TEMPORAL AND COMPARATIVE ANALYSIS OF GENES THAT PLAY A ROLE IN BRAIN VASCULAR DEVELOPMENT

by

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TEMPORAL AND COMPARATIVE ANALYSIS OF GENES THAT PLAY A ROLE IN BRAIN VASCULAR DEVELOPMENT

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ABSTRACT

TEMPORAL AND COMPARATIVE ANALYSIS OF GENES THAT PLAY A ROLE IN BRAIN VASCULAR DEVELOPMENT

Arteriovenous malformations (AVMs) are direct abnormal connections of arteries and draining veins without a capillary bed. AVMs, in which normal blood flow is interrupted, carry high risk of bleeding throughout life. They are thought to arise during development and there is very limited knowledge on the molecular pathogenesis of the AVMs. To unravel the molecular mechanisms and develop therapy modalities, detailed understanding of the brain vascular development at molecular level is crucial. Besides, today compelling number of evidence suggests that blood vessels and neurons use common signals and mechanisms to shape the nervous systems. Moreover, angiogenesis and some of its key modulators were recently shown to protect the nervous system from neurodegeneration. Thus, the knowledge of the norms of brain vascular development and the genes involved is essential for detailed understanding of the role of angiogenesis in neurodegenerative mechanisms. However, most of what we know about angiogenesis at molecular level is derived from pathological studies. In literature, there are no multi-gene level studies covering the norms of brain vascular development at molecular level. In this study, for the first-time in literature, brain vascular development was investigated using a pathway-specific, low-density microarray system in a systemic manner. Temporal and comparative expression of 113 angiogenesis-related genes was analyzed and genes that play a role in cerebrovascular development were determined. Genes like Bai1, Nudt6, Ctgf, were shown to be expressed in brain development for the first time in this study. The findings are expected to make important contributions to the limited research on brain vascular development and are expected to be an important reference for functional studies in AVM and neurodegeneration; they will also form the basis for further studies in humans.

ÖZET

BEYNİN DAMARSAL GELİŞİMİNDE ROL OYNAYAN GENLERİN ZAMANSAL VE KARŞILAŞTIRMALI ANALİZİ

Arteriovenöz malformasyonlar (AVM) atardamarlar ile toplardamarların arada kılcal damar yatağı olmadan düzensiz olarak birbirine bağlandığı damar yumaklarına denir. AVM'lerin içerisinde sağlıklı kan akışı yoktur, dolayısıyla yüksek kanama riskiyle yaşamsal tehdit oluştururlar. Gelişim döneminde oluşmaya başladığı düşünülen AVM'lerin, moleküler patogenezleri hakkındaki bilgimiz oldukça kısıtlıdır. AVM gelişimindeki moleküler mekanizmaları gün ışığına çıkarmak ve etkin tedavi yaklaşımları geliştirebilmek için, beynin damarsal gelişimini moleküler düzeyde anlamak oldukça önemlidir. Ayrıca, yeni birçok önemli bulgu göstermektedir ki, kan damarları ve nöronlar sinir sistemini oluşturmak ve korumak için ortak sinyal ve mekanizmaları kullanmaktadır. Buna ek olarak, anjiogenezin ve anjiogenezde rol alan önemli faktörlerin merkezi sinir sisteminin nörodejenerasyondan korunmasında etkin olduğu birçok çalışmada gösterilmiştir. Bu nedenle, anjiogenezin nörodejeneratif mekanizmalardaki rolünü iyi anlayabilmek için beyin damarsal gelişiminin normlarını ve bu süreçte rol alan genleri bilmek gereklidir. Ancak, anjiogenez hakkındaki moleküler düzeydeki bilgimizin büyük kısmı patolojik çalışmalardan elde edilmiştir. Literatürde, beynin damarsal gelişiminin moleküler normlarını ele alan bütünsel bir çalışma bulunmamaktadır. Bu çalışma çerçevesinde, yolak-odaklı, düşük-yoğunluklu mikroarray sistemleri kullanılarak, literatürde ilk kez, beynin damarsal gelişimi sistemik olarak incelenmiştir. 113 adet anjiogenez ile ilgili genin ekspresyonu, zamansal ve birbiriyle kıyaslamalı olarak incelenmiş ve serebrovasküler gelişimde rol alan genler belirlenmiştir. Bai1, Nudt6, Ctgf gibi genlerin beyin gelişiminde rol aldığı ilk defa bu çalışma ile gösterilmiştir. Bulguların, çok kısıtlı olan beyin damarsal gelişimi araştırmalarına önemli katkıda bulunması ve AVM, nörodejenerasyon konularında gerçekleştirilecek işlevsel çalışmalar için referans oluşturması beklenmektedir. Bunun dışında, çok bakir bir alanda yapılan çalışmada alınan sonuçların ilerideki insan araştırmaları için de yol gösterici olacağı düşünülmektedir.

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g	Gravity Force
M	Molar
mA	Milli Amper
mg	Milli gram
ml	Milli liter
mM	Milli molar
nm	Nano mter
V	Volt
µg	Micro gram
µl	Micro liter

AIDS	Acquired Immune Deficiency Syndrome
Alk1	Activin Receptor-like Kinase 1
Alk5	Activin Receptor-like Kinase 5
ALS	Amyotrophic Lateral Sclerosis
Ang1	Angiopoietin 1
Ang2	Angiopoietin 2
ANP	Atrial Natriuretic Peptide
APS	Ammonium Persulfate
APSA	Alkaline Phosphatase-conjugated Streptavidin
ARTN	Artermin
AS1	Artificial Sequence 1
AS1R1	Artificial Sequence 1 Related 1
AS1R2	Artificial Sequence 1 Related 2
AVM	Arteriovenous Malformation
B2m	Beta-2 Microglobulin
BAS2C	Biotinylated Artificial Sequence 2 Complementary Sequence
BDNF	Brain-derived Neurotrophic Factor
BNP	Brain Natriuretic Peptide

BSA	Bovine Serum Albumin
^0C	Degree Celcius
CBB	Coomassie Brilliant Blue
cDNA	Complementary Deoxyribonucleic acid
CM	Cavernous Malformations
CNP	type-C Natriuretic Peptide
CNS	Central Nervous System
cRNA	Complementary Ribonucleic acid
Ctgf	Connective Tissue Growth Factor
dH_2O	Distilled Water
Dll4	Delta-like 4
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
dNTP	2'-Deoxynucleoside 5'-triphosphate
DTT	Dithiothreitol
E	Embryonic
EC	Endothelial Cell
ECL	Electrochemiluminescence
ECM	Extracellular Matrix
Edg1	Endothelial Differentiation Sphingolipid G-protein Coupled Receptor-1
EDTA	Ethylenedinitrilo-tetraacetate
Egf	Epidermal Growth Factor
EtOH	Ethyl Alcohol
FAM	6-carboxy-fluo-rescine
Fgf2	Fibroblast Growth Factor 2
Fgfr3	Fibroblast Growth Factor Receptor 3
Flt1	Fms-related Tyrosine Kinase 1
GAPDH	Glyceraldehyde-3-phosphate Dehydrogenase
GC	Guanylyl Cyclase
GDNF	Glial-derived Neurotrophic Factor
HCl	Hydrogen Chloride
HGF	Hepatocyte Growth Factor

HHT	Hereditary Hemorrhagic Telangiectasia
HIF	Hypoxia Inducible Transcription Factors
Hr	Hour
HRP	Horse Radish Peroxidase
Hsp90a1	Heat shock protein 90kDa alpha, class B member 1
IDEA	Image Data Extraction Applet
Igf1	Insulin-like Growth Factor 1
IgG	Immunoglobulin G
Il	Interleukin
Jam1	Junction Adhesion Molecule 1
kDa	Kilo Dalton
Kdr	Kinase Insert Domain Receptor
Mdk	Midkine
MetOH	Methanol
$MgCl_2$	Magnesium Chloride
min	Minute
Mmp	Matrix Metalloproteinase
Mmp1	Matrix Metalloproteinases 1
Mmp2	Matrix Metalloproteinases 2
Mmp3	Matrix Metalloproteinases 3
Mmp9	Matrix Metalloproteinases 9
MND	Motor Neuron Degeneration
mRNA	Messenger RNA
NaCl	Sodium Chloride
Ngf	Nerve Growth Factor
NMJ	Neuromuscular Junction
Npr1	Natriuretic Peptide Receptor 1
Npr2	Natriuretic Peptide Receptor 2
Nrp1	Neuropilin Receptor 1
Nrp2	Neuropilin Receptor 2
NT3	Neurotrophin 3
NT4	Neurotrophin 4
Nudt6	Nudix (Nucleoside Diphosphate Linked Moiety X)-Type Motif 6

OD	Optical Density
OD_{230}	Optical Density at 230 nm
P	Postnatal
p53	53 Kilo Dalton Protein
PA	Plasminogen Activator
PAGE	Polyacrylamide Gel Electrophoresis
Pai1	Plasminogen Activator Inhibitor 1
Pai2	Plasminogen Activator Inhibitor 2
PBS	Phosphate Buffered Saline
PC	Personal Computer
PCR	Polymerase Chain Reaction
PDGF-A	Platelet Derived Growth Factor A
PDGF-B	Platelet Derived Growth Factor B
PDGFR-β	Platelet Derived Growth Factor Receptor-β
Pgf	Placental Growth Factor
Ppia	Peptidylprolyl Isomerase A
Ptn	Pleiotrophin
PVDF	Polyvinylidene Fluoride
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
RefSeq	NCBI Reference Sequences
RI	Ribonuclease Inhibitor
RNA	Ribonucleic Acid
RNAse	Ribonuclease
rpm	Revolutions Per Minute
Rps27a	Ribosomal Protein S27a
rRNA	Ribosomal Ribonucleic Acid
RT	Room Temperature
RT-PCR	Real-Time Polymerase Chain Reaction
SBMA	Spinal and Bulbar Muscular Atrophy
sec	Seconds
Sip1	Sphingosine-1-phosphate-1
SMC	Smooth Muscle Cell
SOD1	Superoxide Dismutase 1

SSC	Saline-Sodium Citrate
TBE	Tris Boric acid EDTA
TBS	Tris Buffered Saline
TBST	Tris Buffered Saline Twen-20
TEMED	N’,N’,N’,N’-Tetramethylethylenediamine
TGF	Transforming Growth Factor
TGF-α	Transforming Growth Factor-α
TGF- β	Transforming Growth Factor- β
Tie1	Tyrosine Kinase with Immunoglobulin-like and EGF-like Domains1
Tie2	Tyrosine Kinase with Immunoglobulin-like and EGF-like Domains2
Timp	Tissue-Inhibitor of Metalloproteinase
tPA	Tissue-type Plasminogen Activator
Tsp1	Thrombospondin-1
TNF-α	Tumor Necrosis Factor α
uPA	Urokinase-type Plasminogen Activator
U	Unit
UPL	Universal Probe Library
UV	Ultraviolet
VEGF	Vascular Endothelial Growth Factor
Vegfr1	Vascular Endothelial Growth Factor Receptor 1
Vegfr2	Vascular Endothelial Growth Factor Receptor 2
Vegfr3	Vascular Endothelial Growth Factor Receptor 3
w/v	Weight/Volume

1. INTRODUCTION

The vascular system, by penetrating every organ and tissue, supplies cells with oxygen and provides circulation of metabolites. The emergence of vascular system is one of the earliest events in embryogenesis and is achieved through two mechanisms: vasculogenesis and angiogenesis. Vasculogenesis is the term used for the *de novo* formation of vascular plexus from angioblasts in the early embryonic development. In contrast to vasculogenesis, angiogenesis is the generation of new blood vessels from pre-existing vessels (Jain, 2003; Carmeliet, 2005; Karamysheva, 2008).

1.1. Embryonic Vascular Development

Embryonic vascular development is a well-programmed, dynamic sequence of events that requires the correct spatial and temporal expression of specific sets of genes. It involves the formation of a nascent vascular network by vasculogenesis and angiogenesis, followed by remodeling and specialization of vessels (Yancopoulos *et al*., 2000).

1.1.1. Vasculogenesis

During gastrulation, mesenchymal stem cells are induced to differentiate to hemangioblasts, leading to formation of "blood islands" in the yolk sac. Hemangioblasts at the center of blood islands are transformed to hematopoietic stem cells, and those that are found in peripherals of islands are transformed to angioblasts, the precursors of endothelial cells (ECs). Angioblasts, while also transforming to the endothelial cells concurrently, connect to each other and construct a primitive tubular network, called "primary capillary plexus", the first vascular-like structures of the developing embryo (Risau and Flamme, 1995; Risau, 1997; Lamalice *et al*., 2007) (Figure 1.1).

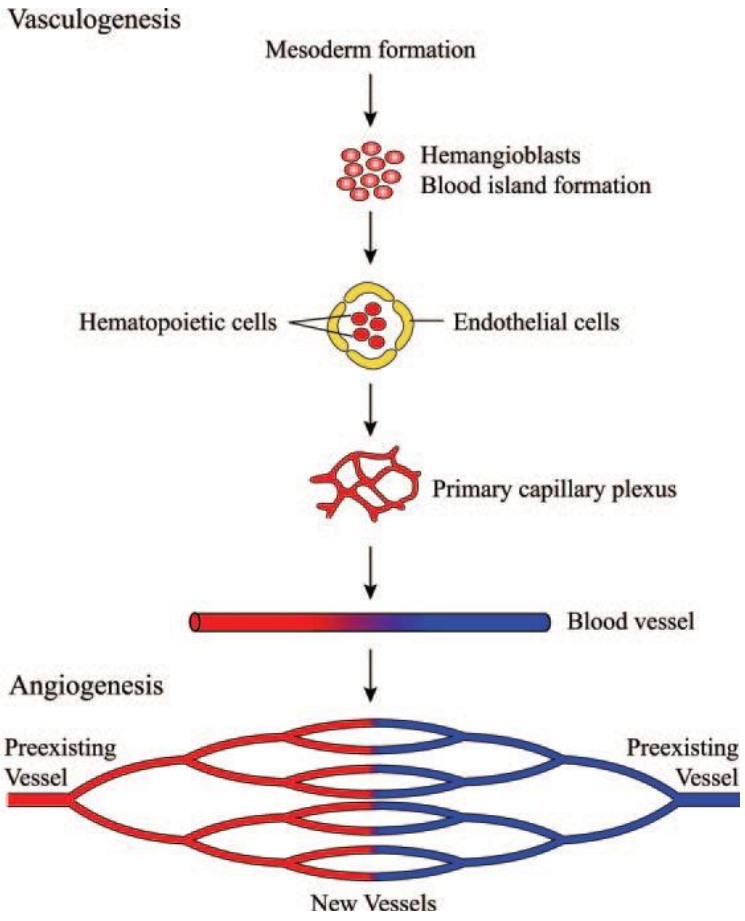

Figure 1.1. Genesis of the vascular system (Lamalice *et al*., 2007)

1.1.2. Angiogenesis

After formation of the vascular plexus, endothelial cell formation increasingly continues, and new capillaries are generated from pre-existing capillaries, by the process called angiogenesis (Risau, 1997) (Figure 1.1.).

In embryonic development, angiogenesis occurs mainly by two mechanisms; sprouting and non-sprouting (intussusceptive) angiogenesis (Burri and Tarek, 1990) (Figure 1.2). Sprouting angiogenesis occurs in both the yolk sac and the embryo, mostly at later organogenesis and particularly in brain development. Sprouting involves the activation of ECs, the proteolytic degradation of the extracellular matrix (ECM), migration and proliferation of the ECs to form the lumen, followed by the functional maturation and specification of vessels. Non-sprouting angiogenesis is a term used for splitting of pre-

existing vessels by transcapillary tissue pillars. It starts with EC proliferation within the vessel, and causes the enlargement of the lumen which can be split and partitioned by insertion of tissue columns. While vascularization of the yolk sac, kidney, thymus, brain, limb and choroid plexus occurs by sprouting angiogenesis, lung and heart are vascularized by both types of angiogenesis (Risau, 1997; Djonov *et al.*, 2000).

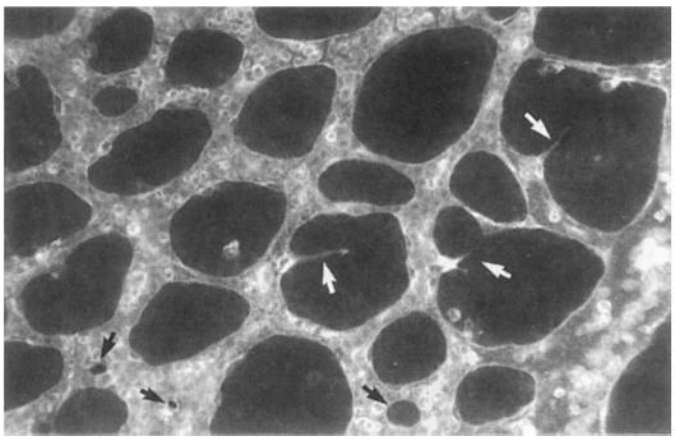

Figure 1.2. Sprouting and non-sprouting angiogenesis in the 3-day-old quail-yolk-sac: Both processes occur simultaneously (black arrows point to the intussusceptive pillars and white arrows indicate tips of sprouts) (Risau, 1997)

It is shown that angioblasts can migrate over long distances from their sites of origin and form a new vascular plexus at a distant site. So although the primary mechanism for establishment of the vascular network is angiogenesis, vasculogenesis is also seen in vascularization of some endodermal organs, such as liver, lungs, pancreas, stomach, intestine and spleen (Dieterlen-Lievre *et al.*, 1999).

1.1.3. Vessel remodeling and specialization

Blood vessels are composed of endothelial cells, pericytes, vascular smooth muscle cells (SMCs) and surrounding elastic lamina and basement membrane. While ECs form the vessel lumen, pericytes and vascular SMCs, having specialized connections with ECM, cover vessels, providing mechanical support and giving visco-elasticity to the vessels (Cleaver and Melton, 2003).

The composition of the vessel wall changes according to vessel function and tissue type (Figure 1.3). Capillaries consist of EC tubes, surrounded by the basement membrane and the sparse layer of pericytes, embedded in ECs. The arterioles and venules have thicker walls, with more mural cells (pericytes, vascular smooth muscle cells) and much connective tissue. The walls of larger vessels consist of three layers: intima, media and adventitia, each with varying composition of cells and ECM. Lymphatic vessels lack pericytes and have totally different vessel walls, specialized for the collection of the intracellular fluid (Jain, 2003; Karamysheva, 2008).

