECAM-1, both classified as endothelial cell surface antigens, were specifically detected in formalin-fixed tissues using mAb Sn6h (197, 276) and mAb JC70A respectively (277) (Figure 2.5). Both antibodies stained endothelial cells of arteries, veins and capillaries in control and patient H9; no other cell type was reactive. Specificity of staining was confirmed by absence of reaction with control murine non-immune IgG1, and differential staining of arteries, veins and capillaries was achieved. Staining with mAb 1A4 to α-SMC actin was also performed to differentiate veins and arteries (data not shown).

We next compared the expression of endoglin and PECAM-1 in lung sections of patient H9 and control (Figure 2.5a). In the control newborn, endoglin and PECAM-1 were observed at equivalent levels on the endothelium of artery (areas A in Figure 2.5a), vein (areas B in Figure 2.5a) and capillaries (areas C in Figure 2.5a). With patient H9, the staining intensity of endoglin, compared with PECAM-1 was much reduced on the artery (areas a, Figure 2.5a), vein (areas b, Figure 2.5a) and capillaries (areas c, Figure 2.5a), compared with control sections. We quantified by image digitization and densitometry analysis, the staining intensity of vessels illustrated in Figure 2.5a and on additional surrounding lung vessels (Table 2.2). PECAM-1 mean density was relatively similar between different vessel types and between patient and control samples, stained in the same experiment. Values range from 107 to 171 in control and 95 to 182 in patient H9. Endoglin levels were also similar between vessel types but clearly lower in patient H9 (30 to 81) than control (106 to 145). To standardize data analysis, the endoglin/PECAM-1 ratio was calculated for each vessel, to allow comparison between patient and control (Table 2.2). The relative endoglin levels (Endoglin/PECAM-1 ratio of patient to control) were then calculated and shown to be reduced in all lung vessels, including 46 to 61% in arteries, between 31 and 52% in veins between 47 and 52% in capillaries (Table 2.2).
Figure 2.5 Vessels of lung and central nervous system from the HHT1 newborn express less endoglin than control, compared with PECAM-1.

Formalin-fixed, paraffin-embedded lung (a) and spinal cord (b) sections from control and patient H9 were stained with mAbs Sn6h to endoglin and JC70A to PECAM-1, followed by an alkaline phosphatase conjugated antibody. (a) Endoglin and PECAM-1 staining on an artery (A, control; a, patient H9), a vein (B, b) and capillaries (C, c) of lung tissues are illustrated for control and patient H9. (b) Sections of dural vessels of spinal cord are shown. Two arteries (D, d; E, e), two veins (F, f; G, g) are represented for age-matched control and patient H9. PECAM-1 was also detected on platelets in vessels with blood infiltrates. Original magnification X 194.
Table 2.2 Densitometry analysis of endoglin and PECAM-1 on endothelial cells of patient H9.

<table>
<thead>
<tr>
<th>Vessel Type</th>
<th>Control</th>
<th>Patient H9</th>
<th>Relative endoglin levels (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Endoglin (E)</td>
<td>PECAM-1 (P)</td>
<td>E/P ratio</td>
</tr>
<tr>
<td></td>
<td>mean density</td>
<td>mean density</td>
<td></td>
</tr>
<tr>
<td>Lung artery A</td>
<td>112 ± 1.7</td>
<td>153 ± 1.6</td>
<td>0.73</td>
</tr>
<tr>
<td>Lung vein B</td>
<td>145 ± 1.4</td>
<td>142 ± 1.4</td>
<td>1.02</td>
</tr>
<tr>
<td>Lung capillaries C</td>
<td>128 ± 1.5</td>
<td>144 ± 2.1</td>
<td>0.89</td>
</tr>
<tr>
<td>Lung artery</td>
<td>125 ± 2.1</td>
<td>171 ± 2.0</td>
<td>0.73</td>
</tr>
<tr>
<td>Lung artery</td>
<td>106 ± 1.8</td>
<td>139 ± 2.1</td>
<td>0.76</td>
</tr>
<tr>
<td>Lung vein</td>
<td>133 ± 1.7</td>
<td>126 ± 2.6</td>
<td>1.06</td>
</tr>
<tr>
<td>Lung capillaries</td>
<td>113 ± 1.9</td>
<td>107 ± 1.8</td>
<td>1.06</td>
</tr>
<tr>
<td>Spinal cord artery</td>
<td>D 123 ± 1.5</td>
<td>179 ± 2.2</td>
<td>0.69</td>
</tr>
<tr>
<td>Spinal cord artery</td>
<td>E 119 ± 1.2</td>
<td>121 ± 1.6</td>
<td>0.98</td>
</tr>
<tr>
<td>Spinal cord vein</td>
<td>F 116 ± 1.4</td>
<td>141 ± 1.8</td>
<td>0.82</td>
</tr>
<tr>
<td>Spinal cord vein</td>
<td>G 137 ± 1.5</td>
<td>147 ± 1.7</td>
<td>0.93</td>
</tr>
<tr>
<td>cerebral AVM</td>
<td>- N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>feeder artery</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cerebral AVM</td>
<td>- N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>venous pouch</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The mean density values were evaluated for selected vessels indicated by corresponding letters in Figures 2.5 to 2.7, as well as additional ones in adjacent tissues, and are expressed as mean ± SEM of 300 measurements per vessel. The relative endoglin levels (%) are estimated as Endoglin mean density / PECAM-1 mean density of patient H9 versus the endoglin level of a newborn control. E/P, Endoglin/PECAM-1 ratio; N.A., not applicable.
To extend our observations to other unaffected vascular beds, we examined dural vessels of spinal cord of child H9 and control. Endoglin and PECAM-1 were detectable on endothelium of all vessels, as shown in Figure 2.5b for selected arteries (areas D, d; E, e) and veins (areas F, f; G, g). Endoglin levels were noticeably reduced, compared with PECAM-1, in patient H9. Mean density values of PECAM-1 and endoglin on endothelial cells in control dural vessels were similar to those observed in lung vessels (Table 2.2). Densitometry analysis (Table 2.2) revealed that the relative endoglin levels of patient H9 were significantly reduced on dural arteries (61% and 69%) and veins (44% and 68). Although not present on the sections analyzed by densitometry, capillaries of the central nervous system showed reduced expression of endoglin in patient H9. Thus in situ arteries, veins and capillaries of normal vasculature of the newborn with HHT1 have significantly reduced endoglin levels.

B. Cerebral AVM

To determine if vascular lesions in HHT1 are associated with further reduction or focal loss of endoglin, we examined by immunostaining, the cerebral AVM from patient H9. Sections from a major feeding vessel to the cerebral AVM, the right middle cerebral artery, were stained for elastin, endoglin and PECAM-1 (Figure 2.6). The elastin stain demonstrates an abnormally large, dilated and tortuous vessel. Mesenchymal cells, connective tissue and collagen fibers were disorganized in the adventitia, whereas a thick and compact layer of smooth muscle cells characterized the media. The intima was separated from the media by a continuous internal elastica lamina (Figure 2.6, dark black line) and a thin subendothelial layer lining the feeding vessel. On the uninterrupted endothelial lining of the intima, the Endoglin/PECAM-1 ratio was estimated at 0.50 (Table 2.2). Therefore, the feeding vessel to the AVM of patient H9, despite its highly abnormal structure, expressed 50% endoglin on the endothelium, as observed with unaffected vessels of this patient.

AVMs consist of a tangled network of vascular channels which form dilatations. In patient H9, the aneurysmic dilatation downstream from the right middle cerebral artery was examined (Figure 2.7). Staining for α-SMC actin revealed a thin and tortuous vessel filled with
Figure 2.6 Endoglin is expressed on the large abnormal feeding vessel of the cerebral AVM in patient H9.

Sections from the right middle cerebral artery feeding the cerebral AVM that ruptured in patient H9 were stained with Van Giesen elastin stain. The adventitia (a), media (m) and intima (i) of the artery are marked. The lumen of this vessel is filled with blood (★). Sections stained for endoglin and PECAM-1 by alkaline phosphatase immunostaining, are shown at higher magnification. The arrow outlines endoglin and PECAM-1 staining of the endothelium. Original magnification X31 and X78.
Figure 2.7 Endoglin is detected on the endothelium and the adventitia of the aneurysmic dilatation of the cerebral AVM.

(a) Sections of the aneurysm of patient H9, downstream from the right middle cerebral feeding artery, were stained for α-smooth muscle cell actin. The ★ represents hemorrhage in the ruptured vessel. Original magnification X22. (b) An area showing intense remodeling of the vessel wall in panel a (★) is shown at higher magnification (X194). The same section stained for endoglin revealed expression on mesenchymal cells (▲). (c) The venous pouch shown in panel a (★) was stained for PECAM-1, endoglin and α-smooth muscle cell actin. The dispersed endothelium is indicated by (▲). Original magnification X194.
blood, at the site of rupture (Figure 2.7a). Intense remodeling of the vessel wall can be seen at higher magnification, where α-SMC actin showed variable thickness and a complex network of disorganized smooth muscle cells (Figure 2.7b). Endoglin is barely detectable on the endothelial layer but can be seen on mesenchymal like cell in the small and very compact adventitia. Such a pattern may reflect the ongoing vascular remodeling process in the malformation. The venous pouch (Figure 2.7c) formed in the aneurysmic dilatation showed an extremely thin layer of endothelium outlined by PECAM-1 staining. There was minimal endoglin left on these endothelial cells. The extreme dilatation leads to dispersed endothelium and to lower mean density values for both PECAM-1 (46 ± 1.5) and endoglin (27 ± 0.8) evaluated by image analysis (Table 2.2). In the dilatation, the endoglin/PECAM-1 ratio (0.58) was not significantly distinct from that observed in the unaffected vessels of the patient (0.47) suggesting that cerebral AVM from HHT1 patients are not associated with a complete absence or a focal loss of endoglin in the vascular lesion.

2.4.4 Immunostaining of pulmonary AVM and adjacent tissues of patient H12 (Family 5)

A. Normal Vasculature

To confirm and extend our observations that endoglin is present at reduced levels on all types of vessels, in HHT1 patients, we studied a 78-year-old patient (H12) who presented with a pulmonary AVM. Surgical specimens of unaffected lung tissues adjacent to the pulmonary AVM were immunostained and compared to those of an age-matched control woman. Endoglin and PECAM-1 were detectable on the endothelium of all vessels, and their mean densities were carefully measured for three arteries and three veins in sections of uninvolved lung in patient H12 and controls. The Endoglin/PECAM-1 ratios ranged from 0.62 to 0.80 for the control and from 0.35 to 0.45 for patient H12 (Table 2.3). The relative endoglin levels in unaffected lung tissues of patient H12 versus control ranged from 52% to 66% on arteries and from 46% to 62% on veins (Table 2.3).
Table 2.3 Densitometry analysis of endoglin and PECAM-1 on endothelial cells of patient H12.

<table>
<thead>
<tr>
<th>Vessel Type</th>
<th>Control</th>
<th>Patient H12</th>
<th>Relative endoglin levels (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Endoglin (E) mean density</td>
<td>PECAM-1 (P) mean density</td>
<td>E/P ratio</td>
</tr>
<tr>
<td>Lung artery</td>
<td>139 ± 2.3</td>
<td>173 ± 2.3</td>
<td>0.80</td>
</tr>
<tr>
<td>Lung artery</td>
<td>106 ± 1.9</td>
<td>158 ± 1.7</td>
<td>0.67</td>
</tr>
<tr>
<td>Lung artery</td>
<td>105 ± 2.3</td>
<td>140 ± 1.9</td>
<td>0.75</td>
</tr>
<tr>
<td>Lung vein</td>
<td>109 ± 1.9</td>
<td>161 ± 1.8</td>
<td>0.68</td>
</tr>
<tr>
<td>Lung vein</td>
<td>90 ± 1.7</td>
<td>146 ± 1.6</td>
<td>0.62</td>
</tr>
<tr>
<td>Lung vein</td>
<td>115 ± 1.8</td>
<td>153 ± 1.8</td>
<td>0.75</td>
</tr>
<tr>
<td>pulmonary</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>AVM</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The mean density values were evaluated and are expressed as mean ± SEM of 300 measurements per vessel. The relative endoglin levels (%) are estimated as Endoglin mean density / PECAM-1 mean density of patient H12 versus that of age-matched control. E/P, Endoglin/PECAM-1 ratio; N.A., not applicable.
B. Pulmonary AVM

The pulmonary AVM located in the lung right middle lobe of patient H12 was stained for elastin, endoglin and PECAM-1 (Figure 2.8). The elastin stain demonstrated an abnormally dilated vascular space, with very dispersed and disorganized mesenchymal cells, connective tissue and collagen fibers in the adventitia. A variable thickness of smooth muscle cells was found in the media of the vessel wall, suggesting attempts at remodeling. The intima was separated from the media by a discontinuous internal elastica lamina (Figure 2.8, dark black line). Elastin was also abundant in the parenchyma, adjacent to the malformation. The endothelial cells of the intima, outlined by PECAM-1 staining, were dispersed as the vessel was highly dilated. Endoglin was still detectable on the endothelium of the pulmonary AVM. Image analysis revealed that the endoglin/PECAM-1 ratio was 0.35 in the lesion, a value not significantly different from that observed on unaffected vessels of this patient (0.40; Table 2.3). Thus the pulmonary AVM is not due to a focal loss of endoglin on the endothelium of the lesion.
Figure 2.8 Endoglin is still detectable on the endothelial cells of pulmonary AVM.
Sections from the right middle lobe of the lung of patient H12, containing a PAVM, were stained with Van Giesen elastin stain. The lung parenchyma (p) and the three layers of the abnormally dilated vessel: adventitia (a), media (m) and intima (i)– are shown. Subsequent sections of the PAVM were stained for endoglin and PECAM-1 by alkaline phosphatase immunostaining. The arrow shows the staining of the endothelial cells with both markers. Original magnifications: X30 and X165.
2.5 Discussion

In the current study, we report a novel endoglin mutation arising in the father of a newborn, who died subsequent to rupture of a cerebral AVM. This mutation was characterized at DNA and protein levels. A duplication of exons 3 to 8 led to expression of a transient mutant protein of higher molecular mass than normal, detectable only by metabolic labeling. Relative levels of endoglin, quantified in several vascular beds of the newborn, were reduced by half, indicating that only the normal allele was expressed on endothelium in situ. Similar results were obtained in the vasculature adjacent to a pulmonary AVM, resected from an elderly patient with a known HHT1 mutation. In both cases, endoglin was still expressed in the AVM, indicating that the lesions were not caused by a loss of heterozygosity.

The End mutation arising in patient H3, and absent from his biological parents, constitutes the first case of a demonstration of new mutation arising in a HHT1 family. Furthermore, such a large insertion has not been reported previously for endoglin. However, we have also observed a duplication of exons 3-8, in another family, of different ethnicity. This novel type of mutation was detected using fragment analysis by QMPCR, which was optimized such that peak height is proportional to the number of copies of each exon present (252). This mutation codes for a mutant monomer of 116 kDa, larger than the normal monomer of 90 kDa, and seen only by metabolic labeling. This mutant form of endoglin was not found at the cell surface, despite the presence of a transmembrane region. It exists only as a transient intracellular species, and not at steady-state level, and is consequently not detectable by Western blot and immunostaining.

In this study, the level of normal endoglin was reduced on activated monocytes of patient H3 (family 2) to 50 ± 4%, and in patient H150 (family 5) to 34 ± 8% of control. Endoglin levels less than 70% are considered reduced, as determined from the analysis of affected members from 67 HHT1 families (230, 252). Reduced levels of endoglin, which were revealed here by in vitro analyses in patients with a novel insertion mutation (Family 2) and with a missense mutation (Family 5), support a model of haploinsufficiency for HHT1, as previously proposed and demonstrated (249, 250).
Data presented in this paper demonstrate that all types of blood vessels, in the two HHT1 patients for which pathological specimens were available (H9 and H12), expressed reduced levels of endoglin in situ. Lung and spinal cord vasculature of newborn H9 were considered normal because they showed no morphological abnormalities. However these normal vessels expressed relative endoglin levels that were reduced to 53%, when compared to age-matched controls. There was no significant difference (95% confidence interval) in the relative endoglin levels on the endothelium of lung and central nervous system blood vessels. In the vasculature of lung tissue adjacent to a pulmonary AVM in the 78-year-old patient, H12, the relative endoglin levels were reduced to 56% of those in age-matched control lung tissue. There was no significant difference (95% confidence) between the relative endoglin levels between arteries, veins and capillaries in these patients. Our findings raise the possibility that an HHT1 affected individual could be identified by estimating the relative levels of endoglin on vessels in normal skin biopsies, which would not require affected vessels such as of telangiectases.

Rupture of cerebral AVMs causes a significant number of cerebral hemorrhages that can be fatal. Cerebral AVMs are mostly seen in children (235, 236, 238-240) and are likely congenital, arising during a period critical to development of brain vasculature (241). Pulmonary AVMs also occur in children, but the majority of them present during adolescence or adult life (230, 270). Common to both angiogenesis and vasculogenesis is the process of remodeling, which occurs in both cerebral and pulmonary AVMs, and involves changes in lumen diameter and vessel wall thickness. In the cerebral AVM of newborn H9, we demonstrated that the feeding artery was highly abnormal but still expressed endoglin on its endothelium, although at reduced levels. In the aneurysmic dilatation, smooth muscle cells were disorganized, leading to a media of variable thickness. No internal elastica lamina was present and the endothelial layer was dispersed and very thin, due to increased pressure. The aneurysmic dilatation of patient H9 was extremely large, with a diameter of 8 to 11 mm, a portion of which is illustrated in Figure 2.7a. The pulmonary AVM of patient H12 was in fact smaller with 6 to 7 mm in diameter in the dilatation shown in Figure 2.8. It demonstrated similar characteristics, with thin-wall dilatation, interrupted elastica lamina, variable thickness of smooth muscle cells and dispersed
and disorganized adventitia. In both lesions, the mean density values of PECAM-1 and endoglin were both reduced because of the larger surface area of the dilated endothelium (Tables 2.2 and 2.3). However the endoglin/PECAM-1 ratio in the AVMs was similar to that observed in normal vessels of the patients. These observations are compatible with an ongoing remodeling process and suggest that cerebral/pulmonary AVMs are not due to a focal loss of endoglin in the lesion.

Other factors/receptors regulating vascular development and integrity, such as VEGF observed in the subendothelial layer and the perivascular spaces (268, 269), and Tie-1 receptor present on the endothelium of cerebral AVMs (269) must contribute to the formation of cerebral and pulmonary AVMs. These molecules are necessary for vascular development and their presence in focal areas of cerebral AVMs suggests active angiogenesis and vascular remodeling (268, 269). The familial form of venous malformations was shown to be associated with an activating mutation in the kinase domain of the Tie-2, expressed on endothelial cells (65). Tie-2 is the receptor for Ang1 (46) a factor that indirectly stimulated the differentiation of smooth muscle cells and plays critical roles during vessel formation (63). Failure to recruit smooth muscle cells may lead to abnormal proliferation of endothelial cells characteristic of venous malformations.

TGF-β1 is another important factor implicated in vasculogenesis/angiogenesis, because it regulates interactions between endothelial cells and both mesenchymal and smooth muscle cells of the vessel wall (4, 18). Endoglin is a component of the TGF-β1/-β3 receptor complex (195) which can modulate several responses to these ligands, as demonstrated in the U-937 monocytic cell line (10). TGF-β3 has been implicated in lung development and altered response to this ligand in an endoglin deficient individual could contribute to the generation of pulmonary AVM (163).

It has been proposed that cerebral AVMs are caused by a defect in early vascular development and are associated with ongoing abnormal vascular remodeling (241). Our results in the newborn would support this idea and suggest that a mutation in endoglin could perturb the regulatory effects of TGF-β on the early development of brain vessels. However, because most cerebral AVMs are not associated with HHT1, we must conclude that endoglin is only one of
several genes regulating brain vasculature. In the case of pulmonary AVMs, 70-80% are found in HHT1 patients (230), suggesting a more frequent contribution of endoglin deficiency in their generation. As TGF-β and other factors are critical for vascular homeostasis, any disruption of their effects by altering their receptors or signaling pathways could lead to abnormal vessel function and subsequent vascular malformations.
2.6 Acknowledgments

We gratefully acknowledge the patients who participated in our study. We thank Dr. D. Masson (Oxford, UK.) for providing mAb JC70A to PECAM-1, Dr. E.A. Wayner (Seattle, USA) for mAb P3D1 and P4A4 and Dr B. Seon for providing mAb Sn6h to endoglin. We thank Dr. C. Smith and Ms I. Diplock and Dr Tessitore, for providing patient samples and excellent paraffin-embedded sections. A. Bourdeau is a recipient of a Studentship from the Medical Research Council of Canada and M. Letarte is a Terry Fox Research Scientist of the National Cancer Institute of Canada.
CHAPTER THREE

Characterization of Mice Lacking Endoglin and Development of a Murine Model of Hereditary Hemorrhagic Telangiectasia

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In this chapter, I performed all experiments including the isolation of genomic murine Endoglin, targeting of embryonic stem cells, and analysis of the phenotype, including the histochemistry. This project was supervised by Drs. Dumont and Letarte.

