PRECLINICAL RESEARCH

Cardiac Mesenchymal Stem Cells Promote Fibrosis and Remodeling in Heart Failure
Role of PDGF Signaling

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VISUAL ABSTRACT

HIGHLIGHTS

- Resident cMSCs comprise a distinct mesenchymal population that is temporally expanded and exhibit pro-fibrotic and pro-inflammatory features in failing hearts.
- cMSCs in failing hearts undergo a phenotypic switch characterized by enhanced pro-inflammatory and pro-fibrotic responses.
- Pro-inflammatory macrophages promote a myofibroblast differentiation fate of cMSCs from failing hearts, in part mediated by PDGF/PDGF receptor β activation.
- Pharmacologic PDGF receptor inhibition in a mouse model of myocardial infarction alleviated long-term cardiac remodeling, inflammation, hypertrophy, and fibrosis and increased myocardial capillary density.
- Inhibiting the pathologic cMSC phenotype, or restoring a normal cMSC phenotype, may combat the progression of cardiac fibrosis and failure after myocardial infarction.

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The authors attest they are in compliance with human studies committees and animal welfare regulations of the authors’ institutions and Food and Drug Administration guidelines, including patient consent where appropriate. For more information, visit the Author Center.

Manuscript received April 21, 2021; revised manuscript received January 11, 2022, accepted January 11, 2022.

ISSN 2452-302X

https://doi.org/10.1016/j.jacbts.2022.01.004
Heart failure (HF) is characterized by progressive fibrosis. Both fibroblasts and mesenchymal stem cells (MSCs) can differentiate into pro-fibrotic myofibroblasts. MSCs secrete and express platelet-derived growth factor (PDGF) and its receptors. We hypothesized that PDGF signaling in cardiac MSCs (cMSCs) promotes their myofibroblast differentiation and aggravates post-myocardial infarction left ventricular remodeling and fibrosis. We show that cMSCs from failing hearts post-myocardial infarction exhibit an altered phenotype. Inhibition of PDGF signaling in vitro inhibited cMSC-myofibroblast differentiation, whereas in vivo inhibition during established ischemic HF alleviated left ventricular remodeling and function, and decreased myocardial fibrosis, hypertrophy, and inflammation. Modulating cMSC PDGF receptor expression may thus represent a novel approach to limit pathologic cardiac fibrosis in HF. (J Am Coll Cardiol Basic Trans Science 2022;7:465–483) © 2022 The Authors. Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
Heart tissue was processed for mononuclear cell isolation and quantitative analysis of cMSCs and immune cells by flow cytometry, or fixed for histologic analyses as previously described. These studies were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee.

**Echocardiography.** After anesthetic induction with tribromoethanol (0.25 mg/g intraperitoneally) and maintenance with 1% to 2% inhaled isoflurane, echocardiography was performed by using a Visual-Sonics Vevo 3100 High-Resolution System and MX400 scan head as previously described. The mice were imaged on a heated bench-mounted adjustable rail system (Vevo Imaging Station) that allowed steerable and hands-free manipulation of the ultrasound transducer.

**Expansion and Characterization of cMSCs.** cMSCs were isolated from sham-operated and HF mice (8 weeks’ post-MI) using primary explant cell outgrowth culture. Cell outgrowths from minced myocardial tissue were cultured in cMSC media (Dulbecco’s modified Eagle medium [DMEM]/Ham’s F-12 culture medium [Thermo Fisher Scientific-Hyclone] supplemented with 0.2 mM L-glutathione, 10 ng/mL leukemia inhibitory factor, 10 ng/mL basic fibroblast growth factor, 10% fetal bovine serum, and 1% penicillin-streptomycin). Lineage-positive (Lin+) cells (CD5+; CD45R+; CD11b+, Gr-1+, TER-119+; Miltenyi Biotec, #130-092-613), endothelial cells (CD31+; BD, #553372), and fibroblasts (discoidin domain receptor 2 positive [DDR2+]; LSBio, #LS-C255961) were depleted from explant cultures by using antibody-conjugated magnetic microbeads. After depletion, Lin CD31 DDR2+ cells were minimally expanded and further sorted by using a specific Sca1 antibody (eBioscience, #25-5981-81) and consequently enriched for Sca1+ cells (Lin CD31 DDR2+Sca1+ cells were minimally expanded and used at cell passages 3 to 5 for all experiments). cMSCs were evaluated for their ability to differentiate into adipogenic, osteogenic, and chondrogenic lineages using standard differentiation protocols. Lineage-specific gene expression was used to confirm successful differentiation as described.

**Immunophenotyping of cMSCs.** cMSCs were evaluated for the expression of previously validated MSC surface markers by flow cytometry using fluorophore-labeled primary antibodies against CD90 (BD, #553006), CD44 (eBioscience, #14-0441-81), CD49 (eBioscience, #13-0495-80), CD29 (eBioscience, #13-0291-82), CD73 (eBioscience, #12-0731-81), and CD105 (eBioscience, #12-1051-81).

**Mouse Inflammation Antibody Array.** The baseline inflammatory protein secretory profile of sham cMSCs and HF cMSCs was determined in conditioned media collected from cultured cMSCs. We used the Mouse Inflammation Array C1 (RayBiotech), which detects 40 murine inflammatory molecules in an antibody-based chemiluminescence detection system. Briefly, after blocking and washing of the antibody array, 1 mL of cMSC-conditioned media was added and incubated overnight at 4°C. After binding and washing, the array was incubated overnight at 4°C with the cocktail of provided biotinylated detection antibodies. Subsequent washing to remove the unbound antibodies was followed by incubation with horseradish peroxidase-conjugated streptavidin and chemiluminescence imaging. Arrays were imaged and analyzed by using Image J software (National Institutes of Health) and reported as arbitrary densitometric units.

