Myofibroblasts and the Vascular Endothelium

Impact of Fibrin Degradation Products and miRNA on Vascular Motility and Function

PEDER FREDLUND FUCHS
Angiogenesis is the formation of new blood vessels from pre-existing vasculature and is important during development as well as wound healing and tissue remodeling. Angiogenesis also occurs during pathological conditions such as diabetic retinopathy and cancer. This thesis is centered on the biology of endothelial cells, lining the blood vessels, and myofibroblasts, important for wound healing.

We investigated an endothelial cell specific gene, ExoC3l2, and its role in VEGFR2 signaling and migration. EXOC3L2 co-localize with members of the exocyst complex, involved in vesicular transport, as well as VEGFR2. Reducing the level of EXOC3L2 in microvascular endothelial cells results in reduced VEGFR2 signaling and subsequently reduced chemotactic response to VEGF-A.

MicroRNA (miRNA) have been shown to be regulators of gene transcription and cell type specific miRNAs have been identified. We investigated two miRNAs, miR-145 and miR-24. miR-145 overexpression reduced chemotaxis in both fibroblasts and endothelial cells, as did suppression of the endogenous miR-145 level in fibroblasts. miR-24 in contrast is expressed by endothelial cells and are able to target Ndst1, important for heparan sulfate (HS) sulfation. Sulfation of HS is important for many processes, amongst them growth factor signaling. Overexpression of miR-24 resulted in lower sulfation of HS chains, decreasing the ability of HS to interact with VEGF-A. Overexpressing miR-24 resulted in disturbed chemotaxis, similar to suppressing Ndst1 using siRNA.

Myofibroblast recruitment is an important step in wound healing. The myofibroblasts contract the wound, synthesize new extracellular matrix and contribute to revascularization by looping angiogenesis. Maturation from resting fibroblast to myofibroblast is dependent on TGF-β. We found that fibrin fragment E (FnE), a degradation product of fibrin, potentiated the response of fibroblasts to TGF-β thus enhancing TGF-β-induced myofibroblast differentiation. FnE was also found to influence the migration of fibroblasts. These responses are dependent on integrins and toll-like receptors.

These findings may serve to further increase the understanding of angiogenesis and wound healing to develop new therapies against pathological conditions.

**Keywords:** Angiogenesis, Vascular biology, Chemotaxis, Endothelial cell, Myofibroblast, Wound healing, miRNA, Heparan sulfate

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ISSN 1651-6206
ISBN 978-91-554-8622-8
urn:nbn:se:uu:diva-196884 (http://urn.kb.se/resolve?urn=nbn:se:uu:diva-196884)
“We live on a placid island of ignorance in the midst of black seas of infinity...”

— H.P. Lovecraft, The Call Of Cthulhu
List of Papers

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


IV Fredlund Fuchs, P., Kreuger, J., Gerwins, P. (2013) Fibrin fragment E stimulates fibroblast migration and enhances TGF-β induced myofibroblast differentiation through an integrin β3-dependent activation of Toll-like receptor 4. *Manuscript*

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Abbreviations

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<tbody>
<tr>
<td>αSMA</td>
<td>Alpha smooth muscle actin</td>
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<tr>
<td>ALK</td>
<td>Activin receptor-like kinase</td>
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<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
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<td>CS</td>
<td>Condroitin sulfate</td>
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<td>EB</td>
<td>Embryoid body</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<td>EMT</td>
<td>Endothelial-mesenchymal transition</td>
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<tr>
<td>ERK</td>
<td>Extracellular signal-related kinase</td>
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<td>FDP</td>
<td>Fibrin degradation products</td>
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<td>FgnE</td>
<td>Fibrinogen fragment E</td>
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<td>FnD</td>
<td>Fibrin(ogen) fragment D</td>
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<td>FnDD</td>
<td>Fibrin fragment DD (D-dimer)</td>
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<td>FnE</td>
<td>Fibrin fragment E</td>
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<tr>
<td>FpA/B</td>
<td>Fibrinopeptide A or B</td>
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<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
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<tr>
<td>HA</td>
<td>Hyaluronan</td>
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<tr>
<td>HS</td>
<td>Heparan sulfate</td>
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<tr>
<td>LAP</td>
<td>Latency-associated peptide</td>
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<tr>
<td>LTBP</td>
<td>Latent TGF-β binding protein</td>
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<tr>
<td>miRNA</td>
<td>MicroRNA</td>
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<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>PDGF-BB</td>
<td>Platelet derived growth factor BB</td>
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<tr>
<td>PG</td>
<td>Proteoglycan</td>
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<tr>
<td>RISC</td>
<td>RNA induced silencing complex</td>
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<td>TGF-β</td>
<td>Transforming growth factor beta</td>
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<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>Smad</td>
<td>Sma/Mad (Small/Mothers against decapentaplegic) homologue</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<tr>
<td>VEGFR</td>
<td>Vascular endothelial growth factor receptor</td>
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<tr>
<td>VSMC</td>
<td>Vascular smooth muscle cell</td>
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</table>
Introduction

A functional vascular system is a necessity for all organisms that are too large to be supplied with oxygen and nutrients by diffusion. The macroscopic structure of the human vascular system has been known for a long time, as exemplified by the illustrations of the blood vasculature by Andreas Vesalius’[1] (Figure 1) and Thomas Bartholin’s[2] and Olof Rudbeck’s [3] investigations of the lymphatic vasculature in the 17th century. The microscopic and molecular aspects have in the last two decades created a great interest and been the subject of numerous investigations due to its involvement in physiological and pathological processes. Not only is the body dependent on the function of the vascular system but also tumors rely on it for their growth and metastatic spread. This thesis investigates different biological aspects of the vascular system with emphasis on angiogenesis and tissue stroma with importance for wound healing, tumor growth and fibrosis.
The human blood vascular system consists of two circulatory systems, the pulmonary and the systemic circulation. In the pulmonary system blood is pumped from the right ventricle of the heart to the lungs for oxygenation and removal of carbon dioxide, finally ending up in the left ventricle. The systemic circulation starts in the left ventricle, from where blood is pumped throughout the body, supplying oxygen and nutrients and removing carbon dioxide.
dioxide. The bloodstream also carries hormones, growth factors and components of the immune system.

Blood vessels are divided into arteries, veins and capillaries. They are all lined with a layer of vascular endothelial cells on the luminal side. The endothelial layer rests on a basement membrane that is composed mainly of collagen IV, laminin, nidogen and heparan sulfate proteoglycans such as perlecan.[4] Endothelial cells are surrounded by supporting cells that in capillaries are a single layer of pericytes and in larger vessels several layers of vascular smooth muscle cells (VSMC). Arteries are more muscular and have a thicker layer of VSMCs as compared to veins. This allows the arteries to regulate blood flow and to maintain pressure and blood flow during diastole.

The lymphatic system is a part of the vascular system but in contrast is open ended. It is believed that the two vascular systems have a common origin and that during early development cells migrate out from the cardinal vein to form lymphatic structures called lymph sacs. The reason for this migration and transdifferentiation is believed to be a gradient of Vascular endothelial growth factor (VEGF)-C produced by nearby mesenchymal cells.[5] The blood and lymphatic systems are usually found close to one another in the tissue, which is easily understandable given their involvement in tissue maintenance and their common origin. On a structural level lymphatic vessels are similar to blood vessels with an inner cell layer composed of lymphatic endothelial cells that are covered by supporting cells. They however lack the surrounding thicker smooth muscle layers and are dependent on the action of nearby skeletal muscles to pump the lymphatic fluid. Intercellular connections between lymphatic endothelial cells are not as tight as those in blood vessels, which allows draining of tissues of excess fluid. Impairment of lymphatic drainage in a tissue causes swelling known as edema. Lymphatic fluid is returned to the blood circulation through a connection to the venous system at the confluence of the internal jugular veins and the subclavian veins. The lymphatic system plays an important role for the immune system facilitating transport of immune cells and it also harbors lymph nodes where antigen presentation and B-cell responses are modulated. Lymphatic vessels also transport fatty acids derived from food digestion and deliver them to the blood.

