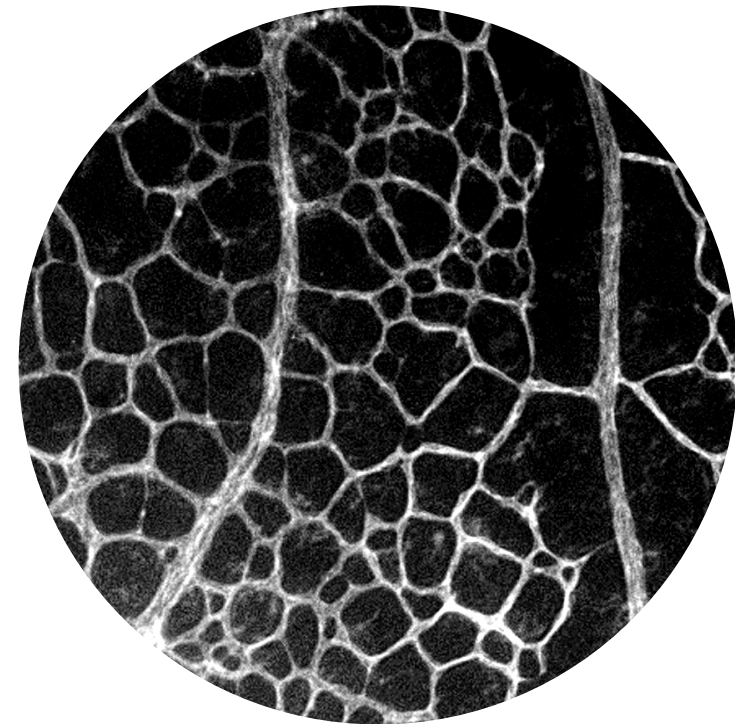


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MIRA ERNKVIST  
CONTROLLING ANGIOGENESIS – FUNCTIONAL STUDIES OF ANGIOMOTIN

# CONTROLLING ANGIOGENESIS – FUNCTIONAL STUDIES OF ANGIOMOTIN



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*In motion*

*The sated day is never first.  
The best day is a day of thirst.*

*Yes, there is goal and meaning in our path -  
but it's the way that is the labour's worth.*

*The best goal is a night-long rest,  
fire lit, and bread broken in haste.*

*In places where one sleeps but once,  
sleep is secure, dreams full of songs.*

*Strike camp, strike camp! The new day shows its light.  
Our great adventure has no end in sight.*

Karin Boye

TO:

MATTIAS AND MY FAMILY



## ABSTRACT

The expansion of a blood circulatory network by the process of angiogenesis is essential for embryonic as well as postnatal growth. Deregulated blood vessel formation may also negatively contribute to the pathogenesis of diseases, e.g. cancer and diabetic retinopathy. Furthermore, compounds that interfere with angiogenesis signalling pathways have shown great promise when used in clinical trials for patients suffering from cancer or macular degeneration.

The formation of a functional blood vessel involves several distinct steps. Cells of the vessel wall, i.e. endothelial cells, extend filopodia that sense pro-angiogenic signals and direct migration and the formation of the vessel network. Circulation is established when two endothelial sprouts fuse and form a continuous lumen. Angiomotin has previously been implicated to play a role in endothelial migration and to be a possible target for anti-angiogenic therapy. The aim of this thesis was to investigate the biological role of angiomotin during vascular development and to elucidate the signalling pathways involved.

In this thesis it is demonstrated that the angiomotin gene is expressed as two isoforms with distinct functions. The shorter isoform, p80-angiomotin, is expressed during the migratory phase of retinal angiogenesis, whereas the other isoform, p130-angiomotin, is expressed during and after vessel maturation. In vitro, these isoforms exhibit opposite functions. For instance, p80-angiomotin promotes cell migration whereas p130-angiomotin promotes actin fibre formation and cell contacts. It is further shown that p80-angiomotin expression removes p130-angiomotin from cell junctions resulting in a migratory switch.

The angiomotin signalling pathway was analysed by identifying binding proteins by two different approaches, peptide pull down and yeast two-hybrid screening. Data are presented demonstrating that the PDZ-binding domain of angiomotin binds to a polarity protein complex as well as to a Rho-GEF, which has previously been shown to mediate endothelial cell migration. These findings argue that angiomotin acts as a scaffold for proteins regulating cell polarity and GTPase activity.

The biological role of angiomotin during mouse and zebrafish embryogenesis was also studied. Genetic ablation of angiomotin in the mouse results in vascular defects in the intersomitic region as well as dilated vessels in the brain and embryonic lethality after E11. Furthermore, knockdown of angiomotin in zebrafish impaired the migration of intersegmental vessels and caused dilation of vessels in the brain, confirming the phenotype found in the mouse. It is further shown that angiomotin deficient endothelial cells have an intact proliferative response to VEGF but exhibit defects in migration.

Taken together, these data indicate that angiomotin is essential for normal embryonic vessel formation and that it may affect directional cell migration by controlling cell polarity and GTPase activity.

# LIST OF PUBLICATIONS

This thesis is based on the following papers, referred to in the text by their Roman numerals:

- I. **Mira Ernkvist**, Karin Aase, Chinwe Ukomadu, James Wohlschlegel, Ryan Blackman, Niina Veitonmäki, Anders Bratt, Anindya Dutta, and Lars Holmgren.  
p130-angiomotin associates to actin and controls endothelial cell shape  
*FEBS Journal* 2006 **273**: 2000-2011
- II. Karin Aase, **Mira Ernkvist**, Lwaki Ebarasi, Lars Jakobsson, Arindam Majumdar, Chunling Yi, Olivier Birot, Yue Ming, Anders Kvanta, Dan Edholm, Pontus Aspenström, Joseph Kissil, Lena Claesson-Welsh, Akihiko Shimono, and Lars Holmgren.  
Angiomotin regulates endothelial cell migration during embryonic angiogenesis.  
*Genes & Dev.* 2007 **21**: 2055-2068
- III. **Mira Ernkvist**, Olivier Birot, Indranil Sinha, Niina Veitonmäki, Staffan Nyström, Karin Aase, and Lars Holmgren.  
Differential roles of p80- and p130-angiomotin in the switch between migration and stabilization of endothelial cells.  
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- IV. **Mira Ernkvist**, Nathalie Luna Persson, Stéphane Audebert, Patrick Lecine, Indranil Sinha, Miaoliang Liu, Anders Bratt, Arie Horowitz, Karin Aase, Arindam Majumdar, Jan-Paul Borg, and Lars Holmgren.  
Angiomotin regulates migration via the Rho-GEF protein syx-1.  
*Submitted.*

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## LIST OF ABBREVIATIONS

AJ	Adherens junction
Ang	Angiopoietin
Amot	Angiomotin
AmotL	Angiomotin like
CEC	Circulating endothelial cell
DLL4	Delta-like 4
E	Embryonic day
EC	Endothelial cell
ECM	Extracellular matrix
EPC	Endothelial progenitor cell
ES	Embryonic stem
FAK	Focal adhesion kinase
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FISH	Fluorescence in situ hybridisation
GAP	GTPase activating protein
GDI	Guanine nucleotide dissociation inhibitor
GEF	Guanine nucleotide exchange factor
JEAP	Junction enriched and associated protein
MAE	Mouse aortic endothelial
MAGI-1	Membrane associated guanylate kinase with inverted domain structure 1
MDCK	Madin-Darby Canine Kidney
MMP	Matrix metalloproteinase
MTOC	Microtubule-organizing centre
NP	Neuropilin
NSCLC	Non-small cell lung cancer
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PECAM	Platelet endothelial cell adhesion molecule
PI3K	Phosphatidylinositol 3-kinase
PIGF	Placenta growth factor
SCID	Severe combined immunodeficiency disease
TGF	Transforming growth factor
TJ	Tight junction
TSP-1	Thrombospondin-1
VE-cadherin	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VPF	Vascular permeability factor

# INTRODUCTION

Cancer development is a multistep process that requires genetic alterations to transform normal cells into tumour cells. Tumours can either be benign (i.e. localised tumours that are non-aggressive) or malignant (i.e. invasive tumours that metastasise).

In 2000, Hanahan and Weinberg suggested that a cell must acquire six traits to become malignant (Hanahan and Weinberg 2000). These “Hallmarks of cancer” comprise the following:

- Self-sufficiency in growth signals.
- Insensitivity to growth-inhibitory (anti-growth) signals.
- Evasion of programmed cell death (apoptosis).
- Limitless replicative potential.
- Sustained angiogenesis.
- Tissue invasion and metastasis.

Each of these changes represents a potential target for cancer therapy since all of them are necessary for the progression of the disease.

In this thesis, I will focus on one of these traits, i.e. sustained angiogenesis, and the role of angiominin in the regulation of blood vessel formation.

## BLOOD VESSEL FORMATION

The vascular system is composed of arteries and arterioles that carry oxygenated blood from the heart to the tissues. Capillaries, from which oxygen and nutrients are released, branch from the arterioles and are the smallest blood vessels. The venules and veins are the vessels responsible for carrying the blood back to the heart and for removing waste products.

The formation of new blood vessels occurs in the developing embryo where the primary vascular tree is established as well as an adequate vasculature for growing and developing organs (reviewed in (Eichmann et al. 2005; Adams and Alitalo 2007).

In the adult, the vascular system is normally quiescent except for during wound-healing and in the female reproductive cycle. There are however several diseases caused or characterised by increased blood vessel formation (i.e. angiogenesis) such as cancer, retinopathy and rheumatoid arthritis.

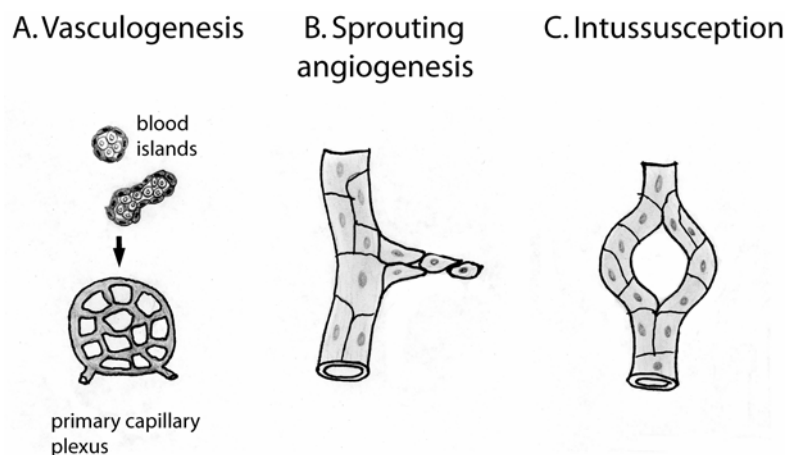
Tissue function depends on a supply of oxygen and nutrients from the blood vessels. Like normal tissues, tumours require an adequate supply of oxygen and metabolites and an effective way to remove waste products in order to grow and metastasise (Papetti and Herman 2002). This was observed as early as 150 years ago, but the research field of tumour angiogenesis essentially did not begin until 1971 when Judah Folkman suggested that tumours cannot grow beyond the size of a few cubic millimetres without inducing new blood vessel growth, and that blocking of angiogenesis could be a strategy to inhibit tumour growth (Folkman 1971).

## The vascular system during embryogenesis and in the adult

The diffusion distances of molecules such as oxygen are limited to approximately 100-200  $\mu\text{m}$ . Therefore it is essential that the vascular system establish early during embryonic development.

Blood vessel formation in the embryo is classically divided into two different processes, namely vasculogenesis and angiogenesis (Figure 1A and B). Vasculogenesis is the de novo formation of blood vessels from differentiating hemangioblasts whereas angiogenesis is the formation of new blood vessels via extension or remodelling of existing blood vessels (Figure 1A and B). Angiogenesis occurs throughout development and in adulthood. Vasculogenesis was originally thought to occur only during a limited period early in embryonic development, however, it can also occur during adulthood as I describe below.

A third way of inducing new blood vessels is that of intussusceptive microvascular growth, which is the splitting of one vessel into two (Figure 1C) which has been observed during the development of the lung after birth (Burri et al. 2004).



*Figure 1. Blood vessels are formed by three different mechanisms; vasculogenesis, angiogenesis, and intussusception. Figure adapted from Adams & Alitalo 2007.*

### Vasculogenesis

Vasculogenesis is the process where new vessels develop from endothelial precursor cells, i.e. the angioblasts (Figure 1A). The angioblast was described as early as 1900 when it was found that cells isolated from embryos were able to give rise to blood vessels. It was also during this early period that it was suggested that something called a hemangioblast – a common precursor for both endothelial cells (ECs) and hematopoietic cells – existed. However, the presence of the hemangioblast was not confirmed until almost 100 years later (Choi 1998).

Vasculogenesis is a highly regulated and complex process that occurs during the embryonic development, which begins in the structures called blood islands (aggregations of mesenchymal cells) in the extra embryonic tissues. The outer cells of

the blood islands are endothelial, whereas the inner cells give rise to the hematopoietic progenitors.