3.1 Abstract

Endoglin, CD105, an accessory protein of the TGF-β receptor superfamily, is highly expressed on endothelial cells. Hereditary Hemorrhagic Telangiectasia type 1 is associated with mutations in the *Endoglin* gene leading to haploinsufficiency. To generate a disease model and ascertain the role of endoglin in development, we generated mice lacking one or both copies of the gene. *Endoglin* null embryos die at gestational day 10.0-10.5 due to defects in vessel and heart development. Vessel formation appears normal until hemorrhage occurs in yolk sacs and embryos. The primitive vascular plexus of the yolk sac fails to mature into defined vessels, and vascular channels dilate and rupture. Internal bleeding is seen in the peritoneal cavity, implying fragile vessels. Heart development is arrested at day 9.0, and the atrioventricular canal endocardium fails to undergo mesenchymal transformation and cushion-tissue formation. These data suggest that endoglin is critical for both angiogenesis and heart valve formation. Some heterozygotes, either inbred 129/Ola or mixed C57BL/6-129/Ola background show signs of HHT such as telangiectases or recurrent nosebleeds. In this murine model of HHT, it appears that epigenetic factors and modifier genes, some of which are present in 129/Ola, contribute to disease heterogeneity.
3.2 Introduction

Hereditary Hemorrhagic Telangiectasia is an autosomal dominant vascular dysplasia that affects 1:10,000 individuals. It is heterogeneous both between and within families in terms of age of onset and severity of clinical manifestations (12). The first symptom of HHT is generally epistaxis, while telangiectases are highly variable. Gastrointestinal bleeding usually occurs later on in life, and 10-25% of patients develop life-threatening complications such as pulmonary or cerebral AVMs. This heterogeneity has been explained in part by the identification of 2 distinct genes. End is mutated in HHT1 (11) and associated with a higher incidence of pulmonary AVMs than HHT2, a milder form of the disease with a later onset and due to mutations in the ALK-1 gene (11, 256, 261, 271, 272). We have shown that mutated endoglin is rarely expressed in HHT1 patients, and when present is found exclusively as an intracellular species (250). The normal copy of endoglin is expressed at the cell surface at half levels relative to normal suggesting that haploinsufficiency is responsible for HHT1 (250, 255).

Human endoglin (CD105) is a homodimeric transmembrane glycoprotein constitutively expressed at high levels on endothelial cells of capillaries, veins and arteries (5, 6). Murine endoglin is found on all endothelial cells and on mesenchymal stromal cells in several tissues (214). Endoglin binds TGF-\(\beta\)1 and -\(\beta\)3 through its association with T\(\beta\)R-II (8, 195, 278); and modulates several responses to TGF-\(\beta\)1 (10, 228). It also binds activin, BMP-7 and BMP-2 by interacting with their respective ligand binding receptors, suggesting that it is in the receptor complex for several growth factors of the TGF-\(\beta\) superfamily (8).

We report here that deficiency in endoglin causes vascular and cardiovascular defects leading to embryonic lethality. Mice heterozygous for the mutation develop normally. However, some heterozygotes show clinical signs of HHT, providing the first animal model of HHT1.
3.3 Materials and Methods

3.3.1 Generation of Endoglin null mice

A murine endoglin genomic clone of 14.8 kb was isolated from a 129 SVJ library by screening with the non-radioactive DIG-system (Boehringer-Mannheim, Montreal, QC) with full length and a 5'-end fragment (583 bp) of murine endoglin cDNA (214). The replacement targeting vector was engineered in three cloning steps and required the pSDKLacZ vector, with a LacZ cassette, and the pPNT vector with neomycin (Neo) and herpes simplex thymidine kinase (tk) cassettes for positive and negative selections. A 1426 bp Smal endoglin fragment, containing the promoter area and terminating 8 bp upstream of the ATG, was used as 5' homology region (5' HR) and subcloned upstream of the LacZ cassette into the pSDKLacZ vector. A Smal-BglIII fragment (5100 bp of intron 1) was used as 3' HR and ligated into the blunted Xbal (BamHI cohesive) site of the pPNT vector, upstream of the tk cassette. The 5' HR and LacZ cassette were then inserted as a SalI fragment into the XhoI site, upstream of the Neo cassette in the pPNT vector. The LacZ and Neo genes thus replaced a region of 609 bp containing the End initiation codon.

The endoglin-targeting construct was linearized with NotI and electroporated into the 129/Ola-derived ES cell line E14. Colonies were subjected to positive and negative selection with G418 and gancyclovir (2 µM) for 10 d. DNA was made from all resistant ES cell clones and digested with Scal. Screening for proper recombination events was achieved by Southern blot analysis using a 32P-labelled 5' external probe (614 bp Smal fragment) and a 1000 bp EcoRI-SacI LacZ internal probe to confirm single integration events, as indicated in Figure 3.1a. Positive ES cell clones were injected into C57BL/6 blastocysts yielding four fertile male chimeras, two of which gave germline transmission.

3.3.2 Breeding of mice

The first generation (F1) of End +/- mice was produced by mating the chimeras with wild type C57BL/6 females (Taconic Farms, Germantown, NY, USA). Eight F1 C57BL/6 End +/- males were crossed with wild type or C57BL/6 End +/- females for four generations. These F1 to F4
mice have a mixed genome of C57BL/6 and 129/Ola. The phenotype of End<sup>−</sup> embryos was analyzed on F1 and F2 intercrosses between C57BL/6 End<sup>−/−</sup> mice. F1 C57BL/6 End<sup>−/−</sup> were also crossed with wild type CD1 (Harlan Sprague Dawley, Indianapolis, IN, USA). These mice with a mixture of CD1, C57BL/6 and 129/Ola genes were maintained by backcrosses with wild type CD1 for three generations. End<sup>−/−</sup> mice have also been generated on an inbred 129/Ola background (same strain as ES cells) by mating of the chimeras with wild type 129/Ola (Harlan UK, Bicester, United Kingdom) and subsequent backcrossing for two generations. All studies were approved by the Ontario Cancer Institute Animal Care Committee.

3.3.3 Genotyping of mice

The mid-day of plug observation was counted as ED0.5. Embryos of equivalent stage and generally from the same litter were used for comparative analyses. Genotypes of embryos were determined using DNA isolated from the yolk sac or a small piece of embryonic tissue; for postnatal DNA, tails were used. A multiplex PCR was designed to amplify 3 sets of primers in a single reaction that can distinguish End<sup>+++</sup>, End<sup>−/−</sup> and End<sup>−/−</sup> alleles. All PCR reactions were run with initial denaturation for 2 min at 94°C, followed by 30 cycles of 94°C for 30 s, 56°C for 35 and 68°C for 2.5 min, with a final extension at 68°C for 5 min. Reactions contained 300 ng DNA, 400 µM dNTP, 2.5 mM MgCl₂, 1 unit AmpliTaq®DNA Polymerase (Perkin Elmer, Roche Molecular System, Branchburg, NJ, USA), 40 pmol of primers ME1F and MEZR and 20 pmol of the others. Selected primer sequences, 5'→3' direction, were: ME1F (TACCTCTGGATACCGGATAAG), ME1R (AAGTTTGGCATCCTATGAAAC), MEZR (AAATGTGAGCGAGTAACAACC), ME2F (ATCTTACCCACTGAGCCATCT) and ME2R (CCCAGTCTACTCCGATTCTTA).

3.3.4 RT-PCR

RNA was extracted from ED9.5 embryos using TRIzol® Reagent (GibcoBRL, Mississauga, ON, Canada), and 1 µg was used for first strand cDNA synthesis using Superscript RT (GibcoBRL). cDNA was amplified for β-actin and endoglin using previously reported primers (214). PCR
conditions were similar to above except for 30 cycles of extension at 72°C for 30 s, and a final one of 5 min, and using 1.5 mM MgCl₂ and 200 μM dNTP.

3.3.5 Whole mount β-galactosidase (β-gal) staining

Embryos or yolk sacs were dissected in PBS, fixed and stained as described (50) except for post-fixation in 10% formaldehyde (Fisher Scientific Co., Nepean, ON, Canada). The embryos were then paraffin-embedded, sectioned (7μm) and counterstained with 0.1% nuclear fast red (Fluka Chemica, Oakville, ON, Canada). Images were acquired using an Olympus BX50 microscope linked to a CCD video camera (Carsen Medical Scientific, Markham, ON, Canada) and digitized on a Power Macintosh G3 computer.
3.4 Results

3.4.1 Generation of Endoglin null mice

A targeting construct was engineered to delete End exon 1 and its initiation codon, replacing it with the LacZ-Neo cassette (Figure 3.1a). Using Southern blot analysis, eight heterozygous (End +/-) embryonic stem (ES) cell clones were identified, yielding a recombination frequency of 1/30. Results obtained for the two ES cell clones that gave germ line transmission, 4A-11 and 4A-36, are illustrated in Figure 3.1b.

Embryos obtained from matings of C57BL/6 End +/- mice were genotyped by multiplex PCR. Results from typing of an ED9.5 litter are shown in Figure 3.1c. Two End +/- endoglin embryos expressed the 476 bp fragment of the recombinant allele, and the 383 bp fragment from normal exon 2. Three wild type (End ++/) embryos showed the normal PCR products for exon 1 (300 bp) and exon 2 (383 bp). Four End +/- embryos and the End +/- mother (#21) showed all three PCR products.

To verify that the targeted mutation inactivated the gene we examined expression of End mRNA in the same embryos analyzed in Figure 3.1c. The 468 bp product corresponding to murine endoglin (exons 7-10) was present in the End +/- and End ++/ embryos, but absent from the two End +/- embryos. β-actin (550 bp) was expressed at equivalent levels in all embryos. Thus, no wild type or truncated endoglin mRNA was detectable in End +/- mice (Figure 3.1d).

3.4.2 End +/- mice die in utero at ED10-10.5

Expression of β-gal, under the control of the endoglin promoter in the targeting vector, recapitulated endogenous expression of endoglin in End +/- mice and facilitated the analysis of the End +/- phenotype in embryos. Examination 103 ED7.5-8.5 live embryos from C57BL/6 End +/- intercrosses revealed no obvious embryonic, placental or yolk sac abnormalities and a normal 1:2:1 Mendelian ratio (Table 3.1). At ED9.0, End ++/ and End +/- embryos were of similar size and their vasculature was revealed by the staining associated with endothelial cells. The heart of several End +/- embryos was enlarged (Figure 3.2). At ED9.5, the End +/- embryos showed signs of growth retardation such as a smaller head, underdeveloped nasal processes and branchial arches.
Figure 3.1 Generation of End<sup>−/−</sup> mice.

(a) Targeting strategy. The homologous recombination event deletes 609bp of the End gene including exon 1 (E1; 66bp), placing the LacZ gene under control of its promoter. The targeting construct contains a 1.4 kb Smal fragment (5' HR) and a 5.1 kb Smal-BglII fragment (3' HR) of the End gene flanking the LacZ-Neo cassette. Proper recombination events were screened by Southern blot analysis using the 5' external and LacZ internal probes indicated. Bm, BamHI; Bg, BglII; El, EcoRI; Sa, SacI; Sc, Scal; Sm, Smal; Xb, XbaI. (b) Identification of targeted ES cell lines. DNA from 2 geneticin and gancyclovir-resistant targeted ES cells (4A-11 and 4A-36) and a wild type clone (WT) were digested with Scal. The 5.4 kb wild type and 7.3 kb recombinant alleles hybridizing with the 5' external probe and the recombinant allele reacting with the internal LacZ probe are shown. (c) Genotyping of embryos by multiplex PCR. DNA prepared from yolk sacs of embryos of an ED9.5 litter derived from a C57BL/6 End<sup>+/−</sup> mating is shown; #21 represents the mother and C, the PCR control reaction without DNA. Primers ME1F and ME1R amplify normal exon 1 (300 bp), ME2F and ME2R amplify normal exon 2 (383 bp), while ME1F and MEZR specifically amplify the recombinant product (476 bp). (d) Absence of endoglin mRNA in End<sup>−/−</sup> embryos. RNA was prepared from the same embryos as in c, and analyzed for endoglin and β-actin mRNA by RT-PCR.
Figure 3.2 Arrest in development in End⁻/⁻ embryos.

β-gal expression driven by the End promoter was analyzed in whole End⁺/+ and End⁻/- embryos. At ED9.0, embryos are of similar size and their vasculature appears normal. The End⁻/- embryo shows an enlarged heart protruding forward (arrowhead). At ED9.5, edematous pericardium (arrowhead) is observed in the End⁻/- embryo, which is growth retarded compared to the End⁺/+ embryo. At ED10.5, the End⁻/- embryo is dead, and much smaller than the End⁺/+ embryo. Bar: 500 μm.
The heart was still beating but edematous pericardium was seen (Figure 3.2). At ED9.0-9.5, 99 live embryos gave a normal Mendelian ratio. Of the 40 embryos analyzed at ED10-10.5, only seven were End $^+$, six were dead with collapsed vasculature, edemic heart, and extensive tissue necrosis and one showed irregular heart beats and extensive edema (Figure 3.2 and Table 3.1). From ED11.5 to 15.5, no End $^-$ embryos were found amongst 30, confirming that deficiency in endoglin leads to embryonic lethality at ED10-10.5 (Table 3.1). From a total of 136 born offsprings, 33% were wild type (End $^{++}$), 67% were End $^{+-}$ and none were homozygous (End $^{-}$), showing conclusively that the Endoglin-null phenotype is lethal (Table 3.1).

3.4.3 Yolk sac defects in End $^-$ mice

The primitive vasculature of the End $^-$ embryos and the early vascular plexus of the yolk sac were normal until ED9.0. This suggested a defect in yolk sac remodeling, essential for the interconnection of embryonic and extraembryonic circulatory systems. Yolk sacs of End $^-$ embryos at ED9.0-9.5 revealed an abnormal vascular plexus. At ED9.0, vascular channels were numerous, lined with an intact endothelium and filled with normal levels of primitive erythroblasts (Figure 3.3, a and b). However, no branching occurred, in contrast to the End $^{+-}$ yolk sac (Figure 3.3, c and d). At ED9.5, vitelline vessels were completely absent from End $^-$ yolk sacs (Figure 3.3, e and f) and were replaced by enlarged and leaky channels that released their contents into the yolk sac cavity (Figure 3.3, g and h). This hemorrhage was evident in unstained embryos, which showed blood accumulating in the yolk sac cavity, between the amnion and the yolk sac membranes (Figure 3.3, i and j). We conclude that in absence of endoglin, primitive vascular channels of the yolk sac did not form large vessels and were susceptible to rupture releasing hematopoietic precursors into the yolk sac cavity.
Figure 3.3 Vascular defects in End⁻/⁻ mice.

Whole mount embryos in their yolk sac were stained for β-gal activity. (a, b, e and f) Direct microscopic examination of the yolk sacs. (c, d, g and h) Sagittal histological sections. At ED9.0, the normal capillary plexus and initiation of branching are seen in the End⁺/⁺ embryo (a and c). The End⁻/⁻ yolk sac also has a highly vascularized plexus but no vessel branching is observed (b and d). At ED9.5, a vitelline vessel (v) is readily detectable in the End⁺/⁻ yolk sac (e); this intact vessel is full of primitive red cells (g). The End⁻/⁻ yolk sac shows a disorganized capillary plexus and no vitelline vessels (f); abnormally dilated blood islands are seen (h), which have ruptured, releasing primitive erythroblasts toward the amnion (arrowhead). (i and j) Unstained ED9.5 embryos, with yolk sac and placenta still attached. Bleeding in the yolk sac cavity (arrowhead) and edematous pericardium (arrow) are observed in End⁺/⁻ embryo (j) compared with a littermate control (i). An unstained ED9.0 End⁺/⁻ embryo (k), dissected away from the yolk sac, is compared to an End⁻/⁻ littermate (l), which shows internal bleeding (arrow). Sagittal sections stained for β-gal demonstrate the presence of blood (arrow) in the peritoneal cavity of the End⁺/⁻ embryo (n), which is absent from the End⁺/⁺ embryo (m).

a, dorsal aorta; pc, peritoneal cavity; u, umbilical vein. Bar: 100 µm (a–h), 500 µm (i and j) and 250 µm (k–n).
Table 3.1 Genotype of Offspring From C57BL/6 End<sup>+&minus;</sup> Matings.

<table>
<thead>
<tr>
<th>Day of gestation</th>
<th>Total embryos</th>
<th>End&lt;sup&gt;++&lt;/sup&gt;</th>
<th>End&lt;sup&gt;+-&lt;/sup&gt;</th>
<th>End&lt;sup&gt;+-&lt;/sup&gt;</th>
<th>Resorbed</th>
</tr>
</thead>
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<tr>
<td>7.5-8.5</td>
<td>108</td>
<td>25</td>
<td>50</td>
<td>28</td>
<td>5</td>
</tr>
<tr>
<td>9.0-9.5</td>
<td>107</td>
<td>23</td>
<td>49</td>
<td>27</td>
<td>8</td>
</tr>
<tr>
<td>10.0-10.5</td>
<td>40</td>
<td>13</td>
<td>20</td>
<td>7&lt;sup&gt;1&lt;/sup&gt;</td>
<td>0</td>
</tr>
<tr>
<td>11.5-15.5</td>
<td>30</td>
<td>9</td>
<td>17</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Adults</td>
<td>136</td>
<td>43</td>
<td>89</td>
<td>0</td>
<td>4&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
</tbody>
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<sup>1</sup> 6 of 7 embryos were present but dead.

<sup>2</sup> Mice who died of unrelated causes, between 1 and 3 months.
3.4.4 Internal hemorrhage and abnormal heart development in End−/− embryos

Direct microscopic examination of unstained whole embryos dissected away from the yolk sac at ED9.0 revealed internal hemorrhages in End−/− embryos (Figure 3.3, k and l), suggesting that vascular defects were not limited to the extraembryonic vasculature. Histological sections of End−/− embryos showed blood accumulating in the peritoneal cavity indicating that endothelium integrity was compromised. The dorsal aorta or the observed dilated umbilical vein could be the source of the extravasated blood (Figure 3.3, m and n). Thus in the absence of endoglin, embryonic vessels are more fragile and susceptible to rupture.

End−/− embryos harvested at ED9.0-10.5 had enlarged hearts and showed edematous pericardium when compared to their End+/+ littermates (Figure 3.2). Sagittal sections revealed major differences in heart development. Normal End+/+ embryos showed signs of a progressively developing endocardium, such as increasing trabeculation and closure of the atrioventricular canal (Figure 3.4). Endoglin expression, visualized by β-gal activity, was also observed on cells undergoing endocardial-mesenchymal transformation, and on the migrating mesenchymal cells that later form the atrioventricular cushion tissue (Figure 3.4). In contrast, the End−/− embryos manifested defects in heart development from ED9.0. The endocardium remained rudimentary in the primitive ventricle, whose lumen failed to close in the atrioventricular area. At ED9.0 some embryos revealed an abnormally dilated endocardium in the common atrial chamber and the bulbus cordis (Figure 3.4). No mesenchymal cells were detected in the heart of the End−/− embryos analyzed, indicating that endocardial-mesenchymal transformation, which leads to valve formation and heart septation did not occur in these embryos. At ED10.5, the heart of the End−/− embryos was arrested, no longer beating, and with extensive necrosis.

