On Vascular Endothelial Growth Factor B
and
Platelet-Derived Growth Factor C

Two members of the VEGF/PDGF family of growth factors

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The main benefit of a theory should be that it guides the experiments without preventing the progress of the true theory when it appears.

James Clerk Maxwell
ABSTRACT

Vascular endothelial growth factors (VEGFs), platelet-derived growth factors (PDGFs) and their receptors are important for normal development. They have also been implicated in many pathological conditions. VEGFs have been shown to play an important role in the development of both blood and lymphatic vessels. PDGFs, on the other hand, are important regulators of the connective tissue cells of both the vascular network and other organs systems.

The focus of the work presented in this thesis has been to elucidate the role of two members of the VEGF/PDGF family of growth factors, namely VEGF-B and PDGF-C. Embryonic analysis at the mRNA and protein level showed that VEGF-B was expressed in several organs, with highest expression in the developing muscles. VEGF-B was not detected in endothelial cells, where its receptor VEGFR-1 is expressed, which suggested that VEGF-B acts in a paracrine way. The expression of the two isoforms, VEGF-B_{167} and VEGF-B_{186} were investigated using techniques that can distinguish the two isoforms. The results showed that the VEGF-B_{167} isoform is predominantly expressed in most tissues. The VEGF-B_{186} isoform is expressed at lower levels and only in a limited numbers of organs. Moreover, the VEGF-B_{186} isoform is upregulated in mouse and human tumour cell lines and primary tumours compared with their corresponding normal tissues. These data suggest a fine genetic control of the expression of the two isoforms of VEGF-B, implying tissue- and cell-specific roles for the two VEGF-B isoforms. To elucidate the function in vivo, a VEGF-B knockout mouse strain was generated. The results showed that VEGF-B is not required for normal development of the cardiovascular system or for angiogenesis in adults. However, adult VEGF-B deficient mice have an atrial conduction abnormality characterised by a prolonged PQ interval in the electrocardiogram; thus VEGF-B appears to be required for normal heart function in adult animals.

The second growth factor, PDGF-C contains a domain structure not present in other members of the VEGF/PDGF family. Following the signal sequence, PDGF-C contains an N-terminal CUB-domain, and in the C-terminus, a VEGF/PDGF homology domain. PDGF-C is synthesised as an inactive precursor protein that has to be proteolytically processed in the N-terminus before it can bind and activate its receptor, PDGFR-\(\alpha\). Expression analysis during mouse development suggests that PDGF-C acts in both paracrine and autocrine ways.
ABBREVIATIONS

Ang         angiopoietin

CUB         complement subcomponents C1r/C1s, urchin EGF-like protein, bone morphogenic protein-1

E           embryonic day

ECG         electrocardiography

ECM         extracellular matrix

Fk          fetal liver kinase

Flt         fms-like tyrosine kinase

Ig          immunoglobulin

HIF         hypoxia-inducible factor

KDR         kinase insert domain-containing receptor

MMP         matrix metalloproteinase

mRNA        messenger ribonucleic acid

NP          neuropilin

PCR         polymerase chain reaction

PDGF        platelet-derived growth factor

PDGFR       platelet-derived growth factor receptor

PIGF        placenta growth factor

RT-PCR      reverse transcriptase PCR

VEGF        vascular endothelial growth factor

VEGFR       vascular endothelial growth factor receptor

vSMC        vascular smooth muscle cells

TGF         transforming growth factor
ORIGINAL PUBLICATIONS
This thesis is based on the following publications, which will be referred to by their roman numerals:


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INTRODUCTION

BUILDING THE CARDIOVASCULAR SYSTEM
A well-working and efficient cardiovascular system is essential for proper development and maintenance of organ systems in the vertebrate, both at the embryonic and adult stages. The blood vessels within this system serve as transport routes for nutrients and oxygen to, and waste products from, the organs. The blood vessels also enable macromolecules, such as hormones and growth factors to reach their targets. This is an intricate and complex system and its development and maintenance is finely regulated, but as is true for all of us also the regulation can go crazy which results in a variety of disorders, such as rheumatoid arthritis, psoriasis, atherosclerosis and tumour growth.

I will in this introduction discuss the construction and morphology of blood vessels and some of the factors involved. In the end, I will mention the roles in both normal and pathological events that these factors play. Most experimental data I refer to are derived from studies using the mouse as a model system.

BLOOD VESSELS
The first event in blood vessel formation is the recruitment of endothelial precursors (angioblasts) from the surrounding mesenchyme. This process is called vasculogenesis and leads to the first primitive tubes both within the embryo and in the extra-embryonic yolk sac. The primitive vascular network is expanded by a process called angiogenesis, which was originally defined as the sprouting of pre-existing vessels (for a review see Flamme et al., 1997). More recently, the term angiogenesis refers to the important task of expanding and modifying the primitive network into a complex network, which involves three different processes: sprouting, splitting (division by transendothelial cells) and intussuception (splitting by ingrowth of periendothelial cells) (Carmeliet, 2000; Patan et al., 1992; Risau W, 1997). However, in order for the naked vessel to survive and function it needs supporting cells; the vascular smooth muscle cells or pericytes (Benjamin et al., 1998; Lindahl et al., 1997a).

As I stated above, the formation of new blood vessels is complex and must be tightly controlled. Some of the assistants in the building and controlling of a vessel are members of the Vascular Endothelial Growth Factor/ Platelet Derived Growth Factor (VEGF/PDGF) family of growth factors. The identity of this family lies within the
VEGF/PDGF homology domain, which contains eight invariant cysteins involved in both intra- and interdisulphide bonding between the two antiparallel subunits (Heldin & Westermark, 1999; Joukov et al., 1997a).

The rest of the introduction will be devoted to these growth factors and their receptors, both at a structural and functional level, with the emphasis on those factors and receptors that are relevant for my work.

**ENDOTHELIUM- GROWTH FACTORS**

In this first part, I will introduce the VEGFs, which have been shown to play a part in the establishment of the inner lining of the blood and lymphatic vessels - the endothelium. The VEGFs and their receptors are illustrated in Fig. 1.

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![Fig. 1 Vascular endothelial growth factors and their receptors. For more detailed information see the text.](image-url)

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1 Cys 2 and Cys 4 are involved in the cystein bonds between the two subunits and the other six serve as interchain stabilisers; Cys 1 pairs with Cys 6, Cys 3 with Cys 7, and Cys 5 with Cys 8. PDGF-D however does not contain the fifth invariant cystein (Bergsten et al., 2001).
Expression of VEGFs and their receptors, and PDGFs and their receptors during mouse embryogenesis and in adult tissues.
VEGF-A

The most potent and well-studied angiogenic factor is Vascular Endothelial Growth Factor A (VEGF-A). VEGF-A was purified by three independent groups and referred to as VEGF or vasculotropin for its ability to specifically promote endothelial proliferation, and vascular permeability factor (VPF) for its ability to induce leakiness of blood vessels (Connolly et al., 1989a; Connolly et al., 1989b; Dvorak et al., 1995; Ferrara, 1993; Ferrara & Henzel, 1989; Keck et al., 1989; Plouet et al., 1989; Senger et al., 1983).

The gene encoding human VEGF-A gives rise to six different transcripts. This is due to differential use of the eight exons by alternative splicing of the mRNA; VEGF-A_{121}, VEGF-A_{145}, VEGF-A_{165}, VEGF-A_{183}, VEGF-A_{189} and VEGF-A_{206} (Houck et al., 1991; Lei et al., 1998; Leung et al., 1989; Poltorak et al., 1997). Four isoforms from the mouse homologue have been found, VEGF-A_{115}, VEGF-A_{120}, VEGF-A_{164} and VEGF-A_{188} (Shima et al., 1996; Sugihara et al., 1998).

The biologically active molecules are glycosylated disulphide-linked dimers, which display different biological properties. For example, the shortest isoforms, VEGF-A_{115} and VEGF-A_{120/121} are freely secreted whereas the others are sequestered on the cell surface or the extracellular matrix (ECM) due to binding to heparin sulfate proteoglycans (Poltorak et al., 2000; Sugihara et al., 1998). The predominant forms of VEGF-A are VEGF-A_{120/121}, VEGF-A_{164/165} and VEGF-A_{188/189}, whereas the others are comparatively rare (for a review see Robinson & Stringer, 2001). VEGF-A mediates its functions by binding and activating receptors located on the cell surface, VEGF receptor-1 (VEGFR-1/ flt-1), VEGFR-2 (flk-1/KDR) and neuropilin-1 and -2 (NP-1 and -2) (for a review see Neufeld et al., 1999).

VEGF-A is expressed in most tissues both in the adult and embryo, such as the heart, skeletal muscle, kidney, brain and lung (Berse et al., 1992; Dumont et al., 1995; Flamme et al., 1995; Olofsson et al., 1996a). The embryonic expression begins already a few days following implantation, suggesting that VEGF-A is important also for the vascularization of the extra-embryonic tissues (decidua, placenta and vascular membranes) (Jakeman et al., 1993). An overview of the expression of the different growth factors and their receptors is given in Table 1. VEGF-A is also expressed by

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2 The number in subscript refers to the number of amino acids in the mature protein.
most tumours, as I will discuss later. VEGF-A is regulated by the availability of oxygen, thus VEGF-A is upregulated under hypoxic conditions and downregulated under hyperoxic conditions (Alon et al., 1995; Shweiki et al., 1992). The upregulation of VEGF-A by hypoxia can occur by three different mechanisms. One way is the binding of the hypoxia–inducible transcription factor-1 (HIF-1) to a site in the promoter region of VEGF-A (Levy et al., 1995). Also an increase in mRNA stability leads to more VEGF-A production (Levy et al., 1996; Levy et al., 1998; Stein et al., 1995). The most recently discovered mechanism is the use of an alternate transcription initiation site with a downstream internal ribosomal entry site (IRES) that mediates cap-independent translation (Stein et al., 1998).

