universität wien

MASTERARBEIT

Titel der Masterarbeit

The role of EphA7 and its binding partner ephrinA5 in angiogenesis

angestrebter akademischer Grad

Master of Science (MSc)

Verfasserin / Verfasser:	Angela Peer, BSc
Studienrichtung (lt. Studienblatt):	A 066 830 Masterstudium Molekulare Mikrobiologie und Immunologie UG 2002
Betreuerin / Betreuer:	Ao. Univ.-Prof. Dr. Erhard Hofer

Wien, am 4.10.2010

Danksagungen

Ich möchste mich herzlichst bei Ao. Univ.-Prof. Mag. Dr. Rainer De Martin bedanken, der es mir ermöglicht hat, als Diplomandin in seiner Arbeitsgruppe am Institut für Gefässbiologie und Thromboseforschung der Medizinischen Universität Wien zu arbeiten, und mir mit Rat und Tat beim Verfassen meiner These zur Seite gestanden ist. Weiterer Dank gilt allen Labormitgliedern, ganz besonders Dr. Martina Hoeth and Dr. Martin Piskacek, die mich als Betreuer meiner Masterarbeit unterstützt haben.

Auch Ao. Univ.-Prof. Dr. Erhard Hofer möchte ich als offiziellem Betreuer meiner Masterarbeit herzlichst danken.

Weiterer grosser Dank gilt allen, die mich davon überzeugt haben, ein fast verloren geglaubtes Studium endlich abzuschliessen.

Abstract

Angiogenese bezeichnet die Entstehung von neuen Blutgefässen aus dem bereits bestehenden Netzwerk und stellt somit einen Schlüsselmechanismus für Entwicklung und Wachstum dar. Ephrine und ihre dazugehörigen Eph-Rezeptoren stellen eine der vier Familien von „Guidance-Molekülen“ für Axone dar, die auch eine sehr wichtige Rolle in der vaskulären Entwicklung und der Differenzierung zwischen Arterien und Venen spielen. Sie bilden die grösste Familie von Rezeptor-Tyrosin-Kinasen und verursachen durch komplexe Interaktion mit anderen Signalmolekülen und Signaltransduktionswegen eine biologische Antwort mit zwei unterschiedlichen Folgen- Repulsion oder Attraktion von Zellen. Die Eph-Ephrin Signalgebung wurde bereits in verschiedensten Aspekten untersucht, unter anderem in neuronalen Zellen, und wurde als potentielle Anti-Tumor Therapie eingestuft.

Das Ziel dieser Arbeit war es, die Reaktion von Endothelzellen auf EphrinA5-EphA7 Signalgebung bezüglich Migration und Proliferation zu untersuchen. Nachdem frühere Untersuchungen gezeigt haben, dass EphrinA5-EphA7 Expression zu Repulsion von neuronalen Zellen führt, wurden ähnliche Antworten in Endothelzellen erwartet.

Die Schwerpunkte der Experimente stellten das Generieren von Ephrin-Fc und monomerischen Ephrin Konstrukten und deren Einsatz in funktionellen Tests mit HUVEC (human umbilical venous endothelial cells) dar. Diese basierten auf dem Vorwissen, dass EphrinA7 ein Zielgen des Transkriptionsfaktors SOX18 ist. Durch transiente Transfektion von HUVEC mit SOX18-IRES/GFP wurde die Expression von EphA7 ausgelöst. HUVEC, die mit SOX18/GFP transient transfektiert wurden, reagierten mit Retraktion auf die Konfrontation durch mit Antikörper geclustertem EphrinA5-Fc. Ein weiterer Ansatz, in dem Zellen mit SOX18/GFP Adenovirus infiziert und EphrinA5-Fc, das mit Protein G Polystyrene Kugeln geclustert wurde, ausgesetzt wurden, führte zu keiner Reaktion. In einem in vitro Wundheilungsversuch, in dem EphrinA5-Fc Fusionskonstrukte, EphrinA5 Monomere und Kontroll-Fc verwendet wurden, wurden keine signifikanten Ergebnisse erzielt.

Zusammenfassend kann man sagen, dass es sich bei den erzielten Daten um die ersten Schritte handelt, um die Rolle von EphA7 in Endothelzellen besser zu beleuchten, die in zukünftigen Studien weiterverfolgt werden sollten.

Abstract

Angiogenesis denotes the generation of new blood vessels from the pre-existing network and is therefore a key mechanism in development and growth. Ephrins and their corresponding Eph receptors form one of four families of axon guidance molecules, which have been revealed to be crucial players in vascular development and arteriovenous differentiaton. They constitute the largest family of receptor tyrosine kinases and evoke by complex interaction with other signalling molecules and signal transduction pathways a biological response with two different outcomes- repulsion or attraction of cells. Eph-ephrin signaling has already been extensively studied in various aspects, including neuronal cells, and has been considered as potential anti-tumor therapy.

The purpose of this work was to investigate the response of endothelial cells to ephrinA5-EphA7 signaling in the context of migration and proliferation. Since previous research has revealed that ephrinA5-EphA7 expression leads to repulsion of neuronal cells, similar responses were expected in endothelial cells. The experiments focused on the generation of ephrin_fc and monomeric ephrin constructs and theirs use in functional assays with HUVEC (human umbilical venous endothelial cells). They were based on previous findings that EphrinA7 is a target gene of the transcription factor SOX18. Expression of EphA7 was driven by transient transfection of HUVEC with SOX18-IRES/GFP. Confronting the cells with antibody-clustered ephrinA5-Fc lead to the expected retraction of the cells.

Whereas HUVEC transiently transfected with SOX18/GFP showed retraction when treated with clustered ephrinA5-Fc, a complementary approach using adenovirus SOX18/GFP infected cells and protein G polystyrene particle-clustered ephrinA5-Fc lacked a response.

A wound healing assay, in which ephrinA5-Fc fusion constructs, ephrinA5 monomers and control Fc have been used, did not show any noticeable results. Therefore, the data obtained so far are a first step towards the elucidation of the role of EphA7 in endothelial cells but need to be followed up in future studies.

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1 Introduction

1.1 The vascular system

The vascular network of blood and lymphatic vessels in the body is built up by the endothelium, which is a specialized form of the epithelium. Among the many functions of the endothelium are the control of blood pressure, blood clotting, trafficking of cells and nutrients, and inflammation. Vascular dysfunctions are involved in artherosclerosis, coagulation disorders, oedema formation and malignant cell growth. Also chronic diseases like diabetes, arthritis, psoriasis and Crohn´s disease are traced back to impaired vessel function.

Endothelial cells form a single layer that surrounds the lumen of the vessel. A basal lamina separates the endothelium from the outer layers of the vessel, which consist of smooth muscle cells, fibroblasts, pericytes, the basement membrane and the extracellular matrix. Capillaries are only loosely covered by pericytes, whereas larger vessels are supported by smooth muscle cells and multiple layers of extracellular matrix.

The endothelium is a highly active organ which displays heterogeneity in various tissues and organs. The high endothelial venules of the lymph nodes, the fenestrated endothelium of the gastrointestinal tract and the kidney glomeruli, and the tight blood-brain barrier are just a few examples of variations of the endothelium.

1.2 Angiogenesis

After formation of endothelial cell precursors, the angioblasts, from the mesoderm in the developing embryo, they generate blood vessels *de novo* which build the early vascular plexus. This process is called vasculogenesis. Angiogenesis describes the generation of further blood vessels from the pre-existing network. Two different processes have been described: sprouting and non-sprouting angiogenesis or intussusception (Risau, 1997). In the process of sprouting angiogenesis, cells migrate and proliferate after the extracellular matrix is proteolytically degraded. The formation of a lumen is followed by the functional maturation of the endothelium. In the process of non-sprouting angiogenesis, which was first described in the embryonic lung, pre-existing vessels are split. When a wide lumen is formed by proliferation of endothelial cells inside a vessel, it can be split by transcapillary

pillars or fusion and splitting of capillaries. Vessels generated by vasculogenesis form typical tissues like the lung, pancreas, spleen, the heart tube and dorsal aorta. Tissues like the kidney and brain are established by angiogenesis.

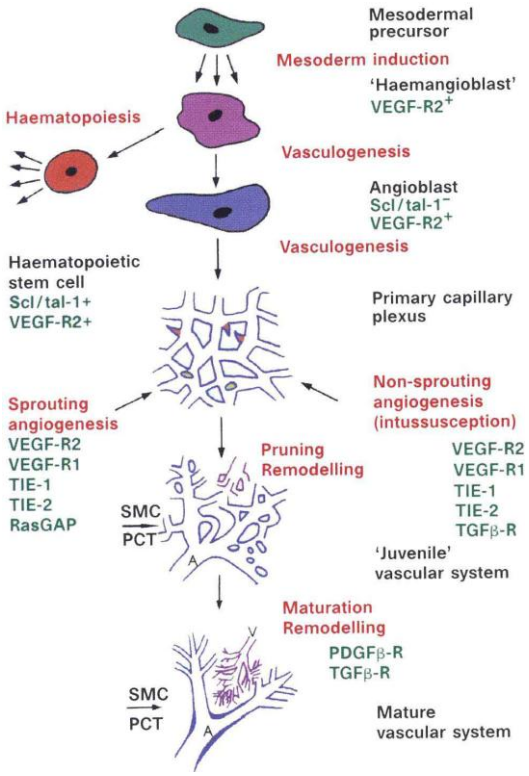


Figure 1. Vascular development. All these processes contain a multitude of key regulators. The existence of a bipotential precursor, the haemangioblast, has been suggested for a long time. Remodelling involves the recruitment of smooth muscle cells and pericytes to the vessels (Risau, 1997).

It was shown that both processes of vascularization, vasculogenesis and angiogenesis, share quite a number of regulatory mechanisms.

Different receptor families are expressed on endothelial cells, which are activated by signals from the target tissues (Mosch et al., 2009). VEGF, Tie and ephrin receptors seem to play a dominant part in angiogenesis (Hofer et al., 2006). Endothelial cells also express receptors not specific for this cell type, such as acidic and basic fibroblast growth factor (aFGF, bFGF), platelet-derived growth factor (PDGF), and epidermal growth factor (EGF) receptors, which are all receptor tyrosine kinases. The transforming growth factor beta (TGF-β) receptor, which has serine/threonine kinase activity, plays a role in vessel matura-

tion. Various other factors have been identified to be involved in vascularization, such as cell-adhesion molecules.

Angiogenesis, which is a multi-step process, is triggered in special physiological conditions, e.g. the ovarian cycle and wound healing, and in pathological conditions. Tumor growth and spreading is supported by the mechanisms of angiogenesis, thus making them an important target for therapies.

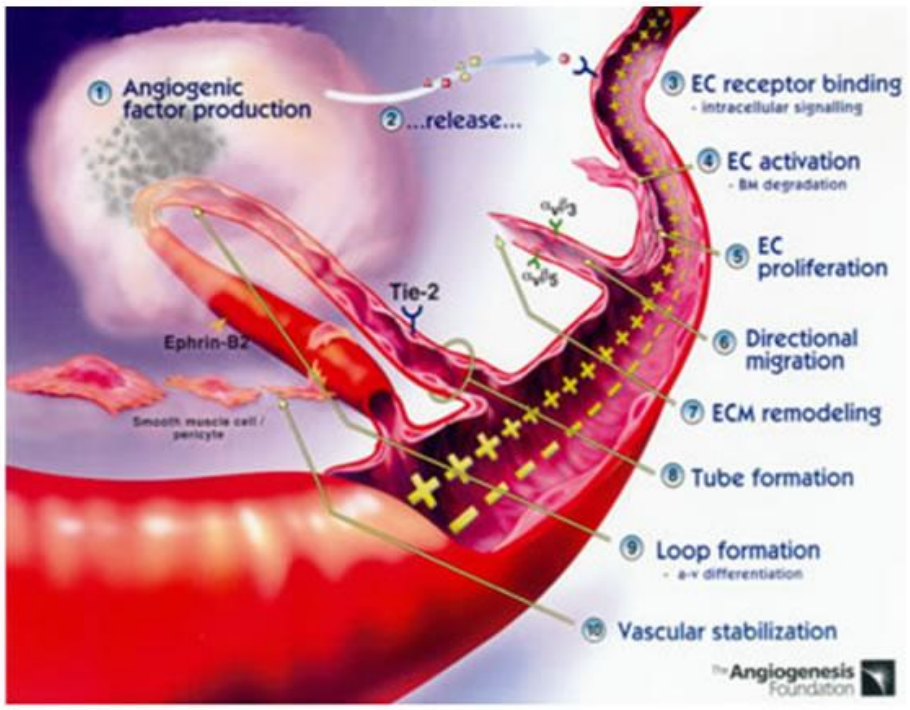


Figure 2. Angiogenesis is a multi-step process. Once the endothelial cells (ECs) are activated, the extracellular matrix is degraded. After proliferation and migration of the ECs, the extracellular matrix is remodeled followed by tube and loop formation. Vascular stabilization is the last step ensuring a save blood flow (The Angiogenesis Foundation. www.angio.org).

1.2.1 VEGF and receptors

VEGFs, which are dimeric glycoproteins, belong to the PDGF family of growth factors and can be divided into VEGF-A, -B, -C, -D and PIGF. Their importance has been shown in knock-out mice, which die *in utero* (Beck et al., 1997). VEGFs are key regulators of angiogenesis, leading to important functions like regulating blood vessel formation during embryonic development and in wound healing. Furthermore, they are important for vessel homeostasis in the adult. VEGF expression is strongly driven in hypoxic reagions, there-

fore resulting in an induction of angiogenesis. Furthermore, it is also induced by various cytokines and growth factors, e.g. TGF α and β, IL 1 and Il 6. VEGF receptors are transmembrane receptor tyrosine kinases with an extracellular domain containing seven immunoglobulin-like domains and a split tyrosine kinase domain in their intracellular part. There are three members of the VEGF receptor family: VEGFR-1 (Flt-1), VEGFR-2 (KDR in humans/ Flk-1 in mice) and VEGFR-3 (Flt-4) (Breier et al., 1996).

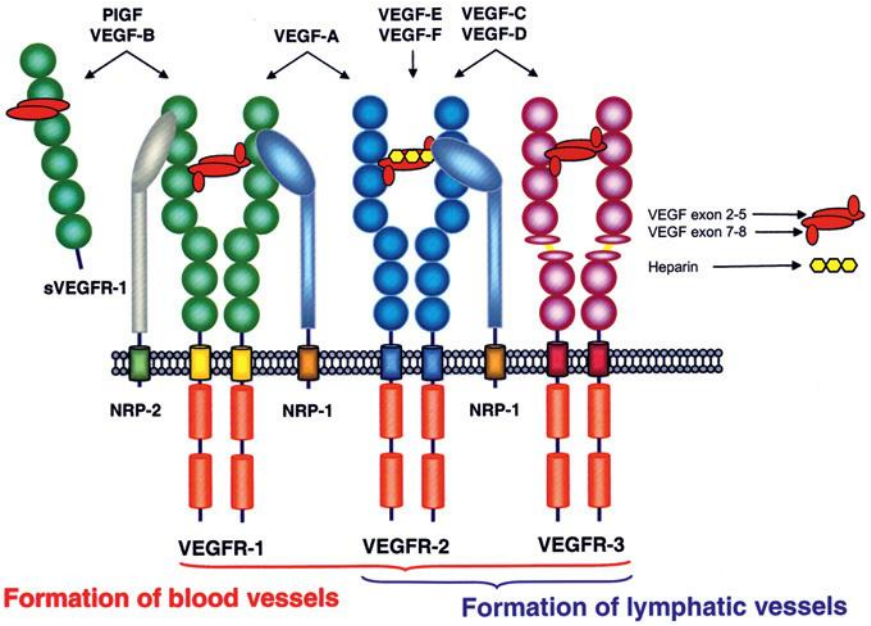


Figure 3. VEGF receptors and their ligands. VEGFR-1 and VEGFR-2 bind VEGF-A, VEGFR-1 binds VEGF-B and PIGF (placenta growth factor), VEGF-C and –D are ligands of VEGFR-3. Related proteins are VEGF-E, which is expressed by poxviruses of the Orf family, and VEGF-F, which was identified in snake venoms. Both ligands bind toVEGFR-2 (Cébe-Suarez et al., 2006).

The several roles of different VEGFRs have also been elucidated in knock-out mice (Cébe-Suarez et al., 2006). VEGFR-1 regulates blood vessel morphogenesis. Most is known about VEGF-A/VEGFR-2 signaling, which is able to induce more than 100 genes. VEGFR-2 is important in angiogenic signaling, regulation of vessel permeability and the organization of the cytoskeleton. Furthermore, it promotes mitogenesis and is essential for cell survival. The related VEGFR-3 is described as a regulator of lymphangiogenesis as it is expressed after development in the lymphatic endothelium.

1.2.2 Tie- Angiopoietin system

Another class of receptor tyrosine kinases preferentially expressed in endothelial cells is formed by Tie1 and Tie2 (Tek) (Mustonen et al., 1995). Both receptors are necessary for differentiation of endothelial cells and establishment of vessel integrity.