**Collagen Gel Contraction Assay.** cMSCs were cultured to ~75% confluency. After trypanosinization, washing, and cell counting, cMSCs (1 × 105 cells/mL) were resuspended in DMEM media containing 10% fetal bovine serum and mixed with a solution containing Type I rat tail collagen (Thermo Fisher Scientific), 5X PBS, and 50 mM sodium hydroxide on ice in a volume ratio of 3:3:3:2:1.3 mL, respectively. Next, 300 μL per well of this mixture (containing 1 × 105 cMSCs) was plated in a 48-well tissue culture plate and incubated at 37°C for 24 hours to induce gelation. After gelation, 300 μL of DMEM + 10% fetal bovine serum was added, and the cells were cultured overnight. For co-culture experiments, RAW 264.7 (ATCC TIB-71) mouse macrophage cells were polarized in vitro either to an M1 phenotype upon treatment with lipopolysaccharide (1 μg/mL) and interferon-γ (4 ng/mL) or to an M2 phenotype upon treatment with interleukin (IL)-4 (10 ng/mL) or IL-10 (10 ng/mL) for 4 hours followed by washing in serum-free DMEM media and cell counting. Polarized macrophages (2 × 105 cells) were then added to the collagen mixture (described earlier). After overnight incubation, the cells were treated as indicated and cultured at 37°C in a 5% carbon dioxide incubator for 4 days with medium change at day 2. In some experiments, cMSC contraction assays were performed with the addition of conditioned media from polarized mouse RAW 264.7 macrophages. In studies of PDGF inhibition, imatinib mesylate (10 μM; Santa Cruz Biotechnology) was added to the regimen after overnight culture of cells in collagen gels. On day 5, the collagen gels were detached from the culture wells by using a pipette tip, and the gel contraction was measured after overnight incubation. Collagen
gels were imaged by using a digital camera, and images were analyzed for total area by using Image J software. The results were expressed as percent change in area relative to the control undifferentiated group.

TRANSWELL MIGRATION ASSAY. Macrophage migration assays were performed by using Transwell inserts (6.5 mm, 8 μm pore size; Corning). Mouse RAW 264.7 (ATCC TIB-71) macrophages were polarized to an M1 phenotype (described earlier). M1-polarized macrophages (7.5 × 10⁴ cells) were added to Transwell inserts containing 100 μL of serum-free DMEM media and placed into chambers containing sham cMSCs and HF cMSCs (1 × 10⁵ cells) that were cultured overnight in cMSC media. Macrophages were allowed to migrate through the Transwell membrane for 2 to 6 hours in a cell culture incubator. In some experiments, macrophage-loaded inserts were instead exposed to conditioned media collected from cMSCs after 24 hours of culture. After co-culture, inserts were removed, washed with PBS, fixed with 3.7% paraformaldehyde for 10 minutes, and stained with wheat germ agglutinin (WGA) and 4’,6-diamidino-2-phenylindole for 30 minutes in the dark. After staining, nonmigrating cells on the upper surface of the inserts were removed with a cotton swab. Migrated cells on the bottom surface of the inserts were imaged and counted in 4 different microscopic fields. Data are expressed as mean ± SD of 2 independent experiments with 4 replicates per group.

RNA INTERFERENCE AND IMMUNOBLOTTING. cMSCs were transfected with 30 nM mouse PDGFβR (or control) small interfering RNA (siRNA) (Santa Cruz Biotechnology) using INTERFERin siRNA transfection reagent (Polyplus Transfection) according to the manufacturer’s instructions. After 96 hours of transfection, cMSCs were evaluated for PDGFβR expression by immunoblotting using standard protocols as previously described. In other experiments, siRNA-transfected cMSCs were trypsinized, washed, and counted 24 hours post-transfection followed by the collagen gel contraction assay as described earlier. Primary antibodies used for immunoblotting were purchased from Santa Cruz Biotechnology, including against PDGFβR (sc-338), PDGFβ (sc-432), alpha smooth muscle actin (α-SMA) (sc-8669), α-tubulin (sc-8035), vinculin (sc-736), and glyceraldehyde-3-phosphate dehydrogenase (sc-32249).

ISOLATION OF MONONUCLEAR CELLS AND FLOW CYTOMETRY. Mononuclear cells were isolated from the heart as previously described. Isolated cell suspensions were incubated for 1 hour at room temperature in a cocktail of fluorophore-labeled antibodies to label cardiac macrophages, resident cMSCs, and cMSC-derived myofibroblasts. The following antibodies (from eBioscience, unless otherwise specified) were used: CD45–605 NC (30-F11), MerTK-APC (FAB5912A, R and D Systems), MHCII-eFluor450 (48-5320-82), Ly6C-PE/Cy7 (HK1.4), F4/80-eFluor 450 (BM8), F4/80-PerCP-Cy5.5 (45-4801-82), C-C chemokine receptor 2 (CCR2)-fluorescein isothiocyanate (FAB538F, R and D Systems), LY-6A/E(Sca-1; 17-5981-81), CD31(BD, #55372), DDR2 (LSBio, #LS-C255961), and α-SMA (Santa Cruz; Sc-58669). CD45 was used to identify leukocytes, and macrophages were considered as CD45⁺MerTK⁺MHCII⁺Ly6C⁺F4/80⁻ cells. Within this population, infiltrating (monocyte derived) and resident macrophages were defined as CCR2⁺ or CCR2⁻ cells, respectively. cMSCs were defined as CD45⁻Sca1⁻CD31⁻DDR2⁻ cells and cMSC-derived myofibroblasts as CD45⁻Sca1⁺CD31⁻DDR2⁻α-SMA⁻ cells.

Data were acquired on an LSRII flow cytometer (BD Biosciences) and analyzed with FlowJo software version 10.0.6 (FlowJo LLC). Cell populations were normalized to the total mononuclear cell population (frequency) or expressed as absolute numbers.

REAL TIME RT-PCR. Quantitative RT-PCR (reverse transcription polymerase chain reaction) to evaluate messenger RNA (mRNA) expression was performed as described previously. Briefly, TRIzol (Life Technologies) extracted total RNA was quantified by using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). Total RNA (250 ng) was subjected to complementary DNA (cDNA) synthesis by using the High Efficiency cDNA Synthesis Kit (Invitrogen). The levels of various mRNA transcripts were determined by using Fast SYBR Green (Life Technologies) and gene-specific forward and reverse primer sets (Table 1) on a ViIA 7 RT-PCR instrument (Life Technologies). Then, 18S ribosomal RNA or β-actin expression was used to normalize mRNA expression levels by using the ΔΔC_T comparative method.

