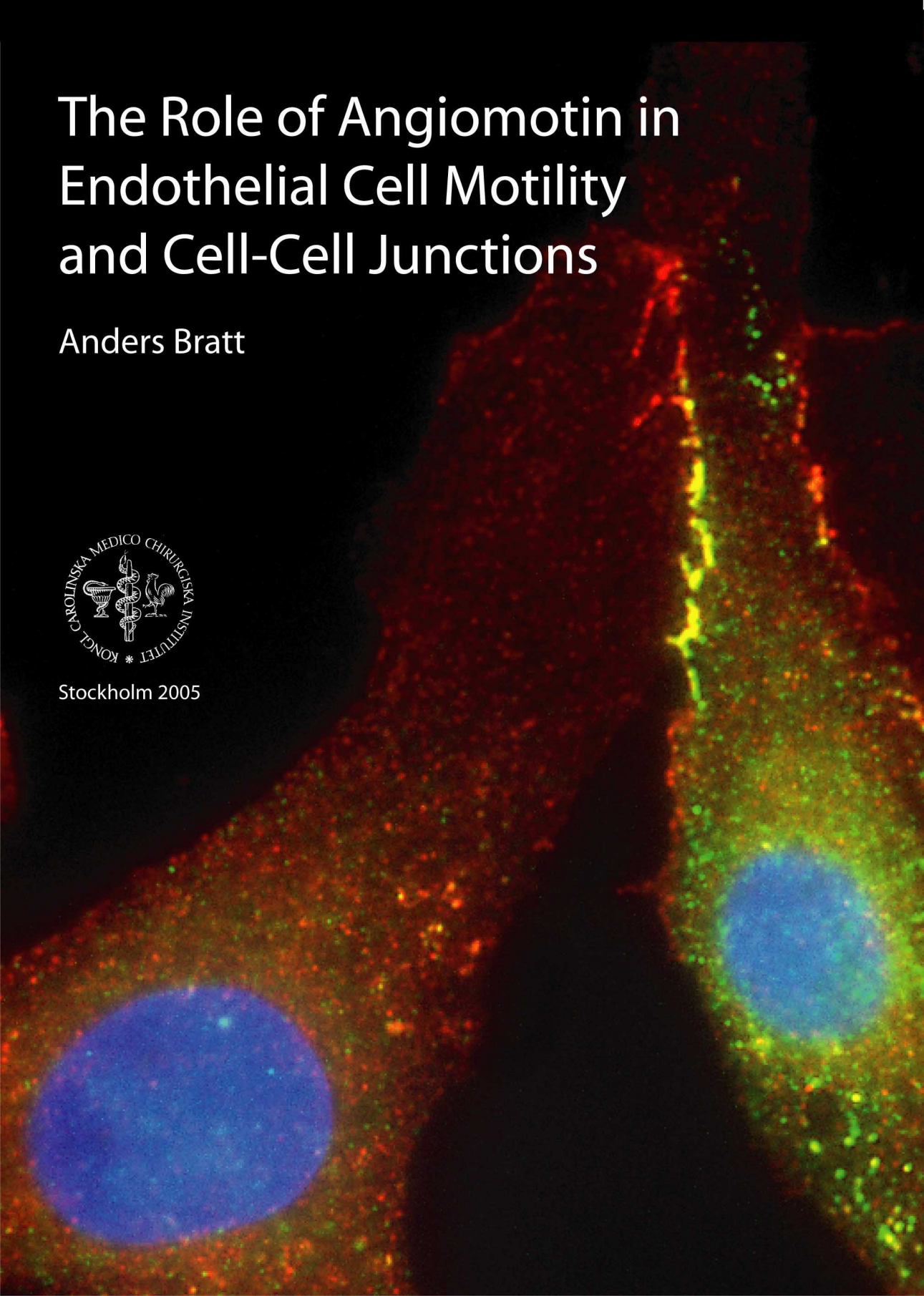
The Role of Angiomotin in Endothelial Cell Motility and Cell-Cell Junctions

Anders Bratt



Stockholm 2005

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Cover design by Peder Fredricson. Photo by Anders Bratt
Front: bovine capillary endothelial cells forming a cell-cell junction. Green: angiomotin, red: ZO-1, blue: DAPI.
Back: A mouse aortic endothelial cell that is forming lamellipodia and spreading. Green: paxillin, red: f-actin.

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ABSTRACT

Clinical as well as pre-clinical data show that tumor growth can be restrained by inhibiting angiogenesis. We have been investigating the mechanism of action of angiostatin, an inhibitor of angiogenesis, in order to design novel therapies against cancer. In line with this we have previously identified angiomotin as an angiostatin-binding protein that controls endothelial migration and tube formation. Angiomotin mediates the effect of angiostatin *in vitro*.

In this study, we first show that angiomotin belongs to a novel protein family with two additional members in human; angiomotin like 1 and angiomotin like 2. The protein family is characterized by a highly conserved coiled-coil motif, a C-terminal putative PDZ binding motif and an N-terminal glutamine rich domain. Database searches show that family members are present in the genomes of metazoa such as mouse and zebra fish.

Previous data show that angiomotin expression promotes cell motility. Here we report that angiomotin promotes endothelial cell invasion of matrigel and collagen *in vitro*, and hemangioendothelioma growth and invasion in a mouse model. Cells expressing angiomotin with a truncation of the PDZ binding motif, however, invaded less than control cells, and tumors formed by these cells remained dormant and were non-invasive. Cell migration, but not cell proliferation or protease activity, accounted for the invasive property of angiomotin-expressing cells. We also present data that suggest that the effects of angiomotin and angiostatin in mouse aortic endothelial cells are mediated by the Rho effector Rho kinase (ROCK).

We show that angiostatin binds angiomotin on the cell surface which provides the rationale for future attempts to design antibodies that are targeted against angiomotin. Furthermore, p80 and p130 isoforms of angiomotin localize to cell-cell junctions *in vivo* and *in vitro*. p130, but not p80 angiomotin, interacts with the tight junction protein MAGI-1. Both angiomotin isoforms reduced paracellular permeability when stably expressed in Chinese hamster ovary cells. Angiostatin reduced angiomotin-mediated migration but not angiomotin-mediated control of permeability. This suggests dual roles for angiomotin in angiogenesis and suggests a therapeutic window for interfering with angiomotin.

In conclusion, this work provides insight into the molecular and cellular biology of angiomotin, which may be important for designing new compounds that interfere with angiogenesis.

LIST OF PUBLICATIONS

I. **Bratt A**, Wilson WJ, Troyanovsky B, Aase K, Kessler R, Van Meir EG and Holmgren L.
Angiomotin belongs to a novel protein family with conserved coiled-coil and PDZ binding domains.
Gene, 2002 Sep 18;298(1)69-77.

II. Levchenko T, **Bratt A**, Arbiser JL and Holmgren L.
Angiomotin expression promotes hemangioendothelioma invasion.
Oncogene, 2004 Feb 19;23 (7):1469-73.

III. **Bratt A**, Narumiya S and Holmgren L.
Rho-kinase dependant control of endothelial migration and tube formation by angiomotin and angiostatin.
Manuscript.

IV. **Bratt A**, Birot O, Sinha I, Veitonmäki N, Aase K, Ernkvist M and Holmgren L.
Angiomotin regulates endothelial cell-cell junctions and cell motility.
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CONTENTS

LIST OF ABBREVIATIONS

AJ	adherens junction
Amotl1	angiomotin like 1
Amotl2	angiomotin like 2
Ang1	angiopoietin 1
Ang2	angiopoietin 2
AVE	anterior visceral endoderm
BAI-1	brain angiogenesis inhibitor-1
BBB	blood-brain barrier
CNS	central nervous system
EC	endothelial cell
ECM	extracellular matrix
ESAM	endothelial cell selective adhesion molecule
FAK	focal adhesion kinase
GAP	GTPase activating protein
GEF	guanine nucleotide exchange factor
HMVEC	human dermal microvascular endothelial cells
HUVEC	human umbilical vein endothelial cells
JAM	junction associated molecule 1
JEAP	junction enriched and -associated protein
MAGI-1	membrane-associated guanylate kinase with inverted domain structure-1
MAE cells	mouse aortic endothelial cells
MMP	matrix metalloproteinase
PDGF	platelet-derived growth factor
ROCK	rho kinase
TJ	tight junction
VEGF	vascular endothelial growth factor A
PECAM	platelet endothelial cell adhesion molecule

1 INTRODUCTION

1.1 THE MOLECULAR BASIS OF CANCER

In their article in Cell in 2000, Douglas Hanahan and Robert Weinberg reviewed twenty-five years of cancer research (Hanahan and Weinberg, 2000). The message, which is now dogma in the cancer research world, was that cancer is not so much a riddle anymore, but can be understood as the selection of cells with certain traits that cause the malignant disease. The traits are the following: 1. self sufficiency in growth signals, 2. insensitivity to anti-growth signals, 3. evasion of apoptosis, 4. limitless replication potential, 5. ability to metastasize, and, 6. sustained angiogenesis.

Each of the six traits are putative targets for therapies against cancer because each is necessary for the progression of the disease into malignancy. This thesis is concerned with one of these properties, angiogenesis, and a certain protein, angiomotin, which is involved in the control of angiogenesis. The overall aim of this work is to investigate angiomotin as a drug target for anti-angiogenic therapy of cancer.

1.2 ANGIOGENESIS

Several investigators since the 19th century have suggested that tumors are dependant on the formation of new blood vessels but it was Judah Folkman who, in 1971, proposed that cancer may be treated by interfering with this process (Folkman, 1971; Kerbel, 2000; Ribatti et al., 1999). A search for "angiogenesis" in PubMed limited to that year yields three (3) hits (including the aforementioned paper, and another paper of which Dr. Folkman is first author). For the year 2004 the search yields 3557 hits. This reflects the enormous increase in interest for the field of angiogenesis.

What is the evidence that tumor growth is angiogenesis dependent? It was observed early (the first report is from 1865) that tumors contained large numbers of vessels that seemed to be growing and budding, and during the 20th century experiments in animal models suggested that tumor implants could induce novel vessel formation.

In 1971 it was first reported that a factor that could induce angiogenesis was isolated from a tumor (Folkman et al., 1971) and basic fibroblast growth factor was identified as a growth factor for endothelial cells in 1984 (Shing et al., 1984). In 1989 the first growth factor specific for endothelial cells (ECs) was identified, namely vascular endothelial growth factor (VEGF). Since then it has been shown that tumors express VEGF, often at high levels, and that expression levels correlates with clinical outcome (the higher the expression, the worse the prognosis). The development of methods to quantify vessel density in histological preparations made it possible to show that high vessel density correlates with poor prognosis (Kerbel, 2000). In addition, a wealth of experimental evidence shows that tumor growth can be modulated by inhibiting angiogenesis in animal models. Finally, in 2004, came the clinical proof-of-concept: it was reported that the use of an antibody against VEGF prolonged survival of cancer patients (Hurwitz et al., 2004).

1.2.1 Vasculogenesis, intusussception and sprouting angiogenesis

The vasculature is crucial for tissue function and cell survival as it carries out transport of nutrients and oxygen to, and catabolites away from, the cells. This makes it necessary for all living tissue to reside within 100 μm - which is the diffusion limit of oxygen - of a capillary blood vessel (Carmeliet and Jain, 2000).

In the adult, neovascularization occurs by two general mechanisms: sprouting angiogenesis and intussusception, which is the splitting of one vessel into two. Intussusception has been observed during development as well as in tumors (Burri et al., 2004; Patan et al., 1996). This thesis is concerned primarily with the more well-characterized sprouting angiogenesis which will usually be referred to simply as "angiogenesis".

During development, blood vessels can be formed by the in situ differentiation of progenitor cells to primitive blood vessels (vasculogenesis). Vasculogenesis is similar to angiogenesis in many ways and conclusions about angiogenesis are sometimes drawn from model systems where vasculogenesis is studied, such as gene ablation in mice and *in vitro* embryonic stem cell assays. In this thesis, vasculogenesis will mostly be discussed in that context.

1.2.2 Pathological angiogenesis

Formation of new blood vessels is a tightly regulated process which, in the adult, normally takes place in a limited number of settings such as wound healing and in the female corpus luteum. Tumor cells are, like all cells, dependent on the circulation for supply of oxygen and nutrients. Therefore, for solid tumors to grow beyond a few millimeters in size, they must be able to induce angiogenesis (Figure 1) (Folkman, 1995; Hanahan and Weinberg, 2000). In the absence of angiogenesis, solid tumors remain confined in a state where cell proliferation is balanced by cell death (Holmgren et al., 1995). Such tumors may be dormant for long periods of time until they gain the capability to switch on neovascularization. This event - the angiogenic switch - leads to the rapid growth of the tumor (Hanahan and Folkman, 1996). In addition, vascularization of primary tumors contributes to the ability of tumor cells to enter the circulatory system and establish distant metastases.

Tumor blood vessels are often leaky, tortuous, abnormal and have sluggish blood flow. This may be caused by aberrant levels and ratios of morphogenic angiogenic factors in the tumor such as VEGF and angiopoietins (see below).

In the absence of formation of novel blood vessels tumors may grow by residing in close proximity to pre-existing vessels. This process, *co-option*, leads to the breakdown of the vessels and, thus, the need for the tumor to induce angiogenesis (Yancopoulos et al., 2000). Also, it has been reported that vascular progenitor cells may be recruited to sites where neovascularization is needed where they can participate in formation of novel blood vessels (Asahara et al., 1997). This will not be discussed further as this thesis is primarily concerned with sprouting angiogenesis.

