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A PDGFR-ALPHA PERSPECTIVE ON PDGF SIGNALLING IN DEVELOPMENTAL AND PATHOLOGICAL PROCESSES

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A PDGFR-ALPHA PERSPECTIVE ON PDGF SIGNALLING IN DEVELOPMENTAL AND PATHOLOGICAL PROCESSES

THESIS FOR DOCTORAL DEGREE (PH.D.)

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"Le hasard ne favorise que les esprits préparés"

Louis PASTEUR

ABSTRACT

Platelet-derived growth factors PDGFs are a family of ligands and receptors that regulate multiple processes of paramount importance during embryonic development. They are also involved in several pathological events, spanning from tumours to fibrotic diseases. PDGFs have been studied for more than 30 years, but the mechanisms, as well as the tissue and cell type specific physiological roles of PDGF biology are still not fully understood.

The work presented in this thesis investigates the role of PDGFR-alpha in cell differentiation during cardiac tissue specification, and the effects of PDGF overexpression in the myocardium during development and in the context of cardiac injury. We also examine the elusive function of the PDGF-A retention motif in PDGFR-alpha signalling during morphogenesis. The adoption of a reporter construct knocked into the PDGFR-alpha locus allowed us to precisely follow its site of expression.

Here we report evidence of a role for PDGFR-alpha signalling in the recruitment and differentiation of second heart field-derived cells during cardiac inflow tract development (paper I).

We also studied of the physiological role of the PDGF-A retention motif and the effects of its ablation. This work points to a preponderant role for the diffusible isoform in PDGFR-alpha signalling (paper II).

The data presented in paper III investigate the effects of PDGF-A and PDGF-B overexpression in the developing mouse myocardium, and complements previously published reports on PDGF-C and PDGF-D overexpression in the same system. Our findings extend the evaluation of the role of PDGFs in myocardial fibrosis induction, and suggest PDGFR-alpha positive cells as the source of excessive extracellular matrix deposition in the heart in response to ectopic PDGF expression.

Ectopic expression of the different PDGF ligands in injured and inflamed cardiac tissue reveals an unexpected role for PDGFs in tissue recovery, that appears to be mediated by PDGFR-alpha signalling cells (paper IV).

Many questions remain open-ended regarding PDGF signalling. The work presented in this thesis points at new and interesting directions for future studies.

LIST OF SCIENTIFIC PAPERS

I. Cardiac malformations in PDGFR-alpha mutant embryos are associated with increased expression of WT1 and Nkx 2.5 in the second heart field.
Noortje A. M. Bax, Steven B. Bleyl, **Radiosa Gallini**, Lambertus J. Wisse, Jennifer Hunter, Angelique A. M. Van Oorschot, Edris A. F. Mahtab, Heleen Lie-Venema, Marie-Jose Goumans, Christer Betsholtz, Adriana C. Gittenberger-de Groot (2010). *Developmental dynamics : an official publication of the American Association of Anatomists* 239**:** 2307-2317

II. Analysis of mice lacking the heparin-binding splice isoform of platelet-derived growth factor A.
Johanna Andrae, Hans Ehrencrona, **Radiosa Gallini**, Mark Lal, Hao Ding, Christer Betsholtz (2013). *Molecular and cellular biology* 33**:** 4030-4040

III. Isoform specific modulation of inflammation induced by adenoviral mediated delivery of PDGF in the adult heart.
Radiosa Gallini, Jenni Huusko, Seppo Ylä-Herttuala, Christer Betsholtz, Johanna Andrae (2014). *Manuscript*

IV. PDGFR-alpha positive cells in PDGF-A and PDGF-B induced cardiac fibrosis in mouse.
Radiosa Gallini, Per Lindblom, Cecilia Bondjers, Christer Betsholtz, Johanna Andrae (2014). *Manuscript*

CONTENTS

LIST OF ABBREVIATIONS

Alpha-SMA	Alpha-smooth muscle actin
cDNA	Complementary deoxyribonucleic acid
CUB	Complement C1r/C1s, Uegf, Bmp1
E	Embryonic day
E-cadherin	Epithelial-cadherin
GFP	Green fluorescent protein
mRNA	Messenger ribonucleic acid
Oligo(dT)	Single-stranded sequence of deoxythymine
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
RALDH2	Retinaldehyde dehydrogenase 2
RT-PCR	Real time-polymerase chain reaction
SH2	Src homology 2
TGF-beta1	Transforming growth factor beta1
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
WT1	Wilm's tumour suppressor gene1
3D	Three dimensional

1 INTRODUCTION

HEART DEVELOPMENT AND NKX 2.5 EXPRESSION

The murine heart development is dependent on many different proteins, the most important of which is perhaps Nkx 2.5. Nkx 2.5 is the vertebrate homologue of Drosophila *tinman* homeobox transcription factor, the first driver of cardiac specification (Bodmer 1993; Lints, Parsons et al. 1993; Chen and Schwartz 1995; Stanley, Biben et al. 2002).

Nkx 2.5 positive cardiac progenitor cells get localized in the lateral anterior mesoderm during late gastrulation (E7) (Garcia-Martinez and Schoenwolf 1993; reviewed by Schoenwolf and Garcia-Martinez 1995; Tam, Parameswaran et al. 1997). At E7.5 these groups of cells converge at the midline and form the cardiac crescent (Redkar, Montgomery et al. 2001) (Figure 1). The midline has been perceived as a source of induction of gradient (Davis, Edwards et al. 2001; Kelly, Brown et al. 2001), with Notch signalling as key regulator of fate restriction (Raffin, Leong et al. 2000; Rones, McLaughlin et al. 2000).

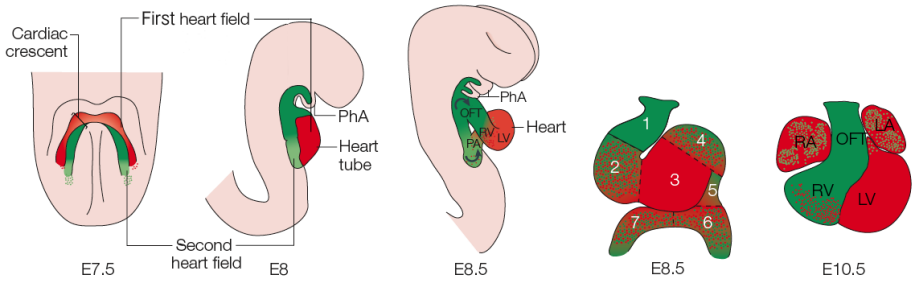

Figure 1: Two sources of myocardial cells in the developing heart. Myocardial cells in the mouse embryo. The location and contribution of the second heart field is shown in green, compared with the myocardial cells that are derived from the first heart field (shown in red). The lineage contributions to the heart at E8.5 are summarized with the same colour coding. LA, left atria; LV, left ventricle; OFT, outflow tract; PA, primitive atria; PhA, pharyngeal arches; RA, right atrium; RV, right ventricle; 1, outflow region; 2, primitive right ventricle; 3, primitive left ventricle; 4/5, atrioventricular region; 6/7, primitive left and right atria (modified, with permission, from Stainier 2001).

At the cardiac crescent stage, between E7 and E8, Notch signalling resolves the fate of the progenitors, and we can witness the heart morphogenetic fields formation (Rones, McLaughlin et al. 2000). The first heart field progenitors are located ventrally, and they keep on expressing Nkx 2.5; they represent the main myogenic source for the left ventricle. The second heart field is located dorsally, and is characterized by Isl1 expression. These cells will be a second source of myocardium for the outflow tract and inflow tract, for the atria, and for the right ventricle

(Webb, Brown et al. 1998; Kelly, Brown et al. 2001; Mjaatvedt, Nakaoka et al. 2001; Waldo, Kumiski et al. 2001; Zaffran, Kelly et al. 2004; Moretti, Caron et al. 2006) (figure 1). Beside myocardium, second heart field progenitors can give rise to endothelial and smooth muscle cells (Moretti, Caron et al. 2006). The presence of common ontological mechanisms giving rise to inflow and outflow tract at the opposite poles of the heart primordia seems to be confirmed by many conotruncal defects common to the two regions in pathological situations (Bajolle, Zaffran et al. 2009).

At around E8 the primordial heart tube closes, and the heart mesoderm splits into inner endocardial tube (endothelial cells) and surrounding myocardial cells. The process is regulated by Notch signalling, fibronectin gradient interaction, retinoic acid concentration, and endoderm differentiation itself (Linask and Lash 1993; Eisenberg and Bader 1995; Narita, Bielinska et al. 1997; reviewed by Stainier 2001; reviewed by Harvey 2002).

At E8.5 the left ventricular myocardium expands, the heart loops (reviewed by Eisenberg and Markwald 1995; Christoffels, Habets et al. 2000; Christoffels, Hoogaars et al. 2004) and the endocardium forms the cardiac cushion by epithelial to mesenchymal transition (Markwald, Fitzharris et al. 1977). By E10.5 the heart has acquired its four chambers structure, and by E14.5 it has septa and is divided in four separate chambers (Christoffels, Habets et al. 2000; Moorman, Schumacher et al. 2000; reviewed by Harvey 2002). The curvature of the heart tube seems to be an inducer of transcriptional regulation (Manner, Seidl et al. 1993; reviewed by Harvey 2002), and, once again, Nkx 2.5 seems to be a key player in the process (Lyons, Parsons et al. 1995; Biben and Harvey 1997; Tanaka, Chen et al. 1999; Palmer, Groves et al. 2001), together with matrix components (Camenisch, Spicer et al. 2000). The complex valves and septa patterning seems to follow ontological sequential differentiation processes that recapitulate the phylogenetic development of the cardiac structures (Cai, Liang et al. 2003; Meilhac, Esner et al. 2004; Zaffran, Kelly et al. 2004).

Around E8.5 also the proepicardial serosa develops from extracardiac tissue characterized by coelomatic markers like WT1 and RALDH2 (Moore, McInnes et al. 1999; Carmona, Gonzalez-Iriarte et al. 2001). This tissue is the source of epicardial cells and all the cellular elements of the cardiac connective tissue (subepicardial and intermyocardial cells) (Manner 1993). The progenitors of all these cell types (smooth muscle cells and fibroblasts) share the same coelomatic markers (Mikawa and Gourdie 1996; Vrancken Peeters, Gittenberger-de Groot et al. 1999; reviewed by Manner, Perez-Pomares et al. 2001; Cai, Liang et al. 2003; Zhou, Ma et al. 2008). A contribution to the coronary endothelium is controversial (Mikawa and Gourdie 1996; Guadix, Carmona et al. 2006; reviewed by Riley and Smart 2011). The epicardium provides key signalling required for correct myocardial development (Chen, Chang et al. 2002; Perez-Pomares, Phelps et al. 2002; Stuckmann, Evans et al. 2003; Kang, Gu et al. 2008) (figure 2).

The fundamental importance of Nkx 2.5 expression during heart development has been highlighted by loss of function studies, which demonstrate a nuance of defects depending on mutation site and time of induction. The defects include septation abnormalities between atria (Schott, Benson et al. 1998), looping failure (reviewed by Harvey 1996), and conduction system dysfunction (Schott, Benson et al. 1998; Pashmforoush, Lu et al. 2004). Moreover, Nkx 2.5 needs

to be downregulated for the correct development of the venous pole (Christoffels, Mommersteeg et al. 2006; Gittenberger-de Groot, Mahtab et al. 2007; Douglas, Mahtab et al. 2009; Mahtab, Vicente-Steijn et al. 2009).

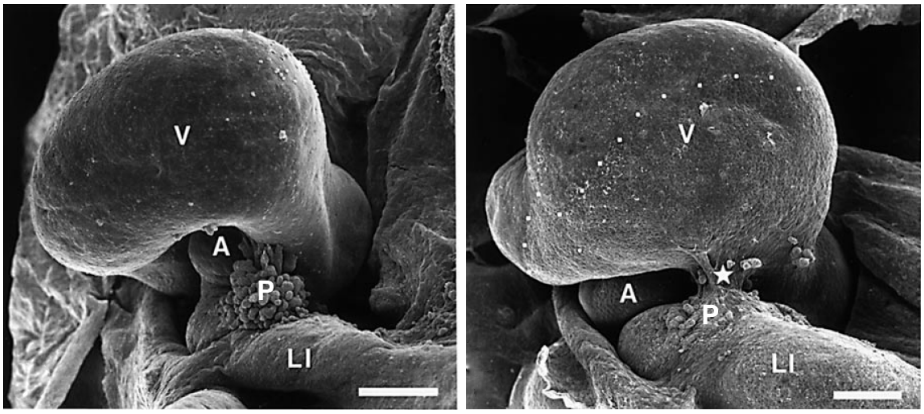

Figure 2: Proepicardial organ. Scanning electron microscope micrographs showing the heart loop and the right horn of the sinus venosus from caudal direction. From the proepicardial organ, the primitive epicardium spreads as a continuous epithelial sheet over the naked myocardial surface (asterisk). A = Primitive atrium; V = ventricular loop; P = proepicardial organ; LI = liver. Scale bars = 200 μm (modified, with permission, from Manner, Perez-Pomares et al. 2001).

There has been growing evidence pointing to a role for PDGF signalling in heart development. The initial studies of PDGF expression (Van Den Akker, Lie-Venema et al. 2005) and PDGF loss of function (Leveen, Pekny et al. 1994; Soriano 1997; Hellstrom, Kalen et al. 1999; Tallquist and Soriano 2003; Bjarnegard, Enge et al. 2004) related the heart defects observed to impaired contribution of neural crest cells for PDGFR-alpha signalling (Soriano 1997; Tallquist and Soriano 2003), and to coronary arteries maturation for PDGFR-beta signalling (Leveen, Pekny et al. 1994; Hellstrom, Kalen et al. 1999; Bjarnegard, Enge et al. 2004). The molecular mechanisms remained unclear until the cardiac defects seen in PDGF-B/PDGFR-beta loss of function were traced back to to impairment in the contribution of epicardial-derived cells (Van den Akker, Winkel et al. 2008). The key role for PDGFR-alpha signalling in epicardium development and epicardial-derived cell maturation remained undetected until our contribution to the field (Bax, Lie-Venema et al. 2009; Bax, Bleyl et al. 2010).

PDGF FAMILY SIGNALLING

The first Platelet-derived growth factor ligands were identified through their proliferative effect on fibroblasts (Balk 1971). The main source of these growth factors has been identified as platelets (from which they derive their name), and their mitotic effect on fibroblasts (Kohler and Lipton 1974), smooth muscle cells (Ross, Glomset et al. 1974) and glial cells (Westermark and Wasteson 1976) emerged very quickly. After the first purification of PDGFs (Heldin, Westermark et al. 1979; Antoniades 1981), two peptides were identified: PDGF-A and PDGF-B (Johnsson, Heldin et al. 1982; Betsholtz, Johnsson et al. 1986). It was soon discovered that PDGF-A existed in two different splice variants (Bonthron, Morton et al. 1988; Rorsman, Bywater et al. 1988), with different relative abundance in different cell types (Matoskova, Rorsman et al. 1989). Other two ligands have been discovered more recently, in a quest for VEGF growth factor homologues: PDGF-C (Kazlauskas 2000; Li, Ponten et al. 2000) and PDGF-D (Bergsten, Uutela et al. 2001; LaRochelle, Jeffers et al. 2001).

The first PDGF receptor identified was PDGFR-alpha (Heldin, Westermark et al. 1981). PDGFR-beta was identified by sequence similarity (Matsui, Heidaran et al. 1989). The structure analysis revealed their Tyrosine Kinase Receptor nature and the ligands interaction mechanisms (Claesson-Welsh, Eriksson et al. 1989).

The ligands are secreted as dimers, linked by disulfide bonds, in 5 possible combinations: PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC and PDGF-DD (reviewed by Betsholtz, Karlsson et al. 2001; reviewed by Andrae, Gallini et al. 2008). Their expression pattern is regulated differently in the various tissues and cell types, and the expression of PDGF-A and PDGF-B does not overlap (reviewed by Hoch and Soriano 2003). PDGF-A and PDGF-B are released as active dimers, while PDGF-C and PDGF-D require proteolytical processing of their CUB domains in order to bind the receptors (Li, Ponten et al. 2000; Bergsten, Uutela et al. 2001).

Upon ligand binding, the receptors dimerize and are auto-phosphorylated at their intracellular domain (Kelly, Haldeman et al. 1991). The intracellular phosphorylation creates docking sites for signal transduction mediator proteins, most of which bind the phospho-tyrosines through their SH2 domains. The downstream signalling can influence cytoskeleton organization and gene transcription (reviewed by Heldin, Wasteson et al. 1985; Kazlauskas and Cooper 1989; reviewed by Claesson-Welsh 1994; reviewed by Kazlauskas 1994; Fambrough, McClure et al. 1999; reviewed by Heldin and Westermark 1999; reviewed by Betsholtz, Karlsson et al. 2001). The ligands bind the different receptors with different affinity, and *in vitro* studies have demonstrated that PDGFR-alpha can bind to PDGF-A, PDGF-C and PDGF-B with high affinity, while PDGFR-beta can bind with high affinity to PDGF-B and PDGF-D (Claesson-Welsh, Eriksson et al. 1989; Matsui, Heidaran et al. 1989; reviewed by Heldin and Westermark 1999; Kazlauskas 2000; Li, Ponten et al. 2000; Bergsten, Uutela et al. 2001; LaRochelle, Jeffers et al. 2001). *In vivo*, only the interactions between PDGF-A, PDGF-C and PDGFR-alpha, and PDGF-B and PDGFR-beta have been confirmed, by loss of function studies (Morrison-Graham, Schatteman et al. 1992; Leveen, Pekny et al. 1994; Soriano 1994; Bostrom, Willetts et al. 1996; Lindahl,

Johansson et al. 1997; Lindahl, Karlsson et al. 1997; Soriano 1997; Hellstrom, Kalen et al. 1999; Karlsson, Lindahl et al. 2000; Price, Thielen et al. 2001; Bostrom, Gritli-Linde et al. 2002; Hamilton, Klinghoffer et al. 2003; Ding, Wu et al. 2004; reviewed by Andrae, Gallini et al. 2008) (figure 3).

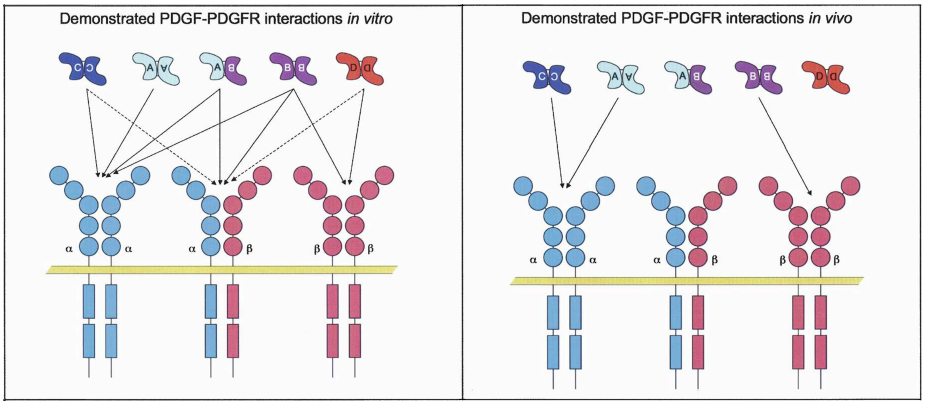

Figure 3: PDGF–PDGFR interactions. The first panel shows the interactions that have been demonstrated in cell culture. Hatched arrows indicate weak interactions or conflicting results. The second panel shows interactions proven to be of importance in vivo during mammalian development (modified, with permission, from Andrae, Gallini et al. 2008).

The expression patterns and loss of function phenotypes of growth factors and receptors have been extensively studied *in vivo*. PDGFR-alpha is expressed by mesenchymal cells in general, by mesenchymal progenitors in lung, intestine, skin, heart, and by oligodendrocyte precursors (Morrison-Graham, Schatteman et al. 1992; Orr-Urtreger, Bedford et al. 1992; Orr-Urtreger and Lonai 1992; Palmieri, Payne et al. 1992; Schatteman, Morrison-Graham et al. 1992; Soriano 1997; Price, Thielen et al. 2001; Bax, Lie-Venema et al. 2009; Smith, Baek et al. 2011). The PDGFR-alpha loss of function phenotype displays cleft face, sub-epidermal blistering, apoptosis of neural crest-derived mesenchyme, spina bifida, skeletal, vascular and cardiac defects (Morrison-Graham, Schatteman et al. 1992; Soriano 1997; Price, Thielen et al. 2001; Bax, Bleyl et al. 2010; Smith, Baek et al. 2011). PDGF-A and PDGF-C are expressed very early during embryonic development (blastocyst), and, in general, remain expressed by epithelial cells, muscle cells and neuronal progenitors (Mercola, Wang et al. 1990; Yeh, Ruit et al. 1991; Orr-Urtreger and Lonai 1992; Palmieri, Payne et al. 1992; Ding, Wu et al. 2000; Durand and Raff 2000; Tallquist, Weismann et al. 2000). Loss of function studies evidenced specific roles for these two ligands: PDGF-A deletion reduced paraxial mesoderm development and dermal mesenchyme in skin, originates villus dys-morphogenesis in gut and emphysema pathologies in lungs, defects in testis, vertebrae, ribs, oligodendrocyte precursors population expansion and nerves myelination (Bostrom, Willetts et al. 1996; Lindahl, Karlsson et al. 1997; Calver, Hall et al. 1998; Fruttiger, Karlsson et al. 1999; Karlsson, Bondjers et al. 1999; Gnessi, Basciani et al. 2000; Karlsson, Lindahl et al. 2000; Bostrom, Gritli-Linde et al. 2002); PDGF-C deletion originates cleft palate and spina bifida (Ding, Wu et al. 2004), and cerebellar defects (Andrae et al., unpublished).

PDGFR-beta expression is observed in mesenchymal cells in tight contact with endothelial cells (vascular smooth muscle cells and pericytes) (Hellstrom, Kalen et al. 1999). Loss of function of this receptor results in cardiovascular complications like oedema, haemorrhages, heart and blood vessels dilation, abnormal kidney glomeruli, and hypoplasia of smooth muscle cells (Soriano 1994; Hellstrom, Kalen et al. 1999). PDGF-B is expressed by vascular endothelial cells and megakaryocytes, especially in sprouting endothelia, in capillaries and in growing arteries (Lindahl, Johansson et al. 1997; Hellstrom, Kalen et al. 1999). Deletion of this growth factor produces the same phenotype observed in PDGFR-beta loss of function (Leveen, Pekny et al. 1994; Lindahl, Johansson et al. 1997; Hellstrom, Kalen et al. 1999). PDGF-D expression can be detected in fibroblasts and smooth muscle cells (Bergsten, Uutela et al. 2001; LaRochelle, Jeffers et al. 2001), but loss of function experiments for this ligand didn't show any major phenotype (Eriksson, personal communication).

The extracellular diffusion of the PDGF growth factors is strictly regulated: by proteolysis of the retention motif in PDGF-B (Ostman, Andersson et al. 1991), by alternative splicing for PDGF-A (Ostman, Andersson et al. 1991; Pollock and Richardson 1992; Andersson, Ostman et al. 1994; Fager, Camejo et al. 1995; Lustig, Hoebeke et al. 1996; Feyzi, Lustig et al. 1997), and probably by CUB domain processing for PDGF-C and PDGF-D (Li, Ponten et al. 2000; Bergsten, Uutela et al. 2001; reviewed by Betsholtz, Karlsson et al. 2001; reviewed by Andrae, Gallini et al. 2008). The possible function of these highly conserved extracellular-binding domains is the generation of gradients, a very important feature for growth factors signalling (Raines and Ross 1992; Kelly, Sanchez et al. 1993; reviewed by Betsholtz, Karlsson et al. 2001; reviewed by Andrae, Gallini et al. 2008; reviewed by Rogers and Schier 2011). The functional importance of the retention motif is well documented for PDGF-B (Abramsson, Lindblom et al. 2003; Lindblom, Gerhardt et al. 2003; Abramsson, Kurup et al. 2007), but evidence for a specific role of the two splice variants of PDGF-A had been reported only for amphibians (Damm and Winklbauer 2011), until our study was published (Andrae, Ehrencrona et al. 2013).

PDGFs IN WOUND HEALING AND FIBROSIS

When a tissue gets injured, the epithelial and endothelial cells release inflammatory mediators and trigger a coagulation cascade. The thrombocytes circulating in the blood stream are activated by the interaction with exposed extracellular matrix components, like collagen and von Willebrand factor, present in the sub-endothelial layer. Prothrombin is then cleaved by proteolysis and generates thrombin, an enzyme that converts fibrinogen into its insoluble form of fibrin strands. Fibrin assists the thrombocytes in clumping, and together they form a fibrin clot that helps restoring haemostasis. The activated thrombocytes degranulate and release growth factors (like PDGF and TGF-beta1), cytokines and chemokines, which induce recruitment and proliferation of leukocytes and endothelial cells. The endothelial cells secrete matrix metalloproteinases that disrupt the basement membrane and increase the permeability of the tissue, facilitating the inflammatory cells migration to the injury site. The leukocytes recruited secrete growth factors

and cytokines that promote the amplification of the inflammatory reaction. Release of PDGFs and TGF-beta1 growth factors stimulates fibroblast proliferation and matrix deposition. TGF-beta1 promotes also mesenchymal transition of epithelial and endothelial cells, which increases the fibroblast population. In the inflammatory context, fibroblasts become activated myofibroblasts, which express alpha-SMA and release cytokines in the wound, contributing to inflammation enhancement. The myofibroblasts migrate into the wounded tissue along the fibrin matrix, and assist wound contraction, pulling the edges of the injury towards the centre. In a normal situation the wound heals, with the epithelial and endothelial cells that proliferate and migrate over the basal layers to regenerate the damaged tissue, forming new blood vessel, and remodelling the extracellular matrix. If the inflammation persists and remains chronic, the accumulation of extracellular matrix components and the release of metalloproteinases inhibitors exceed the production of matrix metalloproteinases, and we can observe permanent fibrotic scarring and consequent organ malfunction. This unspecific and highly conserved healing process is common to all the different tissues, and it has been extensively recapitulated and reviewed in the last 30 years, thus I will refer the readers only to some of the most recent reviews on the topic (reviewed by Reinke and Sorg 2012; reviewed by Wynn and Ramalingam 2012; reviewed by Honda, Park et al. 2013) (figure 4).

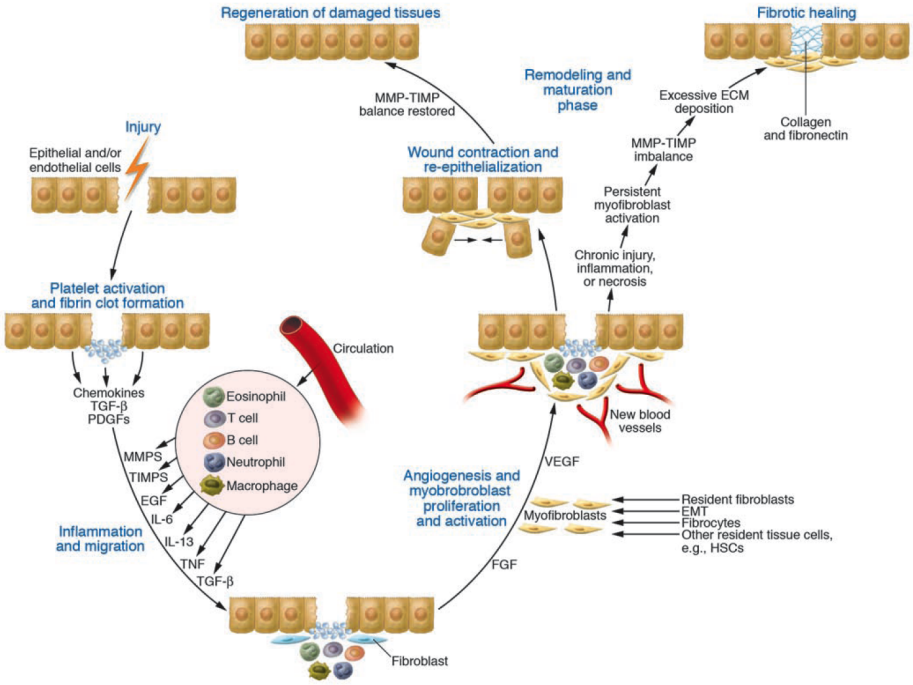

Figure 4: Outcomes of wound healing: tissue regeneration or fibrosis MMPS = matrix metalloproteinases; TIMPS = tissue inhibitors of metalloproteinases; EGF = epithelial growth factor; IL-6 = interleukin 6; IL-13 = interleukin 13; TNF= tumour necrosis factor; FGF = fibroblast growth factor; EMT = epithelial to mesenchymal transition; HSCs = hepatic stellate cells (with permission, from Wynn 2007).

The role of PDGF ligands and receptors has been investigated in fibrotic pathologies of many different tissues. In dermal fibrosis like scleroderma, increased expression of PDGFs and their receptors has been observed (Klareskog, Gustafsson et al. 1990; Kawaguchi, Hara et al. 1999; Distler, Jungel et al. 2007; Sonnylal, Denton et al. 2007; Olson and Soriano 2009). PDGFR-alpha signalling-dependent mesenchymal proliferation (Fellstrom, Klareskog et al. 1989) and PDGF-D upregulation (Ostendorf, van Roeyen et al. 2003; Ostendorf, Rong et al. 2006) have been linked to renal fibrosis, where inhibition of PDGF receptors has been reported to reduce inflammation (Chen, Chang et al. 2011). Hepatic fibrosis has been associated to upregulation of PDGFR-beta and PDGF-B (Pinzani, Milani et al. 1996; Borkham-Kamphorst, van Roeyen et al. 2007). An increase in PDGF-C expression has been detected in pulmonary fibrosis (Homma, Nagaoka et al. 1995; Rice, Moomaw et al. 1999; Zhuo, Zhang et al. 2004; Abdollahi, Li et al. 2005), where a reduction in fibrosis has been obtained with PDGF signalling inhibitors (Rhee, Lee et al. 2011). A key role for PDGFR-beta in vessel maturation and for PDGFR-alpha in fibroblast activation has been described in post infarct myocardial injury (Zymek, Bujak et al. 2006). Increased expression of PDGF-A, PDGF-B, PDGF-C and PDGF receptors has been reported for deoxycorticosterone-induced cardiac fibrosis (Ma, Li et al. 2012; Fan, Ma et al. 2013; Fan, Ma et al. 2014).

Gain of function studies in pulmonary and cardiac tissues have demonstrated that PDGF-B overexpression originates focal areas of emphysema-like airspace expansion, inflammation and fibrosis in lung (Hoyle, Li et al. 1999), that PDGF-C and PDGF-D overexpression causes general and focal fibrosis respectively, in the myocardium (Ponten, Li et al. 2003; Ponten, Folestad et al. 2005), and that adenoviral-driven expression of PDGF-A, PDGF-C and PDGF-D accelerates fibrosis in cardiac allografts (Tuuminen, Nykanen et al. 2006). Our studies extend the investigation on the effects of ectopic expression of PDGFs in the cardiac tissue, adding the characterization of PDGF-A and PDGF-B overexpression in the myocardium, and a comparative study of the focal expression of all the PDGF ligands in cardiac injury (Gallini, Lindblom et al., unpublished, paper III in this thesis; Gallini, Huusko et al., unpublished, paper IV in this thesis).

2 THESIS AIM

The aim of the work presented is to, literally, shed some more light (green and fluorescent) on the role of PDGFR-alpha signalling in developmental and pathological contexts. To do that, we utilized animals where a histone H2B-GFP fusion protein reporter construct was knocked into the PDGFR-alpha locus. This fusion protein allowed us to illuminate the PDGFR-alpha expression pattern, at a single cell resolution, in each of the studies presented in this thesis.

In paper I we investigate the role of PDGFR-alpha signalling in the inflow tract and the second heart field-derived structures, in a PDGFR-alpha loss of function developmental study.

In paper II we examine the physiological role of the evolutionarily conserved PDGF-A retention motif, and inquire how its ablation affects PDGFR-alpha signalling.

Paper III explores the ability of PDGF-A and PDGF-B overexpression to cause fibrosis during cardiac development, as described for PDGF-C and PDGF-D. We inspect which of the two PDGF receptors is responsible for the signalling that leads to excessive extracellular matrix deposition.

Paper IV looks into the involvement of the PDGF ligands in cardiac fibrosis in adult myocardium, and explores the role these growth factors play in cardiac injury.

3 METHODS

PDGFR-alpha knock-in tool: we used mice expressing histone H2B-GFP fusion protein reporter construct knocked into the PDGFR-alpha locus (Hamilton, Klinghoffer et al. 2003), in order to identify and follow cells expressing PDGFR-alpha.

Generation of transgenic mice: the DNA we wanted to modify was subcloned and modified by site-specific mutagenesis and enzymatic restriction. The DNA constructs to integrate into the genome by homologous recombination were either microinjected into fertilized oocytes pronuclei (paper III), or transfected by electroporation into ES cells (paper II). The ES cells were then injected into blastocysts. The oocytes or the blastocysts were in the end transplanted into pseudopregnant females to generate gene-targeted mice.

Echocardiography: this technique applies high frequency sound waves to create an image of the heart, and, by Doppler effect application, allows the determination of blood flow speed and direction. Estimation of the heart performance can be obtained from cardiac output, ejection fraction and diastolic function data. We used a high-resolution imaging system especially developed for small animal research. The transducer was placed on one side of the mouse thorax after shaving, while the animals were anaesthetized with isoflurane (paper IV).

Adenovirus injections: the adenoviruses encoding for the PDGF ligands were injected into the ventricle wall of the mice under high-resolution echocardiography guidance. This method uses ultrasound imaging of the mouse heart to guide the injections, and allows the procedure to be performed on closed chest, avoiding the need for surgical visualization of the organ.

Harvesting of embryos: in order to stage the embryos we crossed the animals overnight and checked for vaginal plugs the next morning, which was set as E0.5. The pregnant females were sacrificed at the desired embryonic day, the embryos were harvested and dissected under a dissection microscope, and fixed right away. We genotyped the embryos by PCR on yolk sacs or tail fragments, with specific primers (papers I, II, III).

Immunohistochemistry: light microscopy can be used to examine the histology of different organs. To visualize specific molecules, paraffin-embedded samples are exposed to antibodies conjugated to enzymes that can catalyse a colour-producing reaction. The tissues are usually counterstained to provide contrast and identify cell nuclei and cellular bodies.

Quantification of lung alveolar density: in paper II we evaluated the number and perimeter of the open areas in lung's alveolar network on bright field images of paraffin embedded tissues.

Immunofluorescent staining: in this technique fluorescent-labelled antibodies are used to detect and visualize the location and distribution of the antigen-molecules in the tissue sample.

Confocal microscopy: this technique uses laser point illumination of the sample and a pinhole in front of the detector to eliminate out of focus signal. Only the light from fluorescence in the optical section of the focal plane can be detected, and the thickness of the optical section is determined by the pinhole. The technique allowed us to obtain high-resolution pictures of tissue morphology, with the help of fluorescent-labelled antibodies, dyes and transgenic expression of fluorescent proteins.

Quantification of cells in spinal cord: we needed to quantify the number of oligodendrocytes in the spinal cord in paper II. Consecutive transverse sections of embryo's spinal cord were analysed by confocal microscopy. The ratio between PDGFR-alpha expressing cells (GFP labelled) and area was evaluated for each section and averaged along the spinal cord.

Scar tissue analysis: in paper IV we evaluated the size of the injured cardiac tissue approximating it to an ellipsoid volume. The length of the axes was obtained by measurements in the two dimension confocal images and by recording thickness and number of the tissue sections. We also evaluated the PDGFR-alpha distribution in the injured area by imaging and counting GFP-expressing cells.

Morphometric analysis of myocardial volume: this is a method used to perform quantitative analyses of tissues. The AMIRA software system allows volumetric analysis in the three-dimensional models, and we used it to evaluate the extension of several cardiac structures in paper I.

Three-dimensional reconstruction of atrial and ventricular myocardium: in paper I we created three-dimensional models of heart tissue from microscope digital images, using the AMIRA software system.

Epicardial cell isolation and cell culture: fragments of epicardial tissue were put in culture in paper I. The epicardial cells were obtained through cells outgrowth from the tissue fragments.

RNA interference: this method uses RNA molecules to inhibit gene expression by post-transcriptional regulation. RNA molecules containing sequences complementary to the target mRNA cause its elimination.

mRNA isolation and real-time PCR analysis: real-time PCR amplifies and quantifies the amount of a targeted DNA molecule. We detected the cDNA during the polymerase chain reaction progression with sequence-specific DNA probes, labelled with fluorophores that emit fluorescence only after hybridization. The amplification is carried out in special thermal cyclers that can illuminate the samples with specific wavelengths and detect the fluorescence emitted by the fluorophore. We used relative quantification methods to analyse gene-expression. We obtained the cDNA by reverse-transcription of mRNA. Our mRNA was prepared by spin column purification, where the mRNA extract is placed in a column and bound to its silica gel membrane by centrifugation. The gel membrane is then washed and eluted. The mRNA was retro-transcribed using random and oligo(dT) primers combined.

Northern blotting: this method detects mRNA and determines its amount. The purified mRNA is separated by size by an electrophoresis gel run, and then transferred to a filter membrane and crosslinked. After blocking, the nucleic acid is detected by hybridization of a probe complementary to the target sequence.

Western blotting: this method detects a protein and determines its size. The protein extract is loaded on polyacrylamide gel and size-fractioned by electrophoresis. The proteins are then transferred to a membrane by blotting, and detected by antibody staining after blocking.

Analysis of albuminuria: albuminuria is a type of proteinuria where albumin is present in urine. The urine spots collected were loaded on polyacrylamide gel where the proteins were separated by electrophoresis. The gel was stained with a dye (Coomassie blue) that turns blue when bound to proteins, and the albumin band was identified and quantified in relation to bovine serum albumin standard (paper II).

Statistics: the statistical significance of the experimental measurements was evaluated with independent sample (papers I, II, III) or paired (paper IV) Student's t-test.

Ethical considerations: some of the experiments have been performed abroad. The animals kept in Sweden were bred and handled in accordance with Swedish animal welfare legislation, and all experiments were approved either by the Committee for Animal Ethics in Göteborg and in Stockholm North, or by the Animal Experiment Provincial Office in South Finland, or by the American Association for Laboratory Animal Science.

4 RESULTS AND DISCUSSION OF PRESENTED STUDIES

PAPER I

PDGFR-ALPHA IN SECOND HEART FIELD DEVELOPMENT

When I made the first observations on PDGFR-alpha as a possible epicardial-derived cell marker in 2007, the outflow tract and septation defects reported for PDGFR-alpha null mutants and PDGF-A and PDGF-C double mutants had been ascribed to a failure in neural crest cells contribution (Schatteman, Motley et al. 1995; Ding, Wu et al. 2004; reviewed by Reigstad, Varhaug et al. 2005; Richarte, Mead et al. 2007). The research on epicardium and epicardial-derived cells was focusing on PDGFR-beta, at that time (Van Den Akker, Lie-Venema et al. 2005; Mellgren, Smith et al. 2008; Van den Akker, Winkel et al. 2008). Shortly after my preliminary observations, a role for PDGF-A/PDGFR-alpha signalling in epicardial cells proliferation has been reported (Kang, Gu et al. 2008), and we started a collaboration with prof. Adriana Gittemberger-de Groot's group to investigate the specific role of PDGFR-alpha in second heart field contribution. The first detailed analysis of the expression patterns of PDGF-A, PDGF-C and PDGFR-alpha was performed in chicken embryos (Bax, Lie-Venema et al. 2009), and confirmed my observations in mouse embryos.

In the paper that I include in this thesis, we focus our attention on the inflow tract defects that originate in the absence of PDGFR-alpha signalling. Embryos carrying a PDGFR-alpha null mutation (knock-in with histone H2B-GFP fusion protein Hamilton, Klinghoffer et al. 2003) have been analysed from 9.5 to 14.5 days of embryonic development. The mutants show hypoplasia of the proepicardial organ, of the ventricular myocardium, and of the sinoarial node. Defects in ventricular and atrioventricular septation are also present, and the pulmonary vein fails to develop with the correct orientation. The epicardial-derived cells miss to efficiently migrate into the myocardium in the absence of PDGFR-alpha expression. The detailed analysis of Nkx 2.5 expression revealed that this transcription factor remains expressed in the sinoatrial node, where it should be downregulated in order to allow the correct differentiation of the sinus venosus and the development of its pacemaker function (Pashmforoush, Lu et al. 2004; Blaschke, Hahurij et al. 2007; Espinoza-Lewis, Yu et al. 2009).

Our discoveries indicate a key role for PDGFR-alpha in myocardial addition to the venous pole and in sinoatrial pacemaker development. The hypoplasia observed could be due to a direct role of PDGFR-alpha in second heart field's epicardial cells differentiation (Prall, Menon et al. 2007), it could be ascribed to the lack of trophic signals from the epicardium to the myocardium (Perez-Pomares, Phelps et al. 2002; Merki, Zamora et al. 2005; Kang, Gu et al. 2008; Lie-Venema, Eralp et al. 2008), or it could depend on partial heart tube looping failure (reviewed by Lie-Venema, van den Akker et al. 2007; Snarr, O'Neal et al. 2007; Snarr, Wirrig et al. 2007), as the pulmonary vein orientation and the mesenchymal cap and protrusion would suggest.

The ectopic expression of Nkx 2.5 infers a role for PDGFR-alpha in the homeodomain transcription factor repression. The misregulation in WT1 expression that we detected would also point in this direction, since WT1 has been shown to participate in epicardial-derived cells epithelial to mesenchymal transition through Snail (Martinez-Estrada, Lettice et al. 2010). The crucial role of PDGFR-alpha in epicardial to mesenchymal transformation has been described in relation to a specific subset of transcriptional inducers of epithelial to mesenchymal transition active in cardiac development (Smith, Baek et al. 2011), and this would explain why we observed differential regulation of the transcription factors that we analysed (alpha 4-integrin, VCAM and E-cadherin).

Since defects in sinoatrial node and pulmonary vein connection are often related to conduction abnormalities (Korbmacher, Buttgen et al. 2001), it would be interesting to perform conduction and arrhythmia studies in hearts of epicardial specific PDGFR-alpha mutants.

PAPER II

PDGFR-ALPHA SIGNALING IN PDGF-A EXON6 KO MICE

PDGF-A mRNA is transcribed into two alternative splicing isoforms: PDGF-A_L includes exon 6, which encodes for an 18 amino acids long carboxy-terminal heparin-binding motif; PDGF-A_S does not include the transcript from exon 6, and its carboxy-terminal sequence is limited to the 3 amino acids encoded by exon 7 (Betsholtz, Johnsson et al. 1986; Bonthron, Morton et al. 1988; Rorsman, Bywater et al. 1988). The presence of these isoforms is conserved across vertebrates, and the ability to bind heparin and heparan sulphate proteoglycans is common to many extracellular signalling proteins, where the presence of a retention motif has been linked to the ability to form gradients and boundaries (reviewed by Lin 2004; reviewed by Hacker, Nybakken et al. 2005; reviewed by Lindahl and Li 2009; reviewed by Rogers and Schier 2011).

In this paper we investigated the physiological role of PDGF-A_L. From our expression analysis, it emerged that PDGF-A_S is the main component of secreted PDGF-A, with a minimal percentage of PDGF-A_L. The highest absolute amount of PDGF-A_L is present in lungs and intestine. With the approach applied to VEGF-A and PDGF-B retention motif studies in mind, we generated mutant mice homozygous for the deletion of the splice acceptor site in exon 6 of the *pdgfa* gene. These mice fail to produce the long isoform of PDGF-A, which binds to the extracellular matrix, and they only express the short, freely diffusible isoform PDGF-A_S. These mutants have been named PDGF-$A^{\Delta ex6/\Delta ex6}$. The mutation results in viable and fertile animals, that don't show any obvious phenotype. We tried to challenge the mutants and limited the signalling trough PDGFR-alpha, combining the PDGF-$A^{\Delta ex6}$ mutation with PDGF-C null mutation PDGF-$C^{-/-}$, or with heterozygous knocked-in receptor PDGFR-$alpha^{GFP/+}$. PDGF-$A^{\Delta ex6/-}$; PDGFR-$alpha^{GFP/+}$ mutants develop progressive lung alveoli loss, and PDGF-$A^{\Delta ex6/\Delta ex6}$; PDGF-$C^{-/-}$ animals display vertebral and intestinal defects.

We could not rule out a hypomorphic effect of the PDGF-$A^{\Delta ex6}$ mutation, and the observed phenotypes could be the outcome of a dosage-dependent effect rather than being range dependent.

What emerges from our study is that PDGFR-alpha signalling is probably a long range signalling in most of the physiological situations, and the main receptor/ligand interaction is carried out by the short isoform of PDGF-A. This interpretation would confirm what has been observed about PDGFR-alpha signalling in Xenopus gastrulation, where PDGF-A_S is specifically required to guide directional migration of cells in the precordal mesoderm (Nagel, Tahinci et al. 2004; Damm and Winklbauer 2011). It would also comply with the fact that we can't detect myelination defects on our mutants, in spite of the key role of PDGFR-alpha signalling on oligodendrocyte development (Calver, Hall et al. 1998; Fruttiger, Karlsson et al. 1999). The presence of PDGF-A_S in our mutants would ensure the presence of a long range PDGFR-alpha signalling.

Our approach of eliminating the long isoform of PDGF-A was inspired, as I mentioned, by the results obtained in the studies on PDGF-B and VEGF-A retention motifs, two growth factors whose short range signalling has fundamental implications in vessel morphogenesis and maturation (Ruhrberg, Gerhardt et al. 2002; Abramsson, Lindblom et al. 2003; Gerhardt, Golding et al. 2003; Lindblom, Gerhardt et al. 2003; Abramsson, Kurup et al. 2007). It would be interesting to study mutants lacking the possibility to splice out exon 6 of the *pdgfa* gene, and expressing only the long isoform of PDGF-A. Comparison of those mutants with PDGF-A null mutants could shed more light on the specific role of PDGF-A_L and on the reasons for its conservation.

PAPER III - PAPER IV

PDGFR-ALPHA RESPONSE TO ECTOPIC EXPRESSION OF PDGF LIGANDS IN THE CARDIAC TISSUE

PDGF signalling has been correlated to the onset of fibrosis in many different systems. These growth factors are documented mitogens and chemotactic agents for fibroblasts, and activate extracellular matrix deposition (Canalis 1981; Blatti, Foster et al. 1988; Schonherr, Jarvelainen et al. 1991; Fruttiger, Calver et al. 1996). In particular, they have been associated with cardiac fibrotic responses (Tuuminen, Nykanen et al. 2006; Ma, Li et al. 2012; Fan, Ma et al. 2013; Fan, Ma et al. 2014).

We analysed the effect of ectopic expression of PDGF-A and PDGF-B during heart development in paper III, and the role of the different PDGF ligands in injury and inflammatory context in paper IV.

In paper III we have generated and analysed transgenic mice, where PDGFs were overexpressed under the Myosin heavy chain-promoter, specific for cardiomyocyte development. The stable

and homogeneous expression of PDGF-A and -B in myocardium produces severe fibrosis, extensive hypertrophy and cardiac failure for PDGF-A gain of function, and focal fibrosis, correlated with moderate myocardial hypertrophy, for PDGF-B gain of function. These phenotypes can be directly compared to the effects of PDGF-C and PDGF-D gain of functions generated in the same transgenic system (Ponten, Li et al. 2003; Ponten, Folestad et al. 2005). In general we see fibroblast activation and abundant extracellular matrix deposition for each of the PDGF ligands ectopic expression (paper III, Ponten, Li et al. 2003; Ponten, Folestad et al. 2005).

In paper IV we injected adenoviruses expressing the different PDGF isoforms into adult hearts of PDGFR-alpha$^{GFP/+}$ mice. The focal and transient expression of the PDGF ligands by viral cDNA in such an inflammatory context has unpredicted effects: PDGFR-alpha agonists reduce the injury extension and recruit PDGFR-alpha positive cell population; PDGFR-beta agonists give rise to opposite reactions, with PDGF-B exacerbating inflammation without PDGFR-alpha cells recruitment, and with PDGF-D heavily reducing injury extension and inflammation. In each of the PDGF stimulation conditions we also witness Nkx 2.5 expression alteration.

Our expression analysis in these systems suggests that PDGFR-alpha is a constant target for the signalling, and, possibly, the main receptor responsible for the final phenotypic outcomes, since we see enrichment in PDGFR-alpha positive cells, and they do not seem to co-express PDGFR-beta. In line with this hypothesis, transgenic overexpression of PDGF-A gives a stronger fibrotic reaction than PDGF-B overexpression (paper III), and we detect more PDGFR-alpha positive cells recruited to the injury site in the virus-driven transient expression of PDFR-alpha agonists PDGF-A and PDGF-C (paper IV). PDGF-B has also been proved a potent PDGFR-alpha agonist *in vitro* (Hart, Forstrom et al. 1988; Heldin, Backstrom et al. 1988), but its transgenic expression in paper III gives rise to a milder fibrotic effect than PDGF-A and PDGF-C, and very similar to the effect of the PDGFR-beta agonist PDGF-D (Ponten, Folestad et al. 2005).

The Nkx 2.5 expression responses in paper IV point at a role for PDGF signalling in cell fate determination, and it has been related to cardiac extracellular matrix remodelling (Sullivan, Quinn et al. 2014).

In the virus-mediated focal expression of PDGFs in paper IV we observe opposite effects of the PDGFR-beta agonists PDGF-B and -D, and this could lead to speculation on different receptor specificities. In this system, the availability of PDGFR-alpha responding cells is not limiting: the epicardium is a stable source (Weeke-Klimp, Bax et al. 2010; Smith, Baek et al. 2011). We, however, fail to detect cells co-expressing PDGFR-alpha and PDGFR-beta, and the effect on PDGFR-alpha positive cell recruitment is absent in PDGF-B stimulation, whilst the effect on injury extension goes in the opposite direction compared to the other PDGFR-alpha agonists. Considering that PDGF-B includes a retention motif, and that its effect could be diffusion restricted, there is the possibility that the growth factor would not be surrounded by PDGFR-alpha responding cells. We wonder if the explanation for PDGF-B ectopic expression reaction should be sought with the recently demonstrated effect of PDGFR-beta signalling in inflammatory reactions (Olson and Soriano 2011; Stark, Eckart et al. 2013). In this perspective, the focal fibrotic effect displayed by the PDGF-B transgenic mutants in paper III could be secondary to the strong inflammatory reaction that seems to be mediated by PDGF-B in our

virus-driven ectopic expression system in paper IV (mechanisms are reviewed by Kong, Christia et al. 2014).

It is very surprising that PDGF-B and -D have such opposite effects when overexpressed in an inflamed tissue (paper IV). Other studies have indicated a contradictory effect of the two PDGFR-beta agonists in another cardiac fibrosis model (Fan, Ma et al. 2013) and a specific role for PDGF-D in fibroblast differentiation, as well as in metalloproteinases and TGF-beta expression (Ponten, Folestad et al. 2005; Zhao, Zhao et al. 2013). The data available on PDGF-D is still limited, and we don't know if the two PDGFR-beta agonists compete with each other on the same receptor, directing the signalling to different downstream effectors.

It is not the first time that PDGF signalling is known to have a positive effect on wound healing and tissue recovery (Zymek, Bujak et al. 2006), and our study in paper IV would suggest PDGF-D as possible target for future therapeutic developments in injury-driven inflammation treatments.

Our experimental design was based on previous experience on VEGF overexpression (Huusko, Merentie et al. 2010), and, given the short life of the viral cDNA, we decided to analyse the samples after two weeks from expression induction. The inflammatory reaction is still ongoing at this time point, and it would be very interesting to analyse the samples at a later stage, after the inflammation resolution, to record the final fibrotic outcome of the injuries.

5 CONCLUDING OBSERVATIONS

The work presented in this thesis adds a small share of intelligence to the complex field of PDGF signalling.

The results obtained during the course of the studies reported identify a key role for PDGFR-alpha in second heart field-derived cells differentiation and fate determination, during cardiac development. With the examination of the physiological function of the retention motif of PDGF-A, we were able to determine the prevalence of a long range signalling for PDGFR-alpha, unlike other receptors of the family, PDGFR-beta and VEGFR2. Our data recognizes PDGFR-alpha positive cells as the main players in cardiac fibrosis induced by excessive PDGF signalling during myocardial development. PDGFR-alpha signalling appears to be responsible for fibroblast recruitment and involved in extracellular matrix deposition. When stimulated in an inflammatory context, on the other hand, PDGFR-alpha positive cells seem to play an interesting role in tissue recovery.

The interpretation of the data has sometimes been elusive, especially when the outcome of the experiments contrasted with previous knowledge and results. The tools at our disposal, however, made the observations solid enough to start some very interesting discussions, often raising more questions than they answered. It seems that the more we dig into PDGF signalling, the more we grasp the extent of our lack of knowledge in the field. This is probably true for the majority of the biological fields, and it's what keeps us interested and engaged.

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