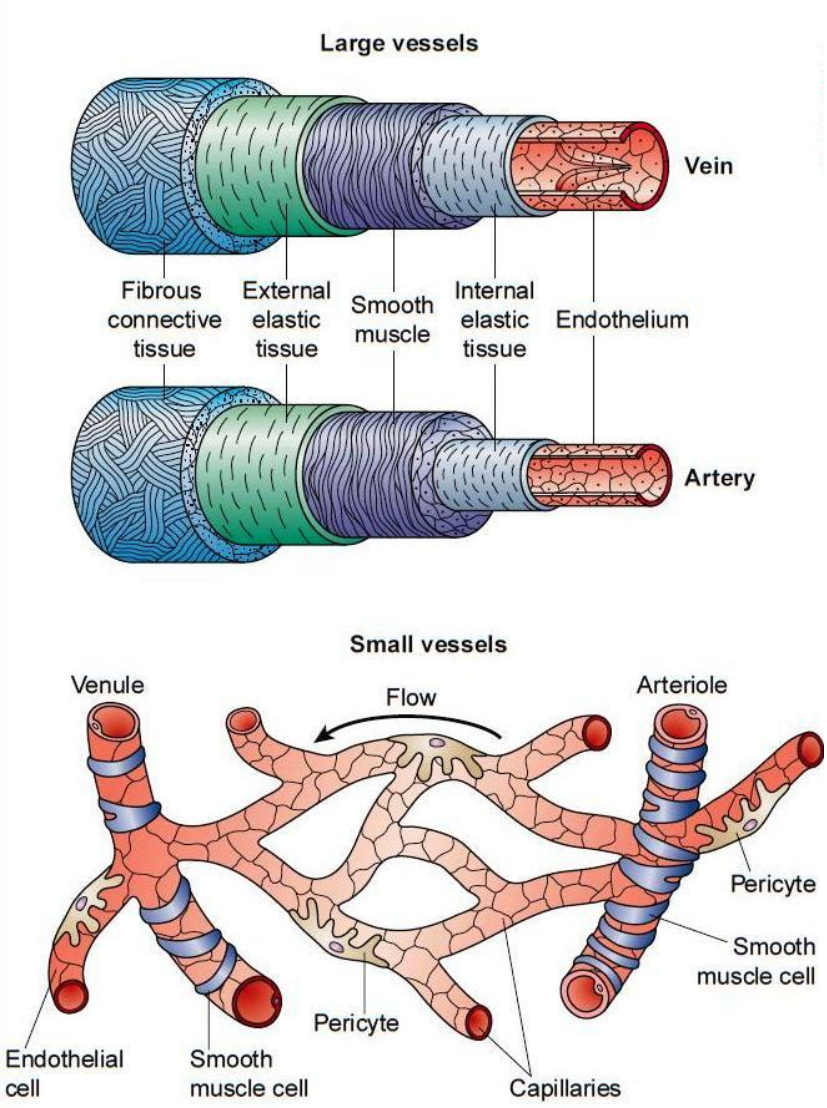

Figure 1.3. The wall composition of large versus small vessels (Cleaver and Melton, 2003)

Newly formed nascent tubules remodel and specialize, according to the needs of tissue they are found in, and vessel type they become (i.e. arterio-venous determination). Every tissue is different from the other, and thus, blood vessels, especially capillaries, exhibit tissue-specific features (Hynes, 2002).

Remodeling of vessels is achieved partly through vigorous interaction of ECs with the surrounding ECM. ECM does not function just as a supporting material, but it also anchors many signals that direct ECs and mural cells during proliferation (Jain, 2003). Especially integrin receptors have a major role in branching of vessels that mediate communication of ECs with ECM components (Hynes, 2002).

Beside ECM, the endothelial cell itself also plays a determinant role in tissue-specificity of blood vessels. In spite of their elongated, thin and fragile structures, ECs interconnect to each other to form stable channels and perfectly carry and deliver blood to trillions of cells. Moreover, although they can remain in dormancy for several years, if stimulated, they can proliferate immediately and form new vessels in a coordinated fashion. ECs posses these qualifications, mostly due to their outstanding capabilities of interaction with each other and with neighboring cells. They are equipped with a certain set of molecules that make them sense the blood pressure and hemodynamic changes; they also help ECs to dynamically cooperate with the internal cytoskeleton and the surrounding ECM. Endothelial specific cadherins, claudins, occludins and junction-adhesion molecule-1 (JAM1) are examples of these molecules, which function as “mechanical zippers” and transmit crucial environmental signals to ECs (Carmeliet *et al*., 1999; Carmeliet, 2003a). Furthermore, there are many different types of ECs, each determines its own way of behavior in different tissues. The genetic mechanisms underlying this heterogeneity are unknown (Ribatti, 2006).

The arterio-venous fate of vessels was thought, for a long time, to be the result of hemodynamic forces. But studies on zebrafish showed that before even the blood starts to flow, ECs fate is determined by the ligand Ephrin-B2 and its receptor Ephrin-B4. Ephrin-B2 is expressed in arterial ECs and vascular SMCs, and the Ephrin B4 receptor is expressed in venous ECs. After this discovery, several other markers for arteries and veins

were also reported: Neuropilin receptors 1 and 2 (NRP1 and NRP2) and several members of the Notch family (Adams, 2003).

1.2. Regulation of Angiogenesis

Playing a fundamental role during embryonic vascular development, angiogenesis remains quiescent in adulthood; but it can become rapidly active in some physiological and pathological events, like in wound healing, tissue regeneration, uterine cycle, placenta formation, tumor growth, stroke, chronic inflammation and in many vascular anomalies (Carmeliet, 2005).

Angiogenesis is a dynamic and complex process that depends on cellular interactions with numerous regulatory factors, including growth factors and their receptors, chemokines, ECM molecules, cell-cell and cell-matrix proteins. It involves; i) activation of ECs, ii) proteolytic degradation of ECM, followed by iii) cell migration, proliferation and lumen formation, and finally iv) maturation to functional blood vessels by mural cells (Liekens *et al*., 2001).

1.2.1. Angiogenic Switch: Activation of Endothelial Cells

Angiogenesis becomes inactive after embryonic development and is not switched on, unless activated by angiogenic signal. Angiogenesis can be re-stimulated by a number of signals, such as metabolic stress (hypoxia, low pH, hypoglycemia), mechanical stress (pressure generated by proliferating cells, shear stress), inflammatory response and genetic defects, e.g. mutations in genes that play a role in angiogenesis. The “angiogenic switch” is regulated by a tight balance between pro- and anti-angiogenic molecules. For example, synthesis of hypoxia-inducible transcription factors (HIFs) by surrounding tissues upon hypoxia, leads to vascular endothelial growth factor (VEGF) expression increase, up to 30-fold in a minute. Secreted VEGF strictly regulates angiogenesis in a dose-dependent manner. When sufficient neovascularization is achieved, pro-angiogenic signals are down-regulated and inhibitors are activated, consequently ECs become re-quiescent and the angiogenic switch is turned off (Carmeliet and Jain, 2000; Pugh and Ratcliffe, 2003).

Up to now, many angiogenic stimulatory molecules have been discovered. Some of these molecules have direct and specific actions on ECs, like VEGF family members and angiopoietins. Some others are acting directly, but not specifically (meaning that beside ECs, they have roles in many other functions in different cells); the most prominent examples of this group are fibroblast growth factor-2 (Fgf2) and the ephrin family. The third group consists of the unspecific and indirect-acting molecules, which act through direct acting molecules, mostly through VEGF. The two mostly studied examples of this group are tumor-necrosis-factor-α (TNF-α) and transforming growth factors (TGFs) (Chiarugi *et al*., 1998; Klagsbrun and Moses, 1999; Liekens *et al*., 2001).

VEGF, playing a pivotal role in angiogenesis, stimulates the degradation of the ECM, proliferation, migration and tube formation of ECs and the permeability of vessels (Liekens *et al*., 2001). There are six members of the VEGF family; VEGF-A (or VEGF), placental growth factor (Pgf), VEGF-B, VEGF-C, VEGF-D and VEGF-E (Veikkola and Alitalo, 1999). The loss of only a single allele leads to embryonic lethality in VEGF knock-out mice, which explains the indispensible role of VEGF during embryonic vascular development (Carmeliet *et al*., 1996; Ferrara *et al*., 1996). There are three VEGF receptors on endothelium, vascular endothelial growth factor receptor-1 (VEGFR-1), previously also known as Fms-related tyrosine kinase 1 (Flt1), vascular endothelial growth factor receptor-2 (VEGFR-2), previously called kinase-insert-domain-receptor (Kdr) and the vascular endothelial growth factor receptor-3 (VEGFR-3) (Yancopoulos *et al*., 2000). Homozygous deletion of VEGFR-1 or of VEGFR-2 results in embryonic lethality. Several studies showed that while VEGFR-2 is crucial for VEGF functioning, the role of VEGF and VEGFR-1 interaction is still unclear (Shalaby *et al*., 1995; Fong *et al*., 1999). Later it was found, that VEGF can bind also to NRP1 and NRP2, but how they transmit their signal is still unknown. Finally, VEGF levels are equal in angiogenically active and quiescent tissues, which may imply that VEGF also plays a role as a survival factor (Ferrara, 1999; Liekens *et al*., 2001).

Angiopoietin-1 (Ang1) and angiopoietin-2 (Ang2) are other important angiogenic signals, and their receptors Tie1 and Tie2 (Tie for tyrosine kinase with immunoglobulin-like and EGF-like domain) are specifically expressed in ECs. According to knock-out studies, angiopoietins seem to be VEGF`s most important partners by playing a role in the

regulation of endothelial cell stabilization and quiescence, and their integration with supporting cells (Suri *et al*., 1996; Puri *et al*., 1999; Liekens *et al*., 2001). While the exact function of Ang2 is still unknown, it may be a natural antagonist of Ang1, thereby destabilizing the new vessels, which are necessary for subsequent remodeling (Maisonpierre *et al*., 1997).

Beside its role in many other biological processes, Fgf2 plays a key role in angiogenesis. Fgf2 leads to activation of many angiogenic processes, including proliferation of ECs, proteolysis and tube formation. It is expressed in any tissue and cell type examined, which implies its fundamental roles inside the cell (Liekens *et al*., 2001). Ephrins and their receptors are other crucial players that are involved in arterio-venous specialization of ECs and mediate interactions of ECs with surrounding cells (Tang and Conti, 2004). Integrin receptors, which mediate cell-cell and cell-matrix interactions, are also important for EC survival and vascular development. Integrins can bind to the variety of ECM molecules, such as fibronectin, vitronectin, collagen, laminin, von Willebrand factor, fibrinogen, thrombospondin and osteopontin. Such high interaction capacities make integrins important players in the entire angiogenesis process, especially in EC stability after proliferation. Knock-out studies on several integrin members result in embryonic or postnatal lethality with severe vascular defects (Rupp and Little, 2001; Chavakis and Dimmeler, 2002).

Platelet-derived growth factors (PDGF), epidermal growth factors (EGF), transforming growth factors α and β (TGF-α and TGF-β) and insulin-like growth factor 1 (IGF1) are other important angiogenic signals, secreted by cells that lead to increase of VEGF levels and activation of angiogenesis (Karamysheva, 2008).

1.2.2. Proteolytic Degradation of Basement Membrane and ECM

To initiate the formation of new tubules, ECs have to degrade the vessel basement membrane, and the surrounding tissue stroma. This proteolytic degradation process is achieved by cooperated activities of the plasminogen activator (PA) system, matrix metalloproteinases (MMPs) and several other proteases (Mignatti and Rifkin, 1996; Liekens *et al*., 2001).

There are two types of plasminogen activators; urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA), that convert inactive plasminogen to active plasmin by proteolytic cleavage. Activated plasmin has a broad range of substrate specificity and can degrade many ECM molecules, including fibronectin, fibrin, laminin and proteoglycans. They also can trigger activation of MMP1, MMP2, MMP3, and MMP9. There are 16 different MMPs, which are capable of degrading almost all ECM components. MMPs are secreted in inactive form and become active when their pro-domain is protealytically cleaved upon secretion (Westermarck and Kahari, 1999; Liekens *et al*., 2001).

The strict control of proteolytic activities of the PA system molecules and MMPs is crucial for preserving normal tissue structure. This control is achieved at three levels; i) their activation is regulated by angiogenic growth factors, ii) they are secreted in inactive form and can simply get active by proteolytic cleavage and iii) they are secreted together with their inhibitors: tissue inhibitor of metalloproteinases (TIMP), α_2-antiplasmin and plasminogen activator inhibitors-1 and -2 (PAI1 and PAI2) (Bajou *et al*., 1998; Blavier *et al*., 1999; Liekens *et al*., 2001).

1.2.3. Endothelial cell migration, proliferation and lumen formation

Preserving the present blood vessel integrity during the endothelial cell migration is crucial for proper maintenance of the blood supply of the tissue. This is achieved by "leader" or "tip" endothelial cells, found on the vessels that are specialized to firstly migrate upon signal and then lead to the remaining cells. So, not all cells react equally to the signal and do not start to migrate at the same time (Figure 1.4). Members of Notch family receptors, Notch 1 and Notch 4 and their ligand Delta-like 4 (Dll4), play a key role in such versatile behavior of ECs. Upon VEGF stimulation, leader cells, express Dll4, gain invasiveness, secrete proteolytic enzymes and start to migrate to the degraded matrix. They are followed by proliferating ECs, which express Notch receptors, are stimulated by a variety of growth factors, some of which are released from the degraded ECM (Hellstrom *et al*., 2007; Karamysheva, 2008).

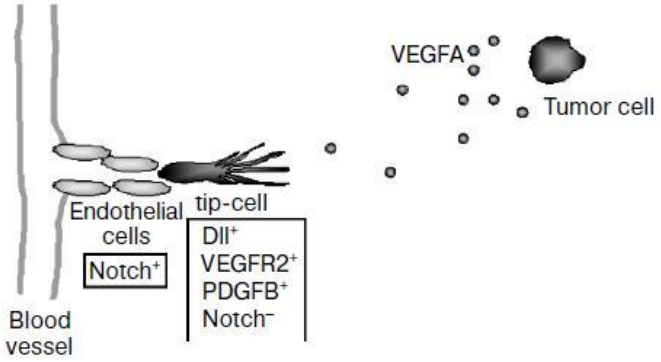

Figure 1.4. Formation of a new capillary (Karamysheva, 2008)

During migration, ECM contacts transiently disappear, but after formation of tubular structures by proliferating cells, they reappear and get enrolled in proper functioning of new vessels. Newly formed tubules are not stable, and to become functional they should mature by mural cells (Carmeliet *et al*., 1999; Carmeliet, 2003a).

1.2.4. Maturation to Functional Vessel

Maturation of nascent vessels includes recruitment of mural cells, generation of ECM and elastic laminae, and tissue- and function-specific remodeling of the vessel. All these processes are orchestrated by spatio-temporal and tight regulation the expressions of many molecules in a dose-dependent manner (Jain, 2003). Some of these regulatory molecules act in the following way:

PDGF-B is secreted by ECs after VEGF stimulation and recruits mural cells. PDGF-B binds to the platelet-derived growth factor receptor β (PDGFR-β) on mural cells and triggers proliferation and migration of mural cells (Hellstrom *et al*., 2001). As it is previously mentioned, the Ang1/Tie2 system, by playing a role in vessel stabilization, regulates vessel leakage, which is important for communication between EC and mural cells (Loughna and Sato, 2001). Another important couple of molecules, which play a role in vessel maturation, are sphingosine-1-phosphate-1 (Sip1) and its receptor, the endothelial differentiation sphingolipid G-protein coupled receptor-1 (Edg1). Edg1 is expressed by mural cells and plays a role in EC-mural cell interaction (Cho *et al*., 2003). TGF-β, a multifunctional cytokine, also plays a central role in vessel maturation, by stimulating ECM production and mural cell differentiation. TGF-β functions in angiogenesis through

its receptor endoglin and two downstream molecules, activin receptor-like kinase 1 and 5 (Alk1 and Alk5). Through its action on Alk1, it leads to induction of proliferation and migration of cells. By acting through Alk5, it induces vessel maturation, by stimulating the production of PAI and thus stopping the ECM degradation (Pepper, 1997; Jain, 2003).

1.3. Pathological Angiogenesis

Angiogenesis is a dynamic and complex process that is regulated by a tight balance between numerous pro- and anti-angiogenic molecules. Disruption of this balance results in many important health problems, such as cancer, ischemia, hemangioma, venous anomalies, cerebral cavernous malformations, arterio-venous malformations and several inflammatory diseases (Buysschaert *et al.*, 2008) (Table 1.1 and Table 1.2).

Table 1.1. Diseases characterized or caused by abnormal or excessive angiogenesis (adapted from Carmeliet, 2003)

Organ	**Disease in mouse or humans**
Numerous organs	Cancer , infectious diseases, autoimmune disorders
Blood vessel	Arteriovenous malformations, other vascular malformations, DiGeorge Syndrome, HHT, cavernous hemangioma, atherosclerosis, transplant arteriopathy
Adipose tissue	Obesity (angiogenesis is induced by fatty diet)
Skin	Psoriasis, warts, allergic dermatitis, scar kleoids, pyogenic granulomas, blistering disease, Kaposi sarcoma in AIDS patients
Eye	Persistent hyperplastic vitreous syndrome, diabetic retinopathy, retinopathy of prematurity, choroidal neovascularization
Lung	Primary pulmonary hypertension, asthma, nasal polyps
Intestines	Inflammatory bowel and periodontal disease, ascites, peritoneal adhesions
Reproductive system	Endometriosis, uterine bleeding, ovarian cysts, ovarian hyperstimulation
Bone, joints	Arthritis, synovitis, osteomyelitis, osteophyte formation

Table 1.2. Diseases characterized or caused by insufficient angiogenesis or vessel regression (adapted from Carmeliet, 2003)

Organ	Disease in mice or humans	Angiogenic mechanism
Nervous system	Alzheimer disease	Vasoconstriction, microvascular degeneration and cerebral angiopathy
	Amyotrophic lateral sclerosis	Impaired perfusion and neuroprotection, causing motor neuron degeneration
	Stroke	Correlation of survival with angiogenesis in brain; arteriopathy
Blood vessels	Atherosclerosis	Impaired collateral vessel development
	Hypertension	Microvessel rarefaction due to impaired vasodilation or angiogenesis
	Diabetes	Impaired angiogenesis in ischemic limbs, but enhanced retinal neovascularization
	Restenosis	Impaired re-endothelialization after arterial injury
Gastro-intestinal system	Gastric or oral ulcerations	Delayed healing due to production of angiogenesis inhibitors by pathogens
	Crohn disease	Characterized by mucosal ischemia
Skin	Hair loss	Retarded hair growth by angiogenesis inhibitors
	Skin purpura, telangiectasia and venous lake formation	Age-dependent reduction of vessel number and maturation due to EC telomere shortening
Reproductive system	Pre-eclampsia	EC dysfunction resulting in organ failure, thrombosis and hypertension
	Menorrhagia (uterine bleeding)	Fragility of SMC-poor vessels due to low Ang1
Lung	Neonatal respiratory distress	Insufficient lung maturation and surfactant production in premature mice
	Pulmonary fibrosis, emphysema	Alveolar EC apoptosis upon VEGF inhibition
Kidney	Nephropathy	Age-related vessel loss due to TSP-1 production
Bone	Osteoporosis, impaired bone fracture healing	Impaired bone formation due to age dependent decline of VEGF-driven angiogenesis

1.3.1. Tumor Angiogenesis

Mammalian cells need oxygen and nutrients for their survival. To grow beyond the diffusion limit of oxygen, which is 100 to 200 µm, mammalian cells induce new blood vessel formation (Carmeliet and Jain, 2000). Tumor growth is a multi-step process that starts with an uncontrolled rapid growth of cells, forming the small spheroid structure. As the tumor volume increases, cells lose contact to blood vessel cells, which results in dying of cells due to lack of oxygen and nutrients. At this stage, growth may enter to steady-state, which may continue for a long time, e.g. several months or even years. Then cells switch to the angiogenic phenotype and induce the formation of new capillaries (Liekens *et al*., 2001). Beside hypoxia, mutations in tumor suppressor genes and proto-oncogenes can start the angiogenic switch in tumors (Dachs and Tozer, 2000). Tumor vessels have many abnormalities; these are leaky, unstable, loosely interconnected, disorganized with irregular diameters and defective wall structures (Tang and Conti, 2004) (Figure 1.5).

