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Studies of the Novel PDGFs, focusing on PDGF-D

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När du tittar på dina båda tassar, behöver du bara bestämma
dig för vilken som är den högra, sedan kan du vara säker på
att den andra är den vänstra
Nalle Phu

Cogito, ergo sum
Descartes

Populärvetenskaplig sammanfattning

Högre organismer, som exempelvis människan, är uppbyggd av miljontals celler. Otroligt nog härstammar alla dessa celler från en och samma ursprungscell. Allt startar vid embryots första celldelning och fortsätter sedan upprepade gånger under en individs hela utveckling. Det finns över 200 olika celltyper som alla har unika egenskaper och grupperas i vävnader, vilka bildar organ med olika funktioner. För att förmå cellerna att bilda en hel organism från en enda ursprungscell är tre olika processer viktiga; cellproliferation, celldifferentiering och celldöd. Cellproliferation innebär att cellerna växer och delar sig. Den processen ser till att det finns tillräckligt många celler. Celldifferentiering innebär att varje cell utvecklas till en speciell sorts cell, en viss celltyp med specifika egenskaper. Med celldöd menar man den så kallade programmerade celldöden, vilken innebär att celler som inte kan utföra sin uppgift programmeras att dö. För att kunna förstå individens normala biologiska utveckling och uppkomsten av olika sjukdomstillstånd är det viktigt att förstå hur cellproliferation, celldifferentiering och celldöd regleras. Cancer t.ex är en sjukdom där cellproliferationen, celldifferentieringen och celldöden har rubbats ur sin balans, vilket leder till en totalt okontrollerad celltillväxt och celldelning. Det är våra gener som styr de tre olika processerna. Några av dessa gener ger upphov till en grupp av kroppsegna proteiner som kallas för tillväxtfaktorer. Denna grupp av proteiner är ofta inblandad i cellprolifereringen. Idag finns det över femtio kända tillväxtfaktorer. Dessa påverkar celler att bli aktiva och dela sig genom att binda till specifika receptorer, vilka återfinns på cellens yta. Trots intensiv forskning för att kartlägga vår biologiska utveckling och de processer som styr den, finns det många nya bitar att upptäcka i det enorma pussel av bitar som skall passa ihop. För några år sedan upptäckte vår forskargrupp två nya bitar till detta pussel; två nya tillväxtfaktorer. Dessa två tillväxtfaktorer, som kallas PDGF-C och PDGF-D, tillhör PDGF/VEGF-familjen (platelet-derived growth factor/vascular endothelial growth factor). Denna familj är mest känd för att var inblandad i nybildandet av blodkärl – angiogenes. Våra blodkärl är uppbyggda av flera lager av celler. En tillväxtfaktor som kallas VEGF-A är viktig för rekryteringen av det innerst lagret av celler som kallas endotelceller, vilka bygger upp själva röret i blodkärlet. PDGF-A och PDGF-B, vilka är nära besläktade med PDGF-C och PDGF-D, lockar till sig de celler som stabiliserar blodkärlen, de så kallade glatta muskelcellerna.

Den här avhandlingen handlar om de två nyupptäckta tillväxtfaktorerna PDGF-C och PDGF-D, men är fokuserad på PDGF-D. Artikel I beskriver hur PDGF-D upptäcktes och några av egenskaperna hos PDGF-D. Här visas också vilken specifik receptor som PDGF-D binder till och aktiverar. Artikel II handlar om på vilka kromosomer de gener som kodar för PDGF-C och PDGF-D proteinerna är lokaliserade. I Artikel III visas att om man får för mycket av PDGF-D, påverkas de glatta muskelcellerna att dela på sig mer än vad de normalt gör. Det leder till att man får tjockare blodkärl. Slutligen, i artikel IV undersöks om PDGF-D kan påverka cellproliferering och celldifferentiering av muskelstamceller som sen utvecklas till muskelceller vilka bygger upp muskelfibern.

Abstract

Thirty years ago the classical platelet-derived growth factors (PDGFs), PDGF-A and PDGF-B were discovered. For a long time they were thought to be the only PDGF isoforms to exist, but recently two novel PDGF molecules were identified, namely PDGF-C and PDGF-D. This finding was unexpected and indicates that the PDGF signalling system is much more complex than was previously thought. The four PDGF chains form five different disulphide-linked homo- and heterodimers; PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC and PDGF-DD, which exert their biological effects by binding to, and signalling through two receptor tyrosine kinases, PDGFR-α and PDGFR-β. The biological effects of PDGF-A and PDGF-B have been extensively studied. They are known to be major mitogens for several cell types of mesenchymal origin and have been implicated in several pathological conditions, such as atherosclerosis and fibrotic diseases. The discovery of two new members of the PDGF family makes it important to further characterize the players in the PDGFR signalling system in order to understand their different biological functions.

Part of this work describes the identification of the most recent found member of the PDGF-family, PDGF-D. In a computer-assisted search, a cDNA clone encoding PDGF-D was found. The full-length cDNA encoded a polypeptide of 370 amino acids. The secreted protein turned out to be a disulphide-linked homodimer, which displays a two-domain structure similar to that found in PDGF-C, with an N-terminal CUB domain and a C-terminal PDGF/VEGF homology domain. Secreted PDGF-DD was found to be a latent protein, which requires proteolytic removal of the CUB domains for activity. Proteolytically processed PDGF-DD preferentially binds to and signal through PDGFR-β. Further, the human genes for PDGF-C and PDGF-D were found to be located on chromosomes 4q32, and 11q22.3 to 23.2, respectively. Characterization of the genes showed that the *PDGFC* gene contained 6 exons, while the *PDGFD* gene contained 7 exons.

The biological role(s) and the *in vivo* responses of the novel PDGFs are less well known. Data presented herein suggest that PDGF-D might have a role in cardiac fibrosis. This was indicated when PDGF-D was overexpressed in the heart of transgenic mice. Overexpression of PDGF-D induced cardiac fibrosis and hypertrophy, which subsequently caused cardiac failure. In addition, vascular changes, with dilated microvessels, and proliferation of the smooth muscle cells leading to thickening of arterial walls, was seen.

The protease uPA has recently been reported to activate PDGF-DD, and in a study where skeletal muscle injury was experimentally induced in uPA deficient mice, uPA was found to be required for efficient regeneration of damaged muscle. Data presented herein establish a role of PDGF-D in skeletal muscle development and regeneration. Using cultured myoblasts, PDGF-DD was found to stimulate proliferation and migration of the myoblasts *in vitro*, as well as inhibiting their differentiation.

In summary, the findings presented in this thesis improve our understanding of the biological function of PDGF-D and enhance the knowledge of the complexity of the PDGF/PDGFR signalling system.

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List of publications

This thesis is based on the following papers. In the text the roman numbers below are used to refer to each paper:

I **PDGF-D is a specific, protease-activated ligand for the PDGF β-receptor**
Bergsten E*, Uutela M*, Li X, Pietras K, Östman A, Heldin CH, Alitalo K, Eriksson U
Nat Cell Biol., 3:512-516, 2001

II **Chromosomal location, exon structure, and vascular expression patterns of the human *PDGFC* and *PDGFD* genes**
Uutela M, Laurén J, Bergsten E, Li X, Herelli-Kuitunen N, Eriksson U, Alitalo K
Circulation, 103:2242-2247, 2001

III **Platelet-derived growth factor D induces cardiac fibrosis and proliferation of vascular smooth muscle cells in heart-specific transgenic mice**
Pontén A*, Folestad Bergsten E*, Pietras K, Eriksson U
Circ Res., 97:1036-1045, 2005

IV **Platelet-derived growth factor DD stimulates proliferation and migration of mouse myoblasts**
Folestad Bergsten E, and Eriksson U
Manuscript

*equal contribution

List of abbreviations

ApoE	apolipoprotein E
α-MHC	alpha myosin heavy chain
cDNA	complementary deoxyribonucleic acid
CUB	complement subcomponents C1r/C1s, urchin EGF-like protein, bone morphogenic protein-1
EC	endothelial cells
ECM	extracellular matrix
ER	endoplasmatic reticulum
kDa	kilo dalton
LRP	low density lipoprotein receptor-related protein
MRNA	messenger ribonucleic acid
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
SMC	smooth muscle cell
tPA	tissue plasminogen activator
uPA	urokinase plasminogen activator
uPAR	urokinase plasminogen activator receptor
VEGF	vascular endothelial growth factor
vSMC	vascular smooth muscle cell

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1 Introduction

Incredibly, a whole organism is generated by cell division from one single cell. During development, this cell divides repeatedly to produce many different cells building a tightly regulated puzzle. Higher organisms such as humans contain approximately 10^{14} cells. The cells in the human body are divided into about 200 different cell types, which collaborate with each other to form different tissues, arranged into organs performing widely varied functions. Higher forms of life can be described as large "multicellular cities", whose individual inhabitants are directed by complex systems of communication[1]. The mechanisms for communication are essential to warrant proper cell functions, such as proliferation, differentiation and death. A flow of extracellular signalling molecules, which are produced by cells, is important for maintaining the communication. These extracellular signalling molecules can be secreted proteins such as growth factors, which bind to specific cell-surface receptor(s), inducing cascades of intracellular events that lead to different outcomes for the cell. Many growth factors are known to induce positive effects on cell growth and cellular differentiation. One of the first growth factors to be identified was platelet-derived growth factor (PDGF). PDGF was identified as a serum factor that stimulated smooth muscle cells (SMCs) to grow in culture[2]. The growth promoting activity found in platelets of blood serum were indicated to reside in two components[3]. Further studies showed that PDGF consisted of two different polypeptide chains, the classical PDGF-A and PDGF-B chains[4,5]. These two PDGFs were thought to be the only PDGF family members to exist, but a few years ago a significant progress in PDGF biology occurred with the identification of two new members in the PDGF family, PDGF-C and PDGF-D. The main aim with the work described in this thesis was to increase the understanding of the biological function of the novel PDGFs, with a focus on PDGF-D, whose discovery and cloning is described in one of the articles in this thesis.