PlGF

The second growth factor in the VEGF-family was named Placenta Growth Factor (PIGF), since it was isolated from a human term placenta cDNA library (Maglione et al., 1991). Just like VEGF-A, PIGF is a glycosylated, secreted disulphide-linked dimer that comes in different lengths called: PIGF-1 (PIGF_{131}), PIGF-2 (PIGF_{152}) and PIGF-3 (PIGF_{221}) (Cao et al., 1997; Hauser & Weich, 1993; Maglione et al., 1993). Only PIGF-2 binds heparin with high affinity suggesting that only this isoform is sequestered on the cell surface/ ECM (Hauser & Weich, 1993). So far, only the PIGF-2 homologue has been detected in mouse (DiPalma et al., 1996). All variants of PIGF bind VEGFR-1, but only the heparin binding PIGF-2 bind NP-1 (Migdal et al., 1998; Park et al., 1994). None of the isoforms bind VEGFR-2 (Park et al., 1994).

PIGF is, as its name implies, highly expressed in the placenta and only low levels are detected in the adult, e.g. in lung, skeletal muscle, testis and thyroid (see Table 1 and for a review Persico et al., 1999). PIGF overlaps in expression with VEGF-A both in normal tissues, and in many tumours, in fact naturally existing heterodimers of PIGF•VEGF were purified from a rat glioma cell line (DiSalvo et al., 1995). Later, recombinant heterodimers were also purified and shown to induce binding to the VEGF-A receptor, VEGFR-2 (Cao et al., 1996). In contrast to VEGF-A, PIGF has been shown to be downregulated under hypoxic conditions (Gleadle et al., 1995, and this is reviewed in Ahmed et al., 2000).
VEGF-B

VEGF-B or VEGF-related factor (VRF) was also discovered by independent groups (Grimmond et al., 1996; Olofsson et al., 1996a). The mouse and human VEGF-B genes are almost identical and are composed of seven exons (Olofsson et al., 1996b). There are two isoforms of VEGF-B, VEGF-B_{186} and VEGF-B_{167}, caused by differential use of an alternative splice acceptor site in exon 6. This leads to an insertion of 101 base pairs and a subsequent shift in the reading frame. This frame shift gives the two isoforms different carboxy-terminals that affect their biochemical and biological features. The shorter variant contains a cell-associating, heparin-binding, carboxy-terminus resembling that of VEGF-A, whereas the carboxy-terminal region of the longer variant is hydrophobic and freely secreted. VEGF-B shares many features of VEGF-A, such as its disulphide-linked dimerisation and binding to the VEGFR-1 and NP-1, but unlike VEGF-A, VEGF-B is not affected by hypoxia, is not N-linked glycosylated and does not bind VEGFR-2 (Enholm et al., 1997; Mäkinen et al., 1999; Olofsson et al., 1998).

I will not dwell on this factor any longer, as it is one of the proteins this thesis is about, hence it will be discussed extensively in papers I-III.

VEGF-C and VEGF-D

VEGF-C and VEGF–D differs in many aspects from VEGF-A, PlGF and VEGF-B. They are mainly thought of as being important for lymphatic endothelium and lymphangiogenesis and constitute a subgroup based on their unique amino- and carboxy-terminal regions flanking the VEGF/PDGF homology domain (Joukov et al., 1996; Lee et al., 1996; Orlandini et al., 1996; Yamada et al., 1997). Their carboxy-terminal regions contain a repetitive pattern of cystein residues with homology to a component in the silk produced by the silk larvae, Chironomus tentans (Chilov et al., 1997). VEGF-C and VEGF–D are synthesised as pre-proproteins where the carboxy-terminal parts are cleaved off upon secretion, but remain bound via disulphide bonds. The proproteins bind to VEGFR-3 (flt-4), a receptor exclusively expressed on lymphatic

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3 VEGF-B_{186} has to be proteolytically processed before it can bind NP-1 (Mäkinen et al., 1999).
4 VEGF-B_{186} is O-linked glycosylated in its unique carboxy-terminus (Olofsson et al., 1996b).
endothelium, whereas binding to VEGFR-2 requires proteolytic processing of the amino-terminal regions (Joukov et al., 1997b; Kukk et al., 1996).

During development, VEGF-C is strongly expressed in regions destined to be rich in lymphatic vessels (Kukk et al., 1996) (Table 1). In adult, expression of VEGF-C mRNA is found in heart, placenta, ovary and small intestine (Kukk et al., 1996). VEGF-D expression in the developing embryo is detected in several regions, such as heart, lung, kidney, liver and skin (Avantaggiato et al., 1998). In the adult, VEGF-D is found in lung, heart, small intestine, skeletal muscle, colon and pancreas (Yamada et al., 1997).

There is another member of the VEGF-family, called VEGF-E, which is a collective name for a protein product from three strains of the Orf virus; NZ2, NZ7 and D1701 (Lyttle et al., 1994). As VEGF-E are virus proteins I will not devote any time to it in this introduction, other than to say that it binds VEGFR-2 and is angiogenic as shown in both in vitro and in vivo assays (Meyer et al., 1999).

**ENDOTHELIUM-RECEPTORS**

Growth factors exert their functions via binding to cell surface receptors and thereby transducing their signals into the cell (Fig. 1). The VEGF family bind to two different types of receptors, the VEGF receptors (VEGFR) and neuropilins (NP). VEGFRs are protein-tyrosine kinase receptors that consist of an extracellular domain of seven immunoglobulin-like (Ig) repeats, a hydrophobic transmembrane domain that anchors the receptor in the cell membrane, a juxtamembrane domain and a protein kinase domain that is split into two halves. The dimeric VEGFs bind two receptors simultaneously; thus forming a stable receptor dimer that becomes activated by the subsequent "autophosphorylation", i.e. one receptor subunit phosphorylates the other. The phosphorylation sites (phosphorylated tyrosine residues) serve both to increase the kinase activity and to create docking sites for downstream signal transduction molecules containing SH2 domains (for a review see Heldin, 1995).

Neuropilin is a transmembrane glycoprotein without any catalytic domains; in fact its cytoplasmic part is only 39 amino acids long (Kolodkin et al., 1997).

**VEGFR-1**

VEGFR-1, or flt-1 (fms-like tyrosine kinase), is the receptor for VEGF-A, PIGF and VEGF-B (de Vries et al., 1992; Olofsson et al., 1998; Park et al., 1994; Shibuya et al.,
VEGF-A binding to VEGFR-1 has a K_d value of 10 pM (as compared to 400-900 pM when VEGF-A binds to VEGFR-2) and 100-200 pM when PlGF binds (Waltenberger et al., 1994), for a review see Shibuya (1999). The determination of the K_d value of VEGF-B has been hampered due to difficulties in purifying soluble active protein, but the affinity was estimated to be half that of VEGF-A (Olofsson et al., 1998).

The second Ig loop of VEGFR-1 has been shown to specify the binding to VEGF-A, and since VEGF-A can compete for binding to VEGF-B, it is likely that they use the same binding domain on the receptor (Davis-Smyth et al., 1996; Olofsson et al., 1998). Even though VEGF-A also can compete for binding to PlGF, there is some discrimination residing in charged residues within the second and third Ig loop, of which some are important for PlGF binding, but not for VEGF-A binding (Barleon et al., 1997; Davis-Smyth et al., 1998). The fourth Ig loop has been shown to be essential for stabilising the dimer (Barleon et al., 1997). The fifth and sixth Ig loops of VEGFR-2 have been demonstrated to be important for VEGF-A retention after binding, and the first loop for regulating ligand binding (removal of Ig loop one improved VEGF-A binding) (Shinkai et al., 1998). The loops of VEGFR-1 might display similar functions, but have not yet been studied in detail.

The kinase activity of VEGFR-1 is much lower than that of other fms tyrosine kinases and the receptors for the PDGFs (Sawano et al., 1996; Shibuya et al., 1999). The weak kinase activity could be a result of unstable VEGFR dimers, tight regulation by tyrosine phosphatases or few phosphorylation sites (Shibuya et al., 1999). So far, five phosphorylation sites (docking sites for the downstream signalling molecules) have been identified (Ito et al., 1998; Sawano et al., 1997). Due to the weak kinase activity, a detailed study of the signalling events initiated by the receptor has been hindered. However, a number of signal-transduction molecules have been found to bind the specific phosphorylation sites. The end result of VEGFR-1 signalling is endothelial cell morphogenesis, monocyte migration and induction of tissue factor (TF) production (Shibuya et al., 1999). Just as many of the VEGFs, the VEGFR-1 gene gives rise to two different isoforms; a full-length receptor and an extracellular form called soluble VEGFR-1 (sVEGFR-1) (Kendall et al., 1996). This soluble receptor is abundantly expressed in the placenta and can form heterodimers with VEGFR-2 (Kendall et al., 1996).