Angiopoietin 1-4 are the ligands for Tie2, whereas opinions differ concerning the ligands for Tie1. It was suggested that Ang1 and Ang4 can activate Tie1 and its interaction with Tie2. Tie receptors play a role in later stages of vascularization compared to VEGF signaling. They are involved in remodeling and maturation of the vessels.

1.3 Guidance of endothelial cells

The vascular and nervous systems share quite a number of similarities. Both build a branching network and often display a similar pattern in tissues. Axons have to find their correct way and migrate in the right direction. A sensory structure at the tip of the axon, called growth cone, allows reaction to guidance cues and defines the direction of growth.

Studies have shown that there are also specialized endothelial cells called tip cells, reacting to attractive and repulsive cues and therefore contributing to the navigation of blood vessels. Four families of axon guidance molecules have been discovered to also regulate vascular development (Carmeliet et al., 2005; Suchting et al., 2005).

These families are the Netrins with their DCC and UNC5 receptors, Slits with their Robo receptors, Semaphorins with their Neuropilin (NRP) and Plexin receptors, and Ephrins with their Eph receptors.

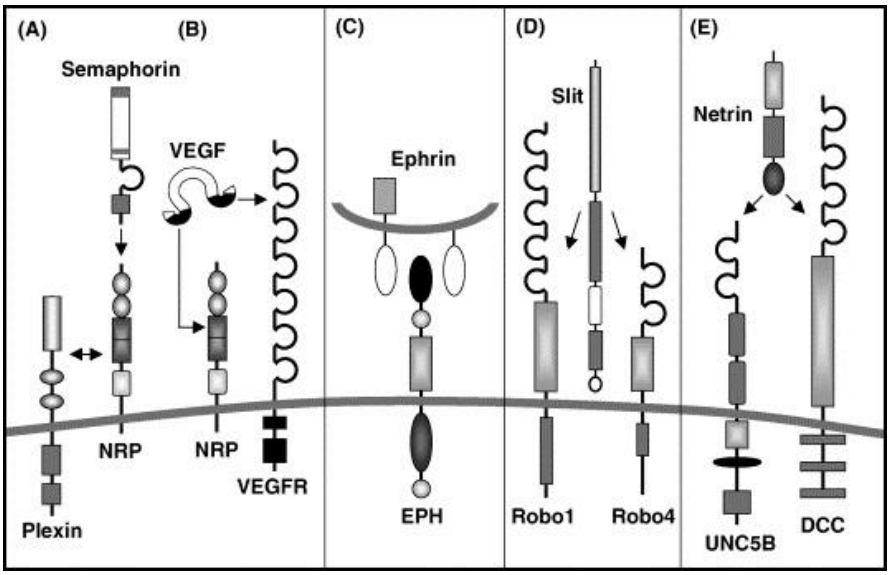


Figure 4. Four families of receptors and ligands involved in axon guidance and blood vessel development. Additionally to semaphorins, also VEGF binds to neuropilins (Klagsbrun et al., 2005).

Through expression gradients of these signaling molecules, offering either attractive or repulsive signals to the tip cells, correct movement of vessels is ensured.

To study endothelial guidance cells, the zebrafish has proven to be a useful model organism (Suchting et al., 2005). The correct patterning of intersomitic vessels (ISV) is abolished in knockdown of the guidance molecules PlexinD1, UNC5B and Robo4.

ISV have to follow both attractive cues to sprout from the dorsal aorta and grow dorsally in the intersomitic space, and repulsive cues not to invade the somites. Only when the complex interplay of several signaling molecules works, correct guidance of the vessels can be ensured.

One family of guidance cues is formed by Netrins, which are ligands for DCC and UNC5 receptors. Netrins where shown to guide axons to the midline of the brain, offering them an attractive signal by binding to DCC receptors. The interaction with UNC5 receptors, which can form heterodimers with DCCs to mediate a long-range signal, results in axon repulsion. UNC5b, which is also expressed in mammalian endothelial cells along with 3 other UNC5 receptors, was revealed to regulate blood vessel guidance.

Slits function as long- or short- range cues important for guidance of axons at the midline dependent of the expression level of their Roundabouts (Robo) receptors. Slits have been

identified to be necessary for repulsion of axons from the midline. Four Robos have been identified in vertrebrates, including Robo4, which shows a different structure than the other receptors from this family and seems to be highly expressed by endothelial cells.
Semaphorins also provide guidance cues for axons and are important players in neural wiring processes. Whereas membrane-bound Semaphorins bind to Plexins, secreted Semaphorins bind to Neuropilins, which are co-receptors for Plexin signaling.
Studies have shown that Sema3A is not the only binding partner of Neuropilin-1 (NP1), but also binds to $VEGF_{165}$. Both molecules compete for binding to the receptor, leading to different signal responses. Competition between Sema3F and $VEGF_{165}$ was also demonstrated in binding to NP2. These examples show again, how complex the network around the endothelial guidance molecules is and that lots of other different factors are involved. Experiments in mice and zebrafish have elucidated the crucial role of semaphorins and their receptors as regulators of vessel guidance and branching.

1.4 Ephrins

Eph receptors (erythropoietin-producing human hepatocellular carcinoma) and their ligands, the ephrins (Eph family receptor interacting proteins), which have originally been identified as neuronal pathfinding molecules and regulators of nervous system patterning (Frisén et al., 1997) and development of the vertebrate brain, were – like the other three families described above- shown to be important players in regulation of vascular assembly and differentiation in development and of vascular homeostasis in the adult (Augustin et al., 2003). In the last few years, the various roles of Eph-ephrin signaling have been elucidated (Kullander et al., 2002).

1.4.1 Structure and Function

Ephrin receptors turned out to constitute the largest family of receptor tyrosine kinases (RTKs). The glycosylated extracellular domain of Eph receptors consists of an N-terminal ephrin binding domain, a cysteine rich domain and two fibronectin type III motifs. It is connected via a single transmembrane spanning domain with the intracellular part, containing a tyrosine kinase domain and a sterile alpha motif (SAM) and PDZ-binding motif, both for protein interactions.
Ephs and their ligands are divided into A and B subfamilies, dependent on the structural features of the ligands (Figure 5). Ligands of the EphA receptors are plasma membrane

molecules with a glycosyl-phosphatidyl inositol (GPI) anchor. Ligands of the EphB receptors were identified as transmembrane molecules, containing a short cytoplasmic reagion and a PDZ domain for protein interactions in their intracellular part. In the human genome, nine EphA receptors and corresponding 5 ephrinA ligands, and five EphB receptors together with 3 ephrinB ligands have been identified. Binding within the A or B subfamilies happens preferentially, but it was also shown that crosstalk can occur. EphA4 and EphB2 are well-known exceptions, which also bind to ephrinB2 and ephrinA5 (Pasquale, 2010; Miao et al., 2009). Eph-ephrin signaling is dependent on cell-cell contact and is able to proceed bidirectionally. Forward signaling into the receptor expressing cell needs the Eph kinase activity, leading to autophosphorylation of intracellular tyrosine residues and further to activation of downstream signalling molecules. Reverse signaling into the ligand expressing cell is triggered by phosphorylation of tyrosine residues in the cytoplasmic reagion of ephrinB ligands by Src (Sarcoma) kinases. Also ephrinA ligands are capable of reverse signalling, as it was proven in *C. elegans*. It was shown, that Eph receptors and ephrins are also able to signal independently from each other by interaction with other signaling systems.

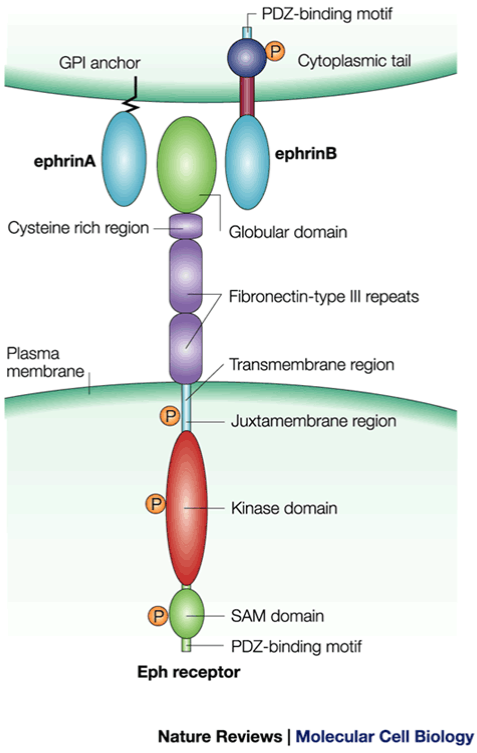


Figure 5. The structure of Eph receptors and their ligands (Kullander et al, 2002).

Several experiments have demonstrated, that clustering of ephrins is crucial for activating the receptor, since soluble ephrins do not evoke signaling in the receptor (Davis et al., 1994; Mellitzer et al., 2000).

An active Eph-ephrin complex consists of at least two receptors and two ligands, which undergo dimerization. The degree of clustering of the ligands affects the degree of Eph-ephrin clustering, resulting in a corresponding strength of biological effects (Frisén et al., 1999). Interactions via different protein binding domains in the structure of Ephs and ephrins may also lead to oligomerization of the complex (Poliakov et al., 2004). Interestingly, also non-activated receptors are being recruited to the complex and phosphorylated. The multimeric structure of the Eph-ephrin complexes allows phosphorylation of the involved molecules, stimulating further signaling cascades through other signal transduction pathways.

1.4.2 Signaling pathways involved in Eph-ephrin signaling

1.4.2.1 The role of adaptor and scaffolding proteins

A large number of signaling molecules and complex signaling pathways are involved in linking Ephs and ephrins to the cellular responses (Figure 6).

Several phosphotyrosines on the Eph receptors have been identified as binding sites for adaptor proteins like SH2 (Scr Homology 2) domain proteins that initiate signal transduction pathways (Mellitzer et al., 2000). Among these are the cytoplasmic tyrosine kinases Fyn, Src, Nck and Crk, RasGAP, LMW-PTP (low molecular weight phosphotyrosine phosphatase), PI3K (phosphatidyl-inositol-3 kinase) and other adaptor proteins like Grb2, Grb10 and SLAP (Src-like adaptor protein). It was shown, that LMW-PTP and Nck are capable of leading to cell attachment by interacting with integrins. Activated EphB4 receptors were shown to interact with PI3K and as a result trigger endothelial cell migration and proliferation (Surawska et al., 2004). Eph receptor bound ephrinB ligands becomes phosphorylated by Src family kinases, which leads to the onset of further downstream signaling. The tyrosine kinase Abl (Abelson) and Arg (Abl-related gene) have also been identified as binding partners of EphB2 and EphA4 receptors.

Since ephrinB ligands feature a cytoplasmic PDZ domain, a number of PDZ domain proteins, such as GRIP1 (glutamate-receptor-containing protein 1), GRIP2 and PHIP (ephrin-

interacting protein) is able to bind there. GRIP proteins were suggested to act as scaffolding proteins for the signaling complex, since they are found together with Ephs and ephrins in membrane rafts, promoting interaction with downstream signaling pathways.
Binding of proteins to Ephs and ephrins is not necessarily dependent on phosphorylation, as it was shown for GEFs (guanine exchange factors) for Rho family proteins and PDZ domain proteins.
Eph-ephrin signaling is capable of triggering changes in the actin cytoskeleton when mediating repulsion or adhesion. It could be shown, that Eph receptors modulate the activity of integrins, which transduce signals between the extracellular matrix and the actin cytoskeleton.
To add even more complexity, the extent of receptor clustering determines the cellular response as the degree of the clustering affects which signaling proteins are being activated. Experiments with EphB2 receptor expressing cells have provided interesting results. In case of EphB2 overexpression, cell adhesion was inhibited. As SHEP1 (SH2 domain-containing Eph receptor-binding protein 1) links EphB2 to R-Ras, a tyrosine residue of R-Ras gets phosphorylated and therefore abolishes the binding to Raf-1. As a consequence, integrin-mediated adhesion is downregulated. It was demonstrated in various other experiments, that low-degree clustering of Ephs and ephrins supports integrin-mediated adhesion, whereas high-degree clustering causes de-adhesion.

1.4.2.2 G proteins link Eph-ephrin signalling and the cytoskeleton

Eph-ephrin signaling regulates a variety of different signaling pathways in a complex manner. G proteins, like members of the Ras and Rho family of GTPases, constitute the biggest groups of affected signaling molecules (Poliakov et al., 2004). It is very important to elucidate the link between Eph-ephrin signaling and the Ras and Rho family members, because they play a huge role in regulation of the cytoskeleton, cell migration, proliferation and adhesion. They are activated by GEFs (guanine nucleotide exchange factors), which promote the exchange of GDP to GTP. Several studies have revealed, that a number of GEFs are either directly bound by Eph receptors or indirectly activated, as Ephs and ephrinB ligands induce PI3K signalling. GAPs (GTPase-activating proteins) reverse the action of GEFs and are also regulated by Eph-ephrin signaling. The three Rho family GTPases Cdc42, Rac1 and RhoA are key regulators of reorganization of the actin cytoskeleton.

Among the multitude of players in downstream signaling of Ephs and ephrins, RhoA was identified as an important member. When stimulating EphA3 expressing cells with ephrinA5, RhoA is activated and leads to deadhesion and rounding up of the cells by reorganization of the cytoskeleton. Lawrenson and her colleagues have made the discovery, that ephrinA5-EphA3 signalling modulates RhoA activity and therefore leads to reorganization of the cytoskeleton. It was shown in melanoma cell lines, that ephrinA5 causes rounding and detachment of EphA3 expressing cells. The adaptor protein CrkII is binding to tyrosine-phosphorylated EphA3 and as a result, transiently activates RhoA.

It has been shown in neurons that ROCK (Rho kinase) is an important component downstream from RhoA (Noren et al., 2003) and therefore also plays a role in the cellular response to ephrinA5. Furthermore, the guanine nucleotide exchange factors Ephexin (Eph-interacting exchange protein) and Vms-RhoGEF could be identified as a link between EphA receptors and the activation of RhoA. Ephexin is not only expressed in the nervous system, but also in liver and kidney (Kullander et al., 2002). It was demonstated in vitro, that it activates RhoA, but inhibits Cdc42 and Rac1.
EphB receptors activate Rac1 and Cdc42 by binding the exchange factors Intersectin and Kalirin. It was further suggested, that Dishevelled could be a link both between EphBs and RhoA, and ephrinB ligands and RhoA.
Brantley-Sieders et al. (2003) have pointed out the important role of EphA2 in context of endothelial cell migration and assembly. Upon activation of the receptor with ephrinA1, Rac1 is activated, which was shown to be essential for EphA2 mediated cell responses. Further experiments have revealed that the p85 regulatory subunit of PI3K is associated with activated EphA2. Therefore, PI3K is a crucial link between EphA2 and Rac1.
It is now well-known, that Eph receptors can either positively or negatively regulate Rho GTPase activity by interacting with GEFs or GAPs.

1.4.2.3 Negative regulation of the MAPK pathway

As mentioned above, Eph-ephrin signaling also regulates the activity of Ras family proteins, which are key players in the MAPK (mitogen-activated protein kinase) pathway. In most cases, Eph receptors were shown to inhibit this pathway. The MAPK pathway causes cellular responses in the presence of growth factors, like PDGF, VEGF, EGF and angiopoietin1. Activation of Ras (mostly H-Ras), which happens when the Grb2 (growth factor

receptor bound protein 2)/Sos (Son of sevenless) complex is recruited, triggers a phosphorylation cascade, leading to cell proliferation and differentiation.

Dail and her colleagues (2005) have elucidated the mechanisms of Eph-mediated regulation of R-Ras. The active form of R-Ras is part of signaling pathways leading to cell adhesion and migration. When it is negatively regulated, inhibition of these responses was observed. Several experiments have lead to the conclusion, that both activated EphA and EphB receptors inactivate R-Ras using two different mechanisms. First, through GTP hydrolysis by binding of p120RasGAP, a GTPase-activating protein acting on Ras, rendering Ras inactive. Second, through phosphorylation of Ras in the effector domain.

Another interesting approach by Parri et al. (2005) lead to the conclusion, that LMW-PTP (low molecular weight protein-tyrosine phosphastase) negatively regulates EphA2-ephrinA1 mediated repulsive response in cells. LMW-PTP, which only associates with activated EphA2, regulates the phosphorylation level of the receptor and therefore its function. As it was already demonstrated before, binding of p120RasGAP to Eph receptors leads to a downregulation of the MAPK pathway. LMW-PTP inhibits binding of p120RasGTP and as a result, blocks inhibition of the MAPK pathway.

