Posterior heart field and epicardium in cardiac development : PDGFRα and EMT

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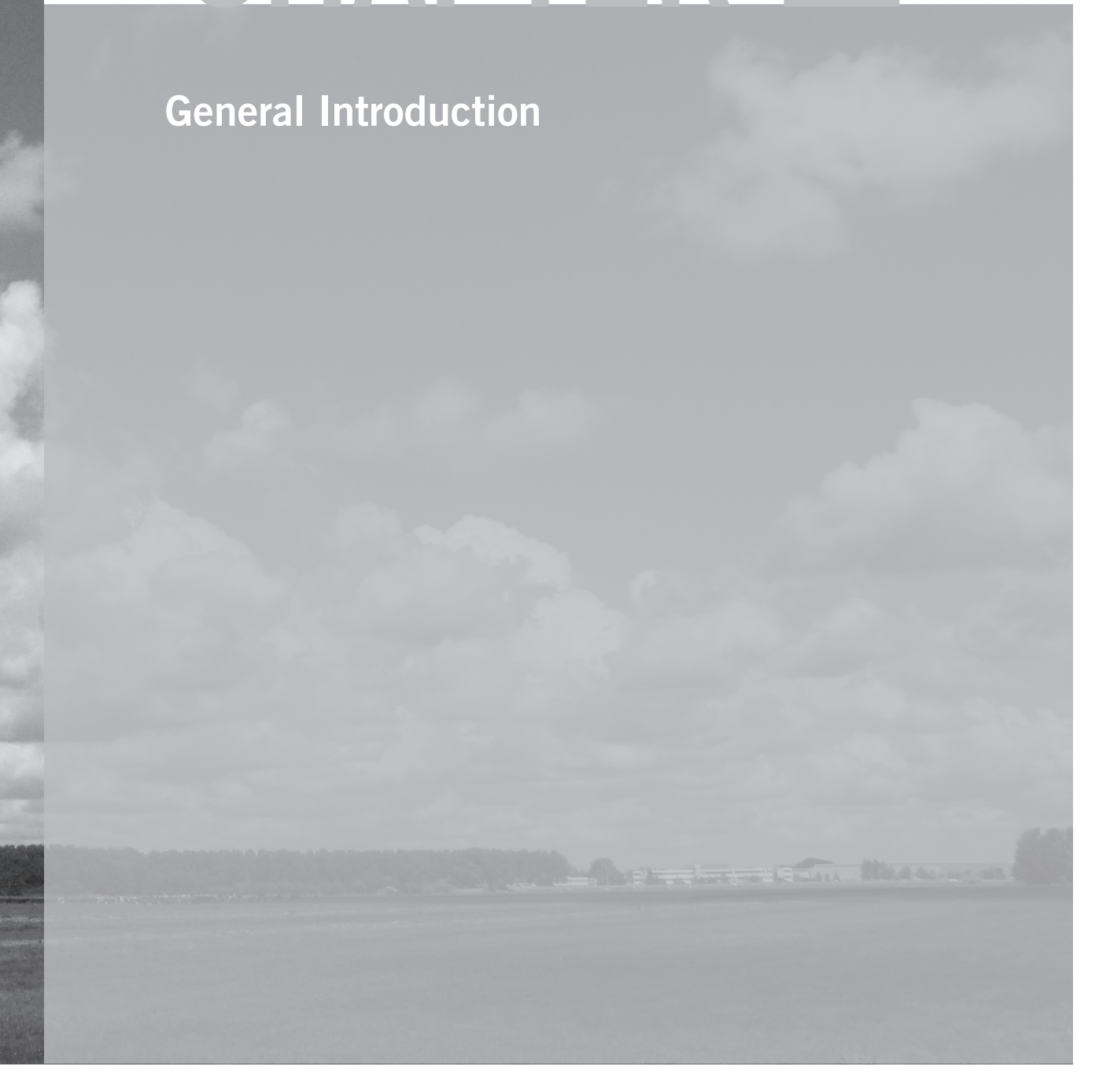
CHAPTER 1

General Introduction



AIM AND OUTLINE OF THE THESIS

The aim of this thesis was twofold. In the first part, the line of investigation concentrates on the role of Platelet-derived growth factors (PDGFs) in the development of second heart field-derived cardiac structures, especially at the venous pole of the heart. For this purpose we have described the expression pattern of PDGF-A, -C and their receptor PDGFR-α in the avian heart. Additionally, we studied mouse embryos in which the *Pdgfrα* gene was mutated.