**Angiogenesis**

Growth of the embryo and the cellular need for oxygen cannot be supported by diffusion of oxygen alone and at a certain point a functional circulatory system is required. Formation of the first vascular plexus occurs through a process called vasculogenesis whereby angioblasts create clusters of vascular endothelial cells that form tube like structures. Vessels then sprout from this primary vascular plexus into the surrounding tissues which results in
formation of a closed system of blood vessels.[6] This formation of new branches from existing vessels is called angiogenesis.

Sprouting angiogenesis is one of the most investigated forms of angiogenesis although there are still unanswered questions regarding this form of neovessel formation. Classically, a tip cell that directs the sprout heads the sprouting vessel. It has lately also been proposed that the tip cell is not absolute and that different cells at the edge of the sprout compete for the role of tip cell in a dynamical fashion.[7] Sprouting is initiated with an endothelial cell receiving a cue, for instance in the form of a gradient of a growth factor like vascular endothelial growth factor A (VEGF-A). The reacting endothelial cell will respond by degrading the basement membrane and migrate out into the surrounding tissue while still maintaining contact with other cells in the vessel. As the sprout grows the stalk cells divide in order to allow the sprout to elongate. When sprouts meet they fuse to finally form a single lumenized vessel.

A second mechanism of neovessel formation is intussusceptive angiogenesis, which can be viewed as splitting a vessel longitudinally. This form of angiogenesis will in the end yield two parallel vessels from a single original. Intussusceptive angiogenesis is initiated by mural cells that start to form an invagination by pushing down through the vessel. Once the endothelial cells make contact with the endothelial cells on the other side they will rearrange into two separate but smaller vessels.

Recently a third mechanism of angiogenesis was described in healing wounds where functionally perfused vessels are pulled into the wound by mechanical tension that is created by myofibroblasts during wound contraction. This form of angiogenesis has been termed looping angiogenesis. Recruitment of functionally perfused vascular loops by vessel translocation has the advantage of establishing circulation fast in contrast to sprouting angiogenesis where blunt ended sprouts have to fuse with another sprout or vessel before perfusion is established. In looping angiogenesis there is no initial need for proliferation of mural and endothelial cells to allow vessel expansion. Instead redistribution of existing endothelial and mural cells from the growing pre-existing vessels initially allows elongation of pre-existing vessels and cell proliferation will only be necessary at later time points to allow neovessels to extend over larger distances. Looping angiogenesis does not exclude sprouting or intussusceptive angiogenesis but instead these different mechanisms might be complementary.[8]

Clotting

Clotting or coagulation of blood is an important mechanism to stop bleedings from injured vessels. Coagulation can be initiated by two pathways; the intrinsic and the extrinsic (Figure 2). The intrinsic pathway is initiated upon
blood contact with a negatively charged surface[9] (such as glass[10]) whereas the extrinsic pathway is initiated by tissue factor[11] that is produced by cells in connective tissue. Initiation of either of these pathways results in activation of coagulation cascades that converge with activation of Factor X generating Factor Xa which in turn converts prothrombin into thrombin together with Factor Va. Thrombin is a protease that converts circulating fibrinogen to a network of fibrin that is also cross linked to form a covalently bound matrix of fibrin molecules. This tight mesh-like matrix together with platelets constitute most of the early thrombus[12], a homeostatic plug which prevents bleeding and excessive fluid leakage.

There is a fine balance of pro- and anti-coagulant factors in the blood. Both positive and negative feedback mechanisms ensure that coagulation is only initiated when needed. Intravascular coagulation is a dangerous condition in which damage to the endothelium of vessels results in thrombus formation. As time progresses this clot can grow further which restricts blood flow or it can detach from the vessel wall and block smaller vessels as it travels along the vascular tree.

![Figure 2. Simplified illustration of the blood coagulation cascade. The intrinsic pathway starts with surface activation whereas tissue factor is the initiator of the extrinsic pathway. Both pathways converge at the generation of Factor Xa and result in the generation of a cross-linked fibrin clot. Note that there are many positive and negative feedback loops not shown here, for instance thrombin’s role in activating Factors VIII and V. Thrombin also activates inhibitors of the cascade. Thick arrows indicate activation of factors, thin arrows indicate activity upon another factor.](image-url)
Fibrinogen and Fibrin

Fibrinogen is a 340 kDa glycoprotein produced in the liver that circulates in the blood. The concentration of fibrinogen in the blood of healthy human individuals are usually in the range of 2-4.5 mg/ml.[13] Fibrinogen consists of six polypeptide chains, linked by in total 16 disulfide bonds. Fibrinogen is composed of two identical aggregates each containing one α-, β- and γ-chain, arranged in a manner so the N-terminals of all chains are collected in the central region of the molecule[14] which makes up the E-domain that is flanked on both sides by a D-domain. The structure of human and chicken fibrinogen can be seen in Figure 3. Despite only ~60% sequence homology between human and chicken fibrinogen the overall protein structure is very similar, with the E-domain showing a higher degree of homology than the C-terminal ends.[15]

Fibrinogen is converted to fibrin upon thrombin-mediated cleavage of N-terminal region of the α- and β-chains. This cleavage generates fibrinopeptide A (FpA) and B (FpB), originating from the α- and β-chains respectively. Releasing these 16 (FpA) and 14 (FpB) amino acid residue peptides results in an aggregation of the fibrin monomers. This aggregation is arranged in an overlapping fashion, one E-domain interacting with two D-domains from two adjacent fibrin molecules. Factor XIIIa, that is generated from circulating Factor XIII by thrombin activity, crosslinks the γ-chains of two adjacent fibrin monomers creating a fibrin polymer chain[16] and these chains branch and form a mesh like structure that capture blood cells that creates the clot.

The fibrin molecule contains numerous sites for interaction with growth factors such as fibroblast growth factor 2 (FGF-2)[17], VEGF-A[18] and interleukin-1β (IL-1β) [19]. Fibrin also contains specific binding sites for several cell types, the most well known being platelets primarily via the αIIbβ3 integrin[20], but also for other cell types via among others the αVβ3, αXβ2 and αMβ2 integrins[21].
Figure 3. “Top” view of the crystal structure of human (top) and chicken (Gallus gallus, bottom) fibrinogen with fibrinoeptide A and B projecting outward from the image. The central E-domain is flanked by D-domains. α-chains depicted in green, β-chains in blue and γ-chains in red. Adapted from Kollman et al. Biochemistry 2009 48(18), 3877-3886[22].

Fibrin degradation products

During the wound healing the fibrin clot is degraded and the void filled with granulation tissue. One of the major enzymes that digest fibrin is plasmin. Plasmin is generated from circulating plasminogen primarily by tissue plasminogen activator (tPa) activated in the wound area[23]. Plasmin cleaves fibrin (as well as fibrinogen) in the region between the E- and D-domains and also generates further truncation of the β-chain, removing the β15-42 region.[24] Plasmin cleavage generates two distinct types of fibrin degradation products (FDP), the ~50 kDa fibrin fragment E (FnE) generated from the central E-domain and the ~180 kDa D-dimer (FnDD) consisting of two cross-linked D-domains.[14, 24, 25] Clinically D-dimers are used as a diagnostic tool to detect e.g. deep venous thrombosis[26] and pulmonary embolism[27]. Plasmin digestion of fibrinogen generates fibrinogen fragment E (FgnE) closely resembling FnE, however since thrombin has not acted on this fragment FpA is still present.[24] Since soluble fibrinogen is a monomer, plasmin cleavage of fibrinogen results in generation of a D-fragment (FnD/FgnD) rather than a dimer.