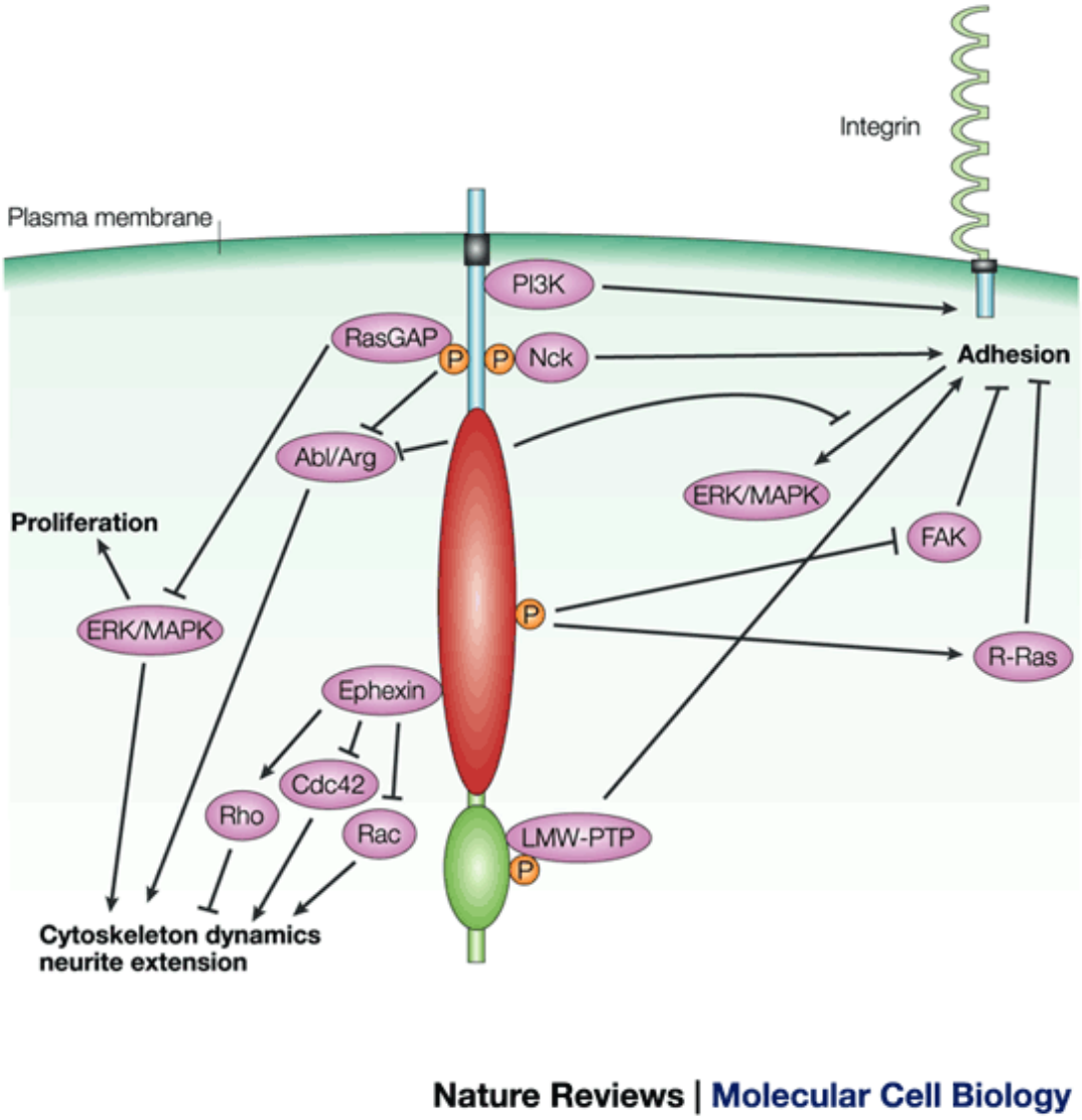


Figure 6. Overview over the interaction of an activated Eph-receptor with various adaptor proteins. (Kullander et al., 2002)

Recently, it was shown that Ephs and ephrins are also capable of regulating heterotrimeric G proteins. Interestingly, ephrinB ligands can also be bound by PDGF (platelet derived growth factor) and FGF (fibroblast growth factor) receptors, showing that crosstalk between different signaling networks plays a role.

1.4.3 Repulsion versus adhesion

As already described above, both the degree of clustering of Eph receptors and ephrin ligands and the level of Eph phosphorylation influence the sigalling outcome and the cellular response. The complex interactions between Eph signaling and a big number of other signaling pathways can result either in repulsion or adhesion. Various mechanisms have been discovered, how the balance between repulsion and adhesion is maintained (Halloran et al., 2006; Poliakov et al., 2004).

One way to allow separation of cells is by either uni- or bidirectional transendocytosis of the EphB-ephrinB signaling complex. Activated EphB receptors trigger Ras-dependent cytoskeletal assembly, allowing endocytosis of the EphB-ephrinB complex (Wilkinson, 2003). Is has not been elucidated yet, if Eph-ephrin signaling continues inside the cell. However, it seems possible, since the Ephs end ephrins are endocytosed as full-length proteins. Blocking of endocytosis, which happens when receptor or ligand, or both are truncated, results in adhesion of the cells. The question, if EphA-ephrinA complexes are also internalized, is still not solved.

The proteolytic cleavage of extracellular domains of ephrins is another mechanism leading to detachment of cells. It is best described for ephrinA ligands. The metalloprotease ADAM10 (A disintegrin and metalloprotease 10)/kuzbanian associates in *cis* with EphA3 and is not active, before ephrinA5 binds to its receptor. The cysteine-rich domain of ADAM10 recognizes EphA3-ephrinA5 complexes, which activates the enzyme function. Consequentially, ephrinA5 is cleaved in *trans* (Figure 7).

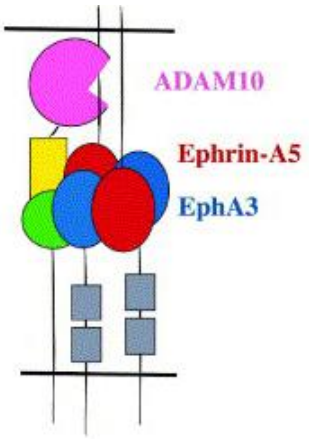


Figure 7. The mechanism of ADAM10 metalloprotease-mediated ephrinA5 cleavage (Janes et al, 2005)

In most cases, forward signaling results in a repulsive response, whereas reverse signaling leads to adhesion. Some mechanisms have been discovered, which shift the response toward adhesion by blocking forward sigalling (Figure 8).

When truncated versions of Eph receptors, possessing no kinase domain, are co-expressed with full-length Ephs, adhesion is favored. It was already suggested by Holmberg et al. (2000), that different splice forms of an Eph receptor determine either repulsion or adhesion. In the absence of ephrinA5 and EphA7, neural folds in mice are not able to fuse at the dorsal midline. It could be shown, that three different splice variants of EphA7 receptor are expressed at the edges of neural folds- one full length (FL) receptor and 2 truncated (T1 and T2) versions without kinase domain. Expression of EphA7-FL and ephrinA5 in endothelial cells lead to repulsion between receptor and ligand expressing cells. When EphA7-T1 was co-expressed, this response could not be observed. EphA7-T1 was shown to reduce ligand-induced tyrosine phosphorylation of EphA7-FL and as a result to trigger adhesion, not repulsion.

Cis expression of ephrinA ligands to EphA receptors abolishes signaling of EphA, since ephrinA binds to one of its fibronectin III domains and as a result, prevents phosphorylation of the receptor. This mechanism can be abolished, if EphAs and ephrinAs are expressed in different membrane domains, not allowing their *cis* interaction.

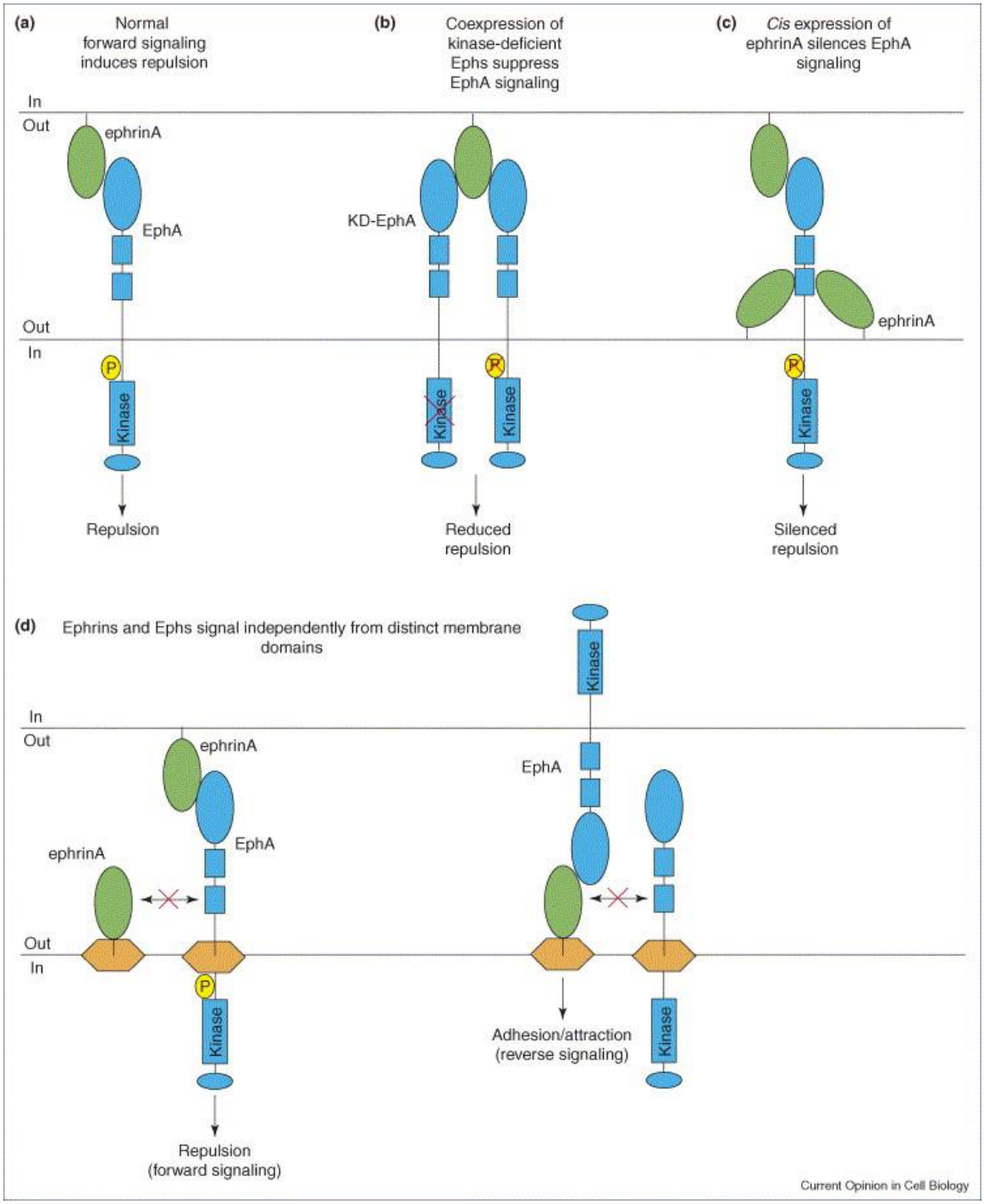


Figure 8. Mechanisms in altering the signaling outcome. (Halloran et al., 2006)

1.4.4 Expression of Ephs and ephrins in different cell types

Eph-ephrin signaling became highly interesting, since it was shown that it is crucial both in neuronal and vascular development. Ephs and ephrins are expressed in nearly all embryonic tissues and in a huge variety of other cells in adult vertebrates, e.g. neuronal cells, endothelial cells, smooth muscle cells, epithelial cells, circulating leukocytes, bone marrow cells and tumor cells (Miao et al., 2009; Poliakov et al., 2004). Here, just some of the many implications of this complex signaling system in biological systems, will be illustrated.

1.4.4.1 Neuronal pathfinding molecules

As described above, Eph receptors and ephrin ligands have been, together with three other families of signalling molecules, first described as crucial players in neuronal development and in the adult brain. One of the first experiments on the quest for identifying neuronal guidance molecules was leading to the conclusion, that axons were repelled by ephrinA5 as well as by ephrinA2 (Frisen et al., 1999). Zimmer and her colleagues (2008) have demonstrated the importance of the repulsive effect of ephrinA5 on migrating cortical interneurons expressing EphA4 to ensure a safe and correct pathway from the ganglionic eminiscences into the neocortex.

A correct axonal pathway is critical for formation of topographic connections between the neuronal cell bodies and their targets in other tissues. Ephs and ephrins of the A family are required for anterior-posterior mapping, whereas EphB receptors and ephrinB ligands are important players in dorsal-ventral mapping. Ephs and ephrins show a graded expression in the nervous system, making offering of re-und propulsive cues possible. Lower expression leads to lower clustering of receptors and ligands, resulting in axon growth. High clustering leads to repulsive signaling. Studies with knock-out mice have shown that EphA8, EphB2 and EphB3 are essential for correct axonal crossing of the midline (Frisen et al., 1999).

Ephs and ephrins are involved into the topographic mapping of the retinotectal system as well (Mellitzer et al., 2000). Retinal axons show a graded expression of EphA3, whereas ephrinA2 and ephrinA5 are gradually expressed in the tectum, enabling regulation the growth and the direction of the axonal projections from the retina to the tectum.

Eph-ephrin signalling can also increase cell adhesion, as shown by Sela-Donenfeld et al. (2005). They have demonstrated that Eph-ephrin signaling is able to sharpen boundaries in

the complex organisation of the hindbrain into rhombomeres. EphA4 mediates both repulsion to restrict intermingling of cells at interfaces and adhesion of cells within rhombomeres.

As one of many other examples, ephrinA5 was shown to play a role in neural tube closure, which was quite surprising because its interaction with EphA7 was known to mediate repulsion in *in vitro* experiments with axons. EphrinA5 is expressed at the edges of the dorsal neural folds together with the EphA7 receptor. In the absence of EphA7 and ephrinA5, neural tube fusion is impaired (Holmberg et al., 2000).

1.4.4.2 Expression in endothelial cells

Eph-ephrin signalling controls vascular development, vessel guidance and morphogenesis. EphB receptors and their ligands have been shown to be essential mediators in arteriovenous differentiation during embryonic angiogenesis (Augustin et al, 2003; Adams et al, 2000). Two crucial players in establishing vascular borders are ephrinB2 and EphB4. EphrinB2 is expressed by arterial endothelial cells, whereas EphB4 is a marker of venous endothelial cells. Their pro- and repulsive activities establish the boundary between arteries and veins by preventing cell intermingling. EphrinB2-deficient mice die because of the inability to form a proper network of arterial and venous endothelial cells (Mellitzer et al., 2000). The asymmetric expression of EphB4 and ephrinB2 was also shown in the adult, suggesting that their interaction also plays a role in controlling vascular homeostasis. *In vitro,* EphB4 receptors activated by ephrinB2 cause endothelial cell proliferation and migration (Mosch et al., 2009). Even more players in vascular patterning have been identified in different experiments, making the picture more complex. EphB3, EphB4 and ephrinB1 are expressed by venous cells. Arterial endothelial cells express EphB3, EphB4, ephrinB1 and ephrin B2.

An important pair of the A-class of Ephs/ephrins involved in angiogenesis is EphA2 and ephrinA1. EphrinA1 was the first identified Eph receptor ligand and showed to be induced by VEGF. Tang et al. (2005) did not only demonstrate, that ephrinA1 enhanced vascular endothelial migration and induced tube formation, but also that green tea catechin epigallocatechin gallate (EGCG) is able to inhibit that response by targeting ERK-1/2 activation. The authors suggest that the consumption of green tea may be able to reduce the risk of tumour development, since it is tightly dependent on angiogenesis.

There are also some examples for Eph-ephrin signaling between endothelial cells and other cell types. For proper vascular development, Eph-ephrin signaling also has to take place between endothelial cells and the surrounding mesenchymal cells (Adams et al., 2000). EphB2 receptors are expressed my mesenchymal cells. Additionally, Eph-ephrin signaling plays a role in recruitment of smooth muscle cells towards endothelial cell channels (Carmeliet et al., 2005).
Salvucci and her colleagues (2008) have shown that ephrinB2 reverse signaling is crucial for assembly of endothelial cells and pericytes. Experiments, in which ephrinB2 expression was silenced, resulted in the failure of endothelial cells and bone marrow derived mesenchymal stem cells to assemble with each other in cordlike structures.

1.4.4.3 The role of Eph-ephrin signalling in cancer

Ephs and ephrins were also identified to play a crucial part during tumorigenesis (Surwaska et al., 2004; Campbell et al., 2008; Mosch et al., 2009; Pasquale, 2010). To enable tumor growth, the supply of nutrients, oxygen and growth factors has to be guaranteed. Therefore, angiogenesis has to be switched on. Eph receptors and ephrin ligands were found to be both up- and downregulated in human carcinomas and in the tumor vasculature (Figure 9). As some of many examples, EphA2 and EphB4 are upregulated in malignant cancers, whereas EphA1 and EphB6 are downregulated.
The dysregulation of the ratio between Ephs and ephrins affects a long chain of signaling events, leading to severe changes in cell survival, motility, proliferation and organization of the cytoskeleton. Activation of EphA2 reduces focal adhesion kinase (FAK) phosphorylation, leading to inhibition of integrin-mediated cell adhesion. Therefore, integrins are important players in invasion and metastasis.
Trying to explain how the disbalance of Ephs end ephrins is established, the binding of destabilizing mRNA to Eph/ephrin transcripts and chromosomal alterations was proven in tumor cell lines. Furthermore, mutations in the genes encoding all Eph receptors were identified. Hypoxia upregulates several Ephs and ephrins, together with other factors like tumor necrosis factor-α (TNFα) and VEGF.