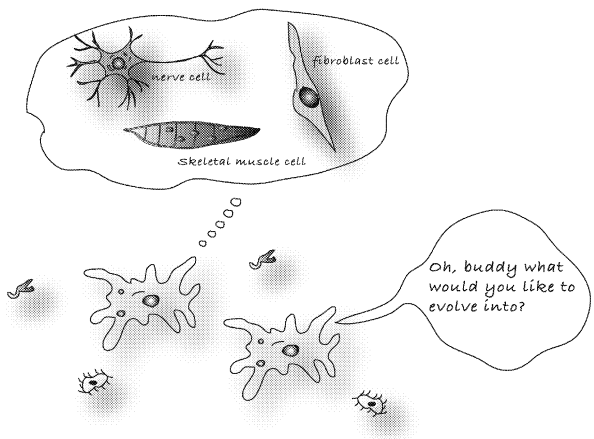

Figure 1. Drawing of precursor cells and some differentiated cell types. Precursor cells evolve into different "differentiated" cells, which build up the tissues in the human body.

1.1 PDGF family

Since PDGF was one of the first growth factors to be identified and characterized, it has been used as a model in understanding the mechanism of many other growth factors. Today a large number of growth factors are known and they tend to occur as members of larger families of structurally related proteins. The PDGFs constitute a subfamily in the PDGF/vascular endothelial growth factor (VEGF) super family. All PDGF/VEGF family members contain a cysteine-knot motif, which is characterized by eight conserved cysteine residues. These cysteines are located in their PDGF/VEGF homology domains and are involved in intra- and inter-chain disulphide bond formation[6].

1.1.1 PDGF isoforms

The PDGF family consists of disulphide-bonded homo- and heterodimers. The four PDGF polypeptide chains form five different isoforms PDGF-AA, PDGF-BB, PDGF-AB, PDGF-CC and PDGF-DD (Paper I[7] and[5,8,9]). All PDGFs contain the structurally well-conserved PDGF/VEGF homology domain. In addition, the novel PDGF-C and PDGF-D chains possess an N-terminal CUB domain, which is not found in the classical PDGF-A and PDGF-B chains. These two domains are separated by a stretch of amino acids called the hinge region (Paper I[7] and[8]). The CUB domain was originally named after the first three proteins that were identified to contain this domain, namely **C**omplement subcomponents C1r/C1s, **U**rchin EGF-like protein and **B**one morphogenic protein-1. A CUB domain has approximately 110 amino acids, and it is found in different extracellular proteins with diverse functions. CUB domains are known to be involved in protein-protein and protein-carbohydrate interactions[10,11]. In the novel PDGFs, the CUB domains are known to block receptor binding and activation (Paper I[7] and[8]). The CUB domain thus has to be proteolytically removed to release the active fragments of PDGF-C and PDGF-D. The proteolytic processing of the PDGFs is further discussed in section 1.2.

PDGF-A exists in two isoforms due to alternative splicing, with and without the exon 6-encoded sequence. The more abundantly expressed isoform is the shorter form (PDGF-A_S), which is a polypeptide of 196 amino acids, while the longer isoform (PDGF-A_L) contains 211 amino acids[12]. PDGF-B and PDGF-C respectively, are expressed in one isoform each and PDGF-B is secreted as a polypeptide of 241 amino acids, while the PDGF-C polypeptide is 345 amino acids[6]. PDGF-D is synthesized as two different polypeptides of 364 (isoform 2) and 370 (isoform 1) amino acids, respectively (Paper I[7] and[9,13]). PDGF-D isoform 2 uses an alternate in-frame splice site in the 5' coding region, resulting in a 6 amino acid shorter splice isoform. Not much is known about PDGF-D isoform 2 and henceforth when discussing PDGF-D it is isoform 1 that is intended. An additional splice variant of the PDGF-D isoform has been identified in mice, where exon 6 is deleted, which results in a truncated protein where most of the PDGF/VEGF homology domain is missing. Thus the protein lacks mitogenic activity[14].

The mature monomers of PDGF-A and PDGF-B have molecular weights of around 15 kDa[5], whereas full-length PDGF-C and PDGF-D are found as species of 50-55 kDa, respectively. Full-length PDGF-C and PDGF-D, as mentioned above, are secreted as inactive proteins that need proteolytic removal of their CUB domains to become active. Proteolytically processed monomers of PDGF-C and PDGF-D have molecular weights of around 20 kDa[7-9].

1.1.2 PDGF genes

Genes located on four different chromosomes encode the four human PDGFs. The *PDGFA* and the *PDGFB* genes are located on chromosome 7 and 22, respectively[15-17], while the *PDGFC* and the *PDGFD* genes are located on chromosome 4 and 11, respectively (Paper II[18] and[9]). These four genes are organized in a similar manner, where *PDGFA*, *B* and *D* genes have seven exons, while the *PDGFC* gene only have six exons (Paper I[18]). Exon 1 encodes the signal sequence. In the *PDGFA* and *PDGFB* genes exons 2 and 3 encode the precursor sequence, exons 4 and 5 encode the PDGF/VEGF homology domain, which is the largest part of the mature protein, and exon 7 is mainly non-coding (reviewed in[5]). In the *PDGFC* and *PDGFD* genes exons 2 and 3 encode the CUB domain. In the *PDGFC* gene exon 4 encodes the hinge region, while exons 4 and 5 encode the same region in the *PDGFD* gene. Exons 5 and 6 in the *PDGFC* gene, and exons 6 and 7 in the *PDGFD* gene, encode the PDGF/VEGF homology domain (Paper II[18] and[6]).

The sizes of the introns show a remarkable difference between the four genes. *PDGFA* and *PDGFB* genes span approximately 20 kb of genomic DNA, while the *PDGFC* and *PDGFD* genes are more then ten times larger[6].

Comparing the four gene products revealed that the sequence similarity between PDGF-A and PDGF-B chains where about 50%, while the sequence similarity between PDGF-C and PDGF-D chains where about 43%. In addition when comparing the PDGF/VEGF homology domains between all four members the sequence similarity is only about 20% (Paper I[7]).

1.2 Proteolytic activation of PDGFs

Proteolytic activation is an important regulatory step for many proteins. The physiological roles of proteases are very diverse, ranging from digestive functions, the removal of damaged proteins, as well as the involvement in protein maturation, to the precise processing of proteins from a latent into the biologically active state. Proteases are known to play key roles in many signal transduction pathways by regulating and controlling the levels of critical components that signals through the pathway[19].

The members of the PDGF family have to be proteolytically processed to mature and to become active. The differences in structure and proteolytic processing segregate the PDGF ligands into two subfamilies. The classical PDGF-A and PDGF-B chains undergo intracellular proteolytic activation, while the novel PDGF-C and PDGF-D chains undergo extracellular activation[6].

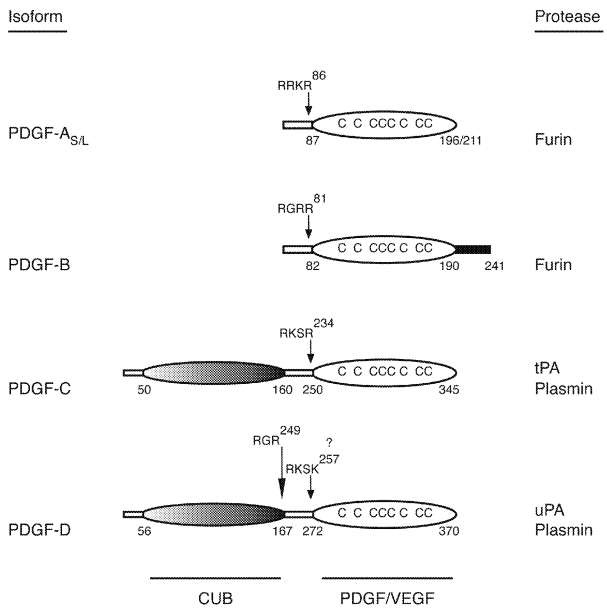

Figure 2. Schematic illustration of the four PDGFs and their modes of proteolytic activation. The suggested basic cleavage sites located N-terminal of the growth factor domain are indicated with arrows. The candidate proteases to the respective PDGF isoform are listed to the right. The number refers to the amino acids in the full length proteins.

1.2.1 Classical PDGFs

The classical PDGF-A and PDGF-B are expressed as precursor molecules and to generate the mature proteins they have to be proteolytically digested intracellularly in the amino terminus[5,20]. Synthesized proPDGF-A and proPDGF-B chains dimerize in the ER, and the dimeric complexes transit through the Golgi apparatus towards the trans-Golgi Network, where the dimers are proteolytically cleaved in their N-terminals at the sequence RRKR^{86}↓ and RGRR^{81}↓, respectively (illustrated in Fig. 2). The activated PDGF-A and PDGF-B dimers are carried in vesicles to the cell surface where they are released by exocytosis. The protease responsible for the cleavage of both PDGF chains was recently identified to be furin, which is a dibasic-specific proprotein convertase (PC)[21,22].