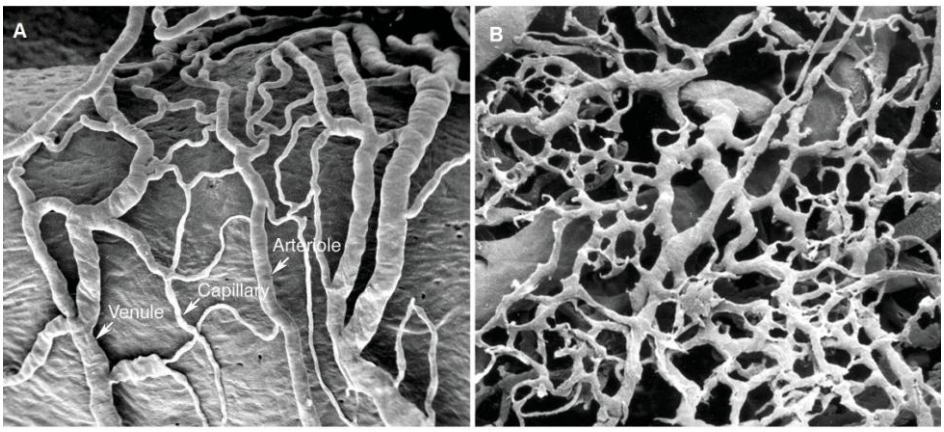

Figure 1.5. Scanning electron microscopic images of a) healthy (vasa vasorum of rat carotid sinus) and b) tumor vessels: tumor vessels lack conventional hierarchy of blood vessels, they posses disorganized capillaries, arterioles and venules (McDonald and Choyke, 2003)

After speculation that tumor growth and metastasis can be inhibited by anti-angiogenic agents, by Folkman in 1971, numerous studies have been performed investigating this topic. Many drugs have been approved up to now and several others are in trial (Folkman, 1971; Kilic *et al*. 2000a; Ferrara and Kerbel, 2005).

1.3.2. Angiogenesis and Neurodegeneration

Until recently, angiogenesis and neurodegeneration were thought as two distinct phenomena. New intriguing findings on many well-known angiogenic molecules revolutionized this view. Genetic studies reveal that insufficient production of some angiogenic signals can lead to neurodegeneration. Moreover, many angiogenic signals are shown to have neurotrophic and neuroprotective roles. Likewise, some molecules, that are prominent for their neuroregenerative and neuroprotective roles, can also regulate angiogenesis. The current developing idea suggests that blood vessels and neurons use similar signals and mechanisms to differentiate, grow, and connect with their targets. Actually, it shouldn't be surprising that millions of blood vessels criss-crossing the nervous system might not be the silent bystanders of the neurodegeneration process (Zacchigna *et al*., 2008).

VEGF is discovered as an angiogenic mitogen in 1983, since then, thousands of studies have mentioned its roles in angiogenesis and related events, but its neuronal actions were not known until the study by Sondell *et al*., in 1999. This and many other functional studies showed that VEGF plays a role as a neurotrophic factor (Senger *et al*., 1983; Sondell *et al*., 1999). In 2001, Oosthuyse *et al*. opened a new era in understanding neurodegenerative processes. They showed that transgenic mice with reduced VEGF expression (by deleting the hypoxia response elements in the promoter region of VEGF and inducing hypoxia in mice), surprisingly develop muscle weakness and atrophy due to motor neuron degeneration (MND), which is a characteristic of amyotrophic lateral sclerosis (ALS) (Oosthuyse *et al*., 2001). In similar studies, conducted by the same group, on SOD1-deficient mice (a mouse model for human ALS), it was demonstrated that while reduction of VEGF levels increased the severity of the disease, increasing the number of VEGFR receptors in motor neurons' surface, attenuated the disease (Lambrechts *et al*., 2003; Storkebaum *et al*., 2005). Although SOD1 animal models have taught us many lessons, the molecular pathogenesis of ALS is still obscure, but these studies have opened a new and unexpected era in ALS research (Bruijn *et al*., 2004).

VEGF was also found to play a role in other motor neuron and neurodegenerative disorders, like in spinal and bulbar muscular atrophy (SBMA), Alzheimer's and

Parkinson's Diseases. It is plausible to say that VEGF is "rediscovered" with its roles in neurons and motor neuron disorders (Sopher *et al*., 2004; Yang *et al*., 2004; Yasuhara *et al*., 2005; Bogaert *et al*., 2006).

There are arguments to whether death of motor neurons is a result of VEGF's direct actions on neurons, or whether it is because of vascular abnormalities due to insufficient VEGF. It seems that both processes are effective in motor neuron pathogenesis. Cell culture studies show that; i) VEGF receptors are expressed in neural cells, ii) VEGF increases the survival of motor neurons, iii) VEGF decreases the serum deprivation, oxidative stress or hypoxia-induced motor neuron death iv) VEGF protects the SOD1-transfected cells from death. Other studies in *C. elegans* (which lacks a vascular system) and *D. melanogaster*, expression of VEGF in neurons implies that, VEGF directly acts on neurons and has more roles beyond being just a vascular factor (Oosthuyse *et al*., 2001; Li *et al*., 2003; Van Den Bosch *et al*., 2004; Bogaert *et al*., 2006; Tarsitano *et al*., 2006).

Today it is known that, VEGF, beside its numerous angiogenic functions, plays also roles in neurogenesis, differentiation, survival, migration and proliferation of neurons, axon extension and branching, synaptic plasticity, as well in neuromuscular-junction innervations and astrocytes and glial cells (Zacchigna *et al*., 2008) (Figure 1.6). These findings make VEGF and other angiogenic molecules potential candidates in treatment of neurodegenerative diseases (Greenberg and Jin, 2005).

There is compelling evidence that angiogenesis and neurogenesis are two integrated phenomena that shape the nervous system and together protect it from disease, cross-talking with each other (Greenberg and Jin, 2005). They are not distinct events and are shaped by many common molecules. Beside VEGF, there are some molecules that are originally known as angiogenic, but their direct roles on neurons were later discovered. Likewise, today a number of molecules, formerly reported through their specific roles in neurons, are linked to angiogenesis. Fgf2, PDGF and angiogenin are examples to the first group; and nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT3) and neurotrophin 4 (NT4), the ephrin family and members of the glial-derived neurotrophic factor (GDNF), all of which are vital developmental neurotrophic factors, are examples to the second group (Huang and Reichardt, 2001;

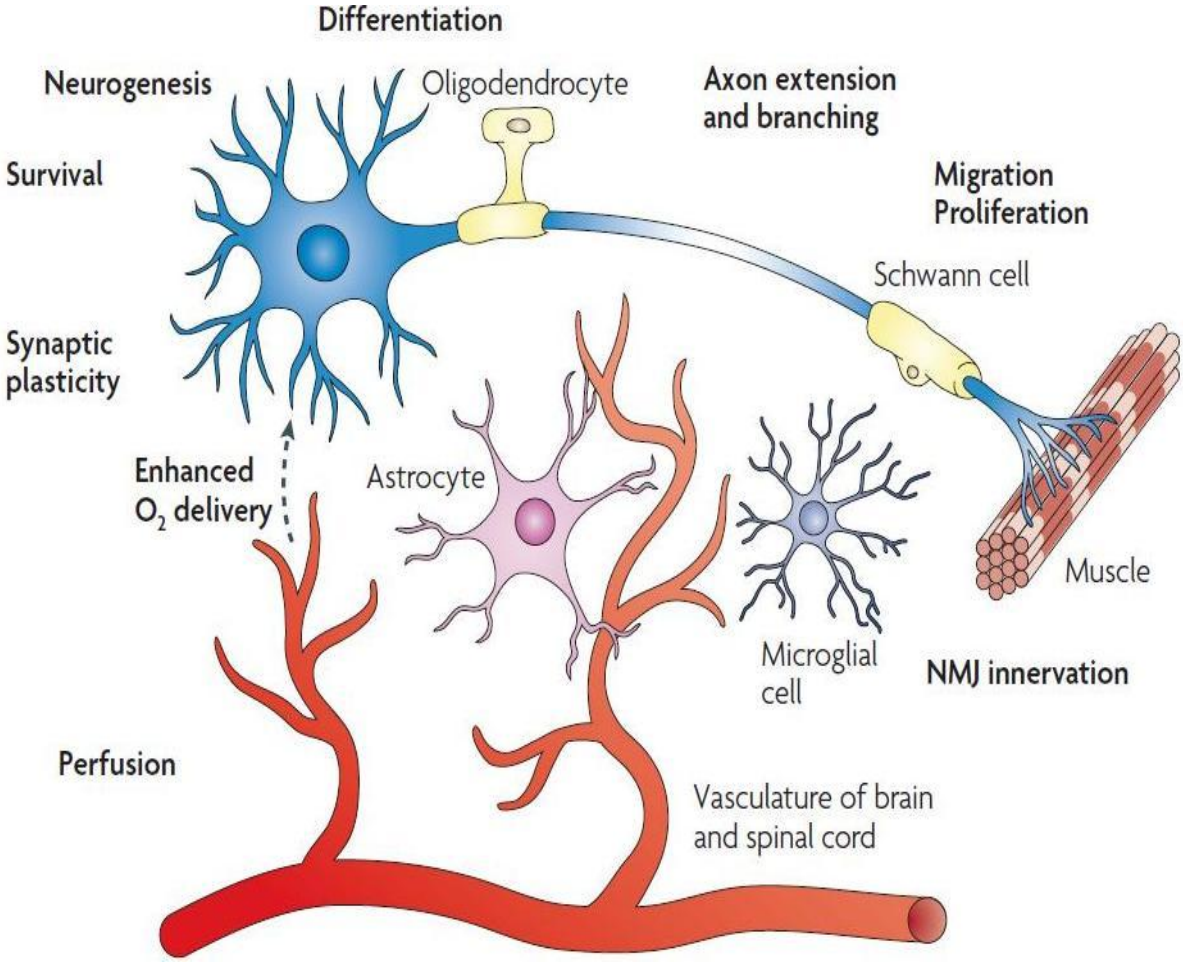

Figure 1.6. VEGF as a multi-tasking neuronal factor (Zacchigna *et al*., 2008)

Louissaint *et al*., 2002; Sun *et al*., 2003; Greenway *et al*., 2006; Zhao *et al*., 2007; Zacchigna *et al*., 2008). Also there is a third group that contains pleiotropic molecules, like EGF, TGFβ1, IGF, hepatocyte growth factor (HGF) and erythropoietin, that are known to have roles both in angiogenesis and in neurons (Tesseur *et al*., 2006; Zacchigna *et al*., 2008). Therefore, it can be claimed that, angiogenesis and central nervous system development (CNS) are two interconnected phenomena and should be considered together in health and disease of the CNS (Carmeliet, 2003b; Rosenstein and Krum, 2003; Greenberg and Jin, 2005; Zacchigna *et al*., 2008) (Figure 1.7).

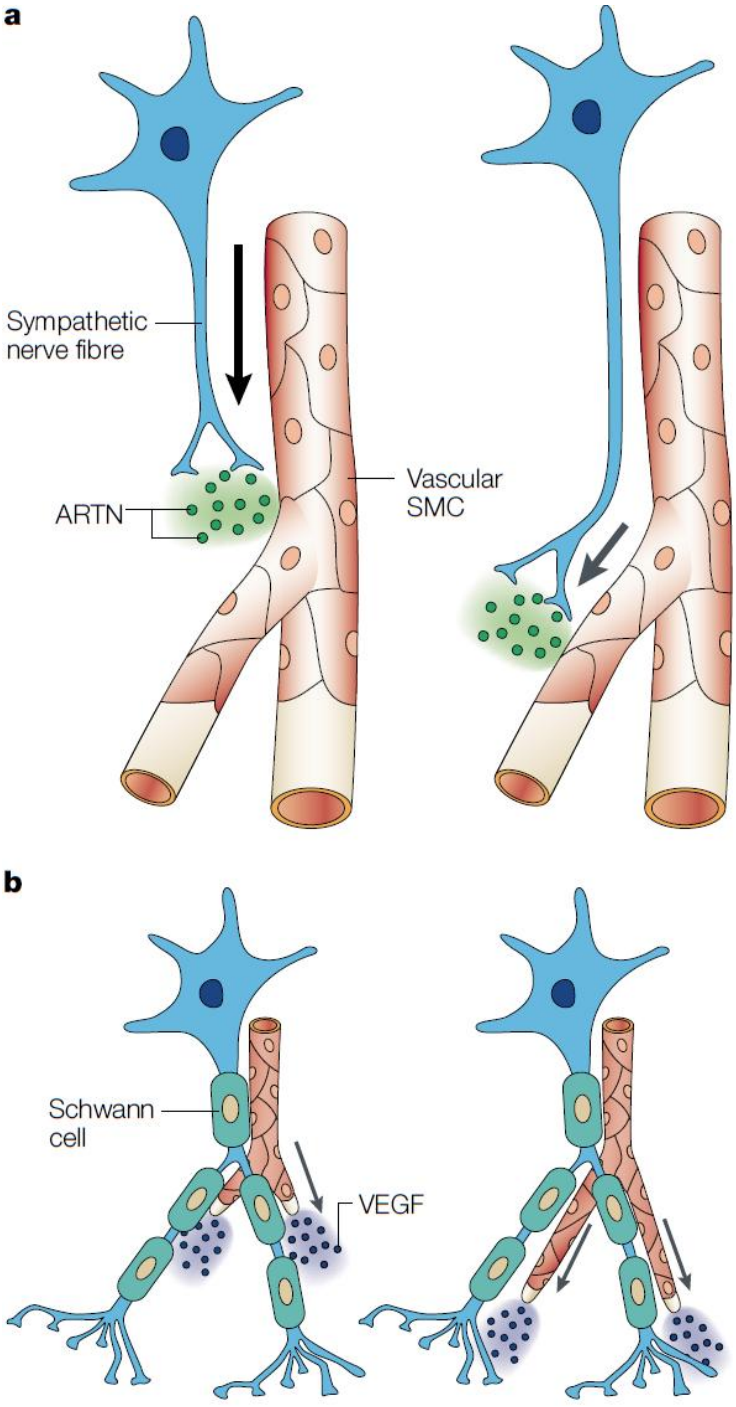

Figure 1.7. Coordinated patterning of nerves and blood vessels: a) artermin (ARTN) is a vessel-derived neurotrophic guidance signal for sympathetic nerve axons; ARTN gradually shifts distally and thereby guides the nerve to the target organ. b) VEGF is a nerve-derived signal that induces arteries to track alongside the nerve fibers (Carmeliet, 2003b)

1.3.3. Angiogenesis and Brain Vascular Malformations

Vascular malformations of the central nervous system (CNS) are generally considered to be congenital disorders, resulting from defects in embryonic vascular development. They bear a morphological resemblance to vascular plexus seen in CNS development (Yasargil, 1987). According to McCormick classification, there are four groups of intracranial vascular malformations: arteriovenous malformations (AVMs), cavernous malformations (CMs), venous angiomas and capillary telangiectasia (McCormick, 1985).

AVMs consist of high-flow tangles of malformed arteries and veins without an intervening capillary bed. CMs consist of dilated thin-walled vascular sinusoids (caverns) that are full of slow-flow blood. They have a single layer of endothelium surrounded with varying amounts of ECM tissue. Venous angiomas are tangles of abnormally enlarged veins and capillary telangiectasias are clusters of abnormally dilated capillaries (Hanjani, 2002).

1.4. Arteriovenous Malformations

Among cerebrovascular malformations, AVM is the most dangerous, with a high risk of intracranial hemorrhage (Perret and Nishioka, 1966). Mortality rates associated with hemorrhage are approximately 15 % (Hanjani, 2002). The vast majority of AVM cases are sporadic; nearly no familial association is observed so far (Matsubara *et al*., 2000). They come to attention between the ages 20 and 50, by hemorrhage (%50), by seizures (%25) and by headache and progressive neurological deficits (25%) (Hanjani, 2002). AVM diagnosis can be performed through angiography (Figure 1.8). Endovascular embolization, radiosurgery and surgical resection are therapy modalities of AVM. It can affect both sexes equally. There are controversial reports on prevalence of AVMs due to the presence of undiagnosed cases, but the prevalence is approximately 0.01-0.02 % in the population (Forsting *et al*., 2008).

AVMs are characterized with direct joints of arteries and draining veins without a capillary bed. Arteries and veins are intertwined and have abnormally developed and

dilated structure. The absence of a normal capillary network results in low resistance and high flow-rate, which cause dilation and tortuosity in vessels, especially in veins (Challa *et al*., 1995; Matsubara *et al*., 2000; Takagi *et al*., 2000). Features of spontaneous hemorrhage, recurrence, growth and regression strongly suggest that AVMs are angiogenically active lesions (Kilic *et al*., 2000b).

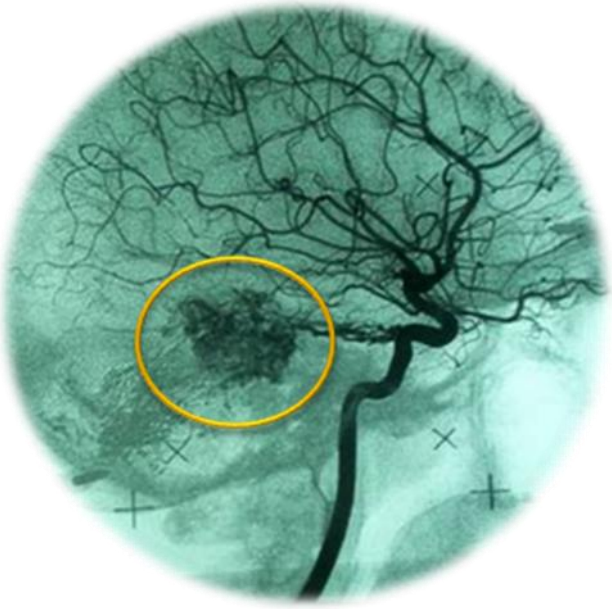

Figure 1.8. Magnetic resonance (MR) angiographic image of AVM: lesion is indicated in yellow circle (Kilic T., unpublished)

1.4.1. Genetics of AVM

Since AVM cases are not hereditary, genetic linkage analysis is not possible, therefore, very little is known about the genetic factors contributing to AVM. AVM is thought to arise during vascular specification of primary capillary plexus; however there is no genetic data available (Hanjani, 2002).

Some studies on surgically excised tissue specimens have given some clues about the molecular pathology of AVM. Differential expression in some angiogenic (VEGF, Fgf2, TGF-α) and structural (collagen IV, collagen III, smooth muscle actin, fibronectin, and laminin, integrin) molecules were reported (Kilic *et al*., 2000a; Seker *et al*., 2006). The angiopoietin-Tie system has been shown to be problematic in AVM samples, with

abnormal Ang2 concentrations (Hashimoto *et al*., 2001). Carlson *et al*. showed that constitutive expression of Notch 4 in adult endothelium leads to vessel enlargement and arterio-venous shunting, which are characteristics of AVM (Carlson *et al*., 2005).

Although familial AVM cases are extremely rare, fortunately, there is an inherited disorder with an AVM like phenotype; this is hereditary hemorrhagic telangiectasia (HHT), also called Osler-Weber-Rendu syndrome. HHT is an autosomal dominant genetic disorder with an incidence of 1 in 10.000 (Abdalla and Letarte, 2006). Small dilated vessels (telangiectases) and AVMs in the lung, liver and brain are characteristics of HHT (ten Dijke *et al*., 2008).

Genetic studies on HHT patients showed that loss-of-function mutations in Alk1 gene can lead to HHT (Johnson *et al*., 1996). Alk1 encodes a receptor for TGF-β superfamily of growth factors (ten Dijke *et al*., 1994). Transgenic mice lacking Alk1 develop large shunts between arteries and veins, downregulate arterial Efnb2 and fail to confine intravascular haematopoiesis to arteries, thus resulting in embryonic lethality in mid-gestation (Urness *et al*., 2000). Other genetic studies on variants of HHT showed that mutations in endoglin and Smad4 can also cause HHT (McAllister *et al*., 1994; Gallione *et al*., 2004). Animal and cell culture models of genes detected in genetic analyses of familial HHT may help to better understand AVM genetics.