Vasculogenesis occurs both within the extraembryonic and intraembryonic compartments. In both cases, hemangioblasts at the border of the blood islands will start to differentiate and connect into a primitive vascular plexus that expands throughout the embryo. The first sign of mammalian blood vascular development occurs at mouse embryonic day (E)7.5 in the yolk sac. The intraembryonic angioblasts appear at E7.5 in the head region where they develop a primitive capillary plexus similar to that in the yolk sac at E8-8.5. The dorsal aorta and cardinal veins are formed directly from aggregating angioblasts without first forming a capillary plexus, whereas local vascular plexuses develop and slowly remodel into major vessels and capillary beds in other areas of the embryo (reviewed in (Coultas et al. 2005; Ferguson et al. 2005).

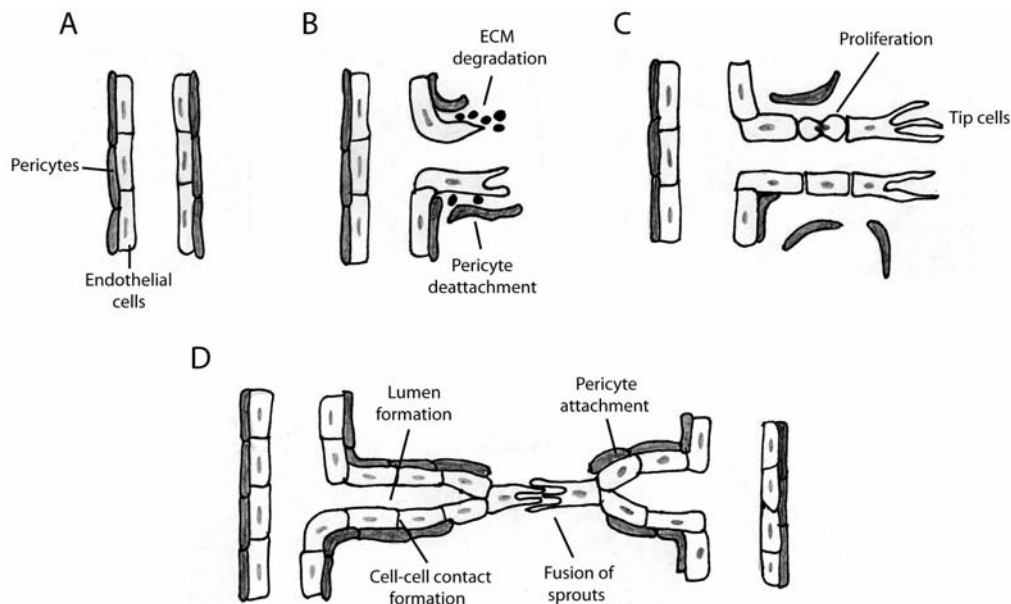
Initially, it was thought that vasculogenesis occurred only in the developing embryo. However, adult vasculogenesis, i.e. differentiation of circulating endothelial progenitor cells (EPCs), has also been shown to play a role in the formation of new blood vessels in the adult (reviewed in (Khakoo and Finkel 2005; Kopp et al. 2006). The cells that are essential to this process, the EPCs, may be comparable to the embryonic angioblasts, since they circulate, proliferate, and participate in the development of a vascular network by differentiating into mature ECs (Rafii 2000). However, the phenotypic characterisation of EPCs remains controversial. Efforts to accurately characterise these cells have been confounded by the presence of other circulating ECs (CECs) in the peripheral circulation. Unlike EPCs, CECs are thought to derive from mature vascular endothelium and have a markedly reduced ability to participate in postnatal vasculogenesis compared with EPCs. Several studies have shown that EPCs play a role in physiological and pathological vessel growth in the adult and that they are involved in processes such as myocardial ischemia (Balsam and Robbins 2005; Yoon et al. 2005), retinal neovascularisation (Grant et al. 2002), atherosclerosis (Sata et al. 2002; Dimmeler and Zeiher 2004), and tumour vascularisation (Davidoff et al. 2001; Nolan et al. 2007).

### *Angiogenesis*

After a primitive vascular plexus has formed through vasculogenesis in the embryo, the blood vessels will start to remodel, undergo proliferation and regression, and branch and migrate into different regions of the embryo, a mechanism known as angiogenesis. It is worth noting that many of the events that occur during the normal progression of vascular development in the embryo are repeated during events of neoangiogenesis in the adult (Carmeliet 2003).

The molecular characteristics of angiogenesis occur in a stepwise progression (Figure 2). Sprouting angiogenesis is induced by hypoxia, which upregulates the expression of a number of genes required for vessel formation (e.g. VEGF and angiopoietin-2). The vessel then starts to dilate in response to nitric oxide and become leaky in response to VEGF. Pericytes are then removed from the branching vessel and the EC basement membrane and extracellular matrix (ECM) are degraded and remodelled by specific proteases such as matrix metalloproteinases (MMPs) (Moses 1997) (Figure 2B). Over 20 MMPs have been described and implicated in angiogenesis, tumourigenesis, and cell proliferation (Nelson et al. 2000). After the degradation, the ECs starts to sprout from

the existing vessel, i.e. migrate and proliferate in response to VEGF and other endothelial cell mitogens until sufficient divisions have occurred (Figure 2C). It is the tip cell that guides the vessel in the correct direction (Gerhardt et al. 2003). The tip cell moves as a single cell in front of the stalk cells and, in contrast to the stalk cells, the tip cell is non-proliferative. When the ECs have reached their target, they arrest and form a tube-like structure (Figure 2D).



*Figure 2. The steps involved in sprouting angiogenesis. A. A blood vessel consists of endothelial cells covered by mural cells. B. The basal membrane and ECM are degraded, pericytes detach, and ECs will start to migrate. C. ECs proliferate and are guided by tip cells. D. ECs adhere to each other and form a lumen. Pericytes are recruited to the surfaces of the tubes. Figure adapted from Bergers & Benjamin 2003.*

The newly formed sprouts then anastomose to form vascular loops and networks. Mural cells (which are pericytes in the microvasculature and smooth muscle cells in the larger vessels) are then recruited to the outer surface of the tubes whilst vessels that are not covered by pericytes regress. Finally, blood flow is established in the new vessel (reviewed in (Carmeliet 2000; Conway et al. 2001; Papetti and Herman 2002; Jain 2003; Adams and Alitalo 2007).

Angiogenesis is a frequent event during embryogenesis, but also occurs in adults, for example during the female reproduction cycle, repair and regeneration of tissues, and wound healing (Folkman 1995).

### Pathological angiogenesis

More than 70 disorders have been implicated as “angiogenesis-dependent diseases” so far.

The best known conditions where angiogenesis is switched on are cancer, ocular and inflammatory disorders, but angiogenesis may play a role in many other diseases, such

as obesity, asthma, diabetes, endometriosis, and autoimmune diseases (see Table 1) (Carmeliet 2003; Carmeliet 2005; Folkman 2007).

In diseases such as ischemic heart disease, too few new blood vessels are formed. This leads to inhibited re-vascularisation, EC dysfunction, and/or vessel malformation or regression (for a more complete list of diseases, see Table 1) (Carmeliet 2003; Carmeliet 2005; Folkman 2007).

Table 1. Angiogenesis-dependent diseases.

<b>Organ</b>	<b>Diseases<sup>a</sup> characterised or caused by excessive angiogenesis</b>	<b>Diseases<sup>a</sup> characterised or caused by insufficient angiogenesis</b>
Multiple organs	Cancer and metastasis Infectious diseases Auto-immune disorders, e.g. multiple sclerosis (Kirk and Karlik 2003; Kirk et al. 2004) and Sjögrens disease (Ohno et al. 2004).	
Blood vessels	DiGeorge syndrome (Stalmans et al. 2003)	Hypertension (Sane et al. 2004) Atherosclerosis (Moulton 2006)
Adipose tissue	Obesity (Rupnick et al. 2002)	
Eye	Diabetic retinopathy Age-related macular degeneration (Gariano and Gardner 2005; Nowak 2006; Hernandez and Simo 2007).	
Nervous system		Alzheimers disease (de la Torre 2004; Zlokovic 2005) ALS (Storkebaum et al. 2004)
Heart		Ischemic heart disease (Shiojima et al. 2005)
Skin	Psoriasis (Leong et al. 2005)	Hair loss (Yano et al. 2001)

<sup>a</sup>For a more complete list of diseases, see (Carmeliet 2005).

### *Tumour angiogenesis*

Tumour blood vessels are architecturally different from their normal counterparts (reviewed in (Bergers and Benjamin 2003; Jain 2003; Baluk et al. 2005; Hillen and Griffioen 2007)). They are organised in a chaotic way as evidenced by the fact that they

are irregularly shaped, dilated, twisted, and have widely variable diameters and dead ends (Hashizume et al. 2000). The blood vessels within tumours are often leaky (Hashizume et al. 2000), partly due to overproduction of VEGF. Also, it has been shown that blood vessels within tumours are compromised not only of ECs. Instead, cancer cells can be integrated into the vessel wall, giving rise to mosaic blood vessels (note that the integration of tumour cells into vessels is termed “vasculogenic mimicry”) (Chang et al. 2000; Folberg et al. 2000).

Recent research has also shown that ECs within tumours are not genetically stable. Fluorescent in situ hybridization (FISH) analysis has demonstrated that tumour ECs are aneuploid and have abnormal multiple centrosomes (Hida et al. 2004). Another study demonstrated that a large number of ECs in B-cell lymphomas contain lymphoma-specific chromosomal translocations (Streubel et al. 2004). New data from the Holmgren group shows that fibroblasts and ECs are capable of acquiring and replicating tumour DNA by the mechanism of horizontal gene transfer provided the apoptotic tumour cells contain the SV40 large T antigen. In addition, a significant number of ECs within tumours are tumour-host hybrids that display endothelial specific markers that are capable of forming functional vessels (Ehlfors. On the mechanism and consequences of cell to cell DNA transfer, PhD thesis 2007).

It has also been shown that pericytes covering the ECs in tumour vessels are abnormal. They express atypical markers, are loosely associated with the ECs and exhibit cytoplasmic processes that invade the tumour parenchyma (Morikawa et al. 2002; Ozawa et al. 2005).

The most studied mechanism of new blood vessel formation is sprouting angiogenesis (described on pages 3-4). Sprouting angiogenesis is not only important for the growth of primary tumours, but is also involved in the formation of metastases since tumour cells can travel to distant organs through the blood vessels (Hanahan and Weinberg 2000). The process of sprouting angiogenesis is tightly controlled by positive and negative regulators, as will be described later.

A variant of tumour angiogenesis is intussusceptive angiogenesis where one pre-existing vessel is split into two vessels by the formation of a transvascular tissue pillar into the lumen of the vessel (Djonov et al. 2003; Burri et al. 2004).

It is now generally accepted that new blood vessels can also be formed by recruited EPCs, as described above (see page 3). EPCs are also involved in neovascularisation of tumours, as described for the first time by (Asahara et al. 1999). However, the reported contribution of EPCs to blood vessels within tumours varies between studies. For instance, it has been shown that the contribution of EPCs to blood vessels is as high as 50% (Lyden et al. 2001) or as low as 5% (Peters et al. 2005), or in some cases, not detected at all (Gothert et al. 2004; Ziegelhoeffer et al. 2004).

Tumour cells do not have to induce an angiogenic response. Instead, they can grow along already existing vessels, a process defined as vessel co-option. This was first observed in metastases of the lungs after subcutaneous injection of Lewis lung carcinoma into C57BL6/J mice (Holmgren et al. 1995) and two years later in non-small cell carcinoma (Pezzella et al. 1997). Later, it was also shown that the growing glioma can co-opt host vessels (Holash et al. 1999a). Another study has demonstrated that there is no evidence of direct vessel growth into human and murine melanoma. Instead, the tumours grow by incorporating the vascular plexus into the peritumoural connective tissue (Dome et al. 2002).

Several factors are involved in the angiogenic switch. A thorough description of each lies beyond the scope of this thesis so only those most relevant to my studies will be described below.

## **Vascular effectors**

The angiogenic process is a highly complex and dynamic process that is regulated by a number of growth factors. Angiopoietins, ephrins, FGFs, Notch ligands, PDGFs, TGF- $\beta$  and VEGFs, for instance, all play essential roles in this process.

### *VEGFs and VEGF receptors*

One of the major pathways involved in promoting embryonic as well as tumour angiogenesis is the vascular endothelial growth factor (VEGF) family of proteins and receptors (reviewed in (Ferrara et al. 2003; Byrne et al. 2005; Hicklin and Ellis 2005; Olsson et al. 2006). In mammals, the VEGF family consists of five members, VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placenta growth factor (PlGF) (Figure 3). VEGF-A was first identified as a protein that increased the vascular permeability and was therefore referred to as vascular permeability factor (VPF) (Senger et al. 1983). In 1989, Ferrara et al identified a growth factor produced by ECs that they cloned and named VEGF (Leung et al. 1989), which was found to be the same protein as VPF. VEGF-A undergoes alternative splicing which gives rise to different isoforms. The human isoforms are VEGF-A<sub>121</sub>, VEGF-A<sub>145</sub>, VEGF-A<sub>165</sub>, VEGF-A<sub>189</sub>, and VEGF-A<sub>206</sub> (Houck et al. 1991; Tischer et al. 1991). VEGF-A<sub>121</sub> is a freely diffusible protein whereas VEGF-A<sub>189</sub> and VEGF-A<sub>206</sub> are sequestered in the ECM and require cleavage by proteases for their activation. VEGF-A<sub>165</sub>, the most predominant isoform, is secreted, but a significant amount remains bound to the ECM.