The cleavage of the classical PDGFs is essential for their biological function. It has been shown that introduction of mutations in the processing site of PDGF-A, prevents processing and subsequently abolish binding and activation of its receptor[23,24].

Both the less abundantly expressed PDGF-A_L chain, and the PDGF-B chain contain a carboxy terminal retention motif, which keeps the proteins associated to the extracellular matrix (ECM). PDGF-B can be further processed in the C-terminus[20,22]. It is possible that matrix-bound PDGF-B is proteolytically processed and released from the ECM, which creates a more diffusible form of PDGF-B[20]. However, matrix association of PDGF-B is of high importance. Deleting the PDGF-B retention motif by targeted mutagenesis in mice causes severely impaired recruitment of pericytes to the microvessels[25,26]. The C-terminal retention motif plays an important role in regulating the bioavailability of PDGF-B, by promoting the formation of gradients, and facilitating presentation of the ligand to the relevant target cell. It is possible that the bioavailability of the matrix-bound form of PDGF-A also is regulated by additional proteolytic processing to be released from the ECM. However this has not been reported yet. Identification of the extracellular binding components for these retention motifs will enhance our understanding of PDGF biology[25].

1.2.2 Novel PDGFs

The novel PDGF-C and PDGF-D undergo extracellular proteolytic processing to generate active proteins. The first indications of protease induced activation of the novel PDGFs were observed when expressing recombinant PDGF-CC and PDGF-DD in tissue cultures, and a component was present in the normal fetal calf serum that could activate both factors[6,9,27]. Plasmin turned out to be able to activate both PDGF-CC and PDGF-DD. However, due to the wide substrate specificity of plasmin it is probably not the physiologically relevant protease (Paper I[7] and[8]). Recently, tissue plasminogen activator (tPA) was identified as a specific protease activating PDGF-CC[28], and urokinase plasminogen activator (uPA) was found to activate PDGF-DD[29].

It has been suggested that the protease cleavage sites in the novel PDGFs are located within the hinge region where a highly conserved tribasic site is found. This is the site where the classical PDGFs are proteolytically processed (see above). In PDGF-CC and PDGF-DD these sites are RKSR^{234} and RKSK^{257}, respectively (Paper I[7]

and[8])(illustrated in Fig. 2). In PDGF-CC, tPA was found to actually cleave at this site[28,30]. Investigating the uPA-mediated cleavage of PDGF-DD revealed that another site is important in the cleavage of PDGF-D, $R^{247,249}$, which is located upstream of the predicted site[29]. This site has also been suggested by others to be the relevant cleavage site[9]. Further studies are needed to determine what role the tribasic site might have in the activation of PDGF-DD. In the processing of PDGF-CC it has been found that tPA interacts with both CUB and the PDGF/VEGF homology domain to mediate the cleavage[28,30]. In the case of uPA-mediated cleavage of PDGF-D it is not established whether this kind of interaction is necessary. The identification of tPA and uPA as the proteases activating the novel PDGFs was surprising, since these two proteases are mostly known for their function in fibrinolysis, where they are important in the conversion of plasminogen to plasmin[31]. Today it is established that these two proteases regulate the bioavailability of the novel PDGFs by extracellular cleavage. Whether the bioavailability of the novel PDGFs is further regulated by other factors is not well understood. The PDGF-C and PDGF-D lack recognizable retention motifs in their primary structures, which can keep the proteins cell associated. However, instead the CUB domains have been suggested to mediated binding to cellular structures.[8] This has to be further investigated. Furthermore, uPA is known to exert its biological effect by creating a complex with its cell surface receptor, urokinase plasminogen activator receptor (uPAR)[31]. The possibility that uPAR might interact with PDGF-D as an adaptor protein that feeds ligand to the protease has to be investigated. Interestingly, uPAR has been suggested to function as a coreceptor to PDGFR-β[32], further discussed in section 1.3.2. Characterization of the different factors that regulate the bioavailability is important to further understand the biological role of the novel PDGFs

1.3 PDGF receptors

A few years after the identification of the classical PDGFs, binding of ^{125}I-labeled PDGF to human foreskin fibroblasts indicated the presence of membrane receptors to theses ligands[4]. Later evidence suggested that at least two different PDGF receptors existed[33]. Today it is well known that the PDGFs exert their biological responses by binding to, and activating, two structurally related tyrosine kinase receptors, named PDGFR-α and PDGFR-β.

1.3.1 Structure of the PDGF receptors

The two human PDGF receptors are encoded by two genes that have been located on chromosomes 4 and 5, respectively[34,35]. The PDGFR-α polypeptide chain contains 1089 amino acids, while the PDGFR-β polypeptide chain contains 1106 amino acids. After maturation, the α- and β-receptors are found as cell surface proteins that have molecular sizes of about 170 and 180 kDa, respectively.

The two PDGF receptors consist of an extracellular part containing five immunoglobulin(Ig)-like domains, a transmembrane domain, and an intracellular split tyrosine kinase domain[5,35,36]. The three N-terminal Ig-like domains are involved in ligand binding, whereas the fourth Ig-like domain stabilizes the receptor-receptor complex and the function of the fifth Ig-like domain is unknown (reviewed in [37])(illustrated in Fig. 3).

The PDGFs bind with different specificities to the two receptors. PDGF-AA, PDGF-BB, PDGF-AB and PDGF-CC bind to PDGFR-α homodimers, whereas PDGF-BB and PDGF-DD bind to PDGFR-β homodimer. In addition, all dimer ligands except PDGF-AA can bind heterodimers of PDGFR-αβ (Paper I[7], reviewed in[5] and[8,9]). In the case of PDGF-DD there are contradictory results regarding its ability to bind to PDGFR-αβ heterodimers, which is further discussed in section 3.1.

1.3.2 Signalling from the PDGF receptors

Dimerization is a key event and a general mechanism underlying the activation of receptor tyrosine kinases[38,39]. PDGF ligand binding induces receptor dimerization by simultaneously binding to two receptors creating a stable receptor dimer. Contact between the intracellular parts of the receptor dimers allows autophosphosrylation of tyrosine residues, which take place by one receptor molecule phosphorylating the other in the dimer. The autophosphorylation of tyrosine residues takes place in two different phases in the intracellular domain. In the first phase the conserved residues Tyr^{849} in PDGFR-α and Tyr^{857} in PDGFR-β become phosphorylated. Both residues are located within the kinase domain and phosphorylation induces increased kinase activity[37,40]. In the second phase, tyrosine residues located outside the kinase domain are autophosphorylated. These residues serve as binding sites for different intracellular signalling proteins, which themselves become activated upon binding,

thus initiating signal transduction. The activation of the PDGF receptors triggers responses such as cell proliferation, migration, differentiation and cell survival. The signalling molecules that interact with phosphotyrosine residues contain specific domains called Src homology 2 (SH2) domains, which recognize phosphorylated tyrosines. A few specific amino acids downstream of the phosphorylated tyrosine residues determine the specificity in the SH2-domain binding. Different SH2-domain containing molecules bind to individual phosphorylated tyrosine residues in a specific manner, thus initiating different signalling pathways[5,37]. SH2 domain-containing signalling molecules known to be involved in PDGFR signalling include phosphatidylinositol 3'kinase (PI_3-kinase), Phospholipase C-γ (PLC-γ) and Src. The enzyme PI_3-kinase induces actin reorganization, chemotaxis, as well as stimulation of cell growth and inhibits apoptosis. The enzyme PLC-γ is involved in proliferation of certain cell types. Members of the Src family are themselves tyrosine kinases, which are implicated in the mitogenic response of PDGF.

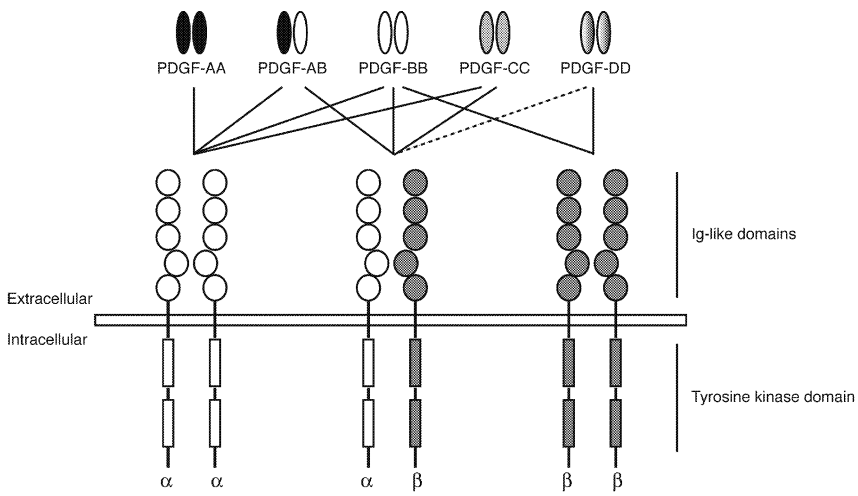

Figure 3. Structure of the PDGF receptor dimers and binding of the different PDGF ligands to the receptors. The PDGFR-α and PDGFR-β, which contain five extracellular Ig-like domains, a transmembrane domain and an intracellular split tyrosine kinase domain. Binding of the different dimeric PDGF ligands to the three first Ig-like domains induce dimerization of the receptors, and the fourth Ig-like domain mediates stabilization of the ligand-receptor complex. The dimerization directs the phosphorylation of the intracellular tyrosine residues, which leads to activation and a cascade of intracellular events.