3.3.5 End+/- mice develop clinical signs of HHT

Four generations of C57BL/6 End+/- mice (a total of 227) were examined, over a period of 1 to 12 mo, for clinical manifestations of HHT. Sick animals were first recognized by ruffled fur, weight loss and visible ear telangiectases. We observed 14 affected End+/- mice, 13 of the 20
Figure 3.4 Heart deficiency in End−/− mice.
Transverse sections from whole mount embryos stained for β-gal activity are shown. The ED9.0 End+/− embryo shows staining associated with the endocardium in the ventricular compartment (v) and tightly delineated in the atrioventricular region (av). The endocardium of the End−/− embryo is rudimentary, with an abnormally large lumen in the common atrial chamber (a) and the bulbus cordis (b). At ED9.5, increased trabeculation is observed in the ventricle of the End+/− embryo; a few β-gal stained mesenchymal cells (arrowhead) are seen adjacent to the atrioventricular canal. The heart of the End−/− embryo is much larger than control and devoid of trabeculae; mesenchymal cells are absent. At ED10.5, cushion tissue mesenchyme (arrowhead) from the End+−/− embryo stains strongly positive for β-gal; in contrast, the heart of the End−/− embryo shows edema and signs of necrosis. Bar: 100 μm.
with a light coat color and only 1 of the 207 with a dark coat color (Table 3.2). Some animals also had neck and tail telangiectases and bled from nose or mouth once or twice a wk (Table 3.2). Two mice died with extensive disease manifestations such as difficulty to breath and internal hemorrhage. These C57BL/6 End "" mice (F2-F4) still have a mixed genetic background with 129/Ola, from which the light coat color is derived. To determine if the 129/Ola strain was associated with the disease phenotype, we generated End "" mice on this inbred background. We also bred the mixed C57BL/6-129/Ola F1 to CD1 mice, also of light coat color.

None of the 51 End "" CD1 showed signs of disease, when observed over 3-9 mo. However, 5/10 End "" inbred 129/Ola mice showed visible telangiectases on the ears and 3 had nosebleeds, when observed over a period of 7 mo (Table 3.2). Male 55.5 had its first nosebleeds at 7 wks and thereafter at a frequency of 2-3 times per wk. Dried blood was noticeable around the nose after each bleeding episode (Figure 3.5). At the End of a bleeding episode spanning 18-24 h, the hematocrit value was dramatically reduced relative to control (45 versus 67) but was returned to normal four days later. Female 69.1 had nosebleeds at least once a wk, starting at 10 wks (Table 3.2). Its hematocrit measured on a day with epistaxis was comparable to that of a female control (55 versus 52). Hematocrit values were normal for twenty C57BL/6 End "" male (58-70) and female (49-63) mice tested.

An ear telangiectasia in the 129/Ola End "" affected male 55.5 is shown in Figure 3.5. Direct microscopic examination of ear biopsies showed large dilated vessels at the periphery of the ear lobe, not seen in the normal control (Figure 3.6, a and b). These small vascular lesions generally lasted for 2-3 d and then ruptured and bled, often leading to partial ear necrosis. The β-gal-stained sections revealed the presence of abnormally dilated vessels, much larger than the capillaries normally seen in the ear (Figure 3.6, c and d). One such dilated vessel corresponding to a telangiectasia is shown at higher magnification along with several normal capillaries (Figure 3.6e). Female 102.6, progeny of male 55.5, showed several tail telangiectases and tail bleeding, frequent nosebleeds and slower development, and died at 7 wks.
Table 3.2 Clinical Signs of HHT in End \(^{+-}\) mice.

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>Total #</th>
<th>Nosebleeds</th>
<th>Telangiectases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td># of mice</td>
<td>Age of onset (wks)</td>
</tr>
<tr>
<td>C57BL/6 WT (dark coat color)</td>
<td>45</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>C57BL/6 End (^{+-}) mixed 129/Ola (F1-F4)</td>
<td>227</td>
<td>4</td>
<td>-</td>
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<tr>
<td>dark coat color (^1)</td>
<td>207</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>light coat color (^2)</td>
<td>20</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>(\sigma) (F2, F3)</td>
<td>11</td>
<td>3</td>
<td>9-23</td>
</tr>
<tr>
<td>(\varphi) (F3, F4)</td>
<td>9</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>CD1 WT (light coat color)</td>
<td>20</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>CD1 End (^{+-}) mixed C57BL/6 (F1-F3)</td>
<td>51</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>dark coat color</td>
<td>19</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>light coat color</td>
<td>32</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>129/Ola WT (light coat color)</td>
<td>20</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>129/Ola End (^{+-}) (light coat color)</td>
<td>10</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>(\sigma) 55.5 (F1)</td>
<td>12</td>
<td>2-3</td>
<td>Ear, Neck, Tail</td>
</tr>
<tr>
<td>(\varphi) 69.1 (F1)</td>
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<td>1</td>
<td>Ear</td>
</tr>
<tr>
<td>(\varphi) 70.3 (F1)</td>
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<td>-</td>
<td>Ear</td>
</tr>
<tr>
<td>(\varphi) 55.83 (F1)</td>
<td>-</td>
<td>-</td>
<td>Ear</td>
</tr>
<tr>
<td>(\varphi) 102.6 (F2)</td>
<td>2</td>
<td>2</td>
<td>Tail, Ear</td>
</tr>
</tbody>
</table>

\(^1\) Black or brown

\(^2\) White, beige or gray
Figure 3.5 *End*<sup>+/−</sup> 129/Ola mice have nosebleeds and telangiectases characteristic of HHT. (a) A 129/Ola *End*<sup>+/−</sup> male mouse with recurrent nosebleeds and telangiectases is shown on the right of a female littermate with no disease manifestation. (b) Telangiectases are present on the upper part of both ears. (c) A side view provides a closer look at the affected nasal area.
Figure 3.6 Microscopic examination of a telangiectasia from a 129/Ola End<sup>+/−</sup> mouse.

Ear lobe biopsies from an End<sup>+/−</sup> mouse and normal littermate were left unstained (a and b) or were stained for β-gal activity (c and d). (a) A dilated vessel (arrow), engorged with blood, is seen in the affected End<sup>+/−</sup> ear lobe, together with a necrotic area (arrowhead) from a previously ruptured telangiectasia. (b) The corresponding area from an unaffected control 129/Ola mouse shows much smaller vessels. (c) A small telangiectasia (arrow), close to the edge of the affected ear, appears as a dilated vessel distinct from the adjacent network of capillaries. (d) Normal capillary network from an unaffected ear. (e) A cross-section of the resected ear segment from the affected 129/Ola End<sup>+/−</sup> mouse was stained for β-gal. An enlarged vessel filled with blood (arrow) corresponding to a telangiectasia is seen. The endothelial cells of this vessel and of several normal small capillaries are stained; a layer of cartilage (ca) is seen across the section. Bar: 500 μm (a–d) and 100 μm (e).
3.5 Discussion

The endoglin promoter-driven expression of β-gal activity allowed us to establish that endoglin is expressed normally on the endothelium of both End⁺ embryos and End⁺ extraembryonic structures such as yolk sac and placenta until ED9.0. This implies that in situ differentiation of endothelial cells from mesodermally derived precursors, their assembly into the primary capillary network of both yolk sac, placenta and embryo, and the generation of primitive vessels connected to the primordia of the heart, do not require endoglin. These early steps of vasculogenesis are thus normal. Subsequent events in vascular development include proliferation and migration of endothelial cells which lead to growth and remodeling of the initially homogeneous capillary network by branching to form small and large vessels (3). Our results suggest that endoglin plays an important role in these angiogenic processes.

The primary capillary plexus of the yolk sac expressed endoglin as detected by β-gal activity. In the End⁺ yolk sacs, the blood islands at ED9.0-9.5 dilated and fused to generate abnormal and irregular channels that lysed and released their primitive erythroblasts into the yolk sac cavity; the normal vitelline vessels were not formed. Li et al. (158) have observed that End⁺ embryos died in utero at ED11.5, and that no large vessels were observed in the yolk sac. It was suggested that angiogenesis was defective because of a scarcity of vascular smooth muscle cells in yolk sac and embryonic vessels, occurring prior to the cardiogenesis defects, which were not described (158). These mice, like ours, were crossed into C57BL/6 but the ES cells were of 129/SVJ origin rather than 129/Ola. Although these two strains are highly related, this might explain the delayed phenotype observed in their studies. In our case, both yolk sac and heart defects were observed simultaneously at ED9.0, and all mice died by ED10.0-10.5.

Contact between endothelial and mesenchymal cells in a developing vessel was proposed to activate TGF-β1, which induces differentiation of mesenchymal cells into pericytes and smooth muscle cells stabilizing the endothelial tubes (4). Mice deficient in TGF-β1 or TBR-II, die at ED10.5 of an inadequate yolk sac capillary network showing poor adhesiveness between endothelial and mesothelial layers (18, 19). In vitro and in vivo mosaic studies with End⁺ cells will be required to assess if the impaired structural integrity of vessels is endothelial cell
autonomous or due to lack of production of PDGF-B, for example, required to recruit pericytes (4, 71).

Ang1 and its endothelial receptor, Tie-2/Tek, are also implicated in regulation of interactions between endothelial and mesenchymal cells and their extracellular matrix (48-50, 63). Embryos, deficient in Tie-2, die at ED9.5-E10.5 with a related but distinct phenotype from those lacking endoglin. Endothelial cell number in the yolk sac of Tie-2<sup>−/−</sup> embryos is reduced while normal in End<sup>−/−</sup> embryos both mutant show distended vascular channels, lack of vitelline vessels, hemorrhaging and arrested heart development. An activating mutation of human Tie-2 tyrosine kinase leads to venous malformations, composed of dilated serpiginous channels with a variable thickness of smooth muscle cells (65). In HHT, telangiectases start as dilated post-capillary venules that eventually fuse with arterioles and show disorganized smooth muscle cells (231). Thus endoglin and Tie-2 might have complementary roles in regulating cellular interactions in the vessel wall.

The pattern of endoglin expression in the developing heart was very striking and allowed us to visualize the endothelial-mesenchymal transformation in the region of the atrioventricular canal, since both cell types express endoglin. Endoglin is present on the endocardium throughout human heart development and is also transiently up-regulated on cushion tissue mesenchyme of both the atrioventricular canal and the outflow tract at 5-8 wks of gestation, suggesting an important role in valve formation and heart septation (7). TGF-β1 deficient mice also show abnormal heart development at E10.5 (18, 279). All three isoforms of TGF-β, and TβR-II, have been implicated in cushion tissue formation in vitro (280, 281). Endoglin is detected on chicken mesenchymal cells undergoing transformation and migrating into the cushion tissue, closely following the expression of the TGF-β3 isoform (274). It was proposed that betaglycan might be the component of the TGF-β receptor system responsible for endocardial cell transformation (282). However our previous work on embryonic human heart and the data presented here collectively suggest that endoglin is essential for heart valve formation and septation.

Disease manifestations were observed in some mice expressing of a single copy of End confirming that HHT1 is associated with a loss of function of the mutated allele (250). Our
observations that 14/20 mixed C57BL/6-129/Ola with light coat color and 5/10 129/Ola End +/- inbred mice showed clinical signs of HHT, suggest that disease is associated with the 129/Ola background. This implies that other gene(s) are contributing to the severity and heterogeneity of HHT. In the 129/Ola background, one such gene might be linked to coat color. It has been reported that 70-80% of 129/Ola inbred mice have dramatic alterations in liver and lung vasculature such as portal shunting and reduction and truncation of peripheral vessels, when compared to C57BL/6 and CBA/Ca mice (283, 284). This phenotype might predispose mice with a single End copy, to express HHT manifestations. The identification of modifier genes in the 129/Ola background is critical to our understanding of this complex vascular disorder and its underlying pathology.

The absence of clinical manifestations in half of 129/Ola End +/- mice, and variation in age of onset and frequency of nosebleeds and telangiectases, resemble the human disease with heterogeneity even within a family. This suggests that epigenetic factors such as environment, blood pressure, oxygenation, shear forces and hormonal levels must influence clinical manifestations (230). More detailed analysis of a large number of affected End +/- mice might reveal severe complications of HHT such as pulmonary, hepatic and/or cerebral AVM. Monitoring these vascular malformations by emerging techniques such as MR microscopy (285) would give us a better understanding of their origin and progression.

Our studies demonstrate that endoglin plays a crucial role in vascular and cardiovascular development as its absence leads to death of murine embryos in utero at ED10.0-10.5. Further analysis of endothelial and mesenchymal cells isolated from End +/- mice might allow us to identify the precise role of endoglin in modulating effects of TGF-β1 and other members of this superfamily on blood vessel and heart development. A murine form of HHT was generated by ablating the expression of a single End allele, confirming that haploinsufficiency leads to this disorder. The observations that the 129/Ola background is more susceptible to disease suggest that additional genes contribute to this complex vascular disorder. This mouse model will be invaluable in understanding the underlying pathophysiology of HHT and in testing potential therapeutic strategies.
3.6 Acknowledgments

We would like to thank W. Khoo, R. Sarao, M. Bissasseur, and N. Chan for their expert technical assistance and L. Morikawa, for invaluable help in preparing sections from embryos and the staff of the animal colony at the Ontario Cancer Institute. This research was supported by grant #NA3434 from the Heart and Stroke Foundation of Ontario (ML) and by grants from the Medical Research Council of Canada (ML and DJD). AB is recipient of a studentship from the Medical Research Council of Canada, and ML is a Terry Fox Research Scientist of the National Cancer Institute of Canada.
CHAPTER FOUR

Heterogeneity of Vascular Defects in Endoglin Heterozygous Mice with Hereditary Hemorrhagic Telangiectasia suggests Involvement of Modifier Genes

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³Department of Laboratory Medicine and Pathobiology, University of Toronto Health Network, M5G 2C4, Canada

I was responsible for the generation of all mice described. I was the first to recognize the HHT phenotype in our endoglin deficient animals. In this chapter I characterized the phenotype of End⁺⁻ mice that spontaneously developed clinical signs of HHT. I performed daily observations to document external signs. Autopsies described were done with the help of Dr. Letarte. Tissue sections were obtained from Pathology and I set up the conditions and for immunohistochemistry and performed the staining on the sections. Analysis of the sections were done in collaboration with Dr. Wanless, pathologist. The validation of our murine model and comparison with the human disease was done with Dr. Faughnan, respirologist and director of the Toronto HHT Centre. Dr. Letarte supervised this project.

4.1 Abstract

Hereditary Hemorrhagic Telangiectasia is an autosomal dominant disorder associated with mutations in either *End* (HHT1) or *ALK-1* (HHT2) genes and with haploinsufficiency. To generate an animal model of HHT, we engineered mice with a single allele of *Endoglin* on two inbred background strains: C57BL/6 and 129/Ola. Of a total of 171 such mice observed over one year, 50 developed clinical signs of HHT. Disease prevalence was high in 129/Ola strain (72%), intermediate in C57BL/6 intercrosses (35%), and low in C57BL/6 backcrosses of first and second generations (7%). Age of onset varied from 1-50 wks but was earlier in the first two groups. Disease manifestations were heterogeneous but most mice first presented with an ear telangiectasia and/or recurrent external hemorrhage. One-third of mice with HHT showed severe vascular abnormalities such as dilated vessels, hemorrhages, liver and lung congestion, and/or brain and heart ischemia. Disease sequelae included stroke, hydrocephalus, fatal hemorrhage and congestive heart failure. Our results demonstrate that the HHT mouse model reproduces well the varied clinical manifestations of the human disease. The data suggest that in addition to reduced expression of endoglin, modifier genes, such as may be present in 129/Ola strain, contribute to heterogeneity and severity of HHT.
4.2 Introduction

Hereditary hemorrhagic telangiectasia, also known as Rendu-Osler-Weber syndrome, is an autosomal dominant disorder that affects blood vessels and has a prevalence of about 1:8000 (286). HHT is primarily associated with epistaxis (recurrent nosebleeds), telangiectases and internal vascular lesions (229). Patients can develop life-threatening complications such as severe gastrointestinal bleeds and arteriovenous malformations of the liver, lung or brain. Shunting of blood through pulmonary or cerebral AVMs can lead to hypoxemia, stroke, brain abscess, heart failure and fatal hemorrhage (230).

Clinical manifestations of HHT are highly heterogeneous between families as well as within a given family. Genetic and epigenetic factors have been postulated to account for this diversity. Two genes are responsible for HHT, End mutated in HHT1 (11) and ALK-1, mutated in HHT2 (261). HHT1 is associated with a higher incidence of pulmonary AVMs than HHT2, which generally has a later onset (256). Severity of HHT is not correlated with the type of mutation or its position (255). Mutated endoglin is rarely expressed in HHT1 patients and only as an intracellular species (250). The current model for HHT1 is haploinsufficiency, due to reduced levels of functional endoglin at the surface of endothelial cells (209, 250, 252, 253). ALK-1 haploinsufficiency appears to be associated with HHT2 (264).

Both endoglin and ALK-1 are components of the TGF-β superfamily of receptors, which are predominantly expressed on vascular endothelium. Endoglin cannot bind ligands of the TGF-β superfamily by itself. However it binds TGF-β1, TGF-β3, activin-A, BMP-7 and BMP-2 via association with their respective ligand binding receptors (8). Endoglin modulates several cellular responses to TGF-β1 but its role in regulating effects of other ligands has yet to be demonstrated (10, 227). ALK-1 is a type I receptor recently shown to bind TGF-β1 in endothelial cells (136). Thus the receptor complex for TGF-β1 on vascular endothelium contains endoglin associated with the ligand binding receptor, TβR-II and signaling via the type I receptors ALK-1 or ALK-5.

Recently a crucial role for endoglin in angiogenesis was demonstrated in End deficient mice which showed multiple vascular and cardiac defects leading to death in early embryos (158,
From ED9.0, the primitive vascular plexus of yolk sac failed to remodel into mature vessels causing vascular channel dilation, rupture and hemorrhage. Internal bleeding was also observed in the embryo implying vessel fragility. Endocardial cushion formation, essential for valve development and heart septation did not occur. Pericardial edema was also observed (287). The yolk sac defects in End null embryos were similar to those observed in mice lacking ALK-1, TGF-β1, TβR-II and Smad5 (18, 19, 136, 160). We also observed that some End+− mice developed external signs of HHT such as telangiectases and bleeds (287).

We now report the full characterization and validation of the murine model of HHT. Phenotypic heterogeneity, including severe visceral involvement, is described for 50 End+/− mice with external signs of disease. Our data suggest that endoglin haploinsufficiency and effects of modifier genes are responsible for heterogeneity and severity of HHT.
4.3 Materials and Methods

4.3.1 Generation and breeding of End⁺⁺

Mice expressing a single copy of End were generated by homologous recombination using embryonic stem cells of 129/Ola origin (287). The first backcrosses (N1) were produced by mating male chimeras with wild type 129/Ola (Ola) (Harlan UK, Bicester, UK) or C57BL/6 (B6) females (Taconic Farms, Germantown, New York, USA). The N1 Ola backcrosses (Olaₙ) are already inbred, as well as all their subsequent generations. Backcrosses onto C57BL/6 mice (B₆ₙ) have a mixed genome: N1 (50% B₆:50% Ola), N2 (75% B₆:25% Ola). Intercrosses (B₆; F1 and F2) were produced amongst N1 End⁺⁺ B₆ₙ and also have a mixed (50% B₆:50% Ola) genome. End⁺⁺ mice were also backcrossed to a parent of mixed genotype (End⁺⁺ B₆ₙ N1 and N2) suspected of disease. These animals also have a mixed genome (B₆ₙ; on average 60% B₆:40% Ola). Mice were kept in ventilated racks in a germ-free facility and all protocols were approved by the Ethics Committee of the Hospital for Sick Children Laboratory Animal Services.

4.3.2 Genotyping of mice

The genotype of each mouse was first assessed by β-gal staining made possible by the presence of a LacZ reporter gene driven by the endoglin promoter in the targeting construct (287). At weaning time (3 wks), an ear punch from each pup was washed in PBS, fixed in 0.25% glutaraldehyde for 15 min, washed 3 times for 5 min, stained overnight at 30°C in X-gal solution and observed under a microscope. Blue vessels are associated with End⁺⁺ genotype as all End⁺⁺ mice die in utero at ED10-10.5. The genotype was often confirmed by multiplex PCR using tail DNA (287).

4.3.3 HHT phenotype assessment

Wild type, End⁺⁺⁺ and End⁺⁺ mice were observed daily for any signs of HHT. They were given a score based on the age of onset of external signs: telangiectases (location, frequency/wk), interstitial bleeding (location, severity, frequency/wk), breathing capacity, mobility, weight loss,
moribund state, and other manifestations such as ear or tail loss. Internal signs were also carefully examined during autopsy. Each mouse was dissected to score dilation of inner skin vessels, interstitial bleeding and its origin. Organs including spleen, liver, intestine, kidneys, lungs, heart and brain were scored based on parameters such as size, color, edema, effusion, prominence of vessels, and presence of telangiectases and hemorrhages (focal or diffuse). Other severe consequences from hemorrhage such as hydrocephalus and stroke were also noted.

