Targeted Ablation of Periostin-Expressing Activated Fibroblasts Prevents Adverse Cardiac Remodeling in Mice

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Rationale: Activated cardiac fibroblasts (CF) are crucial players in the cardiac damage response; excess fibrosis, however, may result in myocardial stiffening and heart failure development. Inhibition of activated CF has been suggested as a therapeutic strategy in cardiac disease, but whether this truly improves cardiac function is unclear.

Objective: To study the effect of CF ablation on cardiac remodeling.

Methods and Results: We characterized subgroups of murine CF by single-cell expression analysis and identified periostin as the marker showing the highest correlation to an activated CF phenotype. We generated bacterial artificial chromosome–transgenic mice allowing tamoxifen-inducible Cre expression in periostin-positive cells as well as their diphtheria toxin-mediated ablation. In the healthy heart, periostin expression was restricted to valvular fibroblasts; ablation of this population did not affect cardiac function. After chronic angiotensin II exposure, ablation of activated CF resulted in significantly reduced cardiac fibrosis and improved cardiac function. After myocardial infarction, ablation of periostin-expressing CF resulted in reduced fibrosis without compromising scar stability, and cardiac function was significantly improved. Single-cell transcriptional analysis revealed reduced CF activation but increased expression of prohypertrophic factors in cardiac macrophages and cardiomyocytes, resulting in localized cardiomyocyte hypertrophy.

Conclusions: Modulation of the activated CF population is a promising approach to prevent adverse cardiac remodeling in response to angiotensin II and after myocardial infarction. (Circ Res. 2016;118:1906-1917. DOI: 10.1161/CIRCRESAHA.116.308643.)

Key Words: extracellular matrix ■ fibroblast ■ heart failure ■ myocardial infarction ■ phenotype

Cardiac fibroblasts (CF) are the most abundant noncardiomyocyte cell population present within the heart; their main function is the balanced synthesis and degradation of extracellular matrix (ECM).1,2 The cardiac ECM serves many purposes: it provides structural support to cardiac myocytes, helps to distribute mechanical force, supports cardiomyocyte growth and differentiation, and, last but not least, forms the cardiac skeleton, that is, the fibrous connective tissue that electrically isolates the atria from the ventricles, the 4 valves, and valvular anchorage tissue.3,4 Although CF are highly active during organ development, CF of the adult heart are quiescent and maintain only a low level turnover of ECM.5,6 However, in response to various forms of cardiac damage, no matter whether ischemic, mechanical, or inflammatory, fibroblasts rapidly reacquire an activated synthetic phenotype, resulting in increased matrix deposition and fibrosis.2-7 This is often accompanied by an upregulation of contractile proteins such as α smooth muscle actin (αSMA), giving rise to the term myofibroblast for activated fibroblasts.2,7 Also, the overall number of fibroblasts increases rapidly under conditions of damage, both because of proliferation of existing CF and because of transdifferentiation from hematopoietic cells, endothelial cells, or epicardial cells.8,9 The profibrotic response may be initially beneficial and help to reinforce the structurally damaged myocardium, but excess deposition of ECM results in myocardial stiffening and functional deterioration.10,11 Containment of CF activation was therefore suggested as a therapeutic strategy in cardiac diseases,12 but whether suppression of CF,
or subgroups thereof, truly improves cardiac function is unclear. This uncertainty is partly because of a lack of selective CF markers, hampering the identification or selective targeting of these cells. Typical fibroblast marker genes such as vimentin or collagen isoforms are expressed in all CF, but also label extracardiac fibroblasts and other cells of mesodermal origin, such as vascular smooth muscle cells. α-SMA was suggested as a marker of activated ventricular fibroblasts, but a recent study showed that only 15% of collagen-positive cells express α-SMA in pressure overload–induced cardiac fibrosis. In addition, α-SMA is constitutively expressed in vascular smooth muscle cells. Other putative markers for subgroups of CF are the collagen receptor discoidin domain–containing receptor 2 (Ddr2), the calcium-binding protein S100-A4 (also known as fibroblast-specific protein-1), or thymus cell antigen-1 (Thy1, also known as CD90). Unfortunately these markers lack specificity, because fibroblast-specific protein-1 and Thy1 are expressed in leukocytes and endothelial cells, and Ddr2 is found on smooth muscle cells and leukocytes. Recent studies also suggested platelet-derived growth factor receptor α (Pdgfra) and the ECM protein peristin (Postn) as CF markers, but both proteins are also expressed in other fibroblasts and cells of mesenchymal origin, in particular during development and in response to injury.

Whether the aforementioned CF markers identify separate or overlapping CF populations has not been analyzed to date, and it is not known how these subpopulations contribute to fibrosis in vivo. It is also unclear which of these markers may be best suited to selectively identify activated CF, and how intervention within such an activated CF population would affect heart function under both physiological and pathological conditions. Especially, the question whether inhibition of fibrosis in the infarcted heart can be achieved without compromising wall stability is still unanswered. To address these issues, we analyzed the expression of putative CF marker genes on the single-cell level under healthy and diseased conditions and identified Postn as the marker with the best correlation to an activated CF phenotype. Using a newly generated mouse line that allows tamoxifen-inducible, Cre-mediated recombination in Postn-expressing cells, we show that ablation of this cell population prevents excess fibrosis and preserves ejection fraction both in chronic angiotensin II (AngII) exposure and after myocardial infarction (MI) without reducing scar stability.

Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AngII</td>
<td>angiotensin II</td>
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<tr>
<td>β-gal</td>
<td>β-galactosidase</td>
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<tr>
<td>CF</td>
<td>cardiac fibroblast</td>
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<tr>
<td>Ddr2</td>
<td>discoid domain receptor 2</td>
</tr>
<tr>
<td>DTR</td>
<td>diphtheria toxin receptor</td>
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<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>MI</td>
<td>myocardial infarction</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>Pdgfra</td>
<td>platelet-derived growth factor receptor α</td>
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<tr>
<td>Postn</td>
<td>peristin</td>
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<tr>
<td>SMA</td>
<td>smooth muscle actin</td>
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<tr>
<td>Thy1</td>
<td>thymus cell antigen 1</td>
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In this study, we investigated the function of Postn in CF and its role in the development of cardiac fibrosis after myocardial infarction (MI) or chronic angiotensin II (AngII) exposure. To this end, we generated tamoxifen-inducible Cre-transgenic mice expressing a tdTomato reporter under the control of the Postn promoter and analyzed their cardiac phenotype. We found that ablation of Postn-expressing cells prevented excess fibrosis and preserved ejection fraction both in chronic AngII exposure and after MI. These findings provide new insights into the role of Postn-expressing cells in cardiac fibrosis and suggest that targeted ablation of this cell population offers a promising strategy for the treatment of cardiac fibrosis.

Methods

Chemicals and Antibodies

Angiotensin II (A9525), tamoxifen (T5648), and diphtheria toxin (D0564) were from Sigma-Aldrich (St Louis, MO); X-Gal (2315.4) was from Carl Roth (Karlsruhe, Germany). Antibodies directed against peristin (ab14041) and β-galactosidase (ab9361) were from Abcam (Cambridge, UK). Anti-carcinoembryonic antigen-1 (Thy1, also known as CD90) was from Biolegend (San Diego, CA). All other reagents were from Sigma-Aldrich.

Animals

The study was approved by the Institutional Animal Care and Use Committee of the Regierungspräsidium Darmstadt in accordance with Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.
Histological Analyses
Freshly isolated tissues were fixed in 4% paraformaldehyde and embedded in paraffin after dehydration. Paraffin-embedded tissues were sectioned at 10-µm thickness and further stained with picrosirius red or hematoxylin/eosin according to standard protocols. For immunostaining, antigen retrieval was performed on paraffin sections using a standard citrate buffer protocol, and sections were stained further with indicated antibodies. For β-galactosidase (β-gal) staining, 10 µm cryosections were prepared and stained according to standard protocols, followed by eosin or picrosirius red counterstaining. For assessment of the degree of cardiac fibrosis, NIH ImageJ software was used (6–10 randomly chosen sections per sample).

Magnetic Resonance Imaging
Cardiac MRI measurements were performed on a 7 T Bruker Phasmacan, equipped with a 300 mT/m gradient system, using a custom-built circularly polarized birdcage resonator and the Early Access Package for self-gated cardiac Imaging (Bruker, Ettlingen, Germany). The mice were measured under volatile isoflurane (2.0%)/anesthesia. The measurement is based on the gradient echo method (repetition time=6.2 ms; echo time=1.6 ms; field of view=2.20 cm; slice thickness=1.0 mm; matrix=128×128; repetitions=100). The imaging plane was localized using scout images showing the 2- and 4-chamber view of the heart, followed by acquisition in short-axis view, orthogonal on the septum in both scouts. Multiple contiguous short-axis slices consisting of 9 or 10 slices were acquired for complete coverage of the left ventricle. Magnetic resonance imaging data were analyzed using Qmass digital imaging software (Medis, Leiden, Netherlands).

Single-Cell Quantitative Real-Time PCR
For single-cell expression analysis, cardiac ventricles were minced into small pieces and digested while shaking in digestion mix for 80 to 90 minutes at 37 °C (Digestion mix: collagenase II, 2 mg/mL; elastase I, 0.04 mg/mL; and Dnase I, 5 U/mL in PBS). After filtering and washing with prewarmed PBS, cells were plated for 1 hour at 37 °C and 5% CO2 in DMEM containing 10% fetal bovine serum, l-glutamine, sodium pyruvate, and penicillin/streptomycin. Nonadherent cells were collected and washed with PBS before staining with MF20 antibody, and labeled cells were then sorted with JSAN-sorter (Bay Bioscience) for further analysis. Adherent cells were trypsinized and washed thoroughly with PBS before loading onto the microfluidic-C1 Single-Cell Auto Prep System (Fluidigm) followed by RNA isolation and cDNA synthesis. cDNAs derived from chambers containing no cell or >1 cell were excluded from further analysis.

High-throughput quantitative PCR of harvested cDNA from single cells was performed on 96.96 Dynamic Array IFC with a BioMark system (Fluidigm) using Sso-Fast EvaGreen Supermix low ROX (BioRad, Hercules, CA) and Delta Gene primer assays for mouse genes designed by Fluidigm as listed below:

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Only single-cell cDNAs negative for lineage markers Cd45, myosin heavy chain (Myh), or Pprrc and positive for Collα1 and Gapdh (ct<20) were included into statistical analyses. For Collα1 cDNA samples were potentially contaminated with genomic DNA, intron-spanning primer design was used for all genes. For EGFP, no intron-spanning design was possible, and a weak background amplification (ct>20) was observed even in the absence of Cre, most likely because of genomic DNA amplification. For EGFP analysis, therefore, only cells with ct<20 (genomic background) were evaluated. The limit of detection for the BioMark HD System has been estimated to be at ct value of 24 cycles (limit of detection Ct); all sample ct values were therefore subtracted from the (limit of detection) Ct using the formula: gene expression=2^(limit of detection ct-measured ct).