Several signal transduction molecules bind to both PDGFR-α and PDGFR-β and initiate overlapping signal transduction pathways. Signalling through the two receptors also gives rise to distinctly different effects on target cells, which can be explained by differential interactions with SH2-domain containing proteins such as Crk that binds only to the α-receptor, and GAP that binds only to the β-receptor[41]. In addition, some small differences have also been described comparing the signalling from homo- and heterodimeric receptor complexes in cells that express both α- and β-receptors[5]. However, *in vivo* studies where the cytoplasmic domains of PDGFR-α and PDGFR-β were swapped, demonstrated that the β-receptor signal transduction can substitute for the α-receptor signals during embryogenesis. Surprisingly, swapping the cytoplasmic domains the other way around generated several abnormalities in the development of vSMCs, which indicates that PDGFR-β has a unique function in these cells. Another *in vivo* study where neither the PDGFR-α nor the PDGFR-β were capable of binding PI_3-kinase, revealed that PI_3-kinase activity is a major transducer of α-receptor signalling, but that during certain circumstances the β-receptor can be used as a transducer of this activity (reviewed in[42]). The *in vivo* roles of the PDGF-receptors both during development and adulthood are further discussed in section 1.4.2.

However, the different descriptions implying that intracellular signal transduction consists of a number of linear signalling pathways that mediate the different cellular effects of PDGF give a simplified picture. The PDGF receptor signalling is a complex system and extensive crosstalk between the intracellular signals and the network of interacting signalling components occurs, and these pathways are carefully controlled by inhibitory feedback mechanisms[43]. The receptor signalling is also regulated by the different and temporal expression of the receptors. To make it more complex, recent reports have implicated several co-receptors for PDGFR-β signalling. Low-density lipoprotein recepor-related protein (LRP) have been reported to function as a co-receptor, interacting with PDGF-BB, thus inducing phosphorylation of both PDGFR-β and LRP on fibroblasts and SMCs.[44]. In addition uPAR has been reported to interact as a co-receptor for PDGFR-β. A recent report suggested that uPA-mediated activation of uPAR induces PDGFR-β activation by phosporylation of cytoplasmic tyrosine kinase domains and receptor dimerization inducing vSMC migration and proliferation[32]. This is of particular interest in light of that PDGF-D is a specific PDGFR-β ligand that recently was described to be activated by uPA. However, the role of PDGF-D in uPAR/PDGFR-β induced signalling has to be further investigated.

1.4 Functions of the PDGFs and their receptors

The functions of the classical PDGFs and their receptors are well investigated, whereas less is known about the novel PDGFs. PDGF signalling is known to be involved in many different cellular responses, and the PDGFs are often described as the major mitogen for cells of mesenchymal origin, including smooth muscle cells and fibroblasts[5].

1.4.1 Expression and regulation of the PDGFs and their receptors

To be able to understand the different biological functions of the PDGFs it is essential to consider all factors that can be of importance. Expression of both receptors, and each of the four PDGF chains, is under independent control, allowing the PDGF/PDGFR system a high degree of combinatorial flexibility. Examination of the PDGFs and the PDGF receptors respective expression profiles may reflect some of the sites where they have biological implications. Other factors, such as different expression levels of both the PDGF ligands and their receptors by different cell types are also important. In addition, the expression levels are often influenced by external stimuli, such as proteolytic processing, inflammation, exposure to low oxygen or stimulation from other growth factors[5].

Comparing the mRNA expression patterns of the four genes in normal human tissues revealed that PDGF-A, PDGF-B and PDGF-C are expressed in most human tissues with highest expression of PDGF-A in heart, pancreas and skeletal muscle, PDGF-B in heart and placenta, and PDGF-C in heart, kidney and placenta, whereas PDGF-D expression is more restricted with high expression in heart, pancreas and ovary (also discussed in section 3.1). The expression pattern showed that each gene is expressed in a unique, but partially overlapping manner (illustrated in Fig 4.) (Paper I[7] and[6]).

Studies of the expression profiles of the classical PDGFs in different cell lines revealed that most cells expresses both A- and B-chains, but they are independently regulated both at the transcriptional and posttranscriptional levels. PDGF-A and PDGF-B are expressed by normal cells such as fibroblasts, vSMCs, kidney mesangial cells, vascular endothelial cells and macrophages, whereas myoblasts and astrocytes only express PDGF-A (reviewed in[5]). Another important way of regulating the classical PDGFs is the binding to the ECM by their retention motifs, which creates gradients that present the ligand to the relevant target cell. Furthermore, the classical PDGFs can interact with soluble proteins such as α_2-macroglobulin, which regulates the amount of PDGF available for interaction with the PDGF-receptors[45].

The expression level of the receptors is also a factor that can differ. Studies of the expression profile of the two PDGF receptors in cell cultures revealed that fibroblast and smooth muscle cells express both PDGFR-α and PDGFR-β, but they generally expresses higher levels of β-receptors. Some cell types display one receptor type, but not the other. Cell types known to only express PDGFR-α are O-2A glial precursor cells, human platelets, liver endothelial cells and astrocytes.

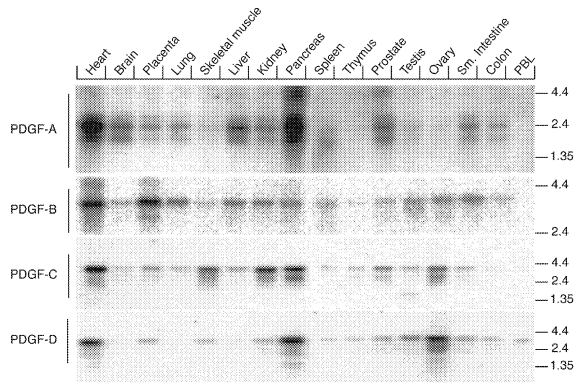

Figure 4. Multiple tissue Norhtern blot. Expression analysis of the four PDGF chains in normal adult human tissue. The Northern blot showing that all four PDGF chains are widely expressed, but that each chain displays a unique expression pattern. Picture adopted from[6].

Other cells types such as Itoh cells of the liver, myoblasts, capillary endothelial cells, pericytes and macrophages only express PDGFR-β (reviewed in[5]).

During embryonic development, PDGF-A and PDGFR-α are produced at earlier stages, and are more abundantly produced than PDGF-B and PDGFR-β. PDGF-A has been detected as a maternal transcript in the oocyte[46]. Furthermore, the embryonic expression of PDGF-A is found in epithelial cells, while the PDGFR-α is expressed in the adjacent mesenchymal cells[47]. In addition, PDGF-B is expressed in both epithelial and endothelial cells, whereas PDGFR-β is expressed in adjacent mesenchymal cells, such as pericytes and vSMCs[48].

The expression profiles of the novel PDGFs are less investigated. Both PDGF-C and PDGF-D are expressed by SMCs, fibroblasts, ECs and myoblasts (Paper II[18] and IV), while platelets only express PDGF-C[49]. The embryonic expression of PDGF-C is wide spread and found in the epithelium of several organs, similar to PDGF-A expression[50]. The expression of PDGF-D is more restricted. High expression of PDGF-D is found in heart, kidney skeletal muscle and in SMCs of some blood vessels (Paper I[7], III[51] and IV).

The novel PDGFs are also dependent on the expression of the highly specific proteases that activates them. Since they are activated extracellularly, factors such as colocalization of protease and substrate are of importance. tPA and PDGF-C, is coexpressed during embryonic development in at least kidney and surface ectoderm, and coexpression has also been found in myoblasts (Paper IV and[28])

In the case of PDGF-D and uPA, less is known but we report that they are coexpressed in myoblasts (Paper IV).

Considering that the PDGFs and their receptors are expressed in numerous tissues and cell types both during embryonic development and in the adult it seems that the

PDGFs can act by both paracrine and autocrine signalling. The different expression profiles indicate that the PDGFs have different biological roles. The exact physiological and pathological roles of the PDGF/PDGFR signalling system has to be studied in other ways than just by investigating the expression profiles. The physiological and pathological outcomes of PDGF signalling are further discussed below.

1.4.2 Physiological roles

The PDGFs, especially the classical PDGFs, have been extensively characterized in culture-based assays, where they have been shown to drive cellular responses including proliferation, survival, and migration. Advances in the ability to generate knockout mice have made it possible to understand the *in vivo* function(s) of the different genes. Genetic deletion of the classical PDGFs and their receptors has shown that they are important during development, where the PDGFs are mitogenic to, and drive proliferation of undifferentiated mesenchyme, and some progenitor cell populations. Conditional knockouts, and small mutations in the PDGF and PDGFR genes have provided further insight into the roles of the PDGF signalling system during postnatal development. During these stages, PDGF signalling has been implicated in processes such as tissue remodelling and cellular differentiation. The picture of PDGF biology is starting to take shape, but it is still incomplete. The biological function of the novel PDGFs is indistinct, even though the PDGF-C knockout was reported recently[25,52,53].

