Role of Extracellular Retention of Platelet-derived Growth Factor-B

Functions in Development and Disease

Per Lindblom

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Role of Extracellular Retention of Platelet-derived Growth Factor-B

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Per Lindblom

Department of Medical Biochemistry
Box 440, SE-405 30, Göteborg, Sweden

Abstract

During development, the cell secretes growth and differentiation factors (GDFs) to the surrounding microenvironment. These factors are often key regulators of organogenesis and embryogenesis. Several GDFs carry sequences that mediate specific interaction with molecules of the extracellular matrix (ECM) that surrounds the cell. The deposition of factors in the matrix can theoretically result in (1) reservoirs of growth factors, (2) in spatially restricted action range of the factor or (3) in the critical growth factor concentrations or gradients needed for the factor to elicit specific cellular responses i.e. to act as a morphogen. So far, few attempts have been made to analyse the functional importance of GDF-ECM interactions. Several platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) family members display basic amino acid motifs at the C-terminus, which confer retention of the factor in the extracellular milieu surrounding the producing cell. To address the role of PDGF-B retention *in vivo*, we deleted the retention motif in mice by a gene targeting approach. This resulted in reduced recruitment and defective investment of pericytes in the micro-vessel wall, and in delayed formation of the glomerular mesangium. Long-term effects of lack of PDGF-B retention included reactive gliosis in the CNS, severe retinal detoriation, glomerulosclerosis and proteinuria. Several tumours express PDGF-B and the cognate receptor PDGFR-β. To investigate the effects of altered PDGF-B distribution in a pathological situation, we analysed the vasculature in a tumour model. In tumours transplanted to PDGF retention deficient mice, pericytes were fewer and partially detached, leading to significantly increased vessel diameter and haemorrhaging. Tumour specific over-expression of PDGF-B increased the pericyte density in both control and in PDGF-B retention deficient mice, but could not correct the defective pericyte integration in the vascular wall. To analyse developmental effects of PDGF over-expression, we generated transgenic mice that over-expressed different PDGF isoforms specifically in the heart. This caused pathological changes ranging from severe and generalised cardiac fibrosis and early postnatal lethality to focal fibrosis in the adult heart. Thus, PDGF signalling seems sufficient to induce cardiac fibrosis. In conclusion, the alteration of PDGF distribution or increased/decreased PDGF levels, have detrimental effects during development, as well as in the adult. The results point to an important role for PDGF in cancer and on retinal, glomerular and myocardial diseases.

Key words: PDGF, pericyte, cancer, fibrosis, ECM, HSPG, cell retention, endothelial
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Department of Medical Biochemistry
Göteborg University

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Per Lindblom

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Abbreviations

BM	basement membrane
CUB	domain found in complement urchin-like EGF protein and bone morphogenetic protein-1
dally	division abnormally delayed
E	embryonic day
EC	endothelial cell
ECM	extracellular matrix
EGF	epidermal growth factor
FGF	fibroblast growth factor
GAG	glucosaminoglycan
GDF	growth and differentiation factor
GDNF	glial-derived neurotrophic factor
GlcA	glucuronic acid
GlcNAc	N-acetylglucoseamine
HSPG	heparan sulfate proteoglycan
MHC	myosin heavy chain
MTC	Masson-tri-chrome
NDST	N-deacetylase/N-sulfotransferase
P	postnatal day
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
PlGF	placenta growth factor
Shh	sonic hedgehog
SH2	src homology 2 domain
SMA	α-smooth muscle actin
SPARC	secreted protein, acidic, and rich in cysteine
SSV	simian sarcoma virus
TGF	transforming growth factor
TNF	tumour necrosis factor
VEGF	vascular endothelial growth factor
vSMC	vascular smooth muscle cell
Wg	wingless

Introduction

Embryonic development has fascinated us and been subject for study since ancient times. It was not until the late 19th century, when classical anatomical embryology, experimental embryology and genetics began to address the common and central question of how a multicellular organism can develop from a single primordial cell, the zygote. Although there was a split between classical genetics and embryology in the 1920´s, the recent decades have merged them again and it is now apparent that the two disciplines are closely linked and dependent on each other.

The completion of the human genome sequence project in 2001 set a milestone in the genetic research. The three billion nucleotide mammalian genome contains about 40,000 genes and we are just beginning to realise the complex nature of its regulation. We are also only at the start of addressing the specific function(s) of each individual gene. One of the most powerful inventions that have helped us study gene functions in the context of the living organism *in vivo* is gene targeting. This approach allows us to introduce specific alteration in any gene of interest. The effect of the mutated gene can be analysed in the developing or adult organism and during normal and pathological conditions. Gene targeting in mice has been utilised in the work described in this thesis. Firstly, we have introduced a mutation in the *platelet-derived growth factor-b* (*pdgf-b*) gene, which changes the way the PDGF-B protein distributes in the cellular interstitium. We analysed the consequences of this mutation in several organs, including kidney and retina. Secondly, we analysed the consequences of this mutation in a pathological situation. We chose a well-characterised tumour model and

studied its progression in the background of the PDGF mutation. Thirdly, by a classical transgenic approach we over-expressed three forms of PDGFs under the control of a heart-specific promoter and analysed resulting pathological responses.

The tools that we have applied and the way we are now able to analyse the genome, demonstrate how closely linked genetics and developmental biology have become. However, while it is now possible to manipulate the genotype with high precision, it is still difficult to interpret the phenotypic consequences of such manipulations. To reach mechanistic insight, it is necessary to do careful analyses of gene expression patterns and protein distribution. In addition, it is as necessary to follow the time course progression of the phenotypic consequences, in order to discern primary from secondary effects. This will be very clear in the first part of the thesis, which deals with one of the most complex component of our bodies, the extracellular matrix (ECM). I initially discuss the main components of the ECM to illustrate the dynamic environment that surrounds the cell and what milieu the growth factors face upon secretion. The first part also exemplifies several proteins potentially important for the interactions between the ECM and PDGF. While condensed and far from comprehensive, this general description of the ECM is necessary for the subsequent discussion of my results. The description also serves as an illustration to the recent advances and limitations of gene-targeting experiments.

General background

The extracellular matrix

The ECM was previously referred to as "connective tissue", which is a misleading term that implies a stable and uniform mesh with a simple function; to connect and scaffold cells in our bodies. Data accumulated over the last two decades show the contrary: the ECM is a highly dynamic complex of substances, which shows unique composition in different organs and functional states, and plays a number of critical roles in the regulation of cell function. The ECM has major impact on development and on disease processes such as diabetic nephropathy, cancer and fibrotic conditions. These diseases will be further discussed in the sections relating to papers I, II and III, respectively. The ECM can affect the differentiation state of the individual cell, and thereby also the factors that the cell delivers to its environment. Moreover, the ECM composition influences the function and distribution of certain growth and differentiation factors (GDFs). The ECM distributes between two major domains, the basement membranes (BM) and the interstitial matrix. The BM is located adjacent to e.g. epithelial and endothelial cells, adipocyte and muscle cells. The BM serves both as a unit that separates cells, or as a substrate, on which the cell can migrate, proliferate and differentiate.

Collagen and the basement membrane

Collagen constitutes 50% of the basement membrane, which additionally contains approximately 50 different proteins. The dominant collagen isoform varies depending on the tissue. Collagen I is widely expressed (except for

cartilage), while expression of collagen II is restricted to cartilage and the eye. So far, more than 20 genetically distinct types of collagen have been identified. Expression of collagen is localised to cells present in the tissue stroma (fibroblasts, chondrocytes, osteoblasts, myofibrobroblasts). However, certain epithelial and endothelial cells also produce collagens e.g. collagen type IV. All collagens are characterised by a repetitive amino acid sequence, in which every third amino acid is a glycine (so called Gly-X-Y motif). Collagen subtypes I, II, III, V and XI carry long such stretches and have the ability to form fibrils, which act as supportive elements of tendons, cartilage, bone and skin. Fibril formation takes place in the ECM, where specific metalloproteinases cleave the procollagen. Some collagens are not able to form fibrils, but rather form net-like structures. Collagen IV is also known as "network forming collagen" and is the major component of basement membranes. Collagen type VI, IX, XII and XIV associate with fibril structures, and finally collagen type XIII is transmembranous (reviewed in Kalluri 2003).

Extracellular glycoproteins

The ECM consists of several other proteins besides collagen. One of the main components is laminin. Laminins exist in several isoforms that, in different combinations, form the functional protein. The mature laminins consist of three disulphide-linked polypeptide chains. Production of the isoforms is tissue-specific. Different laminins induce different effects in cells, including differentiation, adhesion and cell migration. Laminins interact with cells mainly through integrins. Metalloproteinases (MMPs) are able to cleave laminins, enabling cell detachment from the ECM and cell migration. Laminins are able to bind collagens, which is important for the

establishment of the ECM. In a tumour, aberrant laminin expression often correlates with defective interaction between invading tumour cells and the ECM. Hence, altered laminin expression is thought to be an important feature in cancer progression (reviewed in Bosman and Stamenkovic 2003).

Fibronectin (FN) is another large ECM protein (460 kDa), which is synthesised both in normal and pathological conditions. FN is mainly produced by fibroblasts, chondrocytes, endothelial cells, macrophages and certain types of epithelial cells. FN functions as an adhesive molecule by binding to integrins, but it also binds collagen, thereby contributing to the organisation of the ECM. FN forms a substrate for migrating cells. For example, germ cells that migrate from the yolk sac to the developing gonads depend on interactions with FN. FN has heparin and heparan sulfate proteoglycan (HSPG) binding sites. The interaction with HSPGs may affect cell adhesion, and the presentation of HSPG-bound growth factors to the cell (reviewed in Pankov and Yamada 2002).

Tenascin has a complex domain structure and can bind to several molecules on the cell surface, including integrins, Ig cell adhesion molecules (CAMs) and phosphacan (a chondroitin sulphate proteoglycan). Expression of tenascin at sites of ongoing matrix rearrangement coincides with the expression of MMPs. MMPs may cleave tenascin and thereby affect the cellular functions dependent on tenascin interactions with the ECM. Tenascin is normally not expressed in the adult, however, re-expression is detected during neovascularisation and tumour growth (reviewed in Jones and Jones 2000).

Similar to tenascin, SPARC (secreted protein acidic and rich in cysteine) is ubiquitously expressed during development. SPARC is down-regulated in the adult and re-expressed at sites of ongoing remodelling and wound healing. SPARC and PDGF-B co-localise in platelet α–granules and show a pH dependent interaction. This may influence the release of PDGF in e.g. wound healing. Moreover, SPARC has an inhibitory effect on vascular endothelial growth factor, fibroblast growth factor and PDGF-A signalling. The spectrum of SPARC-growth factor interactions suggests that SPARC may have a regulative rather than a structural function in the ECM (Raines et al. 1992; reviewed in Brekken and Sage 2001).

