Infarct Fibroblasts Do Not Derive From Bone Marrow Lineages

Short Communication

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Rationale: **Myocardial infarction is a major cause of adult mortality worldwide. The origin(s) of cardiac fibroblasts that constitute the postinfarct scar remain controversial, in particular the potential contribution of bone marrow lineages to activated fibroblasts within the scar.**

Objective: **The aim of this study was to establish the origin(s) of infarct fibroblasts using lineage tracing and bone marrow transplants and a robust marker for cardiac fibroblasts, the Collagen1a1-green fluorescent protein reporter.**

Methods and Results: **Using genetic lineage tracing or bone marrow transplant, we found no evidence for collagen-producing fibroblasts derived from hematopoietic or bone marrow lineages in hearts subjected to permanent left anterior descending coronary artery ligation. In fact, fibroblasts within the infarcted area were largely of epicardial origin. Intriguingly, collagen-producing fibrocytes from hematopoietic lineages were observed attached to the epicardial surface of infarcted and sham-operated hearts in which a suture was placed around the left anterior descending coronary artery.**

Conclusions: **In this controversial field, our study demonstrated that the vast majority of infarct fibroblasts were of epicardial origin and not derived from bone marrow lineages, endothelial-to-mesenchymal transition, or blood. We also noted the presence of collagen-producing fibrocytes on the epicardial surface that resulted at least in part from the surgical procedure. (*Circ Res*. 2018;122:583-590. DOI: 10.1161/CIRCRESAHA.117.311490.)**

Key Words: bone marrow ■ fibroblasts ■ fibrosis ■ heart diseases ■ myocardial infarction

Myocardial infarction (MI) results in massive myocyte loss that severely compromises cardiac function. The adult myocardium lacks regenerative capacity, and lost myocardium is replaced by a fibrous scar. Although this scar provides vital chamber structural integrity, it often results in adverse myocardial stiffening and deleterious effects on cardiac function. Therefore, modulation of scar formation could have potential beneficial effects on post-MI remodeling and cardiac function, and understanding the sources of fibroblasts in the context of MI may assist future therapeutic approaches targeting the fibrotic process.

Editorial, see p 540
In This Issue, see p 533

Cardiac fibroblasts are the main cell type responsible for extracellular matrix deposition in the heart.[1] During development, a majority of cardiac fibroblasts are derived from epicardium although a subset enriched in the interventricular septum derives from endothelium/endocardium.[2,3] Fibroblasts play a key role after infarction as the outcome depends on the generation of a fibrous scar comprised largely of collagen.[4] The origins of collagen-producing fibroblasts after infarction are controversial. A subset of fibroblasts is produced by epithelial-to-mesenchymal transition of adult epicardium after infarction.[5] Furthermore, adult endothelial-to-mesenchymal transition (EndoMT) has also been reported to contribute to scar formation[6] although this has been recently challenged.[7,8] Several studies have reported the presence[9,10] or absence[7,8,11] of circulating fibroblast progenitors that make a significant contribution to the postinfarct fibroblast population.

Previously, we described cardiac fibroblast origins during development and in the context of a mouse model of pressure

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Novelty and Significance

What Is Known?

- Cardiac fibroblasts produce the extracellular matrix, including collagen type I, required for scar formation after myocardial infarction.
- Multiple sources of fibroblasts have been reported, including epicardium, endothelium, and bone marrow–derived lineages.
- Whether or not bone marrow–derived lineages make a major contribution to scar formation post-myocardial infarction is unclear.

What New Information Does This Article Contribute?

- Using genetic lineage tracing and bone marrow transplantation, we showed that collagen-producing fibroblasts in the heart post-myocardial infarction were not derived from hematopoietic or bone marrow–derived lineages.
- Fibrocytes, collagen-producing cells of hematopoietic lineage, were at the surface of the heart, near the suture placed on the coronary artery to produce myocardial infarction.
- Collagen-producing fibroblasts in the infarct area were not derived from adult endothelium and were essentially of epicardial origin.

After myocardial infarction, dead myocardium is replaced by scar tissue that ensures structural integrity of the left ventricular chamber. Fibroblasts play an essential role in scar formation, primarily by producing extracellular matrix. Studies using bone marrow transplantation or genetic lineage tracing have produced conflicting conclusions as to contribution of bone marrow–derived fibroblasts to scar formation after myocardial infarction. By using both of these approached, in combination with a murine Collagen-GFP reporter line in which collagen-producing fibroblasts are faithfully labeled, we observed that collagen-producing cells of bone marrow origin located to the surface of hearts of sham-operated and infarcted mice but were not observed within the healthy myocardium or in the infarct area. We found no evidence for adult endothelial contribution to fibroblasts and observed that the vast majority of the fibroblasts in the infarcted area were of epicardial origin. Understanding how fibroblasts are recruited for scar formation might have implications for treatment of patients with myocardial infarction and development of therapies to modulate the fibrotic process.

