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

Bax, N.A.M.

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PART 1

Platelet-derived growth factors (PDGFs) in the development of second heart field-derived cardiac structures

CHAPTER 2

Platelet-derived growth factor is involved in the differentiation of second heart field-derived cardiac structures in chicken embryos

Noortje A.M. Bax[1], Heleen Lie-Venema[1], Rebecca Vicente-Steijn[1,2], Steven B. Bleyl[3], Nynke M.S. van den Akker[1,4], Saskia Maas[1], Robert E. Poelmann[1], Adriana C. Gittenberger-de Groot[1]

1 Department of Anatomy and Embryology, Leiden University Medical Center, Leiden, The Netherlands.
2 Department of Cardiology, Leiden University Medical Center, Leiden, The Netherlands
3 Department of Pediatrics and Department of Neurobiology and Anatomy, University of Utah School of Medicine, Salt Lake City, Utah, USA.
4 Current address: Department of Cardiology, CARIM, Maastricht University, Maastricht, the Netherlands.

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ABSTRACT

For the establishment of a fully functional septated heart, addition of myocardium from second heart field-derived structures is important. Platelet-derived growth factors (PDGFs) are known for their role in cardiovascular development. In this study, we aim to elucidate this role of PDGF-A, PDGF-C and their receptor PDGFR-α. We analyzed the expression patterns of PDGF-A, -C and their receptor PDGFR-α during avian heart development. A spatiotemporal pattern of ligands was seen with colocalization of the PDGFR-α. This was found in second heart field-derived myocardium as well as the proepicardial organ (PEO) and epicardium. Mechanical inhibition of epicardial outgrowth as well as chemical disturbance of PDGFR-α support a functional role of the ligands and the receptor in cardiac development.

INTRODUCTION

The establishment of a fully septated heart is dependent on the addition of cells from a second heart cell lineage to the primary heart tube [1-3]. The second heart field or second lineage is located in the splanchnic mesoderm and can be divided into two fields related to the craniocaudal axis of the primary heart tube. From the second heart field myocardium is added at the outflow tract (OFT) or arterial pole (anterior heart field (AHF)) [2]. Myocardium added to the inflow tract (IFT) or venous pole is also derived from the second heart field, and has been referred to as posterior heart field (PHF) [3,4]. The outermost layer of the heart, the epicardium, develops initially as a cauliflower-like mesothelial primordium, the proepicardial organ (PEO), which is located near the sinus venosus [5-7]. In our view, the PEO is part of the PHF-derived structures [4]. Cells from the PEO traverse the pericardial cavity and spread out over the myocardial surface. Soon after the heart is covered, a subepicardial space develops between the epicardium and myocardium, which becomes populated by epicardium-derived cells (EPDCs). Subsequently, a subpopulation of EPDCs migrates into the myocardial interstitium to form interstitial fibroblasts, and smooth muscle cells and fibroblasts of the coronary vasculature [8-10]. Furthermore, EPDCs migrate into the atrioventricular (AV) endocardial cushions where they are involved in the differentiation of the atrioventricular valves [9-11].

Proper outgrowth of the PEO and the epicardium, as well as normal interaction between EPDCs and developing cardiomyocytes are indispensable for normal cardiogenesis [11-13]. Several signalling pathways involved in EMT, migration and differentiation of the epicardium and EPDCs in normal heart development have been described [7]. The initiation of EMT is regulated by many transcription factors, including Slug and Wilm's Tumor1 (WT1), and by retinoic acid (RA)-synthesizing enzyme RALDH2. These factors are also important for epicardial-myocardial interaction [14-16]. Recently a role for podoplanin and RhoA in this EMT process has been described [17].

The platelet-derived growth factor (PDGF) family comprises regulators involved in epicardial-myocardial signalling and have been investigated in relation to coronary vascular development [18,19]. In our current study, we have extended this investigation to epicardial-myocardial interaction in pre-vascular stages. We also investigated the, in chicken cardiovascular development hitherto undescribed, PDGF-C expression and the expression of the ligand PDGF-A and their receptor PDGFR-α. The PDGF family consists of five dimeric isoforms, PDGF-AA, -AB, -BB, -CC and –DD. The PDGF receptor-α (PDGFR-α) and receptor-β (PDGFR-β), are receptor tyrosine kinases that can form homo- and heterodimers upon ligand binding [20-23]. Evidence for a functional role of PDGFs in avian heart development was provided by our description of the PDGF and PDGFR expression patterns in proepicardial quail/chicken chimeras. The spatiotemporal localization of

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 [18]. Recent data in mice revealed that PDGFR-β is required for epicardial cell migration and development of coronary vascular smooth muscle cells [24].
The localization of PDGF-A and its α-receptor showed that PDGF-A signalling through PDGFR-α was important for remodelling of the myocardium [18], possibly, like in the mouse, through epicardial-myocardial interactions [25,26]. The role of PDGF-A and -C during development has been investigated in several null-mutant and over-expression mouse models. Phenotypical features of the *Pdgfc*$^{-/-}$ mice largely overlap with those of *Pdgfa*$^{-/-}$ mice, without a cardiac phenotype [27-29]. The hearts of the *Pdgfc*$^{-/-}$*Pdgfa*$^{-/-}$ double mutants, however showed lack of atrial septum formation [28,29].
Patch mutants, with a spontaneous mutation of the PDGFR-α gene, showed severe heart malformations, including hypoplastic hearts and malformed valves. The number of adventitial fibroblasts in the coronary vessels was decreased and a locally thinned myocardium was observed in both models [22,30,31]. Thin compact myocardium is additionally observed in several models with defects in epicardial development. In these models there is disturbed or delayed outgrowth of the PEO, either by mechanical [12,13,32] or by genetic intervention [4,14,33,34].
PDGF-C plays a role as a fibroblast mitogen. In the heart, it induced strong proliferation of myocardial interstitial fibroblasts [29]. Expansion of the cardiac interstitium in transgenic *Pdgfc* overexpression mice caused an altered myocardial architecture and an increased thickness of the ventricular walls [35,36].
We hypothesize that PDGF-C, a recently discovered ligand for the PDGFR-α, is also involved in avian heart development. Therefore, we analysed the expression patterns during development of PDGF-A, PDGF-C and their receptor PDGFR-α in the avian heart. We observed that their expression was spatiotemporally related to the development of the second heart field-derived myocardium and to the proepicardium and epicardium. Analysis of embryos with mechanical inhibition of epicardial outgrowth as well as pharmaco-chemical disturbance of Pdgfrα-signalling support a functional role of the ligands and the receptor in development of proepicardial derivates and in cardiac development.

MATERIALS AND METHODS

Chicken embryos

Fertilized eggs of White Leghorn chicken (*Gallus domesticus*) were incubated at 37°C and 80% humidity. Embryos were isolated as described earlier [37] and staged according

to the criteria of Hamburger and Hamilton (HH;[38]). Controls consisted of a developmental series of normal chicken embryos from stage HH17 to HH35 (n=3 per stage).
Inhibition of outgrowth of the proepicardial organ (PEO) in chicken embryos (HH15 to HH18) was achieved by placing a piece of egg shell membrane between the PEO and the heart tube, as described earlier [13,39]. After reincubation, embryos were isolated at stages HH20 to HH28 (n=5 per stage). The embryos were fixed in 20% dimethylsulfoxide (DMSO) and 80% methanol for immunostaining with PDGFR-α or in 4% paraformaldehyde (PFA) for 24-48 hrs for all other staining protocols. Subsequently, they were embedded in paraffin and 5 µm serial sections were mounted on egg white/glycerine-coated microscope slides (Menzel-Gläser).