It has been proposed that FDPs are not only degradation products but also have biological activities. In vitro studies have shown that FnE regulates migration [28-30], proliferation and differentiation[30] in the nM range using endothelial cells[30] and vascular smooth muscle cells[28, 29, 31, 32]. Both FnE and FnD have been shown to inhibit ingrowth of smooth muscle cells into fibrin gels.[31] FnE has also been shown to induce apoptosis in the μM-mM range[30, 33]. Interestingly FgnE does not have the FnE specific apoptosis inducing effect[33] and has been suggested to have an inhibitory effect on migration and tubule formation[34] as well as migration of vascular endothelial cells[34] in vitro. In vivo treatment of guinea pigs with an anti-
FnE antibody caused regression of line-10 hepatoma tumors implanted in the dermis.[35]

**Fibroblast and myofibroblasts**

Fibroblasts are cells of mesenchymal origin dispersed throughout tissues of the body. These cells can be activated and further differentiated into myofibroblasts. In their resting state fibroblasts are mainly participating in production and modulation of extracellular matrix (ECM) components such as collagen which contributes to tissue homeostasis.[36] Embedded in the ECM they are shielded from mechanical stress.[37] When the tissue is damaged this shielding is lost and fibroblasts are exposed to mechanical tension, which has been shown to mediate conversion of fibroblasts into activated fibroblasts so called protomyofibroblasts.[38, 39] The entire process of myofibroblast differentiation is not fully understood, but transforming growth factor-β1 (TGF-β1) has been shown to induce myofibroblastic characteristics in fibroblast in vitro.[40] Myofibroblasts are characterized by alpha smooth muscle actin (αSMA) expression that confers contractile properties to the cell and also by the presence of gap junctions. Gap junctions are contacts between cells containing small pores that allow passage of molecules.[41] Neither gap junctions nor stress fibers are found in resting fibroblasts in vivo,[42], but stress fibers lacking αSMA can be seen in cultured fibroblasts.[43] Myofibroblast differentiation appears to be a gradual process in which the activated fibroblasts not yet incorporating αSMA into their stress fibers are called proto-myofibroblasts.

While it is believed that fibroblasts are the main source of myofibroblasts[44] it is possible that other cells like vascular smooth muscle cells and pericytes can acquire myofibroblast like properties. It has also been suggested that circulating fibroblast precursor cells, known as fibrocytes, can end up at the injury site and differentiate into fibroblasts and myofibroblast. Markers for circulating fibrocytes include CD34, CD45 as well as vimentin and collagen I and III.[45] The myofibroblasts are highly contractile due to their expression of αSMA and they also interact tightly with the surrounding ECM by focal adhesions were the actin cytoskeleton is connected to the ECM via integrins.[46] Because of theses focal adhesions and αSMA stress fibers myofibroblasts have the ability to contract the wound[47]. Platelet derived growth factor (PDGF) BB is an important chemotactic factor for myofibroblast recruitment and by blocking PDGF receptor signaling myofibroblast recruitment can be inhibited.[48]
Wound healing

Activity in the wound after clot formation is high, with multiple cell types acting to facilitate healing. The initial activation of platelets and leucocytes results in release of growth factors that stimulate ingrowth and differentiation of proto/myofibroblasts into the wound area a few days after injury.[49] Myofibroblasts are important for reconstituting damaged tissue and it has been suggested that myofibroblasts mediate revascularization and formation of granulation tissue. This allows rapid formation of functionally perfused vessels. In contrast sprouting angiogenesis is dependent on the relatively slow process of cell migration and proliferation and blunt ended sprouts need to fuse with another vessel before circulation is established. It is therefore likely that looping angiogenesis is responsible for a major part of revascularization while intussusceptive and sprouting angiogenesis serve to fine tune the vascular network.[8]

In some instances the wound does not heal in a correct way. This can be the result of excessive proliferation, defective migration and overactive myofibroblasts that deposit excessive amounts of ECM. This leads to excessive scarring and potential keloid formation which results in a tissue with less flexibility and tensile strength.[49] Keloid is a type of scar expanding past the borders of the original injury.[50] It is therefore important that once the myofibroblasts have served their purpose they are dedifferentiated into resting tissue fibroblasts or removed by apoptosis.

Although important myofibroblasts are neither the only nor the first cells recruited to a wound site. As mentioned previously platelets are the initial source of growth factors, releasing e.g. PDGF and TGF-β. Cells following in quick succession are components of the innate immune system such as neutrophils and monocytes. These cells do not only sustain production of factors such as VEGF-A, PDGF-BB and TGF-β but also remove foreign elements such as bacteria from the wound. Re-epithelialization starts shortly after injury and is needed in order to restore the protective barrier against foreign material and organisms.[49]

Cancer

As the average life span increases so do cancer incidence and the World health organization (WHO) predicts that the increase will continue.[51, 52] Cancers show great heterogeneity[53] and depending on their origin they are dependent mostly on environmental factors but also genetic factors contribute. Solid tumors are not able to grow larger than approximately 1-2 mm in diameter without recruiting blood vessels to support further growth.[54] The transition from an avascular to a vascularized tumor is called the angiogenic switch. This process, although thoroughly studied, is still not fully understand-
stood. What is clear, however, is that vessels of the tumor often display aberrant morphology[55] with poor function. Tumor vessels are leaky[56], most likely a result of the chaotic environment they exist in where they are often exposed to high levels of different growth factors like VEGF-A which is a potent inducer of vascular permeability.

Cancers originate from most cells in the body and their classification is dependent on their origin, e.g. sarcoma is formed by cells of mesenchymal origin while carcinoma originates from epithelium. Initiation of cancer growth is thought to be a result of mutations distorting cell cycle control, sensitivity to apoptosis, signaling[57, 58] and immortalization[59]. Epithelial-mesenchymal transition (EMT) is essential during development of multicellular organisms, however this process is believed to be an important event for metastatic potential and infiltration capacity for tumors of epithelial origin. The normal epithelium rests on the basement membrane and will not degrade and infiltrate the underlying matrix under normal conditions. During the process of EMT epithelial cells gradually acquire the phenotype of a mesenchymal fibroblast like cell.[60] Due to these newly acquired abilities they are able to move through the tissue and if they have undergone cancerous transformation may extravasate into a blood vessel and travel to distant parts of the body to initiate metastasis.[53] TGF-β is implicated as a major factor in EMT; the involvement is, however, dual in nature. At early stages TGF-β inhibits tumor formation whereas at later stages it promotes tumor growth.[61]

Tumors do not only consist of cancer cells, but also inflammatory cells and fibroblast or myofibroblasts. The latter often called cancer-associated fibroblasts (CAFs) or tumor associated fibroblasts (TAFs). CAFs are ill defined and lack molecular markers to cover all CAFs. Similar to myofibroblasts, αSMA is often used for identification, however, there are subpopulations that do not express αSMA.[62] The origin of the CAFs is not a single cell type as evidence of CAFs originating from fibroblast[63], bone marrow derived[64] or even of epithelial[65] or endothelial[66] cells have been presented. The ECM found in and around solid tumors is often highly dense and fibrotic in nature due to an overproduction of ECM components by the CAFs.[67] CAFs are also producers of growth factors such as TGF-β, VEGF, EGF and FGF believed to contribute to tumor growth.[62, 67] The poorly functioning vasculature causes a leakage of fibrinogen resulting in a fibrin deposition associated with the tumor[68]. Parallels have because of these reasons been drawn between tumors and wounds in the past and tumors have been referred to as “wounds that do not heal”.