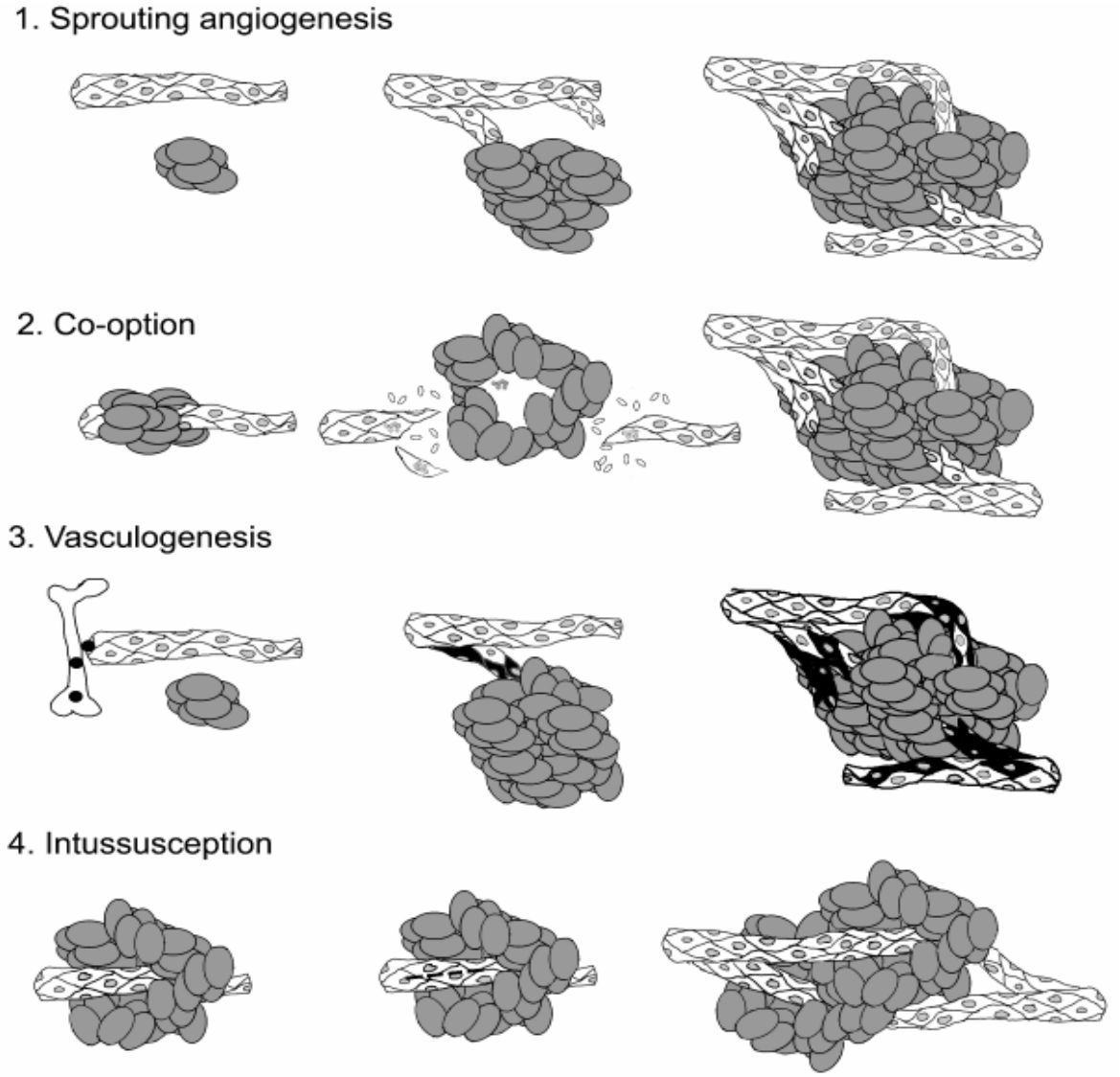

Figure 1. *Establishment of blood supply to tumors. 1. Sprouting angiogenesis. 2. Co-option of a blood vessel leads to breakdown of the vessel and the need for angiogenesis. 3. Vasculogenesis by recruitment of endothelial stem cells from the bone marrow. 4. Intussusception - the splitting of a vessel into two vessels - has also been reported to play a role in the formation of tumor circulation. Illustration kindly provided by Niina Veitonmäki.*

Several diseases besides cancer are associated with excessive angiogenesis. For example, age-related macular degeneration is a major cause of blindness, for which there has been no therapy. Recently, however, an anti-angiogenic RNA molecule, Macugen (Pegaptanib), that binds directly to the VEGF peptide, was approved for the treatment of this disease (van Wijngaarden et al., 2005). Other diseases that are associated with excessive angiogenesis are arthritis and psoriasis (Folkman, 1995). An understanding of angiogenesis may also lead to better therapies for diseases associated with a *lack* of angiogenesis, such as coronary artery disease.

1.2.3 The current model of sprouting angiogenesis

Angiogenesis is controlled by factors that may promote (i.e. angiogenic factors) or inhibit (i.e. angiogenesis inhibitors) angiogenesis. A shift in the balance towards more pro-angiogenic factors induces blood vessel formation (Hanahan and Folkman, 1996).
During sprouting angiogenesis endothelial cells (ECs), which are usually quiescent and do not divide, begin to proliferate. VEGF is a mitogen for ECs but also increases the permeability of the vessels. This allows the leakage of plasma which lays down a scaffold of fibrin for migrating endothelial cells. For the ECs to be able to colonize the surroundings they also have to break down or remodel the basement membrane, which largely is composed of collagen, laminin and proteoglycans. For this purpose, they secrete various proteases, of which matrix metalloproteinases (MMPs) and urokinase plasminogen activator (uPA) play important roles (Carmeliet, 2000; Stupack and Cheresh, 2004) (Figure 2).

The ECs form a sprout of migrating cells led by a specialized cell, the so called tip cell. VEGF isoforms bound to the matrix guides the tip cell (Ruhrberg et al., 2002) which extends filipodia that appear to help the cell to feel its way forward. The tip cell does not divide; proliferation takes place mainly in the stalk of the sprout (Gerhardt et al., 2003). Proliferating ECs specifically express the $\alpha v\beta 3$ integrin which is important for EC migration and survival (see section 1.4.3, below).

Often, ECs first assemble into a cord that only subsequently forms a lumen (Carmeliet, 2000). This can take place by the coalescence of vesicles within one cell and then between cells (Lubarsky and Krasnow, 2003).

Cell-cell contacts play an important role during angiogenesis. First, cell-cell contacts need to be loosened in order for cells to migrate and participate in angiogenesis (Carmeliet, 2000). But on the other hand, cell-cell contacts are needed to achieve a functional vessel (see section 1.5, below). During maturation of the vessel pericytes are recruited that stabilize the vessel and new basement membrane is synthesized. Finally, the vessels are pruned, i.e superfluous vessels that are not needed regress.

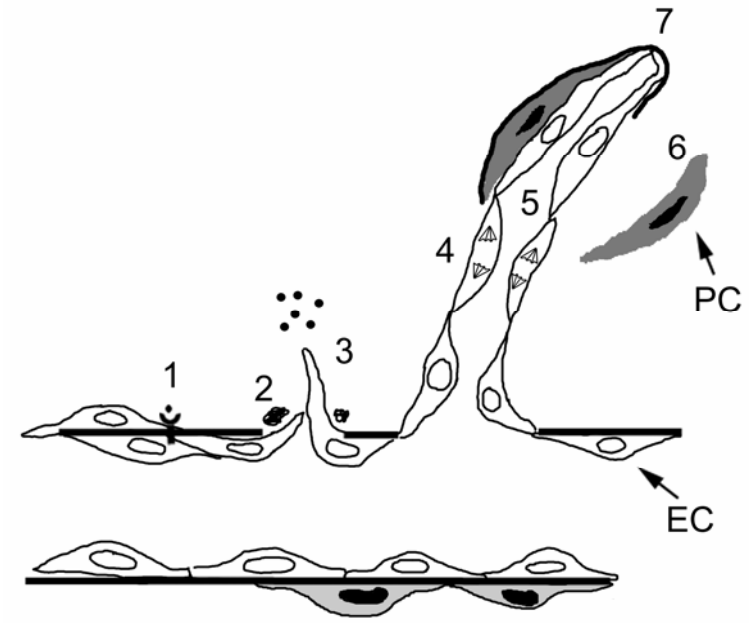

Figure 2. *Sprouting angiogenesis. 1. Binding of angiogenic factors to receptors on endothelial cells. 2. Proteases remodel and break down the basement membrane 3. Cells migrate in response to angiogenic factors. 4. Endothelial cell proliferation. 5. Tube morphogenesis. 6. Recruitment of pericytes. 7. Construction of a new basement membrane. Illustration kindly provided by Niina Veitonmäki.*

1.2.4 Angiogenic factors

1.2.4.1 Vascular endothelial growth factor

The prototypic angiogenic factor is vascular endothelial growth factor A (VEGF), a peptide that plays crucial roles in vasculogenesis and angiogenesis. VEGF was initially discovered by Dvorak and coworkers as a factor that could increase vascular permeability (Senger et al., 1983). By binding to specific receptors, VEGF controls EC survival, proliferation, and migration. VEGF gene ablation in the mouse leads to an embryonic lethal phenotype with impaired vessel formation. In fact, already the heterozygote knock-out is lethal (Carmeliet et al., 1996; Ferrara et al., 1996). VEGF receptors are receptor tyrosine kinases that activate several signaling pathways among which are ones important for proliferation and migration, such as PI3 kinase, PLC gamma and focal adhesion kinase (FAK) (Matsumoto and Claesson-Welsh, 2001). Mice that lack the primary VEGF receptor, VEGF receptor 2, fail to produce ECs and die at embryonic day 8.5 (Shalaby et al., 1995).

VEGF is frequently expressed by tumors and VEGF-dependant angiogenesis is an important tumor driving factor. For that reason, great efforts have been directed at interfering with VEGF signaling. The most successful concept so far has been the VEGF

antibody bevacizumab which has shown efficacy against tumors in animal models and, most importantly, promising results in cancer patients. Among colon cancer patients with metastatic disease, the addition of bevacizumab to a chemotherapy regiment improved median survival from 15 to 20 months (Hurwitz et al., 2004). As a single agent bevacizumab improved time to progression in metastatic renal cancer patients (Yang et al., 2003), with limited side effects. Also, at the 2005 ASCO meeting efficacy against breast and lung cancer was reported. This confirms one of the anticipated and hoped-for effects of anti-angiogenic therapy: that it should not be cancer-type specific but block the growth of all kinds of solid tumors.

The VEGF receptors have been considered to be exclusively expressed by ECs. However, recently, VEGF receptor expression was detected in neurons (Storkebaum et al., 2004), which raises questions regarding the long-term safety of anti-VEGF therapy.

1.2.4.2 The angiopoietins and platelet derived growth factor

The role of angiopoietins in controlling angiogenesis is somewhat complex. Angiopoietin-1 (Ang1) and angiopoietin-2 (Ang2) both binds to the receptor tyrosine kinase Tie-2, which is exclusively expressed by ECs.

First of all, Ang1 is an angiogenic factor; mice deficient in Ang1 are unable to form a complex vascular network and die early during embryogenesis (Suri et al., 1996). Ang1 stimulates EC survival and migration, induces EC maturation, and causes the recruitment of pericytes (Metheny-Barlow and Li, 2003). Furthermore, Ang1 reduces vessel permeability by controlling the formation of tight junctions (TJs) in the blood-brain barrier (BBB) (Gamble et al., 2000; Lee et al., 2003).

Ang2 is an antagonist of Ang1 as determined by *in vitro* assays. Also, mice overexpressing Ang2 have a phenotype similar to that of Ang1 knock-out mice (Maisonpierre et al., 1997; Metheny-Barlow and Li, 2003). This suggests that Ang2 is an inhibitor of angiogenesis. However, that is not the case. On the contrary, Ang2 is necessary for the early stages of angiogenesis where it works in concert with VEGF to loosen cell-cell contacts and increase permeability. In addition, Ang2 is more frequently overexpressed in human tumors than Ang1, whereas expression of Ang1 may actually be downregulated in malignancies. The fact that tumor vessels are often leaky, and sometimes lack pericyte coverage and are constantly renewed could be explained by overexpression of Ang2. Knock-out experiments show that Ang2 is dispensable for embryonic vascular development, but necessary for certain post-embryonic angiogenesis, which indicates that Ang2 is necessary in order to permit VEGF-induced angiogenesis from pre-existing vessels (Metheny-Barlow and Li, 2003).

Platelet derived growth factor B (PDGF B) plays an important role for the recruitment of pericytes to newly formed blood vessels. PDGF B is secreted by ECs, which attracts pericytes. In PDGF B knock-out mice, vessels dilate and form aneurysms because the lack of pericytes makes the vessels unstable. A model has been proposed where Ang1 binding to ECs induces expression of PDGF B, which leads to the recruitment of pericytes (Lindahl et al., 1998). It has been confirmed that PDGF B secreted by ECs promotes tumor angiogenesis and tumor growth (Abramsson et al., 2003; Furuhashi et al., 2004). Pharmacological inhibition of PDGF receptor signaling inhibits tumor growth in the RipTag mouse model of cancer (Bergers et al., 2003).

1.2.5 Inhibitors of angiogenesis

Angiogenesis is controlled by a balance between the aforementioned growth factors and endogenous inhibitors of angiogenesis (Hanahan and Folkman, 1996). In general, discovery of endogenous inhibitors of angiogenesis began later than pro-angiogenic factors and they are still less understood at the molecular level. A number of angiogenesis inhibitors have been identified (Folkman, 2004).

1.2.5.1 Angiostatin

Occasionally, the removal of a primary tumor may cause rapid growth of metastases. In an effort to understand the mechanism behind this O'Reilly et al. purified an anti-angiogenic factor that was produced by a primary tumor, the Lewis lung carcinoma. The factor, which was named angiostatin, was found to be a 38 kDa internal cleavage fragment of plasminogen (from residue 98 to residue 465) (Figure 3). Plasminogen is the precursor of plasmin which normally is involved in controlling blood clotting. When angiostatin produced by degradation of plasminogen was administered to mice it could inhibit angiogenesis and inhibit growth of metastases (O'Reilly et al., 1994). Systemic administration of angiostatin could inhibit the growth of several types of experimental tumors by sustaining dormancy (Holmgren et al., 1995; O'Reilly et al., 1996), a state where, in the lack of a functional vasculature, tumor cell proliferation is balanced by tumor cell apoptosis (Holmgren et al., 1995). Angiostatin could also prevent bFGF-induced neovascularization in the mouse cornea assay when administered systemically (O'Reilly et al., 1996). It is not known if the dormancy of micometastases in patients is caused by angiostatin.

Intense effort has been guided towards understanding the mechanism of action of angiostatin. It was initially discovered as being able to inhibit cell number increase of bovine capillary endothelial cells (O'Reilly et al., 1994). This suggested that angiostatin could inhibit cell division or induce apoptosis. Further analysis indicated that angiostatin could, indeed, induce apoptosis in ECs, and signaling trough FAK has been implicated (Claesson-Welsh et al., 1998; Lucas et al., 1998). However, other studies indicate that angiostatin inhibits EC motility, a crucial step of angiogenesis (Ji et al., 1998; Levchenko et al., 2003; Troyanovsky et al., 2001). A study has shown no effect on proliferation but only on migration, although without seeing any change in activation of the motility proteins Rac or Pak (Eriksson et al., 2003). However, Gupta et al. has showed that angiostatin can mediate activation of Rho as measured by membrane-association of this GTPase (Gupta et al., 2001).

How is angiostatin produced? Angiostatin can be generated by proteolytic cleavage of plasmin or plasminogen by different enzymes, but is not completely clear how the primary tumor causes its production. It has been suggested that tumor cells do not produce angiostatin themselves but instead create a microenvironment that increases proteolysis of plasminogen to angiostatin, maybe by secreting a protease. Tumors may secrete urokinase-type plasminogen activator (uPA), which cleaves plasminogen to plasmin which may then be further processed to angiostatin. MMPs may generate angiostatin (Dong et al., 1997;

Lijnen et al., 1998; O'Reilly et al., 1999; Patterson and Sang, 1997) and angiostatin in the original Lewis Lung Carcinoma model was generated by MMP-2 (O'Reilly et al., 1999). Also, plasmin, which is a serine protease itself, may undergo self-cleavage to angiostatin if the disulfide bonds in the kringle domains are reduced. The reducing enzyme may be phosphoglycerate kinase which can be secreted by tumor cells (Lay et al., 2000).

There are a number of different forms of angiostatin. Initially kringle domains 1-4 of plasminogen was named angiostatin but some groups have reported activity of kringle 1-4.5 (Wang et al., 2004), kringle 1-5 (Cao et al., 1999) or kringle 5 alone (Cao et al., 1997). The various activities of angiostatin seem to be harbored in different kringle domains (which are not identical). Kringle 4, for example, efficiently blocks EC migration but not proliferation, whereas the opposite is true of kringle 1-3 (Ji et al., 1998).