4.3.4 Immunohistochemical staining

Organs from End™ and control mice were immediately embedded in OCT, frozen on isopentane/dry ice. Cryosections (7µm) were fixed 10 min in acetone, washed briefly in TBST (0.01 mol/L Tris, pH 7.4, 0.16 mol/L NaCl, 0.2% Tween 20), dipped 5 sec in 0.1N HCl to remove endogenous alkaline phosphatase and washed thoroughly in TBST. Sections were then blocked with 5% normal rabbit serum (Dako, Mississauga, ON, Canada) for 20 min, blocked sequentially with Avidin and Biotin solution (Vector Labs, Burlington, ON, Canada) for 20 min, and washed. Sections were incubated at 4°C for 2h with optimal concentrations of primary antibodies. These were mAb JC7/18 to endoglin (CD105; purified IgG, 2µg/ml, Pharmingen, Mississauga, ON, Canada), mAb MEC13.3 to PECAM-1 (CD31; purified IgG, 5µg/ml, Pharmingen), mAb 1A4 to α-smooth muscle cell (SMC) actin (ascites, diluted 8000-fold, Sigma, Oakville, ON, Canada) and non-immune rat IgG (5µg/ml, Sigma). Slides were washed and incubated for 1h at 4°C with biotinylated rabbit anti-rat IgG (diluted 400-fold; Vector Labs). For α-SMC actin detection, biotinylated polyclonal antibody from the LSAB kit was used (Dako). The streptavidin-alkaline phosphatase amplification system (StreptABC/AP, Dako) was used and the enzymatic reaction was performed as described (253). Some sections were counterstained with 5% neutral red (Sigma). Tissue morphology was assessed with both frozen and paraffin-embedded sections stained with hematoxylin and eosin (H&E) and/or Masson’s trichrome.
4.4 Results

4.4.1 End+/- mice develop HHT

Mice with a single copy of the End gene were found to spontaneously develop clinical signs of HHT. To characterize onset, progression and mechanism of disease, we studied 171 End+/- mice for a minimum of 25 wks and a maximum of 52 wks. Mice who developed early onset of disease and died prior to 25 wks were also included. A diagnosis of HHT was made in 50 mice based on their End+/- genotype and at least one of these criteria: the presence of telangiectases on the ears, skin, tail or genitals, external bleeds from nose/mouth, ears, tail, genitals or intestine, and/or vascular abnormalities in viscera such as lungs, brain, liver and intestine. In 90% of cases, telangiectases were the first signs of disease while 52% of HHT mice experienced external bleeds (Figure 4.1a). Telangiectases on the ears, and bleeding from nose/mouth and ears were the most frequent external HHT signs in the End+/- mice (Figure 4.2). Disease severity was highly variable; some mice had a mild phenotype while others (32%) reached an agonal phase due to rupture of major vessels that caused fatal internal hemorrhage (Figures 4.1a and 4.2). 72% of the Ola mice observed for 41.8 ± 12.6 wks, 32% of B6, observed for 43.0 ± 8.9 wks, and 37% of B6m observed for 40.9 ± 15.0 wks developed HHT while only 7% of B6b did when observed for an even significantly longer time of 48.7 ± 14.3 wks (p=0.002). The age of onset was highly variable. It ranged from 1-37 wks in Ola, 14-43 wks in B6, 2-27 wks in B6m and 37-51 wks in B6b (Figure 4.1b). These data indicate an earlier onset and higher susceptibility to HHT in the Ola than in B6b suggesting that the 129/Ola background contributes disease modifier genes. This is further supported by intermediate disease prevalence and age of onset in intercrossed mice.

4.4.2 Gastrointestinal bleeds in End+/- mice with HHT.

To examine visceral involvement, 22/50 End+/- mice with life-threatening signs of HHT and/or at an advancing age were sacrificed. Direct microscopic examination of the small intestine mesenteric surface revealed a telangiectasia, seen as a network of dilated vessels, in an End+/- HHT mouse but not in the End+/- littermate control (Figure 4.3, a and b). Antibodies to endoglin
Figure 4.1 Prevalence and signs of HHT in End<sup>+/−</sup> mice.

Four groups of mice were analyzed: End<sup>+/−</sup>-mice backcrossed onto 129/Ola wild type (129/Ola wt; n=18; 100% Ola), intercrosses of End<sup>+/−</sup> C57BL/6 mixed 129/Ola (n=41; 50% B6:50% Ola), backcrosses to a parent of mixed genotype suspected of disease (n=54; on average 60% B6:40% Ola), and backcrosses onto wt C57BL/6 (n=58; 50-75% B6:50-25% Ola). These mice were observed for a period of at least 25 wks (except those dying of HHT prior to this period) and up to 52 wks. Of those mice, 50 developed HHT. (a) Graph representing the % of these 50 mice with HHT presenting with telangiectases or external bleeds as first signs of disease. The % of mice with HHT dying of the disease is also shown. (b) Graph representing the age of onset of HHT based on external signs in each group of End<sup>+/−</sup> mice. * p=0.004, ** p=0.0001 compared to 129/Ola backcrosses.
Figure 4.2 Autopsy findings in 22 End<sup>+</sup> mice with HHT.
The external and internal signs of HHT were systematically assessed for 22 of 50 mice. The percent C57BL/6 (B6) and 129/Ola genes (Ola) is shown for each animal. External signs such as telangiectases (red spot on skin or mucosal surfaces) and bleeding were examined daily and internal signs were determined at autopsy and by histologic analysis of liver, lungs, heart and brain. Tissue sections stained with H&E, Masson's trichrome or with antibodies to endoglin and PECAM-1 were examined. Various parameters were scored as present or absent. Additional important features concerning each animal are also noted.
<table>
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<th>Onset of HHT (wks)</th>
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<th>Internal signs</th>
<th>Others</th>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Telangiectases</td>
<td>Dilated vessels</td>
<td>Hemorrhage</td>
</tr>
<tr>
<td>54.78</td>
<td>F</td>
<td>0:100</td>
<td>36</td>
<td>O</td>
<td>X</td>
<td></td>
</tr>
<tr>
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<td>M</td>
<td>0:100</td>
<td>13</td>
<td>O</td>
<td>X</td>
<td></td>
</tr>
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<td>O</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>95.1</td>
<td>M</td>
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<td>35</td>
<td>O</td>
<td>X</td>
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</tr>
<tr>
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<td>7</td>
<td>O</td>
<td>X</td>
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**Intercrosses of N1 C57BL/6**

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**Backcrosses onto parent of C57BL/6-129/Ola mixed genome suspected of disease**

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**Backcrosses onto wt C57BL/6**

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**Legend:**

- **Ears**
- **Skin**
- **Nose/ mouth**
- **Genitals**
- **Gastrointestinal**
- **Tail**
- **Liver**
- **Lungs**
- **Heart**
- **Brain**
and PECAM-1, specifically stained endothelial cells of arteries, veins and capillaries in the submucosa in control and End<sup>−/−</sup> HHT mice (Figure 4.3, c-f). Abnormally dilated arteries and veins were noted within the submucosa and serosa of the HHT mouse (Figure 4.3, d and f). Levels of expression of endoglin and PECAM-1 were similar in vessels of the control intestine (Figure 4.3, c and e). However, in the HHT mouse, endoglin staining intensity was much reduced, compared to PECAM-1 (Figure 4.3, d and f). This lower level of endoglin on endothelial cells of End<sup>−/−</sup> mice confirms the expression of a single allele. We randomly tested 20/50 HHT mice for fecal occult blood in their stools and found 11 positive ones including mouse #441 described in Figures 4.2 and 4.3. Though we could not exclude that positive tests were caused by ingested nose, mouth or ear bleeds, there were no external bleeds observed in mouse #93.3 and 3 others with HHT (Figure 4.2 and data not shown). These findings demonstrate the presence of gut telangiectases and intestinal bleeds in some End<sup>−/−</sup> HHT mice.

### 4.4.3 Liver lesions in End<sup>−/−</sup> mice with HHT.

Upon autopsy, gross morphology revealed that 48% of End<sup>−/−</sup> HHT mice had liver abnormalities, including prominent vessels and/or telangiectases in one or several segments, hepatomegaly, or focal or severe hemorrhage (Figure 4.4a). However, histologic examination revealed 80% of dissected mice with vessel enlargement, hepatic congestion (increased red blood cells in the sinusoids of the liver) and/or hemorrhage (Figure 4.2). Endoglin was detected on endothelial cells of all types of vessels including central veins and sinusoidal endothelium in End<sup>−/−</sup> control mice as illustrated in Figure 4.4b. Liver sections from an End<sup>−/−</sup> HHT mouse showed dilated central veins when compared to control, and signs of hepatic congestion in a case of mild disease (Figure 4.4c). At lower magnification, a marked increase in the number of vessels was noticeable, especially in the subcapsular region in mild cases of disease (Figure 4.4e) compared to a littermate control (Figure 4.4d). Sinusoidal dilation and hepatocellular atrophy (gradual destruction of hepatocytes) were seen near the central veins in severe cases of HHT (Figure 4.4f).
Figure 4.3 Intestinal telangiectases in HHT mice.

(a) Microscopic examination of small intestine mesenteric surface showing normal ramification of vessels in an End<sup>+/+</sup> mouse. (b) In the HHT mouse (#44.1), a collection of abnormally dilated vessels forming a telangiectasia is shown (arrow). Cryosections were stained for endoglin (c and d) and PECAM-1 (e and f). (c) Endoglin is expressed on vessels of the submucosa (sm) in the normal mouse. (d) It is also seen on dilated vessels in the muscularis propria (mp) and the serosa (sr) in an End<sup>+-</sup> mouse. (e) PECAM-1 serves as a positive control for endothelial cells in normal vessels. (f) It is also expressed at normal levels on the dilated vessels of the End<sup>+-</sup> mouse. Bar: 1000µm (a and b), Bar: 200µm (c-f).
Figure 4.4 Dilation of vessels and focal hemorrhage in the liver of HHT mice.
Upon dissection, gross morphology was assessed (a) and cryosections of several lobes of liver were immunostained for endoglin (b-f). (a) Liver of mouse #44.1 with HHT showing focal sites of hemorrhage (arrows) and dilated vessels (arrowheads). (b) Liver of a control mouse showing endoglin expression on a vein and on normal sinusoidal endothelium. (c) In an animal with mild disease (#54.78), dilation of veins and congestion of the sinusoids disrupts the normal tissue organization. (d) Edge of a liver lobe showing normal lobular architecture with small vessels. (e) Liver of mouse #54.78 with congestion and increased number of vessels at the edge of a lobe. (f) In a more severe case of HHT (#91.10), more dilation of vessels, reduced endoglin staining and atrophy of hepatocytes are seen. Bar: 100μm (b and c), 500μm (d-f).
4.4.4 Vascular anomalies and hemorrhage in lungs of End<sup>−/−</sup> HHT mice

Pulmonary involvement was seen by gross morphological examination in 33% of sacrificed HHT mice while microscopic analysis revealed abnormalities in 50% of cases (Figures 4.2 and 4.5). Gross changes included dilated vessels, visible telangiectases and hemorrhages ranging in severity from focal to diffuse (Figure 4.5, a-c). Histologic sections demonstrated abnormally large vessels with increased thickness of the adventitial layer in lungs of End<sup>−/−</sup> HHT mice compared to littermate controls (Figure 4.5, d and e). At higher magnification, congestion of the alveolar capillaries was apparent (Figure 4.5, f and g).

4.4.5 Cerebral phenotype in End<sup>−/−</sup> HHT mice

Most of the End<sup>−/−</sup> HHT mice first showed external signs of disease between 7 and 43 wks, as described in Figure 4.2. Animals 77.5, 111.3 and 111.140 were exceptions as they seemed unwell and developed cephalic changes at 2-4 wks of age, prior to appearance of telangiectasia or external bleeds. These affected mice developed dome-shaped head, limb weakness, kyphosis, lethargy, drowsiness and emaciation (Figure 4.6a). Upon cranial exposure, severe subarachnoid hemorrhage was found, accompanied by an expanded calvarium and underlying brain with hydrocephalus (Figure 4.6b). Bilateral enlargement of the ventricles with thinning of the cerebral cortex is shown on a cross-section stained with α-smooth muscle cell actin (Figure 4.6c). Cortical atrophy was likely due to necrosis or apoptosis resulting from elevated intracranial pressure. A higher magnification shows normal smooth muscle cell distribution and endoglin expression on small cerebral vessels (Figure 4.6, d and e). A fourth case, among the 22 dissected End<sup>−/−</sup> HHT mice, experienced a subdural hemorrhage followed by hydrocephalus (Figure 4.2).

Other brain manifestations were seen in 45% of End<sup>−/−</sup> HHT mice after histologic examination (Figure 4.2). These included focal subdural, subgaleal, and subarachnoid hemorrhage as well as brain infarction. Two HHT mice (#95.1 and 75.71) had a stroke manifested by hemiplegia, facial flaccidity and ptosis. Brain histology revealed a localized subarachnoid hemorrhage with blood infiltration into the cortex, not seen in an unaffected
Figure 4.5 Pulmonary abnormalities in HHT mice.

Gross morphology of several lungs was assessed (a-c) and tissue sections were stained with Masson’s trichrome (d-g). (a) Pink colored lung of a healthy mouse. (b) Lung of HHT mouse #95.1 revealing a focal site of hemorrhage in the right upper lobe (arrow). (c) Lung of HHT mouse #91.10 with telangiectases (arrowheads) and severe hemorrhage. (d) Littermate control lung showing size of normal vessels relative to bronchi. (e) Dilated pulmonary arteries and veins in the lung of HHT mouse #75.73. (f) Littermate control illustrating normal lung architecture at higher magnification. (g) In mouse #75.73, congestion is noted by an increase in red blood cell number. Bar: 500μm (d and e), 100μm (e and f).
Figure 4.6 Cerebral lesions in mice with HHT.
(a) An End$^{+/}$ mouse (#111.140) showing signs of hydrocephalus. (b) Massive hemorrhage within the subarachnoid space is revealed upon lifting the skin over the cranium. (c) Cryosection immunostained with α-smooth muscle cell (SMC) actin shows severe bilateral dilatation of the ventricles with compression and atrophy of the surrounding brain. (d-e) Higher magnification view showing normal expression of SMC actin and endoglin in the cerebral vasculature. (f) Formalin-fixed section of a normal brain region from HHT mouse #75.71 (with a stroke), stained with Masson's trichrome adjacent to (g) an area with subarachnoid hemorrhage (arrows). (h) Higher magnification shows the abnormal vessels with fibrinoid necrosis and lymphocytic infiltration in the lesion of this HHT mouse. Bar: 1000μm (c), 500μm (d and e), 200μm (f and g), 50μm (h).
adjacent region (Figure 4.6, f and g). At higher magnification, lymphocytic infiltration and inflamed vessels with fibrinoid necrosis of the media were seen in the hemorrhagic area. A 3 fold thickening of adventitia and loosening of muscle bundles were also observed. The neuropile was vacuolated and early traces of infarction were seen (Figure 4.6h).

4.4.6 Cardiac changes in End⁺⁻ mice with HHT.

50% of the dissected End⁺⁻ HHT mice had cardiac abnormalities, ranging from mild to severe. At autopsy, 9/22 hearts were enlarged by 50-500% in HHT mice compared to littermate controls (Figure 4.2). Sections demonstrated biventricular hypertrophy with dilatation especially of the left atria, and of the coronary arteries (Figure 4.7). Organizing thrombi such as shown in Figure 4.7b were seen in the atrium of 4 mice with HHT. Ischemic regions associated with muscle necrosis were noted in 3 animals (Figure 4.7, a and b). Immunostaining of endoglin confirmed vascular hypertrophy seen in some animals and revealed large dilated coronary vessels (Figure 4.7, c and d).
Figure 4.7 Cardiac abnormalities in End<sup>+/−</sup> mice with HHT.

Tissue sections from End<sup>+/+</sup> and End<sup>+/−</sup> mice were stained with Masson’s trichrome (a-b) and anti-endoglin (c-d). (a) Normal heart from an End<sup>+/+</sup> mouse. (b) Abnormal heart from mouse #75.73, showing right and left ventricular hypertrophy, and left atrial enlargement with thrombus formation (arrow). (c) Coronary vessels stained for endoglin in a normal littermate control. (d) Dilation of coronary vessels in the heart of HHT mouse #75.2. Bar: 1000μm (a and b), 200μm (c and d).
4.5 Discussion

We have developed the first animal model of HHT. The mice in this model have a single copy of the End gene and show external signs and visceral involvement very similar to the human disease, supporting haploinsufficiency as the underlying cause of HHT. This murine model displays a phenotypic heterogeneity similar to that seen in human HHT, suggesting that additional genes contribute to the severity of this complex disorder.

Cutaneous telangiectases were found in 90% of End^{+/-} HHT mice and most frequently on the ears. 80-90% of people with HHT also have telangiectases, mostly on nasal and labial mucosae, and on skin of face and hands (286). Both murine and human telangiectases increase in size over time and can bleed easily (288). Gut telangiectases give rise to gastrointestinal bleeds in approximately 25% of older HHT patients (233) and can lead to severe anemia, often requiring transfusions (232). The prevalence of GI bleeding was difficult to assess in the HHT mice because of the low sensitivity of the guaiac test employed. Nevertheless, fecal blood was found in some mice as early as 9 wks, and correlated with the presence of microscopic telangiectases on the intestinal surface. Hematocrit values were normal even in older mice with HHT, unlike in elderly human patients where intestinal bleeds lead to chronic anemia.

Liver abnormalities were seen in 80% of HHT mice. This high prevalence is based on histological examination. In human HHT, clinical liver involvement is seen in one third of patients (245, 289). The hepatic congestion, increased number of blood vessels, and dilated sinusoids and larger vessels observed in mice have been described in patients with HHT (290, 291). We were unable to find in End^{+/-} HHT mice cirrhosis, irregular portal tracts, fibrosis, and regenerative nodules reported in some patients with liver involvement (290-292). Liver AVMs in HHT patients have been diagnosed on the basis of a large shunt through the hepatic circulation and are seen in <20% of patients (245). Most patients with clinically detected shunts in fact have numerous small liver telangiectases but there might very well be patients with fewer liver telangiectases who remain undiagnosed due to small shunts. The murine model suggests that liver involvement may be underestimated among HHT patients. However, it should be mentioned that the majority of wild type 129/Ola mice have reduced numbers of peripheral
vessels in liver and lung and were shown to exhibit natural hepatic shunts (283, 284). This might in part explain the higher susceptibility of this strain to hepatic manifestations of HHT.

Lungs are frequently affected in HHT, with more than 30% of patients developing pulmonary AVMs. These range from diffuse telangiectases to large AVMs, and are predominantly located in the lower lobes. The right-to-left shunt can give rise to significant hypoxemia and permit the passage of emboli, leading to cerebral infarction or abscess (230). Although pulmonary AVMs were not directly visualized in End+/- HHT mice, multiple abnormally dilated vessels along with focal and diffuse hemorrhage mostly on the ventral aspect of the upper lobes were found in 50% of these animals, strongly suggesting that vascular malformations were indeed present. These findings suggest that pulmonary lesions tend to develop more often in the dependent areas of both affected human (lower lobes) and murine lungs (upper lobes). Several End+/- HHT mice had difficulty breathing, manifested by tachypnea and marked inspiratory effort. This respiratory distress was likely related to hypoxia, secondary to a right-to-left shunt.

Intracranial hemorrhage was observed in approximately 30% of End+/- HHT mice. 3/22 mice had a subarachnoid hemorrhage at 2-4 wks of age, prior to any external sign of HHT. Similarly, in humans, fatal hemorrhage due to rupture of a cerebral AVM has been reported in newborn infants and children (235, 236). Hydrocephalus seen in 4 mice, has been reported in only one human case of HHT (293). As intrathecal injection of TGF-β1 in C57BL/6 mice was shown to induce hydrocephalus (294), we cannot exclude the possibility that it was caused in our mice by an accumulation of TGF-β1 in cerebral spinal fluid, subsequent to a dysregulation caused by reduced endoglin expression. However it is more likely secondary to the massive subarachnoid hemorrhage, as documented for several human cases (295, 296). Cerebral manifestations of HHT in two older mice included hemorrhagic stroke with associated neurological deficits, including paralysis, tics, and seizures. These are reminiscent of neurological defects observed in human HHT with cerebral involvement (235, 238).

