Dissertation zur Erlangung des Doktorgrades
Der Fakultät für Chemie und Pharmazie
Der Ludwig-Maximilians-Universität München

The role of Src-homology 2 domain containing tyrosine phosphatase 2 in growth factor dependent endothelial signalling and angiogenesis

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Vallentuna, Schweden

2007

Erklärung

Diese Dissertation wurde im Sinne von § 13 Abs. 3 bzw. 4 der Promotionsordnung vom 29. Januar 1998 von Herrn Prof. Ulrich Pohl betreut und von Herrn Prof. Ernst Wagner vor der Fakultät für Chemie und Pharmazie vertreten.

Ehrenwörtliche Versicherung

Diese Dissertation wurde selbständig, ohne unerlaubte Hilfe erarbeitet.

München, am ……

……

Unterschrift des Autors

Dissertation eingereicht am	…09.03.2007……...
1. Gutachter	…Prof. Ernst Wagner..
2. Gutachter	…Prof. Ulrich Pohl……
Mündliche Prüfung am	…24.05.2007…….

CONTENTS

1. INTRODUCTION

“The important thing in science is not so much to obtain new facts as to discover new ways of thinking about them.”

~Sir William Bragg~

1.1. THE PROCESS OF ANGIOGENESIS

The formation of new blood vessels is subdivided into vasculogenesis and angiogenesis. Vasculogenesis is the formation of blood vessels *de novo,* in the absence of any pre-existing vascular system, from endothelial cell precursors called hemangioblasts. In vasculogenesis the endothelial cell precursors proliferate and migrate into avascular areas starting to form a primitive tubular network. These hemangioblasts later differentiate into endothelial cells [1-3]. This process was until recently believed to only occur during embryogenesis. However, new findings show that endothelial precursor cells exist in the bone marrow in adults [4] and it has further been shown in animal models that they are able to participate in both physiological and pathological neovascularization [5]. These findings indicate that vasculogenesis not only occurs in the early embryo but also contributes to new vessel formation in adults.

Once the primary vessel plexus has been formed, it is remodelled. Vessels fuse to form larger vessels or divide to create a network of smaller vessels. This sprouting of endothelial cells from already existing vessels to further expand the vascular tree is referred to as angiogenesis and occurs not only in the embryo but is repeated many times in the adult during wound healing and the reproductive cycle [1-3]. In addition to the angiogenesis belonging to daily life, several pathological conditions can induce this process, such as tumour growth, ischemia, chronic inflammatory diseases and diabetic retinopathy [6,7].

Angiogenesis is a complicated highly regulated process dependent on the interplay and balance between several angiogenic and anti-angiogenic factors favouring or inhibiting angiogenesis respectively. Angiogenesis is comprised of different steps, as pointed out below, such as vasodilation and permeability of the already existing vessel, vessel destabilization and matrix degradation, endothelial cell proliferation and migration and finally lumen formation and vessel stabilization. Figure 1.1 contains a summarized description of the angiogenesis process and the factors influencing it.

Vasodilation and endothelial permeability

Vasodilation of the pre-existing blood vessel initiates angiogenesis and is mediated by nitric oxide (NO) which stimulates the relaxation of the smooth muscle cells surrounding the vessel by the formation of cyclic guanosine monophosphate (cGMP) [8]. To increase the permeability of endothelial cells, intercellular clefts and/or vesiculo-vacuolar organelles (VVO) are formed. The latter are grape like clusters of vesicles and vacuoles which can span from the luminal to the abluminal side of the plasma membrane. Both mechanisms permit macromolecules, such as fibrinogen and plasminogen, to pass through the endothelial layer [9]. The extravasation of these molecules constitutes a temporary framework for the later migrating endothelial cells. Both vasodilation and increased permeability are induced and initiated by vascular endothelial growth factor (VEGF), thus being the most critical factor for vessel formation [10-12].

Vessel destabilization and matrix degradation

To allow for endothelial cells to sprout from the existing vessel, the mature vessel needs to be destabilized. For this the intercellular and pericellular contacts need to be disrupted. Angiopoetin 2 may be of importance here by detaching the surrounding smooth muscle cells or pericytes and by loosening the matrix [8]. Further, the endothelial cell basement membrane must be degraded and the perivascular stroma has to be remodelled. This is achieved by different proteinase members of the plasminogen activator, matrix metalloproteinases, chymase and heparanase families. The endothelial cells secrete most of these extracellular matrix (ECM) degrading enzymes upon growth factor stimulation, such as VEGF and basic fibroblast growth factor (bFGF) [13]. The degradation of the ECM also releases growth factors such as bFGF, VEGF and insulin growth factor 1 (IGF-1) further stimulating angiogenesis [2,8].

Endothelial cell proliferation and migration

Once the path has been cleared, proliferating endothelial cells migrate into the remodelled perivascular space along a chemotactic gradient established by angiogenic growth factors. Important factors driving proliferation and migration are members of the VEGF- and FGF family, epidermal growth factor (EGF), insuline like

growth factor 1 (IGF-1), angiopoetin 1 and 2 (Ang1 and 2), angiogenin, the integrins $\alpha_v\beta_3$ and $\alpha_5\beta_3$ and C-X-C chemokines (chemokines with paired cysteines, C, separated by a different amino acid) such as interleukin 8 (IL-8). Whereas VEGF and the angiopoetins are specific for the endothelium, the other proangiogenic factors can also induce proliferation and migration of other cell types [2].

Tube formation and vessel stabilization

When the endothelial cells have reached the site of supposed neovascularization, they organize themselves into a monolayer to form tube-like structures. By intercalation and thinning of the endothelial cells and fusion of already existing vessels, the new vessel increases its diameter and length [2,8]. Some isoforms of VEGF and Ang1 combined with VEGF are responsible for the increase of lumen diameter. Essential factors for endothelial cells to adhere to each other and so extending the new vessel are cell surface receptors such as the endothelial cell adhesion molecule (PECAM-1) and vascular endothelial (VE)-cadherin [1]. The formation of tube-like structures is not only regulated by different growth factors, adhesion molecules and their respective receptors but also by the extracellular matrix (ECM). Whereas endothelial cells grown on collagen in two-dimensional (2D) culture dishes are in a proliferative state, endothelial cells grown in a three-dimensional (3D) collagen gel seize to proliferate and exhibit a more differentiated phenotype to finally form tube-like structures [14,15]. The integrin family is one of many to mediate cell-extracellular matrix interactions and function as receptors for the ECM on endothelial cells to promote cell motility or maintain vascular stability [16]. The integrins $\alpha_v\beta_3$, which is a vitronectin receptor, and $\alpha_1\beta_1$, a collagen receptor, are examples of integrins involved in lumen formation [2,3,17]. Once the endothelial cells have interconnected this network needs to be strengthened. This is accomplished by the basement membrane laminin-binding integrins such as $\alpha_6\beta_1$ and $\alpha_3\beta_1$ [17]. Finally, the premature vessel needs to be further stabilized. For this mesenchymal cells proliferate and migrate along the newly formed endothelial tube where they then differentiate into pericytes (lining small vessels) or smooth muscle cells (lining larger vessels) [2,3,13]. Angiogenic factors inducing these mechanisms are platelet derived growth factor BB (PDGF-BB), angiopoetin 1 and VEGF. VEGF contributes probably not only by inducing VEGF dependent cell signalling but also by stimulating the release of PDGF thereby recruiting mural cells. This recruitment is also promoted by tissue factor. Ang1

stabilizes the interaction between mural cells and the endothelial tube and induces branching [8]. Another important agent is tumour necrosis factor β (TGFβ), which inhibits endothelial cell growth and migration thereby enabling stabilization and maturation of the vessel. In addition, TGFβ controls the differentiation of vascular smooth muscle cells and stimulates new extracellular matrix production to further strengthen the interaction between endothelial and mural cells [2,3,8,13]. Other factors stimulating this process include heparin-binding epidermal-growth-factor like factor and endothelin-1.

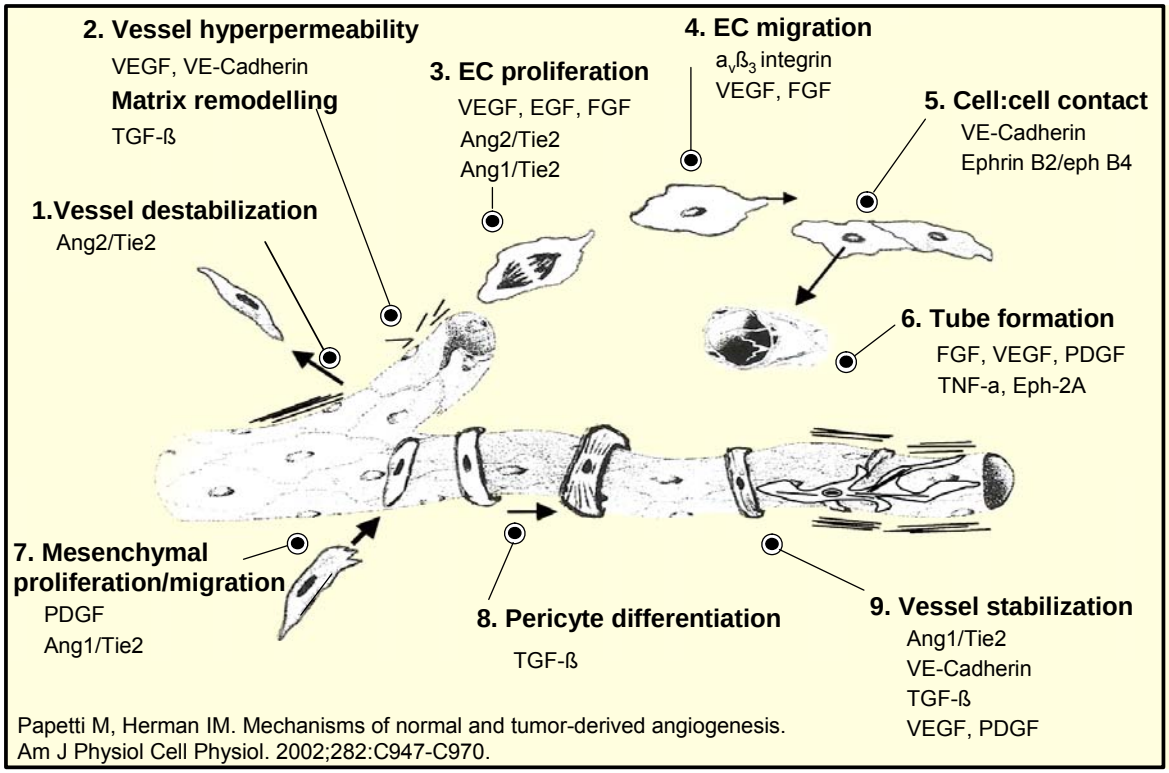


Fig. 1.1 The process of angiogenesis

Angiogenesis is initiated by vessel dilation, permeabilisation and destabilization. This is followed by proliferation of endothelial cells, which migrate into the avascular space where they form cell-cell contacts for subsequent tube formation. Finally the new vessels become stabilised by pericytes or smooth muscle cells. This process is influenced by different factors.

1.1.1. VEGF and angiogenesis

In 1983 Senger et al. identified a 34-42 kDa protein in tumour ascites fluids from guinea pigs that increased microvascular permeability. This protein was named tumour vascular permeability factor (VPF) [18]. In 1989 Ferrara et al reported the finding of a growth factor having mitogenic effects exclusively on endothelial cells. The factor was therefore named vascular endothelial growth factor (VEGF) [19]. Later the same year, cloning of both VPF [20]and VEGF [21] revealed that it was the same protein. Meanwhile the human VEGF family is known to consist of VEGF-A, -B, -C, -D, and placental growth factor (PlGF). The members of the VEGF-family are dimeric glycoproteins belonging to the platelet derived growth factor (PDGF) superfamily and bind with different affinities to three related receptor tyrosine kinases: VEGF receptor 1 (VEGFR-1 or Flt-1), VEGF receptor 2 (VEGFR-2 or KDR) and VEGF receptor 3 (VEGFR-3 or Flt-4). VEGF-A binds to VEGFR-1 and -2 as well as to receptor heterodimers. VEGF-A also binds to Neuropilin-1, a neuronal receptor for the collapsin/semaphorin family also expressed on endothelial cells. Neuropilin-1 interacts with VEGFR-2 and thus enhances the binding of VEGF-A to VEGFR-2 [22,23]. VEGF-B and PlGF only bind VEGFR-1, while VEGF-C prefers binding to VEGFR-2 and-3. Although VEGFR-1 is more abundant in adult tissue and binds VEGF with a higher affinity than VEGFR-2 [2], it has been shown that VEGFR-2 is mainly responsible for VEGF signalling [24].

In contrast to the other VEGF isoforms, which seem to exhibit tissue specificities, VEGF-A is expressed in most organs in humans and at least five different isoforms of the human VEGF-A exist due to alternative splicing of a single pre-mRNA [2,7,13,22,24]. The different isoforms are denoted after their amount of amino acids ($VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$, $VEGF_{206}$) and exhibit different signalling properties as well as different expression patterns. Isoforms consisting of 148, 162 and 183 amino acids have also been reported as well as the isoform VEGF-A_{165}b which seems to have opposing effects to VEGF-A_{165}. VEGF-A_{145}, VEGF-A_{189}, VEGF-A_{206} are bound to the cell surface due to heparin-binding domains whereas VEGF-A_{121} and VEGF-A_{165} are diffusible. The latter two isoforms are the most abundant ones but VEGF-A_{165} seems to be the most potent promoting angiogenesis as it stimulates endothelial DNA synthesis and proliferation, has anti-apoptotic effects and induces endothelial cell migration. VEGF induced endothelial cell proliferation is mainly

mediated by the Ras-Raf-MEK-ERK pathway [2,24,25] but also by the formation of NO, while endothelial cell survival is maintained by activation of the PI3-Kinase/Akt pathway as well as through interaction with several integrins. Migration of endothelial cells upon VEGF stimulation is driven by p38-MAPK signalling, the focal adhesion kinase (FAK) and RhoA GTPase among others [24,25].

VEGF has a prominent role in angiogenesis. Mice lacking a VEGF allele die in utero between day 11 and 12, probably due to defective vascularization [23,24]. Deletion or mutation of VEGFR-2 is also embryonically lethal due to impaired development of both endothelial and hematopoietic cells and the complete absence of blood vessels [2,10,23,24]. Deletion of VEGFR-1 leads to failure of formation of an organized vascular network. Interestingly, studies where the tyrosine kinase domain but not the intracellular domain of the VEGFR-1 was absent showed no defects in vessel formation. This led to the proposition that VEGFR-1 may compete with VEGFR-2 for substrate during angiogenesis and an inability to do so would lead to elevated levels of available VEGF for VEGFR-2 and so to an excessive VEGFR-2 signalling. This could be responsible for the impairment in normal vessel growth in the VEGFR-1 knock-out model [2,10,23,24]. VEGF is not only crucial for embryonic angiogenesis but it is also essential for angiogenesis in adults as administration of soluble VEGF receptors, which block VEGF signalling, in juvenile mice results in suppression of blood vessel invasion into bone growth plates leading to reduced femur length [2,3]. As mentioned earlier, VEGFR-1 does not seem to be the primary mediator of VEGF signalling during development. This may be due to its poor kinase activity and it has further been reported that only weak proliferative and migratory responses are mediated through this receptor. VEGFR-1 has recently been shown to be more important in pathological angiogenesis and wound healing by facilitating VEGFR-2 signalling. VEGFR-2 on the other hand regulates endothelial cell proliferation, migration, differentiation, survival and increases vessel permeability and dilation [23]. VEGFR-3 does not seem to be essential for the vasculogenesis in embryos, but is more important for the remodelling of the primitive vascular plexus as mice with a disrupted VEGFR-3 fail to remodel the plexus and die after day 9.5 due to cardiac failure. The receptor appears to be responsible for the formation of blood as well as for lymphatic vasculature in the embryo but is constricted to the lymphatic vessels in adults. In addition to the VEGFR-1-3, the VEGF receptor Neuropilin-1 also seems to

be essential for normal vessel formation as Neuropilin deficient mice show incomplete vessels and vascular network development [2] (For a schematic illustration of the role of VEGF in angiogenesis, see fig. 1.1).

1.1.2. FGF and angiogenesis

20 distinct fibroblast growth factors (FGF1-20) and four tyrosine kinase receptors (FGFR1-4) have been identified so far. Among these 20 different FGFs, acidic FGF (aFGF) or FGF-1 and basic FGF (bFGF) or FGF-2 are the best described in angiogenesis. They are secreted from a variety of different cell types and can bind to all four receptors [2,26]. bFGF has been shown to induce endothelial cell proliferation by binding mainly to the FGFR-1 thereby activating the MAPK pathway [25,27]. Further it has been demonstrated that bFGF is important for endothelial cell migration and differentiation as well as for endothelial cell survival in combination with VEGF by activating the PI3-K pathway [28]. In addition, bFGF has been shown to stimulate endothelial cells to form tube like structures in a 3D collagen matrix [13,29]. Moreover, it was also observed that bFGF induced the production of urokinase-type plasminogen activator (u-PA) in endothelial cells, which relates well to the invasive properties of endothelial cells during angiogenesis [29]. Further, bFGF increases the expression of $\alpha_v\beta_3$ integrins on endothelial cells [2,26] thereby probably facilitating migration. The VEGF expression is also upregulated by bFGF and it induces angiogenesis in the chicken chorioallantoic membrane (CAM) assay [28]. Recently, it was shown that also aFGF is capable of inducing sprouting of new vessels in the CAM assay [30,31]. Astonishingly, vascular development is normal in mice deficient in aFGF and bFGF [2,13,26]. On the other hand, wound repair seemed to be delayed in bFGF and bFGF/aFGF knock-out mice [2,26]. FGFs are stored not only in endothelial cells but also in the ECM by binding to heparin. Mechanical injury disrupts the ECM and endothelial cells thus release bFGF. Mechanical force has also been proposed to stimulate the endothelium to release FGF. This suggests that FGF *in vivo* may be more important in angiogenesis in the context of tissue repair rather than in all angiogenic responses [13,26] (Fig. 1.1 shows the overall role of FGF in angiogenesis).

1.1.3. PDGF and angiogenesis

PDGF exist in homodimers (PDGF-AA, PDGF-BB, PDGF-CC and PDGF-DD) and heterodimers (PDGF-AB). The PDGF receptors exhibit different binding affinities

depending on the isoforms. PDGF-AA forms αα-receptor homodimers, PDGF-AB induce the formation of αα-receptor homodimers and αβ-receptor heterodimers. PDGF-BB is able to form all three compositions (αα, αβ and ββ) while PDGF-CC and –DD mainly form αα- and ββ-receptor homodimers [13,32]. Whereas extensive research has been performed on VEGF and FGF in angiogenesis, less is known about the exact role of PDGF in this context. Capillary endothelial cells express PDGF β-receptors and PDGF-BB has been found to stimulate endothelial cell proliferation and tube formation *in vitro* [33] as well as to increase the vessel density in the chicken chorioallantoic membrane [34], whereas PDGF-AA seemed to elicit less angiogenic effects both *in vitro* and *in vivo* [33-35]. The angiogenic potency of PDGF-BB is further strengthened by the finding that the movement of entire endothelial cord structures is increased when exposed to PDGF-BB [36]. Blockage of the PDGF β-receptor in glomerular endothelial cells led to apoptosis, indicating a role for PDGF in endothelial cell survival [37]. PDGF-B knock-out mice die during late gestation by an abnormal renal development and failure of pericytes to migrate to the new blood vessels. Finally, the role of PDGF in angiogenic processes is highlighted by its potency in mediating tumour angiogenesis. PDGF released from tumour cells stimulate the migration and proliferation of both vascular smooth muscle cells and endothelial cells. Furthermore, PDGF has been shown to also indirectly stimulate angiogenesis by inducing the transcription and secretion of VEGF [35].

1.2. ANGIOGENIC PATHWAYS

1.2.1. The PI3-Kinase pathway

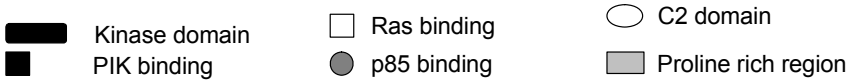
Anti-apoptotic signals are necessary for angiogenesis to occur. Normally, when cells are detached from the extracellular matrix, they undergo programmed cell death, apoptosis. This form of apoptosis which is induced by the lack of attachments to ECM is also called anoikis and preserves the organism from dysplastic growth of cells [38,39]. During angiogenesis however, the ECM is degraded and the endothelial cells loose their attachments to the ECM without undergoing apoptosis. This is achieved by repression of the apoptotic signals by certain anti-apoptotic or cell survival signalling pathways. Not only is the anti-apoptotic effect important in this particular step of angiogenesis but it is vital through the whole angiogenic process as

well as for preservation of the quiescent endothelium preventing vascular regression. There are several factors promoting cell survival and thus inhibiting apoptosis, such as the survival factors bFGF and VEGF, NO, integrin-adhesion to the ECM and interendothelial connections through VE-Cadherin. They all transduce their signals through different intracellular signalling molecules but almost all of the signalling pathways activated seem to merge at one point, they induce the activation of the phosphoinositide 3-Kinase (PI3-Kinase)/Akt pathway. However, the PI3-Kinase/Akt pathway not only suppresses apoptosis but also promotes cell proliferation as well as migration and is involved in vesicular trafficking, differentiation and cytoskeletal rearrangements [33,40].

In mammals there are three classes of PI3-kinases, namely class I, class II and class III based on their structures and substrate specificity (for a more detailed description of the different members of the three classes, see fig. 1.2). The best studied group is the class I, which is further divided into class IA and IB. The members of these classes phosphorylate phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$) thereby producing phosphatidylinositol-3,4,5-trisphosphate ($PI(3,4,5)P_3$). The class IA and IB enzymes all form heterodimeric complexes consisting of a catalytic subunit of 110-120 kDa and a non catalytic regulatory subunit or adaptor of 55 or 85 kDa. The class I catalytic subunits include p110α, -β, -δ and -γ and all, except p110γ, contain a p85 subunit binding domain at the N-terminus. They further contain a Ras binding domain, a phosphoinositide kinase domain and a C-terminal catalytic domain [41]. They all exhibit serine/threonine protein kinase activity used to phosphorylate the regulatory subunit p85 on a serine residue. This phosphorylation decreases PI3-Kinase activity [41-43]. Whereas the p110α and -β are ubiquitously expressed, the p110δ is predominantly expressed in leucocytes and the p110γ is found in pancreas, skeletal muscle, liver and heart. The regulatory subunits of the class IA are encoded by at least three different genes which give rise to three isoforms p85α, -β and p55 γ [41,44]. The subunits p85α and -β contain two Src- homology 2 (SH2) domains which allow the subunit to bind to phospho-tyrosines. Interaction with p110 is mediated by the region situated between the two SH2 domains. In addition they exhibit a Src- homology 3 (SH3) domain at the N-terminus directing protein interaction to polyproline motif regions [40,41,45]. The class IB regulatory subunit is a 97 kDa protein called p101 [41]. It is the regulatory subunit that controls the recruitment

of PI3-Kinase to the membrane, enabling it to associate with membrane associated signalling complexes, and brings it close to its lipid substrate [41,44]. In quiescent cells the degradation of the p110 subunit is prevented and the lipid kinase activity is inhibited by the regulatory subunit. This suppression is relieved when the SH2 domains of p85 bind tyrosine phosphorylated proteins such as tyrosine kinase receptors or receptor adaptor proteins with the amino acid sequence pTyr-X-X-Met [40,41]. Recently it has been shown that tyrosine phosphorylation of the p85 subunit on Tyr^{688} by Src kinases also alleviate the inhibitory effect of p85 on p110 thus regulating PI3-Kinase activity [40,46]. A negative regulator of p85 has also been identified, the Src homology domain 2 containing tyrosine phosphatase 1 (SHP-1). It has been shown to bind to tyrosine phosphorylated p85 and expression of a phosphatase inactive form of SHP-1 increases PI3-Kinase activity [40].

Class	Catalytic subunit	Schematic representation	Substrate specificity	Adaptor/ Binding partner
I_A	p110α p110β p110δ		PtdIns, PtdIns (4)P, PtdIns(4,5)P_2	p85α p85β p55γ/$p55^{PIK}$
I_B	p110γ			p101
II	PI3-K-C2α/ mcpk/p170 PI3-K-C2β PI3-K_68D/cpk		PtdIns, PtdIns (4)P (PtdIns(4,5)P)	Unknown
III	Vps34p/ PtdIns 3-kinase		PtdIns	Vps15p/p150



Domin J, Waterfield MD. Using structure to define the function of phosphoinositide 3-kinase family members. FEBS Lett. 1997;410:91-95.

Fig. 1.2 Classification of mammalian PI3-Kinase family members

The catalytic subunits are divided into classes according to the sequence homology of the catalytic domain between the subunits. $p55^{PIK}$ is specific for mouse, whereas p55γ is bovine. The Class III consists of the PI3-Kinase yeast homologue (Vps34p).

Active PI3-kinase produce PI(3,4,5)P_3 to which Akt, a serine/threonine protein kinase also referred to as protein kinase B (PKB), bind thereby recruiting Akt to the membrane. Akt is then subsequently phosphorylated at a serine and a threonine residue by 3-phosphoinositide-dependent protein kinase 1 and 2 (PDK1 and 2) to become fully active. In mammals three Akt isoforms exist: Akt 1, 2 and 3 whereof Akt 1 is highly expressed in endothelial cells. In cell survival, Akt exhibits a major role as a regulatory enzyme. For example, Akt inhibits proapoptotic proteins such as Bad, Bax and caspase-9 [25,47]. By inhibiting Bad, Akt also increases the amount of active Bcl-2 which is an anti apoptotic protein. By activating Inhibitor of nuclear factor kappa-B kinase (IKK) thereby favouring the dissociation of nuclear factor kappa-B (NFκB) from its inhibitor IκB leading to the nuclear translocation of NFκB and transcription of anti-apoptotic proteins such as the inhibitor of apoptosis proteins (IAPs) Akt further promotes cell survival [25]. Finally, Akt increases the production of NO by stimulating NO synthase [48,49]. The NO produced is in turn involved in caspase inactivation[47].

As well as regulating cell survival in endothelial cells, the PI3-Kinase / Akt pathway also mediates endothelial cell migration. It is not fully clear how but several studies indicate that Akt is required for Rho family GTPases dependent migration by either activating Rac or by itself being activated by Rac and Cdc 42. Both Rac and Cdc 42 drive endothelial cell migration by inducing membrane ruffling, extension of lamellipodia and membrane protrusions [47].

Several stimuli activate the PI3-Kinase / Akt pathway. Both VEGF and FGF have also been shown to influence endothelial migration [50,50,51] and DNA synthesis [50] through the PI3-Kinase/Akt pathway as well as angiogenesis in the chorioallantoic membrane (CAM) assay [50,51]. Whereas very little is known about the precise signalling pathways initiated by the FGFs in endothelial cells, there are more studies involving VEGF in endothelial signalling. It has been demonstrated that VEGF together with integrin mediated adhesion to the ECM activates Akt. This leads to activation of the focal adhesion kinase (FAK) and the formation of focal adhesions thus preventing anoikis [25,47]. The proliferative response to PI3-Kinase activation upon VEGF stimulation has been shown to be mediated by MAP kinase activation [45].

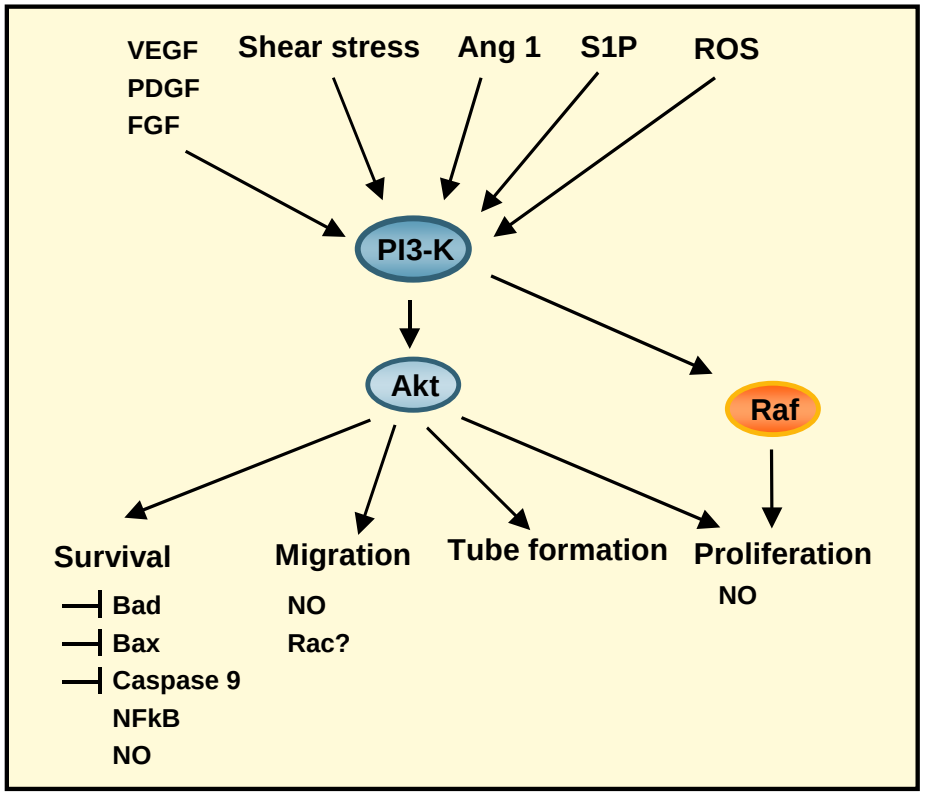


Fig. 1.3 Schematic illustration of the PI3-Kinase regulated pathways

Many different stimuli have been shown to activate the PI3-Kinase, such as different growth factors, shear stress, ROS and sphingosine-1-phosphate (S1P). Most of the effects caused by a PI3-Kinase activation are transduced through Akt, but some have also been shown to be mediated by Raf. The different effects are stimuli specific.

In addition to VEGF and FGF, PDGF can also activate PI3-Kinase. It has been shown that PI3-Kinase directly binds to phosphorylated tyrosines on the PDGF receptor (PDGFR) upon PDGF stimulation in fibroblasts [52]. It has further been shown that the association between the PDGFR and PI3-Kinase is of high stoichiometry so that almost all available PI3-Kinase molecules are occupied by the receptor [53]. Moreover, it has been established that the binding to the PDGFR is important for PI3-Kinase activity [53] and Akt activation [54] as well as for PDGF induced DNA synthesis [53]. Experiments in fibroblasts and HepG2 cells with mutant PDGFR which cannot bind PI3-Kinase and which are unable to activate Ras showed that although the receptors bound PI3-Kinase they were unable to activate the enzyme. This led to the assumption that PDGF induced PI3-kinase activity not only depends on the receptor/PI3-Kinase complex but also on the activation of Ras [55]. The exact role of

PDGF in PI3-Kinase/Akt signalling in endothelial cells remains to be elucidated (for a schematic illustration of the PI3-Kinase pathway in angiogenic signalling, see fig. 1.3). Other stimuli for activation of the PI3-Kinase / Akt pathway in endothelial cells, which will not be further discussed here, are IGF, Ang-1, sphingosine-1-phosphate, fluid shear stress, estrogen, reactive oxygen species and corticosteroids [47].

1.2.2. The Raf-MEK-ERK Pathway

Another signalling pathway of critical importance for the angiogenic process is the Raf-MEK-ERK pathway also referred to as the mitogen activated protein kinase (MAPK) pathway. This pathway has repeatedly been shown to protect cells from apoptosis [56,57] and be essential for proliferation and cell cycle progression [58,59]. Moreover, this pathway has also been demonstrated to be involved in extracellular matrix degradation, endothelial cell migration as well as differentiation [25].

Raf is a serine/threonine protein kinase (also called the mitogen activated protein kinase kinase kinase, MAPKKK) and three Raf isoforms exist in higher eucaryots, namely Raf-1 or c-Raf, A-Raf and B-Raf. They all share three domains: 1) CR1, which has a binding site for Ras, 2) CR2, which renders the kinase inactive when phosphorylated on a serine residue and 3) CR3, which when phosphorylated on a serine and two tyrosine residues positively controls Raf activity [58,59]. Although all Raf isoforms share this homology, they differ in action. Maximal activation of Raf-1 and A-Raf requires both Ras and Src activity whereas B-Raf only needs Src. Moreover, it has been shown that VEGF and FGF induces robust activation of Raf-1 while B-Raf was only weakly activated by FGF and not activated at all by VEGF in endothelial cells [56]. It has been shown in NIH 3T3 cells that A-Raf promotes cell cycle progression, whereas B-Raf and Raf-1 caused cell cycle arrest. Intriguingly, in hemotopoetic cells the activation of Raf-1 and A-Raf induced cell proliferation while B-Raf activity resulted in apoptosis [59]. Furthermore, the tissue distribution of the Raf isoforms also differ. Raf-1 is expressed ubiquitously while the strongest A-Raf expression is found in urogenital tissues and B-Raf predominates in neuronal tissues. Interestingly, B-Raf knockout mice showed severe defects in vascular endothelial cell

survival and differentiation leading to embryonic death and A-Raf knock-out mice died shortly after birth with gastrointestinal and neurological defects. Raf-1 knock-out mice however, died embryonically and had defects in the development of skin, lung and placenta [59].