Clinical studies suggest that angiostatin is often present in the plasma or urine of patients with different types of cancer (Sten-Linder et al., 1999; Volm et al., 2000; Yabushita et al., 2002) and that the presence of angiostatin is associated with longer survival (Volm et al., 2000).

Angiostatin is one of a number of endogenous angiogenesis inhibitors that are cleavage fragments of larger proteins. Other examples are endostatin (O'Reilly et al., 1997) and tumstatin which are fragments of collagens XVIII and IV, respectively. Both these fragments seem to exert their anti-angiogenic properties by binding to integrins (Bix and Iozzo, 2005). It has been shown that tumstatin induces apoptosis in ECs by inhibiting cap-dependant protein synthesis after binding to the $\alpha v\beta 3$ integrin (Maeshima et al., 2002). This probably makes tumstatin the most well-understood endogenous angiogenesis inhibitor.

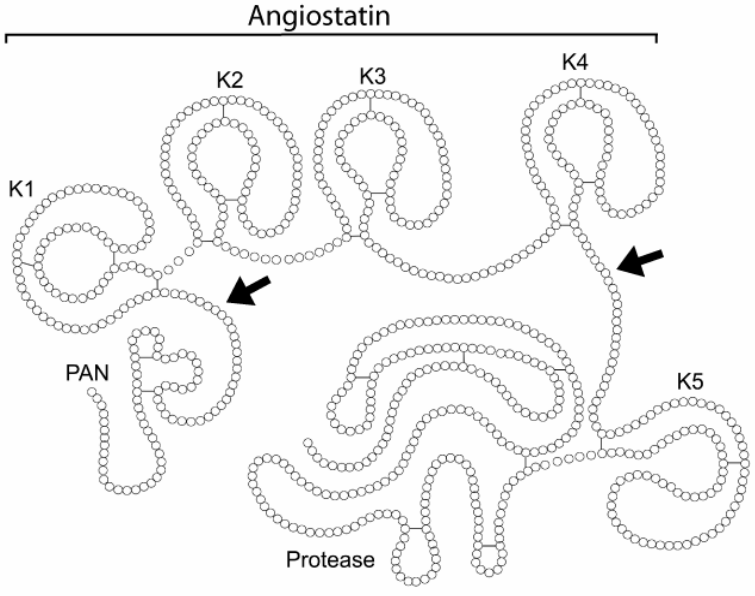

Figure 3. *Primary structure of angiostatin. Angiostatin consists of the first four kringle domains (K) 1-4 of plasminogen. In addition plasminogen consists of a plasmin protease domain, a PAN domain and kringle 5. Approximate cleavage sites for the formation of angiostatin are indicted by arrows. Disulfide bonds are indicated by lines between circles.*

1.3 ANGIOMOTIN - AN ANGIOSTATIN BINDING PROTEIN

Since angiostatin was identified in 1994 intense efforts have been guided towards understanding the mechanisms of its anti-angiogenic properties. Specifically, the identification of a receptor that is exclusively expressed on ECs would be an important step.

The yeast two-hybrid screen can be used not only for finding intracellular interactions but also receptor-ligand interactions (Ma et al., 2001; Nguyen et al., 2004). In 2001, Troyanovsky et al. carried out a yeast two-hybrid screen to identify a putative receptor for angiostatin. Three positive clones were identified and they all contained the same insert, a cDNA sequence that coded for a 134 amino acid sequence that bound to angiostatin *in vitro*. The entire gene, initially referred to as angiostatin binding protein 1 (ABP-1), was cloned and named angiomotin, for its ability to induce motility when transfected into ECs (Troyanovsky 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.

HeLa cells expressing p80 angiomotin bound and internalized FITC-labeled angiostatin whereas control cells did not. RT-PCR analysis and immunostaining show expression of angiomotin in blood vessels of the human placenta and in Kaposis sarcoma, which is a tumor of endothelial origin. In addition, angiomotin was expressed in human dermal microvascular endothelial cells (HMVEC) and human umbilical vein endothelial cells (HUVEC) but not in cells lines of non-endothelial origin. In addition, immunostaining showed expression in cytotrophoblasts. These cells, which invade the uterine interstitum during the formation of the human placenta, express endothelial markers such as VE-cadherin, PECAM and the $\alpha v\beta 3$ integrin (Bazzoni and Dejana, 2004). Immunofluorescence studies show that angiomotin localizes to the lamellipodia of migrating cells (Troyanovsky et al., 2001). Together, these data indicated that angiomotin was an endothelial-specific receptor for angiostatin. Interestingly, expression in angiostatin-sensitive neutrophils has also been detected (Benelli et al., 2002).

Mouse aortic endothelial (MAE) cells are ECs that do not express endogenous angiomotin. MAE cells retrovirally transfected with p80 angiomotin become more motile in the Boyden chamber assay for cell migration. However, this effect can be blocked by angiostatin (Ernkvist et al., 2005; Levchenko et al., 2003; Levchenko et al., 2004; Troyanovsky et al., 2001). Thus, angiostatin inhibits migration of angiomotin-transfected cells in the Boyden chamber assay and blocks tube formation of angiomotin-transfected cells in the matrigel tube formation assay.

The mechanism by which angiomotin promotes cell motility has not been defined but *in vivo* and *in vitro* evidence shows that the C-terminal domain, which is predicted to bind to a protein with a PDZ domain, plays an important role. Cells that express angiomotin with a three amino acid truncation of the PDZ-binding motif ($\Delta 4$ angiomotin) are defective in migration and tube formation. Transgenic mice that express this mutant form of angiomotin under the endothelial-specific TIE-promoter die at embryonic day 9.5. The embryos displayed impaired blood vessel formation in the brain and in the intersomatic blood vessels, leading to bleedings and aneurysms. Histological analysis suggested that the phenotype was caused by impaired migration of endothelial cells (Levchenko et al., 2003). These data indicate that angiomotin may act as a promoter of angiogenesis by enhancing cell motility and migration and that angiostatin is an antagonist of angiomotin.

Recently, a splice form named p130 angiomotin was identified. p130 angiomotin contains identical domains as p80 angiomotin, and in addition an N-terminal extension of 409 amino acid residues that mediates the localization of angiomotin to actin stress fibers. Expression of p130 angiomotin in MAE cells leads to increased stress fiber formation and changes in cell morphology (Ernkvist et al., 2005).

Mice deficient for both p80 and p130 angiomotin isoforms have been generated. These were born at a lower than expected ratio as approximately 70% of angiomotin-deficient embryos died, suggesting an incomplete penetrance of the phenotype. At day 5 in embryogenesis, cells of the anterior visceral endoderm (AVE) migrate from a position on the distal tip of the embryo to a more proximal position where they subsequently participate in gastrulation and give rise to the internal organs. Migration of the AVE takes place in the form of active cell migration across the surface of the epiblast with formation of filipodia in the direction of migration (Srinivas et al., 2004). Angiomotin knock-out embryos displayed defective migration of these cells, which led to morphological abnormalities and early embryonic death (Shimono and Behringer, 2003). The endothelial phenotype of surviving animals is yet to be reported.

1.3.1 Other angiostatin binding proteins

Besides angiomotin, other receptors for angiostatin have been identified. ATP synthase, which is usually thought of as exclusively located in the membrane of mitochondria where it catalyzes the formation of ATP, was identified as an angiostatin binding protein by Moser et al. It was suggested that ATP synthase in the outer cell membrane produced extracellular ATP that could later diffuse back into the ECs and provide energy. This was blocked by angiostatin (Moser et al., 1999). Later it was proposed that angiostatin blocked formation of extracellular ATP that hypothetically serves as a pro-angiogenic factor (Moser et al., 2001). Binding of angiostatin to ATP synthase on the cell surface induces apoptosis in a caspase dependent manner (Veitonmaki et al., 2004).

The $\alpha v\beta 3$ integrin is important for angiogenesis (see section 1.4.3, below) and it has been shown that angiostatin can bind to this integrin in a manner similar to tumstatin. It was suggested that this interferes with cell-matrix interactions and blocks integrin survival signalling (Tarui et al., 2001). Other angiostatin binding proteins include the hepatocyte growth factor receptor c-met (Wajih and Sane, 2003) and annexin II (Tuszynski et al., 2002).

How, and if, the different proposed mechanisms interplay is not known.

1.4 CELL MOTILITY

Cell motility plays an important role during development, for the immune system and in pathological settings. During angiogenesis, cell motility is important as cells migrate when forming new vessels. In the field of cancer, cell motility is also crucial for understanding tumor invasion and metastasis.

It can be useful to think of cell migration as divided into different subprocesses: protrusion, polarization, attachment, contractility and deattachment (Lauffenburger and

Horwitz, 1996; Ridley et al., 2003). Cell migration involves the coordinated action of these processes (Figure 4).

Protrusion: The cell extends protrusions in the form of lamellipodia (i.e. sheet-like structures observed *in vitro* that are parallel to the surface to which the cell is attached) and filipodia (i. e. thin finger-like extensions that the cell probably uses to probe its surroundings with). Polymerization of actin drives these processes.

Polarization: For cells to migrate in a particular direction they need to have a front and a back. Cells extend lamellipodia and filipodia in the direction of movement with the Golgi apparatus usually positioned in front of the nucleus. Proteins that are important for motility can be shuttled towards the front of the cell.

Attachments: Integrins are the major class of proteins that mediate attachments of the cell to the matrix (see section 1.4.3, below). These are often clustered into focal adhesions where their cytoplasmic tails are attached to stress fibers and signaling proteins such as FAK and paxillin. Newly formed protrusions form smaller attachment sites, i. e. focal complexes, that can mature into focal adhesions as the cell moves forward.

Contractility: When the cell has extended protrusions and formed attachments it can pull itself forward by applying tension trough contraction of actin-myosin stress fibers.

De-attachment: The rear part of the cell must release its attachments from the substratum as the cell pulls itself forward.

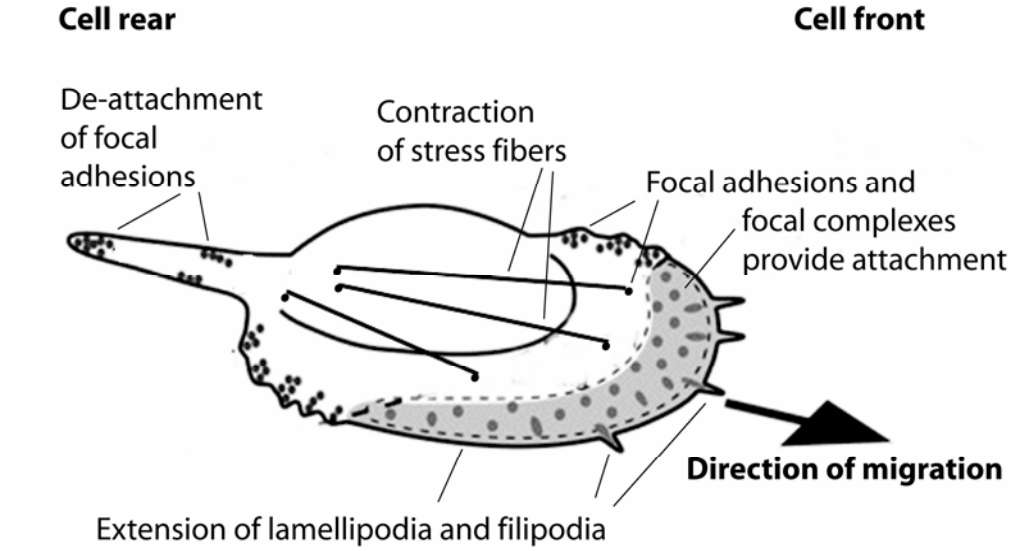

Figure 4. *Cell motility. Adopted from (Ballestrem et al., 2001).*

1.4.1 The Rho GTPases control cell motility

Several, if not all, of the above-mentioned processes, are controlled by the Rho family of GTPases, a family of proteins that are similar to oncogenic Ras and can, just like Ras, cycle between an active GTP-bound state and an inactive GDP-bound state. The importance of Rho GTPases was revealed in the beginning of the 1990s by Alan Hall and coworkers. At the time it was already known which amino acids that were crucial for the activity of onogenic Ras. By mutating the same residues in expression plasmids with Rho GTPase family members Rho, Rac and Cdc 42 and microinjecting the DNA into fibroblasts, the investigators found that active Rho resulted in the formation of stress fibers and focal adhesions (Ridley and Hall, 1992), active Rac resulted in lamellipodia (Ridley et al., 1992), whereas active Cdc42 caused extensive formation of filipodia (Nobes and Hall, 1995). The Rho family of GTPases has since then been found to control every aspect of cell morphology and motility. Examples include phagocytosis, establishment of polarity of epithelial cells, axon and dendrite growth in neurons and migration of leukocytes (Etienne-Manneville and Hall, 2002). In the case of ECs the Rho family of GTPases controls endothelial cell permeability (Etienne-Manneville and Hall, 2002; Wojciak-Stothard et al., 2001), EC migration *in vitro* (Aepfelbacher et al., 1997; Hoang et al., 2004; Soga et al., 2001) and angiogenesis *in vivo* (Hoang et al., 2004).

1.4.2 Upstream and downstream of the Rho GTPases

The activation of Rho, Rac and Cdc42 must be closely controlled in space and time and the understanding of how this occurs has increased enormously during the last ten years. The activation state is controlled by GEFs (guanine nucleotide exchange factors), which activate the GTPase and GAPs (GTPase activating proteins) that induce GTP hydrolysis and deactivation of the GTPase. By way of example, stimulation of cells with chemotactic agents or by growth factors that bind to cell surface receptors leads to activation of PI3 kinase, which enhances localized phosphorylation of the lipid PIP_2 to PIP_3. PIP_3 in turn may activate GEFs for Rac, which can induce lamellipodia (Ridley et al., 2003). Recently a novel mechanism for controlling Rho activity has been identified: localized degradation of Rho by targeting this protein for proteolysis, which causes membrane protrusion (Wang et al., 2003).

When Rho GTPases are bound to GTP they are active and interact with downstream effectors, which may be kinases or lipid-modifying enzymes that relay the signals to the cytoskeleton. For example, downstream of Rac, WAVE proteins are stimulated to activate the Arp2-3 complex, which leads to branched actin polymerization and formation of lamellipodia (Ridley et al., 2003).

p160 ROCK or Rho kinase (ROCK) is a kinase that is activated by Rho. It is believed that binding of Rho to ROCK liberates the kinase domain of ROCK from a state where it is bound to the C-terminal inhibitory domain of ROCK itself (Riento and Ridley, 2003). ROCK is thought to control the cytoskeleton mainly by two different pathways (Figure 5). Firstly, ROCK can directly phosphorylate myosin light chain (MLC) (Amano et al., 1996). ROCK also achieves increased phosphorylation of MLC by inhibiting MLC phosphatase. The phosphorylation of MLC, in turn, leads to the activation of myosin and increased contractility of actin-myosin fibres (Kimura et al., 1996). Contraction of stress fibers

creates tension in the cell and enables the cell to move forward, but also correlates negatively with membrane protrusion (Worthylake and Burridge, 2003). Secondly, active ROCK can phosphorylate LIM kinase which in turn can activate cofilin by phosphorylation. Cofilin can directly influence actin polymerization by severing actin filaments (Maekawa et al., 1999). Severing actin (maybe counter intuitively) leads to increased F-actin by creating more polymer ends which serve as starting points for actin polymerization.