Pulmonary and hepatic congestion were observed concurrently in 9/22 End+/- HHT mice along with hypertrophy of the myocardium suggesting that congestive heart failure had occurred
(297). The cause of cardiac hypertrophy could not be determined with certainty, but evidence of multiple myocardial infarcts in HHT mice indicated that ischemic injury was involved. Ischemia might have occurred via two different mechanisms. First, liver vascular anomalies can lead to high output heart failure. Second, coronary vessels were abnormally dilated, suggesting that impaired heart perfusion may have contributed to tissue ischemia and infarction. Atria were dilated and often contained visible thrombi, which may have resulted from turbulent blood flow within the abnormally dilated atria, and/or from regurgitant blood flow across the mitral and tricuspid valves following ventricular dilatation (298). Together with pulmonary vascular anomalies, atrial thrombi may have caused cerebral infarction (299), observed in at least 4 HHT mice, but possibly occurring more frequently.

Our findings suggest two major causes of death in ENtH/ HHT mice. One is massive hemorrhage occurring after rupture of a major vessel, including the abdominal aorta, renal artery, and cerebral arteries. The other major cause of death is chronic congestive heart failure, occurring as a result of high output failure due to pulmonary, hepatic or coronary vascular abnormalities.

Although direct connections between larger veins and arteries (AVMs) were not detected in mice with HHT, the multiple vascular anomalies and their sequelae suggest that they might be present. These would not have been detected in our studies by microscopic examination of random histologic sections. Imaging techniques, such as computed tomography scan and angiography, are needed to detect AVMs in HHT mice. In humans, histology of AVMs reveals dilated, tortuous and thin walled vessels with variable thickness of smooth muscle cells and disorganized adventitia (253). Active angiogenesis, and ongoing vascular remodeling are associated with these lesions and would be expected in affected murine vessels. An increased number of blood vessels was observed in the liver of several HHT mice and dilated vessels were seen in almost every organ. Some of these enlarged vessels showed dispersed smooth muscle cell nuclei and increased adventitial thickness indicative of remodeling. Improper interactions between intimal, medial and adventitial layers of vessels are the likely cause of vascular enlargement and damage. TGF-β1 is known to regulate the interaction between endothelial cells
and both mesenchymal and smooth muscle cells of the vessel wall (4, 18). A deficiency in endoglin, which modulates several responses to TGF-β1 (10), could lead to destabilization of cellular interactions in the vessel wall and generation of vascular anomalies.

The observations that 72% of End+/− generated by backcrosses onto wild type 129/Ola have HHT, while only 7% of End+/− backcrossed to wild type C57BL/6 mice develop disease, strongly suggest that additional genes from the 129/Ola strain contribute to disease severity. This is further supported by an intermediate disease prevalence in mice with a mixed genome generated by inter-breeding such as End+/− B6, (50% B6 : 50% Ola) and End+/− B6m (on average 60% B6: 40% Ola). Thus, a single endoglin allele may not be sufficient to cause disease, since a large proportion of End+/− mice do not display clinical signs, when observed for one year. Our data suggest that HHT is a genetically complex trait, influenced by multiple segregating loci and environmental factors. Thus, the co-inheritance of endoglin and other genes, referred to as modifier genes, is necessary for generation of the vascular lesions associated with HHT. It is reasonable to speculate that distinct modifier genes might contribute to liver, lung and brain involvement in HHT. Modifier genes which modulate phenotypic outcome in a strain-dependent manner have been reported for several genes including TGF-β1 (300).

Murine HHT mimics the human disorder and provides an excellent model for studying pathogenesis and novel therapeutic approaches. We have demonstrated that endoglin is essential for maintenance of vascular homeostasis as expression of a single allele can lead to abnormal vessels. Furthermore, the murine model suggests that disease heterogeneity is due to modifier genes that contribute to the severe clinical manifestations of HHT. Identification of these modifier genes by quantitative trait linkage analysis should increase our understanding of the molecular and physiological mechanisms leading to abnormally dilated vessels and associated vascular lesions.
4.6 Acknowledgments

We thank S. Kaw, M.-L. McDonald for technical assistance and L. Morikawa, for invaluable help in tissue section preparation. We are grateful to the staff of the Lab Animal Services at the Hospital for Sick Children who helped in the daily assessment of the mouse HHT phenotype. We thank Dr. M.G. Sirois for allowing use of microscopes and computers for data analysis. This research was supported by grants #NA3434 from the Heart and Stroke Foundation of Ontario and #MT-6247 from the Medical Research Council of Canada (ML). AB is a recipient of a studentship from the Medical Research Council of Canada. MF is supported by the Nelson Arthur Hyland Foundation and the Squires Club of Toronto. ML is a Terry Fox Research Scientist of the National Cancer Institute of Canada.
CHAPTER FIVE

Endoglin Heterozygous Mice show B and T cell defects

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This study was initiated from a preliminary experiment with Dr. Yeung revealing that End\(^{wt}\) splenocytes were hypereactive to SEB stimulation. I then undertook the characterization of the potential immune defects in End\(^{ht}\) mice and performed all experiments for the analysis of thymocytes and peripheral T and B cells as well as immunohistochemistry on the spleen. Mr. Trop contributed to all experimental design, isolated thymocytes subsets to study endoglin expression and helped with the flow cytometry analysis of populations. Ms McDonald performed immunohistochemistry on the thymus while Drs Lu, Wu and Zuniga-Pflucker are involved in the ongoing analysis of the bone marrow phenotype. This project was supervised by Dr. Letarte.

5.1 Abstract

Endoglin is a member of the TGF-β receptor superfamily expressed at high levels on vascular endothelium but also found in several hematopoietic cells in humans. As expression of a single Endoglin allele leads to a vascular disorder in both human and mouse, we investigated if this defect altered the immune system of End<sup>-/-</sup> mice. We first show that endoglin is induced via pre-T cell receptor signaling at the CD4<sup>+</sup>CD8<sup>-</sup> to CD4<sup>+</sup>CD8<sup>+</sup> transition, and remained at low levels on mature T cells in both End<sup>+/-</sup> mice and littermate controls. Thymic cellularity was decreased by 3-fold in End<sup>-/-</sup> mice but the distribution of CD4/CD8 subsets was normal. Histologic examination of thymic sections revealed abnormally dilated blood vessels and a decreased number of capillaries in End<sup>-/-</sup> mice. Endoglin was not detectable on peripheral B cells. Splenic cellularity was normal in 6-16 wk old End<sup>+/-</sup> mice, but the CD4/CD8 ratio progressively increased starting at 8 wks. A 2.5 fold increase in both T and B cells was observed by 18 wks. There was also an increased number of B cells with an IgM<sup>+</sup> I-A<sup>b</sup> CD24<sup>int</sup> phenotype, characteristic of cells in transition from immature to mature stages. Peripheral T and B cells from End<sup>-/-</sup> mice were hyper-responsive to stimulation with SEB and LPS, when compared to littermate controls. Flow cytometry analysis revealed that endoglin was present on 3% of bone marrow cells and that distribution of hematopoietic subsets was normal in End<sup>+/-</sup> mice. Our results suggest that reduced expression of endoglin on vascular endothelium and/or hematopoietic cells is responsible for the observed immune defects. Endoglin, likely via modulation of TGF-β1 effects, is thus necessary for T and B lymphocyte homeostasis.
5.2 Introduction

Endoglin (CD105) is known as an endothelial cell marker (6), which plays a crucial role in vascular development (158, 159, 287). End null mice, fail to undergo vessel maturation and die at ED10.0 of massive hemorrhage (287). Consequently, endoglin function in hematopoiesis was difficult to assess. However, the expression of endoglin on several subsets of human hematopoietic cells suggests a possible role in the development of the immune system. Endoglin is present on pre-B ALL and AML (13, 207). In fetal bone marrow, endoglin is found on early B-lineage progenitors (CD19+, CD34+) and pro-erythroblasts (CD71+, glycophorin A+) (14, 15). In adult bone marrow, it is on pro-erythroblasts (15) and not on B, T, NK, and myeloid cells (14). Endoglin is also found on stromal cells derived from fetal and adult bone marrow (15, 208). In peripheral blood, endoglin is absent from human B and T cells and from monocytes. However, endoglin is up-regulated upon activation of monocytes in culture, and is present on circulating and tissue macrophages (16, 209).

A more recent study reported the expression of endoglin (CD105) in 20-35% of circulating CD34+ progenitors (210). The CD34+ cells were capable of restoring hematopoiesis in autologous and allogeneic recipients after myeloablation (211, 212). The CD34+ CD105+ population was enriched in proliferating progenitors with high cloning efficiency and LTR potential (210, 213). Endoglin is thus expressed in early human hematopoietic development. The CD34+CD105+ population was shown to produce autocrine TGF-β1, a property which was lost in CD34+ CD105− precursors (301), suggesting that the presence of endoglin was required for TGF-β1 responsiveness.

Endoglin is a component of the TGF-β1/β3 receptor complex, known to modulate several responses to these ligands (8, 10, 195). Since we demonstrated that a single allele of endoglin can lead to the vascular disorder Hereditary Hemorrhagic Telangiectasia in human and mouse, we hypothesize that it may also dysregulate development and function of hematopoietic cells and alter immune responses. We first determined the expression pattern of endoglin in murine lymphoid tissues. We analyzed hematopoietic subset distribution in thymus, spleen and bone marrow. We estimated the total cell number and the tissue architecture of thymus and
spleen and measured the mitogenic response of T and B cells in *endoglin* heterozygous mice. We demonstrate that mice with a single functional *endoglin* allele show impaired homeostasis in thymus and peripheral lymphoid organs.
5.3 Materials and Methods

5.3.1 Mice

For isolation of thymocyte mRNA for RT-PCR, timed-pregnant female CD1 mice were purchased from Charles River Canada (Saint-Constant, QC, Canada), and fetuses were obtained at day 14 of gestation. RAG-2" CD45.1 congenic mice (302) were kindly provided by Dr. P. Poussier (University of Toronto, Toronto, ON, Canada). End"/" mice were generated by homologous recombination using ES cells of 129/Ola origin (287). The first backcrosses (N1) were produced by mating male chimeras with wild type 129/Ola (Harlan UK, Bicester, United Kingdom) and C57BL/6 (Taconic Farms, Germantown, New York, USA) females. End"/" and End"/" 129/Ola mice are inbred, and thus have 100% 129/Ola genes. End"/" and End"/" C57BL/6 mice, produced by backcrosses (N1 and N2) and intercrosses (F1 to F3) have a mixed genome, ranging from 50% to 87.5% C57BL/6 genes, and 50% to 12.5% 129/Ola genes. Mice were genotyped by β-gal staining and multiplex PCR as described in chapters 3 and 4. Mice without external signs of HHT were used in the current study. Littermate End"/" mice were used as controls. Mice were kept in ventilated racks in a germ-free facility, and all protocols were approved by the Ethics Committee of the Hospital for Sick Children Lab Animal Services.

5.3.2 Flow Cytometry

FITC-, PE-, APC- or biotin-conjugated antibodies were purchased from Pharmingen (Mississauga, ON, Canada). Flow cytometry was performed as previously described (303). For detection of apoptosis, single cell suspensions were prepared from the thymus of End"/" or End"/" C57BL/6 mice, and stimulated with immobilized anti-CD3ε mAb (5 µg/ml) for 24 h, or left untreated. Cells were analyzed for surface expression of CD4 and CD8, and for annexin binding to exposed phosphatidylserine using the FACS Annexin V-FITC apoptosis detection kit (R&D Systems, Minneapolis, MN). Data analysis was performed using the CELLQuest software (Becton Dickinson, San Jose, CA); where appropriate, live cells were gated by size and lack of propidium iodide uptake.
To obtain purified subsets of double negative (CD4-CD8-;DN) thymocytes, single cell suspensions from day 14 CD1 fetal thymic lobes were stained with anti-CD25-FITC, anti-CD117-PE and biotinylated lineage markers (CD3ε, CD4, CD8, B220, Gr-1, Mac-1, pan-NK cell), followed by APC-conjugated streptavidin. Purified double positive (DP) and single positive (SP) thymocytes were obtained by staining single cell suspensions from adult CD1 thymus with anti-CD4-FITC, anti-TCRβ-PE and anti-CD8-APC; DP cells were defined by a CD4+CD8+TCRβint phenotype, and SP cells were defined by a CD4+CD8 TCRβhi or CD4+CD8+TCRβhi phenotype. For isolation of RAG-deficient thymocytes, newborn RAG2−/− mice were injected intraperitoneally with anti-CD3ε mAb (Pharmingen), and thymocytes were harvested at day 10 after birth. Injections were given 36 h or 72 h prior to harvest to obtain CD25lo or immature SP (ISP) and DP thymocytes, respectively (304). Cells were sorted using a Coulter Elite flow cytometer (Coulter Electronics, Montréal, QC, Canada); in all cases, sort purity was >99%.

5.3.3 RT-PCR

Total RNA was prepared from purified thymocyte populations using the RNeasy Mini kit (QIAGEN, Mississauga, ON, Canada). cDNA was produced with the Omniscript RT kit (QIAGEN) using random hexamers. The cDNA was amplified for β-actin and endoglin using reported primers (214). PCR was performed as described (287).

5.3.4 Immunohistochemical staining

Spleens and thymi from End−/− and littermate control mice were immediately embedded in OCT and frozen on isopentane/dry ice. Cryosections (7μm) were obtained from the Pathology Department, Hospital for Sick Children, Toronto. Sections were dried, fixed 10 min in acetone, washed briefly in Tris-buffered saline-T 0.01 mol/L Tris, pH 7.4, 0.16 mol/L NaCl, 0.2% Tween 20 (TBS-T), dipped 5 sec in 0.1N HCl to remove the endogenous alkaline phosphatase and washed thoroughly in TBS-T. Sections were then blocked with 5% normal rabbit serum (Dako, Mississauga, ON, Canada) in TBS-T for 20 min, washed, blocked sequentially with Avidin and
Biotin solutions (Vector Labs) for 20 min and washed. Tissues were then incubated at 4°C for 2 h with optimal concentrations of primary antibody. These were mAb JC7/18 to endoglin (CD105; 2µg/ml, Pharmingen), mAb RA3-6B2 to CD45R/B220 (3µg/ml, Pharmingen), mAb RM4-5 to CD4 (3µg/ml; Pharmingen). Slides were washed and incubated for 1 h at 4°C with biotinylated rabbit anti-rat IgG (diluted 400-fold; Vector Labs). The streptavidin-alkaline phosphatase amplification system (StreptABC/AP, Dako) was used. The enzymatic reaction was performed as described (253) and sections were counterstained with 5% neutral red (Sigma, Mississauga, ON, Canada). Thymus and spleen sections were stained with hematoxilin & eosin (H&E) to assess morphology.

5.3.5 Proliferation Assay

Spleen cell suspensions were plated onto 96-well plates in Iscove's modified Dulbecco's medium supplemented with 10% FCS, 5 x 10^{-5} M β-mercaptoethanol, and 0.01% penicillin/streptomycin. For stimulation with Staphylococcus enterotoxin B (SEB), 5 x 10^5 cells per well were stimulated with the indicated concentration. For stimulation with Phorbol 12-myristate 13-acetate (PMA) and ionomycin, 1 x 10^5 cells per well were cultured with the indicated concentration of PMA and 0.5 μM ionomycin, with or without IL-2. For stimulation with Lipopolysaccharides (LPS), 1 x 10^5 cells were incubated with the indicated concentration, for 1 to 4 days, as indicated. Cells were pulsed with 1 μCi[^3]H-thymidine for 16 h, and incorporation was quantified. All proliferation assays were done in triplicate wells.
5.4 Results

5.4.1 Endoglin is expressed on T but not B cells

Endoglin is expressed by several human hematopoietic precursors, suggesting it might play a role during the early stages of lymphocyte development. However, little is known about its pattern of expression in murine lymphoid tissues. To determine whether endoglin is expressed during T cell development, we fractionated thymocytes according to CD4 and CD8 expression. The more immature CD4CD8⁻ subset was further subdivided on the basis of CD25 and CD117 expression into DN₁ (CD25⁺CD117⁻), DNII (CD25⁺CD117⁺), DNIII (CD25⁺CD117⁻) and DNIV (CD25⁺CD117⁻). RT-PCR analysis of these subpopulations showed that endoglin is first expressed at the DNIII stage, and is then present on the subsequent stages of T cell maturation (Figure 5.1a).

The transition from the DNIII to the DNIV stage is known to be regulated by signaling through the pre-T cell receptor (pre-TCR) (305). To determine whether endoglin expression might be regulated by pre-TCR-derived signals, we used the RAG-deficient mouse model. In these mice, thymocyte development is arrested at the DNIII stage due to an inability of the cells to rearrange their TCR genes. Pre-TCR signals can be mimicked by stimulation with anti-CD3ε mAb. Following CD3 crosslinking, RAG-deficient thymocytes gradually lose CD25 expression (CD25⁻) as they mature to the DNIV stage. Subsequently, they acquire expression of CD4 and CD8 to become CD4⁺CD8⁻ or CD4⁺CD8⁺ immature single-positive (ISP) thymocytes and, ultimately, CD4⁺CD8⁺ (DP) thymocytes. Endoglin expression was assayed on each of these subsets by RT-PCR (Figure 5.1b). Whereas endoglin was undetectable in unstimulated RAG⁻ thymocytes (CD25⁻), it was rapidly up-regulated in CD25⁺ thymocytes, immediately following stimulation with anti-CD3ε mAb. These data show that endoglin expression is regulated by pre-TCR signaling during T cell development.

Endoglin expression was confirmed by flow cytometry in DP and SP thymocytes of healthy End⁺⁺ and End⁺⁻ C57BL/6 mice (Figure 5.1c). A decreased level of endoglin was noted in End⁺⁻ mice, reflecting the absence of a functional allele. We also examined endoglin expression in peripheral T and B cells in lymph nodes and spleen and found that it remained
Figure 5.1 Endoglin expression in T and B lymphocyte lineages.

(a) Thymocytes from CD1 fetal thymic lobes or adult thymus were fractionated according to surface expression of CD25 and CD117 (DN-I-DNIV), or CD4 and CD8 (DP and SP), as described in the Experimental Procedures. cDNAs were prepared from total RNA, and analyzed for expression of endoglin and β-actin mRNA by RT-PCR. As control (H₂O), RT-PCR reactions lacking template were amplified simultaneously. (b) Newborn RAG-2⁻/⁻ mice were injected intraperitoneally with anti-CD3ε mAb to obtain CD25⁺, ISP and DP thymocytes. CD25⁺ thymocytes were obtained from untreated RAG-2⁻/⁻ mice. Endoglin and β-actin expression was analyzed by RT-PCR. (c) Single cell suspensions were prepared from thymus, lymph nodes and spleen of End⁺/+ mice (thin line) and End⁻/⁻ mice (thick line), stained for surface expression of CD4, CD8, B220, and endoglin (CD105) as indicated, and analyzed by four-color flow cytometry. As negative control (dashed line), cells stained with an isotype control IgG were analyzed.
present at low levels at the surface of peripheral CD4 and CD8 T cells. The levels of expression were again decreased in End<sup>−/−</sup> mice relative to littermate controls. Endoglin could not be detected at the surface of mature B cells (Figure 5.1c).

5.4.2 Thymic cellularity is decreased in End<sup>−/−</sup> mice

Since endoglin is expressed in the thymus, we assessed by flow cytometry the frequency of thymic lymphocyte subsets in End<sup>+/+</sup> and End<sup>−/−</sup> mice (Figure 5.2a). No significant differences were noted in the distribution of CD4 and CD8 thymocytes between these mice. Thymic cellularity was determined in two strains of mice, 129/Ola and C57BL/6, expressing one or two functional endoglin alleles (Figure 5.2b). In End<sup>−/−</sup> mice of both strains, thymi were smaller compared to littermate controls. The absolute number of thymocytes within each subset (CD4<sup>+</sup>CD8<sup>−</sup>, DN; CD4<sup>+</sup>CD8<sup>+</sup>, DP; CD4<sup>+</sup>CD8<sup>−</sup> and CD4<sup>+</sup>CD8<sup>+</sup>; SP) was reduced by 3-fold. This ratio was maintained for mice ranging from 6 to 24 wks of age. The decreased number of thymocytes in End<sup>−/−</sup> mice could not be explained by increased frequency of apoptotic cells, as measured by staining with propidium iodide (PI) and annexin V, in resting T cells, and following TCR stimulation by anti-CD3 mAb-mediated crosslinking (data not shown). Cell cycle analysis was normal in End<sup>−/−</sup> mice indicating that reduced thymic cellularity was not due to increased apoptosis or altered cell proliferation kinetics.

