

ORIGINAL RESEARCH



Leptin Receptor Fibroblasts Are Preferential Contributors to Cardiac Fibrosis

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BACKGROUND: Cardiac fibrosis, a hallmark of heart failure and an unmet clinical need, arises from pathological activation of preexisting cardiac fibroblasts (CFs), but the contribution of CF heterogeneity to this process remains unclear.

METHODS: Murine models were used to lineage trace or deplete a specific sub-population of CFs at baseline and after myocardial infarction. Transcriptional and epigenetic differences between fibroblast subsets were assessed using next-generation sequencing. Conservation in humans was evaluated through single-cell RNA-seq data sets and histological examination.

RESULTS: In mice, fibroblasts were the sole cardiac cell type expressing the signaling-capable isoform of the LepR (leptin receptor). LepR+ CFs emerged neonatally, occupied a defined niche in the coronary adventitia, exhibited enhanced hedgehog signaling, and responded to leptin. After myocardial infarction, LepR-Cre+ CFs proliferated more than interstitial CFs, became a predominant fibroblast lineage in the scar, and their genetic ablation reduced fibrosis while improving function. LepR+ CFs were also detected in the human heart, where they were embedded in an adipocyte-rich niche.

CONCLUSIONS: These findings identify adventitial fibroblasts as key drivers of pathological remodeling and demonstrate that fibroblasts, rather than cardiomyocytes, are the principal responders to leptin in the heart, redefining how this major endocrine pathway influences cardiac remodeling and disease.

GRAPHIC ABSTRACT: A [graphic abstract](#) is available for this article.

Key Words: adipocytes ■ adventitia ■ fibroblasts ■ fibrosis ■ leptin ■ myocardial infarction

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Despite advances in clinical care, heart failure remains a leading cause of mortality worldwide.¹ Cardiac fibrosis, resulting from excessive deposition of extracellular matrix by activated cardiac fibroblasts (CFs), is a hallmark of most types of heart failure and an unmet clinical need.^{2,3} Several studies have shown that most activated CFs arise from preexisting quiescent CFs^{4–7}; however, it remains unclear whether all preexisting CFs contribute equally to fibrotic processes. In the context of hypertension, fibrosis often develops in the vicinity of large vessels (perivascular fibrosis).⁴ Recent efforts to target fibrosis with CAR T cells have shown efficient

elimination of interstitial fibroblasts, while sparing fibroblasts located in the adventitia of large coronary vessels.⁸ These observations suggest that CFs from the adventitia of large coronary vessels might possess distinct molecular features that enable them to play a pivotal role in pathological remodeling. Yet, this hypothesis remains unproven, largely due to the lack of tools that allow selective labeling and tracing of specific CF subsets.

Obesity is widely recognized as a major risk factor for heart failure.⁹ Beyond serving as an energy reservoir, adipose tissue is a key endocrine organ, releasing a broad range of adipokines with systemic effects.^{10,11} Among

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Novelty and Significance

What Is Known?

- Cardiac fibrosis results from pathological activation of preexisting fibroblasts.
- The heart contains distinct fibroblast subsets, but their functional relevance is poorly understood.
- Leptin has cardiac effects, but its target cell type(s) in the heart remain unclear.

What New Information Does This Article Contribute?

- In the mouse heart, leptin receptor expression is restricted to fibroblasts.
- LepR+ (leptin receptor) fibroblasts reside in the coronary adventitia and are conserved in the human heart.
- LepR+ fibroblasts preferentially drive fibrotic remodeling, and their ablation reduces fibrosis and improves cardiac function postinjury.

The heart contains anatomically and molecularly distinct fibroblast subsets, yet the functional relevance of cardiac fibroblast heterogeneity remains poorly

defined. Leptin is an adipocyte-derived hormone that exerts important cardiac effects, largely interpreted from a cardiomyocyte-centered perspective. Here, we identify fibroblasts as the sole cardiac cell type expressing the signaling-competent LepR isoform in the mouse heart. We further define a spatially restricted LepR+ fibroblast subset residing in the coronary adventitia that is conserved in human hearts, underscoring its translational relevance. Functionally, LepR+ fibroblasts preferentially contribute to fibrotic remodeling after cardiac injury, and their genetic ablation reduces fibrosis and improves cardiac function. Together, these findings establish adventitial fibroblasts as key mediators of pathological remodeling, demonstrate that fibroblast heterogeneity has functional consequences in the injured heart, and support the concept that selective targeting of pathogenic fibroblast subsets may represent a strategy to limit excessive fibrosis and preserve cardiac function.

Nonstandard Abbreviations and Acronyms

CF	cardiac fibroblast
DEG	differentially expressed gene
DT	diphtheria toxin
DTR	diphtheria toxin receptor
LepR	leptin receptor
LepR-Cre+ _CFs	cardiac fibroblasts labeled by LepR-Cre
STAT	signal transducer and activator of transcription
TGF-β	transforming growth factor-β
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

these is leptin, a 16-KDa hormone secreted by adipocytes and primarily recognized by its capacity to act on hypothalamic LepR (leptin receptor)-expressing neurons, controlling food intake and energy expenditure.¹¹ Alternative *Lepr* splicing generates at least 6 distinct isoforms,¹² but only the long isoform (*Leprb*) is capable of intracellular signaling, primarily via JAK-STAT (Janus kinase - signal transducer and activator of transcription).^{11,12} In the cardiovascular system, leptin has been implicated in promoting cardiac hypertrophy and regulating vascular tone and inflammation.¹⁰ Early studies showed that leptin can induce hypertrophy of cultured rat¹³ or human¹⁴ ventricular cardiomyocytes. Subsequent cardiomyocyte-specific

LepR ablation studies in mice suggested that leptin is essential for cardiomyocyte energy homeostasis.^{15,16} The role of leptin in noncardiomyocyte populations has been less explored, but leptin has been linked to cardiac fibrosis.¹⁷ Studies using cultured CFs suggested this is mainly explained by direct regulation of genes encoding extracellular matrix molecules and matrix-degrading proteases.¹⁷ However, whether these in vitro findings accurately reflect in vivo processes, and whether all CF subsets possess the molecular machinery necessary to sense leptin, remains largely unknown.

We found that, contrary to previous reports, LepR expression in the murine heart is restricted to fibroblasts and identified a previously unrecognized lineage of LepR-Cre+ CFs that occupy a well-defined niche in the adventitia of large coronary arteries. LepR expression in this lineage emerged in the neonatal period, coinciding with the onset of white adipogenesis and a peak in circulating leptin. Using both constitutive and inducible LepR-Cre models, we showed that following myocardial infarction (MI), LepR-Cre+ adventitial CFs proliferated more than interstitial CFs and became the dominant CF population within the scar. Ablation of LepR-Cre+ adventitial CFs before MI reduced fibrosis and improved cardiac function, and LepR+ adventitial CFs were also detected in human hearts. Together, these findings challenge current models of leptin signaling in the heart and define a novel lineage of fibroblasts that act as preferential progenitors during post-MI remodeling, highlighting them as a potential therapeutic target for limiting pathological fibrosis.

METHODS

Data Availability

Sequencing data sets were deposited in Zenodo (17305938) and GEO (GSE253551). Postmapping count matrices used for differential gene expression analysis and uncropped western blot images are provided in the [Supplemental Material](#). All other data supporting the findings of this study are available from the corresponding author on reasonable request.

Animal Models

Procedures involving mouse models conformed to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes or the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Experiments were authorized by the Regierungspräsidium Darmstadt, Hessen, Germany (FU/2041) or the University of California, San Diego Institutional Animal Care and Use Committee. The following alleles were used: *LepR-Cre*,¹⁸ *Col1a1-GFP*,¹⁹ *Tie2-Cre*,²⁰ *Gli1-CreERT2*,²¹ *Rosa-tdTomato*,²² *Rosa-iDTR*,²³ *LepR-CreERT2*,²⁴ and *Leptin-LacZ* (Knockout Mouse Project). Male and female animals were used in all experiments for characterization of the *LepR-Cre* lineage in uninjured hearts. Male animals were used for experiments involving MI models. Detailed methods are described in the [Supplemental Material](#).

Human Subjects

Analyses involving human samples followed the principles outlined in the Declaration of Helsinki. Portions of the left ventricular free wall containing large segments of the LAD were dissected postmortem and immediately placed in 4% formaldehyde for overnight fixation, followed by routine protocols for paraffin histology. Detailed protocols and subject information are provided in the [Supplemental Material](#).

Isolation of Murine CFs

CFs were isolated from adult and neonatal murine hearts using a modified version of a previously described dissociation protocol.⁴ A full description of the procedure is provided in the [Supplemental Material](#).

RNA-Seq

FACS-sorted CFs were collected in Qiazol (Qiagen 79306), and mRNA was purified using the miRNeasy Micro Kit (Qiagen 217084) with on-column DNase digestion. Quality control was done using TapeStation (Agilent). Samples with RNA integrity number between 6.7 and 9.4 were used for library preparation using the TruSeq Stranded mRNA library Prep kit (Illumina 20020594) and sequenced (single read 50 protocol) on an Illumina HiSeq 4000 System.