Interestingly, Eph receptors expressed in tumor cell lines show a low level of activation by ephrins. When triggering forward signaling, proliferation, invasion and metastasis can be inhibited by repulsive meachanisms and increased adhesion between tumor cells. There-

fore, cancer cells try to avoid forward signaling using mechanisms like low expression of ephrins, dephosphorylation of the receptor or inefficient interaction of receptor and ligand. Reverse signalling seems to have the same capability in some contexts.
However, both forward and reverse signalling have been shown in many cases to support tumour development and angiogenesis. Eph receptors can also be used by other oncogenic signalling pathways, making them independent from ephrins.

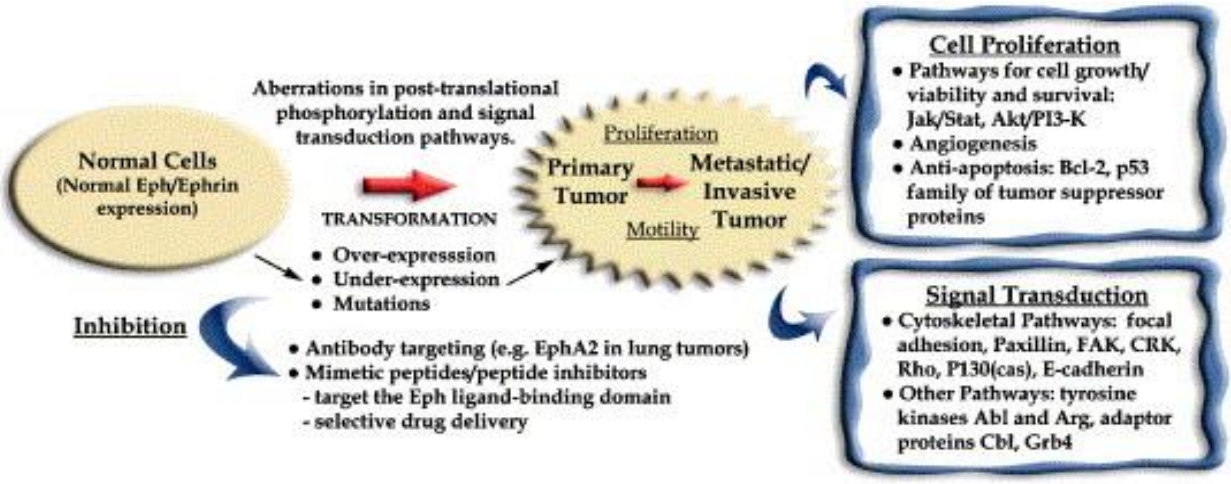


Figure 9. Implications of Eph/ephrin expression in tumorigenesis. Aberrant expression of Ephs and/or ephrins results in transformation of normal cells into tumor cells (Surawska et al., 2004).

Elucidating the mechanisms causing the dysbalance in expression of these signaling molecules and their aberrant signalling activities offers the possibility to find putative targets for anti-angiogenic cancer therapies. A promising approach is to impair Eph-ephrin signalling, which can be achieved in different ways. There have been attempts to use monoclonal antibodies against Eph receptors, which resulted in inhibition of tumor growth. The same result has been achieved by blocking signaling with soluble forms of Ephs or ephrins or other peptides. It was shown in the literature that soluble EphA2-Fc or EphA3-Fc receptors inhibit tumor angiogenesis by competively binding available ligands (Brantley et al., 2002).
Other possibilities of intervening are the intracellular use of inhibitors of tyrosine kinases, small interfering RNA or antisense oligonucleotides. There have also been attempts to interfere with other pathways regulating cell proliferation and growth. The complexity of Eph-ephrin signaling makes it quite difficult, to establish successful targeting strategies without side effects, making it necessary to invest further work into this topic.

1.4.5 EphA7 as a target gene of SOX18

Previous work in the group has identified EphA7 as a target gene of the transcription factor SOX18. HUVEC were transduced with an adenovirus expressing SOX18-IRES-EGFP. The mRNA was isolated and used for microarray analysis and real-time PCR. Follow-up experiments have shown that the EphA7 promoter contains several SOX18 binding sites. Activation of the EphA7 promoter by SOX18 was verified by reporter gene assays (Hoeth et al., unpublished). Therefore, we decided to investigate the functional consequences of EphA7 expression in endothelial cells in the context of angiogenesis.

1.5 SOX18

SOX18 is a transcription factor, which is expressed in endothelial cells during embryonic development and adult neovascularization. Studies have shown that it is an important regulator of vascular development and a trans-activator of gene expression (Downes et al., 2001; Hosking et al., 2000). SOX18 belongs to the SOX (Sry-related HMG box) family of proteins. Together with SOX7 and SOX17, it forms the SOX Group F. Redundant roles of SOX17 and SOX18 were demonstrated previously (Matsui et al., 2006).

1.5.1 Structure

The HMG box of SOX18 recognizes a heptameric DNA consensus sequence (WWCAAWG; W= A/T) to which it binds. This binding is believed to recruit other cofactors or transcription factors. The HMG and transactivation domain which were identified in SOX18 are conserved between mouse and human. Bioinformatic studies in our lab have previously revealed, that three highly conserved blocks are found in the C-terminus of SOX18, which were termed proline, charged and serine block due to the occurrence of the respective amino acids. Working with mutant constructs of SOX18 showed, that the charged block is essential for transcriptional activity. Furthermore, the C-terminus is believed to mediate interactions with other proteins.

1.5.2 Expression and roles of SOX18

Studies in mice and chickens have shown that SOX18 is expressed during vasculogenesis and angiogenesis. The role of SOX18 in vascular development has been proven by the

analysis of ragged mutant mice that carry naturally-occuring mutations in SOX18 and which show defects in cardiovascular and hair follicle development (Pennisi et al., 2000).

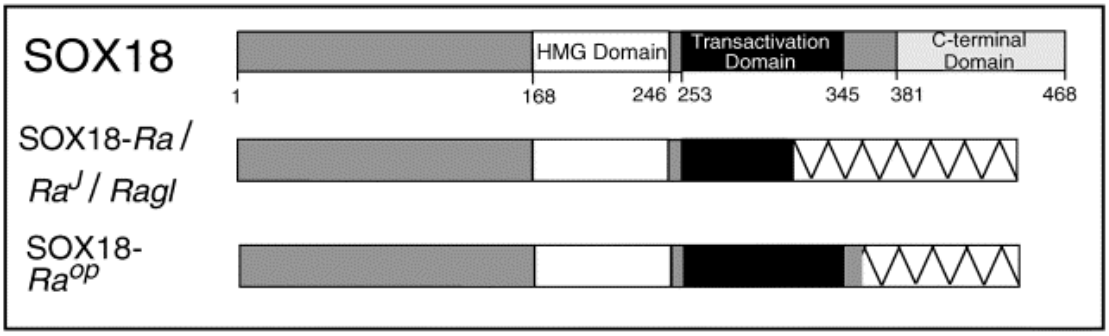


Figure 10. Normal form and mutations of SOX18. In case of the Ra, Ra^J and Ragl phenotypes, mutations are located in the transactivation domain. The Ra^{op} mutation lies outside (Downes et al., 2001).

The naturally occurring mutant ragged (Ra) and the three allelic mutatations ragged-Jackson (Ra^J), ragged-opossum (Ra^{op}) and ragged-like (Ragl) result in translational frame-shifts which leads to the expression of truncated proteins. Therefore, SOX18 dysfunction impairs normal vessel development. In constrast to ragged mice, SOX18 knockout mice show normal vascular development and are viable. A small reduction in pigmentation is the only abnormal phenotype found in these mice. This suggests that other SOX proteins may supplant the functions of SOX18. This function is inhibited in the case of ragged mice. Mutant forms of SOX18 have been suggested to act in a dominant-negative manner by blocking the binding to DNA.

The expression of SOX18 in the adult mouse was detected by RT-PCR in the lung tissue and in the skin after wounding, and at lower levels in heart and skeletal muscle, intestine, spleen and kidney.

Human SOX18 is expressed in various tissues including the heart, brain, skeletal muscle, spleen, kidney, liver, small intestine, placenta, lung, colon and thymus (Pennisi et al., 2000; Saitoh et al., 2002). The hypotrichosis-lymphedema-telangiectasia (HLT) syndrome, a hereditary disease characterized by sparse or totally absent hair growth, transparent skin on hands and feet and the onset of lymphedema, is caused by a mutation in SOX18 (Irrthum et al., 2003). Several SOX genes were found to be involved in human disorders. Because SOX18 is expressed in Flt-1 (VEGFR-1) knock-out embryos but not in Flk-1

(VEGFR-2) null embryos, is has been placed into the VEGF pathway downstream from VEGF and Flk-1 (Downes et al., 2001).

However, signaling pathways of SOX18 still have to be elucidated to get a better insight into its functions in vasculogenesis and the maintanance of blood and lymphatic vessels.

2 Results

Ephs and ephrins are not only neuronal pathfinding molecules as demonstrated in the past (Frisén et al., 1997; Sela-Donenfeld et al., 2005; Holmberg et al., 2000), they also control vascular development and vessel guidance as they are expressed in endothelial cells (Augustin et al., 2003; Adams et al, 2000; Carmeliet et al., 2005). EphA7, a receptor for ephrinA5, was identified as a target gene of SOX18 by microarray analysis and real-time PCR in our lab previously. Furthermore, it was shown with bioinformatic tools as well as experimentally, that the EphA7 promoter contains several SOX18 binding sites.
These findings and the previous finding, that neuronal cells expressing EphA7 avoided cells expressing ephrinA5 (Holmberg et al., 2002), lead to the decision to investigate the consequences of expression of EphA7 and ephrinA5 in endothelial cells in context of cell migration and proliferation. Furthermore, we wanted to elucidate if Eph-ephrin signaling evokes comparable responses in endothelial cells as in neuronal cells. The main aim of the project for my diploma thesis was to generate ephrinA-Fc fusion proteins and ephrinA monomers, to have them ready as tools for further experiments. Furthermore, I was also able to collect some preliminary data in *in vitro* assays of endothelial cell migration.

2.1 Generation of ephrinA1-A5 – Fc fusion constructs

As it is known from the literature that soluble ephrins do not activate their receptors unless they are artificially clustered (Mellitzer et al., 2000), we decided to generate ephrin-Fc fusion constructs. The vector pIGplus, featuring an IgG Fc tail as a functional site, was chosen as cloning vehicle. This leads to expression of ephrin-Fc fusion proteins secreted from the cells, which dimerize and are therefore expected to activate the EphA7 receptor. The ephrins of the A subfamily, which are GPI anchored membrane molecules, were cloned without their GPI anchor. When expressed, they are secreted from the cell and can be harvested as soluble proteins suitable for further experiments after purification via their Fc part. A fragment of human ephrinA5 encoding 203 amino acids was amplified via PCR from an image clone (Gene Service) and then cloned into the vector pIG plus (K. Alitalo) (NheI, BamHI).

Human ephrinA1 and A3 and murine ephrinA2 and A4 (number of amino acids: see list below) were cloned with the same strategy into pIGplus (KpnI, BamHI).

Protein	*Image Clone Number*	*Number of amino acids*	*UniProtKB/Swiss-Prot entry*
ephrinA1h	AT54-G10	1-182	P20827
ephrinA2m	AV105-D7	1-184	P52801
ephrinA3h	AT20-G1	1-214	P52797
ephrinA4m	D730009E04	1-175	O08542
ephrinA5h	10097-I05	1-203	P52803

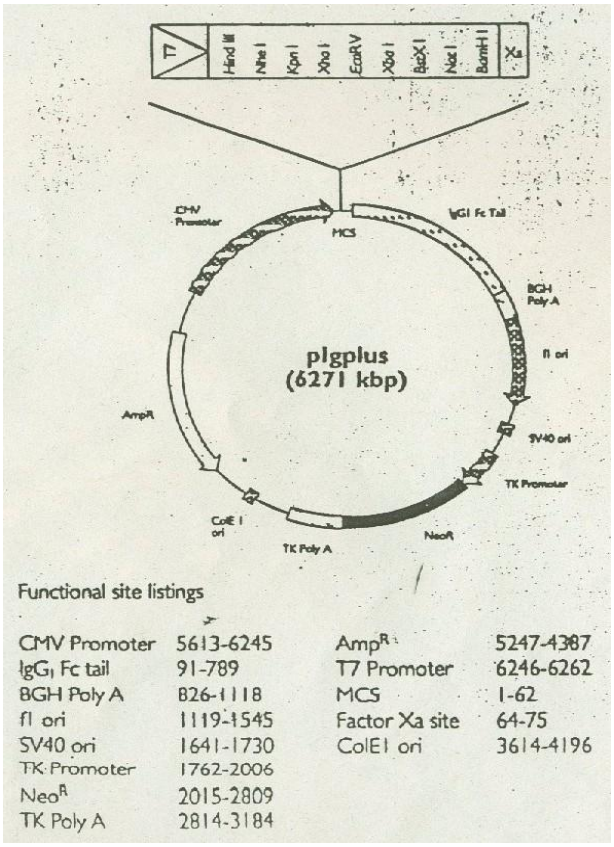


Figure 11. pIGplus. The gene cloned into the multiple cloning site is expressed as a Fc- fusion protein.

2.2 Expression of soluble human ephrinA5- Fc fusion protein and Fc

Because we expected a too low yield of protein after transient transfection, we decided to generate stable cell lines expressing soluble human ephrinA5-Fc and Fc for control experiments. Cells were cultured in a 10 cm dish until they reached a confluency of about

60% and were then transfected with ephrinA5-Fc or the vector Signal pIGplus (Fig. 12) using the calcium-phosphate method.

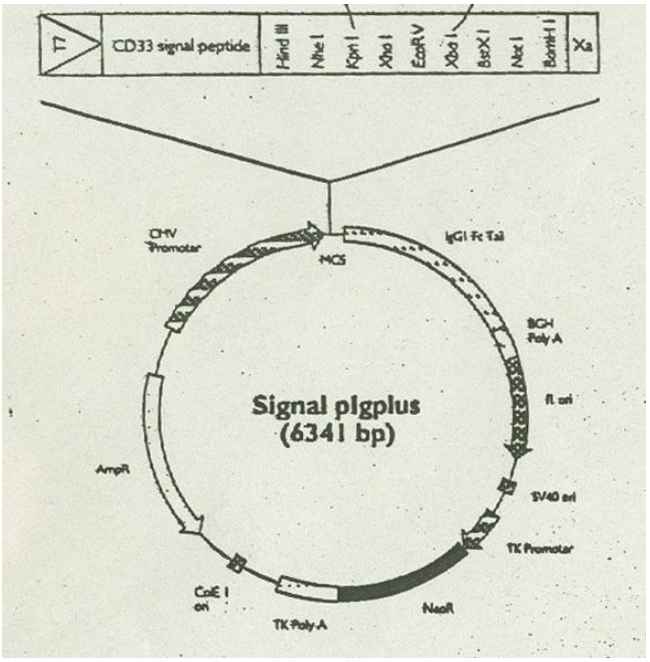


Figure 12. Signal pIGplus. Due to the presence of a CD 33 signal peptide, the Fc tail is expressed from this vector.

After selecting for stably transfected cells (10-14 days) with Neomycin (starting concentration: 800 μg/mL), clones were picked and seeded into a 48 well plate. They were further cultured in a 10 cm dish. After culturing putative stable clones, the expression of ephrinA5-Fc or Fc was verified on Western Blot. Clones with the strongest expression were cultivated further and frozen to be kept in liquid N_2.

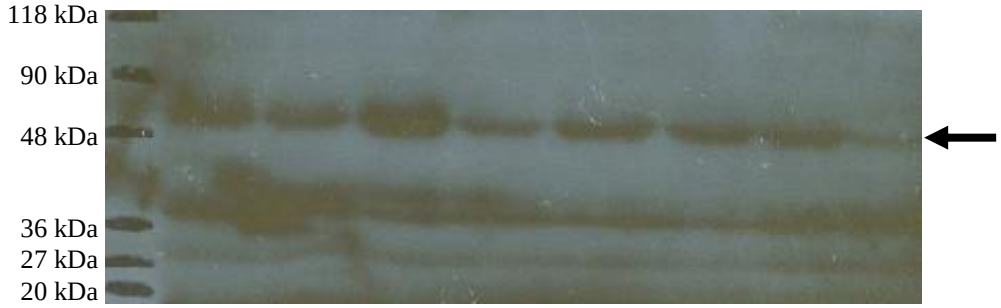


Figure 13. Expression of soluble ephrinA5-Fc from eight different stable cell clones (HEK 293) (antibody: anti- human IgG, Fc specific)

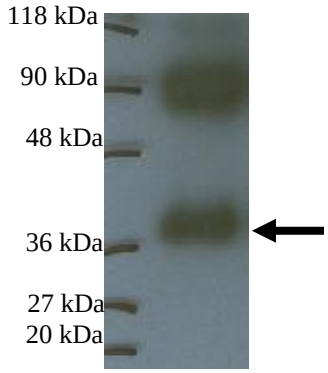


Figure 14. Expression of soluble Fc from stable cell clones (HEK 293). The band above originates from a Fc-dimer (antibody: anti- human IgG, Fc specific).