Pdgfra inhibition in chick

For PDGFRA inhibition experiments [40], the RTK inhibitor Imatinib (STI571/Gleevec, Novartis Pharma AG, Switzerland) was used. Briefly, Imatinib tablets (100 mg) were dissolved in sterile saline with penicillin and streptomycin at room temperature, filtered and diluted to a 0.125 mg/ml working solution. Fertilized eggs were windowed at HH15 and treated with three doses of RTK inhibitors at 8-hour intervals. In separate experiments, 200 µl of Imatinib solution was added directly on top of the vitelline membrane at doses of 45, 75, 90, 150, 275 and 350 µg, while control embryos were treated with saline/antibiotic. For all *in ovo* inhibition experiments, embryos were inspected under a dissecting microscope at each dose for viability, assessing the correct stage of development and abnormalities. After treatment, embryos were reincubated for an additional 48-56 hours *in ovo* until 5 days of development (HH26-HH27). The embryos were fixated in modified Bodian's fixative at 4°C overnight. Subsequently, they were sectioned and stained with Mayer's haematoxylin and eosin.

Western blotting

Previous performed Western blots of PDGF-A and PDGFR-α confirmed the specificity of these antibodies [18]. To confirm the specificity of the polyclonal antibody PDGF-C in chicken embryonic material, we also performed Western blots. In short, total protein was extracted from hearts of normal chicken embryos in 0.1 M Tris HCl with 0.1% Tween (pH7.5). The total protein content of the extracts was determined using the Ecl Advance Detection Kit (Amersham Biosciences). The results showed that the antibody specifically recognized the PDGF-C protein. Although PDGF-C can be secreted in a latent form and for activation cleavage of the N-terminal CUB domain by Tissue plasminogen activator (tPA) is necessary, we assume that regions with expression of PDGF-C are regions with potential activity.

Immunohistochemistry

Serial sections were subjected to standard immunohistochemical procedures [4,18]. Microwave antigen retrieval was applied for the PDGFR-α, cTnI, WT1, Isl-1, Tbx18 and Nkx2.5 staining. Endogenous peroxidase was inactivated with 0.3% H_2O_2 in PBS. Before incubation with the primary antibodies, sections of chicken embryos selected for the PDGF-C staining were blocked with 5% bovine serum albumin (BSA) in 0.05% Tween/PBS for 15 minutes. Sections were incubated overnight with rabbit-α-human PDGF-A (α-PDGF-A, 1:25; SC-128, Santa Cruz Biotechnology), goat-α-human PDGF-C (α-PDGF-C, 1:150, SC-18228, Santa Cruz Biotechnology), goat-α-human PDGFR-α (α-PDGFR-α, 1:50, P2110, Sigma-Aldrich), rabbit-α-human Wilm's tumor suppressor protein (α-WT1, 1:500, CA1026, Calbiochem) goat-α-human Nkx2.5 (α-Nkx2.5, 1:6000, SC-8697 Santa Cruz Biotechnology), goat-α-human Tbx18 (α-Tbx18, 1:800, SC-17867, Santa Cruz Biotechnology), mouse-α-Isl-1 39.4D5 (α-Isl-1, 1:400, Developmental Studies Hybridoma Bank), goat- α-human cardiac Troponin I (α-cTnI, 1:400, SC-8118 Santa Cruz Biotechnology) or anti-atrial myosin light chain 2 (α-MLC-2a, 1:6000, which was kindly provided by S.W. Kubalak, Charleston, SC), followed by incubation with a secondary biotin-labelled antibody for 1 hr. Secondary antibodies used were biotin-labelled goat-α-rabbit (BA-1000, Vector Labs), horse-α-mouse (BA-2000, Vector Labs) and horse-α-goat (BA-9500, Vector Labs) in combination with normal goat serum (S-1000, Vector Labs) or normal horse serum (S-2000, Vector Labs), respectively. Subsequently, the sections were incubated with Vectastain ABC staining kit (PK-6100, Vector Labs) for 45 minutes. Slides were rinsed in PBS and Tris/Maleate (pH 7.6). 3-3'diaminobenzidine tetrahydrochloride (DAB) (D5637, Sigma-Aldrich) was used as chromogen and Mayer's haematoxylin as counterstaining. Finally, all slides were dehydrated and mounted with Entellan (Merck).

In situ hybridization

Sense and anti-sense digoxigenin-labeled riboprobes were used as previously described [41,42]. Probes were synthesized from chick *Pdgfra* clones (ChEST55k2) and from chick *Pdgf-a* ligand clones (ChEST826l7). Sections were examined with a Nikon compound microscope using a QImaging CCD camera with QCapture software (QImaging, Burnaby, Canada).

RESULTS

At subsequent stages of development expression patterns of PDGF-A, PDGF-C and their receptor PDGFR-α were analysed in the chicken heart (Figure 1). In addition, we examined embryos in which we mechanically inhibited epicardial outgrowth as well as embryos treated with Imantinib to block PDGF-signalling. In both experimental designs the role of epicardial-myocardial interaction in relation to PDGF was investigated.

Expression of PDGF-A and PDGF-C

At Hamburger and Hamilton stage (HH) 17, staining of PDGF-A and PDGF-C was present in the complete myocardium of the heart, comprising the sinus venosus, the atrium, the ventricle, and the outflow tract (OFT) (Appendix Figure 1). The PEO as well as the villous protrusions of the PEO connecting to the myocardium were also positive for both ligands.

At stage HH20, the epicardium and the developing atrial septal myocardium had also become positive for PDGF-A and –C (Appendix Figure 1). The expression levels in the PEO were decreased in comparison to HH17.

At HH22, the myocardial wall of the sinus venosus, including the sino-atrial node (SAN) region as well as a cluster of cells medial to the left cardinal vein (LCaV), expressed both ligands comparable to stage HH20. In the atrioventricular (AV) cushions, the mesenchymal cell population contained positive and negative cells for both PDGF-A and –C. The expression pattern in the OFT cushions was comparable to that in the AV-cushions. The ventricular compact and trabecular myocardium could be discerned separately at this stage and both layers were positive for PDGF-A and –C. Less cells were positive in the epicardium and PEO compared to HH20.

At stage HH25, the trabecular myocardium of the ventricle showed more marked expression of PDGF-A and –C compared to the compact myocardium, especially in the inner part of the trabecular myocardium. This phenomenon was more distinct for PDGF-A than for PDGF-C (Appendix Figure 1). This became more clear near the apex. The myocardium of the developing ventricular septum was positive for both ligands. The number of mesenchymal cells in the AV and OFT cushions that stained positive for both ligands was increased compared to previous stages, especially more cells in the subendocardial region of the cushions had become positive. The epicardial cells, fully covering the ventricular myocardium, and the epicardium-derived cells (EPDCs) in the subepicardium showed a mosaic pattern for PDGF-A and –C expression. Compared to previous stages, there was an ongoing decrease in the number of positive cells in these two layers.