VEGFR-1 starts to be expressed in primitive endothelial cells outside the blood islands at embryonic day (E) 7 and within the embryo at E8.5 (Fong et al., 1995; Peters et al., 1990).
During late embryogenesis (E14.5-16.5) VEGFR-1 expression decreases but is upregulated in new-born mice and continues to be expressed in the adult (Peters et al., 1993). However, the expression of VEGFR-1 is not restricted to endothelium, but is also expressed in trophoblasts, renal mesangial cells, and monocytes which migrate towards VEGF-A and PIGF-2 (Barleon et al., 1996; Charnock-Jones et al., 1994; Clauss et al., 1996; Takahashi et al., 1995).

VEGFR-1, but not VEGFR-2 nor VEGFR-3 is upregulated by hypoxia and subsequently a binding site for HIF-1 was identified in the promoter region of VEGFR-1 (Gerber et al., 1997; Sandner et al., 1997). Interestingly, NP-1 is downregulated by hypoxia (Ding et al., 2000b). Both VEGFR-1 and VEGFR–2 bind heparin and are thought to associate with heparan-sulfate proteoglycans on the cell surface (Cohen et al., 1995). Experiments have however, indicated that the two receptors are differentially regulated by heparin, as VEGF-A binding to VEGFR-1 is inhibited by exogenous heparin, whereas binding to VEGFR-2 is stimulated (Terman et al., 1994).

**VEGFR-2**

The second receptor was originally called KDR (kinase insert-domain containing receptor) for the human protein and flk-1 (fetal liver kinase 1) for the mouse homologue (Matthews et al., 1991; Terman et al., 1991). VEGFR-2 binds VEGF-A, VEGF-C and VEGF-D (Achen et al., 1998; Joukov et al., 1996; Terman et al., 1992). The overall structure and binding properties are highly related to VEGFR-1 (Fuh et al., 1998; Shibuya et al., 1999).

VEGFR-2 mRNA is first detected in presumptive mesodermal yolk-sac blood island progenitors at E7 and then in the primitive endothelium surrounding the blood islands, thus VEGFR-2 seems to play an important role during vasculogenesis (Shalaby et al., 1995) (Table 1). Just like VEGFR-1, embryonic expression declines towards the end of gestation, but unlike VEGFR-1 it remains downregulated in quiescent endothelial cells (Millauer et al., 1993). VEGFR-2 is also expressed by hematopoietic stem cells, megakaryocytes and platelets as well as by retinal progenitor cells (Katoh et al., 1995; Yang & Cepko, 1996).
VEGFR-3

VEGFR-3 (flt-4), is a receptor for VEGF-C and VEGF-D and differs from the other VEGF receptors in that it has to be proteolytically processed before it can bind to its ligands (Achen et al., 1998; Joukov et al., 1996; Pajusola et al., 1992; Pajusola et al., 1994).

Early in mouse development, VEGFR-3 is expressed in the cardinal vein and in the angioblasts of the head mesenchyme but as embryogenesis continues VEGFR-3 expression becomes restricted to the lymphatic endothelium (Kaipainen et al., 1995) (Table 1). In adults, VEGFR-3 is found in the lymphatic endothelium of the lung, mesenterium and tonsil, while vascular endothelium is negative (Kaipainen et al., 1995). In spite of the documented restriction to lymphatic vessels, recent data show that VEGFR-3 is expressed both in the blood and lymphatic vessels in the nasal mucosa of human adults and in the fenestrated capillaries of several organs (Partanen et al., 2000; Saaristo et al., 2000).

NP-1 and NP–2

Neuropilins are type I transmembrane receptors with a large extracellular domain, a hydrophobic transmembrane domain, and a short 39 amino acid intracellular domain. The extracellular domain is divided into three unique domains, called a1/ a2, b1/b2 and c. The first domain is composed of two non-catalytic CUB (complement subcomponents C1r /C1s, urchin EGF-like protein, bone morphogenic protein-1) regions, the second is a domain similar to the C1 and C2 domains of coagulation factors V and VIII and the third is a MAM (meprin, A5, µ) domain (Bork & Beckmann, 1993; Fujisawa & Kitsukawa, 1998) (Fig. 1). Due to their short non-catalytic intracellular domains and since CUB domains mediate protein-protein or protein-carbohydrate interactions, neuropilins are thought of as cell surface molecules that mediate cell-cell interaction and not as signalling receptors (Soker et al., 1998; Takagi et al., 1995).

Neuropilins were initially described as the receptors for members of the class III subfamily of the collapsin/ semaphorin family; Sema E, Sema IV and Sema III. Neuropilins have been shown to serve as guidance cues for axon growth (Chen et al., 1997; He & Tessier-Lavigne, 1997; Kolodkin et al., 1997; Takagi et al., 1991, as reviewed in Fujisawa & Kitsukawa, 1998). More recently, NP-1 was found to be a VEGF-

5 Neuropilin-1 was originally called A5 (Takagi et al., 1991).
A165-specific co-receptor to VEGFR-2 that enhances the binding of VEGF-A165 to VEGFR-2 (Soker et al., 1998). Later the heparin-binding form of PIGF (PIGF-2) and both isoforms of VEGF-B were also shown to specifically bind to NP-1 (Makinen et al., 1999; Migdal et al., 1998). A new report showed that NP-1 also interacts with VEGFR-1, but instead of potentiate binding, the interaction inhibits further binding of VEGFR-1 to VEGF-A165 (remember that also heparin plays different roles in potentiating/ inhibiting binding to VEGFR-1 and VEGFR-2) (Fuh et al., 2000). This is very interesting, since hypoxia downregulates NP-1, but upregulates VEGFR-1 and this means that, during hypoxic conditions, VEGF-A-mediated VEGFR-1 signalling is not only potentiated by the upregulation of VEGF-A and VEGFR-1, but also by the decreased inhibition of VEGFR-1 by NP-1. Thus, NP-1 is likely to be a co-receptor for various ligands and the function of these depends on the abundance of NP-1 on the cell membrane. The closely related NP-2 also functions as a receptor for VEGF-A145 and VEGF-A165 (Gluzman-Poltorak et al., 2000). Analogous to VEGFR-1, a naturally occurring soluble form of NP-1 has been identified and shown to act as an antagonist to VEGF-A165 (Gagnon et al., 2000).

During mouse embryonic development, NP-1 is expressed in the cardiovascular system, nervous system, and limbs at particular developmental stages (Kitsukawa et al., 1995) (Table 1). NP-1 is also detected in blood vessel endothelium and in tumours (Soker et al., 1998). Immunohistochemical analysis has shown that the expression pattern of NP-1 resembles that of VEGFR-1 in the developing human heart, with staining in the endothelium of blood vessels (Partanen et al., 1999).

**SUPPORTING CELLS**

Some of the factors involved in the initial step of building a vessel have now been introduced and now it is time for the subsequent morphological step – the recruitment of the supporting perivascular cells, the vascular smooth muscle cells (vSMC) and the pericytes. During development and in the adult organism, the vSMC plays various roles; being either a biosynthetic, proliferative or contractile component of the vessel wall (Hungerford & Little, 1999). The pericytes are comparable to vSMC, but serve as

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6 In the nervous system, NP-1 was detected in neurons when axons were actively growing to form neuronal connections, and was suggested to be involved in growth, fasiculation and targeting for a particular group of axons (Kawakami et al., 1996).
supporting cells for capillaries, whereas vSMC surround larger vessels. There is a gradual phenotypic transition between vSMC and pericytes as the vessels become smaller, with a decrease in e.g. myofilaments (Diaz-Flores et al., 1991). The most reliable marker used to distinguish vSMC from pericytes is the difference in basement membrane. vSMC have their own basal lamina, whereas pericytes share with the endothelial cell (Diaz-Flores et al., 1991). The vSMC and the pericyte originates from mesenchymal cells in the mesoderm or from cranial neural crest cells (Diaz-Flores et al., 1991; Hungerford et al., 1996).

It has been shown that the platelet-derived growth factors (PDGFs) A and B, and their receptors, are important for the recruitment of these supporting cells to the correct location; both within the vascular network and in other organ systems. The PDGFs and their receptors are illustrated in Fig. 2.

![Fig. 2 Platelet-driven growth factors and their receptors. For a detailed description see the text.](image)

**GROWTH FACTORS**

**PDGFs**

Platelet-derived growth factor (PDGF) is the other subfamily in the VEGF/ PDGF family of growth factors. PDGF was originally described as a product of platelets which was able to induce *in vitro* proliferation of fibroblasts, smooth muscle cells, and glia cells (Kohler & Lipton, 1974; Ross *et al.*, 1974; Westermark & Wasteson, 1976). PDGF was also shown to stimulate migration and modulate ECM production. Despite its name,
PDGF is also expressed by endothelial cells, epithelial cells, macrophages, and neurons (reviewed in Betsholtz, 1995). When PDGF was isolated, it was found to be composed of a dimeric molecule of disulphide-bonded A- and B-polypeptide chains (Anatoniades et al., 1979; Heldin et al., 1979; Johnsson et al., 1982; Waterfield et al., 1983a).

The A- and B-chains are produced as precursor molecules that undergo intracellular proteolytical processing in the amino terminus (PDGF-A and PDGF-B) and in the carboxy terminus (PDGF-B). The chains can naturally form heterodimers as well as homodimers, thus forming PDGF-AA, PDGF-AB and PDGF-BB. Both the A- and B-chain contain a carboxy terminal retention motif in, which is similar to the one present in some of the VEGFs, and keeps the secreted proteins on the cell surface or in the ECM. The proteolytical processing of the carboxy terminus in PDGF-B allows the molecule to become diffusible. There is no proteolytic cleavage of the carboxy-terminus of PDGF-A, instead the gene encodes two splice isoforms, one long cell-surface restricted and one freely diffusible, lacking the carboxy-terminus altogether (reviewed in Heldin & Westermark, 1999).

Recently, two other PDGFs were cloned, PDGF-C and PDGF-D (Bergsten et al., 2001; Ding et al., 2000a; Hamada et al., 2000; Li et al., 2000; Tsai et al., 2000). PDGF-C and PDGF-D introduced a new structural subgroup within the PDGF-family. Following the signal peptide, there is a CUB domain followed by a hinge region and the VEGF/ PDGF homology domain (Bork & Beckmann, 1993; Heldin & Westermark, 1999; Joukov et al., 1997a). PDGF-C is the other protein in this thesis (paper IV and V) thus a more detailed discussion will follow below.

**PDGF receptors**

PDGFs bind and activate tyrosine kinase receptors located on the cell surface. There are at present two receptors called PDGFR-α and PDGFR-β (Claesson-Welsh et al., 1988; Claesson-Welsh et al., 1989; Matsui et al., 1989; Yarden et al., 1986). These receptors are related to the VEGFRs, but contain five extracellular Ig domains, not seven as in VEGFR1-3 (compare Figs.1 and 2). PDGFR-α binds PDGF-A, PDGF-B and PDGF-C, whereas PDGFR-β binds PDGF-B and PDGF-D chains (Bergsten et al., 2001; Heldin et al., 1989; Kanakaraj et al., 1991; Li et al., 2000; Seifert et al., 1989). The PDGF receptors form heterodimers upon ligand binding, and as a result PDGF-AA and PDGF-CC induce PDGFR-αα dimers, PDGF-AB induce PDGFR-αα or PDGFR-αβ dimers,
PDGF-BB induce all types of dimers, and PDGF-DD induce only PDGFR-ββ dimers (Eriksson et al., 1992) (Fig. 2).

The signal transduction from the three dimeric receptor-conformations is overlapping but not identical. Most studies have focused on the transduced signals from the homodimeric receptors, but there is increasing evidence that the signals from the heterodimeric αβ-receptor is different from the homodimeric receptors (reviewed in Heldin et al., 1998). The signalling pathways from the PDGF receptors have been extensively studied, but I will just briefly mention two types of SH2-containing molecules binding the receptor complexes (reviewed in Heldin et al., 1998). There are proteins with enzymatic properties, such as PI 3-kinase, that mediates, for example actin reorganisation, chemotaxis, cell growth and antiapoptosis, and there are proteins that serve as adaptors for downstream enzymes, such as Grb2 that mediates binding of Sos, a molecule that activates Ras.

The distribution of PDGF-A and PDGF-B and the α- and β-receptors during embryogenesis suggest that signalling can be autocrine or paracrine, depending on the tissue and the developmental stage of the embryo. PDGF-A and receptor-α expression pattern have been thoroughly investigated, whereas there has been less attention on the β-receptor and PDGF-B expression patterns (Table 1). PDGF-A and PDGFR-α are produced at earlier stages and more widely than PDGF-B and PDGFR-β; in fact PDGF-A is detected as maternal transcript in the oocyte and blastocyst (Rappolee et al., 1988). In summary, PDGF-A and PDGFR-α are produced in separate but adjacent cell layers in the developing mouse embryo, with the receptor in mesenchymal cells and the ligand in the adjacent epithelia (Orr-Urtreger & Lonai, 1992). Also, PDGF-B and PDGFR-β are expressed in adjacent cell layers, with the receptor in mesenchymal cells, and the ligand in this case expressed both in epithelia and endothelia (reviewed in Ataliotis & Mercola, 1997).

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7 Activated Ras initiates a cascade that stimulates cell growth, chemotaxis and differentiation.
OTHER PLAYERS

Other growth factors and receptors important for blood vessel development and maintenance are e.g. the angiopoietins/Tie receptors, the ephrins/Eph receptors and TGFβ-1.

Ang/Tie

The Tie receptors (Tie-1 and Tie-2/Tek), compose a separate class of receptor tyrosine kinases that are specifically expressed in developing endothelial cells and early hemopoietic cells (Batard et al., 1996; Dumont et al., 1993; Dumont et al., 1992; Iwama et al., 1993; Korhonen et al., 1994). As of now, there are four members of the angiopoietin family, Ang1-4 (Davis et al., 1996; Maisonpierre et al., 1997; Valenzuela et al., 1999). All Angs bind Tie-2 but thus far, there is no report of Tie-1 ligands. Ang1 induces phosphorylation of Tie-2 and functions as a remodeller and stabiliser of vessel walls, both by optimising the communication between endothelial cells and its supporting cells and by protecting the adult vasculature from vascular leakage (Suri et al., 1996; Suri et al., 1998; Thurston et al., 2000; Thurston et al., 1999, and reviewed in Yancopoulos et al., 2000). Ang2 was identified based on its homology to Ang1, but was found to be a naturally occurring antagonist to Tie-2 (Maisonpierre et al., 1997). Furthermore, transgenic overexpression of Ang2 resulted in disrupted blood vessel formation in the mouse embryo, a defect also reported for the Ang1 and Tie-2 knockouts, suggesting that Ang2 could act as an antagonist in vivo (Patan, 1998; Suri et al., 1996). Not much is known about Ang3 and Ang4 other than Ang3 works as an antagonist and Ang4 as an agonist (Valenzuela et al., 1999).

ephrin/Eph

In the beginning of the last decade, ephrin-A1 (B61) was shown to be upregulated during in vitro capillary tube formation and also to induce angiogenesis (Holzman et al., 1990; Pandey et al., 1995). However, the main focus of ephrins and the Eph-receptors had been on their role in axonal guidance (reviewed in O'Leary & Wilkinson, 1999). In 1998, ephrin-B2 and Eph-B4 were shown to play a part in the establishment of arteries and veins, thus the roles of ephrins and their receptors in both neural and vascular

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8 Ang3 and Ang4 might represent the mouse and human counterparts of the same gene (Valenzuela et al., 1999).
development became equally interesting. Wang et al. revealed that from the onset of angiogenesis, ephrin-B2 marks arterial endothelial cells and Eph-B4, the receptor, marks veins (Wang et al., 1998). The ephrin-B2 knockout mice displayed defects in angiogenesis involving both vascular remodelling and vessel maturation. Furthermore, the supporting periendothelial cells were poorly associated to the endothelium, a phenotype resembling the one reported from the knockout studies made of Ang1 and its receptor Tie-2 (Sato et al., 1995; Suri et al., 1996; Wang et al., 1998).

Despite the ideal and logical situation, with an ephrin-Eph pair reciprocally expressed on arteries and veins, a recent finding demonstrated the presence of ephrin-B1 on both arteries and veins (Adams et al., 1999). This argues that ehprins and their receptors are important not only for the arterial-venous boundary, but also may regulate the whole vasculature. Adams et al. also showed that Tie-2 could phosphorylate the cytoplasmic domain of ephrin-B2 in vitro, suggesting a cross-talk between the Ang-Tie pathway and the ephrin-Eph pathway, regulating the interactions between endothelial cells and its supporting mesenchymal cells.

**TGF-β1**

Transforming growth factor β1 (TGF-β1) is a member of a large superfamily of cytokines and is secreted from cells in a latent form that has to be activated in the extracellular milieu (Munger et al., 1997). It has been proposed that extracellular matrix proteases release latent TGF-β1, which is then activated by cell-associated plasmin (Munger et al., 1997). The exact mechanism of activation in vivo is still unknown, but recent reports have shown that αvβ6 integrin, matrix metalloprotease-2 (MMP-2) and MMP-9 bind and activate latent TGF-β1 in vitro (Munger et al., 1999; Yu & Stamenkovic, 2000). TGF-β1 binds two types of cell surface receptors TGF-βRI and II. Receptor II exists as an oligomer and the binding of TGF-β1 leads to the recruitment of receptor I and the formation of a stable complex. The kinase activity of both receptors is necessary for the transduction of TGF-β1 signals (reviewed in Pepper, 1997).

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9 TGF-β1 is noncovalently bound to latency associated peptide (LAP), which is a homodimer formed from the propeptide region of TGF-β1. LAP is disulfide linked to another protein, latent TGF-β binding protein (LTBP) that targets latent TGF-β1 to the matrix.
In the mouse embryo, TGF-β1 is expressed in the blood islands at E7 and in all endothelial cells from E9.5, as well as in hematopoietic cells (Akhurst et al., 1990). From E12.5 onwards, TGF-β1 is also expressed in cartilage, bone, teeth, muscle, heart, blood vessels, lung, kidney, gut, liver, eye, ear, skin, and nervous tissue (Pelton et al., 1991). The TGF-βII receptor expression pattern correlated with TGF-β1 in various developing tissues, primarily in the mesenchyme and epidermis (Lawler et al., 1994). Both TGF-β1 and TGF-βRII serve important functions in blood vessel development of the yolk sac (Dickson et al., 1995; Oshima et al., 1996). Inactivation of the genes resulted in similar phenotypes with vessel wall fragility due to disruption of the contacts between the endothelial cells and the mural cells.
NORMAL DEVELOPMENT

VEGF ligands

In the developing embryo, avascular tissues become hypoxic and thereby stimulate VEGF-A production (Forsythe et al., 1996; Shweiki et al., 1992). The upregulation of VEGF-A leads to vasodilation\(^1\) and vascular permeability. The increased permeability leads to leakage of plasma proteins that will serve as a scaffold for migrating endothelial cells. However, before the endothelial cells can start to migrate they must detach from their basal lamina, supporting cells, and the ECM. Proteinases assist in angiogenesis, both by degrading ECM components and by releasing/activating growth factors residing in the matrix (Carmeliet et al., 1998; Hiraoka et al., 1998; Imai et al., 1997). Once the path has been cleared, the endothelial cells migrate and proliferate to distant sites (for a review see Carmeliet, 2000).

The importance of VEGF-A has been demonstrated in several studies. Mice lacking one VEGF-A allele died in utero due to cardiovascular defects (Carmeliet et al., 1996; Ferrara et al., 1996). Partial depletion of VEGF-A\(^1\) in neonatal mice resulted in increased mortality and impaired growth, whereas total depletion in neonatal mice using soluble VEGFR-1(1-3)-IgG, resulted in death due to metabolic failure (Gerber et al., 1999). These findings as well as other reports, show the importance of correct levels of VEGF-A for proper development (Haigh et al., 2000). However, VEGF-A was not essential for growth and survival of juvenile mice, although VEGF-A was required for proper corpus luteum angiogenesis (Ferrara et al., 1998).

Tufró and co-workers demonstrated that in rat embryonic kidney explants, VEGF-A induced nephrogenesis during metanephric development, as well as directed the developing capillaries towards the developing nephrons (Tufro, 2000; Tufro et al., 1999). A study using mice expressing only the freely diffusible VEGF-A\(^1\) has shown that the different isoforms of VEGF-A cannot replace each other, as these mice died shortly after birth due to impaired myocardial angiogenesis and ischemic cardiomyopathy (Carmeliet et al., 1999b). Hearts from the VEGF-A\(^1\) mice also displayed fewer vSMC around the coronary arteries and renal vessels. There were also less retinal pericytes

\(^1\) The dilation is achieved through nitric oxide (NO) induced by VEGF-A (Faller, 1999).
\(^1\) A cre-loxP mouse was used in which only partial VEGF-A inactivation was achieved.
surrounding the capillaries in the retina. The reduction in supporting cells coincided with a reduction in PDGF-B and PDGFR-β, thus the effect could be due to decreased PDGFR-β signalling known to be important for the recruitment of these cells (Hirschi et al., 1998). However, a more recent report shows that VEGF-A induces migration, but not proliferation of vSMC and that VEGFR-1 and VEGFR-2 are expressed on vSMC, thus the observed reduction of vSMC could be caused by the lack of the other VEGF-A isoforms (Grosskreutz et al., 1999). VEGF-A is not only needed for endothelial cell proliferation, but a certain threshold level is needed for the cells to survive and for stabilisation of the newly-formed vessels (Alon et al., 1995; Benjamin et al., 1998). The survival function has been shown to be mediated by a complex consisting of VEGFR-2, β-catenin and VE-cadherin12 (Carmeliet et al., 1999a). The importance of precise levels of VEGF-A was also illustrated in overexpression studies, where for example excess VEGF-A in the skin lead to the development of more, bigger, winding and leaky vessels (Detmar et al., 1998). The use of exogenous delivery of VEGF-A13 has been explored and further demonstrated the necessity of correct concentrations in order to avoid deleterious or even lethal effects (Drake & Little, 1995; Hariawala et al., 1996; Lee et al., 2000; Springer et al., 1998).

There is still some controversy over whether PlGF is angiogenic or not, but a recent study has shown that PlGF plays little or no role in embryonic angiogenesis (Carmeliet, 2001).

The finding that VEGF-C was angiogenic in vivo was unexpected since overexpression in mouse skin resulted in specific lymphangiogenesis with no effect on endothelial cells (Cao et al., 1998; Jeltsch et al., 1997). Moreover, VEGF-C also increases vascular permeability, migration, and proliferation of endothelial cells, thus VEGF-C is likely to play a dual role, both as an angiogenic and lymphangiogenic growth factor; depending on the spatio-temporal expression of its receptors (Cao et al., 1998). Also, VEGF-D has been shown to be angiogenic as well as serving as a mitogen for both fibroblasts and endothelial cells, although it is less potent than VEGF-A (Achen et al., 1998; Marconcini

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12 β-catenin and VE-cadherin are molecules involved in adherens junctions that link endothelial cells together.
13 This was done by injecting: VEGF-A protein, retrovirus expressing VEGF-A, or transduced myoblasts overexpressing VEGF-A into the mouse model.
et al., 1999; Orlandini et al., 1996). As of now, there are no reports of transgenic mice depleted of either VEGF-C or VEGF-D.

**VEGF receptors**

*In vitro* studies have shown that VEGFR-1 and VEGFR-2 respond differently to VEGF-A stimulation. Both receptors induced cell migration, but mitogenic signals were only transduced via VEGFR-2 (Barleon et al., 1996; Seetharam et al., 1995; Soker et al., 1998; Waltenberger et al., 1994; Yoshida et al., 1996). Also, VEGF-A-induced vascular permeability occurs primarily via VEGFR-2 (Hiratsuka et al., 1998; Joukov et al., 1998). *In vivo* studies have shown the importance of both receptors during development. Mice lacking VEGFR-1 were reported to die *in utero* due to a disorganised vasculature (Fong et al., 1995). However, a more thorough analysis showed that an increase in mesenchymal-hemangioblast transition was the primary defect and that the vascular disorganisation was a result of the increased endothelial progenitor density (Fong et al., 1999). In fact, endothelial cells lacking VEGFR-1 could form normal vascular channels when the density was reduced. The deduction made by Fong et al. was that when endothelial cells are too close to one another they form interactions in many different directions which results in disorganised cavities. One interesting fact with VEGFR-1 is that the catalytic cytoplasmic domain is not essential for proper cardiovascular development, thus signalling from VEGFR-1 seems to be insignificant during embryogenesis and the role of VEGFR-1 might be that of a sink, where excess VEGF-A is drained (Hiratsuka et al., 1998). However, VEGF-A- or PIGF-induced monocyte migration was suppressed in mice lacking the intracellular tyrosine kinase domain of VEGFR-1, and Carmeliet and co-workers have shown that VEGFR-1 signalling might be different in pathological contra physiological conditions (see “PATHOLOGICAL CONDITIONS” and Carmeliet, 2001).

Mature endothelial cells and hematopoietic cells were absent in mice depleted of VEGFR-2 which lead to an embryonic lethal phenotype, with no blood island formation or vasculogenesis within the embryo (Shalaby et al., 1997; Shalaby et al., 1995). Contrasting the report from Shalaby et al., another group was able to show that VEGFR-2-deficient embryonic stem cells were able to form hematopoietic and endothelial cells *in vitro*, but that the subsequent migration and proliferation depended on VEGFR-2
signalling (Schuh et al., 1999). They speculated that in VEGFR-2⁻/⁻ embryos, the hematopoietic precursors are present early in development (E7.5), but are absent one day later. The reason for this is believed to be their disability to migrate to the correct site, where they can receive the accurate signals required for blood formation. A recent report have shown that VEGFR-2⁺ cells serve as vascular progenitor cells, inducing both endothelial cells or mural cells depending on whether they are exposed to VEGF-A or PDGF-B (Yamashita et al., 2000).

Neuropilin is, as I stated above thought of as a co-receptor and expressed both in neurons and the cardiovascular system. The importance of NP-1 and NP-2 in nerve fibre guidance was demonstrated in mice deficient of NP-1 or NP-2 (Giger et al., 2000; Kitsukawa et al., 1997). Embryos, homozygous for NP-1 deletion, died in utero around mid-gestation due to defects in the cardiovascular system (Kawasaki et al., 1999). The phenotype of the NP-1⁻/⁻ embryos was not as severe as that observed for VEGFR-1 or VEGFR-2, the two VEGFRs that NP-1 interacts with. Instead, the phenotype suggested that NP-1 is necessary for the maturation and remodelling of the primary vascular network and for early development of the cardiovascular system. In addition, the authors reflect upon the possibility of a cross-talk between the VEGFs and the Semaphorins, regulating both neural development and angiogenesis. For example, SemaIII which is expressed in the embryonic heart, serve as a functional antagonist for VEGF-A₁₆₅ and vice versa (Giger et al., 1998; Miao et al., 1999). Since nerves and blood vessels often follow similar routes and modes of migration during embryogenesis, NP-1 might function as a communication-link between the developing vasculature and the growing axons (Martin & Lewis, 1989; Shima & Mailhos, 2000).

Phenotypes exhibited by transgenic embryos overexpressing NP-1, were excess and dilated vessels, excess and defasciculated nerve fibres and extra digits on the limbs, thus overexpression of NP-1 seemed to enhance effects from preexisting signals (Kitsukawa et al., 1995). The affected organ systems are formed from different developmental processes and Kitsukawa et al. suggested that each process uses one of the three separate extracellular domains of NP-1.

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14 Remember that the affinity for VEGF-A is higher for VEGFR-1 than VEGFR-2 (Waltenberger et al., 1994).
15 Both SemaIII and VEGF-A₁₆₅ bind the coagulation factor domains of NP-1, furthermore SemaIII binds the CUB domain, indicating that not all functions are competitive.
In line with the angiogenic capacity of VEGF-C and VEGF-D, VEGFR-3 was shown to play a role in the development of the cardiovascular system before lymphatic vessels appear (Dumont et al., 1998). Vasculogenesis was not affected in VEGFR-3 deficient embryos, nor was sprouting angiogenesis, but VEGFR-3 signalling was shown to be important for vascular remodelling and maturation of the primitive vascular network.

In order to circumvent the effect on the cardiovascular system, Alitalo and co-workers expressed a dominant negative soluble form of VEGFR-3 under the skin-specific keratin14 (K14) promoter (Mäkinen et al., 2001). The expression from the K14 promoter is not turned on until E14.5-16.5, when the expression of VEGFR-3 is restricted to the lymphatic endothelium, hence not affecting the cardiovascular system (Kaipainen et al., 1995). The expression of the soluble VEGFR-3 lead to inhibition of lymphangiogenesis as well as regression of already made lymphatic vessels, proving that VEGF-C/VEGF-D signalling is important in lymphatic development.

**PDGFs and their receptors**

Both PDGF-A and PDGF-B have been shown to be essential for normal development. As I mentioned above PDGF-A is expressed in the oocyte and blastomere of the early embryo where its initial function is thought to be proliferative through autocrine stimulation via its receptor PDGFR-α. The knockout of PDGF-A revealed a complex phenotype with half of the embryos dying at mid-gestation and the other half postnatally (Boström et al., 1996). Embryos that did not pass the embryonic restriction point probably died due to defects in somite signalling, leading to impaired axial skeleton development (Tallquist et al., 2000). The rescue of the latter half is thought to be due to more maternal PDGF-A transcripts in the early blastomere, thus permitting those embryos pass the restriction point. Pups that pass the embryonic restrictions display several phenotypes. They die from an emphysema-like disorder due to disrupted alveoli formation in the lung most likely caused by the lack of smooth muscle cells (Boström et al., 1996; Lindahl et al., 1997b). Other defects were oligodendrocyte deficiency, skin and hair follicle defects, dysmorphogenesis of intestinal villi, and loss of the Leydig cells in the testis (Fruttiger et al., 1999; Gnessi et al., 2000; Karlsson et al., 1999; Karlsson et al., 2000).

PDGFR-α deficient embryos died *in utero* displaying similar but more severe defects compared to those observed in the PDGF-A⁻/⁻ embryos (Soriano, 1997). The reported
defects were *spina bifida*, cleft face, abnormalities of the rib cage, lateral bleedings, and subepidermal blebs. Since PDGFR-\(\alpha\) also is the receptor for PDGF-B, the discrepancy might be due to impaired PDGF-B signalling via PDGFR-\(\alpha\). But, embryos lacking both PDGF-A and PDGF-B do not display these additional defects (C. Betsholtz, personal communication). The recent finding of an additional PDGFR-\(\alpha\) specific ligand, PDGF-C, suggests that it might be responsible for the observed difference in phenotype (Li *et al*., 2000).

Mice lacking PDGF-B or PDGFR-\(\beta\) exhibit similar phenotypes, suggesting that PDGF-B is the major ligand for PDGFR-\(\beta\) and although PDGF-B binds and activates PDGFR-\(\alpha\), this signalling pathway is not likely to be used during embryogenesis (Levéen *et al*., 1994; Soriano, 1994). Both mutant-types died *in utero* late in gestation displaying severe abnormalities in the kidney glomeruli, heart dilation, haematological disturbances, and haemorrhages. The bleedings in PDGF-B embryos were due to aneurysm formations believed to be caused by a loss of capillary pericytes (Lindahl *et al*., 1997a). It is well established that PDGF-B induces proliferation and migration of vSMC and pericytes. As I mentioned above, also VEGF-A has been shown to induce migration (but not proliferation) of vSMC. Grosskreutz *et al* demonstrated that both PDGF-B and VEGF-A were induced by hypoxia, although VEGF-A faster (1h) and PDGF-B slower (6-12h) (Grosskreutz *et al*., 1999). Thus, VEGF-A and PDGF-B might work in concert with overlapping functions but different timing, to provide a potent hypoxia-enhanced stimulus for vSMC.

**PATHOLOGICAL CONDITIONS**

I will in this section focus on tumour pathology since it is has most relevance for my thesis.

**VEGFs**

The drawback with VEGF-A, a seemingly altruistic molecule, is that it acts on cues delivered from the surroundings - not knowing if it is appropriate to induce angiogenesis or not. The Mr Hyde version of VEGF-A introduces itself in tumour progression. Avascular tumours cannot expand more than a few cubic millimetres, since all cells benign as well as malign, depend on blood vessels for their survival and, they cannot distance themselves more than a few “cell-diameters” from the nearest capillary. Most
tumours display elevated levels of VEGF-A and studies have shown that when VEGF-A signalling is inhibited, tumour angiogenesis, and subsequently tumour growth, is impaired (Kim et al., 1993). VEGF-A not only assists the developing tumour with the ingrowth of new capillaries, but also by inducing permeability of the blood vessels\textsuperscript{16}. The permeability leads to the same scenario that I have discussed under “NORMAL DEVELOPMENT”, with migration of endothelial cells, tumour and stromal cells.

Carmeliet and co-workers have thoroughly investigated the function of PlGF in deficient embryos (Carmeliet et al, 2001). They concluded that although PlGF had no effect in normal development, it stimulated VEGF-A-driven angiogenesis in malignant conditions. Many tumours have an upregulation of PlGF, which leads to more efficient signalling via VEGFR-1.

VEGF-B and VEGF-C are expressed in a variety of tumours (Salvén et al., 1998). VEGF-D on the other hand seems to be expressed at only low levels (Niki et al., 2000; Yamada et al., 1997). Recent reports have demonstrated that both VEGF-C and VEGF-D are involved in the lymphatic spread of tumour metastasis (Skobe et al., 2001; Stacker et al., 2001). The possible role of VEGF-B in tumour growth will be discussed in paper III.

**VEGFRs**

It has been demonstrated that VEGFR-1 is upregulated in endothelial cells undergoing tumour-angiogenesis and that VEGFR-2 and NP-1 are expressed by both endothelial cells and tumour cells (Fiedler et al., 1997; Hatva et al., 1995; Liu et al., 1995; Shibuya, 1995; Soker et al., 1998). Shibuya and Carmeliet with co-workers have independently demonstrated that VEGFR-1 plays a positive role in pathological angiogenesis, thus VEGFR-1 might have a dual function, acting as either a positive or negative regulator of angiogenesis (Carmeliet, 2001; Hiratsuka et al., 2001).

Also the expression of the lymphatic-specific VEGFR-3 is upregulated in the vascular endothelium of some solid tumours (Lymboussaki et al., 1998; Valtola et al., 1999). As I mentioned above, both ligands of VEGFR-3 are implicated in tumour-induced lymphangiogenesis, which is thought to promote the spread of metastasis.

\textsuperscript{16}The permeability is achieved by formation of fenestrations in the blood vessel, but also by the formation of caveolae-like vesicles that form channels across the endothelial cells (Esser et al., 1998).
**PDGFs and their receptors**

PDGF-B was shown to be the human homologue to the transforming gene of simian sarcoma virus (v-sis) and to transform cells *in vitro* as well as cause tumours *in vivo* (Dalla-Favera *et al.*, 1981; Waterfield *et al.*, 1983b; Wong-Staal *et al.*, 1981) and reviewed in Heldin & Westermark (1999). Many tumours express PDGFs and their receptors, although an upregulation of receptor activation has not been studied (Heldin & Westermark, 1999).

PDGFR-α-positive cells are found in all grades of human glioma, whereas its ligand PDGF-A shows a dramatic increase from low/undetectable expression in low-grade tumours to high expression in high-grade tumours, suggesting a role in tumour progression (Hermanson *et al.*, 1996). Increased expression-levels can also be a result of amplification of the gene, which is the case in some glioblastomas overexpressing PDGFR-α (Heldin & Westermark, 1999). In chronic myelomonocytic leukemia, PDGFR-β is translocated and fused to an ets-like gene, resulting in a constitutively active receptor (Heldin & Westermark, 1999). The PDGF-B gene is rearranged by chromosomal rearrangement in some sarcomas, which results in a chimeric protein with transforming activity in culture and PDGF-C has newly been shown to be a transforming factor in Ewing family tumours (Simon *et al.*, 1997; Zwerner & May, 2001). In addition to the autocrine stimulation of the tumour cells in the above examples, PDGF is also involved in paracrine stimulation of stroma cells. In many tumour types, the PDGFs are expressed by the tumour cells and the cognate receptors in the stroma cells (Heldin & Westermark, 1999).

Both atherosclerotic and fibrotic conditions show an over-activity of PDGF (Heldin & Westermark, 1999). In line with the phenotypes displayed by the PDGFR-α knockout embryos (with e.g. *spina bifida*), a recent report has indicated that promoter haplotype combinations of PDGFR-α affect human neural tube defects (Joosten *et al.*, 2001).
PRESENT STUDY

I will in this section discuss each paper separately, which includes a short summary of the results presented in each paper, followed by a discussion of the possible relevance and importance of each finding. In the end, I will try to assemble all results and put them into perspective.

MATERIALS AND METHODS
Apart from the standard molecular biology and biochemical techniques used in my thesis work, the more specific methods are listed below with a reference to the paper in which an extensive description can be found under “Materials and Methods”.