After verification of the expression, ephrinA5-Fc or Fc were purified from the culture medium using Protein G Sepharose beads to use them for future experiments. Further Western Blots, in which the sample was quantified using known amounts of BSA, showed that the yield of protein, compared to the used amounts in the literature, was sufficient for cell assays even after transient transfection of HEK 293 cells.

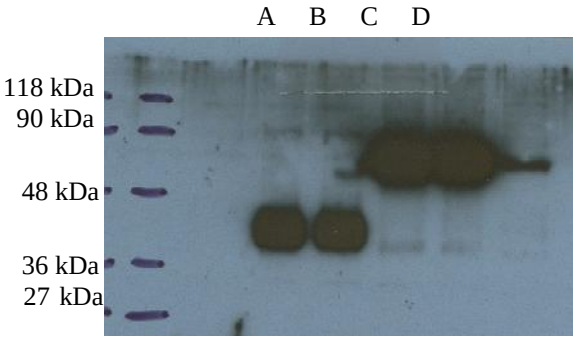


Figure 15. Expression of soluble Fc (A, B) and ephrinA5-Fc (C, D) from transiently transfected HEK 293 cells (antibody: anti- human IgG, Fc specific)**.**

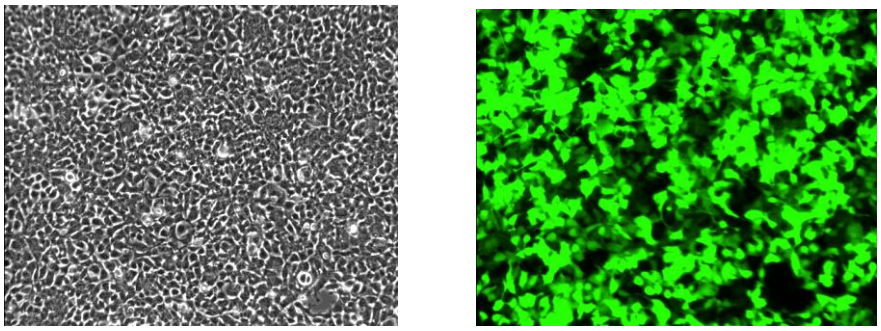
Figure 16. Expression of soluble ephrinA5-Fc from transiently transfected cells (antibody: α-EphrinA5, goat polyclonal IgG).

2.3 Generation of soluble monomeric human ephrinA5

To obtain ephrin monomers, which should block Eph receptors, the Xa protease recognition site between the ephrin and Fc was used. After digestion of ephrinA5-Fc with Xa Protease, the expression of ephrinA5 was checked on Western Blot.

Figure 17. Expression of soluble ephrinA5-Fc eluted from Protein G Sepharose beads (A, B) and expression of soluble monomeric efnA5 (C, D) (antibody: α-EphrinA5, goat polyclonal IgG)**.**

2.4 Expression of the constructs human ephrinA1-Fc and ephrinA3-Fc, and murine ephrinA2-Fc and ephrinA4-Fc



A B

Figure 18. Transient transfection of HEK 293 with ephrinA1-Fc and E-GFP. (A) phase contrast (B) fluorescence.

To verify the expression of the other ephrin-Fc constructs (ephrinA1-A4) by Western Blot, HEK 293 cells were transiently transfected using the calcium-phosphate method. The conditioned medium was then used for isolation of the Fc fusion proteins using Protein G Sepharose beads.

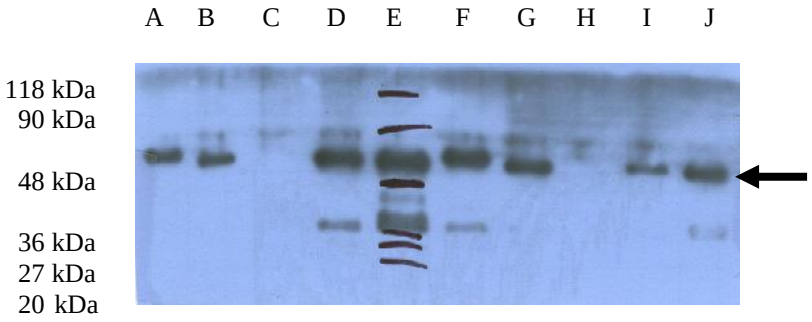


Figure 19. (A-E) Expression of soluble ephrinA1, A2, A3, A4, A5-Fc eluted from with Protein G Sepharose beads. EphrinA3-Fc was not expressed. **(F-J) Remaining ephrinA1, A2, A3, A3, A5-Fc on the beads after elution** (antibody: anti- human IgG, Fc specific).

As it was demonstrated that the ephrin-Fc fusion proteins and Fc are expressed in stably transfected, as well as in transiently transfected cells, *in vitro* cell assays using the isolated proteins could be started.

2.5 Retraction assay I in HUVEC cells

The fact that cells expressing EphA7 and cells expressing ephrinA5 avoided each other (Holmberg et al., 2002) lead to the first approach, the aim of which was to study the consequences of incubating HUVEC with the construct ephrinA5-Fc.

Cells cultured in 6 well plates were transiently transfected with the plasmid EGFP-N1 as control or IRES-SOX18/GFP in order to stimulate expression of EphA7, using PEI. After verification of a successful transfection by checking fluorescence of the cells, 0,3 µg/mL Fc as control or ephrinA5-Fc clustered with 0,5 µL/mL antibody (Alexa Fluor goat anti-human IgG, Molecular Probes) was added to each well. Pictures were taken before and after addition of the protein. The microscope table was kept at a temperature of 37°C to be as close to incubation conditions as possible. In this experiment, cells transfected with EGFP-N1 or IRES-SOX18/GFP did not significantly react to incubation with the control construct Fc.

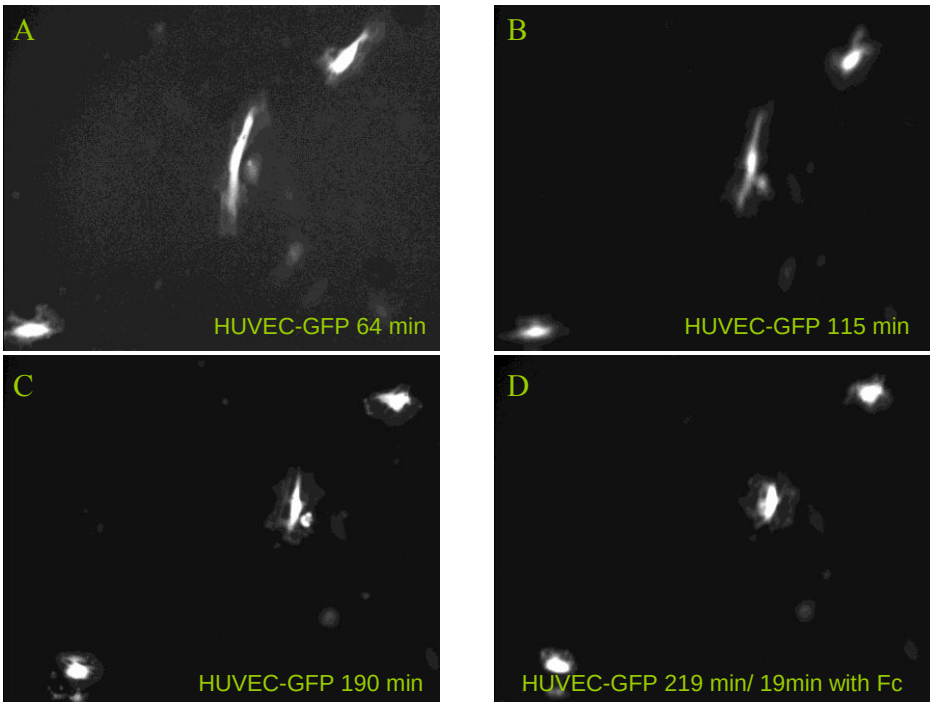
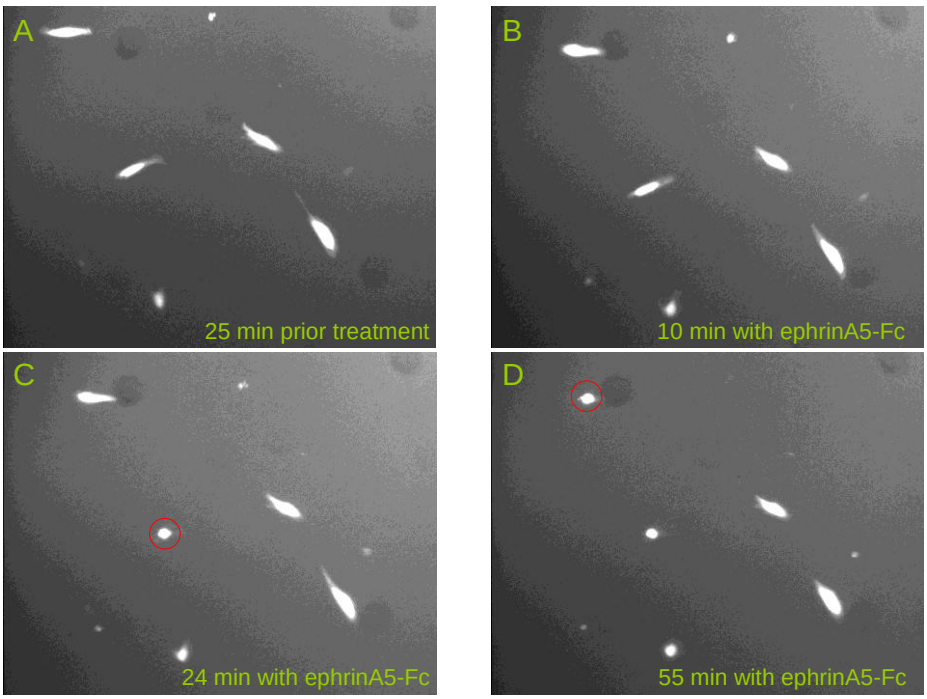


Figure 20. Fluorescent HUVEC transiently transfected with EGFP-N1 before (A-C) and after (D) addition of Fc.

EGFP-N1 transfected cells reacted just as little to ephrinA5-Fc. In contrast, retraction of IRES-SOX18/GFP transfected cells after incubation with ephrinA5-Fc could be observed.



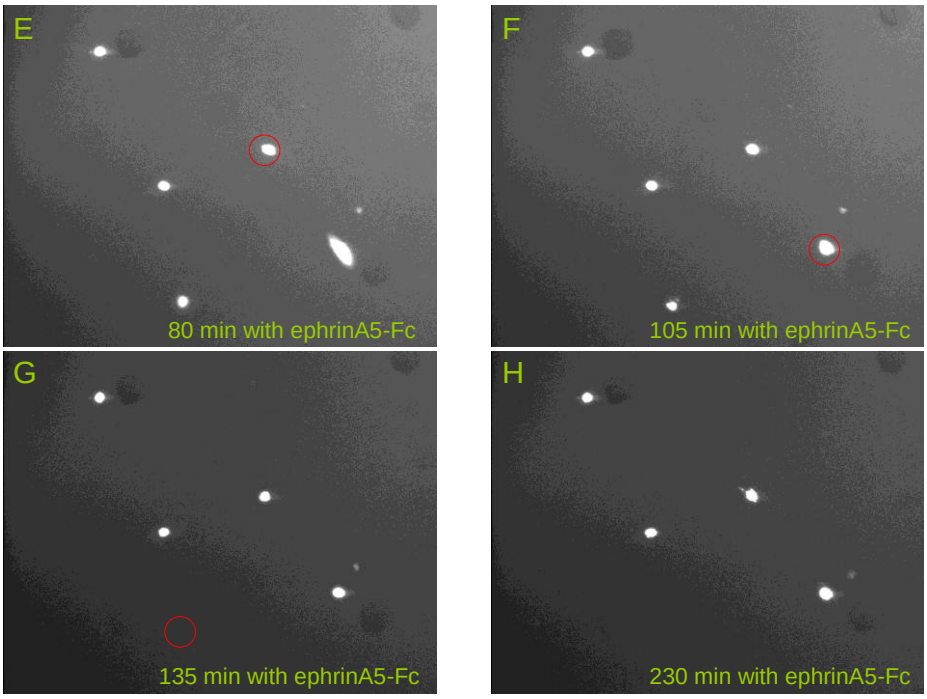


Figure 21. Fluorescent HUVEC transiently transfected with SOX18-IRES/GFP before (A) and after (B-H) addition of ephrinA5-Fc. Red circles mark retracting cells, after 135 min (G) one cell even disappeared.

It can be clearly seen that cells retracted significantly during 105 minutes. The cell which apparently disappeared and has detached from the dish could be an indicator that the cells do not just retract but detach. This is restricted to SOX18 transfected cells and does not affect untransfected cells, as Fig. 22 shows.

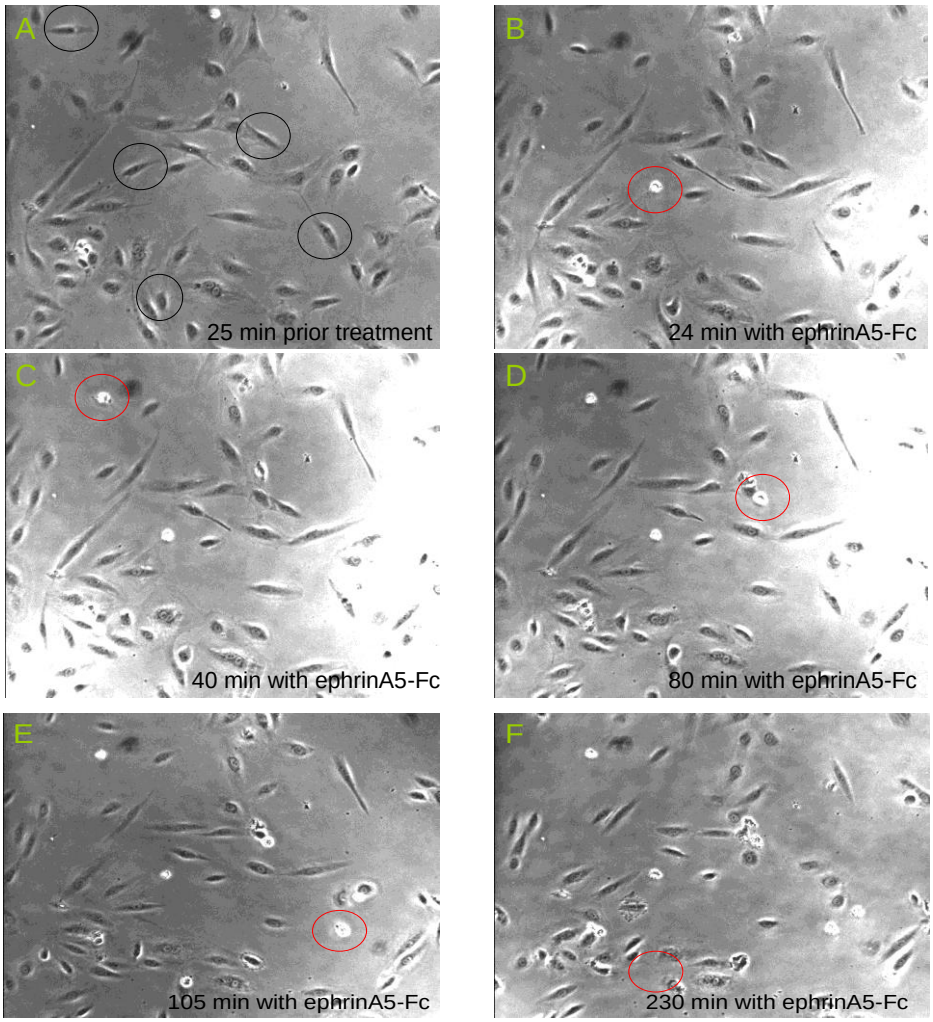


Figure 22. The same HUVEC as above transiently transfected with SOX18-IRES before (A) and after (B-F) addition of ephrinA5-Fc in phase contrast. (a) Black circles mark the transfected cells. (B-F) Red circles mark the same retracting cells as above.

It was shown that the effect of retraction is definitively not concerning all cells and that it can not be interpreted as general cell death. This supports our hypothesis that EphA7-ephrinA5 signaling is resulting in a repulsive response in SOX18 transfected cells. Here it is necessary to mention that EphA7 is not the only Eph receptor activated by ephrinA5 and that other Eph receptors must be involved in evoking that response.

2.6 Retraction assay II in HUVEC cells

In my second approach, my aim was to change the previous experiment slightly. In contrast to PEI transfection of the cells, they were infected with SOX18/GFP adenovirus. Instead of clustering ephrinA5-Fc with an antibody, protein G polystyrene particles (4. 26 μm; Kisker) were used. Infected HUVEC cells were incubated with 5 μL/mL protein G polystyrene particles and 0,3 μg/mL ephrinA5-Fc.

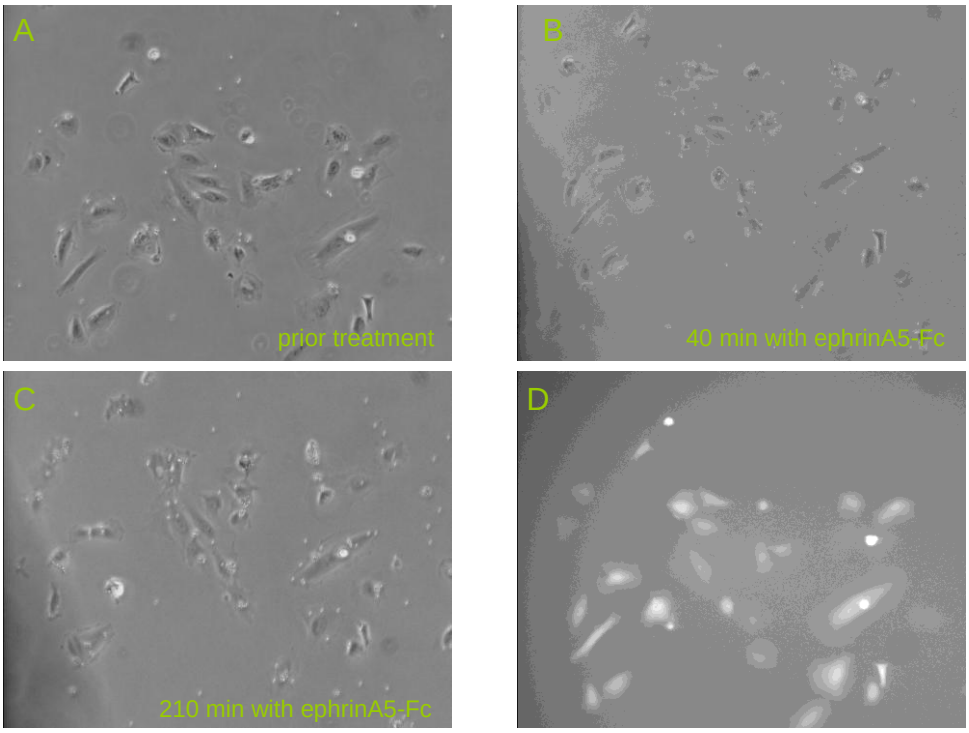


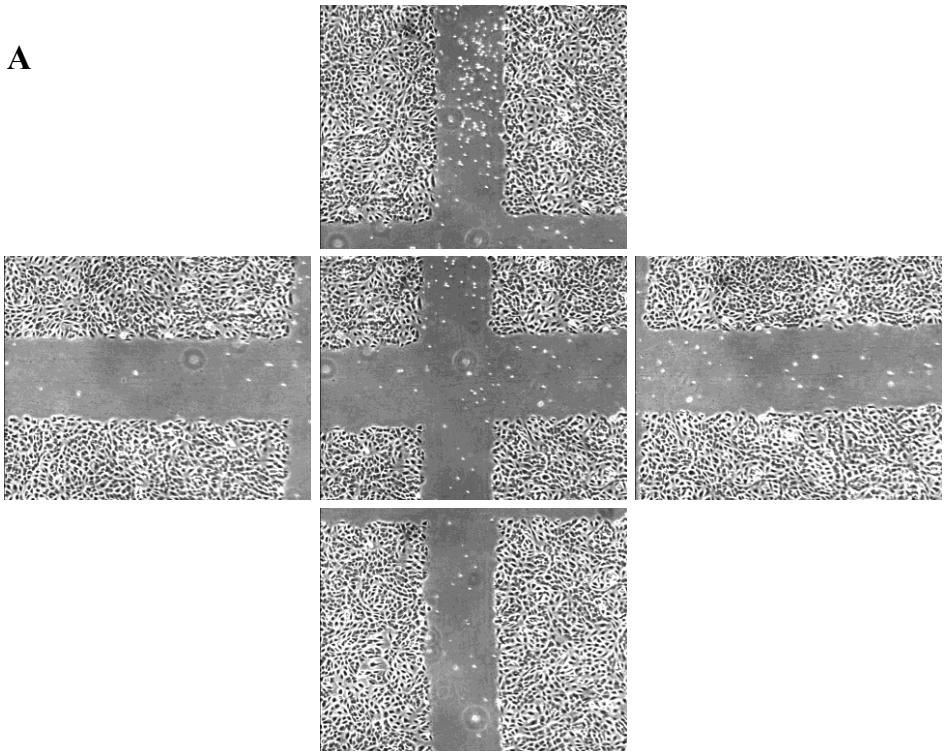
Figure 23. HUVEC infected with SOX18/GFP adenovirus before (A and D) and after (B and C) incubation with ephrinA5-Fc on protein G polystyrene beads. A-C phase contrast. D fluorescence.

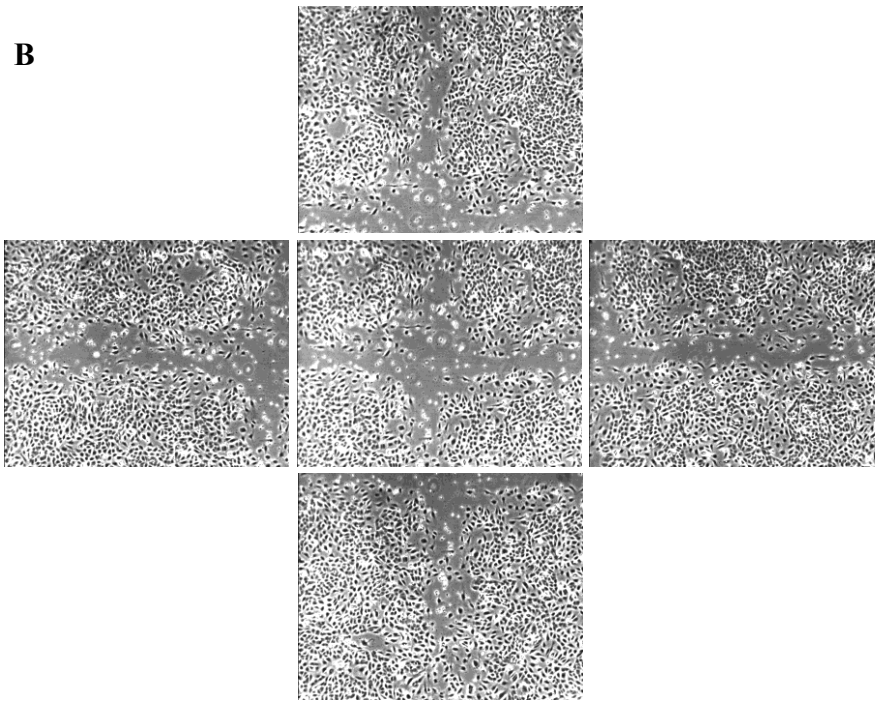
In contrast to the previous experiment, where SOX18/GFP transfected cells completely retracted after 105 minutes, no repulsive response could be observed, even at the latest time point (210 min). This fact will be discussed later.

2.7 Wound healing assay

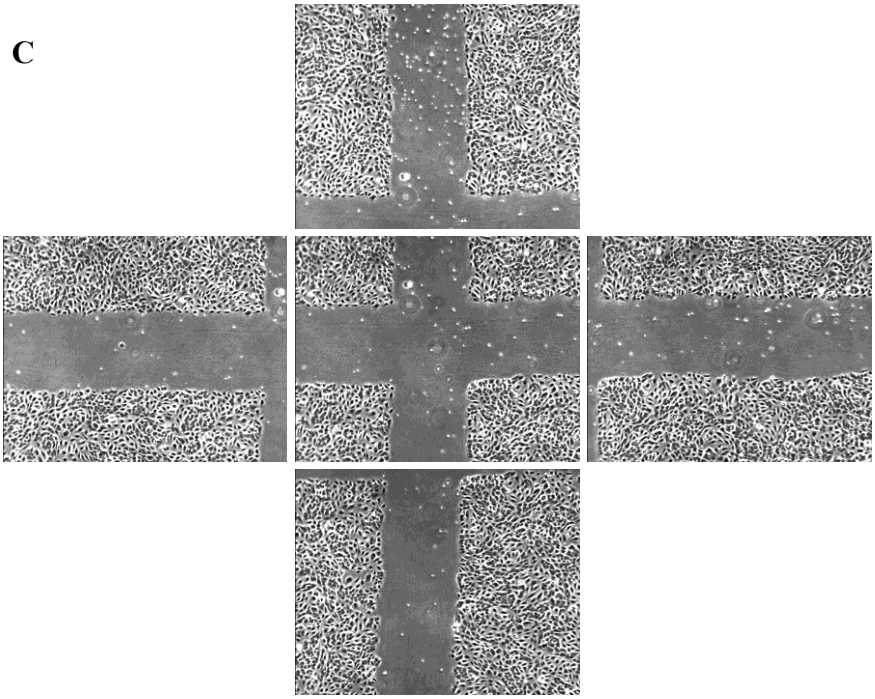
As angiogenesis is also triggered in wound healing, an *in vitro* wound healing assay was designed to make further studies concerning Eph-ephrin signaling in this context. HUVEC

were infected with SOX18/GFP adenovirus and cultivated until they reached confluency. A cross was scratched into the layer of cells to observe further migration (Fig. 24).

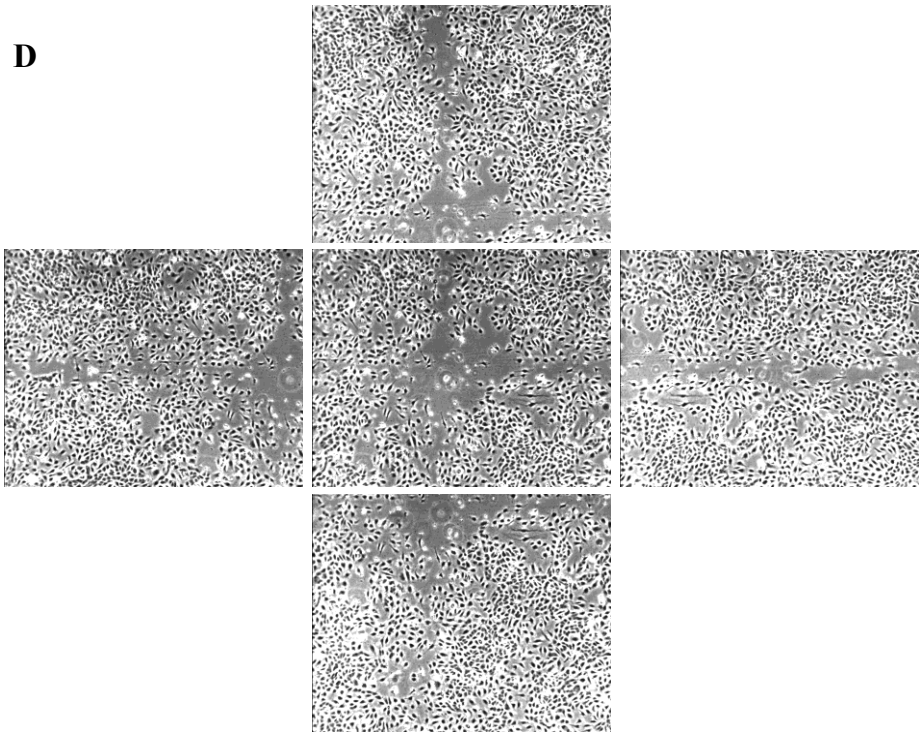




C



D



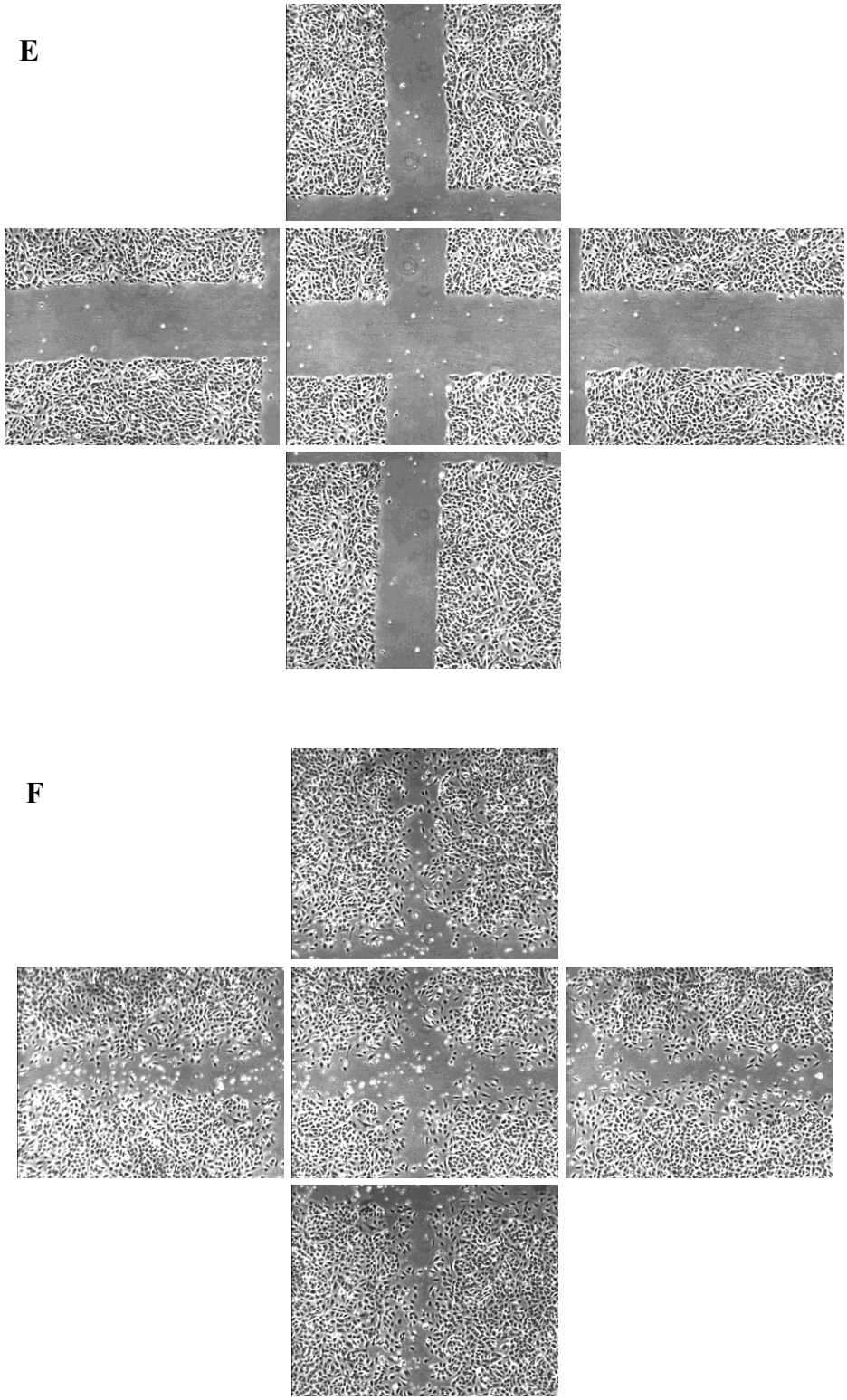


Figure 24. HUVEC infected with SOX18/GFP adenovirus. (A, C, E) Immediately after scratching. (B, D, F) 16 hours after scratching. Addition of Fc (A), ephrinA5-Fc (C) and ephrinA5 monomer (E). Fc, ephrinA5-Fc and ephrinA5 were added at an amount of 0,3 µg/ mL and were clustered with 0,5 µL/mL antibody.

Whereas ephrinA5-Fc should activate and ephrinA5 monomer should block the Eph receptor and make it inaccessible for other ephrin ligands, control Fc fragment should not have any effect on ephrin signaling. In all three cases, no prominent differences in scratching experiments after 16 hours could be observed.

Since effects were observed only in one out of three assays, the results remain controversial, and no definitive conclusions can be drawn at this point. In further studies optimization of the experimental setup will be required.

3 Discussion

3.1 Generating ephrin-Fc fusion proteins

The emphasis of my project was to generate ephrin-Fc fusion proteins and to verify their expression by Western Blot. Since it was shown previously in neuronal cells that the ligands for Eph receptors need to be clustered to evoke receptor activity (Mellitzer et al., 2000; Davis et al., 1994), several groups have used ephrin-Fc fusion proteins in their studies.

EphrinA1-Fc fusion proteins have been used by Brantley-Sieders and his colleagues (2003) to demonstrate that regulation of endothelial cell migration and vascular assembly caused by activation of EphA2 receptor requires activation of Rac1 GTPase by phosphoinositide 3-kinase. Lawrenson et al. (2001) have shown, that ephrinA5-Fc mediated activation of EphA3 in 293T and melanoma cells leads to recruitment of the adaptor protein CrkII to the receptor, resulting in activation of RhoA, leading to cytoskeletal changes.

To proof the repulsive effect of ephrinA5 on migrating cortical interneurons, some of the experiments done by Zimmer and her collegues (2008) included the use of ephrinA5-Fc as well, like the stripe assay.

Vidovic et al. (2007) made an interesting discovery using both Eph- and ephrin-Fc fusion proteins for experiments with adult bees, in which they showed that Eph-ephrin signalling affects learning and memory.

In another interesting approach, Li and his colleagues (2009) were able to show, that treatment of glioma cells with ephrinA5-Fc or EphA2-Fc leads to decreased expression of EGFR (epidermal growth factor receptor), proofing with additional experiments that ephrinA5 acts as a tumor suppressor in glioma.