Histologic examination of thymic sections revealed normal thymic architecture in End<sup>−/−</sup> mice (Figure 5.3). However, immunostaining of endoglin showed abnormally dilated blood vessels at the cortico-medullary junction and a decreased number of capillaries in both cortex and medulla. Those vessels expressed lower levels of endoglin than the End<sup>+/+</sup> controls. Endoglin was detected on thymocytes but at very low levels compared to the endothelium. Thus, in the presence of a single functional endoglin allele, the thymic microvascular environment is altered and lymphoid cellularity is decreased while a normal CD4/CD8 ratio is maintained.
Figure 5.2 Thymic defects in End⁺⁻ mice.
(a) Single cell suspensions were prepared from thymi of 11 wk-old 129/Ola and 10 wk-old C57BL/6 End⁺⁺ and End⁺⁻ mice. Cells analyzed for surface expression of CD4 and CD8 by three-color flow cytometry, showed a normal distribution. (b) Single cell suspensions were prepared from thymi of End⁺⁺ (white circles) and End⁺⁻ (black circles), 6 to 16 wk-old 129/Ola mice (n=2), 6 to 16 wk-old C57BL/6 mice (n=10) and 18 to 24 wk-old C57BL/6 mice (n=4). The frequency of each thymocyte subset was determined by surface staining of CD4 and CD8, followed by three-color flow cytometry analysis. Mean cellularity is indicated by a horizontal line. Comparison of End⁺⁺ and End⁺⁻ cells in each group revealed a significant difference with a p-value < 0.006 except for CD4⁻CD8⁻ 129/Ola thymocytes (p = 0.06).
Figure 5.3 Dilated blood vessels in the thymus of $End^{+/+}$ mice.

Histological staining with H&E of thymic sections from 10 wk old C57BL/6 $End^{+/+}$ and $End^{+/–}$ mice showing normal cortical (c) and medullary (m) areas. Immunostaining of sections with antibodies to endoglin revealed abnormally dilated vessels (arrows) at the cortico-medullary junction and a much reduced density of capillaries in the $End^{+/–}$ mice. Magnification 20X.
5.4.3 Altered CD4/CD8 ratio in the spleen of End⁻/⁻ mice

To investigate if thymic defects lead to abnormal peripheral T cells, we analyzed the frequency of CD4 and CD8 subsets in spleens of End⁺/+ and End⁺⁻ C57BL/6 mice at 8, 12, 16 and 20 wks (Figure 5.4). We noted a progressive decrease in the percentage of T cells, with a corresponding increase in non-T cells. Whereas the percentage of non-T cells in End⁺/+ mice remained constant at these ages (60% ± 5%), their absolute number increased 1.5-fold in End⁺⁻ mice over the age of 12 wks. The ratio of CD4 to CD8 T cells was also altered in End⁺⁻ mice. At 8 wks, this ratio was normal (2.3 and 7.2) in spleens of End⁺/+ and End⁺⁻ and remained constant with increasing age in End⁺/+ mice. However, the percentage of CD8 T cells decreased over time in End⁺⁻ mice, such that the CD4/CD8 ratio in these animals was on average 2.9, after 12 wks. The decrease in the number of CD8 T cells was not due to abnormal function, since CD8 T cells from End⁺/+ and End⁺⁻ mice could lyse P815 target cells with equal efficiency (data not shown). Thus, in mice expressing reduced levels of endoglin, the number of CD8 T cells in the periphery is decreased with respect to the number of CD4 T cells, but the cytotoxic function of CD8 T cells is preserved.

5.4.4 Age-dependent changes in peripheral T and B lymphocytes in End⁺⁻ mice

We next examined the total spleen cellularity in mice ranging from 6 to 24 wks. Lymphocyte counts were similar in 129/Ola and C57BL/6 mice aged 6-16 wks. A mean 2.5-fold increase was noted in End⁺⁻ C57BL/6 mice, aged 18-24 wks, when compared to littermate controls. This increase was due to an expansion of both CD4 and CD8 T cells (2.3-fold and 2-fold increase, respectively), and to proliferation of B lymphocytes (2.4-fold increase) (Figure 5.5a). We next examined the splenic architecture by immunostaining of cryosections with antibodies to endoglin, CD4 and B220 (Figure 5.5b). In End⁺⁻ mice, endoglin was expressed at high levels especially on venous sinuses within the red pulp, and on the central arterioles and capillary network of the white pulp. The expression pattern was similar in End⁺⁻ mice, but the levels of expression were clearly reduced on all blood vessels. In End⁺/+ mice, T cells were present at the
Figure 5.4 Abnormal CD4/CD8 ratio in the spleen of End\textsuperscript{+/-} mice.

Cell suspensions were prepared from the spleen of 8, 12, 16 and 20 wk-old C57BL/6 End\textsuperscript{+/-} mice and littermate controls. Cells were analyzed for surface expression of CD4 and CD8 by three-color flow cytometry. Percentages of cells in each quadrant are shown.
Figure 5.5 The absolute number of T and B lymphocytes is increased in the spleen of End<sup>+/−</sup> mice 18 wks and older.

(a) Single cell suspensions were obtained from the spleen of End<sup>+/+</sup> (white circles) and End<sup>+/−</sup> (black circles), 6 to 16 wk-old 129/Ola mice (n=3), 6 to 16 wk-old C57BL/6 mice (n=11) and 18 to 24 wk-old C57BL/6 mice (n=5). The frequency of each lymphocyte subset was determined by staining for CD4, CD8 and B220, followed by four-color flow cytometry analysis. Mean cellularity is indicated by a horizontal line. Comparison of End<sup>+/+</sup> and End<sup>+/−</sup> cells in the C57BL/6 18-24 wks revealed a significant difference with a p-value < 0.004 in all subsets.

(b) Frozen sections were prepared from the spleen of 18 wk-old C57BL/6 End<sup>+/+</sup> and End<sup>+/−</sup> mice, and immunostained for endoglin, CD4 or B220, as described in Materials and Methods. Magnification 20X.
periphery of B cell follicles. An increased number of T cells was observed in *End*<sup>−/−</sup> mice, with infiltration into the red pulp. B cells were also present in increased numbers and spread outside the boundary of the B cell follicles into the red pulp (Figure 5.5b). Thus, a reduction in the level of endoglin expression on spleen vessels was observed as well as an age-dependent expansion of lymphocyte populations, leading to abnormal B and T cell zones within the follicles.

**5.4.5 Age-dependent B cell defects in *End*<sup>−/−</sup> mice**

We analyzed the distribution of B cell subsets in spleens of *End*<sup>−/−</sup> mice at 8 wks of age, when cellularity was normal, and at 20 wks, when B and T cells were present in increased numbers. The frequency of immature and mature B cells was assessed from the surface expression of IgM and B220 on splenocytes from *End*<sup>+/+</sup> and *End*<sup>−/−</sup> mice (Figure 5.6a). At 8 wks of age, both groups of mice showed identical frequencies of B220<sup>+</sup>IgM<sup>+</sup> cells. In contrast, at 20 wks of age, *End*<sup>−/−</sup> spleens showed a 2 to 2.5 fold increase in B220<sup>+</sup>IgM<sup>+</sup>-I-A<sup>+</sup> cells compared to control, as illustrated in Figure 5.6a. Moreover, the mean fluorescence intensity for IgM was higher, suggesting a more immature phenotype. The IgM<sup>+</sup> B cells over represented in *End*<sup>−/−</sup> mice all express MHC class II (Figure 5.6a).

To further characterize the subsets of B cells in *End*<sup>−/−</sup> mice, we examined the expression of CD24 (heat-stable antigen) on B220<sup>+</sup> cells (Figure 5.6b). In both young and old *End*<sup>−/−</sup> mice, three populations could be distinguished: CD24<sup>lo</sup>, CD24<sup>int</sup> and CD24<sup>hi</sup>, corresponding to mature, immature and transitional B cells, respectively. Although young *End*<sup>−/−</sup> mice also possessed these three distinct populations at a frequency similar to the littermate control, most B cells in old *End*<sup>−/−</sup> mice showed an abnormal CD24<sup>int/hi</sup> phenotype, with a small subset of CD24<sup>hi</sup>. These results were also confirmed in *End*<sup>−/−</sup> mice of 129/Ola background. High levels of IgM and intermediate to high levels of CD24 suggest accumulation of B cells in transition from immature to mature stages of development (Figure 5.6c). A similar B cell phenotype was observed in lymph nodes of *End*<sup>−/−</sup> mice.
Figure 5.6 Immature B lymphocytes accumulate in the spleen of End^{+/−} mice from 18 wks.
(a) Cell suspensions were prepared from the spleen of 8 and 20 wk-old C57BL/6 End^{+/−} mice
and littermate controls, and analyzed for surface expression of B220, and IgM or I-A^b, by three-
color flow cytometry analysis. Percentages of cells in each quadrant are shown. (b) Splenocytes
from 8 and 20 wk-old C57BL/6 End^{+/−} mouse (thick line) and littermate control (thin line) were
analyzed for surface expression of B220 and CD24 by three-color flow cytometry. The CD24
profile of the B220^+ population is illustrated. (c) Scheme of late stages of B cell development
showing the modulation of several surface markers. Adapted from reference 308.
5.4.6 Increased Proliferation of T and B cells in End<sup>−/+</sup> mice

To determine if T cells of End<sup>−/+</sup> mice were more sensitive to superantigens and mitogens, spleen cell suspensions were treated with *Staphylococcus* enterotoxin B (SEB) or Phorbol 12-myristate 13-acetate (PMA) plus ionomycin. The I-E negative mouse strains, which include C57BL/6 and 129/Ola, are low responder strains to SEB, as shown in Figure 5.7a. After 3 days in culture, a 3-fold increase in the response to SEB (0.1μg/ml) was seen in the End<sup>−/+</sup> mice compared to End<sup>+</sup> mice. Dose response curves revealed higher proliferation at all concentrations. This heightened response was seen in both 129/Ola and C57BL/6 End<sup>−/+</sup> young mice (Figure 5.7b). When spleen cells were stimulated with PMA, or PMA plus ionomycin and IL-2, no difference was observed between End<sup>−/+</sup> mice and End<sup>+</sup> mice (Figure 5.7c).

The ability of B cells to respond to mitogenic stimulation by Lipopolysaccharides (LPS) was tested. A significantly higher response was observed in the End<sup>−/+</sup> mice after 3 or 4 days in culture, and at all doses tested (Figure 5.7d). The increased mitogenic response to LPS was seen in 129/Ola and C57BL/6 End<sup>−/+</sup> young mice as well as in older C57BL/6 mice (Figure 5.7e).

5.4.7 Normal distribution of progenitors in bone marrow of End<sup>−/+</sup> mice

Because of the abnormalities seen in splenic B cells, we compared the distribution of hematopoietic cells in the bone marrow of End<sup>−/+</sup> mice to that of End<sup>+</sup> littermate controls (Figure 5.8a). Bone marrow cells were analyzed for CD43, a marker of myeloid lineage cells and of pro-B lymphocytes and for CD24 which stains both myeloid and lymphoid cells. The percentage of CD43<sup>+</sup>CD24<sup>+</sup> cells was similar (57-58%) in End<sup>−/+</sup> and End<sup>+</sup> mice and represented mostly cells of myeloid origin. The CD43<sup>−</sup>CD24<sup>+</sup> which are mostly lymphoid cells, constituted 35% of the bone marrow cells in both End<sup>−/+</sup> and End<sup>+</sup> mice. The proportion of myeloid and lymphoid populations was confirmed using CD11b and Gr-1, which are both myeloid lineage markers. Thus there is no imbalance in the number of bone marrow progenitors in End<sup>−/+</sup> mice.

As endoglin is expressed on several subsets of human hematopoietic cells, we analyzed its expression in murine bone marrow (Figure 5.8b). Endoglin was present at low levels on 2 to 3% of bone marrow hematopoietic cells in both End<sup>−/+</sup> and End<sup>+</sup> mice. Endoglin positive
(CD105*) cells were within the Sca-1- population, indicating that they are not hematopoietic stem cells. Half of CD105* cells were CD117* suggesting early progenitors; the CD117- cells would represent more committed cells. Since 75% of CD105* cells were CD43*, it suggests that both myeloid and pro-B cells express endoglin. This is suggested by the observations that 43% of CD105* cells expressed CD11b while 15% were B220*. Endoglin positive cells did not express CD90, which is low on hematopoietic stem cells and high on T lymphocytes. The precise identification of the CD105* subsets of hematopoietic cells should be confirmed by cell sorting and four color flow cytometry analysis. The level of endoglin expression was reduced in End<sup>−</sup> hematopoietic cells compared to End<sup>+/</sup> mice.
Figure 5.7 Increased mitogen-induced proliferation of T and B lymphocytes from End"+" mice.

Single cell suspensions were prepared from the spleen of End"++" mice (light circles/bars) and End"+" mice (dark circles/bars). (a) Kinetics and dose-response of T cells to stimulation with superantigen. T cells from 7 wk-old C57BL/6 mice were stimulated with 0.1 μg SEB for 1 to 4 d (left panel), or with various concentrations of SEB for 3 d (right panel). Cell proliferation was assayed as described in Materials and Methods. The data are representative of five independent experiments. (b) T cells from 11 wk old 129/Ola and 10 wk old C57BL/6 mice were stimulated with 0.1 μg SEB for 3 d, and cell proliferation was measured. The data are representative of two independent experiments. (c) T cells from 7 wk old C57BL/6 mice were stimulated for 3 d with 0.1 μM PMA and 0.5 μM ionomycin (Io), with or without the addition of IL-2, and cell proliferation was assayed. The data are representative of four independent experiments. (d) Kinetics and dose-response of B cells to stimulation with LPS. B cells from 7 wk-old C57BL/6 mice were stimulated with 20 μg LPS for 1 to 4 d (left panel), or with various concentrations of LPS for 3 d (right panel), and cell proliferation was measured. The data are representative of four independent experiments. (e) B cells from 11 wk old 129/Ola, and 10 or 24 wk old C57BL/6 mice were stimulated with 20 μg LPS for 3 d, and cell proliferation was measured. The data are representative of three independent experiments. Mean values ± SD for triplicate samples are given.
Figure 5.8 Analysis of bone marrow myeloid and lymphoid lineage cells in End<sup>+/+</sup> mice.
Cell suspensions were prepared from the bone marrow of 9 wk-old C57BL/6 End<sup>+/+</sup> mice and End<sup>+/+</sup> littermate controls. (a) Cells were analyzed for surface expression of CD43, CD24, CD11b and Gr-1 by three-color flow cytometry. (b) Endoglin expression was assessed in the bone marrow of wild type mice and compared to Sca-1, CD117, CD43, CD90, B220 and CD11b expression. Percentages of cells in each quadrant are shown.
5.5 Discussion

In this study, we report the expression of endoglin on subsets of hematopoietic cells and immune defects in End−/− mice. During thymic development, endoglin was induced at the late DNIII stage following pre-TCR activation. In peripheral organs, endoglin was present at low levels on mature T cells, but was not expressed by mature B cells. Mice expressing reduced levels of endoglin showed decreased thymic cellularity, but increased numbers of mature T cells in the spleen, with a preferential increase in CD4 T cells. The thymic microenvironment was deficient in capillaries and contained large dilated vessels. An age-dependent accumulation of abnormal B cells expressing a surface phenotype consistent with cells in transition from immature to mature stages of differentiation, was also observed. Both peripheral T and B cells from End−/− mice were hyperresponsive to stimulation by chemical mitogens and superantigens. The distribution of myeloid and lymphoid progenitors was normal in the bone marrow and endoglin was present on only 3% of cells. Taken together, our results suggest that the level of endoglin on vascular endothelium and perhaps on some defined hematopoietic subsets is important for regulation of normal T and B cell function.

The reduced thymic cellularity observed in End−/− mice could not be explained by a decreased number of proliferating cells or an increase in apoptosis, since the percentages of cycling and apoptotic thymocytes were normal. However, the observation that End deficient mice had dilated vessels at the cortico-medullary junction, accompanied by a substantial reduction in the number of capillaries, is highly reminiscent of the vascular phenotype described in non-lymphoid organs of HHT mice (Chapter 4). This is the first description of vascular changes in hematopoietic tissues. Importantly, these changes occur prior to any clinical manifestations of the disease. Since precursor cells colonizing the thymus are presumed to enter at the cortico-medullary junction by migration through the microvasculature (306), the lack of capillaries might impair precursor seeding of the thymus, resulting in the three fold reduction in cellularity seen in End−/− mice. The probability that a decreased number of precursors were generated in the bone marrow is less likely since no abnormalities were seen in the distribution of lymphoid progenitors in End−/− mice. Increased deletion of weak self-reactive T cells due to
increased sensitivity to endogenous superantigens is also unlikely since no difference was observed in the TCR Vβ usage (data not shown).

The induction of endoglin expression upon pre-TCR signaling, and the preferential increase in CD4+ T cells in peripheral lymphoid organs of End+/− mice suggest a potential role for endoglin in thymopoiesis and mature T cell regulation. This function of endoglin might be achieved by its modulation of TGF-β1 effects on T cells. During thymic development, TGF-β1 inhibits the maturation of CD4+CD8− into CD4+CD8lo and CD4+CD8+ thymocytes, indicating that TGF-β1 interferes with pre-TCR signaling. Furthermore in the absence of TGF-β1, DP thymocytes are not efficiently deleted (185), suggesting that TGF-β1 decreases signaling through the TCR and alters the outcome of thymic selection. The acquisition of endoglin during the transition from the DN to DP stage might be necessary for the TGF-β1 to regulate this differentiation process. The additive effects of TGF-β1 and TNF-α on DP thymocytes favor the expansion of CD8 T cells (186). Although thymocyte distribution among the CD4/CD8 subsets was not altered in the thymus of End+/− mice, the CD4/CD8 ratio progressively increased in the peripheral organs of End+/− mice after 12 wks of age. This progressive effect might be due to the persistence of a dysregulated state of response of T cells to TGF-β1 in End+/− mice. TGF-β1 is also immunosuppressive and generally inhibits T cell proliferation (307). We observed that peripheral T cells of End+/− mice were more responsive to superantigen stimulation, suggesting that they might be less responsive to TGF-β1 inhibition, due to reduced expression of endoglin. This possibility will be tested by assessing the ability of TGF-β1 to inhibit T cell proliferation in response to these stimuli.

An accumulation of B cells in transition from the immature to the mature stage was seen in the spleen of End+/− mice, 18 wk and older. Transitional B cells are normally highly sensitive to antigen induced apoptosis following B cell receptor ligation. A strong signal transduced through the B cell receptor induces clonal elimination in transitional B cells while low basal signaling promotes survival of these transitional B cells. Our observations suggest decreased signaling through the B cell receptor in older End+/− mice. This could be tested by measuring apoptosis induced by anti-IgM cross-linking (308). The lack of expression of endoglin on these
cells precludes a direct role for endoglin in regulating their function. Nevertheless, B cells from End+/− mice were hyper-responsive to stimulation with LPS. TGF-β1 is a potent regulator of B cell development from the pre-B cell stage to Ig-secreting plasma cells (189). It inhibits IL-7-mediated stimulation of precursors in the bone marrow, and prevents κ chain expression during the transition from pre-B to mature B cell stages. TGF-β1 is also a potent inhibitor of antigen and mitogen-driven B cell proliferation (309). Provided the defects observed in the B cell lineage of End+/− mice are cell autonomous, a possible explanation for our results might be that endoglin expression is reduced at a critical stage during B cell development, leading to impaired maturation. This notion is supported by the observation that endoglin is expressed on early human B-lineage progenitors (CD19+ CD34+), in fetal bone marrow, and in pre-B ALL (13, 15, 207). Furthermore, our results suggest that endoglin is also expressed at early stages of B cell differentiation in mouse bone marrow. Lower levels of endoglin were observed on these progenitors in End+/− mice which could contribute to the dysregulation observed in peripheral B cells.