Development

Deletion of the genes encoding different PDGF ligands and their receptors showed that they are essential during development. The PDGF-A knockout is invariably lethal, with half of the embryos dying around embryonic day (E) 10, whereas the other half survives until early postnatally[54]. The large phenotypic differences observed in the PDGF-A knockouts are probably connected to the genetic background[25]. The early lethality in PDGF-A deficient mice is not well understood. However, mice surviving until birth display severe lung emphysema due to incomplete alveolar septum formation[54]. Further analysis of the alvegonesis showed that the SMCs, which drives the alveogenesis are missing in the PDGF-A knockouts. The phenotype also includes severely reduced elastin depositions in the lungs[54,55]. *In vitro* as well as *in vivo,* PDGF-AA, is a major mitogen for oligodendrocyte progenitors[56] and the PDGF-A deficient mice showed loss of oligodendrocyte progenitors which causes a severe hypo-myelination[57]. Other defects found in the PDGF-A knockouts are progressive loss of dermal mesenchyme, and reduction in testicular size, as well as loss of Leydig cells[58,59].
The phenotype of the PDGFR-α deficient mice is more severe than that caused by deletion of the PDGF-A gene. All PDGFR-α knockout animals die between E8 and

E16. The phenotype observed includes in addition to the defects found in the PDGF-A knockout mice, cleft face, spina bifida, subepidermal blistering, smaller and abnormally patterned somites, which later affects the vertebrae and ribs, as well as vascular defects[60]. The more severe phenotype of the PDGFR-α deficient mice compared with that of PDGF-A suggests that the α-receptor mediates signalling from another ligand such as PDGF-B or PDGF-C. Since homozygous deletion of both PDGF-A and PDGF-B did not reproduce the phenotype observed in PDGFR-α knockouts, PDGF-C was thus proposed to be the missing link in α-receptor signalling[8]. Expression of PDGF-C in metanephric mesenchyme adjacent to the lost PDGFR-α expressing cells in the kidney of PDGFR-α knockouts, and the fact that loss of mesecnhymal cells was not found in the PDGF-A or PDGF-B deficient mice, further supported a developmental role of PDGF-C[8]. The PDGF-C knockouts showed different phenotypes with perinatal lethality, or postnatal survival depending on genetic background[25,53]. The phenotype observed in mice dying perinatally includes feeding and respiratory difficulties due to cleft palate. The phenotype observed in the 129S1/sv background was less severe than that of the PDGFR-α deficient mice. However, deficiency of both the PDGF-A and the PDGF-C genes phenocopied the PDGFR-α knockouts confirming that PDGF-C is the missing link in the receptor α-signalling during development[53]. Target deletion of the gene encoding the PDGF-C activating protease tPA revealed a mild phenotype. Comparing the reported phenotypes of the PDGF-C deficient mice and the tPA knockout mice did not show any obvious overlap[53,61].

The PDGF-B and PDGFR-β deficient mice show highly similar phenotypes, and they die during later stages of embryonic development due to cardiovascular complications, where numerous vessels are incompletely covered by vSMCs and pericytes[62,63]. Analysis of the PDGF-B and PDGFR-β knockouts showed abnormal kidney glomeruli, capillary microaneurysms, arterial SMC hypoplasia, cardiac muscle hypotrophy, placenta defects and widespread edema and haemorrhages[62-66]. The similar phenotypes of these two knockouts raise the question about the role of PDGF-D in PDGFR-β signalling during development. There are currently no PDGF-D deficient mice available today. Deletion of the gene that encodes the PDGF-D activating protease uPA displayed a very mild phenotype, which possibly could be explained by redundancy from tPA. Double uPA/tPA knockouts showed a more severe phenotype. Even though they survive, they have a markedly reduced fertility and shortened life span[61]. Thus uPA does not seem to be crucial during embryonic development. This reinforces the conclusion that PDGF-D is not important during embryonic development. However, at some sites in PDGF-B knockouts, such as adrenal gland and skeletal muscle, pericytes and SMCs are almost properly recruited, while this was not seen in PDGFR-β deficient mice[66]. Interestingly, PDGF-D expression was observed at these sites indicating that PDGF-D may have a developmental role at these sites (Paper III[51] and Paper IV and[51]). Further investigations of these sites in a future PDGF-D knockout will hopefully provide us with the right answer and tell us what role PDGF-D has during development.

Adult

Significant progress has been made in understanding the roles of at least the classical PDGFs during development, but the physiological role of the PDGFs in the adult is less understood. Studies using PDGFR kinase inhibitors, such as imatinib (Gleevec/Glivec) as a cancer treatment has shown mild side effects even during long-term treatment. This suggests that the PDGF signalling system is not essential for maintaining the adult human organism[67].

However, PDGFR signalling has been found to be important during wound healing, where the PDGFs stimulates mitogenicity and chemotaxis of SMCs, fibroblasts and different types of inflammatory cells. The healing process also involves reepitelialization, angiogenesis, and extracellular matrix deposition. Several growth factors are involve in the different steps in the healing process and PDGFs can be a part of all of them directly or indirectly[5,68]. Recombinant PDGF-BB has been used clinically to stimulate wound healing[69]. Furthermore, PDGFR-β regulates the interstitial fluid pressure, which is important in controlling transport from the vasculature into the extracellular compartment of connective tissue[5,68]. The PDGFs have also been suggested to have a neuroprotective role[5]. The significant developmental role of the PDGFR-β and PDGF-B in blood vessel formation and the fact that the PDGF receptors are expressed on adult capillary endothelial cells, suggests that the PDGF signalling system is involved in angiogenesis during adulthood. Several studies have explored the potency of PDGFs to initiate blood vessel formation both *in vitro* and *in vivo*[70-72]. However, if the angiogenic effect is a direct effect of PDGFs on endothelial cells, or if it is an indirect effect by PDGF induced upregulation of VEGF-A, needs to be further investigated[72,73].

In the work presented in this thesis we suggest that PDGF-D may have a role in skeletal muscle regeneration and growth, which is of importance during repair of skeletal muscle injuries and when the muscle is exercised. The PDGF-D activating protease uPA, has been found to have a significant role in skeletal muscle regeneration *in vivo*[74], and the link between PDGF-D and uPA indicate that PDGF-D may have a role in skeletal muscle regeneration. This is further discussed in section 3.4.

The role of the PDGF/PDGFR signalling in adult physiology needs to be further investigated, and careful analysis of conditional and subtle mutagenesis in mice will be valuable in these studies.

1.4.3 Pathological roles

PDGFs and their receptors have been linked to several diseases, such as atherosclerosis, fibrosis and different malignancies. In all cases enhanced signalling of the PDGF receptors plays an important role. These findings have induced an extensive research in developing agents that block PDGFR signalling[75].

Malignant diseases

Cancer is a disease characterized by uncontrolled proliferation of cells, and the ability of these cells to migrate and invade other tissues. The unregulated growth is caused by different mutations, rearrangement and changes in the gene expression of genes that encode for growth factors that normally are important for cell proliferation. The PDGFs have been linked to several tumour-associated processes. In some tumours the PDGF/PDGFR signalling system is involved in autocrine growth stimulation, where the tumour cells express the PDGFs and the PDGF receptors at high levels. The autocrine growth is in many cases caused by genetic alterations, which cause constitutive activation of the PDGF receptors. In addition, the PDGFs are involved in paracrine stimulation, where the tumour highly expresses the PDGFs, and the PDGF receptors are expressed by the stroma cells. Furthermore, the PDGFs and the PDGFRs are involved in stimulation of tumour angiogenesis and recruitment of tumour fibroblasts[67,76]. The classical PDGFs have been implicated in different cancers such as gliomas, dermatofibrosarcomas, neurofibroma, chronic monomyelocytic leukemia, osteoblastomas and osteosarcomas (reviewed in[77]).

The first indications that the PDGFs are involved in tumorgenesis came with the discovery that the transforming protein of simian sarcoma virus (SSV), is encoded by the v-sis oncogene, which constitutes a virtual homologue of the PDGF-B chain[78]. Today it is established that PDGF-B through PDGFR-β signalling possesses high transforming activity. The novel PDGF-C and PDGF-D were also found to be potent transforming growth factors of NIH/3T3 cells, suggesting that they might play a role in malignancy[79]. This is further indicated by the expression of the novel PDGFs in several primary tumours and tumour cells lines such as breast, prostate and glioblastomas (Paper II[18] and[80]). Further, PDGF-C has been found as an EWS/FLI-induced transforming growth factor[81], and PDGF-D has been implicated in paracrine tumour growth in the B16 mouse melanoma tumour model[81]. PDGF-D has also been linked to prostate tumours, where PDGF-D is active in both an autocrine and a paracrine manner, accelerating early onset of prostate tumour growth and enhancing prostate carcinoma cell interaction with surrounding stromal cells[82].

The presence and upregulation of PDGF receptors in many different tumours provides a therapeutic target. Inhibiting the autocrine stimulation of tumour growth by blocking the PDGF receptors have in different cell lines and xenograft models shown promising results. Therefore a number of PDGFR kinase inhibitors have been developed and have been evaluated in several different tumours. Many of these agents are still at early stages of development and testing. Most of the experience of PDGFR kinase inhibitors comes from studies with the clinically approved drug, imatinib (STI571, Gleevec)[67,83]. Also other PDGF antagonists have been used in tumour treatment. These have to be further evaluated to explore their possibilities[67].

Atherosclerosis and restenosis

In the western world, more people die of the complications of atherosclerosis than of any other cause. Atherosclerosis is a protective, inflammatory, fibroproliferative

response, which is induced by events that take place within the arterial wall, and starts with endothelial dysfunction, leading to recruitment of activated macrophages and lymphocytes, which accumulates in the subendothelial zone. Further, medial SMCs are attracted and migrate into the intimal layer, initiating the atherosclerotic lesion. Subsequently, a plaque consisting of SMCs and macrophages is formed in the intima, followed by the formation of a layer of fibrous tissues creating a fibrous cap surrounding the plaque. Eventually the atherosclerotic vessel becomes clogged, which leads to a thrombotic occlusion that may cause a heart attack, or a stroke[84].

SMC proliferation is a critical process in atherosclerosis, and in an attempt to determine why SMC cells accumulates in atherosclerosis, the growth requirements of arterial SMCs were investigated in culture. This led to the identification of the classical PDGFs, which then were purified based on their ability to stimulate the proliferation of SMC[2]. This fact and the later findings that PDGF receptor signalling is essential during vascular development, suggest that the PDGFs may have a role in the SMC accumulation in atherosclerosis (reviewed in[85]). This is further indicated by the low expression of the classical PDGFs in arteries from healthy adults, whereas the expression of PDGFs is increased when the inflammatory-fibroproliferative response is induced. Moreover, the PDGFR-β expression and activation is increased[5,86]. These findings suggest that PDGFs, secreted by activated macrophages, SMCs, ECs or platelets, stimulate the SMCs in the media, which leads to the formation of lesions in atherosclerosis.

However, eliminating the PDGF-B from circulating cells, or blocking the PDGF receptors in the well-established atherosclerotic mice ApoE -/-, delays lesion progression, but does not prevent SMC accumulation in advanced atherosclerotic lesions. These results suggest that other growth factors can promote SMC accumulation and connective tissue deposition in the atherosclerotic processes[87]. Furthermore, in several studies where restenosis is induced by arterial injury, PDGF-B has been found to be important in attracting SMCs to the neointima formation (reviewed in[5]).

The possible role of the novel PDGFs in atherosclerosis and restenois is poorly investigated. However, PDGF-D has been suggested to be involved in the formation of neointimal hyperplasia induced at vascular injury, and might serve as a target in preventing vascular restenosis[88]. In addition, PDGF-D expression has been found in some neointimal SMCs of atherosclerotic vessels[89]. In this study we report an increased amount of active PDGF-DD in apoE -/- mice (Paper III[51] further discussed in section 3.3). Interestingly, human atherosclerotic lesions have been reported to contain elevated levels of uPA, which is thought to accelerate the atherosclerotic process[90].

Considering the likely involvement of PDGF/PDGFR signalling system in the pathogenesis of atherosclerosis, clinically useful PDGF antagonists are desired. In animal models of restenosis, therapeutic effects have been obtained from PDGF SELEX aptamers[91] and adenoviral-mediated transfer of a gene encoding a soluble form of the PDGFR-β[92]. Several other PDGF antagonist treatments have also been successfully tested, which suggest that blocking of PDGF receptor signalling is a possible treatment of restenosis (reviewed in[76]). The use of PDGF antagonists in the treatment of atherosclerosis is less investigated. The accumulation of SMCs in the

atherosclerotic lesion has been regarded as a negative event promoting the development of occlusive lesions. However, recent data have indicated that this is not a negative event, instead the SMCs stabilize the lesion and protect the fibrous cap from rupture. The prevention of SMC proliferation may therefore be associated with undesirable outcomes. An optimized balance of SMCs is probably the ultimately solution[93].

Fibrotic disease

Fibrosis is a disease that affects millions of people worldwide. The disease includes pulmonary fibrosis (lung fibrosis), scleroderma (thickening of the skin), diabetic retinopathy (fibrotic diseases of the eye), diabetic nephropathy, glomerulosclerosis and IgA nephropathy (causes of kidney failure) and cirrhosis (leading cause of liver fibrosis).

Fibrosis is a pathologic process, which includes scar formation and excessive production of extracellular matrix by the connective tissue, as a response to tissue damage. The molecular process is induced when the normal wound healing response goes wrong. Proliferation of mesenchymal cells that possess a myofibroblast phenotype is induced, accompanied by collagen production. This leads to a faster formation of scar tissue. The excessive production and deposition of collagen results in pathological scarring, and the scar tissue causes organs to become stiff and they cannot perform functions essential to life and health. This progression can lead to organ failure and death[94]. Induction of fibrosis can be triggered by a variety of events including trauma, surgery and infections.

Many factors that are involved in normal tissue repair can also promote fibrogenesis, and several different cell types secrete PDGFs as a response to injury. PDGFs play a major role in stimulating proliferation, migration and survival of myofibroblasts during fibrotic diseases, which then leads to upregulated expression of the PDGF receptors[94].

The roles of the classical PDGFs during the pathogenesis of fibrosis are well characterized and they have been linked to various pulmonary, liver and renal fibrotic conditions[5]. Several studies have also implicated the novel PDGFs in fibrotic processes. Overexpressing PDGF-C induces the development of liver fibrosis, and enhanced levels of PDGF-C have been found in activated hepatic stellate cells[95,96] Further, upregulated PDGF-C protein expression was detected within sclerosing glomerular and fibrosing tubulointerstitial lesions, which suggest that PDGF-C has a role in kidney fibrosis[97]. PDGF-C has also been linked to pulmonary fibrosis and cardiac fibrosis[98,99]. In addition, PDGF-D has been implicated in renal fibrosis, where PDGF-D has been suggested as a major mediator of mesangial cell proliferation, which leads to mesangio proliferative glomerulopathy[100,101]. In paper III we describe that overexpression of PDGF-D in the mouse heart induces cardiac fibrosis. Cardiac fibrosis was also observed when PDGF-C was overexpressed in the mouse heart[51,98]. This is further discussed in section 3.3.

Fibrosis is considered an irreversible process, at least clinically. Current therapies for fibrotic diseases include anti-inflammatory drugs, which alleviate the symptoms at

best, but fail to prevent the fibrotic process, and subsequently the disease progresses. There is a great need of safe and effective anti-fibrotic therapies that delay disease progression, and reduce mortality. However, imatinib has been found to reduce the proliferation of activated hepatic stellate cells, and inhibits the fibrogenesis when treatment is initiated before fibrosis has developed[102].

2 Aims

It was almost thirty years ago since the classical PDGF family members, PDGF-A and PDGF-B were discovered and during these years they have been extensively studied. The finding of two new member of the PDGF family was unexpected, and suggests that signalling through the PDGF/PDGFR system is more complex than was thought. In this study we try to elucidate the complexity of this system. The discovery of PDGF-D is described in one of the papers in this thesis, thus the thesis is mainly focused on PDGF-D. One of the major aims is to characterize the biological function of PDGF-D.

The specific aims of the studies described in this thesis have been:

- To clone the cDNA of the human PDGF-D, and to characterize this novel member of the PDGF family (Paper I)
- To determine the chromosomal location and exon/intron structures of the *PDGFC* and the *PDGFD* genes (Paper II)
- To elucidate the biological function of PDGF-D, by heart-specific overexpression in transgenic mice (Paper III)
- To investigate the role of PDGF-D in skeletal muscle growth and regeneration (Paper IV)

3 Results and discussion

In this section I will summarize the most important findings from each paper and discuss the result. A detailed description of the results, including Material and Methods are found in respective paper.

3.1 PDGF-D is a specific, protease-activated ligand for the PDGF β-receptor (Paper I)

For more than twenty years the PDGF family consisted of only two members. During later years the development of sequence databases for expressed mRNAs has made it easier to discover new genes using bioinformatics tools. We were interested in finding new members of the PDGF/VEGF family. All known members of that family have a PDGF/VEGF homology domain, which contains a structural motif comprising eight well-conserved cysteine residues. The combined PDGF/VEGF homology domains from different known family members were used as a search string in searches of the human and mouse EST databases at NCBI (National Center for Biotechnology Information) using BLAST. These searches resulted in the discovery of two new members of the PDGF/VEGF family. Phylogenetic analysis showed that the novel family members formed a subgroup that clustered together with the PDGFs. The novel PDGF family members were consequently named PDGF-C and PDGF-D. The discovery and characterization of PDGF-C is described in a separate study not presented in this thesis[8].

The finding of one new member of the PDGF family was not completely unexpected. Deletion studies of the genes that encode the classical PDGFs, or their receptors, revealed that mice lacking PDGFR-α showed a more severe phenotype than the PDGF-A knockout, and deletion of both PDGF-A and PDGF-B did not reproduce the defects observed in PDGFR-α. This raised the possibility of the existence of yet another PDGFR-α ligand[103]. Knockout mice, which lack the genes for both PDGF-A and PDGF-C resembles the phenotype of PDGFR-α deficient mice, thus PDGF-C showed to be the missing PDGFR-α ligand[8,53]. However, the discovery of a fourth member of the PDGF family was unexpected, since mice lacking either PDGF-B, or PDGFR-β showed similar phenotypes.

In this paper we describe the cloning of the cDNA for PDGF-D, which encodes a polypeptide chain of 370 amino acids. To examine the biochemical properties of PDGF-D, recombinant full-length PDGF-DD protein was produced in baculovirus infected Sf9 insect cells. Analysis by SDS-PAGE showed that purified PDGF-DD migrated as a 90 kDa species under non-reduced conditions, and as a 55 kDa species under reduced conditions. This indicated that PDGF-DD is secreted as a disulphide-bonded homodimer similar to the other PDGF family members. PDGF-D was found to display a two-domain structure similar to that found in PDGF-C, with an N-terminal CUB domain, and a C-terminal PDGF/VEGF homology domain. We predicted that full-length PDGF-D was secreted as a latent protein, considering that PDGF-C is secreted as an inactive protein requiring proteolytic processing to become

active. The attempts to produce a truncated version of PDGF-D in baculovirus infected SF9 cells with only the PDGF/VEGF homology domain failed. Instead we constructed a version of full-length PDGF-D containing a specific cleavage site for factor Xa, allowing site-specific cleavage and release of the CUB domain. Full-length and cleaved PDGF-DD were tested in receptor binding and stimulation assays. We observed that cleaved PDGF-DD, but not full-length PDGF-DD, was able to preferentially bind to and stimulate PDGFR-β. This finding confirmed that PDGF-D is expressed as an inactive precursor, and that it is necessary to proteolytically remove the CUB domain to release the active growth factor domain. In addition we found that plasmin was able to cleave and activate full-length PDGF-D. However, due to its wide substrate specificity, this protease is unlikely to be a physiologically relevant protease[28]. Recently uPA was identified as an enzyme capable of activating PDGF-D[29]. The discovery of PDGF-DD as a specific PDGFR-β ligand was unexpected considering the findings from the knockout studies. This indicates that the signalling through the PDGFR-β is more complex than was previously thought.

At almost the same time as we discovered PDGF-D, another group independently discovered the same protein[9]. Contradictory to us they have reported that PDGF-DD can signal through PDGFR-α via heterodimers with PDGFR-β. In our hands, and under our standard experimental settings, PDGF-DD does not signal through PDGFR-α. We further confirmed this in Paper III, where PDGF-DD was able to stimulate PDGFR-β, but not PDGFR-α, expressed by primary cardiac fibroblasts[51]. Thus, in our hands PDGF-DD is a specific PDGFR-β agonist.

In a multiple tissue northern blot analysis of human mRNAs the expression pattern of PDGF-D was compared with that of the other PDGFR-β ligand, PDGF-B. The expression pattern of PDGF-D in human tissues is more restricted and lower compared to that of PDGF-B, indicating that PDGF-B and PDGF-D have distinct functions. Further analysis of the tissue expression of PDGF-D during mouse embryonic development revealed high expression in heart, lung and kidney. A more detailed analysis of the kidney suggested that PDGF-D might have a paracrine function in proliferation of PDGFR-β-expressing perivascular cells of the undifferentiated metanephric mesenchyme. However, the PDGF-B and PDGFR-β deficient mice did not reveal any phenotypic differences in the developing kidney, which argues against the suggested paracrine role of PDGF-D in the kidney. To test this a PDGF-D knockout is needed, which has not been reported yet.

In conclusion, the novel member of the PDGF- family, PDGF-D, is expressed as a latent protein that is regulated by protease activation to become a specific PDGFR-β ligand. PDGF-D is suggested to have unique temporal and spatial roles. In addition, PDGF-D might be involved in pathological conditions such as fibrotic disease, which lately has been confirmed in several studies, where PDGF-D has been linked to mesangial proliferative glomerulopathy[100,104]. Furthermore, in Paper III we also show that overexpression of PDGF-D induces cardiac fibrosis[51].

3.2 Chromosomal location, exon structure, and vascular expression patterns of the human *PDGFC* and *PDGFD* genes (Paper II)

The classical PDGFs are well characterized both structurally and functionally, whereas less is known about the novel PDGFs.

To further characterize the two novel PDGFs we analyzed the chromosomal localization and genomic organization of the corresponding genes. By fluorescence in situ hybridization we located the human *PDGFC* and *PDGFD* genes to chromosome 4q32 and 11q22.3 to 23.2, respectively. The exon/intron structures of the two genes were determined by sequencing from genomic DNA clones. The results showed that the *PDGFC* gene has 6 exons, while the *PDGFD* gene has 7 exons. Both PDGF-C and PDGF-D chains are arranged in a two-domain structure with an N-terminal CUB domain and a C- terminal PDGF/VEGF homology domain, where exons 2 and 3 encode the CUB domain in both genes. In the *PDGFC* gene exons 5 and 6 encode the PDGF/VEGF-homology domain, while in the *PDGFD* gene the growth factor domain is encoded by exons 6 and 7. The putative proteolytic cleavage site consisting of a stretch of basic amino acids is located in exon 4, and exon 5 in the *PDGFC* and the *PDGFD* genes, respectively. This site has been identified as the cleavage site where tPA activates PDGF-C[30]. In addition, uPA activates PDGF-D, but it is still unclear which site is utilised. A site upstream of the predicted site ($R^{247, 249}$) has been reported to be necessary for the uPA-mediated cleavage of PDGF-D[29].

We also investigated the expression of PDGF-C and PDGF-D in human vascular cells and in some tumour cell lines. Northern blot analysis demonstrated that human umbilical vein endothelial cells (HUVEC) and human microvascular endothelial cells (HMVEC) express both PDGF-C and PDGF-D mRNAs. Human coronary artery smooth muscle cells (HCASMC) abundantly express PDGF-C mRNA, whereas human fetal lung fibroblasts (Wi-38) abundantly expressed PDGF-D mRNA. Several tumour cell lines such as the breast tumour (BT-474) and the prostate tumour (PC-3) cell lines expressed both PDGF-C and PDGF-D mRNA, while the fibrosarcoma (HT-1080) cell line only expressed PDGF-C mRNA. Comparing the expression profiles of the four PDGF mRNAs in different tumour cell lines indicated that the four genes are independently regulated. Further, we localized the novel PDGFs in the arterial wall by immunostaining of sections of the suprarenal artery, which showed weak staining of PDGF-C in the SMC layer, whereas staining for PDGF-D was found in the adventitial connective tissues layer. Both PDGF-CC and PDGF-DD were also found to stimulate proliferation of cultured SMCs.

In conclusion, the novel *PDGF* genes have similar genomic structures, which resemble the reported structures in the *PDGFA* and the *PDGFB* genes. Furthermore, considering the expression of PDGF-C and PDGF-D in several tumour cell lines and the role the classical PDGFs have in malignancy indicates that inappropriate expression of PDGF-C and PDGF-D may contribute to certain cancers. Several other studies have subsequently linked PDGF-C and PDGF-D to different malignancies (reviewed in[105]). Further expression of PDGF-C and PDGF-D in the arterial wall and culture vascular cells suggests that they are involved in PDGFR signalling inducing

proliferation and migration of SMCs. In addition other studies revealed that PDGF-D is able to induce proliferation of arterial SMCs *in vivo*[51], which is further discussed below.

3.3 Platelet-Derived growth factor D induces cardiac fibrosis and proliferation of vascular smooth muscle cells in heart-specific transgenic mice (Paper III)

It is well known that the classical PDGFs induce proliferation and migration of cells of mesecnhymal origin, such as smooth muscle cells and fibroblasts. In addition the PDGFs have also been implicated in fibrotic diseases, as well as atherosclerosis[5]. Less is known about the novel PDGFs, during physiological and pathological conditions.

Here we further investigated the expression pattern and potential pathological role of PDGF-D. The expression analysis showed high expression of PDGF-D in heart, and thus we focused the study on the cardiovascular system. In embryonic and adult heart PDGFR-β was expressed in blood vessels and microvessels, while PDGF-D was expressed in the adjacent myocardium. In addition, in some developing arterial SMCs, expression of PDGF-D colocalized with PDGFR-β expression. These observations suggest that PDGF-D may provide both paracrine and autocrine signalling in PDGFR-β-expressing cells

We addressed the potential pathological effect(s) of excess PDGFR-β signalling by generating transgenic mice overexpressing active PDGF-DD in the heart. Transgenic overexpression of PDGF-D caused cardiac fibrosis followed by dilated cardiomyopathy and consequently cardiac failure. A heart-specific transgenic mice overexpressing full-length PDGF-C was recently reported to also induce cardiac fibrosis[98]. Comparing these two transgenes showed that PDGF-D induced a similar, but more severe phenotype as compared to full-length PDGF-C, including proliferation of interstitial fibroblasts, extensive deposition of collagen, vascular remodelling such as vessel dilation, locally decreased capillary density, and increased number of SMC-coated vessels. PDGF-D also induced proliferation of arterial vSMCs, which caused thickening of the vessel wall. This was not observed in the PDGF-C transgenic mice. In most PDGF-D transgenic mice, the expression of the protein was restricted to distinct areas where a strong local accumulation of interstitial fibroblasts was observed, and only in these areas thickening of the arterial walls was found. The differences observed in the PDGF-C and PDGF-D transgenic mice may be due to the fact that different receptors are activated, thus different downstream signals are induced. The PDGF-D transgenic mice showed enhanced activation of PDGFR-β. In the SMCs of enlarged arterial walls expression of the β-receptor was detected, while no expression of PDGFR-α was found at these sites. The PDGF-C transgenic mice showed enhanced PDGFR-α activation. In a study where the cytoplasmic domain of PDGFR-β was exchanged to that of PDGFR-α the development of vSMCs showed several abnormalities, which indicates that PDGFR-β has a unique function in the development of SMCs (reviewed in[42]). Summarising the

above findings indicates that PDGF-D is more potent than PDGF-C in stimulating proliferation of SMCs, due to its capacity to specifically signal through PDGFR-β. However, the less mitogenic effect of PDGF-C on SMCs in the transgenic mice might be due to the fact that PDGF-C is limited by having to be proteolytically activated, while PDGF-D was directly expressed in its active form in the transgenic mice. Considering that PDGF-C can activate PDGFR-β via αβ heterodimers, thus PDGF-C should be able to induce a similar effect on SMCs as PDGF-D.
Further, we demonstrated that PDGF-DD has a direct effect on proliferation of the cardiac interstitial fibroblasts by specifically stimulating PDGFR-β expressed on these cells. In Paper II we also show that PDGF-D, as well as PDGF-C, is a potent mitogen of cultured SMCs[18]. Taken together these results suggest that PDGF-DD is potent mitogen for SMCs and fibroblasts.
To strengthen the pathological relevance of the transgenic model and to investigate the possible involvement of PDGF-D in cardiovascular disease, we investigated the expression of PDGF-D in hearts from the well-known cardiovascular disease model the apoE -/- mice. These animals spontaneously develop atherosclerosis. The proliferation SMCs is a critical process in the development of atherosclerosis, where vSMCs accumulate in the subendothelial zone of larger vessels[85]. The results showed that PDGF-D expression is upregulated in the hearts of apoE-/- mice. In addition we found increased amounts of active PDGF-DD in apoE-/- mice, as well as increased expression of uPA. In a related cardiovascular disease process, others have suggested that PDGF-DD has a role in intimal hyperplasia at vascular injury[88]
In conclusion, this study has provided novel insight into the functional significance of PDGF-D, and suggests that PDGF-D is a potent mitogen for fibroblasts and vSMC both *in vitro* and *in vivo*. Furthermore, we found that PDGF-D may be involved in the pathogenesis of cardiovascular diseases, such as atherosclerosis and cardiac fibrosis.

3.4 Platelet-derived growth factor DD stimulates proliferation and migration of mouse myoblasts

Although it was some years ago since the specific PDGFR-β ligand, PDGF-D, was discovered, the biological function is still obscure. In an expression analysis of PDGF-D during embryonic mice development we observed expression in different tissues including blood vessel of the skeletal muscle. A study where PDGFR-β chimaeric embryos were investigated revealed that PDGFR-β has a role in the development of all muscle lineages (smooth, cardiac, skeletal and pericyte)[106]. In addition, comparing embryos of PDGFR-β and PDGF-B deficient mice revealed similar phenotypes, but one of the few sites that showed some differences in the phenotypes was the skeletal muscle[66]. Furthermore, in a recent study, where skeletal muscle injury was experimentally induced in adult uPA deficient mice, uPA was found to be required for efficient regeneration of damaged muscle[74]. The fact that uPA recently has been reported as the enzyme that activates PDGF-D, and the importance of PDGFR-β in muscle development, as well as the observed phenotypic differences in skeletal muscle of PDGFR-β and PDGF-B, suggest a possible role for

PDGF-D in the skeletal muscle development. Based on these facts we hypothesized that PDGF-D has a role in the skeletal muscle regeneration process. To explore possible biological function(s) of PDGF-DD in skeletal muscle, we investigated its role in skeletal muscle development and regeneration. We demonstrated expression of PDGF-DD in the skeletal muscle fibers of developing mouse embryos from E13.5 and onwards. The expression of PDGF-D during later stages of mouse development indicates that PDGF-D may have a role in the formation of fetal muscle fibers. We also examined the expression profile of the PDGFs and PDGFRs in the well-known myoblast cell line, C2C12. Reverse transcriptase PCR analysis of cDNA from undifferentiated and differentiated cells interestingly showed abundant expression of PDGF-D, PDGFR-β and uPA, while very low levels of PDGF-B and PDGFR-α were observed. Considering the expression profile observed in the C2C12 cells, the PDGF ligand has to primarily signal through PDGFR-β. The low expression levels of the other PDGFR-β ligand, PDGF-B, proposes that PDGF-D is the PDGFR-β signalling ligand in the myoblast cells, thus playing a role in skeletal muscle regeneration. Furthermore, undifferentiated C2C12 cells were found to cleave and activate latent PDGF-DD. In addition, we demonstrated that PDGF-DD was able to stimulate PDGFR-β expressed by C2C12 cells. Based on our observations we suggest that PDGF-DD may create an autocrine loop, where expressed PDGF-DD is activated by uPA, and active PDGF-DD then induces stimulation of the PDGFR-β expressed by the myoblasts.

To verify that PDGF-DD is an important mitogen for myoblasts, we stimulated the C2C12 myoblasts with recombinant active PDGF-DD. This showed that active PDGF-DD induces both proliferation and migration of myoblast cells, as well as inhibits differentiation of the myoblasts into muscle fibers. These results suggest that PDGF-DD is involved in skeletal muscle regeneration and growth through induction of proliferation and migration of muscle progenitor cells.

To summarize our findings, we suggest the following (proposed mechanism illustrated in Fig. 5): After muscle injury, satellite cells become activated, and start to produce PDGF-DD as a locally expressed chemoattractant. The muscle satellite cells also produce uPA, which activates PDGF-DD that induces receptor activation of PDGFR-β positive satellite cells, thus stimulating proliferation and migration of these cells. Furthermore, PDGF-DD recruits macrophages to the site of injury. In turn, the activated macrophages produce factors such as uPA, PDGF-BB and possibly also PDGF-DD (unpublished observation), which further stimulates the skeletal muscle satellite cells to divide. In addition, PDGF-DD inhibits the differentiation of satellite cells, but at a certain point, when enough cells have accumulated at the sites of injury, the expression of growth factors is down regulated, and muscle specific genes are switched on, inducing differentiation and fusion of the satellite cells into fully developed multinucleated muscle fibers.

In conclusion, we have demonstrated that PDGF-DD is a potent growth factor for myoblasts *in vitro*, and our study provides novel insight into the biological role of PDGF-DD in skeletal muscle regeneration and growth.

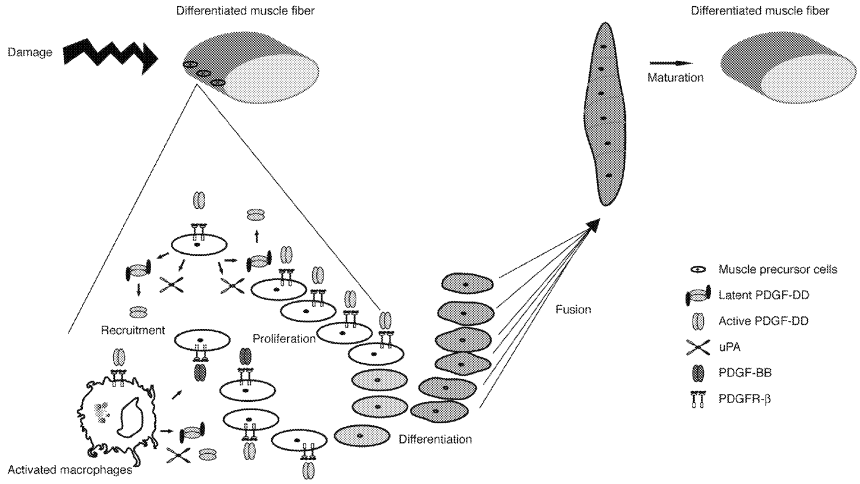

Figure 5. Hypothesized mechanism for PDGF-DD stimulation of muscle progenitor cells. Muscle progenitor cells are positioned between the plasma membrane and the surrounding basal lamina of differentiated muscle fibers. The muscle progenitor cells respond to injury or exercise by starting to proliferate. The cells divide a limited number of times and then they differentiate and fuses into multinucleated myotubes, which mature into fully differentiated muscle fibers. PDGF-DD, PDGFR-β and uPA are expressed from the muscle progenitor cells. PDGF-DD is activated by uPA. Activated PDGF-DD stimulates PDGFR-β expressed on the progenitor cells and they start to proliferate. Activated PDGF-DD also recruits macrophages, which becomes activated and start to produce PDGF-BB, PDGF-DD and uPA, which further stimulates the muscle progenitor cells to divide.

4 Future perspectives

To summarize, the studies presented in this thesis describe the discovery of one of the novel PDGFs, PDGF-D, which has contributed to an increased understanding of PDGF biology. The presence of a fourth PDGF ligand indicated that signalling through the PDGF receptors is more complicated than was previously thought. Although the novel PDGFs have been known for some years, their exact biological function is still obscure. Therefore it is important to further characterize the *in vivo* function of the novel PDGFs. In the case of PDGF-D there is no knockout mouse available yet. Analysis of the phenotype of PDGF-D deficient mice would hopefully enhance the understanding of the biological function of PDGF-D. The fact that PDGF-D is expressed as a latent growth factor, which is activated when the specific protease is present, represents a strategy by which growth factor activity might be regulated by adjacent or distant cell populations dependent on protease expression, recognition and cleavage. These different issues have to be further investigated and above all it is important to characterize the protease that activates PDGF-D. uPA has recently been reported to activate PDGF-D. The protease uPA is best known for its function in fibrinolys and tissue remodelling where uPA directs plasmin to facilitate degradation of extracellular components. One of the projects in our group is to characterize the role of uPA in PDGF-D activation. It is important to establish why PDGF-D is expressed as a latent factor? Which cellular processes regulate the latency?

In addition, uPA has a role in skeletal muscle regeneration *in vivo*. In the last paper some interesting findings about skeletal muscle regeneration and PDGF-D was observed (discussed in section 3.4). In another ongoing study where we have overexpressed PDGF-C and PDGF-D by using adenoviruses that were injected in to the ears of nude mice, we made some interesting observations in the injected muscle layer. The main aim with the study was to investigate the angiogenic properties of PDGF-C and PDGF-D. We found that both PDGF-C and PDGF-D exhibit angiogenic properties, where PDGF-C stimulates the angiogenesis and PDGF-D promoted arterializations. Interestingly, we observed that both factors upregulated smooth muscle actin in the dermal skeletal muscle. Furthermore in the skeletal muscle an increased cellularity was also observed corresponding to an unidentified cell population that surrounded each muscle fiber. This cell population might be proliferating skeletal muscle progenitor cells. However, these findings have to be further investigated. To identify the cell population and to further investigate the role of PDGF-D in skeletal muscle regeneration we will use the PDGF-D adenoviruses (full-length PDGF-D and active PDGF-D) and inject them into the muscle of both wild type and uPA deficient mice. The questions we hope to be asked in this study are; which cell type proliferates? is it a direct effect of the PDGF-D? how is this regulated in the uPA deficient mice?

To conclude this thesis, the discovery of PDGF-D “stirred things up” in the PDGF world and showed that there are always new things to discover!

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