There is a variety of vectors, allowing the expression of Fc-fusion proteins. The gene of choice is cloned into the plasmid in frame with the Fc part of an immunoglobulin. This strategy allows easy protein purification using protein A or protein G sepharose beads and results in the desired clustering of the protein. There is a variety of applications for Fc-fusion proteins (Flanagan et al., 2007).

The plasmid chosen for my project was pIGplus, into which ephrinA1 to A5 were cloned. As illustrated in the Results section, I was able to express the desired soluble Fc-fusion

proteins as well as the control Fc protein alone both from stably and transiently transfected cells (HEK 293T). After purification using Protein G Sepharose beads, the new tools for *in vitro* assays were ready. Therefore the main aim of my project has been reached by establishing a new technique and providing the ephrin-Fc fusion proteins to the lab for further investigation.

3.2 Generating monomeric ephrinA5 protein

Since dimerized or clustered ephrins are needed to activate their receptor and evoke a response, Ephs blocked by ephrin monomers show a lack of response (Mellitzer et al., 2000; Lawrenson et al., 2000).
For obtaining monomeric ephrin A5, the previously generated Fc- fusion proteins were cleaved by the Xa Protease as described previously (Zimmer et al., 2008). Successful expression of soluble, monomeric ephrin A5 was shown in Western Blot (see the Results section) and allowed therefore further usage of the protein.

3.3 *In Vitro* Assays

In addition, I decided to try some preliminary studies using the ephrinA5-Fc fusion proteins. To study the effect of Eph-ephrin signalling on cell proliferation and migration, different approaches were chosen.

3.3.1 Retraction assays

In the first of two different retraction assays (as described in the Results section), retraction of SOX18-IRES/GFP transiently transfected HUVEC was observed when they were incubated with ephrinA5-Fc clustered with an antibody (as recommended in the literature- see Brantley-Sieders et al., 2003; Zimmer et al., 2008). In control experiments not only non-transfected cells but also cells transfected with EGFP did not change morphology after addition of ephrinA5-Fc.

This result is consistent with data from Lawrenson and his colleagues (2001), who showed that w/t 293T cells do not react significantly to treatment with ephrinA5. In comparison,

addition of ephrinA5 to cells transfected with the corresponding receptor (EphA3) lead to cell rounding and detachment. An effect comparable with my results was shown by Parri et al. (2005) in various experiments, in which cells expressing EphA2 retracted when stimulated with ephrinA1-Fc. In this assay I also demonstrated that the control construct Fc did not lead to a noticeable response of HUVEC transfected with EGFP or SOX18/GFP.

Taken together, these findings and the fact that EphA7 is a target gene of SOX18 as it was shown in our lab, further support the theory that EphA7- ephrinA5 signalling evokes a repulsive response in cells as already described in literature (Holmberg et al., 2002).

It is important to mention that also other Ephs of the A subfamily are receptors for ephrinA5, not to forget the crosstalk between the A and the B subfamily. Cheng Q. et al (2003) reported that ephrinA5 caused morphological changes in PC-3M cells (a prostate epithelial cell line) and observed rounding up of the cells. In this article it is not mentioned which Eph receptors are expressed by the used cell line, so it is still unclear which Eph receptors are involved in the interaction resulting in retraction of the cells caused by ephrinA5.

In the second retraction assay, HUVEC were not transiently transfected like in the previous experiment, but infected with SOX18/GFP adenovirus. Clustering of ephrinA5-Fc was achieved by using protein G polystyrene particles instead of an antibody like in the first assay. No changes of cell morphology could be observed during incubation of the cells with ephrinA5-Fc, which was first hard to believe after the previous approach lead to such an interesting result. The used samples and incubation conditions were the same as before. One theoretical problem could have been that the polystyrene particles may allow less clustering as compared to the use of antibodies. However, this technique has been described in the literature (Wimmer-Kleikamp et al., 2004).

Another important point is that transiently transfecting or infecting the HUVEC with adenovirus could also cause a difference in the cell response to the added protein. So it may be speculated that the transfection agent or the procedure of transfection might influence the biological system. This may have lead to the result that SOX18/GFP transfected cells retracted in contrast to SOX18/GFP adenovirus infected cells during incubation with ephrinA5-Fc.

3.3.2 Wound healing assay

The wound healing assay, which has been described by Miyoshi et al. (2004) and Parri et al. (2005), was chosen as another approach to study cell migration and proliferation. HUVEC were infected with SOX18/GFP adenovirus, and then incubated either with ephrinA5-Fc, which should activate the Eph receptors, or with ephrinA5 monomer, which should block the receptors, or with control Fc, which should have no effect. However, in all three cases no changes in cell migration could be observed. In this experiment, again an antibody was used for clustering ephrinA5-Fc or Fc as in the first retraction assay. It has to be mentioned, that migration of the cells would be easier to observe, if the proliferation was stopped, which would be possible by starving them before the experiment in serum-free medium as it was done by Parri and his colleagues (2005).
I suppose that in this experiment no significant migration of the cells could be noticed because proliferation predominated over migration.

3.4 Conclusions

First, it is important to mention that we did not check the ephrinA5-Fc fusion protein, the ephrinA5 monomer or Fc for activity in terms of binding to HUVEC, so we do not know if these proteins are biologically active. Without this knowledge, the fact that the major part of the *in vitro* assays did not provide the expected data cannot be interpreted. This binding of the recombinant proteins to the cells will need to be confirmed by FACS analysis in future experiments.
Furthermore it is known from the literature, that different splice forms of the EphA7 receptor exist, which determine whether repulsion or adhesion/ propulsion is mediated (Holmberg et al., 2002). As it is not known which splice form is expressed in case of my experiments, it is very difficult to draw a conclusion. This question should be elucidated by real-time PCR of HUVEC cells using appropriate primers that cover the respective regions of the mRNA.
Furthermore, incubation conditions during the retraction assays were not optimal. I tried to work at a stable temperature, so far this was possible with a microscope table heated to 37 degrees. For doing reproducible experiments, doing time-lapse microscopy under stable conditions for the cells (37 degrees, 5% CO2) would definitely be an advantage.

To try a different approach, ephrinA5 was not just expressed as soluble Fc fusion protein without GPI anchor, but also as membrane molecule with GPI anchor. Two cell lines, one which constitutively expresses ephrinA5 and another with Ecdysone-inducible expression (Invitrogen) of ephrinA5, have been established and co-cultured with HUVEC. It was observed that none of the cells showed any significant reaction.

All these results show that additional work will be necessary to elucidate the interactions between Eph receptors and their ligands, both as soluble constructs and membrane molecules, and their consequences in the context of cell migration and/or proliferation. A more systematic approach will be necessary to continue the research on this topic, including first steps like verification of binding of the harvested proteins to the cells and confirmation of a putative effect of transfection (agent) on cell behavior.

Furthermore, it is necessary to study the outcome of incubation of HUVEC with the other ephrin ligands of the A subfamily, which have already been cloned as Fc fusions, to gain a better insight into Eph-ephrin signalling.

Eph-ephrin signalling has been described in the literature as very complex, including activation or modification of a variety of other signaling molecules, leading to transduction cascades which trigger cellular responses. Different expression patterns of Eph receptors and their ligands were shown to even result in a graded outcome of cell repulsion (Mellitzer et al., 2000). Also the degree of multimerization of the ephrins activating their receptor has an effect on the nature and the strength of the cellular response (Mosch et al., 2009).

From these and a number of other findings it can be concluded, that a well-considered approach is necessary to answer some of the many questions appearing in the topic Eph-ephrin signalling.

4 Material and Methods

4.1 DNA techniques

4.1.1.DNA digestion with restriction enzymes

In our lab, all digestions are made with enzymes and buffers from Roche. One unit of restriction enzyme is needed to digest 1 μg of DNA in 1 hour at 37°C. There are also exceptions concerning temperature (e.g. SmaI). Usually, the DNA is digested in a total volume of 15 μL.

Standard reaction mix:
- x μg DNA
- 1,5 μL 10x buffer
- y μL enzyme
- A.d. to 15 μL

4.1.2 Dephosphorylation of linearized plasmid DNA

To prevent religation of the linearized vector, 5 units of Alkaline Phosphatase (Roche) are used to remove 5´ phosphate groups of 5 μg plasmid DNA in a reaction volume of 50 μL. The sample is incubated for 1 hour at 37°C.

Standard reaction mix:
- 5 μg plasmid DNA
- 5 μL 10x buffer
- 5 μL Alkaline Phosphatase
- A.d. to 50 μL

4.1.3 DNA ligation

After digestion of DNA, fragments with compatible ends can be ligated. The ratio plasmid:insert has to be optimised dependent on the size of the fragments. From my experience, it can vary from 1:1 to 1:3. The sample is incubated for 4 hours at room temperature or overnight at 16°C. 3-5 μL is used for transformation of E.coli.

Standard reaction mix: x μg plasmid DNA
y μg insert DNA
1 μL 10x buffer
1 μL T4 DNA Ligase (New England Biolabs)
A.d. to 10 μL

4.1.4 Transformation after ligation

The required amount of competent cells is thawed on ice. 100 μL competent cells and at least 3 μL of the required DNA are mixed on ice and incubated there for 30 minutes. After 1,5 minutes heat shock (42°C) and 1-2 minutes incubation on ice, 1 mL SOC or LB medium is added. The mix is incubated for 1 hour (200 rpm, 37°C) and pelleted (5 min, 3 krpm). After decanting 1 mL of the supernatant, the pellet is resuspended in the remaining supernatant. The suspension is spread onto a LB- Agar plate containing the appropriate antibiotic. After incubation overnight (37°C), colonies can be picked.

4.1.5 Re- Transformation

100 μL competent cells and 1 μL of the required DNA are mixed and treated just like above. 100 μL of the mix, which is incubated for 45 minutes (200 rpm, 37°C), is then spread onto a LB- Agar plate containing the appropriate antibiotic.

SOC Medium

300 mL LB-Medium
3 mL 1 M $MgSO_4$
3 mL 1 M $MgCl_2$
autoclaved
180 μL 20% Glucose
sterile filtered

4.1.6 Preparation of plasmid DNA from E.coli (Mini-Preps)

Bacterial colonies are picked from LB- Agar plates containing the appropriate antibiotic and used to inoculate 5 mL of LB Medium. After incubation overnight (37°C, 200 rpm), the bacterial suspension is pelleted (10 min, 3000 rpm). The supernatant is decanted by turning the tubes completely upside down. After resuspending the pellet in 250 μL P1 buffer, 250 μL P2 buffer is added and the tube is inverted 5-7 times. After 5 min of incubation at room temperature, 250 μL P3 buffer is added and again, the tube is inverted 5-7 times. The tube is centrifuged for 10 min (14 krpm, 4°C) and the supernatant transferred into a new tube. After adding 750 μL isopropanol to precipitate the plasmid DNA, the tube is vortexed and incubated for 20 min at room temperature. Then, the plasmid DNA is pelleted (30 min, 14 krpm, 4°C) and the supernatant removed. The pellet containing plasmid DNA is washed with 100 μL 70% cold ethanol and cetrifuged for 5 min (14 krpm, 4°C). After the supernatant is decanted, the pellet is air-dried at room temperature and resuspended in 50 μL TE buffer.

Resuspension buffer P1 (Qiagen)

50 mM Tris-HCl pH 8,0
10 mM EDTA
100 μg/mL RNase A

Lysis buffer P2 (Qiagen)

200 mM NaOH
1% SDS (w/v)

Neutralization buffer P3 (Qiagen)

3 M potassium acetate pH 5,5

TE buffer pH 8,0

10 mL 1 M Tris pH 8,0 (Trishydroxymethylaminomethane Ultrapure, USB)

2 mL 0,5 M EDTA pH 8,0
H_2O to 1 L

0,5 M EDTA

18,6 g Titriplex III (Ethylendinitrotetraessigsäure Dinatriumsalz Dihydrat, Merck)
H_2O to 70 mL
10 M NaOH to pH 8,0
H_20 to 100 mL

4.1.7 Preparation of plasmid DNA from E.Coli (Maxi/Midi-Prep)- QIAGEN

Bacterial colonies are picked from LB- Agar plates containing the appropriate antibiotic and used to inoculate 250/100 mL of LB Medium. After incubation overnight (37°C, 200 rpm), the bacterial suspension is pelleted (15 Min, 4000 rpm). The pellet is resuspended in 10/4 mL buffer P1 and incubated for 5 minutes at room temperature. After addition of 10/4 mL buffer P2, the tube is inverted 6 times and the lysate incubated for 5 minutes at room temperature. Then, 10/4 mL buffer P3 is added, the tube is inverted 6 times and incubated for 20 minutes on ice. After centrifugation (5 min, 4 krpm), the sample is filtrated through a Whatman filter. In case the sample is further used for transfections of cells, endotoxin removal buffer is added (1/10 of the volume of the filtrate) and the sample incubated for 30 minutes on ice. A Qiagen-tip 500/100 is equilibrated with 10/4 mL buffer QBT and the sample applied. After washing the tip with 2x 30/10 mL buffer QC, the DNA is eluted with 15/5 mL buffer QF into a Corex tube. The DNA is precipitated with 10,5/3,5 mL (0,7 volumes) isopropanol. The tube is mixed and centrifuged (30 min, 9 krpm, 4°C). After decanting the supernatant, the DNA pellet is washed with 5/2 mL 70% ethanol and centrifuged (5 min, 13 krpm, 4°C). Then, the ethanol is taken off, the DNA allowed to air- dry and resuspended in 100/50 µL 1x TE buffer (pH 8,0) or TE endotoxinfree buffer. Eventually, the DNA sample can be precipitated once more after the isopropanol step with sodium-acetate and ethanol abs.. (see: precipitation of DNA)

4.1.8 Preparation of plasmid DNA from E.Coli (Maxi-Prep)- INVITROGEN

Usage	*Instead of buffer..*	
Resuspension	P1	R3
Lysis	P2	L7
Neutralization	P3	N3
Equilibration	QBT	EQ1
Washing	QC	W8
Elution	QF	E4

The column is equlibrated with 30 mL EQ1. After addition of N3 to the sample, it is centrifuged (10 min, 5000 rpm) and then filtrated. When the sample is applied to the column, it is washed with 60 mL W8. The other steps are exactly the same as above.

4.1.9 Precipitation of DNA

1/10 of volume 3 M sodium-acetate (pH 2,5) and 2,5 volumes ice-cold ethanol abs. are added and the sample centrifuged (30 min, 13 krpm, 4°C). After washing it with 70% ethanol, it is centrifuged (5 min, 13 krpm, 4°C). The ethanol is removed and the pellet allowed to air-dry. The DNA is resuspended in a suitable volume of 1x TE buffer (pH 8,0) or TE endotoxinfree buffer.

4.1.10 Isolation of genomic DNA from HEK293 cells

HEK293 cells are cultivated in a 10 cm dish until they reached confluency. After addition of 3 mL lysisbuffer and 150 µL Proteinase K (final conc. 0,5 mg/mL; Sigma), the lysate is incubated for 4 hours at 55°C and then transfered into 4 eppendorf tubes. They are mixed for 5 minutes (14 krpm) and then, 250 µL 6M NaCl is added to each tube. After shaking the tubes for 5 minutes by hand, they are centrifuged (7,5 min; 13,2 krpm). 750 µL of supernatant is transfered to a fresh tube and mixed with 500 µL isopropanol. DNA now becomes visible and the samples are incubated for 5 minutes at room temperature. After centrifugation (2 min, 2000 rpm), supernatants are taken off and the pellets washed with 70% ethanol. Each pellet is resuspended in 50 µL 1x TE (pH 8,0) and the samples are pooled.

Lysisbuffer

10 mL 0,5 M Tris-HCl pH 8,0
20 mL 0,5 M EDTA pH 8,0
10 mL 1 M NaCl
5 mL 20% SDS (Sodium Dodecyl Sulfate Ultra Pure, MP Biomedicals)
H_2O to 100 mL

4.1.11 Determination of DNA concentration

100 µL A. dest. is used as blank. 1 µL of the DNA sample is mixed with 99 µL A. dest and its absorption measured at 260 nm / 280 nm with a spectrophotometer (Pharmacia Biotech). The DNA concentration is calculated with the following formula:

[$OD_{260\ nm}$ x 50 (specific factor for dsDNA) x 100 (dilution factor)] / 1000 =

= DNA concentration [µg/µL]
The ratio is an index for the purity of the DNA sample with an optimum between the values 1,8 and 2.

4.1.12 Agarose gel

DNA fragments are separated according to their size on an agarose gel. Usually (dependent on the expected fragment lenght), a 1% gel is made. For that, 1g agarose (Agarose MP, Roche) is melted in 100 mL 1x TAE buffer. After addition of 5 µL ethidium bromide (Invitrogen; final conc. 0,5 µg/mL), the gel is poured and the comb inserted.
Aliquots of the samples are mixed with DNA gel-loading buffer and loaded onto the gel. 7 µL marker (GeneRuler 1kb DNA Ladder, Fermentas) is used for determination of fragment size. DNA is visualized under UV light.

50x TAE buffer

242 g Tris base
57,1 mL Glacial acetic acid
100 mL 0,5 M EDTA pH 8.0
H_2O to 800 mL
HCl to pH 8,5
H_2O to 1 L

4.1.13 Purification of DNA from agarose gels (GFX PCR DNA & Gel Purification Kit, Amersham)

The slice of agarose containing the DNA of interest is cut out with a razorblade and weighed in a tube. After adding capture buffer (10 μL for 10 mg), the sample is incubated at 60°C until melting of the agarose (about 5 min). When the sample is applied onto the column, it is incubated for one minute at room temperature. Then it is centrifuged (30 s, 14 krpm) and the collection tube emptied. After adding 500 μL washing buffer, the sample is centrifuged as above. The column is placed into a fresh tube and 30 μL 1x TE (pH 8,0) are added. After incubation for one minute at room temperature, the sample is centrifuged. (1 min, 14 krpm).

4.1.14 Sequencing DNA samples

Samples from our lab are sent to MWG Biotech (www.mwg-biotech.com).
5 μg DNA is precipitated and centrifuged (30 min, 13 krpm, 4°C). The supernatant is removed and the pellet allowed to air-dry.
If special primers are needed, 5 μL primer [100 ng/ μL] is sent with the sample.

4.2. Cell culture

4.2.1 Used cell lines

HEK (human embyonic kidney) 293T cells are purchased from ATCC.
HUVEC (human umbilical venous endothelial cells) cells are isolated from umbilical cords (Warren, J.B., 1990, The endothelium, Wiley-Liss New York, p. 263) in our lab.

4.2.2 Culture conditions

All cells are cultured at 37°C and treated under sterile conditions. All dishes (except flasks for HEK) are coated with 1% gelatine/PBS.
HEK 293 cells are grown in DMEM complete and HUVEC cells in M199 complete.

4.2.3 Media

DMEM complete (for HEK 293)

500 mL DMEM (HyQ DMEM/High Glucose Dulbecco´s Modified Eagles Medium, HyClone)
10% FCS (Gibco)
5 mL Glutamine (200 mM)
5 mL Penicillin/Streptomycin (Bio Whittaker, 10 mg/mL)
sterile filtered 0,2 μm

M199 complete (for HUVEC cells)

500 mL M199 (BioWhittaker)
10 mL PSFG (Bio Whittaker)
20% FBS (PAN Biotech)
30 μg/mL ECGS (Techno Clon)
5U/mL Heparin (Roche)
sterile filtered 0,2 μm

Serum-free medium (for HEK 293)

100 mL medium (Ex-Cell 293, SAFC Biosciences)
3 mL Glutamine (200 mM)
3 mL Penicillin/Streptomycin (10 mg/mL)

4.2.4 Passaging cells

After removing the medium, cells are washed with PBS (BioWhittaker) and Trypsin (Trypsin EDTA, BioWhittaker) is added. When the cells round up, the Trypsin is stopped by adding complete medium. Therefore, the cells are resuspended in the required volume of medium and seeded into a new dish.

4.2.5 Freezing and thawing cells

For freezing cells, they are washed twice with PBS, trypsinized and resuspended in complete medium. After centrifugation (5 min, 1000 rpm), the supernatant is taken off and the cell pellet resuspended in medium [M199 or DMEM + same volume serum, 10% DMSO (Dimethyl Sulphoxide Hybri-Max, Sigma)]. Pre-cooled cryogenic vials (Nalgene) are filled with 1 mL of cell suspension, put into a cellfreezer (Cell Freeze Container, Nalgene) and then placed at –80°C overnight to achieve cooling –1°C/min. Then, the vials are stored in a liquid nitrogen container.
For thawing cells, the cryogenic vials are placed into a waterbath (37°C). As soon as there is hardly any ice left, cells are immediately transferred into prewarmed medium and further cultivated.

4.2.6 Calcium- phosphate transfection of HEK 293 cells (transient)

Cells are cultured in a 6 well plate (2 mL medium/ well) until they are 60-70% confluent. In a 4 mL polypropylene tube, 59 µL A. dest. is mixed with 4 µg DNA. After addition of 9 µL 2 M $CaCl_2$, 72 µL 2x HeBS (Hepes buffered saline) is added drop-wise. The tube is

shaken and after 3-4 minutes of incubation, the mixture is added drop-wise to one well. After 24 hours, medium is exchanged. After 48 hours, cells are harvested.

2 M $CaCl_2$

$CaCl_2$ x 2 H_2O (Merck)

sterile filtered through 0,2 μm filter

2x HeBS

400 mL H_2O

8 g NaCl

0,107 g Na_2HPO_4 (Merck)

6,5 g Hepes (Sigma)

H_2O to 800 mL

HCl to pH 7,05

H_2O to 1 L

sterile filtered through 0,2 μm filter

4.2.7 Reporter gene assay

Cells are cultured in a 12 well plate and transiently transfected in triplicates. After 48 hours, they are lysed with 50 μL lysisbuffer (Passive Lysis Buffer 5x, Promega) and shaken for 30 minutes at room temperature. The lysate is then used for measuring luciferase activity. 5 μL is mixed with 350 μL reaction mix containing ATP and the sample is measured in a luminometer (Lumat LB 9501, Berthold). For each sample, 100 μL injection mix containing luciferin is injected.

For measuring β- galactosidase expression, a 96 well plate is used for mixing 5 μL lysate with 95 μL CPRG. After a change of color is noticed (time varies for different amounts of β- gal), absorbance is measured at 570 nm in an ELISA plate reader (SLT 340 ATTC).

All data are expressed as RLU (relative light units)/ β-gal. Additionally, the fold induction is calculated, which shows expression levels in comparison to mock- expression levels.

Injection Mix

9 mL 25 mM Glycylglycine pH 7,8 (Sigma)
1 mL 20 mM ATP pH 7,4 (Sigma)
0,1 mL 1 M $MgSO_4$

Reaction Mix

4 mL 25 mM Glycylglycine pH 7,8
1 mL 1 mM Luciferin (BioThema)
Luciferin is only soluble in A. dest., when NaOH is added.

CPRG (Roche)

0,5 mg/mL in 1x PBS def. + 0,1% BSA (Sigma)

4.2.8 PEI transfection of HUVEC cells

Cells are cultured in 6 well plates until they are 40-80% confluent. In a 4 mL polypropylene tube, 2 μg DNA is mixed with 125 μL HBS. After mixing in a second tube 2-10 μL PEI and 125 μL HBS, the PEI solution is added drop-wise to the DNA solution. The mix is incubated for 20 minutes at room temperature.
After washing the cells with PBS, 800 μL Optimem (OptiMEM I, Gibco) is added to each well. The PEI-DNA solution is added drop-wise to the medium and after 2-4 hours, 2 mL M199 complete is added to each well. After 24 hours, cells are harvested.

2x HBS: see 2x HeBS
HCl to pH 7,4

PEI (Sigma-Aldrich)

4.3 Bacterial techniques and media

4.3.1 Generating chemical competent cells

2 mL of a bacterial preculture is inoculated to 200 mL LB medium. Cells are incubated at 37°C (200 rpm) until the OD of 0,4 (595 nm) is reached. After centrifugation (15 min, 4 krpm, 4°C), the pellet is resuspended in 20 mL ice-cold TSS buffer on ice and 5 mL 87% glycerol is added. Aliquots of 250 µL are made and frozen in liquid N_2. Storage –80°C.

TSS buffer

10 g PEG
1 mL 1 M $MgCl_2$
1 mL 1 M $MgSO_4$
93 mL LB-Medium
5 mL DMSO
sterile filtered

4.3.2 Preparing glycerol- stocks

1 mL of the bacterial overnight- culture is mixed with 1 mL 30% glycerol and then put on –80°C.

4.3.3 Media

LB Medium

20g/L LB Broth Base (Invitrogen)

LB Agar plates

15g agar (Bacto Agar, Difco)/ L LB medium

Used antibiotics for bacteria

Antibiotic (Sigma)	*Stock concentration*	*Working concentration*
Ampicillin	50 mg/mL	100 μg/mL
Kanamycin	10 mg/mL	50 μg/mL

4.4 Special protein techniques

4.4.1 Isolation of ephrin-Fc fusion proteins/ Fc fragments with Protein G Sepharose beads

HEK 293 cells are cultured for two days after transfection in serum-free medium. Conditioned medium from the cells secreting the ephrin-Fc fusion protein or the Fc- fragment is collected and centrifuged in a Falcon tube (5 min; 4,5 krpm). After incubating the supernatant with Protein G Sepharose beads (Protein G Sepharose 4 Fast Flow, GE Healthcare; for 40-50 mL medium 50 μL beads) for 15 minutes at 4°C, it is centrifuged (2,5 min; 500 rpm). The supernatant is taken off and the beads are washed twice with diluted 1x PBS (1:3) at a centrifugation speed of 1 krpm for 1 minute. After taking off the supernatant, 200 μL elution buffer (0,1 M glycine pH 2,5) is added to the beads. The tubes are mixed by inverting them for 10 minutes at 4°C and then centrifuged (1 min, 14 krpm). The supernatant is transfered into a new tube containing 23 μL neutralisation buffer (0,4 M Tris-HCl pH 8,8). 150 μL 1 M Tris-HCl pH 8,0 and 400 μL 87% glycerol are added and the sample is stored at –20°C.

4.4.2 Digestion of ephrin-Fc fusion proteins with Xa protease

When the sepharose beads from the previous steps are washed with diluted 1x PBS for the first time, about one third of the prep is used to incubate the beads with Xa Protease reaction buffer. After taking off the supernatant, 100 μL reaction buffer is added to the beads. The sample is incubated with 3 μL Xa Protease (Factor Xa Protease 400 units, Qiagen) and 1 μL Heparin overnight at 37°C. Then, the sample is centrifuged to collect the remaining

beads at the bottom of the tube and the supernatant is mixed in a new tube with 75 µL 1M Tris-HCl pH 8,0 and 200 µL 87% Glycerin. The sample is stored at –20°C.

Xa Protease Reaction buffer

4 mL 500 mM Tris-HCl pH 6,8
1 mL 5 M NaCl
0,1 mL 1 M $CaCl_2$

4.4.3 Removal of Xa protease after digestion

150 µL Xa Removal Resin (Qiagen) is centrifuged (5 min, 1 krpm). After taking off the supernatant, 750 µL reaction buffer is added to the beads and centrifuged. The supernatant is taken off and the sample from the previous step (after incubation overnight) is added. It is incubated for 10 minutes at 4°C and then centrifuged (5 min, 1 krpm). The supernatant is stored.

4.5 Verification of gene expression

4.5.1 SDS- Page (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis)

This technique is used to separate proteins according to their size.
After pouring the separation gel (the chosen percentage is dependent on the molecular weight of the protein), it is overlaid with butanol and allowed to polymerize. Then, the gel is washed with distilled water, the stacking gel poured and the comb inserted. After polymerization, the whole gel is inserted into the chamber of the apparatus containing running buffer. After mixing the samples with gel-loading buffer and heating them for 10 minutes on 95°C, aliquots are loaded onto the gel. On a single lane, 7 µL of Prestained Protein Molecular Weight Marker (Fermentas; 0,2 mg/mL of each protein) is loaded. Electrophoresis is done at a voltage of 70V.
To visualize all proteins after SDS-Page, a Coomassie staining can be done. A Western Blot is necessary to detect your specific protein of interest and for determining its size.

Gel percentage	protein size range
10%	21 – 100 kDa
12%	10 – 40 kDa

Separation gel	*10%*	*12,5%*
A. dest.	4,1 mL	3,3 mL
Solution A	2,5 mL	2,5 mL
Solution B	3,4 mL	4,2 mL
10% APS	50 µL	50 µL
TEMED	10 µL	10 µL

Stacking gel	
A. dest.	3,1 mL
Solution Ast	1,25 mL
Solution B	0,65 mL
10% APS	50 µL
TEMED	10 µL

Solution A

181,5 g Tris
4 g SDS
HCl to pH 8,8
A.d. to 1 L

Solution B

75 mL 40% Acryl- Bis-acrylamide (29-1 Mixture 40% Solution, Qbiogene)
25 mL A.d.

Solution Ast

60 g Tris
4 g SDS
400 mg Phenolred
HCl to pH 6,8
A.d. to 1 L

10% APS

N, N, N´, N´- Ammonium Persulfate, International Biotechnologies

TEMED

Tetramethylethylenediamine, Fluka

10x Running buffer

30 g Tris
144 g Glycine (Fluka)
10 g SDS
A.d. to 1 L

2x SDS gel-loading buffer

150 mM Tris-HCl pH 6,8
300 mM DTT
6% SDS
0,3% Bromphenolblue (Fluka)
30% Glycerol

4.5.2 Western Blot

The components for the „blot sandwich“ are soaked with blotting buffer and then put into the blotting apparatus as follows: black- sponge- 2 whattman papers (9 x 7 cm)- gel- membrane (size according to gel. Hybond-C Extra, Amersham)- 2 whattman papers- sponge-white. Blotting is carried out for 1,5 hours at 300 mA. Afterwards, the membrane is washed with PBS Tween to remove the methanol and incubated for a few minutes in Ponceau S solution. After washing it three times with water, the protein bands become visible. Then, the membrane is blocked with 5% skim milk (Merck) in PBS Tween + 0,02% NaN_3 for 30 minutes to avoid unspecific binding of the antibodies and washed 3 times with PBS Tween. The membrane is incubated overnight at 4°C with the primary antibody (in PBS Tween + 1% BSA + 0,02% NaN_3). Next day, the membrane is washed 3 times with PBS Tween and incubated with the secondary antibody (in PBS Tween) for one hour at room temperature. This antibody binds to the primary antibody and is coupled to the enzyme horse raddish peroxidase (HRP). After washin with PBS Tween 3 to 4 times (each at least

5 minutes), the membrane is incubated for 5 minutes with the substrate (SuperSignal West Pico Chemiluminiscent Substrate, Pierce). The light generated in this reaction is captured on an X-ray film (Fujifilm Super RX) giving a black band representing the protein of interest, whose size is determined according to the marker.
In case a directly conjugated antibody is used, membranes are blocked, washed and then incubated with the antibody for one hour at room temperature.

Blotting buffer

100 mL 10x running buffer
200 mL methanol
700 mL A.d.

10x PBS (phosphate-buffered saline)

80 g NaCl
2 g KCl
17,8 g Na_2HPO_4 x 2 H_2O
2,4 g KH_2PO_4
H_2O to 800 mL
HCl to pH 7,4
H_2O to 1 L

PBS Tween 0,1%

1 mL Tween (Plus One Tween 20, Amersham Biosciences)
1 L PBS

4.6 Used antibodies

Antibody	*Company*	*Dilution*
Anti- human IgG, Fc specific (Peroxidase Conjugate)	Sigma	1:2000
EphrinA5, goat polyclonal IgG	Santa Cruz	1:200
Donkey anti-goat IgG- HRP	Santa Cruz	1:5000

In case the ephrin A5 antibody is used, additional blocking (1 hour) after the last washing step is necessary.

4.7 Primers for cloning

Name	*Sequence*	*Restriction sites*
ephrinA1h forward	ATGGGGTACCACCATGGAGTTCCTCTGGGCC	KpnI
ephrinA1h reverse	AACGGGATCCATACTGTGAGCGATGCTATGTAG	BamHI
ephrinA2m forward	ATGGGGTACCACCATGGCGCCCGCGCAGCGC	KpnI
ephrinA2m reverse	AACGGGATCCATGTTACTGGTGAAGATGGGCTC	BamHI
ephrinA3h forward	ATGGGGTACCACCATGGCGGCGGCTCCGCTG	KpnI
ephrinA3h reverse	AACGGGATCCATCCCGCTGATGCTCTTCTCAAG	BamHI
ephrinA4m forward	ATGGGGTACCACCATGCGGCTGCTGCCCCTG	KpnI
ephrinA4m reverse	AACGGGATCCATGCTTTCTCCAGGACTCCCAAC	BamHI
ephrinA5h forward	AACTAGCTAGCATGTTGCACGTGGAGATG	NheI
ephrinA5h reverse	AAGCGGGATCCAAGTTCTCGCCGCGGGAT	BamHI

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CURRICULUM VITAE
Angela Maria PEER

Geboren am: 15.2.1984 (Horn, NÖ)
Wohnhaft in: Kulmgasse 44/39, 1170 Wien

Sept. 1990 – Juni 1994	Besuch der Volksschule in Langenlois
Sept. 1994 - Juni 2002	Besuch des Bundesgymnasiums Piaristeng. 2, 3500 Krems
10.6.2002	Reifeprüfung mit Auszeichnung
Okt. 2002	Beginn des Studiums Biologie, Studienzweig Genetik-Universität Wien
19.3.2004	Abschluß des ersten Studienabschnittes (1. Diplomprüfung)
30.6.2006	Beginn des praktischen Teiles der Diplomarbeit am Institut für Gefäßbiologie und Thromboseforschung der Medizinischen Universität Wien
30.6.2007	Beendigung des praktischen Teiles der Diplomarbeit
seit 1.7.2007	Technische Assistentin/ Research Assistant im IMBA (Institute of Molecular Biotechnolgy of the Austrian Academy of Scienes), Arbeitsgruppe Dr. Jürgen Knoblich
1.3.2010	Erwerbung des Grades Bachelor of Science