At HH28, the expression of both ligands in the atrial wall was comparable to earlier stages. There was a slightly more intense PDGF-A staining in the atrial septum (Figure 2d), while the expression of PDGF-C was not altered (Figure 2a,g). The cell population of the AV and OFT cushion mesenchyme showed more positive cells near the subendocardial border compared to stage HH25. A more marked expression of PDGF-C in the ventricular trabecular myocardium was observed compared to the compact myocardium (Figure 2b,h). The expression of PDGF-A decreased in both layers of the myocardium, being still more distinct in the trabecular myocardium (Figure 2e). There was also a decrease in expression of both ligands in the OFT myocardium at HH28 (Figure 2c,f,i). The PEO had become negative for PDGF-A and –C. The subepicardium and epicardium, referred to hereafter as (sub)epicardium, showed less positive cells for both ligands compared to HH25.

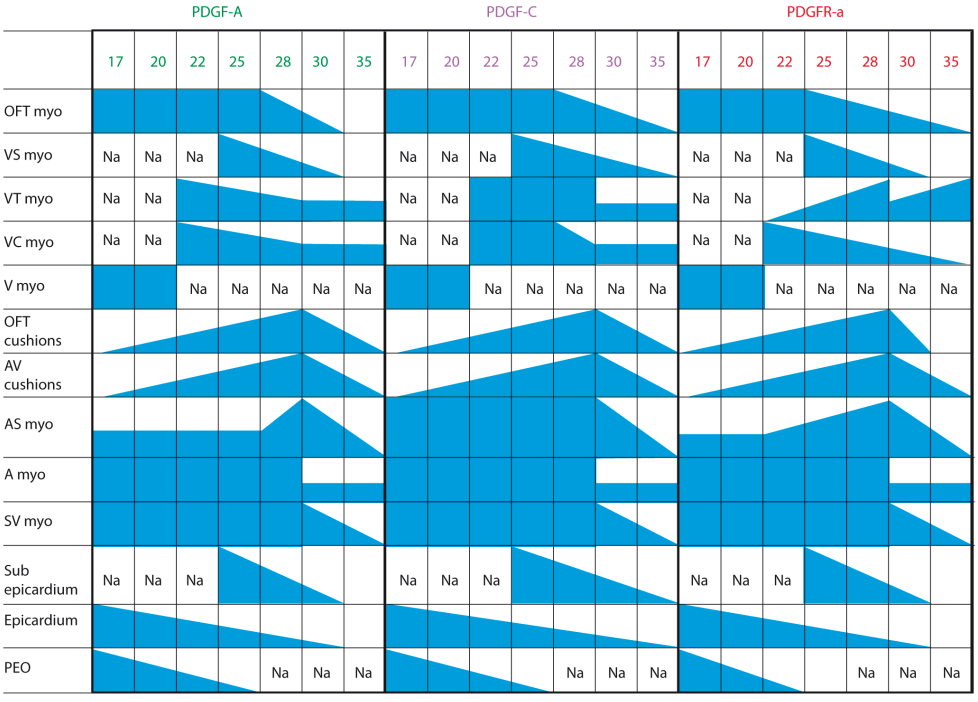


Figure 1. Summary of the spatiotemporal protein expression of PDGF

Platelet-derived growth factor (PDGF)-A, PDGF-C and their receptor PDGFR-α are expressed in the avian heart. This table displays alterations in protein expression per heart area at different Hamburger and Hamilton (HH) stages. Filled squares represent maximum intensity at that stage for the specific compartments. Partially filled squares represent increase and decrease of expression. A, atrial; AS, atrial septum; AV, atrioventricular; myo, myocardium; Na, not applicable; OFT, outflow tract; PEO, proepicardial organ; V, ventricular; VC, ventricular compact; VS, ventricular septum; VT, ventricular trabecular, SV, sinus venosus.

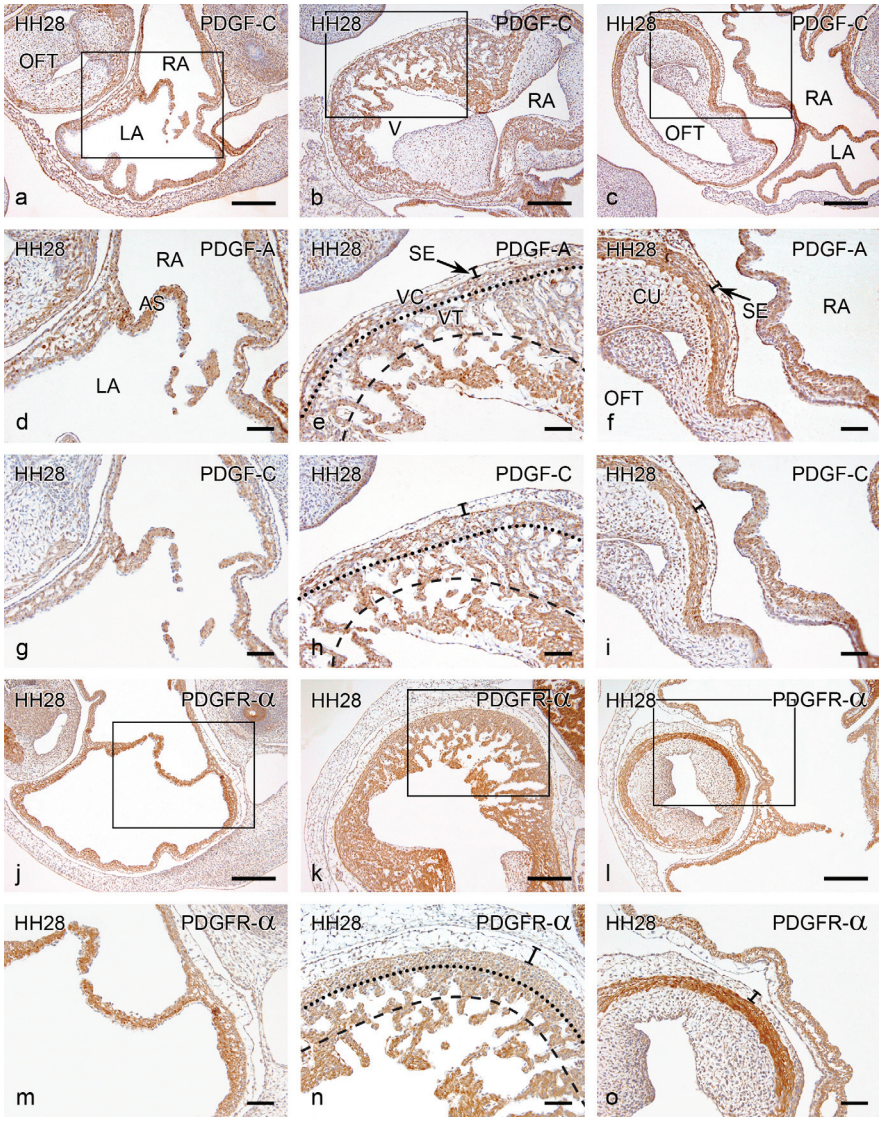


Figure 2. Expression of platelet-derived growth factor (PDGF)-A, -C and PDGFR-α in chicken hearts of Hamburger and Hamilton (HH) stage 28