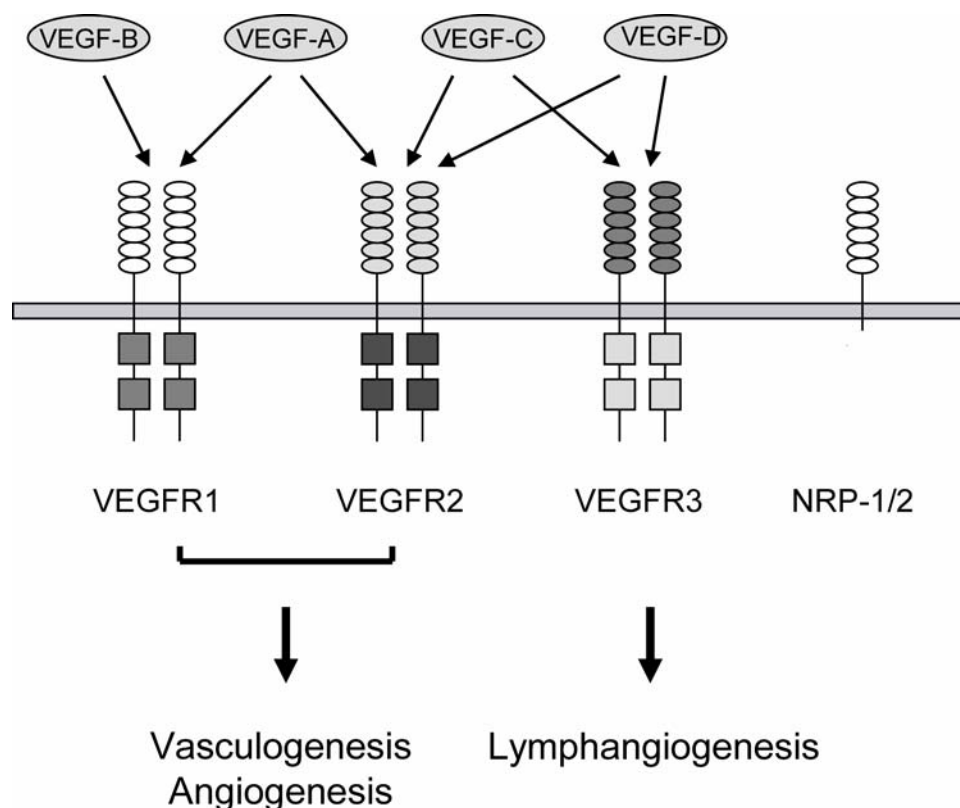
Homozygote deletion of VEGF-A is embryonic lethal (at E9.5-10.5) due to severe defects in vascular development (Carmeliet et al. 1996). Heterozygote deletion of VEGF-A is also embryonic lethal, but at a later stage (E11-12), also due to defective vascular development (Carmeliet et al. 1996; Ferrara et al. 1996). VEGF-B null mice display no defects during embryogenesis; however, the adult mice display a reduced heart size, dysfunctional coronary vasculature and mild cardiac conduction defects (Bellomo et al. 2000; Aase et al. 2001). Homozygous deletion of VEGF-C in mice is embryonic lethal due to a lack of lymphatic vessels resulting in fluid accumulation in tissues (Karkkainen et al. 2004). Heterozygote mice develop cutaneous lymphatic hypoplasia and lymph oedema (Karkkainen et al. 2004). Mice deficient in VEGF-D are healthy and fertile. The only phenotype they display is a slight reduction of lymphatic vessels adjacent to lung bronchioles (Baldwin et al. 2005).

The VEGF ligands mediate their angiogenic effects via several different receptors. Two tyrosine kinase receptors were originally found to be expressed on ECs, i.e. VEGFR1 (Shibuya et al. 1990) and VEGFR2 (Terman et al. 1991). Later, an additional VEGFR (i.e. VEGFR3), which is primarily involved in lymphangiogenesis, was identified (Pajusola et al. 1992; Pajusola et al. 1994; Kaipainen et al. 1995) (Figure 3). All VEGF-A isoforms bind to both VEGFR1 and VEGFR2, whereas VEGF-B binds specifically



to VEGFR1. VEGF-C and VEGF-D interact with both VEGFR3 and VEGFR2 (Figure 3).

VEGFR1 is essential for developmental angiogenesis since VEGFR1 null mice die in utero between E8.5 and 9.5. Null mice embryos form ECs but their vascular channels are abnormal due to an increase of EPCs (Fong et al. 1995; Fong et al. 1999). Interestingly, knock-out mice lacking the tyrosine kinase domain of VEGFR1 without affecting the ligand binding region develop normal vessels and survive (Hiratsuka et al. 1998). The ligand binding domain together with the transmembrane domain of VEGFR1 is therefore sufficient to allow embryonic development with normal angiogenesis. The reason for this is most likely that VEGFR1 traps VEGF-A to absorb excessive VEGF-A, thereby regulating the amount of VEGF that binds to VEGFR2 (Hiratsuka et al. 2005). However, it has also been shown that VEGFR1 plays a role in activating VEGFR2 and thereby angiogenesis by binding PIGF which is highly upregulated during pathological conditions. PIGF displaces VEGF from VEGFR1 and makes more VEGF available to bind and activate VEGFR2. Also, PIGF activates VEGFR1, which leads to intermolecular transphosphorylation of VEGFR2. PIGF and VEGF can also form heterodimers, which activate and transmit angiogenic signals through a VEGFR1/VEGFR2 heterodimer receptor complex (Autiero et al. 2003).



*Figure 3. Ligand binding leads to receptor dimerisation and signalling events resulting in cellular responses important for vasculogenesis, angiogenesis and lymphangiogenesis occur. Receptor heterodimers of VEGFR1/VEGFR2 or VEGFR2/VEGFR3 take place but their functions are unclear. Figure adapted from Olsson et al 2006.*

A truncated form of VEGFR1, named soluble VEGFR1 (Kendall and Thomas 1993) has been found to be abnormally high and suggested as the causative agent for the development of preeclampsia (Maynard et al. 2003; Levine et al. 2004).

VEGFR2 is the primary mediator of VEGF signalling. It plays an important role during vasculogenesis since homozygote and heterozygote embryos die in utero (E8.5 to 9.5) due to defects in blood island formation and vasculogenesis (Shalaby et al. 1995; Shalaby et al. 1997). VEGFR3 is expressed in embryonic ECs, but during development, its expression by blood vessels decreases and becomes restricted to the lymphatic endothelium (Kaipainen et al. 1995). Homozygote knock-out mice die in utero before E12, and before the formation of lymphatic vessels, due to defects in blood island formation and vascular development (Dumont et al. 1998). A naturally occurring soluble form of VEGFR2 has also been described (Ebos et al. 2004).

Neuropilin-1 (NRP-1) was originally identified on neuronal cells as a receptor for the semaphorin/collapsin family of proteins that serve as neuronal guider mediators (Fujisawa and Kitsukawa 1998). NRP1- and NRP-2 have been shown to serve as co-receptors for VEGF. Both NRP-1 and NRP-2 lack intracellular tyrosine kinase domains and must therefore act in conjugation with other receptors to mediate VEGF signalling. The role of NRP-1 in vascular development was recognised in mouse models where NRP-1 knock-out embryos exhibit various types of vascular defects, as well as defective neuronal patterning, and deletion of the gene is embryonic lethal (Kawasaki et al. 1999). NRP-2 null mice show a severe reduction in lymphatic vessels and capillaries during development (Yuan et al. 2002). If both NRP1- and NRP-2 are deleted, the embryos die in utero at E8.5 due to defective vascular development (Takashima et al. 2002).

It is clear that the VEGF family plays an important role during vascular development, but what is the function of VEGF in ECs? I will present just a brief overview of the events that are activated when VEGF interacts with its tyrosine kinase receptor and the signal is transmitted to various downstream proteins.

VEGF was originally discovered by its ability to induce permeability (Senger et al. 1983). The increase in permeability in tumours results in the leakage of several plasma proteins. This transforms the normally antiangiogenic stroma of normal tissues into a proangiogenic environment.

VEGF stimulates DNA synthesis and proliferation via VEGFR2, which activates Erk1/2. It was first shown to act as a survival factor for retinal ECs (Alon et al. 1995) and it has now been shown to promote survival in several ECs, both in vitro and in vivo. It binds to VEGFR2 and inhibits apoptosis by activating the PI3K-Akt pathway (Gerber et al. 1998).

VEGF acts as a chemo attractant for ECs, which suggests that it plays a role in migration and invasion. It induces cell migration by activating factors such as focal adhesion kinase (FAK) and paxillin, leading to focal adhesion turnover and actin filament organisation. VEGF also induces a variety of enzymes and proteins important in the process of degrading the basement membrane, and helping the ECs to migrate and invade.

### *FGFs and FGF receptors*

The fibroblast growth factors (FGFs) were among the first angiogenesis molecules identified and since then they have been found to stimulate EC proliferation, migration, and differentiation in vitro and in vivo (Bikfalvi et al. 1997).

The FGFs belong to a family of structurally similar proteins and this family consists to date of 23 heparin-binding polypeptides. FGF-1 and FGF-2 were the first isolated FGFs and they share about 55% sequence identity (Folkman et al. 1988). They exert their biological activities by binding to tyrosine kinase receptors (i.e. FGFRs) on the surface of target cells. FGFR1 is the main FGFR expressed on ECs in vitro and it has also been detected on ECs in vivo. Small amounts of FGFR2 have also been found in ECs (Nakamura et al. 2001) whereas the expression of FGFR3 or FGFR4 has never been reported in the endothelium. In capillary EC lines, stimulation of FGFR1 induces proliferation, migration, protease production, and tubular morphogenesis, whereas activation of FGFR2 increases only motility.

FGF-2 knock-out mice are viable and phenotypically impossible to differentiate from wild type mice. However, they exhibit altered vascular tone and neuronal defects (Dono et al. 1998; Zhou et al. 1998). The fact that the knock-out mice do not exhibit a defective vascular phenotype suggests a functional redundancy during developmental vasculogenesis and angiogenesis. Gene knock-out of FGFR1 leads to embryonic death prior to gastrulation and the embryos display severe growth retardation and defects in mesodermal structures (Deng et al. 1994; Yamaguchi et al. 1994).

In the adult, FGFs are thought to be involved in tissue repair, wound healing, and neuronal migration.

### *PDGFs and PDGF receptors*

The platelet-derived growth factors (PDGF) consist of four different proteins encoded by four different genes. The PDGF-A and -B isoforms are denoted classical PDGFs and were discovered almost three decades ago (Westermarck and Wasteson 1976; Antoniades et al. 1979; Heldin et al. 1979; Deuel et al. 1981). More recently the PDGF-C and -D chains were identified (Li et al. 2000; Bergsten et al. 2001; LaRochelle et al. 2001). The four PDGF chains form dimers and five different combinations have been identified so far, namely PDGF-AA, -BB, -CC, -DD, and the heterodimer PDGF-AB (Fredriksson et al. 2004).

PDGFs bind to two tyrosine kinase receptors, i.e. PDGFR- $\alpha$  and PDGFR- $\beta$ . PDGF-AA, -BB, -AB, and -CC have a high affinity for binding to PDGFR- $\alpha$  whereas PDGF-BB and -DD have a high affinity for binding to PDGFR- $\beta$ .

The most important PDGF for angiogenesis is a component of the PDGF-BB/ PDGFR- $\beta$  pathway, which is critical in recruiting perivascular cells to blood vessels. Tip cells express high amounts of PDGF-B (Gerhardt et al. 2003). This creates a gradient of PDGF-BB, which allows for proliferation, directed migration and attachment of pericytes to the growing vessel. PDGF-B or PDGFR- $\beta$  knock-out mice die shortly before birth due to haemorrhage, oedema and defects in the kidney glomeruli (Leveen et al. 1994; Soriano 1994). The haemorrhage is due to defects in the expansion and migration of perivascular cells whereas the initial development of pericytes is not affected since the number of pericytes covering the aorta is normal (Lindahl et al. 1997).

A number of cellular responses such as proliferation, migration and cell survival, are activated after PDGF stimulation (Claesson-Welsh 1994). The PDGFRs are activated in a similar way compared to the VEGFRs and signalling molecules such as Src, PI3K and Ras are engaged.

#### *Angiopoietins and Tie receptors*

The human angiopoietin family consists of four members, Ang1, Ang2, Ang3, and Ang4, which function as ligands for the Tie1 and Tie2 tyrosine kinase receptors. The role of the angiopoietins in angiogenesis is rather complex. Both Ang1 and Ang2 bind to Tie2, which is mainly expressed in ECs, but their functions differ. Ang1 is an angiogenic factor that promotes the structural integrity of blood vessels. Mice deficient in Ang1 are unable to form a vascular complex network and die during embryogenesis (Davis et al. 1996; Suri et al. 1996). Functional Ang1 is essential for EC survival, vascular branching and pericyte recruitment (Metheny-Barlow and Li 2003). Ang2 functions as a naturally occurring antagonist of Ang1, either promoting or inhibiting vessel growth depending on the levels of other growth factors such as VEGF-A. Mice overexpressing Ang2 exhibit a phenotype similar to that of Ang1 knock-out mice (Maisonpierre et al. 1997; Holash et al. 1999b).

