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PDGF-A and PDGF-B induces cardiac fibrosis in transgenic mice

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ABSTRACT

Platelet-derived growth factors (PDGFs) and their receptors (PDGFRs) contribute to normal heart development. Deficient or abnormal expression of *Pdgf* and *Pdgfr* genes have a negative impact on cardiac development and function. The cellular effects of PDGFs in the hearts of *Pdgf/Pdgfr* mutants and the pathogenesis of the resulting abnormalities are poorly understood, but different PDGF isoforms induce varying effects. Here, we generated three new transgenic mouse types which complete a set of studies, where all different PDGF ligands have been expressed under the same heart specific alpha-myosin heavy chain promoter.

Transgenic expression of the natural isoforms of *Pdgfa* and *Pdgfb* resulted in isoform specific fibrotic reactions and cardiac hypertrophy. *Pdgfa* overexpression resulted in a severe fibrotic reaction with up to 8-fold increase in cardiac size, leading to lethal cardiac failure within a few weeks after birth. In contrast, *Pdgfb* overexpression led to focal fibrosis and moderate cardiac hypertrophy. As PDGF-A and PDGF-B have different affinity for the two PDGF receptors, we analyzed the expression of the receptors and the histology of the fibrotic hearts. Our data suggest that the stronger fibrotic effect generated by *Pdgfa* overexpression was mediated by *Pdgfr*α in cardiac interstitial mesenchymal cells, i.e. the likely source of extracellular matrix depostion and fibrotic reaction. The apparent sensitivity of the heart to ectopic PDGFRα agonists supports a role for endogenous PDGFRα agonists in the pathogenesis of cardiac fibrosis.

1. Introduction

Cardiac fibrosis is characterized by excessive production of extracellular matrix proteins such as collagens and fibronectin deposited by activated fibroblasts (a.k.a. myofibroblasts). These cells accumulate at sites of injury or inflammation in response to locally released fibrogenic mediators. The origin of cardiac myofibroblasts is unclear but may potentially involve multiple sources, such as cardiac fibroblasts, fibroblast progenitors, vascular mural cells, epicardial epithelium and endothelial cells (reviewed by [1,2]). Accumulation of extracellular matrix proteins in the cardiac interstitium causes myocardial stiffness and ventricular dysfunction. Organ failure due to fibrosis is indeed the major cause of death from inflammatory diseases. Unfortunately, therapies directly targeting fibrosis or its pathogenesis are still limited (reviewed by [3,4]).

Several molecular mediators are active during cardiac fibrosis, one of them being the platelet-derived growth factors (PDGFs). PDGF signalling has been implicated in fibrosis of different organs, such as lung, liver, skin, kidney and heart (reviewed by [5]). PDGFs affect multiple cellular functions, such as cell proliferation, differentiation, cytoskeletal rearrangements and cell migration including chemotaxis. In normal vertebrates, members of the PDGF family are widely expressed throughout the body and play roles both during organogenesis and during disease processes. To-date, four PDGF ligands have been identified (PDGF-A, -B, -C and -D), which form four homodimers (AA, BB, CC and DD) and one heterodimer (AB) that bind to and activate two different tyrosine kinase receptors (PDGFRα and -β) with different affinity. A wide variety of potential ligand-receptor interactions have been demonstrated in vitro, but not all have been confirmed in developmental in vivo studies of knockout mice (reviewed by [5]). In general, PDGF-A and -C bind to PDGFRα, and PDGF-B and -D bind to PDGFRβ in vivo.

All PDGFs have been reported to influence heart development. Endothelial cells express PDGF-B and -D, whereas vascular mural cells (smooth muscle cells and pericytes) express PDGFRβ. Genetic loss-of-function of PDGF-B or PDGFRβ in mice lead to a hypoplastic myocardium that lack vascular smooth muscle cells [6,7], whereas deletion of PDGF-D causes only a mild vascular phenotype in the heart

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[8]. PDGF-A and -C are both expressed by myocardial cells, whereas PDGFRα-positive interstitial cells have been identified in the epicardium, myocardium and endocardium. PDGFRα signalling is needed during the establishment of the second heart field-derived structures, such as ventricular septum, epicardial cells, epicardial-derived fibroblasts, second heart field-derived myocardium, epicardial-mesenchymal derivates, cardiac neural crest cells, sinus venosus and outflow tract [6,9–13].

Several reports point to a surprising complexity in the cardiac responses to PDGF/PDGFR signalling, suggesting that fibrogenic responses to PDGFs may be both model- and context-dependent. A primary general increase in PDGFRα activation in mice leads to multi-organ fibrosis, including in the heart [14], whereas increased PDGFRβ activation does not [15]. In a heterotypic heart transplantation model in rats, administration of adenoviruses expressing PDGF-A, -C and -D led to accelerated cardiac fibrosis and chronic rejection [16]. Likely, myocardial injection of adenoviruses expressing different PDGFs results in an increased inflammatory reaction through PDGFRα activation. Moreover, PDGFRα and -β neutralizing antibodies were shown to attenuate the response to myocardial infarction, including decreased collagen deposition and impaired neovessel maturation [17]. By administrating adenoviruses expressing different PDGF isoforms to the heart of adult mice, we recently described that whereas PDGF-B aggravated the adenovirus-induced inflammation PDGF-D attenuated it, suggesting that different modes of activation of the same receptor may result in seemingly opposite effects [18].

Overexpression of PDGF-C or -D from the α-myosin heavy chain promoter (α-MHC) induces cardiac fibrosis in transgenic mice [19,20]. These mice were viable but developed hypertrophic hearts with signs of dilated cardiomyopathy, proliferation of interstitial fibroblasts and increased deposition of extracellular matrix. In addition, they developed malformed vascular networks with decreased capillary density and dilated blood vessels with increased α-smooth muscle actin (ASMA) expression. Here, we have generated transgenic mice overexpressing PDGF-A_{short}, PDGF-A_{long} and PDGF-B from the same α-MHC promoter and phenotypically characterized their hearts. We also analyzed the expression of PDGF receptors in developing and adult mouse hearts. Our results confirm a PDGF isoform-specific effect to induce cardiac fibrosis, and further suggest a major role for PDGFRα-positive cells in pathological cardiac fibrotic responses.

2. Materials and methods

2.1. Ethics statement

This study was carried out in strict accordance with applicable standards. The protocols for this study were approved by the Committees on the Ethics of Animal Experiments in Gothenburg (permit numbers 81/97, 64-2000) and Stockholm North (permit numbers N33/10, N15/12). Diet and water were provided ad libitum, and standard light cycles were used. All efforts were made to minimize animal suffering.

Transgenic mice were monitored daily, and mice that showed any signs of disease were sacrificed. When the first mouse was suddenly found dead, our daily observation was intensified. One mouse showed clinical symptoms, and was euthanized and sacrificed with perfusion fixation through the heart. Anesthesia was administered with i.p. injection of avertin. The remaining mice were judged healthy, until they all suddenly were found dead. As all mice belonged to the founder generation, they were principally different and we had no reasons to believe that they were all going to die within a few days. Criteria for humane endpoints followed an internal template based on the following documents; NIH Guidelines for Pain and Distress in Laboratory Animals: Responsibilities, Recognition and Alleviation, ARAC – 03/08/00, Morton DB; A systematic approach for establishing humane endpoints. [21]; Guidelines on the recognition of pain, distress and discomfort in experimental animals and an hypothesis for assessment. [22]; Recognizing and assessing pain, suffering and distress in laboratory animals: a survey of current practice in the UK with recommendations, P. Hawkins, Laboratory Animals (2002); 36.

3. Generation of transgenic mice

Transgenic mice were produced by pronuclear injection of the DNA constructs schematically outlined in Fig. 2. The α-MHC promoter [23] was cloned together with the full cDNA clones for PDGF-A_{short} (clone 13.1, [24]), PDGF-A_{long} (clone D1, [25]) and PDGF-B [26]. DNA constructs were excised from the vector backbone, purified using the Qiaex II gel extraction kit (Qiagen) and injected into fertilized C57BL6/CBA oocytes, which were subsequently cultured until two-cell stage, and transplanted into pseudo-pregnant B6 females. For screening/genotyping by Southern Blot, tail biopsies were lyzed in 500 μl lysis buffer (50 mM Tris, pH 8; 100 mM EDTA; 100 mM NaCl; 25 μl 20% SDS and 25 μl 10 mg/ml proteinase K) and DNA was purified by phenol/chloroform extraction and ethanol precipitation. Southern blot was performed with standard techniques using PDGF-A and PDGF-B human cDNA as probes [25,27,28].

4. Genotyping of mice

For PCR genotyping of transgenic founders, tail biopsies were lysed in 100 μl lysis buffer (67 mM Tris, pH 8.8; 6.7 mM $MgCl_2$; 0.5 mM β-mercaptoethanol; 6.7 mM EDTA; 0.5% Triton-X100 and 500 μg/ml Proteinase K). The following PCR primers were used: *Pdgfa* fwd 5′-CTAAGGGATGGTACTGATTTTCGC-3′; *Pdgfa* rev 5′-AGGAATCTC GTAAATGACCGTCC-3′; *Pdgfb* fwd 5′-ATAGACCGCACCAACG-CCAACTTC-3′; *Pdgfb* rev 5′- AATAACCCTGCCCACACACTCTCC-3′. This resulted in a 411 bp product for both PDGF-A_{long} and PDGF-A_{short}, and 486 bp for PDGF-B.

The $Pdgfra^{GFP/+}$ mice were genotyped by their strong GFP expression under a ultraviolet light, or with PCR using the following primers: 5′-CCCTTGTGGTCATGCCAAAC-3′; 5′-GCTTTTGCCTCCA-TTACACTG G-3′; 5′-ACGAAGTTATTAGGTCCCTCGAC-3′ generating a 242 bp GFP-band and a 451 bp wt-band.

5. Fixation of heart tissue

Pdgf transgenic mice were perfusion fixed through the heart, or when mice were found dead hearts were dissected out and immersion fixed. All hearts were fixed in 4% paraformaldehyde (PFA) for 12 h at 4 °C and washed with PBS, before embedding in paraffin.

Hearts from $Pdgfra^{GFP/+}$ knock-in mice in C57Bl/6J background [29] were analyzed from embryonic day 11.5 (E11.5) until postnatal day 150 (P150). A minimum of 4 animals per timepoint was analyzed. Embryos (E11.5, E12.5, E14.5, E15.5, E17.5) and postnatal mice (P0, P1, P2, P6, P7, P15) were sacrificed by decapitation or with CO_2. Adult mice (P150) were perfusion fixed through the heart. All hearts were fixed in 4% paraformaldehyde (PFA) for 12 h at 4 °C, washed with PBS and soaked in a sucrose gradient (10–30% in PBS) before embedding in OCT. Cryo sections (25–30 μm) were collected on poly-L-lysine-coated slides and post fixed in 4% PFA for 10 min at RT.

6. Immunostaining of heart tissue

Immunohistochemistry on paraffin embedded hearts from transgenic mice was performed using antibodies directed against α-smooth muscle actin (ASMA, DAKO, U7033) and PECAM-1 (PharMingen) as previously described [30]. Collagen staining was performed using a Masson Trichrome/aniline blue staining kit, according to manufacturer's instructions (Bio-Optica, Milan).

For immunofluorescent stainings of OCT embedded hearts from $Pdgfra^{GFP/+}$ mice, the sections were permeabilized and blocked in

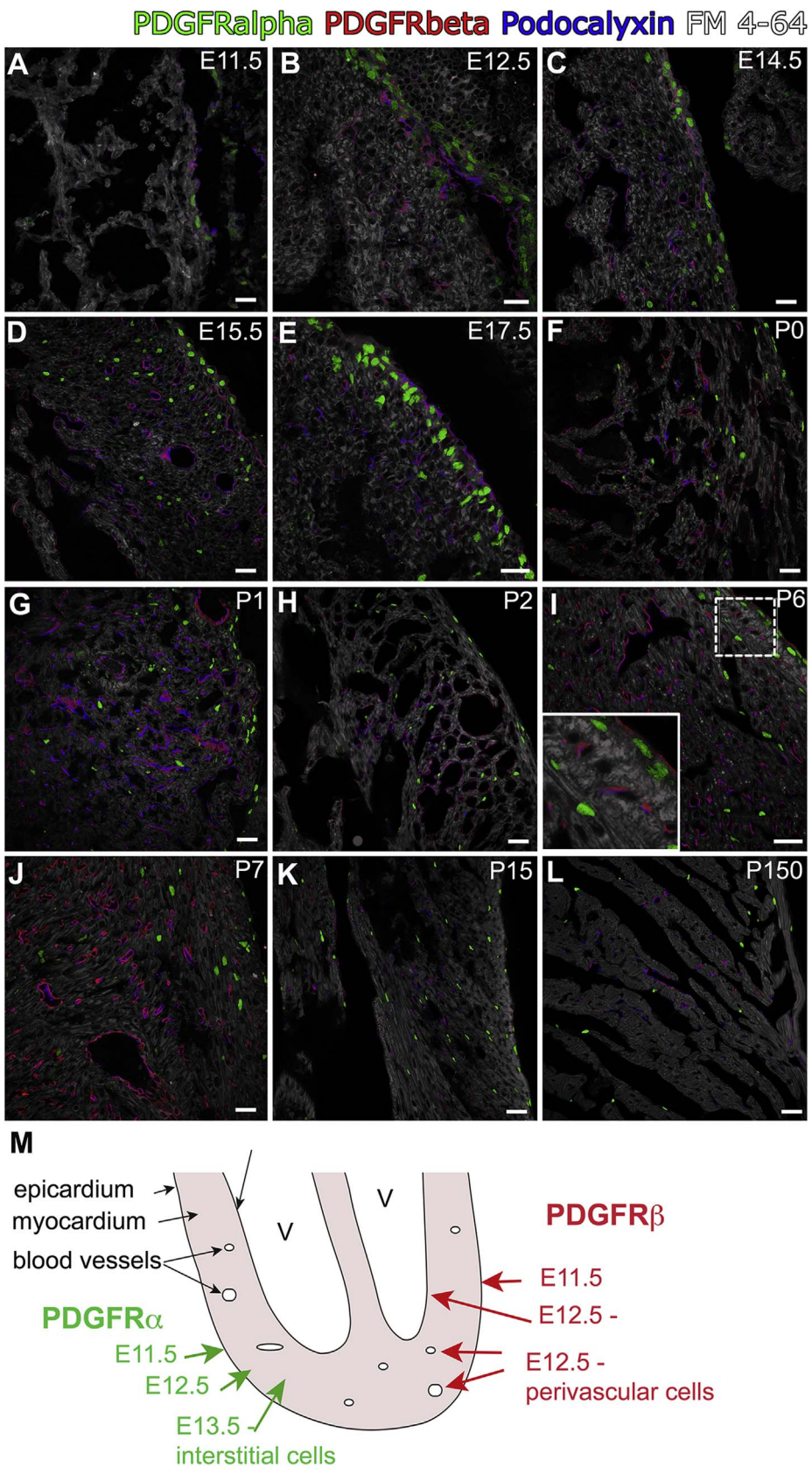


Fig. 1. Expression of PDGFR*α* and PDGFR*β* during normal heart development. Cardiac ventricular tissue from subsequent embryonic and postnatal stages of *Pdgfra*$^{GFP/+}$ mice, stained with immunofluorescence for PDGFRβ (red) and the endothelial marker podocalyxin (blue). The membrane marker FM4-64 (white) is included to visualize the heart tissue. Scalebar is 30 μm. (A) Epicardial expression of both PDGFRα and -β at E11.5. (B) Expansion of epicardial PDGFRα positive cells, whereas PDGFRβ and podocalyxin positive cells appear in the myocardium at E12.5. (C–E) Between E14.5-E17.5, PDGFRα positive cells migrate from the epicardium into the myocardium, and PDGFRβ expression remains perivascular. (F–L) Postnatally, PDGFRα positive cells are found in the interstitium of the myocardium and PDGFRβ expression remains perivascular. PDGFRα and -β were not co-expressed by the same cells, and PDGFRβ expression was always in close proximity to endothelial podocalyxin expression around blood vessels (insert in i). (M) Schematic summary of PDGF receptor localization at the different stages. V–ventricle.

0.5% Triton-x 100 in 0.1% BSA. All antibodies were diluted in 0.1% BSA. Primary antibodies used: PECAM-1 (553370, www.bdbiosciences.com) 1.25 μg/ml; ASMA Cy3-conjugated (C6198, www.sigmaaldrich.com) 2.8 μg/ml; PDGFRβ (CD140b, 14–1402-82, www.ebioscience.com) 2.5 μg/ml; Podocalyxin (AF1556, www.rndsystems.com) 1 μg/ml. Secondary antibodies used: Alexa Fluor 647 goat-anti-rat IgG (A-21247, www.invitrogen.com) 2 μg/ml; DyLight-conjugated donkey-anti-goat (705-475-147, www.jacksonimmuno.com) 3.75 μg/ml; Cy3-conjugated goat-anti-rat IgG (112-165-167, www.jacksonimmuno.com) 7.5 μg/ml. Sections were counter stained with 5 μg/ml FM 4-64 FX membrane dye (F34653, www.invitrogen.com), and imaged with confocal microscopy (Zeiss 700 ZEN), where optical sections were acquired with depth 0.684 μm.

7. Statistical analysis of interstitial cells and capillary density

Histological sections were imaged with a Zeiss Apotome microscope. The number of cells in the interstitial space between cardiomyocytes was recorded and divided by the number of intact cardiomyocytes. The number of cross-sectioned capillaries was recorded and divided by the analyzed area. The results were compared with unpaired T-test between transgenic and control animals, and standard deviation is shown with error bars in graphs.

8. Results

Overexpression of platelet-derived growth factors has been shown to induce cardiac fibrosis in several animal models. Here, we present three new transgenic mouse models that develop cardiac fibrosis as a response to forced *Pdgf* expression in the heart; either by one of the two splice isoforms of PDGF-A (PDGF-A_{short} or PDGFA_{long} [24,25]) or by PDGFB. PDGF-A_{long} carries a C-terminal proteoglycan-binding domain, which limits the solubility of the growth factor in vitro, and presumably its ability to diffuse in a tissue in vivo. It is hypothesized that PDGF-A_{long} remains localized close to its cellular source of secretions [5], whereas PDGF-A_{short} that lacks the proteoglycan-binding domain is more diffusible in the tissue interstitium.

Transgenic expression of the different PDGF isoforms led to fibrosis of varying severity, a variation that we hypothesized to depend on the PDGF receptor subtype that was activated. To identify cell types that were potential target cells for PDGFs, we first performed a thorough characterization of the expression patterns of the two PDGF receptors (PDGFRα and -β) in hearts from embryonic, early postnatal and adult mice.

9. Cardiac expression of PDGFRα and PDGFRβ

PDGFRα and PDGFRβ expression was analyzed in cryo-sectioned hearts of $Pdgfra^{GFP/+}$ knock-in mice [29] from E11.5 until adulthood. $Pdgfra^{GFP/+}$ mice carry a H2B-GFP reporter cassette targeted to the *Pdgfra* locus, which generates a bright nuclear fluorescence in all PDGFRα-positive cells. The targeted allele is null for PDGFRα protein expression; hence $Pdgfra^{GFP/+}$ mice are equivalent to *Pdgfra* heterozygous knockouts. PDGFRβ expression was identified by immunofluorescent staining.

At 11.5 days post fertilization (E11.5), epicardial cells surrounding the forming heart expressed both PDGFRα and PDGFRβ (Fig. 1A). No co-expression of the two receptors was detected in any cell within the myocardium. Between E12.5-E14.5, PDGFRα was still mainly expressed by cells in the epicardial area, but a few single PDGFRα positive cells were identified in the myocardium. PDGFRβ expression, on the other hand, was observed in both the myocardium and endocardium, where it was associated to perivascular cells (Fig. 1B and C). From E14.5 onwards, the epicardial PDGFRα positive cell population expanded and migrated into the myocardium. By E17.5, this cell population had reached a distribution pattern identical to that observed for PDGFRα positive cells in the adult heart, i.e. expression by a proportion of the interstital cells in the myocardium (Fig. 1E–L).

At all stages, PDGFRβ expression was localized to perivascular cells in close association to endothelial cells, identified by podocalyxin expression (Fig. 1I, inset). The PDGFRβ perivascular cells were most likely pericytes. We were at no time point able to identify any cells co-expressing PDGFRα and PDGFRβ. Observations are schematically summarized in Fig. 1M.

10. Generation of α-MHC-Pdgf transgenic mice

The cardiac fibrosis-inducing potential of PDGF-A and -B was investigated in transgenic mice, generated to express either of the two PDGF-A isoforms (PDGF-A_{short} and PDGF-A_{long}) or PDGF-B, re-

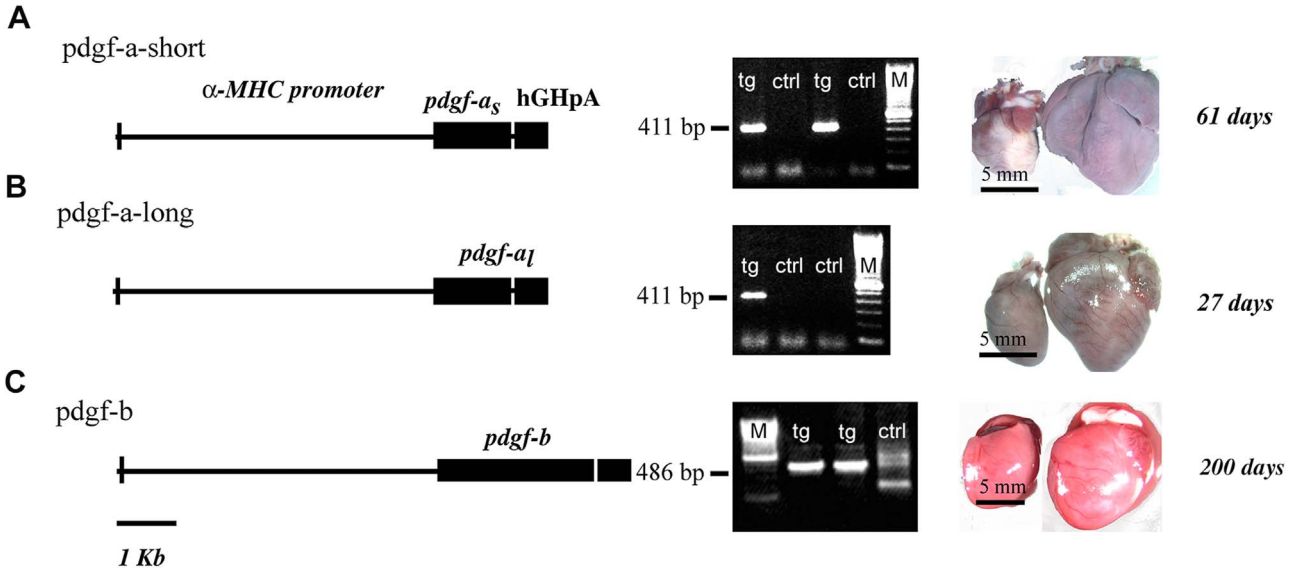
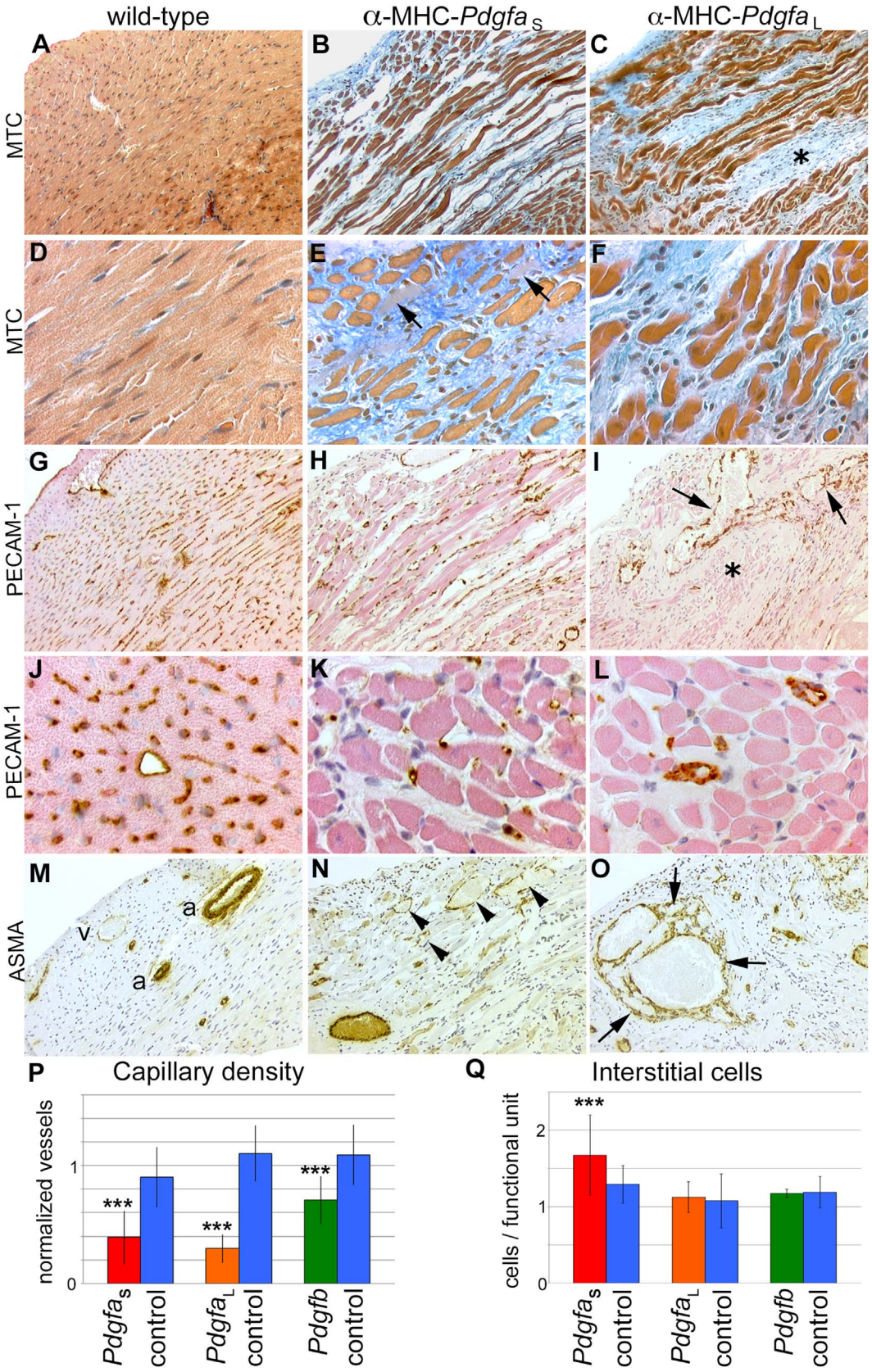


Fig. 2. Transgenic constructs, genotyping and cardiac enlargement in transgenic mice. Design of transgenic constructs to express *Pdgfs* in cardiac myocytes under the α-MHC promoter, PCR genotyping and whole mount images of enlarged hearts from transgenic mice. Control littermates are to the left. (A) $Pdgfa_{short}$ construct, PCR product of 411 bp confirms transgene expression, and enlarged heart at P61. tg: transgene carriers. ctrl: non-transgenic littermates. M: DNA ladder. (B) $Pdgfa_{long}$ construct, PCR product of 411 bp confirms transgene expression, enlarged heart at P27. (C) *Pdgfb* construct, PCR product of 486 bp confirms transgene expression, enlarged heart at 7 months.


wild-type
α-MHC-Pdgfa S
α-MHC-Pdgfa L
A
B
C
MTC
D
E
F
MTC
G
H
I
PECAM-1
J
K
L
PECAM-1
M
N
O
ASMA
a
v
a
P
Capillary density
normalized vessels
1
0
***
***
***
Pdgfa S
control
Pdgfa L
control
Pdgfb
control
Q
Interstitial cells
cells / functional unit
2
1
0
***
Pdgfa S
control
Pdgfa L
control
Pdgfb
control

spectively. The transgenic constructs were designed as previously reported [19,20], with expression driven by the heart specific α-myosin heavy chain (α-MHC) promoter (Fig. 2). In the ventricular myocardium, the promoter activity initiates at birth and maintains a high expression during adulthood [23]. Before birth, the promoter activity was restricted to the atrium.

11. Severe fibrosis and cardiac hypertrophy induced by PDGF-A

Two different *Pdgfa* transgenic constructs were engineered; one expressing the long isoform PDGF-A_{long} and the other expressing the short isoform PDGF-A_{short}. Four + four *Pdgfa* transgenic founders were obtained, all of which developed severe cardiac hypertrophy. All PDGF-A_{short} founders died within 2 months after birth. Three died spontaneously at around 6 weeks of age, and one was euthanized for ethical reasons. These mice displayed an extensive cardiac hypertrophy (Fig. 2A) that also caused deformation and compression in surrounding anatomical structures. Three (out of 4) PDGF-A_{long} founders died within 5 weeks after birth, also displaying hypertrophic hearts (Fig. 2B). Those hearts were enlarged up to 8-fold, which in turn affected the development of the pleural cavity and the rib cage. The 4th founder was perfectly healthy, but was later identified as a non-expressing founder.

In all *Pdgfa* transgenic mice there was an extensive increase in extracellular matrix in the ventricular myocardium (Fig. 3A–F). Collagen was accumulated in the interstitium between the muscle fibers, but was also concentrated in focal areas, especially in PDGF-A_{long} transgenics (Fig. 3C, asterisk). In some areas cardiomyocytes appeared damaged, according to morphology (Fig. 3E, arrows).

The heart vasculature of the *Pdgfa* transgenic mice had an abnormal appearance, including a reduced density of PECAM-1 positive capillaries in comparison with wildtype control mice (Fig. 3G–L). On average, there was a more than 50% reduction of capillaries surrounding the cardiomyocytes. The phenotype was more pronounced in PDGF-A_{long} mice (> 70% loss, Fig. 3P). In the fibrotic tissue of PDGF-A_{long} transgenic mice, there was also an enrichment of large, dilated and irregular blood vessels with a glomeruloid appearance (Fig. 3I, arrows). These vessels where positive for ASMA (Fig. 3O, arrows), which is more abundant in arteries. However, the layer of mural cells was thin, resembling the morphology of veins (Fig. 3M and N). In PDGF-A_{short} mice, a small but significant increase (30%) in the number of interstitial cells per intact unit of cardiomyocytes was observed in fibrotic areas. No differences in number of interstitial cells per intact unit was present in the hearts of neither PDGF-A_{long} nor PDGF-B transgenic mice (Fig. 3Q).

12. Local fibrosis and moderate hypertrophy induced by PDGF-B

Using the α-MHC-*Pdgfb* transgenic construct we obtained four founders, which all surivived until adulthood. They were euthanized at 7 months of age for analysis. Similar to the *Pdgfa* transgenic mice, all *Pdgfb* transgenic founders exhibited cardiac hypertrophy (Fig. 2C) and focal accumulations of collagen enriched extracellular matrix in the ventricular myocardium (Fig. 4A–F). However, in contrast to the *Pdgfa* transgenic mice, collagen deposition in the *Pdgfb* transgenic mice was mostly concentrated around intramyocardial branches of coronary arteries (Fig. 4B) with a milder phenotype around cardiomyocytes (Fig. 4D and F). In contrast to *Pdgfa* transgenic mice, the microvascular morphology in the *Pdgfb* mice appeared normal (Fig. 4G and H), although small changes in capillary density were observed in fibrotic areas (30% decrease, Fig. 3P). There was no change in number of interstitial cells per intact unit of cardiomyocytes in the *Pdgfb* transgenic mice (Fig. 3Q).

13. Discussion

PDGF is known as a mitogen and chemotactic agent for fibroblasts and smooth muscle cells and an inducer of extracellular matrix protein synthesis, including fibronectin [31], proteoglycans [32] and collagens [33]. Forced overexpression of PDGFs in mice induce proliferative and fibrotic pathology in multiple organs, including retina [34–37], lens [38], lung [39–41], brain [42,43] and liver [44]. Likewise, constitutive activation of the PDGFRα leads to multi-organ fibrosis [14]. In the present study, we focused on the ability of the classical PDGFs, i.e. PDGF-A and PDGF-B, to induce cardiac fibrosis when expressed transgenically in the mouse heart.

Beyond confirming that PDGF overexpression leads to cardiac fibrosis, our study addresses the different abilities of different PDGFs to induce fibrosis. PDGF-C and PDGF-D were previously shown to induce heart fibrosis and vascular remodeling when expressed under the α-MHC promoter in transgenic mice [19,20]. To extend this comparison to all known PDGF ligands, we generated transgenic mice overexpressing PDGF-A (both splice variants) and PDGF-B, respectively, using the same α-MHC promoter [19,20]. All new founder mice developed heart hypertrophy and cardiac fibrosis. Therefore, we conclude that overexpression of all PDGF isoforms in ventricular myocardial cells lead to cardiac fibrosis, but that extent and localization of the fibrotic reactions vary depending on PDGF ligand expressed (Table 1).

The most severe pathology was generated by PDGF-A overexpression, where all expressing founder mice died before 6 weeks of age. As a consequence, no α-MHC-*Pdgfa* germline transgenic mouse strain could be established, and all analyses were performed directly on the founder generation, limiting postmortem analysis to a single timepoint. The phenotypic differences between founders carrying the same transgenic construct was small, in spite of the fact that each founder mouse represented a new integration site of the transgene. Hence, it is not likely that phenotypic changes between the different transgenic contructs depended on variations in expression level. Thus, we conclude that the data obtained from the different α-MHC-*Pdgf* transgenic mice can be compared with good reliability, although the number of mice expressing each PDGF ligand was limited.

We propose that differences in severity of the fibrotic reactions generated by different PDGF ligands depend on the type of PDGF receptor that was activated, and the cells carrying those receptors. Differential fibrogenic effects of different PDGF isoforms have previously been analyzed in the mouse lung, when PDGF-A, -B and -C were expressed in the distal lung epithelium under control of the surfactant protein-C promoter [39–41]. These studies suggested that differences in PDGFR activation dictate the different fibrogenic out-

Fig. 3. Histological analysis of hearts from α-MHC-*Pdgfa*$_{short}$ and α-MHC-*Pdgfa*$_{long}$ transgenic mice. (A–O) Histological analyses of paraffin sectioned hearts from wildtype (A, D, G, J, M), α-MHC-*Pdgfa*$_{short}$ (B, E, H, K, N) and α-MHC-*Pdgfa*$_{long}$ (C, F, I, L, O) mice. (A–F) Masson TriChrome staining (MTC) visualizes collagen (blue), nuclei (black), cytoplasm, keratin, muscle fibers, fibrin (red). (A–C) Hearts at low magnification. Asterisk in (C) marks focal fibrosis lacking cardiomyocytes. (D–F) Hearts at high magnification. Arrows in (E) point at dead cardiomyocytes. (G-L) PECAM-1 positive endothelium (brown) counterstained with haematoxylin/eosin. (G-I) Dilated vessels in transgenic hearts at low magnification. In (I), arrows point at glomeruloid vascular structures, and asterisk marks area of complete focal loss of microvessels. (J-L) Decreased capillary density in hearts of transgenic mice, at high magnification. (M-O) Smooth muscle cells marked with alpha-smooth muscle actin (ASMA) (brown) and counterstained with hematoxylin. Arrow heads in (N) point at thin vein-like vessels, arrows in (O) point at glomeruloid vascular structures. a – artery, v – vein. (P) Graph showing the capillary density in transgenic hearts vs. control littermates, normalized to the area of analysis. The capillary density decrease was highly significant in all three types of transgenic mice. (Q) Graph showing the number of interstitial cells in relation to number of intact cardiomyocytes in transgenic hearts vs. control littermates. Hearts from α-MHC-*Pdgfa*$_S$ mice showed a significant increase in interstitial cells. Error bars in (P, Q) represent standard deviation.

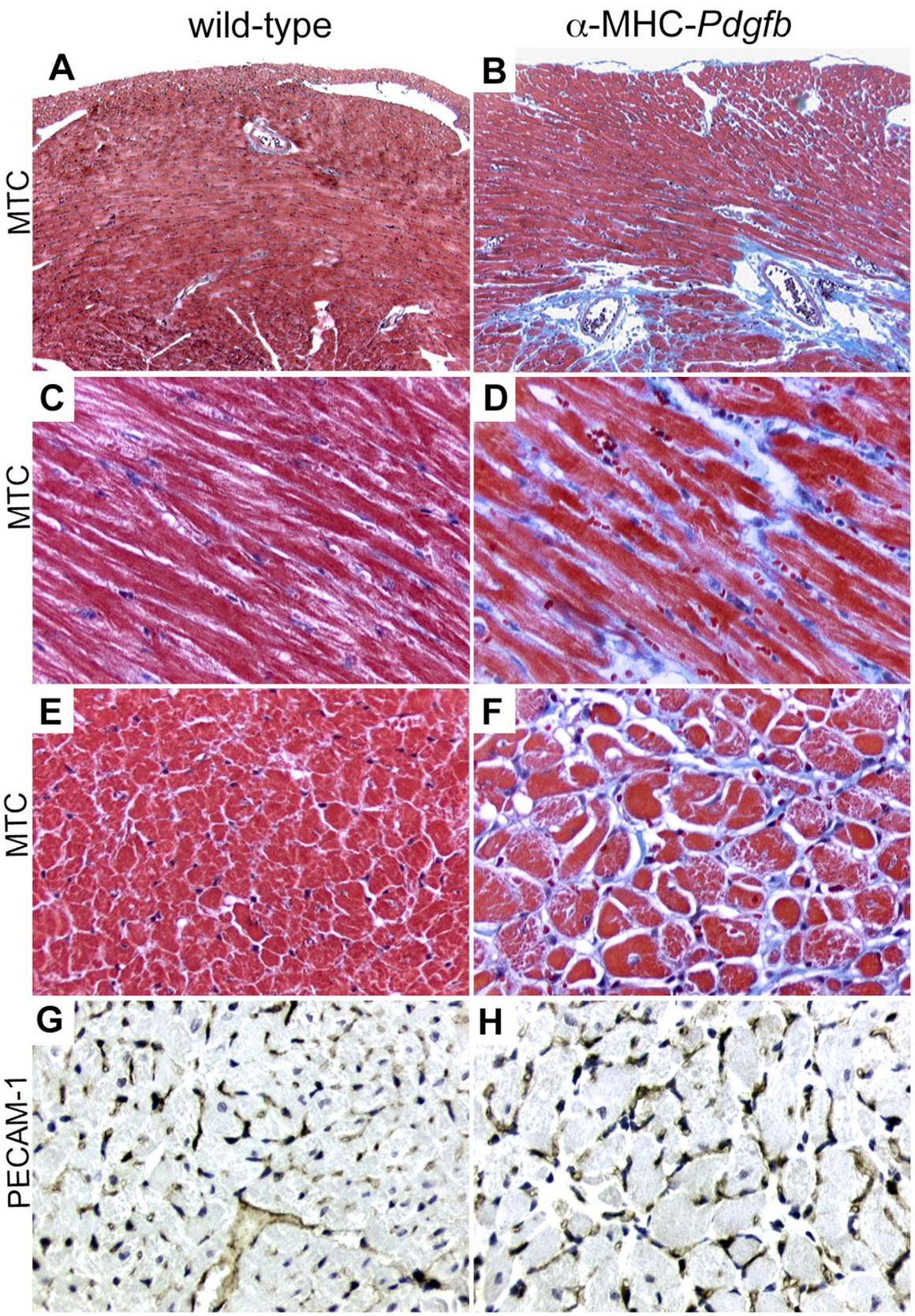


Fig. 4. Histological analysis of hearts from α-MHC-*Pdgfb* transgenic mice. (A-F) Masson TriCrome staining of paraffin sectioned hearts from wt (A, C, E) and α-MHC-*Pdgfb* (B, D, F). (A, B) Hearts in low magnification, perivascular collagen deposition (blue) in α-MHC-*Pdgfb*, but not in wt. (C, D) High magnification of sections longitudinal to the cardiomyocytes shows collagen deposition along the cells in α-MHC-*Pdgfb* mice. (E, F) High magnification of sections perpendicular to the cardiomyocytes shows deposited collagen surrounding the cells in α-MHC-*Pdgfb* mice. (G, H) Immunohistochemistry for PECAM-1 (brown) visualizes a subtile difference in vessel morphology between wt (G) and α-MHC-*Pdgfb* (H).

Table 1
Summary of the phenotypes of α-MHC-*Pdgf* transgenic mice in comparison to wildtype control mice.

transgene	Life span	Fibrotic reaction	Capillary density	Interstitial cells	Reference
$Pdgfa_{short}$	< 2 months	Diffuse	56% less	30% more	
$Pdgfa_{long}$	< 1.5 months	Diffuse	73% less	No change	
Pdgfb	> 7 months	Local	36% less	No change	
Pdgfc	> 7 months	Diffuse	25% less	More	[19]
Pdgfd	< 2 months	Local	Less	More	[20]

come. Expression of the two PDGFRα ligands (PDGF-A and -C) resulted in perinatal lethality due to mesenchymal cell overgrowth and abnormal differentiation of the lung epithelium. In contrast, expression of the PDGFRβ ligand PDGF-B was not lethal, but caused fibrosis, inflammation and emphysema-like airway enlargement. These results conform with our data in the heart where PDGF-A leads to a more severe fibrogenic response and earlier lethality in transgenic mice.

Under normal circumstances PDGF signalling works in a paracrine way. Here we hypothesized that interstitial cardiac fibroblasts expressing PDGFRα were the main target cell type for the transgenic PDGFs expressed by the cardiomyocytes. In order to test this, and to characterize potential target cells, we performed a thorough expression analysis of PDGFRα and -β expression in the ventricular heart tissue at different developmental stages, ranging from embryonic development to adulthood. Others have shown (by immunoprecipitation) that primary mouse cardiac fibroblasts in culture express both PDGF receptors [20]. However, we could not detect co-expression of PDGFRα and -β by overlapping reporter expression and immunostaining in tissue sections. At early cardiogenesis (E11.5), both receptors were detected in the epicardium, but thereafter, their expression patterns diverged. Whereas PDGFRβ-positive cells were always localized to perivascular areas, PDGFRα-positive cells coincided with interstitial fibroblasts in the myocardium, which has also been reported in chicken [11] and mouse [13]. These data concur with observations that PDGFRβ is important for the development of coronary mural cells, whereas PDGFRα is important for the development of cardiac interstitial fibroblasts [6,45].

In α-MHC-*Pdgf* transgenic mice some cardiomyocytes displayed severe morphologic abnormalities. A highly interesting connection that we can only speculate around is whether fibrosis generated cardiomyocyte death or if damage to the cardiomyocytes generated fibrosis. In fact, fibrosis and cardiac cell death can both be drivers in the "fibrosis-cell death cycle" during heart failure (reviewed by [46]).

Taken together with previous publications [19,20], our data do not support a model where all different PDGF ligands can activate the same type of cell through different receptors. Out of all PDGFs, the most severe phenotype was generated by overexpressing PDGF-A in the heart. Because PDGF-A is a high affinity ligand for PDGFRα, but does not bind PDGFRβ, it is reasonable to assume that the PDGFRα-positive cardiac fibroblasts were involved in the fibrotic process. PDGF-C is also a high affinity ligand for PDGFRα, but the phenotype of the α-MHC-*Pdgfc* mice was less severe [19]. A likely explanation might be the fact that PDGF-A is secreted as an active protein, whereas PDGF-C is secreted as an inactive precursor protein that requires proteolytic cleavage in the extracellular space to become biologically active [47]. In α-MHC-*Pdgfc* mice, the vast majority of PDGF-C detected in the heart had the size of the full-length inactive form [19]. In a parallell study, we have also analyzed the effects of all different PDGF ligands on adenovirus induced fibrosis in mouse hearts [18]. The two studies support each other by showing PDGF ligand-specific effects.

The phenotypically different focal fibrosis in proximity to blood vessels observed in α-MHC-*Pdgfb* and in α-MHC-*Pdgfd* mice [20] is difficult to explain by PDGFRα activation. PDGF-B and -D are both high affinity ligands for PDGFRβ, which has several reported key roles in inflammatory responses in association with vessels [15,48,49]. Indeed, PDGF-B has affinity for PDGFRα in vitro [50,51], but no such affinity has been reported for PDGF-D [52]. It should, however, be kept in mind that PDGF ligand-receptor interactions during adult homeostasis and pathology have not been extensively analyzed and remain poorly understood.

Together with the observed perivascular expression of PDGFRβ, it seems plausible that stimulation of PDGFRβ-positive perivascular cells caused the fibrotic events observed in α-MHC-*Pdgfb* (and α-MHC-*Pdgfd* [20]) mice. These presumably vascular mural cells (pericytes and/or vascular smooth muscle cells) might themselves transform into a myofibroblast phenotype, or elicit a local inflammation that triggers nearby non-mural mesenchymal cells, e.g. interstitial fibroblasts, to assume a myofibroblast phenotype. Irrespective of scenario, the localization of the fibrosis correlated spatially with the normal myocardial distribution of PDGFRα and PDGFRβ−positive cells.

Taken together, our study and previously published work [19,20] show that all known PDGF isoforms are capable of generating cardiac fibrosis and hypertrophy when overexpressed from cardiomyocytes in transgenic mice. However, the degree and location of fibrosis vary between the different ligands, which are likely a result of differential activation of the two PDGF receptors, which show largely non-overlapping patterns of expression in the heart.

Due to the sudden and unexpected death of α-MHC-*Pdgfa* founder mice it was not ethical to generate more mice for further analysis. There are, however, still several open paths to follow up for which new strategies with inducible promoters should be considered.

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References

[1] J. Baum, H.S. Duffy, Fibroblasts and myofibroblasts: what are we talking about?, J. Cardiovasc. Pharmacol. 57 (2011) 376–379. http://dx.doi.org/10.1097/FJC.0b013e3182116e39.
[2] P. Kong, P. Christia, N.G. Frangogiannis, The pathogenesis of cardiac fibrosis, Cell. Mol. Life Sci. 71 (2014) 549–574. http://dx.doi.org/10.1007/s00018-013-1349-6.
[3] T.R. Ramalingam, T.A. Wynn, Mechanisms of fibrosis: therapeutictranslation for fibrotic disease, Nat. Med. 18 (2012) 1028–1040. http://dx.doi.org/10.1038/nm.2807.
[4] T.A. Wynn, Cellular and molecular mechanisms of fibrosis, J. Pathol. 214 (2008) 199–210. http://dx.doi.org/10.1002/path.2277.
[5] J. Andrae, R. Gallini, C. Betsholtz, Role of platelet-derived growth factors in physiology and medicine, Genes Dev. 22 (2008) 1276–1312. http://dx.doi.org/10.1101/gad.1653708.
[6] C.L. Smith, S.T. Baek, C.Y. Sung, M.D. Tallquist, Epicardial-derived cell epithelial-to-mesenchymal transition and fate specification require PDGF receptor signaling, Circ. Res. 108 (2011) e15–e26. http://dx.doi.org/10.1161/CIRCRESAHA.110.235531.
[7] N.M.S. Van Den Akker, L.C.J. Winkel, M.H. Nisancioglu, S. Maas, L.J. Wisse, A. Armulik, et al., PDGF-B signaling is important for murine cardiac development: its role in developing atrioventricular valves, coronaries, and cardiac innervation, Dev. Dyn. 237 (2008) 494–503. http://dx.doi.org/10.1002/dvdy.21436.
[8] H. Gladh, E.B. Folestad, L. Muhl, M. Ehnman, P. Tannenberg, A.-L. Lawrence, et al., Mice Lacking Platelet-Derived Growth Factor D Display a Mild Vascular Phenotype, PLoS One 11 (2016) e0152276. http://dx.doi.org/10.1371/journal.pone.0152276.
[9] M.D. Tallquist, P. Soriano, Cell autonomous requirement for PDGFRalpha in populations of cranial and cardiac neural crest cells, Development 130 (2003) 507–518.

[10] J. Kang, Y. Gu, P. Li, B.L. Johnson, H.M. Sucov, P.S. Thomas, PDGF-A as an epicardial mitogen during heart development, Dev. Dyn. 237 (2008) 692–701. http://dx.doi.org/10.1002/dvdy.21469.
[11] N.A.M. Bax, H. Lie-Venema, R. Vicente-Steijn, S.B. Bleyl, N.M.S. Van Den Akker, S. Maas, et al., Platelet-derived growth factor is involved in the differentiation of second heart field-derived cardiac structures in chicken embryos, Dev. Dyn. 238 (2009) 2658–2669. http://dx.doi.org/10.1002/dvdy.22073.
[12] J.J.H. Chong, H. Reinecke, M. Iwata, B. Torok-Storb, A. Stempien-Otero, C.E. Murry, Progenitor cells identified by PDGFR-alpha expression in the developing and diseased human heart, Stem Cells Dev. 22 (2013) 1932–1943. http://dx.doi.org/10.1089/scd.2012.0542.
[13] N.A.M. Bax, S.B. Bleyl, R. Gallini, L.J. Wisse, J. Hunter, A.A.M. Van Oorschot, et al., , Cardiac malformations in Pdgfralpha mutant embryos are associated with increased expression of WT1 and Nkx2.5 in the second heart field, Dev. Dyn. 239 (2010) 2307–2317. http://dx.doi.org/10.1002/dvdy.22363.
[14] L.E. Olson, P. Soriano, Increased PDGFRalpha activation disrupts connective tissue development and drives systemic fibrosis, Dev. Cell. 16 (2009) 303–313. http://dx.doi.org/10.1016/j.devcel.2008.12.003.
[15] L.E. Olson, P. Soriano, PDGFRβ signaling regulates mural cell plasticity and inhibits fat development, Developmental Cell. 20 (2011) 815–826. http://dx.doi.org/10.1016/j.devcel.2011.04.019.
[16] R. Tuuminen, A. Nykänen, M.A. Keränen, R. Krebs, K. Alitalo, P.K. Koskinen, et al., The effect of platelet-derived growth factor ligands in rat cardiac allograft vasculopathy and fibrosis, Transplant. Proc. 38 (2006) 3271–3273. http://dx.doi.org/10.1016/j.transproceed.2006.10.056.
[17] P. Zymek, M. Bujak, K. Chatila, A. Cieslak, G. Thakker, M.L. Entman, et al., The role of platelet-derived growth factor signaling in healing myocardial infarcts, J. Am. Coll. Cardiol. 48 (2006) 2315–2323. http://dx.doi.org/10.1016/j.jacc.2006.07.060.
[18] R. Gallini, J. Huusko, S. Ylä-Herttuala, C. Betsholtz, J. Andrae, Isoform-Specific Modulation of inflammation induced by Adenoviral Mediated Delivery of Platelet-Derived Growth Factors in the Adult mouse heart, PLoS One 11 (2016) e0160930. http://dx.doi.org/10.1371/journal.pone.0160930.
[19] A. Pontén, X. Li, P. Thorén, K. Aase, T. Sjöblom, A. Östman, et al., Transgenic overexpression of platelet-derived growth factor-C in the mouse heart induces cardiac fibrosis, hypertrophy, and dilated cardiomyopathy, Am. J. Pathol. 163 (2003) 673–682. http://dx.doi.org/10.1016/S0002-9440(10)63694-2.
[20] A. Pontén, E.B. Folestad, K. Pietras, U. Eriksson, Platelet-derived growth factor D induces cardiac fibrosis and proliferation of vascular smooth muscle cells in heart-specific transgenic mice, Circ. Res. 97 (2005) 1036–1045. http://dx.doi.org/10.1161/01.RES.0000190590.31545.d4.
[21] D.B. Morton, A systematic approach for establishing humane endpoints, ILAR J. 41 (2000) 80–86.
[22] D.B. Morton, P.H. Griffiths, Guidelines on the recognition of pain, distress and discomfort in experimental animals and an hypothesis for assessment, Vet. Rec. 116 (1985) 431–436.
[23] A. Subramaniam, W.K. Jones, J. Gulick, S. Wert, J. Neumann, J. Robbins, Tissue-specific regulation of the alpha-myosin heavy chain gene promoter in transgenic mice, J. Biol. Chem. 266 (1991) 24613–24620.
[24] F. Rorsman, M. Bywater, T.J. Knott, J. Scott, C. Betsholtz, Structural characterization of the human platelet-derived growth factor A-chain cDNA and gene: alternative exon usage predicts two different precursor proteins, Mol. Cell. Biol. 8 (1988) 571–577.
[25] C. Betsholtz, A. Johnsson, C.H. Heldin, B. Westermark, P. Lind, M.S. Urdea, et al., cDNA sequence and chromosomal localization of human platelet-derived growth factor A-chain and its expression in tumour cell lines, Nature 320 (1986) 695–699. http://dx.doi.org/10.1038/320695a0.
[26] K. Forsberg, I. Valyi-Nagy, C.H. Heldin, M. Herlyn, B. Westermark, Platelet-derived growth factor (PDGF) in oncogenesis: development of a vascular connective tissue stroma in xenotransplanted human melanoma producing PDGF-BB, Proc. Natl. Acad. Sci. USA 90 (1993) 393–397.
[27] T. Collins, D. Ginsburg, J.M. Boss, S.H. Orkin, J.S. Pober, Cultured human endothelial cells express platelet-derived growth factor B chain: cDNA cloning and structural analysis, Nature 316 (1985) 748–750.
[28] B. Matoskova, F. Rorsman, V. Svensson, C. Betsholtz, Alternative splicing of the platelet-derived growth factor A-chain transcript occurs in normal as well as tumor cells and is conserved among mammalian species, Mol. Cell. Biol. 9 (1989) 3148–3150.
[29] T.G. Hamilton, R.A. Klinghoffer, P.D. Corrin, P. Soriano, Evolutionary divergence of platelet-derived growth factor alpha receptor signaling mechanisms, Mol. Cell. Biol. 23 (2003) 4013–4025.
[30] M. Hellström, M. Kalén, P. Lindahl, A. Abramsson, C. Betsholtz, Role of PDGF-B and PDGFR-beta in recruitment of vascular smooth muscle cells and pericytes during embryonic blood vessel formation in the mouse, Development 126 (1999) 3047–3055.
[31] S.P. Blatti, D.N. Foster, G. Ranganathan, H.L. Moses, M.J. Getz, Induction of fibronectin gene transcription and mRNA is a primary response to growth-factor stimulation of AKR-2B cells, Proc. Natl. Acad. Sci. USA 85 (1988) 1119–1123.
[32] E. Schönherr, H.T. Järveläinen, L.J. Sandell, T.N. Wight, Effects of platelet-derived growth factor and transforming growth factor-beta 1 on the synthesis of a large versican-like chondroitin sulfate proteoglycan by arterial smooth muscle cells, J. Biol. Chem. 266 (1991) 17640–17647.
[33] E. Canalis, Effect of platelet-derived growth factor on DNA and protein synthesis in cultured rat calvaria, Metab. Clin. Exp. 30 (1981) 970–975.
[34] M. Fruttiger, A.R. Calver, W.H. Krüger, H.S. Mudhar, D. Michalovich, N. Takakura, et al., PDGF mediates a neuron-astrocyte interaction in the developing retina, Neuron 17 (1996) 1117–1131.
[35] M.S. Seo, N. Okamoto, M.A. Vinores, S.A. Vinores, S.F. Hackett, H. Yamada, et al., Photoreceptor-specific expression of platelet-derived growth factor-B results in traction retinal detachment, Am. J. Pathol. 157 (2000) 995–1005. http://dx.doi.org/10.1016/S0002-9440(10)64612-3.
[36] H. Yamada, E. Yamada, A. Ando, M.S. Seo, N. Esumi, N. Okamoto, et al., Platelet-derived growth factor-A-induced retinal gliosis protects against ischemic retinopathy, Am. J. Pathol. 156 (2000) 477–487. http://dx.doi.org/10.1016/S0002-9440(10)64752-9.
[37] K. Mori, P. Gehlbach, A. Ando, G. Dyer, E. Lipinsky, A.G. Chaudhry, et al., Retina-specific expression of PDGF-B versus PDGF-A: vascular versus nonvascular proliferative retinopathy, Investig. Ophthalmol. Vis. Sci. 43 (2002) 2001–2006.
[38] L.W. Reneker, P.A. Overbeek, Lens-specific expression of PDGF-A in transgenic mice results in retinal astrocytic hamartomas, Investig. Ophthalmol. Vis. Sci. 37 (1996) 2455–2466.
[39] G.W. Hoyle, J. Li, J.B. Finkelstein, T. Eisenberg, J.Y. Liu, J.A. Lasky, et al., Emphysematous lesions, inflammation, and fibrosis in the lungs of transgenic mice overexpressing platelet-derived growth factor, Am. J. Pathol. 154 (1999) 1763–1775. http://dx.doi.org/10.1016/S0002-9440(10)65432-6.
[40] J. Li, G.W. Hoyle, Overexpression of PDGF-A in the lung epithelium of transgenic mice produces a lethal phenotype associated with hyperplasia of mesenchymal cells, Dev. Biol. 239 (2001) 338–349. http://dx.doi.org/10.1006/dbio.2001.0441.
[41] Y. Zhuo, G.W. Hoyle, B. Shan, D.R. Levy, J.A. Lasky, Over-expression of PDGF-C using a lung specific promoter results in abnormal lung development, Transgenic Res. 15 (2006) 543–555. http://dx.doi.org/10.1007/s11248-006-9007-5.
[42] A.R. Calver, A.C. Hall, W.P. Yu, F.S. Walsh, J.K. Heath, C. Betsholtz, et al., Oligodendrocyte population dynamics and the role of PDGF in vivo, Neuron 20 (1998) 869–882.
[43] K. Forsberg-Nilsson, A. Erlandsson, X.-Q. Zhang, H. Ueda, K. Svensson, M. Nistér, et al., Oligodendrocyte precursor hypercellularity and abnormal retina development in mice overexpressing PDGF-B in myelinating tracts, Glia 41 (2003) 276–289. http://dx.doi.org/10.1002/glia.10191.
[44] K.J. Riehle, M.M. Johnson, F. Johansson, R.L. Bauer, B.J. Hayes, D.G. Gilbertson, et al., Tissue-type plasminogen activator is not necessary for platelet-derived growth factor-c activation, Biochim. Biophys. Acta. (2013). http://dx.doi.org/10.1016/j.bbadis.2013.11.013.
[45] A.M. Mellgren, C.L. Smith, G.S. Olsen, B. Eskiocak, B. Zhou, M.N. Kazi, et al., Platelet-derived growth factor receptor beta signaling is required for efficient epicardial cell migration and development of two distinct coronary vascular smooth muscle cell populations, Circ. Res. 103 (2008) 1393–1401. http://dx.doi.org/10.1161/CIRCRESAHA.108.176768.
[46] A. Piek, R.A. de Boer, H.H.W. Silljé, The fibrosis-cell death axis in heart failure, Heart Fail Rev 21 (2016) 199–211. http://dx.doi.org/10.1007/s10741-016-9536-9.
[47] X. Li, A. Ponten, K. Aase, L. Karlsson, A. Abramsson, M. Uutela, et al., PDGF-C is a new protease-activated ligand for the PDGF alpha-receptor, Nat. Cell Biol. 2 (2000) 302–309. http://dx.doi.org/10.1038/35010579.
[48] C. He, S.C. Medley, T. Hu, M.E. Hinsdale, F. Lupu, R. Virmani, et al., PDGFRβ signalling regulates local inflammation and synergizes with hypercholesterolaemia to promote atherosclerosis, Nat. Commun. 6 (2015) 7770. http://dx.doi.org/10.1038/ncomms8770.
[49] K. Stark, A. Eckart, S. Haidari, A. Tirniceriu, M. Lorenz, M.-L. von Brühl, et al., Capillary and arteriolar pericytes attract innate leukocytes exiting through venules and “instruct” them with pattern-recognition and motility programs, Nat. Immunol. 14 (2013) 41–51. http://dx.doi.org/10.1038/ni.2477.
[50] C.E. Hart, J.W. Forstrom, J.D. Kelly, R.A. Seifert, R.A. Smith, R. Ross, et al., Two classes of PDGF receptor recognize different isoforms of PDGF, Science 240 (1988) 1529–1531.
[51] C.H. Heldin, G. Bäckström, A. Ostman, A. Hammacher, L. Rönnstrand, K. Rubin, et al., Binding of different dimeric forms of PDGF to human fibroblasts: evidence for two separate receptor types, EMBO J. 7 (1988) 1387–1393.
[52] E. Bergsten, M. Uutela, X. Li, K. Pietras, A. Ostman, C.H. Heldin, et al., PDGF-D is a specific, protease-activated ligand for the PDGF beta-receptor, Nat. Cell Biol. 3 (2001) 512–516. http://dx.doi.org/10.1038/35074588.