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

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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 [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 [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 [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 [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.
signs of disease were sacrificed. Positive cells in pathological cardiac mouse hearts. Our results confirmed that expression of PDGF-Ashort, PDGF-Along and PDGF-B from the same MHC promoter and phenotypically characterized their hearts. We also showed that 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 arrhythmias such as ventricular septum, epicardial cells, epicardial-derived macrophages, neutrophils and natural killer cells, which were subsequently cultured until two-cell stage, and transplanted into pseudo-pregnant B6 females. For screening and 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.

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-Abshorth (clone 13.1, (24)), PDGF-Bshorth (clone D1, (25)) and PDGF-B (26). DNA constructs were excised from the vector backbone, purified using the Qiagen gel extraction kit (Qiagen) and injected into fertilized C57BL/6J/CBA oocytes, which were subsequently cultured until two-cell stage, and transplanted into pseudo-pregnant B6 females. For screening and 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.

4. Genotyping of mice

For PCR genotyping of transgenic founders, tail biopsies were lyzed in 100 µl lysis buffer (67 mM Tris, pH 8.8; 6.7 mM MgCl2; 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′-CTAAGGATGATGACTACTTTCG-3′; Pdgfa rev 5′-AAGAATCTCTGTTAAGGACCTGTC-3′; Pdgfb fwd 5′-ATAGACCGCAGACCGACACCACACTGTCG-3′; Pdgfb rev 5′-AATTACCCGGCACAACCTCTCTCC-3′. This resulted in a 411 bp product for both PDGF-Abshorth and PDGF-Bshorth and 486 bp for PDGF-B. The Pdgfrα GFP/+ mice were genotyped by their strong GFP expression under a ultraviolet light, or with PCR using the following primers: 5′-CCTTGTGGTGATGTTTCCAG-3′; 5′-GTGTTTGCTCTCA-TTACACTG G-3′; 5′-ACGAGTTTATTAGTCCCCAGCAG-3′ generating a 242 bp GFP-band and a 451 bp wt-band.

5. Fixation of heart tissue

Pdgfa 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 Pdgfrα 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 CO2. 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 PECA1 (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 Pdgfrα GFP/+ mice, the sections were permeabilized and blocked in
Fig. 1. Expression of PDGFRα and PDGFRβ 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. Scale bar 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.
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-Ashort or PDGFAlong [24,25]) or by PDGFB. PDGF-Ashort 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-Along remains localized close to its cellular source of secretions [5], whereas PDGF-Ashort that lacks the proteoglycan-binding domain is more diffusive 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 PdgfraGFP/+ knock-in mice [29] from E11.5 until adulthood. PdgfraGFP/+ 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 PdgfraGFP/+ 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 interstitial cells in the myocardium (Fig. 1E–I).

At all stages, PDGFRβ expression was localized to perivascular cells in close association to endothelial cells, identified by podocalyxin expression (Fig. 1L, 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-Ashort and PDGF-Along) or PDGF-B, re-
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-Along and the other expressing the short isoform PDGF-Ashort. Four + four Pdgfa transgenic founders were obtained, all of which developed severe cardiac hypertrophy. All PDGF-Ashort 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-Along 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-Along 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–I). On average, there was a more than 50% reduction of capillaries surrounding the cardiomyocytes. The phenotype was more pronounced in PDGF-Along mice (> 70% loss, Fig. 3J). In the fibrotic tissue of PDGF-Along transgenic mice, there was also an enrichment of large, dilated and irregular blood vessels with a glomeruloid appearance (Fig. 3I, arrows). These vessels were 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-Ashort 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-Along 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 survived 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 constructs 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. 4. Histological analysis of hearts from α-MHC-Pdgfb transgenic mice. (A-F) Masson Trichrome 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 subtle difference in vessel morphology between wt (G) and α-MHC-Pdgfb (H).
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]).

Table 1

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<tr>
<th>transgene</th>
<th>Life span</th>
<th>Fibrotic reaction</th>
<th>Capillary density</th>
<th>Interstitial cells</th>
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<td>Pdgfa&lt;sub&gt;short&lt;/sub&gt;</td>
<td>&lt;2 months</td>
<td>Diffuse</td>
<td>56% less</td>
<td>30% more</td>
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<tr>
<td>Pdgfa&lt;sub&gt;long&lt;/sub&gt;</td>
<td>&lt;1.5 months</td>
<td>Diffuse</td>
<td>73% less</td>
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<tr>
<td>Pdgfb</td>
<td>&gt;7 months</td>
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<td>36% less</td>
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<tr>
<td>Pdgfc</td>
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<tr>
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<td>&lt;2 months</td>
<td>Local</td>
<td>Less</td>
<td>More</td>
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Taken together with previous publications [19,20], our data do not 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 PDGFD [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 isofoms 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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