Nonstandard Abbreviations and Acronyms

αSMAα	smooth muscle actin
EndoMT	endothelial-to-mesenchymal transition
GFP	green fluorescent protein
LAD	left anterior descending artery
MI	myocardial infarction
Thy1	thymus cell antigen 1

overload.[2] Fibroblasts were identified with a Collagen1a1-GFP (green fluorescent protein) reporter that has superior specificity when compared with fibroblast-specific protein 1, αSMA (α smooth muscle actin), vimentin (Vim), or thymus cell antigen 1 (Thy1) stainings.[2,8,12] Here, using genetic lineage tracing and complementary bone marrow transplant experiments, we provide strong evidence that fibroblasts within the infarcted fibrotic area of the left ventricle do not arise from hematopoietic lineages or infiltrating bone marrow–derived cells, or from EndoMT, but rather are principally of epicardial origin. However, although never within the infarct scar itself, collagen-producing blood-derived fibrocytes were observed at the epicardial surface of the infarcted area. Our results provide strong evidence that endogenous fibroblasts are the primary critical target for therapeutic targeting of post-infarct fibrosis.

Methods

The data that support the findings of this study are available from the corresponding author on reasonable request. Expanded methods are presented in the Online Data Supplement.

Results

Collagen1a1-GFP Comprehensively Labels Fibroblasts of the Infarcted Area

We previously reported that cell types within the myocardium, including endothelial cells, resident immune cells, vascular smooth muscle cells, and pericytes, do not express appreciable levels of Collagen1a1-GFP relative to fibroblasts.[2] The infarcted area of hearts from Collagen1a1-GFP$^{+/-}$ mice was imaged at distinct stages after MI induced by permanent left anterior descending (LAD) coronary artery ligation, and by 21 days post-surgery, fibroblasts had completely invested the infarcted area (Figure 1A). Quantification of the Collagen1a1-GFP^{+} area of the left ventricular free wall showed that, compared with 2.8±0.4% of the myocardium in sham-operated controls, fibroblasts occupied 2.5±0.3% (2 days), 10±2% (4 days), 13.5±4% (7 days), and 30±6% (21 days) in infarcts (Figure 1B). Infarcts represented 40±3% and 45±5% of the left ventricular circumference at 7 and 21 days, respectively (Figure 1C; Online Figure IA). Echocardiography revealed that left ventricular function was significantly reduced in MI groups (Online Figure II; Online Movie I). Trichrome staining with fluorescence imaging of adjacent sections clearly showed colocalization of collagen and Collagen1a1-GFP signal in infarcts (Online Figure IB). Markers including αSMA, Thy1, and platelet-derived growth factor receptor α have been commonly used to identify activated fibroblasts within the infarcted area.[7,8,10] We found that a subset of Collagen1a1-GFP fibroblasts in the scar coexpressed αSMA. This population was significantly higher at 21 days (23%) compared with 7 days (19%) post-infarct (Figure 1D and 1E). Thy1 was highly expressed in immune cells and endothelium and was expressed in only a subset of fibroblasts (Online Figure III). However, virtually all Collagen1a1-GFP fibroblasts expressed the mesenchymal marker platelet-derived growth factor receptor α (Figure 1F).

No Evidence for Fibroblasts of Hematopoietic Origin Within Infarct Tissue

To investigate a contribution of hematopoietic lineages to fibroblasts of the infarct, we generated Vav-Cre$^{+/-}$;Rosa$^{tdT/+}$; Collagen1a1-GFP$^{+/-}$ mice and subjected them to LAD coronary artery ligation. As previous studies demonstrated a maximal contribution of bone marrow–derived cells to infarct fibroblasts at day 7 post-infarct, we harvested and examined