BULK cMSC RNA-SEQUENCING AND ANALYSIS. Live mononuclear cells isolated from chronic HF (or sham-operated) hearts (8 weeks post-MI) were stained with SYTOX (live/dead staining) and CD45 and Sca1 antibodies. SYTOX CD45⁻Sca1⁻ cells were sorted on a BD FACS Aria flow cytometer. Using the SMART-Seq v4 Ultra Low Input RNA Kit (Takara Bio USA Inc), 250 cells were collected in lysis buffer at room temperature, pelleted, and processed for cDNA library preparation according to the kit protocol. Briefly, mRNA in cell lysates were reverse transcribed, and cDNA was prepared for amplification by template switching and extension. cDNA was amplified with 21 PCR cycles
and purified for Illumina Nextera DNA library construction and sequencing. STAR (version 2.5.3a) was used to align the raw RNA-sequencing FASTQ reads to the reference genome from Gencode. Cufflinks was used on the STAR-aligned reads to assemble transcripts, estimate their abundances, and test for differential expression and regulation. Cuffmerge was used to merge the assembled transcripts to a reference annotation and track Cufflinks transcripts across multiple experiments. Groups were compared by using an unpaired Student’s t-test to assess statistical significance and adjusted P value corrected for multiple hypothesis testing with the Benjamini-Hochberg procedure. DESeq2 analyzed data were analyzed on the Ingenuity Pathway Analysis platform (QIagen) to identify differentially regulated pathways. Heat maps were prepared by using log2-transformed normalized read counts using the heatmap package within the R software (R Foundation for Statistical Computing, version R 4.0.0) in the Catalina build).

**HISTOLOGY AND IMMUNOHISTOCHEMISTRY.** Formalin-fixed, paraffin-embedded hearts from sham and HF mice were sectioned (5 μm), deparaffinized, and rehydrated. Immunohistological staining was performed as previously described. Masson trichrome staining was used to quantify fibrosis while Alexa Fluor 488-conjugated WGA (Invitrogen) and 4',6-diamidino-2-phenylindole staining were used to delineate cardiomyocytes and cell nuclei for measurement of cross-sectional area.

**STATISTICAL ANALYSIS.** Analyses were performed by using GraphPad Prism 8.0 (GraphPad Software). All data are expressed as mean ± SD. Two-group comparisons were made by using an unpaired Student’s t-test for normally distributed variables. For comparisons of >2 groups, data were first assessed for normality by using the Shapiro-Wilk test. For normally distributed data, comparisons among multiple groups were evaluated by using 1- or 2-way analysis of variance, with Tukey’s or Sidak’s post hoc test analysis for multiple comparisons. For non-normal distribution, the data sets were log-transformed, and if normality was satisfied, analysis of variance and Tukey’s post-test were then performed. Changes in cardiac structure and function after imatinib treatment were evaluated by paired Student’s t-test analysis. A P value <0.05 was considered statistically significant.

**RESULTS**

**CHARACTERISTICS OF MOUSE RESIDENT cMCSs.** cMCSs were isolated as cellular outgrowths from enzyme-perfused adult mouse hearts. Primary unsorted cMCSs were negative for c-kit expression but exhibited high expression (>70% of cells) of MSC markers Sc1a, CD90, CD29, CD49, and CD44, while also expressing low levels (~2% of cells) of the fibroblast marker DDR2 and the endothelial cell marker CD31 (Figure 1A). After multiple rounds of depletion of hematopoietic cells (Lin−, CD45−), endothelial cells (CD31+), and fibroblasts (DDR2+), and enrichment for Sc1a expression, cMCSs (Sc1a−Lin+CD45+CD31−DDR2+) were minimally expanded and evaluated for in vitro capacity to differentiate into mesodermal lineages. Isolated cMCSs were able to differentiate into adipogenic, osteogenic, and chondrogenic lineages, as assessed by cell staining and gene expression markers (Supplemental Figure 1).

Next, we assessed cMCSs for the expression of fibroblast gene markers and performed myofibroblast phenotypic functional evaluation by using collagen gel contraction assays. As shown in Figure 1B, baseline expression of collagen-I, collagen-III, fibronectin, and α-SMA was substantially lower in cMCSs than in primary fibroblasts. Primary unsorted cMCSs were negative for c-kit expression but exhibited high expression (>70% of cells) of MSC markers Sc1a, CD90, CD29, CD49, and CD44, while also expressing low levels (~2% of cells) of the fibroblast marker DDR2 and the endothelial cell marker CD31 (Figure 1A). After multiple rounds of depletion of hematopoietic cells (Lin−, CD45−), endothelial cells (CD31+), and fibroblasts (DDR2+), and enrichment for Sc1a expression, cMCSs (Sc1a−Lin+CD45+CD31−DDR2+) were minimally expanded and evaluated for in vitro capacity to differentiate into mesodermal lineages. Isolated cMCSs were able to differentiate into adipogenic, osteogenic, and chondrogenic lineages, as assessed by cell staining and gene expression markers (Supplemental Figure 1).

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compared with mouse cardiac fibroblasts. Mouse cardiac fibroblasts were isolated and cultured from adult wild-type mouse hearts as described by Kishore et al.\textsuperscript{34} Moreover, cMSCs did not induce contraction of collagen gels; cMSC-imbued gels exhibited greater area than gels with the prostate myofibroblast cell line WPMY-1 (ATCC CRL-2854). Collectively, these data establish cMSCs as a distinct MSC population in the heart that do not harbor key features of fibroblasts or myofibroblasts at baseline.

**cMSCs ARE EXPANDED IN CHRONIC HF.** Using flow cytometry, we assessed cMSC abundance in C57BL/6 mouse hearts at early and late time points after a large nonreperfused MI. As previously shown, a progressive phenotype of HF with reduced ejection fraction (EF) is reliably produced in mice after non-reperfused MI,\textsuperscript{2,22,27} which was confirmed by echocardiography. Figure 2 depicts representative flow cytometry gates of cardiac mononuclear cells and corresponding quantitation of Sca\textsuperscript{+}DDR2\textsuperscript{+} fibroblasts, total Sca\textsuperscript{+} cells, and Sca\textsuperscript{+}CD31 DDR2\textsuperscript{+} cMSCs in mouse hearts after acute MI (1-day post-MI) and with chronic HF (8 weeks’ post-MI). Acutely after MI (Figure 2A), there was a significant ~2-fold increase in cardiac fibroblasts compared with sham-operated hearts but comparable levels of total Sca\textsuperscript{+} cells and Sca\textsuperscript{+} cMSCs. In contrast, chronically failing hearts (Figure 2B) exhibited robust expansion of both total Sca\textsuperscript{+} cells (~8-fold increase) and Sca\textsuperscript{+}CD31 DDR2\textsuperscript{+} cMSCs (~5.5-fold increase), but no difference in cardiac fibroblast number, compared with naive hearts.

Failing hearts are characterized by progressive fibrosis accompanied by myofibroblast proliferation. Although cardiac fibroblasts are considered their main cellular precursors,\textsuperscript{35} MSCs can also differentiate into myofibroblasts.\textsuperscript{31,127,36} Hence, we next...
quantiﬁed the abundance of α-SMA-expressing, Sca1+DDR2+ myoﬁbroblasts, which we considered as derived primarily from cMSCs, in the chronically failing heart. As illustrated in Figure 2C, compared with naive hearts, failing hearts exhibited markedly increased numbers of α-SMA+DDR2+Sca1+ myoﬁbroblasts (~20-fold increase), along with comparable robust increases in total α-SMA+ and total α-SMA+DDR2+ cells. As shown Supplemental Figure 2, immunostaining revealed signiﬁcant expansion of Sca1+CD31- DDR2+ cMSCs in failing hearts, predominantly in border zone myocardium and also in the infarct scar, where they were found together with Sca1+CD31- DDR2+ cells. Collectively, these results suggest that expanded cMSCs are an important source of myoﬁbroblasts in the failing heart.

HF-DERIVED cMSCs EVIDENCE A PRO-INFLAMMATORY PHENOTYPE. Resident cMSCs were isolated from sham-operated and failing hearts (8 weeks' post-MI), expanded in culture, and used for experimentation between passages 3 and 5. cMSC secretory proﬁles were deﬁned in conditioned media pooled from 3 cell isolates after 24 hours of culture using antibody-based arrays. HF cMSCs exhibited a pro-inﬂammatory secretome with increased levels of stromal cell-derived factor 1, C-C motif chemokine ligand (CCL) 11 (eotaxin-1), IL-6, and granulocyte-colony stimulating factor (Figure 3A). Furthermore, basal gene expression of chemokines CCL2, CCL6, CCL7, and CCL12 that recruit innate and adaptive immune cells was increased in HF cMSCs compared with sham cMSCs (Figure 3B).

We next determined the capacity of the cMSC secretome to polarize RAW 264.7 (RAW) macrophages in vitro. As shown in Figure 3C, RAW cells exposed to HF cMSC-conditioned media for 24 hours signiﬁcantly upregulated genes associated with both M1 (inducible nitric oxide synthase, tumor necrosis factor, and Toll-like receptors 2 and 4) and M2 (CD206 and arginase) polarities vs exposure to either control or sham cMSC-conditioned media. cMSC-driven macrophage chemotaxis was evaluated by using a Transwell Migration assay. Compared with sham cMSCs, HF cMSCs signiﬁcantly augmented M1-polarized RAW 264.7 macrophage migration.
Findings that were recapitulated by HF cMSC–conditioned media (24 hours) in place of HF cMSC chemotactic stimulus. Quantitative transcriptional profiling was performed on flow-sorted primary CD45 Sca1+ cells from sham-operated and HF hearts (8 weeks’ post-MI) by using RNA-sequencing analysis. As illustrated in Figure 4A, CD45 Sca1+ cells from failing hearts exhibited marked alterations of their transcriptomic profile, with ~400 differentially expressed genes compared with sham CD45 Sca1+ cells. Ingenuity Pathway Analysis of differentially expressed genes supported a profibrotic and pro-inflammatory phenotype of HF cMSCs with greater predicted activation of transforming growth factor-β (TGFβ), IL-6, and nuclear factor-κB signaling pathways (Figure 4B). Collectively, these data suggest that cMSCs undergo a phenotypic switch in the failing heart milieu that promotes tissue fibrosis and inflammation, and facilitates the infiltration and activation of pro-inflammatory macrophages.

MACROPHAGES PROMOTE MYOFIBROBLAST DIFFERENTIATION OF cMSCs IN THE FAILING HEART. The failing heart is characterized by both expanded tissue macrophages and fibrosis. Hence, we evaluated whether macrophages modulate myofibroblast differentiation of cMSCs. TGFβ-induced myofibroblast differentiation was confirmed in naive HF-Derived cMSCs Exhibit a Pro-inflammatory Phenotype

(A) Cell-free conditioned media from untreated sham and HF cMSCs cultured for 24 hours was analyzed for chemokine content using antibody arrays. cMSC media was used as control. Array controls (positive and negative), blanks, and some of the most dramatically up-regulated chemokines are indicated by numbered boxes. (B) Quantitative polymerase chain reaction analysis of baseline gene expression from sham and HF cMSCs for genes involved in monocyte and T-cell recruitment. Data are represented as mean ± SD of 3 to 4 independent experiments done in duplicate or triplicate. Statistical comparisons (unpaired Student’s t-test) in B were performed after logarithmic data transformation to satisfy the normality assumption as described in the Methods. *P < 0.05, **P < 0.005. (C) Quantitative polymerase chain reaction analysis of genes involved in macrophage polarization after 24 hours of co-culture of mouse RAW246.7 macrophage cells and conditioned media collected from untreated sham and HF cMSCs. Results are expressed as mean ± SD of 3 independent experiments done in duplicate or triplicate. *P < 0.05, **P < 0.005. (D) Results from Transwell migration assay showing representative images and the group data of the migrated M1-polarized mouse RAW 246.7macrophages when co-cultured with either sham or HF cMSCs (upper panel) or the conditioned (Cond) media collected from untreated sham and HF cMSCs (lower panel) as chemotactic in the bottom wells of the Transwell plates. cMSC media was used as control. Each image is a stitched composite of 4 separate overlapping images. The yellow dotted circle demarcates the boundary of the Transwell. Four independent areas of observation were counted from each Transwell. Data are expressed mean ± SD of 2 independent experiments with multiple replicates. *P < 0.05 vs sham groups and control cMSC media. Statistical comparisons in C and D were analyzed by 1-way analysis of variance followed by Tukey’s post hoc analysis. CCL = C-C motif chemokine ligand; G-CSF = granulocyte-colony stimulating factor; IL-6 = interleukin-6; iNOS = inducible nitric oxide synthase; SDF-1 = stromal cell-derived factor 1; TNF = tumor necrosis factor; TLR = Toll-like receptor; other abbreviations as in Figures 1 and 2.
cMSCs by α-SMA gene expression, immunostaining, and immunoblotting (Figure 5A). Collagen gel contraction assays revealed comparable TGFβ-induced myofibroblast differentiation (indexed by collagen gel contraction) of both sham and HF cMSCs (Figure 5B); nonetheless, gene expression of fibrotic markers collagen-I, collagen-III, and fibronectin, and the myofibroblast marker α-SMA, was augmented ~3- to 7-fold in TGFβ-differentiated HF cMSCs vs sham cMSCs (Figure 5C).

We next co-cultured naive RAW 264.7 macrophages with either sham or HF cMSCs in collagen gels. As shown in Figure 5D, macrophage co-culture robustly increased the myofibroblastic phenotype of HF cMSCs but not sham cMSCs. Macrophages were subsequently polarized in vitro to an M1 or an M2 phenotype before co-culture with either cMSCs or National Institutes of Health 3T3 fibroblasts (ATCC CRL-1658) in collagen gels. As illustrated in Figure 5E, M2 macrophages (but not M1 macrophages) induced myofibroblast differentiation of 3T3 fibroblasts. In contrast, both M1 and M2 macrophages induced myofibroblast differentiation of HF cMSCs, with a much more marked contraction response to M1 macrophages, whereas neither induced myofibroblasts from sham cMSCs. These results were recapitulated by substitution of macrophage-conditioned media in place of macrophages for the gel contraction studies (data not shown). Together, these results indicate that pro-inflammatory macrophages induce profound, and perhaps inappropriate, differentiation of HF-derived cMSCs into functional myofibroblasts, and suggest that a macrophage-cMSC interaction can drive fibrosis in the failing heart.

**HF cMSCs INCREASE PDGFRβ EXPRESSION.** PDGFR activation enhances fibroblast proliferation and collagen synthesis. We measured baseline expression of the 2 PDGFR isoforms in cMSCs. As seen in Figure 6A, sham cMSCs exhibited predominant PDGFRα expression (~4-fold higher than HF cMSCs), whereas HF cMSCs exhibited predominant PDGFRβ expression (~3-fold higher than sham cMSCs). The switch in PDGFR expression from alpha to beta in HF cMSCs was additionally validated by immunoblotting.
analysis of PDGFR protein. HF cMSCs also exhibited increased baseline mRNA levels of the PDGFRβ ligands PDGF-B, and PDGF-D (Figure 6B). Notably, exposure to conditioned medium from M1-polarized RAW 264.7 macrophages increased HF cMSC expression of PDGF-A and PDGF-B, and exposure to either M1 or M2 macrophage-conditioned medium increased expression of PDGF-D, compared with sham cMSCs (Figure 6C).

The role of PDGFR in macrophage-facilitated differentiation of HF cMSCs to myofibroblasts was probed in collagen gel contraction assays by using the PDGF inhibitor imatinib mesylate (10 μM). Co-culture of HF cMSCs with conditioned media from M1-polarized macrophages (but not M2-polarized macrophages) induced collagen gel contraction (Figure 6D). Imatinib treatment profoundly attenuated this response. Parallel experiments showed that a 4-day exposure to imatinib also significantly decreased α-SMA gene and protein expression in HF cMSCs. Identical collagen contraction gel assays were observed upon knockdown of PDGFRβ expression in HF cMSCs using PDGFRβ siRNA (30 nM) compared with control siRNA transfection, confirming specificity of the response. Notably, PDGFRβ siRNA-transfected HF cMSCs exhibited >95% loss of PDGFRβ protein expression over a period of 4 days. Collectively, these results support a key role for PDGF-PDGFRβ signaling in the fibrotic response linked to cMSCs and their interactions with macrophages.

**Imatinib Treatment in Vivo Attenuates Post-MI LV Remodeling and Fibrosis.** In view of the aforementioned results with isolated cMSCs, we...
FIGURE 6  HF cMSCs Exhibit Increased Expression of PDGFRβ That Promotes Their Myofibroblast Differentiation

A

cMSC Baseline PDGFR Expression

B

Baseline cMSC PDGFRβ Ligand Expression

C

cMSC Polarized Macrophage Co-Culture and PDGFR Ligand Expression

D

Conditioned Media

HF cMSCs

+ Imatinib

Control siRNA  PDGFRβ siRNA

Vinculin

PDGFRβ (Demisometric Units)

Control siRNA  PDGFRβ siRNA

Transfected HF-cMSCs

Transfected HF-cMSCs

Contraction (% of Undiff)

Undiff

M1

M2

Control + Imatinib

α-SMA

GAPDH

Control

Cont + Imatinib

Contract siRNA PDGFRβ siRNA

Contract siRNA PDGFRβ siRNA

Contract siRNA PDGFRβ siRNA

Contract siRNA PDGFRβ siRNA

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evaluated the in vivo effects of PDGFR inhibition on cardiac remodeling in the post-MI failing heart. To avoid suppression of essential early inflammation and wound healing, and to focus the evaluation on chronically remodeled hearts after nonreperfused MI, we administered either imatinib (30 mg/kg) or PBS vehicle intraperitoneally daily to mice from 2 to 5 weeks’ post-MI, and to sham-operated control mice (Figure 7A). Echocardiography was performed at 2 weeks’ post-MI to ensure comparable degrees of post-MI LV remodeling and dysfunction before treatment randomization.

As shown in Figures 7A and 7B, HF mice in both treatment groups had similar LV end-diastolic (EDV) and end-systolic volume (ESV) and EF before starting treatment. PBS-treated HF mice exhibited progressive LV dilatation (greater EDV and ESV) and systolic dysfunction (lower EF) over the treatment period. In contrast, imatinib-treated HF mice exhibited no further deterioration of LV size or function, indicating abrogation of LV remodeling. Analysis of changes in EDV, ESV, and EF revealed significantly smaller ∆EDV and ∆ESV, and a trend toward smaller ∆EF, in imatinib-treated HF mice vs PBS-treated HF mice (Figure 7C). In contrast, in a parallel group of similarly treated sham-operated mice, neither imatinib nor vehicle affected LV size or systolic function over the 3-week treatment period (Supplemental Figure 3).

Gravimetric analysis (Figure 8A) revealed smaller normalized heart weight in imatinib-treated HF mice compared with PBS-treated HF mice, with no differences in normalized spleen or wet lung weights. Histologic assessment (Figure 8B, Supplemental Figure 4) revealed that imatinib-treated failing hearts exhibited significantly smaller cardiomyocyte area (~30% decrease), less border zone fibrosis (~60% decrease), and higher capillary density compared with PBS-treated hearts. There were no differences in remote zone fibrosis (data not shown). Flow cytometric evaluation revealed comparable levels of cardiac CD45+ Sca1+ cMSCs, as well as total CD45+ leukocytes, total MerTK+ MHCIId71+Ly6C+F4/80+ macrophages, and CCR2+ resident macrophages between the treatment groups (Figure 9). However, compared with PBS-treated HF mice, imatinib-treated HF mice exhibited significantly fewer CCR2+ infiltrating macrophages in the heart. No differences in the abundance of either CCR2+ or CCR2+ macrophages were observed between sham-operated hearts treated with imatinib or PBS (Supplemental Figure 5). However, as expected, we observed ~9-fold higher (P = 0.047) total cardiac macrophages, ~152-fold higher (P = 0.029) CCR2+ macrophages, and ~7.6-fold higher (P = 0.054) CCR2+ macrophages in PBS-treated HF mice vs PBS-treated sham mice. Collectively, these data suggest that imatinib-mediated PDGFR inhibition alleviated the progression of chronic post-MI cardiac remodeling, inflammation, and fibrosis.

**DISCUSSION**

The current study uncovered a novel pro-fibrotic axis in HF mediated by alterations in the phenotype and function of resident cMSCs. There are several key findings. First, a distinct resident Sca1+CD31 DDR2+ cMSC population significantly expands and develops a pro-inflammatory and pro-fibrotic profile in the failing heart, potentially serving as an additional source of myofibroblasts. Second, mutual interactions between cMSCs and macrophages...
promote pro-fibrotic and pro-inflammatory effects, such that HF cMSCs polarize and attract activated macrophages, whereas M1 macrophages induce myofibroblast differentiation of HF cMSCs. Third, PDGF-PDGFR signaling contributes importantly to the pro-fibrotic and pro-inflammatory responses of cMSCs in HF, likely related in large part to PDGFRβ activation, as this PDGF isoform, and its corresponding ligands, were differentially up-regulated in cMSCs from failing hearts. Fourth, receptor tyrosine kinase inhibition with imatinib inhibited macrophage-induced cMSC myofibroblast differentiation and improved LV fibrosis, function, remodeling, and monocyte-derived macrophage infiltration in vivo during post-MI HF, underscoring a potential approach to clinical translation. Hence, cMSCs in the failing heart channel a maladaptive myofibroblastic and pro-inflammatory fate, and this phenotypic switch is sustained, at least in part, by tissue macrophages and PDGFR signaling. Conceptually, our findings suggest that restoring or maintaining a normal cMSC phenotype may represent a novel approach for combating pathologic fibrosis in the failing heart. Given that the PDGFR inhibitor imatinib is clinically approved for human use,38,39 our studies provide rationale for testing this drug as a primary therapeutic in HF.

Multiple studies have characterized stem cells from mouse hearts based on surface markers. We isolated cMSCs based on selective depletion of Lin− hematopoietic cells, CD31+ endothelial cells, and DDR2+ fibroblasts, and enrichment of Sca1-expressing cells. Cells derived with this approach robustly expressed MSC markers such as CD90 but lacked fibroblast and myofibroblast markers, and did not exhibit surface c-Kit expression. Importantly, however, upon appropriate stimulation, cMSCs were

![FIGURE 7 Imatinib Treatment Post-MI Attenuates LV Remodeling and Preserves Systolic Function](image-url)

(A) Top: Schematic of the experiment protocol. Following 2 weeks of permanent coronary artery ligation (or sham surgery), wild-type C57BL/6 mice were randomized to receive daily injections of either 200 µL phosphate-buffered saline (PBS) vehicle or imatinib (30 mg/kg) for 3 weeks before being reassessed by echocardiography and subsequent euthanasia. Bottom: Representative long-axis B-mode serial echocardiographic images at end-diastole from HF mice at 2 and 5 weeks’ post-MI after treatment with either PBS or imatinib as described in A. The dotted line marks the left ventricular (LV) cavity. (B) Corresponding paired echocardiographic group data from HF mice at 2 and 5 weeks’ post-MI after treatment with either PBS or imatinib as described in A. n = 11 to 14 per group. (C) Echocardiographic group data depicting changes (Δ) in end-diastolic volume (EDV) and end-systolic volume (ESV) and ejection fraction (EF) from 2 to 5 weeks’ post-MI with treatment with either PBS or imatinib as described in A. n = 11 to 14 per group. Data were analyzed by using either paired (B) or unpaired (C) Student’s t-test. *P < 0.05, **P < 0.005. Abbreviations as in Figures 1 to 4.
capable of differentiation in vitro to broader mesenchymal (adipogenic, osteogenic, and chondrogenic) lineages as well as to myofibroblasts. Hence, Sca1\(^+\) cMSCs represent a resident cMSC population distinct from MSC-like cardiosphere-derived cells that grow as self-assembling clusters in suspension cultures, and exhibit low c-Kit and varying CD90 expression, both of which negatively correlate with therapeutic benefits induced by cardiosphere-derived cells.\(^{40}\)

cMSCs are significantly expanded in chronically remodeled failing hearts post-MI (eg, ischemic cardiomyopathy) and exhibit a pathologic pro-fibrotic and pro-inflammatory profile at transcriptional, translational, and functional levels. Indeed, whereas (Sca1\(^+\)) DDR2\(^+\) fibroblasts\(^{41}\) expanded acutely after MI, consistent with their indispensable role in post-MI wound healing and scar formation,\(^{42}\) these cells, surprisingly, were not increased in the chronically failing heart. In contrast, Sca1\(^-\)CD31\(^-\)DDR2\(^-\) cMSCs were not increased acutely after MI but were robustly expanded \(~6\)-fold in chronic HF and accompanied by a nearly 20-fold increase in \(\alpha\)-SMA\(^-\)DDR2\(^-\)Sca1\(^+\) myofibroblasts. Moreover, the transcriptomic profile of primary sorted cMSCs in HF indicated heightened pro-fibrotic TGF-\(\beta\) pathway activation. These results suggest that phenotypically altered cMSCs are important contributors to pathologic fibrosis in the chronically failing heart. However, given the overlap of surface markers between MSCs, fibroblasts, and pericytes, more extensive transcriptomic and proteomic studies of cMSCs will be needed to definitively establish their biological properties.

Our observations regarding cMSCs are consistent with prior studies establishing that perivascular\(^{12}\) and epicardial\(^{43}\) MSC-like cells in the heart are important cellular sources of myofibroblasts and organ fibrosis in nonischemic and ischemic models of cardiac injury, respectively. Although fibroblasts are considered the main effector cells of cardiac fibrosis, the role of chronic fibroblast activation in the pathogenesis of ischemic cardiomyopathy is unknown.\(^{44}\) Our data suggest differential roles for cardiac fibroblasts and...
Cardiac MSCs, Fibrosis, and HF

Hamid et al

JACC: BASIC TO TRANSLATIONAL SCIENCE VOL. 7, NO. 5, 2022
MAY 2022:465-483

FIGURE 9 Imatinib Treatment Attenuates Post-MI Myocardial Inflammation

(A) Representative flow cytometry scatter plots and corresponding group quantification of cMSCs (CD45<sup>–</sup>Sca1<sup>+</sup>) in HF hearts after treatment with either PBS or imatinib (30 mg/kg per day) for 3 weeks after 2 weeks of MI. (B) Representative flow cytometry gating strategy and quantitation of total leukocytes (CD45<sup>+</sup>), total macrophages (CD45<sup>+</sup>MerTK<sup>–</sup>MHCII<sup>–</sup>Ly6C/F4/80<sup>+</sup>), infiltrating macrophages (CD45<sup>+</sup>MerTK<sup>–</sup>MHCII<sup>–</sup>Ly6C/F4/80<sup>–</sup>CCR2<sup>+</sup>), and resident macrophages (CD45<sup>+</sup>MerTK<sup>+</sup>MHCII<sup>+</sup>Ly6C/F4/80<sup>–</sup>CCR2<sup>–</sup>) from experimental groups as in panel A. n = 4 to 7 per group. Data in A and B were analyzed by using unpaired Student’s t-test (statistical comparisons in B were performed after logarithmic data transformation to satisfy the normality assumption as described in the Methods). Data are presented as cells per heart. *P < 0.05. FSC-A = forward scatter-area; FSC-W = forward scatter-width; MHC-II = major histocompatibility complex-II; SSC-A = side scatter-area; other abbreviations as in Figures 1 to 4 and 7.

cMSCs as effectors of fibrosis during post-MI cardiac remodeling, with cardiac fibroblasts as drivers of wound healing and scar formation in the acute and subacute stages, and cMSCs as mediators of pathologic fibrosis during chronic remodeling and failure. Tissue fibrosis in chronic diseases such as HF is closely interlinked with inflammation and immune cell activation.1,13 MSCs are well known to be broadly immunoregulatory, with the capacity to either suppress or promote immune responses in response to the microenvironment.35,46 A key finding of our study is that in addition to pro-fibrotic features, cMSCs from the failing heart are distinctly pro-inflammatory and harbor a reciprocal relationship with M1-type macrophages that may further amplify fibrotic and inflammatory tissue responses. At the transcript level, HF cMSCs displayed activation of IL-6- and nuclear factor-κB-linked inflammatory pathways, and up-regulation of multiple chemokines, including CCL2, CCL7, and CCL12, the ligands for CCR2, a key marker of monocyte-derived tissue-infiltrating macrophages in the heart.47 Moreover, the HF cMSC secretome was sufficient to induce both polarization of naive macrophages and chemotaxis of M1-type macrophages. In turn, macrophages, especially M1 macrophages, selectively induced myofibroblast differentiation of HF cMSCs (but not sham cMSCs). Given that HF is characterized by pro-inflammatory cardiac macrophage expansion,1 this suggests a feed-forward mechanism promoting tissue fibrosis involving a macrophage–cMSC interaction in the failing heart. We propose that cMSCs in failing hearts lose immunomodulatory properties, which then serves to trigger and sustain chronic inflammation and fibrosis.

The PDGF signaling axis is an important stimulator of fibrosis in various organ pathologies, including those of the heart,15-17,48 and hence we explored its potential role in altering the cMSC phenotype in HF. PDGF signals via 2 transmembrane tyrosine kinases receptors, PDGFRα and PDGFRβ.49 Whereas PDGFRα has been proposed as an MSC marker,50 the role of PDGFRβ in MSCs is poorly defined. We found that failing heart cMSCs switch from predominant PDGFRα to PDGFRβ expression, and up-regulate their corresponding ligands PDGF-B and PDGF-D. Moreover,
PDGFRβ ligand expression further augments upon exposure to macrophages, especially M1 macrophages. Thus, with augmented cMSC-macrophage interactions occurring in the failing heart, cMSCs would constantly be exposed to a PDGF-rich microenvironment that sustains chronic PDGFRβ activation and subsequent downstream pro-fibrotic responses. Analogous inflammation-driven PDGF expression switching has also been described in hepatic satellite cells in chronic liver fibrosis and airway fibroblasts in asthma.51,52

Imatinib is a potent inhibitor of the receptor tyrosine kinases Bcr-Abl (in human chronic myelogenous leukemia), PDGFα and PDGFβ, and c-kit.53 We used imatinib to probe the functional role of PDGFRβ activation in the pro-fibrotic phenotype of cMSCs and cardiac remodeling in HF. In vivo administration of imatinib to mice with established cardiac remodeling and HF abrogated progression of LV dysfunction and improved remodeling at both the chamber and tissue level, including salutary effects on border zone fibrosis, capillary density, and CCR2+ monocyte-derived macrophage infiltration. In vitro, imatinib markedly attenuated macrophage-induced myofibroblast differentiation of HF cMSCs, which may partly underlie its beneficial in vivo impact on myocardial fibrosis and function. As noted earlier, imatinib was given systemically and is not a specific inhibitor of PDGFRβ. Therefore, the observed beneficial effects of imatinib may not be solely caused by effects on cMSCs, and this must be acknowledged as a study limitation. Nonetheless, although imatinib is not a selective PDGFR inhibitor, these results are wholly consistent with a meaningful and important effect on cMSC-localized inactivation of PDGF-β signaling. PDGFRs are primarily localized to interstitial and vascular cells in the heart,54 with age-dependent cardiomyocyte PDGFRβ inactivation starting by 2 weeks following birth.55 Our FACS analysis further indicated that Sca1+ cMSCs rather than DDR2+ fibroblasts are the primary mesenchymal cell type expanded and sourcing myofibroblasts in the failing heart, and that these cMSCs exhibit PDGF-β signaling predominance. Finally, we observed no c-Kit expression in cardiac mesenchymal cells. Hence, our results strongly suggest that altered cMSC PDGF signaling plays a key role in sustaining the pro-fibrotic and inflammatory responses observed in the failing heart, and that inhibiting this axis may represent a potential therapeutic approach for reducing cardiac fibrosis and inflammation in HF.

Our findings with imatinib in chronic LV remodeling and failure extend prior studies showing imatinib-mediated improvements in cardiac fibrosis in murine models of reperfused MI, β-adrenergic activation, and myocarditis.19,21 Notably, however, the salutary effects of imatinib early after reperfused MI seem related to PDGFα inactivation, as specific antibody-mediated PDGFRβ inhibition led to impaired scar and vasculature maturation, and prolonged inflammation.21 In humans, imatinib has been suggested to be cardiotoxic.56 However, several subsequent clinical studies of imatinib-treated patients have reported a low incidence of cardiac dysfunction comparable to the expected population incidence.57,58 Importantly, the imatinib dose we used was substantially lower than the dose used for leukemia treatment in humans, as determined by a dose-conversion formula,26 suggesting that low doses which avoid imatinib toxicity in humans may be considered in HF. Obviously, this concept would need to be tested and validated in clinical studies.

Our results also have implications for cMSC-based cell therapy to affect cardiac repair. MSCs have been considered an ideal stem cell type for regenerative therapy in view of their immunomodulatory properties.59 To date, however, autologous transplantation of MSCs (and other stem cell types) in HF has had very limited clinical success60,61 whereas animal models have reported low cell survival, myocardial engraftment, and persistence.62-64 Our results suggest that an important factor limiting cMSC therapy may be a cardiac microenvironment that pathologically channels cMSCs away from immunomodulation and toward fibrosis and inflammation, thus severely limiting benefit. Although not directly tested, our results further support the idea that PDGFR inhibition at the time of cMSC delivery and the peri-transplantation period may be a fruitful adjunctive approach to improve therapeutic efficacy.

**CONCLUSIONS**

We have shown that a cardiac resident Sca1+CD31-DR2- MSC population, which does not exhibit fibroblast or myofibroblast characteristics at baseline, significantly expands and contributes to myofibroblast abundance in the failing heart and exhibits a pro-inflammatory and pro-fibrotic profile. Mutual cell-to-cell interactions between cMSCs and
macrophages, especially pro-inflammatory macrophages, promote and amplify the pro-fibrotic and pro-inflammatory responses in HF. Activated PDGF-PDGFR signaling, and a pathologic isoform switch to PDGF-β expression in cMSCs, plays a key role in the altered cMSC phenotype. Pharmacologic PDGFR inhibition robustly improves macrophage-induced cMSC myofibroblast differentiation, and LV fibrosis, function, inflammation, and remodeling in established HF post-MI HF, highlighting a potential approach for restoring normal cMSC phenotype as a primary therapeutic in HF.

ACKNOWLEDGMENT The authors thank Dr Suresh Verma at the University of Alabama at Birmingham (UAB) for providing adult mouse cardiac fibroblast RNA samples.

FUNDING SUPPORT AND AUTHOR DISCLOSURES

This work was supported by National Institutes of Health grants R01HL137046 (Dr Hamid) and R01HL125735 (Dr Prabhu), a VA Merit Award 101BXX002706 (Dr Prabhu), and a pilot grant from the UAB Comprehensive Cardiovascular Center (Dr Hamid). The authors have reported that they have no relationships relevant to the contents of this paper to disclose.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: HF is characterized by chronic inflammation and pathologic fibrosis that promote disease progression. Although activated fibroblasts are classically considered primary mediators of fibrosis, to date strategies aimed at modulating fibroblast activation in HF have yet to translate to clinical practice. Our studies establish that resident cMSCs expand and exhibit pro-inflammatory and pro-fibrotic characteristics in HF, suggesting that along with fibroblasts, cMSCs contribute meaningfully to cardiac fibrosis. Furthermore, interaction between cMSCs and pro-inflammatory macrophages, which are also expanded in failing hearts, augments their pro-fibrotic fate and thereby may contribute to pathologic remodeling and fibrosis in HF. Attenuation of cMSC expansion or modulation of the myocardial pro-inflammatory microenvironment in HF may therefore be feasible approaches to prevent progressive remodeling and failure.

TRANSLATIONAL OUTLOOK: For clinical translation, these observations in mice should be confirmed in large animal models. These studies uncover a novel pro-fibrotic axis in HF related to enhanced PDGFRβ activation in cMSCs. PDGFRβ activation limits the immunomodulatory properties of cMSCs and channels a myofibroblast differentiation fate that promotes fibrotic and inflammatory responses. Restoring or maintaining a normal cMSC phenotype in HF may thus represent a promising therapeutic approach for reducing fibrosis and inflammation. Indirectly, our studies also suggest that circumscribed pharmacologic PDFR inhibition at the time of exogenous cMSC cell transplantation could be an effective strategy to improve the therapeutic efficacy of cMSC-mediated cardiac repair.
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**KEY WORDS** cardiac remodeling, fibrosis, heart failure, mesenchymal stem cells, myocardial inflammation, myofibroblasts, platelet-derived growth factor receptor

**APPENDIX** For supplemental figures, please see the online version of this paper.