The second part of this thesis is based on *in vitro* investigations in which we focus on epicardial cells and epicardium-derived cells (EPDCs), which also originate from the second heart field at the venous pole of the heart. In this part we studied the role of EPDCs in the differentiation of the myocardium. Furthermore, the process of epithelial-to-mesenchymal transformation (EMT) in human adult epicardial cells and the effect of this process on electrical conductivity will be outlined.

Chapter Outline

Part I is entitled "Platelet-derived growth factors (PDGFs) in the development of second heart field-derived cardiac structures".

Chapter 2 describes the cardiac expression pattern of PDGF-A, -C and their receptor PDGFR-α during several stages of cardiac development in the avian embryo.

Chapter 3 shows the effect of *Pdgfrα* mutation on heart development and more specifically the development of second heart field-derived cardiac structures at the venous pole of the heart.

Chapter 4 provides insight in the development of total anomalous pulmonary venous return (TAPVR) after dysregulation of the *Pdgfrα* gene in combination with abnormalities in the development of the pulmonary vein.

Part II is entitled "EPDCs in epithelial-to-mesenchymal transformation and cardiomyocyte differentiation".

Chapter 5 describes how embryonic EPDCs affect cardiomyocyte organization into cellular arrays, providing involved mechanisms underlying the induction of myocardial cytoarchitectural organization.

Chapter 6 demonstrates which growth factors are involved in the process of EMT in human adult epicardial cells, which are an interesting target for assisting myocardial regeneration due to endogenous reactivation after myocardial injury.

Chapter 7 describes the electrophysiological aspects of human adult epicardial cells, especially the regulation of connexins Cx40, Cx43 and Cx45 and ion channels Kir2.1, SCN5a and CACNA1C during the process of EMT.

Finally, *Chapter 8* will reflect on the data presented in this thesis in a concluding general discussion.

In the following paragraphs an overview of cardiac development and the role of PDGFRα and EMT in this development will be given.

The onset of cardiogenesis

The heart is the first organ to form during organogenesis, although in the pregastrula stage, prospective heart cells reside in the posterior region of the lateral epiblast or primitive ectoderm [1,2]. The epiblast is derived from the inner cell mass or embryoblast and lies above the hypoblast (primary endoderm). Gastrulation commences with the appearance of the primitive streak in the epiblast and epiblast cells undergo EMT and ingress into the subepiblast space to form the third germ layer, the mesoderm [3]. Prospective heart cells including future endocardial, myocardial and pericardial cells, occupy the anterior half of the primitive streak (PS) (Figure 1). Epiblast cells destined for the heart co-localize with cranial mesenchyme to the distal region of the newly formed mesoderm at the primitive-streak stage [2]. This newly formed mesoderm transiently expresses the earliest markers for cardiac progenitors MESP1 and MESP2 (mesoderm posterior 1 and 2) [4,5]. MESP1 and MESP2 are expressed in the early mesoderm that is destined to become the extraembryonic and cranial-cardiac mesoderm. These transcription factors are required for the prospective heart cells to leave the primitive streak and migrate anterolaterally and condense in the left and right anterior lateral plate mesoderm. In absence of these two genes, the embryos show failure of development of the heart, gut or somites [4]. The anterior lateral mesoderm divides dorsoventrally to form the somatic mesoderm and splanchnic mesoderm, between which the pericardial coelom is established [6]. Bilateral primary heart fields or first heart

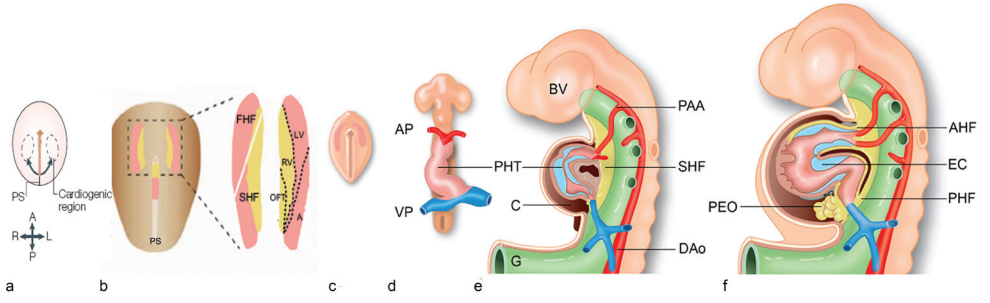


Figure 1. Cardiac development

Schematic figure depicting the myocardial progenitors originate in the primitive streak (PS), from where they migrate to the anterior (a) were they contribute to the bilateral formation of the cardiac crescent (b,c), which are derived from the splanchnic mesoderm. The bilateral plates fuse and form the primary heart tube (PHT) (pink) (d,e). Myocardium is added from the second heart field (SHF) (yellow) to both the arterial (AP) and venous pole (VP) of the heart (e). The subset of the SHF that contributes to the AP is called the anterior heart field (AHF) and that contributing to the VP the posterior heart field (PHF). The proepicardial organ (PEO) develops as mesenchymal contribution of the PHF and contributes to heart development by covering the myocardium of the heart tube (f). C, coelomic cavity; DAo, dorsal aorta; EC, endocardial cushions; FHF, first heart field; PAA, pharyngeal arch arteries. Adapted from Poelmann and Gittenberger-de Groot [70, 9] and Buckingham [5].

fields (FHF) are formed in the splanchnic mesoderm on both sides of the midline [5] and fuse at the midline to form the cardiac crescent (first heart field) in the anterior region of the embryo [7]. The cardiac crescent is patterned with the expression of the cardiac markers Nkx2.5, Gata4 and Tbx5 (T-box 5). Transforming growth factor beta (TGFβ) is also expressed in the precardiac mesoderm comparable to Nkx2.5 [8]. The cardiac crescent remodels into the primitive heart tube (Figure 1c,d), located in the pericardial cavity, with separate outflow (or arterial) and inflow (or venous) poles and the heart starts beating [3,7,9]. The primitive heart tube consists of an outer myocardial layer and an inner endocardium and is segmented into the left ventricle (LV), the atrioventricular canal (AVC) and the primitive atrium (Figure 1c,d) [10].

Subsequently, the straight primitive heart tube will undergo a process which is named cardiac looping. Multiple factors are involved in cardiac looping and will involve coordination of multiple pathways [11]. Onset of looping is marked by bending of the right lateral margin of the straight heart tube, forming a c-shaped tubular structure. This phase of looping is called dextral-looping and leads to establishment of the left-right asymmetry of the ventricular component of the heart. The c-shaped loop is transformed into an s-shaped loop thereby shortening the distance between the fixed arterial and venous pole. The last phase of looping is characterized by leftward shift of the proximal part of the outflow tract (OFT) and by the appearance of the anlagen of the arterial trunks in the distal part of the OFT. After looping, the cardiac chambers will further differentiate [11]. Transcription factors like MEF2c, Nkx2.5, Tbx5 and retinaldehyde-dehydrogenase2 (RALDH2), involved in retinoic acid signalling, are necessary for the completion of heart looping and the expansion of the specific cardiac chambers [12].

Second heart field

The primitive heart tube grows and elongates not only as a result of expansion of the first heart field-derived tissue but also from progressive addition of heart progenitor cells to both the arterial and venous pole from the surrounding mesoderm [7,13,14] which is also referred to as second heart field (SHF) [15] or second lineage [15,16]. The second lineage contributes to the outflow tract (OFT) and all other heart regions except the primitive LV and AVC (Figure 1e, Figure 2).

The presence of a second heart lineage was confirmed by analysis of the gene encoding the LIM homeodomain transcription factor Islet-1 (Isl1) [15]. Progenitor populations that express Isl1 will give rise to the OFT, right ventricle (RV), a major part of the atria and small part of the LV [15] (Figure 2). Undifferentiated Isl1-expressing progenitors reside throughout the anteroposterior extent of splanchnic mesoderm dorsal to the heart and

migrate in at the arterial and venous pole [15]. Other markers of the SHF are already present at the cardiac crescent stage and are localized medially from the primary heart field (Figure 1b) [12]. At the arterial pole the SHF includes the secondary or anterior heart field [13,17] while at the venous pole the posterior heart field is distinguished (Figure 2) [14].

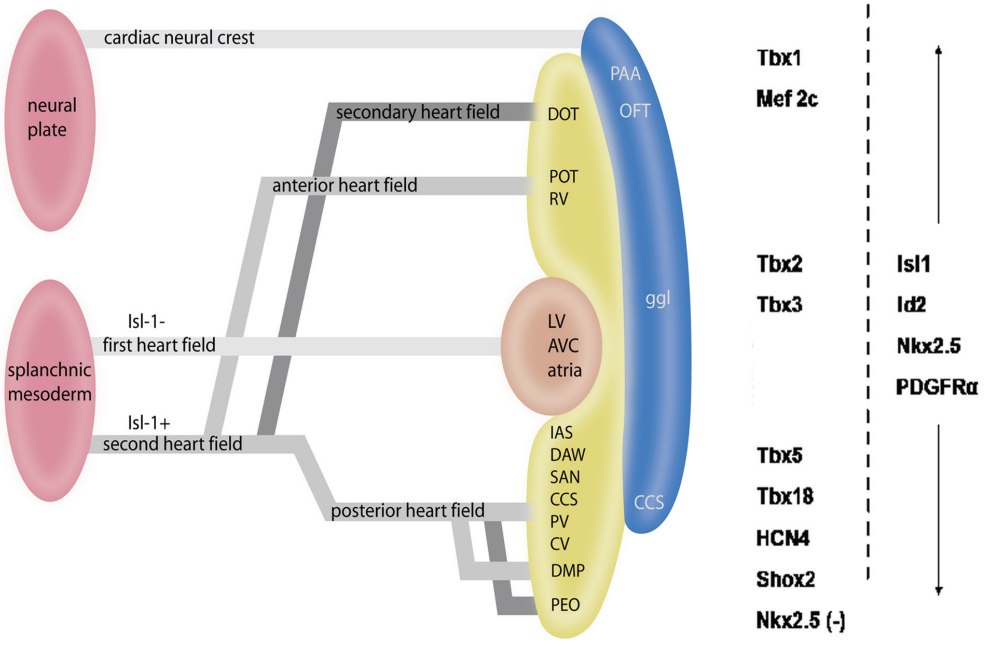


Figure 2. Heart fields
Schematic representation of the heart fields including genes and proteins expressed in the second heart field. The primary heart tube (brown) consists of the left ventricle (LV), atrioventricular canal (AVC) and part of the atria and is derived form Isl1 negative precursors in contrast to the second heart field (SHF). The second heart field can be divided into the anterior and secondary heart field at the arterial pole of the heart which are involved in the development of the outflow tract (OFT) and right ventricle (RV). The part of the SHF at the venous pole of the heart is called the posterior heart field and includes the sinus venosus myocardium and consists of the sinoatrial node (SAN), the atrial septum (AS) and dorsal atrial wall (DAW) as well as the myocardium of the wall of the pulmonary (PV) and cardinal veins (CaV). The PHF also includes the mesenchyme of the dorsal mesenchymal protrusion (DMP) and the proepicardial organ (PEO). CCS, cardiac conduction system; DOT, distal outflow tract; ggl, cardiac ganglia; PAA, pharyngeal arch arteries; POT, proximal outflow tract. Adapted from Jongbloed [71].

Secondary or anterior heart field

During the process of looping, myocardium is recruited to the OFT. Experimental manipulation of chicken embryos and analysis of transgenic and mutant mice, support the evidence of a specific precursor population at the arterial pole of the heart. Due to different experimental approaches, the precise location of this second source of progenitors, is described as secondary heart field and as anterior heart field [5]. Prepharyngeal mesoderm caudal to the OFT, which is defined as the secondary heart field, contributes to the base of the great arterial vessels, which contains the distal myocardial part of the OFT and the most proximal smooth muscle that forms the tunica media of the arterial trunk [17,18] (Figure 2). Cells of the secondary heart field express Nkx2.5 and Gata4 comparable to the primary heart field [17]. Growth of the distal OFT is driven by a balance between fibroblast growth factor (FGF) driving progenitor-cells proliferation and the bone morphogenetic protein (BMP) driving differentiation-promoting signals [17].
The anterior heart field (AHF) is the region defined by FGF-10-lacZ expression that is restricted to the right ventricle and proximal part of the OFT myocardial progenitors [13] (Figure 2). These progenitors are recruited not only from the prepharyngeal mesoderm but also from the lateral and more anterior splanchnic mesoderm [13,19]. Next to the cardiac markers Nkx2.5 and Gata4, also MEF2c and Tbx1 are present in the AHF [20,21]. MEF2c (myocyte enhancer factor 2c), also known as RSRF (related to serum response factor), directs transgene expression to the second heart field [22]. MEF2c is a cardiac lineage marker that is expressed shortly after Nkx2.5 and GATA4 and transcriptionally regulates anterior heart field development [22]. Tbx1 is a member of the T-box family of transcription factors including also Tbx5 and cell fate analysis using the *Tbx1* enhancer suggests that *Tbx1*-expressing cells contribute extensively to the right ventricular myocardium as well as to the OFT. The cardiac phenotype of *Tbx1* mutant mice is similar to that of the *FGF8* hypomorph [5]. *FGF8* which is co-expressed with FGF10 in the pharyngeal mesoderm is a driver in AHF deployment [16]. The basic helix-loop-helix (bHLH) protein Hand2 is another transcription factor required for proper development of the OFT and RV.

Posterior heart field

Recruitment of myocardium at the venous pole of the heart, which was referred to by our lab by the new position term: posterior heart field (PHF), is complementary to the anterior heart field [14] (Figure 2). Progenitors recruited from the PHF form a restricted myocardial population contributing to the sinus venosus region as well as a mesenchymal population [14,23].

Myocardial component

The myocardial component of the PHF contributes to the sinoatrial node (SAN), the atrial septum (AS), the sinus venous (SV), pulmonary veins (PV), cardinal veins (CaV) and the components of the cardiac conduction system (CCS) [14,23,24]. Myocardium recruited from the PHF is characterized by the expression of various proteins e.g. MLC-2a, podoplanin, Shox2, Tbx5 and Tbx18. The expression of Nkx2.5 is remarkably absent in the SAN and wall of the CaV and is mosaic in the venous valves (VV), atrial septum (AS) and myocardium around the PV [14 25,26].
Sinus venosus myocardium also expresses HCN4, while the primary heart tube is negative. The expression of HCN4 is first detected throughout the cardiac crescent and as development progresses expression becomes confined to the most caudal portion of the heart. At later stages, the SAN, the wall of the PV and CaV express HCN4 [27].

Mesenchymal component

Isl1-expression at the venous pole was observed in a discrete set of mesenchymal cells closely related to the dorsal mesocardium. Furthermore, the mesenchymal progenitor population surrounding the sinus horns fails to express Nkx2.5 and the majority of the precursors lack Isl1 expression before their differentiation into myocardium. Instead, this mesenchyme uniquely expresses Tbx18 [26]. The mesenchymal component of the posterior heart field contributes to the development of the dorsal mesenchymal protrusion and the proepicardial organ, the epicardium and EPDCs [28].

Dorsal mesenchymal protrusion

Extracardiac mesenchyme associated with the dorsal mesocardium contributes the venous pole of the heart (Figure 2). Historically, this mesenchyme has been referred to as spina vestibuli, vestibular spine or more recently as dorsal mesenchymal protrusion (DMP) [29,30]. The DMP, which is positive for Isl1 and MEF2c and negative for Nkx2.5 [31], is continuous with the mesenchymal cap on the primary atrial septum and the AV cushion mesenchyme [30], resulting in the formation of the AV mesenchymal complex [30]. The cap, which is positive for MEF2c, but negative for both Nkx2.5 and Isl1 [32] initially forms a small cushion-like tissue on the anlagen of the primary atrial septum. As the septum is growing, the cap becomes a prominent mesenchymal ridge on the myocardial part of the septum [33]. As the septum elongates and descends into the atrial cavity, the cap supports the closing of the primary atrial foramen and thereby left and right atrium are separated [33]. The development of the cap is suggested to be regulated by factors from the atrial septal

myocardium. The DMP mesenchyme undergoes a mesenchymal to myocardial differentiation [31], thereby forming the myocardial base of the primary atrial septum [34]. The DMP is important for proper atrial septation and the development of the pulmonary veins [31,32]. Perturbed development of the mesenchyme from the cardiac inflow region corresponding to the DMP leads to atrioventricular septal defects (AVSDs) [29].

Proepicardial organ

The proepicardial organ (PEO) originates from the coelomic lining where the sinus venosus enters the pericardial cavity. The PEO expresses Tbx18, which is specific for mesenchyme at the venous pole, suggesting that the PEO is a PHF-derived cardiac structure (Figure 2) [26,35]. The PEO is a cauliflower-like protrusion which can differentiate, by BMP- and FGF-signalling into a migrating mesothelium which is distinct from the myocardial cells of the sinus venosus wall [36,37]. Cells of the PEO will migrate across the pericardial cavity and attach to the myocardial surface. Factors that are important for migration and adhesion of the epicardium to the underlying myocardium are vascular cell adhesion molecule (VCAM-1), α4-integrin and Tbx5 [38-41]. After the heart is completely covered by the epicardial sheet, epicardium-derived cells (EPDCs) are formed by EMT and they will invade the subepicardial space [35,42]. EMT of epicardium is regulated by homeobox transcription factors Slug and Snail [43,44], the transcription factor Wilm's tumor suppressor protein WT1 [45], the adhesion molecules E-cadherin [43], α4-integrin [40] and growth factors such as transforming growth factor beta (TGFβ) [46] and platelet-derived growth factor (PDGF) [47]. Another important factor for epicardial development is retinaldehyde-dehydrogenase2 (RALDH2), a key enzyme in the synthesis of retinoic acid and expressed in the primitive epicardium [45].

After EMT, a subpopulation of EPDCs migrates into the myocardium to form interstitial fibroblasts, and smooth muscle cells and fibroblasts of the coronary vasculature [42,48]. EPDCs have also a regulatory role in the differentiation of the AV valves and the development of the ventricular myocardium [42,49,50].

The essential function of the epicardium and the EPDCs seems not to be restricted to embryonic development. In the adult heart, the epicardium is a squamous epithelium which functions as a smooth surface on which the heart slides in the pericardial cavity during contractions [51]. Adult epicardial cells have a regulatory effect on adult cardiomyocyte phenotype and function, which is dependent on cell-cell interactions between epicardial cells and cardiomyocytes [52]. Recent studies have also shown that adult epicardium is reactivated after a myocardial infarction and that this reactivated adult epicardium re-expresses embryonic epicardial markers such as *raldh2*, *Tbx18* and Wilm's tumor 1 (WT1) [53-57]. The re-activation of endogenous epicardium and the migration of these cells into the myocardium, suggests therapeutic potential of the endogenous epicardium.

Platelet-derived growth factor

The platelet-derived growth factor (PDGF) family consists of four ligands, PDGF-A,-B,-C and -D, and two receptors, PDGFR-α and -β [58]. All PDGF ligands are members of the cystine knot family of proteins and can form five dimeric isoforms, PDGF-AA, -AB, -BB, -CC and –DD. The cystine knot region is a strongly conserved homologue domain of PDGF/VEGF proteins and may be partially characterized by a local sequence motif CXGXC [59]. The PDGFs show high sequence identity with the vascular endothelial growth factors (VEGF). Both are receptor tyrosine kinases that can form homo- and heterodimers upon ligand binding [58,60-62].

Evidence for a role for *Pdgfrα* in the development of SHF-derived cardiac structures was suggested as it was already expressed in the precardiac region and early cardiac crescent [63]. At late crescent stages, *Pdgfrα* was also strongly expressed in caudal SHF progenitors specifically in the dorsal mesocardium [63].

A stimulatory role for PDGFs in avian heart development was further provided by our description of the PDGFs and PDGFRs expression patterns in proepicardial quail/chicken chimeras [64]. The spatiotemporal localization for PDGF-B and its receptors suggested that PDGF-B signalling through PDGFR-β was important for EPDC-related maturation of the coronary system and atrioventricular valves [64,65]. Recent data in mice revealed that PDGFR-β is required for epicardial cell migration and development of coronary vascular smooth muscle cells [66]. The localization of PDGF-A and its α-receptor showed that PDGF-A signalling through PDGFR-α was likely important for remodelling of the myocardium [64], possibly like in the mouse through epicardial-myocardial interactions [67].

The specific role of *Pdgfrα* in cardiac development was elucidated with the use of the *Patch* mouse with a spontaneous deletion of the PDGFR-α gene. The mutant phenotype is similar to the milder one of the *Pdgf-α*$^{-/-}$ mouse [61,68], surviving up to E17 while the *Patch*-mutation is lethal at E11. Furthermore, *Patch* and *Pdgfrα* mutants show severe heart malformations, including hypoplastic hearts and malformed valves. The number of adventitial fibroblasts in the coronary vessels is decreased and a thin myocardium, although not uniformly through the heart, was observed in both models [61,68,69].

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