Endoglin was also expressed on some myeloid progenitors in mouse bone marrow. Recent studies have reported a CD34+CD105+ population, enriched in circulating human proliferating progenitors with high cloning efficiency, LTR potential, and producing autocrine TGF-β1 (210, 213, 301). The loss of CD105 expression in this cell population abrogated their ability to respond to TGF-β1, suggesting that endoglin expression might be correlated with the ability of TGF-β1 to inhibit cell cycle progression of primitive precursors (213). Although our data suggest that murine bone marrow hematopoietic stem cells (Sca-1+) were CD105+, we cannot rule out the existence of CD105+ peripheral stem cells, similarly regulated by TGF-β1. Future analysis of the differentiation capacity of CD105+ progenitors, in the presence or absence of TGF-β1, should help clarify the role of endoglin in murine hematopoiesis.

The bone marrow microenvironment, including stromal and endothelial cells, is essential for hematopoiesis. Since a defect in thymic microvasculature was seen in End+/− mice, staining of bone marrow endothelial cells might also reveal dilated vessels and/or reduced numbers of capillaries. Experiments are currently in progress to establish whether the immune phenotype
observed in $End^{+/-}$ mice is due to the microvascular environment, or to reduced expression of endoglin on hematopoietic cells. To determine whether the hematopoietic defects observed in $End^{+/-}$ mice can be transferred to host mice, $End^{+/-}$ donor cells (CD45.2) have been injected into congenic CD45.1 recipients. The reciprocal experiment will also be performed to assess the contribution of endoglin expressed on endothelium to the immune defects.

A hematopoietic cell autonomous defect would imply a crucial role for endoglin expressed on these cells in the development of the immune system. A vascular defect in lymphoid organs leading to altered lymphopoiesis, as well as abnormal B and T cell function, would underscore the importance of the endothelium in regulating the differentiation and maturation hematopoietic cells. The immune defect observed in $End^{+/-}$ mice and caused by reduced endoglin on hematopoietic cells or vascular endothelium of lymphoid organs, might be a contributing factor to the progression of HHT in humans.
5.6 Acknowledgments

We would like to thank Mr. Martin Bissasseur and Mr. Sigmund Kaw for technical assistance, Ms. Lily Morikawa for preparing spleen sections and Ms Gisele Knowles for expertise and advise on flow cytometry analysis. We acknowledge the help of Drs. Ruey Su and Richard Miller of the Ontario Cancer Institute for performing the cytotoxicity assays. We are also grateful to the staff of the Lab Animal Services at the Hospital for Sick Children. This research was supported by grant #NA3434 from the Heart and Stroke Foundation of Ontario and Medical Research Council of Canada (ML). AB is a recipient of a studentship from the Medical Research Council of Canada, ML is a Terry Fox Research Scientist of the National Cancer Institute of Canada.
The primary goal of this thesis is to define the role of endoglin in neovascularization occurring both in normal and pathological situations as well as its implication on the generation, expansion and response of hematopoietic cells. The derivation and characterization of End null mice defined a function for endoglin in vessel growth and remodeling required for embryonic angiogenesis. Furthermore, we have demonstrated that endoglin is essential for maintenance of vascular homeostasis as expression of a single allele can lead to abnormal vessels. Thus we propose a model for the generation of vascular lesions in HHT patients that would both implicate the presence of modifier genes as well as differential use of signaling pathways in the TGF-β superfamily. We also describe the immunological phenotype of End heterozygous mice, which may represent autonomous cell defects of hematopoietic cells expressing endoglin or be caused by abnormal vascular microenvironment.

6.1 Role of endoglin in angiogenesis

In early development vasculogenesis gives rise to the primary vascular plexus. In the yolk sac, these vascular channels form new capillaries by angiogenesis involving vessel sprouting from pre-existing ones or intussusceptive growth in response to VEGF-A. Remodeling of vessels into a mature vascular network is achieved by branching, pruning, recruitment and differentiation of mesenchymal cells into pericytes. Binding of Ang1 with its endothelial receptor Tie-2 leads to interaction of pericytes with the endothelial layer. This contact activates TGF-β1 that inhibits EC proliferation, induces differentiation of PC and ECM deposition necessary for vessel wall assembly (see Figure 1.3).

In End null mice, vasculogenesis is normal giving rise to primitive vessels with intact endothelium and regular size lumen. However, angiogenesis is impaired (Figure 6.1).
Figure 6.1 Angiogenic defects in Endoglin null mice.

The primary vascular plexus is formed by vasculogenesis. The subsequent phase of vascular development involves the expansion of the primary network via two distinct mechanisms of angiogenesis: sprouting and intussusceptive growth. In Endoglin null mice, the primary capillary plexus of the yolk sac dilates and fuses to generate abnormal and irregular channels that bleed. We propose that the phenotype observed is due to reduced sprouting and absence of intussusceptive growth. It may also be due to defects in remodeling which inhibit the recruitment and differentiation of pericytes in the Endoglin null mice. This imbalance in vessel wall components would contribute to weakening of vessels in the Endoglin null mice.
lack of endoglin decreases the extent of sprouting, a process that requires expression of VEGF-R1, VEGF-R2, Tie-1 and Tie-2 on endothelial cells. Non-sprouting angiogenesis, i.e. growth of vessels by intussusception, appears to be lacking in End null mice, leading to dilated vascular channels that fuse into enlarged aneurysmic-like structures. This requires the additional expression of TβR-II on endothelial cells. The remodeling phase of angiogenesis does not take place in mice lacking End, such that recruitment and differentiation of mesenchymal cells is defective. Receptors needed for maturation of vessels include PDGF-R, TβR-II and Tie-1. The primitive and fragile vessels, without any endoglin, are unable to sustain blood flow and consequently rupture, leading to hemorrhage (Figure 6.1).

Since the major difference between sprouting and non-sprouting angiogenesis, appears to be a requirement for TβR-II, it suggests that regulation by TGF-β is defective in the End null mice. Endoglin is thus necessary for the mediation of this effect and for the division of a large vessel into two smaller capillaries. TGF-β is also needed for remodeling of the vessels. Poor adhesiveness between endothelial cells and pericytes is observed in yolk sac of mice deficient in TGF-β1, TβR-II and End leading to vessel destabilization. If sprouting and branching occurred to a certain degree in the End null mice, failure in recruitment and differentiation of smooth muscle cells in the remodeling phase of angiogenesis would lead to vessel fragility and death of the embryo from hemorrhage at mid-gestation.

6.2 Endoglin deficient mice, a unique model to study Hereditary Hemorrhagic Telangiectasia

Murine HHT mimics the human disorder and its heterogeneity. This is illustrated by comparing a series of End−/− mice with HHT to a group of clinically diagnosed patients as shown in Figure A.4 and discussed in Appendix I.

The majority of mice with HHT had external manifestations of disease, such as mucocutaneous telangiectases, epistaxis and other external bleeding. The prevalence of telangiectases and external bleeding may have been somewhat underestimated in the mouse due to difficulties in detection. Visceral manifestations were reported for autopsied mice only and
appear more frequent in mice with HHT than in the human population. The prevalence of 33% of pulmonary disease was similar to that in humans. However, the prevalence of gastrointestinal, hepatic and cerebral disease was somewhat greater in mice than has been observed in people. There are several possible explanations for this. The most obvious one is that mice were autopsied, and therefore more disease would be detected. The prevalence of liver AVMs might be underestimated in the human population since only patients with a large shunt would have been diagnosed with the current screening procedures. Since liver AVMs in patients with HHT are not always macroscopic AVMs but rather multiple small telangiectases, a large number is probably required before a significant shunt is detectable. Another potential explanation is that all mice studied have an endoglin mutation, whereas most patients have not been genotyped and therefore may have an endoglin or ALK-1 mutation. Disease severity may be diluted by the presence of ALK-1 families, who generally have a milder phenotype. The increased prevalence of liver disease might also be related to the 129/Ola background, since 70-80% of the wild type 129/Ola mice were reported to have large intrahepatic connections between portal and hepatic veins as well as reduced number and extent of peripheral vessels (283, 284).

The murine model is exciting as it reproduces most of the clinical manifestations of HHT. Although AVMs were not detected in the mice, the extent of liver, lung and brain involvement and their sequelae such as stroke, suggests that they are present. Future angiography studies should allow us to determine if AVMs can be detected in mice with HHT. The ability to isolate multiple vessels from HHT mice and study their vascular tone and its regulation should also increase our understanding of the mechanisms responsible for abnormal blood vessel dilation. Novel therapeutics modalities can also be tested in mice with HHT with potentially important implications for patients with this chronic disease.
6.3 Potential mechanisms for the generation of vascular lesions in HHT

Both murine and human HHT show phenotypic heterogeneity. In human, this is explained in part by mutations in two distinct genes, Endoglin and ALK-1. Expression studies have shown that endoglin haploinsufficiency is the mechanism responsible for HHT1 (209,230,250,252,253,255). HHT2 also appears to be associated with haploinsufficiency in ALK-1 (262,264). These results indicate that disease heterogeneity cannot be explained by position and type of mutation. All mutant proteins studied to date cannot reach the cell surface, and thus cannot interfere with the normal function of endoglin. In the case of ALK-1, a loss of function can be due to either reduced protein expression or reduced enzymatic activity. Not all mice expressing a single allele of endoglin develop HHT. It is very dependent on the genetic background of the inbred strain. Our data strongly suggest that additional genes, so-called modifier genes, such as contributed by the 129/Ola strain, are necessary to generate the vascular abnormalities associated with HHT. The identification of these genes in mice should lead to the recognition of their human counterparts, which might be responsible for the generation of AVMs. Thus a mutation in endoglin (or ALK-1) and the co-inheritance of modifier genes predispose to the formation of AVMs as illustrated in Figure 6.2a.

The majority of vessels in HHT patients appear to develop normally. However, most patients have some dilated vessels (telangiectases) suggesting that vessels with a reduced functional level of endoglin are more susceptible to dilation and remodeling. Epigenetic factors such as altered blood flow, shear stress and hormonal changes, for example during pregnancy, are likely implicated in the generation of vascular lesions. This would explain the focal localization of telangiectases and AVMs in HHT patients (Figure 6.2a). The combination of modifier genes and precipitating epigenetic factors would be responsible for severe disease, associated with large AVMs or hundreds of telangiectases leading to extensive hemorrhage.

How can we explain at the molecular level the formation of AVMs? It has been suggested that a telangiectasia arises from the dilation of a post-capillary venule which results in direct fusion with an arteriole, bypassing the capillary network (231). This would suggest that
Figure 6.2 Mechanisms leading to the formation of arteriovenous malformations in patients with HHT.

(a) Mutations in the Endoglin gene are associated with HHT1 and lead to haploinsufficiency (loss of function). However AVMs are not associated with focal loss of endoglin, ruling out loss of heterozygosity as the cause of these lesions (Chapter 2). We propose that the co-inheritance of mutated endoglin and other genes, referred to as modifier genes, predisposes to severe manifestations including the formation of AVMs. In the absence of modifier genes, mild disease can occur. Epigenetic factors such as environment, blood pressure, oxygenation, shear forces could influence for example, the location of telangiectases. Factors such as increased blood volume and cardiac output combined with hormonal changes observed in pregnancy can precipitate the growth of AVMs. Thus epigenetic factors would influence both mild and severe forms of disease. (b) AVMs are believed to arise during embryonic development but may remain microscopic for many years. Telangiectases are in fact small AVMs since they represent a direct connection between a venule and an arteriole, without intervening capillaries. As these small lesions grow in size, the vessels dilate further and become tortuous. Ongoing abnormal vascular remodeling occurs and leads to disorganized and variable thickness of smooth muscle cells, particularly in the veins. These become arterialized in an attempt to adapt to blood flow at arterial pressure, due to direct shunting. Elastin is seen in these vessels as well as an abnormal adventitial layer. Increased angiogenesis, seen by the presence of multiple vessels within a large lesion, is also occurring. Several factors such as TGF-β are thought to be implicated in the formation of these vascular lesions.
Single functional endoglin allele

+ 

co-inheritance of modifier genes

No

→ Mild disease

↓

Epigenetic factors

Influence on location of telangiectases

Yes

→ Predisposition to severe disease

↓

Formation of AVM

Influence on location

b

NORMAL VESSEL

Artery

Fibroblasts

SMC

EC

ECM

Remodeling defect

ARTERIOVENOUS MALFORMATION

Vein

TGF-β1

VEGF-A

Tie-1

Artery
50% expression of endoglin in these venules is associated with their dilation (stretching of endothelial cells) rather than their normal branching into capillaries. This implies that the mechanisms regulating the normal angiogenic process of vessel branching are altered. Disorganized smooth muscle cells were observed in both telangiectases (231) and large AVMs (Chapter 2) implying that the interactions between vessel wall components were abnormal. The major factors implicated in the regulation of vessel assembly are Ang1 and TGF-β1. Ang1 is primarily responsible for PC recruitment and TGF-β1 for synthesis of the ECM required to maintain proper interactions between endothelial, muscular and adventitial layers. Since SMC are present in both telangiectases and AVMs, there does not appear to be a defect in their recruitment (Figure 6.2b). However we cannot rule out that a reduction in Ang1 might be partially responsible for vessel destabilization in HHT. The disorganized array of SMC in smaller lesions and their uneven thickness in arterializing veins of AVMs, suggest that regulation by TGF-β1 is altered. The remodeling of the lesions to generate large complex AVMs containing multiple tortuous vessels is associated with increased angiogenesis, VEGF and Tie-1. Proliferation of adventitial fibroblasts, a process mediated by TGF-β1, is also occurring in these lesions (199) (Figure 6.2b).

6.4 Participation of endoglin and ALK-1 in TGF-β signaling pathways likely critical to vascular development.

How can reduced levels of functional endoglin, and/or ALK-1, on vascular endothelium lead to abnormally dilated vessels and the generation of arteriovenous malformations?

Presently, very little is known about the mechanism through which endoglin mediates its function. However, its association with receptors for TGF-β suggests a role in regulating biological responses to this growth factor. TGF-β has multiple effects in development and homeostasis of the vascular system. It regulates endothelial cell proliferation, production of extracellular matrix, vascular tone and interactions between endothelium and smooth muscle cell layers needed for vessel wall assembly and vascular remodeling.
Signaling is initiated by binding of TGF-β to TβR-II, which induces the phosphorylation of ALK-5 (Figure 6.3). Activated ALK-5 initiates downstream signaling by recognizing and phosphorylating Smad2 or 3. Endoglin can only bind TGF-β1 or -β3 in association with TβR-II and is known to modulate several responses. *In vitro* experiments demonstrated that overexpression of endoglin in monocytes and fibroblasts inhibits several of the effects of TGF-β1 while treatment of endothelial cells with anti-sense oligonucleotides to endoglin potentiates its effects. These observations would predict that a reduction in endoglin should be associated with increased responses to this growth factor. We favor the view that endoglin is a regulatory component of the TGF-β receptor complex and that its reduction leads to an imbalance in TGF-β function. The absence of endoglin was associated with an embryonic lethal phenotype highly reminiscent of TGF-β1,TβR-II and ALK-1 null mice, suggesting a defective receptor system.

How are endoglin, ALK-1, TβR-II and TGF-β1 functionally related in normal and pathological HHT vessels? ALK-1 was recently shown to bind TGF-β1 in endogenous endothelial cells and was found in a receptor complex in association with TβR-II and endoglin. Once phosphorylated, ALK-1 signals through Smad1 or 5. Thus ALK-1 is capable of mediating a TGF-β1 signal. Furthermore, overexpression of ALK-1 inhibited ALK-5 mediated TGF-β1 responses (136). These results suggest that ALK-1 signaling negatively regulates TGF-β1 signaling through ALK-5. Thus a balance between TGF-β1 activation of ALK-5 and ALK-1 and their respective downstream signaling Smads is required for normal angiogenesis. Endoglin which, can be present in either complex, could also modulate responses to TGF-β1 in endothelial cells via ALK-5 or ALK-1 (Figure 6.3). Thus both endoglin and ALK-1 can be present in a TGF β1 receptor complex likely critical to the pathophysiology of HHT. Signaling through ALK-1 pathway could be defective in dilated vessels of HHT patients. Studies of receptor and Smad activation by TGF-β1 in normal as well as in endoglin or ALK-1 deficient endothelial cells might elucidate specific alterations in HHT1 and/or HHT2. These findings will be informative in the elucidation of the angiogenic mechanisms altered in dilated vessels and AVMs. They may also lead to the development of novel therapeutic interventions.
Figure 6.3 Endoglin and ALK-1 functions in the TGF-β receptor complex.
Endoglin can bind TGF-β1 via its association with TβR-II, which then recruits either ALK-5 or ALK-1 to the receptor complex. Endoglin can thus modulate both ALK-5 and ALK-1 mediated pathways. When ALK-5 is phosphorylated, it transmits signals through Smads 2 or 3 while activated ALK-1 signals via Smads 1 or 5. It has been proposed that these two pathways would have complementary functions (136). Signaling through Smads 2/3 could activate genes "A" for example, while signalling through Smads 1/5 could inhibit these genes. We also hypothesize that certain genes "B" could be stimulated by both pathways while genes "C" and "D" would be specific for either Smads 2/3 or Smads 1/5. In HHT1 patients with a generally more severe phenotype, both ALK-1 and ALK-5 pathways could be affected, while in HHT2 patients only ALK-1 mediated signals could be altered.
6.5 A role for endoglin in modulating effects of TGF-β on hematopoietic cells.

We demonstrated alterations in T and B cell homeostasis in End<sup>+</sup> mice. What is unclear at the present time is whether this imbalance is due to reduced expression of endoglin on vascular endothelium or on hematopoietic subsets. We have two working hypotheses to explain our experimental observations. The first one is that decreased expression of endoglin on the endothelium of lymphoid tissues influences the differentiation and maturation of progenitors and immune subsets leading to impaired immune response. The second hypothesis is that reduced expression of endoglin on hematopoietic subsets is responsible for the observed imbalance in mature T and B cells. Since TGF-β1 acts on multiple cell types to regulate their growth and differentiation and is critical for vascular and hematopoietic development, we propose that the effects of endoglin are due to modulation of TGF-β1 responses.

Since reduced endoglin expression on vascular endothelium leads to HHT, and dilated vessels in multiple non-lymphoid organs, our observations of a thymic microvascular environment deficient in capillaries and showing enlarged vessels would argue in favor of an endothelial effect. The concept of the vascular network of the thymus influencing the migration of precursors and their subsequent maturation is exciting and has been poorly investigated. In spleen, the sinusoidal endothelium is most abundant and surrounds the lymphoid follicles. Since endothelial cells secrete TGF-β1 that can act on B and T cells, less endoglin on endothelium would lead to impaired responses to this potent immunosuppressive agent. In bone marrow, differentiation of multiple lineages occurs in close proximity with the sinusoidal endothelium. Furthermore, angiogenic factors and cytokines produced by the endothelium stimulate proliferation and/or differentiation of hematopoietic cells (310). Thus an endoglin deficient endothelium in lymphoid organs is likely to be altered in its interactions with progenitor and mature cells of multiple lineages.

Endoglin is expressed on human pre-B cells and activated monocytes (16, 207, 224) and on murine T cells and 3% of bone marrow cells, as demonstrated in Chapter 5. Endoglin was shown to modulate several responses to TGF-β1 in human monocytic cell lines (10) and is likely able to do so in murine hematopoietic cells. Since most progenitors and cells of the immune
system are regulated by TGF-β1, reduced levels of endoglin could influence their differentiation and activation state. This would explain the defects seen in peripheral T and B cells.

Bone marrow reconstitution experiments should clarify whether vascular micro-environment or progenitors are responsible for the immune defects seen in our mice. It is also possible that the complex immune phenotype seen in End⁺/⁻ mice is due to the compounded effects of reduced endoglin on both endothelium and hematopoietic cells. It is now very interesting to determine if immune functions are altered in HHT patients and could contribute to disease progression particularly in elderly patients, as suggested by hyperreactive T and B cells in older mice. Infections such as brain abscess and endocarditis have been seen in HHT patients and ascribed to lack of capillary blood filtration due to AVMs. However immune defects, similar to those seen in End⁺/⁻ mice might contribute to increased susceptibility to infection in these patients.

We have demonstrated that endoglin is essential for normal blood vessel development since Endoglin null mice die at mid-gestation of severe vascular and cardiovascular defects. We have also shown that critical levels of endoglin are necessary for maintenance of vascular homeostasis since both human and mice with a single endoglin allele can develop Hereditary Hemorrhagic Telangiectasia. Furthermore, we have gathered evidence that reduced levels of endoglin lead to abnormal T and B cell function.
REFERENCES


APPENDIX

Endoglin Deficient Mice, a Unique Model to Study Hereditary Hemorrhagic Telangiectasia

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We were invited to write this review for Trends in Cardiovascular Medicine. I report here my analysis of both external and visceral involvement of murine HHT. In collaboration with Dr. Faughnan, respirologist and director of the Toronto HHT Centre, we compared the murine phenotype to a group of human patients seen at the Toronto HHT clinic. This project was supervised by Dr. Letarte.