Statistical Analyses
Data are presented as means ± SEM. Comparisons between 2 groups were performed with unpaired Student t test. The correlation between expression of individual genes in single cells was determined using Spearman rank correlation coefficient. P values are indicated as follows: *P<0.05; **P<0.01; ***P<0.001.

Results
We used single-cell quantitative reverse transcription PCR to study gene expression in CF under basal conditions and after continuous AngII infusion, which promotes cardiac remodeling both through induction of arterial hypertension and through direct effects on cardiomyocytes and CF. To enrich CF, cell suspensions from digested hearts were allowed to adhere to cell culture plastic for 1 hour, and single-cell expression analyses were performed with adherent cells only. Forty percent of individual adherent cells expressed lineage markers for endothelial cells, myeloid cells, cardiomyocytes, or smooth muscle cells and therefore were excluded from the analysis (Online Figure IA). Sixty percent of adherent cells were negative for these lineage markers, and those which expressed α1 type I collagen were putative CF (linα1 colα1 CF). In the unchallenged heart, linα1 colα1 CF were transcriptionally quiescent, but 6 to 10 days of AngII infusion resulted in a clear upregulation of various fibrosis-associated genes, prominently among them the collagen isoforms Collα1, Collα2, Collα3, and Collα1a1, fibronectin (Fn1), connective tissue growth factor (Ctgf), insulin-like growth factor (Igfl), protein-lysine 6-oxidase (Lox), or transforming growth factor β1 (Tgb1; Figure 1A). Also, putative CF marker genes such as Acta2, Ddr2, Postn, Pdgfra, S100a4 or Thy1 were strongly
upregulated: although only a minority of lin\(^{−}\)colpos CF from healthy hearts were CF marker positive, AngII treatment resulted in a strong upregulation both with respect to individual strength of expression (Figure 1B) and percentage of expressing cells (Figure 1C). However, although Acta2, S100a4, or Thy1 were expressed in only 32% to 53% of cells after 6 to 10 days of AngII treatment, markers Ddr2, Pdgfra2, or Postn were present in 86% to 94% of lin\(^{−}\)colpos CF (Figure 1C). Expression of Ddr2, Pdgfra2, or Postn was largely, but not fully overlapping (Online Figure 1B), whereas expression of Acta2, S100a4, or Thy1 showed no clear associations (Online Figure IC). To identify the marker gene with the highest correlation to an activated CF phenotype, we performed Spearman rank correlation coefficient analyses between CF marker genes and the 10 fibrosis-associated genes most strongly upregulated in response to AngII treatment. These analyses showed the strongest correlation with an activated CF phenotype for Postn, closely followed by Ddr2 and Pdgfra (Figure 1D and 1E).

On the basis of the finding that Postn expression is highly correlated with the activation state of an individual CF, we set out to generate genetic tools allowing selective manipulation of Postn-expressing cells in the adult mouse heart. To avoid confounding effects generated by embryonic expression of Postn, we chose a tamoxifen-inducible approach that allows...
temporally controlled expression of the recombinase Cre under control of the Postn promoter. Using Red/ET recombination, we inserted the tamoxifen-regulated Cre recombinase CreERT2 into the start codon of the Postn gene, thereby replacing exon 2 and parts of exon 1 (Online Figure IIA–IIC). To investigate the inducibility and specificity of CreERT2 expression, we crossed Postn-CreERT2 founder mice (short PnCreERT2) with the B6;129S4-Gt(Rosa)26Sortm1Sor/J reporter line, which expresses the LacZ gene encoding β-gal on Cre-mediated recombination (Cre-inducible LacZ, short i-LacZ). Cre-mediated recombination was induced in PnCreERT2; i-LacZ mice by intraperitoneal injection of 1 mg tamoxifen on 5 consecutive days, and 1 week later, the degree of recombination was analyzed by β-gal staining in various tissues. In healthy adult mice, β-gal staining was observed in atrioventricular and semilunar valves, although no staining was detected in the atrial or ventricular walls (Figure 2A) or other organs such as kidney, liver, lung, or aorta (Online Figure IID). To exclude potential effects of PnCreERT2 expression on cardiac function, we performed magnetic resonance imaging but did not find differences between PnCreRT2-positive and PnCreERT2-negative mice before and 4 weeks after tamoxifen treatment (Online Figure IIE), and also histological analysis was normal (data not shown). Also, directly after tamoxifen treatment, no differences between PnCreRT2-positive and PnCreRT2-negative mice were observed (Online Figure IIF). We next investigated whether the PnCreERT2 mouse line was able to induce Cre-mediated recombination in activated CF in response to AngII exposure. PnCreERT2; i-LacZ double-positive mice and corresponding i-LacZ–positive control mice received chronic infusion of AngII or saline through an implanted osmotic minipump, as well as tamoxifen injections on 5 consecutive days starting 1 day after pump implantation. Twenty-eight days after pump implantation, we observed β-gal staining in ventricular walls of PnCreERT2; i-LacZ mice that had received both AngII and tamoxifen but not in vehicle-treated mice (Figure 2B). β-gal+ cells localized to intercardiomyocytic spaces, which could be identified by picrosirius red staining as fibrotic areas (Figure 2C). We did not observe AngII-induced expression of PnCreERT2 in other tissues (Online Figure IIG). To quantify Cre-mediated recombination in the AngII-activated CF population, we performed co-staining with an antibody directed against periostrin and found that the majority of periostrin-positive cells co-stained for β-gal (Figure 2D). To further characterize the degree of recombination in PnCreERT2 mice, we investigated recombination efficiency on the single-cell level in Gt(Rosa)26Sortm4(Actb-tdTmato,-EGFP) mice, which express EGFP on Cre-mediated recombination (Cre-inducible EGFP, short i-EGFP). We found that 6 days after AngII exposure, 84.6% of Postn expressing lin−;col− CF-expressed EGFP (Figure 2E).

To study the relevance of Postn-expressing fibroblasts under both physiological and pathophysiological conditions, we crossed PnCreERT2 mice to the C57BL/6-Gt(Rosa)26Sortm1(HBEGF)Awai/J mouse line, which expresses the DTR on Cre-mediated recombination, rendering Cre-expressing cells sensitive to diphtheria toxin (Cre-inducible DTR, short i-DTR). To selectively ablate Postn-expressing cells in the unchallenged adult heart, Cre-mediated recombination was induced by intraperitoneal tamoxifen injection on 5 consecutive days, followed by treatment with diphtheria toxin for another 7 days (Figure 3A). Studies in mice carrying the Cre-dependent lacZ reporter showed that this treatment resulted in a significant reduction of β-gal–positive valvular cells (Figure 3B), although cardiac morphology (Figure 3C and 3D) and function (Figure 3E) were not altered 4 and 16 weeks after ablation.

We next determined the consequences of ablation of Postn-expressing activated CF (Postn-ablated) during AngII-induced fibrosis (Figure 4A). Flow cytometric analysis on day 14 of AngII exposure showed that the population of putative CF, here defined as being CD45/CD31−;Pdgfrα+;β-gal− was clearly reduced (Figure 4B; Online Figure IIIA and IIIB). Single-cell expression analysis of the remaining CF showed that on day 14 of AngII exposure, the Postn-expressing population was reduced from 100% to 40% (Figure 4C). The CF population in ablated hearts showed not only a strong reduction of Postn expression but also a generally reduced profibrotic activity, as well as altered expression of matrix-modifying enzymes (Figure 4D). In line with this, we observed a clear reduction of periostin-immunoreactive cells in fibrotic areas of hearts from PnCreERT2;DTR mouse (Figure 4E), and the total fibrotic area in ablated hearts was clearly diminished (Figure 4F). Also, proteomic analysis confirmed the reduction of fibrosis (Online Figure IIIIC). Importantly, reduced fibrosis in ablated hearts was associated with an improved output function, as indicated by higher ejection fraction, fractional shortening, and cardiac index in hearts of ablated mice (Figure 4H and 4I). Cardiac hypertrophy as judged by the heart weight/ body weight ratio or expression of hypertrophy-related genes in isolated cardiomyocytes did not differ between the groups, nor did gene expression differ in individual cardiac macrophages (Online Figure IIIID–IIIF).

We next investigated how ablation of Postn-expressing activated CF would affect cardiac fibrosis and function in a mouse model of MI. MI was induced by ligation of the left anterior descending artery, followed by tamoxifen induction and diphtheria toxin treatment (Figure 5A). Surgery-associated mortality did not differ between the experimental groups (data not shown). Single-cell expression analysis of CF showed that in control hearts the percentage of Postn–positive CF increased to 60% at day 14 after infarction and that diphtheria toxin–mediated ablation reduced this population to 17% (Figure 5B). We compared the expression of fibrosis-associated genes in activated CF from infarcted hearts and found them to be significantly decreased in the ablated group (Figure 5C). Twenty-one days after surgery, immunohistochemical analysis showed that periostrin deposition was reduced in scars of ablated mice, although not fully lost (Figure 5D). Systematic histological analysis revealed that especially in the more apical sections, the fibrotic area was significantly reduced in hearts of Postn-ablated mice, and also the total fibrotic area across all planes was decreased (Figure 5E and 5F). Remote interstitial fibrosis did not differ significantly between the genotypes (Online Figure IVA). Magnetic resonance imaging performed 20 days after infarction showed a significantly better ejection fraction in Postn-ablated mice than in control mice, as well as reduced
end-diastolic and end-systolic volumes (Figure 5G–5J). In line with the assumption that myocardial damage should be equal before onset of DiphTox-mediated ablation, we did not detect significant differences in ejection fraction on day 10 after MI (Online Figure IVB). Furthermore, observation ≤6 weeks after MI showed that the ablated hearts maintained...
their improved ejection fraction (Online Figure IVB), indicating that the local increase in hypertrophic and inflammatory markers did not result in long-term adverse effects. We also investigated whether application of Tamoxifen/DiphTox before and during MI would reproduce the beneficial effect but found here only nonsignificant changes (Online Figure IVC–IVF).

Interestingly, we did not find evidence for reduced ventricular wall stability in Postn-ablated mice (Figure 6A); on the contrary, scars of ablated mice contained more cardiomyocytes compared with control hearts (Figure 6B). In addition, cardiomyocytes in scar-adjacent areas showed in ablated hearts an increased cross-sectional area, indicative of enhanced hypertrophy (Figure 6C). The heart weight/body weight ratio, however, was not changed (Online Figure IVG). Single-cell expression analyses in cardiomyocytes, here identified by the expression of myosin heavy chain 6 (\(\text{Myh6}\), encoding \(\alpha\)MHC), showed an increased expression of hypertrophy-associated genes such as \(\text{Myh7}\) (encoding \(\beta\)-MHC), \(\text{Acta1}\) (encoding skeletal muscle \(\alpha\)-actin), or \(\text{Acta2}\) (encoding \(\alpha\)SMA) in ablated hearts (Figure 6D). What is more, cardiomyocytes from ablated hearts expressed higher levels of messenger RNA for hypertrophic growth factor transforming growth factor \(\beta\)1 (TGF\(\beta\)1) and showed a trend to increased interleukin-6 production (Figure 6D). Because also immune cells importantly contribute to the remodeling process,26–28 we extended our analysis to cardiac macrophages, here identified by the expression of Integrin \(\alpha\)M (CD11b). Also, these cells showed significantly increased expression of genes encoding prohypertrophic factors such as TGF\(\beta\)1 and platelet-derived growth factor B (PDGF-B), as well as trends to increased expression of genes encoding PDGF-A and TGF\(\beta\)2 (Figure 6E). Also, the gene encoding angiotensin-converting enzyme, a key regulator of the endogenous production of the prohypertrophic/profibrotic mediator AngII, was increased in macrophages from ablated hearts (Figure 6E). These findings indicate that ablation of Postn-expressing cells in the subacute phase of MI not only reduces CF activation and consecutive fibrosis without affecting scar stability but also causes secondary changes in the activation state of other cardiac cell populations. Together, reduced fibrosis and enhanced cardiomyocyte hypertrophy give rise to a significant preservation of cardiac function in Postn-ablated hearts after MI.

**Discussion**

Most cardiac pathologies, including ischemic and rheumatic heart disease, myocardial inflammation, hypertrophy, or infarction, are associated with activation of CF and consecutive fibrotic remodeling.2,4–6,8,29,30 Selective targeting of fibroblast subpopulations that are involved in pathological, but not homeostatic, remodeling has been proposed as a therapeutic approach,5,6,31 but whether such populations exist and how they may be targeted remains unclear. We describe here for the first time a genetic model allowing inducible and selective ablation of Postn-expressing activated CF in the adult heart and show that this results in a significant improvement of cardiac function after MI.

Our single-cell expression analysis showed that under basal conditions, the lin\(^{neg}\);colpos ventricular CF population is transcriptionally quiescent, with only a minority of cells expressing one or more putative CF markers such as Acta2,
Figure 4. Consequences of diphtheria toxin-mediated ablation of Postn-expressing cells during angiotensin II (AngII)–induced fibrosis. 

**A**, Schematic diagram of the experimental design. B, Flow cytometric analysis of the percentage of CD45/CD31\(_{-}\);Pdgfra\(_{+}\)putative cardiac fibroblast (CF) in adherent cells from control and ablated hearts after 14 days of AngII infusion (n=4). 

**C** and **D**, Single-cell expression analysis in linn\(_{-}\);colpos CF obtained 14 days after onset of AngII infusion from left ventricles of tamoxifen- and diphtheria toxin-treated i-DTR mice (control) and PnCreERT2; i-DTR mice (ablated). 

**E**, Left ventricular sections from AngII-, tamoxifen-, and diphtheria toxin-treated i-DTR mice (control) and PnCreERT2; i-DTR mice (ablated) were stained with antibodies directed against periostin (green), sarcomeric myosin (MF20, red), and DAPI (4′,6-diamidino-2-phenylindole) after 28 days of AngII infusion (n=4). 

**F**, Picrosirius red staining for fibrotic areas in hearts from AngII-, tamoxifen-, and diphtheria toxin-treated i-DTR mice (control) and PnCreERT2; i-DTR mice (ablated) after 28 days of AngII infusion (left: overview, right: left ventricular wall ×40; n=9). 

**G**, Quantification of **F**. 

**H** and I, Left ventricular function was assessed by MRI before and 4 weeks after AngII infusion and Tam/DiphTox treatment in i-DTR mice (control) and PnCreERT2; i-DTR mice (ablated) (n=7–10). CI indicates cardiac index; DiphTox, diphtheria toxin; ED/ES, enddiastolic/endsystolic; and Tam, tamoxifen. All data are means±SEM; comparisons between groups were performed using 2-tailed t test. *P<0.05; **P<0.01; ***P<0.001. 

Gene names: as in Figure 1, and *Mmp2/3*, matrix metalloproteinase-2/3; *Timp1/2/3*, tissue inhibitor of metalloproteinase-1/2/3.
Ddr2, Pdgfra, Postn, S100a4, or Thy1. These values are significantly lower than those reported based on flow cytometric analyses, in which 26.2% of adult cardiac cells stained positive for Ddr2, 32.39% for Thy1.12,33 This difference is most likely because of the vigorous exclusion of any lineage-positive cells from our analysis, because both Ddr2 and Thy1 are known to be expressed in various non-CF populations.12,34

The single-cell analysis furthermore showed that the percentage of Postn expressing lin<sup>−</sup>col<sup>−</sup> ventricular cells rises in response to AngII treatment from 13% to 94%, and in response to MI to 60%. In line with this, a robust upregulation of Postn was observed in various murine models of cardiac diseases as well as in patients with acute MI and heart failure.35,36 Periostin is a member of the fasciclin family of ECM proteins and has in vitro been implicated in cell adhesion and migration;1,38 whether it also affects cardiomyocyte proliferation is under debate.39,40 Previous studies showed that periostin is highly expressed during embryonic development of cardiac valves and outflow tract18 and that inactivation of periostin results in various abnormalities.
including dwarfism and disturbed valve development.\textsuperscript{41,42} In adult mice, periostin deficiency reduces cardiac fibrosis in different cardiac pathologies.\textsuperscript{43–45}