Upon growth factor receptor activation the adaptor proteins Shc and Grb2 pull the GDP/GTP exchanger protein Sos (son of sevenless) towards the cell membrane where it activates Ras [58-60]. This causes a conformational change of Ras which reveals a high affinity binding site for Raf that results in the translocation of Raf to the cell membrane. This in turn enables other proteins such as Rac1, p21 activated kinase (PAK), Src kinase and protein kinase C (PKC) to thereby activate Raf [58-61]. It has been demonstrated that VEGF induced activation of the Raf-MEK-ERK pathway occurs mainly through PKC following its own activation by phospholipase C γ (PLCγ), and to a lesser extent through the Ras dependent pathway [62]. Furthermore, this route of Raf-MEK-ERK pathway activation is the strongest inducer of proliferation upon VEGF [25]. In either way, by phosphorylation of two serine residues Raf activates the MAPK kinases (MAPKK) MEK 1 and MEK 2. Following MEK activation, Raf activity is abrogated by phosphorylation by protein kinase A (PKA) [58,59]. There are some studies reporting an inactivation of Raf caused by Akt [63,64], whereas others claim PKA is the main inhibitor of Raf [58,59]. Raf inhibition by Akt primarily seems to depend on the cell type and the differentiation stage of the cell [59,63,64].

For MEK 1 and 2, the extracellular signal regulated kinase ERK 1/2 is the only target and it activates ERK1/2 (also called p42 and p44) by phosphorylation of a tyrosine and a threonine residue [65]. ERK transduces the signal initiated by Raf by phosphorylation of several nuclear transcription factors such as Ets-1, c-Jun and c-Myc. In addition, it indirectly activates the transcription factor CREB. Activation of the above mentioned proteins lead to transcription of genes which positively regulate cell cycle progression and cell survival. Furthermore, by phosphorylating IKK, ERK indirectly activates NFκB and can so induce expression of genes important for immune responses, proliferation, prevention of apoptosis and angiogenesis. Mainly though, Raf activation can also lead to NFκB activation independently of ERK, by activation of MEKK 1 [59]. It has been demonstrated by Cheresh et al. that VEGF was able to activate Raf-1 through Src and this rescued endothelial cells from stress

mediated apoptosis (intrinsic pathway) while bFGF enhanced Raf-1 activity by phosphorylation of PAK thereby preventing endothelial cells from undergoing receptor mediated apoptosis (extrinsic pathway) [56].

Besides affecting proliferation and cell survival, the Raf-MEK-ERK pathway is also a mediator of cell migration upon VEGF receptor and integrin activation. In this context it is activated by FAK which regulates the assembly of focal adhesions and the organization of actin. In addition, Raf is activated by the small GTPase Rac which drives actin polymerization and formation of lamellopodia that are also important for cell migration [25]. Another important step of angiogenesis, where the Raf- MEK-ERK pathway also seems to be highly involved is extracellular matrix degradation. Upon FGF or VEGF stimulation this pathway is activated by the Grb2/Sos/Ras complex resulting in the expression of Ets-1 and matrix metalloproteinases [25].

The last steps of the angiogenic process which involves endothelial cell differentiation also includes the Raf-MEK-ERK pathway. Not much is known about the precise signalling mechanisms regulating this process, but FGF and VEGF seem to activate Raf either through PI3-Kinase or PLC respectively. Thus, the Raf-MEK-ERK pathway is involved in almost all steps of angiogenesis and can be activated in many different ways by several different stimuli (for a schematic picture of the Raf-MEK-ERK pathway in angiogenesis, see fig. 1.4).

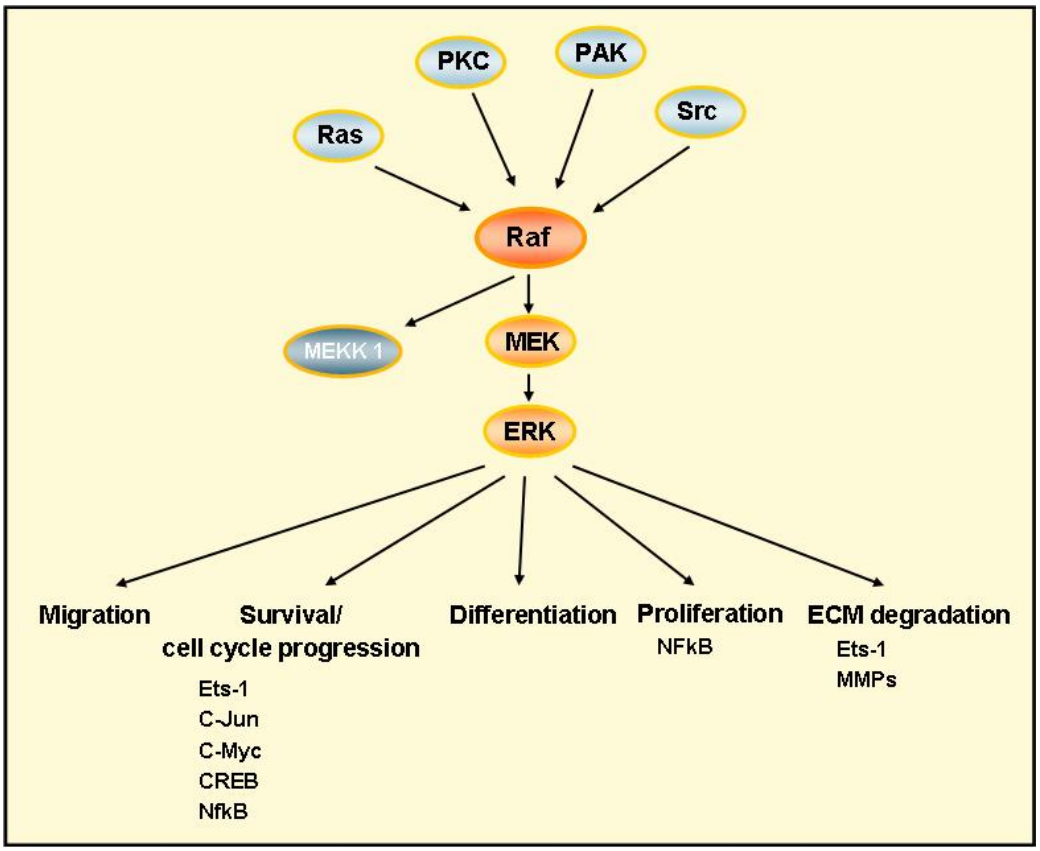


Fig. 1.4 The Raf-MEK-ERK pathway and its effects on angiogenesis.

The Raf-MEK-ERK pathway is responsible for many important features in the angiogenic process, such as cell migration, survival, differentiation, proliferation and degradation of the extracellular matrix (ECM) by activation of several transcription factors such as Ets-1, c-Myc, NFkB, CREB and c-Jun. Most of the effects are mediated through Raf dependent activation of MEK and ERK but NFkB activation has also been shown to be mediated by Raf dependent MEKK 1 activation. Several kinases have been shown to activate Raf and the activation of these upstream regulators seem to be dependent on the stimulus given.

1.3. THE SRC-HOMOLOGY 2 DOMAIN CONTAINING TYROSINE PHOSPHATASE 2

Cellular responses to the environment are mediated by intracellular signalling pathways. They can be initiated by growth factors, cytokines, hormones, cell adhesion molecules and extracellular matrix components. The different transduction pathways monitor several fundamental cellular activities like proliferation, migration, differentiation and survival. These processes are in turn dependent on and regulated by protein phosphorylation and dephosphorylation carried out by protein kinases and protein phosphatases respectively. Protein phosphorylation or dephosphorylation is responsible for protein activation, inhibition and direct interaction between proteins, which are very important for transducing the original message and dysregulation of the signalling pathways causes malfunctioning of the cells and may eventually lead to disease. Many extracellular stimuli, which are important for cellular homeostasis, activate a signal pathway by inducing phosphorylation of tyrosine residues on their receptors and the activities of several of the intracellular proteins transmitting the signal further also depend on tyrosine phosphorylation. Protein tyrosine kinases (PTK) and protein tyrosine phosphatases (PTP) thus play critical roles in the control of cellular behaviour. Until a few years ago it was believed that tyrosine phosphorylations were mainly regulated by protein tyrosine kinases but there are now many studies showing that protein tyrosine phosphatases are equally important. However, the knowledge about their signalling mechanisms is not as well profound as with tyrosine kinases. The PTP superfamily consists of three families: classical PTPs, which dephosphorylate tyrosine residues, dual specificity phosphatases dephosphorylating serines, threonines and tyrosine residues within the same protein substrate and atypical PTPs lacking activity against conventional PTP substrates. The classical PTPs consists of transmembrane PTPs and non-transmembrane PTPs [66-68]. In vertebrates, two non transmembrane Src homology domain containing tyrosine phosphatases exist, namely Src homology 2 domain containing tyrosine phosphatase 1 and – 2 (SHP-1 and SHP-2 respectively). This study concentrates on SHP-2.

1.3.1. The biochemistry of SHP-2

Mammalian SHP-2 (also called SH-PTP2, SH-PTP3, PTP2C, PTP1D or Syp) was first discovered and cloned in 1992 by Neel et al [69] and has homologues in both *Drosophila* (Corkscrew, shortened Csw) and *C. elegans* (Ptp2). SHP-2 is an ubiquitously expressed protein with the highest levels being expressed in heart, brain and skeletal muscle [70]. As stated by the name, SHP-2 contains two tandem Src homology 2 (SH2) domains at the N-terminus of about 100 amino acids each (N-SH2 and C-SH2). The SH2 domains mediate the binding of SHP-2 to phosphorylated tyrosine residues on other molecules and thus control the specific protein-protein interaction between SHP-2 and its substrates [71,72]. The SH2 domains also regulate the phosphatase activity. It has been shown that binding of a phosphotyrosyl peptide to the N-SH2 domain stimulates activation of the phosphatase domain (PTP), which is situated at the C-terminus and exhibits a low basal activity. In addition, biphosphorylated ligands (ligands that contain two phosphorylated tyrosine residues) that bind both SH2 domains further stimulate catalysis [71,73]. Interestingly enough, deletion of the N-SH2 part of the protein, but not the C-SH2, also leads to an enhanced activation of SHP-2 implying that the N-SH2 domain is critical for inactivation of the phosphatase. The crystal structure of SHP-2 revealed that the N-SH2 domain is inserted into the catalytic cleft of the phosphatase domain in the basal state and so sterically hinders phosphatase activity. At the same time, the p-Tyr binding pocket on the N-SH2 domain is hidden away from other substrates. There are two suggested mechanisms for SHP-2 activation (for a schematic picture see Fig. 1.5). In the first suggested mechanism two tyrosine residues (Y542 and Y580) in the C-tail of SHP-2 are phosphorylated and can therefore bind to the N-SH2 and C-SH2 domains respectively. This binding leads to the exposure of the catalytic cleft of the PTP domain now able to exert its phosphatase activity. The tyrosine residue at position 542 has been shown to be the major *in vivo* phosphorylation site on SHP-2 [74]. In the second way of activation, which is supported by both crystal structure and enzymological studies [73], the C-SH2 domain is left open (because only the N-SH2 domain is captured by the phosphatase domain) and can freely bind tyrosine phosphorylated ligands. Binding of a ligand with two p-Tyr sites to the C-SH2 results in a local increase of the ligand concentration which is enough for the engaged N-SH2 domain to overcome the allosteric inhibition and bind the second

p-Tyr site on the substrate, thus rendering the protein active. SHP-2 ligands or substrates are tyrosine phosphorylated receptors or tyrosine phosphorylated adaptor molecules and it is believed that by either binding to receptors or other adaptor proteins, the SHP-2 is translocated from the cytosol to the plasma membrane where it exerts its action.

The closest homologue to SHP-2 is the Src homology 2 domain containing tyrosine phosphatase 1 (SHP-1), which in contrast to SHP-2 is mainly expressed in hematopoetic cells. Nevertheless, both proteins share extremely high sequence identity. The SH-2 domains at the N-terminus of both proteins have 65% and 52% similarity respectively and the catalytic domains are to 60% identical [75]. Moreover, the tertiary structures of the catalytic domains are almost superimposable, which makes it very complicated to develop selective inhibitors targeting the PTP domains.

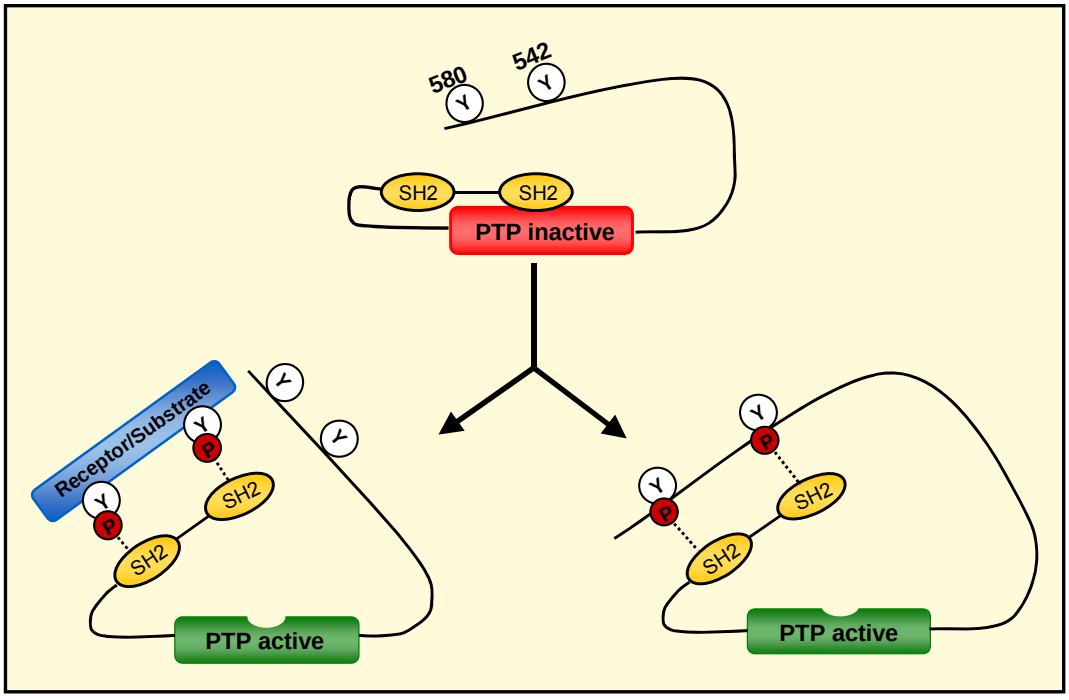


Fig. 1.5 Mechanisms of SHP-2 activation

Two mechanisms of SHP-2 activation have been shown so far. The phosphatase is kept inactive by steric blockage of the PTP domain by the N-SH2 domain. Upon SH2 domain binding to phosphorylated tyrosine residues on receptors or adaptor molecules the catalytic cleft in the PTP domain is left open and can so dephosphorylate tyrosine phosphorylated residues on other molecules (left mechanism). This way of activation is supported by enzymological and crystal structure studies. In the other suggested mechanism of SHP-2 activation the two tyrosine residues (Y542 and Y580) in the C-tail of SHP-2 are phosphorylated and therefore bind to both SH2 domains. This binding exposes the catalytic cleft of the PTP domain now able to exert its phosphatase activity (right mechanism).

1.3.2. SHP-2 and disease

One of the best defined diseases where SHP-2 plays an outstanding role is the developmental disorder Noonans syndrome (NS) first described by two pediatric cardiologists Jacqueline Noonan and Dorothy Ehmke. NS is an autosomal dominant disorder defined by short stature, facial dysmorphism, skeletal anomalies and cardiovascular abnormalities such as congenital heart disease [76,77]. The prevalence of this disorder is estimated between 1:1000 and 1:2500 live births, although disease incidence seems to be higher than prevalence, since fetal loss has been documented. Approximately 50% of the NS cases are caused by germline missense mutations situated at or close to the interface between the exons coding for N-SH-2 and PTP domains of SHP-2 [76]. This suggests that the inhibitory N-terminal SH-2 domain interaction with the PTP domain is destabilized resulting in a gain of function of SHP-2 (Fig. 1.6) [76]. This model is supported by data obtained from NS-causative SHP-2 mutants, where an increased basal phosphatase activity was detected. Furthermore, functional studies on these mutants in vitro under basal conditions or upon EGF stimulation show that the destabilization of the inactive form of SHP-2, thus favouring the shift in equilibrium towards the active state, results in a prolonged ligand-dependent activation of ERK-2. Moreover, the binding of SHP-2 to Grb-2 associated binder-1 (Gab-1) was increased [77]. Mice with a NS-causative mutation (*Ptpn11*$^{D61G/\,D61G}$) in the gene for SHP-2, Ptpn11, die in utero of extensive edema and bleeding. *Ptpn11*$^{D61G/+}$ embryonic mouse fibroblasts show features which resembles those observed in NS and exhibit characteristics indicating a myeloproliferative disease. An enhanced SHP-2 activity was also observed in this mouse model [76].

Patients with NS have an increased incidence of different hematologic abnormalities such as transient monocytosis, thrombocytopenia and juvenile myelomonocytic leukaemia (JMML). The latter is characterized by excessive proliferation of myeloid progenitor cells which are hypersensitive to granulocyte macrophage colony stimulating factor (GM-CSF) due to defective Ras inhibition. 20-25% of the cases are due to somatic SHP-2 mutations [71,76] and some of these mutations occur at the same spots as mutations in NS. Interestingly, JMML and NS mutations often result in the incorporation of different amino acids at these positions suggesting that different levels of SHP-2 activity determine whether to cause NS or leukemia [71]. This finding raised the question if mutations in Ptpn11 would have a significant impact on

cancers. Mutational analyses of several different tumours revealed that Ptpn11 mutations occur at low frequency in some human cancers [76]. Further, somatic Ptpn11 mutations have been detected in children and adolescents with acute lymphoblastic leukaemia (ALL) [76] and an increased expression and thus activity of SHP-2 has been found in neutrophils from patients with severe congenital neutropenia (Kostmann´s syndrome) indicating that SHP-2 also is important for cell differentiation [78].

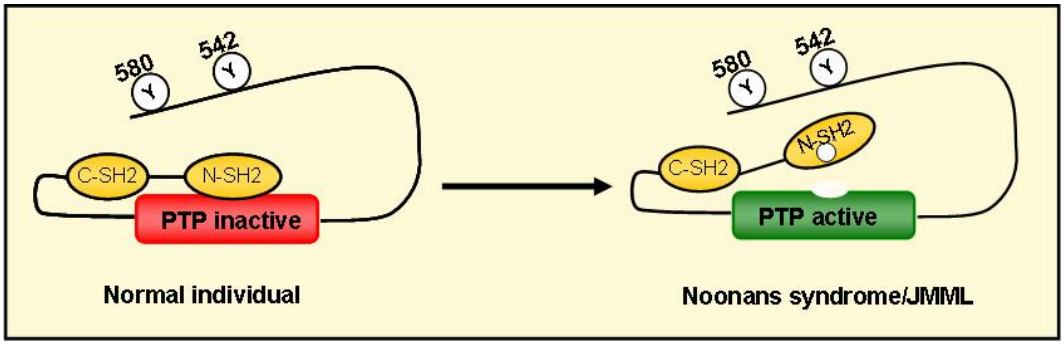


Fig. 1.6 Mutation in SHP-2 responsible for Noonans syndrome

Whereas the N-SH2 domain of SHP-2 sterically hinders the catalytic activity of the PTP domain in normal individuals, missense mutations situated at or close to the interface between the exons coding for N-SH-2 and PTP domains of SHP-2 results in a gain of function of SHP-2 in 50% of the patients diagnosed with Noonans syndrome.

Another developmental disorder related to mutations in Ptpn11 is the LEOPARD syndrome (LS), which, likewise to NS, also is an autosomal dominant condition. It is characterized by some of the features seen in NS, such as congenital heart disease, short stature and facial dysmorphism, but also by café-au-lait spots, abnormal genitalia in male and deafness to mention a few. Since the two diseases show similar characteristics and because the missense mutations in LEOPARD syndrome are situated in the exon encoding the phosphatase domain of SHP-2, the mutations in LS are so predicted to also result in a gain of function of SHP-2 [79]. Recent data however, show that LS is characterized by a decreased SHP-2 activity [80]. How these clinically similar syndromes can result from the opposing effects of these SHP-2 mutants remains to be clarified.

SHP-2 has also been shown to be involved in both insulin dependent- (IDDM) and non insulin dependent (NIDDM) diabetes mellitus [70,81]. In streptozotocin-treated rats,

which are insulin deficient resembling IDDM, levels of SHP-2 protein in fat and liver tissue were decreased [81]. Transgenic mice expressing a dominant negative mutant SHP-2 were found to be insulin resistant and exhibited an impaired insulin signalling, such as phosphorylation and thus activation of the insulin receptor substrate 1 (IRS-1), PI3-K and Akt [82]. Several studies of the role of SHP-2 in insulin signalling exist [83-86] and it has been shown that SHP-2 associates with IRS-1 upon insulin stimulation. Further, it has been demonstrated in cells overexpressing the insulin receptors that SHP-2 seems to be important for the insulin dependent MAPK- and PI3-Kinase activity [85,86].

1.3.3. SHP-2 and growth factor signalling

SHP-2 has been implicated in a variety of different signalling pathways initiated by several different growth factors, such as PDGF, EGF, IGF, FGF and cytokines such as IL-1-3, IL-5-6, GM-CSF, erythropoietin (EPO) as well as interferon and insulin. SHP-2 has been demonstrated to be involved in the Ras-Raf-MEK-ERK, PI3-Kinase, Jak-Stat and NFκB pathways. Another feature of SHP-2 is its dual role in signalling; although most of its effects are tyrosine phosphatase dependent, SHP-2 can probably also function as an adaptor molecule due to its SH2-domains and it has been shown to interact with several signalling intermediates such as Grb2, FRS-2, Jak 2, the p85 subunit of the PI3-Kinase, IRS-1 and Gab-1 and -2 [71,72] as well as directly with receptors such as the PDGFR [87], EGFR and the VEGFR [88,89]. In addition, SHP-2 has also been found to both positively and negatively enhance cellular signalling. The exact mechanism of this involvement differs somewhat depending on the cell type and stimulus. While it has been shown that SHP-2 negatively regulates the Jak-Stat pathway, it seems to positively influence the Raf-MEK-ERK and PI3-Kinase pathways [71,72], which are probably the most investigated pathways in the context of SHP-2. Although numerous studies showing a positive role for SHP-2 in these pathways exist, the exact mechanism of how SHP-2 activates these pathways remains to be elucidated. SHP-2 was shown to dephosphorylate the PDGFR on position Y771, which is the binding site of the GTPase activating protein RasGAP [90]. This suggests a limited Ras activation in the absence of SHP-2, thus coupling the phosphatase to Ras-Raf-MEK-ERK signalling upon PDGF stimulus.

However, this model cannot account for the entire extent of SHP-2 dependent ERK activation, since studies with overexpression of the Grb2 associated binder-1 (Gab-1) resulted in an increased association of Gab-1 to SHP-2 as well as enhanced ERK activation upon PDGF stimulation [91]. A study using a Gab-1/SHP-2 fusion protein suggested that SHP-2 mediates its positive effects on ERK signalling through activation of Src and that Gab-1 is necessary for targeting SHP-2 to the cell membrane and thus to the vicinity of its targets [92]. Furthermore, SHP-2 has also been suggested to activate the Ras-Raf-MEK-ERK signalling pathway by preventing recruitment of the negative Src regulator Csk [93]. Another potential SHP-2 target is the Sprouty protein, initially found to negatively regulate FGF signalling in Drosophila [71]. An additional target for SHP-2 in FGF dependent cell signalling is the FGF receptor substrate 2 (FRS2). Upon FGF stimulation FRS2 associates with SHP-2 which then becomes phosphorylated and binds Grb2. A failure of forming this protein complex resulted in a weak and transient MAPK activation in embryonic kidney cells [94] and myoblasts [95].

SHP-2 has also been shown to be important for cell survival by regulating the PI3-Kinase/Akt pathway [96,97]. One study indicates that SHP-2 controls activation of PI3-Kinase by promoting the association of the docking protein Cbl, which is phosphorylated by Src, and the p85 subunit of PI3-Kinase [96]. Furthermore, SHP-2 has been shown to directly associate with the p85 subunit of PI3-Kinase leading to its activation after EGF and IGF stimulation in glioblastoma and mouse fibroblast cells [97,98]. Intriguingly, upon EGF stimulation in fibroblasts, SHP-2 has been demonstrated to both positively [98] and negatively [99] regulate PI3-Kinase activity. Furthermore, it was shown that the EGF dependent association between Gab-1 and PI3-Kinase was enhanced in SHP-2 mutant fibroblasts suggesting that SHP-2 negatively regulates PI3-Kinase activity by dephosphorylating p85 binding sites on Gab-1 [99]. SHP-2 binding to Gab-1 upon EGF stimulation linking it to PI3-kinase has also been shown to mediate NFκB activation [100]. However, the exact mechanism of SHP-2 regulation of PI3-Kinase remains to be investigated.

SHP-2 has also been demonstrated to regulate signalling initiated by tyrosine kinase receptors *in vivo*, as primary cells from SHP-2 mutant embryos showed a prolonged MAP Kinase activity upon PDGF stimulation whereas aFGF and bFGF stimulation of

these cells resulted in a decreased and transient MAP Kinase activation in comparison to wild type cells [101]. In addition, cells from Shp-2 $^{-/-}$/wild type chimeric mice showed diminished EGF-R signalling, such as reduced activation of PI3-Kinase and ERK 1. Apart from exhibiting defects in the formation of mesoderm during development, the phenotype of the Shp-2 $^{-/-}$/wild type chimeric animals showed eye and skin abnormalities as well as immature lung development, which indicates an impairment in EGF signalling [102].

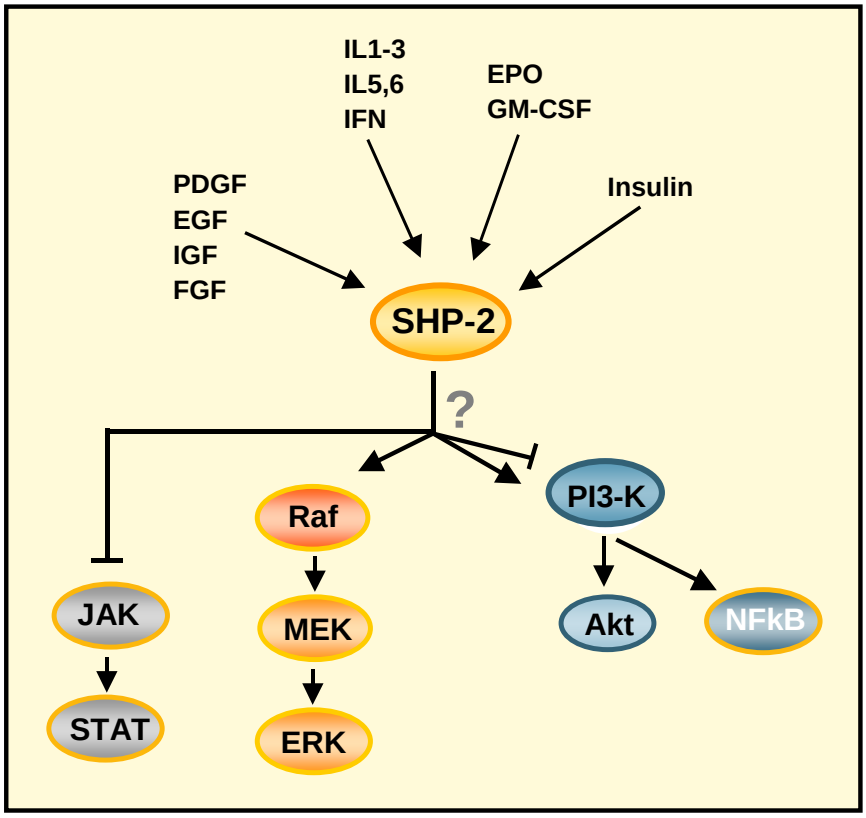


Fig. 1.7 Growth factor dependent SHP-2 signalling

SHP-2 has been shown to take part in signalling initiated by several different factors. In most cases SHP-2 positively regulates PI3-Kinase and the Raf-MEK-ERK pathways, whereas it exhibits a negative role in the Jak-Stat pathway. However, SHP-2 has also been found to negatively regulate PI3-Kinase activity. The path and way of regulation chosen seem to depend on the stimulus and the cell type. The direct target for SHP-2 remains to be fully elucidated.

1.3.4. SHP-2 and endothelial physiology

In endothelial cells, SHP-2 has been found to interact with adhesion molecules such as VE-Cadherin and platelet endothelial cell adhesion molecule (PECAM). It has been found to be associated with VE-Cadherin in quiescent endothelial cells and to dissociate from the Cadherin complex upon thrombin stimulation [103] and shear stress [104]. Furthermore, it has been shown that association of SHP-2 to PECAM is important for dephosphorylation of β-catenin, a protein tightly bound to both VE-Cadherin and PECAM, since cells lacking PECAM-1 showed a sustained phosphorylation of β-catenin. This was conjoined with an increase in cell permeability [105]. Thus, these findings suggest that SHP-2 has a role in cell-cell junction assembly and that dissociation of SHP-2 from these adhesion molecules upon stimulation with a vasoactive substance or shear stress promotes endothelial permeability. In addition, SHP-2 has been shown to bind to PECAM-1 and thereupon become active in confluent HUVEC, whereas this association is decreased in migrating endothelial cells. According to these results it is suggested that PECAM directs SHP-2 in influencing proteins important for maintaining a confluent cell layer [106]. In contrast, the observation that wound-induced migration stimulates PECAM-1 tyrosine phosphorylation and SHP-2 association leads to the assumption that SHP-2 may instead promote cell motility [107]. A positive role for SHP-2 in endothelial cell migration is supported by another study showing a defective cell migration towards PDGF when SHP-2 is unable to associate to the PDGFR-β. Endothelial cells expressing a mutant PDGFR lacking the binding site for SHP-2 not only exhibit a reduced cell motility but also show a diminished activation of the focal adhesion kinase (FAK) [108], which regulates the formation of focal adhesions important for cellular migration.

Endothelial SHP-2 also seems to be involved in the inflammatory response, as it has been shown to be important for proper intercellular adhesion molecule 1 (ICAM-1) signalling and thus neutrophil migration along the endothelium [109]. In addition, SHP-2 is probably important for E-Selectin transmembrane signalling in the activated endothelial cell at sites of inflammation [110].

In spite of these insights, to date, the data about the different signalling pathways influenced by SHP-2 in endothelial cells are limited. In addition, the functional consequences of SHP-2 signalling remain to be investigated.

1.4. A NOVEL STRATEGY OF INHIBITING SHP-2

1.4.1. Posttranscriptional gene silencing

Posttranscriptional gene silencing (PTGS) defines a mechanism that serves to protect the genome from foreign DNA or RNA by sequence specific destruction of the nucleic acids. PTGS has been shown to play a critical role in normal cellular functions [111] and it appears to be a natural defence mechanism against viruses and transposable elements [112,113]. It was first discovered in plants into which a transgene that was supposed to enhance the expression of the product encoded by the transgene was introduced, but instead led to the suppression of itself and of a homologous endogenous gene [114]. Thus, this process can be induced by the introduction of exogenous nucleic acids which target cellular mRNA homologous to its sequence for cleavage, thereby silencing endogenous genes specifically [115]. Antisense oligonucleotides (AS-ODN) and short interfering RNAs (siRNA) are examples of exogenous nucleic acids that switch on PTGS. However, there are other molecules that trigger specific mRNA cleavage such as ribozymes and which are also used for experimental purposes [115,116].

Ribozymes, like the hammerhead ribozyme, are RNA enzymes that can be used for gene knock-down. They consist of a catalytic domain flanked by sequences which are complementary to the target mRNA enabling selective binding. The target mRNA is cleaved directly by the intrinsic activity of the ribozyme (Fig. 1.8). However, the RNA backbone makes these molecules susceptible for degradation from RNases and thus biological unstable in vivo. Deoxyribozymes, which are catalytic DNAs, can also be applied for selective gene silencing. They bind to their target RNA and directly cleave it in the same way as the ribozymes do. In contrast to the ribozymes, no natural occurring deoxyribozyme is known to exist and they exhibit a greater biological stability due to their DNA backbone [117,118].

Antisense oligonucleotides (AS-ODN) consist of approximately 15-20 desoxynucleotides and bind to the pre-mRNAs or the mRNAs homologous to its sequence [116]. The AS-ODN can then either occupy critical sites of the mRNA thereby disturbing translation or target the mRNA for degradation [113,116]. By constructing AS-ODNs that are targeted to the 5´terminus of the mRNA, the ribosome is blocked by steric hindrance and translation can not be completed [116,117]. Most widely used is the mechansim of RNase H cleavage, which is induced by AS-ODNs that are negatively charged (Fig. 1.8). RNase H hydrolyses RNA strands when bound to a DNA strand and therefore causes the degradation of mRNA [116,117]. Since naked DNA is rapidly degraded by exo- and endonucleases in the cell, the AS-ODN can be modified in different ways to increase its stability. The most common protection is the replacement of one of the non bridging oxygen atoms in the phosphate group with a sulfur group, giving a so called phosphorothioate oligonucleotide [115,116,118]. This modification gives the AS-ODN a half-life in human serum of about 9-10 hours compared to approximately 1 hour for unmodified AS-ODN. In addition, these molecules are negatively charged and therefore water soluble and activate RNase H. A disadvantage with these molecules is that they exhibit a slightly reduced affinity towards complementary RNAs, but instead a greater specificity of hybridization compared to unmodified AS-ODN. Another disadvantage of phosphorothioate AS-ODN is the ability to bind to certain proteins, such as heparin-binding proteins. However, this shortcoming was proven to be of advantage in *in vivo* situations, where AS-ODNs bound plasma proteins which protected them from filtration and increased their serum half-life [116].