1.4.2. Molecular Pathology of AVM

Many clinical and molecular studies imply that AVMs arise during embryonic vascular specialization, but we have very limited knowledge on the molecular pathogenesis of the disease. Functional studies are needed to unravel the mechanisms, but there is no *in vivo* or *in vitro* model of AVM. There are many immunohistochemistry studies which attempt to show expression differences of some angiogenic molecules in AVM and healthy vessels, but their results do not explain the real causes of AVM.

There are two gene expression microarray studies in AVM. Hoshimoto *et al*. conducted their research on six AVM and five control brain specimens and using 12,625 probes, found that 1,781 genes were differentially expressed in AVMs. However, their

results were not conclusive and they speculated that integrin may be worth to further investigate (Hashimoto *et al.*, 2004). In another study, Sasahara *et al*. scanned 17,086 genes in five AVM tissues. Their results are totally controversial to previous immunohistochemistry data. They did not find any expressional differences on many suspected angiogenic molecules, including VEGF, angiopoietins and ephrin family members, except ephrin A1, whose expression was described to be 2-9 times higher (Sasahara *et al*., 2007).

2. PURPOSE

Brain arteriovenous malformations, as the most dangerous cerebrovascular malformations are thought to arise during development and carry a high risk of bleeding throughout human life. Unfortunately, our knowledge about the molecular pathogenesis of AVMs is very restricted. To understand AVM pathogenesis and to develop effective treatment modalities, the elucidation of the mechanisms of how angiogenic genes are orchestrated in brain vascular development is crucial. Additionally, proper establishment of brain vasculature and maintenance of its key regulators are also important in protecting the central nervous system from neurodegeneration. Thus the knowledge of the norms of brain vascular development and genes involved is essential for the detailed understanding of the role of angiogenesis in neurodegenerative mechanisms. However, the classic literature provides descriptive knowledge about the embryonic vascular development, and most of what we know about angiogenesis at molecular level is derived from pathological studies performed in adult organisms. In literature, there are no multi-gene level studies covering the norms of brain vascular development at molecular scale.

In the framework of this thesis, we aimed to investigate the temporal and comparative expression analysis of angiogenesis-related genes in mice brain vascular development, using angiogenesis-specific low density microarray systems.

The study is expected to shed light on the:

- norms of brain angiogenic development at molecular level in a systematic manner
- molecular pathogenesis of brain vascular malformations, particularly arteriovenous malformations
- common mechanisms and factors involved in angiogenesis and neurogenesis
- interaction between angiogenesis and neurodegenerative mechanisms
- studies in humans
- mechanisms and treatment of any pathology in which angiogenesis is involved.

3. MATERIALS

3.1. Animals

Forty pregnant mice were purchased from TUBITAK-MAM-GMBE, Transgenic and Experimental Animals Laboratory, Gebze, Turkey.

3.2. Equipments

Facilities of the NDAL Laboratory at the Department of Molecular Biology & Genetics at Boğaziçi University and the Laboratory of Molecular Neurosurgery, Institute of Neurological Sciences at Marmara University were used (Table 3.1).

3.3. Buffers and Solutions

All chemicals and solutions used in this study (Table 3.2) were purchased from Merck (Germany), Fluka Chemica (Germany), Roche (Germany) and Sigma (USA), unless otherwise stated in the text.

3.4. Kits

MagnaPure Compact RNA Isolation Kit	:	Roche, Germany
High Fidelity RNA Isolation Kit	:	Roche, Germany
Array Grade Total RNA Isolation	:	SABiosciences, USA
Transcriptor High Fidelity cDNA synthesis Kit	:	Roche, Germany
cDNA Synthesis Kit (True Labeling-Amp 2.0)	:	SABiosciences, USA
In vitro transcription Kit (True Labeling-Amp 2.0)	:	SABiosciences, USA
ArrayGrade cRNA Clean up Kit	:	SABiosciences, USA
Chemiluminescent Detection Kit	:	SABiosciences, USA
UPL Mouse GAPD Gene Assay	:	Roche, Germany
Light Cycler TaqMan Master Hybprobe	:	Roche, Germany

Table 3.1. Equipment used in the study

Autoclaves	Model MAC-601, Eyela, Japan
	Model ASB260T, Astell, UK
Balances	440-47N, Kern, France
Centrifuges	Labnet, Korea
	2-16K, Sigma, USA
	5415, Eppendorf, Germany
	Universal 16R, Hettich, Germany
	Allegra X22-R, Beckman Coulter, USA
Deep Freezers	2021D (-20 C), Arçelik, Turkey
	Sanyo (-70 C), Sanyo, Japan
Documentation Systems	GelDoc Documentation System, BIO-RAD, USA
	Stella, Raytest, Germany
Electrophoretic Equipment	Minicell Primo E320, Thermo, USA
	Wide Sub Cell GT BIO-RAD, USA
	Mini-PROTEAN 3 Cell, BIO-RAD, USA
	Mini Trans-Blot, BIO-RAD, USA
Scanner	Scanjet 3670, HP, USA
Heat Block	Thermostat Heater 5320, Eppendorf, Germany
Homogenizer	MagNa Lyser, Roche, Germany
Light Cycler	LightCycler 2.0, Roche, Germany
Magnetic Stirrer	Chiltern Hotplate Magnetic Stirrer, HS31, UK
Microscope	Zeiss, Germany
Nitrogen Tanks	TP100, Air Liquide, France
	TR7, Air Liquide, France
Nucleic Acid Isolation	MagNA Pure Compact, Vs. 1.0, Roche, Germany
Ovens	Hybrigene, Techne, UK
	MD 554, Microwave Oven, Arçelik, Turkey
	EN 400 (37°C), Nuve, Turkey
	BD53 (56°C), Binder, Germany
Water baths	1083 Shaking Water Bath, GFL, Germany
Power Suppliers	EC 135-90 Thermo, USA, Model 200, BRL, USA
Refrigerators	4250T, Arçelik, Turkey
Spectrophotometers	NanoDrop ND-1000, Thermo, USA
	SmartSpec 3000, BIO-RAD, USA
Shakers	DuoMax 1030, Heidolph, Germany
Thermal Cyclers	TC 312, Techne, UK
	Techgene, Progene, UK
	Techne, Progene, UK
Vortex	Reax Top, Heidolph, Germany
Water Purification	WaTech Water Technologies, Turkey

Table 3.2. Buffers and solutions used in the study

Homogenization	Lysis buffer for RNA isolation (pH 6.6)	4.5 M guanidine-HCl 50 mM Tris-HCl 30% Triton X-100 (w/v)
	Lysis buffer for protein analysis in western blot (2X)	100 mM Tris (pH 7.6) 300 mM NaCl 4 mM EDTA 0.4% NP-40 Protease cocktail(1 µl/20 mg tissue)
	DNAse I	10 KU lyophilized DNase I
	Protease Inhibitor Cocktail	Commercial Solution (AEBSF, pepstatinA, E-64, bestatin, leupeptin, and aprotinin)
Gel Electrophoresis	10X TBE buffer (pH 8.3)	0.89 M Tris-Base 0.89 M Boric Acid 20 mM Na_2EDTA
	6X DNA Loading Dye	10 mM Tris-HCl (pH 7.6) 0.03 per cent Bromophenol Blue 0.03 per cent Xylene Cyanol FF 60 per cent glycerol 60 mM EDTA (Fermentas, Lithuania)
	2X RNA Loading Dye	295% formamide 0.025% SDS 0.025% bromophenol blue 0.025% xylene cyanol FF 0.025% ethidium bromide 0.5 mM EDTA
	Ethidium Bromide (EtBr)	10 mg/ml
	DNA Ladder	100 base pair (bp) (Fermentas, Lithuania)
	RNA ladder	200-6000 base RNA ladder (Fermentas, Lithuania)
Array Experiments	Alcohol	Absolute Ethanol
	Bitoinylated-dUTP	10 mM biotin 16-dUTP
	Hybridization solution	Proprietary formulation (SABiosciences, USA)
	Wash Solution 1	2X SSC 1 % SDS
	Wash Solution 2	0.1X SSC 0.5 % SDS
	20X SSC buffer pH 7.0	175.3 g/L NaCl 88.2 g/L sodium citrate dihydrate
	20% SDS	200 g/L sodium dodecyl sulfate

Table 3.2. Buffers and solutions used in the study (continued)

Western Blotting	Bradford BSA standards	Serial dilutions: 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 mg/ml
	Bradford Reagent	1X commercial solution (Bradford Quick Start, BIORAD, USA)
	Sample Buffer, SDS-reducing buffer	%35. 5 (v/v) deionized water 1/8 (v/v) 0.5 M Tris-HCl, pH 6.8 1/4 (v/v) glycerol 1/5 (v/v) 10% (w/v) SDS 1/50 (v/v) 0.5% bromophenol blue 1/20 (v/v) β-Mercaptoethanol
	10x Electrode Buffer, pH 8.3, (Running)	30.3 g/L Tris base 144.0 g/L Glycine 10.0 g/L SDS
	Acrylamide:Bisacrylamide (37.5:1)	29.2 g/100 ml acrylamide 0.8 /100 ml N`N`-bis-methylene-acrylamide
	Separating Gel Buffer	1.5 M Tris-base (pH 8.8)
	Stacking Gel Buffer	0,5 M Tris-base (pH 6.8)
	SDS	10 % (w/v) Sodium Dodecyl Sulfate
	Ammonium Persulfate	10 % APS (w/v) in dH2O
	TEMED	N,N,N,N-tetramethylethylenediamine
	Coomassie Blue Solution	1x Comassie Blue Dye
	De-staining solution	10% EtOH 10% Acetic acid 80% distilled water
	Transfer Buffer (pH 8.3)	25 mM Tris 192 mM Glycine 20% MetOH 0.05 % SDS
	Ponceau Solution	10X commercial solution
	10X TBS Buffer (pH 7.5)	500 mM Tris-HCl (78.8 g/L) 1.5 M NaCl (87.66 g/L)
	1X TBST Buffer	100 % TBS 0.001 % Tween20
	MetOH	Methanol Extra Pure
	Luminol Reagent	1X commercial solution (Santa Cruz, USA)
	PBS	10X Phosphate buffered saline
	Protein Ladder	10 different-sized protein 10-190 kDa (Invitrogen, USA)
Chemiluminescense	Developer	Commercial Solution (Kodak, USA)
	Fixer	Commercial Solution (Kodak, USA)

3.5. Fine Chemicals

3.5.1. Primers

Sequences of the primers, used in the qRT-PCR, are given in Table 3.3; primers were purchased from Elips Health Products Ltd., Istanbul.

Table 3.3. Oligonucleotide primer pairs used in qRT-PCR

Gene	Primer Sequence
Bai1 F	Forward: 5'-GGCCAAGAATGAGAACGTG-3'
Bai1 R	Reverse: 5'-CCAGTTCTGCATACCGTGATT-3'
Fgf2 F	Forward: 5'-AGCGGCTCTACTGCAAGAAC-3'
Fgf2 R	Reverse: 5'-CGTGTGGGTCGCTCTTCT-3'
Npr1 F	Forward: 5'-TGGAGACACAGTCAACACAGC-3'
Npr1 R	Reverse: 5'-CGAAGACAAGTGGATCCTGAG-3'
Nudt6 F	Forward: 5'-GACTCTGTGGCTGGGAGAAG-3'
Nudt6 R	Reverse: 5'-TCCTGGTGCTAACATCAAATACA-3'
Pdgf-B F	Forward: 5'-CGGCCTGTGACTAGAAGTCC-3'
Pdgf-B R	Reverse: 5'-GAGCTTGAGGCGTCTTGG-3'

3.5.2. UPL Probes for qRT-PCR

Table 3.4. UPL probes used in qRT-PCR

Gene	RefSeq	Probe #	Sequence
Bai1	ENSMUST00000042035.8	50	TCTGGAGC
Fgf2	NM_008006.2	4	CTTCCTGC
Npr1	NM_008727.5	60	TGGGGAAG
Nudt6	NM_153561.2	3	GACCCAG
Pdgf-B	NM_011057.3	32	GGGAGCAG

3.5.3. Antibodies

Table 3.5. Antibodies used in Western Blot analysis

Antibody	Source	Commercial Code
Bai1 (H-270)	rabbit polyclonal	sc-66815
Npr1 (H-125)	rabbit polyclonal	sc-25485
Actin (H-196)	rabbit polyclonal	sc-7210
Anti- Rabbit IgG-HRP	Goat	sc-2004

3.5.4. Other Materials

- MagNa Lyser Green Ceramic Beads (Roche, Germany) were used for homogenization using the MagNa Lyser instrument.
- Angiogenesis-specific membrane microarrays, designed for mouse, (OMM-024, SABiosciences, USA) were used in array experiments.
- PVDF nylon membranes (BIO-RAD, USA) were used in Western Blot assay.

4. METHODS

4.1. Animal Experiments

4.1.1. Breeding of Animals

Forty male and female balb-c type adult mice were mated at TUBITAK-MAM-GMBE Transgenic and Experimental Animals Laboratory. Pregnancies of the female mice were tested every 12 hrs; observation of vaginal plaque formation in the mice by an expert eye was taken as proof for pregnancy. Animals that formed plaques were labeled as Day 0 of their pregnancies. Regular diets were applied and healthy maintenance of pregnancies was followed by daily controls.

Animal Ethical Council permission is present (Marmara University, 01.10.2007-27.2007.mar).

4.1.2. Obtaining Brain Samples

Pregnant animals were euthanized by an overdose of ether treatment. After assuring the unconsciousness of the animal, the peritoneum was incised using a surgical blade (#12), and the placenta excised. After dissection of the embryos (8-14 embryos) from the placenta, craniotomy was applied to each embryo (#11, #21 blades) by using a surgical microscope. The same craniotomy procedure was applied to new born mice after an overdose of ether treatment.

Brains were obtained from the animals at 18 different developmental stages between embryonic day 12 and postnatal day 20: E12, E13, E14, E15, E16, E17, E18, E19 and E20, a last day of pregnancy and P1, P3, P5, P7, P9, P11, P13, P20 and adult. Immediately after excision, brain samples were put into the labeled cryo-stable tubes and stored in liquid nitrogen (HABAS, Turkey).

4.2. Array Experiments

The arrays used in this study are, unlike classic high-density microarrays, low-density and pathway-specific membrane arrays that contain probes for 113 angiogenesis-related genes (OMM-024 Mouse Angiogenesis Microarray, Oligo GEArray, SABiosciences, USA). The genes present on the array are pre-selected by the company by literature scanning, and include many growth factors, receptors, adhesion molecules, proteases, inhibitors, matrix proteins, transcription factors, cytokines, chemokines and some other angiogenesis-related genes. Beside these 113 angiogenesis-related genes, the array contains house-keeping genes as positive controls, and also some additional positive and negative control artificial sequence spots (Table 4.1).

Table 4.1. List of genes present on the mouse angiogenesis array: blue: target genes; red: house-keeping genes; purple: negative control sequences; green: artificial positive sequences to confirm the success of chemiluminescense

Gapdh	Adra2b	Angpt1	Angpt2	Akt1	Angptl3	Angptl4	Anpep
Bai1	Ccl11	Ccl2	Cdh5	Col18a1	Col4a3	Csf3	Ctgf
Cxcl1	Cxcl10	Cxcl11	Cxcl2	Cxcl5	Cxcl9	Ecgf1	Edg1
Efna1	Efna2	Efna3	Efnb2	Egf	Eng	Epas1	Ephb4
Ereg	F2	Fgf1	Fgf2	Fgf6	Fgfr3	Figf	Flt1
Fzd5	Gna13	Hand2	Hgf	Hif1a	Ifna1	Ifng	Igf1
Il10	Il12a	Il18	Il1b	Il6	Itgav	Itgb3	Jag1
Kdr	Lama5	Lect1	Lep	Mapk14	Mdk	Mmp19	Mmp2
Mmp9	Notch4	Nppb	Npr1	Nrp1	Nrp2	Nudt6	Pdgfa
Pdgfb	Pecam1	Pgf	Plau	Plg	Plxdc1	Pofut1	Prok2
Pten	Ptgs1	Ptgs2	Ptn	Serpinf1	Sh2d2a	Smad5	Sphk1
Stab1	Stab2	Tbx1	Tbx4	Tek	Tgfa	Tgfb1	Tgfb2
Tgfb3	Tgfbr1	Thbs1	Thbs2	Timp1	Timp2	Timp3	Tmprss6
Tnf	Tnfaip2	Tnfrsf12a	Tnfsf12	Tnfsf15	Tnnt1	Vegfa	Vegfb
Vegfc	Wasf2	PUC18	Blank	Blank	AS1R2	AS1R1	AS1
Rps27a	B2m	Hspcb	Hspcb	Ppia	Ppia	BAS2C	BAS2C

Array experiments include the homogenization of tissues, RNA isolation, cDNA synthesis, cRNA synthesis, hybridization, image acquisition and data analysis steps (Figure 4.1).

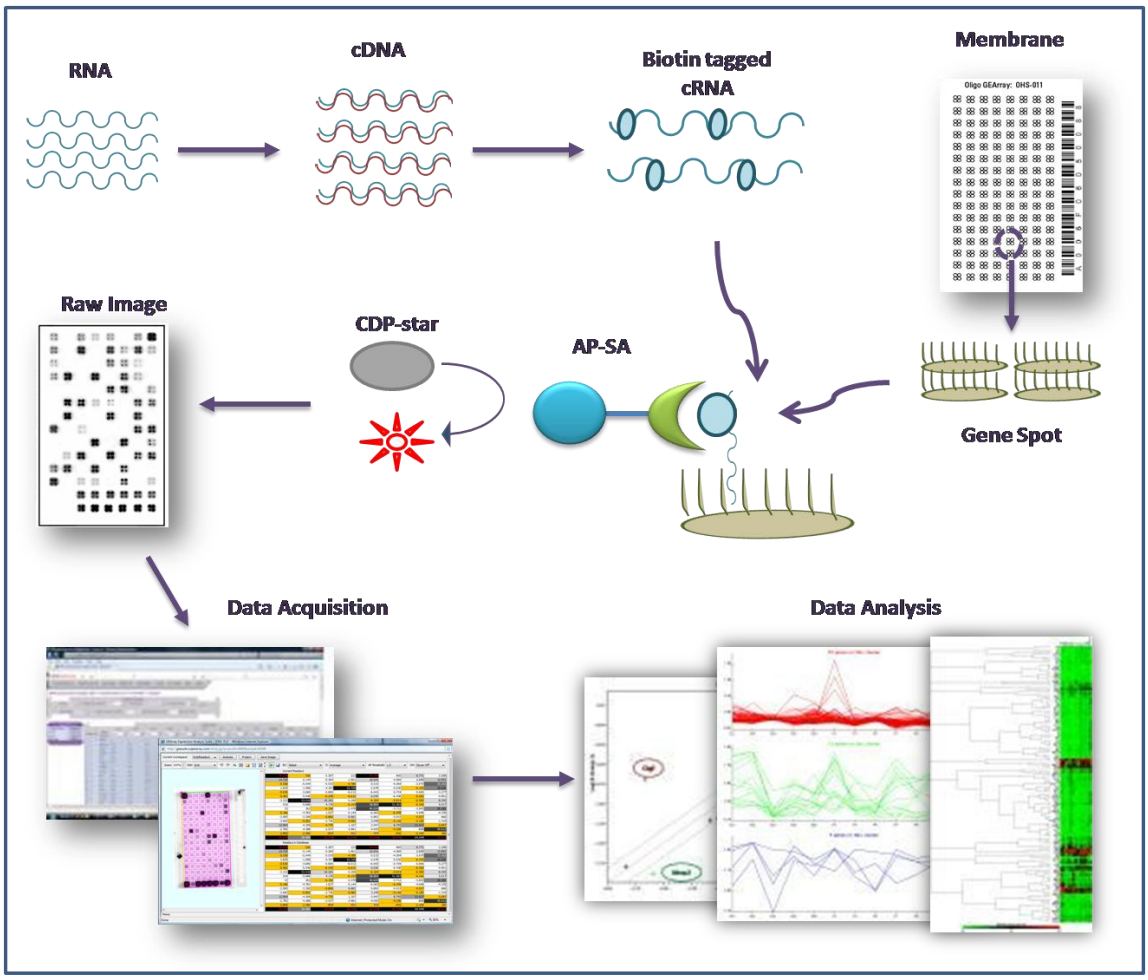

Figure 4.1. Array experiment steps: RNA isolation from homogenized tissues, cDNA synthesis, biotin-tagged cRNA synthesis by in vitro transcription, hybridization, acquisition of chemiluminescense and analysis of data (AP-SA: alkaline phosphatase conjugated streptavidin)

4.2.1. Homogenization

Single brains derived from days E12, E14, E16, E18, E20, P1, P3, P5, P7, P9, P11, P13 and adult-stage animals, were removed from liquid nitrogen and kept in ice for 5 min.; approximately 20 mg of tissue was taken by weighing. One ml lysis buffer was added to

the bead-containing MagNa-Lyser tubes. Without allowing them to melt, the tissues were put into tubes and homogenized for 50 sec at 6500 rpm by the MagNa-Lyser Homogenizer. Then samples were cooled for 1 min at 4 ^{o}C and kept at RT for 30 min.