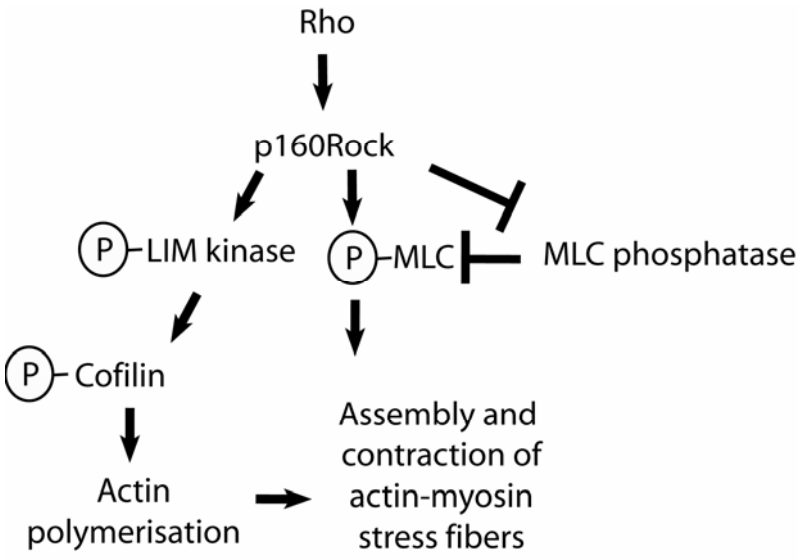

Figure 5. *Signalling from Rho to ROCK and further to the cytoskeleton. Adopted from (Bishop and Hall, 2000)*

1.4.3 Integrins

In order to migrate, cells need to constantly form and break contacts with the surrounding extracellular matrix (ECM). Integrins are transmembrane proteins that are the principal mediators of attachment to the ECM. Integrins consist of one alpha subunit and one beta subunit. There are at least 24 different alpha and 8 different beta subunits and these can combine into integrin pairs that have specific affinity for different matrices. Integrin binding to the ECM provides traction to the cell during cell motility. In addition, binding of integrins to the ECM is a pro-survival signal, in the absence of which cells undergo apoptosis (Giancotti and Ruoslahti, 1999). Under the influence of Rho activity and stress fiber contraction the integrins cluster into larger focal adhesions.

The cytoplasmic tail of integrins are attached to the actin cytoskeleton and signaling proteins that mediate signals that control cell survival and cell motility. One prominent cytoplasmic component of focal adhesions is FAK. Cells deficient in FAK show impaired motility and enhanced formation of focal adhesions, suggesting that FAK is necessary for

focal adhesion turnover (Ilic et al., 1995). FAK can be recruited to focal adhesions by the multidomain adaptor protein paxillin, which can be phosphorylated by FAK. FAK, in turn, can bind PI3 kinase and it is likely that this plays a part in mediating survival signals from integrins (Giancotti and Ruoslahti, 1999).

For angiogenesis, the αvβ3 integrin is usually singled out as especially important. This integrin is expressed exclusively by ECs participating in angiogenesis. It is one of the most promiscuous integrins and binds to a wide variety of matrix components. Rac activity induces recruitment of activated high-affinity integrins to the advancing lamellipodia of ECs (Kiosses et al., 2001) where they can cluster into focal complexes. An antibody, LM609, that binds to this integrin blocks angiogenesis (Brooks et al., 1994) by inducing EC apoptosis (Stromblad et al., 1996), and the humanized form of this antibody, Vitaxin, is currently in clinical trials for the treatment of cancer (Stupack and Cheresh, 2004). Somewhat surprisingly, mice lacking this integrin are viable and have no vascular defect (Hodivala-Dilke et al., 1999). Other integrins that are important for angiogenesis include αvβ5 and α5β1 (Stupack and Cheresh, 2004).

1.5 CELL-CELL JUNCTIONS

Cell-cell adhesion structures were first identified and classified in epithelial cells where at least three different types of contacts were identified by electron microscopy; tight junctions, adherens junctions and desmosomes, where tight junctions are the most apical and desmosomes the most basolateral. The nomenclature has carried over to ECs where it may not be appropriate because in ECs, tight junctions and adherens junctions are intermingled and less distinguishable from each other and possibly intermingled (Ruffer et al., 2004; Schulze and Firth, 1993). Desmosomes, which connect intermediate filaments in epithelia, are not present in the endothelium.

1.5.1 Tight junctions

Tight junctions (TJs) are responsible for forming an impermeable barrier between cells that enable the body to control the flux of solutes and macromolecules across epithelial layers (in for example the kidney and glands) and endothelium (blood- and lymphatic vessels). It has also been suggested that TJs play an important role in maintaining polar distribution of membrane proteins by acting as a "fence", but it seems that this notion is currently being questioned (Bazzoni and Dejana, 2004).

TJs were first identified by electron microscopy as electron-dense (i.e. "dark") areas between cells in close proximity. In freeze-fracture analysis TJs appeared as punctuate structures, like pearls on a string. On the molecular level, TJs consist of pairs of transmembrane proteins, one from each cell, that homodimerize through their extracellular domains (Figure 6). The first of these proteins to be identified was occludin. Occludin is expressed by epithelial and endothelial cells that have low permeability, such as in the blood-brain barrier (BBB) of the central nervous system (CNS). Occludin transfected into fibroblasts, which normally do not form TJs, induces cell aggregation and localizes to cell-cell contacts. Surprisingly, occludin-deficient mice are viable and seem to have normal TJs

(Saitou et al., 2000). This has put the role of occludin in question and suggests that there may be redundancy among TJ molecules.

In 1998 the claudin family of proteins was identified (Furuse et al., 1998). There are around 20 different claudins. Claudins can, like occludin, form TJs when transfected into fibroblasts. The claudins have no homology to occludin and have a tissue-specific expression pattern, with claudin 5 being the EC-specific form. Mice deficient in this gene have normal vasculogenesis and seem to have normal TJs as judged by electron microscopy. These mice, however, die shortly after birth from brain hemorrhage. It turned out that the BBB of these mice has become permeable to small molecules (<800Da). However, the TJs were still impermeable to large molecules, probably due the expression of claudin-12 in the BBB (Nitta et al., 2003).

The junction associated molecule (JAM) family of proteins is a group of transmembrane proteins that mediate homodimerization through immunoglobulin-like domains. JAM A localizes to TJs, mediates cell aggregation and controls permeability (Martin-Padura et al., 1998). However, JAM A knock-out mice seem to have no TJ or endothelial defect, again indicating gene redundancy at tight junctions (Cera et al., 2004). 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). ESAM deficient mice have normal vasculature but exhibited slower growth of implanted tumors accompanied by low microvascular density. Also, vascularization of matrigel implants was lower in knock-out animals, suggesting that ESAM plays a role in angiogenesis. Interestingly, ESAM -/- cells showed lower migration in the Boyden chamber assay, suggesting a link between TJ formation and control over cell motility (Ishida et al., 2003). JAM has also been implicated in control of bFGF-induced cell motility during angiogenesis (Naik et al., 2003; Naik et al., 2003).

Transmembrane tight junction proteins often decrease paracellular permeability when transfected into cells that previously do not express the protein. This has been shown for TJ proteins claudin-1 (Inai et al., 1999) and JAM (Martin-Padura et al., 1998).

A number of proteins bind to the cytoplasmic side of TJs. Several of these mediates interactions between transmembrane proteins and the cytoskeleton, while others are signaling proteins or transcription factors. This suggests that TJs could be important sites for signaling, much like focal adhesions (Tsukita et al., 2001).

The best characterized of these adaptor proteins is ZO-1. The ZO proteins are members of the MAGUKs (membrane associated guanylate kinases), a family of large, membrane associated proteins that are characterized by several PDZ domains, SH3 domains and a guanylate kinase domain. ZO-1 is associated to the actin cytoskeleton through its carboxy-terminal half, and can also interact with transcription factors and signaling molecules (Bazzoni and Dejana, 2004). The PDZ motif of ZO-1 binds directly to the C-terminal of claudins (Itoh et al., 1999). The C-termini of claudins gathered at TJs are thought to protrude into the cell in a "tooth-brush"-like manner, where binding of ZO-1 and other proteins creates a huge molecular complex (Tsukita et al., 2001) (see also section 1.6.1 on PDZ domains). ZO-1 has also been reported to transiently localize to adherens junctions (AJs) during their formation (Itoh et al., 1997).

A subset of MAGUKs is the MAGIs (membrane-associated guanylate kinase with inverted domain structure) which differ from conventional MAGUKS by having WW domains instead of SH3 domains. MAGI-1 can be recruited to TJs by ESAM, probably by interaction between the C-terminal of ESAM and the PDZ-domain of MAGI-1 (Wegmann et al., 2004). MAGI-1 associates with the actin cytoskeleton by binding alfa-

actinin 4 and synaptopodin (Patrie et al., 2002). Interestingly, MAGI-1 is indirectly implicated in the control of angiogenesis as it has also been identified as a protein that can bind to the cytoplasmic region of brain angiogenesis inhibitor 1 (BAI-1), which is a seven-spanning transmembrane protein (Shiratsuchi et al., 1998). BAI-1 has extracellular domains that are homologous to the angiogenesis inhibitor thrombospondin and was identified as a gene that, just like thrombospondin, can be upregulated by p53. The thrombospondin-similar domains of BAI-1 have anti-angiogenic activity *in vivo* (Kaur et al., 2005; Nishimori et al., 1997).

The assembly of cell-cell contacts is closely connected to the control of the cytoskeleton and is regulated by the Rho GTPases. Recently it has been shown that Par3 and Par6 members of the Par (partitioning-defective) proteins play important roles in controlling assembly of TJs upstream of Rho and Rac (Chen and Macara, 2005; Ozdamar et al., 2005).

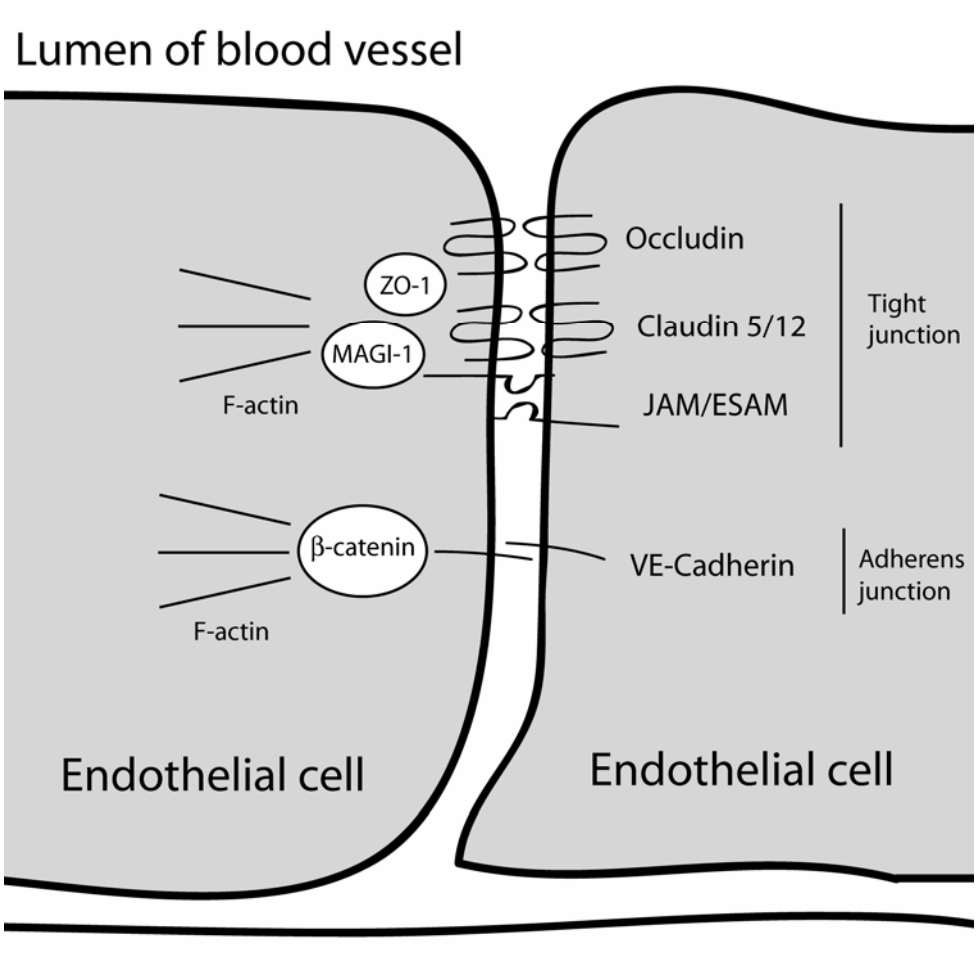

Figure 6. *Cell–cell contacts between endothelial cells.*

1.5.2 Adherens junctions

Adherence junctions (AJs) are crucial for cell recognition and aggregation and formation of tissue architecture. AJs are assembled by homodimers by one distinct class of proteins, the cadherins. The expression pattern of cadherins is tissue specific, with VE-cadherin/cadherin 5 (VE-cadherin) being the endothelial specific protein (Breviario et al., 1995; Lampugnani et al., 1995; Lampugnani et al., 1992; Suzuki et al., 1991).

Knock-out of VE-cadherin is lethal at embryonic day 9.5 with the embryo showing vascular defects: vessels collapse, regress and large hemorrhages occur. Surprisingly, ECs can assemble into an immature vascular plexus. However, the subsequent remodeling and maturation to blood vessels is disturbed, probably due to impaired survival of ECs (Carmeliet et al., 1999).

Endothelial cells also express N-cadherin which is not localized to adherens junctions but is diffusely distributed along the basolateral side where it probably serves as a link between endothelial cells and pericytes (Bazzoni and Dejana, 2004).

The cytoplasmic domains of cadherins are connected to the actin cytoskeleton through adaptor proteins and bind to signaling proteins, notably β–catenin which can, when receiving Wnt-signaling, translocate to the nucleus where it controls gene expression. Through this pathway, VE-cadherin may control cell growth, apoptosis and differentiation. VE-cadherin has also been implicated in VEGF signaling.

From a general cancer point of view it is interesting to note that the epithelial specific E-cadherin is often downregulated in metastatic tumors, indicating the important role for cadherins in maintaining an organized tissue structure (Christofori and Semb, 1999).

PECAM/CD31 is an adhesion molecule that is expressed on endothelial cells, platelets and certain leucocytes. It mediates adhesion between cells but its localization is not restricted to a specific type of adhesion structure (Vestweber, 2000). It is often used as a histological marker for endothelial cells.

1.5.3 Vascular permeability

One of the most important functions of the endothelium is the control of flux of solutes from the lumen of the vessel to the surrounding tissue. There are two principal manners by which solutes and plasma may cross the endothelium; either between cells (paracellular pathway), or through cells (transcellular pathway). In the CNS, the flux of molecules from the blood is restricted to the transcellular pathway because of the BBB which comprises well-developed TJs. In other tissues, the endothelium is more permeable.