The use of short interfering RNAs (siRNA) to silence protein expression is expanding rapidly. This technique takes advantage of the process of RNA interference (RNAi), which was first discovered in *Caenorhabditis elegans*, *Drosphila* and plants [119] but has also been observed in different vertebrate systems [120] such as in early mice embryos, *zebrafish* and *Xenopus* [119]. Naturally occurring RNAi is triggered by dsRNA that targets single-stranded RNA homologous in sequence to the dsRNA for degradation [119,120].

It has been shown that dsRNA is a potent inducer of nonspecific gene silencing in mammalian cells due to activation of the interferon response. These nonspecific responses have for quite a time disturbed the detection of RNAi in somatic mammalian cells [119,120]. A breakthrough came when the nonspecific gene silencing of dsRNA in mammalian cells was shown to be length-dependent [119] and that this can be overcome by introduction of short interfering RNAs with less than 30 nucleotides, that can be either expressed endogenously (short hairpin RNA) or delivered exogenously [117,120]. Endogenously expressed hairpin RNA (shRNA) is cut into shorter RNAs, by the cytosolic RNAse III enzyme Dicer [120]. The short interfering RNAs (siRNA) assemble with the RNA-induced silencing complex (RISC) and target mRNA homologuous in sequence for degradation by RISC [120]. Due to its helicase activity, RISC separates the two mRNA strands, thereby enabling the siRNA to bind to the target mRNA. RISC also exhibits an endonuclease activity which then hydrolyses the mRNA at the site of siRNA binding [121] (Fig. 1.8). Although reported to be the most potent antisense technology discovered so far, a major disadvantage with this technique is the off-target effects, i.e the non specific silencing of other proteins [122]. This can partly be overcome by lowering the siRNA concentration, however at the expense of efficiency.

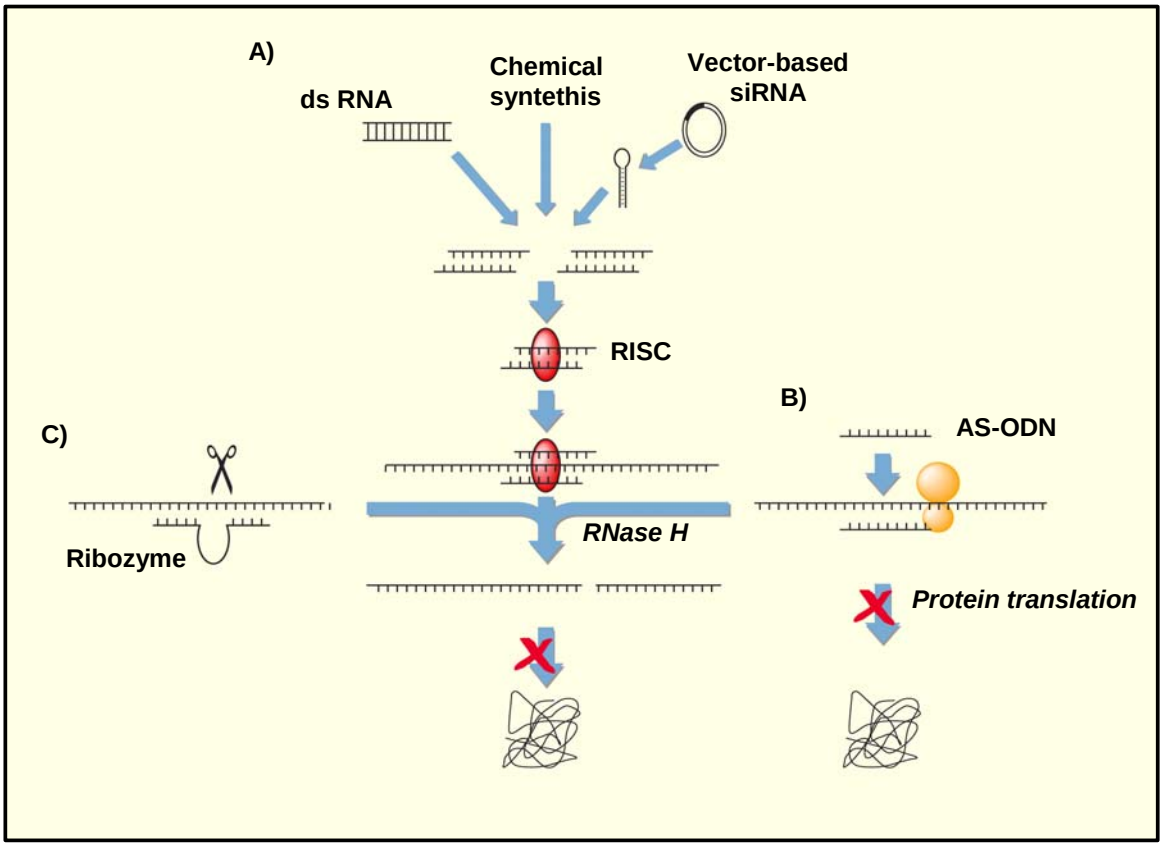


Fig. 1.8 Strategies to suppress protein translation

Short interfering RNA (siRNA) can be produced by either cleavage by Dicer from longer dsRNA (A, top left), chemical synthesis (A, top middle) or transcription from a vector containing the target sequence (A, top right). In all cases, siRNA assembles with RISC and directs mRNA cleavage. AS-ODN binds to mRNA homologous in sequence and can either prevent translation by blocking the ribosome or induce mRNA cleavage by a RNase H dependent mechanism (B, bottom right). Ribozymes and Deoxyribozymes also bind mRNA homologous in sequence but exhibit an intrinsic catalytic activity and so silence the gene product (C, bottom left).

1.4.2. Magnetofection- a highly efficient tool for transfection of endothelial cells

The challenge for all antisense based technologies is the efficient delivery of these molecules into cells, tissues or organs as the negative charge of RNA and DNA molecules limits the cellular uptake. A new method of introducing gene vectors into cells is the so called magnetofection method, invented by Plank and colleagues at the Institute of Experimental Oncology at the Technical University of Munich, Germany, where magnetic nanoparticles associated with vector DNA are transfected

into cells by the influence of an external magnetic field [123] (Fig. 1.9). This technique has been shown to enhance the efficiency of both viral or non viral vectors up to several thousand times [124].

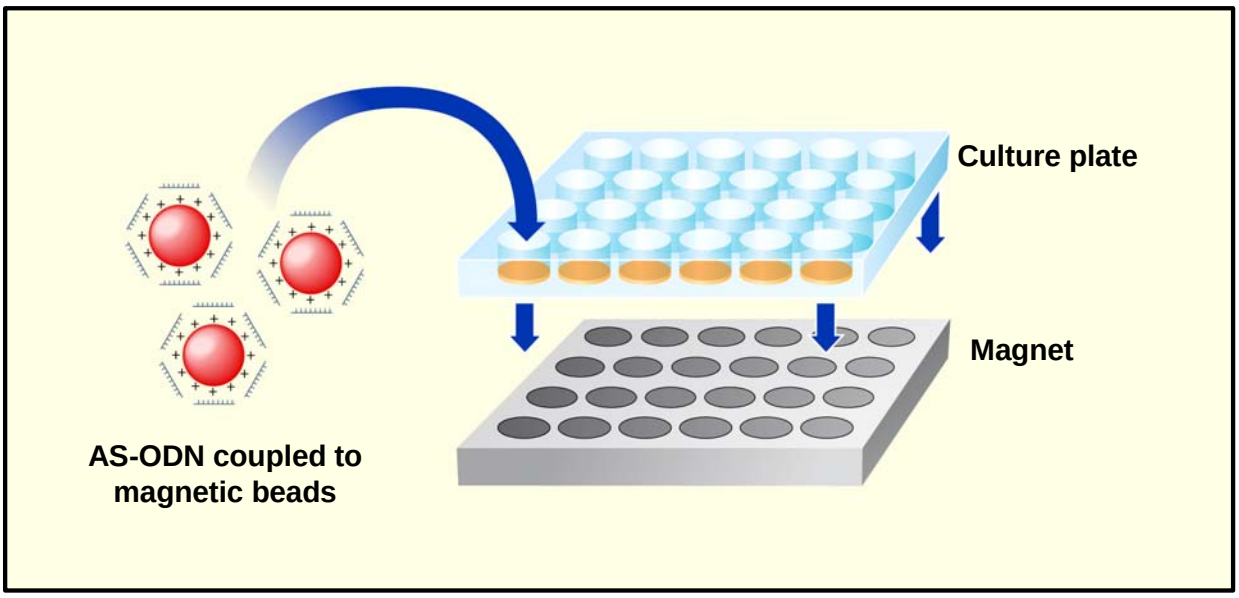


Fig. 1.9 The method of magnetofection

Magnetic nanoparticles coated with Polyethylenimine bind to DNA due to their positive charge. This complex is applied to and transfected into the cells by a short time (15 minutes) influence of an external magnetic field.

There are several advantages of using this transfection method. When performing transfection *in vitro* this method provides a quick and easy way to introduce DNA into cells. The incubation time of the DNA-nanoparticle complexes with the cells is greatly reduced (from standard 2-4 hours to 15 minutes) with a vast increase in vector concentration at the cell surface as the consequence. This in turn means that the vector dose needed for yielding a satisfying transfection level also can be reduced [124-126]. Further, since these magnetic particles do not rely on receptors or other cell membrane bound proteins for cell uptake, it is possible to transfect cells that normally are non permissive [124]. Importantly, the magnetofection method in combination with a standard lipid transfection reagent has been shown to successfully deliver AS-ODN to primary human umbilical vein endothelial cells (HUVEC), which are known to be transfected with difficulties, with transfection efficiencies of over 80%. Furthermore, this was associated with a low cellular toxicity [125].

The possibility of directing the transfection towards a specific area using an external magnetic field gives this approach many advantages for *in vivo* use [124,125,127]. Combining drugs with nanoparticles would reduce systemic side effects and yield a higher concentration of the drug at the target site [127]. Administration of a lower amount of the drug would also probably be sufficient (as for the *in vitro* situation), further reducing systemic side effects. Experiments of delivering anti tumour agents in this way have already been performed successfully in humans [128].

1.5. OBJECTIVES OF THIS STUDY

SHP-2 has been implicated in several different signalling pathways initiated by a variety of growth factors and cytokines. Pathways shown to be influenced by SHP-2 are the Ras-Raf-MEK-ERK, PI3-Kinase, Jak-Stat and NFκB signalling cascades. Although numerous studies showing a positive role for SHP-2 in these pathways exist, the exact mechanism of how SHP-2 activates these signalling cascades remains to be elucidated. Furthermore, apart from its phosphatase activity, SHP-2 can probably also function as an adaptor molecule due to its SH2-domains and has been shown to interact with signalling intermediates [71,72] as well as directly associate with receptors [87,88,89]. Apart from this, SHP-2 has also been shown to influence cellular signalling both positively and negatively depending on the cell type and stimulus [71,72]. Most studies involving SHP-2 so far have been performed in mouse fibroblasts or other equivalent cell lines. Limited studies of SHP-2 signalling in primary human endothelial cells exist and the actual functional effects of SHP-2 in endothelial signalling remain to be investigated. In addition, the possible role of SHP-2 in the angiogenic process is so far unknown. Therefore, this study is aiming at investigating the following questions:

1.5.1. SHP-2 and angiogenesis initiation

- ✓ Is SHP-2 important for the initial steps of angiogenesis, such as growth factor induced proliferation?
- ✓ Is a functional SHP-2 a prerequisite for angiogenesis initiation by preventing endothelial apoptosis?

1.5.2. SHP-2 and vessel formation

- ✓ Is SHP-2 required throughout the angiogenic process, by affecting the differentiation of endothelial cells into tube like structures?
- ✓ Is SHP-2 important for proper vessel formation, such as vessel sprouting and branching?

1.5.3. SHP-2 and endothelial signalling

- ✓ Do angiogenic growth factors, such as FGF, VEGF and PDGF, activate SHP-2 in endothelial cells?
- ✓ Does SHP-2 influence known anti-apoptotic and proliferation enhancing signalling pathways such as the PI3-Kinase/Akt and the Raf-MEK-ERK pathway in endothelial cells?
- ✓ If so, is the effect of SHP-2 on these pathways of positive or negative manner upon VEGF, FGF and PDGF stimulation?
- ✓ Which are the signalling intermediates linking SHP-2 to these pathways in endothelial cells?

To be able to answer these questions, we chose to silence the expression of the SHP-2 protein by antisensedesoxynucleotides (AS-ODN) using the magnetofection technique. For this the following parameters had to be determined:

- ✓ The transfection efficiency achieved by the magnetofection method of endothelial cells
- ✓ Cell cytotoxicity after magnetofection of endothelial cells
- ✓ The kinetic of SHP-2 knock-down

2. MATERIALS AND METHODS

“No amount of experimentation can ever prove me right,
a single experiment can prove me wrong”

~Albert Einstein~

2.1. CHEMICALS

Endothelial growth media was purchased from PromoCell (Heidelberg, Germany), new born calf serum and fetal calf serum were purchased from Biochrom (Berlin, Germany). Accutase was purchased from PAA (Graz, Austria). Collagenase A was provided by Roche (Mannheim, Germany). Collagenase I and the polyacrylamide:bisacrylamide solution in SDS-PAGE was from Genaxxon Biosciences (Biberach, Germany). Collagen G was purchased from Biochrom AG (Berlin, Germany). All other cell culture reagents were obtained from Sigma-Aldrich (Taufkirchen, Germany). Membranes used for sterilization of cell culture media and solutions were from MembraPure GmbH (Bodenheim, Germany). Leupeptin, aprotinin and pepstatin used in lysis buffers and the chemiluminescence detection kit for horseradish peroxidase were ordered from Applichem (Darmstadt, Germany). The BCA (bicinchoninic acid) protein assay reagent kit was provided by Pierce (Bonn, Germany). MACS separation columns and µMACS Protein G Microbeads used for immunoprecipitations were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). The Annnexin V Apoptosis detection kit 1 and growth factor reduced Matrigel matrix used for the capillary like sprouting and aortic ring sprouting assays were purchased from BD Biosciences (Heidelberg, Germany). The nitrocellulose membrane for the blotting of proteins in Western blots was obtained from PeqLab (Erlangen, Germany) and the non fat dry milk powder used in blocking buffers for western blots was from Saliter (Obergünzburg, Germany). The growth factor bFGF was purchased from Tebu-bio (Offenbach, Germany) and the SHP-2 inhibitor Ptp inhibitor IV was provided by Calbiochem (Darmstadt, Germany). Sodium pentobarbital (Narcoren) for euthanizing the mice for the aortic ring sprouting assay were ordered from Merial GmbH (Hallbergmoos, Germany) and the Tutofusin Voll-E solution used to rinse the dissected mouse aortas was obtained from Baxter Deutschland GmbH (Unterschleissheim, Germany). Effectene transfection kit was purchased from Qiagen (Hilden, Germany) and the superparamagnetic iron oxide nanoparticles coated with the polycation polyethylenimine (TransMAG^{PEI}) were kindly provided by Chemicell (Berlin, Germany). All other chemicals were purchased from Sigma-Aldrich (Taufkirchen, Germany).

2.2. CELL CULTURE AND CELL LINES

Media and solutions

HUVEC growth medium (20%)

M199	
+ New born calf serum (NBCS)	20%
+ Endothelial growth media	10%
+ Penicilline/Streptamycine	1%
pH 7.4	

HUVEC starvation medium (1%)

M199	
+ New born calf serum (NBCS)	1%
+ Penicilline/Streptamycine	1%
pH 7.4	

HMEC growth medium (10%)

M199	
+ Fetal calf serum (FCS)	10%
+ Endothelial growth media	10%
+ Penicilline/Streptamycine	1%
pH 7.4	

HMEC starvation medium (1%)

M199	
+ Fetal calf serum (FCS)	1%
+ Penicilline/Streptamycine	1%
pH 7.4	

PAEC growth medium (10%)

Dulbeccos´s modified Eagle´s medium (DMEM)	
+ Fetal calf serum (FCS)	10%
+ Penicilline (10 000 U) / Streptamycine (10 mg)	1%
+ L-Glutamine	200 nM
pH 7.4	

PAEC starvation medium (1%)

Dulbeccos´s modified Eagle´s medium (DMEM)	
+ Fetal calf serum FCS	1%
+ Penicilline (10 000 U) / Streptamycine (10 mg)	1%
+ L-Glutamine	200 nM
pH 7.4	

Sterilization of media was performed by first filtering the solutions through a glas filter (C5) with a pore size of 0.45 µm followed by filtration through a second filter (Memfil CA) with a pore size of 0.2 µm.

HUVEC freezing media

Dimethylsulfoxid (DMSO)	10%
in new born calf serum	

HMEC and PAEC freezing media

DMSO	10%
in fetal calf serum	

Phosphate buffered Saline

	PBS^+	PBS^-
NaCl	8 g	8 g
KCl	0.20 g	0.20 g
Na_2HPO_4	1.15 g	1.42 g
KH_2PO_4	0.20 g	0.20 g
$MgCl_2$	0.04 g	
$CaCl_2$	0.50	
Distilled H_2O	ad 1000 ml	ad 1000 ml
pH 7.4		

Collagenase A solution

Collagenase type I (1700 U/mg)	1g
PBS^+	ad 1000 ml

The solution was passed through the filter Membrex 25 CA with a pore size of 0.2 µm for sterilisation.

2.2.1. Isolation and cultivation of human umbilical vein endothelial cells

The human umbilical cords were kindly provided by the obstetrical department of the Ludwig Maximilians University hospital of Munich. Written and informed consent was obtained from all patients. To isolate human umbilical vein endothelial cells (HUVEC) the vein in the umbilical cord was first rinsed with warm phosphate buffered saline supplemented with Ca^{2+} (PBS^+). To detach the endothelial cells from the vessel wall,

the umbilical cord vein was incubated with 1 mg/ml collagenase A solution in a humidified incubator for 10 minutes. The primary endothelial cells were then washed out of the vessel with HUVEC growth medium (20%). Following collection of the cells, they were pelleted by centrifugation at 1200 rpm (Heraeus Megafuge 1.0R from Kendro, Langenseldbold, Germany) for 5 minutes at room temperature and subsequently washed with M199 medium. Finally, the purified primary endothelial cells were obtained in standard culture flasks in endothelial growth medium and left to grow at 37 °C in a humidified incubator with 5% CO_2. When reaching confluency, the cells were passaged and maintained in HUVEC growth medium (20%) at 37 °C with 5% CO_2 in a humidified incubator. The primary HUVECs were used until the third passage only.

2.2.2. Isolation and cultivation of porcine arterial endothelial cells

Isolation of porcine arterial endothelial cells (PAEC) was performed as previously described by Gloe et. al [129]. Briefly, fresh aortas from pig were kindly provided from the local slaughterhouse and kept in PBS^+ until final preparation. Before dissection of the aorta, fat together with connective tissue was removed. The aorta was then cut longitudinally to expose the luminal surface followed by washing in PBS^+. With the luminal side facing upwards, the vessels were positioned into a frame and incubated with a sterile collagenase A solution (1 mg/ml) for 20 min at 37 °C in a humidified incubator. Lastly, the endothelial cells were washed out of the vessel with culture medium and further cultivated in standard plastic culture dishes. The cells were maintained in PAEC growth medium (10%) at 37 °C with 5% CO_2 in a humidified incubator.

2.2.3. Cultivation of human microvascular endothelial cells

Human micro vascular endothelial cells (HMEC) were provided by Ades et al [130] and cultured in HMEC growth medium (10%). The cells were maintained at 37 °C with 5% CO_2 in a humidified incubator.

2.2.4. Passaging of cells

Confluent cells were washed once with warm PBS- followed by incubation with trypsin-EDTA for approximately 2 minutes to allow the majority of the cells to detach from the cell culture dish. The enzymatic activity of trypsin was inhibited by application of the respective media containing at least 10% serum. Non attached cells were gently scraped off the dish with a rubber scraper and the cell suspension diluted in the respective media followed by seeding onto new culture dishes.

2.2.5. Freezing of cells

To be able to always keep a stock of endothelial cells, a fraction was always kept frozen. For this, confluent endothelial cells (HUVEC, HMEC or PAEC) were incubated with trypsin (1 ml) for approximately 2 minutes. The enzymatic reaction was inhibited by the addition of their respective freezing media (1 ml). The cells were then gently but quickly scraped off the cell culture dish with a rubber scraper and collected in cryo-vials. The cells were kept at -80 ˚C for 24 hours before final storage in liquid nitrogen.

2.3. MOLECULAR BIOLOGY

2.3.1. AS-oligodesoxynucleotide transfer

Solutions

Transfection solution

Table 1: Mixing scheme for the transfection solution

Chemicals	Conc.	Amount / well in a 24 well plate	Amount in a 60 mm culture dish	Amount in a 100 mm culture dish	Ratio Chemical: AS-ODN
AS-ODN	1 µg/µl	0.6 µl (0.6 µg)	1 µl (1 µg)	7 µl (7 µg)	
Enhancer Solution	1 mg/ml	4.8 µl	8 µl	56 µl	8 µl / µg AS-ODN
TransMAGPEI	1 mg/ml	1.2 µl	2 µl	14 µl	2 µl / µg AS-ODN
EC Buffer		25.4 µl	89 µl	123 µl	
Effectene	1 mg/ml	6 µl	10 µl	70 µl	10 µl / µg AS-ODN
Media		162 µl	890 µl	1730 µl	
Media added to cells before transfection		200 µl	1 ml	2 ml	
Total volume		400 µl	2 ml	4 ml	

HMEC growth media (10%)

See section 2.2

HMEC starvation media (1%)

See section 2.2

HUVEC growth media (20%)

See under section 2.2

Experimental procedure

To achieve a knock-down of SHP-2 protein, phosphorothioate modified oligodesoxynucleotides (ODN) were transferred into endothelial cells using the previously described magnetofection technique [125] together with the Effectene transfection kit. In detail, DNA oligos were mixed with Enhancer Solution,

TransMAG^{PEI} and EC buffer according to table 1. After short vortexing and incubation at room temperature for 2-5 minutes, Effectene was added according to table 1 and the solution was incubated at room temperature for another 10 minutes. HUVEC growth medium (20%) or HMEC growth medium (10%) were then applied to the cells to be transfected in the following amounts: 200 µl / well in a 24 well plate, 500 µl for a 60 mm cell culture dish and 1 ml for a 100 mm cell culture dish. HUVEC growth medium (20%) or HMEC growth medium (10%) were mixed with the transfection solution (according to table 1) before applying the solution to the cells. For AS-ODN delivery into the cells, the cell culture plate was immediately put upon a neodymium-iron boron magnet in the humidified incubator for 15 minutes. The cells were then rinsed gently three times with PBS^{+} and left in fresh medium. For experiments the cells were kept in culture, in HUVEC growth medium (20%), HMEC growth medium (10%) or HMEC starvation medium (1%), as far as not otherwise stated, for 6-24 hours following transfection. Oligonucleotide sequences designed according to GenBank entry number XM_293497 were as follows: SHP-2 AS-ODN: 5´ctccgcgatgtcatgttcct 3´, Rdm ODN: 5´cccttatttactactttcgc 3´ (both from MWG Biotech, Ebersberg, Germany). The GC/AT ratio was 55% and 40% respectively. Both ODNs had phosphorothioate added to the first 4 nucleotides on the 5´end and to the five last nucleotides on the 3´end to protect them from degradation. Blast-searches through the NCBI database were done with the sequences to ensure no alignment with other mRNAs of mouse or human kind.

2.3.2. Assessment of transfer efficiency

Solutions

Propidium iodide staining solution

Propidium iodide	0.6 mg
in 50 ml HEPES buffer	

Formaldehyde solution

Formaldehyde	2%
in HEPES	

Sodium citrate solution

Sodium citrate (NaCi)	15 mM
KCl	135 mM

Other reagents

Cy-3 labelled Rdm ODN	0.6 µg / well in a 24 well plate format

Experimental procedure

HMEC were transfected using the magnetofection method as described in section 2.3.1. ODN uptake into endothelial cells 4 hours post transfection with a Cy-3 (carbocyanin) labelled ODN was quantified by FACScan flow cytometer (BD Biosciences, Heidelberg, Germany) after enzymatic removal of the cells with trypsin-EDTA from the culture dish. The cells were washed with PBS^+ followed by fixation in 2% formaldehyde for 10 minutes at room temperature. Photos of cells transfected with Cy-3 labelled ODN were taken 4, 16 and 24 hours post transfection with an invert laser scanning microscope (LSM 410, Zeiss, Jena, Germany) using a 570 nm longpass emission filter and exciting at 543 nm. Cytotoxicity was assessed 24 hours following AS-ODN transfection by propidium iodide staining. Cells were detached from the cell culture dish nonenzymatically with sodium citrate, incubated with the propidium iodide solution for 20 minutes in the dark followed by washing three times with PBS^+. Finally, the cells were fixated in a 2% formalin solution. Cells

staining positive for propidium iodide, as analysed by flow cytometry, were assumed to be non viable.

To evaluate the efficiency of SHP-2 AS-ODN to degrade SHP-2 mRNA and so detain SHP-2 protein expression in the experiments in this study, the SHP-2 protein level was detected using the western blotting technique (described in detail in section 2.4.1) 6, 24 and 48 hours post AS-ODN transfection. As a control for loading the SDS-PAGE with equal amounts of protein, the membranes were incubated with an α-Actin antibody after stripping (see section 2.4.1). The protein bands were detected using a bioluminescence detection system attached to a digital camera (Sequoia, Hamamatsu, Herrsching am Ammersee, Germany). The density of the protein bands were measured as intensity/pixels in regions of interests (ROI) with the software WASABI version 5.1 to reveal the extent of inhibition of SHP-2 protein translation. The ratio of the SHP-2/Actin protein band was calculated to enable a comparison between western blots.

2.3.3. Cell extracts for immunoprecipitations, western blot analysis and SHP-2 activity measurement

Buffers and solutions

Lysis buffer

KH_2PO_4	20 mM
Ethylenediaminetetraacetic acid (EDTA)	1 mM
Pefablock	1 mM
Leupeptin	1 µM
Pepstatin	1 µM
NaF	50 mM
$Na_40_7P_2$	40 mM
Na_3VO_4	2 mM
pH 7.3	

Before usage of the buffer, Triton X-100 was added to a final concentration of 1%.

Lysis buffer for SHP-2 activity measurement

Tris	20mM
EDTA	1mM
Pefablock	1mM
Leupeptin	1µM
Pepstatin	1µM
Aprotinin	1µM
NaF	50mM
$Na_40_7P_2$	40mM
pH 7. 35	

Before usage of the buffer, Triton X-100 and sodium desoxycholate (DOC) was added to a final concentration of 1% and 0.5% respectively.

Sodium orthovanadate (Na_3VO_4) 200 mM

Sodium orthovanadate is a non specific inhibitor for tyrosine phosphatases. To achieve maximal inhibition of protein tyrosine phosphatases, the sodium orthovanadate has to be activated. Depolymerisation and so activation of sodium metavanadate ($NaVO_3$), converting it into Na_3VO_4, was achieved through the following procedure:

1) $NaVO_3$ was solved in distilled H_2O to a final concentration of 200 mM and the pH was set to 10. Under these conditions the solution is yellow.
2) The solution was boiled in a water bath until it was colourless
3) The solution was left to cool down to room temperature before the pH was again set to 10.
4) The steps 2 and 3 were repeated until the pH stabilized at 10 and the solution remained colourless independent of temperature.

Aliquots were kept at -20 ˚C until further usage.

Experimental procedure

To obtain protein lysates the cells were washed once with PBS^+ and immediately put on ice and lysed for 10 minutes using ice-cold lysis buffer. For cells appointed for SHP-2 activity measurement, the cells were lysed with SHP-2 activity lysis buffer. The cells were then scraped off the dish with a rubber scraper and passed repeatedly through a 29 g needle. The debris was removed from the lysate by centrifugation at 10 000 g for 10 minutes and at a temperature of 4 °C. The protein concentration for the remaining solution was determined using BCA (bicinchoninic acid) protein assay reagent kit according to the manufacturer's protocol (see section 2.3.4). The protein lysates were either directly analysed or kept frozen at -20 °C until further handling.

2.3.4. Assessment of protein concentration

Materials

BCA protein assay reagent kit

Bovine Serum albumin solution 2 mg/ml in distilled water

Experimental procedure

The BCA (bichinconinic acid) protein assay reagent kit is based on the biuret reaction where Cu^{2+} is reduced to Cu^{1+} by proteins in an alkaline medium. Addition of bichinconinic acid leads to a purple-coloured end product which can be detected colometrically at 562 nm. This end product is formed by the chelation of two molecules of BCA with one Cu^{1+} and is almost linear with increasing protein concentrations. 10 µl of protein lysate was added to a well in a 96 well plate formate prior to application of 200 µl BCA solution (mixed according to the supplier´s protocol). Every sample was measured in triplicates with SpectraFluor (Tecan, Crailsheim, Germany). A dilution series with BSA was run alongside with the protein lysates for the establishment of a standard curve. The mixture was incubated for 30 minutes at 37 ˚C prior to measurement at 550 nm. The protein concentration was calculated according to the following formula:

$$f(x) = m\,x + b$$

where **x** has to be dissolved to reveal the protein amount in µg and **m=** gradient of the standard curve; **b=** axis intercept of the standard curve and **f(x)=** the absorbance data obtained.

2.4. PROTEIN CHEMISTRY

2.4.1. Western Blot analysis

Reagents and solutions

SDS-PAGE

Separation gel (10%):

Distilled H_20	20 ml
Tris 1.5 M pH 8.8	12.5 ml
Sodeum dodecyl sulfate (SDS) 10%	0.5 ml
Acrylamide/Bisacrylamide 30% / 0.8% (w/v)	16.6 ml
Ammoniumpersulfate 10% (w/v)	0.25 ml
TEMED	0.025 ml

Stacking gel (4%):

Distilled H_2O	6 ml
Tris 0.5 M pH 8.8	2.5 ml
SDS 10% (w/v)	0.1 ml
Acrylamide/Bisacrylamide 30% / 0.8% (w/v)	1.3 ml
Ammoniumpersulfate 10% (w/v)	0.05 ml
TEMED	0.01 ml

For both gel solutions, H_2O, Tris, Acrylamide/Bisacrylamide were mixed and degassed before addition of SDS, Ammoniumpersulfate and TEMED. Upon pouring the separation gel, a layer with butanol was added on top of the gel to prevent it from drying while polymerising. The butanol layer was meticulously washed off with distilled water before pouring the stacking gel on top of the separation gel.

Loading dye (4 x)

Tris HCl pH 6.8	0.25 M
SDS	8%
Glycerine	40%
Bromphenolblue	0.02%
Mercaptoethanol	400 mM

Running buffer (5 x)

Tris Base	123.8 mM
Glycine	959.1 mM
SDS	17.3 mM

Transfer buffer:

Glycine	39 mM
Tris Base	48 mM
SDS	0.037%
MeOH	10%

Tris buffered saline (TBS) (10 x)

Tris	24.2 g
NaCl	58.44 g
Distilled H_2O	ad 1000 ml

Tris buffered saline with Tween (TBS/T)

TBS	1 x
Tween 20	0.1%
pH 7.6	

Blocking Buffer A

NaCl	200 mM
Tris pH 7,5	50 mM
Bovine serum albumine (BSA)	3%
Tween 20	0.05%
Horse serum	10%

Blocking Buffer B

Tris	500 mM
NaCl	1.5 M
Non fat dry milk	5%
pH 7.5	

Blocking buffer C

Tris	500 mM
NaCl	1.5 M
Non fat dry milk	3%

Blocking buffer D

Non fat dry milk powder	3% in TBS/T

Blocking buffer E

Bovine serum albumine (BSA)	5% in TBS/T

Stripping buffer

Glycine	0.2 M
NaCl	0.5 M
in PBS	
pH 2.9	

Primary antibody solutions

Table 2: Primary antibodies used in western blot (MC: monoclonal, PC: polyclonal)

Antibody	Host	Dilution and buffer	Company
Anti-SHP-2 (B-1)	Mouse (MC)	1:500 C	Santa Cruz, Heidelberg, DE
Anti- p85 (B-9)	Mouse (MC)	1:500 D	Santa Cruz, Heidelberg, DE
Anti-p42/44 (Thr202/Tyr204)	Mouse (MC)	1:1000 A	Cell signalling, Frankfurt a. Main, DE
Anti-pRaf (Ser338)	Rabbit (MC)	1:1000 E	Cell signalling, Frankfurt a. Main, DE
Anti-pAkt 1/2/3 (Thr308)	Rabbit (PC)	1:500 A	Santa Cruz, Heidelberg, DE
Anti-GAPDH	Mouse (MC)	1:10 000 D or E	Chemicon, Hamshire, GB
Anti-Actin (I-19)	Goat (PC)	1:500 B	Santa Cruz, Heidelberg, DE
Anti-pTyr (clone 4G10)	Mouse (MC)	1: 1000 A or E	Biomol, Hamburg, DE
Anti-Gab-1	Rabbit (PC)	1:1000 E	Cell signalling, Frankfurt a. Main, DE
Anti-pSHP-2 (Tyr 542)	Rabbit (PC)	1:1000 D	Biomol, Hamburg, DE

Secondary antibody solutions

Table 3: Secondary antibodies used in western blot

Antibody	Host	Dilution	Company
Anti rabbit	Goat	1:10 000	Calbiochem, Schwalbach, DE
Anti mouse	Goat	1:10 000	Calbiochem, Schwalbach, DE
Anti goat	Rabbit	1:10 000	Calbiochem, Schwalbach, DE

MC: monoclonal, PC: polyclonal

Experimental procedure

Proteins were thawed on ice and 25-40 µg were mixed with 1 x loading dye (0.25 M Tris pH 6.8, 8% SDS, 40% Glycerine, 0.02% bromphenolblue, 400 mM mercaptoethanol), denaturated at 100 °C for 5 minutes before separated on a 10% SDS-PAGE consisting of a 4% stacking gel and a 10% separation gel covered in 1 x running buffer. The current was set to 35 mA and the proteins were left to run until the loading dye front was approximately 1 cm from the edge of the gel. The separated proteins were then transferred onto a nitrocellulose membrane by electrophoresis using semi-dry blotting. For this, the nitrocellulose membranes together with filter papers were soaked in transfer buffer. The membrane and filters were stacked as a “sandwich” in the following order: filter paper, membrane, gel and filter paper. To check for equal amounts of protein and successful blotting, the membrane was incubated with Poinceau S solution (Sigma-Aldrich, Taufkirchen, Germany) which was removed by washing before further handling. To prevent unspecific binding of the antibodies the membrane was blocked with a blocking buffer depending on the antibody to be used (see table 2) for 30 minutes prior to a 1 hour incubation with the primary antibody diluted in their respective blocking buffer (according to table 2) at 37 °C or at 4 °C over night. Unbound antibody was removed by washing 3 x 5 minutes with TBS/T. The membrane was then incubated for 1 hour at room temperature or at 4 °C over night with a secondary antibody conjugated with horseradish peroxidase. After washing three times as described above, the enzyme activity was detected upon addition of chemiluminescence detection kit for horseradish peroxidase according to the supplier’s protocol with a bioluminescence detection system attached to a digital camera (Sequoia).