Photomicrographs of representative transverse sections show expression of PDGF-A, -C and their receptor in the atrial wall and atrial septal (AS) myocardium (a,d,g,j,m). Boxed regions in lower magnification panels correlate to higher resolution images. The expression of PDGF-A and its receptor is more distinct in the AS myocardium compared to the myocardium of the atrial wall (d,j,m). PDGFR-α and its ligands are expressed more prominently in the ventricular trabecular (VT) myocardium compared to the ventricular compact myocardium (VC) (delineated with a dotted line) (b,e,h,k,n), especially in the inner part of the VT (delineated with a dashed line). Staining of PDGFR-α is more marked in the outflow tract (OFT) myocardium in comparison to the atrial myocardium (l,o). For the ligands the staining is comparable between the OFT and atrial myocardium (c,f,i). LA, left atrium; RA, right atrium; SE, subepicardium; CU, cushions. Scale bars: 200 μm (a-c, j-k), 60 μm.

At stage HH30, a clear decrease in PDGF-A and –C expression in the complete heart was observed. In the ventricular septal myocardium staining of PDGF-A had become weak in comparison to the ventricular compact and trabecular myocardium. In the AV and OFT cushion mesenchyme expression of both ligands was diminished. The majority of the cells of the (sub)epicardium were negative at this stage.
At the last stage we analysed, HH35, the expression of both ligands in the wall of the sinus venosus including the SAN region was decreased compared to HH30. The expression in the atrial wall was comparable to the previous stage, while the expression of both PDGF-A and –C was diminished in the atrial septum. The decrease of expression of both ligands in the AV and OFT cushion mesenchyme continued at this stage. The expression patterns of the ventricular compact and trabecular myocardium had not changed compared to stage HH30. The ventricular septum was negative for PDGF-A, while the expression of PDGF-C in the ventricular septum had decreased at this stage. A similar observation was made for the OFT myocardium. All the cells of the (sub)epicardium were negative for PDGF-A, while some cells were still positive for PDGF-C.

Cardiac expression of PDGFR-α

To investigate the relation between PDGF-A and PDGF-C in heart development, we analyzed the protein expression of their common receptor PDGFR-α at the same developmental stages. At stage HH17 and HH20 the receptor colocalized with its ligands spatiotemporally in all previously described parts of the heart (Figure 1 and Appendix Figure 1).
At stage HH22, colocalization of PDGFR-α with both ligands was seen in the myocardial wall of the sinus venosus including the SAN region, the atrial myocardium, the cells of the AV and OFT cushion mesenchyme, both the compact and trabecular myocardium of the ventricles and the OFT myocardium. At this stage, the expression of the receptor was increased in the atrial septum compared to HH20. In the PEO and the epicardium the expression of PDGFR-α colocalized to both PDGF-A and –C and was decreased in comparison to previous stages.
At HH25, receptor expression was further increased in the atrial septum (Appendix Figure 1). The expression pattern of the receptor in the mesenchymal cells of the AV and OFT cushions was comparable to that of both ligands. In the ventricular myocardium, decreasing PDGFR-α levels in the compact myocardium coincided with increasing levels in the trabecular zone compared to HH22. The myocardium of the developing ventricular septum was positive. In general, the staining signal of the receptor was more dense in the OFT myocardium in comparison to that of the atrial myocardium, while for the ligands the staining levels were comparable between the OFT and atrial myocardium (Appendix Figure 1). The expression of receptor-α was decreased in the OFT myocardium compared

to HH22. At this stage, the PEO was negative for PDGFR-α. The (sub)epicardium showed both cells that were positive or negative like the staining for the ligands.
At stage HH28, the staining of PDGFR-α was comparable to HH25, as there was still a marked expression of receptor-α staining in the atrial septum compared to the atrial wall (Figure 2j,m). More cells in the subendocardial region of the AV and OFT cushion mesenchyme were positive compared to stage HH25. In the ventricular compact myocardium the expression of PDGFR-α decreased compared to the increasing expression in the trabecular myocardium (Figure 2k,n). There was also a decrease of expression in the OFT myocardium The staining signal of the receptor was still more marked in the OFT myocardium in comparison to the atrial myocardium (Figure 2l,o), while for the ligands the expression levels were still comparable between the OFT and atrial myocardium at this stage (Figure 2c,f,i). In the (sub)epicardium less cells were positive for the receptor compared to HH25.
Comparable to the expression patterns of PDGF-A and –C at stage HH30, there was a decrease in PDGFR-α expression in the complete heart. The contrast between PDGFR-α expression in the OFT myocardium and atrial myocardium had disappeared. Furthermore, the contrast between ventricular septal myocardium and both layers of the ventricular myocardium was comparable to PDGF-A at this stage. In the AV and OFT cushion mesenchyme the decrease in expression was comparable to both ligands. The majority of the cells of the (sub)epicardium were negative at this stage.
At stage HH35, the expression of PDGFR-α in the wall of the sinus venosus including the SAN region, AV and OFT cushion mesenchyme decreased compared to HH30. The expression pattern of the atrial wall and in the atrial septum was comparable to both ligands. In the (sub)epicardium PDGFR-α was absent at this stage, comparable to PDGF-A. The expression levels in the trabecular myocardium were increased compared to those of stage HH30, while the expression in the compact myocardium decreased. Furthermore, the ventricular septum was negative comparable to PDGF-A. A decrease in PDGFR-α staining was also observed in the OFT myocardium.

PDGF expression related to the developing venous pole

The expression of PDGFs in the OFT myocardium and in the myocardial wall of the sinus venosus suggested a role of PDGFs in the development of the second heart field. To further investigate expression in the SHF in more detail, we focussed on the expression of PDGFR-α and the ligands PDGF-A and -C, specifically in the venous pole region. As myocardium at the venous pole region was characterized by the presence of MLC-2a and absence of Nkx2.5, we stained serial sections of HH22 embryos with antibodies against these proteins. WT1 staining was used to identify epicardium and EPDCs that are derived from the PEO, developing in this region in

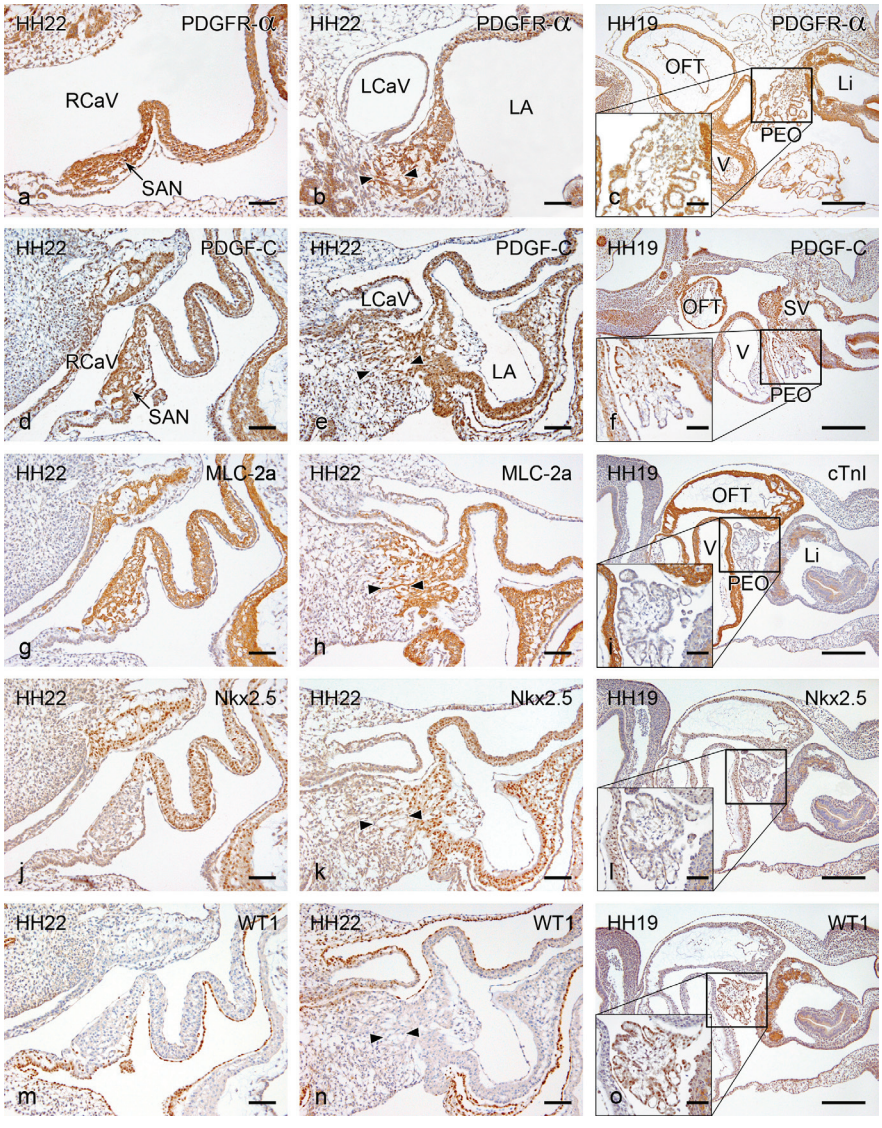


Figure 3. Platelet-derived growth factor (PDGF) is expressed in myocardium derived from the venous pole area of the heart

Photomicrographs show representative transverse sections of the left and right cardinal vein (LCaV, RCaV), sinoatrial node (SAN) region, proepicardial organ (PEO) and epicardium. Panels d-f show expression of PDGF-C and panels a-c the expression of its receptor PDGFR-α. Panels g-h show expression of MLC-2a in the myocardium surrounding the LCaV, RCaV, developing SAN and sinus venosus myocardium (marked by arrowheads). The PEO and epicardium are marked by WT1 expression (o). There is no expression of the myocardial marker cTnI (i) and of Nkx2.5 in the PEO (l). At stage HH19 the PEO and epicardium (c,f) are positive for PDGFR-α and its ligand PDGF-C. Panels j-l show negative Nkx2.5 staining in the wall of the LCaV, RCaV and developing SAN. Arrowheads show the Nkx2.5 negative zone of the sinus venosus (SV) myocardium (k). OFT, outflow tract; V, ventricle; LA, left atrium; SV, sinus venosus; Li, liver. Scale bars: 60 μm (a-b,d-e,g-h,j-k,m-n), 20 μm in magnification box (c,f,i,l,o).

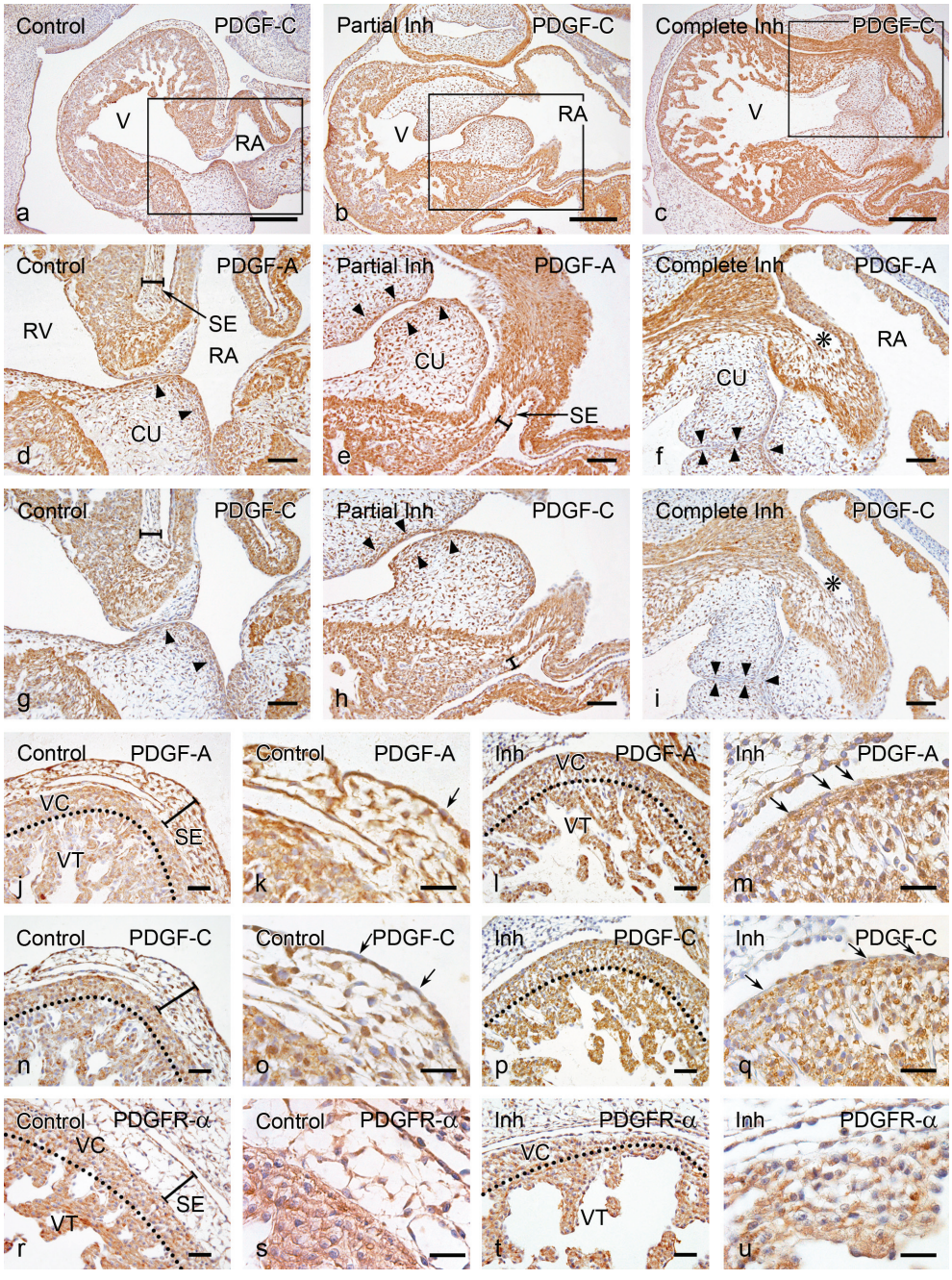
HH19 embryos. In the myocardial wall of the sinus venosus including the developing SAN, both ligands and their receptor were present in MLC-2a positive (Figure 3a,b,d,e,g,h), and Nkx2.5 (Figure 3j,k) negative areas. In the sinus venosus myocardium and in the wall of the cardinal veins including the developing SAN (data not shown), Isl-1 positive cells were also observed. The developing SAN also expressed Tbx18 (data not shown). WT1 positive cells of the PEO were also positive for PDGFR-α and its ligands (Figure 3c,f,o). Furthermore, the cells of the PEO lack both the myocardial markers cTnI and Nkx2.5 (Figure 3i,l). In situ hybridization data show that the PEO and the outgrowing epicardium expressed higher levels of *Pdgf-a* and *Pdgfra* compared to the surrounding tissues (Appendix Figure 2).

Effect of mechanical inhibition of epicardial outgrowth on PDGF expression

The expression of PDGF-A and PDGF-C in the PEO and in the epicardium suggested a possible role in development of the compact myocardium of the ventricles. Therefore, we performed inhibition of epicardial outgrowth at stage HH15-HH18 embryos, to investigate the effect on the expression patterns of both ligands and the receptor at several timepoints (HH20-HH28). Investigation of the collected embryos revealed that there were no marked morphological differences between controls and embryos with inhibited epicardial outgrowth at all timepoints until HH25 (data not shown). At stage HH25 and HH28, subepicardium covering the ventricular myocardium was absent in nine embryos. As the two stages were phenotypically comparable, we described the expression of PDGF-A, -C and their receptor PDGFR-α at stage HH28.

Partial inhibition of epicardial outgrowth was observed in 5 hearts. These hearts showed absence of subepicardium covering the ventricular myocardium, but have some subepicardium in the sulcus area (Figure 4b,e,h). Using immunohistochemistry, the expression of PDGFR-α was investigated in two hearts with partial inhibition of epicardial outgrowth. Expression of the receptor in the compact and trabecular layer of the ventricular myocardium of these embryos was less intense in comparison to the control embryos (Figure 4r-u),

Two hearts had a complete inhibition of epicardial outgrowth, as there was no subepicardium in the sulcus area (Figure 4c,f,i) and covering the ventricular myocardium, and these were used to investigate expression of the ligands. In the subendocardial region of the AV cushions of embryos with a complete inhibited outgrowth of epicardium, there was a decrease in number of cells that expressed PDGF-A and PDGF-C (Figure 4c,f,i). This decrease was more marked for PDGF-C compared to PDGF-A. In the AV cushions of hearts with a partial inhibition of epicardial outgrowth this decrease in expression was not observed (Figure 4b,e,h).


Control
PDGF-C
V
RA
a
Partial Inh
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b
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c
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d
Partial Inh
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Complete Inh
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There was no subepicardium covering the ventricles of embryos with either partial or complete inhibition of epicardial outgrowth (Figure 4l,m,p,q). Expression of PDGF-A and –C in the compact and trabecular layer of the ventricular myocardium of these embryos was more intense in comparison to the control embryos (Figure 4j-q), showing also less cells forming the lining epicardium. The few remaining epicardial cells were mostly negative for the ligands, compared to controls presenting more positive epicardial cells (Figure 4k,m,o,q).

Pharmacochemical inhibition of PDGF-signalling

As PDGFs were expressed in the PEO and (sub)epicardium and PDGFs were altered with mechanical inhibition of epicardial outgrowth, we examined the effect of pharmacochemical inhibition of PDGF-signalling using Imantinib to elucidate a functional role of PDGFs in epicardial-myocardial interaction.

At stage HH25-27, Imantinib treated embryos demonstrated absence of subepicardium (33%) covering the ventricular myocardium, comparable with the embryos with inhibition of epicardial outgrowth (Appendix Figure 3). The epicardial layer was in several areas not properly attached to the myocardium (Appendix Figure 3). The dose-dependent incidence of other defects, comparable with defects in *PDGFR*-α deficient mice [22,44] including neural tube defects and subepidermal hemorrhages, in these Imantinib-treated embryos suggested that PDGF-signalling was being inhibited.

Figure 4. Expression of PDGF–A, and -C at stage HH28

Transverse sections show control chicken hearts (a,d,g,j,n,r) and of hearts with partial (b,e,h) and complete inhibition of epicardial outgrowth at stage HH28 (c,f,i,j,l,m,p,q,t,u). Boxed areas in pictures a,b,c correlate to magnified areas in pictures d-i. Photomicrographs show representative sections of the atrioventicular (AV) groove, the atrioventricular cushions (CU), right ventricle (RV) and right atrium (RA). Subepicardium (SE) in the AV-groove is absent in the heart of embryos with a complete inhibited epicardial outgrowth (asterisk). In the control embryos and embryos with a partial inhibition of epicardial outgrowth the AV-groove is covered with SE (marked with thickness bar). The expression of PDGF-A (e) and –C (b,h) is located specifically in cells of the subendocardial region of the atrioventricular cushions mesenchyme in controls and embryos with a partial inhibition. In contrast, in the embryos with complete inhibition of epicardial outgrowth we observe a decrease in expression of PDGF-A (arrowheads in e) and PDGF-C (arrowheads in k) in this region. Subepicardium covering the ventricles is absent in embryos with either partial or complete inhibition of epicardial outgrowth (l,m,p,q,t,u). In these embryos less cells formed the lining epicardium and relatively fewer cells are positive for PDGF-A and –C (arrows in panels m,q). In the compact (VC) and trabecular (VT) layer of the ventricular myocardium, delineated with a dotted line, of the embryos with either partial or complete inhibition of epicardial outgrowth we observe a higher expression of PDGF-A (l,m) and –C (p,q) and less expression of PDGFR-α (t,u) compared to the control embryos (j,k,n,o,r,s). Scale bars: 200 μm (a-c) 60 μm (d-i), 30 μm (j,l,n,p,r,t) and 20 μm (k,m,o,q,s,u).

DISCUSSION

We described cardiac expression patterns of PDGF-A, -C and their receptor PDGFR-α in the developing avian embryo, highlighting stages HH17-HH35. Furthermore, we have demonstrated that inhibition of epicardial outgrowth alters expression of both ligands and their receptor in the ventricular myocardium. We also showed abnormal epicardial development after PDGF inhibition supporting a functional role for PDGFs in epicardial development.

At the venous pole, the sinus venosus myocardium is derived from a specific area of the second heart field, for which we reserved the term posterior heart field (the PHF) as opposite to anterior pole [3]. The myocardial component of this specific venous pole area is characterized by the expression of the myocardial marker MLC-2a combined with lack of staining for Nkx2.5 [3,43]. The expression of MLC-2a colocalized with both ligands and their receptor in areas that were Nkx2.5 negative. An important role for PDGF in the development of the venous pole and the sinus venosus myocardium-derived structures is supported by the *Patch* (spontaneous mutation of the PDGFR-α gene) mutant mice showing abnormalities of the cardinal veins [18,44] and atrial septal defects [30]. Atrial septal defects are also observed in *Pdgfc*$^{-/-}$*Pdgfa*$^{-/-}$ double mutant mice [30,45]. We showed increase in expression of PDGFR-α in the developing atrial septum from stage HH22 onwards, further supporting the hypothesis that PDGFR-α is important herein.

A role for PDGFs in the development of the AV-cushions is suggested by the staining for both of the ligands and their receptor in these structures. PDGFR-α is crucial, because *Patch* mutant mice fail to develop AV-cushions [44]. We also see a diminished expression of PDGF-A and –C in the AV-cushions of our embryos with complete inhibition of epicardial outgrowth. This is probably caused by impaired migration and subsequent impaired cell contribution of EPDCs to the AV-cushions [11]. Normally, EPDCs invade the cushion mesenchyme directly and may influence EMT of the endocardium [9-11,14]. As embryos with partial inhibition of epicardial outgrowth have a subepicardium in the AV region and EPDCs are still present, this might explain why in these embryos the expression patterns of PDGF-A and –C are not affected.

The venous pole area not only provides myocardial progenitor cells to the heart, but most probably contributes mesenchymal progenitor cells to the PEO and subsequent epicardium and EPDCs [4]. Although the PEO and epicardium are positive for PDGF-A and –C, it is most likely that both ligands were not required for this contribution from the venous pole area as no alterations in PEO and epicardium development have been described or investigated in *Pdgfa*, *Pdgfc* and *Pdgfc*$^{-/-}$*Pdgfa*$^{-/-}$ double mutants [45]. PDGFR-α was also expressed in the PEO and the epicardium until HH25. A role for PDGFR-α in epicardial development is supported by the finding that embryos treated with Imantinib have no

subepicardium covering the ventricular myocardium. Furthermore, *Patch* mutants show a thin myocardium and malformed AV-cushions [30]. Impaired PDGF-B/PDGFR-α-signalling could be at the basis of this altered epicardial outgrowth, because PDGF-B mutants have extreme myocardial hypoplasia [19] and PDGF-B is able to induce epicardial EMT *in vitro* [46]. A role for PDGF-B/Pdgfrβ-signalling cannot be ruled out for altered epicardial outgrowth, because Imantinib also blocks other RTKs including PDGFR-β and the KIT proto-oncogene [40].

The expression of PDGF-A, -C and receptor-α in the epicardium and the ventricular compact myocardium, supported by the fact that PDGF-A is an epicardial mitogen during heart development [26], suggest that the ligands induce cardiomyocyte proliferation and proliferation of the epicardium in a paracrine and/or autocrine fashion. *Pdgfc*$^{-/-}$ *Pdgfa*$^{-/-}$ double mutant mice revealed that PDGF-A and PDGF-C were not required for normal ventricular compact zone development [45]. In contrast, PDGFR-α knockout mice show hypoplastic hearts probably due to decreased proliferation-rate of cardiomyocytes possibly through the lack of PDGF-B/Pdgfrα-signalling [19]. Cardiomyocyte proliferation alone is probably insufficient for proper ventricular myocardial development, it is suggested that interstitial fibroblasts are necessary for establishing the myocardial architecture [4,7,34]. PDGF-A is a potent mitogen for cardiac fibroblasts [47] and overexpression of PDGF-C in mice at 3 months of age upregulated PDGFR-α mRNA and increased fibroblast proliferation leading to cardiac fibrosis and hypertrophy [36]. These data combined with the expression patterns of PDGF-A and –C and their receptor in the epicardium suggest a functional role for epicardium-dependent development of the ventricular myocardium for both ligands and their receptor. This is supported by our experimental data, in which the expression of both ligands and their receptor is altered in trabecular and compact ventricular myocardium of embryos with inhibited epicardial outgrowth as well as by the absence of subepicardium in Imantinib treated embryos.

We observed that both ligands and their receptor lose their subepicardial expression over time, during normal heart development when the subepicardium is formed between the epicardium and myocardium. In embryos with an inhibited epicardial outgrowth the subepicardium covering the ventricles was absent and a higher myocardial expression of ligand-A and –C and a lower myocardial expression of PDGFR-α was observed compared with control embryos. In the embryos treated with Imantinib there was no subepicardium. Therefore, in both experiments EPDCs were not able to migrate into the ventricular myocardium, resulting in abnormal interaction between epicardium and myocardium, which in turn could influence expression of both ligands and the receptor.

EPDCs that migrate into the myocardium are WT1 positive [14] and transient transfection assays demonstrated a WT1-mediated-repression of PDGF-A. The absence of WT1 positive EPDCs in the myocardium could therefore explain the increase of PDGF-A expression in

embryos with inhibited epicardial outgrowth [48]. Recent data in mice showed that PDGFR-α is expressed by epicardial cells after they have migrated into the myocardium [24]. We suggest that in chicken embryos lacking subepicardium the altered expression of the receptor in the ventricular myocardium is caused by a markedly diminished number of EPDCs in the ventricle.

In the ventricular trabecular myocardium expression of PDGF-A and receptor-α became more restricted during subsequent stages [18]. Stimulation of trabecular formation by PDGF-A/PDGFR-α is suggested by impaired trabecular formation in the *Patch* mouse [44]. Expression of PDGF-A and –C in the ventricular septum suggests an activating role for PDGFR-α in the contribution of myocardium to the developing septum as *Patch* mice suffer from ventricular septal defects [30].

The PDGF-expression patterns in the OFT myocardium and cushion mesenchyme indicate the recruitment of cells from the second heart field. These expression patterns correlate with preferential sites of cardiac malformations in the *Patch* mouse [30,44]. At chicken stage HH28 when expression of ligand-A and -C are decreased, we postulate that the addition of cells to the OFT myocardium and cushion mesenchyme from the second heart field seems to be completed. Alternatively, the role of PDGF in this contribution had become less important.

In conclusion, spatiotemporal expression patterns of PDGF-A and PDGF-C indicate that these ligands are overlapping and probably redundant in heart development. Expression of PDGF-A and PDGF-C and their receptor PDGFR-α suggests an activating role in remodelling of the myocardium of both outflow and inflow tract. Furthermore, PDGF-A, -C and PDGFR-α are involved in the remodelling of the ventricular compact and trabecular myocardium through epicardial-to-myocardial interaction. There might be an EPDC-related contribution of PDGF to the development of the AV-cushions. As a consequence, PDGF-A, -C and their receptor PDGFR-α are important in several processes of cardiac tissue remodelling during heart development.

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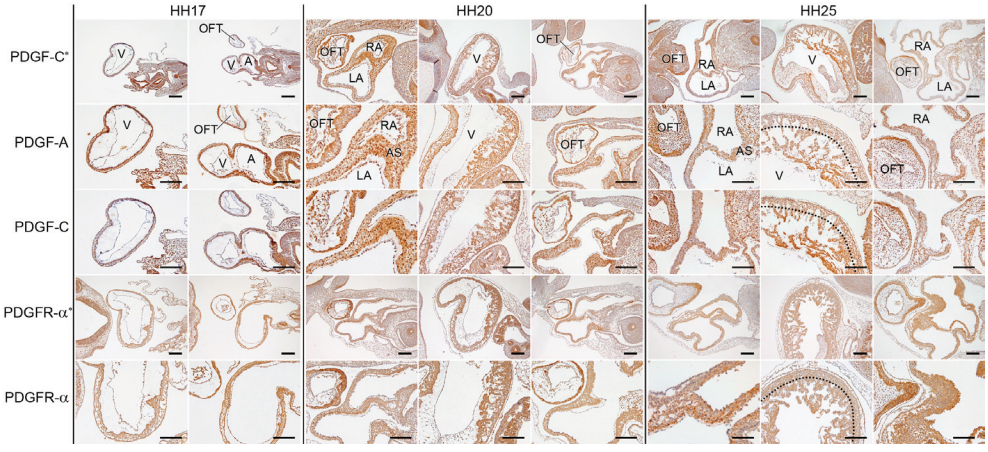
APPENDIX

RESULTS

In the current study we have investigated the protein expression of PDGF-A, -C and their receptor PDGFR-α during several stages of development in the chicken heart (Appendix Figure 1). Furthermore, we have performed in situ hybridizations of stage HH18 to show expression of PDGF-A and its receptor in the PEO and epicardium (Appendix Figure 2). To elucidate the role of PDGFR-α in the development of the compact myocardium of the ventricles, PDGFR-α was inhibited with the use of the receptor tyrosine kinase (RTK) inhibitor Imantinib (ST1571) (Appendix Figure 3).

Expression of PDGF-A, PDGF-C and PDGFR-α

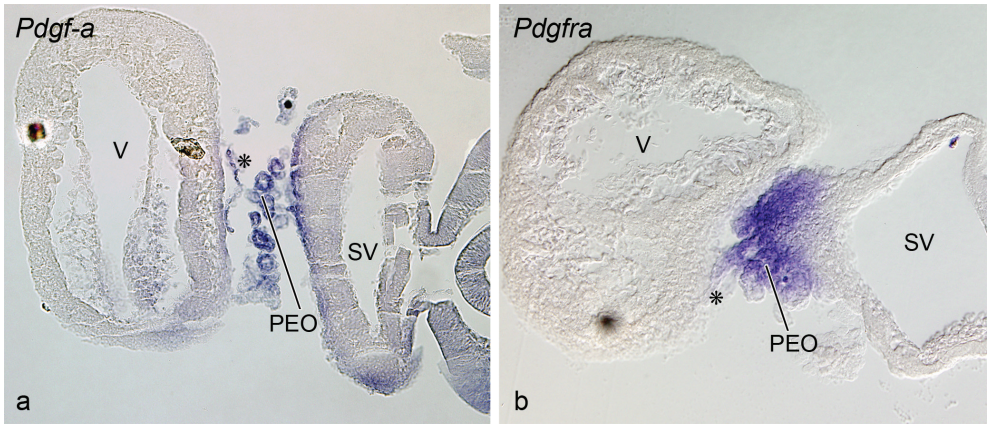
Photomicroscopic representations of protein expression of PDGF-A, -C and PDGFR-α in chicken hearts of stages HH17, HH20 and HH25 (Appendix Figure 1). These pictures support the summary of the spatiotemporal protein expression of both ligands and their receptor in the avian represented in Figure 1 in the manuscript.



Appendix Figure 1. Expression of PDGF-A, -C and PDGFR-α in chicken hearts of stage HH17, HH20 and HH25
Photomicrographs of representative transverse sections show expression of PDGF-A, -C and their receptor at the region of the atrial septum (AS) myocardium, the OFT myocardium and in the ventricular myocardium. The dotted line in HH25 represents the boundary between ventricular compact and trabecular myocardium. A, atrium; AS, atrial septum LA, left atrium; OFT, outflow tract; RA, right atrium; V, ventricle. Scale bars: 200 μm (PDGF-C* and PDGFR-α*) and 60 μm (PDGF-A, PDGF-C, PDGFR-α).

In Situ Hybridization

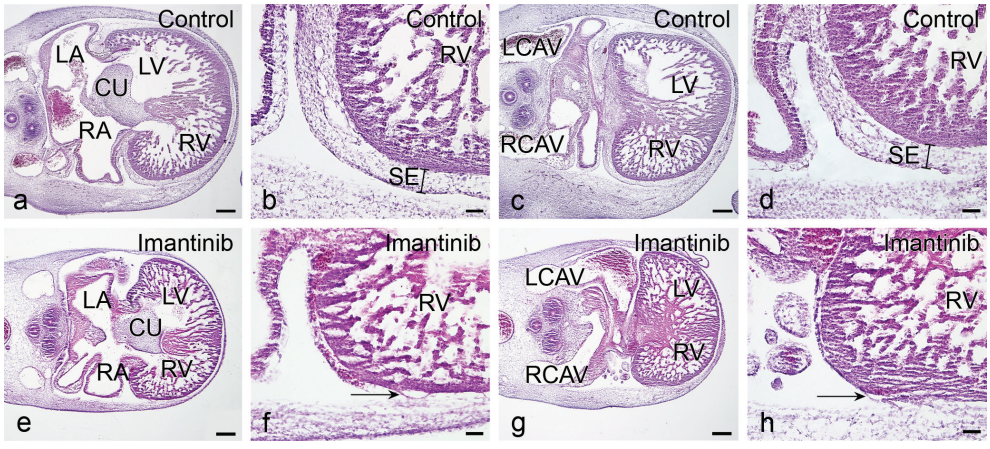
We examined expression of *Pdgfra* and its ligand *Pdgf-a* in the PEO and epicardium of chicken embryos of stage HH18. Both *Pdgfra* (Appendix Figure 2a) as well as *pdgf-a* (Appendix Figure 2b) were expressed in the PEO and outgrowing epicardium.



Appendix Figure 2. PDGF-signalling in the PEO and epicardium
At stage HH18 the PEO and outgrowing epicardium (asterisk) express *Pdgf-a* (a) and *Pdgfra* (b). PEO, proepicardial organ; V, ventricle; SV, sinus venosus. Scale bars: 50 µm.

PDGF-blockade is associated with altered epicardial development

Chicken embryos were treated with Imantinib to inhibit PDGFR-α during cardiac development. In 33% of the embryos treated with Imantinib the subepicardium covering the ventricular myocardium was absent. Also in several areas the epicardium was not attached properly to the myocardium compared to the controls (Appendix Figure 3b,d,f,h).



Appendix Figure 3. Altered epicardial development with Pdgfra blockade

In ovo blockade of *Pdgfra* with Imantinib causes altered epicardial development in 33% of the embryos (e-h) compared to the controls (a-d). Altered epicardial development displays absence of subepicardium covering the ventricular myocardium and epicardium which is not properly attached to the myocardium (arrows). CU, cushions; LA, left atrium; LV, left ventricle; RA, right atrium; RV, right ventricle; SE, subepicardium. Scale bars: 200 µm (a,c,e,g) and 50 µm (b,d,f,h).