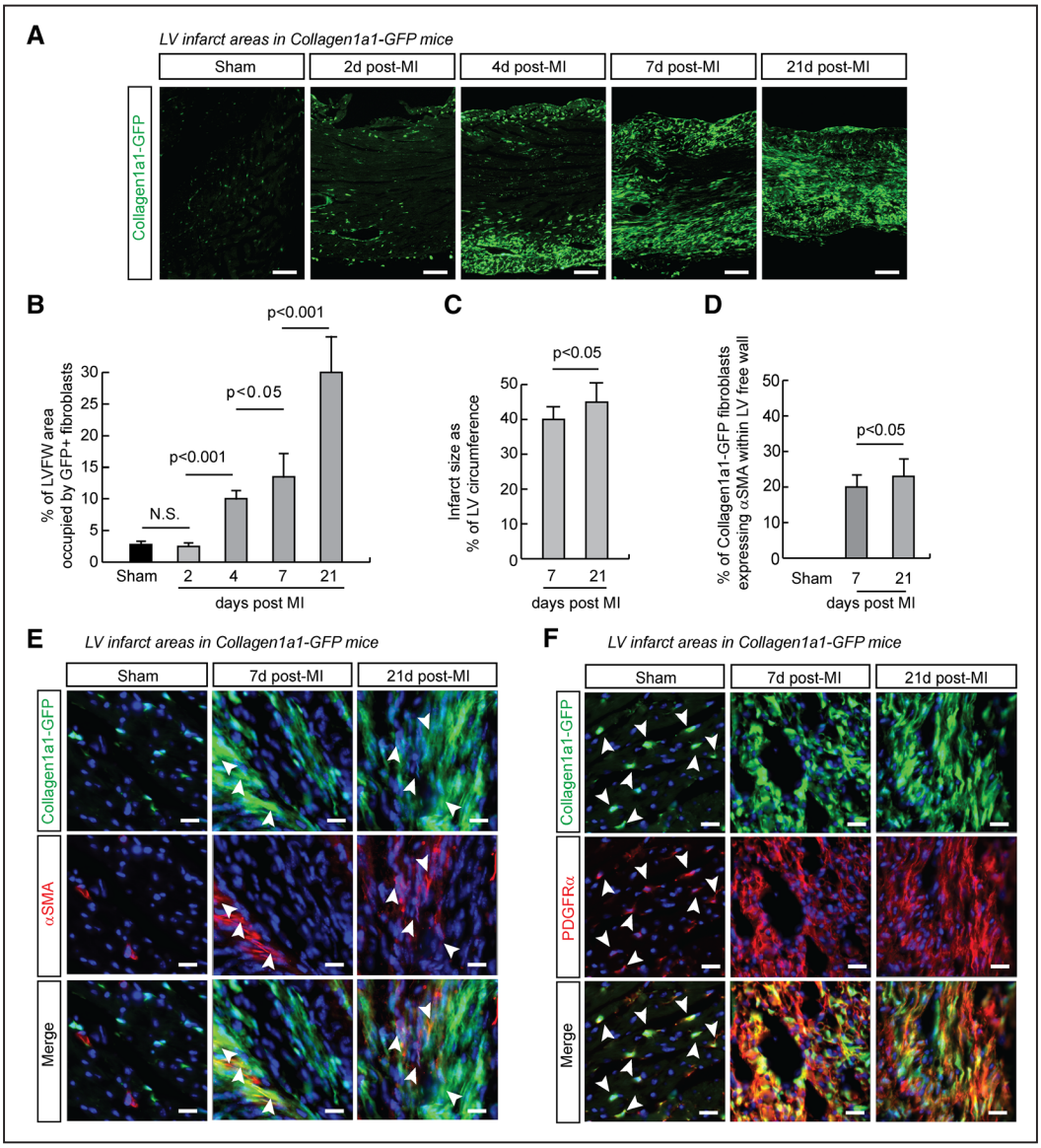


Figure 1. Collagen1a1-GFP (green fluorescent protein) comprehensively labels infarct fibroblasts. **A**, Collagen1a1-GFP expression marks activated fibroblasts successively invading the infarcted area. The epicardial surface is in the lower part of the images. **B**, Quantification of the percentage of the left ventricular free wall (LVFW) area occupied by Collagen1a1-GFP^{+} fibroblasts in sham-operated (n=4) and infarcted hearts at 2-d (n=3), 4-d (n=3), 7-d (n=4), and 21-d (n=4) post-infarction. ANOVA with Bonferroni post hoc test. **C**, Quantification of infarct size as the percentage of left ventricle (LV) circumference from trichrome-stained sections (n=5 hearts per time point). Unpaired, 2-tailed Student *t* test. **D**, Quantification of αSMA (α smooth muscle actin)$^{+}$ fibroblasts in sham LV and infarcts 7 and 21 d after surgery (n=3 hearts per time point). Unpaired, 2-tailed Student *t* test. **E**, Immunofluorescence images showing a subset of myofibroblasts expresses Collagen1a1-GFP and αSMA (arrowheads). **F**, Fibroblasts coexpress Collagen1a1-GFP and platelet-derived growth factor receptor α (PDGFRα) in sham-operated (arrowheads) and infarcted hearts. DAPI (4',6-diamidin-2-phenylindol), blue. Scale bars are 20μm, except in (**A**) 100μm. MI indicates myocardial infarction.

hearts at day 7 and day 21 post-MI.[9,10] We were unable to detect any Collagen1a1-GFP$^{+/-}$ fibroblasts that were labeled by Vav-Cre$^{+/-}$;Rosa$^{tdT/+}$ within infarcted myocardium 7 or 21 days post-infarct. In fact, all Vav-Cre labeled cells expressed the leukocyte marker CD45 (Figure 2A).