A.1 Abstract

Hereditary Hemorrhagic Telangiectasia is a genetic vascular disorder characterized by dilated vessels and arteriovenous malformations. Phenotypic heterogeneity, such as age of onset, severity of disease and organ involvement, is explained in part by two genes being mutated, endoglin (HHT1) and ALK-1 (HHT2). Haploinsufficiency is the mechanism responsible for HHT. This implies that position and type of mutations cannot explain heterogeneity, since mutant proteins are not expressed at the cell surface and consequently cannot interfere with normal function. Based on this model, we generated mice expressing only one allele of endoglin, but in two different inbred strains, 129/Ola and C57BL/6. Phenotypic heterogeneity was also observed amongst the HHT mice and was very dependent on the genetic background. Our data strongly suggest that additional genes, contributed by the 129/Ola strain, are responsible for the vascular anomalies associated with HHT. The murine model is faithful to the human disease and should allow us to identify the modifier genes of HHT as well as to test potential therapeutic interventions.
A.2 Hereditary Hemorrhagic Telangiectasia, a genetic disorder affecting blood vessels

Hereditary Hemorrhagic Telangiectasia, also known as Rendu-Osler-Weber syndrome, is a vascular disorder inherited in an autosomal dominant fashion with an estimated prevalence of 1:8000. In many patients, the first sign of HHT is frequent nose bleeds (epistaxis), caused by telangiectases in the nasal mucosa (1, 2). A telangiectasia is a dilated blood vessel, believed to originate by focal dilation of a post-capillary venule that eventually fuses directly with a dilated arteriole, bypassing the capillary network (3). Dilation of larger vessels can produce arteriovenous malformations known to occur in the lung, brain and liver of HHT patients. AVMs are tortuous thin-walled vessels with interruption of the elastica lamina, variable thickness of smooth muscle cells as well as dispersed and disorganized adventitia that suggest ongoing vascular remodeling (4). The shunting of blood through these lesions can lead to serious complications such as hypoxemia, stroke, brain abscess, heart failure and fatal hemorrhage (2, 5).

Clinical manifestations of HHT are highly heterogeneous within and between families, as illustrated in Figure A.1. Genetic and epigenetic factors have been postulated to account for this diversity. There are currently two distinct genes responsible for HHT, *Endoglin* mutated in HHT1 (6) and *ALK-I*, mutated in HHT2 (7). A higher prevalence of pulmonary AVMs is associated with HHT1 families (8), whereas generally a milder phenotype and later onset are seen in HHT2 families (7). To date 41 mutations have been reported for *Endoglin* (2, 4, 9, 10) and 21 for *ALK-I* (7, 11, 12), distributed throughout both genes. Although every family appears to have a distinct mutation, it is unlikely that the type of mutation or its position will affect the clinical outcome, as haploinsufficiency is the underlying mechanism for HHT. It has been demonstrated in eight families with HHT1, including some with null alleles, that disease severity was not correlated with the type of mutation (13).

This is further illustrated in Figure A.1 with two HHT1 families with different mutations. Family 46, expresses a previously undescribed missense mutation, a C to T substitution at nucleotide 14 in exon 1 of *Endoglin*. This converts threonine 5 of the leader peptide to methionine, and prevents its expression, as confirmed by metabolic labeling studies. The phenotype of this family is considered mild. In the three generations with known disease,
Figure A.1 Clinical heterogeneity within human HHT1 genotype illustrated by two distinct families.

Family 46 (a) has a mild phenotype, with a prevalence of pulmonary AVMs of 1/6 (17%) affected individuals. Phenotype was based on clinical examination and systematic screening in 6 individuals, while family history obtained from these individuals provided information for the remaining family members. Family 114 (b) has a more severe phenotype with a prevalence of pulmonary AVMs of 9/20 (45%) affected individuals. Phenotype was based on clinical examination and systematic screening in 8 individuals, while family history obtained from these individuals provided information for the remaining family members. Patients diagnosed with HHT are indicated by grey filling, those with pulmonary AVMs by black filling. Squares represent males, circles females and hexagons are groups of males and females. Deceased patients are indicated with a slash.
15 individuals are descendants of a parent with a diagnosis of HHT and therefore have a 50% risk of having the disease. Of these 15 individuals, 6 (40%) have been clinically diagnosed with HHT. Of these 6 individuals with HHT, 4 (67%) have mild disease consisting of epistaxis and mucocutaneous telangiectases, 1 (17%) has a history of gastrointestinal bleeding and 1 (17%) was diagnosed with pulmonary AVMs.

Family 114 has a previously unreported mutation, a CA deletion at nucleotide 657 in exon 5 of Endoglin which creates a frameshift and leads to a truncated protein, also not expressed by metabolic labeling. The phenotype of this family is much more severe. In the five generations with known HHT disease, 43 individuals are descendants of a parent diagnosed with HHT. Of these 43 at risk-individuals, 20 (47%) have been clinically diagnosed with HHT. Pulmonary AVMs were found in 9/20 (45%) of these patients and cerebral AVMs were present in one patient (5%). Though the difference in prevalence of serious complications between the two families is not statistically significant, likely due to small sample size, there is clearly a range in phenotype despite both families having an endoglin mutation.

We have shown that mutant endoglin proteins, including missense mutants, are transient intracellular species that cannot reach the cell surface (14). This leads to levels of normal endoglin that are reduced by 50% on endothelial cells and activated monocytes in vitro and on normal vessels and AVMs of HHT1 patients in vivo (4, 9, 10, 14). Thus, haploinsufficiency now demonstrated in close to 100 families, is the underlying mechanism of HHT1, since mutant proteins are not expressed at the cell surface and consequently cannot act as dominant negatives. Reduced levels of functional ALK-1 are also associated with HHT2 suggesting a similar mechanism (12).

A.3 Important role for endoglin in vascular development and homeostasis

How can reduced levels of functional endoglin, and/or ALK-1, on vascular endothelium lead to abnormally dilated vessels and HHT? Human endoglin (CD105) is a homodimeric transmembrane glycoprotein constitutively expressed at high levels on endothelial cells of capillaries, veins and arteries (15, 16). Endoglin associates with ligand binding receptors for
several members of the transforming growth factor beta (TGF-β) superfamily (17). Endoglin binds TGF-β1 and TGF-β3 isoforms, but not by itself. It requires association with the TβR-II, a serine/threonine kinase which initiates signal transduction by phosphorylating ALK-5. Endoglin was shown to modulate several responses to TGF-β1 (18), and is in our view, a regulator of TGF-β1 action in endothelial cells.

TGF-β1 has multiple effects in development and homeostasis of the vascular system. It regulates endothelial cell proliferation, production of extracellular matrix, vascular tone, and interactions between endothelium and smooth muscle cell layers in the vessel wall. It is also implicated in vascular remodeling. ALK-1 is a serine/threonine receptor type I of the TGF-β superfamily, expressed mostly on endothelium and recently shown to bind TGF-β1 in endogenous endothelial cells (19). Thus both endoglin and ALK-1 can be present in a TGF-β1 receptor complex likely critical to vascular development and homeostasis and defective in HHT.

Human and mouse endoglin are 76% identical at the protein level. Murine endoglin is found on all endothelial cells and on stromal cells in several tissues (20). Targeted inactivation of the End gene results in vascular and cardiovascular defects in early mouse embryos (21-23). From ED 9.0, the primitive vascular plexus of the yolk sac fails to form mature structures leading to vessel dilation, rupture and hemorrhage. The vasculature of the null embryos is also very fragile and bleeds, demonstrating a crucial role for endoglin in remodeling required for angiogenesis. Heart defects are also observed in Endoglin null mice. Cushion tissue formation, essential for valve development and heart septation, does not occur and pericardial edema is observed. These vascular developmental anomalies lead to death at ED10-10.5 (22) and illustrate the important contributions of endoglin to the development of vascular and cardiovascular systems.

A.4 Development of a murine model of HHT

The haploinsufficiency associated with HHT1 implies that mutation at a single allele leads to a loss of function and that engineering Endoglin hemizygosity in mice, could yield an animal model of HHT. End<sup>−/−</sup> mice were produced by backcrosses, starting with chimeric founder
animals, onto 129/Ola and C57BL/6 inbred strains (22). The End\textsuperscript{+/-} 129/Ola mice are inbred while the End\textsuperscript{+/-} C57BL/6 mice are of mixed genotype (C57BL/6 and 129/Ola). Mice of mixed genome were also generated by inter-breeding of C57BL/6 End\textsuperscript{+/-} mice. All mice were kept in a germ-free environment and observed for a period ranging from 6 to 18 months.

Several mice were noticeably unwell as judged by a rough coat and low body weight. The first sign of HHT, in many cases, was ear telangiectasia (Figure A.2a). It appeared as a small dilated vessel, increased in size and often ruptured after a few days, causing a small hemorrhage and sometimes partial tissue necrosis. Telangiectases were also found on the back, neck, tail, and genitals. A fraction of HHT mice had recurrent bleeds with a variable onset and frequency (up to 2-3 times/wk) from the nose, mouth, ears, tail, or genitals (Figure A.2b). An early and severe onset of HHT is shown for an End\textsuperscript{+/-} 129/Ola mouse which had abundant nosebleeds at one wk of age as well as tail telangiectases that bled and resulted in partial tail loss and death within a wk (Figure A.2c). Disease severity in HHT mice generally increased with age. Several older mice (>8 months) developed severe bleeding, marked weight loss and often reduced mobility. Figure A.2d, shows a stroke victim, with hemoplegia and ptosis. The stroke was subsequent to a subarachnoid hemorrhage, illustrated in Figure A.2j.

Approximately 30% of mice with HHT end-up in an agonal phase of disease due to rupture of major vessels. We performed 33 autopsies looking at gross morphology of each organ and histological analysis of liver, lungs and brain. The liver was the most affected organ with manifestations ranging from mild to severe. Figure A.2e shows the normal pattern of expression of endoglin on an artery, a vein and the sinusoidal endothelium of an End\textsuperscript{+/-} mouse. In an End\textsuperscript{+/-} HHT mouse with mild disease, congestion was noticeable by an increase in red blood cells and a marked dilation of vessels, primarily veins (Figure A.2f). In more severe cases of HHT, further dilation of vessels leading to destruction of hepatocytes was observed (data not shown). Congestion and marked dilated veins and arteries were also seen in the lungs of HHT mice compared to their littermate controls (Figure A.2, g-h). Cerebral hemorrhages (subdural, subgalial or subarachnoid) were also observed in several HHT mice as illustrated in Figure A.2j; an unaffected adjacent brain region is shown for comparison (Figure A.2i).
Figure A.2 Clinical manifestations of HHT in $End^{+/c}$ mice.

External signs of HHT are revealed by pictures of the mice (a-d) while internal signs are illustrated with sections of liver immunostained for endoglin (e and f) and sections of lungs and brain stained with Masson's trichrome (g and j). (a) An $End^{+/c}$ HHT B6$_{in}$ mouse, with light coat color and an ear telangiectasia (arrow). (b) A dark coat color $End^{+/c}$ HHT C57BL/6 backcross mouse (N2) with a rough coat and severe bleeding from ears and nose (arrows). (c) An $End^{+/c}$ 129/Ola mouse with partial tail loss resulting from repeated bleeds from telangiectases (arrows). (d) An $End^{+/c}$ HHT C57BL/6 intercross showing flaccidity and ptosis as a consequence of a stroke (arrows). (e and f) Liver of an $End^{+/c}$ HHT B6$_{in}$ mouse showing congestion and dilation of vessels compared to $End^{+/c}$ littermate control. (g and h) Dilated arteries and veins in lungs of an $End^{+/c}$ HHT C57BL/6 intercross compared to littermate control. (i and j) Normal brain region from an $End^{+/c}$ HHT C57BL/6 intercross adjacent to the area with a subarachnoid hemorrhage. Bar: 200$\mu$m (e and f), 500$\mu$m (g and h) and 100$\mu$m (i and j).
A.5 Murine HHT is strain dependent and suggests modifier genes effects

HHT manifestations were highly heterogenous in mice. We observed a population of 202 End\textsuperscript{\textasciitilde} mice, all potentially disease prone because of their mutated Endoglin allele, but only 59 developed HHT. The age of onset, judged by external signs, ranged from 1 wk to 18 months with the majority developing HHT before 9 months. Of the 202 End\textsuperscript{\textasciitilde} mice, only 22 were inbred 129/Ola backcrosses as this strain breeds poorly; 75 were C57BL/6 backcrosses of the first and second generations with a mixed background (50-75% C57BL/6, 25-50% 129/Ola). 105 End\textsuperscript{\textasciitilde} mice were generated by inter-breeding of C57BL/6 backcrosses, and are thus also of mixed genome. Figure A.3a shows that 68% End\textsuperscript{\textasciitilde} 129/Ola mice developed HHT while only 9% of the End\textsuperscript{\textasciitilde} C57BL/6 backcross mice did; the intercrosses were intermediate with 34% having HHT. The difference in prevalence of HHT was significant among these 3 groups of mice. This pattern of distribution between parental strains and intercrosses suggests very strongly that modifier genes of 129/Ola origin are contributing to severity and heterogeneity of HHT (Chapter 4).

Organ involvement was assessed at autopsy in the HHT mice (Figure A.3b). A substantial proportion of animals, in the 3 groups, had liver (39-57%), lung (28-50%), and/or brain (0-33%) manifestations, such as described in Figure A.2. Several End\textsuperscript{\textasciitilde} HHT mice also showed gastrointestinal telangiectases and bleeding, detected by fecal occult blood. The frequency of ruptured major vessels (mean 33%) was a reflection of animals dissected in agonal phase of disease, which comprised about 40% of our experimental group. Except for brain not being affected in the C57BL/6 backcrosses, visceral involvement was similar in the 3 groups (Figure A.3b). This suggests that it is indeed the modifier gene(s), contributed by the 129/Ola background, that are responsible for severe organ involvement. It is interesting to speculate that distinct modifier genes might contribute to liver, lung and/or brain involvement in HHT.
Figure A.3 Frequency of HHT in End+/− mice and visceral involvement in dissected mice.

Germ line transmission of the End+/− phenotype was established by backcrossing in the 129/Ola strain (n = 22; Ola₀; 100% 129/Ola) and in the C57BL/6 strain (n = 75; B6₀; N1 and N2 generations still containing 50% and 25% respectively of 129/Ola genome). Inter-breeding of these latter mice, were generated by intercrosses B6₁ and backcrosses to a parent suspected of disease B6₂ (n = 105). Animals were observed for at least 6 months and up to 18 months.

(a) Histogram representing the % of End+/− mice that developed HHT in each group, as determined by external signs. * p<0.01 compared to 129/Ola backcross; ** p<0.0001 compared to 129/Ola backcross. (b) Histogram showing the frequency of liver, lung, brain, and intestinal involvement in dissected HHT mice from each group.
A.6 Human and Murine HHT; similarities and differences

We report a group of patients seen at the Toronto HHT Clinic who were diagnosed clinically and compare these to End$^{+/+}$ mice with HHT (Figure A.4). Of the 197 patients with HHT, the majority had mucocutaneous telangiectases (93%) and recurrent spontaneous nose bleeds (85%); 38% had pulmonary AVMs, 7% had cerebral AVMs, 9% had liver AVMs and 17% had chronic GI bleeding. The expression of disease in our HHT population is similar to that in another previously published large series (24). The phenotype of the End$^{+/+}$ mice was similar to that of the human population, including mucocutaneous telangiectases, external bleeding and involvement of the liver, lungs, brain and GI tract.

The majority of mice with HHT had external manifestations of disease, such as mucocutaneous telangiectases, epistaxis and other external bleeding. The prevalence of telangiectases and external bleeding may have been somewhat underestimated in the mouse due to difficulties in detection. Visceral manifestations were reported for autopsied mice only and appear more frequent in mice with HHT than in the human population. The prevalence of 33% of pulmonary disease was similar to that in humans. However, the prevalence of gastrointestinal, hepatic and cerebral disease was somewhat greater in mice than has been observed in people. There are several possible explanations for this. The most obvious one is that mice were autopsied whereas patients were not, and therefore more disease would be detected in the mice. The prevalence of liver AVMs might be underestimated in the human population since only patients with a large left-to-right shunt would be have been diagnosed with the current screening protocol and clinical exam. Since liver AVMs in patients with HHT are not actually macroscopic AVMs but rather a mass of telangiectases, a large number is probably required before a large left-to-right shunt is detectable. In other words, many more patients would likely have liver telangiectases if autopsied. Another potential explanation is that all mice studied have an endoglin mutation, whereas most patients have not been genotyped and therefore may have endoglin or ALK-1 mutation. Disease severity may be diluted by the presence of ALK-1 families, who generally have a milder phenotype. The increased prevalence of liver disease
Figure A.4 Comparison of human and murine HHT.
(a) Analysis of 197 patients with a clinical diagnosis of HHT. All patients were seen at the Toronto HHT Clinic at St. Michael’s Hospital. Of the 304 consecutive patients seen between February 1997 and April 2000, 197 (65%) had a definite or probable diagnosis of HHT, based on the current clinical criteria (27). The mean age was 40 years (range 15 to 87) and 125 (64%) were female. All patients had a full history and physical examination and were screened for pulmonary AVMs (chest radiography and contrast echocardiography or oxygen shunt test), cerebral AVMs (brain MRI); 90 patients were screened for liver AVMs by doppler ultrasound (J.A. Clark, personal communication). (b) The 59 mice diagnosed with HHT on the basis of external signs were analyzed for expression of telangiectases and visible bleeds from either nose, ear, mouth, tail and/or genitals. The 33 HHT mice dissected because of severe illness or advanced age, were examined for visceral involvement, such as organomegaly, telangiectases and hemorrhage and for dilated vessels by histopathology.
might also be related to the 129/Ola background, since 70-80% of the wild type 129/Ola mice were reported to have large intrahepatic connections between portal and hepatic veins as well as reduced number and extent of peripheral vessels (25, 26).

A.7 How useful is the HHT mouse?

Clinical studies of human HHT revealed phenotypic heterogeneity, which is explained in part by two genes, Endoglin and ALK-1. Expression studies showed that haploinsufficiency was the mechanism responsible for HHT, indicating that disease heterogeneity cannot be explained by position and type of mutations. As all mutant proteins studied to date cannot reach the cell surface, they cannot interfere with the normal function of the normal surface protein. A proportion of the mice expressing a single allele of endoglin, did develop HHT. However it was very dependent on the genetic background of the inbred strain. Our data strongly suggest that additional genes, so-called modifier genes, such as contributed by the 129/Ola strain, are necessary to generate the vascular anomalies associated with HHT. The identification of these genes in mice should lead to the recognition of their counterparts in human.

The murine model is exciting as it reproduces most of the clinical manifestations of HHT. Although AVMs were not detected in the mice, the extent of liver, lung and brain involvement and their sequelae such as stroke, suggests that they are present. Future angiography studies should allow us to determine if AVMs can be detected in mice with HHT. The ability to isolate multiple vessels from HHT mice and study their vascular tone and its regulation should also increase our understanding of the mechanisms responsible for abnormal blood vessel dilation. Novel therapeutics modalities can also be tested in mice with HHT with potentially important implications for patients with this chronic disease.
A.8 Acknowledgments

We would like to acknowledge all patients who participated in the studies. We thank Ms. Urszula Cymerman and Sonia Vera for performing mutation analysis and protein expression studies and Ms. Shelley Kennedy for help with genetic counselling and preparation of pedigrees. We acknowledge the help of Dr. Ian Wanless for interpretation of the pathological murine specimens. We thank Mr. Sigmund Kaw and Ms. Merry-Lynn McDonald for technical assistance and Ms. Lily Morikawa, for her invaluable help in preparing sections from the various murine tissues. We are grateful to the staff of the Lab Animal Services at the Hospital for Sick Children who helped in the daily assessment of the HHT phenotype in the animals. This research was supported by grant #NA3434 from the Heart and Stroke Foundation of Ontario and the Medical Research Council of Canada (M.L.). A.B. is a recipient of a studentship from the Medical Research Council of Canada, M.E.F. was supported by a fellowship from the Medical Research Council of Canada/Canadian Lung Association and by the Nelson Arthur Hyland Foundation and The Squires Club. M.L. is a Terry Fox Research Scientist of the National Cancer Institute of Canada.
A.9 References