4.2.2. RNA Extraction by MagNA Pure Compact

MagNA Pure Compact is a fully automated nucleic acid extraction system that uses the specifically designed MagNA Pure Compact nucleic acid isolation kit. The extraction principle of this system is based on the affinity of magnetic beads for DNA and RNA. During extraction, nucleic acids bind to magnetic glass particles, and in the elution step, they are removed from the beads.

Two hundred and ten µl of lysate was added to 290 µl of lysis buffer (10 mg tissue/350 µl lysis buffer) and centrifuged for 5 min at 17,000 x g. The supernatant was removed and the standard isolation procedure of the system was applied, with an additional 20 µl of DNAse. At the end of this step, 50 µl of RNA was obtained from each tissue sample.

4.2.3. Analysis of Extracted RNA Samples

4.2.3.1. Agarose Gel Electrophoresis. The quality and quantity of the RNA was evaluated by running the samples on a 2% (w/v) agarose gel, which was prepared by boiling two grams of agarose in 100 ml 0.5X TBE buffer. After cooling to 50^{0}C, ethidium bromide was added to the mixture in a final concentration of 0.5 mg/ml, the solution was poured onto a gel plate, and the combs were inserted. When the gel solidified, the combs were removed and the plate was placed into an electrophoresis tank, containing 0.5X TBE buffer. Each RNA sample was mixed with 2X RNA loading dye to a final concentration of 1X, and the samples were loaded into the slots of the gel with a micropipette. The gel was run at 90 V for 30 minutes, and the RNA bands were visualized under UV light. The quality and quantity of the samples were determined by observing the 18S and 28S rRNA bands.

4.2.3.2. Spectrophotometric Measurement of RNA. The exact amounts and concentrations of the RNA samples were determined by spectrophotometric measurement, using a

NanoDrop Spectrophotometer. In the NanoDrop Spectrophotometer, a pulsed xenon flash lamp provides the light source, and the spectrometer is used to analyze the light after passing through the sample. The instrument is controlled by a PC-based software, and the data are logged in an archive file on the PC.

One µl of RNA was pipetted into the end of the receiving fiber optic cable of the NanoDrop Spectrophotometer, and the source fiber optic cable was then brought into contact with the liquid sample, causing the liquid to bridge the gap between the fiber optic ends. Optical density of the RNA was measured at 230 nm (OD_{230}).

4.2.4. cDNA Synthesis

The SABiosciences cDNA Synthesis Kit was used for cDNA synthesis.

4.2.4.1. Preparation of the Annealing Mixture. RNA samples were heated to denature their 3-D structures. Varying volumes of RNA samples, corresponding to 1 µg of RNA, were taken and mixed with 1 µl component G1, the volume was adjusted to 10 µl by RNAse-free H_2O. This annealing mixture was incubated for 10 min at 70 ^oC, then immediately cooled in ice.

4.2.4.2. Preparation of cDNA Synthesis Master Mix. cDNA synthesis mixtures of each reaction includes 4 µl RNAse-free H_2O, 4 µl 5x cDNA Synthesis Buffer (G3), 1 µl RNAse inhibitor (RI) and 1 µl cDNA synthesis Enzyme Mix (G2). All ingredients were added up to a final volume of 10 µl.

4.2.4.3. cDNA Synthesis Reaction. The annealing and master solutions were mixed to a final volume of 20 µl. The reaction was performed for 50 min at 42 ^oC, then the enzyme was inactivated for 5 min at 72 ^oC and later cooled to 37 ^oC.

4.2.5. cRNA synthesis by *in vitro* Transcription

4.2.5.1. cRNA Synthesis, Labeling with Biotin and Amplification (by SABiosciences TrueLabeling-AMP 2.0 Kit). The following PCR components (Table 4.2) were mixed and incubated overnight at 37 ^{o}C.

Table 4.2. Ingredients of cRNA synthesis

Components	**Volume (µl)**
RNA Polymerase Buffer, 2.5X (G24)	16
Biotinylated-UTP, 10 mM	2
RNA polymerase enzyme	2
cDNA synthesis mix	20
TOTAL	40

4.2.5.2. cRNA purification. After the cRNA synthesis, the reaction mixture contains many ingredients that should be removed from the solution for proper array hybridization. Purification of cRNA was performed by SABiosciences ArrayGrade cRNA Cleanup Kit. 50 µl RNase-free H_2O, 315 µl lysis and binding buffer (G6) and 315 µl ACS-Grade ethanol (100 %) were added to each reaction mixture one by one and mixed at each step. This solution was loaded to each sample's spin column and centrifuged for 30 sec at 8,000 x *g*. The flow-through was discarded and the column was installed into a clean tube. Six hundred µl washing buffer (G17 with ethanol) was applied and centrifuged for 30 sec at 8,000 x *g*, the step was repeated with 200 µl washing buffer for 1 min at 11,000 x *g*. At the final washing step, in order to make sure that all of the material was eluted from the column filter, the column was rotated 180^{o} and centrifuged for 2 min at 11,000 x *g*. Then 50 µl Tris elution buffer (10 mM, pH 8) was added to the column, and after incubation for 2 min at room temperature, the column was centrifuged again for 1 min at 8,000 x g, to obtain pure cRNA in a final volume of 50 µl.

4.2.5.3. cRNA quantification. Concentration of the cRNA was determined by using the NanoDrop Spectrophotometer, as described earlier (Section 4.2.3.1).

4.2.6. Hybridization to Membranes

The hybridization of the above prepared cRNA samples (stages E12, E14, E16, E18, E20, P1, P3, P5, P7, P9, P13 and adult) consisted of three main steps: Prehybridization, hybridization and washings.

4.2.6.1. Prehybridization. Before hybridization of the cRNAs to the membranes, the membranes had to be activated for hybridization. For this purpose, 5 ml dH_2O was added to the membrane tube, which was left at inverted position for 5 min. After discarding the water, 2 ml of pre-warmed and completely dissolved (at 60 ^oC) GEAhyb hybridization solution was added and briefly vortexed. The tubes were then pre-hybridized in the hybridization oven for 2 hrs at 60 ^oC with continuous but slow agitation at 5-10 rpm.

4.2.6.2. Hybridization. The target hybridization mix was prepared by putting together the corresponding volume of 2 µg of biotin-labeled cRNA target and 0.75 ml pre-warmed GEAhyb hybridization solution. After discarding the pre-hybridization solution, the target hybridization mix was added to the membrane, which was incubated o/n at 60 ^oC at 5-10 rpm.

4.2.6.3. Washing. To avoid unspecific binding, membranes were washed with washing solution (WS) of different stringencies. After removing the target hybridization mix, 5 ml of WS1, pre-warmed to 60 ^oC, was added to the membranes by brief vortexing, which were then rotated for 15 min at 60 ^oC (at 20-30 rpm) in hybridization oven. Following the first wash, the second wash was performed using 5 ml WS2 at 60 ^oC for exactly, 15 min again at 20-30 rpm. To avoid the membranes to dry out, the chemiluminescense detection step was initiated immediately.

4.2.7. Image Acquisition (by Chemiluminescent Detection Kit, SABiosciences, USA)

To detect the hybridized spots on the membrane, alkaline phosphatase-conjugated streptavidin (AP-SA) and the CDP-star method was used. RNAs on the membrane were blocked by adding 2 ml GEAblocking Solution Q, pre-warmed to 37 ^oC and incubating for 40 min at 20 rpm. Then the GEAblocking solution was discarded from the tubes and 2 ml

AP-SA buffer was added, followed by incubation for exactly 10 min at 5-10 rpm. Membranes were washed four times with 4 ml 1X buffer F for 5 min with gentle agitation. After the last wash, membranes were rinsed twice with 3 ml Buffer G.

1 ml CDP-Star, a substrate for alkaline phosphatase, was added to each hybridization tube. Tubes were rotated for 5 min at RT in the hybridization oven to evenly cover the membrane with the CDP-Star. The excess CDP-star solution was removed by absorbent paper, while taking care of not touching the surface of the array. Without allowing the membranes to dry out, they were placed on a 20x20 cm glass plate which was then covered with a thin stretch-film followed by removing bubbles.

Image acquisition was performed by either of two ways: i) using Raytest-Stella Image Acquisition System and Xstella 1.0 software, in which the camera inside the machine detects chemiluminescense emanating from the membrane and transforms it to the digital image, or ii) using the classic X-ray filming method; after a 2-min exposure of the films to the membranes in dark room, the color was developed in 2-3 min (Kodak, USA, developing solution). Development was stopped by immersing the films into tap water, followed by a 2-min treatment in the fixer (Kodak, USA). The films were then thoroughly rinsed and the acquired images were digitalized by a flat-bed scanner.

4.2.8. Data Analysis

The array images were analyzed by a web-based software; GEArray Expression Analysis Suite (GEAsuite) (SABiosciences, USA).

4.2.8.1. Data Acquisition. By using Image Data Extraction Applet (IDEA), captured array images were uploaded to the system and prepared for data analysis (See Figure 4.2 for IDEA snapshot). Cropping, color and threshold balancing of images were performed, then spots on the array were aligned to the template grid. Proper orientation of each spot to grid was checked, and those that not fit the grid, were manually aligned by sensitive tuning options. After the grid and image were aligned, “readout” was generated, in which a numeric value was assigned to each spot on the array. Finally, fine-tune settings were optimized on the readout; “background correction” was set according to the minimum

value on the array (not according to the blank spots, or local spot rectangle, or global grid value of the image), density was chosen as average of four circles on each spot (not as a sum of four circles), and absent/present threshold of the gene was chosen as 1.5, and clover mode was on off. This process was applied to each array being analyzed.

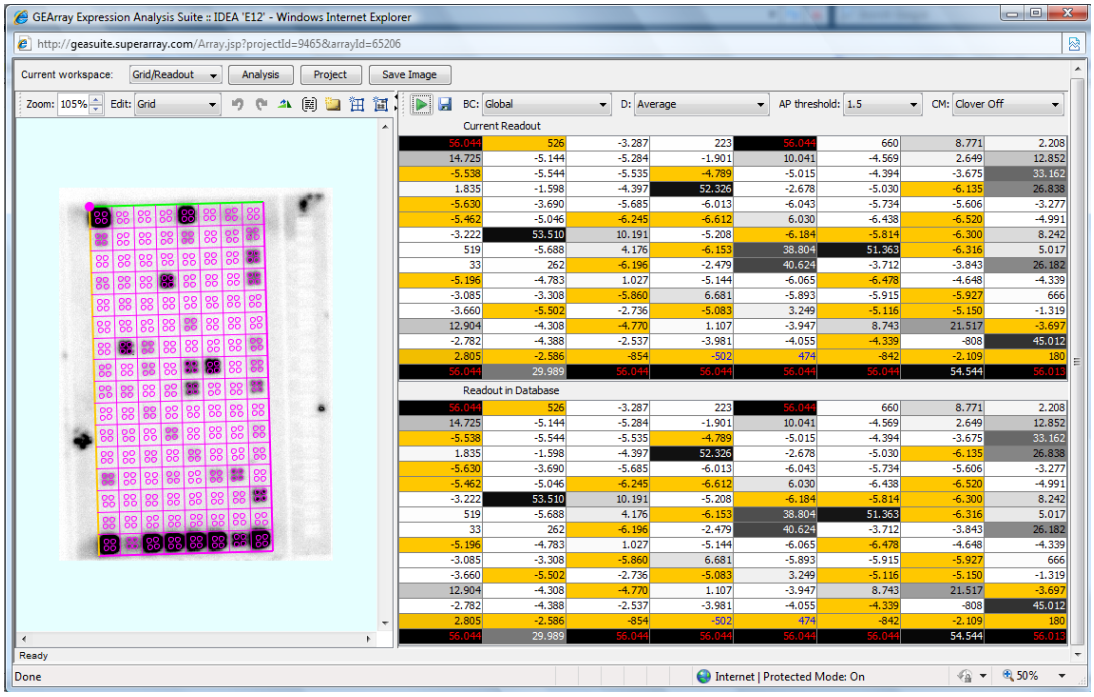

Figure 4.2. Screenshot of the IDEA program interface

4.2.8.2. Determination of Dataset Parameters. Acquired data from arrays by IDEA were transferred to the pre-created project and dataset parameters were determined. While deciding which gene/genes to be used for normalization, many trials were done, before the final optimum selection was chosen. Since GAPDH was fully expressed and had a deep dark appearance in all arrays, it was not sufficient for normalization, and other genes had to be selected, too. In addition to GAPDH, other housekeeping genes, some artificial positive and negative spots were also included for normalization, the final reference spots were: GAPDH, Rps27a, B2m, Hsp90ab1, Ppia as house-keeping genes; PUC18, a couple of blank spots and AS1 as negative controls, BAS2C as artificial positive control. Clover mode was off, background correction was global and density was according to the average of spot circles. For further analysis, arrays were classified into two groups and labeled; group 1 for embryonic stage arrays and group 2 for postnatal stage arrays.

4.2.8.3. Analysis. Array data were analyzed using different methods in GEAsuite web based software.

- First, the clustergram method was applied, in which genes are correlated between different arrays and diagrammed as clusters. A clustergram was constructed for both arrays as separate items and for comparison of groups (embryonic and postnatal), with the parameters of one-dimensional, minimum join type, and sample color-coded.
- By examining the clustergram diagram, captured raw array image and IDEA software output, genes which are expressed were determined.
- K-means cluster analysis was performed to classify the genes with similar expression patterns.
- Scatter plot analysis was conducted to determine differentially expressed genes between embryonic and postnatal stages.

4.3. Selection of Genes for Further Studies

Since it would extend the scope of this thesis to focus to all 113 genes in detail, several target genes were selected for further studies. Selection of genes was based on comprehensive literature search for the genes which seemed to be expressed in the array data. Selection criteria were as follows:

- Direct or indirect involvement of genes in angiogenesis
- Presence of known roles in embryonic development, vascular development, and in brain development
- Presence of knock-out models and results of the gene interruption on vascular system
- Established functions in cell culture studies.

By considering all these criteria, three novel genes were defined worth to study at the first instance, thus were selected for further analysis.

4.4. Quantitative Real-Time PCR Experiments (qRT-PCR)

To validate array data and to obtain more precise temporal expression analysis, qRT-PCR was carried out for the genes selected. Expression levels were determined for the developmental days; E12, E13, E14, E15, E16, E17, E18, E19, E20, P1, P3, P5, P7, P9, P11, P13, P20 and adult.

Universal Probe Library (UPL) (Roche, Germany) system was used in qRT-PCR experiments. This system is based on amplification of short specific-sequences of the genes of interest by appropriate primers, and hybridization of specifically designed fluorescent probes to the amplicon. Hybridization of probes, labeled at the 5'-end with fluorescein (FAM) and at the 3'-end with a dark quencher dye, gives intensive shining as the cycles of PCR increase. The instrument reads the increasing fluorescence, assigns a numerical value and plots the graph.

4.4.1. Homogenization

For three repeat sets of qRT-PCR experiments, in addition to previously homogenized tissues for array experiments, two more tissues were isolated for each developmental day. The previously not homogenized, E13, E15, E17, E19, E20 tissue samples were also homogenized. The homogenization was performed as previously described in Section 4.2.1.

4.4.2. RNA Isolation

RNA isolation was performed using Roche High Pure RNA Tissue Kit. Four hundred µl of supernatant was taken into an Eppendorf tube and mixed with 200 µl ethanol. Filter and collection tubes were combined and the lysate-ethanol mix was applied to the filter. The mixture was centrifuged at 13,000 x g for 30 sec, and the flow-through was discarded. Master DNAse mix, containing 90 µl DNAse incubation solution and 10 µl DNA working solution, was prepared for each tube and applied onto the filter, followed by incubation at 20 ^{o}C for 15 min. After incubation, 500 µl of wash buffer I was added, which was then centrifuged for 15 sec at 8,000 x g; the flow-through was discarded. In the second wash,

500 µl of wash buffer II was added, followed by centrifugation for 15 sec at 8,000 x g; again the flow-through was discarded. In the last wash, 300 µl of wash buffer II was added, followed by centrifugation for 2 min at 13,000 x g. The filter was carefully removed and put into a sterile Eppendorf tube. Finally, 100 µl elution buffer was applied to the filter and centrifuged for 1 min at 8,000 x g. Concentration of the eluted RNA was determined by the NanoDrop Spectrophotometer, as previously described (Section 4.2.3.1). The RNA was stored at -80 ^{o}C for further use.

4.4.3. cDNA Synthesis

cDNA synthesis was performed for each sample by using the Roche High-Fidelity cDNA Synthesis Kit. For each reaction tube, RNA corresponding to 2 µg was mixed with 1 µl of 50 µM oligo-dT primer, and the total volume was completed to 11,4 µl by PCR-grade water. The prepared mix was incubated at 60 ^{o}C for 10 min to ensure denaturation of RNA secondary structures.

For cDNA synthesis reaction, the PCR components shown in Table 4.3 were prepared and mixed with pre-heated RNA-primer mix. Reaction was performed at 50 ^{o}C for 30 min and reverse transcriptase was inactivated by heating the mixture to 85 ^{o}C for 5 min. After measurement of concentrations on the NanoDrop spectrophotometer, 1/5 dilutions were prepared from stock cDNA samples and stored at -20 ^{o}C.

Table 4.3. PCR components of the cDNA synthesis reaction

Components	**Volume (µl)**	**[Stock]**	**[End]**
Preheated RNA-primer mix	11.4	-	-
dNTP	2	10 mM	1 mM
RNAse inhbitor	0.5	40 units/ µl	20 units
DTT	1	100 mM	5 mM
Buffer with $MgCl_2$	4	5X (40 mM $MgCl_2$)	1X (8 mM $MgCl_2$)
Reverse Transcriptase	1.1	10 units/µl	11 units
Total	20	-	-

4.4.4. Reaction Conditions for qRT-PCR

qRT-PCR experiments were performed for the selected genes by using the Roche LightCycler 2.0 and the Roche UPL system. Probes and primers were designed by using the ProbeFinder Vs. 2.43 software. GAPDH was used as reference gene and adult sample cDNA was used as standard sample to construct the curve. Color compensation calibration was done for the Light Cycler to be able to perform multiplex RT-PCR, which means both the GAPDH reference gene and the target gene reactions could be run in the same capillary.

4.4.4.1. Preparation of Standards. For each reaction, a new double (replica) standard set was prepared as reference quantification. Four different standard DNA concentrations were prepared as shown in Table 4.4.