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RESULTS AND DISCUSSION

ANALYSIS OF VEGF-B

I have in my introduction emphasised the potency and importance of VEGF-A, both during normal and pathological conditions, so you can appreciate the sensation when two additional VEGFs were identified. VEGF-B and VEGF-C were both identified in 1996 and the findings made it exciting to elucidate their roles during embryonic development and adult life. In addition, it was interesting to know if there were any pathological conditions related to these molecules. VEGF-B was the molecule that had been cloned and partially characterised in the lab where I started as a PhD-student and my goal was to investigate it both during embryogenesis and in tumour development.

VEGF-B expression during mouse development (Paper I)

The expression pattern of a ligand and its cognate receptor(s) can help us make hypotheses about its possible role(s). We focused on the pattern of VEGF-B expression during development, both at the mRNA- and protein-level. As I stated in the introduction both VEGFR-1 and NP-1 bind VEGF-B. VEGFR-1 is mainly expressed in the endothelium of blood vessels, but also by e.g. macrophages. NP-1 can be found both in the endothelium of blood vessels and in the parenchyma of organs. Previous studies had shown that in adult, VEGF-B is expressed in e.g. heart, skeletal muscle, brain, and kidney (Olofsson et al., 1996a).

VEGF-B expression was restricted to the tissue parenchyma of e.g. heart, skeletal muscle, brown fat, adrenal gland, and pancreas as well as SMC of the larger arteries. No expression was detected in the endothelial cells of the blood vessels. The adjacent expression of VEGF-B and VEGFR-1 suggested that VEGF-B functions in a paracrine manner. However, NP-1 is expressed in endothelia as well as in e.g. cardiomyocytes, and reports have demonstrated the expression of VEGFR-1 on vSMC, suggesting that VEGF-B could act in an autocrine way as well (Kitsukawa et al., 1995; Couper et al., 1997; Grosskreutz et al., 1999).

The expression of VEGF-B in organs, such as the heart, muscles, brown fat, and perichondrium of developing bones, indicated that VEGF-B could play a role in angiogenesis. The heart, muscles, and brown fat are tissues that have a high metabolic rate and subsequently high capillary density. The perichondrium serves as a matrix for invading capillaries during bone formation. The source of VEGF-B at these sites
suggested that VEGF-B might be involved in the angiogenic process and/or function as a maintenance factor in these tissues. However, contradicting the interpretation that VEGF-B has a function in angiogenesis, at least in capillary sprouting, was the lack of VEGF-B in areas of active angiogenesis, such as the brain and the lungs.

However, remodelling of the capillary network occurs also via intussusceptive growth and increasing evidence indicates that intussusceptive growth is the dominant angiogenic mechanism during middle and late embryogenesis in most vascular systems (Djonov et al., 2000; Patan et al., 1992). The extended capillary surface area and volume in the lung that occurs during the first postnatal weeks is mainly accomplished by intussusceptive growth (Kauffman et al., 1974). But, data from mice deficient of VEGF-B indicate that VEGF-B is not likely to play a part in intussusceptive growth either (see next section, paper II). Recently Tie-1 and Tie-2 were proposed to be part of the regulation of intussusceptive growth (Patan, 1998).

VEGF-A and VEGF-B are co-expressed in many organs during development (e.g. heart, adrenal, kidney, bone and muscle) and in vitro studies have demonstrated that VEGF-A and VEGF-B can form heterodimers when co-expressed (Breier et al., 1995; Dumont et al., 1995; Olofsson et al., 1996b). This suggests that VEGF-A•VEGF-B heterodimers form during development, and analogously to PI GF•VEGF-A heterodimers, binding to VEGFR-2 might be induced (Cao et al., 1996). Yet, there is no experimental data to support this hypothesis to this date.

Experiments have clearly demonstrated the importance of both VEGF-A and its receptors during development (see section “NORMAL DEVELOPMENT”) and paper I suggested that also VEGF-B could be important for normal development. This brings us to the next goal, finding the in vivo function of VEGF-B!

**Function of VEGF-B in vivo – studies of a knock out mouse (Paper II)**

Having both the expression studies of VEGF-B, as well as the knockout studies of VEGF-A in mind, we set out to find the in vivo function of VEGF-B. The pups born from the first heterozygous crossing were analysed and, much to our surprise, we had an almost perfect Mendelian ratio between wild type (wt), heterozygous (VEGF-B+/−) and homozygous (VEGF-B−/−) pups. We devoted the following years to find a difference between the normal and knockout mice.
The heart is the major site for VEGF-B production, but thorough histological analyses did not display any anomalies in the VEGF-B deficient hearts. At last, we found that VEGF-B is needed for normal heart function in adult mice. Analysis by electrocardiography (ECG) revealed that VEGF-B is needed for a normal conduction between the atrium and the ventricle of the heart. More specifically, the VEGF-B−/− mice had a 10-15% prolonged P-Q interval (which measures the time of contraction from the atrium to the ventricles). A similar condition in humans is called first-degree atrioventricular (AV) block and is not enough to cause arrhythmia or any other known heart-associated symptom. There could be several causes for the increased P-Q interval, e.g. enhanced vagal tone, defects in the conduction system, electrolyte disturbances, or ischemia. We found no support for any of these possible causes, thus the mechanism(s) by which VEGF-B affects the conduction system of the heart is still unknown.

By RNase protection analysis (RPA), we found that two genes were upregulated in the VEGF-B deficient hearts, VEGF-C and PDGF-B. It is known that PDGF-B is upregulated in immature endothelium during embryonic development, and that reciprocal interactions between endothelial cells and periendothelial structures are essential for normal blood vessel development and maintenance (Folkman & D'Amore, 1996; Hellström et al., 1999). These data and our observations suggested that VEGF-B-dependent regulation of PDGF-B expression may be one component of such reciprocal signalling network. Or, this might indicate that VEGF-B has a similar function as PDGF-B. VEGF-B and VEGFR-1 are both expressed in the vSMC and could be involved in proliferation and/or migration of vSMC (Aase et al., 1999; Couper et al., 1997; Grosskreutz et al., 1999). Compare these data with the VEGF-A120/120 mice that displayed reduced number of vSMC, which might be caused by the lack of the other VEGF-A signalling isoforms (“NORMAL DEVELOPMENT” and Carmeliet et al., 1999b).

Experiments have shown that adult cardiac myocytes induce a microvascular expression of PDGF-B, leading to PDGF-AB heterodimer formation which has been shown to induce expression of VEGF-A and VEGFR-2 (Edelberg et al., 1998). The upregulation of VEGF-C might be caused by this PDGF-AB heterodimer signalling in order to develop and maintain the vasculature.

To summarise the findings, VEGF-B deficient mice were indistinguishable from their normal littermates with respect to growth, fertility and life span, but showed a minor defect in the conduction system of the heart.
Do the two VEGF-B isoforms have differential roles? (Paper III)

Analysis of VEGF-B expression using RT-PCR, in situ hybridisation or immunohistochemistry did not pay any attention to the possible difference in expression of the two isoforms VEGF-B_{167} and VEGF-B_{186}. RNase protection analysis (RPA) gave us a tool to investigate if there were any differences in isoform expression during embryonic development, in adult tissues, and in tumours.

To our surprise, the longer isoform was hardly present during development or adult life, whereas the shorter 167 amino acid long isoform, accounted for more than 80% of the total VEGF-B transcripts (Table 3). Moreover, the 186-isoform was absent in some tissues that expressed the 167-isoform, such as thymus and adrenal gland. These results suggested that during physiological angiogenesis and development VEGF-B signals in a “pericrine” way, i.e. only short distance signalling due to the cell surface adherence of VEGF-B_{167}. In primary tumours and tumour cell lines, however, the freely diffusible 186-isoform was upregulated (Table 3), which could mean that during pathological conditions VEGF-B signals in a more paracrine manner. Although we haven’t found any evidence for VEGF-B expression in endothelial cells during development, others have found expression of VEGF-B_{186} in human adult microvascular endothelial cells (Yonekura et al., 1999). This further supports our hypothesis that VEGF-B_{186} can act in a more paracrine way, whereas VEGF-B_{167} might function in the immediate surrounding of the producing cell.

Pericellular proteolysis during cell migration and tissue remodelling occurs in normal, as well as in pathological conditions, including angiogenesis in tumour progression (Carmeliet & Collen, 1997). The upregulation of VEGF-B_{186} in tumours and the report that VEGF-B_{186} has to be proteolytically processed in the C-terminus before it can bind NP-1, suggests that active pericellular proteases, during for example tumourigenesis, process diffusible VEGF-B_{186} and thereby induce binding to NP-1 (Mäkinen et al., 1999).

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17 The RT-PCR we performed of the two isoforms during mouse development was not quantitative, and although both isoforms look equally expressed a more sophisticated method was needed to confirm the relative expression.
Table 3

<table>
<thead>
<tr>
<th>Tissues</th>
<th>VEGF-B&lt;sub&gt;167&lt;/sub&gt;</th>
<th>VEGF-B&lt;sub&gt;186&lt;/sub&gt;</th>
<th>% 186</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>27</td>
<td>7.3</td>
<td>21</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>27</td>
<td>3.2</td>
<td>11</td>
</tr>
<tr>
<td>Diaphragm</td>
<td>16</td>
<td>3.0</td>
<td>16</td>
</tr>
<tr>
<td>Colon</td>
<td>7</td>
<td>1.7</td>
<td>20</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Eye</td>
<td>6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Kidney</td>
<td>4</td>
<td>1</td>
<td>20</td>
</tr>
<tr>
<td>Brain</td>
<td>4</td>
<td>0.6</td>
<td>13</td>
</tr>
<tr>
<td>Adrenal gland</td>
<td>4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lung</td>
<td>3</td>
<td>0.45</td>
<td>13</td>
</tr>
<tr>
<td>Cerebrum</td>
<td>3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Liver</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Testis</td>
<td>0.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Small intestine</td>
<td>0.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Thymus</td>
<td>0.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Peripheral blood cells</td>
<td>0.2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>T241</td>
<td>12</td>
<td>7.4</td>
<td>40</td>
</tr>
<tr>
<td>B16</td>
<td>9</td>
<td>4.5</td>
<td>33</td>
</tr>
<tr>
<td>BT4C</td>
<td>8</td>
<td>6.3</td>
<td>44</td>
</tr>
<tr>
<td>LLC</td>
<td>6</td>
<td>3.2</td>
<td>35</td>
</tr>
</tbody>
</table>

Expression levels of VEGF-B isoforms in mouse normal adult tissues and transformed cell lines. The values in columns 2 and 3 are arbitrary units of VEGF-B expression after normalisation against the internal control. The 4<sup>th</sup> column represents percentage VEGF-B<sub>186</sub> of total VEGF-B transcript.

In a parallel manner, the different isoforms generated from the VEGF-A gene<sup>18</sup> have been shown to be genetically regulated and display different biological functions. In all tissues but the lung, the 164-isoform was the most abundant transcript in adult mouse as well as during embryogenesis (Ng et al., 2001). VEGF-A<sub>188</sub> displayed the highest relative level in the lung, but showed abundant expression also in the heart and liver. The heart, lung and liver are organs that are initially vascularised by vasculogenesis, as opposed to the organs that displayed high levels of VEGF-A<sub>164</sub>, which are vascularised primarily by

<sup>18</sup>The differential splicing of VEGF-A generates in humans: 121, 145, 165, 183, 186, 206 and in mouse: 115, 120, 164 and 188 amino acids long isoforms.
angiogenesis. The authors speculate that due to its cell association, VEGF-A\textsubscript{188} functions as a maintenance and/or specialisation factor. Support for this notion comes from the observation that VEGF-A\textsubscript{188} fails to recruit systemic blood vessels to the tumour vasculature (Grunstein \textit{et al}., 2000). In addition, blood vessels within tumours that overexpress VEGF-A\textsubscript{188} are stable and non-leaky in contrast to the unstable and leaky vessels within VEGF-A\textsubscript{120} or\textsubscript{164} overexpressing tumours (Cheng \textit{et al}., 1997).

**Is there a role for VEGF-B in development or adulthood?**

I have now concluded the VEGF-B-part of this thesis, but before I continue I would like to discuss what we have learned from these studies. Although promising from the start, VEGF-B has developed into an elusive molecule whose function I have doubted several times. What we have found is that VEGF-B is closely related to VEGF-A, with a homology of about 43% (Olofsson \textit{et al}., 1996a). VEGF-B binds two receptors, VEGFR-1 and NP-1 (Mäkinen \textit{et al}., 1999; Olofsson \textit{et al}., 1998). VEGFR-1 is also an enigmatic protein with respect to its function. It has been shown that the extracellular part is essential for normal development, whereas the intracellular kinase-containing part is indispensable during embryogenesis, but important for VEGF-A-induced monocyte migration (Fong \textit{et al}., 1995; Hiratsuka \textit{et al}., 1998). This lead to the “sink hypothesis”, but I don’t think the intrinsic signalling of VEGFR-1 should be dismissed, since a number of signal-transduction molecules have been found to bind specific phosphorylation sites (Ito \textit{et al}., 1998; Sawano \textit{et al}., 1997). Moreover, Carmeliet and co-workers, and Shibuya and co-workers have indicated different functions for VEGFR-1 during embryonic angiogenesis and pathological angiogenesis (Carmeliet, 2001; Hiratsuka \textit{et al}., 2001).

NP-1 is not a signalling molecule in itself, but has been shown to be important for correct development of the cardiovascular system (Kawasaki \textit{et al}., 1999). Inactivation of the gene encoding VEGF-B did not bring us closer to understand its (possible) function during embryogenesis, but if the function of VEGF-B is analogous to PIGF, we have to focus on the pathological consequences of loss of VEGF-B.

Despite the fact that the VEGF-B deficient mice are normal, I don’t think we should exclude a physiological function of VEGF-B. The tools we use for determining roles and functions for specific factors are still quite crude and I think that the “VEGF-B enigma” demands a more sophisticated investigation with specific questions. A wise man once said to me: “All proteins have a function, but sometimes we are too stupid to understand it”.

40
**ANALYSIS OF PDGF-C**

A partial amino acid sequence with homology to VEGF-A was found in the expressed-sequence tag (EST) databases at the National Center for Biotechnology Information (NCBI). Due to its homology to VEGF-A, we originally named the new protein VEGF-F, but as we continued our work with receptor specificity, we soon found out that we had found a new member of the PDGF-family – PDGF-C

**Cloning of PDGF-C, receptor specificity and overexpression studies (Paper IV).**

Both human and mouse PDGF-C amino acid sequences were isolated and displayed a new domain structure not previously found in VEGF/PDGF family members (Fig. 3). Following the signal sequence there was a CUB domain, followed by a hinge region and, most C-terminal, the VEGF/PDGF homology domain.

The spacing of the cysteins, involved in the disulphide bridges within the VEGF/PDGF homology domain, is different from the other family members; three extra amino acids have been inserted between cystein four and five. Moreover, four extra cysteins located between cysteins 3 and 4, 5 and 6 and 6 and 7 and beyond cystein 8 are found.

In this paper we showed that a truncated form of PDGF-C, which contains only the VEGF/PDGF homology domain, binds and activates PDGFR-α, whereas the full-length protein was unable to do so. Proteolytic processing of the full length PDGF-C, removing the N-terminus, induces binding and activation of the receptor. The processed N-terminus contains the CUB-domain and it is possible that this domain hinder PDGF-C binding by sterically blocking the binding site of the receptor.

Overexpressing PDGF-C in the mouse heart, using the α-myosin heavy chain (α-MHC) promoter, resulted in progressive cardiac hypertrophy with subsequent death in adulthood. The hypertrophy was a result from excess proliferation of interstitial cells,
such as the cardiac fibroblast. The hypertrophy was not apparent during embryonic development, but became increasingly severe the older the mice got.

**Expression of PDGF-C during mouse development (Paper V)**

We did a brief study of the distribution of PDGF-C mRNA in the developing embryo in paper IV, and in this paper we continued with a detailed analysis both at the transcriptional and translational level.

We found that PDGF-C shows a partially overlapping expression pattern with both its competing ligand, PDGF-A, but also with its receptor, PDGFR-α. This suggested that PDGF-C act in both paracrine and autocrine ways. The most striking difference between PDGF-A and PDGFR-α knockout mice is the skeletal abnormalities observed only in the PDGFR-α deficient mice (Soriano, 1997). We found that PDGF-C is expressed in sclerotome, bones, and cartilage, which indicates that lack of PDGF-C signalling at these sites might be the cause of the observed difference in phenotype between PDGF-A and PDGFR-α.

Our analysis revealed that PDGF-C is expressed in a very dynamic and “guiding-like” pattern. A “guiding” pattern was seen in epithelia that will end up in epidermal openings, such as the mouth, ears and nose. The PDGF-C expression in the developing cardiovascular system showed a dynamic pattern with positive staining in SMC of the dorsal aorta and vena cava as they bend, fuse or enter the heart. This suggested that PDGF-C could be linked to the morphogenic process which forms these structures. Two molecules involved in the remodelling of the developing embryo are matrix metalloproteinase 2 (MMP-2) and its activator membrane-type MMP (MT-MMP) (Holmbeck et al., 1999; Reponen et al., 1992). Recent studies have shown that PDGF-A can regulate MMP-2 and MT-MMP expression, and we have results indicating that PDGF-C upregulates MMP-2 (Robbins et al., 1999). Furthermore, MMP-2 and MT-MMP are co-expressed with PDGFR-α in most mesenchymal tissues (Ataliotis & Mercola, 1997; Kinoh et al., 1996). Taken together this suggests that PDGF-C is involved in the remodelling pathway of specific structures in concert with MMP-2. Moreover, *in vitro* data have also shown that PDGF-C can be activated by MMP-2, which indicates that PDGF-C is involved in its own activation in a positive feedback loop.
CONCLUDING REMARKS

I have during this thesis study tried to understand the role of two growth factors of the VEGF/PDGF family of growth factors, VEGF-B and PDGF-C. The results from experiments which try to explain the function of VEGF-B have indicated that VEGF-B is indispensable for normal development, but might have a function in a pathological setting.

The initial results from the studies with PDGF-C are much more promising, although we don’t know the function of the protein during embryogenesis yet. Overexpressing PDGF-C in the heart led to hypertrophy due to excessive proliferation of fibroblasts, however this condition was not lethal until the mice were middle-aged. Our expression studies during mouse development indicated that PDGF-C might be the missing link in the PDGFR-\(\alpha\) signalling pathway.
ACKNOWLEDGEMENTS

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Reine and Ilma for reminding me of what is important in life!

Last of all I would like to thank myself for never giving up.

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19 Gotcha! If you don’t understand what I mean it shows that you haven’t read my thesis, go to page 40 and you’ll find out what my wise man said.
REFERENCES


PDGF-A signaling is a critical event in lung alveolar myofibroblast development and alveogenesis. *Cell*, 85, 863-73.