Previous studies reporting the presence of bone marrow–derived fibroblasts in infarcts have relied on bone marrow transplantation rather than a genetic lineage tracing approach.[9,10] As bone marrow transplants include both hematopoietic and bone marrow stromal cells that might contribute to infarct scar fibroblasts, we also performed bone marrow transplant experiments (Online Figure IVA–IVC). To investigate potential mobilization of cells expressing Collagen1a1-GFP from the bone marrow to the heart post-infarct, 2 months after recovery from transplant, mice that had received bone marrow from Collagen1a1-GFP$^{+/-}$ mice were subjected to permanent LAD coronary artery ligation. Histological analyses of cardiac tissue from recipient mice post-infarct demonstrated that no Collagen1a1-GFP–labeled fibroblasts were detected within infarcted myocardium of chimeric mice at either 7 or 21 days post-infarct (Figure 2B). Thus, infarct fibroblasts were not recruited from bone marrow.

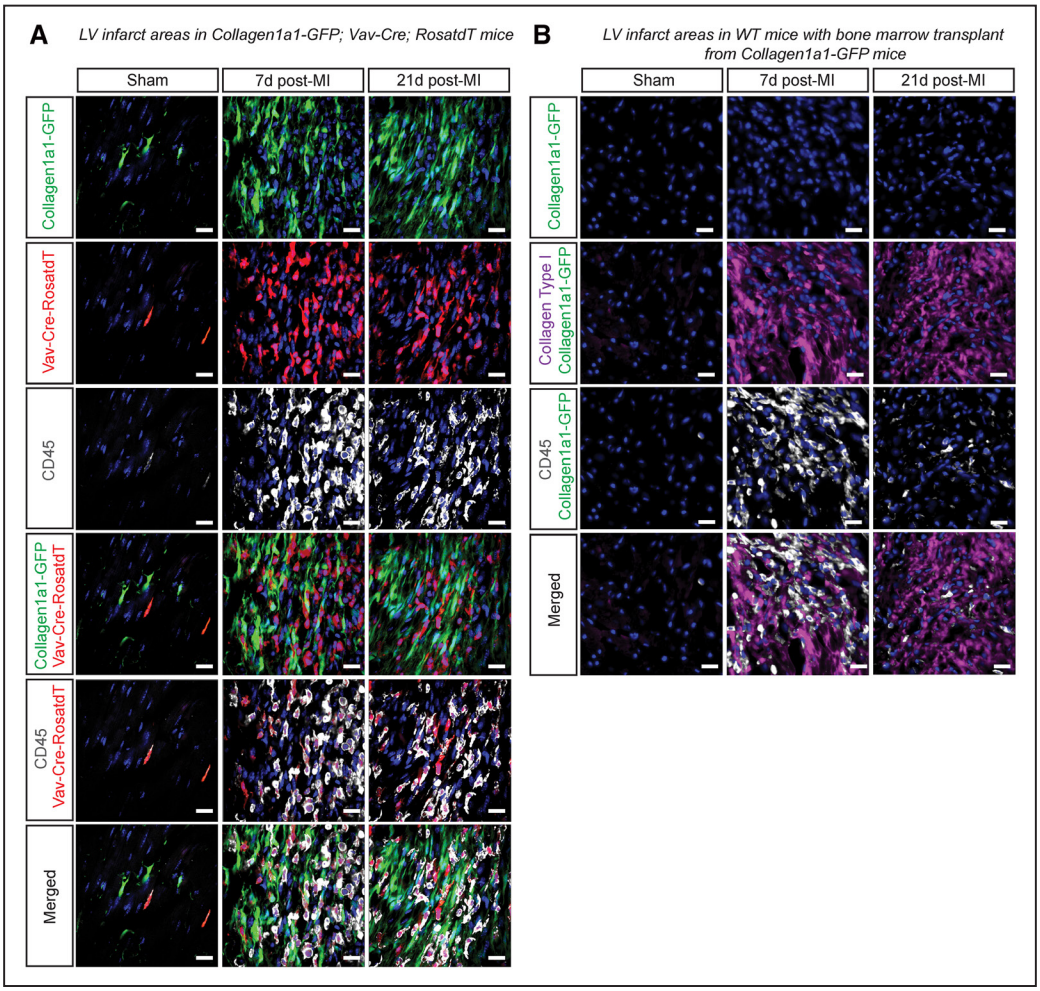


Figure 2. Bone marrow–derived cells do not give rise to infarct fibroblasts. A, Seven and 21 d after infarction, Vav-Cre–labeled cells in infarcted hearts were CD45$^+$ hematopoietic cells and not Collagen1a1-GFP (green fluorescent protein)$^+$ fibroblasts. Scale bars are 20 μm. **B**, No Collagen1a1-GFP$^+$ bone marrow–derived cells were found in the infarct area 7 and 21 d after infarction in wild-type (WT) recipients of bone marrow from Collagen1a1-GFP mice. Collagen type I and CD45 staining indicate infarct area of left ventricle. DAPI (4',6-diamidin-2-phenylindol), blue. Scale bars are 20 μm. LV indicates left ventricle.

Fibroblasts of Hematopoietic Origin (Fibrocytes) Locate to the Epicardial Surface After Infarct

Intriguingly, in wild-type mice that had received a bone marrow transplant from Collagen1a1-GFP$^{+/-}$ mice, although we did not observe any Collagen1a1-GFP$^+$ cells within the infarct scar, we did observe a small population of Collagen1a1-GFP$^+$ cells at the surface of infarcted hearts that coexpressed CD45 (Figure 3A). Collagen1a1-GFP$^+$/CD45$^+$ cells were also observed at the surface of infarcted hearts of Collagen1a1-GFP$^{+/-}$ transgenic mice, indicating that the presence of these cells was not because of the bone marrow transplant procedure (Figure 3A). Further investigation revealed that these cells were not observed in sham-operated mice that were generated by opening of the chest cavity and pericardium but were observed in sham-operated mice that were generated by opening of the chest cavity and pericardium followed by placement of an inert suture around the LAD (sham-plus-suture; Figure 3B). This suggested that, in infarcted mice, recruitment of Collagen1a1-GFP$^+$/CD45$^+$ cells to the surface of the heart was triggered by coronary ligation surgery or the presence of a suture. Indeed, fibrocytes were found proximal to the suture in both sham-operated and infarcted hearts (Online Figure V). Examination of hearts from Vav-Cre$^{+/-}$;Rosa$^{tdT/+}$; Collagen1a1-GFP$^{+/-}$ mice revealed that the Collagen1a1-GFP$^+$/CD45$^+$ cells observed at the surface of infarcted hearts were Vav-Cre lineage traced and expressed the fibrocyte marker CXCR4, suggesting that these cells were fibrocytes, cells of hematopoietic origin expressing CXCR4 and collagen[13] (Figure 3C). The potential functional significance or relevance of these fibrocytes remains to be addressed.

Fibroblasts of Epicardial Origin, Rather Than From EndoMT, Contribute to Infarct Formation

EndoMT is considered a potential source of fibroblasts in fibrosis and has been suggested to contribute to postinfarct fibroblast accumulation.[6] To investigate this possibility, we generated Tie2-Cre$^{+/-}$;Rosa$^{tdT/+}$;Collagen1a1-GFP$^{+/-}$ mice that were subjected LAD coronary artery ligation. As previously reported, in sham-operated hearts, we observed a population of Tie2-Cre lineage-traced fibroblasts (Figure 4A).[2,3] Quantitative analyses demonstrated that this population

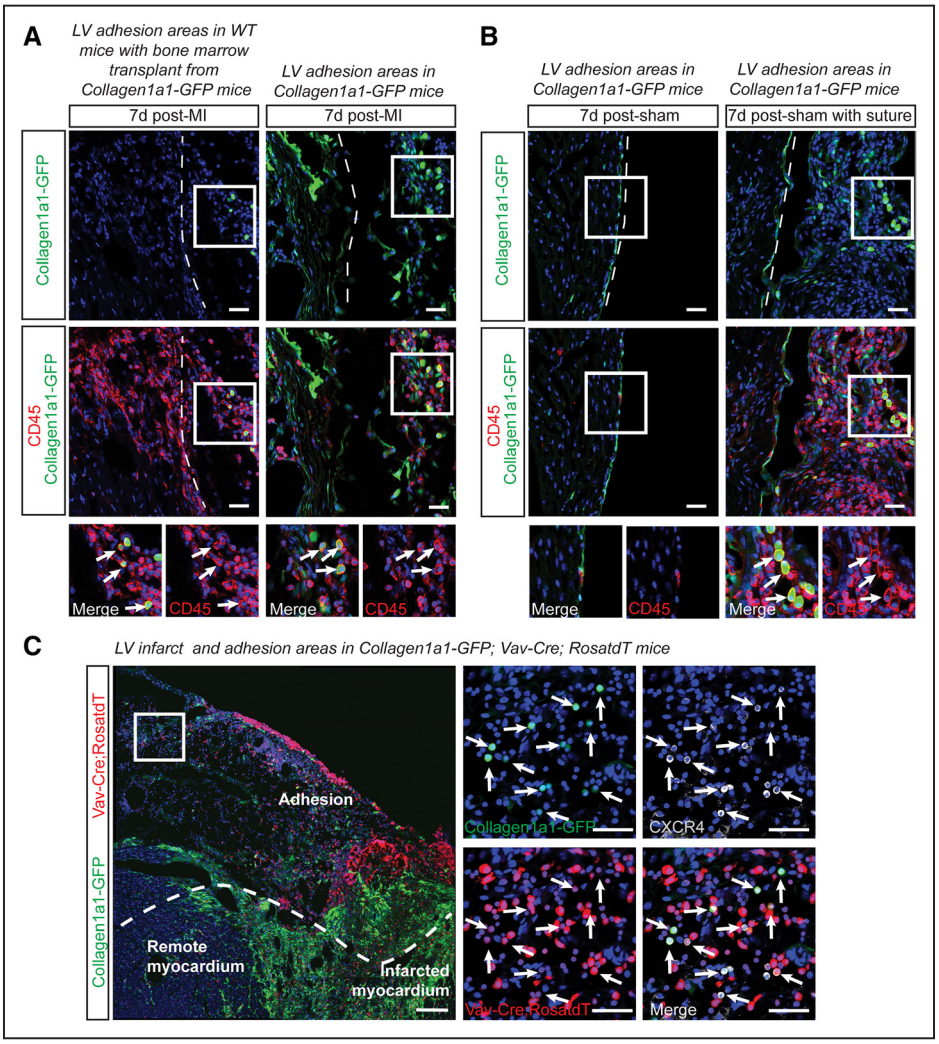
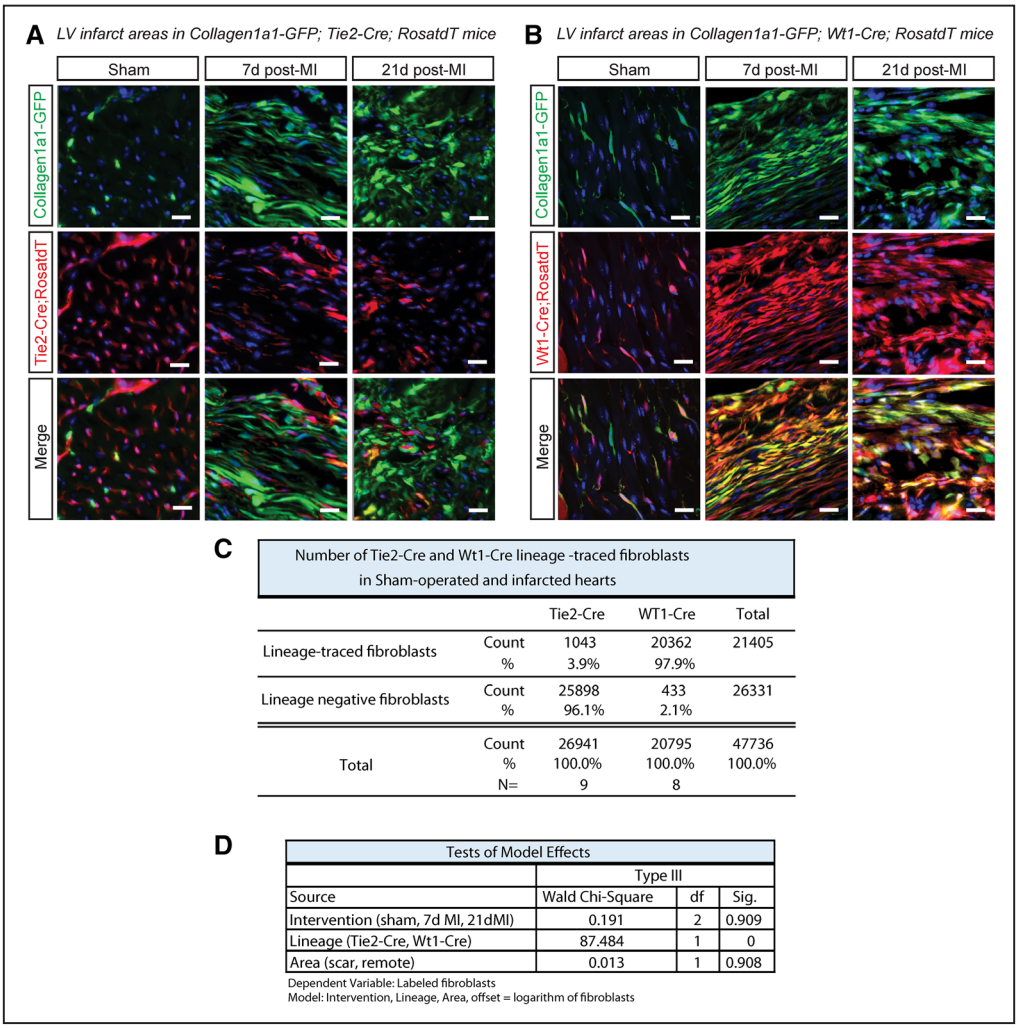


Figure 3. Fibroblasts of hematopoietic origin (fibrocytes) locate to the epicardial surface after infarct or sham operation with suture. **A**, In wild-type (WT) recipients of Collagen1a1-GFP bone marrow, Collagen1a1-GFP^{+} cells were spherical CD45^{+} fibrocytes found outside the infarcts 7-d post-infarction. Collagen1a1-GFP^{+};CD45^{+} cells were also found outside infarcts of Collagen1a1-GFP mice 7-d post-infarction. Similar results were obtained 21-d post-infarction. **B**, Collagen1a1-GFP^{+};CD45^{+} cells were not found on the surface of hearts from Collagen1a1-GFP sham-operated mice 7-d post sham operation when the sham was performed by opening the chest and pericardium of anesthetized mice without needle punch or suture around the left anterior descending (LAD). In contrast, when the sham operation was performed in a more invasive manner, also including a needle punch and suture around the LAD, Collagen1a1-GFP^{+};CD45^{+} cells were found on the surface of hearts 7-d post-operation. Similar results were obtained 21-d post-sham or post-sham with suture surgery. Scale bars represent 50μm. **C**, Collagen1a1-GFP^{+};CD45^{+} cells on the surface of hearts from infarcted Collagen1a1-GFP$^{+/-}$;Vav-Cre$^{+/-}$;Rosa$^{tdT/+}$ mice coexpressed CXCR4 and were labeled by Vav-Cre. The dashed lines represent the epicardium. DAPI (4',6-diamidin-2-phenylindol), blue. Scale bars are 150 and 50 μm (insets). LV indicates left ventricle.

represented 3.8% (n=3) of fibroblasts in the left ventricular free-wall of sham-operated mice. Importantly, similar proportions of Tie2-Cre$^{+/-}$;Rosa$^{tdT+/-}$ Collagen1a1-GFP–expressing fibroblasts was observed 7 days (3.7%; n=3) and 21 days (4.3%; n=3) post-infarction (Online Table I). Collagen1a1-GFP intensity within fibroblasts was not correlated with lineage origin (Figure 4A), suggesting that the cellular context rather than developmental origin determines the level of collagen production. Furthermore, the presence of αSMA^{+} Tie2-Cre$^{+/-}$;Rosa$^{tdT+/-}$ fibroblasts suggested that this subpopulation was able to acquire a myofibroblast phenotype (Online Figure VIA).

Greater than 92% of fibroblasts of the left ventricular free wall derive from the epicardium during development.[2] To investigate whether fibroblasts within the infarct area are also of epicardial origin, we generated Wt1-Cre$^{+/-}$;Rosa$^{tdT/+}$;Collagen1a1-GFP$^{+/-}$ mice and subjected them to permanent LAD coronary artery ligation. Histological analyses of hearts from these mice demonstrated that Collagen1a1-GFP$^{+/-}$ fibroblasts within the infarct area in the left ventricular free wall were almost exclusively Wt1-Cre lineage traced, representing >96% of Collagen1a1-GFP^{+} fibroblasts in the left ventricular free wall and remote myocardium of sham-operated or 7 or 21 days infarcted left ventricle (Figure 4B; Online Table I).

A negative binomial regression was run to statistically assess the main effect of the Cre-lineage (WT1-Cre, Tie2-Cre), intervention (sham, 7 days post-MI, 21 days post-MI), and sampling area (infarct versus remote) on numbers of the lineage-traced fibroblasts (Figure 4C and 4D). Only the



C

Number of Tie2-Cre and Wt1-Cre lineage -traced fibroblasts in Sham-operated and infarcted hearts

		Tie2-Cre	WT1-Cre	Total
Lineage-traced fibroblasts	Count	1043	20362	21405
	%	3.9%	97.9%	
Lineage negative fibroblasts	Count	25898	433	26331
	%	96.1%	2.1%	
Total	Count	26941	20795	47736
	%	100.0%	100.0%	100.0%
	N=	9	8	

D

Tests of Model Effects

	Type III		
Source	Wald Chi-Square	df	Sig.
Intervention (sham, 7d MI, 21dMI)	0.191	2	0.909
Lineage (Tie2-Cre, Wt1-Cre)	87.484	1	0
Area (scar, remote)	0.013	1	0.908

Dependent Variable: Labeled fibroblasts
Model: Intervention, Lineage, Area, offset = logarithm of fibroblasts

Figure 4. The great majority of fibroblasts within infarcted areas display an epicardial signature. **A**, Tie2-Cre–labeled fibroblasts were relatively scarce at 7- or 21-d post-infarction, representing a similar fraction of fibroblasts to that observed in sham-operated hearts. **B**, Wt1-Cre–labeled fibroblasts represent the vast majority of infarct fibroblasts at 7- or 21-d post-infarction. **C**, Quantification of Tie2-Cre and Wt1-Cre lineage-traced fibroblasts in sham-operated and infarcted hearts. Data are presented as absolute counts and percentages. **D**, A negative binomial regression analysis was run to statistically assess the main effect of the lineage (Tie2-Cre, WT1-Cre), intervention (sham, 7-d post–myocardial infarction [MI], 21-d post-MI) and sampling area (scar vs remote) on numbers of the lineage-traced fibroblasts. Only the Cre-lineage, but not intervention or area of sampling, had a significant effect on the number of labeled fibroblasts. DAPI (4',6-diamidin-2-phenylindol), blue. Scale bars represent 20 μm.

Cre-lineage, but not intervention or area of sampling had a significant effect on number of labeled fibroblasts (Figure 4D). Hence, although absolute numbers of fibroblasts increased by several fold after MI (Online Table I), the lack of significant variation in the relative numbers of fibroblasts derived from endothelial and epicardial lineages suggested that EndoMT was not occurring to an appreciable extent in the infarcted or remote myocardium and was therefore not significantly contributing to extracellular matrix deposition and scar formation. Rather, our observations suggest that the vast majority of infarct fibroblasts, including αSMA$^+$ myofibroblasts (Online Figure VIB), arose from epicardial lineages.

Discussion

In this study, using the Collagen1a1-GFP reporter, a robust cardiac fibroblast marker,[2,12] we specifically addressed the contribution of bone marrow and blood/endothelial lineages to the infarct scar in the permanent LAD-occlusion model. By lineage tracing and bone marrow transplant experiments, we could find no evidence for contribution of hematopoietic or Endo-MT–derived fibroblasts to the infarct scar. Collagen-producing fibrocytes of hematopoietic origin were found on the epicardial surface of the infarct area but were not observed to an appreciable extent within infarcts.

Imaging fibroblasts in infarcted heart has proven challenging, notably because of the promiscuity of commonly used markers, such as αSMA or Thy1.[14] The Collagen1a1-GFP reporter faithfully labels cardiac fibroblasts[2,12] and has enabled us to visualize fibroblast invasion of the infarcted area. We found that in the context of infarction, the mesenchymal marker platelet-derived growth factor receptor α also labels all fibroblasts as we and others have reported previously in healthy and hypertrophic heart.[2,12] Hence, these markers are suitable for analyzing the lineage origins of fibroblasts constituting the

infarct scar, a question that has remained controversial. Perhaps not surprisingly, we observed that 19% and 23% of fibroblasts expressed αSMA 7 and 21 days post-infarct, respectively, which is markedly more than what we previously observed in the context of pressure overload–induced cardiac hypertrophy.[2] The presence of more numerous αSMA-expressing cells after infarction as compared with hypertrophy has previously been observed and could be because of increased mechanical stress on fibroblasts in infarcts.[15]

Previous studies have reported a significant contribution of bone marrow–derived fibroblasts to infarcts in mice. Notably, van Amerongen et al[10] found a strong luciferase signal associated with infarcts of mice that had received bone marrow transplants from a Collagen1a2-luciferase reporter line.[10] However, the in vivo imaging used did not allow for determination of whether these collagen-producing bone marrow–derived cells were present within the infarct itself or surrounding the heart. In the event of the latter, this observation would be consistent with our own observation that Collagen1a1-GFP$^+$ fibrocytes accumulated at the surface of the heart post-surgery. Our Vav-Cre lineage tracing studies demonstrated that these fibrocytes were of hematopoietic origin and thus confirmed that fibrocytes observed after bone marrow transplant were derived from bone marrow hematopoietic cells and not bone marrow mesenchymal cells. However, we also observed these cells in sham-operated animals in which a suture had been placed around the LAD, suggesting that their presence at the surface of infarcted hearts was at least partially a response to the presence of the unligated suture or the surgical procedure directed at the epithelial/epicardial surface of the heart. Indeed, fibrocytes have been shown to be recruited in various cases of wound healing involving damage to epithelium.[16]

Another potential source of fibroblasts after infarction is EndoMT.[6] We did not observe an increase in the relative number of Tie2-Cre lineage-traced fibroblasts at baseline, in sham, or in infarcted hearts, arguing against a meaningful contribution from endothelium/endocardium to fibroblasts within the infarct.

Our data showed that >96% of fibroblasts present in the infarct scar of mice subjected to permanent ligation of the LAD were of epicardial origin. It should be noted that these observations are limited to the model used in this study. Previous studies looking at ischemic reperfusion injury or nonischemic heart failure have reported direct fibrocyte contribution to myocardial fibrosis.[17,18] A previous study noted that, post-infarction, epithelial-to-mesenchymal transition of adult epicardial cells contributed to fibroblasts in regions directly underlying the epicardium,[5] suggesting that it is likely that most fibroblasts within the scar derive from resident fibroblasts in the vicinity of the infarct, including the border zone and subepicardial and subendocardial layers. Using Thy1 as a fibroblast marker, another study reported an epicardial origin for the vast majority of fibroblasts of the postinfarct scar.[7] However, Thy1 labels many cell types, including immune cells,[19] lymphatic endothelium,[20] and pericytes.[21] Furthermore, we previously found that approximately a third of Collagen1a1-GFP fibroblasts did not express Thy1,[2] that was recently also reported by another group.[22] A more recent study reported that infarct myofibroblasts were derived from Tcf21$^+$ resident fibroblasts and not from monocytes/macrophages.[8] Hence, by combining the robust Collagen1a1-GFP fibroblast marker with epicardial, endothelial, and pan-hematopoietic genetic lineage tracing and bone marrow transplants, our study clearly demonstrates that infarct fibroblasts derive from the epicardial lineage and not from bone marrow or hematopoietic lineages or from EndoMT. However, we did observe CXCR4$^+$;CD45$^+$;Collagen1a1-GFP$^+$ fibrocytes at the surface of hearts in which a suture was positioned around the LAD which is likely to resolve some seemingly contradictory data in the field.

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Disclosures

None.

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