Proteoglycans

Proteoglycans consist of a protein core with connected sugar polymers glycosaminoglycans (GAGs). The GAGs are attached in a linear fashion to serine residues on the protein core and are subject to specific modifications, other glycoproteins carry unmodified sugar chains that are often branched. The sugar polymers consist of multiple and distinct disaccharide units. Heparan, for example, consists of glucuronic acid (GlcA) and N-acetylglucosamine (GlcNAc), whereas chondroitin consists of repetitive disaccharides of N-acetylgalactosamin and GlcA. Heparan sulfate proteoglycan (HSPG) is a form of proteoglycans that carries sulfated GAG chains. The sulfate groups provide the GAG chains with a negative charge, mediating interaction with positively charged domains of many proteins, for example PDGF/VEGF family members. The relative amount of HSPG versus collagen determines the plasticity/rigidity of the ECM. The localisation of HSPGs to the cell surface likely makes them important for cell-cell interaction. Recently, HSPGs have been shown to be important for

growth factor signalling during development. Despite being such a complex family of molecules, the genetics of the HSPGs is fairly simple. All members of the five different classes; *syndecan*, *glypican*, *perlecan*, *agrin* and *collagen type XVIII*, are encoded by a total of 13 separate genes. These genes are thought to account for at least 95 % of the total numbers of proteoglycans in the ECM (reviewed in Iozzo 2001). On the other hand, HSPGs display an enormous heterogeneity, mostly depending on the multiple serine residues present on the core proteins where the GAGs can bind. Alternative splicing of the genes, proteolytic processing and different modifications of the GAG chains further increase the complexity of the HSPGs. The physical nature of the HSPGs enables them to span a distance of several hundred nanometers, making it possible for them to reach over an entire basement membrane and contact adjacent cells. Obviously, it is interesting to consider the multiple potential implications of such features for growth factor distribution and action. Accordingly, presence of HSPGs has been shown to be required for activation of the fibroblast growth factor receptor (FGFR) by FGF (Yayon et al. 1991a). The heparan sulfate structure does not depend on the core protein. Rather, a given cell produces heparan sulfates of similar structure that may become attached to different core proteins. HSPGs contact the ECM in different ways (outlined in figure 1). The four syndecans are transmembranous proteins with the heparan sulfate chains near the extracellular tips and chondroitin sulfate chains near the cell surface. The six members of the glypicans attach to the cell membrane through a glycosylphosphatidylinositol (GPI)-anchor. Perlecan, agrin and the HSPG/collagen type XVIII are located in the pericellular space.

Synthesis of proteoglycans involves several enzymatic steps. It starts with the addition of a tetrasaccharide linker to a serine residue on the core protein. Xylosyltransferase adds a single xylose to the serine residue, which constitutes the first piece of the linker. Next follows the addition of two galactose units, catalysed by two different galactosyltransferases. Finally, a single GlcNAc is added, which completes the formation of the tetrasaccharide linker (Lander and Selleck 2000; Esko and Lindahl 2001).

Next, two transferases initiate the polymerisation reaction by the addition of GlcNAc-GlcA disaccharide residues. These polymerisation enzymes are encoded by the mammalian EXT gene family. The disaccharides can subsequently be modified e.g. by sulfation, such as N-deacetylation/N-sulfation (removal of the acetyl group from the N atom, followed by sulfate addition to the same N) and O-sulfation (addition of a sulfate group to one of the hydroxyl groups of the sugar ring). Sulfation is by far the most important reaction for generating diversity within the proteoglycans. N-sulfation is performed by four members of the N-deacetylase/N-sulfotransferase (NDST) family.

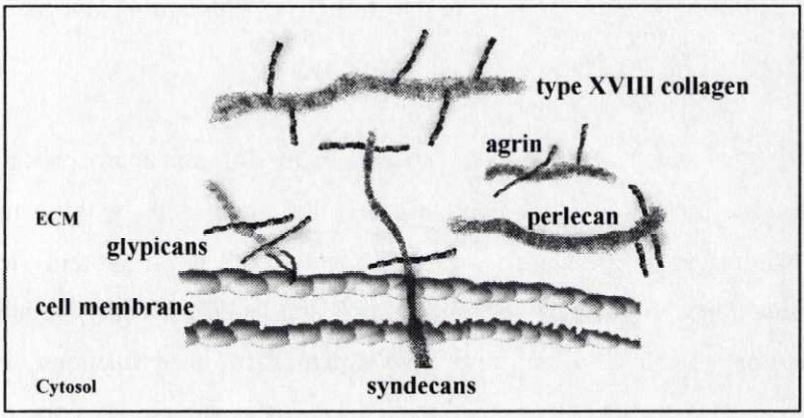

Figure1. A schematic depiction of heparan sulfate proteoglycans. Syndecans are transmembrane proteoglycans, while glypican core proteins are linked via a phosphoinositol anchor to the cell membrane. Collagen type XVIII, agrin and perlecan are secreted proteins, but are retained in close vicinity to the producing cell and are components of the basement membrane. Branches indicate glycosaminoglycan side chains; e.g., perlecan usually carries three HS chains near the N-terminus.

Functional studies on ECM molecules

The high concentration of HSPGs in the ECM, their extraordinary half-life numbers, their negative charge, and their ability to interact with GDFs of numerous families, together make the HSPGs unique among the ECM components. The HSPGs interaction with GDFs enables formation of gradients, necessary for morphogenetic processes during development (Perrimon and Bernfield 2000). Also, GDF signalling properties may depend on HSPGs. For example, FGF depends on the presence of HSPGs on the responding cell surface for optimal receptor binding and activation (Yayon et al. 1991b). Several attempts have aimed at pinpointing the function(s) of HSPGs in the intact organism. The most advanced and productive studies

have utilised mutation screens in the fruit fly *Drosophila melanogaster* and gene targeting in mice.

For genetic analyses on HSPGs, two principally different approaches may be considered. Firstly, the core protein may be inactivated. In this situation, similar HS side chains might still be present on other core proteins, leading to functional redundancy or compensation and lack of phenotypic outcome. The core protein itself can also have structural or other functions, without associated HS side chains, so the phenotypic outcome of core protein ablation may be related to the functions of HSPGs. One might therefore need to compare the result of core protein ablation with the phenotypic outcome of specific mutations of the HS attachment sites. Secondly, enzymes involved in the HS polymerisation or modification processes may be targeted by mutagenesis. This may, however, lead to very complex phenotypes, since it would affect the modifications on all core proteins.

HSPGs core protein mutants in *Drosophila*

In vertebrates, the HSPGs include *glypican*, *perlecan*, *agrin*, *collagen* and *syndecan*. However, the *Drosophila* genome contains only one *syndecan*, two *glypicans* and one *perlecan* homologue. The two *glypicans* are named *dally* (*division abnormally delayed*) (Nakato et al. 1995) and *dally-like* (Khare and Baumgartner 2000), while syndecan is known as *Dsyndecan* (Spring et al. 1994). *Dally* has been inactivated in flies through mutation or RNA interference. Interestingly, *dally* mutants phenocopy the *wingless* (*Wg*) mutant. Wg is a GDF important for patterning processes in *Drosophila*. Several studies have confirmed that the levels and expression patterns of dally and dally-like protein have pronounced positive effect on Wg signaling

(Lin and Perrimon 1999; Tsuda et al. 1999; Baeg et al. 2001). This was one of the first pieces of compelling evidence for a specific function for HSPGs.

HSPGs modification enzyme mutants in *Drosophila*

Drosophila mutants have also been studied for the homologue of UDP-D-glucose dehydrogenase, which produces UDPGlcA, and the NDST homologue. These mutations affect polymerisation (*sugarless*) and sulfation (*sulfateless*), respectively. Alteration of these enzymes has pronounced negative effect on the signalling pathways of Wg, FGF and Hedgehog (Hh) (Binari et al. 1997; Hacker et al. 1997; Lin et al. 1999). Consequently, the severe phenotypes associated with these mutations mimic complete loss of the signalling pathways induced by these factors, highlighting the importance of HSPGs during development.

The interaction between GDFs and HSPGs can be very specific, as demonstrated by fibroblast growth factor-2 (FGF-2) and hepatocyte growth factor (HGF). FGF-2 needs three consecutive trisulfated disaccharides units to bind efficiently to HS (Faham et al. 1996). HGF, on the other hand, requires a pentasaccharide to bind to heparin (Ashikari et al. 1995). A mutation in one EXT homologue (polymerisation enzyme) further enhances the view that proteoglycans interact with growth factors more specifically than previously expected. One would expect this mutant (*tout velu*) to have similar consequences as *sulfateless* or *sugarless*. However, only the Hh signalling pathway seems to be affected. This specificity is surprising, since no core proteins would be expected to carry HS side chains (Bellaiche et al. 1998; The et al. 1999). However, one should bear in mind that the most sensitive and non redundant pathways are most likely to generate a

phenotype when disturbed. Hence, these results do not rule out a function for HS side chains in Wg or FGF signalling.

HSPGs core protein mutants in mouse

The studies referred to above demonstrates the challenge involved in interpreting *Drosophila* phenotypes, into specific biochemical interactions, due to the complexity of the intact *Drosophila* larvae. Thus, the challenge to interpret phenotypic alterations in mice might be expected to be even larger, in particular considering the higher complexity of the mouse genome. For example, the mouse has four NDST and six *glypican* genes, compared to the fly, which has one NDST (*sulfateless*) and two *glypican* genes (*dally and dally-like*) (Table 1). Additionally, the mouse genome contains more GDFs and, most likely, produces a more complex pattern of protein isoforms. However, several core proteins have been genetically targeted in mice and the phenotypes vary from embryonic lethality, e.g. deletion of perlecan (Arikawa-Hirasawa et al. 1999; Costell et al. 1999) to situations where no abnormalities are observed e.g. deletion of glypican-2 (Lander and Selleck 2000).

In mice, the core proteins perlecan, agrin and collagen XVIII, have been genetically ablated. Perlecan-deficient mice display severe abnormalities, not resembling any of the mutants of the enzymes involved in GAG polymerization or modification pathways (reviewed in Forsberg and Kjellen 2001). The perlecan mutant passes through two critical time points. At embryonic day (E) 10-12, many of the mutants die due to defective organisation of the cardiac basement membranes and subsequent rupture. Survivors of this first critical stage die at birth, due to exencephaly or similar

malformation. As previously discussed, this may reflect a function of the perlecan core protein without attached HS side chains. Perlecan interacts both with the ECM components collagen type IV and nidogen I, but it can also bind directly to e.g. FGF-7 (Mongiat et al. 2000). In a recent elegant study, exon three of *perlecan* was specifically altered abolishing the initiation sites for three HS side chains (Rossi et al. 2003). These mice developed normally and were fertile, but their lenses degenerated postnatally. This phenotype was restricted to the eyes and became worse in the background of a *collagen XVIII* mutation, suggestive for overlapping functions of perlecan and collagen XVIII HSPGS in the lens.

Agrin homozygous null mice display defects in presynaptic differentiation, intramuscular nerve branching and other nerve-specific alterations (Gautam et al. 1996). Mice carrying a null mutation in the collagen XVIII gene show delayed regression of the hyaloid vessels (hyaloid vessels normally nurture the lens during development and regress after birth, when lens growth has ceased). These mice also display irregular sprouting of the endothelial cells from the optic disc into the retina. The retinal defect might be secondary to lower levels of VEGF, due to increased oxygen pressure in the retina as a result of persisting hyaloid vasculature (Fukai et al. 2002). For description of retinal development, see the section below relating to paper I.