Assay for Transposase-Accessible Chromatin Using Sequencing

Per replicate, 75000 CFs were lysed in ice-cold lysis buffer (10 mmol/L TrisHCl, pH 7.4, 10 mmol/L MgCl₂, 0.1% Igepal CA-630) and stored at -80°C in storing buffer (10 mmol/L Hepes pH 7.5, 2 mmol/L MgCl₂, 25 mmol/L KCl, 250 mmol/L

sucrose, 1 mmol/L dithiothreitol, 1 mmol/L PMSF, 70% glycerol) until further processing. Libraries were prepared using the ActiveMotif assay for transposase-accessible chromatin using sequencing kit (53150).

Bioinformatic Analyses

Next-generation sequencing data sets were analyzed in R (4.2.1) using standard pipelines. Unless otherwise stated, significance was defined as an absolute $\log_2\text{FC}$ ($|\log_2\text{FC}|$) ≥ 0.5 and an adjusted $P \leq 0.05$. Detailed procedures are available in the [Supplemental Material](#).

Quantitative and Statistical Analyses

Statistical analyses were performed in GraphPad Prism. Parametric tests were applied only when the number of replicates was ≥ 10 , and data satisfied the assumption of normality (Shapiro–Wilk test). For analyses performed using the Kruskal–Wallis test, posthoc multiple-testing correction was performed with the Dunn test.

RESULTS

A Novel Lineage of Adventitial *LepR-Cre*+ CFs

Leptin, adiponectin, and resistin are adipocyte-specific cytokines. To assess whether any of these can directly signal to the heart, we evaluated expression of their cognate receptors in a cell-type-resolved cardiac RNA-seq data set.²⁵ Receptors for all 3 adipokines were detectable in the heart. Whereas receptors for adiponectin and resistin were ubiquitous, the receptor for leptin (*Lepr*) was selectively expressed by CFs (Figure 1A). To validate this highly specific expression pattern, we performed lineage tracing studies employing a *LepR-Cre* that uses an internal ribosome entry site sequence to drive Cre expression under the control of the long, signaling-capable, isoform of *LepR* without disrupting the endogenous transcript.¹⁸ By combining this allele with 2 reporter strains (*Rosa26-tdTomato*²² and *Col1a1-GFP*¹⁹), we generated triple transgenic mice in which all cells with a history of *LepR* expression were permanently marked by tdTomato, while all fibroblasts were labeled by GFP (green fluorescent protein). Flow cytometry confirmed that expression of the long isoform of *LepR* was confined to CFs, with more than 99% of tdTomato+ cells coexpressing GFP (Figure 1B and 1C). Histological analyses revealed that CFs labeled by *LepR-Cre* (*LepR-Cre*+ CFs) were not uniformly distributed throughout the heart, but instead accumulated in the adventitia of large coronary vessels, which extended from the ventricular base to near apical regions (Figure 1D). Confocal microscopy (63 $\times$ magnification) revealed that, in images containing large coronary vessels, *LepR-Cre*+ CFs constituted 64% of all CFs and accounted for 100% of CFs located within the immediate adventitial niche. By contrast, only 8% of interstitial CFs were *LepR-Cre*+ (Figure 1E). Immunostaining with

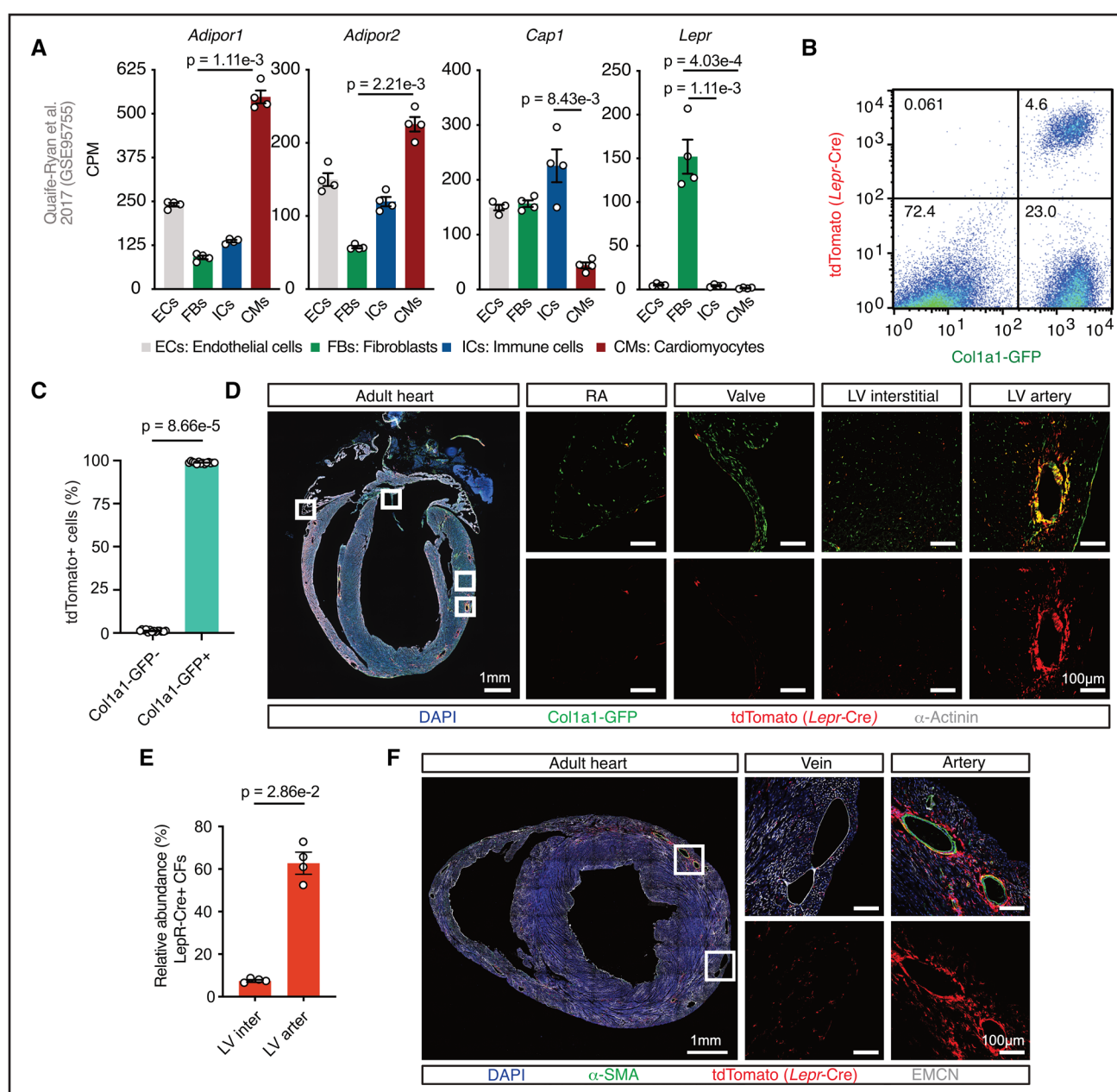


Figure 1. Identification of a lineage of cardiac fibroblasts labeled by *LepR-Cre* (*LepR-Cre*+ CF) occupying the adventitia of large coronary arteries.

A, Expression of adipokine receptors in RNA-seq of isolated cardiac cell types. Whereas most receptors were broadly expressed, *Lepr* was selectively expressed in fibroblasts (N=4, mean $\pm$ SEM, Kruskal-Wallis test with Dunn correction). **B** and **C**, Representative flow cytometry (**B**) and corresponding quantification (**C**) of cells from *LepR-Cre*/WT; *Rosa26-tdTomato*/WT; *Col1a1-GFP*+ hearts showing that the vast majority of cells with a history of expression of the long *LepR* isoform (*tdTomato*+) were fibroblasts (GFP [green fluorescent protein]+; N=14, mean $\pm$ SEM, Mann-Whitney *U* test). **D**, Histological analysis and (**E**) corresponding quantification (N=4, mean $\pm$ SEM, Mann-Whitney *U* test) revealing that *LepR-Cre*+ CFs (GFP+, *tdTomato*+, yellow/orange) accumulated in the adventitia of large coronary vessels. Boxed areas are shown at higher magnification. **E**, Immunofluorescence for Endomucin (gray, marker of endocardium and venous endothelium) and α SMA (alpha smooth muscle actin; green, marking smooth muscle cells in coronary arteries) showing *LepR-Cre*+ CFs localized specifically around large arteries.

specific markers revealed that *LepR-Cre*+ CFs localized preferentially around large arteries, identified by the presence of α SMA (alpha smooth muscle actin)+ smooth muscle cells, rather than around veins, marked by Endomucin (Figure 1F). Sex-stratified analyses showed no differences between males and females in

the frequency or distribution of *LepR-Cre*+ fibroblasts in adult hearts (Figure S1A).

Together, these findings define a previously unrecognized lineage of *LepR*-expressing CFs residing in the adventitia of coronary arteries and establish *LepR-Cre* as a tool to specifically label this CF subset.