Knock-out studies in mice show that a common characteristic of deleting Tie1 (Sato et al. 1995), Tie2 (Dumont et al. 1994; Sato et al. 1995), Ang1 (Suri et al. 1996) or Ang2 (Gale et al. 2002) is that vasculogenesis proceeds normally, but remodelling and maturation of the blood vessels are defective.

#### *Delta-like 4 and Notch receptors*

Several articles describing Delta-like 4 (DLL4) and its role during angiogenesis have recently been published. DLL4 belongs to a family of ligands comprised of five members, i.e. Delta-like1, Delta-like 3, Delta-like 4, Jagged1 and Jagged2. These ligands interact with four Notch receptors, i.e. Notch1 – Notch4 (Roca and Adams 2007).

DLL4 was cloned in 2000 and found to be expressed in the vascular endothelium and in the endothelium of tumour blood vessels. High levels of DLL4 were also detected in the eye and lung at E15.5 (Rao et al. 2000; Shutter et al. 2000; Mailhos et al. 2001). DLL4 is required for normal vascular development and correct arterial formation in mice (Duarte et al. 2004; Gale et al. 2004; Krebs et al. 2004). Also, DLL4 is the only example except VEGF that shows haploid embryonic lethality (Duarte et al. 2004; Gale et al. 2004; Krebs et al. 2004). Data produced from knock-out mice has been confirmed by studies in the developing retina and zebrafish. Three studies have shown that DLL4 regulates vessel branching during development by inhibiting endothelial tip cell formation (Hellstrom et al. 2007; Lobov et al. 2007; Suchting et al. 2007). Deletion of one DLL4 allele gives rise to increased numbers of tip cells and EC proliferation, resulting in the formation of a denser and more highly interconnected capillary plexus (Hellstrom et al. 2007; Lobov et al. 2007; Suchting et al. 2007). Also, the extensions of the filopodia in retinal vessels heterozygote for DLL4 require VEGF, showing that DLL4 is induced by VEGF as a negative feed-back regulator (Hellstrom et al. 2007; Lobov et al. 2007; Suchting et al. 2007). DLL4 is necessary to restrict the angiogenic cell behaviour of tip cells in the intersegmental vessels in the zebrafish and when DLL4 signalling is defective, the tip cells and ECs continue to migrate and proliferate when

they should normally stop these processes (Leslie et al. 2007; Siekmann and Lawson 2007).

DLL4 has also been shown to be of importance for tumour angiogenesis. Studies have shown that blockade of DLL4-Notch reduces tumour growth from 50% to more than 90%, depending on the tumour model (Noguera-Troise et al. 2006; Ridgway et al. 2006). Surprisingly, the reduced tumour growth was associated with enhanced angiogenic sprouting and branching. However, the blood vessels were non-functional as shown by poor perfusion and increased hypoxia. (Noguera-Troise et al. 2006; Ridgway et al. 2006). These results indicate that inhibiting DLL4 could be an approach to preventing tumour growth.

## **Inhibition of angiogenesis**

Inhibiting angiogenesis may present one approach to stabilise disease in patients with angiogenesis-dependent disorders such as cancer and age-related macular degeneration. Today, 10 new drugs in which anti-angiogenic activity is considered to be central for their therapeutic mechanism have been approved by the Food and Drug Administration (FDA) in the United States and at least 43 other drugs that have varying degrees of anti-angiogenic activity are at this time in clinical trials in the United States (Folkman 2007).

A brief background to some of the synthetically produced angiogenesis inhibitors that have been approved for clinical use or are in clinical trials are listed below. In addition, several endogenous inhibitors that have been discovered will be discussed.

### *Synthetic angiogenesis inhibitors*

There are several ways to inhibit angiogenesis and a number of pharmaceutical companies are trying to develop and test new angiogenesis inhibitors.

Bevacizumab, or Avastin (Genentech), is a humanized anti-VEGF monoclonal antibody that neutralises all VEGF-A isoforms and bioactive proteolytic fragments, but it does not neutralise other members of the VEGF family, such as VEGF-B or VEGF-C (Presta et al. 1997). The terminal half-life of bevacizumab in humans is 17-21 days and so far, no evidence of antibody response to bevacizumab has been found in any clinical trials, verifying that the humanization of the original mouse antibody has been successful. Preclinical studies showed that bevacizumab inhibited the growth of human tumour cell lines in nude mice (Presta et al. 1997).

The first phase 1 clinical trial (where a small group of healthy volunteers are selected and the safety, tolerability, pharmacokinetics and pharmacodynamics of the drug are tested) with bevacizumab started in April 1997. These studies showed that bevacizumab was relatively non-toxic as a single agent and it did not aggravate the chemotherapy-associated toxicities if used in combination with standard chemotherapy (Gordon et al. 2001; Margolin et al. 2001). In 1998, five phase 2 studies (performed on a larger group of patients where the efficacy and toxicity of a drug is studied) in different tumour types were initiated. The best results were seen when bevacizumab was combined with chemotherapy in colorectal cancer (Kabbinavar et al. 2003) and non-small cell lung cancer (NSCLC) (Johnson et al. 2004), and when used as a single agent in renal-cell cancer (Yang et al. 2003). In 2004, bevacizumab was approved by

the FDA for colorectal cancer after a phase 3 clinical trial (randomised controlled multicenter trials on large patient groups) showed that bevacizumab in combination with irinotecan, bolus fluorouracil and leucovorin (IFL) gave statistically significant and clinically meaningful improvement in survival among patients with metastatic colorectal cancer (Hurwitz et al. 2004). Today, bevacizumab is approved for clinical use in colorectal cancer, breast cancer and NSCLC and it is in clinical trials for several other tumour diseases and the results look very promising.

Two drugs targeting VEGF have been approved for clinical use in patients suffering from age-related macular degeneration, i.e. Pegaptanib, or Macugen, (OSI Pharmaceuticals) and Ranibizumab, or Lucentis, (Genentech). They have both been shown to improve the vision of patients.

Sorafenib, or Nexavar (Bayer Pharmaceuticals) and Sunitinib, or Sutent (Pfizer Inc.), are broad-spectrum receptor tyrosine kinase inhibitors that inhibit for example VEGFR1-3, PDGFR $\beta$ , c-KIT etc. These drugs have shown anti-tumour activity in patients with metastatic renal cell cancer. Complete response is not common, but the drugs promote disease stabilisation, increase progression-free survival, and they are approved for clinical use (Grandinetti and Goldspiel 2007).

### *Endogenous inhibitors*

A number of endogenous antiangiogenic factors have been described (Folkman 2004; Nyberg et al. 2005). Many of them are proteolytic fragments of larger precursor proteins, and they can be divided into two major classes, matrix-derived inhibitors and non-matrix derived inhibitors (Nyberg et al. 2005). The first endogenous angiogenesis inhibitor that was recognised as a naturally occurring angiogenesis inhibitor was thrombospondin-1 (Good et al. 1990). Since then, endogenous inhibitors such as angiostatin, endostatin, and tumstatin have been identified.

Angiostatin was purified from the urine and serum of tumour bearing mice in 1994 and was discovered to be a 38 - 45 kDa fragment of plasminogen (O'Reilly et al. 1994). Different proteolytic enzymes can cleave plasminogen and generate angiostatin. In vitro experiments have shown that angiostatin is a relatively specific molecule for ECs and EPCs, and that it inhibits their proliferation. However, the proliferation of other cell types including tumour cells is not affected (O'Reilly et al. 1994; O'Reilly et al. 1996; Ito et al. 1999). It also inhibits EC migration, invasion, and tube formation and induces apoptosis (O'Reilly et al. 1994; Gately et al. 1997; Claesson-Welsh et al. 1998; Lucas et al. 1998). In vivo experiments have shown that angiostatin inhibits both primary and metastatic tumour growth (O'Reilly et al. 1994; O'Reilly et al. 1996; Gately et al. 1997). Angiostatin has been shown to interact with several molecules. For instance, a study by Moser et al showed that angiostatin binds to the alpha/beta-subunits of ATP synthase on the cell surface and that this interaction mediates the antiangiogenic effects of angiostatin and down-regulates EC proliferation and migration (Moser et al. 1999). Angiostatin can also interact with integrin  $\alpha v \beta 3$ , a protein that has been shown to be critical for angiogenesis (Brooks et al. 1998; Tarui et al. 2001).

In 2001, the Holmgren group showed that angiomin, a cell surface protein, binds to angiostatin (Trojanovsky et al. 2001), which is discussed in more detail on page 22.

Thrombospondin-1 (TSP-1) was the first protein to be recognised as a naturally occurring inhibitor of angiogenesis (Good et al. 1990). It regulates cell proliferation, migration and apoptosis in a variety of physiological and pathological settings, such as wound healing, inflammation, angiogenesis and neoplasia (Chen et al. 2000). Overexpression of TSP-1 in mice suppresses wound healing and tumour angiogenesis, demonstrating that TSP-1 is a potent angiogenesis inhibitor (Streit et al. 1999; Rodriguez-Manzaneque et al. 2001). Expression of TSP-1 has also been inversely correlated with malignant progression in breast and lung carcinomas and melanomas (Zabrenetzky et al. 1994).

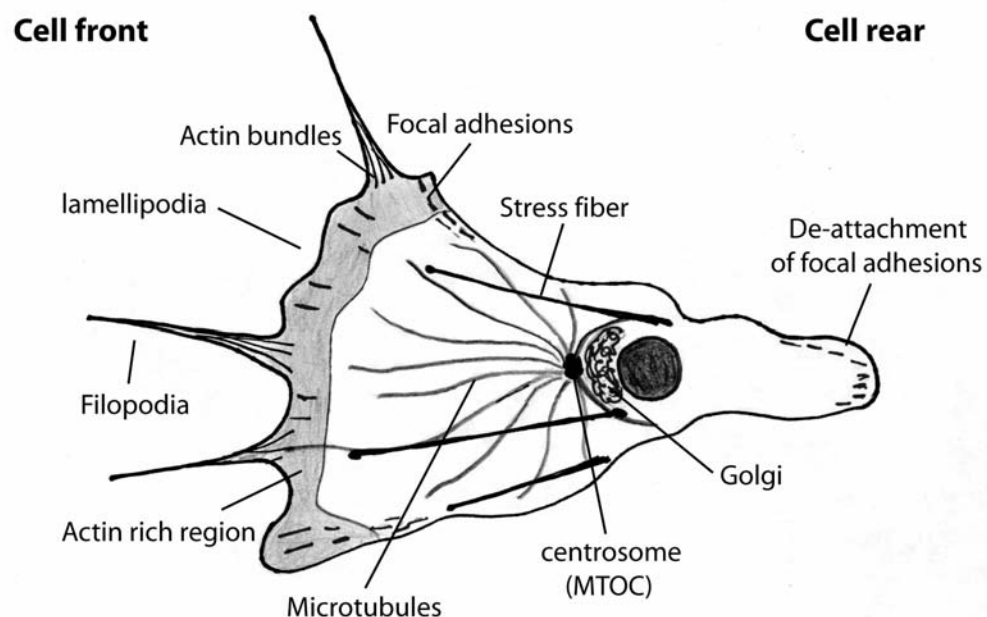
Endostatin is an endogenous angiogenesis inhibitor derived from type XVIII collagen and was identified and purified from a murine hemangioendothelioma cell line (O'Reilly et al. 1997). Recombinant endostatin has been shown to efficiently block angiogenesis and suppress primary tumour growth and metastasis in experimental animal models without any apparent side effects, toxicity or development of drug resistance (Boehm et al. 1997; O'Reilly et al. 1997). Interestingly, it has also been shown that individuals with Down's syndrome have a very low incidence of solid tumours (Yang et al. 2002), which could be a result by the fact that they have significantly higher levels of circulating endostatin, due to the presence of three copies of collagen XVIII on chromosome 21 (Zorick et al. 2001).

## CELLULAR EVENTS DURING ANGIOGENESIS

In order to fully exploit inhibition of angiogenesis as a treatment for cancer, it is critically important to understand the cellular events that occur during this process. Several molecular events are known to be required for angiogenesis, which influence processes including ECM degradation, tube formation, and EC proliferation. Three of these events, namely cell motility, polarisation and cell-cell contact formation, and the proteins involved, i.e. the Rho-GTPases, are of relevance to my studies of angiominin. These events and the proteins involved are therefore discussed below.

### Cell motility

Cell motility (also known as cell migration) is a complex process that is essential for embryonic development and homeostasis. Migration also contributes to tissue repair and immune surveillance in the adult, as well as being involved in several pathological processes such as vascular disease (as described above), osteoporosis, chronic inflammatory disease, and tumour formation. Furthermore, migration occurs during metastasis when some tumour cells migrate out of the primary tumour into the circulation and move to new locations where they form a secondary tumour (Ridley et al. 2003).