Mouse mutants for the cell surface proteoglycans include syndecan-1,-4, and glypican-2, -3. Syndecan-1, -4 and glypican-2 deficient mice develop normally (reviewed in Forsberg and Kjellen 2001). Glypican-3 null mutants die perinatally and display developmental overgrowth, cystic and dysplastic kidneys and abnormal lung development (Cano-Gauci et al. 1999).

Interestingly, a later study linked the kidney phenotype, in the glypican-3 mice, to the loss of BMP-2 inhibitory effect on kidney tubular branching (Grisaru et al. 2001).

HSPG modification enzyme mutants in mouse

Mice carrying targeted mutations in the genes coding for enzymes involved in polymerisation and modifications of the HS side chains include two NDST isoforms, one polymerase enzyme (EXT-1) and a 2-O-sulfotransferase. Mice heterozygous for an EXT-1 null allele (homologous to *Drosophila tout velu*), show reduced HS production, whereas homozygotes die during gastrulation and show abnormally organised mesoderm and extraembryonic tissues (Lin et al. 2000). Thus, the removal of most of the HS side chains from all core proteins resulted in a more severe phenotype than the removal of any of the core proteins.

NDSTs are necessary for the sulfation of the side chains and are homologous to Drosophila *sulfateless*. Mouse mutants have been produced for NDST-1 and -2. NDST-1 mutants show, as expected, reduced levels of N-sulfated HS. The mutants display defective production of surfactants in the lungs, which leads to death shortly after birth. They also show skeletal and eye defects. NDST-2 mice show a defect in the connective tissue type mast-cells. The mast-cells were almost devoid of granules, which normally contains e.g. heparin, proteases and histamine that are released during an inflammatory state. The phenotype was in line with previous data; no compensation could occur since NDST-1 is absent in these cells. However, the embryonic lethality of double NDST-1 and –2 mice suggests that NDST-2 is partially redundant with NDST-1 (Forsberg and Kjellen 2001). For complete

description of NDST mutants see (Forsberg et al. 1999; Humphries et al. 1999).

	Drosophila	Mice
Core proteins		
glypican	**Dally, Dally-like**	**glypican-1, -2, -3, -4, -5, -6**
syndecan	**Dsyndecan**	**syndecan- 1, -2, -3, -4**
Enzymes		
UDP-D-glucose dehydrogenase	**sugarless**	**ugdh**
N-deacetylase/N-sulphotransferase	**sulfateless**	**NDST-1, -2, -3, -4**
GlcNAc/GlcA polymerase	**tout velu**	**Ext 1, Ext 2**

Table 1. An outline of the described core proteins, polymerisation/modification enzymes and mice/Drosophila homologues

In summary, the ECM is a dynamic complex of many different types of molecules. Its interaction with GDFs is critically important in developmental processes and may be more specific than previously expected. Investigations of different mutants of core proteins and HS side chains producing enzymes are ongoing and will most likely add more clues to the functions of HSPGs. This introduction will now be followed by a section describing some of the factors secreted into and interacting with the ECM. PDGF is one of many protein families where certain isoforms carry specific amino acid residues that interact with HSPGs. The exact HSPG binding partners for PDGFs *in vivo* have, however, not yet been identified.

Introduction to Platelet-derived growth factor

The use of the term growth and differentiation factors (GDFs), rather than growth factors, throughout this thesis better reflects the functional diversity of this large group of proteins. Many of the individual GDF names originate

from the source of purification or the first cell type known to be affected. Later on, with improved technology and knowledge, we have realised that most GDFs are highly pleiotropic in their functions. Thus, factors like EGFs, PDGFs, Hh, FGFs and TGFs can affect migration, differentiation, apoptosis/survival, proliferation and other cellular functions. Several of these factors conduct an intrinsic interaction with the molecules in the ECM. GDFs can either bind directly to the core protein, such as FGF-7 and TGF-β (Andres et al. 1989; Mongiat et al. 2000) or interact with the heparan sulfate side chains, such as PDGF, VEGF and FGF-2 (Moscatelli 1988; Houck et al. 1992; Pollock and Richardson 1992; Kelly et al. 1993). In some cases, the GDF-ECM interaction is necessary for optimal receptor activation. In other cases, the interaction mediates retention of the factor close to the cell. To date, only few studies have addressed the functional importance of GDF-ECM interactions *in vivo*. This thesis includes one of the first attempts to analyse the importance of such interactions in vertebrates. Because my work has been based on PDGFs, I provide an introduction to this family of growth factors below.

Platelet derived growth factor - general background

PDGF was originally identified as a factor within blood serum plasma (Kohler and Lipton 1974; Ross et al. 1974; Westermark and Wasteson 1976). It was subsequently purified from human platelets (Antoniades et al. 1979; Deuel et al. 1981). Early *in vitro* experiments showed that PDGF could induce mitosis (Cochran et al. 1983), induce collagen synthesis (Canalis 1981), act as neurotrophic agent (Smits et al. 1991), affect the differentiation on mesenchymal cells (Noble et al. 1988) and induce chemotaxis (Grotendorst et al. 1982a; Seppa et al. 1982; Kundra et al. 1994).

A breakthrough in the PDGF field, which attracted much attention, involved the identification of the transforming gene product of Simian Sarcoma Virus (SSV). This product, the v-sis protein, turned out to be a virally transduced homologue of PDGF-B (Devare et al. 1983; Doolittle et al. 1983; Waterfield et al. 1983).

The *in vitro* stimulatory properties of PDGF on fibroblasts, smooth muscle cells, and inflammatory cells suggested a role for PDGF in wound healing. Similarly, the homology to the v-sis product implied a role in tumour progression. However, the wide expression of PDGFs, both during development and in disease implied additional features (see below). The PDGF superfamily of growth factors now comprises PDGF-A and -B, and the recently discovered PDGF-C and -D. These form, together with the members of the vascular endothelial growth factor family (VEGF-A, -B, -C, -D, -E and placenta growth factor), a PDGF/VEGF super-family of structurally related GDFs.

Expression of PDGFs during development

In mice, PDGF-A and PDGFR-α mRNA is present in pre-implantation oocytes, as well as during early post-implantation stages (Rappolee et al. 1988; Mercola et al. 1990). At E7.5, the embryonic and extraembryonic ectoderm expresses PDGF-A, whereas the mesoderm expresses the α-receptor. At E10-12, adjacent cells express the PDGF-A ligand and α-receptor. For example; the surface ectoderm expresses PDGF-A, while the underlying dermatome expresses PDGFR-α. Similarly, the myotome expresses PDGF-A and the surrounding loose mesenchyme expresses

PDGFR-α (reviewed in Betsholtz 1995). Later, cells in the hair follicles, the lung and in the gastrointestinal tract show a similar paracrine expression (Lindahl et al. 1997b; Karlsson et al. 1999; Karlsson et al. 2000).

In contrast to PDGF-A, only endothelial cells and megakaryocytes express PDGF-B during mouse development (Lindahl et al. 1997a). Adjacent cells, vascular smooth muscle cells/pericytes or their progenitors, express PDGFR-β (Hellstrom et al. 1999). A LacZ reporter gene insertion under the control of the PDGFR-β promoter suggests that the heart, the somites and limb bud mesenchyme express PDGFR-β transiently at E10 (Soriano 1994).

The myotome, the epidermis, and the kidney express PDGF-C. However, the expression differs to some extent from PDGF-A and occasionally overlap with PDGFR-α (Ding et al. 2000; Aase et al. 2002).

The vasculature, the heart and the kidney express PDGF-D. PDGF-D is one of the newly discovered PDGFs and the expression pattern is not extensively mapped (Bergsten et al. 2001; Uutela et al. 2001; Changsirikulchai et al. 2002).

In conclusion, expression of the ligands and receptors indicate paracrine signalling between epithelial/endothelial and mesenchymal cell types. Deletion of the PDGF-A or –B affects smooth muscle cell lineages in e.g. lung and kidney, respectively, thus being demonstrative for such signalling.

Genomic organisation of the PDGFs

The seven PDGF-B exons encompass 24 kb of genomic sequence. The first exon contains untranslated sequences and a sequence that translates into a signal peptide for secretion. Exons two and three code for the amino terminal propeptide and the very first amino acids in the mature polypeptide. Exons four and five code for the major part of the mature protein, and thereby also account for the receptor binding capabilities. Exon six codes for a stretch of basic amino acids, which confer retention of the factor within and/or adjacent to the producing cell (see paper I and section below) and the last exon is non-coding. The genomic structure of PDGF-A is highly similar, though an alternate usage of the sixth exon results in two isoforms. The PDGF-C and –D genes consist of six and seven exons, respectively. The second and third exons encode a CUB-domain, which is unique for PDGF-C and –D within the PDGF/VEGF superfamily. The CUB domain is suggested to inhibit signalling and needs to be proteolytically removed for growth factor activation (Bergsten et al. 2001; LaRochelle et al. 2001). The fifth and sixth exons in PDGF-C encode the core domain. Similarly, the sixth and seventh exons encode the core domain in PDGF-D (see figure 2). PDGF-C and –D have no region homologous to the retention sequence found in

PDGF-A and -B (Heldin et al. 2002).

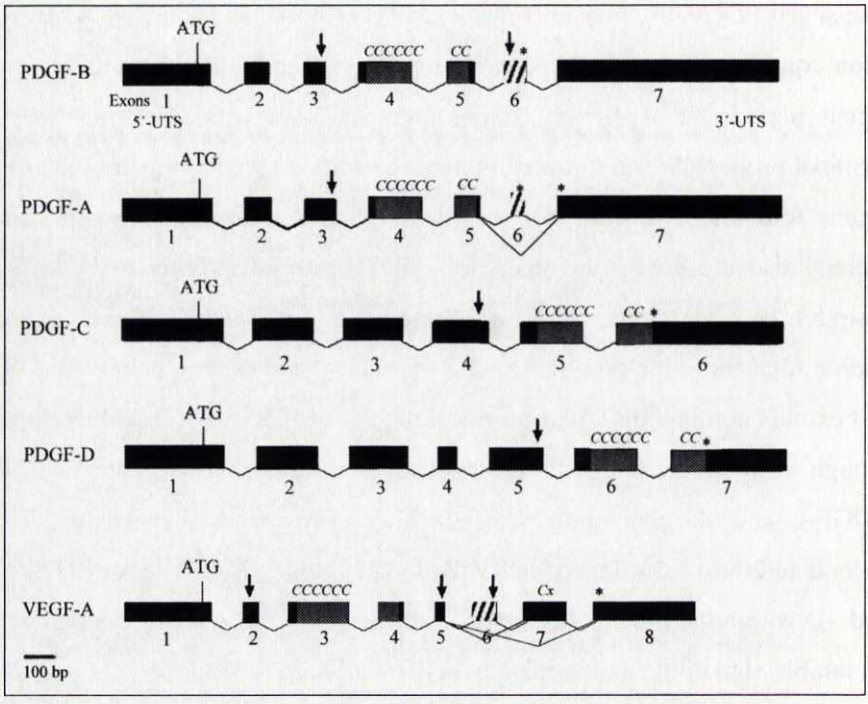

Figure 2. Outline of the genomic organisation of PDGF/VEGF gene family members. The genes are located on the following chromosomes: ch.7 *pdgf-a*; ch.22 *pdgf-b*; ch.4 *pdgf-c*; ch.11 *pdgf-d*. 5´and 3´ untranslated sequences are shown for PDGF-B. Numbered *black boxes* indicate exons; *lines*, splicing pattern; *grey boxes*, sequences involved in receptor binding; *arrows*, known and hypothesised proteolytic processing sites; *asterisks*, translational stop codons; *striped boxes*, retention sequences encoding part of HSPG-binding region; *C*, conserved cysteine residues; *Cx*, cysteine rich area. The CUB domains are encoded by exon two and three in PDGF-C and –D. Note that exon one is not drawn to scale in any gene (Dalla-Favera et al. 1982; Betsholtz et al. 1986; LaRochelle et al. 1991; Raines and Ross 1992; Chilov et al. 1997; Keck et al. 1997; Robinson and Stringer 2001; Uutela et al. 2001).