The LepR-Cre⁺ Lineage Arises and Expands in Neonatal Stages

We next investigated the developmental timing of LepR-Cre⁺_CF emergence. E16.5 hearts were devoid of any LepR-Cre⁺ cells, but by postnatal day 1 (P1), a discontinuous layer of lineage-traced CFs appeared around developing arteries (Figure 2A). This lineage expanded rapidly after birth, and by P7 coronary arteries were fully encircled by LepR-Cre⁺_CFs, as seen in adulthood (Figure 2A). Unlike the adult heart, which showed sparse labeling of interstitial CFs (Figure 1C and 1D), neonatal hearts contained no lineage-traced cells in interstitial regions. Flow cytometry confirmed that the LepR-Cre⁺_CF lineage expanded during the neonatal period, increasing from 2% of all ventricular fibroblasts at P1 to 14% by weaning (Figure 2B). As in adulthood, in neonatal stages, there were no sex-specific differences in the frequency of LepR-Cre⁺_CFs (Figure S1B). Proliferation rates of LepR-Cre⁺ and LepR-Cre[−] CFs were comparable at P1 (25%), P7 (8%), and P19 (3%; Figure 2C), suggesting that lineage expansion was driven by de novo *Lepr* expression rather than differential proliferation. RNA-scope analyses supported this, revealing minimal *Lepr* expression at E16.5, but robust expression of both the long (*Leprb*) and short (*Lepra*) LepR isoforms in adventitial but not interstitial CFs or any other cardiac cell type at P1 and P7 (Figure 2D through 2G). In the ventricular free walls, most CFs have an epicardial origin, but in the septum, a subset of CFs derives, during embryogenesis, from endocardial progenitors.⁴ Analyses in *Tie2-Cre* lineage traced hearts revealed that, in regions containing endocardial-derived CFs, the pattern of *Lepr* expression was the same as the one observed in the ventricular free walls: CFs occupying the adventitia of the septal coronary artery expressed *Lepr*, whereas interstitial CFs did not (Figure S1C through S1I). Together, these findings demonstrate that LepR-Cre⁺_CFs are not a distinct embryonic lineage, but instead arise during early neonatal life through de novo activation of *Lepr* in adventitial CFs of both epicardial- and endocardial-origin.

LepR⁺_CFs Express Genes Associated With Progenitor Potential

The neonatal activation of *Lepr* expression in adventitial CFs coincided with the onset of leptin production. At birth, mammals predominantly contain multilocular adipocytes with little to no leptin expression, as shown using a *Leptin-LacZ* reporter (Figure S2). During the first postnatal week, subcutaneous adipose depots transition to a unilocular white phenotype and markedly increase leptin expression, leading to a surge in circulating leptin.²⁶ An ELISA time course (Figure 3A) confirmed that, in mice, circulating leptin was undetectable at birth, but rose sharply during the first neonatal week,

peaking between P7 and P9 at values >4-fold higher than in lean adults (10 ng/mL). This temporal overlap between leptin production in fat and LepR expression in adventitial CFs suggested a previously unrecognized interorgan signaling axis between adipocytes and this CF subset.

To explore molecular features distinguishing adventitial from interstitial CFs during the neonatal leptin peak, we performed RNA-seq on LepR-Cre⁺ and LepR-Cre[−] CFs FACS-sorted from P7 hearts (Figure 3B). A total of 288 differentially expressed genes (DEGs) were identified, with the strongest differences corresponding to genes expressed at higher levels in LepR-Cre⁺ than in LepR-Cre[−] fibroblasts, including *Lepr* itself (Figure 3C; Table S1). Reactome analysis of genes that had higher expression in LepR-Cre⁺_CFs (Figure 3D) revealed enrichment of transducers of hedgehog signaling, a pathway involved in the maintenance of tissue-resident stem cells and previously shown to be active in the neonatal coronary adventitial niche.^{27,28} The most upregulated gene in LepR-Cre⁺_CFs was *Hhip* (6.27 logFC), a canonical hedgehog target and marker of pathway activation.²⁹ Also elevated were multiple forkhead transcription factors (*Foxd1*, *Foxl1*, *Foxd2*, *Foxc2*, *Foxs1*) known to work downstream of hedgehog signaling.^{30–32} Additional top upregulated genes included *Gdf10* (a member of the BMP [bone morphogenetic protein]/TGFβ [transforming growth factor-β] superfamily³³) and *Esm1* (Endocan), an endothelial tip cell marker³⁴ recently identified as a marker of bone marrow LepR⁺ mesenchymal stem cells.²⁴ Finally, LepR⁺_CFs displayed higher expression of *Il33* and *Cd200*, 2 key immunomodulatory molecules. Selected DEGs were validated via immunostaining and RNAscope (Figure S3A through S3C).

Collectively, these findings demonstrate that transcriptional differences between interstitial and adventitial CFs extend well beyond *Lepr*, with expression of stemness-associated genes being a distinguishing feature of adventitial fibroblasts.

LepR-Cre⁺_CFs Have a Unique Epigenetic Landscape

To identify regulatory regions underlying gene expression differences between adventitial and interstitial CFs, we performed assay for transposase-accessible chromatin using sequencing (ATAC-seq) on LepR-Cre⁺ and LepR-Cre[−] CFs FACS-sorted from P7 hearts. From over 21 000 peaks of accessible chromatin (the majority farther than 50kb from the nearest TSS), 1084 were more accessible in LepR-Cre⁺_CFs (log₂FC ≥ 0.5, FDR ≤ 0.05; Figure 3E and 3F; Table S2). GREAT³⁵ analysis linked these differential peaks to 1433 putative target genes (Table S2). Cross-referencing with RNA-seq results revealed that nearly half of the genes

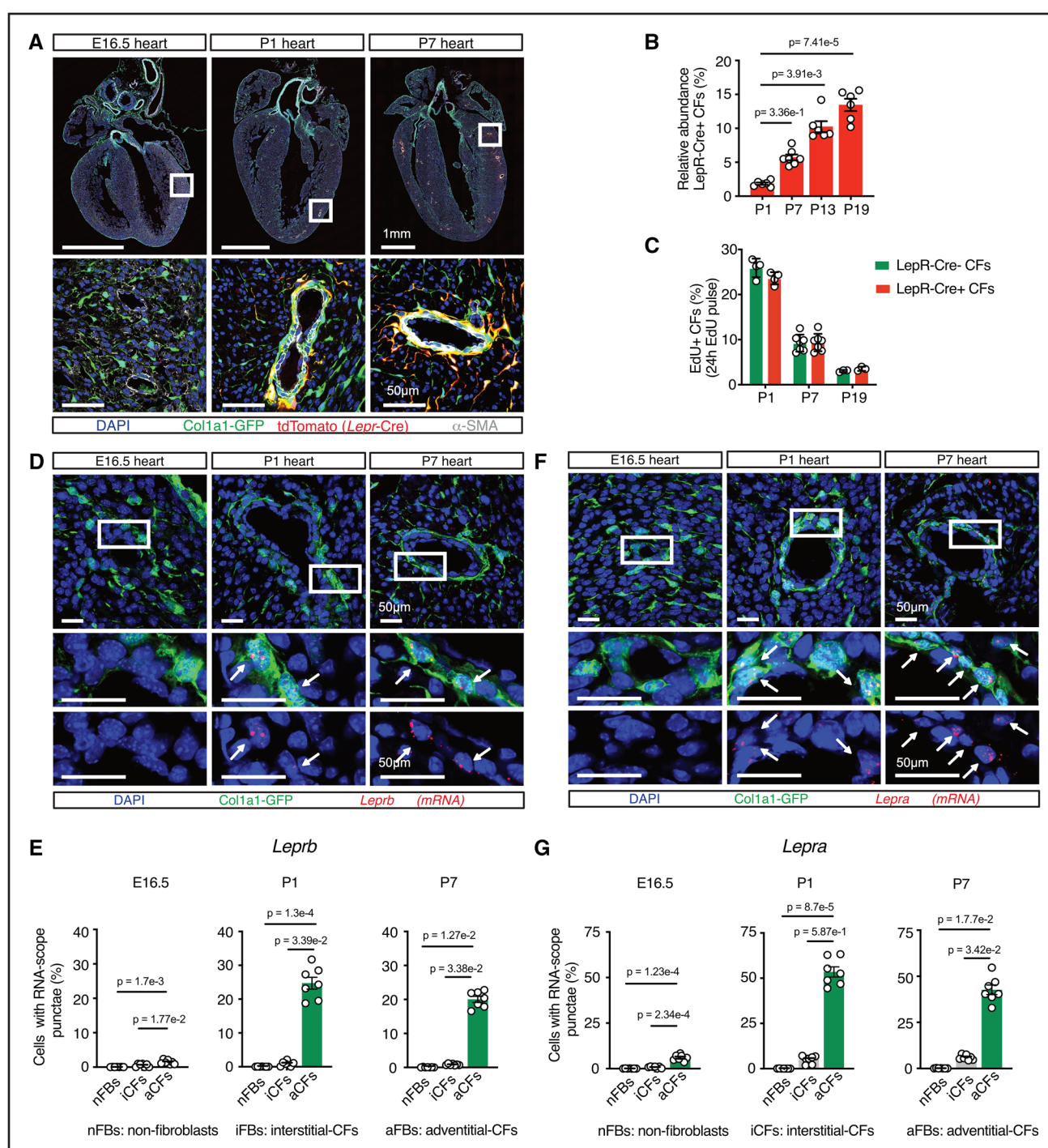


Figure 2. The LepR (leptin receptor)-Cre+ lineage of cardiac fibroblasts emerges and expands rapidly in the neonatal period.