To study the role of activated fibroblasts in health and disease, we generated mice allowing diphtheria toxin-mediated ablation of \textit{Postn}-expressing cells. In the healthy heart, \textit{Postn} expression is restricted to valvular fibroblasts, and ablation of these cells did not affect cardiac function or morphology. Exposure to AngII, which is known to induce cardiac fibrosis not only through pressure overload but also through direct activation of CF,\textsuperscript{2,24,25} led to massive upregulation of various profibrotic and proinflammatory genes in CF. Diphtheria toxin-mediated depletion of \textit{Postn}-expressing activated CF resulted in a significant impairment of profibrotic gene expression and a clear reduction of interstitial fibrosis. Most intriguingly, also ablation of \textit{Postn}-expressing CF after MI resulted in reduced fibrosis but did not compromise scar thickness or stability. Application of tamoxifen and diphtheria toxin before and during infarction, in contrast, was not able to significantly improve cardiac function, which is in line with the finding that the \textit{Postn} promoter is not active in healthy adult CF and therefore cannot mediate diphtheria toxin receptor expression.

Many studies, among them those in constitutive periostin-knockout mice, suggested that disturbance of CF functions or disruption of ECM in the infarcted myocardium bears the risk of aneurysm formation or cardiac rupture.\textsuperscript{36,43–45} The critical factor for achieving reduced fibrosis without compromising scar stability is here certainly the time course of CF ablation, because our model does not generally prevent fibroblast activation, it merely ends it prematurely. Diphtheria toxin–induced cell death can occur only if a given CF has undergone a series of events triggered by the reactivation of the \textit{Postn} promoter: expression of the PnCreERT2 transgene, tamoxifen-induced nuclear translocation of the transgene, Cre-mediated activation of DTR expression, and finally diphtheria toxin entry and consequent translational inhibition. The exact time frame of these events is unknown, but it can be safely assumed that a newly activated CF is able to exert some profibrotic actions before its death. Also, any CF that upregulates \textit{Postn} expression after the end of tamoxifen application will not express the diphtheria toxin receptor and will therefore not be affected

\textbf{Figure 6. Cardiomyocyte and cardiac macrophage functions in Postn-ablated mice after myocardial infarction.} A and B, Morphometric analysis of the thinnest portion of scar tissue (chosen from sections 0.9–1.9 mm from apex) from tamoxifen- and diphtheria toxin-treated i-DTR mice (control) and PnCreERT2; i-DTR mice (ablated): Scar thickness (A) and proportion of cardiomyocytes within the thinnest part of the scar (B) were analyzed in picrosirius red-stained sections. Cardiomyocytes were identified by their fibrillary structure and absence of picrosirius red staining or by staining with myosin heavy chain specific antibody MF20 (fibrotic areas counterstained with wheat germ agglutinin, WGA) (n=5). C, Exemplary photomicrograph (left) and statistical evaluation (right) of the cardiomyocyte cross sectional area in scar-adjacent regions from control and ablated mice (n=5). D and E, Single-cell expression analysis in myosin heavy chain 6 (\(\alpha\)MHC)-positive cardiomyocytes (CMC) and integrin \(\alpha\) M (CD11b)-positive macrophages (MP) obtained 14 days after left anterior descending artery ligation from left ventricles of control and ablated mice. Cell numbers for control and ablated, respectively: cardiomyocytes: n=34 and 47; macrophages: n=46 and 34 (from 4 mice each). All expression data are calculated as follows: gene expression=2\(^{-}\Delta\Delta \text{Ct}\), limit of detection \(\text{Ct}\) was set to 24. DiphTox indicates diphtheria toxin; MI, myocardial infarction; and Tam, tamoxifen. All data are means±SEM; comparisons between groups were performed using 2-tailed t test. *P<0.05; **P<0.01; ***P<0.001. Gene names: as in Figure 1, and Ace, angiotensin-converting enzyme; Acta1, \(\alpha\) 1 skeletal muscle actin; Edn1, endothelin-1; Itgam, integrin \(\alpha\) M; Myh6, myosin heavy chain-6 (\(\alpha\)MHC); Myh7, myosin heavy chain-7 (\(\beta\)MHC); Tnf, tumor necrosis factor; Pdgfa/b, platelet-derived growth factor-A/B; Vegfa, vascular endothelial growth factor-A.
by Diphtox treatment. In line with this notion, we found that periostrict staining was reduced, but not lost, in scars of ablated hearts and that the population of Postn-expressing CF was diminished but not abrogated.

It is in this context highly interesting that ablation of the Postn-expressing population not only reduced fibrosis but also enhanced the proportion and size of cardiomyocytes in the scar area. Single-cell expression analysis revealed a hypertrophic phenotype in cardiomyocytes from ablated hearts, and an increased expression of growth factors such as TGFβ, PDGF, or interleukin-6 both in cardiomyocytes and cardiac macrophages. Because these growth factors, as well as AngII generated by angiotensin-convertase enzyme, are not only inducers of hypertrophy but also of fibrosis, it might be speculated that these changes in nonfibroblast populations represent a compensatory mechanism to make up for the loss of activated CF. We therefore hypothesize that the improved ejection fraction of Postn-ablated hearts is a consequence of both reduced fibrotic stiffening and increased cardiomyocyte hypertrophy and regeneration. How loss of activated CF is detected by nonfibroblast populations in the ablated heart is unclear. One potential explanation is that altered matrix production in ablated hearts exposes cardiomyocytes to increased mechanical stress, resulting in enhanced hypertrophy. Because various ECM components induce integrin-dependent signaling cascades in cardiomyocytes, it is also possible that the altered ECM composition in ablated hearts affects cardiomyocyte functions. However, it also cannot be excluded that mediators released from CF undergoing diphtheria toxin–induced cell death affect neighboring cardiomyocytes or macrophages. It is also interesting to note that locally increased cardiomyocyte hypertrophy was only observed in the infarction model, but not in the AngII model. The trend to even reduced heart weight/body weight ratio in AngII-treated ablated hearts and that the population of Postn-expressing CF population does not affect basal heart function but significantly reduces fibrosis and improves cardiac function in murine models of prolonged AngII exposure and MI. Because no adverse effects on scar stability were detected, strategies allowing selective inhibition of the Postn-positive CF population seem a promising target for future therapies.