*Figure 4. A migrating cell polarises and forms a front and a back. Many different events and proteins are involved in the process of migration, as described in the text. Figure adapted from Etienne-Manneville, 2004.*



Migration can be envisaged as a cyclic process (Figure 4) (Lauffenburger and Horwitz 1996; Ridley et al. 2003). It begins when a cell responds to a migratory promoting agent by polarising and extending a protrusion in the direction of migration. These protrusions can either be large, sheet-like structures called lamellipodia, which contain a very dense actin network and only few microtubules, or spike-like extensions called filopodia, which contain long, parallel actin fibres. The lamellipodia and filopodia always form in the direction of movement with the Golgi apparatus and centrosome positioned in front of the nucleus so that proteins important for motility can be shuttled towards the front of the cell (Etienne-Manneville and Hall 2001). Adhesion complexes are continuously formed and their function is to stabilise the protrusion by attaching it to the ECM. These adhesions serve as traction sites for migration and initiate signals that regulate adhesion dynamics and protrusion formation. Contraction moves the cell body forward and releases the attachments at the back of the cell, as the cell retracts, which completes the cycle (Figure 4).

Described above are the steps observed most distinctly in slow moving cells such as fibroblasts. However, these steps are less obvious in other cell types. For example leukocytes, which are rapidly migrating cells, glide over the substratum by protruding and retracting smoothly without forming obvious attachments.

Actin and myosin are important components of the migratory machinery. Actin-myosin contractility at the front of the cell serves to pull the cell body forward. The release of adhesions at the rear of the cell and the retraction of the tail are mediated by myosin (Etienne-Manneville 2004a).

Rho-GTPases are central to all of the events that occur during cell migration and are therefore described in detail below.

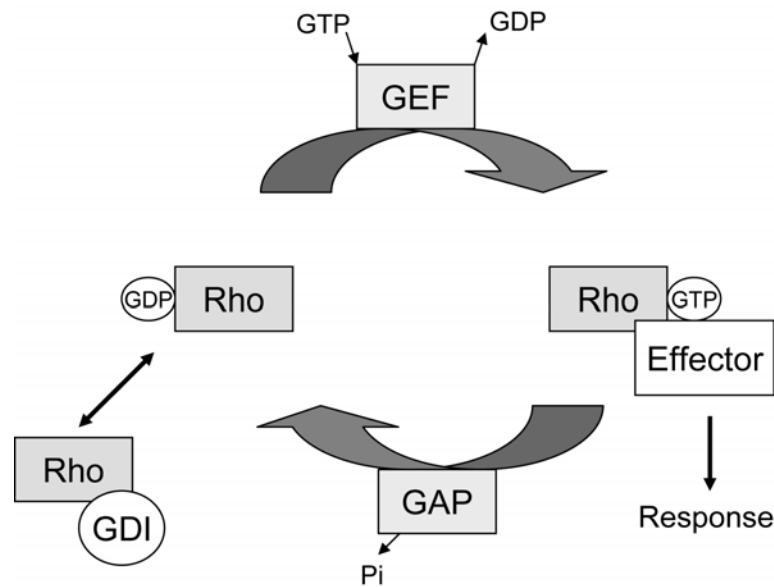
## **Rho-GTPases**

Rho-GTPases are expressed in all eukaryotic organisms and regulate many aspects of cell behaviour, such as cell polarity and motility, through their effects on the cytoskeleton, membrane trafficking and cell adhesion (Jaffe and Hall 2005). Rho-GTPases belong to the Ras family of small GTPases and, to date, 22 human members of the Rho family have been identified. The most studied members of the family are RhoA, Rac1 and Cdc42.

In the early 1990s the group of Alan Hall showed that RhoA, Rac1 and Cdc42 displayed different roles in Swiss 3T3 cells. Using this cellular model system, they demonstrated that RhoA regulates the formation of contractile actin-myosin filaments and focal adhesions in response to a variety of extracellular stimuli (Ridley and Hall 1992). In contrast, Rac1 was demonstrated to induce actin polymerisation at the cell front to produce lamellipodia (Ridley et al. 1992), and Cdc42 was found to promote actin filament assembly and filopodia formation (Kozma et al. 1995; Nobes and Hall 1995).

Rho-GTPases do not only regulate pathways linked to the actin cytoskeleton. They also participate in the regulation of cell polarity, gene transcription, G1 cell cycle progression, microtubule dynamics, and vesicular transport pathways, to name but a few.

Rho-GTPases cycle between an active state when they are bound to GTP and an inactive state when they are bound to GDP (Figure 5). The cycling between GDP and GTP bound states is primarily controlled by two classes of regulatory proteins, i.e. GTPase activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs). GAPs catalyse GTP hydrolysis, converting the Rho proteins to the GDP bound inactive conformation whereas GEFs catalyse the exchange of GDP for GTP. A third set of regulatory proteins, i.e. the guanine nucleotide dissociation inhibitors (GDIs), sequester Rho GTPases in the cytosol in a GDP bound state (Figure 5).



*Figure 5. Rho GTPases exist in an inactive (GDP-bound) or an active (GTP-bound) conformation. In the active state, they interact with effector proteins to induce a cellular response. Figure adapted from Schmidt and Hall 2002.*

More than 70 Rho-GEFs and 80 Rho-GAPs are encoded in the human genome. A remarkable characteristic of Rho-GEFs is that they outnumber their target GTPases by a factor of three, which suggests that multiple GEFs can activate the same GTPase. Furthermore, many GEFs can activate more than one GTPase (Schmidt and Hall 2002; Rossman et al. 2005).

#### *Syx1/GEF720/Tech*

Bioinformatic analysis of all Rho-family GEFs encoded in the human genome shows that 26 out of 70 Rho-GEFs contain a putative PDZ-binding domain in their C-terminus (Garcia-Mata and Burrige 2007). Several interactions between Rho-GEFs and PDZ-domain proteins have been reported recently (Fabre et al. 2000; Dobrosotskaya 2001; Penzes et al. 2001; Park et al. 2003; Radziwill et al. 2003; Audebert et al. 2004; Liu and Horowitz 2006). In the examples studied, the interaction between the Rho-GEF and the PDZ-domain protein played a role in targeting the Rho-GEF to a specific location in the cell. The PDZ proteins that interact with Rho-GEFs are usually multi-domain proteins

that can simultaneously interact with several binding partners, promoting the formation of multiprotein signalling complexes.

One of the Rho-GEFs that contain a PDZ-binding domain is Syx1, which is targeted to the plasma membrane in a synectin-dependent manner (Liu and Horowitz 2006). Syx1 was first reported in 2001 as a brain specific RhoA exchange factor and was called GEF720 (De Toledo et al. 2001). In 2005, the gene was renamed Tech and it was shown to be selectively expressed in cortical and hippocampal neurons (Marx et al. 2005). In this thesis, I will refer to this protein as Syx1. It is important to note that Syx1 is expressed not only in neuronal cells, but in most cell types tested, e.g. epithelial cells and ECs.

The expression of Syx1 in ECs increases migration and tube formation, suggesting that the synectin-dependent targeting of Syx1 is critical to its contribution to these EC functions. A shorter variant of Syx1, named Syx2, which lacks two amino acids in the C-terminal domain and therefore harbours a disrupted PDZ-binding domain, is diffusely distributed in the cytoplasm and its expression does not enhance EC migration or tube formation (Liu and Horowitz 2006).

## **Cell Polarity**

Cell polarisation occurs as a result of the establishment of apico-basolateral cell polarisation in epithelial cells. Another example of polarisation is observed in migrating cells in response to a gradient of signals. One protein in the Rho-GTPase family, namely Cdc42, stands out as playing a central role in establishing cell polarity.

In multicellular organisms, cell polarity is determined primarily by external stimuli. ECM and cell-cell contact receptors such as integrins and cadherins, as well as receptors for soluble ligands, allow individual cells to sense their environment and organise polarity accordingly. This is controlled by Cdc42 and the localised recruitment and activation of Cdc42 is likely to be a key event leading to cell polarisation (Etienne-Manneville and Hall 2002; Etienne-Manneville 2004b).

Polarity is important during morphogenesis. One well-studied example of this is the formation of epithelial sheets, when the development of cell-cell contacts is the first step in the establishment of cell polarity, which generates distinct apical and basolateral surfaces (Rojas et al. 2001). This is initiated by adhesion proteins such as nectin and E-cadherin whose engagement induces Cdc42 activation (Takai et al. 2003).

When cells sense a chemoattractant gradient, they dramatically change their shape, i.e. they polarise in the direction of the gradient. As described above (in the Cell motility chapter), the part of the cell closest to the chemoattractant gradient forms a leading edge that protrudes forward. This response is mediated by F-actin assembly and the process is regulated by Rac and Cdc42, acting through the WASp/SCAR/WAVE proteins that activate the Arp2/3 complex (Etienne-Manneville and Hall 2002; Pollard and Borisy 2003). One pathway important for Cdc42 and Rac activation and recruitment at the leading edge of polarised cells is the PI3K pathway.

Polarised cell migration can also be induced by the release of physical constraints. For example, *in vitro* models of scratch-induced migration have shown that cells polarise in a direction that is at a 90 degree angle to the wound. The actin machinery faces the front, the microtubule system is aligned along the direction of migration, and the centrosome and Golgi are reoriented in the front of the nucleus. Cdc42 is involved in all

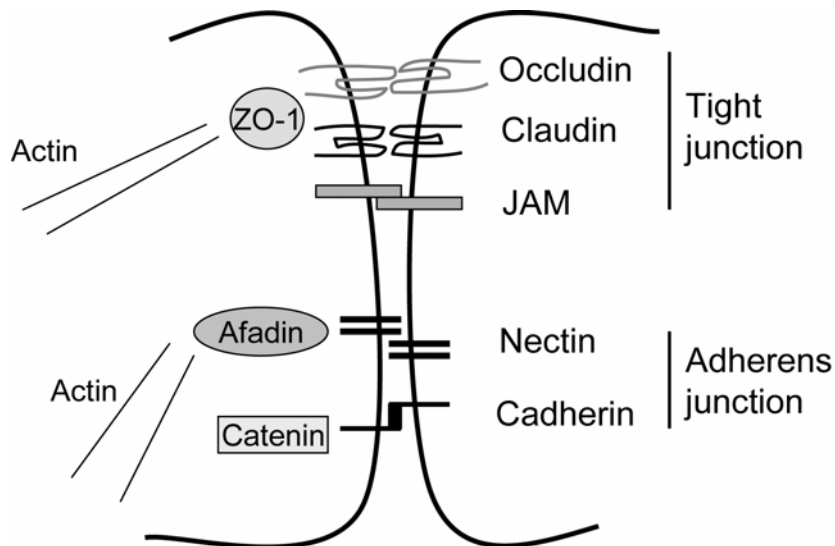
of these polarisation events, and its activity is induced by integrin-matrix interactions at the front of the cell as a consequence of wounding (Nobes and Hall 1999; Etienne-Manneville and Hall 2001; Palazzo et al. 2001).

The Golgi apparatus and microtubule-organising centre (MTOC) are good markers for cell polarisation, because in polarised, migrating cells, secretory traffic and recycling endosomes are directed towards the leading edge. One important step in the redirecting of traffic is the translocation of the MTOC and Golgi apparatus to the anterior side of the nucleus, so that they face the direction of forward movement (Gotlieb et al. 1981; Kupfer et al. 1982). In this way, motile cells can set up a direct line of supply to the front of the cell. The leading edge relies on this constant feeding, and treatments that inhibit traffic from the Golgi to the membrane block cell migration (Prigozhina and Waterman-Storer 2004).

### Cell-cell contacts

Intercellular junctions in epithelial cells are well studied and four distinct kinds of junctions have been observed by electron microscopy, namely tight junction (TJ), adherens junction (AJ), desmosome and gap junction.

In epithelial cells, TJs are concentrated at the apical side of the intercellular cleft whereas AJs form continuous adhesion belts near the apical end of the cell, just below TJs (Figure 6). However, in ECs, TJs are frequently intermingled with AJs all the way along the cleft (Ruffer et al. 2004).



*Figure 6. Composition of TJs and AJs. TJs are composed of transmembrane proteins linked to the actin cytoskeleton via cytoplasmic ZO proteins. AJs are composed of the nectin-afadin system and the cadherin-catenin system. Figure adapted from Miyoshi and Takai 2005.*

TJs mediate adhesion and communication between neighbouring cells. In particular, TJs are responsible for regulating paracellular permeability and maintaining cell polarity. TJs are composed of both transmembrane and intracellular molecules. Three distinct types of transmembrane proteins localise to TJs, namely occluding (Furuse et al. 1993), claudins (Furuse et al. 1998) and junctional adhesion molecules (JAMs) (Martin-Padura et al. 1998) (Figure 6).

Occludin is exclusively expressed at the TJ of epithelial and endothelial cells. It is not essential for the formation of TJs since embryonic stem cells lacking both alleles of the occludin gene still develop a normal network of TJ strands between adjacent epithelial cells of differentiated embryonic bodies (Saitou et al. 1998).

Claudins are detected in both epithelial and endothelial cells in all tissues that bear TJs, and form a complex with occludin and JAMs. More than 20 members of claudins have been identified in humans and some claudins are limited to certain cell types, e.g. claudin-5 expression is restricted to ECs.

JAMs can be found in both TJs and AJs and not only in epithelial and endothelial cells, but also in all lineages of haematopoietic cells (Liu et al. 2000). Endothelial cell selective adhesion molecule (ESAM) is a member of this family that is specifically expressed by ECs (Hirata et al. 2001; Nasdala et al. 2002).