Table 4.4. Standard cDNA dilutions for qRT-PCR

Standard	Sample (adult)	Water (µl)
1/1	10 µl of Undiluted cDNA	0
1/10	2 µl of Undiluted cDNA	8
1/100	2 µl of 1/10 diluted sample	8
1/1000	2 µl of 1/100 diluted sample	8

4.4.4.2. Master Mix Preparation. Since RT-PCR reactions were multiplex, both target gene and reference gene probes and primers were prepared in the same mix (Table 4.5). Wavelengths for target and reference genes were 530 and 560 nm, respectively.

4.4.4.3. Experimental Set Up. In each experimental set, the particular target gene was analyzed for 18 different developmental days. Two negative controls, four standards with replicas, 18 target samples were used in each set (Table 4.5). Target and standard capillaries' information was entered into the LightCycler software system (Version 4.0). Absolute and relative quantification options were chosen, wavelengths for target and reference genes were set as 530 and 560 nm, respectively. The following PCR conditions were applied with the PCR components shown in Table 4.6.

Initial Denaturation	:	95°C	10 minutes	
Denaturation	:	95°C	10 seconds	45-50 cycles
Annealing	:	60°C	30 seconds	
Extension	:	72°C	01 seconds	
Cooling	:	40°C	30 seconds	

Table 4.5. Multiplex real-time reaction master solution

Component	**Volume (µl)**
Target gene UPL probe	0,4
Reference gene UPL Probe	0,4
Primer Forward target gene (20 mM)	0,4
Primer Reverse target gene (20 mM)	0,4
Primer reference	0,4
TaqMan Master (buffer with enzyme)	4
Water	9
Total	**15**

4.4.5. Data Analysis.

After each reaction, standard curves for both target and reference genes were checked, and those reactions with error value outside the range of 0 ±0.2 and efficiency value outside the range of 2±0.5 were excluded from the study and repeated. In some reactions with problematic 1/1000 standard values, the standard curve was plotted by using three standards (1/1, 1/10, 100). Ratio data obtained from relative quantification (concentration of target gene/concentration of reference gene) was accepted as normalized data and transferred to Microsoft Excel to construct the graph.

Table 4.6. Experimental Setup for qRT-PCR

Tube	Description	Master mix	Sample
1	Negative control	15 µl	5 µl (water)
2	Negative control	15 µl	5 µl (water)
2	Standard 1	15 µl	5 µl (1/1)
3	Standard 1	15 µl	5 µl (1/1)
4	Standard 2	15 µl	5 µl (1/10)
5	Standard 2	15 µl	5 µl (1/10)
6	Standard 3	15 µl	5 µl (1/100)
7	Standard 3	15 µl	5 µl (1/100)
8	Standard 4	15 µl	5 µl (1/1000)
9	Standard 4	15 µl	5 µl (1/1000)
10	E12	15 µl	5 µl
11	E13	15 µl	5 µl
12	E14	15 µl	5 µl
13	E15	15 µl	5 µl
14	E16	15 µl	5 µl
15	E17	15 µl	5 µl
16	E18	15 µl	5 µl
17	E19	15 µl	5 µl
18	E20	15 µl	5 µl
19	P1	15 µl	5 µl
20	P3	15 µl	5 µl
21	P5	15 µl	5 µl
22	P7	15 µl	5 µl
23	P9	15 µl	5 µl
24	P11	15 µl	5 µl
25	P13	15 µl	5 µl
26	P20	15 µl	5 µl
27	Adult	15 µl	5 µl

4.5. Western Blot Analysis

Two out of three selected genes were analyzed also at protein level by Western Blot Analysis and actin was used as control. Four embryonic (E12, E15, E18, E20), four postnatal (P1, P5, P9, P13) and one adult sample were run on the same gel.

4.5.1. Sample Preparation

4.5.1.1. Homogenization. Since lysis buffer, previously used in homogenization for RNA isolation, contains the protein denaturant guanidium thiocyanate, homogenization was re-performed for sample preparation in Western blotting with new lysis buffer, containing proteinase inhibitor cocktail. All steps were exactly the same as in the procedure described in Section 4.2.1, but Western lysis buffer had different ingredients.

4.5.1.2. Bradford Assay. After preparation of lysates, Bradford assay was performed to determine the protein concentration in the samples. 10 mg/ml stock solution, 1 mg/ml ready-to-use BSA solutions and seven replica standard BSA solutions with different concentrations were prepared from this stock solution (Table 4.7).

Ready-to-use BioRad Bradford reagent: Quick Start™ Bradford Protein Assay was used for analyses, and lysates were diluted to 10:1. For each standard measurement, 20 µl standard was added to 1 ml of Bradford reagent, and for sample measurement, 2 µl sample and 18 µl water were mixed (1/10 dilution), which were then added to 1 ml of Bradford solution in an Eppendorf tube. After 5 min. measurements were taken on a Bio-Rad spectrometer, using the Bradford Assay program.

4.5.1.3. Preparation of Samples. After calculations of lysate concentrations, aliquots with equal concentrations were prepared by using lysis buffer. 1X sample buffer was added to each aliquot with a final concentration of 1.25 µg/µl (for 20 µl sample to be loaded to the gel; 25 µg protein). The samples were heated to 95 ^{o}C for 4 min and then immediately transferred to ice.

Table 4.7. Preparation of BSA standards for Bradford Assay

Standard Concentration (mg/ml)	1 mg/ml BSA (ml)	Water (ml)
0,0	0,0	1,0
0,2	0,2	0,8
0,4	0,4	0,6
0,6	0,6	0,4
0,8	0,8	0,2
1,0	1,0	-
1,2	0,12 (from 10 mg/ml stock)	0,88

4.5.2. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

A single SDS gel was prepared for nine samples and the Bio-Rad Mini-PROTEAN 3 cell system was used for casting and running of gels.

4.5.2.1. Gel Preparation. Two separating gels, with different percentages were prepared; 8% for actin and 10% for the selected target protein. The concentration of the stacking gel was 4 % for both gels (Table 4.8).

Table 4.8. Gel formulations used for SDS-PAGE (for 10 ml)

Gel %	H_2O (ml)	Acrylamide:Bisacrylamide (37.5:1) (ml)	Buffer	10 % SDS (w/v) (ml)
4 %	6.1	1.3	2.5 (stacking gel buffer)	0.1
8 %	4.7	2.7	2.5 (seperating gel buffer)	0.1
10 %	4.1	3.3	2.5 (seperating gel buffer)	0.1

For 10 ml separating gel solution, 50 µl APS (10%, w/v) and 5 µl TEMED and for 10 ml stacking gel solution, 50 µl APS (10%, w/v) and 10 µl TEMED were used. After preparing the gel cassette sandwich, the separating gel was poured and left for 20 min for completion of the polymerization reaction. To prevent evaporation during polymerization and for smoothening of the upper surface of the separating gel, 50 ml butanol was poured on top of the separating gel. Once the polymerization was complete, the stacking gel was prepared and poured onto the separating gel. After insertion of the combs, the stack of gel was allowed to polymerize for 45 minutes.

4.5.2.2. Sample Loading and Gel Running. By using a Hamilton syringe, the protein marker (5 µl) and samples (20 µl: containing 25 µg protein) were loaded onto the gel and run for 45 min at 200 V.

4.5.2.3. Coomassie Brilliant Blue Staining. Proteins were visualized on the gel by Coomassie Brilliant Blue (CBB) staining. The gel was placed in 1X CBB dye solution for 15-20 min until bands could be seen. Destaining was performed first for 20 min, then for one hour, then o/n, by changing the destaining solution each time.

4.5.3. Blotting

The electrophoretic transfer process was performed using a Bio-Rad Mini Trans-Blot, Electrophoretic Transfer Cell system. The sandwich was prepared according to the instructions of the manufacturer. The fiber pad was wetted with transfer buffer, then two pieces of equal-sized wet filter paper were placed on the pad, the gel was wetted in transfer buffer and placed on filter papers, by carefully removing any bubbles. The PVDF nylon membrane, activated by MetOH and equilibrated in transfer buffer, was carefully placed on the gel. Care was taken to not touch the surface. Again, two pieces of pre-wetted filter paper and fiber pad were placed on the membrane and the sandwich cassette was firmly closed. The cassette was placed in the electrode module. After placing the electrode module into the chamber, the Bio-ice cooling unit was placed, and the chamber was filled with transfer buffer. To help maintain an even ion and temperature distribution in the tank during the blotting process, a magnetic stirrer was used. Blotting was performed at 100 V

and 350 mA for 60-90 minutes. The success of the transfer process was tested by Ponceau Red Solution.

4.5.4. Staining of Proteins

The membranes were stained with corresponding antibodies of selected genes. For this purpose, the membrane was blocked with TBST, containing 5% (w/v) milk powder. Then it was incubated in 5 ml of primary antibody solution for 1 hr, which contains 1:500 diluted (optimized after trials) target gene antibody (polyclonal rabbit), 1 % (v/w) BSA and 5 ml of TBST. Excess and unspecific binding was washed off in TBST for 10 min (3 times). Then, the membrane was incubated with secondary antibody solution for 45 min, which contains 1:1000 diluted HRP-conjugated anti-rabbit secondary antibody, 1 % (v/w) BSA and 5 ml of TBST. Again, excess and unspecific binding was discarded by washing the membrane 3X in TBST for 10 minutes.

4.5.5. Color Development

The color was generated on the membrane by using Horseradish Peroxidase (HRP) and its substrate ECL luminol solution. The HRP catalyzes the reaction in which Lumigen PS-3 is converted into luminol, which gives luminescence.

After the last washing step, the membrane was incubated with TBS solution for 5 min, then incubated with 4 ml of luminol solution, which was prepared by mixing 2 ml of solution A and B, for 1 min. Without allowing the membranes to completely dry out, they were placed on a 20x20 cm glass which then was covered with a thin stretch-film, and bubbles were removed. Chemiluminescense was detected by two different methods, as described in Section 4.2.7.

5. RESULTS

5.1. Expression Analysis of Angiogenic Genes: Array Results

5.1.1. RNA Isolation

RNA was isolated from brain samples belonging to 13 different developmental stages and run on 2 % agarose gels. The intensity of the 28S rRNA was twice the intensity of 18S rRNA (Figure 5.1).

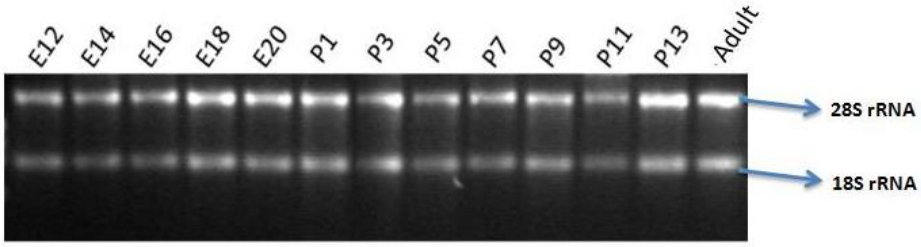

Figure 5.1. 18S and 28S rRNA bands on a two percent agarose gel

5.1.2. Hybridization

Isolated RNA samples were hybridized to membrane arrays and the genes expressed were determined for each day (Figure 5.2).

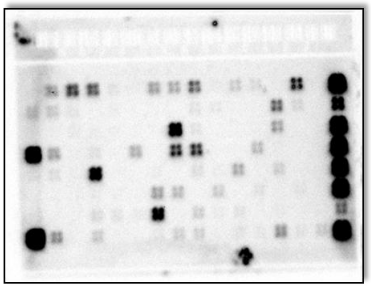

Figure 5.2. Raw array image of the E12 sample after hybridization

5.1.3. Analysis of Array Results

Arrays were analyzed by several methods in GEAsuite software. Expression levels of each gene on the arrays was determined and documented as clustergram image (Figure 5.3). Some genes were totally missing, some had low expression, and some others were constitutively expressed during brain development. When expressed genes were determined by in parallel examination of IDEA output, clustergram image and raw array images, 62 out of 113 genes were shown to be expressed in brain development (Table 5.1). They were classified according to their cellular functions (Table 5.2).

Table 5.1. Expressed genes on the array: blue: expressed genes; light blue: unexpressed genes; red: house-keeping genes; purple: negative control sequences; green: artificial positive sequences

Gapdh	Adra2b	Angpt1	Angpt2	Akt1	Angptl3	Angptl4	Anpep
Bai1	Ccl11	Ccl2	Cdh5	Col18a1	Col4a3	Csf3	Ctgf
Cxcl1	Cxcl10	Cxcl11	Cxcl2	Cxcl5	Cxcl9	Ecgf1	Edg1
Efna1	Efna2	Efna3	Efnb2	Egf	Eng	Epas1	Ephb4
Ereg	F2	Fgf1	Fgf2	Fgf6	Fgfr3	Figf	Flt1
Fzd5	Gna13	Hand2	Hgf	Hif1a	Ifna1	Ifng	Igf1
Il10	Il12a	Il18	Il1b	Il6	Itgav	Itgb3	Jag1
Kdr	Lama5	Lect1	Lep	Mapk14	Mdk	Mmp19	Mmp2
Mmp9	Notch4	Nppb	Npr1	Nrp1	Nrp2	Nudt6	Pdgfa
Pdgfb	Pecam1	Pgf	Plau	Plg	Plxdc1	Pofut1	Prok2
Pten	Ptgs1	Ptgs2	Ptn	Serpinf1	Sh2d2a	Smad5	Sphk1
Stab1	Stab2	Tbx1	Tbx4	Tek	Tgfa	Tgfb1	Tgfb2
Tgfb3	Tgfbr1	Thbs1	Thbs2	Timp1	Timp2	Timp3	Tmprss6
Tnf	Tnfaip2	Tnfrsf12a	Tnfsf12	Tnfsf15	Tnnt1	Vegfa	Vegfb
Vegfc	Wasf2	PUC18	Blank	Blank	AS1R2	AS1R1	AS1
Rps27a	B2m	Hspcb	Hspcb	Ppia	Ppia	BAS2C	BAS2C

a) b)

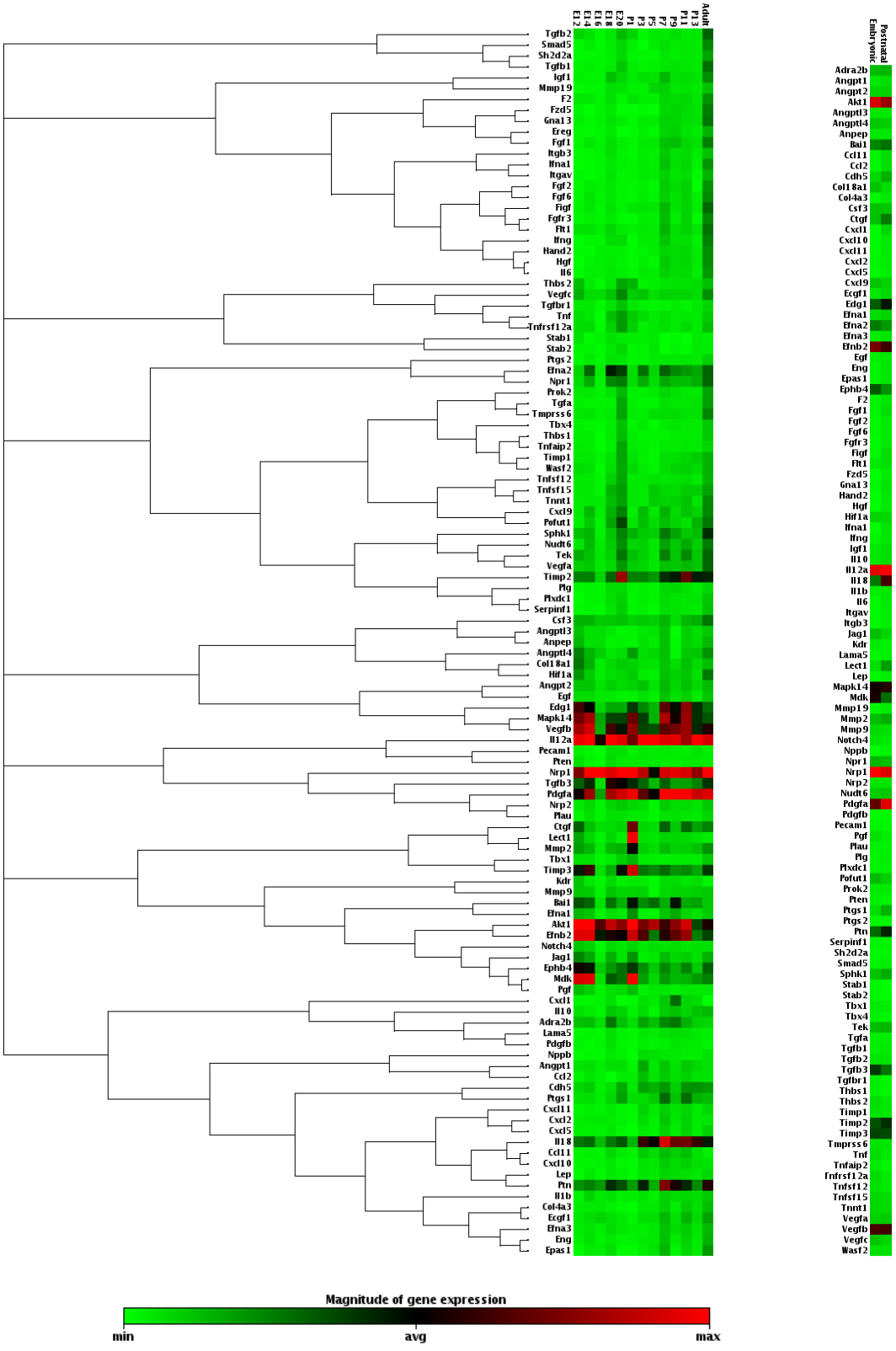

Figure 5.3. Clustergram analysis of array results; a) expressions of 113 genes according to the days of development, b) comparison of gene expression in embryonic and postnatal days

Table 5.2. List of expressed genes according to functional groups: Average of (house-keeping genes-normalized) expression values for each gene were used in the Table. Some genes may have more than one cellular function and may be found in more than one group. Expression value for Gapdh was 1,08 after normalization by other house-keeping genes

Growth factors and receptors			**Growth factors and receptors (Indirectly involved)**			**Transcription factors and others molecules**	
Gene	**Average Expression**		**Gene**	**Average Expression**		**Gene**	**Average Expression**
Nrp1	0,86		Pdgfa	0,75		Akt1	0,76
Vegfb	0,57		Edg1	0,37		Efnb2	0,60
Il18	0,44		Mdk	0,36		Mapk14	0,50
Bai1	0,24		Ptn	0,34		Efna2	0,21
Ctgf	0,19		Tgfb3	0,30		Sphk1	0,14
Tek	0,14		Ephb4	0,25		Lect1	0,14
Jag1	0,11		Npr1	0,14		Ptgs1	0,12
Vegfa	0,10		Igf1	0,06		Pofut1	0,11
Vegfc	0,10		Nrp2	0,06		Nudt6	0,11
Col18a1	0,10		Tgfb2	0,06		Angpt2	0,09
Angpt2	0,09		Notch4	0,06		Hif1a	0,09
Tnfrsf12a	0,06		Egf	0,05		Efna1	0,08
Flt1	0,06		Tgfbr1	0,04		Notch4	0,06
Fgf1	0,06					Efna3	0,06
Pgf	0,06					Tbx1	0,05
Figf	0,06					Pten	0,04
Prok2	0,05						
Kdr	0,05						
Adhesion Molecules			**Cytokines and chemokines**			**Proteases, inhibitors and other matrix proteins**	
Gene	**Average Expression**		**Gene**	**Average Expression**		**Gene**	**Average Expression**
Il18	0,44		Il12a	0,87		Timp2	0,35
Ctgf	0,19		Csf3	0,12		Timp3	0,33
Cdh5	0,12		Cxcl9	0,10		Mmp2	0,14
Col18a1	0,10		Il10	0,07		Angptl4	0,10
Thbs2	0,07		Tnf	0,06		Mmp9	0,07
Tnfrsf12a	0,06		Ccl2	0,05		Ecgf1	0,07
Nrp2	0,06					F2	0,05
Pecam1	0,03					Plau	0,04

Genes were classified according to their expression patterns by K-means clustering (Figure 5.4.). Optimal cluster number was decided as four, in which 95 genes fall into the first cluster, which also contains all of the unexpressed genes, 11 genes into the second cluster, five genes into the third and two genes fall into the fourth cluster (Table 5.3).