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Disclosures

None.

References


We found that ablation of periostin-expressing cardiac fibroblasts resulted in improved cardiac performance during angiotensin II–induced fibrosis and myocardial infarction.

Activated cardiac fibroblasts (CF) contribute to myocardial stiffening and heart failure development, but it is unclear whether inhibition of activated CF improves cardiac performance. Several marker genes have been proposed to identify and target CF, but none of them is exclusive for activated CF. Using single-cell expression analysis, we identified periostin as the most suitable marker to target activated CF and generated a mouse model which allows selective inactivation of these cells. We found that ablation of activated CF resulted in reduced fibrosis and improved cardiac performance both after chronic angiotensin II exposure and myocardial infarction. Interestingly, scar stability was not compromised in the infarction model, suggesting that pharmacological inhibition of periostin-expressing cells is a promising therapeutic approach.
Targeted Ablation of Periostin-Expressing Activated Fibroblasts Prevents Adverse Cardiac Remodeling in Mice
Harmandeep Kaur, Mikito Takefuji, C.Y. Ngai, Jorge Carvalho, Julia Bayer, Astrid Wietelmann, Ansgar Poetsch, Soraya Hoelper, Simon J. Conway, Helge Möllmann, Mario Looso, Christian Troidl, Stefan Offermanns and Nina Wettschureck

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Correction

For the Circulation Research article by Kaur et al (“Targeted Ablation of Periostin-Expressing Activated Fibroblasts Prevents Adverse Cardiac Remodeling in Mice.” Circ Res. 2016;118:1906–1917. DOI:10.1161/CIRCRESAHA.116.308643), the y-axis in Figure 5J (LVESV) was mislabeled: the ticks should be marked 80-60-40-20-0, not 120-90-60-30-0.

The authors apologize for the error.

This correction has been made to the current online version of the article, which is available at http://circres.ahajournals.org/content/118/12/1906.full.
Online Figure I: A, Expression of lineage marker genes in rapidly adhering cells obtained from healthy or AngII-stimulated hearts (expression of more than one marker possible): endothelial cell marker VE-Cadherin (Cdh5), myeloid leukocyte marker CD11b (Itgam), cardiomyocyte marker myosin heavy chain 6 (Myh6), or smooth muscle marker myosin heavy chain 11 (Myh11) (n=417 cells). B, C, Proportional Venn diagrams displaying unique and intersecting expression of putative CF marker genes in lin<sup>neg</sup>.col<sup>pos</sup> cells from AngII-stimulated hearts (n=134) for markers Postn, Ddr2 and Pdgfra (B) as well as markers Thy1, S100a4, and Acta2 (C). Numbers indicate counts of individual CF.
**Online Figure II: Generation of PnCreERT2 mice.**

**A.** The cDNA encoding CreERT2 was inserted into the start codon of exon 1 of the *Postn* gene on BAC RP23-480C1 by RedE/T cloning, thereby replacing exon 2. Filled boxes indicate exons of the *Postn* gene, DNA sequences used as the 5′ and 3′ arms for homologous recombination are shown as black boxes. Red triangles indicate frt sites for Flp-mediated recombination.

**B, C.** Correct recombination (B) and Flp-mediated deletion of the Ampicillin resistance cassette (AmpR) (C) was analyzed by restriction analysis of wildtype (wt) BAC and recombined (rec) BAC; expected changes in the restriction pattern are indicated by arrows.

**D.** Extracardiac tissues from tamoxifen-treated PnCreERT2; i-LacZ reporter mice were sectioned and stained for β-gal activity (n=5-6).

**E.** MRI-based analysis of cardiac function in tamoxifen treated control mice and PnCreERT2-positive mice 4 weeks after tamoxifen induction (n=8).

**F.** MRI-based analysis of cardiac function in PnCreERT2-positive and -negative littermate mice 1 day after end of tamoxifen treatment (n=8-9).