Among the intracellular TJ proteins, ZO-1 is the most studied molecule. The ZO proteins belong to the family of membrane-associated guanylate kinases (MAGUK) and the protein ZO-1 was named because of its localisation at the zonula occludens of several epithelial and endothelial cell types (Stevenson et al. 1986). ZO-1 is associated to the actin cytoskeleton through its C-terminal half and it can also interact with transcription factors and signalling molecules (Bazzoni and Dejana 2004). It is not only expressed in TJs but also in AJs and in cells that do not form TJs at all, such as astrocytes and dermal fibroblasts (Howarth et al. 1992).

AJs are central for cell recognition, aggregation, and formation of tissue architecture. The most important molecular components of AJs are the cadherin-catenin complexes (Figure 6). The cadherin family consists of over 80 members. E-cadherin is primarily expressed in epithelial cells and ECs express a specific cadherin called vascular endothelial (VE)-cadherin, which is essential for development since VE-cadherin deficient mice die at E9.5 due to impaired remodelling and maturation of vascular plexi (Carmeliet et al. 1999). ECs do not only express VE-cadherin, but also express N-cadherin, which is not localised to AJs. Instead, N-cadherin is diffusely distributed on the cell membrane (Navarro et al. 1998). The cytoplasmic tail of VE-cadherin binds to  $\beta$ -catenin and  $\gamma$ -catenin, which link to  $\alpha$ -catenin, which in turn anchors the complex to actin.  $\alpha$ -catenin can also bind  $\alpha$ -actinin (Knudsen et al. 1995) and vinculin (Watabe-Uchida et al. 1998), which may further stabilise AJ anchorage to actin.

The nectin-afadin complex has been described in epithelial cells (Figure 6). The proteins are also present in ECs, although very little is known regarding their role in these cells. The nectin family consists of four members and each member has two or three isoforms generated by alternative splicing (Takai and Nakanishi 2003). Nectin binds to afadin through its cytoplasmic tail, which in turn connects nectin to the actin cytoskeleton (Takahashi et al. 1999).

In Table 2, several of the key proteins involved in cell-cell contacts and polarity are listed. These proteins are of relevance to paper IV.

Table 2. Proteins involved in cell-cell contacts and polarity.

<b>Protein</b>	<b>Number of PDZ-domains</b>	<b>Function</b>	<b>References</b>
Mupp1, Multiple-PDZ-domain protein 1	13	Scaffolding protein that links different protein complexes due to its 13 PDZ domains.	(Ullmer et al. 1998; Hamazaki et al. 2002).
Patj, Protein associated to tight junctions	10	Forms a complex with Pals1, associates to claudin-1 and is recruited to TJ by ZO-3. Involved in TJ formation, cell polarisation and directional migration.	(Lemmers et al. 2002; Roh et al. 2002a; Roh et al. 2002b; Shin et al. 2005; Shin et al. 2007).
Pals1, Protein associated to Lin-7	1	Interacts with Lin-7 and PATJ. Involved in TJ formation and cell polarity.	(Kamberov et al. 2000; Straight et al. 2004).
Lin-7	1	Forms a complex with cadherin and $\beta$ -catenin. Binds PALS1 and PATJ. Involved in TJ formation.	(Borg et al. 1998; Kaech et al. 1998; Perego et al. 2000; Straight et al. 2000; Straight et al. 2006).

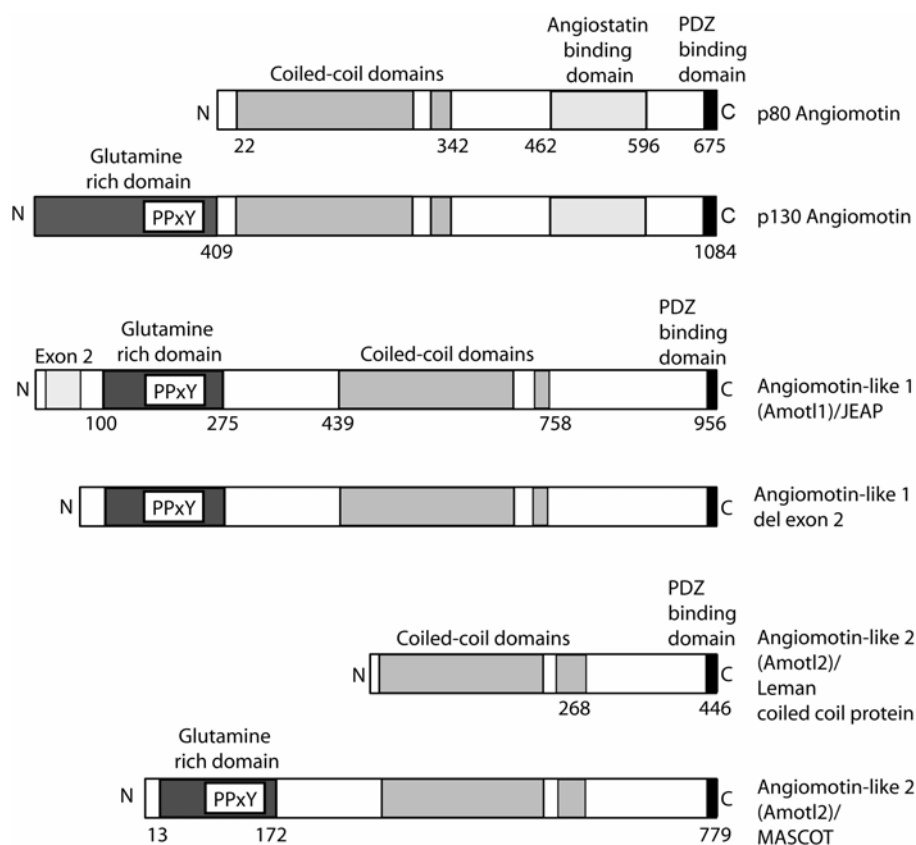
## THE ANGIOMOTIN FAMILY

Angiomotin belongs to the angiomotin family of proteins (Bratt et al. 2002) (Figure 7). The angiomotin family comprises three members, i.e. angiomotin, angiomotin-like 1 (AmotL1), which is also called JEAP (Nishimura et al. 2002), and angiomotin-like 2 (AmotL2), which is also called MASCOT (Patrie 2005).

As we and others have reported, the angiomotin family has alternative splice sites, giving rise to proteins with different isoforms (Moreau et al. 2005; Ernkvist et al. 2006). Today, we know that angiomotin, AmotL1 and AmotL2 are all expressed as two isoforms, one longer and one shorter, and that the functions of the isoforms differ. All the proteins in the angiomotin family of proteins contain a coiled-coil domain and a PDZ-binding domain, and the longer isoforms also contain a glutamine rich domain with no known function (Bratt et al. 2002; Ernkvist et al. 2006).

Studies of AmotL1 and AmotL2 have revealed that AmotL1 localises to TJs where it overlaps with ZO-1 and occludin (Nishimura et al. 2002), and that AmotL2 also localises to TJs where it binds to MAGI-1 (Patrie 2005). It has been reported that AmotL1 and AmotL2 form homo-oligomers, most likely via their coiled-coil domains

(Patrie 2005). However, AmotL1 and AmotL2 do not form hetero-oligomers (Patrie 2005), indicating that the coiled-coil domains have a high degree of specificity. Ongoing work in our lab has confirmed that AmotL1 localises to cell-cell contacts. In contrast, we have not been able to find AmotL2 in cell-cell contacts. Instead, we find AmotL2 in vesicles in the cytoplasm of cells. Recently, it has also been shown that zebrafish AmotL2 is an FGF-responsive gene and that it is essential for cell movement in vertebrate embryos (Huang et al. 2007).



*Figure 7. The angiomotin family of proteins. Illustration kindly provided by Anders Bratt.*

## ANGIOMOTIN

In 2001, the Holmgren group published an article describing the identification of a novel receptor for angiostatin using a yeast-two hybrid screening approach (Trojanovsky et al. 2001). The gene was cloned and named angiomotin (Latin motus = motility) since it induces motility when transfected into ECs (Trojanovsky et al. 2001). This protein is now referred to as p80-angiomotin to distinguish it from the longer isoform p130-angiomotin that was recently discovered (the cloning of p130-angiomotin comprises part of this thesis, i.e. paper I).

Immunohistochemical analysis of human placenta and Kaposi's sarcoma sections showed expression of angiomotin in blood vessels (Trojanovsky et al. 2001) and immunofluorescent staining showed that angiomotin localised to the lamellipodia of migrating cells (Trojanovsky et al. 2001). More recent studies have shown that

angiomin also localises to cell-cell contacts where it overlaps with ZO-1 (Bratt et al. 2005; Wells et al. 2006).

Several studies have revealed that p80-angiomin promotes migration and that this effect is reversed by angiostatin (Trojanovsky et al. 2001; Levchenko et al. 2003; Bratt et al. 2005; Ernkvist et al. 2006). Also, angiostatin inhibits tube formation of p80-angiomin transfected cells in the matrigel formation assay (Trojanovsky et al. 2001; Levchenko et al. 2003), indicating that angiostatin antagonises angiomin function. p80-angiomin has also been shown to stabilise tubes formed on matrigel. Control tubes started to regress after 72 hours whereas p80-angiomin tubes were stable for over 30 days (Levchenko et al. 2004). In vivo injection of MAE-cells infected with p80-angiomin into severe combined immunodeficiency disease (SCID) mice showed that p80-angiomin promoted tumour growth and invasion into surrounding muscle tissue, indicating that p80-angiomin may support angiogenesis by both stimulating invasion as well as stabilising established tubes (Levchenko et al. 2004).

Investigation of the domain structure of p80-angiomin showed that p80-angiomin contains a C-terminal PDZ-binding domain (amino acids EYLI), and a putative coiled-coil domain (Bratt et al. 2002). About two years ago, a model of the topology of p80-angiomin was published that suggested that the angiostatin binding domain is localised on the cell surface whereas the C-terminal PDZ-binding domain and the coiled-coil region are intracellular (Bratt et al. 2005).

How angiomin promotes cell motility remains unknown, but both in vivo and in vitro experiments have shown that the C-terminal domain of p80-angiomin plays an important role. Cells expressing p80-angiomin with a deletion of the last three amino acids in the C-terminal PDZ-binding domain exhibit defective migration and tube formation (Levchenko et al. 2003). Transgenic mice expressing this mutant form of p80-angiomin under the EC specific TIE promoter die around E9.5 due to severe bleeding in the brain as well as from intersomitic vessels (Levchenko et al. 2003). Whole-mount immunostaining using antibodies against the EC marker PECAM1 showed that the vessels in the transgenic embryos do not align properly and fewer capillaries than in the control embryos were observed in the neuroepithelium (Levchenko et al. 2003).

A clinical study comparing breast cancer tissues with normal mammary tissue showed that angiomin is expressed at significantly higher levels in the breast cancer tissues (Jiang et al. 2006). The levels of angiomin were also expressed at significantly higher levels in Bloom-Richardson grade 2 and grade 3, and metastatic disease, compared to grade 1 (Jiang et al. 2006), indicating that angiomin could be a potential therapeutic target.

So far, two studies have been published using two different approaches to inhibit angiomin. In the first study, angiomin was targeted by DNA vaccination (Holmgren et al. 2006). Mice were vaccinated with cDNA encoding p80-angiomin which resulted in a blockage of angiogenesis in the matrigel plug assay and a prevention of growth of transplanted tumours for up to 150 days. Furthermore, a combination of DNA vaccines encoding p80-angiomin and Her-2 (a tyrosine kinase receptor overexpressed in 25-30% of breast cancer patients), inhibited breast cancer progression and impaired tumour vascularisation in Her-2/neu transgenic mice (Holmgren et al. 2006). In the second study, antibodies directed towards the extracellular domain of angiomin were generated (Levchenko et al. 2007). These antibodies reduced the number of endothelial filopodia and inhibited vessel migration during retinal



angiogenesis *in vivo*. In addition, matrigel plugs containing the antibodies showed an almost complete inhibition of angiogenesis compared to control plugs (Levchenko et al. 2007). These studies show that targeting angiomin may be a promising approach to inhibit pathological angiogenesis.

Shimono and Behringer have generated angiomin deficient mice that lack both p80- and p130-angiomin (Shimono and Behringer 2003). The disruption of angiomin in mice resulted in complete elimination of angiomin expression, but the penetrance of the angiomin mutant phenotype is sensitive to genetic background (Shimono and Behringer 2003). Embryos from crosses of mice with a 129/SvEv-background exhibited morphological defects and death of approximately 70% of the angiomin deficient embryos around E7.5. However, backcrossing once into the C57/B6 (129B6 mixed) background resulted in approximately 50% dead embryos at the same time point (E7.5) (Shimono and Behringer 2003). The angiomin knock-out embryos died due to a migratory defect in the anterior visceral endoderm (Shimono and Behringer 2003). However, as shown in this thesis, we have backcrossed the mice further into the C57/B6 background (up to six backcrosses) and in contrast to the previous findings, we discovered that angiomin deficient embryos survived at a near Mendelian ratio after E7.5. Nonetheless, the number of live angiomin deficient pups was only approximately 25% of the expected ratio, indicating that a majority die *in utero*. We investigated the embryos further, and found that they showed vascular defects, as will be discussed in more detail in paper II.

In an attempt to identify modulators of Cdc42, Wells and co-workers identified Rich1 as a Cdc42 GAP (Wells et al. 2006). Immunoprecipitation analysis showed that angiomin associates with Rich1 through its coiled-coil domain, and that it also interacts with Pals1 and Patj. Wells et al also demonstrated that angiomin associates with proteins that were not detected in the Rich1 complexes, including Mupp1, AmotL1 and AmotL2, and proposed that Rich1 and angiomin maintain epithelial polarity by the coordinated regulation of Cdc42 and trafficking of specific polarity proteins at TJs (Wells et al. 2006).

The observation that angiomin associates with Mupp1 and Patj was verified by Sugihara-Mizuno et al., who further demonstrated that the PDZ-binding domain of angiomin is an essential requirement for these interactions (Sugihara-Mizuno et al. 2007).

# PRESENT INVESTIGATION

## AIM OF THE THESIS

New anti-angiogenic drugs and other pharmaceuticals that target specific molecules have succeeded in clinical trials recently. This indicates that we are entering a new era where cancer patients can be treated with more personalised drugs for their specific disease and/or new combinations of therapeutic agents. However, the anti-angiogenic therapies are still not able to stabilise the disease entirely. Therefore, novel targets for treating cancer are urgently required.

The overall aim of this thesis was to characterise the function of angiomin during blood vessel formation and to investigate the molecular function of angiomin. This work is critical to enable validation of angiomin as a candidate for anti-angiogenic therapy.

The specific aims were:

- To clone and investigate the function of a new splice isoform of angiomin (i.e. p130-angiomin) (Paper I).
- To investigate the role of angiomin during embryonic blood vessel formation (Paper II).
- To assess the different roles of the two angiomin isoforms during blood vessel formation (Paper III).
- To identify binding proteins to the PDZ-binding domain of angiomin (Paper IV).

## RESULTS AND DISCUSSION

### **Paper I. Identification of p130-angiomin, a new splice isoform of angiomin**

The aim of this study was to verify the function of a new splice isoform of angiomin, the p130-angiomin.

When angiomin was first identified by yeast-two hybrid screening in 2001, it was the 80 kDa form of angiomin that was cloned (Trojanovsky et al. 2001) and the data in the following publications up until 2005 were based only on p80-angiomin (Bratt et al. 2002; Levchenko et al. 2003; Levchenko et al. 2004). Nevertheless, the first article published that described angiomin identified two bands of different sizes by northern

blot analysis when the expression of angiominin in foetal and adult human tissues was investigated (Trojanovsky et al. 2001), indicating that an alternative transcript for p80-angiominin existed.

In 2005, the group of Fernandes published a study that indicated that alternative splicing occurred within the angiominin protein family (Moreau et al. 2005). Shortly after, we published an article describing the identification of a novel isoform of angiominin, i.e. p130-angiominin (Ernkvist et al. 2006).

We know that Angiominin, AmotL1 and AmotL2 are expressed both as short and long isoforms, but cannot exclude the possibility that more alternative isoforms of the angiominin protein family exist. When using angiominin antibodies, we sometimes detect a third band on Western blot analysis with a size around 100 kDa, indicating that a third isoform of angiominin may exist.

In this study, we found that the p130-angiominin splice isoform was identical to p80-angiominin with the exception that it contains an extended N-terminal domain with a glutamine rich domain of unknown function.

Although the two angiominin isoforms contain identical domains, the specific N-terminal domain of p130-angiominin makes the localisation and function of p130-angiominin different from p80-angiominin. As described above, in the chapter on angiominin, p80-angiominin localises to the leading edge of the lamellipodia and induces migration of ECs. p130-angiominin, on the other hand, co-localises with actin in a punctuated pattern, inhibits migration of ECs, increases the amount of actin fibres and enlarges the size of the cell. Also, p130-angiominin did not appear to respond to angiostatin treatment since angiostatin did not affect cell shape or actin organization in p130-angiominin expressing cells. This indicates that the signalling pathway from the extracellular angiostatin domain differs between the two isoforms.

An interesting difference between the full length p130-angiominin and the specific N-terminal domain of p130-angiominin is that the full length protein overlaps with actin only in the central part of the cell and in a punctuated pattern. In contrast, the N-terminal domain overlaps with actin all over the cell in a fibre-like pattern. The conclusion we can draw from this observation is that the N-terminal domain acts as the actin targeting domain (which was also confirmed by the cytochalasin B experiments) whereas the domain identical to p80-angiominin binds to one or several so far unknown proteins that induce the punctuated pattern. We have tested several different proteins that are found in the cell in similar patterns to see if they overlap with p130-angiominin, such as  $\alpha$ -actinin, tensin (fibrillar adhesions) and paxillin (focal adhesions), but none co-localised with p130-angiominin. We cannot draw the conclusion that the N-terminal domain of p130-angiominin binds directly to actin, since the link to actin could be via another protein. Therefore, it would be interesting to identify the proteins that bind to the N-terminal domain of p130-angiominin since this domain is the only one that discriminates p130-angiominin from p80-angiominin. At the time we published this study describing p130-angiominin, we had already shown that p130-angiominin also localises to cell-cell contacts and that the N-terminal domain associates with MAGI-1B, a TJ protein (Bratt et al. 2005). However, I believe that there must be more N-terminal specific proteins than just MAGI-1B. The finding that p130-angiominin also localises to TJs indicates that p130-angiominin localises to different areas in the cell. However, we do not know what determines this localisation. Is it cell confluence or external or internal stimuli? It would also be interesting to verify what

kinds of signals induce actin fibre formation in the p130-angiotensin expressing cells. Can it be explained by RhoA activation?

It is obvious that the two isoforms of angiotensin have different functions, but what are their individual roles during angiogenesis? Since we published paper I, we have learned more regarding p130-angiotensin, and some of the new data about the individual roles of the two isoforms during angiogenesis will be presented and discussed in paper III.

Conclusions from paper I:

- p130-angiotensin is a splice isoform of angiotensin that contains a cytoplasmic N-terminal extension.
- p130-angiotensin localises to actin fibres in a punctuated pattern and induces actin fibre formation and changes cell shape.
- In contrast to p80-angiotensin, p130-angiotensin does not promote cell migration and does not respond to angiostatin.

## **Paper II. Angiotensin is crucial for blood vessel formation during zebrafish and mouse embryogenesis.**

The overall aim of this paper was to study the role of angiotensin during physiological angiogenesis and to investigate its function at a cellular level, thereby providing an understanding of the role of angiotensin during angiogenesis.

To do that, we used three *in vivo* models (i.e. zebrafish, knock-out mice and the choroidal neovascularisation (CNV) model) and several *in vitro* assays.

In 2003, Shimono and Behringer showed that the disruption of the angiotensin gene in mice with a 129/SvEv background was lethal in 70% of the embryos around E7.5 due to morphological defects. At E7.5, vasculogenesis and angiogenesis have not started, indicating that we could not analyse the role of angiotensin during angiogenesis. However, when the mice were backcrossed into a C57/B6 background (129B6 mixed), only 50% of the embryos died at E7.5. We therefore decided to further backcross the same knock-out mice into the C57/B6 background to investigate whether the early lethality was dependent on the mouse strain background.

We found that 80% of the embryos six backcrosses into a C57/B6 background died between E11 and E11.5. Our data support a role for angiotensin in the vascular reorganisation during blood vessel formation since the cause of death was most likely due to the vascular defects found in the yolk sac, brain, and the capillary network in the intersomitic vessels. However, no haemorrhages were found, all the large blood vessels, such as the aorta, were formed, and the blood vessels were functional since nucleated blood cells were found in all vessels. But still, the embryos died. Why? We speculate that they might die due to a lethal change in blood pressure since several vessels exhibited increased diameter and also lagoon-like structures. Also, we cannot exclude the possibility that the embryos displayed defects other than those affecting the

vasculature since we only analysed the blood vessel phenotype and the placenta, and only observed the general appearance of the embryos.

Interestingly, we could not detect any obvious defects in the few surviving angiomin deficient mice. Therefore, it would be of interest to analyse the surviving adult mice, especially to investigate if pathological angiogenesis is affected. Angiomin most likely plays a role during pathological angiogenesis since angiomin vaccination (Holmgren et al. 2006) and therapeutic antibodies targeted against angiomin (Levchenko et al. 2007) show promising results.

Knockdown of angiomin in zebrafish results in a vascular phenotype similar to that observed in knock-out embryos, confirming the blood vessel defects found in the angiomin deficient embryos. The phenotype in the zebrafish indicates that the vessels display polarisation and migration defects. The vascular phenotype in angiomin knock-down zebrafish could be rescued by angiomin mRNA, but also by AmotL1 mRNA, indicating that the signalling pathway of angiomin and AmotL1 overlaps (which is also shown in paper IV). Therefore, AmotL1 may rescue the phenotype of some of the angiomin deficient mice, resulting in surviving pups.

In the embryoid body assay, it was apparent that angiomin deficient ECs displayed migratory defects. The number of ECs was the same in the wild-type and knock-out bodies, indicating that angiomin does not affect differentiation of ES cells into ECs. Also, the angiomin deficient ECs were able to proliferate and form small tubes, although their migration towards VEGF was impaired, indicating that they were not able to respond to external growth factor signals. A similar result was also obtained in the CNV model. We can therefore draw the conclusion that angiomin is important for EC migration.

To try to understand the vascular phenotype that angiomin deficiency gives rise to in the mouse and zebrafish, and the migratory defects displayed by the embryoid body assay and the CNV model, we immortalized ECs from the embryoid bodies by infecting them with the Polyoma middle T (PmT) virus and analysed them in several different assays. From these experiments we could conclude that angiomin is critical for the organisation of actin and focal adhesions, for growth-factor mediated migration, for polarisation, and for regulating Rac activity. Angiomin deficient ECs extend multiple protrusions instead of a broad lamellipodia and contained an increased number of focal adhesions that were smaller and shorter than their wild-type counterparts. These results indicate that angiomin is important for cellular structure.

The migrational defects displayed by the angiomin deficient PmT-ECs were also displayed by bovine capillary endothelial (BCE) cells transfected with angiomin siRNA.

We and others have shown that angiomin interacts with TJ proteins such as MAGI-1B and Mupp1 but is not essential for TJ formation (Bratt et al. 2005; Wells et al. 2006). This is consistent with the results presented here. Both wild-type and knock-out PmT-ECs form TJs. Furthermore, no haemorrhages were observed in the angiomin deficient embryos, indicating that TJs are also formed efficiently *in vivo*.

Recent findings have shown that angiomin functions as a scaffolding protein for polarity proteins as well as the GAP protein Rich-1 (Wells et al. 2006). Here we have shown that Rich-1 is expressed in ECs where it localises to the leading edge of migrating cells. However, Rich-1 failed to localise to lamellipodia in the PmT-ECs lacking angiomin. We therefore speculate that Rich-1 is not able to turn off the activity of the GTPase, which it normally tightly regulates, leading to hyperactive cells

with several extensions. The local deregulation of GTPase activity could also explain the polarity defects, displayed by the distribution of the Golgi apparatus. Given that angiotensin is involved in regulating polarity, it is tempting to hypothesise that the disorganised blood vessel network found in the angiotensin knock-out embryos is due to polarity defects.

In the absence of external activation the total activity of Rac determine whether a cell displays directional or random migration. Low levels of Rac leads to directional persistent migration, whereas higher levels promote the formation of peripheral lamellae that mediate random, exploratory migration (Pankov et al. 2005). Maybe the increased Rac-1 activity not only caused the excess of cell protrusions, but also switched the cells to a lasting exploratory mode, insensitive to the guiding gradient of growth factors?

Together, the morphological and migrational defects exhibited in the angiotensin deficient ECs are consistent with the vascular defects. Our finding that angiotensin is crucial for vascular formation suggest that targeting angiotensin would affect migration but not EC proliferation or differentiation.

Conclusions from paper II:

- Angiotensin is crucial for physiological blood vessel formation in mouse and zebrafish embryos.
- Angiotensin deficient cells lack the chemotactic response to VEGF.
- Angiotensin controls Rac1 activity and is important for endothelial polarisation during migration.

### **Paper III. The two angiotensin isoforms have different functions during blood vessel formation.**

Paper I clearly showed that the two angiotensin isoforms exert different cellular functions. The aim of Paper III was therefore to investigate the differences between the two angiotensin isoforms comprehensively and to examine if they exhibit diverse functions during angiogenesis.

We used retinal angiogenesis in neonatal mice as a model to investigate the spatiotemporal expression of the two angiotensin isoforms during distinct phases of neovascularisation. Interestingly, only p80-angiotensin was expressed between P3 and P5, the migratory phase when ECs migrate towards the periphery of the retina. In contrast, p130-angiotensin was expressed from P7 and onwards, when ECs stabilise and mature. p80-angiotensin has been shown to induce migration (Trojanovsky et al. 2001; Levchenko et al. 2003) whereas p130-angiotensin reduces migration and stabilises actin fibres (paper I). Our retina data support the hypothesis that p80-angiotensin and p130-angiotensin are involved in different stages during angiogenesis. When working on paper I, we discovered that MAE-cells stably infected with p130-angiotensin also express small amounts of p80-angiotensin. We took advantage of that

finding and generated several different MAE cell clones expressing p130-angiomin with various amounts of p80-angiomin that we analysed in the Boyden chamber assay. To our surprise, only 10% of p80-angiomin expression relative p130-angiomin was enough to induce migration two fold compared to control vector cells. This result therefore indicates that a small amount of p80-angiomin is sufficient to dominate EC function and to induce a migratory phenotype.

When different constructs of angiomin were transfected into Madin-Darby Canine Kidney (MDCK) cells, we observed that the localisation of the isoforms differed. Also, the morphology of the cells correlated with their proposed functions. The amount of migratory cells were 30% higher in p80-angiomin expressing cells compared to p130-angiomin expressing cells, demonstrating that p80-angiomin induces a migratory phenotype, even in confluent, stabilised and polarised cells like MDCK cells.

The calcium switch experiments, where formation of cell-cell contacts is analysed, showed that p80-angiomin is dependent on its PDZ-binding domain. However, p130-angiomin localised to cell-cell contacts even when lacking its PDZ-binding domain, indicating that its N-terminal domain binds to one or several TJ proteins. Therefore, we speculate that the two isoforms may associate to different protein complexes even though they are localised to the same compartments in the cell. p80-angiomin localises to the proteins, which will be discussed in paper IV, via its PDZ-binding domain, whereas p130-angiomin most likely also binds to several other proteins through its N-terminal domain.

It has been shown AmotL1 forms homo-oligomers through its coiled-coil domain (Patrie 2005). We therefore speculated that the switch from a migratory phenotype to a stabilised phenotype in newly formed blood vessels could be regulated by homo-oligomerisation and hetero-oligomerisation between the isoforms. The results showed that p80-angiomin forms homo-oligomers and hetero-oligomers with p130-angiomin whereas no homo-oligomerisation of p130-angiomin could be detected. This indicates that the homo-oligomerisation of p80-angiomin favours EC migration and that the switch from migration to stabilisation of ECs could be mediated by the hetero-oligomerisation of the two isoforms.

Interestingly, when co-transfecting p80-angiomin and p130-angiomin, p130-angiomin disappeared from TJs and it was instead found within the cytoplasm. This result shows that p80-angiomin displaces p130-angiomin and indicates that p80-angiomin function is dominant over p130-angiomin. This data correlates well with the Boyden chamber data where the migratory function of p80-angiomin is also dominant.

In summary, these data implies that two isoforms of the same protein play different roles during blood vessel formation. Very little is understood about the mechanisms underlying the switch from migration to stabilised cells or vice versa, but here we present evidence that hetero-oligomerisation between two isoforms of the same protein could play an important role in regulating such a migratory switch.

Conclusions from paper III:

- During retinal angiogenesis, p80-angiomin is expressed during the migratory phase whereas p130-angiomin is expressed during the period of blood vessel stabilisation and maturation.

- The N-terminal domain of p130-angiomin serves as the dominant targeting domain responsible for the localisation of p130-angiomin to TJ and actin.
- The expression levels of p80-angiomin and p130-angiomin regulate a switch between a migratory and non-migratory cell phenotype. We consider that homo-oligomerisation and hetero-oligomerisation are responsible for this regulation.

#### **Paper IV. The PDZ-binding domain of angiomin binds to several TJ and polarity proteins and to a Rho-GEF**

The aim of this paper was to identify proteins that bind to the PDZ-binding domain of angiomin.

The PDZ-binding domain plays a critical role for angiomin function since cells expressing a C-terminal mutant form lacking the three last amino acids of the PDZ-binding domain display migratory defects (Levchenko et al. 2003). All the members of the angiomin family contain a C-terminal PDZ-binding domain, indicating that they might bind to similar proteins through that domain (Bratt et al. 2002; Nishimura et al. 2002; Patrie 2005). Therefore, we used the same strategy to identify PDZ-binding proteins for all the proteins within the angiomin family.

We found that angiomin, AmotL1, and AmotL2 bind to TJ and polarity proteins, indicating that they associate with similar protein complexes. During the preparation of this manuscript, two articles were published showing the same link between the angiomin protein family and TJ and polarity proteins (Wells et al. 2006; Sugihara-Mizuno et al. 2007), thereby confirming our data. In addition to the polarity proteins, we also identified a Rho-GEF protein named Syx1 that associated with all the proteins in the angiomin family. The link between Syx1 and angiomin has not been published by others, so we decided to focus our studies on Syx1.

Syx1 and angiomin co-localises in lamellipodia, but the localisation of Syx1 was not dependent on angiomin compared to Rich-1, which required angiomin for its correct localisation (Paper II). The reason could be that Syx1 contains a PDZ-binding domain and not a PDZ domain (Liu and Horowitz 2006), implying that the association between angiomin and Syx1 is mediated by another protein. A pull-down assay revealed that the PDZ-binding domain of Syx1 associates with Mupp1, Pals1 and Lin7, indicating that one of them connects Syx1 and angiomin. Since angiomin binds directly to Mupp1 which contains multiple PDZ domains (Hamazaki et al. 2002), it is very tempting to speculate that Mupp1 serves as the link. Another explanation for the localisation of Syx1 in angiomin deficient cells could be that AmotL1 or AmotL2 target Syx1 to the correct cellular location since AmotL1 and AmotL2 also associate with Syx1.

We consider that angiomin plays an important role in regulating directional migration by acting as an adaptor protein for GTPase regulating proteins (e.g. Syx1 and Rich-1) and polarity proteins. The FRET analysis of PmT-ECs supports this hypothesis since angiomin deficient cells display active RhoA all around the cell in an unpolarised pattern whereas the wild-type cells show a polarised pattern where RhoA exhibits high



activity only in the lamellipodia. The unpolarised phenotype displayed by angiomin deficient cells could be due to overactive RhoA and absent Rich-1 in the lamellipodia. It is interesting to note that according to previous published results, Syx1 is a GEF for RhoA (Marx et al. 2005; Liu and Horowitz 2006) whereas Rich-1 is a GAP for Cdc42 (Richnau and Aspenstrom 2001; Wells et al. 2006) and Rac1 (Richnau and Aspenstrom 2001), indicating that Syx1 and Rich-1 do not regulate the same Rho-GTPases. However, these activity studies are usually performed on isolated recombinant proteins in test tubes and may not always reflect the complex situation in a cell. Therefore, it is possible that Syx1 and Rich-1 also regulate other Rho-GTPases, and maybe even the same GTPases.

The zebrafish results presented in this paper showing knock-down of Syx1 are still very preliminary. The Syx1 knock-out embryos showed an impaired migration of the intersegmental vessels, similar to the phenotype exhibited by angiomin knock-down zebrafish (paper II), indicating that Syx1 and angiomin are involved in the same signalling pathway. However, further studies are required to elucidate this phenomenon.

The conclusions that we can draw from this study are that angiomin binds to a complex of polarity proteins and to Syx1. Angiomin probably acts as a scaffolding protein in this complex since deletion of angiomin induces a severe phenotype, both in vivo and in vitro. The results also indicate that Syx1 might be a potential therapeutic target for anti-angiogenic therapy.

Conclusions from paper IV:

- The angiomin protein family binds to a protein complex containing polarity proteins and Syx1 via their PDZ-binding domains.
- Syx1 could be the candidate protein explaining why the PDZ-binding domain is crucial for the migratory function of angiomin.
- Knock-down of Syx1 in zebrafish impairs the migration of intersegmental vessels, resulting in a similar phenotype to that of angiomin knock-down in zebrafish.

## **CONCLUDING REMARKS AND FUTURE PERSPECTIVES**

This thesis represents the most comprehensive investigation to date of the role of angiomin during angiogenesis. When I started this work almost five years ago, it was known that angiomin belonged to a new protein family, that angiostatin binds to angiomin on the surface of ECs and that the PDZ-binding domain of angiomin plays an important role during cell migration (Trojanovsky et al. 2001; Bratt et al. 2002; Levchenko et al. 2003; Levchenko et al. 2004). Today, we know much more about angiomin and its function, partly thanks to the work that I have presented in this thesis and partly thanks to work performed by other members of the Holmgren group and by international groups that have contributed with their knowledge.

This thesis provides evidence that angiominin is essential for blood vessel development during embryogenesis, that the two isoforms of angiominin exhibit distinct functions during angiogenesis, and that angiominin acts together with several proteins required for its function via interactions with its PDZ binding domain. However, more work is required to achieve a broader understanding of angiominin and the angiominin protein family.

In future studies, a logical continuation of paper II would be to knock-out AmotL1 and AmotL2 in the mouse and zebrafish. Currently, we know very little about the involvement of AmotL1 and AmotL2 in angiogenesis, therefore *in vivo* studies are critical to help us understand their role. Furthermore, analysis of adult angiominin deficient mice to examine if they display any defects with respect to pathological angiogenesis would be of interest, especially since angiominin vaccination data and antibodies towards angiominin have shown promising results (Holmgren et al. 2006; Levchenko et al. 2007).

Further study of AmotL1 and AmotL2 may reveal what this novel and exciting family of proteins have in common and what differentiates them. It will also be important to determine if more isoforms of these proteins exist and, if so, characterise their functions.

Another interesting question that requires answering is what are the differences between p80- and p130-angiominin? Even though a large part of this thesis is devoted to exploring this question, more work is required to thoroughly characterise these proteins. For instance, what kind of proteins does the N-terminal domain of p130-angiominin associate with? So far, we have only investigated the proteins that bind to the PDZ-binding domain of angiominin, but there are other interesting domains that could bind to several other proteins. Does the coiled-coil domain interact with proteins other than Rich-1 (Wells et al. 2006) or AmotL1 (paper IV)? And could proteins other than angiostatin bind to the extracellular domain of angiominin, thereby regulating its function? Also, is angiominin involved in regulating transcription of specific genes?

The finding that the PDZ-binding domain associates with Syx1 is interesting. However, we still do not know how angiominin regulates the function of Syx1. Further study of Syx1 will help us to determine how angiominin induces migration of ECs. In addition, the possibility of targeting Syx1 as an anti-angiogenic therapy for cancer should be investigated.

Very little is known about the processes regulating polarity in ECs. Polarity is likely to be of crucial importance for angiogenesis since it is required for migration, tube formation and lumen formation. Therefore, a deeper understanding of the proteins and the signalling pathways involved in controlling polarity will contribute to our knowledge of angiogenesis in general.

In conclusions, this study partly elucidates several of the mechanisms critical for physiological and pathological angiogenesis. This work of course contributes to only a small part of knowledge, but the more pieces that are collected, the more we will understand about how cancer arises, which in turn may facilitate the development of new rational drugs.

## POPULÄRVETENSKAPLIG SAMMANFATTNING

De flesta organismer är beroende av syre för att överleva. För att alla våra celler ska få tillgång till syre och näringsprodukter så har vårt hjärt- och kärlsystem utvecklats. Syret vi andas in levereras till våra organ och celler via blodet som sedan även transporterar bort avfallsprodukter. En viktig celltyp för mina studier är endotelcellerna som täcker insidan av blodkärlen.

Nya blodkärl bildas främst under fosterutvecklingen men också hos vuxna människor vid sårhäkning och menstruation. Vid många sjukdomar bildas det också nya blodkärl vilket kan ha en negativ effekt på sjukdomsförloppet. Ett exempel på en sådan sjukdom är cancer. Nya blodkärl som växer in till en tumör förser den med syre och näring vilket gör att tumören kan växa och bli större. Via blodkärlen kan även tumörcellerna sprida sig till andra organ och där bilda nya tumörer, så kallade metastaser. Att störa eller helt hämma ny blodkärlsbildning är därför ett sätt att behandla cancer på. I andra fall, som t.ex. efter en hjärtinfarkt, är det en brist på funktionella blodkärl och där finns då ett behov av att nya blodkärl bildas. Om man skulle kunna stimulera nya blodkärl till att växa in i den skadade vävnaden så skulle det innebära ett sätt att behandla sjukdomen.

I den här avhandlingen har vi undersökt hur ett protein som heter angiomin påverkar blodkärlsbildning. Vi har visat att det finns två former av detta protein som har helt olika funktioner. Den ena formen som kallas p80-angiomin, stimulerar endotelceller att röra på sig och bilda nya kärl medan den andra formen av proteinet som kallas p130-angiomin, stabiliserar endotelcellerna och hindrar dem från att vandra iväg. Vi har också tagit reda på vilka andra proteiner som angiomin interagerar med eftersom den informationen hjälper oss att förstå hur angiomin påverkar endotelcellerna. Det visade sig att angiomin binder till flera proteiner som har stor betydelse för en cells polaritet, dvs att en cell får en framsida och en baksida (eller svans). Om en cell inte kan bli polariserad så kan den inte heller röra sig i en rak riktning utan snurrar bara runt. Det visade sig att endotelceller som saknar angiomin inte kunde polarisera sig. Därför kunde vi dra slutsatsen att angiomin är viktig för denna mekanism.

I avhandlingsarbetet har vi också använt oss av så kallad knock-out teknik (ett sätt att ta bort gener på) i mus och zebrafisk. Vi har knockat ut angiomin och sedan undersökt om det påverkade blodkärlsbildningen i embryon från möss och zebrafiskar. Dessa studier visade att angiomin spelar stor roll för blodkärlen eftersom de var missformade i de möss och zebrafiskar där angiomin genen saknades.

Sammfattningsvis så har arbetet i den här avhandlingen lärt oss mer om hur nya blodkärl bildas. Eftersom angiomin har visat sig vara så viktig vid denna process så skulle ett läkemedel riktat mot angiomin kunna fungera som behandling av sjukdomar där man behöver hämma nya blodkärl från att växa, t.ex. cancer.

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