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Cell migration and chemotaxis

The ability of a cell to migrate is of paramount importance in many processes in the body, especially during embryonic development during the establishing of organs and tissues.[69, 70] The importance of this process however does not stop with developmental processes, but is also critical during e.g. wound healing and immune responses. It is also a main feature associated with cancer growth and metastatic spread and in EMT. It is believed that cancer cells that possess high motility and ability to degrade the ECM can leave the original tumor, extravasate into the vasculature and establish a metastasis at a remote location.[71]

The ability to physically move alone is not enough for most of these processes; the cells also need to know where to go. The process of directed migration towards a soluble factor is known as chemotaxis[72] whereas the migration toward an increasing concentration of an immobilized factor is known as haptotaxis[73]. Some factors are also chemokinetic, which is to say that they increase the overall motility of the cell.

How cells migrate in the body is not fully understood and different types of cells might utilize different modes of action when they migrate. The two major models of cell migration are described by the membrane flow model and the actin-remodeling model. In the membrane flow model[74], vesicles are continuously shunted from the rear to the leading edge (front in the direction of migration) of the cell. This trafficking will expand the membrane at the front and push the cell forward. The actin-dependent model is based on the contractility and flexibility of the actin cytoskeleton. In this model actin fibers are polymerized at the front of the cell and depolymerized at the rear and sides creating a force forward. [75]

The exocyst complex

In order for the cell to function properly it needs to be able to shuttle proteins to and from the plasma membrane. Internalization of growth factor receptors after ligand binding is commonly seen and newly synthesized membrane bound proteins also need to reach the outside of the cell in order for the cell to function properly. These proteins are transported from the Golgi in secretory vesicles. In order for the delivery of vesicles to function in an ordered fashion it needs to dock with appropriate proteins located at the membrane.[76] The vesicle is transported along the actin cytoskeleton toward its destination. Associated with the vesicle are six members (EXOC2-6 and 8) of the exocyst complex, two other (EXOC1 and 7) are associated with e.g. members of the Rho family at the membrane.[77, 78] The assembly of the full hetero-octameric complex, the exocyst complex, at the membrane allows v-SNAREs (on the vesicle) and t-SNAREs (on the membrane) to come into
contact resulting in the fusion of the vesicle with the membrane[79]. Mutations in exocyst complex components may disturb the assembly of the SNARE complex.[80] Despite the name the exocyst is not a singular complex as its components may vary for different vesicles.[81] In addition to the exocysts role in shuttling post-Golgi vesicles it appears to be involved in recycling of endosomes.[82] The importance of the exocyst has been shown in cell migration, where it accumulates at the leading edge[83], as well as in establishing of cell polarity[84] and in the process of tumor metastasis/invasion[85]. Although the exocyst complex is important for the polarity of cells it is not necessary for all types of vesicle transport and exocytotic events.[84, 86]

MicroRNA

MicroRNAs (miRNA) are short (~18-25 nucleotides) RNA oligonucleotides that are believed to be important for modulation of translation. miRNAs were first discovered in the form of a small RNA derived from the lin-4 gene of Caenorhabditis elegans.[87] miRNAs are found intragenic, as is the case of most mammalian miRNAs, or intergenic often clustered with a single promoter driving transcription of several miRNAs. Whether found inter- or intra-genically the miRNA is generated by sequential excision from larger RNA transcripts. The first step in miRNA biogenesis (Figure 4) is the excision of a shorter pre-miRNA from the primary miRNA (pri-miRNA) transcript (in the case of intergenic miRNAs) or the host transcript (if found intragenically). The enzyme excising the pre-miRNA from the pri-miRNA or host transcript is called Drosha.[88] If found intragenically the miRNA excision may occur before or after splicing of the mRNA transcript.[89] The product of Drosha cleavage is the stem-loop structured pre-miRNA that is further truncated into a smaller double stranded RNA by Dicer[90, 91]. One strand of this miRNA duplex is then loaded onto the RNA induced silencing complex (RISC)[92]. The miRNA together with RISC inhibit translation of transcripts to which it binds. The binding sites are most often found in the 3’ untranslated region (UTR) in animals but may be located throughout the entire transcript in plants.[93] miRNAs are able to decrease the amount of protein produced even without lowering the level of the corresponding mRNA.[87] The mechanism of translational repression is unclear but certain transcripts are also degraded through the slicing endonuclease activity of the RISC.[94] It is believed that the level of complimentarily to the target transcript is what decides if translation is suppressed or the transcript is sliced, however total complementarily is not needed for slicing to occur. The action of the miRNA is dependent on the RISC, the same machinery involved in small interfering RNA (siRNA) mediated silencing of gene expression. In contrast to siRNAs that target the same transcript from which itself is gener-
ated, miRNAs are targeting transcripts of other genes. Some mRNA transcripts may harbor target sites for several different miRNAs as well as multiple target sites for single miRNAs. A particular miRNA may also have targets in many different mRNAs.

Figure 4. miRNA biogenesis. Transcription of inter or intergenic miRNA by RNA polymerase II. Drosha excises the pri-mRNA, which is then exported to the cytoplasm and further truncated by Dicer into a mature miRNA. One strand is incorporated into the RISC and localizes target sequences in a mRNA transcript, inhibiting translation or promoting cleavage of the transcript.

It has recently been shown miRNA produced in one cell can be delivered to other cells in exosomes or other micro vesicles [95] and gap junctions[96, 97]. This ability to deliver miRNA to other cells in the proximity or distally through the blood is believed to be involved in the development of cancer.
miRNAs are also dysregulated in many cancers and there appears to exist both oncogenic[98] and anti-oncogenic[99] miRNAs. Members of the same miRNA clusters have been implicated to act both in an oncogenic and anti-
oncogenic fashion depending on the cell type in which they are expressed.[100, 101] Dysregulation of miRNAs in cancers is proposed to occur both through overexpression of miRNAs targeting tumor suppressor genes and by down regulating miRNAs targeting oncogenes.[102] It has recently been shown that certain cancer cells take advantage of nearby immune cells by producing exosomes containing miRNA triggering a response via Toll-like receptors leading to production of cytokines promoting cancer growth and metastatic potential.[103]

Proteoglycans and glycosaminoglycans

Proteoglycans (PGs) are macromolecules composed of a core protein with covalently attached glycosaminoglycan (GAG) chains. They can be found throughout most tissues and on virtually every cell in vertebrates, their type and composition however are diverse. The most studied forms of PGs are heparan sulfate (HS) PGs, chondroitin sulfate (CS) PGs and dermatan sulfate (DS) PGs, but there is also other types such as keratin sulfate (KS) PGs.

The saccharide structure of the HS and CS GAG chains are an alternating sequence of hexosamine, D-glucosamine (GlcN) in HS or D-galactosamine (GalN) in CS, and hexuronic acid, either L-iduronic acid (IdoA) or D-glucuronic acid (GlcA). Depending on the major type of hexuronic acid in the GalN containing PGs it can be referred to as CS, with a majority of GlcA, or DS (formerly called CS-B) containing more IdoA. [104]

Synthesis of HS/CS GAG chains is initiated by formation of a linkage region covalently bound to the core protein. The tetrasaccharide linkage region has the same sequence composition in both HS and CS, consisting of xylose-
galactose-galactose-glucuronic acid. Priming of the linkage region for synthesis of either HS, initiated by addition of N-acetylglucosamine by glyco-
syltransferases of the EXTL family[105], or CS, initiated by addition of β-N-
acetylglactosamine by the CSGALNAC-transferase, determines what spe-
cies of GAG will be synthesized at a given position. A single core protein can carry both HS and CS/DS chains, although it appears that there is a prefer-
ence for a particular type of GAG at a given position CS/DS can take the place of HS, if HS synthesis is impaired or disrupted. Elongation of the HS
chain is carried out by the EXT1 and EXT2 co-polymerases, adding alternating GlcN and GlcA, whereas chondroitin synthase[106] is responsible for the elongation of the CS chain.