A, Immunofluorescence time-course showing absence of LepR-Cre+ cells in embryonic hearts (E16.5), appearance as a discontinuous monolayer around developing arteries at P1, and complete encirclement of coronary arteries with projections into adjacent tissue by P7 (N=6). **B**, Flow cytometry quantification showing progressive increase in the relative abundance of LepR-Cre+ fibroblasts (proportion of LepR-Cre+ CFs among all cardiac fibroblasts) from birth to weaning (N=6 P1; 7 P7; 6 P13; 6 P19, mean $\pm$ SEM, Kruskal-Wallis test with Dunn correction). **C**, EdU incorporation analysis revealing no significant difference in proliferative rates between LepR-Cre+ and LepR-Cre- neonatal fibroblasts (N=4 P1; 7 P7; 3 P19, mean $\pm$ SEM, individual Mann-Whitney *U* test for each of the 3 stages). **D** through **G**, RNA-scope analyses (**D** and **F**) and corresponding quantification (**E** and **G**) revealing expression of *Leprb* and *Lepra* (red dots) exclusively in adventitial fibroblasts (arrows; N=7, mean $\pm$ SEM, Kruskal-Wallis test with Dunn correction). Boxed areas shown at higher magnification.

showing increased expression in LepR-Cre+ CFs were associated with at least 1 differential ATAC-seq peak (Figure 3G; Table S3), including key hedgehog pathway

genes (*Ptch1*, *Ptch2*, *Boc*, *Gli1*; Figure 3H). We also identified a 1160 bp region located 32 kb upstream of the *Lepr* locus that may account for its selective expression



in adventitial fibroblasts (Figure 3I). Although most DEGs were associated with a single differential regulatory region (Figure S4A), several of the genes showing the strongest differential expression were associated with multiple differential elements—*Foxl1* (4.0 log₂FC, 9 regions), *Foxd1* (5.4 log₂FC, 5 regions), and *Gdf10* (4.5 log₂FC, 4 regions; Figure S4B; Table S3). Motif analysis of the 1084 peaks enriched in LepR-Cre+ CFs identified a top de novo motif resembling a forkhead binding domain (Figure 3J). Restricting the analysis to peaks that had both greater accessibility in LepR-Cre+ CFs and were associated with genes expressed at higher levels in these cells revealed a top motif that contained a perfect match to the sequence recognized by STAT transcription factors, known nuclear mediators of leptin-LepR signaling.^{11,12}

Together, these findings highlight specific regulatory elements that distinguish adventitial from interstitial CFs and suggest a model in which forkhead transcription factors promote the opening of specific regulatory regions and subsequent binding of STAT factors (elicited by leptin/LepR or other transduction pathways) promotes upregulation of target genes.

Leptin Signaling in LepR-Cre+ CFs

In addition to expressing *Lepr* transcripts, adventitial CFs also exhibited robust LepR protein levels (Figure S5), suggesting they might be responsive to leptin. To test this, we FACS-sorted both LepR-Cre+ CFs and LepR-Cre− CFs and stimulated them with leptin in vitro (Figure 4A). Western blot and immunocytochemistry revealed leptin-induced phosphorylation and nuclear accumulation of STAT3 exclusively in LepR-Cre+ CFs, but not in their interstitial LepR-Cre− counterparts, confirming that adventitial CFs are leptin-responsive (Figure 4B through 4D; Figure S6). We next examined whether leptin influences the transcriptome of adventitial CFs by performing RNA-seq on LepR-Cre+ CFs FACS-sorted from wildtype animals or leptin KO/KO littermates at P7 (Figure 4E). The leptin null background resulted in modulation of 284 genes in LepR-Cre+ CFs (107 down, 177 up; Figure 4F; Table S4). Downregulated transcripts were enriched in genes related to TGFβ signaling, a major profibrotic pathway (Figure 4F). Consistently, *Thbs1* and *Ccn2* (encoding CTGF [connective tissue growth factor]) were among the most downregulated transcripts. Other downregulated processes included response to shear stress (transcription factors *Klf2* and *Klf4*) and negative regulation of signaling by smoothed. Conversely, genes upregulated in LepR-Cre+ CFs from leptin null mutants were associated with ATP synthesis and myogenic development. Importantly, expression of hedgehog pathway components and forkhead transcription factors—key features distinguishing LepR-Cre+ CFs from their interstitial counterparts—remained unchanged in

Leptin mutants, indicating that leptin is not required for maintenance of these lineage-defining transcriptional programs. Collectively, our data show that in neonatal adventitial CFs, leptin restrains myoblastic transcriptional programs and augments TGF-β target-gene expression as well as transcription factors linked to shear-stress sensing.

LepR-Cre+ CFs Are Preferential Contributors to Fibrosis

LepR+ mesenchymal cells have been extensively studied in bone marrow, where they act as tissue-resident progenitors that expand after injury to generate most newly formed bone.^{36,37} Cardiac LepR-Cre+ CFs share features with these progenitors, including rapid neonatal expansion and expression of common markers (*Lepr*, *Esm1*),²⁴ leading us to hypothesize that they might also function as progenitors during cardiac remodeling.

To test this, we subjected our triple transgenic animals to a surgical model of MI. Macroscopic analyses 28-day post-surgery revealed that, in contrast to the perivascular tdTomato pattern observed in sham hearts (arrows), in MI hearts, lineage-traced cells massively colonized the fibrotic scar (Figure 5A). A histological time-course revealed that LepR-Cre+ CFs persisted in the infarcted area early post-MI (indicating that the ischemic shock does not trigger their death) and expanded rapidly after injury (Figure 5B). Quantification revealed that their relative abundance (LepR-Cre+ CFs as a fraction of all fibroblasts) doubled in the border zone within 2 days post-MI (Figure 5C) and tripled in the scar 1-week post-MI (Figure 5D). This increase in the relative abundance of LepR-Cre+ CFs suggested that, similarly to what happens in bone marrow,³⁶ in the heart, LepR-Cre+ cells also expand preferentially to make a major contribution to tissue remodeling after injury. Supporting this, EdU incorporation assays demonstrated higher proliferation rates in LepR-Cre+ CFs compared with their LepR-Cre− counterparts at 2-, 4-, and 7-days post-MI (Figure 5E; Figure S7).

The higher mitotic rates of LepR-Cre+ CFs explain the increase in their relative abundance postinjury, yet, because the *LepR-Cre* is constitutively active, lineage expansion could also reflect de novo *Lepr* activation postinjury. However, this possibility was refuted by analyses of published RNA-seq data sets^{25,38} and RNAscope demonstrating that *Lepr* expression declines after MI, with transcripts undetectable in scar fibroblasts (Figure S8). These findings indicate that postinjury expansion of the LepR-Cre+ lineage reflects proliferation of preexisting LepR-Cre+ CFs rather than new *Lepr* activation. To directly address this, we performed time-controlled lineage tracing using a tamoxifen-inducible *LepR-CreERT2*.²⁴ Neonatal tamoxifen induction of triple transgenic animals (*LepR-CreERT2+*; *Col1a1-GFP+*;

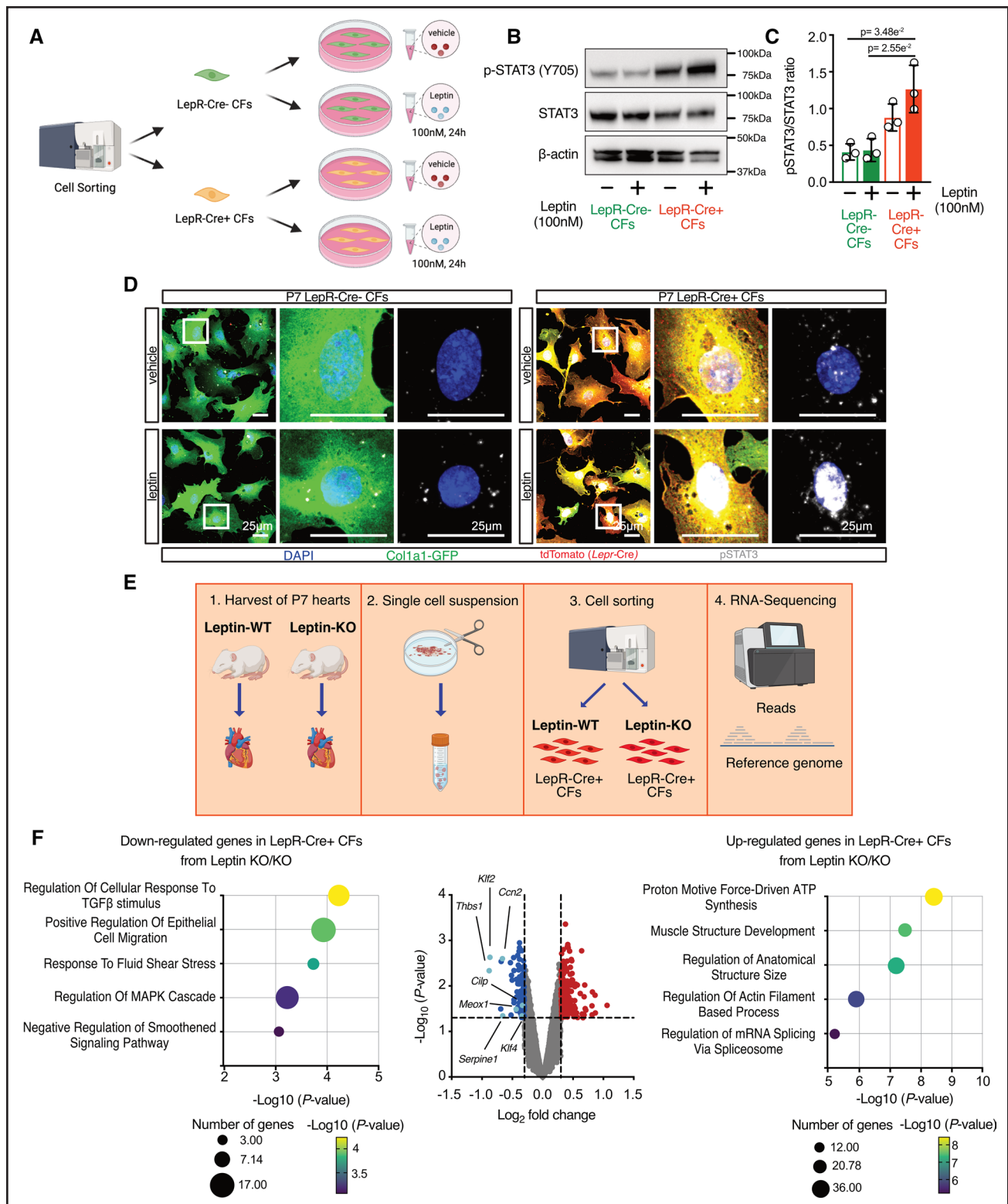


Figure 4. Cardiac fibroblasts labeled by LepR-Cre (LepR-Cre+ CFs) sense and respond to leptin.

A, Experimental design for ex vivo stimulation of CF subsets with leptin. **B** and **C**, Western blot and respective quantification showing increased STAT3 phosphorylation in LepR-Cre+ CFs after leptin stimulation (N=3, mean±SEM, Kruskal-Wallis test with Dunn correction). **D**, Immunofluorescence showing nuclear accumulation of pSTAT3 (gray) specifically in LepR-Cre+ CFs after leptin stimulation (N=3). **E**, Experimental design to assess transcriptional alterations in LepR-Cre+ CFs isolated from leptin-deficient mice. **F**, Volcano plot and functional annotation analyses of differentially expressed genes in LepR-Cre+ CFs from leptin mutants vs wild-type littermates (N=4, |log2FC| ≥ 0.3).

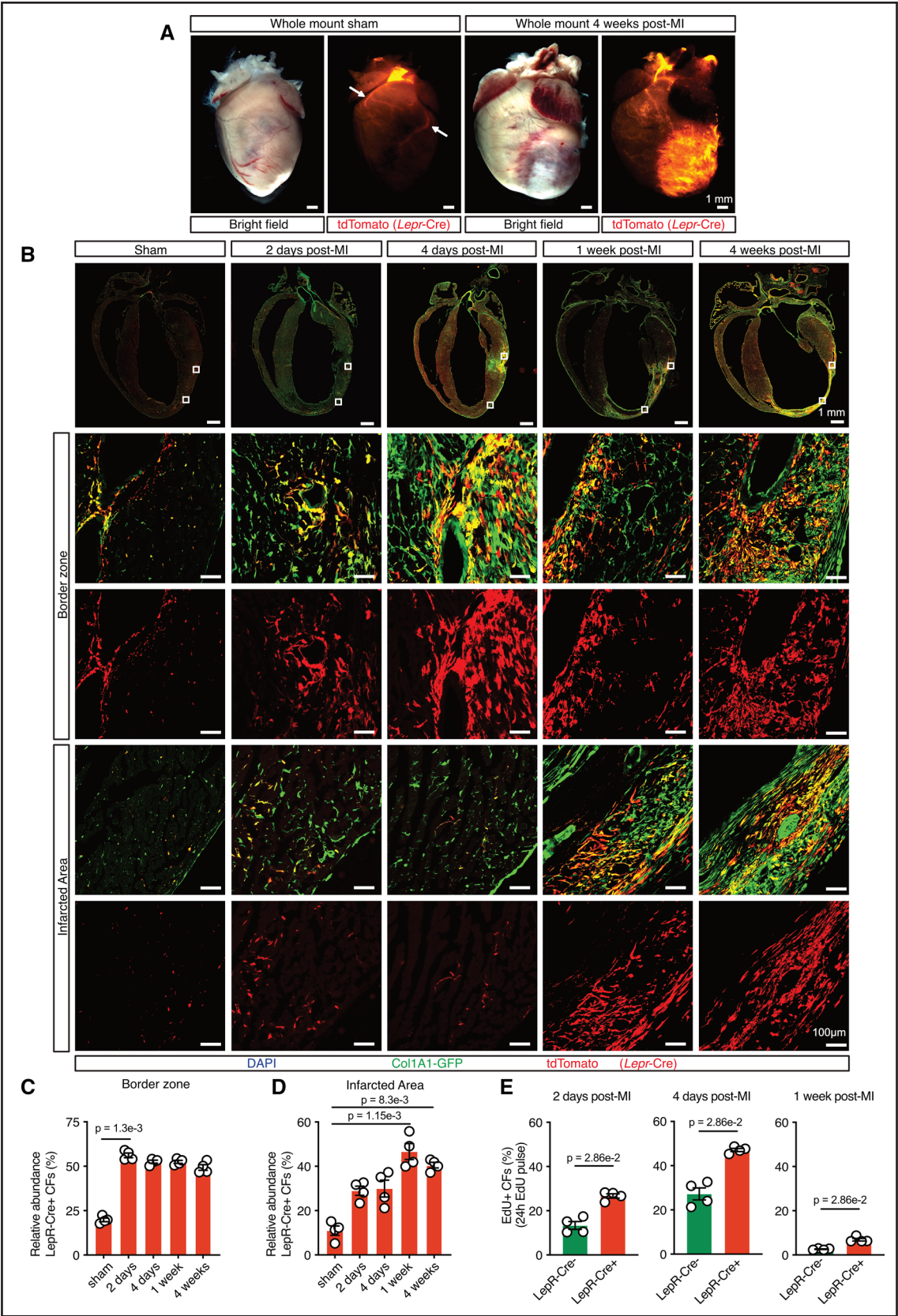


Figure 5. Adventitial cardiac fibroblasts labeled by LepR-Cre (LepR-Cre+ CFs) are preferential contributors to fibrotic remodeling after myocardial infarction (MI).
A, Whole mount views of lineage-traced hearts showing the distribution of LepR-Cre+ CFs (tdTomato+). In sham hearts, labeled cells were confined to the adventitia of large coronary arteries (arrows), but post-MI, they colonized the entire scar. **B**, Histological analysis of (Continued)

Rosa-tdTomato/WT) efficiently labeled adventitial CFs (Figure 6A and 6B; Figure S9A), whereas adult induction produced weak labeling (Figure S9B), consistent with lower adult *LepR* expression observed via RNAscope (Figure 2 versus Figure S8B and S8C). *LepR-CreERT2* also labeled a small subset of cardiomyocytes (particularly evident in the atria), likely due to its lack of isoform specificity, unlike the constitutive *LepR-Cre* (Figure S9A). When neonatal-induced animals were subjected to MI 8 weeks later (Figure 6A), no new labeling could occur (Figure S9C), yet the relative abundance of *LepR-CreERT2*+ CFs (*LepR-CreERT2*+ CFs as a fraction of all fibroblasts) still increased postinjury, both in the border zone and scar, to a similar extent as observed with the constitutive *LepR-Cre* (Figure 6C through 6E). These results confirm that preexisting adventitial *LepR-Cre*+ CFs are preferential contributors to fibrotic remodeling following MI.

Finally, to test whether this preferential contribution extends beyond MI, we subjected *LepR-Cre* lineage-traced mice to trans-aortic constriction, a model of cardiac hypertrophy and fibrosis without cardiomyocyte death.⁴ As in MI, adventitial *LepR-Cre*+ CFs were also preferential contributors to fibrotic remodeling posttrans-aortic constriction (Figure S10).

Collectively, these findings establish that the preexisting lineage of *LepR-Cre*+ adventitial fibroblasts preferentially expands after cardiac injury and acts as a major progenitor pool driving fibrotic remodeling across distinct pathological contexts.

LepR-Cre+ CFs Versus Other Fibroblast Progenitors

Previous studies have described additional fibroblast progenitor lineages in the heart, including *Pdgfra*+/*Ly6a*+ cells,³⁹ *Peg3*+ cells,⁴⁰ *Hic1*+ cells,⁴¹ and *Gli1*+ cells.⁴² Neither *Ly6a*, *Peg3*, nor *Hic1* were differently expressed between adventitial *LepR-Cre*+ CFs and their interstitial counterparts, indicating that the *LepR-Cre*+ lineage is distinct from these populations. However, the strong enrichment of Hedgehog pathway components in *LepR-Cre*+ CFs raised the possibility that they might overlap with *Gli1-CreERT2*+ cells. To test this, we compared *LepR-Cre* and *Gli1-CreERT2* labeling profiles across multiple organs and observed strikingly different patterns (Figures S11 and S12). In the heart, *Gli1-CreERT2*, previously reported to label perivascular cells,⁴² primarily marked fibroblasts. Unlike *LepR-Cre*, which was largely

confined to the adventitia of large arteries, *Gli1-CreERT2* labeled fibroblasts throughout the heart, including adventitial and interstitial fibroblasts in the ventricles, as well as fibroblasts in the atria and valves (Figure S11). These results demonstrate that the 2 lineages are distinct and suggest *LepR-Cre*+ CFs represent a well-defined subpopulation within the broader pool of CFs labeled by *Gli1-CreERT2*.

Improved Post-MI Outcomes on Depletion of LepR-Cre+ CFs

Our lineage tracing experiments revealed that *LepR-Cre*+ CFs preferentially expand during fibrotic remodeling of the heart. To test the functional relevance of this population, we used *LepR-Cre; Rosa26-iDTR/WT* mice, in which the constitutive *LepR-Cre* drives DTR (DT [diphtheria toxin] receptor) expression specifically in *LepR-Cre*+ CFs, enabling their inducible ablation.²³ Because no other cardiac cell type exhibits *LepR-Cre* activity (Figure 1), cardiac DTR expression, and consequently sensitivity to DT, is restricted to this fibroblast population. DT was administered before MI or sham surgeries (Figure 6F), and echocardiographic analyses revealed no baseline differences between groups (Figure 6G; Figure S13). The day after MI, both genotype groups exhibited similar average reductions in ejection fraction (Figure 6H), revealing comparable injury size. However, by 1-week post-MI, animals lacking *LepR-Cre*+ CFs displayed significantly better ejection fraction than controls without cell ablation (38.99% versus 29.22%; Figure 6I). Histological analyses further demonstrated that ablation of *LepR-Cre*+ CFs reduced scar size, as reflected by a decreased percentage of fibrotic area (21.8% of left ventricle area in animals with *LepR-Cre*+ CF depletion versus 32.2% in controls; Figure 6J and 6K).

These results demonstrate that *LepR-Cre*+ CFs play a key role in adverse remodeling after MI, and their depletion confers both structural and functional benefits.

Transcriptional and Epigenetic Features Distinguishing Adult LepR-Cre+ CFs

To investigate transcriptional features underlying the preferential contribution of *LepR-Cre*+ CFs to the post-MI scar, we performed RNA-seq on *LepR-Cre*+ and *LepR-Cre*− CFs FACS-sorted from baseline adult hearts (P56; Figure 7A). Gene expression differences

Figure 5 Continued. *LepR-Cre*+ CFs in distinct areas of the left ventricle before and after infarction. Boxed areas are shown at higher magnification. **C** and **D**, Quantification of histological data presented in **(B)**, showing significant increases in the relative abundance of *LepR-Cre*+ CFs (proportion of *LepR-Cre*+ CFs among all fibroblasts) post-MI in the border zone (**C**) and scar (**D**; N=4, mean±SEM, Kruskal-Wallis test with Dunn correction). **E**, Quantitative analyses of EdU incorporation (histology in Figure S7) revealed higher proliferation rates in *LepR-Cre*+ CFs compared with *LepR-Cre*− counterparts at all time points post-MI (N=4, mean±SEM, Mann-Whitney U test).

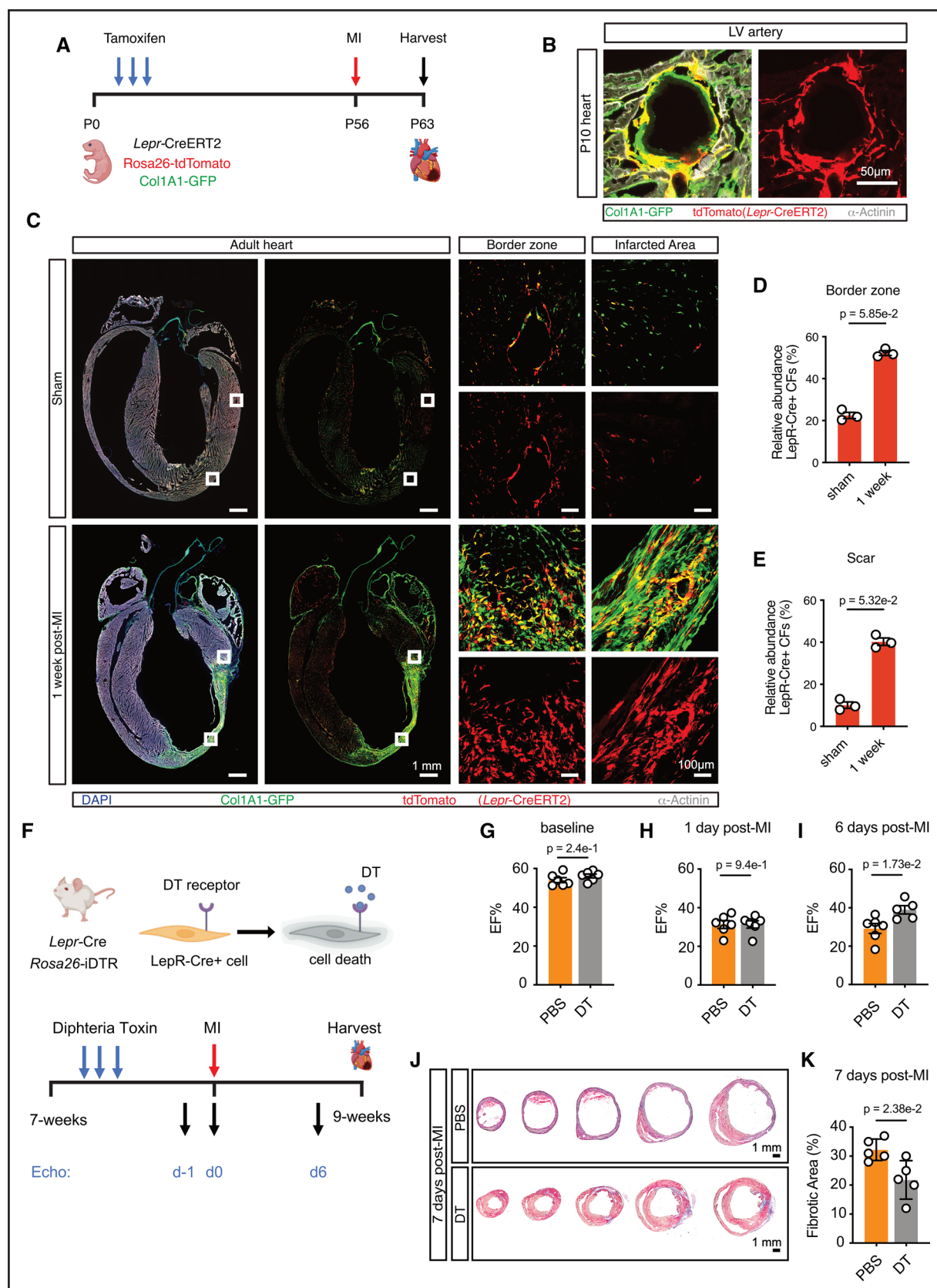


Figure 6. *LepR-CreERT2*⁺ fibroblasts expand postmyocardial infarction (MI), and depletion of *LepR-Cre*⁺ fibroblasts improves outcomes post-MI.

A, Experimental timeline for time-controlled lineage tracing. *LepR-CreERT2* was induced neonatally (P1–P3) and MI was induced 8 weeks later. **B**, Immunofluorescence at P10 showing robust labeling of adventitial CFs by *LepR-CreERT2* (N=3). **C**, Histological analysis of (Continued)

between adult CF subsets were less pronounced than at P7 (106 DEGs at P56 versus 288 DEGs at P7, Figures 7B and 3C; Table S5). Functional annotation of DEGs suggested that adventitial LepR-Cre+ CFs retained a more naïve transcriptome, consistent with a progenitor-like state (Figure 7C and 7D). Interstitial CFs (LepR-Cre−) expressed higher levels of WNT inhibitors, including *Wif1* and *Dkk3*, 2 genes known to mark CFs in an intermediate activation state but absent from CF progenitors⁴³ (Figure 7B and 7C). Interstitial CFs also showed elevated expression of scleraxis (*Scx*), a transcription factor driving CF activation,⁴⁴ as well as *Comp* and *Acta2*, two TGF-β1-inducible genes linked to fibroblast activation.⁴⁵ These patterns support that interstitial CFs are more activated than adventitial LepR-Cre+ CFs. Conversely, adventitial LepR-Cre+ CFs retained higher expression of molecules involved in hedgehog signal transduction, as previously observed at P7 (Figure 7B and 7D). However, transcriptional targets indicative of active hedgehog signaling (*Hhip* and most of the forkhead transcription factors) were no longer differentially expressed in adulthood, revealing reduced adventitial hedgehog activity with age, consistent with previous reports.²⁸ Nevertheless, adult adventitial LepR-Cre+ CFs retained all the machinery necessary to sense hedgehog ligands, positioning them in a privileged position to respond to increased hedgehog ligand availability in the heart post-MI.⁴⁶ Intersection analyses (Figure 7E and 7F) identified a core set of genes consistently enriched in LepR-Cre+ CFs at both P7 and P56 including *Lepr*, hedgehog-sensing components, *Esm1*, *Gdf10* and *I133*. To complement transcriptomic data, we performed ATAC-seq on FACS-sorted adult CFs (Table S6). Joint analyses with neonatal data sets revealed large changes in chromatin accessibility between P7 and adulthood, with almost 9000 differential peaks between these 2 stages in LepR-Cre+ CFs (Figure 7G; Table S6). As with transcriptomics analyses, chromatin differences between adventitial (LepR-Cre+) and interstitial CFs (LepR-Cre−) were less pronounced in adulthood. Multiple peaks that were more accessible in LepR-Cre+ CFs at P7 trended toward higher accessibility in adulthood, but did not reach statistical thresholds for significance (Figure S14).

Together, transcriptomic and epigenetic analyses reveal that adult adventitial LepR-Cre+ CFs were in a less activated state than interstitial CFs, maintaining

progenitor-like features that may underlie their preferential contribution to cardiac remodeling.

The Heart as a Potential Source of Leptin

In the regeneration-competent adult zebrafish heart, one of the 2 leptin paralogs is the most upregulated gene after injury.⁴⁷ In humans, MI triggers a peak in circulating leptin,⁴⁸ and previous reports suggested that cardiomyocytes in mice⁴⁹ and rat^{50,51} may upregulate leptin expression after injury. These observations raised the possibility that, following MI, LepR-Cre+ CFs could sense leptin produced by neighboring cardiomyocytes.

To directly test for cardiac leptin production, we subjected *Leptin-LacZ/WT* mice to sham or MI surgery. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining revealed no leptin expression in any cardiac cell type at baseline (Figure 8A). The only detectable signal was in brown/beige adipocytes surrounding the aortic and pulmonary arteries, which was markedly weaker than in visceral white adipocytes (Figure 8A). Importantly, this pattern remained unchanged after MI, with no evidence of leptin expression in cardiomyocytes or other cardiac cells at either 1 or 4 weeks postinjury (Figure 8A). Analysis of published RNA-seq data sets on purified cardiac cell types^{25,38} confirmed these findings. These results revealed that the peak of circulating leptin post-MI is not caused by production of this hormone in the heart, revealing differences between murine models and the regeneration-competent zebrafish heart.

Unlike the largely fat-free mouse heart, the human heart contains abundant adipocytes surrounding large coronary arteries (Figure 8E). Thus, if adventitial LepR+ CFs also exist in humans, they would be directly embedded in an adipokine-rich environment. To investigate this, we analyzed published single-cell RNA sequencing data sets of the human heart. In data sets from interstitial myocardium (areas devoid of large coronary arteries), *LEPR* was absent from CFs, but, depending on the source laboratory, was detectable in endocardial cells^{52–54} (Figure S15). In contrast, reanalysis of a data set specifically probing coronary arteries⁵⁵ allowed us to identify *LEPR* as a defining feature of adventitial fibroblasts, mirroring our murine findings (Figure 8B through 8D). To further validate this observation, we performed RNAscope analyses of human heart sections containing large segments of the LAD and confirmed abundant expression of the

Figure 6 Continued. LepR-CreERT2+ CFs in different regions of the left ventricle preinfarction and postinfarction. Boxed areas are shown at higher magnification. **D** and **E**, Quantification of data in **(C)**, showing a significant increase in the relative abundance of LepR-CreERT2+ CFs (LepR-CreERT2+ CFs as a fraction of all fibroblasts) post-MI, both in the border zone (**D**) and scar (**E**; N=3, mean±SEM, Mann-Whitney *U* test). **F**, Schematic of the strategy for LepR-Cre+ CF ablation. Adult *Lepr-Cre; Rosa26^{DTR}* mice were injected with PBS or diphtheria toxin (DT) for 3 consecutive days and subjected to MI 5 days later. **G** through **I**, Echocardiographic assessment of left ventricular function showing improved ejection fraction (EF) 6 days after MI in DT-treated animals (N=5–6, mean±SEM, Mann-Whitney *U* test). **J** and **K**, Representative Masson trichrome-stained sections of infarcted hearts and corresponding quantification showing reduced fibrotic area after LepR-Cre+ CF depletion (**K**, N=5, mean±SEM, Mann-Whitney *U* test).

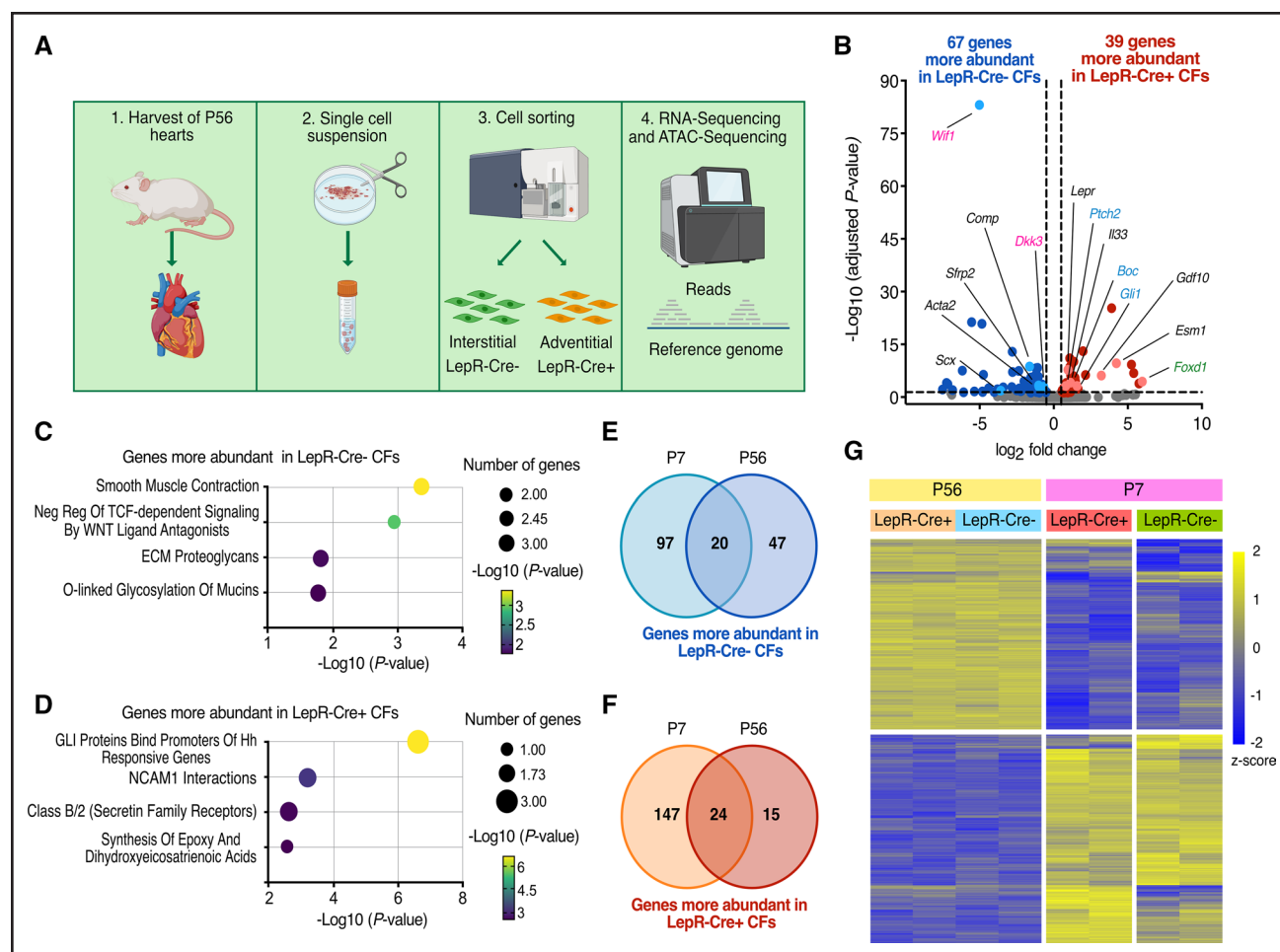


Figure 7. Transcriptomic and epigenomic profiling of adult LepR-Cre+ fibroblasts.

A, Schematic of the multiomics comparison between adult LepR-Cre+ and LepR-Cre- fibroblasts. **B**, Volcano plot displaying transcripts significantly modulated ($|\log_2FC| \geq 0.5$, $FDR \leq 0.05$) in LepR-Cre+ vs LepR-Cre- fibroblasts (N=3). As in, Figure 3C hedgehog signaling molecules are highlighted in blue and forkhead transcription factors in green. **C** and **D**, Reactome analysis of differentially expressed genes showing that cardiac fibroblasts labeled by *LepR-Cre* (LepR-Cre+ CFs) had reduced expression of fibroblast activation markers (**C**) and increased expression of stemness-associated molecules (**D**), consistent with a progenitor-like state. **E** and **F**, Venn diagrams showing genes consistently downregulated (**E**) or upregulated (**F**) in LepR-Cre+ CFs at both P7 and P56. **G**, Heatmap of assay for transposase-accessible chromatin using sequencing data showing that cardiac maturation is associated with large-scale changes in fibroblast chromatin accessibility. Strong differences between LepR-Cre+ and LepR-Cre- fibroblasts at P7 were markedly attenuated in adulthood (N=2).

long *LEPR* isoform specifically in adventitial cells (Figure 8E and 8F).

Together, these findings demonstrate evolutionary conservation of adventitial LepR+ CFs in mammals, refute the idea of cardiomyocytes as a cardiac leptin source, and suggest that adipocytes—rare in mice but abundant in humans—are the sole producers of leptin in the mammalian heart.

DISCUSSION

Understanding cellular and molecular mechanisms underlying cardiac fibrosis, a hallmark of most forms of heart failure, is of paramount importance. Here, using state-of-the-art approaches for gene expression profiling, imaging, and lineage-tracing, we identify a unique

population of CFs localized to the adventitia of large coronary arteries and characterized by LepR expression that serve as preferential contributors to fibrotic remodeling of the heart. Importantly, we also identified LepR+ adventitial CFs in the human heart. Notably, in mice, depletion of LepR+ CFs using an inducible cell ablation strategy that does not affect any other cardiac cell type resulted in improved outcomes post-MI.

In addition to *LepR*, a defining feature distinguishing adventitial CFs from their interstitial counterparts is expression of mediators of hedgehog signaling, a pathway implicated in the maintenance of tissue-resident progenitors.²⁷ These hedgehog-related genes were not modulated in LepR-Cre+ CFs from leptin-deficient mice, indicating that their expression is not regulated by leptin. Although transcriptomic and epigenetic

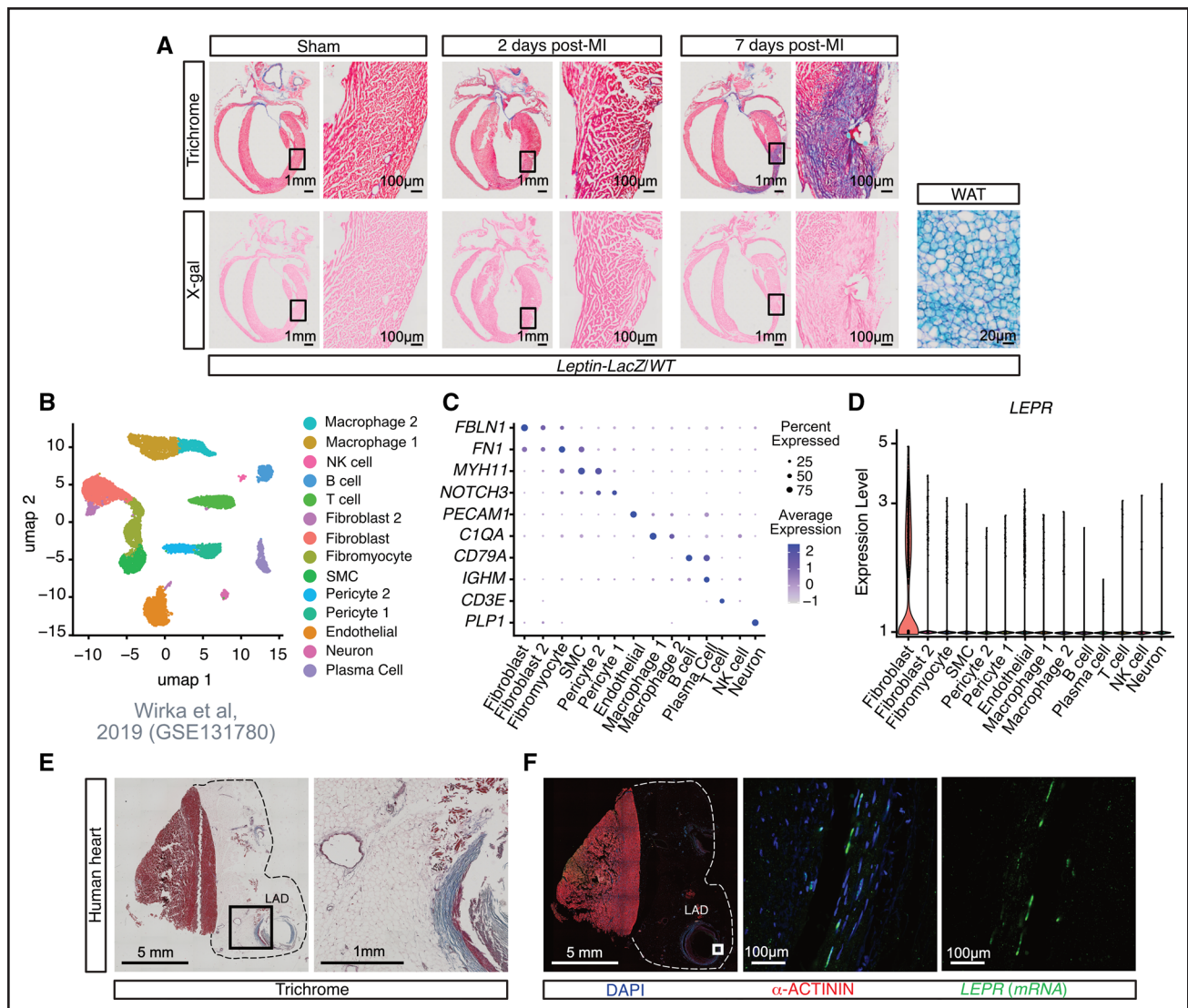


Figure 8. Adventitial fibroblasts in adipocyte-rich coronary niches of the human heart express *LEPR*.

A, Trichrome and X-gal staining of tissues from *Leptin-LacZ/WT* mice. No X-gal-positive cells were detected in the heart before or after myocardial infarction (MI). Peri-gonadal white adipose tissue (WAT) served as a positive control. Boxed areas are shown at higher magnification (N=3). **B**, UMAP plot showing cell types/clusters in single-cell RNA sequencing of cells isolated from the human right coronary artery (N=4). **C**, Dot plot showing expression of top cluster markers. **D**, *LEPR* expression across clusters, highlighting adventitial fibroblasts as the main expressing population. **E** and **F**, Trichrome (**E**) and RNAscope combined with immunofluorescence (**F**) of human heart sections showing an LAD embedded in abundant adipocytes (**E**), with *LEPR* expression (green) detected in adventitial fibroblasts (**F**). Dashed lines in (**E** and **F**) delineate the borders of the epicardial adipose tissue. Boxed areas are shown at higher magnification (N=3).

differences between CF subsets diminish with age, adult LepR-Cre+ CFs retain the machinery required to sense hedgehog ligands, which are strongly induced after MI.⁴⁶ This feature may underlie their preferential contribution to fibrotic remodeling.

The existence of a LepR-Cre+ population of mesenchymal progenitors in the heart is a novel finding, though an analogous population has been recognized in bone marrow.³⁶ Strikingly, in addition to sharing markers (*Lepr*, *Esm1*), these populations also share developmental timing in the neonatal period,^{24,37} coinciding with the onset of leptin production. This parallel suggests that LepR+ mesenchymal progenitors may

emerge simultaneously across multiple tissues, raising the possibility that similar mesenchymal populations exist in additional organs, as supported by our multitissue analyses. It would be of interest to test whether LepR-Cre+ fibroblasts are preferential contributors to fibrotic remodeling in other organs where *Lepr* expression is fibroblast-restricted, such as the lung. In addition to their role in postinjury repair, bone marrow LepR-Cre+ cells also maintain the hematopoietic niche through secretion of trophic factors. Our finding that cardiac LepR-Cre+ CFs express signaling molecules such as IL33 and GDF10 raises the possibility of a similar niche-maintaining role.

Our findings also compel a reappraisal of the role of leptin in cardiac biology. Leptin has been implicated in hypertrophy, which could be a primary effect or an indirect consequence elicited by changes in blood pressure (another known action of leptin).¹⁷ Prior studies, many relying on limited technologies, proposed that leptin acts directly on cardiomyocytes.¹⁷ However, our data—spanning RNA-seq, RNAscope, and genetic lineage tracing—demonstrated that, in the murine heart, CFs are the only cell type expressing the signaling-capable long isoform of LepR. This is seemingly in contrast with previous cardiomyocyte-centered reports,^{13–16} but it is possible that alternative receptors or shorter LepR isoforms with unrecognized signaling capability might be involved. Furthermore, contrary to earlier reports,^{49–51} we found no evidence for cardiac leptin production in mice either at baseline or post-MI. This is in contrast to the regeneration-competent adult zebrafish heart, where leptin is one of the top upregulated genes postinjury.⁴⁷ Given that profibrotic cues in mammals can promote regenerative responses in zebrafish,^{45,56} investigating how leptin is sensed in the zebrafish heart could yield insight into species-specific regenerative mechanisms.

Our study also has several limitations that should be taken into consideration. Transcriptomic analyses were performed with a relatively small number of biological replicates (3 or 4 per experimental group), which may increase the risk of false-positive findings. Although the differential expression of some of the most strongly modulated genes was validated by RNAscope or antibody staining, additional validation will be necessary for individual targets before proceeding with follow-up functional experiments. We used only male mice in MI studies to avoid sex-related confounding factors. Although we cannot exclude different responses in females, the lack of sex-specific differences in LepR-Cre+ CF distribution suggests this is unlikely. The LepR-Cre used in our ablation model is also active in cell types outside the heart; therefore, we cannot fully exclude the possibility that extra-cardiac effects may contribute to the improved function observed post-MI in animals with depletion of LepR-Cre+ CFs. However, this appears unlikely, as ablation of LepR-Cre+ cells in the sham group produced the opposite effect - a modest reduction in ejection fraction 7-day postsurgery (Figure S13), indicating that depletion of LepR-Cre+ cells is beneficial only in the context of fibrotic remodeling following injury, a process in which LepR-Cre+ CFs play a central role.

In conclusion, we identified a new population of adventitial LepR-Cre+ fibroblasts that act as progenitors driving fibrotic remodeling of the heart. Beyond reshaping our understanding of processes underlying cardiac fibrosis, our findings also revise the paradigm of leptin/ LepR signaling in the heart and open new avenues for exploring how this major endocrine pathway intersects with cardiac disease.

ARTICLE INFORMATION

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Disclosures

None.

Supplemental Material

Figures S1–S15
Expanded Materials & Methods
Major Resources Table
Data sets S1–S7

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