The permeability of the endothelium increases during angiogenesis. VEGF was initially identified as a factor that could increase the permeability of blood vessels (Senger et al., 1983). It is well established that this is achieved by inducing gaps, fenestrations or vesiculovascular organelles in the cell wall (transcellular mechanisms) (Bates et al., 2002). Recently it has been shown that paracellular mechanisms are also involved (Fischer et al., 2002; Wang et al., 2001). For example, the TJ proteins occludin and ZO-1 are phosphorylated *in vivo* and *in vitro* upon treatment with VEGF (Antonetti et al., 1999). VEGF-induced increase in permeability is preceded by the rapid phosphorylation of VE-cadherin (Bazzoni and Dejana, 2004). The Rho family of GTPases control vascular

permeability as histamine-induced permeability was shown to be dependant on activity of Rho and ROCK (Wojciak-Stothard et al., 2001).

1.5.4 Cell-cell contacts during angiogenesis

How are cell-cell contacts organized between cells that participate in angiogenesis? Are they completely dissolved or are there still junctional complexes or parts thereof? It seems that this has not been extensively studied. During formation of the *Drosophila* trachea, which is a tube of epithelial cells, AJs are extensively remodeled in an intricate manner but never completely dissolved as the cells "slide" past each other (Ribeiro et al., 2004). It is not known if endothelial cells behave in a similar manner. However, an interfering antibody has been used to show that VE-cadherin is not necessary for endothelial tube formation but rather prevents tube disassembly (Crosby et al., 2005). This argues for a model where AJs are formed at a late "maturation" stage of angiogenesis. In line with this, PECAM but not VE-cadherin, maintained cell-cell localization during tube formation in vitro (Wright et al., 2002), indicating that PECAM-based cell-cell contacts are present during angiogenesis. Interestingly, a possible role for R-cadherin, a cadherin usually expressed by neurons, in angiogenesis has been reported (Dorrell et al., 2002).

It is tempting to speculate that the formation of stable cell-cell contacts is necessary in order for the vessel to mature, just as it is necessary for a newly established vessel to recruit pericytes. In line with this, there is evidence that Ang1 participates in the maturation of vessels by reducing vessel permeability by controlling TJs (Gamble et al., 2000; Lee et al., 2003) and by the fact that VE-cadherin is not necessary for endothelial tube formation but prevents their disassembly (Crosby et al., 2005).

1.6 THE MODULAR NATURE OF PROTEINS

From crystallographic studies of proteins and sequence analysis it has become clear that larger proteins are often composed of smaller sequence domains that can fold independently. The domain is the functional unit of a protein, that is, it always binds to the same partner, or has the same catalytic effect, regardless in which protein it occurs.

Domains can be combined in multiple ways to build proteins. A domain can often fold independently from the rest of the protein and can therefore often be stable by itself (Thornton et al., 1999). Domain borders correlate strongly with exon borders (Liu and Grigoriev, 2004). This has led to the concept of the modular nature of proteins, where domains/exons have been duplicated and shuffled across the genome to build the large multi-domain proteins of eukaryotes. For this reason, protein domains can be thought of as "units of protein evolution".

1.6.1 PDZ domains and PDZ-binding domains

PDZ domains were first recognized as a 90 amino acid repetitive sequence elements in three different proteins: PSD-95(Post-synaptic density protein), the *Drosophila* protein Discs-large and ZO-1. A PDZ domain binds specifically to C-termini with certain motifs. The last three amino acids of the C-terminal are the most important for binding although specificity can sometimes be seen all the way to the -8 position (Songyang et al., 1997). PDZ domains are often repeated several times. One protein, MUPP1, has 13 PDZ domains while ZO-1 has three PDZ domains in a row. PDZ-containing proteins usually use these to cluster other proteins. PDZ binding motifs often occur in membrane spanning proteins that leave their C-termini inside the cell. Thus one protein with several PDZ domains can often function as a scaffold and cluster several transmembrane proteins at the cell membrane (Sheng and Sala, 2001).

1.6.2 Coiled-coil domains

Two alpha-helices may, if they have hydrophobic side chains in a certain repetitive pattern, coil around each other to form a helix. This helix, the coiled-coil, can be the structural element of fibrous proteins such as the intermediate filament protein cytokeratin or the motor protein myosin. However, coiled-coils can also be important protein-protein interaction domains, or as in the case of transcription factors, form a protein-DNA interaction site.

The sequence of coiled-coils are characterized by a seven-residue repeat, (**a**-**b**-**c**-**d**-**e**-**f**-**g**). The first (**a**) and fourth (**d**) position form the dimerization interface between the helices and are generally occupied by hydrophobic amino acids. The other amino acids in the repeat are mostly polar or charged and can form inter- and intrahelical interactions that contribute to the stability and specificity of the complex. The repeat pattern has made it possible to build algorithms - such as *Coils* - that can identify coiled-coil domains in primary sequence (Lupas et al., 1991).

2 THE PRESENT INVESTIGATION

2.1 AIMS OF THE PRESENT STUDY

The success of the VEGF antibody bevacizumab indicates that we are entering the era of targeted angiogenesis therapy for the treatment of cancer. Still, the patient benefit was a modest increase in median survival (see section 1.2.4.1). Basically it works, but not well enough to stabilize the disease. Possibly the effect of blocking VEGF becomes obsolete as the tumor upregulates other angiogenic factors. In any case, other types of anti-angiogenesis drugs should be developed and tried. Obvious candidates for this are the endogenous inhibitors of angiogenesis, such as angiostatin. However, recombinant angiostatin has been difficult to produce in sufficient amounts and must be injected daily by the patient as it has a short half life in circulation. If we could understand the mechanism of angiostatin, we could proceed to design an angiostatin mimetic that is stable and efficiently blocks angiogenesis.

The overall purpose of this thesis is to verify angiomotin as a drug target for anti-angiogenic therapy, specifically:

- To identify similar proteins and protein domains that could help us understand angiomotin (paper I).
- To investigate the role of angiomotin in hemangioendothelioma invasion (paper II)
- To understand how angiomotin is connected to signaling pathways that control the actin cytoskeleton (paper III).
- To investigate if angiostatin binds angiomotin on the cell surface (paper IV).

2.2 RESULTS AND DISCUSSION

2.2.1 A novel protein family (paper I)

A reasonable hypothesis about the function of a protein can often be proposed by comparing its sequence to that of well characterized proteins and by analyzing the domain composition. For that reason, we undertook bioinformatics analysis of the sequence of angiomotin.

It was revealed that angiomotin was not similar to any known protein and could not be classified as belonging to any known protein family. Instead, we found that angiomotin belongs to a protein family of its own, the angiomotin (or, sometimes motin) family of proteins. The motin proteins are large, multi-domain proteins of up to 1000 amino acids (Figure 7).

In human and mouse, where we know the sequence of the entire genomes, we can assert that the angiomotin family of proteins has three members; angiomotin, and two genes that has been named angiomotin like 1 (Amotl1) and angiomotin like 2 (Amotl2) by the HUGO Gene Nomenclature Committee. We identified human Amotl1 by sequencing and searches in GenBank with BLAST.

Sequences similar to the angiomotin family of proteins were identified in several species in addition to human and mouse, including the zebra fish, the *Xenopus* frog and *C. elegans*. No similar sequence was found in yeast, *E. coli* or plants, suggesting that only metazoa (i.e. animals) have these genes.

We reported that a multiple sequence alignment of the most conserved domain, the coiled-coil, of human and mouse sequences for these three proteins (total six sequences) show a 64% *similarity* over 455 residues, using the PAM 250 substitution matrix. However, the sequence *identity* (as opposed to *similarity* as determined by a substitution matrix) for this multiple sequence alignment is 42 % over 300 amino acids.

When discussing sequence homology, it is useful to have an idea about what should be considered a significant homology. One criterion for being "homologous" is having similar high resolution structures. It has been shown, that, when two sequences are between 70-100% identical; they are likely to have identical structures. A sequence identity of between 30% and 70% over 100 residues is intermediate; there is evolutionary conservation but the structures of the proteins may differ. Below 30 % identity is the "Twilight Zone" where it is difficult to know if there is a true similarity or not and where there will be a large number of false positives. When judging alignments, several parameters such as the length of the sequence and the number of gaps have to be considered, but as a rule of thumb, 30% identity is often used as a cut-off for determining homology for pair wise alignments. For the alignments of six sequences, where identity can be expected to be lower than in pair wise alignments, 42% identity over 300 residues must be considered a significant homology. In addition several splice sites were conserved in the angiomotin family, as reported. Thus, there is little doubt that the genes are evolutionarily related.

This was the first publication that reported the sequence of human Amotl1 and analyzed the high similarity between these genes. Since then other groups have also contributed, resulting in mouse Amotl1 also being called junction enriched and -associated protein (JEAP) (Nishimura et al., 2002) and Amotl2 being called MAGI-1-associated coiled-coil protein (MASCOT) (Patrie, 2005).

The most significant similarity within the angiomotin family of proteins is within the central coiled-coil region. It has recently been reported that Amotl1 and Amotl2 self-associates. At least in the case of Amotl2 it has been shown that this dimerization is mediated by the coiled-coil domain. However, Amotl1 and Amotl2 does not bind to each other which shows that the coiled-coil domain, despite the high sequence similarity, has a high degree of specificity (Patrie, 2005).

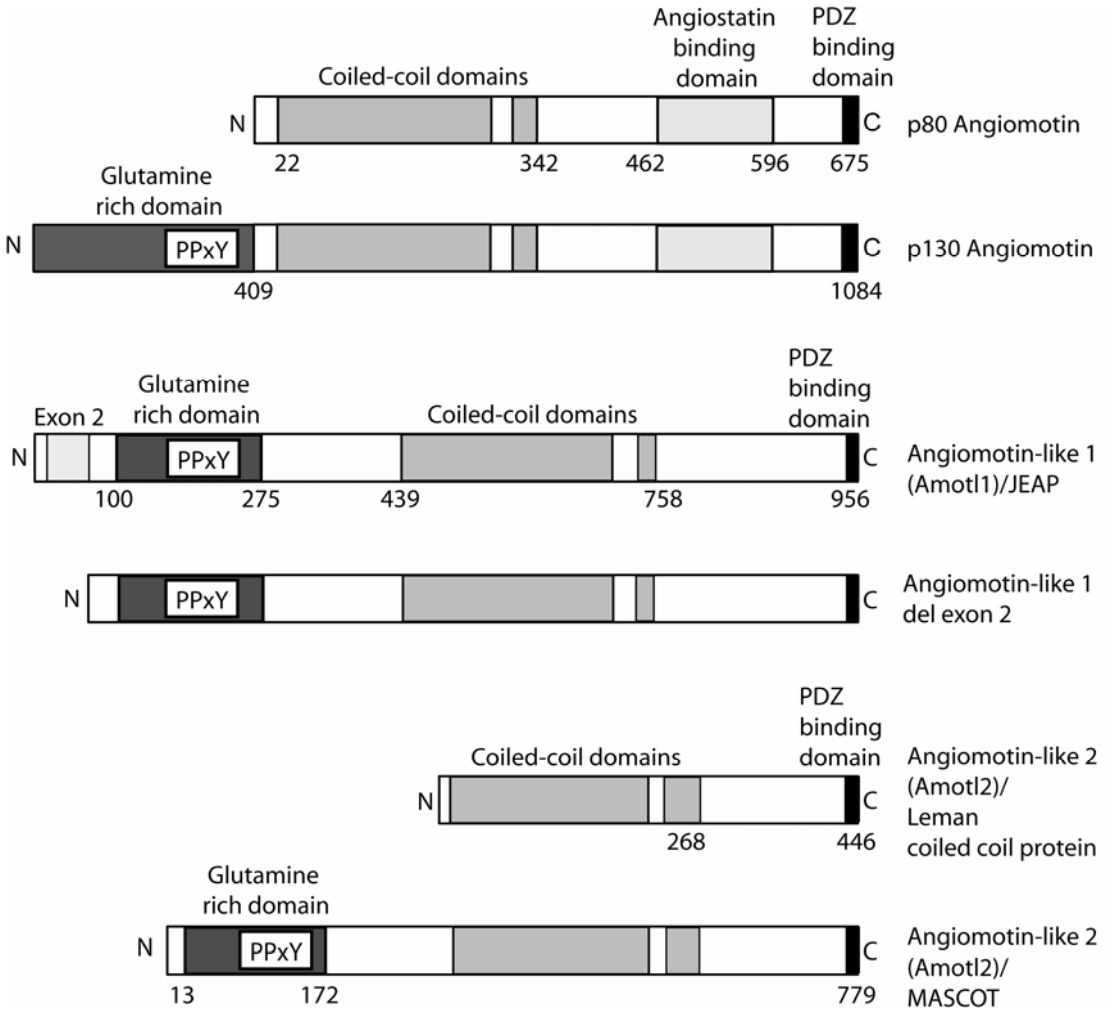

Figure 7. *The angiomotin family of proteins. Numbers indicate domain borders and residue count from the N-terminal. See sections 1.3, 2.2.1 and 2.2.4 for details.*

In addition, the C- and N-termini of the proteins have conserved motifs. The C-terminal is predicated to form a PDZ binding motif and truncation of as little as three amino acids of the C-terminal of angiomotin results in an inhibitory protein (Levchenko et al., 2003).

We also reported that the N-termini of Amotl1 and Amotl2 have several glutamine rich stretches which are conserved. When the p130 angiomotin splice isoform of angiomotin was found we anticipated that such glutamine rich areas would be found in p130 as well. Such a sequence was indeed found. A multiple sequence alignment of the first 243 residues of human angiomotin with human Amotl1 and Amotl2 shows that 58 % are conserved, with five stretches of 8-10 residues having 100 % similarity.

Over time it has become clear that alternative splicing occurs for all three members of this protein family. In the case of angiomotin there is p130 angiomotin which has an N-

terminal extension of 409 amino acids compared to p80 angiomotin (Ernkvist et al., 2005). There exists corresponding isoforms in the case of Amotl2, one long and one short (originally named Leman Coiled Coil Protein). In the case of Amotl1, there is also alternate splicing. One splice form lacks exon 2, resulting in a deletion of 50 amino acids. Another splice form, where both exons 2 and 3 are deleted, was also reported. This mRNA would code for a "p80" Amotl1, if there had not been a frame shift that leads to a premature termination of translation (Moreau et al., 2005).

Conclusions from paper I:

- Angiomotin belongs to a novel protein family with two additional members, Angiomotin like 1 and Angiomotin like 2.
- Members of this novel protein family have highly conserved coiled-coil and PDZ binding domains and glutamine rich domains.