Modification of the HS GAG-chains is carried out sequentially by N-
deacetylase/N-sulfotransferases (NDSTs)[107], adding a sulfate group on the
N-position on the hexosamine, C5-epimerase [108, 109], converting some of
the GlcA to IdoA, and finally by sulfotransferases (2OST, 3OST, 6OST) adding sulfate groups on the 2-O, 3-O and 6-O positions of the hexuronic acid. The primary forms of NDST expressed by most cells in animals are the NDST1 and NDST2, though it appears that only NDST2 is important for heparin production.[110, 111] Because the efficiency of many of the enzymes involved in the HS biosynthesis are dependent on prior modifications of the chain sulfation is not uniform but organized in domains.[112] This results in HS domains of variable length and different degree of sulfation.

![Figure 5](image.png)

Figure 5. A hypothetical proteoglycan (core protein in dark grey), carrying both heparan sulfate (solid black lines) and chondroitin/dermatan sulfate (dashed black line) chains, facilitating the interaction between a growth factor (light grey) and its receptor (white). Both are anchored to the membrane (black). A hypothetical sample of an oligosaccharide sequence is illustrated in the blow up. GlcNAc: N-acetylated glucosamine, GlcA: Glucuronic acid, GlcNSO₃: N-sulfated glucosamine, IdoA2S: 2-O-sulfated iduronic acid.

The sulfation gives the chains a high negative charge enabling it to interact with many growth factors and act in a co-receptor fashion (illustrated in Figure 5). Cell surface HSPGs acting as co-receptors in growth factor signal-
ing have been described for e.g. FGF2[113] and for VEGF-A165[114, 115]. The PG does not necessarily have to be present on the same cell to serve as a co-receptor as it has been shown that they can act in trans.[116] PGs are indispensable for embryonic development and disturbing their synthesis have great and diverse effects.[117, 118] Removal of HSPG GAG chains entirely by knock down of the EXT polymerases surprisingly does not affect in vitro sprouting angiogenesis as much as NDST1/2 deficiency and it appears as if CS can compensate for the lack of HS.[119] PGs are also involved in creating morphogen gradients in vivo.[120, 121]

The sulfation occurs more frequently in certain domains of the chain and the spacing of the domains are of importance for interaction with certain growth factors.[122, 123] While there seems to be a general lack of sequence specificity in this interaction there are cases where the sequence is important. Heparin has a specific anti-thrombin binding pentasaccharide sequence[124] that allows it to bind anti-thrombin and facilitate its interaction with thrombin and several other components of the coagulation cascade[125] thus hindering their activity. Because of this interaction derivatives of heparin are routinely used as an anti-coagulant in a clinical setting.[126] In the laboratory heparin is widely used as a substitute of HSPGs in various experimental settings due to its high degree of sulfation and high availability. In vivo heparin is attached to serglycin, the main PG found in mast cell secretory granules, which is crucial for granule maturation[127].

Some PGs are also components of the ECM, as mentioned perlecan is part of the basement membrane found beneath the endothelial layer in blood vessels and contributes to vessel integrity.[128] Agrin and collagen XVIII are also examples of ECM PGs. Another GAG of the ECM is the polysaccharide hyaluronan (HA), first described in 1934[129]. HA, like unmodified HS, consists of alternating units of GlcNAc and GlcA and is produced directly into the extracellular space by hyaluronan synthases (HAS) localized at the plasma membrane.[130] Unlike other GAG species HA is not attached to a core protein when synthesized. Because HA is synthesized into the extracellular space it lacks modifications such as sulfation and epimerization. In vivo HA molecules can reach sizes millions of Da in magnitude.

HA was previously mainly thought to be a structural component but has since been shown to have receptors, such as CD44[131] and ICAM-1[132], on the cell surface and influence many processes, amongst them cell migration[133]. Its structural role should not be underestimated because of this, as it is integral for hydration of tissues due to its high water binding capacity. Also in the wound-healing context HA is important, the granulation tissue matrix is rich in HA. [134] At early stages of wound healing endothelial cells promote inflammation by producing HA, facilitating adhesion of activated lymphocytes, as a response to inflammatory stimuli such as TNF-α or LPS.[135]
Integrins and mechanotransduction

Sensing of the environment surrounding the cell does not only occur through growth factors or soluble molecules. The adherent cells constantly interact mechanically with the surrounding matrix or other cells. Integrins serves as a link between the surrounding environment to the actin cytoskeleton and the interaction between cell and matrix is predominately dependent on integrins.

Integrins complexes are composed of one $\alpha$ and one $\beta$ subunit, found non-covalently associated and anchored in the plasma membrane.[136] Integrin ligands are frequently but not exclusively components of the ECM e.g. some integrins bind molecules present on the cell surface[137]. Integrins can be found in different conformations, both in a folded, inactive, and in an active conformation. What determines the exact conformation is not fully understood, however, certain growth factor stimuli can shift integrin complexes into an active conformation.[138]

Integrin activated signal transduction is complex and outcomes are diverse. Survival signals are mediated via the PI3K-AKT pathway, proliferative signals via the extracellular signal-related kinase (ERK) pathway and cytoskeletal reorganization via Rho-ROCK pathway.[136] The stiffness of the cell surroundings varies throughout the body, ranging from virtually nonexistent in the blood, to loose connective tissue and hard bone. Cells are able to sense stiffness of the ECM through integrins, a factor of importance for mesenchymal stem cell linage determination.[139] As mentioned previously, alteration of ECM tension is important in the transition from resting tissue fibroblast to activated protomyofibroblast.[39] It should be noted that integrins are not the sole mechanoreceptors present in the cell e.g. ion channels may serve as receptors[140] by being linked to the cytoskeleton and ECM, as may G-protein coupled receptors[141].

Not only do the cell sense its surroundings by integrin signaling, integrins also allow the cells to modulate its surroundings. The cell has the capacity to remodel its matrix depending on integrin expression. Remodeling of collagen fibrils and polymerization of soluble fibronectin[142] are examples of this. This matrix remodeling is an important feature for wound contraction and healing. Integrins are also important for mediating release of latent TGF-$\beta$ deposited in the ECM.[143]

Interaction between growth factor receptors and integrin complexes has been observed on several occasions.[144, 145] Integrins have also been shown to have the capacity to activate growth factor receptors even in absence of growth factor stimulus.[146]

VEGF

The vascular endothelial growth factor (VEGF) family consists of five members, VEGF-A, -B, -C, -D and placental growth factor (PlGF).[147] The
members are predominately found as homodimers, although heterodimeric complexes have been reported. [148-150] VEGF-A is a crucial factor for blood vessel development and maintenance whereas VEGF-C and -D is involved in lymph vessel formation[151, 152].

Several receptors have been reported to bind VEGF-A.[153] VEGFR2 is the canonical receptor through which VEGF-A elicits its effects. VEGFR1 is also phosphorylated when it binds VEGF-A though not to the same degree as VEGFR2. VEGFR1 is thought to be a decoy receptor for VEGF-A and can be shed of the cell surface[154], or produced as a soluble form[155], in order to regulate VEGFR2 signaling by sequestering soluble ligand. This, however, is not the sole role of VEGFR1 as it is also a receptor for PlGF. VEGFR2 phosphorylation results in signaling through several downstream pathways e.g. via PI3K[156], p38MAPK[157] and ERK[158] pathways. Effects of VEGF-A stimulation of endothelial cells include proliferation[159], migration[159] and survival[160].