Table 5.3. List of genes according to K-mean clustering

Cluster	**Gene Number**	**Genes**
1	95 genes	Unexpresed genes and other expressed genes including: Angpt2, Angptl4, Ccl2, Cdh5, Col18a1, Csf3, Cxcl9, Ecgf1, Efna1, Efna3, Egf, F2, Fgf1, Figf, Flt1, Hif1a, Igf1, Il10, Il18, Jag1, Kdr, Lect1, Mmp2, Mmp9, Notch4, Npr1, Nrp2, Nudt6, Pecam1, Pgf, Plau, Pofut1, Prok2, Pten, Ptgs, Sphk1, Tbx1, Tek, Tgfb2, Tgfbr1, Thbs2, Tnf, Tnfrsf12a, Vegfa, Vegfc
2	11 genes	Bai1, Ctgf, Edg1, Efna2, Ephb4, Il18, Mdk, Ptn, Tgfb3, Timp2, Timp3
3	5 genes	Akt1, Efnb2, Mapk14, Pdgfa, Vegfb
4	2 genes	Il12a, Nrp1

Differentially expressed genes between embryonic and postnatal days were determined by scatter plot analysis (Figure 5.3b, Figure 5.5). Means of each gene on days E12, E14, E16, E18 and E20 arrays were used as embryonic expression; means of each gene on P1, P3, P5, P7, P9, P11 and P13 arrays were used as postnatal expression of each gene.

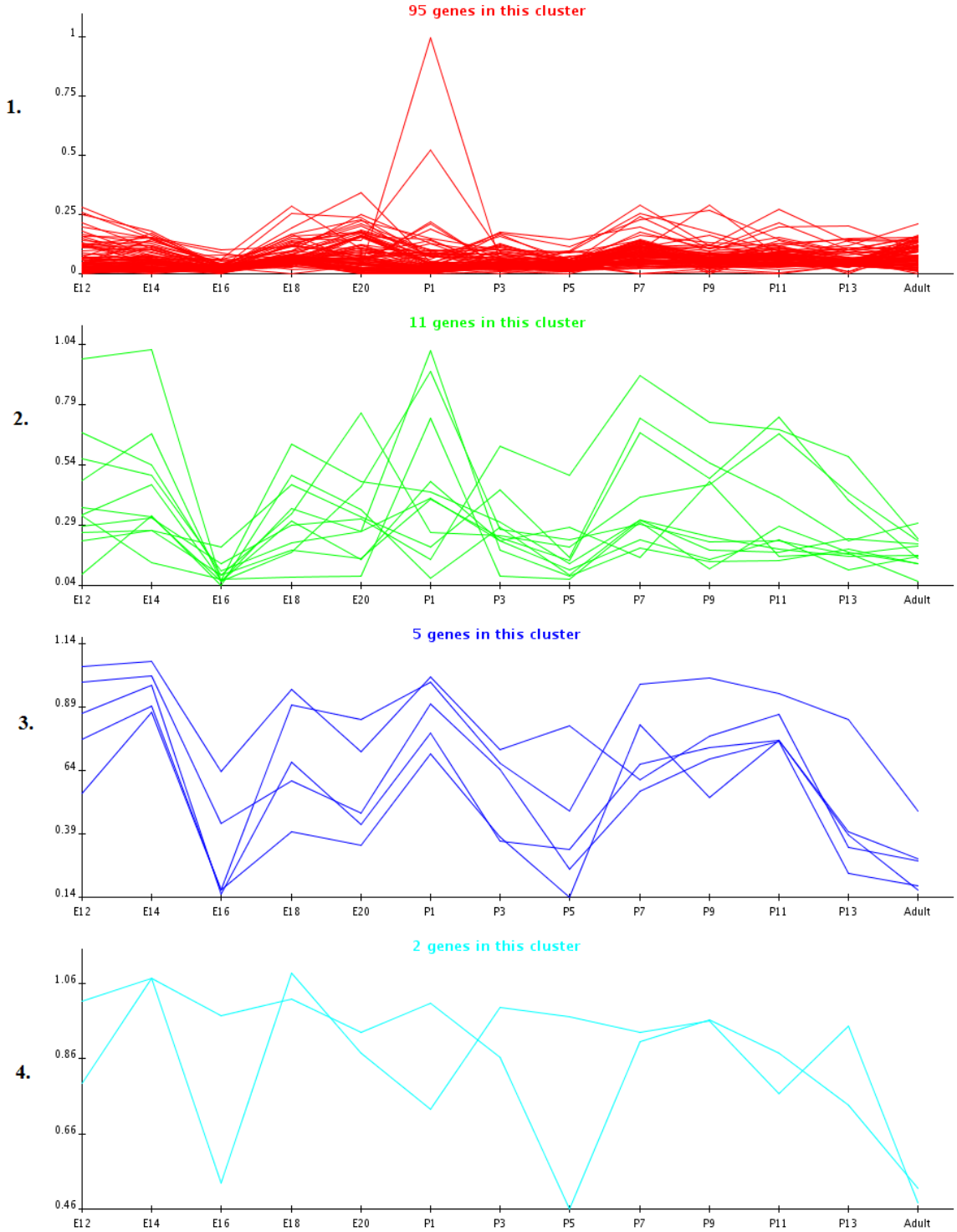

Figure 5.4. K-means clustering of 113 genes according to expression patterns: unexpressed and very low-rate expressed genes fall into the first cluster (Graph 1), and the remaining 18 genes were classified in 3 groups (Graph 2,3,4), (x-axis: days, y-axis: normalized expression levels)

Ctgf and Il18 were found to be overexpressed in postnatal stage, compared to prenatal stage. Mmp2, Efna2, Ephb4, Tgfb3 and Mdk were overexpressed in embryonic stage, compared to postnatal stage (Table 5.4). House-keeping genes and positive control spots were found to be equally expressed in both stages, as expected.

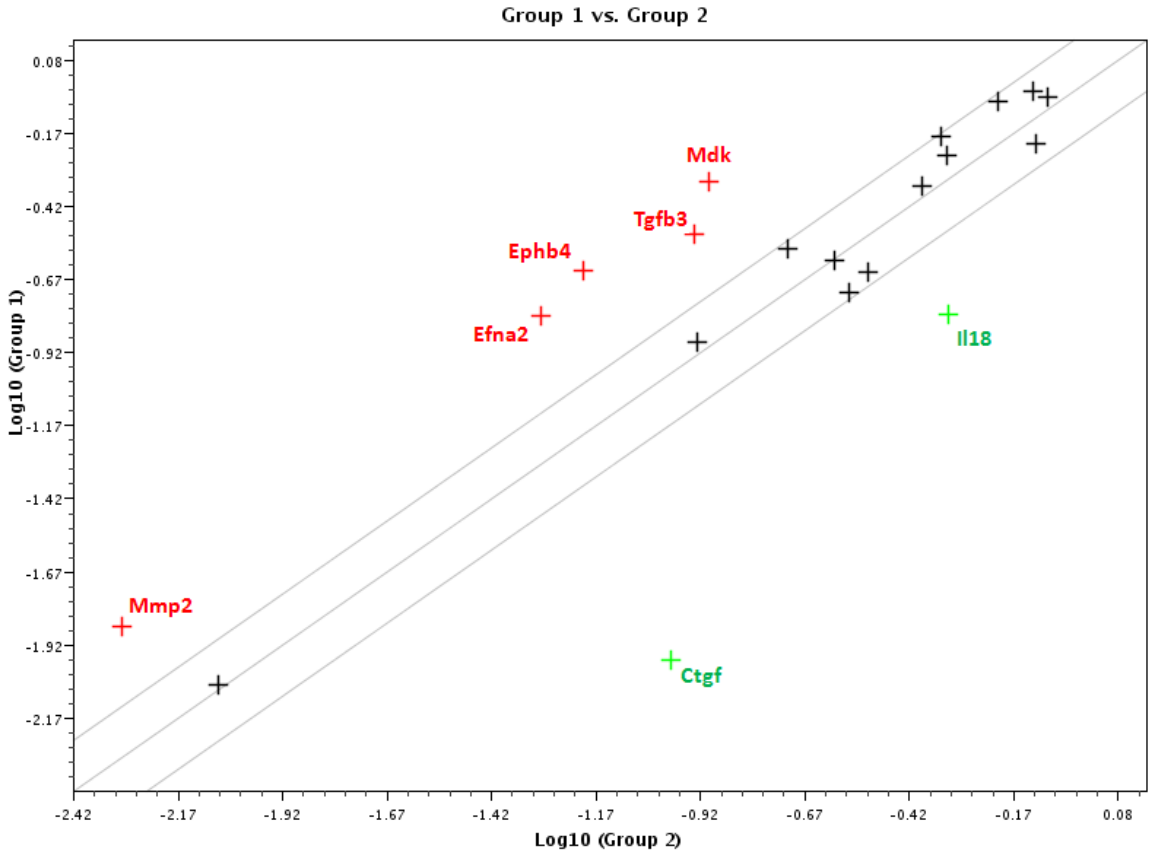

Figure 5.5. Scatter plot analysis of array results: Boundary value was assigned as 1,5 fold. Genes that were 1,5 fold more expressed in embryonic stage, indicated in red, and were located above the upper boundary line. Genes that were 1.5 fold more expressed in postnatal stage, indicated in green, were located below the lower boundary line. Group 1 is embryonic and Group 2 is postnatal stage

Table 5.4. List of differentially expressed genes between two stages of development: Scatter plot analysis

Genes Overexpressed in Postnatal vs. Embryonic Stages		
Array Position	Gene	Postnatal/Embryonic
16	Ctgf	9,54
51	Il18	2,9
Genes Overexpressed in Embryonic vs. Postnatal Stages		
Array Position	Gene	Embryonic/Postnatal
26	Efna2	3,21
32	Ephb4	3,63
62	Mdk	3,69
64	Mmp2	2,79
97	Tgfb3	2,61

5.2. Selected Target Genes for Further Analysis

Comprehensive literature search was performed for the 62 expressed genes and candidate genes were selected for further studies. To gain novel insight, those genes that were previously shown to play a role in any developmental process, especially in vascular development and brain development were not taken. Additionally, those genes that have knock-out models, whose developmental function is already known, were also not selected. Those genes that may have direct roles in angiogenesis, considering their functions, had priority in selection. Each criterion was considered on its own value and three target genes, found most worth to study, thus were selected.

These are;

- Brain specific angiogenesis inhibitor-1 (Bai1)
- Natriuretic peptide receptor-1 (Npr1)
- Nudix (nucleoside diphosphate linked moiety X)-type motif 6 (Nudt6)

5.3. qRT-PCR Analysis for Selected Genes

qRT-PCR experiments were performed for Bai1, Nudt6 and Npr1, using the Roche UPL system. While Bai1 and Nudt6 showed similar expression patterns, Npr1 had a distinct expression pattern. Bai1 showed three peaks on days E15, E18 and P7. Its expression became steady after day 9 and decreased toward the adult stage (Figure 5.6). Nudt6 expression showed a major peak on E15 and minor peaks on days E18, P1 and P7. Its expression level was approximately equal to the Bai1's on each day, and became steady after day 9 and decreased toward the adult stage, too (Figure 5.7). Npr1 expression decreased on day E12 to become steady after embryonic day E13. Its expression was more stable and slightly lower than Bai1 and Nudt6 (Figure 5.8).

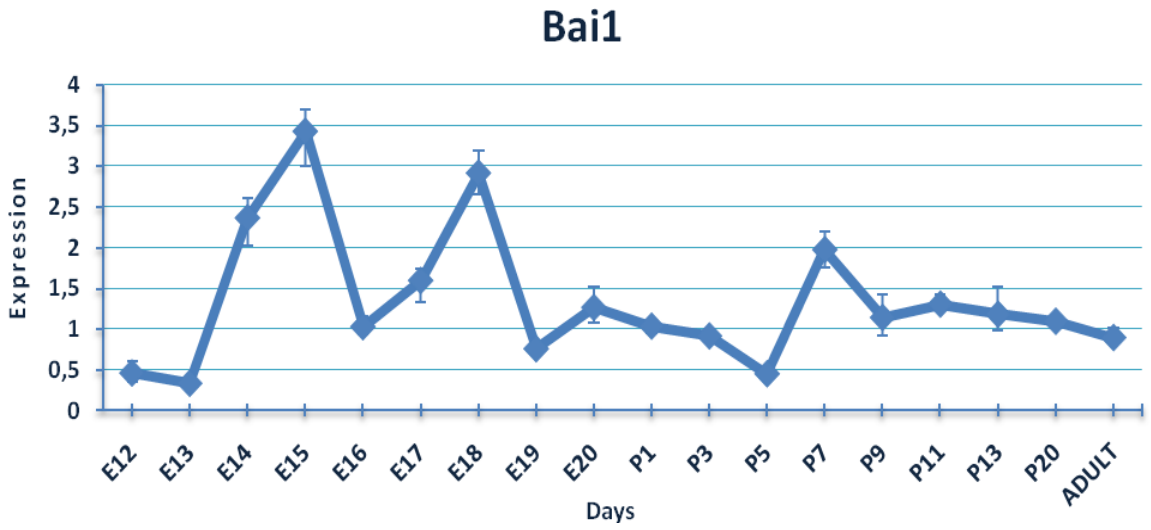

Figure 5.6. Temporal expression analysis of Bai1; qRT-PCR result (each spot indicates the average value of three repeats, GAPDH normalized values are used; positive and negative error values indicate difference of average from the the maximum and minimum values, respectively)

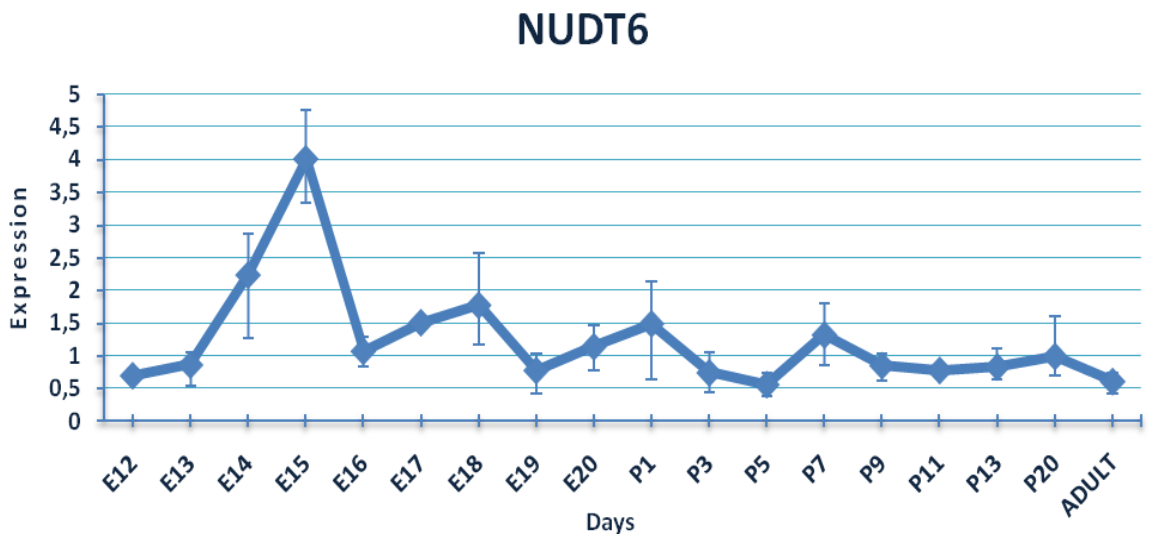

Figure 5.7. Temporal expression analysis of Nudt6; qRT-PCR result (each spot indicates the average value of three repeats, Gapdh normalized values are used; positive and negative error values indicate difference of average from the the maximum and minimum values, respectively)

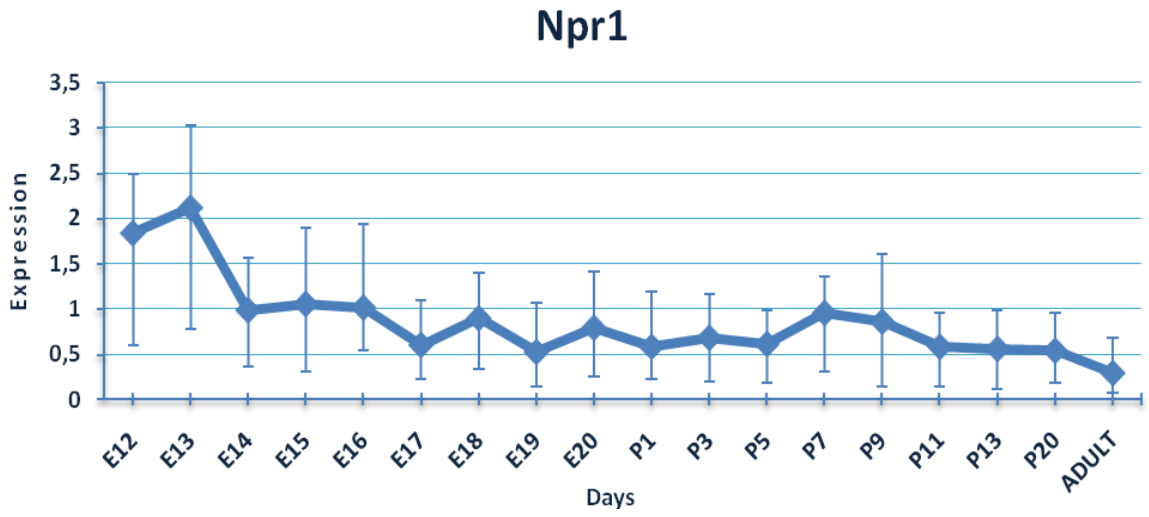

Figure 5.8. Temporal expression analysis of Npr1; qRT-PCR result (each spot indicates the average value of three repeats, Gapdh normalized values are used; positive and negative error values indicate difference of average from the the maximum and minimum values, respectively)

5.4. Western Blot Analysis for Bai1 and Npr1

To verify the RNA expression levels of Bai1 and Npr1 at protein level, Western Blot analysis was performed for samples E12, E15, E18, E20, P1, P5, P9, P13 and adult samples. Both Bai1 and Npr1 proteins were expressed at varying degrees on the days of analysis (Figure 5.9).

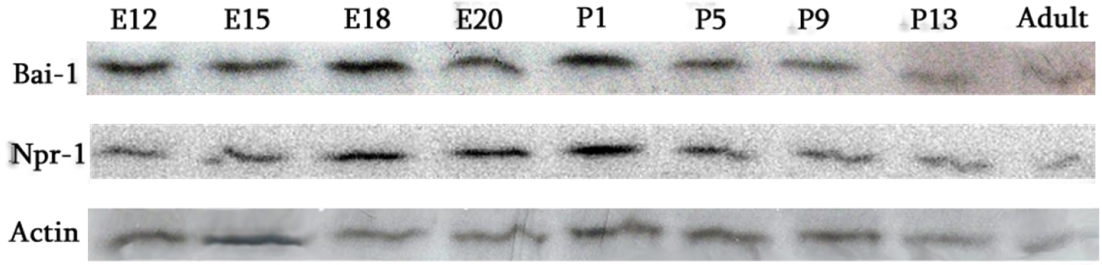

Figure. 5.9. Western Blot analysis of Bai1 and Npr1

Since there is no commercially available Nudt6 antibody for Western Blot analysis, Nudt-6 gene was not analyzed by Western Blot.

6. DISCUSSION

Blood vessels circulate oxygen, metabolites, waste products, hormones and immune system elements to the distal sites within the body. While the function of blood vessels in the body is known for hundreds if not thousands of years, we have only begun to understand the molecular mechanisms underlying the development of the vascular system. Embryonic vascular development is a highly complex order of events that involves a variety of cell-cell interactions and a wide range of functional molecules, including growth factors and receptors, transcription factors, cytokines, chemokines, proteases and their inhibitors, adhesion molecules and many matrix proteins, some of whom have stimulatory and some others inhibitory effects on angiogenesis. Actions of these factors must be well orchestrated in terms of time, space and dose to form a functional vascular network (Yancopoulos *et al*., 2000; Torres-Vazquez *et al*., 2003). Besides, blood vessels have specialized structures and functions in various tissues. ECs, mural cells and ECM (in which they are embedded) determine such distinct tissue-specific properties according to the needs of tissues in which they are found (Carmeliet, 2003a).

The central nervous system vasculature has many special features and functions, too, and therefore should be handled separately from vascular systems of all other tissues. It exclusively develops via angiogenesis, but not via vasculogenesis. Blood vessels of the brain, having epithelial-like tight junctions and a variety of special transport molecules, form a striking barrier between the blood and cells of the brain. They are highly covered with pericytes, dynamically and reciprocally interact with neurons and glial cells and also form a vascular niche for neural stem cells (Pardridge, 2003; Shen *et al*., 2004; Mancuso *et al*., 2008).

The classic literature provides descriptive knowledge about the embryonic vascular development, and most of what we know about the vascular system and angiogenesis at molecular level is mostly derived from the study of pathological events in the adult organisms, like inflammation, tumor growth, tissue repair and variety of diseases with angiogenic defects (Ribatti, 2006). A proper understanding of the brain vascular development is crucial both for understanding normal development, and also for exploring

the CNS disorders, including cerebrovascular malformations and neurodegenerative processes (Rossant and Howard, 2002).

In recent years, an increasing number of angiogenic molecules was shown to be involved in neurogenesis and neuroprotection; conversely, the problems in angiogenic molecules were shown to lead to neurodegenerative disorders, like ALS and Alzheimer's. It is now gradually accepted that healthy maintenance of cerebrovascular system and proper functioning of its key molecules, like VEGF, are crucial to protect the CNS from degeneration (Carmeliet, 2003; Zacchigna *et al*., 2008).

Cerebrovascular malformations arise from abnormal angiogenesis in brain vasculature. Especially arteriovenous malformations (AVMs), the most dangerous of them, carry high risk of bleeding throughout human life. Very little is known about the molecular pathology of AVMs, but at least it is surely known that they are angiogenically active and thought to arise during embryonic development. It is estimated that they are the result of disruption of the balance between angiogenic and anti-angiogenic molecules and/or defects during arterio-venous differentiation of vessels. To find out which genetic factors contribute to AVM pathogenesis, norms of cerebrovascular development should be known at molecular level. Elucidating the mechanisms of how angiogenic genes are orchestrated in vascular development is crucial to understand AVM pathogenesis and to develop effective treatment modalities in clinically different AVM tissues (Kilic *et al*., 2000b; Hanjani, 2002; Seker *et al*., 2006).

In the framework of this thesis, temporal expression of 113 angiogenic genes in mice brain vascular development have been analyzed using an angiogenesis-specific membrane array system.

In literature, a considerable amount of angiogenesis data comes from pathological studies, mostly through tumor angiogenesis. Only a few of these genes' involvements in vascular development has been studied so far. Some other genes' roles in angiogenesis were discovered, accidentally through knock-out studies. As a result, the number of studies, which were specifically designed to find out and analyze the genes that play a role in brain vascular development, is very limited. This study aimed to cover the temporal and

comparative expression analysis of the angiogenesis-related genes in brain development, in order to give new insights into the norms of cerebrovascular development at molecular level.

6.1. Determination of Expressed Angiogenic Genes

Compared to standard microarray chip systems, low-density, pathway-specific arrays are useful devices that are designed for the elucidation of particular biological pathways; thus they provide focused and more interpretable gene expression data. In this study, angiogenesis-specific arrays were used, on which 113 angiogenesis-related target genes' 60-mer oligo probes are fixed. Genes are selected by the manufacturer, by scanning the most recent angiogenesis literature.

Our results showed that 62 of 113 genes analyzed were expressed at varying levels (Table 5.1). These 62 genes contain 31 growth factors and their receptors, eight adhesion molecules, six cytokines and chemokines, eight proteases, inhibitors and some matrix proteins and 16 transcription factors and other signal transduction pathway molecules (some genes fall into more than one cluster). To discuss all genes in detail will exceed the scope of this thesis; some major genes will be dealt in this chapter and some others under further headings.

Nrp1 is a tyrosine kinase receptor for both Vegf-B and semaphorin family members. It was shown to play versatile roles in angiogenesis, axon guidance, cell survival, migration, and invasion. The expression of Nrp1 was shown to be necessary for angiogenesis; mice with damaged Nrp1 gene die during embryonic development. Nrp1 was constitutively expressed throughout brain development, which is consistent with other literature data. Nrp2, another neuropilin receptor, interacts with Vegfr-3 and binds Vegf-C and Vegf-D. It is thought to be involved in angiogenesis, cardiovascular development, axon guidance and tumorigenesis. Its very low expression compared to Nrp1, may indicate that, unlike Nrp1, it has less role in brain and vascular development.

According to our results, Vegf-A, Vegf-B and Vegf-C, three major angiogenic factors, were shown to be expressed during brain development, but Vegf-B was 5-fold

higher expressed than the other two genes (Table 5.2). Knock-out studies showed that blood vessels can develop properly without Vegf-B and recent evidence indicates that, beside angiogenesis, it may also play a major role in neurogenesis (Sun *et al*., 2005; Fischer *et al*., 2008). Our results support the finding that Nrp1, a receptor for Vegf-B, is 15-fold higher expressed than the other angiogenesis-specific Vegf-B receptor Flt1; this means Vegf-B interacting with Nrp1 has other functions than just being an angiogenic factor. Expression levels of Flt1 and Kdr, receptors for the Vegf family and placental growth factor (Pgf/Plgf), were nearly equal, showing similarity with their ligands, Vegf-A, Vegf-C, Pgf.

Angiopoietins (Ang1/Angpt1 and Ang2/Angpt2) and their receptor Tie2 (Tek) are a second major class of signaling molecules in angiogenesis and vascular development, especially during mid-stage of angiogenesis in ECs lumen stability (Liekens *et al*., 2001). Ang2 was expressed at equal amounts with Vegf-A and Vegf-C, but its antagonist Ang1 does not seem to be expressed during brain development according to our results (Table 5.2). As expected, their receptor Tie2 was expressed at significant amounts. Angiopoietins were also shown to play a role in neurodegeneration, so expression of Ang2 indicates the involvement of Ang-Tie system in the maintenance of the healthy brain and its vascular development.

Cytokines including interleukins have pleiotropic effects in the immune and nervous systems. Interleukin-10 (Il10), Il12a, Il18, Il1b and Il6 were previously shown to have roles in pathological angiogenesis (Salven *et al*., 2002; Kohno *et al*., 2003; Huang *et al*., 2004). In this study, while Il12a and Il18 were highly expressed, Il10 had little expression. Il1b and Il6 were not expressed at all during embryonic development (Table 5.2). High and continuous expression of Il12a and Il18 indicates that they have crucial roles in brain development and possibly in angiogenic development. Functional studies are needed to better understand their separate functions in brain development and angiogenesis.

The Eph receptor tyrosine kinases comprise the largest family of growth factor receptors. They were previously known as having central roles in nervous system development, but later it was shown that they have also principal roles in arterio-venous

fate of newly developing blood vessels during early development (Himanen *et al*., 2007). Efna1, Efna2, Efna3, Efnb2 ligands and the receptor Ephb4 were expressed at varying levels (Table 5.2). Their expression was higher in embryonic compared to postnatal stage, which is consistent with the literature (Figure 5.3). Efnb2 expression was 10 times higher than Efna1 and Efna3, and three times higher than Efna2 expression, implicating its pivotal role during the process.

Surprisingly, one of the key regulators of vessel maturation factor, Pdgf-β does not seem to be expressed during brain development, similar to another important factor of angiogenesis, Fgf2 (Table 5.1, Figure 5.3). Both molecules were not expressed in the developing brain according to our results. However the truth may be different; sensitivity of the membrane-array method is limited, therefore, although these two factors might be expressed at very low level, the resolution of the array was not high enough to show such low levels of mRNA. Another possibility is, Fgf2 has 5 isoforms, ranging from 18-34 kDa, and the designed probes for Fgf2 did not match the alternatively spliced isoforms correctly. It is possible that moderate expression of the Nudt6 gene (Table 5.2), an anti-sense gene for Fgf2, may also inhibit the Fgf2 expression or stability, so we were not able to see its expression. On the other hand, in contrast to Pdgf-B and Fgf2; Pdgf-A expression was very high, and Fgf1 was expressed in developing CNS. Lastly, in addition to Fgf2, Fgf6 and Fgfr3 were not expressed either (Table 5.1).

Those genes that seem not to be expressed on the arrays are either not expressed at all (Table 5.2), or expressed in very low amounts, and the array resolution is not high enough to detect their expression. Although arrays are advantageous, because one can analyze more than a hundred genes at one shot, the sensitivity problem is a major drawback of the arrays. On the other hand, the low expression of some angiogenic genes might also be the result of the relatively small volume (0.1%) of vasculature in the brain (Shusta *et al*., 2002). For further analysis on unexpressed genes, more sensitive methods like, qRT-PCR experiments may be needed.

6.2. Genes with Similar Expression Patterns

Expression pattern clustering of genes is a useful method to detect genes with similar patterns (Figure 5.4). To discover new gene regulation pathways and to explore genes regulated with common cellular signals, K-means clustering approach is a helpful way. In our study, four clusters of genes were determined as the optimal cluster number. Those genes that are not expressed or expressed at a very low rate fall into the first cluster, thus discussing their cellular regulation is not reasonable at this point (Table 5.3). When analyzed in detail, it was seen that the program constructed the remaining three groups according to the expression levels of genes, not according to their expression patterns. In other words, genes with the highest expression values fall into cluster four, moderate expressed ones fall into cluster three, and less expressed ones fall into cluster two. As a result, K-mean clustering did not give any clues about the real expression pattern similarities of the genes.

6.3. Differentially Expressed Genes Between Embryonic and Postnatal Stages

In scatter plot analysis, differentially expressed genes between embryonic and postnatal stages were determined (Figure 5.5). To obtain more interpretable results, not all 113 genes, but only some of the expressed genes were included into the analysis. According to scatter plot results, Mmp2, Efna2, Ephb4, Tgfb3 and Mdk genes are overexpressed in embryonic stage, compared to their postnatal expressions.

Although Mmp2 has low expression, it had 2.79 times greater expression (in mean) in embryonic days (Table 5.3). Its partner Mmp-9 seems to play a role in the process, but Mmp-19 was not expressed on any day analyzed (Figure 5.3). The matrix metalloproteinases (MMPs) are a family of extracellular endo-peptidases that selectively degrade components of the extracellular matrix which is needed for proliferation and migration of cells (Jost *et al*., 2006). Mmp2 and Mmp9 are known for their roles in angiogenesis. In the study, higher expression of Mmp2 in embryonic stage may be due to its involvement in neural migration, in addition to endothelial cell migration in angiogenesis. Mmp19 displays unique structural features and tissue distribution among the Mmp family. Its exact role is still unknown, but shown to be negatively regulated in

angiogenesis. Lack of Mmp19 did not affect $Mmp19^{-/-}$ mice viability, fertility and development (Jost *et al*., 2006). This study contributes to the literature showing that, Mmp19 is not being expressed in brain development.

Higher expression of the Ephrin family members, Efna2 and Ephb4 is, as expected, due to their active roles in nervous system development (Table 5.2). Beside their roles in nervous system, Ephrins were also shown to be the determinant factors for arterio-venous differentiation of newly developing vessels. Consequently, their expression values in embryonic development are consistent with previous data.

Transforming growth factors (Tgfb1, Tgfb2, and Tgfb3) are multifunctional peptides that function as cytokines and in control of proliferation, differentiation, and numerous other functions in many cell types, including ECs. Although involvement of Tgfb2 and Tgfb3 is well-known in literature, there are controversial findings on expression of Tgfb1 in the developing brain (Gomes *et al*., 2005). Our findings confirm the expression of Tgfb2 and Tgfb3 in brain development, with five-fold more expression of Tgfb3 (Table 5.2). However, our results showed that Tgfb1 does not play a role in brain vascular development (Table 5.2).

The midkine family consists of two members, midkine (Mdk) and pleiotrophin (Ptn). They show similar biological activities, including anti-apoptotic, mitogenic, transforming, angiogenic, and chemotactic ones. Previously it was shown that during embryogenesis, MK is highly expressed in the mid-gestational period, whereas Ptn expression reaches the maximum level around birth (Kadomatsu and Muramatsu, 2004). Our data are consistent with literature: they were expressed constitutively at similar levels; Mdk was expressed mostly at days E12-E15, and the expression decreased dramatically after birth; and expression of Ptn increased before birth, but stayed high during late-developmental stages (Table 5.2, Figure 5.3).

Connective tissue growth factor (Ctgf) is a major connective tissue mitoattractant secreted by vascular endothelial cells. It mediates heparin-dependent cell adhesion in many cell types including fibroblasts, myofibroblasts, endothelial and epithelial cells. Ctgf can promote endothelial cell growth, migration, adhesion and survival and thus functions in

several stages of angiogenesis. Ctgf is thought to function during development, in which coordinated production and remodeling of the extracellular matrix is essential (Babic *et al*., 1999; Ivkovic *et al*., 2003). To the best of our knowledge, this study shows for the first time that Ctgf functions in brain development. Its expression was moderate and wavy throughout the analyzed days, but higher in postnatal stages, up to nearly 10-fold, in mean (Table 5.2, Figure 5.3).

6.4. Temporal Expression Analysis of Bai1

Brain specific angiogenesis inhibitor-1 (Bai1) is a novel anti-angiogenic molecule that functions as G-protein coupled receptor. It was discovered as a mediator of p53, through its negative regulation in glioblastoma cells (Nishimori *et al*., 1997). Later, its expression was found to be inversely correlated with vascularity and metastasis of colorectal cancer, and it is thought to suppress stromal vascularization of pulmonary adenocarcinoma (Fukushima *et al*., 1998; Hatanaka *et al*., 2000). In following years, functional studies showed that Bai1 overexpression significantly lowers the tumor proliferation in both several cell lines and in tumor transplanted immune-deficient mice, and thus decreases the tumor neovascularization (Duda *et al*., 2002). In the same year, it was demonstrated that Bai1 was expressed in neuronal cells of the cerebral cortex, but not in astrocytes, and Bai1 protein was localized to the cellular cytoplasm and membrane (Mori *et al*., 2002). The anti-proliferative effect of the Bai1 on endothelial cells *in vitro* was shown by Koh *et al*. in 2004, which was the first clear evidence of Bai1's "direct" inhibitory effect on angiogenesis. In the same year, Bai1 expression was analyzed in six different brain tumors and in a total of 51 different tumor samples, and lack of Bai1 expression in brain tumors was correlated with poor clinical outcome (Nam *et al*., 2004). In another study, lipid-mediated delivery of Bai1 gene was shown to reduce the corneal neovascularization in an *in vivo* rabbit model (Yoon *et al*., 2005). The last and only second functional study on Bai1 in literature came out in 2007, in which Bai1 was shown to function as an engulfment receptor in both the recognition and subsequent internalization of apoptotic cells (Park *et al*., 2007).

This study, for the first time in literature, shows that, Bai1 is expressed during murine brain development. According to array results, Bai1 is expressed in a considerably

high level in all developmental processes (Table 5.2). Its expression was 2-3-fold higher - in mean- than Vegf-A and Vegf-B, and 4-5 times greater than Vegf receptors Flt1 and Kdr. According to qRT-PCR results, its expression peaks on days E15, E18 and P7, and become stable after day P9 (Figure 5.6). Western Blotting verified the mRNA level expression analysis of Bai1 at protein level (Figure 5.9).

6.5. Temporal Expression Analysis of Npr1

There are three structurally related natriuretic peptide molecules: atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and the type-C natriuretic peptide (CNP); all of them were shown to be expressed in the brain and in many other organs. The natriuretic peptide receptor-1 (Npr1 or NprA), a single trans-membrane spanning protein that contain guanylyl cyclase (GC) activity, is one of three receptors found for natriuretic peptides (Waschek, 2004).

Natriuretic peptides and their receptors function in development of several organ systems, including heart, nervous system and skeletal growth, in regulation of blood vessel vasodilation and other cardiovascular functions through the actions on the kidney and vascular smooth muscle cells. They are also estimated to have roles in the vascularization of the embryonic brain, establishment of the blood-brain and blood-nerve barriers, and in nerve regeneration. Several studies showed that, natriuretic peptides also function as endogenous inhibitors of VEGF-modulated angiogenesis (Waschek, 2004; Ribatti *et al*., 2007; Kuhn *et al*., 2009).

In this study, Npr1 was shown to be expressed at moderate and varying levels during brain development, confirming the literature data (Figure 5.3, Table 5.2). According to qRT-PCR results, Npr1 expression is high at around day E12-E13, suddenly decreases on day E14 and expression continues steadily until adult stage, at which it slightly decreases again. Western Blot analysis confirmed the expression of Npr1 in brain development at protein level. Comparing these results with previous data indicates that, Npr1 functions in brain development, possibly not just in vascular development, due to its moderately high expression rate, but also in nervous system regeneration. Further

functional studies are needed to exactly understand the molecular pathways is included during brain development.

6.6. Temporal Expression Analysis of Nudt6

Nudt6 is synthesized from the complementary strand of Fgf2, and was implicated to function in post-transcriptional regulation of Fgf2. Studies on Nudt6 are very limited, and its further roles, more than being Fgf2 anti-sense, are still unknown. There are a few studies showing its expression in some pathological conditions, e.g. in leukemic cells. There is no study in literature pinpointing to its expression in any developmental process (Knee *et al*., 1997; Baguma-Nibasheka *et al*., 2007).

This study, for the first time in literature, shows that, Nudt6 is expressed in a relatively high level in brain development (Figure 5.3, Table 5.2). qRT-PCR experiments showed that Nudt6 is continuously expressed throughout brain development, displaying maximum expression at day E15, and minor peaks on days E18, P1 and P7.

Although we do not know whether it has other functions in the cell, we can speculate that, Nudt6 should be involved in vascular development of the CNS, through its effects on Fgf2 expression.

7. CONCLUSIONS

Brain arteriovenous malformations, with their high intracranial bleeding risks, are life-threatening. There is very limited knowledge about the molecular pathogenesis of AVMs, and they are thought to arise during development. To unravel the molecular mechanisms and develop effective therapy modalities, proper understanding of the cerebrovascular development at molecular level is crucial. Moreover, neurodegenerative mechanisms are thought to be linked to problems in the maintenance of the healthy brain vasculature. Exploring the norms of brain vascular development and its involved genes is crucial.

To the best of our knowledge, this thesis, for the first-time in literature, aimed to investigate brain vascular development using a low-density microarray system. The study revealed novel angiogenesis-related genes that play a role in brain vascular development and made the temporal and comparative expression analysis of these genes possible. Our findings are expected to contribute to vascular development research and are further expected to be an important reference for functional studies in AVMs, thus forming a new basis for further studies in humans.

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