Receptor specificity and signal transduction

PDGF stimulates cells of mesenchymal origin that express the receptor tyrosine kinases PDGFR-α and PDGFR-β. PDGF-A and PDGF-C bind the PDGFR-α, while PDGF-D binds the PDGFR-β. PDGF-B is unique in the

sense that it binds and activates both the α and β receptor, either as BB homodimer or as AB heterodimer. PDGF-C and –D do not form heterodimers (reviewed in Hoch and Soriano 2003). Binding of PDGF-B to the receptor, induces PDGFR-β dimerisation and subsequent transphosphorylation of intracellular tyrosine residues. The phosphorylation occurs both within and outside the kinase domain. Following activation, the receptor is internalised and degraded rapidly. Phosphorylation of tyrosine residues within the kinase domain increases the catalytic activity, while phosphorylated residues outside this domain provide binding sites for signal transduction molecules. Several proteins interact with PDGFR-α and PDGFR-β through SH2 domains. The SH2 domain consists of approximately 100 amino acids and is present in downstream signal transduction molecules such as phosphatidylinositol 3´ kinase (PI 3-kinase), phospholipase C (PLC-γ), Scr kinase family members and GTPase activating protein (GAP). Within the PDGFR-β, around 15 specific binding sites have been identified. All the molecules that bind through their SH2 domain can initiate a signalling cascade leading to e.g. cell growth, chemotaxis and actin reorganisation. Downstream signalling molecules include important transducers such as rho and ras (reviewed in Heldin et al. 1998). PDGF-B contributes in the progression of various diseases such as cancer, atherosclerosis and fibrosis. Atherosclerosis is not mentioned or analysed in the papers included in this thesis, and I therefore refer interested readers to other papers, for example Nature Medicine August issue "Special focus on atherosclerosis" 2002.

PDGF in cancer, fibrosis and glomerular disease

Cancer

Several studies support a role for PDGF-B in tumour initiation and/or progression. PDGF-B is a cellular form of the retroviral v-sis product and has the potential to induce cell transformation (Leal et al. 1985; Beckmann et al. 1988). Moreover, injection of SSV into marmosets or mice induces formation of fibrosarcomas and gliomas depending on injection site (reviewed in Heldin and Westermark 1999). PDGF-A and –B as well as their cognate receptors are present in a range of human tumours. In addition, genetic alterations in PDGFR-α (Fleming et al. 1992; Kumabe et al. 1992; Hermanson et al. 1996; Clarke and Dirks 2003), PDGF-B (Simon et al. 1997) and in PDGFR-β (Carroll et al. 1996) may produce active receptors in tumours. Several pre-clinical and clinical trials are ongoing with the aim to inhibit PDGFs and PDGF receptors. For example, imatinib (Gleevec) inhibits PDGFR-α, PDGFR-β, Kit, Abl and Arg tyrosine kinase receptors, and has proven beneficial in certain leukemia and gastrointestinal tumour types (Capdeville et al. 2002). The compound SU11248 that inhibits VEGFR-2, PDGFR-β and Kit, yielded promising results in a phase I clinical trial (Mendel et al. 2003). Chemotherapy is, in part hampered by high interstitial fluid pressure (IFP) in the tumour tissue. A combinatory treatment with a PDGFR-β inhibitor (lowering the IFP) and chemotherapy, resulted in a significantly increased uptake of the chemotherapy agent (Pietras et al. 2002). Other aspects on PDGF and tumour progression are discussed below in conjunction with paper II.

Fibrosis

The ability of PDGFs to stimulate proliferation and chemotaxis of fibroblasts and smooth muscle cells, and chemotaxis of neutrophils and macrophages suggests that it might have a role in fibrotic reactions in different tissues, such as the lung.

Characteristic for idiopathic pulmonary fibrosis is the accumulation of macrophages, neutrofils and mesenchymal cells (fibroblasts, smooth muscle cells and myofibroblasts). This leads to an increased accumulation of matrix components in the interstitial and intra-alveolar space. Vignaud and co-workers showed a three times increase in the percentage of PDGF-B expressing macrophages during pulmonary fibrosis. The up-regulated levels of PDGF-B resulted in an increased number of mesenchymal cells and in increased matrix production (Vignaud et al. 1991). PDGF has been detected in several pulmonary diseases, and is thought to contribute to the progression of e.g. autoimmune-associated pulmonary fibrosis (Deguchi and Kishimoto 1989), histiocytosis X (Barth et al. 1991), acute lung injury (Snyder et al. 1991) and asbestosis (Lasky et al. 1996). Furthermore, in line with the data presented in paper III, lung specific transgenic over-expression of PDGF-B resulted in pulmonary fibrosis (Hoyle et al. 1999). Moreover, expression vectors for *in vivo* gene transfer resulted in a fibrotic response towards PDGF-B. In contrast, gene transfer of the extracellular domain of PDGFR-β, which act as a PDGF trapping agent, markedly reduced bleomycin-induced fibrosis (Yoshida et al. 1995; Yoshida et al. 1999). In analogy to the anti-tumour therapy, using a PDGFR-β specific inhibitor of the tyrphostin class inhibitors (AG1296), mesenchymal cell proliferation

could be blocked and the amount of collagen could be decreased in a rat model of pulmonary fibrosis (Rice et al. 1999).

Glomerular disease

Deletion of either PDGF-B or PDGFR-β in mice results in perinatal embryonic lethality and indistinguishable phenotypes (Leveen et al. 1994; Soriano 1994). There is, however, some discrepancy in the original publications, which report on different cardiac phenotypes. However, in subsequent analyses, identical phenotypes were shown (Mats Hellström and Mattias Kalén pers. comm.). Studies on these mouse mutants have shown that PDGF-B/PDGFR-β is important for recruitment of supporting cells to the vasculature, including mesangial cells to the kidney glomeruli. Loss of mesangial cells led to ballooning of the glomeruli. In human glomerulonephritis, signs of the disease often coincide with abnormal PDGF-B expression. An excessive proliferation of mesangial cells, endothelial cells and neutrophils leads to a disturbed glomerular architecture. The increased number of cells, especially mesangial cells, leads to accumulation of ECM components in the glomerulus, so called mesangial matrix. Consequently, the filtration is disturbed and e.g. proteinuria can be clinically detectable. Yet, the aetiology is often unclear; however, several cytokines and growth factors, e.g. TGF-β and PDGF-B, are likely to play a role. Several studies have detected an increased PDGF expression in mesangial proliferative nephritis (Gesualdo et al. 1991; Iida et al. 1991; Yoshimura et al. 1991). Johnson and co-workers used a rat model in which an antibody (anti Thy 1.1), induces lysis of the mesangial cells. Subsequently, new mesangial cells invade and re-populate the glomerulus. By administrating a neutralising PDGF-B antibody, the proliferative

response could be significantly reduced, together with a broad inhibition of matrix deposition (Johnson et al. 1992). However, large doses of the antibody were used, which could result in an immunoresponse towards the heterologous IgG. Therefore, the study could not continue beyond four days. The second attempt circumvented this fact by designing high affinity oligonucleotide aptamers specific for PDGF-B (Floege et al. 1999). Administration of the aptamer led to a steady decline in the number of mesangial cell mitosis. Eight days after the induction of glomerulonephritis, and six days after initiation of treatment, the number of proliferating mesangial cells had decreased by 95%. Additionally, PDGF-B blockade resulted in significantly decreased levels of fibronectin and collagen type IV. In a follow up study, the analyses continued for 100 days. In this study, PDGF-B aptamer treatment was shown to protect against the proteinuria that developed as a late complication to the anti Thy 1.1 treatment (Ostendorf et al. 2001).

Clearly, increased PDGF-B levels are an important feature in the progression of glomerular disease. Conversely, through mutagenesis or antibody delivery, a few models have been developed providing the opportunity to follow the pathogenesis when a lower level of PDGF-B or impaired receptor signalling is prevalent (Klinghoffer et al. 2001; Sano et al. 2002). As a result, lowered or increased levels of PDGF-B seem to have a similar outcome. See *Present Investigation* and the *Discussion* sections for further details.

Specific background and Present investigation

Paper I "Endothelial PDGF-B retention is required for proper investment of pericytes in the microvessel wall"

Introduction to cellular retention

The PDGFs show a high degree of evolutionary conservation in their genomic organisation and amino acid sequence. The comparison of PDGF-A in human, mouse and frog (*Xenopus laevis*) shows that the mouse amino acid sequence is 94% and 69% homologous to human and frog, respectively (Rorsman and Betsholtz 1992). PDGF-A and -B and VEGF-A contain an evolutionary conserved exon that codes for the retention sequence. The presence of a retention motif was implicated already in early studies of the v-sis and c-cis (PDGF-B) products. A major part of these products remained bound to the cell surface and only small amounts of incompletely processed factor were detected in tissue fluid of transformed cells (Robbins et al. 1985).

Alternative splicing of the PDGF-A gene results in alternate usage of a single exon. The alternative splicing of the sixth exon gives rise to two protein isoforms, namely PDGF-A-long and PDGF-A-short, with similar high affinity receptor binding capabilities (Ostman et al. 1989). These two isoforms are unequally distributed e.g. the two forms are equally expressed in kidney and thymus, but the short form dominates in muscle, heart and brain (Young et al. 1990). In transfected cells, they show a different distribution pattern (Ostman et al. 1991). By inhibiting proteolytic reactions,

LaRochelle and co-workers could show that the long form remains bound to the cell, while the PDGF-A present in the media does not contain the amino acid sequence encoded by exon six (LaRochelle et al. 1991). A similar splicing mechanism is used in the *vegf* gene. Alternative usage of the sixth and seventh exons results in at least four distinct isoforms, which show different distribution profiles upon secretion from the cell (Houck et al. 1991; Tischer et al. 1991). Thus, the presence of these exons in v-sis, PDGF-B, PDGF-A and VEGF mediates similar distribution upon secretion from the cell.

Identification of the cell retention signal

Dimerised PDGF-BB localises to the endoplasmatic reticulum and the Golgi complex, from where it is transferred to lysosymes for degradation or transportation to cell membrane for secretion. A major part, however, remains cell-bound (Robbins et al. 1985; Thyberg et al. 1990; Ostman et al. 1992). Several attempts have aimed to pinpoint by which mechanism the factor associates to the cell. The PDGF-B full-length protein is composed of 241 amino acids. The design of four constructs carrying sequential stop codons positioned at amino acids 185, 211, 227 and 235 mediated functional mapping of the carboxy-terminal (C-terminal). When analysed in transfected cells, each product retained transforming capacity. Moreover, both the 235 and 227 stop mutants were present only in cell lysate, whereas PDGF stop mutants 185 and 211 accumulated in the cell media. Consequently, the sequence responsible for the retention was denoted *retention sequence* and consists of a stretch of basic amino acids within the region 212-226 (LaRochelle et al. 1991). In a similar study, the substitution of the C-terminals of PDGF-A and PDGF-B led to altered protein distributions.

PDGF-A-short/B-COOH, distributed as PDGF-Bwt, whereas PDGF-B/A-COOH distributed as PDGF-Ashort. In the same study, insertion of stop codons, similar to the approach above, at positions 191, 219 and 230, confirmed the presence of the retention sequence to this region, and narrowed it down to positions 219 and 230 (Ostman et al. 1991).

The retention sequence carries potential proteolytic cleavage sites (Raines and Ross 1992; Westermark and Sorg 1993). A cleavage at the C-terminus would release the protein and enable it to diffuse away form the cell. Similarly, PDGF-B without the retention sequence would reach longer distances. This was also the case in an *in vivo* experiment. Keratinocytes transfected with expression vectors for PDGF-B-stop-211 induced a widespread formation of a thick connective tissue in a transplant. Keratinocytes expressing PDGF-Bwt instead induced a local response in the connective tissue. Moreover, cells transfected with PDGF-B-stop-211 released up to 20 times more of PDGF-B (Eming et al. 1999).

mPDGF-B	-**R**	P	P	**K**	G	**K**	**H**	**R**	**K**	F	**K**	**H**	T	**H**
mPDGF-A	-**R**	**R**	E	S	G	**K**	N	**R**	**K**	**R**	**K**	**R**	L	**K**
mVEGF-A	-V	**R**	G	**K**	G	**K**	G	Q	**K**	**R**	**K**	**R**	**K**	**K**
mPlGF-2	-**R**	**K**	T	**K**	G	**K**	**R**	**K**	**R**	S	**R**	N	S	S

Table 2. Alignment of the evolutionary conserved retention sequence in the COOH-terminal in PDGF/VEGF super family members. Basic amino acids in bold. Modified from paper I

The retention sequence found in PDGF-A and –B is similar in both structure and function. Therefore, the homologous sequence found in VEGF would result in an analogous product. Expression of the vegf transcripts in an *in vitro* system showed that VEGF-121 and VEGF-165 accumulated in the cell culture media. The longer isoforms, VEGF-189 and VEGF-206 remained undetected in the media (Houck et al. 1991). In a follow up study, significant amounts of VEGF-165 accumulated in the cell media. However, a part remained extracellularily bound. In contrast, VEGF-121 displayed no signs of cellular retention (Houck et al. 1992). Consequently, the presence of the evolutionary conserved retention sequence in either PDGFs or VEGFs mediates retention of the secreted factor to the producing cell. As mentioned earlier, the importance of such retention may, in theory, include the formation of depots and gradients of importance for spatially restricted responses in developmental and pathological contexts. To test these ideas experimentally is a huge challenge. With recent advances in mouse genetics, however, it has been possible to specifically delete retention motifs in VEGF and PDGF family members. Indeed, published work show that the long retention-motif-containing splice isoforms of VEGF are important for the formation of VEGF gradients and that these gradients, in turn, are necessary for correct patterning of blood vessels (Ruhrberg et al. 2002; Stalmans et al. 2002; Gerhardt et al. 2003). I adopted a similar approach to study the physiological function of PDGF-B retention.

Creating a *pdgf-b retention* deficient mouse model

In order to create an *in vivo* model to investigate the functional importance of the retention motif in the *pdgf-b* gene we set up a project aiming to delete this motif in mice. This rendered us with the possibility to study the

functions during development and in the normal state of an adult organism, as well as in a pathological state. The introduction of a subtle mutation to the *pdgf-b* locus presented us with several concerns. Firstly, we had to clone and further characterise the region coding for the COOH-terminal. Secondly, we needed to introduce a translational stop codon, also containing a diagnostic restriction site, prior to the sequence coding for the retention motif. Thirdly, we had to introduce an antibiotic resistance gene to promote selection of recombinant embryonic stem-cell clones. *LoxP* sequences flanked the selection gene to enable excision by the help of *cre-recombinase*. Fourthly, we had to include enough homology to the target gene in the construct to enable recombination between the targeting construct and the *pdgf-b* locus. The homologous recombination involved the endogenous 5´end upstream of the selection marker, the 3´end directly downstream of the selection marker and the 3´end downstream of the stop-codon and diagnostic restriction site (see figure 3 A, B). By this approach, we managed to create mice whose genome differed from the wt only by eight base pairs in exon six (TAA-stop codon and HindIII-site) and a 34 bp loxp sequence in intron five (after cre mediated excision, see figure 3 C).

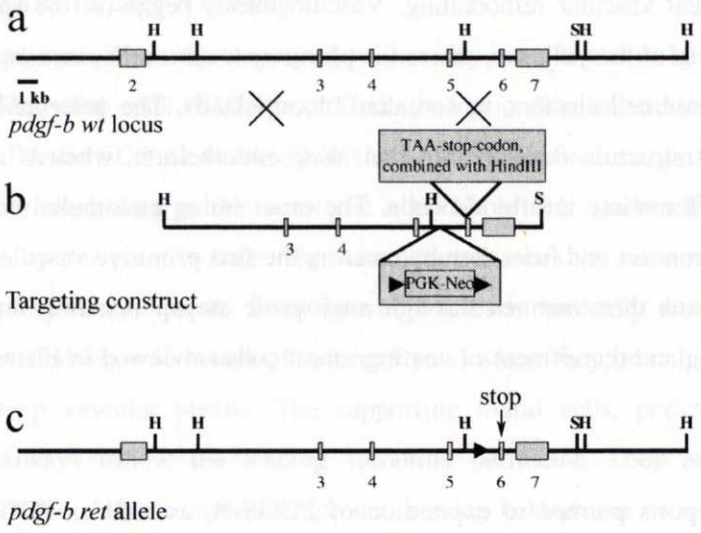

Figure 3. Strategy to delete the pdgf-b retention sequence. A) Pdgf-b wt locus, where exon six codes for the retention sequence. B) Targeting construct including selection marker in intron 5, stop codon and diagnostic HindIII-site in exon six. C) The *pdgf-b ret* allele after cre mediated removal of selection marker. Modified from paper I.

These mice should therefore be able to produce a freely, or at least a more freely diffusible protein upon secretion, based on the data presented above. The characterisation of the *pdgf-b ret/ret* mouse is fully presented in paper I, but the text below is intended to give additional background to the experiments performed and to explain the main findings, in order to set the stage for the discussion section.

Functions of PDGF-B during vascular development

Blood vessels forms through two distinct processes, vasculogenesis and angiogenesis. *De novo* formation of blood vessels is denoted vasculogenesis. Angiogenesis refers to the sprouting from pre-existing vessels and

subsequent vascular remodelling. Vasculogenesis begins in the splanchnic mesoderm of the yolk sac, where the pluripotent stem cells, hemangioblasts, form dense cell clusters, or so called blood islands. The outer cells of this transient structure develop into the lining endothelium, whereas the inner cells differentiate into blood cells. The outer lining endothelial cells (EC) start to connect and fuse, thereby creating the first primitive vascular plexus. The plexus then matures through angiogenic steps, including regression, sprouting and recruitment of coating mural cells (reviewed in Flamme et al. 1997).

Early reports pointed to expression of PDGF-B, as well as PDGFR-β, in endothelial cells during embryonic development (Holmgren et al. 1991; Shinbrot et al. 1994). However, more sensitive detection assays localise PDGF-B in the developing endothelium, whereas the β–receptor is detected in adjacent pericytes, vascular smooth muscle cells (vSMC) and mesangial cells (Lindahl et al. 1997a; Lindahl et al. 1998; Hellstrom et al. 1999). The genetic ablation of this signalling pathway results in identical and remarkably conclusive phenotypes. Organs dependent on angiogenesis such as the brain and the kidney, display severe vascular defects and an almost complete lack of pericytes (in the CNS) and glomerular mesangial cells (in the kidney). This leads to vascular dilatation, oedema, micro-aneurysms, hyperplasia and to ballooning of glomeruli. Organs dependent on vasculogenesis e.g. the liver, display no such alterations. This prompted us to begin our *pdgf-b ret/ret* analyses in organs where PDGF-B or PDGFR-β appeared indispensable.

Retinopathy in *pdgf-b ret/ret* mice

The retinal vasculature develops in the same way in mice and in humans. In mice, endothelial cells (EC) sprout from the optic disc immediately after birth. At P4, the EC have reached half the way to the retinal margin. The EC are closely associated to the astrocytic network, which reaches the retina periphery by this point in time. The vasculature reaches the same margin at P7. Simultaneously, sprouts from the veins spreads vertically downwards, into the retinal inner and outer plexiform layers, where they branch and fuse into a deep vascular plexus. The supporting mural cells, pericytes and vSMC, always follow the leading sprouting perimeter. They are never situated at the absolute foremost sprouts, but lag behind one or a few cell diameters.

Although the pericytes, judged by their location, do not seem instructive for the retina vasculature, compelling evidence has recently emerged that point towards essential features of the pericytes. Mice heterozygous for PDGF-B display a 30% reduction in pericyte numbers, leading to a subtle effect on the vasculature (Hammes et al. 2002). Klinghoffer and co-workers further show that slight alterations in the PDGF-B/PDGFR-β signalling pathway disturb the vascular patterning. Substituting the cytosolic signalling domain of PDGFR-β with that of PDGFR–α on one allele and combining this with a *pdgfr-β* null allele, leads to severely distorted retinal vasculature. The mutants display a reduced number of pericytes, which are unevenly distributed over the retinal surface resulting in focal haemorrhages (Klinghoffer et al. 2001). Conclusions on PDGF functions in the retina had previously been based on either ectopic over-expression (Seo et al. 2000) or

on ectopic injections of PDGF-B protein (Benjamin et al. 1998). Therefore, Klinghoffer's study represents the first genetic evidence of a crucial role for PDGF signalling in the retina. Pericyte recruitment in the retina could also be inhibited by specific blocking of PDGFR-β using antibodies, resulting in a similar distortion of the vasculature (Uemura et al. 2002). Recently, Enge et al presented elegant proof utilising EC-specific cre-recombinase/loxp mediated excision of the *pdgf-b* gene. Since Cre mediated excision is usually not taking place in all cells (of a certain type), Enge and co-workers analysed the phenotypic alterations in a wide range of pericyte depletion states. Up to 90% of pericyte deficiency is compatible with postnatal survival, though such reduction of pericyte density lead to severe retinopathy. Interestingly, there seems to be a threshold at about 50% pericyte reduction. Below this value, a vascular proliferative response occurs. Pericyte reduction above 50% results in focal vascular changes e.g. microanuerysms and vessel regression, however without a proliferative component (Enge et al. 2002) .

Basically, at the same time as these studies emerged, we observed that a fraction of the *pdgf-b* retention deficient mice showed macroscopically distinguishable white opacities in the lens. This led us to initiate a time-course study on the eye and specifically on retinal development. Commencing at the onset of vascularisation from the optic disc, we found that the sprouting was irregular and severely delayed. Moreover, the retina in the *pdgf-b ret/ret* mice had a reduced pericyte density and certain areas were completely devoid of pericytes. Blood vessels were dilated, hyperfused and had no well-defined sprouting region. These abnormalities of early retinal vascularisation progressed and subsequently led to retinal degeneration and

formation of fibroblast-rich areas that spread over the retinal surface. In later stages, secondary alterations of the lens structure occurred and the retinal pigment epithelium invaded the retina. Furthermore, pericytes failed to integrate to the vessel wall in *pdgf-b ret/ret* mice at early stages, which implied that we observed more than a hypo-functional situation. However, this was not surprising, since we would expect a more diffusible protein from the mutation that we had introduced. The *pdgf-b ret/ret* mice display the most advanced type of abnormal retinal patterning among the models described above. This was surprising, since PDGF-B expression was maintained at the sprouting tip, however, at reduced levels. Consequently, correct localisation of the secreted protein is equally important as having a functional signalling pathway.

Kidney and glomeruli development

The vertebrate kidney is formed through an extensive cross-talk between cells of epithelial and mesenchymal origin. The kidney starts to develop as early as E8 in mice and day 22 in humans when inductive signals from the nephric duct reach the adjacent mesenchyme and stimulate the formation of pronephros and subsequently mesonephros. These structures are transient, although a part of the mesonephros persists and contributes to the sperm-carrying ducts in males. Moreover, the mesonephros is a producer of blood cells over a limited time during development (pronephros is a functional kidney in fish, but is not thought to be active in mammals). The metanephros, the functional vertebrate kidney, arises from an outgrowth of the nephric (wolffian) duct. The branch, referred to as the ureteric bud, responds to signals from the metanephrogenic mesenchyme and develops through reciprocal interaction into ureter, renal pelvis and collecting ducts.

The glomerular structure first appears as mesenchymal condensates at the tip of the ureteric branches. In the formation of the glomeruli, the condensates undergo mesenchymal-to-epithelial conversion and subsequently develop through the readily distinguishable comma-, S- and cup-shape bodies. The upper part of the S-shape body gives rise to the proximal tubule, loop of Henle and the distal tubule that connects to the ureteric branch, which generates the collecting ducts. Other parts of comma- and S-shapes bodies develop into glomerular visceral epithelial cells and parietal epithelial cells that constitute the lining of Bowman's capsule. Vascularisation of metanephros takes place when the first ureteric branches form. The vasculature becomes evident in the cleft of the S-shape stage. The origin of the invading mesangial and endothelial cells is unknown, but both vasculogenic and angiogenic processes are suggested. These data will not be discussed here and I refer to (Ricono et al. 2003) and references therein.

Functions of PDGF-B in glomeruli formation

It is clear that PDGF-B/PDGFR-β mutants are affected in organs were angiogenesis is the main process in the establishment of the vascular bed. Evidently, these mutants display abnormal vascular morphogenesis in the kidney glomeruli. PDGF-B is expressed both by the capillaries that grow into the S-shape as well as in afferent/efferent vessels, while expression of PDGFR-β is detected in vessel associated cells in the S-shape (but not in the comma shape). Cell populations located at the core of the glomeruli are positive for both PDGFR-β and the mesangial marker desmin. PDGFR-β *in situ* hybridization also labels a small cluster of cells at the cup-shape state

that, most likely, reflects mesangial cell precursors. In the PDGF-B/PDGFR-β mutants, these cells fail to contribute to the mature glomeruli. Instead one or a few endothelial tufts form that are completely devoid of mesangial cells, which makes the glomeruli appear as a balloon shaped structure filled with erythrocytes (Leveen et al. 1994; Soriano 1994; Lindahl et al. 1998). This suggests that the kidney is sensitive to alterations in the PDGF signalling system and furthermore, that these signals are essential for proper glomeruli development.

Abnormal glomeruli formation in *pdgf-b ret/ret* mice

A correct spatial localisation of PDGF-B might, hypothetically, be critical for proliferation, migration and survival of the mesangial cells. Thus, we undertook an in depth characterisation of the glomeruli in the *pdgf-b* retention deficient mice. We analysed embryos from E15 to E18 using hematoxylin/eosin, pecam1 and α-smooth muscle actin staining. All the embryos analysed phenocopied, to a major extent, the PDGF-B/PDGFR–β mutants. Virtually all mature glomeruli completely lacked, or contained very few, mesangial cells and the single capillary loop within the glomerulus was extensively dilated (ballooning glomeruli). The viability of *pdgf-b ret/ret* mice offered a unique possibility to follow the consequences of the mesangial cell loss at the postnatal stage. Surprisingly, the glomeruli recovered remarkably fast, the contribution of mesangial cells had by P5, increased dramatically, and was normalised at P30. Interestingly, at later stages the picture was again pathological, with several signs of a glomerulosclerotic condition. The individual effect on each glomerulus varied from a single sclerotic loop (focal sclerosis) to fully sclerotic glomeruli, which contained fewer mesangial cells instead replaced by

collagen, fibronectin and other extracellular matrix substances. Not only were the glomerulus tuft affected, but we also detected thickening of the basement membrane in the Bowman's capsule. These findings may, at least in part, explain the increased levels of albumin that were present in the urine from the age of three months. The glomerular structure served as a good model to analyse the effect of varying doses of PDGF-B. Therefore, we compared glomeruli during development in *pdgf-b +/+, pdgf-b ret/+, pdgf-b ret/ret, pdgf-b ret/- and pdgf-b-/-* mice The numbers of recruited mesangial cells were clearly dependent on the theoretical dose of PDGF-B. The *pdgf-b ret/-* and *pdgf-b -/-* were phenotypically indistinguishable at E17 and had their mesangial cell core replaced with a blood filled cavity. *Pdgf-b ret/ret* mice displayed a similar phenotype, but the effect was not as dramatic and as penetrant. *Pdgf-b ret/+* and *pdgf-b +/+* mice showed an equal contribution of mesangial cells to the glomerulus core, as judged by light microscopy. Thus, genetic data pointed towards a *hypomorphic* situation in the *pdgf-b ret/ret* mice. We performed similar analyses in the CNS using a pericyte specific LacZ marker, with comparable results (see paper I).

RNA and protein studies on *pdgf-b ret/ret* mice

In order to achieve mechanistic insight into the morphological disturbances that occurred, both transcript and protein levels, as well as protein localisation, needed to be determined. RNA from *pdgf-b +/+, pdgf-b ret/+* and *pdgf-b ret/ret* P21 mice was prepared and subjected to northern blot analysis, which pointed to an approximately 50% reduction in the mRNA levels in *pdgf-b ret/ret* mice. To analyse the protein distribution, we established primary endothelial cell cultures. However, these primary cells did not survive enough passages required for metabolic labelling. Therefore,

we produced endothelioma cell lines. With these cells we performed several attempts to immunoprecipitate PDGF-B following metabolic labelling, but failed to detect the protein either in wt or in *pdgf-b ret/ret* cells. While disappointing, this was not unexpected, since previously published data on PDGF-B protein detection by similar methodologies have all been performed on transfected cells expressing large amounts of recombinant PDGF-B. To our knowledge, no one has been able detect endogenous mouse PDGF-B satisfactorily by immunological techniques. However, using PDGF-B binding aptamers (Fredriksson et al. 2002), which are able to detect very low amounts of PDGF-B, a five fold reduction of protein content in the *pdgf-b ret/ret* cell line was observed (paper I). This study also showed that, albeit with lower total levels, secretion of PDGF-B into the medium was significantly higher in the *pdgf-b ret/ret* cells compared to the wt. In addition, quantification of PDGFR-β receptor phosphorylation inducing activity had similar outcome. This also supplied an important finding; the PDGF-B ret product had maintained bioactivity.

PDGF-B is required for proper developmental blood vessel formation and is, in addition, present in tumour types of several different origins (reviewed in Heldin and Westermark 1999). Moreover, tumour vessels express PDGF-B. To what extent is the embryonic angiogenesis, and pericyte recruitment, similar to a rapid growing tumour, with a different matrix composition and eventual lack of basement membrane? The *pdgf-b ret/ret* mice could, therefore, be used as a loss-of-function tool to address the role of endogenous PDGF-B in tumour development.

The role of PDGF-B/PDGF-RB in tumour angiogenesis - introduction

In the current view, angiogenic endothelial cells establish a vascular plexus and concomitantly recruit mural cells. A well-studied example is the mouse retina, where the endothelial cells grow towards a source of VEGF and simultaneously recruit coating cells as they reach the retina perimeter. The mural cells are, however, always slightly lagging behind the front sprouting region. Furthermore, the origin of the mural cells is unclear, but the endothelium may induce precursor cells to differentiate and by such means attract pericytes/vSMC. Another scenario is that the endothelial cells induce the pericytes/vSMC to migrate along the vessels. It is most likely that both of these mechanisms occur *in vivo* and the mural cells are, once recruited, essential for normal blood vessel function, as demonstrated by several studies. Unless covered by mural cells, the retinal vasculature is more sensitive to pruning (Benjamin et al. 1998). Moreover, complete absence of brain pericytes, as in PDGF-B/PDGFR–β mutants, leads to endothelial hyperplasia with an approximate 60% increase of endothelial cells (Hellstrom et al. 2001). Similarly, a reduction of pericyte density by 50%, using specific removal of endothelial PDGF-B, results in an endothelial proliferative response (Enge et al. 2002). This finding is in agreement with earlier observations suggesting that pericyte drop-out preceded proliferative diabetic retinopathy (Speiser et al. 1968). Finally, studies on transplanted polymeric scaffolds containing VEGF and PDGF revealed measurable effects on the vessel density. VEGF-A alone induce a higher vessel density, but the vessels are small and not properly formed. PDGF-B, on the other hand, does not produce more vessels, but more mature vessels, as judged by staining for a vSMC marker. Interestingly, a dual simultaneous

administration of PDGF and VEGF, where VEGF is released more rapidly than PDGF-B, results in higher blood vessel density and in more mature blood vessels (Richardson et al. 2001). Thus, pericytes and smooth muscle cells have more functions than just to act as stabilising and supportive compounds on the endothelium, and their presence have implications for the actions of VEGF. Data imply that endothelial cells lacking covering mural cells are more sensitive to VEGF withdrawal. Indeed, in a mouse glioma model and in a primary human prostate cancer ablation of VEGF led to apoptosis of endothelial cells that were immature, in the sense that they were not covered with mural cells (Benjamin et al. 1999). In line with these data, and as described in the general background, several trials have focused on antagonising receptor tyrosine kinases (RTKs). In a study by Gee *et al*, anti-angiogenic treatment decreased the total vessel density, but significantly increased the relative number of vessels with a mural coating (Gee et al. 2003). Recently, Bergers and co-workers provided conclusive data by targeting both PDGFR-β on mural cells and at the same time inhibiting VEGF receptor signalling. Here, tumour vessels were reduced and the combined approach led to regression of late-stage tumours (Bergers et al. 2003). Those data were published during the time we were preparing the paper II manuscript and they support our own conclusions. I will summarize the most important observations and the experiments we performed to be able to understand the mechanism by which PDGF-B and PDGFR–β are involved in pericyte recruitment to the tumour vasculature,

Influence of PDGF-B/PDGFR-β on the tumour vasculature

Wildtype and *pdgf-b ret/ret* mice were transplanted with a mouse fibrosarcoma cell line, T241. We chose the T241 cell line because a comparatively high proportion of the tumour vessels have mural coating and show a relatively normal morphology compared to the tumour vessels in many other models that have been studied by us and others. Tumours grown on *pdgf-b ret/ret* mice were, after 12 days, more haemorrhagic and had a looser consistency upon dissection, but no obvious difference in tumour size was observed. However, the tumour vasculature differed remarkably: vessels were dilated, pericytes were detached and pericyte coverage was significantly reduced. Thus, endogenous PDGF-B was not present in adequate amounts in the pericellular space surrounding the vessels to support "normal" vessel formation in the T241 tumours.

Several described tumours express PDGF-B, although not the T241 cell line. To analyse the effect of tumour-derived PDGF-B on smooth muscle cell recruitment, we used a CMV promoter to drive the expression of human pdgf-b cDNA in T241 cells. This provided us with four types of tumours; T241 on wt, T241 on retention mice, T241-B-transfected on wt and T241-B transfected on retention mice. Briefly, the vessel diameter in the T241-ret tumours were two-fold wider than in T241-wt, but showed no significant change in vessel density. More than half of these vessels had no contact with mural cells. Interestingly, the vessel diameter was normalised in T241-B-ret tumours, reflecting the fact that the pericyte coverage was significantly increased from 4% in T241-ret compared to 15% in T241-B-ret and 17% in T-241 to 36% in T-241-B. In addition, the pericytes that connected to the vessels in T241-ret and T241-B-ret did not invest the vessel wall properly.

For instance, 4% of the mural cells in T241-wt extended further than 10 μm away from the vessel, while the comparable proportion was 80% in T241-ret.

Next, we injected a mixture of T241 cells and cells that originated from wt and PDGFR-β deficient mice, also harbouring a transgenic pericyte marker. This rendered us with the possibility to prove that PDGF-B plays a major role in the recruitment of mural cells to the tumour vessels. No difference was observed between the numbers of recruited mural cells in tumours transplanted to wt or *pdgf-b ret/ret* mice. Thus, the recruitment of exogenously added pericytes was clearly not dependent on PDGF-B retention, but proper integration into the vessel wall still failed. Importantly, we thereby show an absolute requirement for the expression of PDGFR-β on the exogenously added pericytes to enable their recruitment; since no such cells were present adjacent to the endothelium. We further show that loss of PDGF-B retention results in defective integration of pericytes to the micro-vascular wall (in line with the published data in paper I), and affects the tumour vascular patterning.

From published literature and the findings in paper I and II, it is clear that retention of PDGF-B to the pericellular space is indispensable during development and exerts analogous functions during tumour progression. Recruitment of pericytes and vSMC is apparently the most important developmental function for PDGF-B and PDGFR-β. However, gene-targeting experiments only depict fundamental roles for each gene; other roles, which may be important, but lack a morphological consequence might

go undetected. The actions of PDGF-B are important in several fibrotic conditions. PDGF acts through activation of PDGFR-α and PDGFR–β, expressed on fibroblasts and induces mitosis and matrix production. Such signalling is likely to occur e.g. in the heart where PDGF-A is expressed by cardiac myocytes and PDGFR–α by cardiac fibroblasts. The deletion of PDGFs or PDGF receptors has provided very limited information concerning the functions of PDGFs during cardiac development. For example, do the dilated hearts in PDGF-B mutants reflect a direct role for PDGF on cardiac fibroblasts? Or are the cardiac defects secondary to the vascular malformation? Are the actions of PDGFs mainly of developmental importance or are these factors also effective in the adult? With the aim of investigating the effect of PDGF in the heart, we placed the α–myosin heavy chain (α-MHC) promoter in control of the expression of the short, freely diffusible form of PDGF-A, the long heparin binding PDGF-A isoform and PDGF-B, and generated transgenic mice using these constructs.

Paper III "Heart specific over-expression of different platelet-derived growth factor isoforms results in varying degree of cardiac fibrosis"

Derivation of α-MHC-PDGF transgenic mice

The transgenic α–MHC promoter mimics the endogenous myosin heavy chain expression in the heart (Subramaniam et al. 1991). During development, the main MHC isoform is the β–MHC. At birth, a shift towards α–MHC isoform occurs. Therefore, we expected no dominant effect of the trangenes during embryogenesis and the major changes that we could observe occurred postnatally. We designed three similar transgenic

constructs, by joining the α–MHC promoter to human cDNA coding for PDGF-A-long isoform (PDGF-A_L), PDGF-A-short form (PDGF-A_S) and PDGF-B. All constructs terminated in a polyA signal, derived from the human growth hormone 3´end.

Cardiac fibrosis in α-MHC-PDGF transgenic mice

We identified four postnatal founders for each construct. Over-expression of PDGF-A_L resulted in postnatal lethality at around three weeks of age, due to extensive enlargement of the heart. Comparably, over-expression of PDGFA_S caused a similar, but milder, cardiac hypertrophy and consequently, lethality at six to eight weeks. As expected from the time of death, we detected the most severe disturbances in the PDGF-A_L transgenes where an extensive amount of collagen was interspersed between the muscle layers. Moreover, the response to the elevated doses of PDGF-A was not limited to focal sites, but spread over the entire myocardium. Since over-expresssion of PDGF-C has recently been shown to induce cardiac fibrosis accompanied by vascular changes, we stained the vascular compartment in the PDGF-A transgenic hearts with α–smooth muscle actin (SMA) and Pecam1. This revealed decreased capillary density around the myofibres and an increased number of dilated and irregular vascular structures (paper III).

The α–MHC PDGF-B positive founders seemed healthy and viable, and two of them were sacrificed for analysis at seven months of age. They displayed an approximate three-fold increase in the size of the heart, and staining using MTC revealed focal deposits of collagen. The collagen was not present in such high amounts as in PDGF-A_S, which made the muscle layers appear

normal, except for areas were collagen deposits were dense. Similarly, the two other founders analysed at ten months, showed focal fibrosis and collagen accumulation. Since PDGF-A induced the most severe responses; we assumed that the observations were dependent on signalling over the PDGFR-α expressed by cardiac fibroblasts. Confirming this idea, we detected the PDGFR-α in the ventricle and atrium wall by *in situ* hybridisation in wt mice. In PDGF-A_S transgenes, expression of the receptor coincided with focal thickening of the atrium wall, most likely reflecting proliferative cardiac fibroblasts. PDGFR-α was also expressed on one side of the cardiac valves. In conclusion, PDGFs influence cardiac fibroblasts and induce proliferation that eventually leads to myocardial hypertrophy. We can also conclude that each of the PDGF isoforms contribute to various extents to the progression of cardiac fibrosis.

General Discussion

Despite the lack of detailed information of GDF-ECM interaction and their physiological relevance, virtually all biological systems appear to utilise the growth factor retention. There appears not to be one single mechanism governing extracellular GDF distribution, but a variety of means that eventually leads to specific localisation of the factor. In PDGF/VEGF superfamily, a specific motif rich in basic amino acid residues mediates interactions with the ECM and maintains the factor in close vicinity of the producing cell. However, other mechanisms exist that confer cellular retention. Secreted TGF–β, for example, consists of a complex of subunits with different functions. The secreted protein binds to associated proteins

that in turn have matrix- interacting capabilities. Activation of the factor requires proteolytic removal of associating proteins. EGF and TGF-α family members represent a third way. These molecules carry hydrophobic stretches that integrate into the cell membrane. Apparently, this is an evolutionary conserved feature since *Drosophila* homologues to Notch and Delta and the nematode *Caenorhabditis elegans* proteins lin-12 and glp-1 share similar membrane-binding characteristics (Massague 1990). The number of factors utilising similar themes are still growing and now include, apart from the above, FGF, IGF-1, SCF, CSF-1, wnt-1, Hh and HGF family members (reviewed in Gallagher 2001; Iozzo 2001).

Functions of GDF-ECM interaction

The function of pericellular retention is probably dependent on the type of interaction, the growth factor and the intended signal. It is possible to imagine several theoretical scenarios. Since the retention sequence in the PDGF family is evolutionary conserved and considering the actions of PDGF-B, extracellular deposits of the growth factors might be of physiological relevance in response to cellular stress e.g. tissue damage. Rapid release of growth factors from such reservoirs through matrix degradation would be possible without the need to initiate transcription. Thereby, the necessary wound healing response can occur quickly. Another function might involve autocrine stimulation (where the signal activates receptors expressed by the same cell). Autocrine signalling is a debatable feature of PDGF, but several studies imply possible autocrine PDGF-B signalling with implications for tumour progression (Betsholtz et al. 1984; Leal et al. 1985; Beckmann et al. 1988) and for wound healing (Crosby et al. 1999). Furthermore, presentation of a growth factor in close proximity to the

producing cell might facilitate direct cell-to-cell signalling. The membrane bound *notch/delta* and *ephrin/eph* ligand and receptor pairs, demonstrate such signalling. However, the analyses we performed do not rule out the possibility that secreted factors also would benefit from such presentation. We show in both paper I and paper II that pericytes fail to integrate into the vessel wall, which can be explained by altered presentation of PDGF-B on the endothelial cell surface. Furthermore, we detected expression of PDGF-B at the absolute tip of the sprouting retinal endothelium. The restricted expression might reflect the need for precise localisation of the growth factor to this region (see figure 4). Another possibility is that cellular retention is a way for the cell to further control the signal to reach the correct receptor-expressing cell. In a situation where the receptor is widely expressed, the signal must be restricted. For example, the c-ret receptor is expressed along the nephric duct during kidney morphogenesis. The outgrowth and formation of the ureteric bud only takes place close to the metanephrogenic mesenchyme, which produces glial-derived neurotrophic factor (GDNF) (reviewed in Schedl and Hastie 2000). Hypothetically, if GDNF was to diffuse further from the source, the ureteric bud might not form or develop at an ectopic site. A similar function involves the creation of a gradient that results in varying cellular responses, depending on the actual dose the cell receives. For example, the TGF-β superfamily member *decapentaplegic* (*dpp*), patterns the *Drosophila* wing through a gradient (Lecuit et al. 1996). Similarly, formation of a sonic hedgehog (Shh) gradient during neural development differentially influences the neural tissue. Shh, secreted from the neural tube, induces adjacent tissue to differentiate to motor neurons or interneurons depending on the concentration of Shh (Ericson et al. 1996). More speculative, and potentially concurrent with the above, is that the

receptor-expressing cell needs to bind a substantial number of molecules to induce signal transduction. For example, a particular mouse fibroblast cell line carry approximately 25,000 PDGF–α receptors and 40,000 PDGF-βreceptors (Simm et al. 1992) and adult rat cardiac fibroblasts carry approximately 15,000 PDGF-α and 25,000 PDGF-β receptors (Simm et al. 1997). In addition, the induction of a migratory response requires less PDGF-B, than the induction of a proliferative one. (Grotendorst et al. 1982b). Thus, what proportion of these receptors requires activation to induce a full proliferative and/or migratory response? The amount of cellular retained factors that influences patterning and morphogenesis involves numerous GDF families, each with a capacity to influence the state of the cell. It is tempting to speculate that the factors the cell faces during migration or growth supplies the cell with information of the processes that recently occurred. The ECM thereby functions as a "matrix-stored memory". If this was the case, the cell can express proper developmental spatial and temporal signals, or for instance, instruct the cell to end proliferation or to start production of extracellular matrix substances. These hypothetical functions of cellular retention are based on more or less well-founded studies and theoretical arguments, but I have to point out how few published *in vivo* experiments that have aimed at answering such questions. Several studies on ECM molecules in *Drosophila* and mice result in complex phenotypic alterations that could include several pathways and the mechanistics are often unclear.

Functional studies on GDF cell association

The most well-characterised protein in terms of functions and properties of extracellular retention is VEGF-A. As previously described, alternative splicing leads to the formation of at least four VEGF-A isoforms. The shortest form ($VEGF_{120}$) lacks the heparin binding sequence encoded by exon six and seven and is freely diffusible after secretion, while the longer form ($VEGF_{188}$) that contains these sequences accumulates pericellularily. The isoforms containing one of these sequences distribute at an intermediate frequency (Houck et al. 1992). Mice engineered to express only the short form displayed several angiogenic defects that resulted in ischemic cardiomyopathy (Carmeliet et al. 1999), impaired skeletal formation (Maes et al. 2002; Zelzer et al. 2002) and abnormal development of the brain and retinal vasculature (see figure 4) (Ruhrberg et al. 2002; Stalmans et al. 2002; Gerhardt et al. 2003). Collectively, these studies point to a requirement for tight regulation of the VEGF protein distribution. Alterations in the distribution pattern were detrimental in multiple ways. Moreover, these studies provided the first series of evidence for the importance of correct

GDF distribution in a vertebrate system.

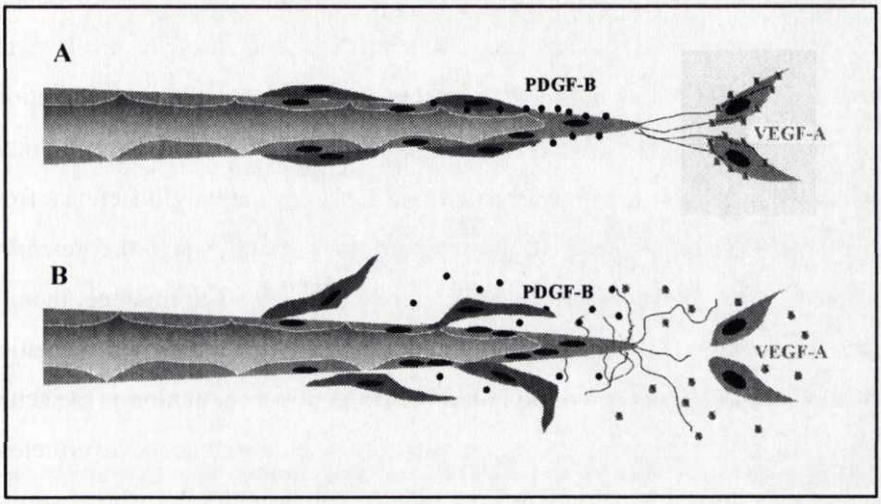

Figure 4. A conceptual illustration of PDGF/VEGF cell retention. A) The endothelium is guided toward a deposit of stored VEGF. PDGF is expressed by the tip cell and is retained in close vicinity to the endothelium, to which pericytes are recruited. B, In the absence of retention sequences, PDGF/VEGF are more diffusible, resulting in defective investment of pericytes to the vascular wall, endothelial hyperplasia, increased number of sprouts and altered guidance of the endothelium. Based on and modified from (Ruhrberg et al. 2002; Gerhardt et al. 2003 and paper I and II).

The analyses I present in this thesis further strengthen this concept. It is also clear that the generation of mouse mutants, carrying subtle alterations of specific exons is time-consuming and require careful technological consideration and morphological analyses. Conversely, altering ECM core protein or heparan sulfate polymerization enzymes by conventional gene targeting is more straightforward, but the consequences of such mutations can result in complex phenotypes or redundancy. It is also possible that redundancy is not as common for highly conserved exons that code for protein distribution signals. However, I favour both approaches and they

should be used in parallel. Evidently, our data are limited, in the sense that we have not been able to detect the PDGF-B protein in tissues despite numerous attempts. Furthermore, the mRNA and protein levels were reduced in *pdgf-b ret/ret* mice, for reasons that are debatable. The alterations of the gene could *per se* alter the expression, which would seem unlikely when we have a 34 bp lox sequence in an intron as the only difference from the wt. A cellular feedback mechanism may exist, since the retention sequence also mediates intracellular retention. It is hard to imagine, though, that loss of intracellular retention should lead to a general down-regulation of PDGF-B expression. We do not know if the down-regulation is the actual *cause* of defective pericyte recruitment, or a consequence. Nevertheless, mice with an endothelial specific *pdgf-b* deletion do not show similar defective pericyte integration to the vessel wall, despite up to 90% reduction in pericyte density. The severity of the retinal phenotype points to more than just a down-regulation. Moreover, the cell culture data pointed to a more diffusible PDGF-B protein in *pdgf-b ret/ret* cells.

The third paper describes how different isoforms of PDGFs can affect the proliferation of cardiac fibroblast at the postnatal stage. Also in this study, differences in the effect mediated by the long heparin binding form differ from those induced by the freely diffusible form. However, the outcome may reflect altered expression levels depending on the genomic integration site. Thus, we do not wish to draw too strong conclusions. Previous attempts to over-express PDGF-A and -B have been limited to lung (Hoyle et al. 1999) and eye (Seo et al. 2000) and the deletion of either PDGF ligands or receptors have not conclusively shown a direct effect of PDGF in the heart. On the one hand, up-regulated levels of PDGFs, either achieved by

transgenic approaches or in pathological models, induce a fibrotic response. This is, most likely, independent of the organ analysed, as long as the expression of ligand and receptor coincides. On the other hand, this thesis shows that a PDGF-B down-regulation has a similar outcome. Thus, physiological levels of available PDGF are maintained within a narrow concentration range and deviations from these levels, either by altered expression or by distribution, are detrimental. Clearly, these data are in line with the attempts to antagonize the effect of PDGFs, by other means than genetical. These attempts show promising results in the treatment of fibrosis and the inhibition of certain tumours.

Conclusions and future perspectives

In this thesis, I have tried to illustrate pieces of the high complexity in the construction of an organism and the functional organs. In addition, I have shown how both subtle as well as general genetic manipulation in the mouse can deepen our understanding of the physiological functions of proteins. This presents us with highly specialised tools to decipher the complex nature of our genome. In conclusion, we show in paper I that retention of platelet-derived growth factor-B is necessary for recruitment of mesangial cells to the developing kidney, for recruitment of pericytes to the central nervous system and for correct development of the retinal vasculature. We showed that retention of PDGF-B is necessary for maintenance of functional kidney glomeruli in the adult and that ablation of the retention sequence led to glomerulosclerosis and proteinuria. In paper II we used this model to study whether the mechanism of pericyte recruitment during tumour progression was similar to developmental angiogenesis. We showed that PDGF-B and

PDGFR-β had pronounced influence on the pericyte recruitment and vascular patterning in a tumour model. To create further understanding of the phenotypic changes several studies are ongoing. Firstly, the *pdgf-b ret/ret* mice are subjected to physiological studies that aim to determine the effect of altered PDGF-B signalling in cardiac, renal and vessel function (Nyström H., Lindblom P., Bergström G unpublished). Secondly, the *pdgf-b ret/ret* mice are used for studying the sequential changes in the kidney gene expression, from the embryonic mesangial cell loss, through a phase of recovery, to the adult stage of glomerulosclerosis (Norlin J., Lindblom P. and Takemoto M. unpublished). This approach allows the discovery of novel genes important for disease progression in the kidney. The approach may also give us insight into the mechanisms of mesangial cell recruitment and function. Thirdly, the use of the retention deficient mice in a femoral artery ligation assay aims to study the involvement of PDGF-B in arteriogenesis (Lindblom P. and Schaper W. unpublished). Fourthly, we aim to continue the use of the retention deficient mice as a model system to analyse mechanisms of sprouting tumour angiogenesis, and to further study the effect of angiogenic inhibitors in presence and absence of pericytes, (Abramsson A. and Lindblom P. unpublished). Other future objectives involve the identification of potential binding substrates for PDGF-B that mediate cell retention. Studies are ongoing to compare defective integration of pericytes to the micro-vascular wall in the *pdgf-b ret/ret* mice and those aberrancies found in the HSPG polymerase NDST-1 null mutants (Gerhardt H. and Abramson A. pers.comm). As described in the introductory part, PDGF interacts with the ECM protein SPARC. We examined the development of the retinal vasculature in SPARC null mutants. However, we did not detect any retinal disturbances (Lindblom and Gerhardt

unpublished). Moreover, it would be interesting to design constructs that allow *in vivo* tracing of PDGF. To combine this with alterations of the distribution, such as deletion of retention sequences, mutagenesis of potential proteolytic processing sites or switching between retention sequences in different genes, would allow further mechanistic insight. Obviously, it is tempting to speculate about the functions of retention in other GDF families.

In paper III, we show that different PDGFs can be involved in the pathogenesis of myocardial diseases by affecting cardiac fibroblasts. This is the first study that over-expressed two PDGF-A isoforms *in vivo*, and we could also detect differences in the responses they induced. From this study, we do not wish to conclude that the cause of the discrepancies is solely due to alterations in the splice forms, but it is tempting to speculate that they can induce different responses. Future experiments should aim to use inducible systems, since potent growth factors often lead to organ failures, which hinder adequate expression studies. The published transgenic lines that over-express PDGF do not recapitulate the endogenous expression. Instead, it would be of high interest to study the effect on vascular smooth muscle cell recruitment in the presence of prolonged and increased PDGF-B expression from endothelial cells. In such experiment, using genes coding for products with altered distribution capabilities could increase the understanding of PDGF-B signalling. So far, embryonic lethality of transgenic founders has hampered our attempts to do so. (Betsholtz and Lindblom unpublished). This clearly highlights the need for inducible systems.

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