Transforming growth factor-β, its receptors and signaling

The human transforming growth factor-β (TGF-β) family includes 33 members. In addition to the TGF-β isoforms (1, 2 and 3) the family also consists of bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs) and activins. Most members are dimeric secreted polypeptides and play an important role in tissue homeostasis in adult tissues, body axis asymmetry and organ morphogenesis during development as well as wound healing and tissue reorganization. [161]

TGF-β1 is a dimeric polypeptide with a molecular weight of 25 kDa. The three isoforms TGF-β1, TGF-β2 and TGF-β3 share a high degree of structural similarity and induce similar effects in vitro but are differently expressed in vivo. TGF-β1 is secreted as a pro-peptide non-covalently associated to the latency-associated peptide (LAP) generated from the N-terminal of the TGF-β precursor by proteolytic processing. This inactive complex is deposited in the ECM together with latent TGF-β binding proteins (LTBPs). As cells degrade the ECM they mediate the release and activation of TGF-β, primarily by matrix metalloproteinase (MMP) activity but also via integrins αVβ6 and αVβ8. It is believed that interaction between these integrins and the RGD domain of LAP triggers exposure and release of mature TGF-β.[143]

The effects of TGF-β on cells are diverse. Mesenchymal cells respond mainly by increased proliferation and differentiation[162] whereas stimulation of vascular endothelial cells elicit different responses depending on the dose[163]. High doses leads to maturation with decreased proliferation whereas low doses promote increased proliferation and migration.[164] The diverse effects of TGF-β are mainly due to the variation in receptors ex-
pressed in different cell types. The TGF-β family receptor (TGF-βR) is a heterotetrameric complex consisting of two type I and two type II receptors. There are seven known type I receptors, also called activin receptor-like kinases (ALKs), and five type II receptors. In TGF-β signaling the type II receptor is the TGF-βRII (TβRII) that can form complexes with several different type I receptors. The receptor complex found in mesenchymal cells is composed of TβRII and ALK5. Vascular endothelial cells have receptor complexes containing ALK5 but also complexes containing ALK1.[161]

Ligand binding to the receptor complex results in phosphorylation and activation of ALK that initiates a signaling cascade. TGF-βR has the ability to activate several different pathways, the most canonical being the Smad pathway (Figure 6). The receptor regulated (R-) Smads associate with common mediator (Co-) Smads and enter the nucleus promoting transcription of a large number of genes. Depending on the type of TGF-βR activated, different Smads are phosphorylated; ALK5 signals via Smad2 and Smad3 while ALK1 signals via Smad1 and Smad5.[165] TGF-βR signaling can in addition also result in phosphorylation and activation of ERK1 (p44, MAPK3) and ERK2 (p42, MAPK1)].[166] This activation can be fast, within 5-10[166] minutes or slow, appearing hours[167] after the initial stimulation. Phosphorylated ERK can inhibit phosphorylation of R-Smads.[168] What determines the kinetics of the ERK phosphorylation is not fully understood.

TGF-β signaling can also be regulated by inhibitory (I)-Smads that inhibits the phosphorylation of R-Smads. The production of the I-Smad Smad7 increases upon TGF-β stimulation and provides a negative feedback loop by blocking the R-Smad binding to the TGF-βR complex.[169]

Another layer of regulation can be accomplished by the expression of the BMP- and activin inhibiting protein (BAMBI), a type-I pseudo-receptor with a short cytoplasmic tail lacking a kinase domain.[170] BAMBI was first identified in Xenopus laevis as an inhibitor of BMP-signaling but has since been demonstrated to inhibit TGF-β mediated signaling and is also conserved in humans. BAMBI interacts with the TGF-β type-I[170] as well as type-II receptors and Smad7[171]. Overexpression of BAMBI has been shown to suppress the Smad2/3 phosphorylation induced by TGF-β stimulation, inversely knockdown leads to sensitization of cells, resulting in higher phosphorylation.
Figure 6. Schematic illustration of canonical TGF-β signaling via Smad 2/3 (A) and two mechanisms of blocking TGF-β signaling (B & C). (A) Upon TGF-β binding the TβRII the complex associates with the type I receptor ALK5 homodimer. Signaling is initiated by TβRII phosphorylating ALK5, which in turn phosphorylates Smad 2/3. A Smad dimer associates with the co-Smad Smad 4 and is translocated to the nucleus to initiate transcription of target genes together with a variety of transcription factors (TF). If the ligand-TβRII complex associated with BAMBI, which lack most of the intracellular domain, no signaling can be initiated (B). I-Smads such as Smad 7 can inhibit Smad 2/3 phosphorylation by interacting with the receptor complex (C).
Toll-like receptors

The human Toll-like receptor (TLR) family is composed of eleven members of transmembrane dimer forming proteins.[172] These members are classically associated with innate immune response and react to a variety of bacterial, viral and parasite components as well as endogenous ligands. TLRs are also referred to as pattern recognition molecules because of their ability to recognize patterns of microbial molecules. Ligand binding induces dimerization of receptors, depending on the ligand both homo-[173] and heterodimeric[174, 175] complexes can be formed. TLRs do not contain an endogenous kinase domain and rely on the activity of other kinases recruited upon receptor-ligand complex formation.[176] One of the major and perhaps mostly intensively studied downstream pathways of the TLRs is the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway, linked to inflammatory response. The TLRs have also been shown to signal through other pathways e.g. p38 and ERK.[177]

Toll-like receptors are associated with defense against microorganisms such as viruses and bacteria. Many TLR complexes react to foreign molecules such as the bacterial lipopolysaccharide (LPS, activating TLR4)[178] or double stranded RNA from viral replication (activating TLR3)[179].

Recently endogenous ligands have been found to activate TLRs.[172] Some molecules such as hyaluronan[180] and fibrinogen[181, 182] bind several TLRs. These new discoveries widen the role of TLRs and TLRs have been implicated to be involvement in several pathological processes such as fibrosis[182] and tumorigenesis[183, 184].
Present investigations

Using a microfluidic device to study cell migration

All of the studies included in this thesis have utilized a microfluidic device[185] to create growth factor gradient in order to study the chemotactic or chemokinetic response of cells in vitro. The device or “migration chamber” uses the principle of laminar flow to create a hill shaped gradient of the factor of interest. Since there is no turbulence in the channel the only thing dispersing the factor is diffusion. A schematic illustration of the chamber layout and examples of frames collected can be seen in Figure 7. In brief, cells are allowed to attach to a gelatin-coated plate and left in serum containing medium for 4-6 h after which they are starved over night. The chamber is applied on the plate and kept in place by vacuum force. The device is made of gas permeable PDMS silicone, enabling control of CO₂-concentration in the system. During the experiments cells are kept at 37 °C and the migration of the cells is recorded using an inverted microscope. The quantification method used throughout this thesis is a measurement from start to end point of cells located in the unsaturated part of the chamber at the start of the experiment. Also recorded is the direction in which the individual cells are moving. This allows for representation in the form of overall movement as well as the average distance moved towards or away from higher concentration of factor. These measurements are exemplified in Figure 7.
Figure 7. Schematic illustration of microfluidic device layout (A) and frames collected during the migration assay (B). (A) Factor of interest (FoI) can be added to the central medium reservoir (white) and serum free medium to the others (black) at the top of the image. A gradient is generated by laminar flow along the channel. Blow-up illustrates a single cell (red dot) migrating from the right hand side (low FoI concentration) to the central part (higher FoI concentration), ending up at the white dot. Measurements collected are distance start-to-end (red line, d) and distance moved towards or away from higher FoI concentration (white bar, x). (B) Images taken at 0 min (top) and 4 h (bottom) of HFL1 fibroblast migrating towards PDGF-BB. Dots mark starting points and lines indicate distance from start to end.

Paper I

Aim

Many factors and genes are known to play important roles in the process of angiogenesis, many, however, are still unknown. In this paper the aim was to identify and investigate novel genes involved in angiogenesis.