**G.** Extracardiac tissues from PnCreERT2; i-LacZ reporter mice that were exposed to tamoxifen treatment and four weeks of AngII infusion were sectioned and stained for β-gal activity (n=5-6). All data are means ± SEM. Ns, not significant.
Online Figure III: Ablation of Postn-expressing CF in the Ang II model. A, B, Flow cytometric analysis of the percentage of CD45/CD31\textsuperscript{neg},Pdgfra\textsuperscript{pos} putative CF in adherent cells from control and ablated hearts after 14 days of AngII infusion. A, Gating strategy: After gating on CD31/CD45 negative cells, the percentage of Pdgfra-positive cells was determined. B, Exemplary histograms displaying the percentage of CD31/CD45-negative,Pdgfra-positive ventricular cells in ventricles of control mice or ablated mice at 14 days after AngII infusion. C, Upregulation of fibrosis-related proteins in AngII/Tam/DiphTox-treated i-DTR mice (control, n=6) and PnCreERT2; i-DTR mice (ablated, n=3) compared to sham-treated mice was determined by quantitative mass spectrometric analysis. D, Heart weight-to-body weight ratio before and 4 wks after AngII infusion and Tam/DiphTox treatment in i-DTR mice (control) and PnCreERT2; i-DTR mice (ablated) (n=7-11). E, F, Single cell expression analysis in myosin heavy chain 6 (Myh6)-positive cardiomyocytes (E) and integrin α M (Itgam)-positive macrophages (F) obtained 14 days after onset of AngII infusion from left ventricles of control and ablated mice. Cell numbers cardiomyocytes: n=40 cells from 4 control mice and 23 cells from 3 ablated mice; macrophages: 20 cells from 4 control mice and 13 cells from 3 ablated mice. All expression data are calculated as follows: gene expression = \(2^{\text{LoD C_t} - \text{sample C_t}}\); LoD C\_t was set to 24. Ang II, angiotensin II; Tam, tamoxifen; DiphTox, diphtheria toxin. All data are means ± SEM; comparisons between groups were done by 2-tailed t-test. **, p < 0.01.
Online Figure IV: Ablation of Postn-expressing CF in the infraction model. A, Remote interstitial fibrosis 3 weeks after induction of myocardial infarction and Tam/DiphTox treatment in i-DTR mice (control) and PnCreERT2; i-DTR mice (ablated). Left: schematic diagram indicating regions analysed; Center: exemplary photomicrographs depicting picrosirius red stained ventricular sections from the different regions; right: statistical evaluation of picrosirius red-stained areas (n=6). B, Ejection fraction (as determined by MRI) in hearts of tamoxifen- and diphtheria toxin-treated i-DTR mice (control) and PnCreERT2; i-DTR double positive mice (ablated) before and on days 10, 21 and 42 after LAD ligation (n=5-6). C-F, Functional consequences of early induction. C, Schematic overview. D-F, Ejection fraction (D) and fractional shortening (E) were determined by MRI 21 days after MI. F, Scar area was determined by picrosirius red staining 21 days after MI. G, Heart weight-to-body weight (HW/BW) ratio 3 wks after myocardial infarction and Tam/DiphTox treatment in i-DTR mice (control) and PnCreERT2; i-DTR mice (ablated) (n=6). (n=5; p= 0.34 and 0.25 for EF and FS).
Supplemental methods

SDS-PAGE and protein digestion
Left cardiac ventricle was grinded and lysed in 4% SDS in Tris-HCl pH 7.6, followed by sonication and boiling at 95°C for 5 min. Lysates were clarified by centrifugation at 16000g for 10 min and protein concentration was measured using Lowry method (DC assay, Bio-Rad). 40 µg of protein was mixed with NuPAGE LDS buffer (Novex) and loaded onto a 4–12% NuPage gel (Invitrogen). Gels were run at 180V and stained with Instant Blue Coomassie (expedeon). Each lane was cut into 10 slices, which were de-stained and digested with trypsin as previously described.1,2 Peptides were extracted from the gel pieces with acetonitrile, loaded onto STAGE tips for storage, and eluted from the tips shortly before MS analysis.3

Mass Spectrometry
By using an EASY- nLC 1000 (Thermo Scientific) LC system, peptides were separated at a flow rate of 400 nL/min on a self-packed 18 cm column (75 µm ID, 1.9 µm Reprosil-Pur 120 C-18AQ beads, Dr Maisch Germany) housed in a custom-built column oven4 at 45°C. The following gradient of buffers A (0.1% formic acid) and B (80% acetonitrile, 0.1% formic acid) was used: 5 min 10% B, 5-55 min 10%-38% B, 55-60 min 38%-60% B, 60-65 min 60%-95%B, 65-70 min 95%B, 70-73 min 95%-5%B, 73-75 min 5% B. The column was interfaced with a Nanospray Flex Ion Source (Thermo Scientific) to a Q-Exactive HF mass spectrometer (Thermo Scientific). MS instrument settings were: 1.5 kV spray voltage, Full MS at 60K resolution, AGC target 3e6, range of 300-1750 m/z, max injection time 20 ms; Top 15 MS/MS at 15K resolution, AGC target 1e5, max injection time 25 ms, isolation width 2.2 m/z, charge exclusion +1 and unassigned, peptide match preferred, exclude isotope on, dynamic exclusion for 20s.

Protein identification and quantification
Mass spectra were recorded with Xcalibur software 3.1.66.10 (Thermo Scientific). Proteins were identified with Andromeda and quantified with the LFQ algorithm embedded in MaxQuant version 1.5.3.175. The following parameters were used: main search max. peptide mass error of 4.5 ppm, tryptic peptides of min. 6 amino acid length with max. two missed cleavages, variable oxidation of methionine, protein N-terminal acetylation, LFQ min. ratio count of 2, matching between runs enabled, PSM and (Razor) protein FDR of 0.01, advanced ratio estimation and second peptides enabled. Spectra were searched against a mouse proteome database containing 50.691 entries from Uniprot (UP000000589). In addition, spectra were searched against a database of common exogenous protein contaminants.

Flow cytometry
Single cell suspension of adherent ventricular cells were prepared as described for single cell quantitative real-time PCR and resuspended in PBS with 2% FBS, 2.5 mM EDTA. The cell suspension was subjected to FACS analysis using a BD FACSCanto II. The following antibodies and corresponding isotype controls were used: anti–CD31-PE, anti–CD45-PE, anti–PDGFRα-APC (clone APA5) (all eBioscience). Data were analyzed using FlowJo software.
Supplemental references: