Cardiac Fibro-Adipocyte Progenitors Express Desmosome Proteins and Preferentially Differentiate to Adipocytes Upon Deletion of the Desmoplakin Gene


Rationale: Mutations in desmosome proteins cause arrhythmogenic cardiomyopathy (AC), a disease characterized by excess myocardial fibroadipocytes. Cellular origin(s) of fibroadipocytes in AC is unknown.

Objective: To identify the cellular origin of adipocytes in AC.

Methods and Results: Human and mouse cardiac cells were depleted from myocytes and flow sorted to isolate cells expressing platelet-derived growth factor receptor-α and exclude those expressing other lineage and fibroblast markers (CD32, CD11B, CD45, Lys76, Ly6c and Ly6s, thymocyte differentiation antigen 1, and discoidin domain receptor 2). The PDGFRα<sup>pos</sup>:Lin<sup>neg</sup>;THY1<sup>neg</sup>;DDR2<sup>neg</sup> cells were bipotential as the majority expressed collagen 1 α1, a fibroblast marker, and a subset CCAAT/enhancer-binding protein α, a major adipogenic transcription factor, and therefore, they were referred to as fibroadipocyte progenitors (FAPs). FAPs expressed desmosome proteins, including desmoplakin, predominantly in the adipogenic but not fibrogenic subsets. Conditional heterozygous deletion of Dsp in mice using Pdgfra-Cre deleter led to increased fibroadipogenesis in the heart and mild cardiac dysfunction. Genetic fate mapping tagged 41.4±4.1% of the cardiac adipocytes in the Pdgfra-Cre:Eye<sup>f</sup>Y:Dsp<sup>wf</sup> mice, indicating an origin from FAPs. FAPs isolated from the Pdgfra-Cre:Eye<sup>f</sup>Y:Dsp<sup>wf</sup> mouse hearts showed enhanced differentiation to adipocytes. Mechanistically, deletion of Dsp was associated with suppressed canonical Wnt signaling and enhanced adipogenesis. In contrast, activation of the canonical Wnt signaling rescued adipogenesis in a dose-dependent manner.

Conclusions: A subset of cardiac FAPs, identified by the PDGFRα<sup>pos</sup>:Lin<sup>neg</sup>;THY1<sup>neg</sup>;DDR2<sup>neg</sup> signature, expresses desmosome proteins and differentiates to adipocytes in AC through a Wnt-dependent mechanism. The findings expand the cellular spectrum of AC, commonly recognized as a disease of cardiac myocytes, to include nonmyocyte cells in the heart. (Circ Res. 2016;119:41-54. DOI: 10.1161/CIRCRESAHA.115.308136.)

Key Words: adipocytes ■ cardiomyopathies ■ genetics ■ heart failure ■ stem cells

Arrhythmogenic cardiomyopathy (AC) is an enigmatic hereditary disease characterized pathologically by excess fibroadipocytes in the myocardium and clinically by ventricular arrhythmias, heart failure, and sudden death. Molecular genetic basis of AC has been partially elucidated. Mutations in genes encoding desmosome proteins, components of the intercalated disks, have been identified as the main causes of AC. Cardiac myocytes (CMs), thus far, are the only cell type in the heart known to express desmosome proteins. However, CMs are differentiated cells and are not expected to switch fate and differentiate to fibroadipocytes. Thus, the molecular genetic discoveries have raised the intriguing question of how do mutations in the desmosome proteins, expressed in the heart, hitherto, only in CMs, lead to the unique phenotype of fibroadipogenesis in the heart. We surmised that cells other than CMs in the heart express desmosome proteins and differentiate to fibroadipocytes in AC.

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The heart is a cellularly heterogeneous organ, composed of many mature and immature cells. Among the mature cells, CMs are the only cardiac cells that are known to express desmosome proteins. Among the resident progenitor cells, KIT pro-to-oncogene receptor tyrosine kinase (KIT<sup>+</sup>) cells have been shown to express junction protein plakoglobin, a component of the desmosomes, and differentiate to adipocytes.
Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>AC</td>
<td>arrhythmogenic cardiomyopathy</td>
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<tr>
<td>CEBPA</td>
<td>CCAAT/enhancer-binding protein α</td>
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<tr>
<td>CFs</td>
<td>cardiac fibroblasts</td>
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<tr>
<td>CMs</td>
<td>cardiac myocytes</td>
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<tr>
<td>COL1A1</td>
<td>collagen 1 α1</td>
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<td>DDR2</td>
<td>discoidin domain receptor 2</td>
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<td>ECs</td>
<td>endothelial cells</td>
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<td>EGFP</td>
<td>enhanced green fluorescent protein</td>
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<td>FAPs</td>
<td>fibroadipocyte progenitor</td>
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<tr>
<td>KIT</td>
<td>KIT pro-to-oncogene receptor tyrosine kinase</td>
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<tr>
<td>MYH6</td>
<td>myosin heavy chain 6</td>
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<tr>
<td>PDGFR</td>
<td>platelet-derived growth factor receptor</td>
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<tr>
<td>PPARγ</td>
<td>peroxisome proliferator-activated receptor-γ</td>
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<tr>
<td>SMCs</td>
<td>smooth muscle cells</td>
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<tr>
<td>qPCR</td>
<td>quantitative polymerase chain reaction</td>
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<tr>
<td>THY1</td>
<td>thymocyte differentiation antigen 1</td>
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However, KIT+ progenitors are scant in the heart and contribute only to a small fraction of the excess adipocytes in AC. It is unknown whether desmosome proteins are also expressed in other resident mature and progenitor cells in the heart.

A subset of resident skeletal muscle progenitor cells, commonly identified by the expression of platelet-derived growth factor receptor-α (PDGFRα), are considered to be fibroadipocyte progenitor (FAPs).\(^6\)\(^\text{--}\)\(^8\) Skeletal FAPs are bipotential cell types that, under physiological states, are quiescent, but on muscle injury, they are activated to facilitate muscle regeneration by the endogenous myogenic stem cells.\(^6\)\(^\text{--}\)\(^8\) Persistent injury or failure of the skeletal muscle to regenerate after injury leads to differentiation of the FAPs to fibroblasts and adipocytes.\(^6\)\(^\text{--}\)\(^8\) Therefore, we surmised that the heart, similar to skeletal muscles, might contain a subset of resident progenitor cells, which could differentiate to adipocytes in AC. However, the presence and characteristics of the cardiac FAPs or their differentiation to adipocytes in the heart have yet to be demonstrated. Such progenitor cells, in the context of AC, have to express the desmosome proteins to differentiate to adipocytes in the presence of the mutant desmosome protein in AC. Alternatively, such cells could differentiate to adipocytes through paracrine mechanisms, emanating from myocytes that express the mutant desmosome protein. This study is designed to identify and characterize cardiac progenitor cells that differentiate to adipocytes in AC and determine the responsible mechanism(s).

### Methods

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. The Institutional Animal Care and Use Committee and Review Board approved the studies. A detailed Material and Methods section is provided in the Online Data Supplement.

### Isolation and Characterization of Human and Mouse Cardiac FAPs

Steps taken to isolate cardiac FAPs are shown in Online Figure I. In brief, noncardiac myocyte fraction of heart cells was sorted to isolate cells that express PDGFRα and exclude those expressing hematopoietic lineage markers CD32, CD11B, CD45, Lys76, Ly-6c and Ly-6c (Lin-\(\text{hi}\)), stem cell and fibroblast marker thymocyte differentiation antigen 1 (THY1), and fibroblast marker discoidin domain receptor 2 (DDR2). The isolated mouse PDGFRα-\(\text{hi}\);Lin-\(\text{hi}\);THY1-\(\text{hi}\);DDR2-\(\text{hi}\) cells were also analyzed for the expression of lineage markers endothelial-specific receptor tyrosine kinase, PDGFRβ, CD146, and KIT antigen by flow cytometry or immunostaining.

### Isolation of Mouse Adult CMs

To isolate CMs, explanted mouse heart was perfused with a calcium-free perfusion media and a digestion buffer containing collagenase II.\(^10\) CMs were isolated from the digested tissue by filtration and low-speed centrifugation and were gradually introduced to calcium at the final concentration of 1.5 mmol/L. CMs were plated in culture dishes or cover glasses coated with laminin and incubated immediately in a 2% CO\(_2\) incubator at 37°C. The isolation procedure is expected to exclude nonmyocyte cardiac cells.\(^10\)

### Isolation and Culture of Smooth Muscle Cells, Endothelial Cells, and Cardiac Fibroblasts

Mouse aortic smooth muscle cells (SMCs) were isolated from the mouse aortic tissues and cultured as previously published.\(^11\) Mouse primary cardiac microvascular endothelial cells (ECs) were purchased from a commercial source and grown on plates precoated with gelatin-based coating solution in M1168 mouse EC growth medium. Cardiac fibroblasts (CFs) were isolated as previously published.\(^12\)

### Immunofluorescence, Immunoblotting, Immunohistochemistry, and Quantitative Polymerase Chain Reaction

Immunofluorescence, immunoblotting, immunohistochemistry, and quantitative polymerase chain reaction (qPCR) were performed, as published.\(^13\)\(^\text{--}\)\(^16\)

### Detection of Apoptosis

Apoptosis was detected by terminal deoxynucleotidyl transferase dUTP nick-end labeling assay, as previously described.\(^17\)

### Lineage Tracer Mice

Pdgfra::EGFP reporter mice were purchased from Jackson Laboratory.\(^18\) These mice express the H2B–enhanced green fluorescent protein (EGFP) fusion protein from the endogenous Pdgfra locus, leading to expression of H2B-EGFP mimicking the expression pattern of the endogenous Pdgfra gene.\(^18\) Myh6-Cre, Dgpr\(^\text{--}\), and R26\(^\text{2-STOP-EYFP}\) mice have been published.\(^19\)\(^\text{--}\)\(^22\) Pdgfra-Cre bacterial artificial chromosome transgenic mice were from Jackson Laboratory. Oligonucleotide primers used in genotyping by PCR are listed in Online Table I.

### Echocardiography

Left ventricular dimensions and function in mice were assessed by B-mode, M-mode, and Doppler echocardiography using a HP 5500 Sonos echocardiography unit equipped with a 15-MHz linear transducer, as published.\(^3\)\(^\text{--}\)\(^5\)\(^,\)\(^14\)\(^,\)\(^15\) Wall thicknesses and left ventricular dimensions were measured from M-mode images using the leading-edge method on 3 consecutive cardiac cycles. Left ventricular fractional shortening and mass were calculated as previously described.\(^3\)\(^\text{--}\)\(^5\)\(^,\)\(^14\)\(^,\)\(^15\)

### Morphometric and Histological Analyses

Ventricular/body weight ratio, H&E, Masson trichrome, Picrosirius red, and Oil Red O staining were analyzed, as published.\(^3\)\(^\text{--}\)\(^5\)\(^,\)\(^14\)\(^,\)\(^15\) To quantify the extent of fibrosis, collagen volume fraction was determined from sirius red–stained thin myocardial sections using ImageTool 3.0 software.

### Induction of Adipogenesis

Isolated cardiac FAPs were treated with an adipogenesis induction medium for 2 to 7 days, as published.\(^3\)\(^\text{--}\)\(^5\)\(^,\)\(^14\)\(^,\)\(^15\)

### Activation of the Canonical Wnt Signaling Pathway

To activate the canonical Wnt signaling pathway, cells were treated with 2 different concentrations (5 and 10 μmol/L) of
6-bromoiridin-3'-oxime, a known activator of the canonical Wnt signaling, as described.12,21,24

Statistical Analysis

Normally distributed continuous variables between the 2 groups were compared by t test or Mann–Whitney U test. Differences among multiple groups were analyzed by 1-way ANOVA or multivariate ANOVA. Pairwise comparisons were performed by Bonferroni multiple comparison test. Differences among the categorical values were compared by Kruskall–Wallis test.

Results

Isolation and Characterization of Cardiac FAPs

A subset of progenitor cells in skeletal muscles, referred to as FAPs, are characterized by the expression of cell surface marker PDGFRA and exclusion of other cell types known to express PDGFRA, such as bone marrow–derived progenitors.8,25,26 In the heart, the presence and characteristics of FAPs and the subset that differentiate to adipocytes are unknown. To isolate cardiac FAPs, human and mouse myocyte–depleted cardiac cells were sorted by flow cytometry to isolate cells that expressed PDGFRA but not the hematopoietic progenitor markers CD32, CD11B, CD45, Lys76, Ly6c, and Ly6e, stem cells and fibroblast marker THY1, or fibroblast marker DDR2 (Figure 1; Online Figure I). The findings were also corroborated by immunofluorescence staining of human and mouse fluorescence-activated cell sorting–isolated PDGFRApos:Lin−:THY1neg:DDR2neg cells for the lack of expression of THY1 and DDR2 (Figure 1). Myocyte-depleted cardiac cells were analyzed for coexpression of PDGFRA and additional lineage markers endothelial-specific receptor tyrosine kinase (a marker for ECs), KIT antigen (a marker for progenitor cells), CD146 (a marker for pericytes), and PDGFRB (a marker for progenitor cells and pericytes). As shown in Online Figure II, <1% of cardiac PDGFRApos cells expressed endothelial-specific receptor tyrosine kinase, KIT, CD146, and PDGFRB lineage markers.

To determine whether isolated human and mouse PDGFRApos:Lin−:THY1neg:DDR2neg cells were bipotential for fibrogenesis and adipogenesis, similar to FAPs in skeletal muscles, they were stained for the expression of collagen type I (COL1A1) and CCAAT/enhancer-binding protein α (CEBPA), markers for fibrogenesis and adipogenesis, respectively. As shown in Figure 2, PDGFRApos:Lin−:THY1neg:DDR2neg cells showed bimodal expression patterns of these 2 markers as the majority predominantly expressed COL1A1 (62.3±10.4% in mice; 66.9±8.5 in humans) and a minority subset (16.8±7.2% in mice; 17.6±3.7 in humans) predominantly expressed CEBPA (Figure 2). These findings collectively suggest that PDGFRApos:Lin−:THY1neg:DDR2neg cells isolated from the heart are bipotential cells, expressing either adipogenic or fibrogenic markers. Henceforth, they were referred to as cardiac FAPs.

Expression of Desmosome Proteins in FAPs

To determine whether cardiac FAPs express desmosome proteins, expressions of desmoplakin, plakoglobin, plakophilin 2, and desmoglein 2 were determined by immunofluorescence and immunoblotting.27 As shown in Figure 3, desmoplakin, junction protein plakoglobin, plakophilin 2, and desmoglein 2 were expressed in cardiac FAPs (Figure 3). To exclude the potential contamination of the isolated FAPs with CMs, hitherto, the only cardiac cell type known to express desmosome proteins, isolated cardiac FAPs cells were examined for the expression of sarcomere proteins myosin heavy chain 6 (MYH6) and myosin-binding protein C3. As shown in Figure 3, MYH6 and myosin-binding protein C3 were not expressed in cardiac FAPs, indicating that the isolates were devoid of contamination with CMs.

Expression of Desmoplakin Protein in Cardiac Adipogenic FAPs

To determine whether both subpopulations of cardiac FAPs expressed desmoplakin, isolated human and mouse PDGFRApos:Linneg:THY1neg:DDR2neg cells were costained for the expression of desmoplakin and CEBPA or COL1A1. As shown in Figure 4, desmoplakin protein was predominantly coexpressed with CEBPA but not with COL1A1.

Expression of PDGFRA in Other Cardiac Cell Types

As a prelude to genetic fate mapping, we determined whether PDGFRA was expressed in other major cardiac cell types, namely, CMs, CFs, SMCs, and ECs. Neither CMs, ECs, nor SMCs expressed PDGFRA protein as detected by immunofluorescence staining of isolated cells and immunoblotting of extracted proteins (Online Figure III). Likewise, Pdgfra mRNA was undetectable in isolated adult CMs (Online Figure III). In contrast, 71.1±20.2% of isolated CFs, identified by the expression of COL1A1, also expressed PDGFRA (Online Figure III), a finding that is in accord with the literature and immunostaining of the FAPs for COL1A1 (Figure 2).8,28–30 In accord with these findings, immunostaining of thin mouse myocardial sections also showed expression of the PDGFRA in CFs but not in CMs, SMCs, or ECs (Online Figure IV).

In a complementary set of studies and to corroborate detection of expression of PDGFRA in cardiac cell types, we determined the expression of the reporter protein EGFP in the heart in the Pdgfra-Egfp reporter mice. In this model, expression of EGFP is transcriptionally regulated by the Pdgfra locus.18 Approximately 51.3±9.8% of CFs were tagged with the EGFP (Online Figure V), a finding that was in accord with the expression of PDGFRA in isolated adult CFs (Online Figure III) and immunostaining of cardiac FAPs for COL1A1 (Figure 2). However, expression of the reporter protein EGFP was not detected in CMs, SMCs, and ECs, indicating that Pdgfra locus was transcriptionally inactive in these mature cardiovascular cells (Online Figure V).

Absence of Expression of Desmplakin in CFs, SMCs, and ECs

To determine whether desmplakin was expressed in CFs, ECs, and SMCs in the heart, isolated murine CFs, SMCs, and ECs were stained for the expression of desmplakin and analyzed by immunoblotting. As shown in Online Figure VI, desmplakin protein was not expressed in isolated CFs, SMCs, and ECs, whereas it was expressed in isolated CMs, as expected.
To determine whether deficiency of desmoplakin affects differentiation of FAPs to adipocytes, Dsp gene was conditionally deleted on expression of cre recombinase in cardiac FAPs. In this strategy, crossing of Pdgfra-Cre deleter with Dsp<sup>F/F</sup>:R26<sup>-STOP-Eyfp</sup> mice led to expression of the cre recombinase in cardiac FAPs, deletion of the floxed exon 2 of the Dsp gene, and deletion of the STOP sequence upstream of the Eyfp gene. The former led to inactivation of Dsp and the

**Figure 1.** Isolation of fibroadipocyte progenitor (FAPs) from human and mouse hearts. A and C, Flow cytometry plots showing the multistep approach used to isolate FAPs from human (A) and mouse (C) hearts: nonmyocyte fraction of cardiac cells was sorted to isolate cells positive for platelet-derived growth factor receptor A (PDGFRA) but negative for the hematopoietic progenitor markers CD32, CD11B, CD45, Lys76, Ly-6c and Ly6c, the stem cell and fibroblast marker THY1, and the fibroblast marker DDR2. Positive gates were set by analyzing signals from negative control samples, which were stained only with the corresponding IgG isotype for each marker. Immunofluorescence panels confirming the lack of expression of THY1 and DDR2 in human (B) and mouse (D) fluorescence-activated cell sorting–isolated PDGFRA<sup><sup><sup><sup>pos</sup></sup></sup></sup>:Lin<sup><sup><sup><sup>neg</sup></sup></sup></sup>:THY1<sup><sup><sup><sup>neg</sup></sup></sup></sup>:DDR2<sup><sup><sup><sup>neg</sup></sup></sup></sup> cells.

**Lineage Tracing Upon Genetic Deletion of Dsp Gene in Cardiac FAPs**

To determine whether deficiency of desmoplakin affects differentiation of FAPs to adipocytes, Dsp gene was conditionally deleted on expression of cre recombinase in cardiac FAPs. In this strategy, crossing of Pdgfra-Cre deleter with Dsp<sup>F/F</sup>:R26<sup>-STOP-Eyfp</sup> mice led to expression of the cre recombinase in cardiac FAPs, deletion of the floxed exon 2 of the Dsp gene, and deletion of the STOP sequence upstream of the Eyfp gene. The former led to inactivation of Dsp and the
latter to expression of EYFP in FAPs in the Pdgfra-Cre;R26-STOP<sup>e</sup>-Eyfp:Disp<sup>+/-</sup> lineage tracer mice (hitherto Pdgfra-Cre:Eyfp:Disp<sup>+/-</sup>; Online Figure VII).

To determine the efficiency of the cre-mediated recombination, FAPs were isolated from the hearts of Pdgfra-Cre:Eyfp:Disp<sup>+/-</sup> lineage tracer mice and analyzed by flow cytometry for the expression of EYFP as an indicator of recombination efficiency (Figure 5A and 5B). Expression of EYFP was detected in ≈84.3±5.6% of the cardiac FAPs (Figure 5). Direct examination of isolated cardiac FAPs under fluorescence microscopy also confirmed the expression of EYFP in 84.8±7.9% of the isolated FAPs (Figure 5). To complement the data on the recombination efficiency, thin myocardial sections from the Pdgfra-Cre:Eyfp:Disp<sup>+/-</sup> lineage tracer...
mice were costained for the expression of PDGFRA and EYFP. Approximately 68.2±9.2% of PDGFRA expressing cells also expressed EYFP (Figure 5).

To determine functional deletion of the Dsp gene in cardiac FAPs, mRNA and protein levels of Dsp were determined by qPCR and immunoblotting, respectively. Transcript levels of Dsp were reduced by 52.8±3.3% and that of desmoplakin protein by ≈56.0±0.7% in cardiac FAPs isolated from the Pdgfra-Cre:Eyfp:DspW/F lineage tracer mice when compared with control mice (Figure 5).

To exclude fortuitous deletion of Dsp in other cardiac cells in the lineage tracer mice, mRNA and protein levels of Dsp gene, as well as expression of EYFP, were determined in CMs isolated from wild-type (WT) and Pdgfra-Cre:Eyfp:DspW/F mice. Surprisingly, 17.9±0.3% of CMs isolated from the hearts of Pdgfra-Cre:Eyfp:DspW/F mice expressed EYFP, suggesting, to our knowledge for the first time, developmental heterogeneity of CMs in the mouse heart (Online Figure VIII). The finding suggests transcriptional activity of the Pdgfra locus in a subset of CMs during development. To determine whether genetic tagging of a subset of CMs under the transcriptional activity of the Pdgfra locus affected the expression levels of Dsp mRNA and protein, their levels were quantified in isolated CMs.

Dsp mRNA and protein levels were not significantly altered in CMs isolated from the lineage tracer mice when compared with control CMs (Online Figure VIII). Likewise, immunofluorescence staining of isolated CMs showed localization of desmoplakin to the junctional areas (Online Figure VIII). Considering that adult CMs do not express PDGFRA (Online Figures III, IV, and V), tagging of a subset of CMs with EYFP in the reporter mice suggests transient transcriptional activity of the Pdgfra locus during CM development but not persistent active transcription.

Expression of EYFP was also analyzed in myocardial sections from the Pdgfra-Cre:Eyfp:DspW/F mice stained for EYFP and specific markers for CFs, SMCs, and ECs. Only CFs but not SMCs or ECs expressed EYFP (Online Figure IX). This finding is in accord with the data in the Pdgfra-Egfp reporter mice (Online Figure V), as well as with the data showing in vitro and in vivo expression of PDGFRA in isolated CFs from WT mice (Online Figures III and IV).

Phenotypic Consequences of Deletion of Dsp in Cardiac FAPs

Echocardiographic data on left ventricular dimensions and function in 9-month-old WT Pdgfra-Cre, and Pdgfra-Cre:Eyfp:DspW/F mice are shown in Online Table II. As shown, Pdgfra-Cre:Eyfp:DspW/F mice exhibited mild cardiac dilatation and dysfunction when compared with WT and Pdgfra-Cre control mice. There was no discernible cardiac dysfunction in the Pdgfra-Cre:Eyfp or R26-STOPF-Eyfp or DspF/F mice.

Ventricular/body weight ratio was modestly increased in the Pdgfra-Cre:Eyfp:DspW/F mice when compared with WT mice or Pdgfra-Cre mice (Online Figure X). Picrosirius staining of thin myocardial sections showed increased myocardial interstitial fibrosis in the Pdgfra-Cre:Eyfp:DspW/F mice, comprising 3.8±1.1% of the myocardium (Online Figures X and XI). Similarly, Oil Red O staining of thin myocardial sections showed a 13±8-fold increase in the number of adipocytes in the heart of the Pdgfra-Cre:Eyfp:DspW/F mice when compared with control mice (Online Figures X and XI). Moreover, the number of cells expressing adipogenic transcription...
factor CEBPA was also increased significantly in the hearts of Pdgfra-Cre:Eyfp:DspW/F mice when compared with the control groups (Online Figures XD, XG, and XI). To further explore fibroadipogenesis, FAPs were isolated from WT and Pdgfra-Cre:Eyfp:DspW/F mouse hearts and along with thin myocardial sections were stained for profibrotic transforming growth factor-β1. As shown in Online Figure XII, transforming growth factor-β1 expression levels were increased in isolated FAPs and myocardial sections from the Pdgfra-Cre:Eyfp:DspW/F mice. Finally, considering that deletion of Dsp in CMs induces apoptosis,17 we determined whether deletion of Dsp in cardiac FAPs also induced apoptosis. The number of cells stained positive in the terminal deoxynucleotidyl transferase dUTP nick-end labeling assay was not significantly different between the WT and Pdgfra-Cre:Eyfp:DspW/F mice (Online Figure XIII).

**Origin of Adipocytes From Cardiac FAPs**

To determine whether excess adipocytes in the heart originated from FAPs, thin myocardial sections were immunostained with antibodies against EYFP and adipogenic transcription factors CEBPA or peroxisome proliferator–activated receptor-γ (PPARG). Approximately 41.4±4.1% of cells in the heart of lineage tracer mice that expressed CEBPA also expressed EYFP (Figure 6). The results were similar for the coexpression of PPARG and EYFP (41.2±2.6%) as shown in Figure 6. These data collectively indicate that close to half of the excess adipocytes in desmoplakin-deficient mouse model originate from FAPs. To determine whether the increased number of adipocytes in the heart of Pdgfra-Cre:Eyfp:DspW/F mice was because of proliferation of the adipocytes, thin myocardial sections were costained for the expression of CEBPA, to mark adipocytes, and Ki67 protein (MKI67), to mark proliferating cells. As shown in Online Figure XIV, although the number of adipocytes was greater in the Pdgfra-Cre:Eyfp:DspW/F mice, percent of adipocytes that were stained positive for the proliferation marker did not differ significantly between the WT and lineage tracer mice.

**Enhanced Differentiation of Dsp-Deficient FAPs to Adipocytes**

To further support differentiation of cardiac FAPs to adipocytes, FAPs were isolated from the hearts of WT and
Pdgfra-Cre:Eyfp:Dsp\textsuperscript{WoF} mice and treated with an adipogenic induction medium, as published.\textsuperscript{5,13} Adipogenesis was analyzed serially at multiple time points by quantifying the number of Oil Red O– and CEBPA-stained cells. The number of Oil Red O– and CEBPA-stained cells (Figure 7) was consistently higher in cardiac FAPs isolated from the Pdgfra-Cre:Eyfp:Dsp\textsuperscript{WoF} mice when compared with cells isolated from the WT mice at all time points. Likewise, quantification
of transcript levels of selected adipogenic genes by qPCR showed marked increases in the transcript levels of *Fabp4*, *Cebpa*, *Pparg*, *Dgat1*, and *Dgat2* in FAPs isolated from the *Pdgfra-Cre:Eyfp:DspW/F* when compared with control mice (Figure 7). Similarly, protein levels of fatty acid binding protein 4, CEBPA, and PPARG were increased in FAPs isolated from the lineage tracer mice (Figure 7). To corroborate the findings based on Oil Red O staining, cells were stained for perilipin, a marker of mature adipocytes. The number of cells expressing perilipin was significantly increased in the *Pdgfra-Cre:Eyfp:DspW/F* when compared with WT mice (Online Figure XV).

To extend the findings in the mouse models to human AC, thin myocardial sections from the hearts of human patients with AC were stained for the expression of PDGFRα and the adipogenic transcription factor PPARG (Figure 7G). Approximately 43.6±0.8% of adipocytes in the human hearts with AC coexpressed PDGFRα and PPARG, suggesting a transitional state of cardiac FAPs to adipocytes in the human hearts with AC.

**Exclusion of a Paracrine Mechanism for Differentiation of Cardiac FAPs to Adipocytes**

Considering that CMs are the main cardiac cells that are known to express desmosome proteins and given that lineage tracing identified about half of the adipocytes as originating from FAPs, a new set of lineage tracing was performed to test for paracrine mechanisms in differentiation of FAPs to adipocytes in AC. According to the paracrine hypothesis, the stimulus has to originate from cells that express desmosome proteins, mainly CMs and target cardiac resident cells that differentiate to adipocytes. To test this hypothesis, *Pdgfra-Egfp* reporter mice, whereby EGFP is expressed under transcriptional regulation of the *Pdgfra* locus, was crossed to the *Myh6-Cre:DspW/F* mouse model of AC. These mice are heterozygous for *Dsp* in CMs. The
Pdgfra-Egfp:Myh6-Cre:DspW/F mice afford the opportunity to test a paracrine effect(s), emanating from the Dsp-deficient CMs and targeting resident EGFP-labeled FAPs for differentiation to adipocytes. Cardiac phenotype in the Pdgfra-Egfp:Myh6-Cre:DspW/F lineage tracer mice was comparable with that published for the Myh6-Cre:DspW/F

Figure 7. Enhanced adipogenesis in cardiac fibroadipocyte progenitors (FAPs) isolated from the desmplakin (DSP) haploinsufficient mice and detection of adipogenic FAPs in the human heart. A and B, Oil Red O staining and CCAAT/enhancer-binding protein α (CEBPA) immunostaining showing accumulation of fat droplets (A) and expression of the adipogenic transcription factor CEBPA (B) in cardiac FAPs isolated from the Pdgfra-Cre:Eyfp:DspW/F lineage tracer mice when compared with wild-type mice, at 4 time points on induction of adipogenesis with insulin, IBMX (3-isobutyl-1-methylxanthine) and DXM (dexamethasone). C and D, Quantitative data showing increased number of mature (Oil Red O+) adipocytes (C) and CEBPA+ cells (D) in the cardiac FAPs from the transgenic mice when compared with wild-type mice at each time point (n=3, ~300 cells counted at each time point for each group, *P<0.05). E, Quantitative polymerase chain reaction data for selected adipogenic genes after 4 days of adipogenesis induction showing marked increase in the transcript levels of Fabp4, Cebpa, Pparg, Dgat1, and Dgat2 in cardiac FAPs isolated from the Pdgfra-Cre:Eyfp: DspW/F mice when compared with wild-type mice (n=3; *P<0.05). F, Immunoblots showing increased protein levels of adipogenic markers FABP4, CEBPA, and peroxisome proliferator–activated receptor-γ (PPARG) in cardiac FAPs isolated from the heart of lineage tracer mice after 4 days of adipogenic induction. G, Immunofluorescence stained panels from a control human heart and a human heart from a patients with arrhythmogenic cardiomyopathy (AC), showing coexpression of platelet-derived growth factor receptor A (PDGFRα) and the adipogenic transcription factor PPARG, suggestive of the presence of FAPs in transition to adipocytes in the human heart with AC.
mice. As would be expected, the phenotype was notable for enhanced fibroadipogenesis and cardiac dysfunction (Online Figure XVI and Online Table III).

To detect whether adipocytes in the heart of Pdgfra-Egfp:Myh6-Cre:DspW/F expressed EGFP, thin myocardial sections were stained for EGFP and CEBPA. The findings are notable for the increased number of adipocytes in the hearts of Pdgfra-Egfp:Myh6-Cre:DspW/F mice (Online Figure XVII), which is in accord with the finding in the Myh6-Cre:DspW/F mice.17 However, the percentage of adipocytes expressing EGFP in the control Pdgfra-Egfp and Pdgfra-Egfp:Myh6-Cre:DspW/F mice was not significantly different (Online Figure XVII). The finding excludes differentiation of cardiac FAPs to adipocytes because of paracrine effects of Dsp-deficient CMs.

Suppression of the Canonical Wnt Signaling as a Mechanism for Enhanced Differentiation of Cardiac FAPs to Adipocytes

Because canonical Wnt signaling, a major determinant of cell fate and differentiation, has been previously implicated in the pathogenesis of adipogenesis in AC, transcript levels of established targets of the canonical Wnt signaling pathways in the heart were analyzed by qPCR. As shown in Figure 8, transcript levels of several canonical Wnt target genes were significantly reduced in the Pdgfra-Cre:Eyfp:DspW/F mice.

To determine the pathogenic role of suppressed canonical Wnt signaling in differentiation of Dsp-deficient FAPs to adipocytes, FAPs were isolated from the hearts of Pdgfra-Cre:Eyfp:DspW/F mice and treated with 6-bromoindirubin-3′-oxime to activate the canonical Wnt signaling.5,24 Treatment with 6-bromoindirubin-3′-oxime rescued adipogenesis in FAPs in a dose-dependent manner as determined by Oil Red O and immunofluorescence staining for CEBPA (Figure 8). Likewise, treatment of cardiac FAPs, isolated from the Pdgfra-Cre:Eyfp:DspW/F mice, with 6-bromoindirubin-3′-oxime normalized transcript levels of several adipogenic genes (Figure 8).

Discussion

A subset of human and mouse resident cardiac progenitor cells, identified as PDGFRA+Lin−THY1+DDR2+ cells, referred to as cardiac FAPs, are a major source of adipocytes in AC caused by Dsp haploinsufficiency. Cardiac FAPs exhibit bimodal expression patterns for the adipogenic transcription factor CEBPA and the fibroblast marker COL1A1. Desmosome proteins, including desmoplakin, are expressed only in a subset of FAPs that predominantly express CEBPA but not in cells expressing COL1A1. Genetic deletion of Dsp in cardiac FAPs leads to their differentiation to adipocytes in the mouse heart through a canonical Wnt-deficient mechanism. Cardiac FAPs give origin to ≈40% of the adipocytes in the heart of a mouse model of AC, a finding that indicates a heterogeneous cellular origin of excess adipocytes in AC. Thus, the finding of the present study by showing expression of desmosome proteins in cardiac FAPs expands the cellular basis of AC, which is conventionally considered a disease of CMs, to include nonmyocyte cells, namely FAPs, in the heart.

Multiple cell surface and lineage-specific markers were used to identify and isolate cardiac FAPs in the human and in the mouse hearts. Likewise, expression of multiple desmosome proteins was detected in cardiac FAPs and confirmed by complementary methods. The data also show 2 distinct subsets of FAPs with regard to expression of the adipogenic and fibrogenic markers, likely serving as progenitors for their respective lineages. Notably, desmosome protein desmoplakin was predominantly expressed in the adipogenic but not the fibrogenic subset of cardiac FAPs. In accord with this observation, desmoplakin was not expressed in CFs and other common cardiac cell types, such as SMCs and ECs, a finding that was confirmed at multiple levels and in vitro studies, as well as in vivo mapping studies using reporter mice. Thus, although a subset of CFs originate from cells transcriptionally regulated by the Pdgfra locus, CFs and a subset of FAPs that predominantly express COL1A1 do not express desmoplakin. Therefore, in the genetic fate mapping studies, expression of the cre recombinase under the transcriptional control of the Pdgfra locus is expected to lead to specific deletion of the Dsp gene in a subset of cardiac FAPs that express desmoplakin but not in CFs and other cell types in the heart.

Because PDGFRA is also considered a fibroblast marker, we excluded cells expressing other fibroblast markers THY1+ and DDR2+ cells. Nevertheless, despite exclusion of such cells, ≈70% of cardiac FAPs also expressed COL1A1, which is a marker for CF lineage. Whether COL1A1+ cells are true fibroblast progenitor cells or mature CFs was not discerned in the presence study, as desmoplakin, which was targeted for deletion, was not expressed in CFs or in the subset of COL1A1+ FAPs. Heterogeneous origin of CFs further cofounds their effective identification and isolation, by a defined set of markers.29 In accord with the data on the developmental heterogeneity of CFs,28–30 genetic fate mapping using the Pdgfra-Cre mice tagged ≈50% of CFs as originating from cells that are transcriptionally regulated by the Pdgfra locus.

Approximately half of cardiac adipocytes in the Dsp heterozygous mice originated from cardiac FAPs. This finding might, in part, reflect an incomplete recombination efficiency, which was estimated to be ≈80%. It also suggests a heterogeneous origin of the excess adipocytes in AC. We have previously shown that a small fraction of cardiac adipocytes originate from cells that express the KIT antigen.2 FAPs are distinct from KIT+ cells as shown in cell sorting and immunostaining data (Online Figure II). Thus, additional cell types, including other mesenchymal progenitor cells, might give rise to excess adipocytes. Alternatively, resident cardiac adipocytes might simply proliferate in response to yet-to-be-defined mechanism(s) in AC. The latter seems unlikely as the number of proliferating adipocytes was not significantly different between the WT and the Dsp-deficient lineage tracer mice. It is also important to note that the mouse models of AC, caused by mutations in the desmosome proteins, do not truly recapitulate the human phenotype because the extent of fibroadipocyte infiltration in the myocardium is rather modest when compared with AC in humans. Incomplete recapitulation of the human phenotype in model organisms is not unusual and rather expected.32,33 Nevertheless, the finding of a subset of adipocytes in the hearts of human patients with AC coexpressing PDGFRA and PPARG offers additional credence to relevance of the findings to human AC.
Figure 8. Suppression of the canonical Wnt and rescue of adipogenesis on activation of the canonical Wnt signaling pathway.

A, Quantitative polymerase chain reaction (qPCR) of the transcript levels of established targets of the canonical Wnt signaling pathway showing reduced levels in the heart of Pdgfra-Cre: Eyfp:Dsp^W/F lineage tracer mice when compared with wild-type control mice (n=3 mice per group). B–E, Rescue of adipogenesis on activation of the canonical Wnt pathway. Oil Red O (ORO)-stained (B) and CCAAT/enhancer-binding protein α (CEBPA)–immunostained (C) panels showing fibroadipocyte progenitors (FAPs) isolated from the heart of wild-type and Pdgfra-Cre:Eyfp:Dsp^W/F mice, subjected to adipogenic stimulation and treated with 2 doses of 6-bromoindirubin-3′-oxime (BIO), a known activator of the Wnt signaling. Quantitative data (D and E) show that activation of the canonical Wnt signaling reduced adipogenesis in a dose-dependent manner (n=3, ≈300 cells for ORO, ≈200 cells for CEBPA-immunofluorescence counted in each experiment for each group). F, qPCR data showing transcript levels of selected genes involved in adipogenesis before induction of adipogenesis, on induction with adipogenic media and after treatment with 2 increasing concentrations of BIO. Treatment with BIO normalized increased transcript levels of the adipogenic genes Fabp4, Cebpa, Pparc, Dgat1, and Dgat2 in FAPs isolated from the heart of Pdgfra-Cre:Eyfp:Dsp^W/F mice in a dose-dependent manner (n=3 for each experiment; *P<0.05).
An intriguing finding of the present study is the developmental heterogeneity of CMs. Accordingly, genetic fate mapping identified a minority fraction of CMs (~20%) that was transcriptionally regulated by the Pdgfra locus sometimes during development. Considering that PDGFRA is not transcriptionally active in adult CM, as shown by multiple sets of data in the present study, the finding indicates transient transcriptional activity of the Pdgfra locus during cardiac development and subsequent silencing of the locus in the adult CMs. It is important to note that despite labeling of a subset of CMs with EYFP, protein and mRNA levels of Dsp gene were unchanged in CMs. This finding might simply indicate that heterozygous deletion of Dsp in ~20% of CMs is not sufficient to reduce levels of Dsp mRNA and protein in the whole heart, particularly considering transcriptional compensation from the WT allele.34 In addition, a modest reduction might not be within the resolution of qPCR and immunoblotting. Nevertheless, the Pdgfra-Cre: EYFP: DspF""”gene exhibited mild cardiac dilatation and dysfunction, which might reflect the role of FAPs in supporting cardiac function or the effects of modest and yet undetectable changes in the expression level of Dsp gene in ~20% of CMs. Biological and functional significance of developmental heterogeneity of CMs, nevertheless, remains to be determined.

The findings also implicate suppressed canonical Wnt signaling in the heart as a mechanism for enhanced differentiation of resident FAPs to adipocytes, which is also in accord with the previous findings.5,13,17 The mechanisms responsible for suppression of the canonical Wnt signaling were not directly tested in the present study but presumably are similar to those published.5,13,17 The second set of genetic fate mapping, whereby EGFP protein was expressed under transcriptional activity of the Pdgfra locus in the background of deletion of Dsp gene in CMs, excluded a possible paracrine mechanism(s) for differentiation of FAPs to adipocytes in the Dsp-deficient mice. Mechanistic studies, however, are not comprehensive of various putative mechanisms that might be involved in the pathogenesis of AC and its perplexing histopathologic phenotypes.

In summary, we have identified a subset of human and mouse resident cardiac progenitor cells, characterized by the expression of PDGFRA but lacking expression of other cell lineage markers, and referred to as cardiac FAPs as a cell source of excess adipocytes in AC. A subset of cardiac FAPs that express adipogenic transcription factor CEBPA also express desmosomal proteins, including desmoplakin, and differentiate to adipocytes in a mouse model of AC caused by Dsp haploinsufficiency, through a mechanism that involves the canonical Wnt signaling pathway. The findings expand the cellular basis of AC to include cardiac FAPs and indicate a heterogeneous cellular basis of the complex phenotype of AC.

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Disclosures

None.

References


We have identified a subset of resident cardiac cells, fibroadipocyte progenitor (FAPs), that express the cell surface marker platelet-derived growth factor \( \alpha \) receptor and exhibit a bidirectional pattern for the expression of either an adipogenic transcription factor CCAAT/enhancer-binding protein \( \alpha \) or a fibroblast marker COL1A1.

- The subtraction of cardiac FAPs that express adipogenic markers also express desmosomal proteins, including desmoplakin.
- Deletion of Dsp gene in FAPs leads to their differentiation to adipocytes.
- FAPs give origin to approximately half of the excess cardiac adipocytes in AC.
- The subfraction of cardiac FAPs that express adipogenic markers also express desmosomal proteins, including desmoplakin.
- A pathological hallmark of AC is excessive fibroadipogenesis in the heart, which contributes to both cardiac dysfunction and arrhythmias.

What New Information Does This Article Contribute?

We found that in addition to cardiac myocytes, desmosomal proteins are expressed in FAPs, and these cells contribute to the pathogenesis of the unique phenotype of fibroadipogenesis in AC.
Cardiac Fibro-Adipocyte Progenitors Express Desmosome Proteins and Preferentially Differentiate to Adipocytes Upon Deletion of the Desmoplakin Gene


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Cardiac Fibro-Adipocyte Progenitors Express Desmosome Proteins and Preferentially Differentiate to Adipocytes Upon Deletion of the Desmoplakin Gene

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ONLINE MATERIAL AND METHODS

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. The Institutional Animal Care and Use Committee approved the studies.

Isolation and culture of FAPs from human hearts: Cardiac myocyte depleted cell fraction was obtained by collagenase type 2 digestion of ~100mg of fresh tissue. In detail, bioptic samples were minced and incubated in a 0.1% collagenase 2 solution (Worthington Biochemical Corp; Lakewood, NJ08701; cat# LS004176) for 45 min at 37 °C under gentle agitation. The collagenase activity was stopped by adding 10 mL of α Modification media (aMEM, Hyclone; cat# SH30265.01) supplemented with 10% stem cell certified Fetal Bovine Serum (ES-FBS, Hyclone; cat# SH30070.03-E) and 1% Antibiotic-Antimycotic solution (Gibco; cat # 15240). The solution was filtered through a 40 mm cell strainer (BD Bioscience cat# 352340) and centrifuged for 5 min at 300g. The cell pellet was washed one time with PBS and re-suspended in complete medium, composed by aMEM supplemented with 20% ES-FBS, and 1% Antibiotic-Antimycotic solution, plated in 60mm 0.1%gelatin coated plates and placed in a 5% CO2 humidified incubator at 37 °C.

After 2 to 3 days in culture, cardiac myocyte-depleted cells were detached by trypsin treatment, resuspended in MACS buffer (Miltenyi Biotec, Bergisch Gladbach, Germany; cat # 130-091-221) and incubated for 45 min at 4 °C in the dark with the following antibodies: anti platelet-derived growth factor receptor A (PDGFRA) (BD Pharmingen, PE Mouse Anti-Human CD140a antibody; cat# 556002), hematopoietic lineage antibody cocktail (BD Pharmingen, FITC Human Lin cocktail; cat #562722), anti thymocyte antigen 1 (THY1) (BD Pharmingen, APC Mouse Anti-Human CD90; cat# 561971) and anti Discoidin domain receptor 2 (DDR2) (Santa Cruz Biotechnology). Unlabeled cells and cells stained with the appropriate isotype IgG controls were included as controls. Unbound antibody was removed by two washes in 2 mL of MACS buffer and cell suspension was passed.
through a 35 μm mesh strainer (BD Bioscience cat# 352235) and sorted through the FACS-Aria flow cytometer (BD Pharmingen, San Diego, CA). A multi-step approach was used (Online Figure I) in order to isolate hematopoietic lineage negative (Lin<sup>-</sup>) cells that expressed PDGFRA but not the stem cell and fibroblast marker THY1 or the fibroblast marker DDR2. FACS-isolated PDGFRA<sup>pos</sup>:Lin<sup>-</sup>:THY1<sup>-</sup>:DDR2<sup>-</sup> cells were seeded onto 0.1% gelatin coated plates at the density of 2.5 x 10<sup>4</sup>/cm<sup>2</sup> in growth medium (αMEM, supplemented with 10% embryonic stem cell certified ES-FBS and 1% Antibiotic-Antimycotic solution) and incubated at 37 °C in a 5% CO2 humidified incubator. The growth medium was changed every day and cultures were expanded by serial passages. Upon reaching 70% confluency, cells were detached by trypsin digestion and split 1:2 ratio. All the experiments were conducted on cells collected at first to third passages. Cells from the experimental groups were matched by the passage number and number of cells.