2.4.2. Immunoprecipitation of p-85 and SHP-2

Materials

Lysis buffer	See under section 2.3.3
Loading dye	See under section 2.4.1
Anti-SHP-2 antibody	0.5 µg/100 µg protein
Anti-p85 antibody	0.5 µg/100 µg protein
Protein G microbeads	50 µl/sample

Cells

HMEC

Experimental procedure

Immunoprecipitations were performed using µMACS Protein G Microbeads and MACS separation columns according to the manufacturer's protocol. Shortly, equal amounts of protein from endothelial cells were incubated for 2 hours at 4°C with a mouse monoclonal p85 or SHP-2 antibody (both 0.5 µg/100 µg protein) followed by 30 minutes incubation with Protein G microbeads (50 µl/ sample) on ice. The precipitates were purified by application onto the separation columns and washed three times with lysis buffer. Finally, the precipitates were detached from the microbeads by application of hot loading dye and the eluate collected in eppendorf tubes. Proteins were detected with western blot (see section 2.4.1) using a mouse monoclonal phosphotyrosine, mouse monoclonal p85 or rabbit polyclonal Gab-1 antibody respectively. As a control for equal amounts of precipitated protein, the precipitated protein itself was detected.

2.5. FUNCTIONAL ASSAYS

2.5.1. Treatment with growth factors and pharmacological inhibitors

Growth factors and concentrations used

VEGF-A_{165} 10 ng/ml

bFGF 10 ng/ml

PDGF-BB 10 ng/ml

Inhibitors

Inhibitor (Name)	Inhibiting protein	Concentration used	Incubation time
Wortmannin	PI3-Kinase	10 nM	30 minutes
GW 5074	Raf-1	5 µM	During the whole experiment
Protein tyrosine phosphatase Inhibitor IV (Ptp I IV)	SHP-2	2 µM	During the whole experiment

Activators

Activator	Activating protein	Concentration used	Incubation time
Phorbol-myristate-acetate (PMA)	Protein kinase C	1 µM	10 minutes

Media

HMEC starvation medium (1%)

HUVEC starvation medium (1%)

PAEC starvation medium (1%)

Experimental procedure

To investigate the effect of growth factors in SHP-2 signalling, HUVEC were starved in HUVEC starvation medium (1%) for approximately 12 hours and HMEC were starved in HMEC starvation medium (1%) for 24 hours and PAEC were starved in PAEC starvation medie (1%) during the whole experiment. bFGF, VEGF-A_{165} and PDGF-BB in starvation media were added to the cells and after incubation in a humidified incubator for 10 minutes, the cells were lysed as described in section 2.3.3. When using pharmacological inhibitors, the cells were starved as described

above, treated with the inhibitors followed by incubation with the above mentioned growth factors. For experiments over 24 hours, the different growth factors and inhibitors were mixed with the respective starvation media and added to the cells every 24 hours.

2.5.2. Protein activity assays

Buffers and solutions

Phosphatase buffer

HEPES	20 mM
NaCl	100 mM
$MgCl_2$	5 mM
pH 6.5	

p-Nitrophenyl phosphate (pNPP) solution

pNPP	5 mg (1 tablet)
in 1.2 ml phosphatase buffer	

Other reagents

Protein G on cross-linked beaded agarose	10 µl/100 µg protein
Mouse monoclonal SHP-2 antibody	0.2 µg antibody/100 µg protein
p-Nitrophenyl phosphate (pNPP)	10 mM
Sodium orthovanadate (Na_3VO_4)	1 mM
bFGF, PDGF-BB and VEGF-A_{165}	10 ng/ml

Cells

HMEC

HUVEC

Experimental procedure

For SHP-2 phosphatase activity measurements, HUVEC were treated with 10 ng/ml bFGF, PDGF-BB and VEGF-A_{165}, washed once with ice-cold PBS^+ and lysed as described in section 3.2.3. To remove proteins binding unspecifically to the protein G

beads, equal amounts of protein were mixed with Protein G on cross linked beaded agarose and incubated at 4 °C for 30 minutes followed by centrifugation at 2500 rpm for 5 minutes at 4 °C. The supernatant was incubated with mouse monoclonal SHP-2 antibody at 4 °C for 2 hours followed by addition of Protein G agarose beads and another incubation at 4 °C for 2 hours. The lysates were then transferred to a column containing 35 µm pore size filter (Mo Bi Tech, Göttingen, Germany) and washed 5 x with lysis buffer without any detergents. The filters were incubated with 10 mM pNPP (a phosphatase substrate producing a yellow end product), for 1 hour at 37 °C in the dark. The pNPP solution was then transferred to a 96 well plate and the colour intensity of the end product read spectrophotometrically at 405 nm with SpectraFluor (Tecan, Crailsheim, Germany) as a measurement for SHP-2 phosphatase activity. To obtain the inducible SHP-2 activity fraction, the filters were incubated with 1 mM Na_3VO_4 for 30 minutes at 37 °C before a second incubation with pNPP and subsequent measurement at 405 nm. The results obtained from this treatment were used as a background and substracted from the data obtained from the first measurement.

For measuring Raf-1, ERK1/2 and Akt activity, the endothelial cells were starved for 24 hours with HMEC starvation medium (1%) followed by treatment with bFGF, PDGF-BB or VEGF-A_{165}, lysed and the lysate submitted to western blotting as described in sections 2.3.3 and 2.4.1 respectively. Membranes were first incubated with the respective phospho-antibodies and after visualisation of the protein bands stripped with stripping buffer (see section 2.4.1) for 1 hour followed by incubation with an anti-GAPDH antibody to ensure equal protein loading. For PI3-Kinase activity, cell lysates were precipitated with a p85 antibody as described in section 2.4.2, subjected to western blotting and subsequently immunoblotted with a phospho tyrosine antibody. The band densities were measured as described in section 2.3.2 and the phospho-protein band normalised to the GAPDH or p85 band respectively for comparison of protein band densities.

2.5.3. *In vitro* wound healing assay

Materials

bFGF, PDGF-BB,VEGF-A_{165}	10 ng/ml
GW 5074	5 µM
Ptp I IV	2 µM
Collagen G	10 µg/ml

Buffers and solutions

HMEC starvation medium (1%)

HUVEC starvation medium (1%)

PAEC starvation medium (1%)

Cells

HMEC

PAEC

Experimental procedure

To evaluate the effect of SHP-2 and Raf inhibition on endothelial wound healing *in vitro*, a standardised scratch assay was used. In detail, HMEC and PAEC were seeded onto Collagen G and following 24 hours of starvation in HMEC or PAEC starvation medium (1%) the cell layer was scratched once from edge to edge of the culture dish using a 0.5 mm pipet tip to obtain a standardized scratch through the otherwise confluent cell layer (Fig. 2.1). The cells were then incubated in HMEC or PAEC starvation medium (1%) to which bFGF, PDGF-BB, VEGF-A_{165} and the Raf inhibitor GW 5074 or the SHP-2 inhibitor (Ptp I IV) had been added. Photos of the scratches were taken at 0, 24, 48 and 72 hours after scratching with a LSM 410 inverted laser scan microscope (Zeiss, Jena, Germany) using a 10 x 0.25 Ph1Var1 objective at room temperature. The level of wound healing was determined by measuring the width of the scratches (in µm) at three different points/photo, to achieve a mean of scratch width, with the software simple PCI.

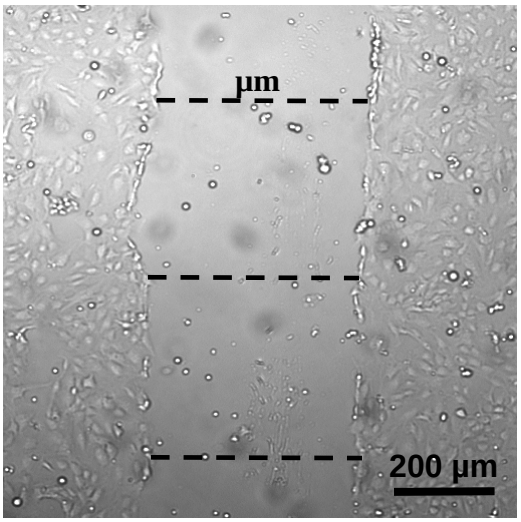


Fig. 2.1 Scratch

A scratch was performed using a 0.5 mm pipet tip on a confluent cell layer. The cell layer was scratched once from edge to edge of the culture dish. Photos were taken immediately after scratching and at 24, 48 and 72 hours. The width of the scratch was measured at three different points (dashed lines) and a mean of scratch width / scratch was calculated.

2.5.4. Cell proliferation assay

Solutions

Colourless DMEM (1%)

Dulbeccos´s modified Eagle´s medium (DMEM) without phenolred	
Fetal calf serum (FCS)	1%
Penicilline (10 000 U) / Streptamycine (10 mg)	1%
L-Glutamine	2 mM

Gelatine solution

Gelatine	10 µg/ml
in PBS $^{-}$	

The solution was autoclaved for sterilization prior to use. To coat the 24 well plates with gelatine, sufficient amounts of the gelatine solution to cover the bottom of each well were added to the well plates. After incubation over night at 4 ˚C or for 30 minutes in a humidified incubator, each well was washed three times with PBS^{+}.

Methylthiazoletetrazolium (MTT) solution

MTT	5 mg/ml
in PBS^+	

For every experiment, the MTT solution was freshly made in sufficient amounts to last for the whole time period of the experiment. Before application to the cells, the MTT solution was diluted further (1:10) in colourless DMEM (1%).

Other reagents

bFGF, PDGF-BB, VEGF-A_{165}	10 ng/ml
GW 5074	5 µM
2 Propanol	100%
SHP-2/Rdm AS-ODN	0.6 µg/well in a 24 well plate format

Cells

HMEC

Experimental procedure

To study their proliferation rate HMEC were seeded onto 24 well plates coated with gelatine and allowed to attach over night. The next day magnetofection was performed (according to table 1 in section 2.3.1) followed by serum starvation for 24 hours prior to stimulation with bFGF, PDGF-BB and VEGF-A_{165} and the t=0 hour measurement. For experiments with GW5074, the cells were plated in medium containing 1% serum and treated with the inhibitor the following day. Proliferation was measured by the reduction of Methylthiazoletetrazolium, MTT, using the method described by Mosmann [131]. Briefly, MTT is reduced to formazan by enzymes in the respiratory chain. Thus, the amount of formazan made is an indirect measure for the number of viable cells. The end product is coloured and can be measured spectrophotometrically at 595 nm. Briefly, cells were incubated with MTT for 2 hours at 37 °C and subsequently washed with PBS^+. The formazan crystals formed by living cells were dissolved with 100% 2-Propanol and transferred to semi-micro cuvettes and measured with a spectrophotometer (Ultrospec 2000, Amersham Biosciences, Freiburg, Germany) at 595 nm and 650 nm. The amount of proliferation was determined by substracting the 650 nm value (background) from the 595 nm value. Data were normalized to the proliferative response, which was set to 100%, at

24 hours following seeding or transfection and before first growth factor treatment (t=0 hour measurement).

2.5.5. Fluorescent-activating cell scanning analyses

A flow cytometer can be used to assess information of the molecular properties of cells. By letting cells pass one at a time through a focused laser beam, the laser light is disrupted and scattered. This scattering of the light gives information about the cell size, so called forward scatter (FSC) and the internal complexity of a cell, such as the extent of granulation, called side scatter (SSC). In addition to scatter, a cytometer is also able to measure fluorescence using three detectors, FL1, FL2 and FL3. By staining cells with fluorescent dyes, such as propidium iodide or fluorochromes coupled to antibodies which recognize and bind cell surface antigens, a defined fraction of cells can be detected. Data obtained in this study using fluorescent-activating cell scanning were all measured with FACScan from BD Biosciences (Heidelberg, Germany). For measurements with endothelial cells, the signal for FSC was multiplied by 0.1 (setting E-1) and the level of amplification was set to 2.86. The detector level for SSC was set to 296 V and the level of amplification to 2.40. Both FSC and SSC were set to a linear data modus. From each sample 10 000 cells were projected through the laser beam and the data obtained were analysed with the CELLQuest software (BD Biosciences, Heidelberg, Germany). For the FACScan measurements in this study, following settings were used:

Assay	Stain (max exc, max em)	Detector	Detector level (V)	Data mode
Cell cycle (Propidium iodide, PI, staining)	PI (530, 620)	FL 3	380	Lin
Apoptosis (Annexin V/PI) staining	FITC (488, 520) PI (530, 620)	FL 1 FL 3	480 447	Log Log
Cell uptake of ODN (cy-3 labelled ODNs)	Cy-3 (545, 560)	FL 2	300	Log
Cell viability (PI staining)	PI (530, 620)	FL 3	288	Log

Lin: linear, Log: logarithmic; exc: excitation (nm), em: emission (nm)

2.5.6. Cell cycle measurement

Buffers and solutions

Propidium iodide solution

Triton X-100	0.1%
Rnase Inhibitor 2	5000 U
Propidium iodide	0.6 mg
HEPES	ad 50 ml H_2O

Other reagents

SHP-2/Rdm AS-ODN 0.6 µg/well in a 24 well plate format

Cells

HUVEC

Experimental procedure

The DNA amount of cells can be used to define the different stages in the cell cycle, as the amount of DNA in a cell corresponds to where in the cell cycle the cell is residing. The cell cycle stage where the cells contain the least amount of DNA in comparison to the other stages is the G1-phase. Cells undergoing apoptosis are subdued to DNA fragmentation and because they also have a disrupted cell membrane these DNA fragments leak out of the cell. When staining cells with propidium iodide, which intercalates into the DNA, the cell membrane needs to be disrupted for the dye to enter the cell. The DNA of viable cells is normally too bulky to escape from the cell, in contrast to the DNA fragments seen in apoptotic cells, and high amounts of propidium iodide can therefore accumulate in the DNA of viable cells. Thus, a cell undergoing apoptosis takes up less amounts of propidium iodide than a cell in the G1-phase of the cell cycle. The apoptotic cell can so be detected in the so called SubG0-fraction. To analyse cell viability, cell cycle states of HUVEC were assessed by flow cytometric measurement of propidium iodide stained DNA as described by Crompton et al [132]. Briefly, 6, 24 and 48 hours after AS-ODN treatment (according to table 1 in section 2.3.1), the cells were detached by trypsination for approximately 2 minutes. The cell suspension was collected and the cells pelleted by

centrifugation at 2000 rpm (EBA 12 centrifuge from Hettich, Tuttlingen, Germany) for 3 minutes at room temperature followed by resuspension in PBS^+ (100 µl) and propidium iodide staining solution (900 µl). After 20 minutes incubation in the dark at room temperature, the DNA content of the cells was analysed by fluorescent-activated cell scanning (FACScan) (for detailed settings see section 2.5.5). Cells detected in the Sub G0 fraction (the fraction with least amount of DNA due to DNA fragmentation) were assumed to include the fraction of apoptotic cells.

2.5.7. Apoptosis measurements

Reagents

SHP-2/Rdm AS-ODN 0.6 µg/well in a 24 well plate format

Annnexin V Apoptosis detection kit 1 containing:

- ✓ Propidium iodide solution
- ✓ Binding buffer (10 x)
- ✓ Annexin V-FITC labelled antibody

Cells

HUVEC

Experimental procedures

To assess the amount of intact, yet apoptotic cells, an Annexin V / Propidium Iodide apoptosis assay kit was used according to the manufacturer's protocol. Shortly, 6, 12 and 24 hours following AS-ODN transfection (according to table 1 in section 2.3.1), HUVEC were washed with PBS^+ and 5 µl propidium iodide solution was added to each well in a 24 well plate followed by incubation at 37 °C for 30 minutes. After rinsing the cells with PBS^+ they were incubated with Accutase until all cells had detached from the plate. The cells were then separated from the solution by centrifugation at 2500 rpm for 3 minutes at room temperature. The pellet was subsequently resuspended in 1 x binding buffer whereupon 5 µl of annexin V-FITC (fluorescein isothiocyanate) labelled antibody was added. After incubation in the dark for 15 minutes and addition of 400 µl binding buffer flow cytometric analysis of cells staining positive for Annexin V but negative for propidium iodide was performed to

identify the fraction of cells undergoing early apoptosis (for detailed settings see section 2.5.5).

2.5.8. Capillary like structure assay

Buffers and solutions

HUVEC growth media (20%)

HUVEC starvation media (1%)

Other reagents

Ptp Inhibitor IV	2 µM
SHP-2 AS-ODN	1 µg/60 mm cell culture dish
Growth factor reduced Matrigel matrix	

Cells

HUVEC

Experimental procedure

To investigate the effect on tube formation, confluent HUVEC were starved with medium containing 1% serum and treated with sham solution or Ptp Inhibitor IV (Ptp I IV) for 6 hours prior to trypsination and collection of the cells. The cells were then resuspended in medium containing 1% serum and Ptp I IV or sham solution and equal amounts of cells were seeded onto wells in a 24 well plate format filled with growth factor reduced Matrigel matrix. For SHP-2 AS-ODN treatment (according to table 1 in section 2.3.1), the cells were left in medium containing 20% serum for 6 hours following transfection prior to seeding equal amounts / well of cells onto matrigel. HUVEC were left to form capillary like structures over night. For analysis, pictures of four predefined sectors (regions of interest) per well were taken the next morning with a LSM 410 inverted laser scan microscope (Zeiss, Jena, Germany) using a 10 x 0.25 Ph1Var1 objective at room temperature. For evaluation the capillary like structures in each sector were counted manually and blindfolded. The sum of capillary like structures in the four sectors was used for further evaluation and statistics.

2.5.9. Aortic ring sprouting

Buffers and solutions

Sodium pentobarbital (Narcoren)

Tutofusin Voll-E®

HUVEC growth medium (20%)

Other reagents

Growth factor reduced Matrigel matrix

Protein phosphatase Inhibitor IV 2 µM

Experimental procedure

To investigate the effect of SHP-2 inhibition on proper vessel formation, the number of new vessels sprouting from a mouse aorta was evaluated. For this, adult Black6 mice of both genders, bred and kept according to the German law of animal protection (TierSchG) paragraphs §1, §2 and §2a, at the animal department of the Institute of Physiology Munich, Germany were used. The mice were euthanized with an overdose of sodium pentobarbital (Narcoren) and the aorta was dissected and cleaned from fat and connective tissue. To be able to wash the lumen of the vessel to remove the remaining blood, the aorta was cannulized using a polyethylene tube before it was cut out of the corpse. After carefully rinsing the aorta with physiological solution (Tutofusin Voll-E®), it was kept in HUVEC growth medium (20%) while being manually cut into small rings. The aortic rings were embedded in growth factor reduced Matrigel matrix, and incubated at 37 °C for 30 minutes to allow the matrigel matrix to become hard. The embedded aortic rings (Fig. 2.2) were then treated with and kept in Ptp I IV diluted in HUVEC growth medium (20%). The aortic rings were left for 72-96 hours before pictures were taken with a LSM 410 inverted laser scan microscope (Zeiss, Jena, Germany) using a a 10 x 0.25 Ph1Var1 objective at room temperature. As a measurement of angiogenesis the bifurcations of the new vessels were counted manually.

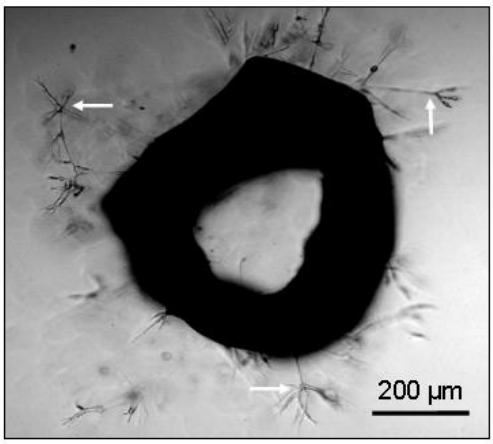


Fig. 2.2 Aortic Ring

A representative photo of an aortic ring 96 hours after embedment in Matrigel. White arrows point at bifurcations of formed sproutings.

2.6. STATISTICAL ANALYSIS

All statistical analyses were performed using Sigma Stat version 2.0. For comparisons of two normally distributed groups of data, the Student's t-test was used. For multiple comparisons of normal distributed data the one-way analysis of variance (one-way ANOVA) was used. The Mann-Whitney Rank sum test was performed when comparing two groups which were not normally distributed, whereas comparisons between several groups not exhibiting normal distribution was achieved by analyzing the data with the analysis of variance on ranks. For descriptive purposes all data are presented as means ± the standard error of means. Results were considered significant at an error probability level of $p<0.05$.

3. RESULTS

The most exciting phrase to hear in science,
the one that heralds new discoveries, is not Eureka! (I found it!)
but rather, “hmm….that´s funny….”

~Isaac Asimov~

3.1. SHP-2 PROTEIN KNOCK-DOWN

3.1.1. Efficiency of AS-ODN transfer

A formulation of antisense- oligodesoxynucleotides (AS-ODN) and the transfection reagent Effectene combined with magnetofection in HMEC showed a transfection efficiency of 86 ± 2% as assessed by transfection of Cy3-labelled Rdm ODN. In contrast, only 26 ± 3% cells had taken up the AS-ODN when a transfection solution without magnetic particles was used. This complied with a 3.3 fold increase of transfected cells ($p<0.01$; n=6-9, grey bar in fig. 3.1A) when using magnetofection. Moreover, cell viability among the transfected cells did not differ significantly between the two groups as assessed by propidium iodide staining 24 hours post transfection. The group treated with the mixture of AS-ODN, Effectene and magnetic particles exhibited 19 ± 4% (n=6-9) non-viable cells as compared to 14 ± 5% (n=6-8) non viable cells among the fraction of cells treated with the transfection mixture but without the influence of magnetic particles (Fig. 3.1A, dot plot).

Cellular distribution and uptake were further confirmed with fluorescence microscopy of cells transfected with Cy-3 labelled Rdm ODN. 4 hours following transfection some of the Cy-3 labelled Rdm ODN could be observed in the cytosol but most of the Rdm ODN seemed to still reside on the cell surface. 16 hours post transfection, the highest amount of Rdm ODN could be observed in the cytosol and to a lesser extent in the nuclei. 24 hours after transfection the highest fluorescence could still be detected in the cytosol, although more Cy-3 labelled Rdm ODN could be observed in the nuclei in comparison to 16 hours following transfection (Fig. 3.1B).

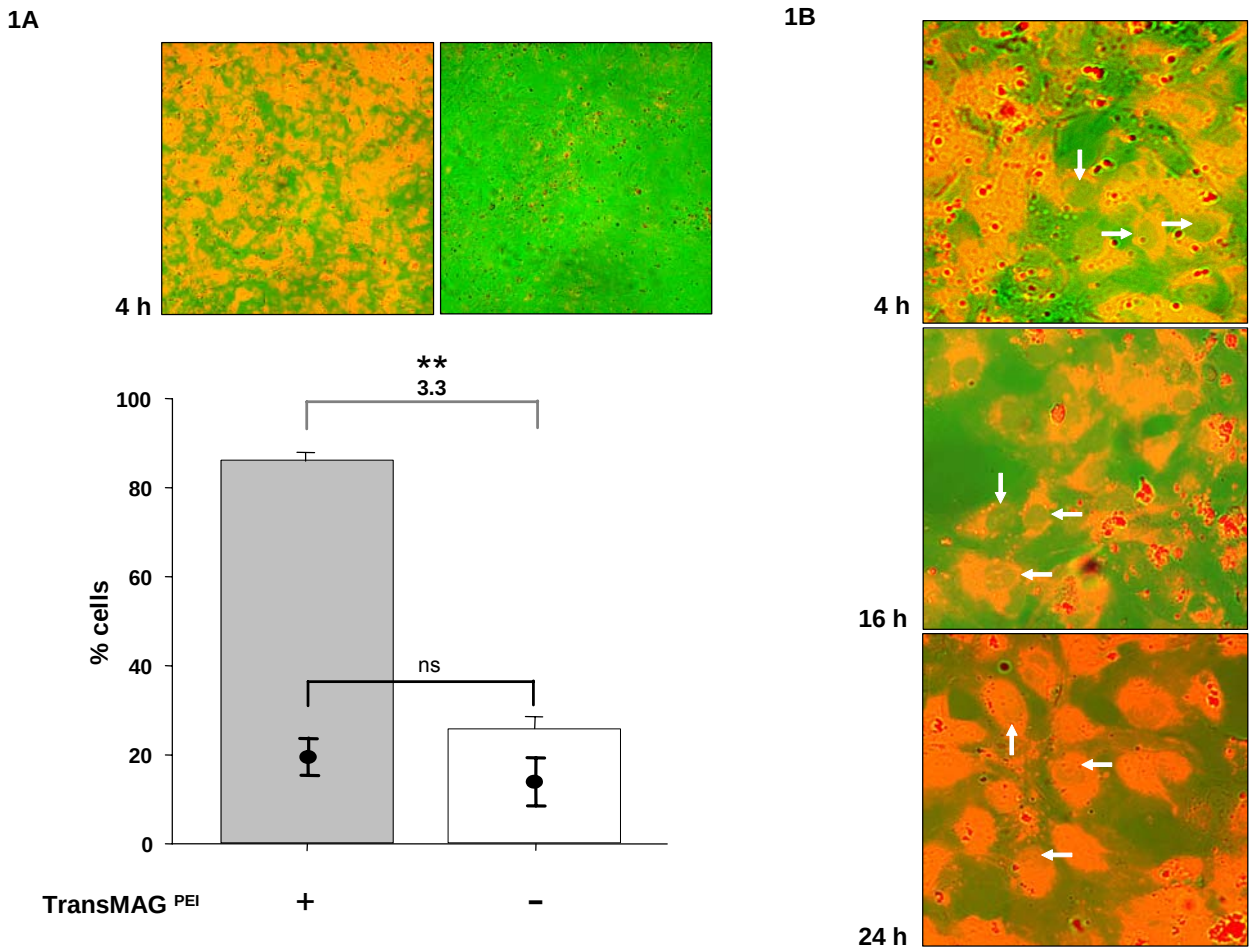


Fig. 3.1 Transfection efficiency and cytotoxicity of antisense magnetofection

Cells were incubated with the Effectene mixtures with (grey bar) or without (white bar) TransMAG^{PEI} particles for 15 minutes. The cells were exposed to the magnetic field and were then washed three times with PBS^{+}, followed by further cultivation in HMEC medium. For FACS analysis of cellular uptake, the cells were detached from the cell culture dish 4 hours post transfection. For cytotoxicity measurement, the cells were detached from the culture dish using sodium citrate 24 hours following transfection and stained with propidium iodide. For FACS analysis cells were fixated in 2% formalin. ***(A)*** *Antisense-ODN uptake was over 80% in cells transfected with TransMAG^{PEI} in comparison to transfection without TransMAG^{PEI} (**p<0.01, n=6-9). Cytotoxicity in both groups was below 20% (dot plots n=6-9, ns: not significant). Photos above bars show AS-ODN (Rdm ODN) accumulation after transfection with (left photo) and without (right photo)TransMAG^{PEI} 4 hours post transfection.* ***(B)*** *Representative photos of cells transfected with Cy-3 labelled AS-ODN in combination with magnetofection (arrows point at nuclei). From top to bottom: 4 hours following transfection, most of the Rdm ODN seem to still be positioned at the cell surface; 16 hours after transfection, the highest fluorescence was detected in the cytosol although some nuclear accumulation could be observed; 24 hours following transfection, a higher nuclear uptake was observed but the highest AS-ODN accumulation seemed to still be in the cytosol.*

3.1.2. Kinetics of SHP-2 knock-down

SHP-2 AS-ODN treatment of endothelial cells (HUVEC and HMEC) resulted in a marked decrease of SHP-2 protein content (normalised to actin which was used as loading control) as soon as 6 hours following magnetofection as compared to Rdm ODN treatment (HUVEC 37 ± 29% inhibition and HMEC 69 ± 15% inhibition, $p<0.05$, both n=3) as shown by detection of SHP-2 protein levels with western blot. This effect was even more explicit after 24 hours (HUVEC 68 ± 18% inhibition, $p<0.01$, n=6, and HMEC 69 ± 10% inhibition, $p<0.01$, n=7) showing that the SHP-2 AS-ODN successfully inhibits SHP-2 expression in both cell types (Fig. 3.2A and 3.2B). However, 48 hours post transfection (n=3), the effect of the AS-ODN could no longer be observed. Therefore, following experiments were performed within or shortly after 6-24 hours following transfection.

To verify the specificity of the SHP-2 AS-ODN, HUVEC and HMEC were transfected with the AS-ODN and the protein level of the Src homology 2 containing tyrosine phosphatase 1 (SHP-1), the closest SHP-2 homologue, was detected by western blot 24 hours post transfection. No reduction in SHP-1 protein expression could be observed after SHP-2 AS-ODN treatment in neither of the cell types, showing that the mRNA targeting by the SHP-2 AS-ODN, in addition to be efficient in degrading SHP-2 mRNA, also is specific (Fig. 3.2C, both n=2).

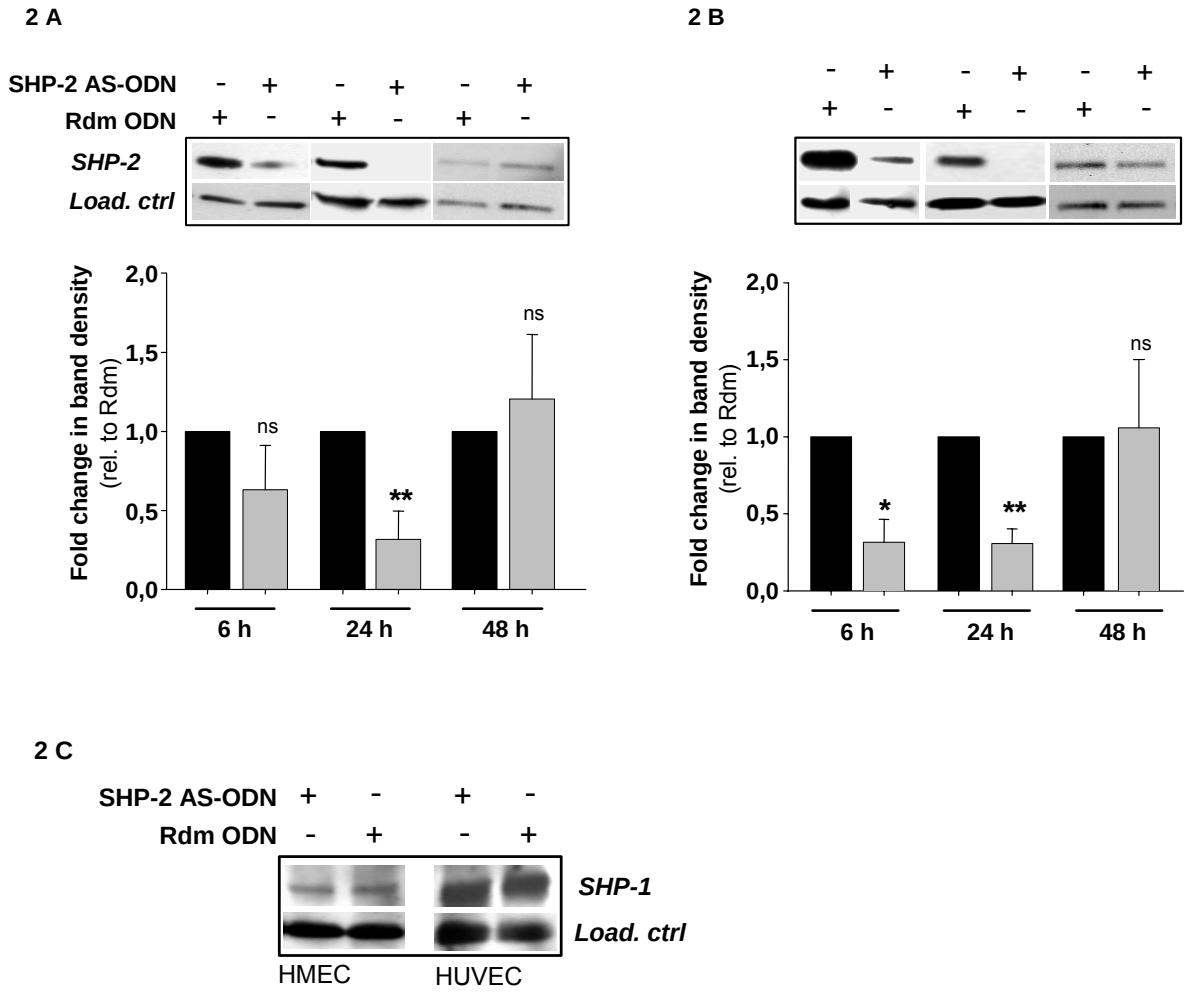


Fig. 3.2 Knock down of SHP-2 protein expression by AS-ODN

Cells were transfected with SHP-2 AS-ODN and Rdm ODN in combination with magnetofection as described in Materials and Methods section 2.3.1 and left in growth medium for 6, 24 and 48 hours respectively. After lysing the cells, SHP-2 protein content was analysed by western blot as described in Materials and Methods section 2.4.1. As a loading control, staining of actin detecting the carboxy terminus of all human isoforms was performed. SHP-2 was successfully knocked down by SHP-2 AS-ODN transfection combined with magnetofection in both HUVEC ***(A)*** *and HMEC* ***(B)*** *6 hours (HUVEC n=3; HMEC n=3, *p<0.05) and 24 hours (**p<0.01, HUVEC n=6, HMEC n=7) following transfection. After 48 hours (both n=3), the inhibition of protein expression could no longer be seen. Representative western blots for independent experiments are shown above the graphs.* ***(C)****The protein level of the SHP-2 homologue SHP-1 was not changed in either HMEC nor in HUVEC when transfecting cells with SHP-2 AS-ODN. The shown blots are representative for each (HMEC and HUVEC) two independent experiments (n=2).*

3.2. SHP-2 AFFECTS IMPORTANT STEPS OF ANGIOGENESIS INITIATION

3.2.1. SHP-2 inhibition suppresses basal endothelial proliferation/migration after wounding *in vitro*

As a first step to elucidate if suppression of SHP-2 has any effect on endothelial angiogenic processes, the ability to migrate over and proliferate into a scratch performed on a confluent cell layer was evaluated. As seen in figure 3.3A and 3.3B untreated cells covered 75 ± 3% (n=3) of the scratch after 48 hours while cells treated with the SHP-2 inhibitor Ptp I IV only covered 41 ± 11% (n=3, $p<0.05$) of the scratch. After 72 hours still, there was a tendency, although not significant, towards a delayed growth over the scratch (both n=3).

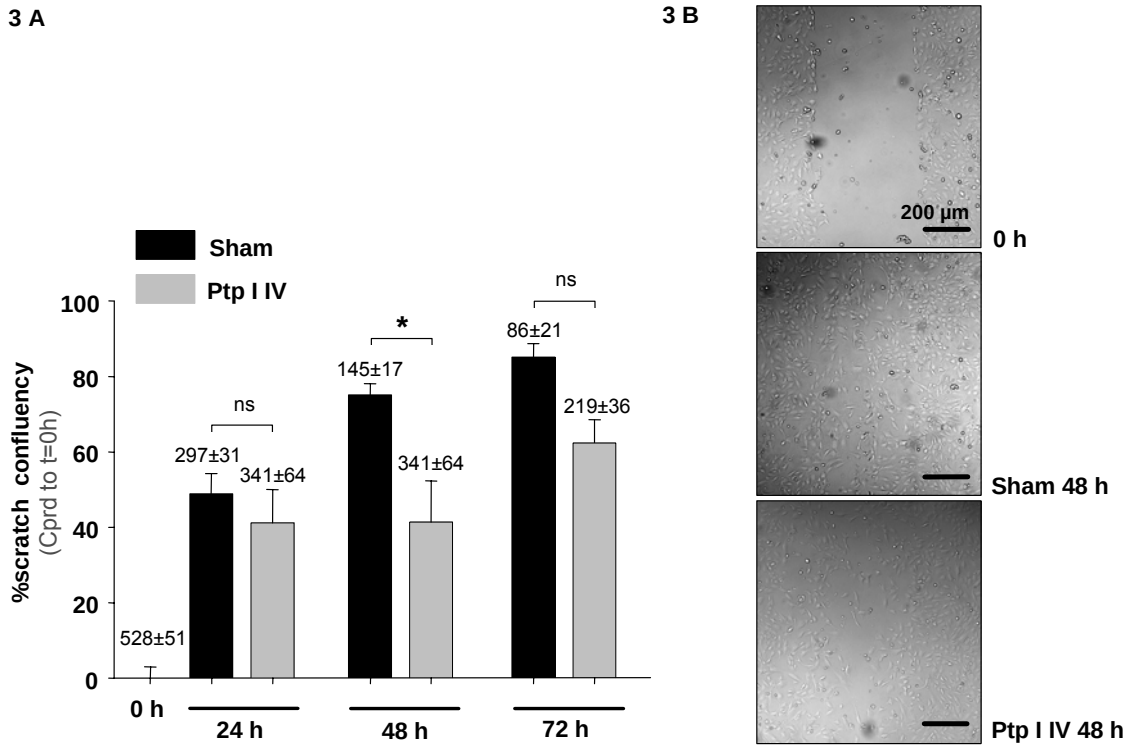


Fig. 3.3 SHP-2 inhibition suppresses basal endothelial proliferation/migration after wounding *in vitro*

Angiogenesis initiation was assessed by a wound healing assay as described in Materials and Methods section 2.5.3. For this HMECs were starved in HMEC starvation media (1%) and treated with the SHP-2 inhibitor Ptp I IV. As control a sham solution was used. ***(A)*** *Inhibition of SHP-2 with the pharmacological SHP-2 inhibitor Ptp I IV (2 µM) significantly delayed the ability of endothelial cells to grow over the scratch (at 48 hours, *p<0.05, n=3). 72 hours after wounding the delay was no longer significant (ns). Numbers above bars show original data of scratch width (µm) as mean ± SED meaning the width of the scratch, which was not covered with cells.* ***(B)*** *Representative photos of the scratches. From top to bottom: photo taken immediately after the scratch (0 h), photo of sham treated cells taken 48 hours later (48 h) and photo of Ptp I IV treated cells 48 hours after scratching (Ptp I IV 48 h).*

3.2.2. SHP-2 protein knock-down inhibits endothelial basal proliferation

Since the previous described scratch assay, during the used time period, does not solely measure the migration of cells, but also their proliferation, we investigated in a next step if SHP-2 AS-ODN had any effects on basal endothelial cell proliferation. For this, the reduction of MTT in HMEC was measured at 0, 24 and 48 hours. SHP-2 AS-ODN treatment, but not Rdm ODN, caused a decline of the proliferation rate by 48% (± 5%; $p<0.01$; n=12.Fig 3.4A) under unstimulated conditions. Moreover, this rate of proliferation was at all time points below the initial value at t=0 hours.

This was confirmed when detecting the DNA content of HUVEC by propidium iodide staining. The amount of DNA in a cell correlates to the different stages of the cell cycle, (for a detailed description, see Materials and Methods section 2.5.6). By measuring the amount of cells situated in the G2/M-phase, the two last phases where G2 is the last check-point for the cells before they enter the M-phase (M, Mitosis) where they divide, an estimation of the proliferative rate can be made. 24 hours post SHP-2 AS-ODN treatment a significantly lower amount of cells in the G2/M-phase of the cell cycle could be observed in comparison with Rdm ODN treatment (Fig. 3.4B, $p<0.01$, n=5). 48 hours after SHP-2 protein knock-down this reduction of cells about to or undergoing mitosis was still there although not significant (Fig. 3.4B, n=3).

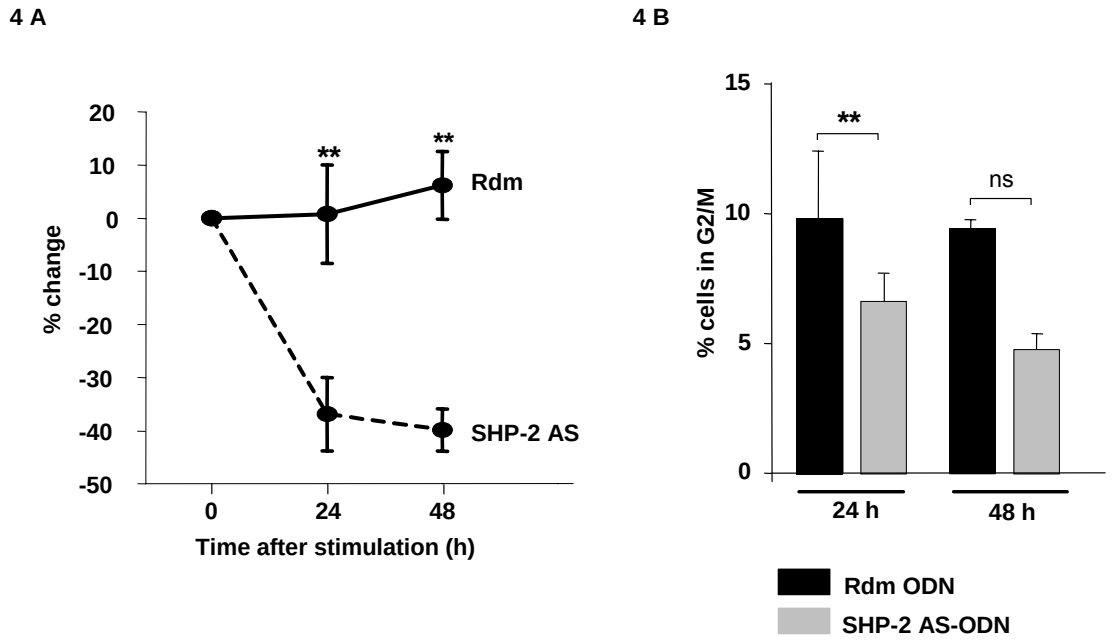


Fig. 3.4 SHP-2 protein knock-down inhibits basal endothelial cell proliferation

Endothelial cell proliferation was assessed by measuring the reduction of MTT as described in Materials and Methods section 2.5.4. For this HMECs were starved in HMEC starvation media (1%) and treated with SHP-2 AS-ODN. As control Rdm ODN was used. The amount of cells in the G2/M-phase of the cell cycle was detected by propidium iodide staining and subsequent FACS analysis as described in Materials and Methods section 2.5.6. For this, HUVEC were transfected with SHP-2 AS-ODN or Rdm ODN and left in HUVEC growth medium (20%) until measurement. ***(A)*** *Treatment with SHP-2 AS-ODN significantly reduced endothelial cell proliferation (**p<0.01; n=12).* ***(B)*** *24 hours following SHP-2 AS-ODN transfection, a lower amount of cells was observed in the G2/M-phase of the cell cycle (**p<0.01; n=5). 48 hours after transfection the reduction was no longer significant (ns, n=3).*

3.2.3. SHP-2 protein knock-down impairs endothelial cell viability and induces apoptosis

To investigate if the decreased proliferation of cells deprived of SHP-2 was due to apoptosis, the DNA content of HUVEC was measured by propidium iodide staining (for a detailed description, see Materials and Methods section 2.5.6). FACS analysis revealed the amount of DNA in the cells, which correlates to the different stages of the cell cycle. The DNA content in the SubG0 fraction, which is the fraction of cells with least amount of DNA due to DNA fragmentation, was measured as an index of apoptosis. Starting at 6 hours after transfection, SHP-2 AS-ODN treated cells gave rise to a non-significant peak in SubG0 compared to Rdm ODN transfected cells (Fig. 3.5A). At 24 hours following transfection this difference reached significance ($p<0.01$; n=6). Treatment with the SHP-2 inhibitor Ptp I IV likewise resulted in a significant higher amount of cells in the SubG0 fraction after 24 hours (Fig. 3.5B, $p<0.05$; n=10). In a further step to verify whether SHP-2 is involved in the control of endothelial apoptosis and to detect the proapoptotic cells, HUVEC were stained with propidium iodide (PI) and against the proapoptotic marker annexin V. The annexin V content was measured with FACScan and cells staining positive for annexin V but negative for PI were assumed proapoptotic. Following SHP-2 knock-down there was a significant increase in Annexin V positive / PI negative cells already 6 hours post transfection compared to Rdm ODN treated cells (by 23 ± 7%, $p<0.05$; n=8, Fig. 3.5C). 12 hours following SHP-2 AS-ODN treatment the amount of annexin V positive / PI negative cells was still higher in comparison with Rdm ODN treatment, although not significantly. Analyses of untreated cells at both time points did not show a higher Annexin V/PI binding than Rdm ODN treated cells (n=9).

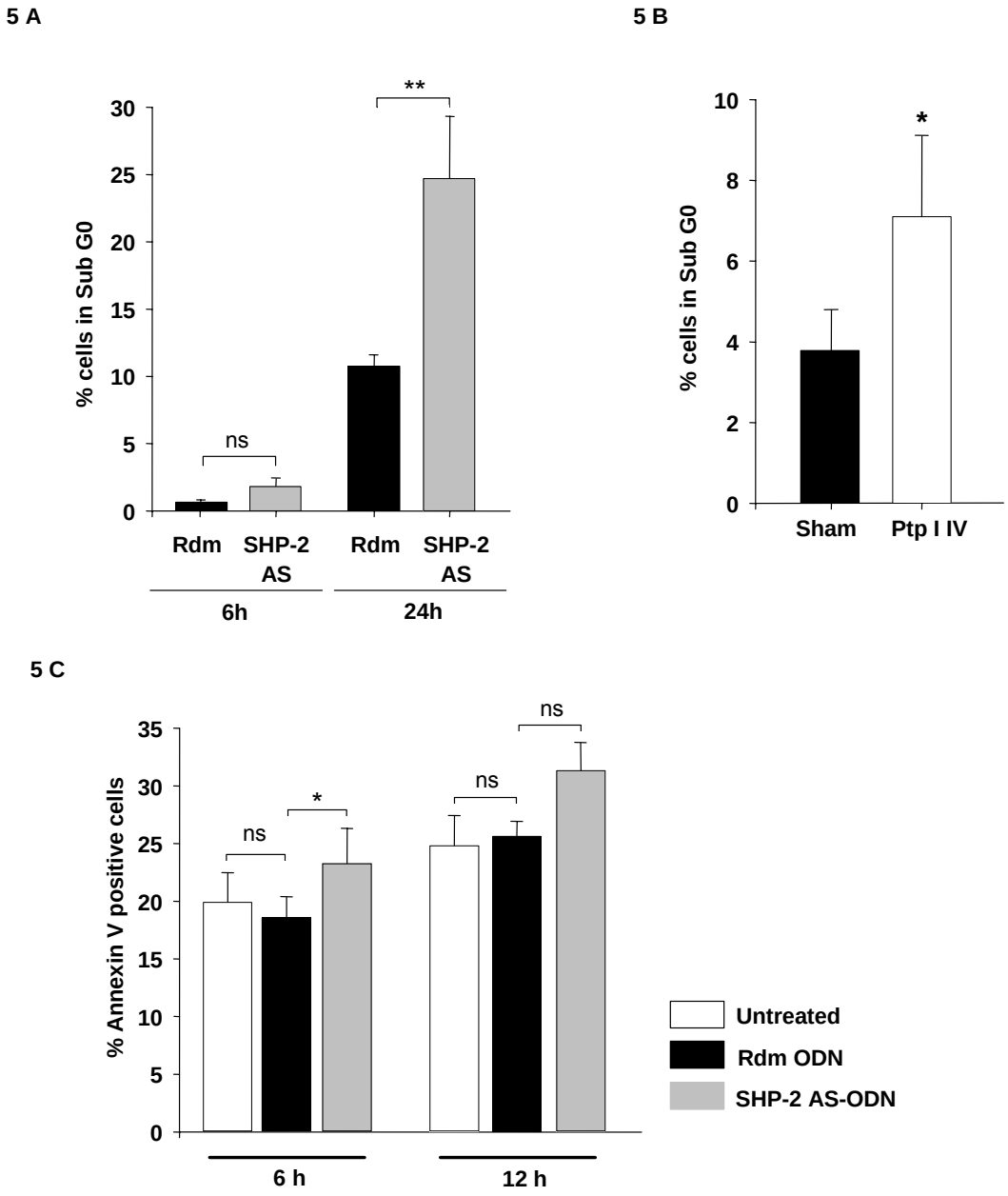


Fig. 3.5 SHP-2 inhibition increases the amount of cells in SubG0 and Annexin V antibody binding

Endothelial cell survival was analysed by propidium staining of HUVEC DNA content as described in Materials and Methods section 2.5.6. The different stages of the cell cycle were visualised by flow cytometry 6 and 24 hours post transfection with SHP-2 AS-ODN and Rdm ODN respectively. Cells detected in the SubG0 fraction were assumed apoptotic. Additional staining against the proapoptotic protein Annexin V as described in Materials and Methods section 2.5.7 and detection of cells positive for Annexin V but negative for propidium iodide stain were assumed proapoptotic. ***(A)*** *Following SHP-2 AS-ODN transfection a significant increase in the subG0 fraction (**p<0.01; n=6, HUVEC, 24h after transfection) was observed compared to Rdm ODN treated cells. (ns: not significant).* ***(B)*** *Treatment with Ptp I IV gave rise to a higher peak in SubG0 in comparison to sham treated cells (at 24 hours, *p<0.05, n=10).* ***(C)*** *Annexin V antibody binding was increased in SHP-2 AS-ODN transfected cells in comparison to Rdm ODN transfected cells (*p<0.05; n=9). Non transfected cells (untreated) showed a similar annexin V/PI binding to Rdm ODN treatment (ns: not significant, n=9).*

3.2.4. Growth factors enhance SHP-2 protein activity and induce phosphorylation of SHP-2

Having observed that SHP-2 inhibition influences endothelial cell proliferation and migration under basal conditions and also affects the cell viability of endothelial cells although cultured in a growth promoting medium (HUVEC growth media 20%), we next questioned if SHP-2 influences cell signalling initiated by distinctive angiogenic growth factors. To investigate this, we choose to study SHP-2 under the influence of VEGF-A, bFGF and PDGF-BB, which are all growth factors critical for events contained in angiogenesis, such as endothelial cell survival, proliferation, migration and ultimately vessel sprouting [13,25,33,34]. Firstly, we assessed the ability of these factors to enhance SHP-2 activity. HUVEC stimulated with VEGF-A, bFGF and PDGF-BB exhibited a higher SHP-2 activity than untreated cells. Stimulation for 10 minutes with bFGF and VEGF-A significantly enhanced the phosphatase activity of SHP-2 ($p<0.05$, n=23 and n=21 respectively. Fig 3.6), whereas PDGF-BB stimulation was increased already after 2 minutes of stimulation ($p<0.05$, n=4). For this reason, a stimulation time of 10 minutes for bFGF and VEGF was used in the following experiments, if not stated otherwise. Although PDGF-BB induced its highest SHP-2 activity after 2 minutes, a stimulation time of 10 minutes was used, as SHP-2 associates with the PDGF receptor but effects further down in the signalling pathway were investigated.

Tyrosine phosphorylation of SHP-2 has been described to be associated with an enhancement of SHP-2 activity upon some, but not all, growth factors [74]. To investigate if SHP-2 is tyrosine phosphorylated by stimulation of VEGF-A, bFGF and PDGF-BB, cell lysates were objected to western blotting and the phosphorylated tyrosine residue 542 (Y 542) was detected. While PDGF-BB and VEGF-A application gave rise to a slightly stronger phosphorylation of Y 542 in comparison to non stimulated cells (both n=5), bFGF stimulation did not (n=5). In fact, bFGF stimulation resulted in an even lesser phosphorylation of Y 542 than observed in untreated cells (Fig. 3.6).

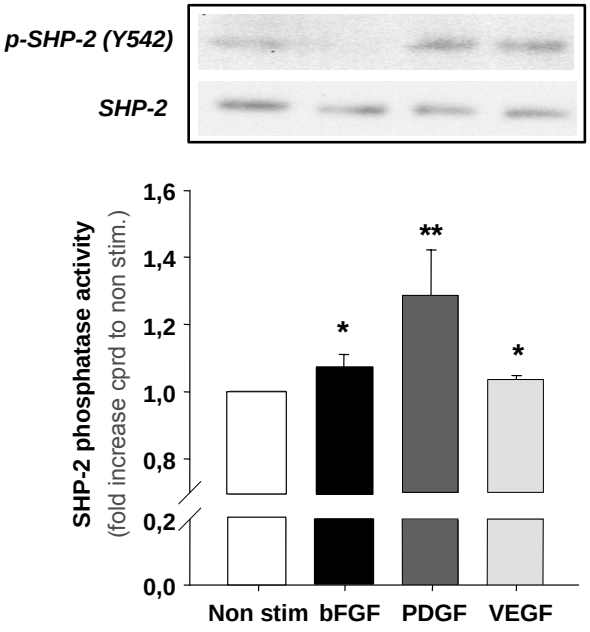


Fig. 3.6 SHP-2 phosphatase activity upon growth factor stimulation

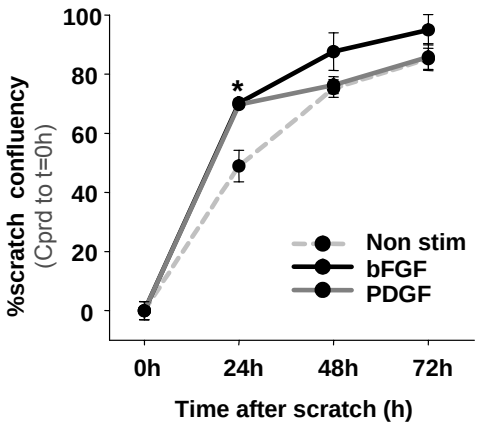
*HUVEC were stimulated with bFGF, PDGF-BB and VEGF-A (each 10 ng/ml), lysed and SHP-2 protein precipitated as described in Materials and Methods section 2.5.2. The activity was measured by addition of pNPP. The phosphorylation of Y 542 on SHP-2 was detected by immunoblot with an α-phospho-Y 542 antibody. Stimulation with bFGF, PDGF-BB and VEGF-A (all 10 ng/ml) enhanced the phosphatase activity of SHP-2 (*p<0.05, **p<0.01; n=21, n=4, n=21 respectively) in comparison to untreated cells (n=27). SHP-2 was tyrosine phosphorylated on position Y 542 upon addition of VEGF-A and PDGF-BB, but not after bFGF stimulation. The western blot over the graph is representative for five independent experiments (n=5).*

3.2.5. Inhibition of SHP-2 impairs growth factor enhanced endothelial proliferation/migration after wounding *in vitro*

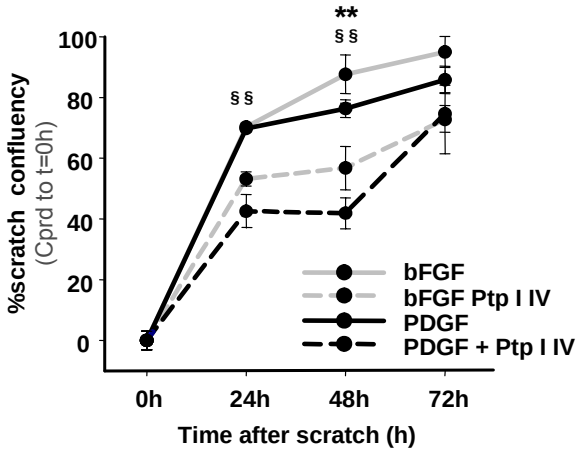
To elucidate if suppression of SHP-2 has any effect on growth factor dependent processes, which are prerequisites for angiogenesis such as endothelial proliferation and migration, a wound healing assay, where the ability of the cells to grow over a scratch in the presence of bFGF or PDGF-BB is evaluated, was used.

In cell culture dishes to which bFGF and PDGF-BB had been added, the wound healing response was accelerated in comparison with non stimulated cells (both bFGF and PDGF-BB $p<0.05$; n=3). After 24 hours, the cell layer in dishes to where bFGF had been applied reached 88 ± 6% confluency and where PDGF-BB was present the cell layer covered 76 ± 3% (both $p<0.05$; n=3, Fig. 3.7A and 3.7C) of the scratch, whereas sham treatment resulted in 49 ± 5% scratch confluency (n=3). At 72 hours the cell layer over the scratch was virtually completely re-established (94 ± 5% and 86 ± 6% respectively; $p<0.05$; n=3). However, treatment with the SHP-2 inhibitor Ptp I IV in the presence of bFGF and PDGF-BB delayed the wound healing response of the endothelial cells. Addition of the growth factors bFGF and PDGF-BB did not change the rate of growth in cells suppressed of SHP-2 as the scratch was still visible and only 52 ± 7% and 42 ± 5% respectively (both $p<0.01$; n=3, Fig. 3.7B and 3.7C) of the scratch had been covered with migrating or proliferating cells after 48 hours. After 72 hours there was still a tendency towards a difference in scratch confluency between cells treated with Ptp I IV in the presence of growth factors and cells stimulated with a growth factor only, although not significant.

7 A



7 B



7 C

	0 h *Scratch width (µm)*	24 h *Scratch width (µm)*	48 h *Scratch width (µm)*	72 h *Scratch width (µm)*
Non stimulated	528±51	297±31	145±17	86±21
bFGF	546±36	173±7	72±37	29±29
bFGF + Ptp I IV	599±21	272±14	252±42	159±66
PDGF-BB	536±5	176±6	137±17	83±26
PDGF-BB + Ptp I IV	661 ±61	334±32	338±30	148±16

Fig. 3.7 SHP-2 affects growth factor induced endothelial proliferation/migration after wounding *in vitro*

Growth factor dependent endothelial cell migration and proliferation was assessed by studying the ability of HMECs to cover a scratch performed on a confluent cell layer under the influence of bFGF and PDGF-BB (each 10 ng/ml) as described in Materials and Methods section 2.5.3. ***(A)*** *Stimulation with bFGF and PDGF-BB accelerated the wound healing of endothelial cells compared to non stimulated cells (both *p>0.05, n=3, 24 hours after scratching). At 48 and 72 hours, the difference in scratch confluency was no longer significant (ns, all n=3).* ***(B)*** *Treatment with Ptp I IV delayed the wound healing response of endothelial cells up to 48 hours following scratching (§§p<0.01 bFGF vs bFGF + Ptp I IV, n=3; **p<0.01 PDGF-BB vs PDGF-BB + Ptp I IV, n=3). After 72h there was no significant difference in confluency between the groups (ns, n=3).* ***C)*** *Table showing original data as mean ± SED of scratch width (µm), meaning the width of the scratched area which was not covered by cells (for evaluation see Materials and Methods section 2.5.3).*

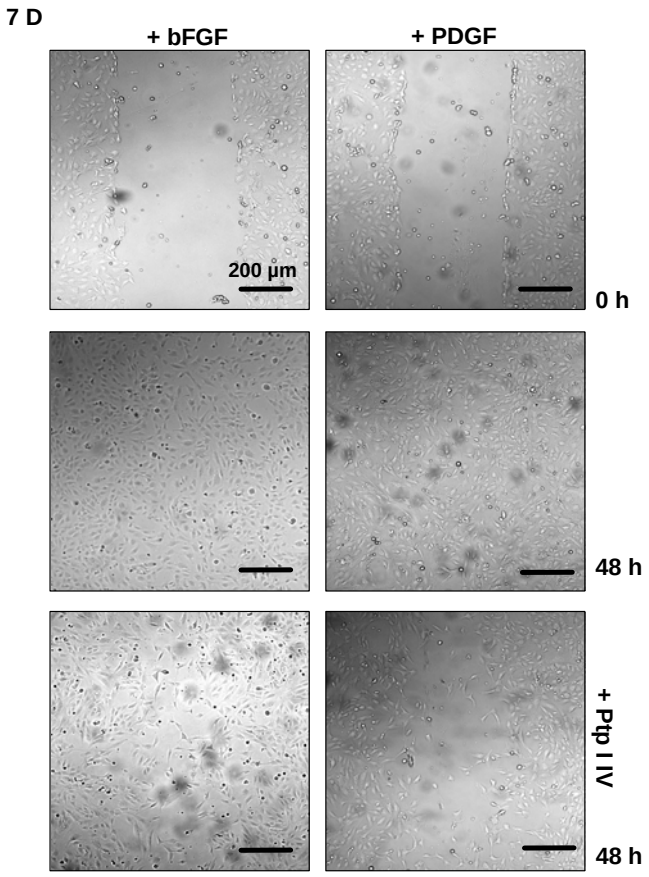


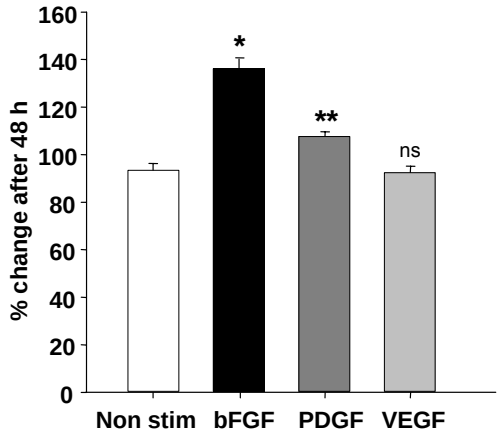
Fig. 3.7 SHP-2 affects growth factor induced endothelial proliferation/migration after wounding *in vitro*

Growth factor dependent endothelial wound healing was assessed by studying the ability of HMECs to migrate and proliferate over a scratch performed on a confluent cell layer under the influence of bFGF and PDGF-BB (each 10 ng/ml) as described in Materials and Methods section 2.5.3. ***(D)*** *Representative photos of scratches. Bars in photos represent 200 µm. From top to bottom: photos taken immediately after scratching (0h), photos of cells stimulated with either bFGF (left photo) or PDGF-BB (right photo) taken 48 hours later (48 h), photos of Ptp I IV and bFGF (left photo) or PDGF-BB (right photo) treated cells 48 hours after scratching (Ptp I IV 48 h).*

3.2.6. Loss of SHP-2 prevents bFGF and PDGF induced endothelial cell proliferation

We investigated in a next step if SHP-2 AS-ODN had any effects on growth factor dependent endothelial cell proliferation by measuring the reduction of MTT in HMEC at 0, 24 and 48 hours. Addition of bFGF and PDGF-BB enhanced the proliferation of endothelial cells by 37 ± 4% ($p<0.05$; n=12, Fig. 3.8A) and 31% ± 2% ($p>0.01$; n=12) respectively compared to sham treated cells. In contrast, stimulation with VEGF-A was not able to induce endothelial cell proliferation at any time point measured in comparison to sham treated cells (Fig. 3.8A, n=12). Treating cells with SHP-2 AS-ODN significantly reduced cell proliferation despite the influence of bFGF (by 41 ± 5% after 24 hours, $p<0.01$; n=11) and PDGF-BB (by 55 ± 7% after 24 hours, $p<0.01$; n=12) in comparison to Rdm ODN (Fig. 4.8B). This rate of proliferation was at all time points below the initial value at t=0 hour.

8 A



8 B

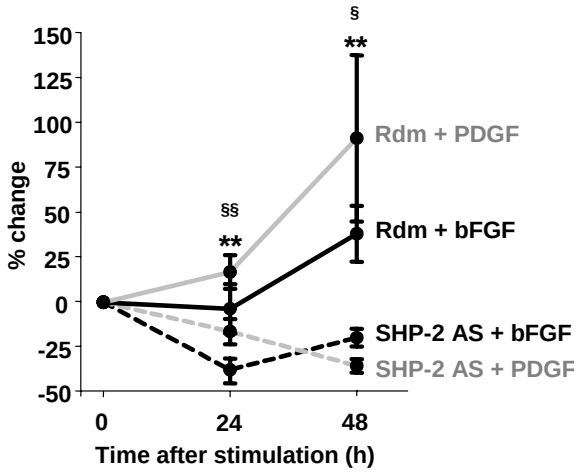


Fig. 3.8 SHP-2 suppresses growth factor induced endothelial cell proliferation

Growth factor dependent endothelial cell proliferation was assessed by measuring the reduction of MTT in HMECs upon SHP-2 AS-ODN and Rdm ODN treatment and addition of bFGF, PDGF-BB and VEGF-A (each 10 ng/ml) as described in Materials and Methods section 2.5.4. ***(A)*** *Stimulation with bFGF and PDGF-BB significantly enhanced the proliferation of endothelial cells after 48 hours (**p<0.01, *p<0.05, n=12), whereas stimulation of VEGF-A was unable to increase the proliferative rate of endothelial cells in comparison with non stimulated cells (ns: not significant versus non stimulated cells, n=12).* ***(B)*** *Treatment with SHP-2 AS-ODN reduced bFGF and PDGF dependent cell proliferation as soon as 24 hours post transfection in comparison to Rdm ODN treated cells (bFGF + SHP-2 AS-ODN: **p<0.01, n=11; PDGF + SHP-2 AS-ODN: §§p<0.01, n=12).*

3.3. SHP-2 IS NECESSARY FOR COMPLETE VESSEL FORMATION

3.3.1. Loss of SHP-2 impairs the ability of endothelial cells to form capillary like structures

To investigate whether the decrease in proliferation and migration and the subsequent increase in apoptosis following SHP-2 deprivation would have an effect on endothelium dependent capillary formation and thus the initiation of new vessel formation, we performed Matrigel assays to assess growth factor dependent formation of capillary like structures from endothelial cells. To do so, cells were either treated with a pharmacological inhibitor (Ptp I IV) of SHP-2 or SHP-2 AS-ODN in separate experiments followed by plating equal amounts of cells onto 3-dimensional Matrigel, which is a proangiogenic matrix. Whereas sham or Rdm ODN treated cells formed organised networks of capillary like structures (Fig. 3.9A and 3.9B, upper photos respectively), cells treated with SHP-2 inhibitor or SHP-2 AS-ODN failed to do so (Fig. 3.9A and 3.9B, lower photos respectively). Instead they detached themselves from the matrix (seen as dark dots) and the capillary structures were incomplete. Inhibition of SHP-2 with AS-ODN showed a 45 ± 13% (3 ± 0.8 capillary structures/mm^2, $p<0.01$; n=8) reduction in the number of capillary like structures compared to Rdm ODN treated cells (7 ± 0.7 capillary structures/mm^2). Treatment with the pharmacological SHP-2 inhibitor also reduced the number of capillary like structures significantly with 55 ± 16% (4 ± 1.6 capillary structures/mm^2, $p<0.05$; n=3) compared to sham treated cells (9 ± 0.5 capillary structures/mm^2, Fig. 3.9C).

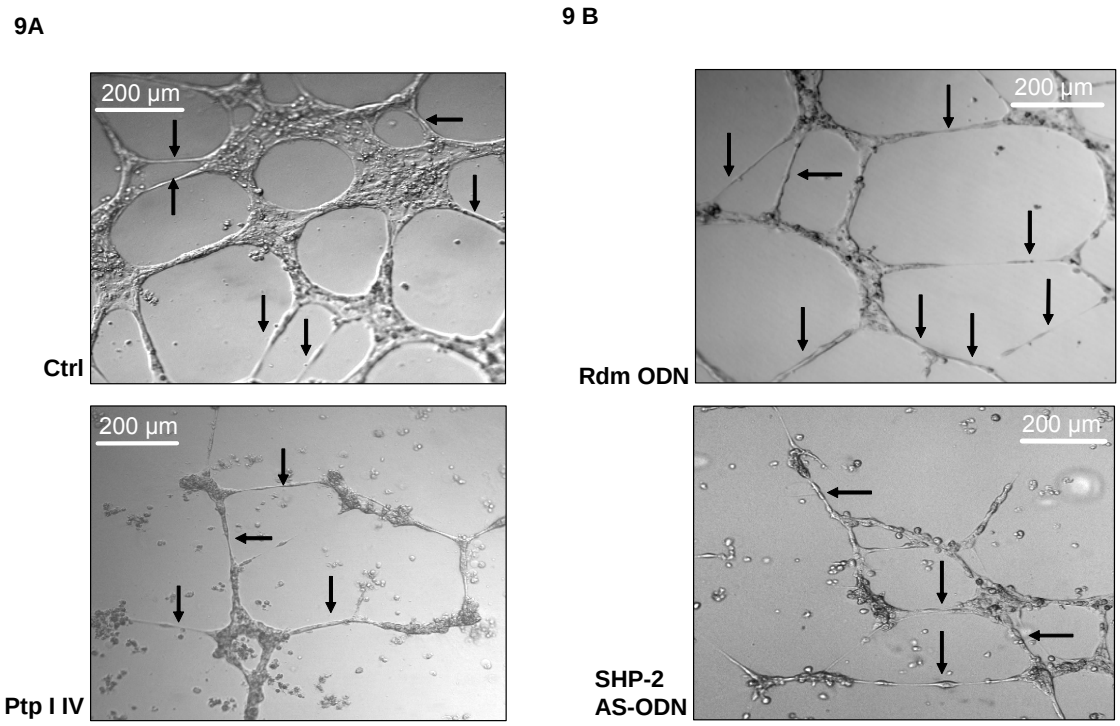


Fig. 3.9 Inhibition of SHP-2 impairs the ability of endothelial cells to form capillary like structures

HUVEC treated with Ptp I IV (2 µM) or SHP-2 AS-ODN were seeded onto a Matrigel matrix and left to form capillary like structures over night as described in Materials and Methods section 2.5.8. The structures were quantified at 16 hours following seeding of the cells. ***(A)*** *Representative photos of capillary like structures (indicated by arrows, black bars in both photos represent 200 µm) taken with a laser scan microscope. From top to bottom: Photo of sham treated cells, photo of cells treated with the SHP-2 inhibitor Ptp I IV.* ***(B)*** *Representative photos of capillary like structures formed by cells transfected with Rdm ODN (upper photo) and cells transfected with SHP-2 AS-ODN (lower photo).*

9 C

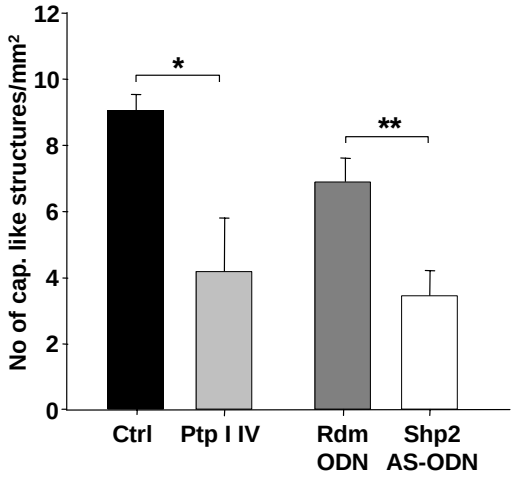


Fig. 3.9 Inhibition of SHP-2 impairs the ability of endothelial cells to form capillary like structures

(C) *The ability to form capillary like sproutings in a human matrix and growth factor rich media was significantly diminished when HUVECs were treated in separate experiments with a specific inhibitor of SHP-2 (Ptp I IV, 2 µM) (*p<0.05; n=3) or SHP-2 AS-ODN (**p<0.01, n=8) compared to sham treated cells or Rdm ODN treatment respectively.*

3.3.2. Inhibition of SHP-2 negatively affects vessel sprouting ex vivo

To further investigate if SHP-2 inhibition directly influences angiogenesis, new vessel sprouting from mouse aortas was used as a model. After dissection, cleaning and cutting of the aorta the aortic rings were embedded in Matrigel and covered with growth factor rich media. Upon sham treatment or treatment with the SHP-2 inhibitor (Ptp I IV), the aortic rings were left to form sproutings for approximately 72-96 hours.

As seen in figure 3.10A and 3.10B SHP-2 inhibition impaired vessel sprouting. The number of bifurcations of the newly formed vessels were remarkably reduced upon SHP-2 inhibition (27 ± 7 bifurcations/aortic ring, $p<0.05$; n=10, Fig 3.10A) in comparison with sham treated aortic rings (59 ± 11 bifurcations/aortic ring, n=8). Furthermore, vessels growing from aortas treated with the SHP-2 inhibitor seemed to be much shorter than sproutings from sham treated aortic rings (Fig. 3.10B).

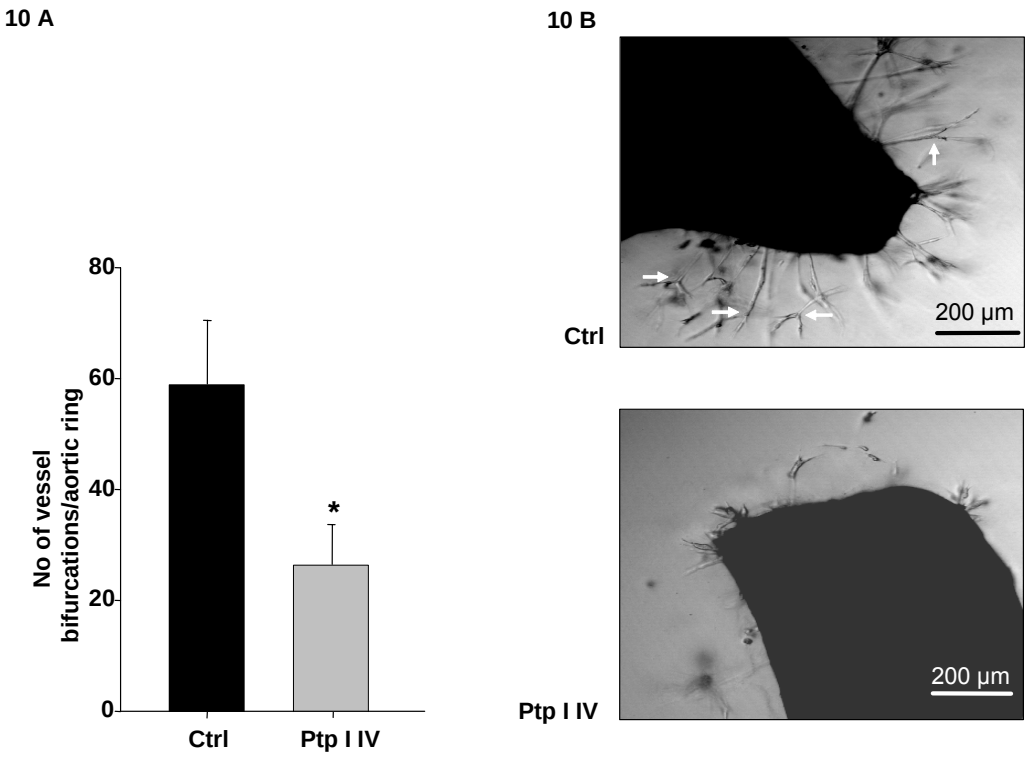


Fig. 3.10 SHP-2 inhibition negatively influences vessel outgrowth *ex vivo*

Dissected mouse aortas were embedded in a Matrigel matrix and treated with Ptp I IV (2 µM) in a growth promoting medium as described in Materials and Methods section 2.5.9. The number of bifurcations of the newly formed vessels sprouting from the aorta was evaluated and used as an indicator of angiogenesis. ***(A)*** *The number of bifurcations on the endothelial sproutings from mouse aortas were significantly lower upon SHP-2 inhibition (*p<0.05, n=10) in comparison to sham treated rings.* ***(B)*** *Representative photos of aortic ring sproutings taken 96 hours post embedment in Matrigel. Bars in both photos represent 200 µm and white arrows point at sprouting bifurcations. The upper photo shows a sham treated (Ctrl) aortic aortic ring and the lower photo shows an aortic ring which has been treated with the SHP-2 inhibitor Ptp I IV.*

3.4. SHP-2 AND ENDOTHELIAL SIGNALLING

3.4.1. SHP-2 inhibition abrogates bFGF, PDGF-BB and VEGF-A induced activation of the PI3-Kinase in endothelial cells

To investigate the signalling steps regulated by SHP-2 during growth factor dependent endothelial cell activation, we assessed whether the PI3-Kinase / Akt pathway, which is a prominent regulator of the cell cycle and influences proliferation in endothelial cells [25,50], is influenced by SHP-2 knock-down. To do so, we first questioned, whether the p85 regulatory subunit of PI3-Kinase is a potential target of SHP-2. Tyrosine phosphorylation of the p85 subunit activates the enzyme, thus PI3-Kinase activity was assessed by immunoprecipitation of p85 followed by immunoblotting against phospho-tyrosine with an α-phospho-tyrosine antibody. The tyrosine phosphorylation of the p85 subunit was induced upon stimulation with bFGF, ($n=4$, $p<0.05$), PDGF-BB ($n=6$, $p<0.01$) and showed a tendency to increase after VEGF-A ($n=6$) stimulation (Fig. 3.11A). However, when inhibiting SHP-2 with AS-ODN this growth factor dependent phosphorylation had the tendency to decrease in comparison to Rdm ODN ($n=3$, $n=3$, $n=2$ respectively, Fig 3.11B).

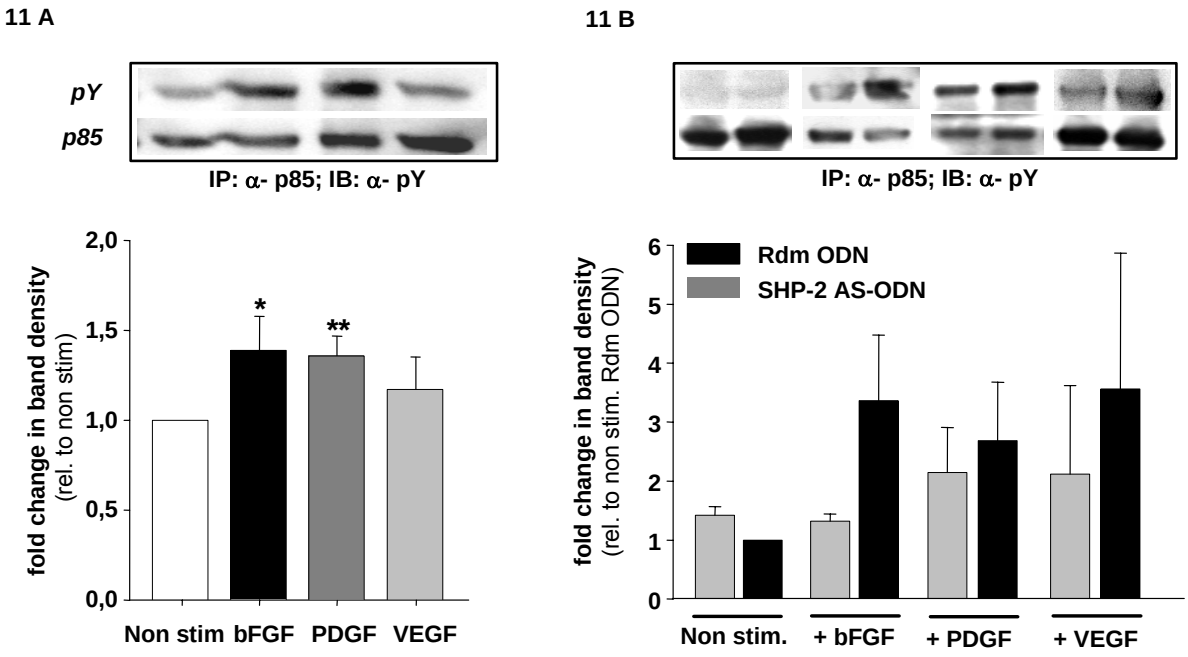


Fig. 3.11 Inhibition of SHP-2 affects the tyrosine phosphorylation of p85

HMEC were lysed as described in Materials and Methods section 3.3.3 and the activity of the PI3-Kinase was assessed by detecting the level of tyrosine phosphorylation of the regulatory subunit p85 through immunoprecipitation with an α-p85 antibody followed by immunoblotting against phospho-tyrosine (Materials and Methods section 2.4.2 and 2.5.2). ***(A)*** *Stimulation with bFGF, PDGF-BB and VEGF-A all resulted in an increased tyrosine phosphorylation of p85 (bFGF *p<0.05, n=4; PDGF-BB **p<0.01, n=6 and VEGF-A n=6) compared to non stimulated cells. Representative western blots are shown above the graphs.* ***(B)*** *Inhibition of SHP-2 protein expression by SHP-2 AS-ODN reduced the growth factor dependent p85 phosphorylation and thus PI3-Kinase activity in comparison with Rdm ODN treatment (AS-ODN/bFGF n=3, AS-ODN/PDGF-BB n=3 and AS-ODN/VEGF-A n=2). Representative western blots are shown above the graphs. The significance between treatment groups in this experiment was not tested due to low number of n´s of western blots (n=2-3), for a more detailed explanation see discussion section 4.4.*

3.4.2. SHP-2 interacts with the p85 subunit of PI3-Kinase and Gab-1 upon growth factor stimulation

Having observed that SHP-2 inhibition suppresses bFGF-, PDGF-BB- and VEGF-A dependent activity of PI3-Kinase through diminished tyrosine phosphorylation of p85, it was of interest to elucidate if SHP-2 may directly interact with the p85 subunit by its phospho-tyrosine residue. To investigate this, SHP-2 protein was immunoprecipitated followed by immunoblotting against p85. This revealed that, upon bFGF, PDGF-BB and VEGF-A stimulation, SHP-2 indeed associated with the p85 subunit of the PI3-Kinase (Fig 3.12, upper blot, n=3).

The Grb-2 associated binder 2 (Gab-1) has been shown to bind growth factor receptors and associate with SHP-2 upon PDGF and EGF stimulation [91,92,99,133]. To investigate whether SHP-2 binds Gab-1 upon growth factor stimulation in our experiments, and thus might form a signalling complex with SHP-2 and p85, SHP-2 was immunoprecipitated and immunoblotting against Gab-1 was performed. After stimulation with bFGF, PDGF-BB and VEGF-A, SHP-2 could be observed as a binding partner of Gab-1 (Fig 3.12, lower blot, n=3).

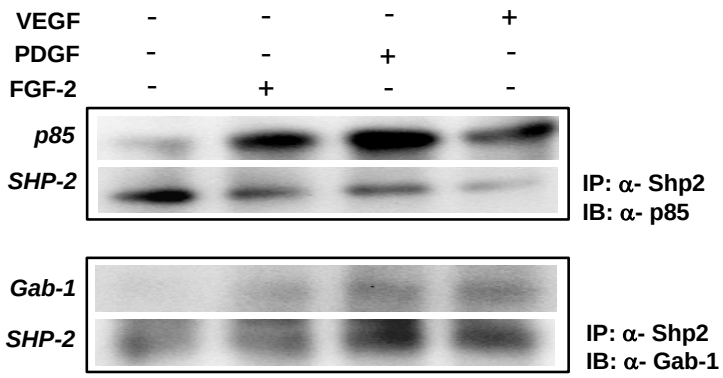


Fig. 3.12 Growth factor induced interaction between SHP-2, p85 and Gab-1

Protein interactions were investigated by precipitation of SHP-2 protein as described in Materials and Methods section 2.4.2, followed by immunoblotting against p85 and Gab-1 respectively. Addition of bFGF, PDGF-BB and VEGF-A resulted in the binding of more p85 and Gab-1 molecules to SHP-2 (western blots are representative for each three experiments, n=3) in comparison to non stimulated conditions.

3.4.3. SHP-2 activates Akt upon bFGF and PDGF-BB but not VEGF-A stimulation

To further investigate if the effects on PI3-Kinase activation seen after SHP-2 protein knock-down actually influences PI3-Kinase dependent signalling, the phosphorylation and thus activation of Akt, one of the downstream targets of PI3-Kinase, was detected using an α-phospho-Akt (pThr308) antibody after stimulation with bFGF, PDGF-BB and VEGF-A. bFGF, PDGF-BB as well as VEGF-A significantly induced the activity of Akt, as seen by a strong enhancement in the phosphorylation level (bFGF $p<0.05$, $n=11$; PDGF-BB $p<0.05$, $n=9$; VEGF-A $p<0.05$, $n=7$; Fig. 3.13A). In contrast, inhibition of SHP-2 seemed to diminish the bFGF ($n=6$, $p<0.05$) and PDGF-BB ($n=5$, $p<0.01$) dependent Akt phosphorylation and hence led to less activation (Fig. 3.13B). Stimulation of Rdm ODN transfected cells with VEGF-A did not seem to enhance the Akt phosphorylation and SHP-2 AS-ODN treatment followed by VEGF-A stimulation did not cause any change in the Akt phosphorylation level in comparison to Rdm ODN ($n=4$).

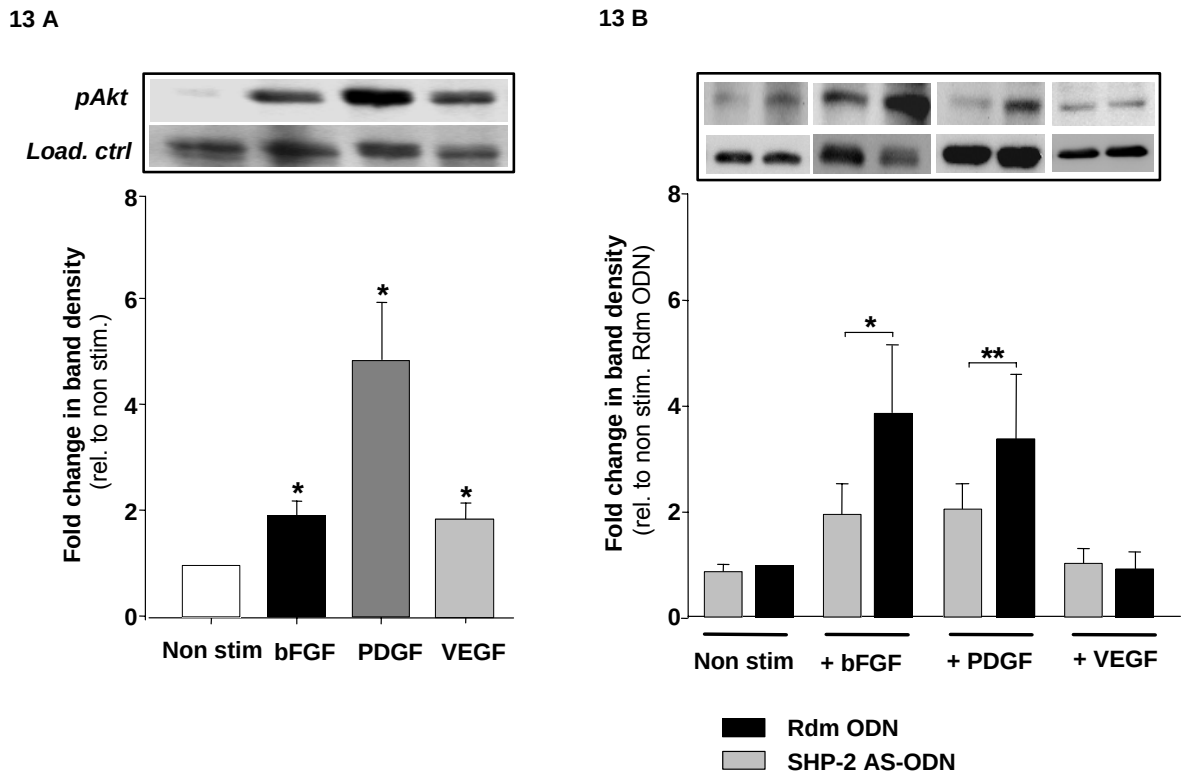


Fig. 3.13 SHP-2 dependent Akt activation

HMEC lysates were subjected to western blotting as described in Materials and Methods section 2.4.1 followed by immunoblotting against the phosphorylated form of Akt. ***(A)*** *bFGF (n=11), PDGF-BB (n=9) and VEGF-A (n=7) stimulation (all 10 ng/ml) signifcantly enhanced the Akt phosphorylation (all *p<0.05) compared to non stimulated conditions. A representative western blot is shown above the graph.* ***(B)*** *While transfection of HMEC with Rdm ODN followed by growth factor stimulation caused an increase in Akt phosphorylation, SHP-2 AS-ODN treatment diminished the bFGF (n=5, *p<0.05) and PDGF-BB (n=5, **p<0.01) dependent phosphorylation of Akt. No change in Akt phosphorylation upon VEGF-A treatment could be observed in Rdm ODN or SHP-2 AS-ODN transfected cells (n=4). Representative western blots are shown above the graphs. The significance between the different groups of this experiment was determined by calculating fold change relative to the respective Rdm ODN (not shown in the graph).*

3.4.4. Raf inhibition impairs endothelial cell proliferation and migration

In addition to the PI3-Kinase / Akt pathway, the Raf-MEK-ERK pathway has also been shown to be essential for endothelial cell proliferation, survival and angiogenesis in vivo [56,57,62,134]. To assess the importance of the growth factor dependent activation of the Raf-MEK-ERK pathway in endothelial cells in our assays, endothelial cell migration and proliferation were evaluated after treatment with a pharmaceutical Raf inhibitor (GW5074) under the influence of bFGF, PDGF-BB and VEGF-A. When inhibiting the Raf-MEK-ERK pathway by using the Raf inhibitor GW5074 and measuring the MTT reduction, the bFGF (Fig. 3.14A) and PDGF-BB (Fig. 3.14B) induced proliferation was markedly diminished at 48 hours (bFGF OD 0.22±0.03 versus bFGF+GW5074 OD 0.20±0.03, $p<0.05$, n=12 and PDGF OD 0.16±0.02 versus PDGF+GW5074 OD 0.13±0.03, $p<0.01$, n=12) and 72 hours (bFGF OD 0.25±0.04 versus bFGF+GW5074 OD 0.14±0.02, $p<0.01$, n=12 and PDGF OD 0.16±0.02 versus PDGF+GW5074 OD 0.11±0.02, $p<0.01$; n=12). Interestingly, in contrast to the inhibition of SHP-2, GW5074 treatment under stimulation with bFGF was not associated with a loss in cell number until after 72 hours, whereas GW5074 treatment under the stimulation of PDGF-BB was associated with a decline in cell number already at 48 hours.

When investigating the effects of Raf suppression on endothelial wound healing by studying the ability of endothelial cells to cover a scratch performed on a confluent cell layer, inhibition of Raf with GW5074 strongly impaired the ability to grow over the scratch in the presence of all three growth factors in comparison to sham treated cells. Cells treated with bFGF, PDGF-BB and VEGF-A re-established the confluency of the scratched area already after 72 hours (by 87 ± 7%, 88 ± 2% and 96 ± 2% respectively, all n=3 compared to t=0 hours), whereas the scratch amongst the cells treated with the Raf inhibitor was visible still after 72 hours (bFGF/ GW 5074 52 ± 6%, PDGF-BB/ GW 5074 29 ± 7 and VEGF-A/ GW 5074 38 ± 2% scratch confluency, all n=3 compared to t=0 hours) despite the presence of growth factors. This difference in scratch confluency between cells treated with growth factors and GW 5074 together with growth factors was significant after 48 hours (VEGF-A/GW 5074 and PDGF-BB/GW 5074, both $p<0.01$, all n=3, Fig. 3.14C and 3.14D) and after

72 hours (VEGF-A/GW 5074, PDGF-BB/GW 5074 and bFGF/GW 5074, all $p<0.01$, all $n=3$, Fig. 3.14C and 3.14D).

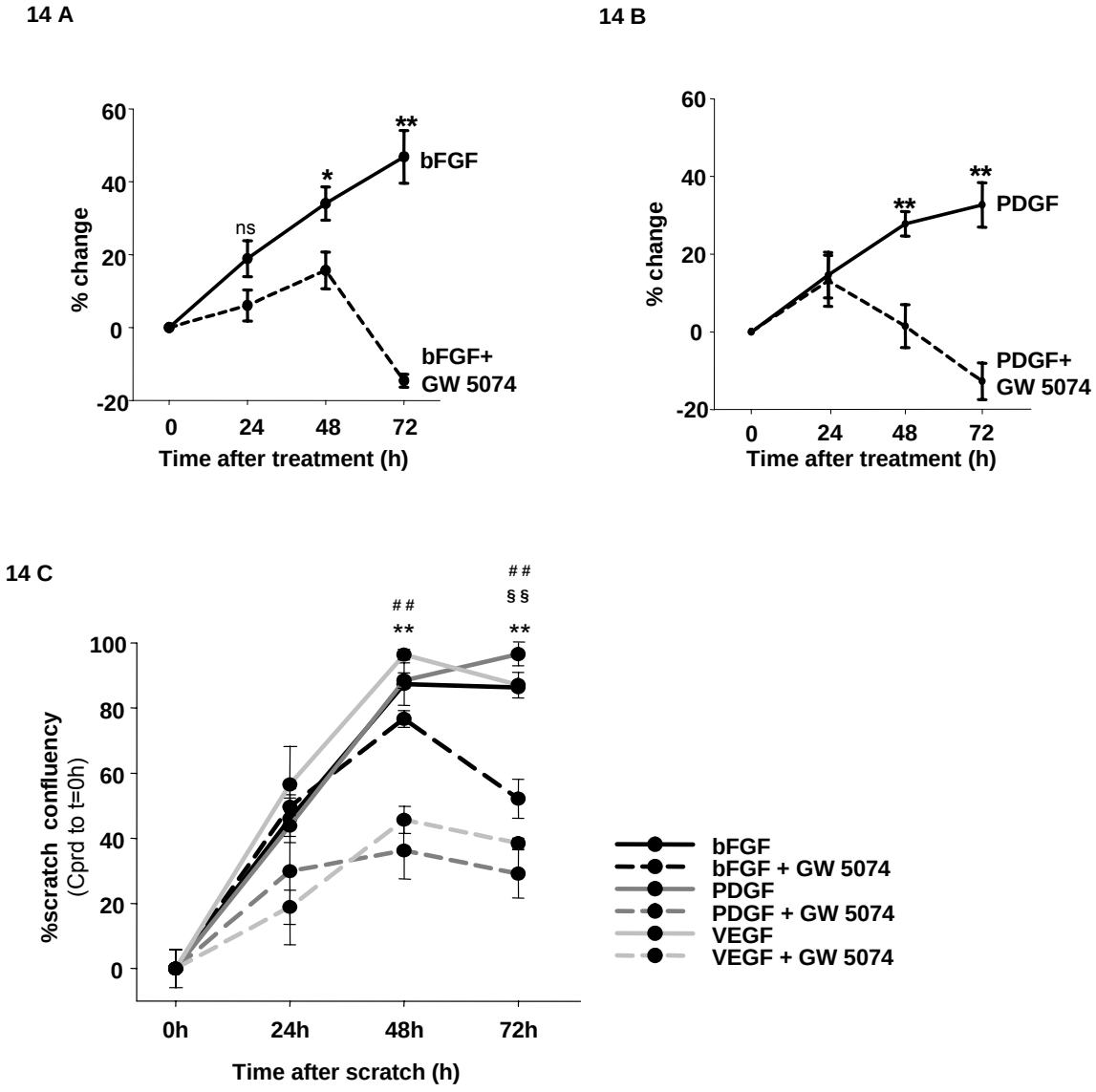


Fig. 3.14 Proliferation and wound healing *in vitro* of endothelial cells is dependent on Raf

HMEC or PAEC were treated with the Raf inhibitor GW 5074 (5 µM) together with either bFGF, PDGF-BB or VEGF-A. Proliferation and wound healing ability of endothelial cells was measured as described in Materials and Methods section 2.5.4 and 2.5.3 respectively. ***(A)*** *Treatment with GW 5074 in the present of bFGF lead to a decreased proliferation rate of endothelial cells (*p<0.05, n=12, HMEC at 48 hours after scratching and treatment) and resulted in a decrease in the cell number after 72 hours (**p<0.01, n=12) in comparison to cells treated with bFGF only.* ***(B)*** *Application of GW 5074 and PDGF-BB resulted in a decreased cell number already after 48 hours (*p<0.05, n=12) and continued to decline as seen at 72 hours (**p<0.01, n=12) compared to cells treated with only PDGF-BB.* ***(C)*** *Whereas cells stimulated with bFGF, PDGF-BB and VEGF-A (all 10 ng/ml) reached confluency again around 48 hours or at 72 hours, cells treated with GW 5074 were not able to cover the scratch at such content (VEGF-A + Ptp I IV $^{\#\#}$p<0.01, n=3; PDGF-BB + Ptp I IV **p<0.01, n=3; bFGF + Ptp I IV §§p<0.01, n=3, PAEC).*

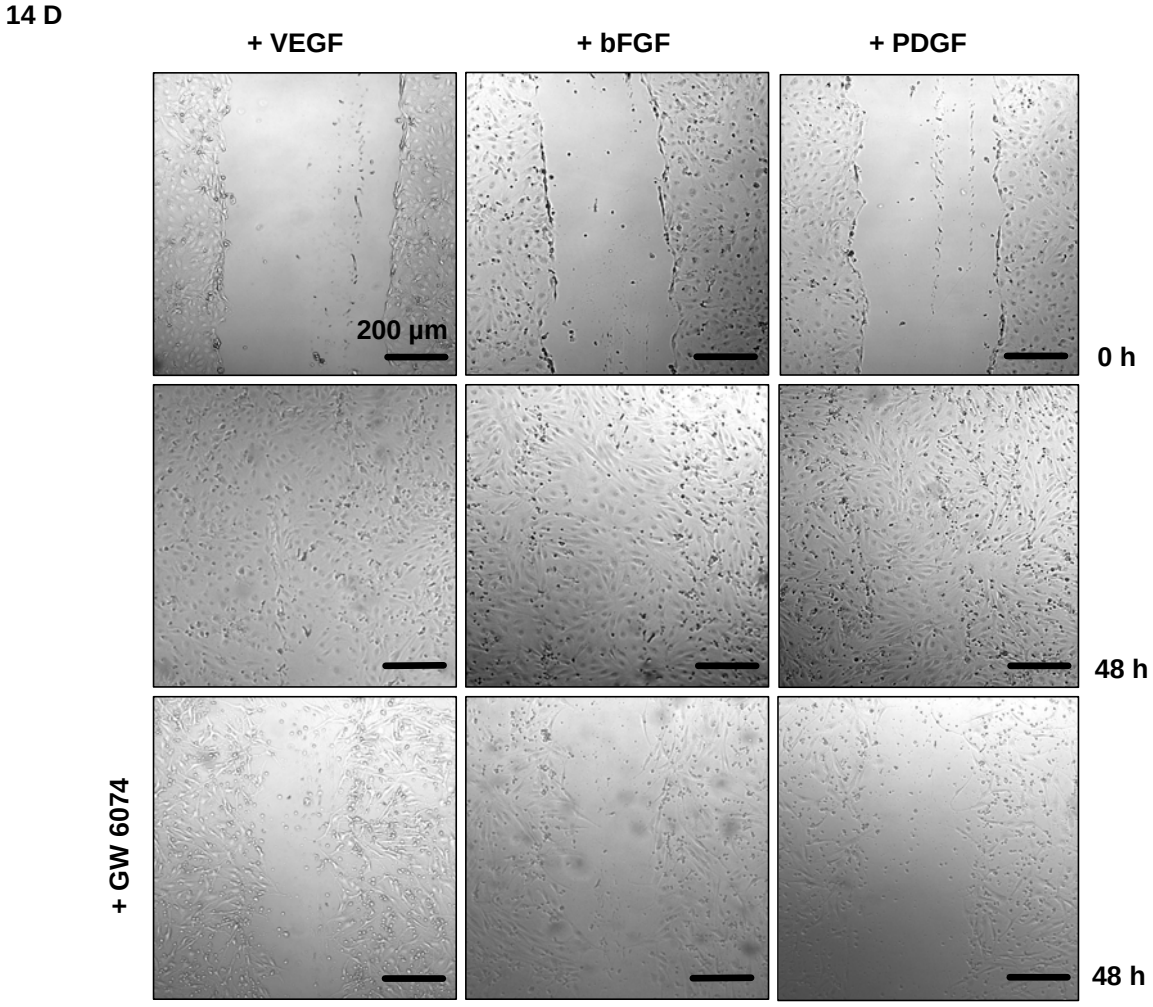


Fig. 3.14 Proliferation and wound healing *in vitro* of endothelial cells is dependent on Raf

(D) *Representative photos of scratches. Bars in photos represent 200 µm. From top to bottom: photos taken immediately after scratching (0 h), photos taken 48 hours after scratching (48 h) and stimulation with growth factors (from left to right: VEGF-A, bFGF, PDGF-BB), photos taken 48 hours after scratching and treatment with GW 5074 and growth factors (from left to right: GW 5074/VEGF-A, GW 5074/bFGF, GW 5074/PDGF-BB).*

3.4.5. Growth factor dependent PI3-Kinase regulation of Raf and ERK activation

Since we observed a marked effect on processes important for angiogenesis by inhibiting Raf in endothelial cells, it was of importance to investigate if there could be a direct link between SHP-2 and the Raf-MEK-ERK pathway. We hypothesized that PI3-Kinase may be an upstream regulator of the Raf-MEK-ERK pathway and thus the target affected by SHP-2 in these experiments. To investigate this, endothelial cells were treated with Wortmannin (10 nM), which inhibits PI3-Kinase activity, and subsequently stimulated with bFGF, PDGF-BB and VEGF-A before staining for phosphorylated Raf-1 and ERK 1/2. Inhibition of the PI3-Kinase by Wortmannin inclined a decrease in the Raf-1 (Fig. 3.15A, bFGF/Wortmannin n=5, PDGF-BB/Wortmannin n=4 and VEGF-A/Wortmannin n=4) and ERK 1/2 (Fig. 3.15B, all n=3) phosphorylation respectively regardless of growth factor stimulation.

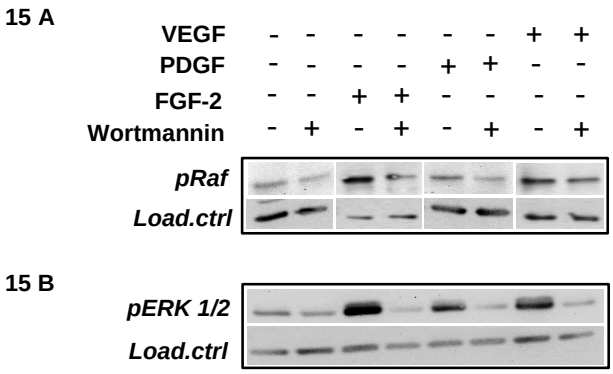


Fig. 3.15 PI3-Kinase dependent Raf and ERK activation

Wortmannin (10 nM, 30 minutes) treated HMEC lysates were subjected to western blotting as described in Materials and Methods section 2.4.1 followed by immunoblotting against the phosphorylated form of Raf-1 and ERK 1/2. ***(A)*** *Inhibition of PI3-Kinase by the application of Wortmannin seemed to reduce the phosphorylation level of Raf despite bFGF (n=5), PDGF-BB (n=4) and VEGF-A (n=4) stimulation. Representative western blots from independent experiments are shown above the graph.* ***(B)*** *Wortmannin treatment also influenced the growth factor dependent activation of ERK 1/2. Western blot is representative for three independent experiments, n=3.*

3.4.6. SHP-2 knock-down reduces growth factor induced ERK activation

Finally, to elucidate if SHP-2 inhibition affects the activity of the Raf-MEK-ERK pathway, the phosphorylation and hence activation of the extracellular regulated kinase 1/2 (ERK1/2) upon growth factor stimulation was assessed by immunoblotting against phospho-ERK 1/2. bFGF, PDGF-BB and VEGF-A all induced a strong phosphorylation of ERK 1/2 (all $p<0.05$, bFGF n=15, PDGF-BB n=13 and VEGF-A n=17, Fig. 3.16A). However, upon transfection with SHP-2 AS-ODN the bFGF (n=7, $p<0.05$), PDGF-BB (n=5, $p<0.05$) as well as VEGF-A ($p<0.05$, n=5) dependent phosphorylation of ERK1/2 significantly decreased in comparison to Rdm ODN treatment (Fig. 3.16B).

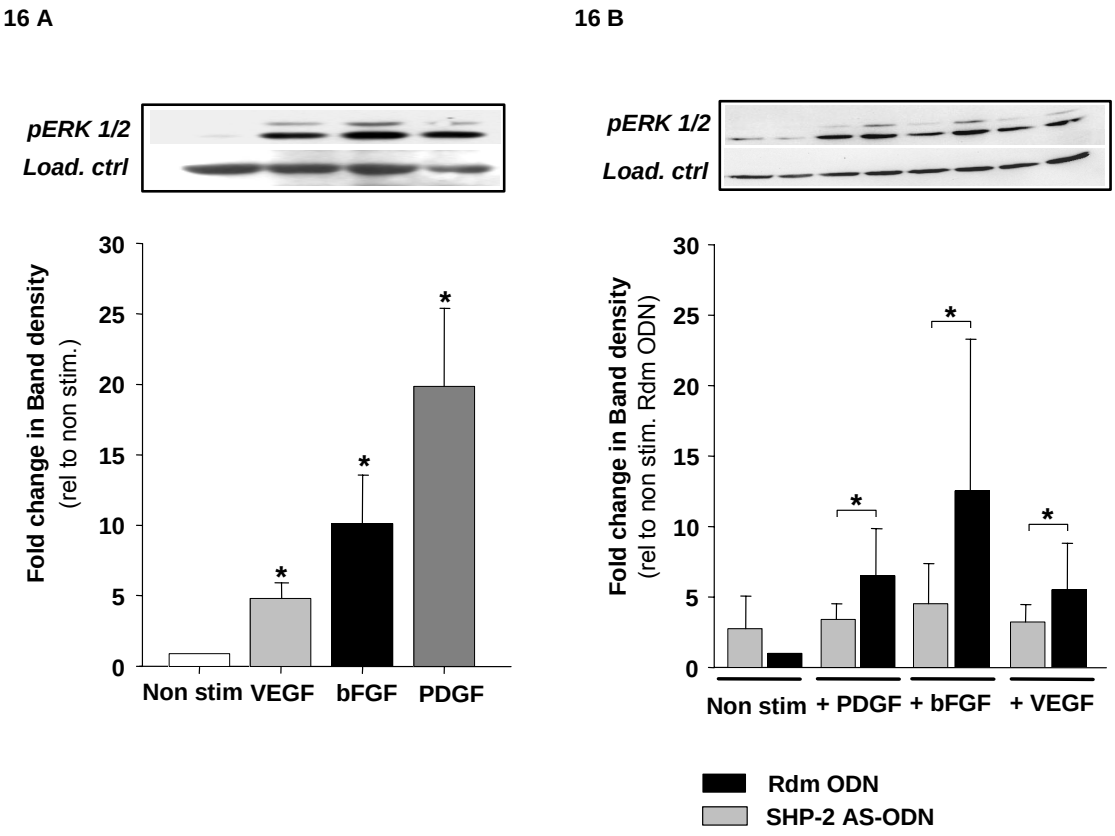


Fig. 3.16 Reduction of growth factor induced ERK phosphorylation by SHP-2 AS-ODN treatment

HMEC lysates were subjected to western blotting as described in Materials and Methods section 2.4.1 followed by immunoblotting against the phosphorylated form of ERK 1/2. ***(A)*** *Stimulation with VEGF-A (n=17), bFGF (n=15) and PDGF-BB (n=13) induced ERK1/2 activity (all *p<0.05). A representative western blot for independent experiments is shown above the graph.* ***(B)*** *SHP-2 knock-down seemed to reduce the bFGF (n=7, *p<0.05), PDGF-BB (n=5, *p<0.05) and VEGF-A (n=5, *p<0.05) dependent ERK phosphorylation. Representative western blot is shown above the graph. The significance between the different groups of this experiment was determined by calculating fold change relative to the respective Rdm ODN (not shown in the graph).*

3.4.7. The effects of SHP-2 knock-down are specific

It was important to ensure that the effects seen following SHP-2 inhibition were specific for SHP-2 knock-down and not just an overall reaction to apoptosis. Since treatment with Phorbol-myristate-acetate (PMA), a direct stimulator of Protein kinase C, for 10 minutes has been shown to activate the Raf-MEK-ERK pathway independent of SHP-2 activation [85], we choose to detect the phosphorylation of ERK1/2 in endothelial cells after SHP-2 AS-ODN treatment and subsequent stimulation with PMA. PMA stimulation of non transfected cells resulted in a robust activation of ERK1/2. In contrast to the bFGF and PDGF-BB dependent ERK 1/2 activation, PMA-induced ERK 1/2 activation was independent of SHP-2, as AS-ODN against SHP-2 had no effect in these experiments (Fig. 3.17, n=4).

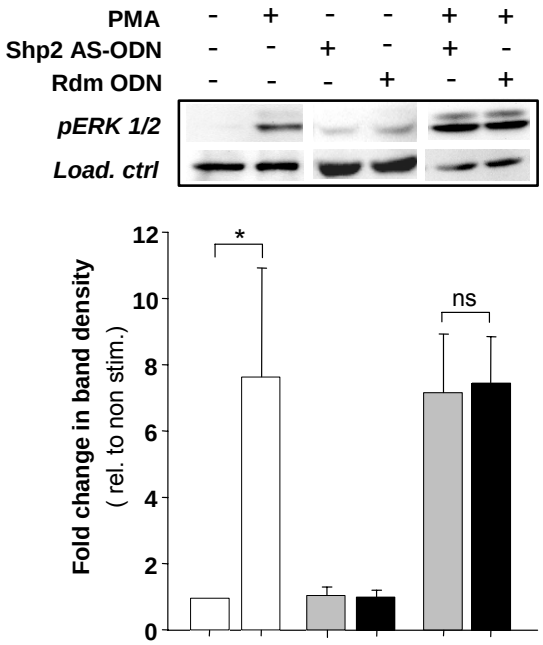


Fig. 3.17 PMA induced activation of ERK1/2 despite SHP-2 AS-ODN treatment

*Non transfected and transfected HMEC were treated with PMA (1 µM, 10 minutes) and the lysates subjected to western blotting as described in Materials and Methods section 2.4.1. ERK phosphorylation was detected using an α-phospho-ERK 1/2 antibody. Stimulation of non transfected cells with PMA resulted in a strong ERK 1/2 phosphorylation (*p<0.05, n=4). SHP-2 protein knock-down did not reduce this PMA dependent ERK 1/2 activation (n=4, ns: not significant). Western blots above graph are representative for four independent experiments (n=4).*

4. DISCUSSION

“Science... never solves a problem without creating ten more.”

~George Bernard Shaw~

The process of angiogenesis is important not only during embryogenesis but also throughout life. Vital processes like wound healing and the reproductive cycle [1,3,135], but also pathological conditions such as tumour growth and ischemic diseases [6,7] relay on angiogenesis. It is dependent on a variety of cytokines and growth factors, such as bFGF, VEGF-A and PDGF-BB important for the initiation of angiogenesis involving the prevention of apoptosis of endothelial cells and the stimulation of endothelial cell proliferation and migration [2] as well as for the later stages of this process involving organization of endothelial cells into tube-like structures followed by the extension and branching of these structures [8]. Two signalling pathways highly involved in these events are the PI3-Kinase / Akt [50,51] and the Raf-MEK-ERK cascade [57]. A tyrosine phosphatase, which has been implicated in the regulation of these pathways, is the Src homology 2 domain containing tyrosine phosphatase 2 (SHP-2) [72]. So far it has been unknown, however, if SHP-2 is involved in angiogenesis. Furthermore, the role of SHP-2 in endothelial angiogenic signalling had not been investigated until now. Finally, the type of regulation of the PI3-Kinase and the Raf-MEK-ERK pathway under the influence of VEGF-A, bFGF and PDGF-BB in human endothelial cells remained to be elucidated. Therefore, in this study we investigated the role of SHP-2 in these processes, which are prerequisites for angiogenesis such as endothelial cell survival and proliferation, but also in events necessary for proper vessel formation such as tube formation and vessel sprouting. Finally, we dissected the roles of SHP-2 in VEGF-A, bFGF and PDGF-BB enhanced signalling in human endothelial cells.

4.1. SILENCING OF SHP-2 PROTEIN

4.1.1. Why AS-ODN?

It is difficult to develop specific inhibitors for tyrosine phosphatases, as these enzymes show a close similarity, particularly the phosphatase domains [70]. The pharmacological inhibitors for SHP-2 existing today all inhibit, at lower or higher concentrations, other phosphatases to some extent. Therefore, to be able to specifically investigate the function of SHP-2, we chose to down regulate SHP-2 posttranscriptionally with either AS-ODN or siRNA. Since not all sequences are

suitable for efficient PTGS, due to the secondary and sometimes the tertiary structure of the mRNA as well as the steric occlusion of a certain stretch of the mRNA by proteins or polycations [117], several different sequences for AS-ODN and siRNA were tried out in this study. Only one AS-ODN sequence was shown to be efficient, which was the reason for performing our experiments using an AS-ODN rather than siRNA.

4.1.2. Specific knock-down of SHP-2 using the magnetofection method

One difficulty with these agents has, so far, been the cellular uptake of the oligonucleotides in cultured cells. The most common method is to use liposomes or charged lipids which encapsulate or form a complex with the antisense molecule [116], but these procedures are time consuming and require a relatively high amount of oligonucleotides. By using the recently developed magnetofection technique, the transfection efficiency is enhanced in comparison to standard lipid transfection reagents [125]. Furthermore, the time of transfection is reduced as well as the vector concentration [123-125], due to effective delivery of the oligonucleotides. The possibility of a rapid delivery of oligonucleotides at a low concentration reduces the cytotoxicity in comparison to other standard transfection reagents, where the cells need to be exposed to the transfection solution for a much longer time [125]. Transfection of a Cy-3 labelled ODN to HMEC using magnetofection in this study resulted in a transfection efficiency of over 80% while only 26% of the cells were observed to have efficiently taken up the Cy-3 ODN when magnetofection was not performed (Fig. 3.1). Furthermore, the cytotoxicity in both groups was similar (approximately 20%, Fig. 3.1), showing that magnetofection is a powerful tool that ensures an efficient delivery of ODN to endothelial cells, which are known to be almost transfection resistant. The positive results obtained from these measurements enabled us to use this technique for PTGS in this study. These results are in accordance to previous work performed by Krötz et al. [125], where magnetofection of AS-ODN into HUVEC also showed a transfection efficiency of over 80% with a cytotoxicity of less than 10%. The explanation for the higher number of non viable cells observed in this study after magnetofection is most likely the higher concentration of ODN that was used. While Krötz et al. transfected cells with 0.2 µg AS-ODN/ well in a 24 well plate format, cells in this study were exposed to 0.6 µg ODN/ well in a 24 well plate format. The

reason for using a higher amount of ODN in these experiments is that the SHP-2 protein was knocked-down reliably at this concentration only. The amount or concentration of the AS-ODN needed to silence the expression of a specific protein relies on the frequency and amount of the particular mRNA expression. Furthermore, the time needed from the point of transfection until a reduction in protein level can be observed depends on how fast the AS-ODNs reach the cytosol after having been deposited on the cell surface but more importantly on the turnover of the actual protein. In our experiments a knock-down of SHP-2 protein could be seen already 6 hours post transfection and reached its maximum 24 hours after transfection (Fig. 3.2). After 48 hours the effect could no longer be seen. This correlates well with the Cy-3 ODN distribution in the cells observed in this study (Fig. 3.1B). 4 hours after transfection the Cy-3 ODN was seen to already reside in the cytosol and nothing or very little was detected in the nuclei. Most of the AS-ODN seemed to still be on the cell surface though, whereas 24 hours post transfection the AS-ODN could clearly be seen both in the cytosol and in the nuclei. The silencing of the SHP-2 mRNA was specific, as detection of SHP-1, which is the closest homologue to SHP-2, did not show any reduction in protein level.

Another item one should take into account is that AS-ODNs have a restricted time of action, due to eventual degradation within the cell, which limits the duration of the silencing. These observations comply with previous data by Vickers et al, who used the endogenous mRNA for Bcl-X as target to investigate the duration of action of oligonucleotides. The highest efficiency of the AS-ODN was detected after 24 hours and was almost lost after 48 hours [121]. Our results showed that AS-ODNs have an efficiency window of about 48 hours and that repeated transfections would be necessary if using this approach therapeutically when a sustained treatment is desired.

4.2. SHP-2 AS A POTENTIAL KEY PROTEIN IN THE ANGIOGENIC PROCESS

4.2.1. Inhibition of SHP-2 suppresses both basal and growth factor induced endothelial response to wound healing *in vitro*

Fibroblast cells lacking a functional SHP-2 have been shown to exhibit an impaired cell spreading and migration associated with an increased number of focal adhesions [136]. In endothelial cells, SHP-2 has been shown to be involved in cellular adhesion processes by interaction with PECAM-1 and VE-Cadherin [103,104,107]. SHP-2 deficiency also associated with a decrease in focal adhesion kinase (FAK) activity [136]. This has led to the suggestion that SHP-2 has a role in endothelial cell motility. Indeed, in our study inhibition of SHP-2 with the pharmacological inhibitor PtpI IV, in comparison to sham treated cells (Fig. 3.3), resulted in an incapability of endothelial cells to re-establish the cell layer after a wound incision, a process where adhesion molecules play an important role. Addition of the angiogenic growth factors PDGF-BB and bFGF could not reverse this effect (Fig. 3.7). Although application of these growth factors to PtpI IV treated cells enhanced the wound healing to some extent, it was still significantly delayed in comparison to cells treated with only PDGF-BB and bFGF. These first findings of this study lead to the assumption that SHP-2 influences processes important for angiogenesis initiation.

4.2.2. SHP-2 knock-down results in a decreased endothelial cell proliferation

Since the above described wound healing assay includes several steps such as endothelial cell migration and proliferation and one cannot distinguish between the two, in a next step the specific effect of SHP-2 on basal and growth factor induced endothelial cell proliferation was investigated. Interestingly, whereas addition of bFGF and PDGF-BB enhanced the proliferation of endothelial cells, addition of VEGF-A_{165} did not (Fig. 3.8A). This was surprising, since VEGF-A has been described as the primary angiogenesis inducer and as a potent stimulator of endothelial cell proliferation [24,25,137]. There are several possible explanations for the inability to detect any increase in VEGF induced endothelial proliferation in this study.

Firstly, the studies showing a VEGF-A induced endothelial proliferation have been performed in HUVEC or PAEC, whereas HMEC were used for this assay in this work. HMEC were chosen for this assay due to their ability to survive in starvation media for a much longer time than HUVEC, which detached from the collagen coated cell dish already after 24 hours when used (data not shown). It is possible, that HMEC, which have been immortalized by the introduction of the large T antigen, exhibit a higher basal proliferative rate than primary endothelial cells and because of this need stronger stimuli, such as bFGF or PDGF-BB, than VEGF-A to accelerate their proliferation rate. Although VEGF-A is endothelium specific [2], it does not appear to be the most potent inducer of endothelial proliferation, as bFGF stimulation has been shown to increase the proliferation of endothelial cells over 2-fold, whereas VEGF treated cells only enhanced the proliferation by approximately 1.5 fold at concentrations ranging from 0.1 to 10 ng/ml [137]. Secondly, the VEGF-R density might vary from primary endothelial cells to HMEC. However, functional effects observed after VEGF-A stimulation, such as ERK1/2 phosphorylation proved that there must be VEGF-R on HMEC. Thirdly, the VEGF-A peptide used in the experiment was only added to the cells every 48 hours, which might have been a too long time span. It is possible, that the peptide was degraded faster than the other growth factors und thus was unable to induce significant proliferation.

Nevertheless, knock-down of SHP-2 showed a decreased proliferation both when measuring the reduction of MTT (Fig. 3.4A) and detecting the amount of cells situated in the G2/M-phase of the cell cycle (Fig. 3.4B). In addition to the diminished basal proliferation, the bFGF and PDGF-BB enhanced proliferation was also reduced when cells were treated with SHP-2 AS-ODN (Fig. 3.8B). Although studies on patients with Noonans syndrome indicate that SHP-2 is important for cellular proliferation, as these patients exhibit a higher risk of developing juvenile myelomonocytic leukaemia [76], which is characterized by a high proliferation rate of myeloid progenitor cells, and recent data show a reduction in self-renewal of SHP-2 heterozygous hematopoetic stem cells [138], these are, to our knowledge, the first data showing a direct role of SHP-2 in bFGF and PDGF-BB induced endothelial cell proliferation.

4.2.3. Loss of SHP-2 impairs endothelial cell viability and induces apoptosis

The reduction of MTT cannot only be used to measure the proliferation of cells but also is an indicator of cell viability, since it is reduced into formazan only in living cells. Whereas the formazan production in cells treated with Rdm-ODN in the absence of growth factors stayed constant throughout our experiments, cells transfected with SHP-2 AS-ODN exhibited a much lower formazan formation and as this was below the initial value at first measurement (Fig. 3.4A), it indicated that the reduction in the proliferative response may be due to an increased cell death. In addition, one important prerequisite for angiogenesis to occur is the prevention of endothelial apoptosis to allow for endothelial proliferation and migration under conditions that normally favours apoptosis, such as loss of contacts to the extracellular matrix. To test whether silencing of SHP-2 indeed would influence endothelial cell viability and thus be a necessary protein in preparing for angiogenesis to take place, the DNA content of endothelial cells was stained with propidium iodide. Cells detected within the SubG0 fraction were assumed apoptotic, as this population contains less DNA than cells situated in the G1 phase of the cell cycle due to DNA fragmentation. 24 hours after treatment with SHP-2 AS-ODN and PtpI IV a significantly higher peak in SubG0 could be detected, which was not seen in Rdm-ODN or Sham treated cells respectively (Fig. 3.5A and 3.5B). Since this assay does not detect the proapoptotic cells, an Annexin V staining was performed. 6 hours after treatment with SHP-2 AS-ODN treatment, an increase in Annexin V positive cells could be observed (Fig. 3.5C), which confirmed our previous results indicating that SHP-2 knock-down leads to an increased endothelial apoptosis. This also led to the conclusion that the reduction in growth factor induced endothelial proliferation most possibly was due to an enhanced apoptosis of endothelial cells, which could not be rescued by the addition of survival factors such as bFGF and PDGF-BB. Even though these results are in concordance with previous work showing a hypersensitivity of SHP-2 deficient fibroblasts towards an apoptosis inducing agent [96,97], they show for the first time the functional consequences of SHP-2 action in primary human endothelial cells. Moreover, these results further confirm our hypothesis that SHP-2 is necessary for events included in angiogenesis initiation, such as endothelial cell proliferation and cell survival.

4.2.4. SHP-2 and new vessel formation

The previous observations pointing at a crucial role of SHP-2 in several of the processes needed for proper vessel formation incited us to investigate the effects of SHP-2 on the formation of capillary like structures by using a Matrigel assay. This is a critical step during angiogenesis where proliferating and migrating endothelial cells organize themselves into stable tubular structures. Treatment with the pharmacological SHP-2 inhibitor PtpI IV as well as with SHP-2 AS-ODN significantly impaired this process by a reduction in the number of capillary like structures with approximately 50% in comparison to sham or Rdm-ODN treated endothelial cells respectively (Fig. 3.9). This observation coincides with the finding that SHP-2 negative mutant endothelial cells failed to organize themselves into a highly vascularized network in the yolk sac of mouse embryos [101].

This assay has been described as one of the most specific tests for angiogenesis and is now widely used. One potential drawback of this assay is that other cells of non endothelial origin have been reported to also respond to Matrigel [139]. Although the Matrigel used in our experiments was reduced of growth factors it still contained a small amount of growth factors such as bFGF, PDGF, EGF and IGF-1. As we wished to study the process of angiogenesis in an environment as close as possible to the in vivo situation, we treated the cells in the Matrigel with growth media supplemented with serum and additional growth media containing growth factors such as bFGF and EGF. As an inhibition of SHP-2 impaired the formation of capillary like structures even in the presence of several different growth factors, SHP-2 might also be important for endothelial signalling induced by other growth factors and may constitute a key position in growth factor dependent angiogenesis.

Not only is the ability to form functional tubes important for later functional blood vessels, but also the capability of the formed tubes to bifurcate and extend. To study this process and to address the question if the effects seen after SHP-2 inhibition or knock-down have physiological consequences, such as impaired vessel sprouting, mouse aortas were cut into rings and embedded in a proangiogenic matrix. The sprouting of new vessels from these rings was impaired when treated with PtpI IV, as observed by the lower amount of sprouting bifurcations (Fig. 3.10). The observation that patients with NS often suffer from congenital heart disease [76] would support a

role for SHP-2 in blood vessel development. Indeed, our findings strongly confirmed the hypothesis that SHP-2 plays a major part, and for the first time dissect its role, in the process of angiogenesis. Moreover, the finding that SHP-2 homozygous knock-out mice die in utero with an incomplete blood vessel development further strengthen our hypothesis [101]. This puts SHP-2 in a completely new functional background. It not only affects the events crucial for angiogenesis initiation such as growth factor dependent endothelial cell survival, proliferation and migration but the impairment of these processes by inhibiting SHP-2 are also of functional significance, as this probably caused the improper vessel formation.

4.3. THE ROLE OF SHP-2 IN ENDOTHELIAL ACTIVATION

4.3.1. Growth factor dependent activation of SHP-2

Having found that SHP-2 seems to have an eminent role in the endothelial angiogenic response, it was of interest to elucidate what pathways could have been involved in the SHP-2 dependent response. To do so, we concentrated on the signalling induced by the three potent angiogenic factors VEGF-A_{165}, bFGF and PDGF-BB. As SHP-2 has been shown to function as both a tyrosine phosphatase but also as an adaptor molecule, we first measured the phosphatase activity of SHP-2 in primary endothelial cells after stimulation with VEGF-A_{165}, bFGF and PDGF-BB respectively (Fig. 3.6). All three growth factors were shown to significantly induce SHP-2 phosphatase activity, with PDGF-BB being the strongest stimulus. This was not surprising, as several others have shown that SHP-2 enhances its phosphatase activity upon PDGF stimulation and that this activity is associated with a tyrosine phosphorylation of SHP-2 [74,140,141]. We could also show that PDGF-BB stimulation of endothelial cells led to a slighty increased phosphorylation of the tyrosine residue 542 on SHP-2 (Fig. 3.6 upper part). SHP-2 has been shown to directly interact with the VEGF-R2 [89] and to become tyrosine phosphorylated upon VEGF-A stimulation but a tyrosine phosphorylation of SHP-2 does not necessary enhance its activation [142] and no study regarding its activity upon addition of VEGF-A has before been performed. In this study, VEGF-A_{165} stimulation also resulted in an increased tyrosine phosphorylation (Y 542) of SHP-2 which correlates well with the measured increase in phosphatase activity. Stimulation with bFGF, however, slightly enhanced

the SHP-2 phosphatase activity but intriguingly enough, we could not detect a phosphorylation of Y 542. It rather seemed to be a dephosphorylation in comparison to untreated cells. Kontaridis et al. showed that bFGF induced tyrosine phosphorylation of SHP-2 in myoblasts and that the catalytic activity of SHP-2 was essential for sustained bFGF dependent ERK activation [95]. The difference between these two studies is that Kontaridis et al. used an antibody detecting unspecific phosphorylated tyrosines, whereas we used an antibody directed against a specific tyrosine residue on SHP-2. It may well be that SHP-2 is tyrosine phosphorylated upon bFGF stimulation, but not on tyrosine residue 542. One hypothetical explanation for the dephosphorylation of Y 542 upon bFGF stimulation may be that SHP-2 functions in the first place as an adaptor molecule under bFGF rather than a phosphatase and that the dephosphorylation of Y 542 is necessary for releasing the binding pocket of one of the SH2-domains to enable the binding to other tyrosine phosphorylated molecules (See Fig 1.5 for mechanisms of SHP-2 activation). This would also explain the small increase in phosphatase activity measured after application of bFGF. The decrease in tyrosine phosphorylation of SHP-2 upon bFGF stimulation may also be time dependent. Our experiments were performed by stimulation with growth factors for 10 minutes; a shorter stimulation might give a different result. As the SHP-2 activity after VEGF-A stimulation was of a similar extent as to bFGF stimulation; it is likely that SHP-2 functions as an adaptor molecule also in VEGF-A signalling. The phosphorylated Y 542 may in this case serve as a binding site for other SH2-domain containing molecules. The hypothesis that SHP-2 also can function merely as an adaptor molecule in some cases is supported by a study where deletion of the N-terminal SH2-domain, responsible for binding of Tyr(P) residues, abrogated EGF induced Akt phosphorylation [98]. Taken together, the results obtained here indicate that the angiogenic growth factors bFGF, PDGF-BB and VEGF-A are able to enhance SHP-2 phosphatase activity, although to different extents.

4.3.2. The role of PI3-Kinase in SHP-2 signalling

The PI3-Kinase / Akt signalling pathway seems to be very important for many processes in endothelial cells. For instance, endothelial migration induced by bFGF and VEGF was impaired when the PI3-Kinase was inhibited with a pharmacological

inhibitor. In addition, FGF and VEGF induced DNA synthesis was decreased when PI3-Kinase was inhibited. Furthermore, PI3-Kinase was shown to be important for the long term stability of endothelial tubes in a collagen gel as well as for FGF and VEGF induced angiogenesis in the chorioallantoic membrane (CAM) assay [50,51]. However, somewhat contradicting results exist about the SHP-2 dependent regulation of PI3-Kinase. Upon EGF stimulation, SHP-2 has been demonstrated to both positively [98] and negatively regulate PI3-Kinase activity in fibroblasts [99,143]. Whereas several studies exist showing a positive role of SHP-2 in PDGF enhanced PI3-Kinase activity [96,99], it had been unknown so far if SHP-2 affects bFGF and VEGF-A induced PI3-Kinase activity. It was therefore of interest to investigate the effects of SHP-2 on PI3-Kinase and Akt signalling in endothelial cells upon bFGF, PDGF-BB and VEGF-A stimulation. All three growth factors enhanced the activation of PI3-Kinase in endothelial cells, as shown by tyrosine phosphorylation of the p85 subunit of PI3-Kinase (Fig. 3.11). This was not surprising since it is known that these factors activate the PI3-Kinase and that it is involved in signalling initiated by these growth factors [40,50,85]. When silencing SHP-2 the PDGF-BB dependent activity of PI3-Kinase tended to be diminished as expected. Treatment of endothelial cells with bFGF and VEGF-A respectively after SHP-2 knock-down also showed a tendency towards a decrease in PI3-Kinase activity. These results indicated that SHP-2 is a positive regulator of bFGF and VEGF-A induced PI3-Kinase activity, in addition to PDGF-BB induced PI3-Kinase activation. In addition, when investigating the activation of Akt, the downstream target of PI3-Kinase which has been implicated in most of the cell survival functions of the PI3-Kinase pathway, the bFGF and PDGF-BB induced Akt activity was reduced upon SHP-2 AS-ODN treatment (Fig. 3.13). This implies that endothelial bFGF and PDGF-BB stimulated Akt activation probably is dependent on SHP-2. Intriguingly, VEGF-A stimulation of Rdm ODN treated cells failed to induce Akt phosphorylation. This was confusing as stimulation of non transfected cells with VEGF-A gave rise to an enhanced Akt phosphorylation. It is unlikely, though, that this inability of Rdm ODN treated cells to induce VEGF-A dependent activation of Akt is caused by the transfection itself, since Rdm ODN transfected cells showed an increase in the VEGF-A dependent phosphorylation levels of p85, Raf and ERK1/2. Nevertheless, there are, to our knowledge, no data concerning the role of SHP-2 in VEGF dependent PI3-Kinase / Akt signalling and our findings indicate that SHP-2 is needed for VEGF induced

PI3-Kinase activation but leaves the question whether SHP-2 is necessary for VEGF induced Akt activation open.

On the other hand, bFGF has repeatedly been shown to be responsible for PI3-Kinase and Akt activation [50,51]. Our data show, for the first time, that SHP-2 acts upstream of the PI3-Kinase and Akt in endothelial cells upon bFGF stimulation thereby positively regulating this pathway. Klinghoffer et al. found that a mutant PDGF-R, which in addition to binding PI3-Kinase also bound SHP-2, was able to fully activate the PI3-Kinase [55]. Furthermore, both PDGF induced PI3-Kinase and Akt activity was reduced in mouse fibroblasts which contained a non functional SHP-2 [98]. These studies support our data that, in addition to bFGF, PDGF induced activation of the PI3-Kinase and its downstream target Akt is regulated by SHP-2. Furthermore, our findings point to a positive role of SHP-2 in bFGF, PDGF-BB and VEGF-A dependent PI3-Kinase activation in endothelial cells as opposed to an inhibitory role as in the case of EGF [97-99].

SHP-2 is believed to be able to mediate its signalling effects in both catalytic-dependent and independent ways [72]. There are data describing a SHP-2 catalytic dependent activation of PI3-Kinase after IGF stimulation in fibroblasts cells [97]. Interestingly, whereas mutations in the N-SH2 domain of SHP-2 led to a decreased EGF dependent Akt activation, mutations in the phosphatase domain rendering SHP-2 catalytically inactive enhanced the EGF dependent Akt activation [98]. This would suggest that SHP-2 is able to, by functioning both as an adaptor protein and as a phosphatase, induce two opposed signalling effects, which in turn may explain the different observations made concerning EGF dependent PI3-Kinase activity and SHP-2. SHP-2 has also been found to associate with p85 after EGF stimulation in glioblastoma cells [98] and it is suggested that SHP-2 and p85 exist in a larger signalling complex but whether SHP-2 in this case functions merely as an adaptor molecule is not clear. The possible adaptor role of SHP-2 will be further discussed below (section 4.3.4).

Taken together, it is likely that SHP-2 mediates parts of its angiogenic effects through the regulation of the anti-apoptotic and proliferation inducing PI3-Kinase / Akt pathway. These findings are supported by data showing a role for SHP-2 in IGF-1

stimulated PI3-Kinase activation and caspase-3 induced apoptosis in fibroblasts [97,98]. The observations made so far in this study strengthen the hypothesis, that SHP-2 influences anti-apoptotic pathways in human endothelial cells.

4.3.3. Involvement of SHP-2 in the endothelial Raf-MEK-ERK pathway

It is known that the MAP-Kinase pathway (Raf-MEK-ERK) strongly affects the angiogenic process by preventing apoptosis and favouring proliferation [57-59]. We also observed this in our assays, as both endothelial cell proliferation and the ability to cover a wound incision in a confluent cell layer was impaired when Raf-1 was inhibited with a pharmacological inhibitor (Fig. 3.14). In both these assays, bFGF seemed to have the highest protective effect, as a loss in cell number was seen first after 72 hours when measuring the MTT reduction, while PDGF stimulated cells treated with the Raf-1 inhibitor started to show a decline in cell number already after 48 hours. Likewise was the wound healing ability in vitro of endothelial cells more severely affected in cells treated with the Raf-1 inhibitor together with either PDGF-BB or VEGF-A in comparison to cells treated with the Raf-1 inhibitor and bFGF. This difference may be explained by the fact that VEGF-A and bFGF, although being responsible for similar functions in most cases such as endothelial proliferation and survival, stimulate the expression of different genes regulating cell growth, survival, migration, apoptosis and oxidative stress [137]. Moreover, as described earlier, although being endothelium specific, VEGF-A does not seem to be the most potent inducer of endothelial dependent processes [57,137]. Furthermore, according to our results, in our assays bFGF stimulation of the Raf-MEK-ERK pathway may be important for endothelial cell proliferation in the first place, as stimulation with bFGF led to a higher increase of proliferation rate than PDGF-BB (Fig. 3.14A and 3.14B). PDGF induction of this pathway, on the other hand, seemed to influence the survival of endothelial cells rather than the proliferation, since the wound healing in vitro was impaired already after 24 hours following treatment with the Raf-1 inhibitor and was kept at a lower level at all times in comparison to cells treated with the Raf-1 inhibitor in the presence of bFGF (Fig. 3.14C). This hypothesis is supported by a study performed by Sano et al., who found that a blockage of the PDGF-Rβ induces endothelial apoptosis [37]. Moreover, recent data showing an

increased apoptosis amongst tumour-associated endothelial cells upon inhibition of the PDGF-R further supports this hypothesis [144]. On the contrary, there are numerous studies confirming the role of PDGF in both endothelial cell survival and proliferation [33,35,37], although it is not elucidated through which pathways these effects are transduced. In the case of bFGF, previous studies have shown that bFGF is a strong inducer of endothelial cell proliferation [13]. Finally, bFGF induces the expression of adhesion molecules, such as the αvβ3 integrin, on the endothelial cell surface [145], which may facilitate wound repair, strongly supporting its role in primarily cell proliferation. On the other hand, Alavi et al. showed that VEGF and bFGF induced Raf activation was important for the prevention of apoptosis [56]. Nevertheless, the bFGF, PDGF-BB and VEGF-A activation of the Raf-MEK-ERK pathway seems to be a possible candidate for the SHP-2 regulated angiogenic response and was therefore investigated further.

To elucidate the role of SHP-2 in the Raf-MEK-ERK pathway in endothelial cells and to address the question if SHP-2 influences pathways activated during the angiogenic process, cells were treated with SHP-2 AS-ODN followed by stimulation with bFGF, PDGF-BB and VEGF-A. The phosphorylation of ERK1/2 was used to measure the activation of the Raf-MEK-ERK pathway. SHP-2 AS-ODN treatment decreased bFGF, PDGF-BB and VEGF-A induced ERK1/2 phosphorylation (Fig. 3.16), indicating that SHP-2 is required for Raf-MEK-ERK activation under the influence of all of these growth factors. Another protein able to activate this pathway is the protein kinase C (PKC) [7,62]. Activation of PKC through stimulation with PMA has been shown to induce a strong ERK1 activation. This activation was not affected by SHP-2 deficiency in mouse fibroblasts [146]. Therefore, we chose this approach to test whether the effect seen on the decreased ERK1/2 phosphorylation by the AS-ODN was specific and not an effect secondary to apoptosis. A robust ERK1/2 phosphorylation upon PMA stimulation of SHP-2 AS-ODN treated endothelial cells could be observed, confirming that the reduction in ERK1/2 activation in these experiments was specific for the inhibition of the Raf-MEK-ERK pathway by SHP-2 AS-ODN (Fig. 3.17).

As we could detect marked deficiencies in the activation of the Raf-MEK-ERK pathway following SHP-2 AS-ODN treatment, it was of significance to investigate if

there may be a factor directly connecting the two. Inhibition of PI3-Kinase with Wortmannin inclined a diminished bFGF, PDGF-BB and VEGF-A induced Raf-1 and ERK1/2 phosphorylation (Fig. 3.15). This led to the assumption that PI3-Kinase does not only function to activate Akt, but also activates the Raf-MEK-ERK pathway promoting endothelial cell survival and proliferation. Furthermore, our findings demonstrate that SHP-2 regulates bFGF, PDGF-BB and VEGF-A dependent ERK1/2 activation probably through PI3-Kinase.

Several studies showing an involvement of SHP-2 in MAP-Kinase signalling exists. For instance, Tang et al. found SHP-2 to function upstream of the Raf-MEK-ERK pathway in FGF signalling being important for mesoderm induction in Xenopus [88]. Furthermore, Shi et al. [146] showed that SHP-2 was required for PDGF and IGF induced ERK1 activation in mouse fibroblasts. However, this is the first time SHP-2 has been demonstrated to be important for angiogenic factor dependent activation of the Raf-MEK-ERK pathway in human endothelial cells. Furthermore, VEGF is one of the best described factors inducing Raf-MEK-ERK activation, but never before has SHP-2 been described as an essential link between VEGF and the Raf-MEK-ERK signalling cascade.

While Zhang et al. [93] suggest that SHP-2 controls ERK activation through Src family kinase activation thereby involving PLCγ and Ras induced ERK activation, Cunnick´s et al. have found, in addition to regulating Src kinase, that a binding of SHP-2 to the Grb 2-associated binder 1 (Gab-1) is necessary for an adequate ERK activation [92] . Our findings are supported by these data and furthermore introduce a new possible target for SHP-2, namely PI3-Kinase, for the activation of ERK1/2 in endothelial cells.

4.3.4. The target of SHP-2 in bFGF, PDGF-BB and VEGF-A dependent endothelial signalling

Such central position of SHP-2 in the regulation of endothelial cell viability points the question towards the potential direct target of SHP-2 in influencing these anti-apoptotic pathways. As PI3-Kinase was shown to regulate both ERK1/2 and Akt activation in endothelial cells in the previous experiments and the fact that the p85

subunit is tyrosine phosphorylated, in order to activate the enzyme, we hypothesized that PI3-Kinase may be the direct target coupling SHP-2 to Akt and ERK1/2 activation and thus may also be the link between SHP-2 and its proliferative and anti-apoptotic effects observed in this study. Indeed, we observed a bFGF, PDGF-BB and VEGF-A dependent association of the p85 subunit of PI3-Kinase with SHP-2 in endothelial cells (Fig. 3.17 upper blot). This is in accordance with other studies showing an interaction between PI3-Kinase and SHP-2 after IGF stimulation resulting in enhanced PI3-Kinase activation in mouse fibroblasts [98]. Others have found that the Grb 2-associated binder 1 (Gab-1) binds SHP-2 upon EGF, PDGF and bFGF stimulation [91,92,99,133] and that this seems to be important for at least EGF and PDGF induced SHP-2 dependent signalling [91,92,99]. Furthermore Gab-1 has been found to bind to the VEGF-R2 in endothelial cells, a receptor to which SHP-2 also was found to bind [89]. Moreover, Ong et al. [133] found that FGF-1 activated PI3-Kinase and Akt through a signalling complex consisting of the FGF-Receptor Substrate 2 (FRS-2), Grb 2 and Gab-1. These findings taken together with ours suggest that PI3-Kinase and SHP-2 could be involved in the same signalling complex together with for instance Gab-1. Indeed, immunoprecipitation of SHP-2 showed an enhanced binding of Gab-1 to SHP-2 upon bFGF, PDGF-BB and VEGF-A stimulation (Fig. 3.17 lower blot), revealing a possible link between SHP-2 and PI3-Kinase / Akt and MAP Kinase signalling in endothelial cells.

Others have reported that SHP-2 regulates ERK1/2 activity through the Src kinase by promoting the disassociation of the negative Src-regulator Csk [93,147]. Src kinases have been found to phosphorylate p85 on a tyrosine residue, thereby inducing the activity of the catalytic subunit p110 [40,46]. We have shown that SHP-2 is necessary for this tyrosine phosphorylation in endothelial cells, which suggests that SHP-2 may even be upstream of Src kinase. Although the exact mechanism remains to be elucidated, one can hypothesize that SHP-2 binds Gab-1 upon growth factor stimulation, which brings SHP-2 close to the cellular membrane. SHP-2 may then positively influence the activity of Src kinase, which then is able to phosphorylate and thereby activate the PI3-Kinase. PI3-Kinase is now able to translocate to the membrane, where it binds Gab-1, which brings it in close proximity to its substrate. This hypothesis is supported by data showing a decreased binding of the p85 subunit to Gab-1 in SHP-2 deficient fibroblasts upon PDGF stimulation, which was

associated with a decreased Akt and ERK phosphorylation [99]. Interestingly, upon EGF stimulation the opposite could be observed by several groups [99,143], showing that SHP-2 does not only function as either a positive or negative regulator of different pathways but the effect on a particularly pathway is highly dependent on the stimulus. For a summary of the role of SHP-2 in the signalling pathways investigated in this study see figure 4.1.

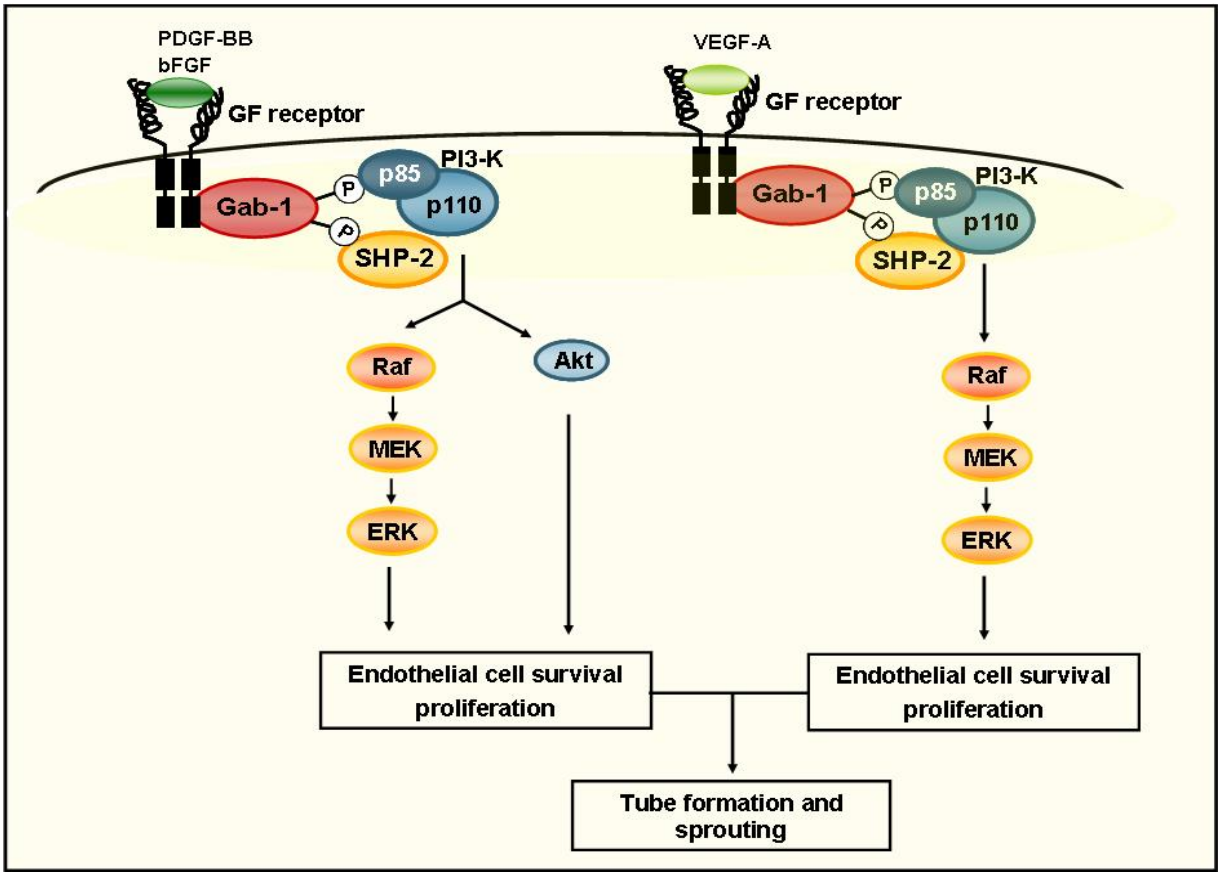


Fig. 4.1 Schematic overview of the mechanisms of SHP-2 function in FGF-2, PDGF-BB and VEGF-A dependent endothelial angiogenic signalling investigated in this study

Upon FGF-2 or PDGF-BB stimulation, the p85 subunit of the PI3-Kinase becomes tyrosine phosphorylated, which renders the kinase active (p110 subunit). This activation seems to be dependent on the presence of SHP-2. Furthermore, SHP-2 associates with p85 and Gab-1, which indicates that SHP-2, PI3-Kinase and Gab-1 are parts of the same signalling complex. SHP-2 dependent PI3-Kinase activation leads to Akt and Raf-MEK-ERK pathway activation in the case of bFGF and PDGF-BB stimulation (left part). VEGF-A application results in only PI3-Kinase dependent ERK activation and is independent of Akt activation (right part). SHP-2 was also shown to be critical for endothelial cell survival, proliferation and migration, which are controlled by the PI3-Kinase / Akt and the Raf-MEK-ERK pathway in endothelial cells. These processes are necessary for tube formation and subsequent vessel sprouting, events which were also influenced by SHP-2 in this study.

4.4. LIMITATIONS OF THE STUDY

While the use of AS-ODN assures a specific and efficient knock-down of the protein of interest, it does not in this case give any information about the need for SHP-2 phosphatase activity in our assays. This would be of interest to investigate, since SHP-2 can function both as a phosphatase and as an adaptor molecule. This would be possible when using a dominant negative phosphatase mutant of SHP-2. To further confirm the results obtained in this study, an over expression of SHP-2 by a constitutively active SHP-2 in endothelial cells would be necessary. Both these approaches would require the transfection of vectors containing the constructs for the SHP-2 mutants into endothelial cells. This could be difficult, as transfer of genes to a practically transfection resistant cell type probably either lacks the necessary efficiency or is hampered by high toxicity. Retroviral techniques may allow for sufficient transfection efficiency without considerable cytotoxicity.

Many but not all experiments were performed with primary HUVEC. In some assays, though, we had to rely on HMEC, due to limited access to umbilical cords. Also, some of the experiments where a longer time in starvation media (over 12 hours) was needed could only be done with HMEC because HUVEC detached themselves from the cell culture dish after 12 hours. Although HMEC share many characteristics with primary endothelial cells, such as cobblestone morphology in monolayer culture and expression of von Willebrand´s factor and form tube like structures in Matrigel [130], it is an immortalised cell line and not primary cells. Furthermore, new research shows that expression of genes involved in cell differentiation and cell cycle regulation does not differ between HMEC and HUVEC, while gene expression regarding cytokines, chemokines and cell surface proteins does [148]. Therefore one should not always refer to endothelial cells of different origins as if they were the same. Some of the experiments were performed in primary PAEC. This could only be done when using inhibitors, as our AS-ODN did not silence porcine SHP-2. Unfortunately, the porcine DNA sequence for SHP-2 was also not available, thus making a SHP-2 AS-ODN synthesis directed against porcine SHP-2 a tricky quest.

A general methodological problem regarded the evaluation of western blots. Although repeated experiments gave rise to the same results (i.e enhancement or reduction in protein phosphorylation levels) it was difficult to mirror this by measuring the protein

band densities of the different blots. This yielded a rather large standard error deviation (SED) due to variability between the blots caused by differences in the cellular expression of the proteins used as loading controls (actin or GAPDH) in the different experiments. This in turn made it difficult comparing ratios between actual protein band and loading control of different western blots. This variance might have been of a minor problem if more experiments (n´s) had been performed, as, according to many statistical textbooks, a testing population greater than the ones used in these experiments would be required for proper statistical testing.

For a more accurate predication of the effects of SHP-2 knock-down in *in vivo*, the results obtained in this study need to be verified by a direct *in vivo* model, such as the chorioallantoic membrane (CAM) assay or the Matrigel plug assay in mice. The CAM-assay, however, would probably not allow for magnet guided transfection of SHP-2 AS-ODN or any other constructs, but would have to rely on inhibitors. Although it probably would be possible to use transfection techniques with the Matrigel plug assay, this could still be troublesome. We experienced difficulties in verifying the transfection of a Cy-3 labelled SHP-2 AS-ODN into the endothelium of the mouse aorts (used for the aortic ring sprouting model), due to auto-fluorescence originating from the mouse itself, and were therefore unfortunately unable to achieve a reliable transfection of the aortae with SHP-2 AS-ODN (data not shown).

Although we could show that SHP-2 is needed for endothelial cell survival and that SHP-2 involves the activation of the Raf-MEK-ERK and PI3-Kinase / Akt pathways, which are important for apoptosis prevention and induction of proliferation in endothelial cells, we have not investigated the exact mechanisms of SHP-2 involvement for these events. Therefore, to further confirm the influence of SHP-2 in the Raf-MEK-ERK and PI3-Kinase / Akt pathways in endothelial cells, it would be of interest to investigate whether SHP-2 affects the activation of proapoptotic proteins, such as Bad, Bax or Caspases, and even the transcription of anti-apoptotic genes as well as transcription of genes important for cell cycle progression.

4.5. THERAPEUTIC POTENTIAL AND FUTURE ASPECTS

The finding that SHP-2 is a positive regulator of angiogenesis may be of interest in the development of strategies aiming at controlling this process. For instance, in ischemic diseases these events are critical to allow for neovascularization of tissue that has suffered from either acute or chronic blood supply loss. An intact physiological function of the SHP-2 dependent regulation of the endothelial angiogenic response may be a prerequisite for the success of any strategy aiming to induce angiogenesis therapeutically. On the contrary, the rate limiting step in tumour development is the ability of the tumour to induce its own vascularisation [149]. Without sufficient blood supply not only is the growth of the tumour inhibited but also the tumours´ ability to spread to other sites is limited. A site directed inhibition of SHP-2, accomplishing an inability of tumour secreted angiogenic factors such as VEGF-A, bFGF and PDGF-BB to activate endothelial cells leading to proliferation and ultimately new vessel sprouting, may impair or even inhibit the tumour induced angiogenesis. This would dramatically reduce the risk of further tumour growth and spreading giving cancer patients a higher chance of survival.

5. SUMMARY

Angiogenesis is a vital process, which continues throughout life. It is dependent on a variety of growth factors, such as basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF-A) and platelet derived growth factor BB (PDGF-BB), which prevent endothelial cell apoptosis, induce endothelial cell proliferation and migration [2] as well as promote the organization of endothelial cells into tube-like structures followed by proper vessel sprouting [8]. The PI3-Kinase/Akt pathway [50,51] and the Raf-MEK-ERK signalling cascade [57] have been shown to be eminent for the angiogenic process. A tyrosine phosphatase, involved in the regulation of these pathways, is the Src homology 2 domain containing tyrosine phosphatase 2 (SHP-2) [72]. The role of SHP-2 in functional angiogenesis and in endothelial angiogenic signalling, however, is unknown. Using antisense oligonucleotide (AS-ODN) magnetofection, a technique where AS-ODNs coupled to superparamagnetic nanoparticles are delivered by magnetic force, or a pharmacological inhibitor of SHP-2 (Ptp I IV) we investigated the role of SHP-2 in angiogenic processes, such as endothelial cell survival, proliferation, tube formation and vessel sprouting as well as in VEGF-A, bFGF and PDGF-BB enhanced signalling in human microvascular- (HMEC) and umbilical vein endothelial cells (HUVEC).

Treatment with PtpI IV resulted in a delayed bFGF and PDGF-BB dependent wound healing response, as assessed by a scratch of a confluent cell layer. Only 52±7% and 42±5% (both $p<0.01$, n=3) of the wound incision was covered again after 48 hours after treatment with PtpI IV together with bFGF and PDGF-BB respectively. Not only basal proliferation, as assessed by MTT reduction, of HMEC was significantly inhibited (by 48±5%; $p<0.01$, n=12) but also bFGF (by 41±5%; $p<0.01$, n=12) and PDGF-BB (by 55±7%; $p<0.01$, n=12) dependent proliferation in SHP-2 AS-ODN treated cells as compared to nonsense (Random) oligonucleotides (Rdm ODN) treatment was impeded. This seemed to be due to enhanced apoptosis, because cell cycle analysis by propidium iodide staining and Annexin V staining revealed a 2.4-fold increase in cells detected in the subG0 fraction ($p<0.01$, n=6) and a significant rise in Annexin V positive cells (23±7%; $p<0.05$; n=8) following SHP-2 AS-ODN treatment compared to Rdm ODN treatment. Moreover, cells lacking a functional SHP-2 exhibited a lower number of capillary-like structures in proangiogenic Matrigel (SHP-2 AS-ODN: $p<0.01$, n=8; PtpI IV: $p<0.05$, n=3). In

addition, when treating aortic rings embedded in Matrigel with the pharmacological SHP-2 inhibitor, sprouting of new vessels from the aortic rings was impeded by 55±12% ($p<0.05$; n=10). Furthermore, SHP-2 knock-down inclined a decreased bFGF, PDGF-BB and VEGF-A induced phosphorylation of the PI3-Kinase regulatory subunit p85 (n=2-3). The bFGF and PDGF-BB induced Akt phosphorylation was also diminished (n=6 and n=5, $p<0.05$ and $p<0.01$ respectively), whereas the VEGF-A induced Akt activation remained unaffected (n=4). Furthermore, SHP-2 AS-ODN but not Rdm ODN transfection led to a diminished bFGF (n=7, $p<0.05$)-, PDGF-BB (n=5, $p<0.05$)- and VEGF-A (n=5, $p<0.05$) dependent phosphorylation of the MAP Kinase ERK 1/2 but not treatment with the protein kinase C stimulator PMA (n=4). Moreover, the growth factor induced ERK 1/2 and Raf-1 activation was suppressed by the PI3-Kinase inhibitor Wortmannin. Lastly, bFGF-, PDGF-BB- and VEGF-A stimulation of endothelial cells led to a direct interaction between SHP-2, p85 and the Grb associated binder-1 (Gab-1) (n=3).

Our results indicate that SHP-2 is necessary for processes involved in angiogenesis, such as endothelial cell survival, bFGF and PDGF-BB dependent endothelial wound healing *in vitro* and cell proliferation. Moreover, SHP-2 is involved in tube formation and vessel outgrowth. Finally, SHP-2 regulates angiogenic growth factor dependent activation of the PI3-Kinase/Akt pathway as well as the MAP kinase pathway in endothelial cells probably by forming a signalling complex with Gab-1 and PI3-Kinase. Thus, our findings may identify SHP-2 as a new angiogenic factor and might be of relevance in situations requiring the control of angiogenesis.

6. APPENDIX

6.1. ABBREVIATIONS

aFGF	acidic Fibroblast Growth Factor
Ang 1 & 2	Angiotensin 1 and 2
ALL	Acute lymphoblastic leukemia
AS-ODN	Antisense desoxynucleotide
bFGF	basic Fibroblast Growth Factor
CAM	Chorioallantoic membrane
cGMP	cyclic guanosine monophosphate
Csw	Corkscrew
Cy-3	Carbocyanin
2D/3D	Two dimensional/ three dimensional
DMSO	Dimethylsulfoxid
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EPO	Erythropoietin
ERK	Extracellular signal Regulated Kinase
FACS	Fluorescent-activating cell scanner
FAK	Focal adhesion kinase
FCS	Fetal Calf Serum
FGFR	Fibroblast Growth Factor Receptor
Flk	Fms-like tyrosine kinase
FRS-2	Fibroblast growth factor receptor substrate 2
Gab-1	Grb-2 associated binder 1
GM-CSF	Granulocyte macrophage colony stimulating factor
Grb2	Growth factor Receptor Bound protein 2
GTPase	Guanosine triphosphatase
HEPES	N-(2-Hydroxyethyl)piperazine-N´-(2-ethanesulfonic acid)
HMEC	Human microvascular endothelial cells
HUVEC	Human Umbilical Vein Endothelial Cells
IAPs	Inhibitor of apoptosis proteins
ICAM-1	Intercellular adhesion molecule 1
IDDM	Insulin dependent diabetes mellitus
IGF	Insulin like growth factor
IKK	Inhibitor of nuclear factor kappa-B kinase
IL	Interleukin
IRS-1	Insulin receptor substrate 1
JMML	Juvenile myelomonocytic leukemia
kDa	Kilo Dalton
KDR	Kinase-insert Domain-containing Receptor
LS	LEOPARD syndrome
MAPK	Mitogen Activated Protein Kinases
MAPKK	Mitogen activated protein kinase kinase
MAPKKK	Mitogen activated protein kinase kinase kinase
MEK	MAPK/ERK kinase
Met	Methionine
MTT	Methylthiazoletetrazolium
NBCS	Newborn Calf Serum
NFκB	Nuclear factor kappa-B

NIDDM	Non insulin dependent diabetes mellitus
NO	Nitric oxide
NS	Noonans syndrome
PAEC	Porcine endothelial cells
PAK	p21 activated kinase
PBS	Phosphate Buffered Saline
PDGF	Platelet Derived Growth Factor
PDGFR	Platelet derived growth factor receptor
PDK1 & 2	3-phosphoinositide-dependent protein kinase 1 & 2
PECAM-1	Platelet endothelial cell adhesion molecule 1
PEI	Polyethylenimine
PI	Propidium iodide
PI3-Kinase	Phosphoinositide 3-Kinase
$PI(3,4,5)P_3$	phosphatidylinositol-3,4,5-triphosphate
$PI(4,5)P_2$	phosphatidylinositol-4,5-triphosphate
PKA	Protein kinase A
PKC	Protein Kinase C
$PLC\gamma$	Phospho Lipase Cγ
PMA	Phorbol-myristate-acetate
pNPP	p-Nitrophenyl phosphate
PTGS	Posttranscriptional gene silencing
PTK	Protein tyrosine kinase
PTP	Protein tyrosine phosphatase
Ptp I IV	Protein tyrosine phosphatase inhibitor IV
Ptpn11	Gene coding for SHP-2
pTyr	Phosphorylated tyrosine
Rdm ODN	Random desoxynucleotide
RISC	RNA Induced Silencing Complex
RNAi	RNA interference
SDS	Sodeum dodecyl sulfate
siRNA	short interfering RNA
SH3	Src- homology 3
SHP-1	Src homology 2 domain containing tyrosine phosphatase 1
SHP-2	Src homology 2 domain containing tyrosine phosphatase 2
Sos	Son Of Sevenless
TGFβ	tumour necrosis factor β
u-PA	urokinase-type plasminogen activator
VE-Cadherine	Vascular endothelial Cadherin
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
VPF	Vascular permeability facto
VVO	Vesiculo-vacuolar organelles
Y542/Y580	Tyrosine residue in position 542/580 respectively

6.2. PUBLICATIONS

Part of this work is published or are in preparation for publication

Poster

SHP-2 as a regulator of endothelial angiogenic signalling
H.Bridell, C. Plank, T. Gloe, H.Y Sohn, U. Pohl, F.Krötz
Poster presentation at the Annual Meeting of Gesellschaft für Mikrozirkulation und Vaskuläre Biologie 2006, Munich, Germany

Lectures

Inhibition of SHP-2 prevents human endothelial cell proliferation by inducing apoptosis
H. Bridell, F. Krötz, C. Plank, T.Gloe, H.Y Sohn, U.Pohl
Young investigator award session at the Annual Meeting of Gesellschaft für Mikrozirkulation und Vaskuläre Biologie 2004, Berlin, Germany

Inhibition of SHP-2 prevents human endothelial cell proliferation and induces apoptosis
H. Bridell, C. Plank, T.Gloe, H.Y Sohn, U.Pohl, F. Krötz
84. Jahrestagung der Deutschen Physiologischen Gesellschaft, Göttingen, Germany
(Abstract published in the European Journal of Physiology, 2005, 449 (1))

Inhibition of SHP-2 prevents bFGF-dependent endothelial cell proliferation and induces apoptosis
H. Bridell, C. Plank, T.Gloe, H.Y Sohn, U.Pohl, F. Krötz
Joint International Meeting of The Physiological Society and FEPS 2005, Bristol, United Kingdom

SHP-2 is involved in bFGF dependent endothelial cell proliferation and prevents apoptosis
H. Bridell, C. Plank, T.Gloe, N. Hellwig, H.Y Sohn, U.Pohl, F. Krötz
3rd European Meeting on Vascular Biology and Medicine 2005, Hamburg, Germany
(Abstract published in the Journal of Vascular Research, 2005, 42, S2)

Original publications

Role of SHP-2 in FGF-2 dependent endothelial angiogenic signalling
H. Mannell, T. Gloe, C. Plank, H.Y Sohn, U. Pohl, F. Krötz
Submitted for publication in Ateriosclerosis, Thrombosis, and Vascular Biology

The Role of SHP-2 in Cell signalling and Disease
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Submitted for publication in Current Enzyme Inhibition

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Acknowledgements

I would like to express my gratitude to **Professor Ulrich Pohl** for giving me the opportunity to perform my PhD dissertation at his institute, for always taking the time to listen to new ideas and problems regarding the lab work and for critical reading of manuscripts as well as for making sure you feel appreciated as a PhD student.

I would like to thank **Professor Ernst Wagner** for taking me on as an external PhD student, thereby enabling the dissertation at the Ludwig-Maximilians-Universität in Munich.

My deepest thanks to **Dr. Florian Krötz** for supporting me and my work through these years, also during and after my pregnancy; for helpful discussions, suggestions and ideas of how to improve my work, for critical reading of manuscripts and last but not least for letting me share his wisdom.

I would also like to thank **Dr. Christian Plank** for giving me the opportunity to stay with the research after I finished my diploma thesis at his lab at the department of Experimental Oncology and Therapy Research at TU, Munich.

Moreover, I am very grateful to the following persons: I am in depth to **Dr. Torsten Gloe** for all the valuable help during my work, for always listening to and always having suggestions of how to solve problems regarding the lab work and experimental set ups. I would like to thank the members of the Krötz/Gloe laboratory including **Kasia Stefanovski, Dorothea Gössel** and **Britta Bscheider** for valuable technical assistance, not the least during my pregnancy when I was not allowed to perform certain lab work due to the circumstances, and for their support during my time in the lab. A special thank to **Nicole Hellwig** with whom I not only shared the office but also who supported me in good and bad times, helped me with valuable experiments for my publication when I was on maternity leave and had fruitful discussions about lab work and so many other things! A big thanks to all the other PhD- and doctoral students **Christina Klarskov, Ronald Gerstl, Carsten Hanft, Barbara Engelbrecht**, **Lena Struthmann** and **Joachim Pirchner** at the Institute of Physiology for good times, nice “Stammtisch evenings” and for valuable exchange of information regarding experiments in the lab. My thanks also go to all the members of the Physiological Institute, who contribute to a relaxing and pleasant working climate and who do not hesitate to help at any time. Finally, I would like to express my deepest gratitude to **my family**, who supported me during these years and who never stopped believing in me. A very special thanks to my husband, who supported me, helped me with the design of figures and schemes and last but not least helped me all those late nights and weekends by taking care of our little daughter so that mummy could work!!