Results

When performing microarray analysis of the mRNA expression from sprouting embryoid body (EB) derived vascular fragments ExoC3L2 was found to be highly expressed. Cultured human microvascular endothelial cells (HMVEC) also expressed ExoC3L2 and expression was further induced by stimulation with Collagen I and VEGF-A or culturing in a 3D collagen matrix. The localization of EXOC3L2 was at the cell periphery in the membrane but could also be detected along the cell actin filaments and in peri-
nuclear vesicles. The endothelial expression of EXOC3L2 could be confirmed by staining of mouse brain. EXOC3L2 was further found to bind EXOC4, a component of the exocyst complex found on certain secretory vesicles. As the name implies EXOC3L2 is related to EXOC3, which is also found in association with other exocyst components on the vesicle. Disrupting the expression of EXOC3L2 resulted in reduction of VEGF-A induced VEGFR2 phosphorylation and reduced VEGF-A induced chemotaxis.

Discussion
In this paper we identified EXOC3L2 as being selectively expressed in endothelial cells and is able to interact with known members of the exocyst complex. Disruption of EXOC3L2 expression also disturbed VEGF-A induced signaling and chemotaxis. Taken together this implicates that EXOC3L2 may be present in or in association with the exocyst. EXOC3L2 partially co-localizes with both EXOC4 and VEGFR2 further supporting its role in vesicular transport of the receptor.

Future perspectives
Although we find that EXOC3L2 is expressed by the endothelium and is involved in the migration of endothelial cells, the precise details are not clear. For in vitro studies, labeling of the endogenous EXOC3L2 with a fluorescent or otherwise traceable marker could give further insights to its function. The mouse ExoC3L2 sequence has, after the publication of this paper, been updated more than once and now appears to be of similar size as the human form. There are however still no publications using EXOC3L2 knock out mice and thus the in vivo effects of ExoC3L2 dysregulation or deficiency is not known. Generation and examination of such a mouse is an important next step on the way to determine the function of EXOC3L2 in general and in angiogenesis, both embryonic and in the adult, in particular.

Paper II
Aim
miRNAs can be specifically expressed in certain cells or tissues and play a role in modulating gene expression. The aims of this paper were to determine miRNAs expressed in the vasculature and to investigate their role(s) in angiogenesis and vessel function.
Results

We used a bioinformatic approach to identify miRNAs selectively expressed in the vasculature and identified miR-145 as being highly expressed in vascularized organs such as the kidney and lung. miR-145 was found in vascular fragments isolated from adult mouse organs, however, it was not detected in fragments isolated from embryonic organs or from blood vessels grown from mouse embryonic stem cell derived embryoid bodies (EBs) *in vitro*. Further experiments revealed that miR-145 was expressed in pericytes and not in the endothelium. The presence of miR-145 in the vascular fragments isolated from tissues, but not from embryonic organs or EBs, was due to the difficulty of fully dissociating the more mature stable vessels embedded in compact ECM of the adult organs. This finding was verified by isolating vascular fragments from PDGF-BB ret/ret mice, which have defects in pericyte recruitment. This reduced pericyte contamination and the level of miR-145 was also reduced. In situ hybridization provided further evidence for the non-endothelial expression. *Fli1*, a transcription factor expressed in endothelial cells, was identified and verified to be a target of miR-145. Overexpression of miR-145 reduced the migration of both endothelial cells towards VEGF-A and fibroblasts towards PDGF-BB. Depletion of miR-145 from fibroblasts had similar effects.

Discussion

miR-145 has previously been described to be expressed in vascular smooth muscle cells and high expression is associated with reduced neointima formation after injury. Here we show that pericytes also express miR-145 and that an increased miR-145 level leads to defective migration. It has previously been speculated that miR-145 disturbs migration because of phenotypes observed in miR-145 knock out mice[186]. miR-145 has targets involved in actin polymerization, some of which inhibit and some stimulate migration. The results from overexpressing or suppressing miR-145 in fibroblast are therefore not very surprising and miR-145 may in these cells act to balance expression of these target genes. The reason for miR-145 targeting *Fli1* is not fully understood. In cases where Fli1 expressing hematopoietic cells differentiate to pericytes[187] miR-145 may have a role establishing this new cell identity. In theory miRNA from the pericyte could be transported to the endothelium via gap junctions or microvesicles. Examples of microvesicles containing miR-145 have been found *in vitro*[188].

Future perspectives

Since our finding that miR-145 disturbs the migration of several cell types others have also shown reduced migratory potential when the level of miR-145 is modulated. Increasing the expression of miR-145 reduced the inva-
siveness and migration of breast cancer cells.[189] Similar results have been reported for glioma cells.[190] These results further support that miR-145 do indeed play a role in migration. What targets of miR-145 that is responsible for this effect is not fully understood and since both increasing and reducing the miR-145 level gives similar result it may prove challenging to fully elucidate its function.

Paper III

Aim

From the bioinformatics analysis performed in paper II we also found miR-24 and miR-23 to be associated with the vasculature. We were able to identify endothelial expression of these miRNAs that were predicted to target Ndst1, thus possibly altering the global sulfation of heparan sulfate (HS). Few previous studies on the effects of miRNA on HS structure have been performed despite the importance of HS as co-receptors in growth factor signaling. Our aim was to investigate if these miRNAs could modulate HS structure and influence angiogenesis.

Results

miR-24 was found to bind to the 3’UTR of Ndst1 mRNA and overexpression reduced N-deacetylation. miR-23a did also bind the 3’UTR but did not affect the N-deacetylase activity. Because of this further investigation was only carried out using miR-24. Analysis of HS and the related CS (chondroitin sulfate) from HUVECs overexpressing miR-24 showed reduced sulfation of HS structures but no change in overall CS sulfation. This reduced sulfation also resulted in a decreased affinity for VEGF-A. Further the level of VEGFR2 was reduced in miR-24 overexpressing cells, resulting in lower downstream signaling induced by VEGF-A. Overexpression of miR-145 as well as reducing Ndst1 mRNA using siRNA resulted in lowered chemotactic response to VEGF-A.

Discussion

It has previously been shown that several miRNAs are selectively expressed in different tissues[191] and cell types[192]. miR-24 overexpression decreases the level of Ndst1 mRNA and N-deacetylation of HS leading to lower overall sulfation of HS. This reduces the affinity between VEGF-A and HS but surprisingly does not reduce the level of VEGFR2 phosphorylation but rather the level of VEGFR2 itself. This could be because there is enough sulfation to facilitate binding of VEGF-A molecules sufficient to activate all receptors, thus it could be the receptor level that is limiting and not the bind-
ing of ligand to HS. Lower doses of VEGF-A might yield different results. An interesting question is why in particular endothelial cells would express a miRNA that downregulates a ubiquitously expressed enzyme involved in a process that is of such importance for many processes. This could be a fine tuning step and other cell types may regulate this process by other miRNAs, however, reduction in miR-24 levels did not increase the N-deacetylase activity implying that in this setting fine tuning by miR-24 may not be necessary. Perhaps miR-24 is regulating other targets as well under normal circumstances and/or serve to safeguard regulation of Ndsl should for some reason the expression increase. Despite the remaining questions this study contributes to expand the knowledge of miRNA and HS biosynthesis regulation, a field in which much remains to be explored.

Future perspectives
Given the complexity involved both on the level of miRNA regulation and HS biosynthesis it may prove difficult to fully understand the mechanisms involved and their interplay. Nonetheless they are both exciting and important fields both in physiological and pathological contexts and may present new possibilities for regulating growth factor signaling for therapeutic means.

Paper IV
Aim
Fibrin degradation products (FDPs) have been shown to have effects on vascular smooth muscle cells and endothelial cells.[28-32] Since the proposal of looping angiogenesis[8] we were interested to investigate whether these FDPs could have an effect on the myofibroblasts important for this process.