**Isolation and culture of FAPs from mouse heart:** Adult mice (3-7 months old) were euthanized and hearts were explanted, washed with cold sterile PBS, and minced into <2 mm pieces. The pieces were incubated in 3 mL of 0.1% type 2 collagenase in α-MEM medium for 20 min at 37°C with gentle agitation. During the incubation, the minced tissue was gently pipetted up and down with a 5 mL pipette every 5 min. The collagenase activity was stopped and the cell suspension was passed through a 40 μm mesh cell strainer to remove debris and centrifuged at 300 g for 5 min at 4 °C. The supernatant was removed and the cell pellet resuspended in MACS buffer and incubated for 45 min at 4 °C in the dark with the following antibodies: anti PDGFRA (eBioscience, San Diego, CA 92121, APC anti-mouse CD140a antibody; cat# 17-1401-81), mouse lineage antibodies cocktail (BD Pharmingen, BD Horizon™ V450 Mouse Lineage Antibody Cocktail, with Isotype Control, cat #561301), anti THY1 (eBioscience, PE-Cy5 Anti-Mouse CD90.2; cat # 15-0902) and anti DDR2. After staining the cell suspension was sorted using the same multipstep approach utilized for human cells. FACS isolated mouse PDGFRA<sup>pos</sup>:Lin<sup>-</sup>:THY1<sup>-</sup>:DDR2<sup>-</sup> cells
were plated onto 0.1% gelatin coated plates in growth medium [α MEM supplemented with 10% ES-FBS, 10 ng/mL mouse basic Fibroblast Growth Factor (bFGF, R&D, Minneapolis, MN; cat #3139-FB), 1000 U/mL of mouse Leukemia Inhibitory Factor (mLIFMillipore; cat # ESG1106) and 1% Antibiotic-Antimycotic solution] and incubated at 37 °C in a 5% CO2 humidified incubator. Cell expansion in culture and experimental conditions were the same used for human cells.

**Characterization of cardiac FAPs.** Myocyte-depleted cardiac cells were analyzed for co-expression of PDGFRA and the following additional lineage markers: TIE2, a marker for endothelial cells; KIT antigen, a marker for progenitor cells; CD146, a marker for pericytes and PDGFRB, a marker for progenitor cells and pericytes. by flow cytometry and immunostaining. The list of antibodies is provided in Online Table I.

**Isolation of mouse adult cardiac myocytes (CMs):** Mice were anesthetized by intraperitoneal (I.P.) injection of pentobarbital (62 mg/Kg) and were anti-coagulated with I.P. injection of 200U of heparin. The heart was then harvested and immediately placed in Ca²⁺ free perfusion buffer [120 mM NaCl, 15 mM KCl, 0.6 mM KH2PO4, 0.6 mM Na2HPO4, 1.2 mM MgSO4 7H2O, 30 mM Taurine, 4.6 mM NaHCO3, 10 mM HEPES, 10 mM 2,3-Butanedione monoxime (BDM), and 5.5 mM Glucose; pH 7.0]. Then, ascending aorta was cannulated with a 22 G blunt needle under a magnifying dissection microscope (Leica, S6D). The cannula was positioned above the aortic valve cusps and was connected to a retrograde perfusion system. The heart was perfused for 2 min with the perfusion buffer at a constant rate of 4 mL/min at 37 °C. Upon washing out the blood, the perfusate was switched to a digestion buffer containing 624 U/mL of collagenase II solution (Worthington, Lakewood, NJ). After 2 min of digestion, 10 ul of 100 mM CaCl2 was added to the CM digestion buffer and the digestion was continued until the heart became pale and spongy upon gentle pinching. The atria was then removed, the heart was disconnected from the cannula, and minced into small pieces using a fine scissors in a 60 mm dish containing 2 ml of the
digestion buffer. The mixture was pipetted up and down gently several times with a sterile plastic transfer pipet (2 mm opening) to dissociate the myocytes. After one min, 8 ml of stop buffer (10% calf serum and 12.5 µM CaCl2 in the perfusion buffer) was added to stop the digestion. The cell suspension was filtered through a 100 µm nylon mesh and transferred to a 15 ml polypropylene conical tube. 100 µl of 200 mM ATP were added to the tube and the myocytes were allowed to sediment by gravity for 2 min. CMs were pelleted by centrifugation at 20 g for 3 min. Following removal of the supernatant, cells were subjected to a four-step calcium reintroduction by resuspending the cells in aliquots of 10 ml of stop buffer containing 2 mM ATP and increasing concentration of CaCl2 at 200 µM, 500 µM, 1 mM and 1.5 mM. At each calcium reintroduction step, the pellet was resuspended in the buffer containing the corresponding incremental concentration of CaCl2 and after 2 min at room temperature the preparation was centrifuged at 20 g for 3 min. The final pellet was resuspended in a CM plating media (MEM media, 1% penicillin-streptomycin, 10% Calf serum, 10 mM BDM, and 2 mM ATP). CMs were plated in culture dishes or cover glasses coated with laminin and incubated immediately in a 2% CO2 incubator at 37 °C. After 1 hour, the plating medium was replaced with fresh medium to remove the unattached CMs. The cells were collected 2 hour later for RNA or protein extraction or fixed in 4% paraformaldehyde for immunostaining.

**Isolation and culture of smooth muscle cells (SMCs), endothelial cells (ECs), and cardiac fibroblasts (CFs).** Mouse aortic SMCs were isolated from the mouse aortic tissues and cultured as previously published (1). In brief, mouse aorta was dissected and adventitia and endothelium were removed under a dissecting microscope. The remaining tissue containing the medial muscle cells was digested by incubation with type II collagenases at 37 °C for about one hour. The digestion was stopped by adding DMEM supplemented with 10% FBS. The cell suspension was centrifuged for 5 min at 300 g and the cell pellet was re-suspended and cultured in DMEM medium.
Mouse primary cardiac microvascular ECs were purchased by Cell-Biologics (2201 West Campbell Park Drive, Chicago, IL 60612; cat# BALB-5024). ECs were grown on cell culture plates pre-coated with gelatin-based coating solution (cat# 6950) in complete mouse endothelial cell growth medium (Cell Biologics, cat# M1168).

Cardiac fibroblasts were isolated as previously published (2). Mouse heart was harvested and immediately washed in cold Base Buffer (1 vial of Krebs-Henseleit buffer powder (Sigma, K3753-10X1L) 0.323 g calcium chloride, 2.02 g sodium bicarbonate, 2.6 g HEPES, and 3.75 g taurine; H2O to final volume of 1L; pH 7.2-7.3). The heart was placed in a sterile dish, covered it with 1 ml Liberase solution [250μL HEPES, 24.15mL HBSS+/-, 500μL Liberase TH Research Grade (Roche, 05401151001), 200μL DNase1 (sigma, D4263)] and quickly minced into small pieces (1 x 1 mm). The minced heart was transferred to a 50 mL tube containing a total of 5 mL of Liberase solution and incubated 5 min at 37°C while stirring at low setting. The suspension was removed from the stirrer and after the tissue settled down, the supernatant was carefully transferred, through a 40 μm cell strainer, into a 50 mL tube containing stop buffer (13.5 mL base buffer, 1.5 mL FBS, 120 μL DNase1) and left on ice while digesting the rest of the heart. An aliquot of 5 ml Liberase solution was added to the undigested tissue, and the solution was incubated for 8 min at 37 °C while stirring. The supernatant was then collected in stop buffer and the same procedure was repeated until the heart was completely digested. At the end of digestion, the cell suspension was centrifuged at 300 g for 5 min at 4 °C and the cell pellet was washed one time in 20 mL of cold Base buffer. After the wash, the cells were resuspended in DMEM/F-12 HEPES (Life Technologies, 11330) medium supplemented with 10% of FBS (GE, SH30070.03) and 1x Antibiotic-Antimycotic (Life Technologies, 15240-062), plated in tissue culture plates and incubated in a 5% CO2 incubator at 37 °C.
**Immunofluorescence (IF):** Thin myocardial sections or isolated cells were immunostained, as published (3-5). In brief, cardiac cross-sections were placed in the OCT compound (Sakura Finetek USA, Inc. Torrance, CA 90501; cat#4583) and frozen in isopentane (2-Methyl Butane, Sigma-Aldrich St Louis, MO, cat #320404). Human myocardial samples were from 2 controls and 2 patients with advanced AC. Samples were fixed in formalin and paraffin embedded. OCT and paraffin embedded tissues were cut into 5-7 µm thin myocardial sections for IF staining. Human myocardial thin sections were deparaffinazed, rehydrated and boiled for 15 min in 10 mM Sodium Citrate (pH 6.0) for antigen retrieval before blocking. For IF staining cells were grown on cover slips. Thin myocardial section and 70 to 80% confluent cells were fixed in 4% paraformaldehyde for 15 min at room temperature. The samples were washed 3 times in PBS and blocked in 5% donkey serum in PBS containing 0.3% Triton X-100 for 1 hour at room temperature. After blocking, samples were incubated with the primary antibody (list of antibodies is available in Online Table I) in 1% BSA and 0.3% Triton X-100 overnight at 4 °C. After 3 washes, samples were incubated with the corresponding secondary antibody in 1% BSA and 0.3% Triton X-100 for 1 hour at room temperature. After 3 rounds of washing in PBS, fluorescence-labeled secondary antibodies were added for 1 hour at room temperature. After 3 more washes in PBS, the samples were stained with a 0.1 mg/mL of 4′, 6-Diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma-Aldrich St Louis, MO; cat# D8417) for 2 min, mounted in fluorescent mounting medium (Dako North America Inc.6392 Via Real, Carpinteria CA 93013, cat# S3023), and examined under fluorescence microscopy (Zeiss, Axioplan Fluorescence Microscope).

**Immunoblotting (IB):** IB was performed per conventional methods (3-5). In brief, total cellular proteins were extracted by homogenizing heart tissues or the collected cells in a RIPA lysis buffer [1X formulation: 25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% Nonidet P-40 (NP-40), 1% Sodium deoxycholate and 0.1 % Sodium Dodecyl Sulphate (SDS); Pierce Biotechnology, Rockford,
IL; cat #89901] in the presence of protease and phosphatase inhibitors (complete protease inhibitor cocktail; Roche Diagnostics, GmbH, Mannheim, Germany; cat # 11-697-498-001; Phosphatase Inhibitor Cocktail 2 and 3; Sigma St. Louis, MO 63103; cat# P0044 and P5726). Protein concentration was measured using the Bio-Rad DC Protein Assay Kit (Bio-Rad Laboratories; cat # 5000111). After denaturation in Laemmli loading buffer at 95 °C for 5 min, 30 µg aliquots of each protein extract were loaded onto SDS-polyacrylamide gels (PAGE), subjected to electrophoresis, and transferred to nitrocellulose membranes. The membranes were blocked in 5% non-fat milk for 1 hour at room temperature and incubated with the primary antibody overnight at 4 °C. After 3 washes in TBS, membranes were incubated with the corresponding horseradish peroxidase (HRP) conjugated secondary antibody for 1 hour and the signal was detected by chemiluminescence. The list of primary and secondary antibodies is provided in Online Table 1. Membranes were stripped by incubation in Restore PLUS Western Blot Stripping Buffer (Thermo Scientific, Hudson, New Hampshire, cat #46430) for 10 min at room temperature, washed in TBS for 3 times and probed with an anti α- tubulin antibody as a loading control.

**TGFB1 Immunohistochemistry:** Expression of transforming growth factor-β1 (TGFB1) was detected by Immunohistochemistry (IHC) as previously published (6) PFA fixed cells and formalin fixed-paraffin embedded thin myocardial sections were incubated with monoclonal mouse anti TGFB1 antibody (R&D System; cat# MAB1835) at the concentration of 10 µg/mL, overnight at 4 °C, followed by staining with an Anti-Mouse HRP-DAB Cell and Tissue Staining Kit (Vector Laboratories, Burlingame, CA 94010; catalog # SK-4100). Signal was detected by peroxidase reaction.

**Detection of apoptosis:** Apoptosis was detected by TUNEL assay, as previously described (7) by using a commercially available kit (In Situ Cell Death Detection Kit, TMR red; Roche cat# 12156792910), per manufacturer instructions. In brief, formalin fixed-paraffin embedded thin
myocardial sections were permeabilized in 0.3% PBS-T for 10 min at RT and subsequently incubated with the TUNEL reaction mixture. Nuclei were countstained with DAPI solution. After washing, the label incorporated at the damaged sites of the DNA was visualized under fluorescence microscopy.

**Lineage tracer mice:** *Pdgfra:Egfp* reporter mice were purchased from Jackson Laboratory (stock No: 007669, Bar Harbor, Maine 04609 USA) (6). These mice express the H2B-eGFP fusion protein from the endogenous *Pdgfra* locus, leading to expression of H2B-eGFP mimicking the expression pattern of the endogenous *Pdgfra* gene (6). *Myb6-Cre, Dsp^F/F* and *R26^F-STOP^F-Eyfp* mice have been published (7-11). *Pdgfra-Cre* BAC transgenic mice (C57BL/6 background) were from Jackson Laboratory (stock No: 013148)(12). The *Pdgfra-Cre* deleter mice were crossed to *Dsp^F/F*:R26^F-STOP^F-Eyfp* mice to generate *Pdgfra-Cre:R26^F-STOP^F-Eyfp:Dsp^W/F* mice (henceforth, *Pdgfra-Cre:Eyfp:Dsp^W/F*). Expression of the Cre recombinase is expected to delete the floxed exon 2 of *Dsp* gene and the LoxP-flanked STOP sequence upstream of the *Eyfp* gene at the Gt(Rosa)26Sor locus, only in cells that are transcriptionally regulated by the *Pdgfra* locus. Mice were genotyped by PCR of tail DNA. Oligonucleotide primers used in PCR reactions are listed in Online Table I. Control age- and sex-matched wild type mice were included in all experiments.

**Echocardiography:** Data were obtained and analyzed without knowledge of the genotype. Cardiac structure and function in mice were assessed by B-mode, M-mode and Doppler echocardiography using a HP 5500 Sonos echocardiography unit equipped with a 15-MHz linear transducer, as published (4, 5, 13). Mice were anesthetized by I.P. injection of sodium pentobarbital (60 mg/Kg). Wall thicknesses and left ventricular (LV) dimensions were measured from M-mode images using the leading-edge method on 3 consecutive cardiac cycles. LV fractional shortening and mass were calculated as previously described (4, 5, 13).
Morphometric and histological analyses: Morphometric and histological analyses were performed without knowledge of the genotype or experimental groups. Ventricular/body weight ratio was calculated in age- and sex-matched mice. Myocardial histology was examined by H&E, Masson Trichrome, Picrosirius Red and Oil red O staining of thin myocardial sections, as published (4, 5, 13). Likewise, isolated cardiac FAPs were stained with Oil Red O to detect their differentiation to adipocytes. In brief, for Oil Red O staining, thin myocardial sections or isolated cells were washed one time with PBS and then fixed in 10% formalin for 15 min at room temperature. After 10 min washing under running water, samples were stained in a modified Mayer’s hematoxylin solution (Richard-Allan Scientific, Kalamazoo, MI; cat # 72804) for 2 min. Samples were then washed for 10 min in water, placed in 100% propylene glycol (Poly Scientific, Bay Shore, NY; cat #s264) for 2 min and then placed in Oil Red O 0.5% solution in propylene glycol (Poly Scientific, Bay Shore, NY; cat #s1848) for 5 hour or overnight at room temperature. The Oil Red O solution was removed and the samples were dipped 2 times in 85% propylene glycol (Poly Scientific, Bay Shore, NY; cat #s264A). After 20 min washing the slides were mounted with aqueous mounting medium. Extent of fibrosis was analyzed by quantification of collagen volume fraction (CVF) of Sirius Red stained thin myocardial sections. Images were analyzed using the ImageTool 3.0 analysis software.

Induction of adipogenesis: Adipogenesis was induced as previously published (14) Cardiac FAPs isolated from the wild type and the lineage tracer mice were plated in individual wells in 24-well plates that contained cover glass coated with 0.1% gelatin in proliferating media. Cells were incubated at 37°C in a 5% CO2 humidified incubator. Upon reaching 100% confluence, media was changed to an Adipogenesis Induction Medium [α-MEM supplemented with 10% FBS, 1% Antibiotic-Antimycotic, 10 μg/mL insulin (Sigma-Aldrich, 3050 Spruce St.; St. Louis, MO 63103; cat # I-0516), 0.5 mM 3-isobutyl-1-methylxanthine (IBMX; Sigma-Aldrich, cat # I-7018), and 1 μM dexamethasone (DXM; Sigma-Aldrich, cat# D-8893)]. The Adipogenesis Induction Medium was
changed every other days for a period ranging from 2 to 7 days. After incubation cells were fixed for Oil Red O staining or IF staining with adipogenic markers. Cells were also collected for RNA extraction and qPCR detection of genes involved in fat metabolism or for protein extraction and IB for selected adipogenic markers.

**Quantitative PCR (qPCR):** Total RNA was extracted using miRNeasy kit (Qiagen, cat # 271004) and cDNA was synthesized using a high-capacity cDNA Reverse Transcription Kit (High-Capacity cDNA Reverse Transcription Kit, Applied Biosystems cat # 4368814). Quantitative PCR was performed using specific TaqMan gene expression assays (Applied Biosystems) per manufacturer’s recommendations. *Gapdh* transcript levels were used for normalization. The ΔCt method was used to calculate the normalized gene expression values.

**Activation of the canonical Wnt signaling pathway:** To activate the canonical Wnt signaling pathway, cells were treated with 2 different concentrations (5 µM, and 10 µM) of 6-bromoindirubin-3'-oxime (BIO; EMD Chemicals-Calbiochem, Gibbstown, NJ 08027; cat # 361550), a known activator of the canonical Wnt signaling, as described (13-15). After 24 hours of incubation with BIO, adipogenesis was induced, as described above, for 7 days in presence of BIO in the culture media. Cells treated with BIO but not subjected to adipogenesis as well as cells induced for adipogenesis but not treated with BIO were included as controls. Media were changed daily. The effects of activation of the canonical Wnt on adipogenesis were determined by quantification the number of cells positive for Oil Red O and CEBPA expression as well as by quantifying transcript levels of the adipogenic genes by qPCR.

**Statistical analysis:** Statistical analysis has been performed using Graph pad Prism 6 (GraphPad Software, Inc., La Jolla, CA 92037) or SPSS version 20 software (IBM North America, New York, NY 10022). Data are expressed as mean ± SD. Normal distribution of the continuous variables was tested using the 1-sample Kolmogorov-Smirnov test. Differences in the continuous
variables between the two groups were compared by t-test or Mann-Whitney U test and among multiple groups by one-way ANOVA or multivariate analysis of variance (MANOVA). Pairwise comparisons were performed by Bonferroni multiple comparisons test. Differences among the categorical values were compared by Kruskall-Wallis test. A p value < 0.05 was considered significant.
ONLINE FIGURE LEGEND

**Online Figure I.** Scheme illustrating the multistep approach used to isolate FAPs from human and mouse hearts:

Cardiac myocyte-depleted cells were sorted to isolate PDGFR\A^{\text{pos}}\cdot\text{Lin}^{\text{neg}} cells. The PDGFR\A^{\text{pos}}\cdot\text{Lin}^{\text{neg}} cells were then sorted in order to exclude the THY1^{\text{pos}} cells. PDGFR\A^{\text{pos}}\cdot\text{Lin}^{\text{neg}}\cdot\text{THY1}^{\text{neg}} cells were further sorted to exclude the DDR2^{\text{pos}} cells. A subset of cardiac PDGFR\A^{\text{pos}}\cdot\text{Lin}^{\text{neg}}\cdot\text{THY1}^{\text{neg}}\cdot\text{DDR2}^{\text{neg}} cells express only the fibrogenic marker COL1A1, but not the adipogenic transcription factor CEBPA and another subset express CEBPA but not COL1A1. The PDGFR\A^{\text{pos}}\cdot\text{Lin}^{\text{neg}}\cdot\text{THY1}^{\text{neg}}\cdot\text{DDR2}^{\text{neg}}\cdot\text{COL1A1}^{\text{pos}} subfraction does not express the desmosome protein desmoplakin (DSP). Only the PDGFR\A^{\text{pos}}\cdot\text{Lin}^{\text{neg}}\cdot\text{THY1}^{\text{neg}}\cdot\text{DDR2}^{\text{neg}}\cdot\text{CEBPA}^{\text{pos}} subfraction expresses desmoplakin and hence is susceptible to cre deletion of $Dsp$ in the following *in vivo* experiments.

**Online Figure II.** Characterization of cardiac FAPs:

A. Flow cytometry analysis of myocyte-depleted cardiac cells from wild type mice for co-expression of PDGFR\A and additional lineage markers: TIE2, a marker for endothelial cells; KIT antigen, a marker for progenitor cells; CD146, a marker for pericytes; and PDGFR\B, a marker for progenitor cells and pericytes. Less than 1% of cardiac PDGFR\A^{\text{pos}} cells expressed TIE2, KIT, CD146 and PDGFR\B. B. Immunofluorescence panels showing absence of TIE2, KIT, CD146 and PDGFR\B in FACS isolated cardiac FAPs, in accord with the flow cytometry data.

**Online Figure III.** Assessment of expression of PDGFR\A in other cardiac cell types *in vitro*:

A. Immunofluorescence staining of isolated adult cardiac myocytes (CMs), smooth muscle cells (SMCs) and cardiac microvascular endothelial cells (ECs), for PDGFR\A and each cell type-specific marker: α-actinin (ACTN2) for CMs, smooth muscle myosin heavy chain II (SM-MHCII) for SMCs
and platelet endothelial cell adhesion molecule-1 (PECAM-1) for ECs, respectively. **B-C.** Absence of expression of PDGFRA in isolated cardiac myocytes as detected by immunoblotting (B, upper panel) and qPCR (C). Lower immunoblots in B depict absence of expression cardiac myocyte sarcomere proteins myosin heavy chain 6 (MYH6) and myosin binding protein C3 (MYBPC3) in FAPs. FACS isolated FAPs were used as positive controls for PDGFRA detection in both experiments. **D-E.** Detection of expression of PDGFRA in isolated cardiac fibroblasts (CFs) identified by the expression of COL1A1 by immunofluorescence (D). Quantitative data (E) showed that PDGFRA was expressed in 71.1 ± 20.2 % of the isolated CFs. (N=3 independent experiments; ~150 cells counted in each experiment).

**Online Figure IV. Assessment of expression of PDGFRA in other cardiac cell types in vivo:**
Co-immunofluorescence of thin myocardial sections from wild type mice for PDGFRA and each specific cell type marker: ACTN2 to identify CMs, COL1A1 for CFs, SM-MHCII for SMCs and PECAM-1 for ECs, respectively. PDGFRA was expressed in CFs but not in CMs, SMCs or ECs, in accordance with the data in isolated cell types.

**Online Figure V. Detection of EGFP in cardiac cell types in the Pdgfra-Egfp reporter mice:**
**A.** Co-Immunofluorescence staining of thin myocardial sections from Pdgfra-Egfp reporter mice for the reporter protein EGFP and the cardiac cell type markers ACTN2, COL1A1, SM-MHCII and PECAM-1 respectively. In this mouse model expression of EGFP is transcriptionally regulated by the Pdgfra locus, hence EGFP is detected in cells expressing PDGFRA. **B.** Quantitative data showing that approximately 51.3 ± 9.8% of CFs was tagged with EGFP while no EGFP expression was detected in CMs, SMCs or ECs. The finding is in accord with the data on isolated cell types and on myocardial
sections from wild type mice after co-immunofluorescence staining for PDGFRA and each cell type marker.

**Online Figure VI. Absence of expression of DSP in CFs, SMCs and ECs:**

A. Immunofluorescence staining of isolated CFs, SMCs and ECs for PDGFRA and each cell type-specific marker. Isolated CMs are included as positive control, B. Detection of DSP by Immunoblot in CFs, SMCs and ECs. Heart lysates are included as positive controls. The data show absence of DSP expression in the cardiac cell types analyzed, except for CMs.

**Online Figure VII. In vivo conditional deletion of Dsp and EYFP labeling of cardiac FAPs:**

\[ Dsp^{+/F} \] and \( R26^{+/F}.STOP^{+/F}.EYFP \) mice were repeatedly crossed in order to obtain \( Dsp^{+/F}:R26^{-/-}.STOP^{+/F}.EYFP \) mice. \( Dsp^{+/F}:R26^{-/-}.STOP^{+/F}.EYFP \) mice were crossed with Pdgfra-Cre deleter mice in order to generate \( Pdgfra-Cre:R26^{-/-}.STOP^{+/F}.EYFP:Dsp^{+/F} \) lineage tracer mice (\( Pdgfra-Cre:EYFP:Dsp^{+/F} \)). In the triple transgenic mice, expression of the cre recombinase leads to deletion of the floxed exon 2 of the \( Dsp \) gene and of the STOP sequence upstream of the \( EYFP \) gene, abolishing expression of DSP and inducing expression of EYFP specifically in PDGFRA positive cells.

**Online Figure VIII. Detection of DSP levels and expression of EYFP in CMs isolated from the hearts of wild type and Pdgfra-Cre:EYFP:Dsp^{+/F} mice:**

A-C. Detection of the reporter protein EYFP by immunofluorescence in isolated CMs from \( Pdgfra-Cre:EYFP:Dsp^{+/F} \) mice after staining for EYFP and the CM specific marker ACTN2 (A). High magnification panels of selected representative areas are included (B). CMs isolated from wild type mice are included as negative controls. C. Quantitative data showing that EYFP was expressed in about 18% of the CMs from the lineage tracer mice (N=3, \( ~1000 \) cells counted per each experiment
in each group). D-G. Exclusion of fortuitous deletion of Dsp in CMs. qPCR (D) and Immunoblotting (E-F) confirming that mRNA and protein levels of Dsp are not significantly altered in CMs isolated from the lineage tracer mice as compared to control CMs. Lower immunoblot panel in E confirms expression of low levels of EYFP in CM protein extracts from the lineage tracer mice. Likewise, IF staining of CMs isolated from the lineage trace mice (G) showed that localization and levels of DSP are not affected in the EYFP\textsuperscript{pos} CM in comparison to the EYFP\textsuperscript{pos} CMs. All the experiments have been repeated in 3 independent CM isolations.

**Online Figure IX. Detection of EYFP in other cardiac cell types in the \textit{Pdgfra-Cre:Eyfp:Dsp}\textsuperscript{W/F} mice:**

A-B. Co-Immunofluorescence staining of thin myocardial sections from wild type (A) \textit{Pdgfra-Cre:Eyfp:Dsp}\textsuperscript{W/F} mice (B) for the reporter protein EYFP and the cardiac cell type markers ACTN2, COL1A1, SM-MHCII and PECAM-1, respectively. As expected, only CFs but not SMCs or ECs expressed EYFP in the heart of the lineage tracer mouse.

**Online Figure X. Cardiac phenotype of the \textit{Pdgfra-Cre:Eyfp:Dsp}\textsuperscript{W/F} lineage tracer mice:**

A. Ventricular/body weight ratio in wild type, \textit{Pdgfra-Cre} and \textit{Pdgfra-Cre:Eyfp:Dsp}\textsuperscript{W/F} mice. Ventricular/body weight ratio was modestly increased in the \textit{Pdgfra-Cre:Eyfp:Dsp}\textsuperscript{W/F} mice as compared to the other 2 groups (N=22 wild type, N=4 \textit{Pdgfra-Cre}, N=11 \textit{Pdgfra-Cre:Eyfp:Dsp}\textsuperscript{W/F} mice; *p< 0.05 as compared with wild type and \textit{Pdgfra-Cre}). B. Picro-Sirius Red stained thin myocardial sections showing increased interstitial fibrosis in the \textit{Pdgfra-Cre:Eyfp:Dsp}\textsuperscript{W/F} mouse hearts, as compared to wild type and \textit{Pdgfra-Cre} deleter mouse. Quantitative data are shown in panel E (N=5 wild type and \textit{Pdgfra-Cre}, N=7 \textit{Pdgfra-Cre:Eyfp:Dsp}\textsuperscript{W/F} mice; *p< 0.05 as compared with wild type and \textit{Pdgfra-Cre}). C. ORO stained thin myocardial sections showing increased number of adipocytes in the heart of the \textit{Pdgfra-Cre:Eyfp:Dsp}\textsuperscript{W/F}
mice. Quantitative data are shown in panel F (N=5 wild type, N=4 Pdgfra-Cre, N=11 Pdgfra-Cre:Eyfp:DspW/F mice; *p< 0.05 as compared with wild type and Pdgfra-Cre). D. Immunofluorescence staining of thin myocardial sections showing increased number of cells expressing the adipogenic transcription factor CEBPA in the hearts of Pdgfra-Cre:Eyfp:DspW/F mice as compared to the control groups. Quantitative data are shown in panel G (N=5 wild type, N=4 Pdgfra-Cre, N=4 Pdgfra-Cre:Eyfp:DspW/F mice; *p< 0.05 as compared with wild type and Pdgfra-Cre).

Online Figure XI. Localization of fibroadiposis in the heart of Pdgfra-Cre:Eyfp:DspW/F mouse hearts: Low magnification pictures of thin myocardial sections from wild type, Pdgfra-Cre and Pdgfra-Cre:Eyfp:DspW/F mice after staining with Sirius Red (SR) for detection of fibrosis and with ORO and an anti CEBPA specific antibody for detection of adipocytes. The lineage tracer mice showed patchy areas of fibrosis, while adipocytes were scattered in the myocardial wall of both left and right ventricles.

Online Figure XII. Detection of TGFB1 expression in the myocardium and in isolated FAPs: A-C. Detection of TGFB1 expression in isolated cardiac FAPs (A) and in thin myocardial sections from wild type and Pdgfra-Cre:Eyfp:DspW/F mice by immunohistochemistry. High magnification panels of selected representative myocardial areas are included (C).TGFB1 expression levels were increased in isolated FAPs and myocardial sections from the Pdgfra-Cre:Eyfp:DspW/F mice.

Online Figure XIII. Detection of apoptosis in the myocardium of the Pdgfra-Cre:Eyfp:DspW/F mice:
A. TUNEL staining to detect apoptotic cells in thin myocardial sections from wild type and Pdgfra-Cre:Eyfp:DspW/F mice. B. Quantitative data showing that the number of cells stained positive in the
TUNEL assay was not significantly different between the wild type and *Pdgfra-Cre:Eyfp:Dsp^W/F* mice (N=3 mice per group, ~1200 nuclei analyzed per each mouse).

**Online Figure XIV. Assessment of adipocyte proliferation in the myocardium of the *Pdgfra-Cre:Eyfp:Dsp^W/F* mice:**

A. Immunofluorescence co-staining of thin myocardial sections from wild type and *Pdgfra-Cre:Eyfp:Dsp^W/F* mice for the expression of CEBPA, to mark adipocytes, and Ki67 to mark proliferating cells. B. Quantitative data showing that percent of adipocytes that were stained positive for the proliferation marker did not differ significantly between the wild type and lineage tracer mice, indicating that number of adipocytes in the heart of the *Pdgfra-Cre:Eyfp:Dsp^W/F* mice was not increased because of proliferation of existing adipocytes (N=3 mice per group, ~200 CEBPA^pos^ cells analyzed for ki67 expression per each mouse).

**Online Figure XV. Increased number of perilipin expressing cells in cardiac FAPs isolated from *Pdgfra-Cre:Eyfp:Dsp^W/F* mice:**

A-B. low (10X, A) and higher (63x, B) magnification pictures at of cardiac FAPs from wild type and *Pdgfra-Cre:Eyfp:Dsp^W/F* mice after immuofluorescence staining for perilipin, a marker of mature adipocytes after 4 days of adipogenesis induction. C. Quantitative data showing that the number of cells expressing perilipin was significantly increased in the *Pdgfra-Cre:Eyfp:Dsp^W/F* as compared to wild type mice (N=3, ~200 cells counted per each group in each experiment).

**Online Figure XVI. Cardiac phenotype of the *Pdgfra-Egfp:Myb6-Cre:Dsp^W/F* mice:**

A. Increased ventricular/body weight ratio in *Pdgfra-Egfp:Myb6-Cre:Dsp^W/F* mice as compared with wild type and *Pdgfra-Egfp* mice (N=10 wild type, N=7 *Pdgfra-Egfp*, N=13 *Pdgfra-Egfp:Myb6-Cre:Dsp^W/F* mice;
*p< 0.05 as compared with wild type and Pdgfra-Egfp mice). B. Masson trichrome (MT) and ORO staining showing enhanced fibro-adipogenesis in the Pdgfra-Egfp:Myh6-Cre:Dsps/F mice as compared with the control groups. Quantitative data are shown in panels C and D (N=4-6 mice per each group; *p< 0.05 as compared with wild type and Pdgfra-Egfp mice).

**Online Figure XVII. Exclusion of a paracrine mechanism for differentiation of FAPs to adipocytes:**

A. Co-expression of the EGFP reporter protein and the adipogenic transcription factor CEBPA in thin myocardial sections obtained from wild type, Pdgfra-Egfp reporter, and Pdgfra-Egfp:Myh6-Cre:Dsps/F lineage tracer mice. High magnification inserts are also shown in B. C-D. Quantitative data. Panel C shows increased number of CEBPA⁺⁺ adipocytes in the heart of Pdgfra-Egfp:Myh6-Cre:Dsps/F mice as compared to the control groups. Quantitative data in panel D show that the percentage of adipocytes expressing EGFP in the control Pdgfra-Egfp and in the Pdgfra-Egfp:Myh6-Cre:Dsps/F mice was not significantly different (N= 5 animals per group. Wild type animal have been included as a negative control for EGFP expression).
### ONLINE SUPPLEMENTARY TABLE I

#### A. Antibodies

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</tr>
<tr>
<td>Anti-goat IgG, Alexa Fluor 488</td>
<td>Life technology</td>
<td>11055</td>
<td>1:1000(IF) 1mg/mL(FC)</td>
</tr>
<tr>
<td>Anti-goat IgG, Alexa Fluor 594</td>
<td>Life technology</td>
<td>11058</td>
<td>1:1000(IF)</td>
</tr>
<tr>
<td>Anti-rat IgG, Alexa Fluor 594</td>
<td>Life technology</td>
<td>11007</td>
<td>1:1000(IF)</td>
</tr>
<tr>
<td>Anti-rat IgG, Alexa Fluor 488</td>
<td>Life technology</td>
<td>21208</td>
<td>1:1000(IF)</td>
</tr>
<tr>
<td>Anti-goat IgG, Alexa Fluor 790</td>
<td>Life technology</td>
<td>11370</td>
<td>1mg/mL(FC)</td>
</tr>
<tr>
<td>APC Rat IgG2a K Isotype Control</td>
<td>eBioscience</td>
<td>17-4321</td>
<td>1mg×10^6cells(FC)</td>
</tr>
<tr>
<td>PE Rat IgG2a, κ Isotype Control</td>
<td>BD Pharmingen</td>
<td>555844</td>
<td>1mg×10^6cells(FC)</td>
</tr>
<tr>
<td>APC Rat IgG2b, κ Isotype Control</td>
<td>BD Pharmingen</td>
<td>553991</td>
<td>1mg×10^6cells(FC)</td>
</tr>
<tr>
<td>AF488 Rat IgG2a, κ Isotype Control</td>
<td>BD Pharmingen</td>
<td>557676</td>
<td>1mg×10^6cells(FC)</td>
</tr>
<tr>
<td>Antibody/Probes</td>
<td>Supplier</td>
<td>Catalog Number</td>
<td>Dilution</td>
</tr>
<tr>
<td>-----------------</td>
<td>----------</td>
<td>----------------</td>
<td>----------</td>
</tr>
<tr>
<td>PE Rat IgG2a K Isotype Control</td>
<td>eBioscience</td>
<td>12-4321</td>
<td>1mg:10^6 cells(FC)</td>
</tr>
<tr>
<td>PE-Cy5 Rat IgG2a K Isotype Control</td>
<td>eBioscience</td>
<td>15-4321</td>
<td>1mg:10^6 cells(FC)</td>
</tr>
<tr>
<td>Anti-Rabbit IgG, HRP linked</td>
<td>Cell Signaling</td>
<td>7074</td>
<td>1:3000(IB)</td>
</tr>
<tr>
<td>Anti-Mouse IgG, HRP linked</td>
<td>Cell Signaling</td>
<td>7076</td>
<td>1:4000(IB)</td>
</tr>
</tbody>
</table>

**B. Taqman gene expression probes (from life technology)**

- **Pdgfra**: Mm004340701_m1
- **CyclinD1**: Mm00432359_m1
- **Ctgf**: Mm01192932_g1
- **Serpine1**: Mm00435860_m1
- **Ankrd1**: Mm00496512_m1
- **Inhba**: Mm00434339_m1
- **Jag1**: Mm00496902_m1
- **Her1**: Mm01342805_m1
- **Faltp4**: Mm00445878_m1
- **Celpa**: Mm00514283_s1
- **Pparg**: Mm01184322_m1
- **Dgat1**: Mm00515643_m1
- **Dgat2**: Mm00499536_m1
- **Gapdh**: Mm99999915_g1
**$D_{sp}$** Mm01351876_m1

**$T_{gf}$** Mm01178820_m1

**C. PCR oligonucleotide primers for mouse genotyping:**

**$P_{dgfra}$:$E_{gfp}$:**

Wild type Forward: CCC TTG TGG TCA TGC CAA AC

Wild type Reverse: GCT TTT GCC TCC ATT ACA CTG G

Mutant Reverse: ACG AAG TTA TTA GGT CCC TCG AC

**Floxed $D_{sp}$:**

Forward: TAAGCTCCCCTCACTTCTCCAG

Reverse: TTCTCTTTTGCTCTTGCATGT

**R26-FSTOPF-Eyfp:**

Rosa26R-1 AAAGTCGCTCTGTGAGTTGTTAT

Rosa26R-2 GCGAAGAGTTTGCTCTCAACC

Rosa26R-3 GGAGCGGGAGAAATGGATATG

**$P_{dgfra}$-Cre:**

Forward: GCG GTC TGG CAG TAA AAA CTA TC

Reverse: GTG AAA CAG CAT TGC TGT CAC TT

Internal Positive Control Forward: CTA GGC CAC AGA ATT GAA AGA TCT

Internal Positive Control Reverse: GTA GGT GGA AAT TCT AGC ATC ATC C

**Myh6-Cre:**

Forward: CCACACCAGAAATGACAGACAGA
Reverse: CGCATAACCAGTGAACAGCAT
### ONLINE TABLE II

Echocardiographic findings in WT, Pdgfra-Cre and Pdgfra-Cre: Eysp: Dsp<sup>W/F</sup> mice

**Abbreviations:** HR, heart rate; bpm, beats per minutes; IVST, interventricular septal thickness; PWT, posterior wall thickness; LVEDD, left ventricular end diastolic diameter; LVEDDi, LVEDD divided for the body weight; LVESD, left ventricular end systolic diameter; FS, fractional shortening; LVMass, left ventricular mass; LVMi, LVMass divided for the body weight.

<table>
<thead>
<tr>
<th></th>
<th>Wild type</th>
<th>Pdgfra-Cre</th>
<th>Pdgfra-Cre: Eysp: Dsp&lt;sup&gt;W/F&lt;/sup&gt;</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>21</td>
<td>15</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>Male/Female</td>
<td>12/9</td>
<td>8/7</td>
<td>13/10</td>
<td>0.972</td>
</tr>
<tr>
<td>Age (months)</td>
<td>9.7±2.5</td>
<td>9.9±3.1</td>
<td>9.2±2.6</td>
<td>0.703</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>31.3±3.6</td>
<td>33.0±5.0</td>
<td>33.5±5.7</td>
<td>0.299</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>563 ± 71</td>
<td>589 ± 65</td>
<td>562 ± 81</td>
<td>0.493</td>
</tr>
<tr>
<td>IVST (mm)</td>
<td>0.97 ± 0.09</td>
<td>0.94 ± 0.10</td>
<td>0.73 ± 0.09&lt;sup&gt;*&lt;/sup&gt;</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PWT (mm)</td>
<td>0.99 ± 0.10</td>
<td>0.91 ± 0.14</td>
<td>0.73 ± 0.11&lt;sup&gt;*&lt;/sup&gt;</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>2.82 ± 0.25</td>
<td>3.17 ± 0.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.81 ± 0.31&lt;sup&gt;*&lt;/sup&gt;</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LVEDDi (mm/g)</td>
<td>0.09 ± 0.01</td>
<td>0.10 ± 0.01</td>
<td>0.12 ± 0.02&lt;sup&gt;*&lt;/sup&gt;</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>0.99 ± 0.21</td>
<td>1.07 ± 0.14</td>
<td>1.78 ± 0.47&lt;sup&gt;*&lt;/sup&gt;</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FS (%)</td>
<td>65±6</td>
<td>66±5</td>
<td>54±10&lt;sup&gt;*&lt;/sup&gt;</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LVM (mg)</td>
<td>86±22</td>
<td>96±17</td>
<td>92±20</td>
<td>0.331</td>
</tr>
<tr>
<td>LVMi (mg/g)</td>
<td>2.8±0.7</td>
<td>2.9±0.5</td>
<td>2.8±0.6</td>
<td>0.716</td>
</tr>
</tbody>
</table>

<sup>*</sup> p ≤ 0.01 vs WT and vs Pdgfra-Cre, # p ≤ 0.01 vs WT by Bonferroni pairwise comparison
## ONLINE TABLE III

Echocardiographic findings in the wild type, *Pdgfra-Egfp, Myh6-Cre:Dsp\[^{W/F}\]*, and *Pdgfra-Egfp:Myh6-Cre:Dsp\[^{W/F}\]* mice

<table>
<thead>
<tr>
<th></th>
<th>Wild type</th>
<th><em>Pdgfra-Egfp</em></th>
<th><em>Myh6-Cre:Dsp[^{W/F}]</em></th>
<th><em>Pdgfra-Egfp:Myh6-Cre:Dsp[^{W/F}]</em></th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>9</td>
<td>7</td>
<td>6</td>
<td>9</td>
<td>0.954</td>
</tr>
<tr>
<td>Male/Female</td>
<td>5/4</td>
<td>3/4</td>
<td>3/3</td>
<td>5/4</td>
<td>0.997</td>
</tr>
<tr>
<td>Age (months)</td>
<td>8.3±1.2</td>
<td>8.4±0.3</td>
<td>8.4±0.4</td>
<td>8.3±0.3</td>
<td>0.318</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>31.8±3.7</td>
<td>37.4±8.2</td>
<td>34.0±4.5</td>
<td>34.5±6.7</td>
<td></td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>546±73</td>
<td>563±56</td>
<td>567±41</td>
<td>599±56</td>
<td>0.309</td>
</tr>
<tr>
<td>IVST (mm)</td>
<td>0.99±0.05</td>
<td>0.96±0.08</td>
<td>0.78±0.09*</td>
<td>0.71±0.07*</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PWT (mm)</td>
<td>1.00±0.09</td>
<td>0.94±0.01</td>
<td>0.71±0.04*</td>
<td>0.74±0.08*</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>2.8±0.1</td>
<td>3.2±0.2</td>
<td>3.7±0.3*</td>
<td>3.9±0.4*</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LVEDDDi (mm/g)</td>
<td>0.09±0.01</td>
<td>0.08±0.01</td>
<td>0.11±0.01*</td>
<td>0.11±0.02*</td>
<td>0.006</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>1.0±0.2</td>
<td>1.2±0.2</td>
<td>2.1±0.6*</td>
<td>2.1±0.6*</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FS (%)</td>
<td>63±5</td>
<td>63±6</td>
<td>44±10*</td>
<td>47±11*</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LVM (mg)</td>
<td>91.8±8.4</td>
<td>98.9±15.6</td>
<td>91.2±18.2</td>
<td>96.3±16.5</td>
<td>0.729</td>
</tr>
<tr>
<td>LVMi (mg/g)</td>
<td>3.0±0.5</td>
<td>2.7±0.7</td>
<td>2.7±0.4</td>
<td>2.8±0.4</td>
<td>0.725</td>
</tr>
</tbody>
</table>

**Abbreviations:** HR, heart rate; bpm, beats per minutes; IVST, interventricular septal thickness; PWT, posterior wall thickness; LVEDD, left ventricular end diastolic diameter; LVEDDDi, LVEDD divided for the body weight; LVESD, left ventricular end systolic diameter; FS, fractional shortening; LVMass, left ventricular mass; LVMi, LVMass divided for the body weight.

* p≤0.05 vs WT and vs Pdgfra-Egfp, by Bonferroni pairwise comparison.
REFERENCES


Myocyte depleted cardiac cells

FACS

PDGFRA

Lin

CD32, CD11B, CD45, Lys76, Ly-6c and Ly6c

Lin neg

THY1

THY1

DDR2

DDR2

COL1A1

CEBPA

DSP

Online Figure I
Online Figure IV
Online Figure V
Online Figure VII
Online Figure XI
Online Figure XIII

A

Wild Type

TUNEL

DAPI

TUNEL: DAPI

Pdgfra-Cre: Eryp:Dsp

TUNEL

DAPI

TUNEL: DAPI

200 µm

B

![Graph showing TUNEL+/1000 nuclei with p=0.535]

Wild type

Pdgfra-Cre: Eryp:Dsp

0

1

2

3

4

TUNEL+/1000 nuclei

p=0.535