2.2.2 Angiomotin promotes invasion of endothelial cells *in vivo* (paper II)

The C-terminal putative PDZ binding motif of angiomotin is important for controlling cell motility and tube formation on matrigel (Levchenko et al., 2003). In this study we found that angiomotin MAE cells invaded matrigel upon prolonged cultivation, and that angiomotin promoted invasion of 3D collagen in a microcarrier bead system. Cells expressing angiomotin with a 3 amino acid truncation of the PDZ binding motif (Δ4 angiomotin), however, invaded less than control cells. When injected into mice, the MAE cells formed slow growing tumors reminiscent of hemangioendothelioma. Angiomotin-expressing tumors grew faster and were more invasive than vector tumors. Importantly, Δ4 angiomotin tumors remained dormant and did not invade. Expression of angiomotin did not affect cell proliferation or proteolytic activity. However, Δ4 angiomotin tumors had a higher apoptotic index. Finally, time lapse microscopy revealed that angiomotin cells migrated more than vector cells which in turn migrated more than Δ4 angiomotin cells.

The results indicate that angiomotin controls signaling pathways that regulate migration, invasion and cell survival. The data also confirms previous findings that expression of Δ4 angiomotin inhibits cell motility (Levchenko et al., 2003). Furthermore, we found that expression of angiomotin stabilized matrigel tubes. Tubes of cells expressing Δ4 angiomotin or vector regressed after 5 days but tubes expressing angiomotin were stable for up to 30 days.

Conclusions from paper II:

- Angiomotin promotes migration and invasion of endothelial cells *in vivo*.
- The C-terminal PDZ-binding motif is crucial for angiomotin-mediated invasion and migration.
- Angiomotin promotes tube stability *in vitro*.

2.2.3 The role of ROCK in angiomotin-angiostatin signaling (paper III).

In this paper, we first show that angiomotin expression promotes cell spreading of MAE cells by a mechanism that is independent of a specific set of integrins and that expression of angiomotin mutated by truncation of the PDZ binding domain (Δ4 angiomotin) results in impaired cell spreading. Inhibiting the Rho effector Rho kinase p160ROCK (ROCK) with compound Y-27632 or dominant negative ROCK increased cell spreading of Δ4 angiomotin expressing cells to the level of cells expressing wild-type angiomotin.

Cells expressing Δ4angiomotin were defective in tube formation on matrigel as were cells expressing wild-type angiomotin when treated with angiostatin. However, both these effects could be reversed by inhibiting ROCK with Y-27632. This indicates that angiostatin treatment as well as the Δ4 mutation inhibits motility of MAE cells by elevating ROCK activity, and suggests that truncation of the C-terminal putative PDZ binding motif of angiomotin mimics the effect of angiostatin.

Attempts to analyze the activity of the Rho family of GTPases in this model were inconclusive, and additional studies are needed to address this issue.

Conclusions from paper III:

- Angiomotin promotes cell spreading and truncation of the C-terminal PDZ binding domain inhibits cell spreading, indicating that angiomotin controls arrangement of the cytoskeleton
- Angiomotin promoted cell spreading on different matrix proteins, indicating that a certain set of integrins is not responsible for angiomotin-mediated cell spreading.
- The effect of angiostatin and the inhibitory mutant of angiomotin is dependant on the activity of ROCK in MAE cells.

2.2.4 Angiomotin is a membrane protein that controls cell-cell junctions (paper IV).

In this study we provide the following evidence that angiomotin can act as a membrane protein and bind angiostatin on the cell surface: 1. Angiomotin was biotinylated by cell-surface labeling of intact transfected, as well as primary, ECs. 2. Parts of the protein is sensitive to trypsin degradation. 3. Angiomotin occurs in the detergent-rich fraction after Triton X-114 partitioning. 4. Angiostatin binds specifically to the surface of angiomotin-transfected cells. 5. Angiomotin and angiostatin could be crosslinked by a crosslinker that does not pass through the cell membrane. 6. Antibodies directed against the angiostatin binding domain bind to angiomotin on the cell surface of transfected cells. 7. Angiomotin and angiostatin interacts *in vitro*. We conclude that angiomotin can act as transmembrane protein and can serve as a receptor for angiostatin.

We provide a model for the transmembrane conformation of angiomotin and we argue that the partly hydrophobic domain of angiomotin forms transmembrane helices and can be inserted into the membrane by unknown mechanisms. Angiomotin is the only

family member with this type of domain, and therefore it is unlikely that Amotl1 or Amotl2 are membrane proteins or bind angiostatin.

We then proceeded to show that angiomotin co-localizes with ZO-1 in endothelial cell-cell junctions *in vitro* and *in vivo*. Interestingly, angiomotin expression is regulated by formation of cell contacts *in vitro*.

Furthermore, using immunoprecipitation, we show that the N-terminal of p130 angiomotin interacts with the TJ protein MAGI-1. It has recently been reported that MAGI-1 interacts with Amotl1 and Amotl2. This is mediated through the WW domain of MAGI-1 (Patrie, 2005). The N-termini of human and mouse sequences of p130 angiomotin, Amotl1 and Amotl2 have a completely conserved PPxY motif that could interact with the WW-motif of MAGI-1 (Figure 7).

p80 angiomotin, which does not bind MAGI-1 also localizes to cell-cell junctions. This indicates that the interaction with MAGI-1 is not necessary for this localization. In line with this, it has recently been reported that the localization of Amotl2 to tight junctions is mediated by the coiled-coil and the C-terminal domains (Patrie, 2005).

To study the functionality of angiomotin in cell-cell contacts we analyzed permeability of cell layers expressing angiomotin. We found that expression of p80 as well as p130 angiomotin reduced cell layer permeability in a manner similar to characterized transmembrane TJ proteins. Since angiostatin has been shown to affect migration of angiomotin-expressing cells, we analyzed if angiostatin could also affect the permeability of such cells. However, no such effect could be found. We concluded that angiostatin controls angiomotin-mediated cell migration but not angiomotin-mediated control of cell permeability.

Several lines of evidence suggest that angiomotin localizes to tight junctions rather than adherens junctions: 1. Angiomotin co-localizes with ZO-1 and may recruit ZO-1 to stress fibers *in vitro*. ZO-1 is a marker for TJs, and has reported to only transiently localize to AJs. 2. Angiomotin co-localizes and binds to MAGI-1, which is a component of TJs. 3. Angiomotin controls permeability. 4. The family member Angiomotin like 1/ JEAP localizes to TJs. However, gold-immuno electron microscopy (EM) is the "golden" standard method to distinguish between tight junctions and adherens junctions, as fluorescence microscopy can not resolve these structures. In immuno-EM, one would expect a TJ protein to be associated with electron dense TJs and localize more apical than AJ proteins such as VE-cadherin. We have carried out such studies, but so far they have been inconclusive.

It might seem contradictory that angiomotin can stimulate EC motility and at the same time induce the formation of cell-cell contacts. However, expression of JAM and ESAM which are transmembrane tight junction proteins also seems to correlate with increased cell motility (Ishida et al., 2003; Naik et al., 2003).

One finding from angiomotin-deficient mice is that the embryos display impaired migration of the anterior visceral endoderm (AVE). This phenotype is consistent with angiomotin promoting motility. But it is also compatible with angiomotin having a role in cell-cell contacts as it has been suggested that direct contact between AVE cells and the epiblast guides AVE migration (Srinivas et al., 2004).

Conclusions from paper IV:

- Angiomotin binds angiostatin on the cell surface.
- Angiomotin localizes to cell-cell contacts.

- p130 angiomotin interacts with the tight junction protein MAGI-1
- Angiomotin controls cell permeability but the effect of angiostatin is limited to inhibiting angiomotin-mediated control of cell migration.
- This work provides the rationale for designing anti-angiomotin antibodies as a starting point for the development of novel anti-angiogenic drugs.

3 FUTURE PERSPECTIVES

Expression of angiomotin correlates with increased cell motility as measured by several different methods. Angiomotin also controls cell morphology and cell-cell contacts. Furthermore, angiomotin localizes to F-actin-rich structures: lamellipodia, stress fibers and cell-cell contacts. These properties suggest that angiomotin binds to, and controls, cytoskeletal proteins.

Therefore, one of the most important future steps in understanding angiomotin is identifying binding partners that can explain these properties of angiomotin, and connect this intriguing protein to a well-known signaling pathway. In particular, the identification of the PDZ –containing protein that binds to the C-terminal of angiomotin could explain the effect of angiomotin on cell motility. One possibility is that the PDZ-containing protein is GEF or a GAP that controls the activation state of Rho. Our data suggest that this may also shed light on the mechanism of angiostatin. Closely related are the questions of how angiomotin controls cell shape and permeability.

Another interesting question is the different roles of p80 and p130 angiomotin. So far our observations suggest that the two isoforms are often expressed together in the same cell but that they can also be expressed alone. Do they interact and can one isoform control the activity of the other? More knowledge about angiomotin like 1 and -2 may show what this novel and interesting family of proteins have in common.

Of academic interest is also the issue of by what mechanism angiomotin is inserted into the membrane. It is possible, for example, that angiomotin is localized to endocytotic vesicles. Also, it is not clear how large a fraction of the angiomotin pool is localized on the cell surface.

From a clinical perspective, the possibility of targeting angiomotin as an anti-angiogenic therapy for cancer should be investigated. As angiomotin has extracellular epitopes, an antibody could be designed to interfere with the pro-angiogenic properties of angiomotin.

Finally, is there a common theme that explains why angiogenesis can be controlled by proteins (angiostatin and endostatin) that are cleavage fragments of larger proteins that are normally not considered as regulators of angiogenesis (plasminogen and collagen)? Is this a mechanism that regulates physiological processes other than angiogenesis?

Molecular biology - in the wide sense - has provided understanding for how cancers arise. The time has now come to convert this knowledge into patient benefit by designing drugs that target tumors. But "rational drug design" which is based on knowledge of the molecular basis of disease, has proven to be difficult. Lately however, molecular biology and rational drug design have started to deliver as some "smart" drugs have been launched. Hopefully, that is just the beginning of a long winning streak against cancer.

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5 REFERENCES

Abramsson, A., P. Lindblom, and C. Betsholtz. 2003. Endothelial and nonendothelial sources of PDGF-B regulate pericyte recruitment and influence vascular pattern formation in tumors. *J Clin Invest*. 112:1142-51.

Aepfelbacher, M., M. Essler, E. Huber, M. Sugai, and P.C. Weber. 1997. Bacterial Toxins Block Endothelial Wound Repair: Evidence That Rho GTPases Control Cytoskeletal Rearrangements in Migrating Endothelial Cells. 17:1623-1629.

Amano, M., M. Ito, K. Kimura, Y. Fukata, K. Chihara, T. Nakano, Y. Matsuura, and K. Kaibuchi. 1996. Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). *J Biol Chem*. 271:20246-9.

Antonetti, D.A., A.J. Barber, L.A. Hollinger, E.B. Wolpert, and T.W. Gardner. 1999. Vascular endothelial growth factor induces rapid phosphorylation of tight junction proteins occludin and zonula occluden 1. A potential mechanism for vascular permeability in diabetic retinopathy and tumors. *J Biol Chem*. 274:23463-7.

Asahara, T., T. Murohara, A. Sullivan, M. Silver, R. van der Zee, T. Li, B. Witzenbichler, G. Schatteman, and J.M. Isner. 1997. Isolation of putative progenitor endothelial cells for angiogenesis. *Science*. 275:964-7.

Ballestrem, C., B. Hinz, B.A. Imhof, and B. Wehrle-Haller. 2001. Marching at the front and dragging behind: differential alphaVbeta3-integrin turnover regulates focal adhesion behavior. *J Cell Biol*. 155:1319-32.

Bates, D.O., N.J. Hillman, B. Williams, C.R. Neal, and T.M. Pocock. 2002. Regulation of microvascular permeability by vascular endothelial growth factors. *J Anat*. 200:581-97.

Bazzoni, G., and E. Dejana. 2004. Endothelial cell-to-cell junctions: molecular organization and role in vascular homeostasis. *Physiol Rev*. 84:869-901.

Benelli, R., M. Morini, F. Carrozzino, N. Ferrari, S. Minghelli, L. Santi, M. Cassatella, D.M. Noonan, and A. Albini. 2002. Neutrophils as a key cellular target for angiostatin: implications for regulation of angiogenesis and inflammation. *Faseb J*. 16:267-9.

Bergers, G., S. Song, N. Meyer-Morse, E. Bergsland, and D. Hanahan. 2003. Benefits of targeting both pericytes and endothelial cells in the tumor vasculature with kinase inhibitors. *J Clin Invest*. 111:1287-95.

Bishop, A.L., and A. Hall. 2000. Rho GTPases and their effector proteins. *Biochem J*. 348 Pt 2:241-55.

Bix, G., and R.V. Iozzo. 2005. Matrix revolutions: "tails" of basement-membrane components with angiostatic functions. *Trends Cell Biol*. 15:52-60.

Breviario, F., L. Caveda, M. Corada, I. Martin-Padura, P. Navarro, J. Golay, M. Introna, D. Gulino, M.G. Lampugnani, and E. Dejana. 1995. Functional properties of human vascular endothelial cadherin (7B4/cadherin-5), an endothelium-specific cadherin. *Arterioscler Thromb Vasc Biol*. 15:1229-39.

Brooks, P.C., A.M. Montgomery, M. Rosenfeld, R.A. Reisfeld, T. Hu, G. Klier, and D.A. Cheresh. 1994. Integrin alpha v beta 3 antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels. *Cell*. 79:1157-64.

Burri, P.H., R. Hlushchuk, and V. Djonov. 2004. Intussusceptive angiogenesis: its emergence, its characteristics, and its significance. *Dev Dyn*. 231:474-88.

Cao, R., H.L. Wu, N. Veitonmaki, P. Linden, J. Farnebo, G.Y. Shi, and Y. Cao. 1999. Suppression of angiogenesis and tumor growth by the inhibitor K1-5 generated by plasmin-mediated proteolysis. *Proc Natl Acad Sci U S A*. 96:5728-33.

Cao, Y., A. Chen, S.S. An, R.W. Ji, D. Davidson, and M. Llinas. 1997. Kringle 5 of plasminogen is a novel inhibitor of endothelial cell growth. *J Biol Chem*. 272:22924-8.

Carmeliet, P. 2000. Mechanisms of angiogenesis and arteriogenesis. *Nat Med*. 6:389-95.

Carmeliet, P., V. Ferreira, G. Breier, S. Pollefeyt, L. Kieckens, M. Gertsenstein, M. Fahrig, A. Vandenhoeck, K. Harpal, C. Eberhardt, C. Declercq, J. Pawling, L.

Moons, D. Collen, W. Risau, and A. Nagy. 1996. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature*. 380:435-9.
Carmeliet, P., and R.K. Jain. 2000. Angiogenesis in cancer and other diseases. *Nature*. 407:249-57.
Carmeliet, P., M.G. Lampugnani, L. Moons, F. Breviario, V. Compernolle, F. Bono, G. Balconi, R. Spagnuolo, B. Oostuyse, M. Dewerchin, A. Zanetti, A. Angellilo, V. Mattot, D. Nuyens, E. Lutgens, F. Clotman, M.C. de Ruiter, A. Gittenberger-de Groot, R. Poelmann, F. Lupu, J.M. Herbert, D. Collen, and E. Dejana. 1999. Targeted deficiency or cytosolic truncation of the VE-cadherin gene in mice impairs VEGF-mediated endothelial survival and angiogenesis. *Cell*. 98:147-57.
Cera, M.R., A. Del Prete, A. Vecchi, M. Corada, I. Martin-Padura, T. Motoike, P. Tonetti, G. Bazzoni, W. Vermi, F. Gentili, S. Bernasconi, T.N. Sato, A. Mantovani, and E. Dejana. 2004. Increased DC trafficking to lymph nodes and contact hypersensitivity in junctional adhesion molecule-A-deficient mice. *J Clin Invest*. 114:729-38.
Chen, X., and I.G. Macara. 2005. Par-3 controls tight junction assembly through the Rac exchange factor Tiam1. *Nat Cell Biol*. 7:262-9.
Christofori, G., and H. Semb. 1999. The role of the cell-adhesion molecule E-cadherin as a tumour-suppressor gene. *Trends Biochem Sci*. 24:73-6.
Claesson-Welsh, L., M. Welsh, N. Ito, B. Anand-Apte, S. Soker, B. Zetter, M. O'Reilly, and J. Folkman. 1998. Angiostatin induces endothelial cell apoptosis and activation of focal adhesion kinase independently of the integrin-binding motif RGD. *Proc Natl Acad Sci U S A*. 95:5579-83.
Crosby, C.V., P.A. Fleming, W.S. Argraves, M. Corada, L. Zanetta, E. Dejana, and C.J. Drake. 2005. VE-cadherin is not required for the formation of nascent blood vessels but acts to prevent their disassembly. *Blood*. 105:2771-6.
Dong, Z., R. Kumar, X. Yang, and I.J. Fidler. 1997. Macrophage-derived metalloelastase is responsible for the generation of angiostatin in Lewis lung carcinoma. *Cell*. 88:801-10.
Dorrell, M.I., E. Aguilar, and M. Friedlander. 2002. Retinal vascular development is mediated by endothelial filopodia, a preexisting astrocytic template and specific R-cadherin adhesion. *Invest Ophthalmol Vis Sci*. 43:3500-10.
Eriksson, K., P. Magnusson, J. Dixelius, L. Claesson-Welsh, and M.J. Cross. 2003. Angiostatin and endostatin inhibit endothelial cell migration in response to FGF and VEGF without interfering with specific intracellular signal transduction pathways. *FEBS Lett*. 536:19-24.
Ernkvist, M., K. Aase, C. Ukomadu, Y. Zhou, J. Wohlschlegel, R. Blackman, N. Veitonmäki, A. Bratt, S. Fisher, A. Dutta, and L. Holmgren. 2005. p130-angiomotin associates to actin and controls endothelial cell shape. *submitted*.
Etienne-Manneville, S., and A. Hall. 2002. Rho GTPases in cell biology. *Nature*. 420:629-35.
Ferrara, N., K. Carver-Moore, H. Chen, M. Dowd, L. Lu, K.S. O'Shea, L. Powell-Braxton, K.J. Hillan, and M.W. Moore. 1996. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature*. 380:439-42.
Fischer, S., M. Wobben, H.H. Marti, D. Renz, and W. Schaper. 2002. Hypoxia-induced hyperpermeability in brain microvessel endothelial cells involves VEGF-mediated changes in the expression of zonula occludens-1. *Microvasc Res*. 63:70-80.
Folkman, J. 1971. Tumor angiogenesis: therapeutic implications. *N Engl J Med*. 285:1182-6.
Folkman, J. 1995. Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat Med*. 1:27-31.
Folkman, J. 2004. Endogenous angiogenesis inhibitors. *Apmis*. 112:496-507.
Folkman, J., E. Merler, C. Abernathy, and G. Williams. 1971. Isolation of a tumor factor responsible for angiogenesis. *J Exp Med*. 133:275-88.

Fong, G.H., J. Rossant, M. Gertsenstein, and M.L. Breitman. 1995. Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature*. 376:66-70.
Furuhashi, M., T. Sjoblom, A. Abramsson, J. Ellingsen, P. Micke, H. Li, E. Bergsten-Folestad, U. Eriksson, R. Heuchel, C. Betsholtz, C.H. Heldin, and A. Ostman. 2004. Platelet-derived growth factor production by B16 melanoma cells leads to increased pericyte abundance in tumors and an associated increase in tumor growth rate. *Cancer Res*. 64:2725-33.
Furuse, M., K. Fujita, T. Hiiragi, K. Fujimoto, and S. Tsukita. 1998. Claudin-1 and -2: novel integral membrane proteins localizing at tight junctions with no sequence similarity to occludin. *J Cell Biol*. 141:1539-50.
Gamble, J.R., J. Drew, L. Trezise, A. Underwood, M. Parsons, L. Kasminkas, J. Rudge, G. Yancopoulos, and M.A. Vadas. 2000. Angiopoietin-1 is an antipermeability and anti-inflammatory agent in vitro and targets cell junctions. *Circ Res*. 87:603-7.
Gerhardt, H., M. Golding, M. Fruttiger, C. Ruhrberg, A. Lundkvist, A. Abramsson, M. Jeltsch, C. Mitchell, K. Alitalo, D. Shima, and C. Betsholtz. 2003. VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. *J Cell Biol*. 161:1163-77.
Giancotti, F.G., and E. Ruoslahti. 1999. Integrin signaling. *Science*. 285:1028-32.
Gupta, N., E. Nodzenski, N.N. Khodarev, J. Yu, L. Khorasani, M.A. Beckett, D.W. Kufe, and R.R. Weichselbaum. 2001. Angiostatin effects on endothelial cells mediated by ceramide and RhoA. *EMBO Rep*. 2:536-40.
Hanahan, D., and J. Folkman. 1996. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell*. 86:353-64.
Hanahan, D., and R.A. Weinberg. 2000. The hallmarks of cancer. *Cell*. 100:57-70.
Hirata, K., T. Ishida, K. Penta, M. Rezaee, E. Yang, J. Wohlgemuth, and T. Quertermous. 2001. Cloning of an immunoglobulin family adhesion molecule selectively expressed by endothelial cells. *J Biol Chem*. 276:16223-31.
Hoang, M.V., M.C. Whelan, and D.R. Senger. 2004. Rho activity critically and selectively regulates endothelial cell organization during angiogenesis. *Proc Natl Acad Sci U S A*. 101:1874-9. Epub 2004 Feb 9.
Hodivala-Dilke, K.M., K.P. McHugh, D.A. Tsakiris, H. Rayburn, D. Crowley, M. Ullman-Cullere, F.P. Ross, B.S. Coller, S. Teitelbaum, and R.O. Hynes. 1999. Beta3-integrin-deficient mice are a model for Glanzmann thrombasthenia showing placental defects and reduced survival. *J Clin Invest*. 103:229-38.
Holmgren, L., M.S. O'Reilly, and J. Folkman. 1995. Dormancy of micrometastases: balanced proliferation and apoptosis in the presence of angiogenesis suppression. *Nat Med*. 1:149-53.
Hurwitz, H., L. Fehrenbacher, W. Novotny, T. Cartwright, J. Hainsworth, W. Heim, J. Berlin, A. Baron, S. Griffing, E. Holmgren, N. Ferrara, G. Fyfe, B. Rogers, R. Ross, and F. Kabbinavar. 2004. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. *N Engl J Med*. 350:2335-42.
Ilic, D., Y. Furuta, S. Kanazawa, N. Takeda, K. Sobue, N. Nakatsuji, S. Nomura, J. Fujimoto, M. Okada, and T. Yamamoto. 1995. Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. *Nature*. 377:539-44.
Inai, T., J. Kobayashi, and Y. Shibata. 1999. Claudin-1 contributes to the epithelial barrier function in MDCK cells. *Eur J Cell Biol*. 78:849-55.
Ishida, T., R.K. Kundu, E. Yang, K. Hirata, Y.D. Ho, and T. Quertermous. 2003. Targeted disruption of endothelial cell-selective adhesion molecule inhibits angiogenic processes in vitro and in vivo. *J Biol Chem*. 278:34598-604.
Itoh, M., M. Furuse, K. Morita, K. Kubota, M. Saitou, and S. Tsukita. 1999. Direct binding of three tight junction-associated MAGUKs, ZO-1, ZO-2, and ZO-3, with the COOH termini of claudins. *J Cell Biol*. 147:1351-63.
Itoh, M., A. Nagafuchi, S. Moroi, and S. Tsukita. 1997. Involvement of ZO-1 in cadherin-based cell adhesion through its direct binding to alpha catenin and actin filaments. *J Cell Biol*. 138:181-92.
Ji, W.R., F.J. Castellino, Y. Chang, M.E. Deford, H. Gray, X. Villarreal, M.E. Kondri, D.N. Marti, M. Llinas, J. Schaller, R.A. Kramer, and P.A. Trail. 1998.

Characterization of kringle domains of angiostatin as antagonists of endothelial cell migration, an important process in angiogenesis. *Faseb J*. 12:1731-8.
Kaur, B., D.J. Brat, N.S. Devi, and E.G. Van Meir. 2005. Vasculostatin, a proteolytic fragment of brain angiogenesis inhibitor 1, is an antiangiogenic and antitumorigenic factor. *Oncogene*. 24:3632-42.
Kerbel, R.S. 2000. Tumor angiogenesis: past, present and the near future. *Carcinogenesis*. 21:505-15.
Kimura, K., M. Ito, M. Amano, K. Chihara, Y. Fukata, M. Nakafuku, B. Yamamori, J. Feng, T. Nakano, K. Okawa, A. Iwamatsu, and K. Kaibuchi. 1996. Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). *Science*. 273:245-8.
Kiosses, W.B., S.J. Shattil, N. Pampori, and M.A. Schwartz. 2001. Rac recruits high-affinity integrin alphavbeta3 to lamellipodia in endothelial cell migration. *Nat Cell Biol*. 3:316-20.
Lampugnani, M.G., M. Corada, L. Caveda, F. Breviario, O. Ayalon, B. Geiger, and E. Dejana. 1995. The molecular organization of endothelial cell to cell junctions: differential association of plakoglobin, beta-catenin, and alpha-catenin with vascular endothelial cadherin (VE-cadherin). *J Cell Biol*. 129:203-17.
Lampugnani, M.G., M. Resnati, M. Raiteri, R. Pigott, A. Pisacane, G. Houen, L.P. Ruco, and E. Dejana. 1992. A novel endothelial-specific membrane protein is a marker of cell-cell contacts. *J Cell Biol*. 118:1511-22.
Lauffenburger, D.A., and A.F. Horwitz. 1996. Cell migration: a physically integrated molecular process. *Cell*. 84:359-69.
Lay, A.J., X.M. Jiang, O. Kisker, E. Flynn, A. Underwood, R. Condron, and P.J. Hogg. 2000. Phosphoglycerate kinase acts in tumour angiogenesis as a disulphide reductase. *Nature*. 408:869-73.
Lee, S.W., W.J. Kim, Y.K. Choi, H.S. Song, M.J. Son, I.H. Gelman, Y.J. Kim, and K.W. Kim. 2003. SSeCKS regulates angiogenesis and tight junction formation in blood-brain barrier. *Nat Med*. 9:900-6.
Levchenko, T., K. Aase, B. Troyanovsky, A. Bratt, and L. Holmgren. 2003. Loss of responsiveness to chemotactic factors by deletion of the C-terminal protein interaction site of angiomotin. *J Cell Sci*. 116:3803-10.
Levchenko, T., K. Aase, B. Troyanovsky, A. Bratt, and L. Holmgren. 2003. Loss of responsiveness to chemotactic factors by deletion of the C-terminal protein interaction site of angiomotin. *J Cell Sci*. 116:3803-10. Epub 2003 Aug 5.
Levchenko, T., A. Bratt, J.L. Arbiser, and L. Holmgren. 2004. Angiomotin expression promotes hemangioendothelioma invasion. *Oncogene*. 23:1469-73.
Lijnen, H.R., F. Ugwu, A. Bini, and D. Collen. 1998. Generation of an angiostatin-like fragment from plasminogen by stromelysin-1 (MMP-3). *Biochemistry*. 37:4699-702.
Lindahl, P., M. Hellstrom, M. Kalen, and C. Betsholtz. 1998. Endothelial-perivascular cell signaling in vascular development: lessons from knockout mice. *Curr Opin Lipidol*. 9:407-11.
Liu, M., and A. Grigoriev. 2004. Protein domains correlate strongly with exons in multiple eukaryotic genomes--evidence of exon shuffling? *Trends Genet*. 20:399-403.
Lubarsky, B., and M.A. Krasnow. 2003. Tube morphogenesis: making and shaping biological tubes. *Cell*. 112:19-28.
Lucas, R., L. Holmgren, I. Garcia, B. Jimenez, S.J. Mandriota, F. Borlat, B.K. Sim, Z. Wu, G.E. Grau, Y. Shing, G.A. Soff, N. Bouck, and M.S. Pepper. 1998. Multiple forms of angiostatin induce apoptosis in endothelial cells. *Blood*. 92:4730-41.
Lupas, A., M. Van Dyke, and J. Stock. 1991. Predicting coiled coils from protein sequences. *Science*. 252:1162-4.
Ma, L., X. Wang, Z. Zhang, X. Zhou, A. Chen, and L. Yao. 2001. Identification of the ligand-binding domain of human vascular-endothelial-growth-factor receptor Flt-1. *Biotechnol Appl Biochem*. 34:199-204.
Maekawa, M., T. Ishizaki, S. Boku, N. Watanabe, A. Fujita, A. Iwamatsu, T. Obinata, K. Ohashi, K. Mizuno, and S. Narumiya. 1999. Signaling from Rho to the actin

cytoskeleton through protein kinases ROCK and LIM-kinase. *Science*. 285:895-8.
Maeshima, Y., A. Sudhakar, J.C. Lively, K. Ueki, S. Kharbanda, C.R. Kahn, N. Sonenberg, R.O. Hynes, and R. Kalluri. 2002. Tumstatin, an endothelial cell-specific inhibitor of protein synthesis. *Science*. 295:140-3.
Maisonpierre, P.C., C. Suri, P.F. Jones, S. Bartunkova, S.J. Wiegand, C. Radziejewski, D. Compton, J. McClain, T.H. Aldrich, N. Papadopoulos, T.J. Daly, S. Davis, T.N. Sato, and G.D. Yancopoulos. 1997. Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. *Science*. 277:55-60.
Martin-Padura, I., S. Lostaglio, M. Schneemann, L. Williams, M. Romano, P. Fruscella, C. Panzeri, A. Stoppacciaro, L. Ruco, A. Villa, D. Simmons, and E. Dejana. 1998. Junctional adhesion molecule, a novel member of the immunoglobulin superfamily that distributes at intercellular junctions and modulates monocyte transmigration. *J Cell Biol*. 142:117-27.
Matsumoto, T., and L. Claesson-Welsh. 2001. VEGF receptor signal transduction. *Sci STKE*. 2001:RE21.
Metheny-Barlow, L.J., and L.Y. Li. 2003. The enigmatic role of angiopoietin-1 in tumor angiogenesis. *Cell Res*. 13:309-17.
Millauer, B., L.K. Shawver, K.H. Plate, W. Risau, and A. Ullrich. 1994. Glioblastoma growth inhibited in vivo by a dominant-negative Flk-1 mutant. *Nature*. 367:576-9.
Moreau, J., M. Lord, M. Boucher, P. Belleau, and M.J. Fernandes. 2005. Protein diversity is generated within the motin family of proteins by alternative pre-mRNA splicing. *Gene*. 350:137-48.
Moser, T.L., D.J. Kenan, T.A. Ashley, J.A. Roy, M.D. Goodman, U.K. Misra, D.J. Cheek, and S.V. Pizzo. 2001. Endothelial cell surface F1-F0 ATP synthase is active in ATP synthesis and is inhibited by angiostatin. *Proc Natl Acad Sci U S A*. 98:6656-61.
Moser, T.L., M.S. Stack, I. Asplin, J.J. Enghild, P. Hojrup, L. Everitt, S. Hubchak, H.W. Schnaper, and S.V. Pizzo. 1999. Angiostatin binds ATP synthase on the surface of human endothelial cells. *Proc Natl Acad Sci U S A*. 96:2811-6.
Naik, M.U., S.A. Mousa, C.A. Parkos, and U.P. Naik. 2003. Signaling through JAM-1 and alphavbeta3 is required for the angiogenic action of bFGF: dissociation of the JAM-1 and alphavbeta3 complex. *Blood*. 102:2108-14.
Naik, M.U., D. Vuppalanchi, and U.P. Naik. 2003. Essential role of junctional adhesion molecule-1 in basic fibroblast growth factor-induced endothelial cell migration. *Arterioscler Thromb Vasc Biol*. 23:2165-71.
Nasdala, I., K. Wolburg-Buchholz, H. Wolburg, A. Kuhn, K. Ebnet, G. Brachtendorf, U. Samulowitz, B. Kuster, B. Engelhardt, D. Vestweber, and S. Butz. 2002. A transmembrane tight junction protein selectively expressed on endothelial cells and platelets. *J Biol Chem*. 277:16294-303.
Nguyen, A.D., S. Itoh, V. Jeney, H. Yanagisawa, M. Fujimoto, M. Ushio-Fukai, and T. Fukai. 2004. Fibulin-5 is a novel binding protein for extracellular superoxide dismutase. *Circ Res*. 95:1067-74.
Nishimori, H., T. Shiratsuchi, T. Urano, Y. Kimura, K. Kiyono, K. Tatsumi, S. Yoshida, M. Ono, M. Kuwano, Y. Nakamura, and T. Tokino. 1997. A novel brain-specific p53-target gene, BAI1, containing thrombospondin type 1 repeats inhibits experimental angiogenesis. *Oncogene*. 15:2145-50.
Nishimura, M., M. Kakizaki, Y. Ono, K. Morimoto, M. Takeuchi, Y. Inoue, T. Imai, and Y. Takai. 2002. JEAP, a novel component of tight junctions in exocrine cells. *J Biol Chem*. 277:5583-7.
Nitta, T., M. Hata, S. Gotoh, Y. Seo, H. Sasaki, N. Hashimoto, M. Furuse, and S. Tsukita. 2003. Size-selective loosening of the blood-brain barrier in claudin-5-deficient mice. *J Cell Biol*. 161:653-60.
Nobes, C.D., and A. Hall. 1995. Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell*. 81:53-62.
O'Reilly, M.S., T. Boehm, Y. Shing, N. Fukai, G. Vasios, W.S. Lane, E. Flynn, J.R. Birkhead, B.R. Olsen, and J. Folkman. 1997. Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. *Cell*. 88:277-85.

O'Reilly, M.S., L. Holmgren, C. Chen, and J. Folkman. 1996. Angiostatin induces and sustains dormancy of human primary tumors in mice. *Nat Med*. 2:689-92.
O'Reilly, M.S., L. Holmgren, Y. Shing, C. Chen, R.A. Rosenthal, M. Moses, W.S. Lane, Y. Cao, E.H. Sage, and J. Folkman. 1994. Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. *Cell*. 79:315-28.
O'Reilly, M.S., D. Wiederschain, W.G. Stetler-Stevenson, J. Folkman, and M.A. Moses. 1999. Regulation of angiostatin production by matrix metalloproteinase-2 in a model of concomitant resistance. *J Biol Chem*. 274:29568-71.
Ozdamar, B., R. Bose, M. Barrios-Rodiles, H.R. Wang, Y. Zhang, and J.L. Wrana. 2005. Regulation of the polarity protein Par6 by TGFbeta receptors controls epithelial cell plasticity. *Science*. 307:1603-9.
Patan, S., L.L. Munn, and R.K. Jain. 1996. Intussusceptive microvascular growth in a human colon adenocarcinoma xenograft: a novel mechanism of tumor angiogenesis. *Microvasc Res*. 51:260-72.
Patrie, K.M. 2005. Identification and characterization of a novel tight junction-associated family of proteins that interacts with a WW domain of MAGI-1. *Biochim Biophys Acta*. 1745:131-44.
Patrie, K.M., A.J. Drescher, A. Welihinda, P. Mundel, and B. Margolis. 2002. Interaction of two actin-binding proteins, synaptopodin and alpha-actinin-4, with the tight junction protein MAGI-1. *J Biol Chem*. 277:30183-90.
Patterson, B.C., and Q.A. Sang. 1997. Angiostatin-converting enzyme activities of human matrilysin (MMP-7) and gelatinase B/type IV collagenase (MMP-9). *J Biol Chem*. 272:28823-5.
Ribatti, D., A. Vacca, and F. Dammacco. 1999. The role of the vascular phase in solid tumor growth: a historical review. *Neoplasia*. 1:293-302.
Ribeiro, C., M. Neumann, and M. Affolter. 2004. Genetic control of cell intercalation during tracheal morphogenesis in Drosophila. *Curr Biol*. 14:2197-207.
Ridley, A.J., and A. Hall. 1992. The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell*. 70:389-99.
Ridley, A.J., H.F. Paterson, C.L. Johnston, D. Diekmann, and A. Hall. 1992. The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell*. 70:401-10.
Ridley, A.J., M.A. Schwartz, K. Burridge, R.A. Firtel, M.H. Ginsberg, G. Borisy, J.T. Parsons, and A.R. Horwitz. 2003. Cell migration: integrating signals from front to back. *Science*. 302:1704-9.
Riento, K., and A.J. Ridley. 2003. Rocks: multifunctional kinases in cell behaviour. *Nat Rev Mol Cell Biol*. 4:446-56.
Ruffer, C., A. Strey, A. Janning, K.S. Kim, and V. Gerke. 2004. Cell-cell junctions of dermal microvascular endothelial cells contain tight and adherens junction proteins in spatial proximity. *Biochemistry*. 43:5360-9.
Ruhrberg, C., H. Gerhardt, M. Golding, R. Watson, S. Ioannidou, H. Fujisawa, C. Betsholtz, and D.T. Shima. 2002. Spatially restricted patterning cues provided by heparin-binding VEGF-A control blood vessel branching morphogenesis. *Genes Dev*. 16:2684-98.
Saitou, M., M. Furuse, H. Sasaki, J.D. Schulzke, M. Fromm, H. Takano, T. Noda, and S. Tsukita. 2000. Complex phenotype of mice lacking occludin, a component of tight junction strands. *Mol Biol Cell*. 11:4131-42.
Schulze, C., and J.A. Firth. 1993. Immunohistochemical localization of adherens junction components in blood-brain barrier microvessels of the rat. *J Cell Sci*. 104 (Pt 3):773-82.
Senger, D.R., S.J. Galli, A.M. Dvorak, C.A. Perruzzi, V.S. Harvey, and H.F. Dvorak. 1983. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science*. 219:983-5.
Shalaby, F., J. Rossant, T.P. Yamaguchi, M. Gertsenstein, X.F. Wu, M.L. Breitman, and A.C. Schuh. 1995. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature*. 376:62-6.
Sheng, M., and C. Sala. 2001. PDZ domains and the organization of supramolecular complexes. *Annu Rev Neurosci*. 24:1-29.

Shimono, A., and R.R. Behringer. 2003. Angiomotin regulates visceral endoderm movements during mouse embryogenesis. *Curr Biol*. 13:613-7.
Shing, Y., J. Folkman, R. Sullivan, C. Butterfield, J. Murray, and M. Klagsbrun. 1984. Heparin affinity: purification of a tumor-derived capillary endothelial cell growth factor. *Science*. 223:1296-9.
Shiratsuchi, T., M. Futamura, K. Oda, H. Nishimori, Y. Nakamura, and T. Tokino. 1998. Cloning and characterization of BAI-associated protein 1: a PDZ domain-containing protein that interacts with BAI1. *Biochem Biophys Res Commun*. 247:597-604.
Soga, N., N. Namba, S. McAllister, L. Cornelius, S.L. Teitelbaum, S.F. Dowdy, J. Kawamura, and K.A. Hruska. 2001. Rho family GTPases regulate VEGF-stimulated endothelial cell motility. *Exp Cell Res*. 269:73-87.
Songyang, Z., A.S. Fanning, C. Fu, J. Xu, S.M. Marfatia, A.H. Chishti, A. Crompton, A.C. Chan, J.M. Anderson, and L.C. Cantley. 1997. Recognition of unique carboxyl-terminal motifs by distinct PDZ domains. *Science*. 275:73-7.
Srinivas, S., T. Rodriguez, M. Clements, J.C. Smith, and R.S. Beddington. 2004. Active cell migration drives the unilateral movements of the anterior visceral endoderm. *Development*. 131:1157-64.
Sten-Linder, M., C. Linder, H. Strander, E. Munck-Wikland, P. Wersall, S. Linder, and B. Wiman. 1999. Angiostatin fragments in urine from patients with malignant disease. *Anticancer Res*. 19:3409-14.
Storkebaum, E., D. Lambrechts, and P. Carmeliet. 2004. VEGF: once regarded as a specific angiogenic factor, now implicated in neuroprotection. *Bioessays*. 26:943-54.
Stromblad, S., J.C. Becker, M. Yebra, P.C. Brooks, and D.A. Cheresh. 1996. Suppression of p53 activity and p21WAF1/CIP1 expression by vascular cell integrin alphaVbeta3 during angiogenesis. *J Clin Invest*. 98:426-33.
Stupack, D.G., and D.A. Cheresh. 2004. Integrins and angiogenesis. *Curr Top Dev Biol*. 64:207-38.
Suri, C., P.F. Jones, S. Patan, S. Bartunkova, P.C. Maisonpierre, S. Davis, T.N. Sato, and G.D. Yancopoulos. 1996. Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. *Cell*. 87:1171-80.
Suzuki, S., K. Sano, and H. Tanihara. 1991. Diversity of the cadherin family: evidence for eight new cadherins in nervous tissue. *Cell Regul*. 2:261-70.
Tarui, T., L.A. Miles, and Y. Takada. 2001. Specific interaction of angiostatin with integrin alpha(v)beta(3) in endothelial cells. *J Biol Chem*. 276:39562-8.
Thornton, J.M., C.A. Orengo, A.E. Todd, and F.M. Pearl. 1999. Protein folds, functions and evolution. *J Mol Biol*. 293:333-42.
Troyanovsky, B., T. Levchenko, G. Mansson, O. Matvijenko, and L. Holmgren. 2001. Angiomotin: an angiostatin binding protein that regulates endothelial cell migration and tube formation. *J Cell Biol*. 152:1247-54.
Troyanovsky, B., T. Levchenko, G. Månsson, O. Matvijenko, and L. Holmgren. 2001. Angiomotin: An angiostatin binding protein that regulates endothelial cell migration and tube formation. *Journal of Cell Biology*. 152(6):1247-54.
Tsukita, S., M. Furuse, and M. Itoh. 2001. Multifunctional strands in tight junctions. *Nat Rev Mol Cell Biol*. 2:285-93.
Tuszynski, G.P., M.R. Sharma, V.L. Rothman, and M.C. Sharma. 2002. Angiostatin binds to tyrosine kinase substrate annexin II through the lysine-binding domain in endothelial cells. *Microvasc Res*. 64:448-62.
Wajih, N., and D.C. Sane. 2003. Angiostatin selectively inhibits signaling by hepatocyte growth factor in endothelial and smooth muscle cells. *Blood*. 101:1857-63.
van Wijngaarden, P., D.J. Coster, and K.A. Williams. 2005. Inhibitors of ocular neovascularization: promises and potential problems. *Jama*. 293:1509-13.
Wang, H., R. Schultz, J. Hong, D.L. Cundiff, K. Jiang, and G.A. Soff. 2004. Cell surface-dependent generation of angiostatin4.5. *Cancer Res*. 64:162-8.
Wang, H.R., Y. Zhang, B. Ozdamar, A.A. Ogunjimi, E. Alexandrova, G.H. Thomsen, and J.L. Wrana. 2003. Regulation of cell polarity and protrusion formation by targeting RhoA for degradation. *Science*. 302:1775-9.

Wang, W., W.L. Dentler, and R.T. Borchardt. 2001. VEGF increases BMEC monolayer permeability by affecting occludin expression and tight junction assembly. *Am J Physiol Heart Circ Physiol*. 280:H434-40.
Wegmann, F., K. Ebnet, L. Du Pasquier, D. Vestweber, and S. Butz. 2004. Endothelial adhesion molecule ESAM binds directly to the multidomain adaptor MAGI-1 and recruits it to cell contacts. *Exp Cell Res*. 300:121-33.
Veitonmaki, N., R. Cao, L.H. Wu, T.L. Moser, B. Li, S.V. Pizzo, B. Zhivotovsky, and Y. Cao. 2004. Endothelial cell surface ATP synthase-triggered caspase-apoptotic pathway is essential for k1-5-induced antiangiogenesis. *Cancer Res*. 64:3679-86.
Vestweber, D. 2000. Molecular mechanisms that control endothelial cell contacts. *J Pathol*. 190:281-91.
Wojciak-Stothard, B., S. Potempa, T. Eichholtz, and A.J. Ridley. 2001. Rho and Rac but not Cdc42 regulate endothelial cell permeability. *J Cell Sci*. 114:1343-55.
Volm, M., J. Mattern, and R. Koomagi. 2000. Angiostatin expression in non-small cell lung cancer. *Clin Cancer Res*. 6:3236-40.
Worthylake, R.A., and K. Burridge. 2003. RhoA and ROCK promote migration by limiting membrane protrusions. *J Biol Chem*. 278:13578-84. Epub 2003 Feb 6.
Wright, T.J., L. Leach, P.E. Shaw, and P. Jones. 2002. Dynamics of vascular endothelial-cadherin and beta-catenin localization by vascular endothelial growth factor-induced angiogenesis in human umbilical vein cells. *Exp Cell Res*. 280:159-68.
Yabushita, H., M. Noguchi, S. Kinoshita, T. Kishida, K. Sawaguchi, and M. Noguchi. 2002. Angiostatin expression in endometrial cancer. *Oncol Rep*. 9:1193-6.
Yancopoulos, G.D., S. Davis, N.W. Gale, J.S. Rudge, S.J. Wiegand, and J. Holash. 2000. Vascular-specific growth factors and blood vessel formation. *Nature*. 407:242-8.
Yang, J.C., L. Haworth, R.M. Sherry, P. Hwu, D.J. Schwartzentruber, S.L. Topalian, S.M. Steinberg, H.X. Chen, and S.A. Rosenberg. 2003. A randomized trial of bevacizumab, an anti-vascular endothelial growth factor antibody, for metastatic renal cancer. *N Engl J Med*. 349:427-34.