Results
Fibrin fragment E (FnE) was found to enhance TGF-β induced α-smooth muscle actin (αSMA) production but did not elicit a αSMA response by itself neither in human fetal lung fibroblasts (HFL1) nor in immortalized human foreskin fibroblasts (BJ-hTert). The αSMA potentiation response was time and dose dependent, but also dependent on the dose TGF-β1. FnE was found to be a chemotactic factor for fibroblasts in vitro whereas other FDPs tested did not produce a chemotactic response at equimolar concentration. D-dimer potentiated αSMA in the same manner as FnE although the potentiation was not as great. FnE also potentiated TGF-β induced collagen IαI-mRNA production in HFL1 cells.
The potentiation as well as chemotaxis was dependent on integrin β3, as siRNA treatment targeting the β3 integrin abolished the effects on αSMA potentiation and directional migration. siRNA targeting integrin β5, however, did not affect potentiation or migration. Stimulation with FnE decreased the mRNA level of the TGF-β pseudoreceptor BAMBI, this effect was evident after 24h and was persistent at 48h. The Toll-like receptor 4 inhibitor CLI-095 greatly inhibited the chemotactic effect of FnE.

Discussion
The notion that a degradation product of fibrin found in a wound would promote the recruitment and differentiation of myofibroblasts that facilitates the healing process is of course appealing. Degradation products of other matrix molecules e.g. hyaluronan have previously been shown to bind TLRs.[180] Fibrinogen has previously been shown to have a proliferative effect in vivo, an effect partly dependent on TLR4[182]. Platelets display the ability to facilitate EMT by activating the NF-κB pathway and sensitizing cells for TGF-β stimulation.[193] The process of EMT is in some aspects similar to the fibroblast to myofibroblast differentiation. LPS, a potent ligand to TLR4 elicits a downregulation of BAMBI in hepatic stellate cells in vivo.[194] Hepatic stellate cells are similar to fibroblasts and the LPS stimulus sensitizes the cells to TGF-β. These observations are similar to our finding that FnE stimulation lowers the expression of BAMBI mRNA and potentiates the TGF-β response. Although not conclusively shown at present, indications point to that TLR4 could be a receptor for FnE. The involvement of integrins is not fully understood at present. Integrin β3 may serve in a co-receptor like fashion or may bind components of the ECM that is required to elicit a response to FnE.

Future perspectives
While TLR4 is a likely candidate receptor for FnE it still remains to be conclusively established. We hope to investigate this interaction using Biacore technology permitting the detection of ligand and receptor interaction. This technique allows immobilization of the receptor and real-time monitoring of its interaction with a potential ligand. This process may not prove so simple if additional components such as co-receptors are required to facilitate proper interaction.

In order to get a functional assay to determine the functionality of the increase in αSMA expression we are in the process of evaluating cell mediated contraction, or wrinkling, of silicone substrates. Cells are seeded into silicone coated glass coverslips before being stimulated to increase αSMA expression. The goal is to investigate if the potentiation in αSMA leads to a functional and improved contractility of the cell.
Fibrinogen knock out mice have been generated and are viable.[195] By performing wound-healing experiments the effect of fibrinogen deficiency (and as a result FDP deficiency) could be studied for parameters such as myofibroblast recruitment. Adding exogenous FDPs to this setting would make it possible to study the role of individual FDPs and their effect on wound healing. Tumor studies could also be initiated in order to determine if FnE or other FDPs do play a role in tumor vascularization and growth.

Vi har undersökt en gen, exoc3l2, vars proteinprodukt EXOC3L2 uttrycks specifikt i endotelceller. Detta protein förefaller vara inblandad i transport av komponenter från cellens insida som ska ut på dess yta. Om cellernas EXOC3L2-mängd minskar så får cellerna svårt att orientera sig när de vandrar. MikroRNA (miRNA) är små RNA-strängar som genom att binda till en mall för proteintillverkning (kallat mRNA) kan minska mängden av det korresponderande proteinet. Vi har undersökt två av dessa miRNA för att se hur de påverkar endotelceller. Det första av dem är miR-145 som har ett mål-mRNA, fli1, som finns specifikt i endotelceller. Om mängden miR-145 ökar så leder det till att cellerna förlorar sin orienteringsförmåga. miR-145 produceras dock normalt inte i endotelcellerna utan i pericytarna. I fibroblaster (som till viss del liknar pericyter och även de producerar miR-145) störs orienteringsförmågan både av mer eller mindre miR-145. Det är sedan tidigare känt att celler kan föra över bland annat miRNA mellan sig, vilket gör att det i teorin är möjligt att pericytarna för över miR-145 till endotelcellerna, även om fli1 inte verkar vara det mål som ger upphov till den observerade effekten.
Det andra miRNAt vi undersökt är miR-24. Detta miRNA uttrycks i endotelcellerna och kan minska mängden NDST1, ett protein som modifierar kolhydratstrukturer på cellen. Dessa kolhydrater är kopplade till proteiner och kallas då för proteoglykaner. NDST1 är inblandad i tillverkningen av en typ heparansulfat. Heparansulfat är inblandat i många mekanismer i kroppen och är viktigt för vår utveckling och överlevnad. NDST1 är viktig för att modifera heparansulfatet så att det blir negativt laddat och därigenom binder många proteiner i kroppen. Om mängden NDST1 minskar så minskar heparansulfatets förmåga att binda dessa. En av funktionerna är att hjälpa cellen att känna viss fattor, t.ex. sådana som är viktiga för orienteringsförmågan. När mängden miR-24 ökar så känner inte cellen av signaler utifrån på samma sätt och därför tappar cellerna även i detta fall förmågan att orientera sig.


Förhoppningen är att dessa upptäckter ska vidga förståelsen kring angiogenes och sårläkning för att bidra till utvecklandet av nya behandlingsmetoder mot patologiska skeenden, exempelvis cancer.
Acknowledgements

This work was carried out at the department of medical biochemistry and microbiology (IMBIM) at the biomedical center (BMC).
I would like to thank the following people:

Firstly, my supervisor Pär Gerwins for the opportunity to work with you. Ideas are never in short supply, even though time may be.

My co-supervisor Johan Kreuger for much assistance and advice as well as for the opportunity to be part of many exciting projects.

My examiner Kristofer Rubin for scientific discussions and sharing much knowledge.

My opponent Arne Östman and the members of the committee; Christian Sundberg, Agneta Siegbahn and Magnus Åbrink, for taking the time to scrutinize this thesis.

Past and present members of the Gerwins group; Ludde for a lot of knowledge of all things possible, Princess Ewa for always being so positive and caring and for bringing me the occasional coffee and snacks during writing of this thesis, Femke and François for bringing new energy into the group.

Past and present members of the Kreuger group; Irmeli for much technical assistance and many pleasant conversations, Johan Heldin for never failing to entertain those around you, intentional or not, Paul for organizing things and many helpful ideas, Zsolt and Seb for nice discussions and assistance in the lab.

My latest roommate Anna for everything, especially the encouragement to write, as well as the many laughs over the years.

Pappa Tamm for entertaining discussions and bad humor at lunchtime and Sara for teaching us that Catalonia was always first… with everything.

Vahid for scientific discussions and motivation for training.
Eva G, the alcohol fairy, you are indispensible in coping with the course labs!

Jimmy for a lot of entertainment.

All other co-authors and collaborators.

Past and present members of other groups at D9:4 and B9:3 for interesting seminars, journal clubs and lending of reagents.

The administration of IMBIM, in particular Barbro, Erika, Susanne L, Rehné, Marianne, Olav and Kerstin.

A special thanks goes out to the coffee brewer in D9:4, I wouldn’t have made it without you!

Dr Do and all of the people at circle for your energy and enthusiasm.

My friends for the wonderful company and exciting activities, no matter if it’s related to playing games, running around in the forest or something completely different.

To the people that I have for some reason forgotten to mention, a sincere thank you!

My family for all the support during these years, all those before and for all those to come. Jag älskar er!

Emelie, för att du på något sätt står ut med mig oavsett hur jobbig jag blir. Tack för allt stöd, det är du som är bäst! Jag älskar dig! ♥
References

51. GLOBOCAN 2008 v2.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 10, 2008: [Internet].


A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine.