Insulin-Like Growth Factor 1 Receptor-Dependent Pathway Drives Epicardial Adipose Tissue Formation After Myocardial Injury

BACKGROUND: Epicardial adipose tissue volume and coronary artery disease are strongly associated, even after accounting for overall body mass. Despite its pathophysiological significance, the origin and paracrine signaling pathways that regulate epicardial adipose tissue's formation and expansion are unclear.

METHODS: We used a novel modified mRNA-based screening approach to probe the effect of individual paracrine factors on epicardial progenitors in the adult heart.

RESULTS: Using 2 independent lineage-tracing strategies in murine models, we show that cells originating from the Wt1$^+$ mesothelial lineage, which includes epicardial cells, differentiate into epicardial adipose tissue after myocardial infarction. This differentiation process required Wt1 expression in this lineage and was stimulated by insulin-like growth factor 1 receptor (IGF1R) activation. IGF1R inhibition within this lineage significantly reduced its adipogenic differentiation in the context of exogenous, IGF1-modified mRNA stimulation. Moreover, IGF1R inhibition significantly reduced Wt1 lineage cell differentiation into adipocytes after myocardial infarction.

CONCLUSIONS: Our results establish IGF1R signaling as a key pathway that governs epicardial adipose tissue formation in the context of myocardial injury by redirecting the fate of Wt1$^+$ lineage cells. Our study also demonstrates the power of modified mRNA -based paracrine factor library screening to dissect signaling pathways that govern progenitor cell activity in homeostasis and disease.

Lior Zangi, PhD
Marcela S. Oliveira, PhD*
Lillian Y. Ye, BS*
Qing Ma, MD
Nishat Sultana, PhD
Yoav Hadas, PhD
Elena Chepurko, PhD
Daniela Später, PhD
Bin Zhou, PhD
Wei Leong Chew, PhD
Wataru Ebina, PhD
Maryline Abrial, PhD
Qing-Dong Wang, MD, PhD
William T. Pu, MD
Kenneth R. Chien, MD, PhD

*Drs Oliveira and Ye contributed equally. See Disclosures for full author contributions.

Correspondence to: Lior Zangi, PhD, Icahn School of Medicine, One Gustave L. Levy Place, Box 1030, New York, NY 10029; William T. Pu, MD, Boston Children's Hospital, Enders 1254, 300 Longwood Ave, Boston, MA 02115; or Kenneth R. Chien, MD, PhD, Karolinska Institutet, 35 Berzelius Vag, Stockholm, Sweden 17117. E-mail lior.zangi@mssm.edu, wpu@pulab.org, or kenneth.chien@ki.se

Sources of Funding, see page 71

Key Words: epicardial adipose tissue ■ epicardium ■ insulin-like growth factor-1 ■ myocardial infarction ■ Wt1

Clinical Perspective

What Is New?

- We show that a novel paracrine factor library screening approach enables in vivo dissection of signaling pathways that govern cardiac progenitor cell activity.
- We demonstrate that insulin-like growth factor 1 signaling is required in combination with the cellular environment created by myocardial infarction to stimulate differentiation of epicardial cells into adipocytes.

What Are the Clinical Implications?

- Whereas stimulation of insulin-like growth factor 1 receptor may be advantageous for survival of cardiomyocytes and some cardiac progenitors, it may promote formation of epicardial adipose tissue in the injured heart.
- These studies provide the foundation for future mechanistic studies on the pathophyosiolgical significance of epicardial adipose tissue.
- Our results suggest that insulin-like growth factor 1 receptor signaling that may be targeted to modulate epicardial adipose tissue formation.

Epicardial adipose tissue (EAT), located between the epicardium and underlying myocardium, constitutes 20% of the mass of the human heart.[1] EAT volume and coronary artery disease are strongly associated, even after accounting for overall body mass.[1–3] The association of EAT volume to heart disease has been attributed to paracrine signaling between EAT and adjacent coronary vessels and myocardium.[2] Despite its pathophysiological significance, the origin and paracrine signaling pathways that regulate EAT's formation and expansion are unclear.

The epicardium is a specialized form of mesothelium, the polarized epithelium that lines the surface of many organs, including the heart, lung, liver, and gut. In the developing heart, epicardial progenitors, marked by the expression of the transcription factor Wt1, undergo epithelial-to-mesenchymal transition (EMT) to form epicardium-derived cells (EPDCs), which migrate into the heart to form fibroblasts, smooth muscle cells, and, possibly, endothelial cells and cardiomyocytes.[4–6] In the adult heart, the epicardium is normally quiescent, but myocardial infarction (MI) reactivates a subset of its fetal program, inducing epicardial thickening, fetal gene reactivation, and differentiation of epicardial progenitors into fibroblasts and myofibroblasts.[7] The behavior and fate of injury-activated epicardium is guided by paracrine signals, as we showed in proof-of-concept experiments in which vascular endothelial growth factor A, delivered to myocardium at the time of myocardial infarction (MI) with modified RNA (modRNA), redirected epicardial progenitor fate by stimulating EPDC expansion, mobilizing their migration into subjacent myocardium, and directing their differentiation into endothelial cells.[8]

Here we used a novel, in vivo, modRNA-based paracrine library screening approach to show that insulin-like growth factor 1 receptor (IGF1R) activation is sufficient to promote EAT formation in the context of MI. In this setting, IGF1R activation enhances the ability of Wt1-lineage cells to differentiate into adipocytes, and IGF1R inhibition or Wt1 inactivation blocked their MI-induced contribution to EAT. Our results establish IGF1R signaling as a key pathway that governs EAT formation and the fate decisions of Wt1-lineage cells in the context of myocardial injury.

METHODS

Please refer to Expanded Methods in the online-only Data Supplement for details.

Mice

All animal procedures were performed under protocols approved by the Boston Children's Hospital Institutional Care and Use Committee. $Wt1^{CreERT2}$, $Wt1^{GFPCre/+}$, $Wt1^{flox/flox}$, $\alpha MHC^{MerCreMer}$, $R26^{Tomato}$, and $R26^{mTmG}$ alleles have been described previously.[6,9–12] Tamoxifen (Tam, 0.12 mg/g body weight) was administered to adult mice twice weekly for 3 weeks to induce CreERT2-mediated recombination. One week after completion of Tam dosing (to allow Tam clearance), the left anterior descending coronary artery was ligated to induce MI.[13] Paracrine factor modRNAs (100 μg/heart, Luciferase [Luc] modRNA serve as control) were injected into the infarct zone myocardium immediately after left anterior descending coronary artery ligation.[8] ModRNA gels were applied to the heart either at the time of left anterior descending coronary artery ligation or 2 weeks before by a lower thoracotomy, as described in the text. Procedures and measurements were performed blinded to genotype and treatment group. All animals that started an experimental protocol and survived to the measurement point were included.

Synthesis of modRNA

ModRNAs were in vitro transcribed from plasmid templates (sequences provided in the online-only Data Supplement Table I) using a custom ribonucleoside blend of 3'-O-Me-m7G(5')ppp(5')G cap analog (6 mM, New England Biolabs), guanosine triphosphate (1.5 mM, USB), adenosine triphosphate (7.5 mM, USB), and 5-methylcytidine triphosphate and pseudouridine triphosphate (7.5 mM, TriLink Biotechnologies), as described previously[8,14,15] RNA was treated with Antarctic Phosphatase (New England Biolabs), quantitated by Nanodrop (Thermo Scientific), precipitated with ethanol and ammonium acetate, and resuspended in 10 mM TrisHCl, 1 mM EDTA. ModRNA was transfected into cultured cells with RNAiMAX (Life Technologies). The transfection mixture was added to cells cultured in DMEM with 2% FBS and 200 ng/mL B18R (eBioscience). ModRNA gel was made by mixing Cre modRNA (10 μL modRNA at 20 μg/μL), Lipofectamine 2000 (30 μL; Life Technologies), and 0.05% polyacrylic acid (10 μL; Sigma). The mixture was incubated for 15 minutes at room temperature to generate the gel, which was painted on the heart surface.

Isolation and Culture of Murine EPDCs and Mesenchymal Stem Cells

Mesenchymal stem cells were isolated from adult (6–8 weeks) CFW femurs as described previously.[16] Isolated cells were cultured in StemXvivo Osteogenic/Adipogenic Base media (R&D Systems) with Penicillin-Streptomycin (1:100). WT1^{+} EPDCs were isolated from heart explants of Wt1$^{GFPCre/+}$ mice 2 days after MI. Cardiac cells (nonmyocytes) were allowed to expand from heart explant cultures. After 2 weeks, green fluorescent protein (GFP^{+}) cells were isolated by fluorescence active cell sorting Aria III and plated in fibronectin-coated (5 ng/mL for 2 hours at 37ºC) wells of a 12-well plate (70 000 cells per well).

For enhancement of adipocyte differentiation in mesenchymal stem cells or EPDCs, culture medium (StemXvivo Osteogenic/Adipogenic Base media; R&D Systems) was supplemented with Adipogenic Supplement (1:20; R&D Systems). ModRNAs were transfected every 3 to 4 days during adipogenic differentiation. For detection of oil droplets, cultures were stained with saturated Oil red O solution (Sigma).[17] To quantitate Oil red O staining, plates were dried and extracted with 1 mL 100% isopropanol. After 10 minutes of incubation with gentle shaking, the OD_{500} was recorded.

Human EPDCs

Human EPDCs were obtained from atria of adult patients undergoing heart operations under a protocol approved by the Medical Ethics Committee of the Leiden University Medical Center. EPDCs were isolated as described and passaged ≤9 times.[18]

Immunostaining

Immunostaining was performed on cryosections of hearts fixed by perfusion with 4% PFA with the antibodies listed in the online-only Data Supplement Table II. Quantification of immunostaining in cardiac sections was performed withg ImageJ Software.

RNA Isolation and Gene Expression Profiling

The peri-infarct zone near the apex was snap-frozen. Total RNA was isolated with the RNeasy mini kit (Qiagen) and reverse transcribed with Superscript III reverse transcriptase (Life Technologies). Real-time qPCR analyses were performed on a Mastercycler Realplex 4 Sequence Detector (Eppendoff) with SYBR Green (Quantitect SYBR Green PCR Kit; Qiagen). Fold changes in gene expression were determined by the ∂∂CT method and were presented relative to *Gapdh* internal control. PCR primer sequences are shown in the online-only Data Supplement Table III.

For microarray gene expression profiling, WT1$^{GFPCre/+}$ mice underwent left anterior descending coronary artery ligation, and 7 days later the hearts were dissociated. GFP^{+} EPDCs were isolated by fluorescence active cell sorting for GFP. RNA was isolated and used to probe Affymetrix Gene 1.0 ST arrays (n=3). Gene expression values were determined with Affy Power Tools, and the distribution of mean values were used to define the detectable gene expression threshold. Genes with gene ontology terms "receptor activity" or "plasma membrane" were manually curated to define a set of cell surface receptors.

Western Blot

Western blotting was performed to measure phosphorylated IGF1R in EPDCs stimulated with IGF1 protein (PeproTech). Samples containing equal amounts of protein were separated by SDS-PAGE, transferred to nitrocellulose, probed with IGFR (pY-1161) antibody (Abcam, 1:1000) and then donkey anti-Rabbit HRP antibody, and visualized by chemo-luminescent detection.

Statistical Analyses

Values are reported as mean±standard error of the mean. Comparisons between groups were made with Welch's 2-tailed *t* test (continuous variables) or Fisher exact test (proportions).

RESULTS

Recent lineage tracing studies indicate that EPDCs are 1 of the cell types that undergo adipogenic differentiation into epicardial fat,[19–21] and MI stimulates EPDC adipogenic differentiation.[20] To gain further insights into this process, we established a lineage-tracing system to measure MI-induced adipogenic differentiation of EPDCs. We used a pulse-labeled genetic lineage-tracing strategy, in which *Wt1* regulatory elements drive epicardial expression of CreERT2[6]. In the presence of the inducing agent tamoxifen, CreERT2 indelibly activates the Rosa26^{Tomato} reporter, so these cells and their descendants express red fluorescent protein.[8,19,20] We refer to these cells durably labeled by Wt1^{CreERT2} as Wt1-lineage cells (WT1LCs). Within the heart, WT1LCs are predominantly EPDCs,[6,7] although Wt1^{CreERT2} also labels other mesothelial cells within the chest, such as the cells of the pericardium and chest cavity. We pulse labeled adult mice by treating them with tamoxifen, performed left anterior descending coronary artery ligation to induce MI, and examined hearts 28 days later. Sham-operated mice did not develop obvious EAT (0/10), whereas 53% (10/19) of MI mice were positive for EAT (Figure 1A, B). Consistent with this observation, myocardial tissue expressed higher levels of adipocyte markers *Fabp4*, *Adiponectin*, *Adipsin*, and *Pparg* (Figure 1C).[22] The observed adipose tissue fulfilled 3 criteria for being EAT (online-only Data Supplement Figure I)[21]: (1) it was perfused from the coronary arteries and not the systemic arteries; (2) it was histologically within the epicardium; and (3) it expressed *Ucp1*, a marker of brown fat, which was not expressed in peri-aortic fat. In Sham-operated mice, immunostaining of tissue sections showed that adipocytes, marked by Perilipin A,[19,20] were infrequent and rarely overlapped with Wt1LCs. In contrast, after MI, adipocytes were more abundant and a subset were Wt1LCs, suggesting that MI stress induces EPDC differentiation into EAT (Figure 1D).

Quantitative analysis showed that 24.1% of Perilipin A^{+} cardiac adipocytes were Wt1LCs (Figure 1E), consistent with reports that some cardiac adipocytes arise from epicardium.[19–21] The partial labeling of adipocytes may reflect incomplete labeling of the *Wt1* lineage by tamoxi-

fen but more likely reflects heterogeneous sources for EAT after MI. However, only a small fraction of Wt1LCs differentiated into adipocytes (Perilipin A$^+$; CD24$^-$; 12%) or preadipocytes (Perilipin A$^-$; CD24$^+$; 3%) (Figure 1F).[19] As a control experiment, we also traced the myocardial lineage with cardiomyocyte-specific Myh6-MerCreMer (Myh6-MCM).[10] Although EAT was induced by MI with equivalent frequency in Myh6-MCM; Rosa26^{Tomato} mice, we did not observe coexpression of the genetic lineage label in adipocytes (Figure 1B, D, E). These data indicate that Wt1LCs, but not cardiomyocytes, differentiate into adipocytes in the context of MI.

Epicardial cells in the fetal heart and injured adult heart acquire plasticity by undergoing EMT to form EPDCs.[7] Epicardial EMT requires *Wt1*.[23,24] Therefore, we hypothesized that EPDC differentiation into adipocytes in the injured adult heart requires epicardial *Wt1* expression. Knockout of a conditional Wt1^{flox} allele[9] by induction of Wt1^{CreERT2} in adults caused death from uncertain mechanisms within a few days,[25] precluding the use of this model to investigate the functional requirement of *Wt1* in EAT formation. To circumvent this problem, we applied Cre modRNA gel[8] to the surface of Wt1$^{flox/flox}$; Rosa26^{Tomato} hearts to locally inactivate *Wt1* and simultaneously label EPDCs. In the heart, the Cre gel selectively labeled the epicardium and its derivatives,[8] although it could also label other cell populations that contact the Cre gel, including cells lining the pericardium, lungs, and chest cavity. We collectively refer to these as Cre gel lineage cells (CGLCs). In Wt1$^{flox/flox}$; R26^{Tomato} mice treated with Cre gel, we continued to observe MI-induced formation of EAT (Figure 1B), consistent with the heterogeneous origin of epicardial fat.[19,20] However, cells marked by Cre gel did not differentiate into adipocytes, indicating that *Wt1* is required for the fate transition of CGLCs into adipocytes (Figure 1D–F). These results provide functional data that confirm the contribution of Wt1$^+$ mesothelium to EAT.

To further establish the adipogenic potential of EPDCs, we studied the capacity of in vitro-cultured primary EPDCs to differentiate into adipocytes. We induced MI in Wt1^{GFPCre} mice, which express GFP-Cre fusion protein from the endogenous *Wt1* locus,[6] and then isolated post-MI EPDCs by fluorescence active cell sorting for GFP. When cultured in adipogenic media, the EPDCs differentiated into adipocytes, as demonstrated by Oil red O staining and upregulation of *Fabp4*, *Adiponectin*, *Adipsin*, and *PPARγ* (online-only Data Supplement Figure II). These results indicate that EPDCs have the potential to differentiate into adipocytes, consistent with our in vivo data and recent reports.[19,20]

To investigate the role of paracrine signaling in MI-induced adipogenic differentiation of EPDCs, we first performed microarray expression profiling of post-MI EPDCs cells (marked by GFP in Wt1^{GFPCre} mice) to identify expressed receptors (Figure 2A). From the microarray data, we selected a panel of 10 canonical candidate receptors expressed in these cells, along with 4 negative controls. To validate the expression of these receptors, we isolated an independent set of post-MI EPDCs. Quantitative reverse transcription polymerase chain reaction (PCR) showed that these cells robustly expressed epicardial markers *Wt1* and *Tbx18*[26] and not cardiomyocyte markers *Myh6*, *Tnni3*, or *Tnnt2* (Figure 2B), confirming that our isolation procedure captured EPDCs. The 10 candidate receptors identified by microarray were robustly expressed in EPDCs, whereas the 4 negative control receptors were not (Figure 2B). We further confirmed expression of these receptors by epicardial cells and their derivatives by immunostaining tissue sections of hearts after MI Wt1^{GFPCre} (Figure 2C). Epicardial cells, marked by GFP, coexpressed the 10 candidate receptors. EPDC expression of these receptors was further validated in cultured primary EPDCs (online-only Data Supplement Figure III).

Having identified a number of EPDC-expressed receptors, we next asked whether overexpression of their cognate ligands at the time of MI would stimulate EAT formation. To efficiently test these factors for adipogenic activity within the bona fide in vivo context of heart injury, we took advantage of the power of modRNAs to induce myocardial expression of a pulse of paracrine factor.[8] We generated a paracrine factor modRNA library encoding the 10 candidate ligands plus vascular endothelial growth factor A, which we previously showed influences EPDC activity,[8] and luciferase (Luc) negative control. We then scored these factors for adipogenic activity when individually delivered to the heart at the time of MI operation. We found that 1 ligand, IGF1, robustly stimulated EAT formation after MI so that EAT was observed in 80% to 100% of hearts, compared with 25% to 50% at baseline or after treatment with other factors (Figure 3A). Unlike IGF1, the other 9 paracrine factors did not increase EAT formation after MI above baseline. It is interesting to note that IGF1 did not stimulate adipogenesis in normal hearts without the stress of MI (online-only Data Supplement Figure IV). Increased IGF1-induced adipogenesis was confirmed by measuring expression levels of adipogenic lineage markers *Fabp4*, *Adiponectin*, *Adipsin*, and *Pparg* (Figure 3B).

Because our data (Figure 1) and prior studies[19–21] indicated that EPDCs can differentiate into adipocytes after MI, we cultured primary EPDCs after MI and treated them with the 10 candidate ligand modRNAs. IGF1, but not the other 9 paracrine factors, stimulated adipogenesis (Figure 3C). Moreover, quantitative reverse transcription PCR comparing cultured EPDCs with or without IGF1 confirmed IGF1 stimulation of EPDC adipogenic differentiation (Figure 3D). To ask whether IGF1 similarly stimulated adipogenesis of human EPDCs, we obtained primary human EPDCs from patients undergoing operation for heart disease. These cells expressed the marker *WT1*, as expected for EPDCs. Consistent with the data

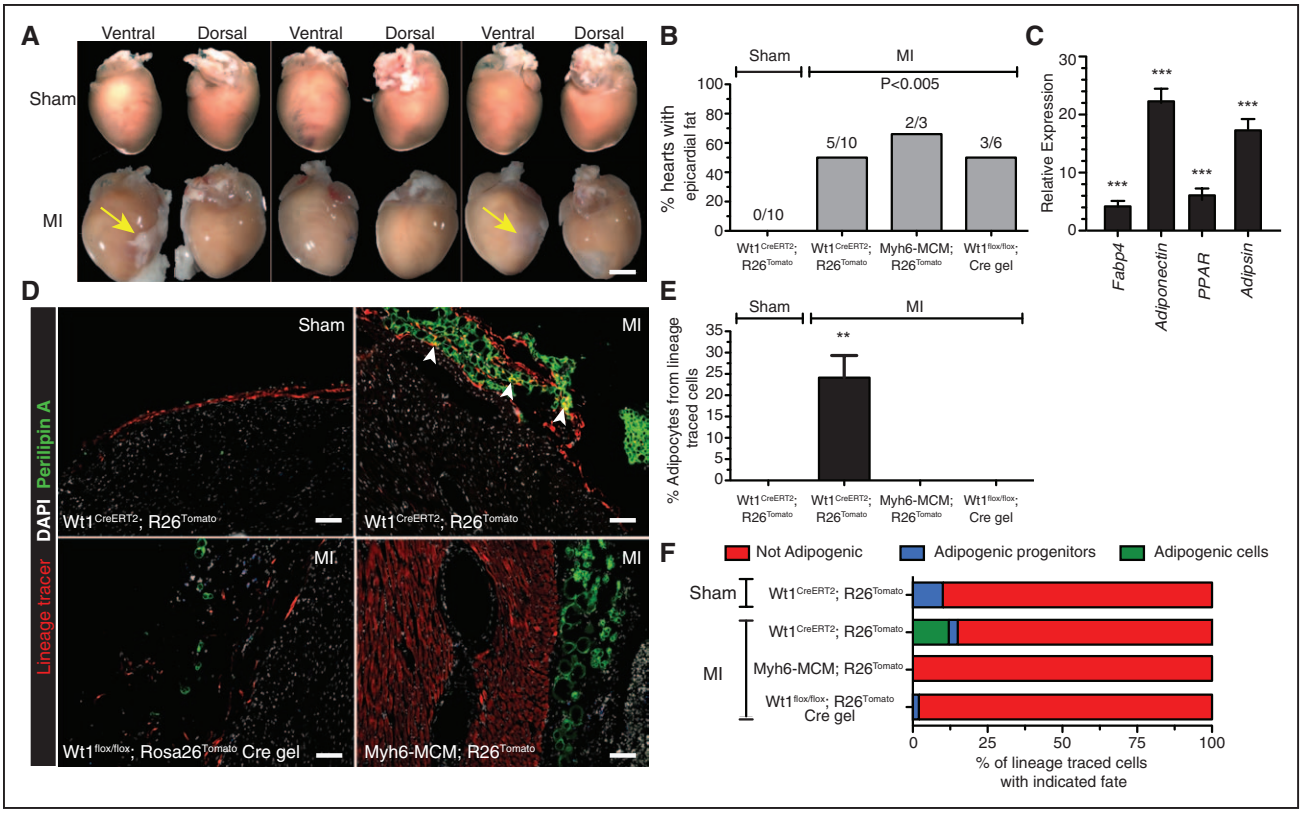

Figure 1. Myocardial infarction (MI) induces epicardium-derived cells (EPDCs) to differentiate into epicardial fat.

Hearts were analyzed 4 weeks after sham or MI operation. **A,** Dorsal and ventral views of 6 representative hearts after sham or MI operation. Arrows indicate examples of epicardial fat. **B,** Percentage of hearts with epicardial fat evident on gross inspection in different experimental models, as scored by a blinded observer. The proportion of hearts with epicardial fat was significantly higher after MI compared with sham ($P<0.005$, Fisher exact test). **C,** Expression of indicated genes relative to sham-operated controls as measured by quantitative reverse transcription polymerase chain reaction (qRTPCR) (n=3; ***$P<0.001$, *t* test). **D** through **F,** Origin of MI-induced adipocytes evaluated by genetic lineage tracing. Representative confocal images are shown in (**D**). Arrowheads point out adipocytes coexpressing the EPDC lineage tracer. Staining was quantified to determine the percentage of Perilipin A$^+$ adipocytes that coexpressed the genetic lineage tracer (**E**) or the percentage of lineage-traced cells that coexpressed markers of adipogenic progenitors (CD24$^+$ Perilipin A$^-$) or mature adipocytes (CD24$^-$ Perilipin A$^+$) (**F**). **E** and **F,** Hearts scoring positive for epicardial fat (panel B) or a minimum of 2 hearts were included. For each heart, a minimum of 5 sections were analyzed (**$P<0.01$, *t* test).

from murine EPDCs, human EPDCs robustly expressed *IGF1R* (Figure 3E). Culture of these cells in IGF1 induced adipogenesis to the same extent as adipogenic media, as determined by Oil red O staining (Figure 3F–G). These data indicate that the IGF1-stimulated EPDC adipogenic pathway is conserved to humans.

We next used in vivo lineage-tracing strategies to directly measure the effect of each candidate paracrine factor on EPDC adipogenesis in vivo. Figure 4A shows representative confocal images of the lineage-tracing data obtained by Wt1^{CreERT2}-mediated pulse labeling. With control Luc modRNA, we again observed that after MI, a small fraction of Perilipin A$^+$ adipocytes coexpressed the genetic marker of Wt1LCs (Figure 4A, top panel). In contrast, IGF1 modRNA strongly increased the frequency of Perilipin A$^+$ cells in Wt1LCs (Figure 4A, bottom panel). Quantitative analysis showed that IGF1 but not the other 9 paracrine factors increased the fraction of adipocytes that originate from Wt1LCs by ≈2-fold (Figure 4B). This finding was independently supported by Cre gel lineage tracing, which showed a similar increase of adipocytes in the Cre-labeled lineage, although the overall extent of contribution to adipocytes was lower likely because the gel labels a lower fraction of epicardial cells. EPDCs are a shared and predominant subset of both Wt1LCs and CGLCs. Thus, in combination with our in vitro data, these data suggest that IGF1 augmented the fraction of adipocyte-derived EPDCs. Although EAT originates from multiple sources, the increase in EPDC-derived adipocytes implies that IGF1 preferentially augments the contribution of EPDCs compared with other sources.

We also analyzed the fraction of EPDCs that express adipocyte or preadipocyte markers. IGF1, but not the other paracrine factors tested, strongly increased the

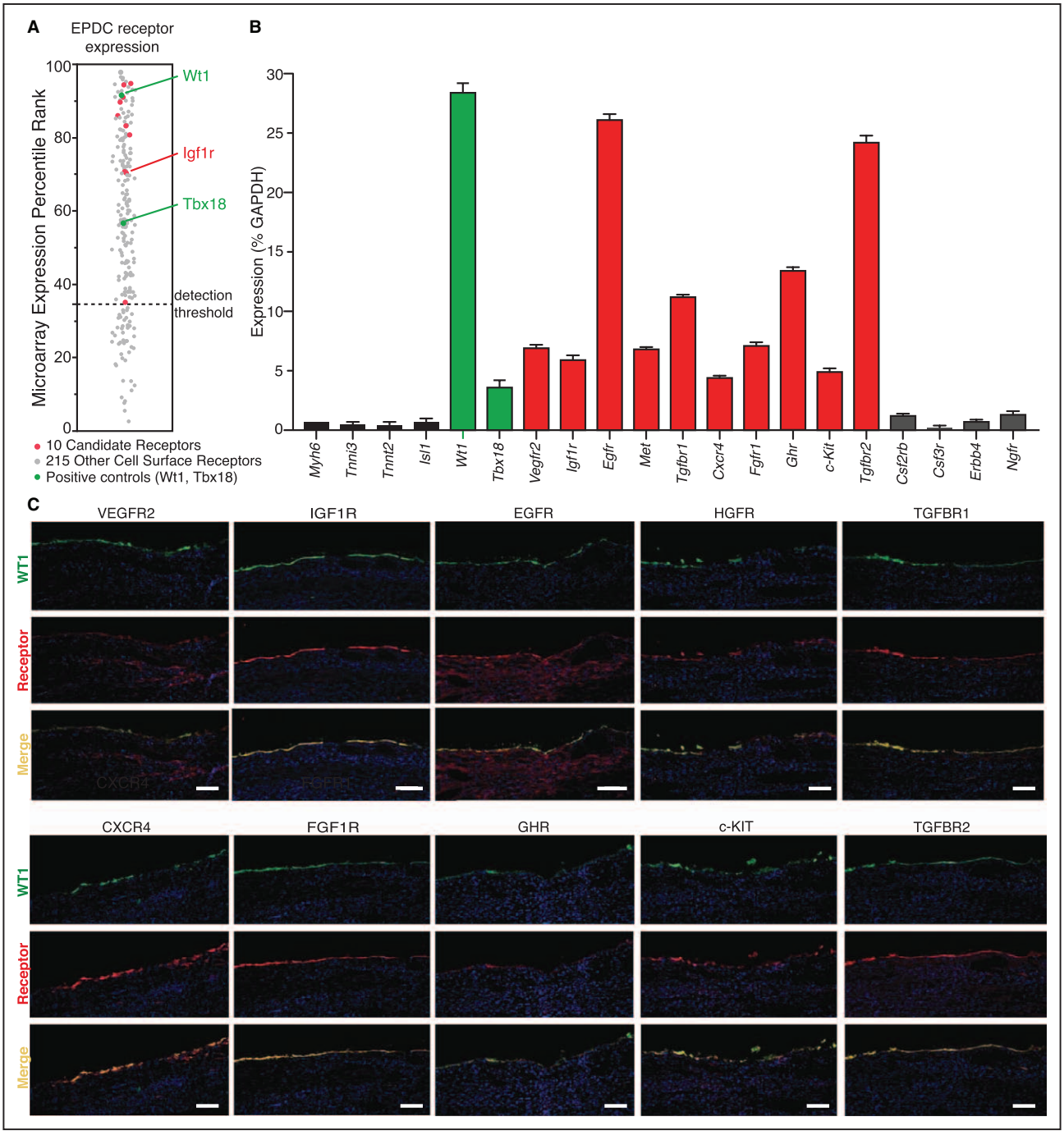

Figure 2. Epicardium-derived cells (EPDCs) paracrine receptor expression.
A, Seven days after Wt1^{GFPCre} mice underwent myocardial infarction (MI) operation, EPDCs were isolated from dissociated hearts by fluorescence active cell sorting for green fluorescent protein (GFP FACS). EPDC RNA was profiled with microarrays. Plot shows the percentile expression rank of selected cell surface receptors and EPDC marker genes *Wt1* and *Tbx18*. 10 canonical paracrine factor receptor further interrogated in this study are indicated in red. **B,** Expression levels of 10 canonical paracrine factor receptors (red) and additional marker genes (black, cardiomyocyte marker; green, EPDC marker; grey, receptors not expressed above microarray threshold) were measured by quantitative reverse transcription polymerase chain reaction (qRTPCR). RNA was isolated from FACS-purified EPDCs 3 days after MI operation. **C,** Immunostaining for 10 canonical paracrine factor receptors, 3 days after MI operation on Wt1^{GFPCre} mice. Bar = 50 μm.

fraction of Wt1LCs that differentiate into preadipocytes and adipocytes (Figure 4D), and this finding was independently supported by the Cre modRNA gel labeling approach (Figure 4E). The enhanced adipogenic differentiation of Cre-labeled progenitors was accompanied by their significantly reduced differentiation into smooth

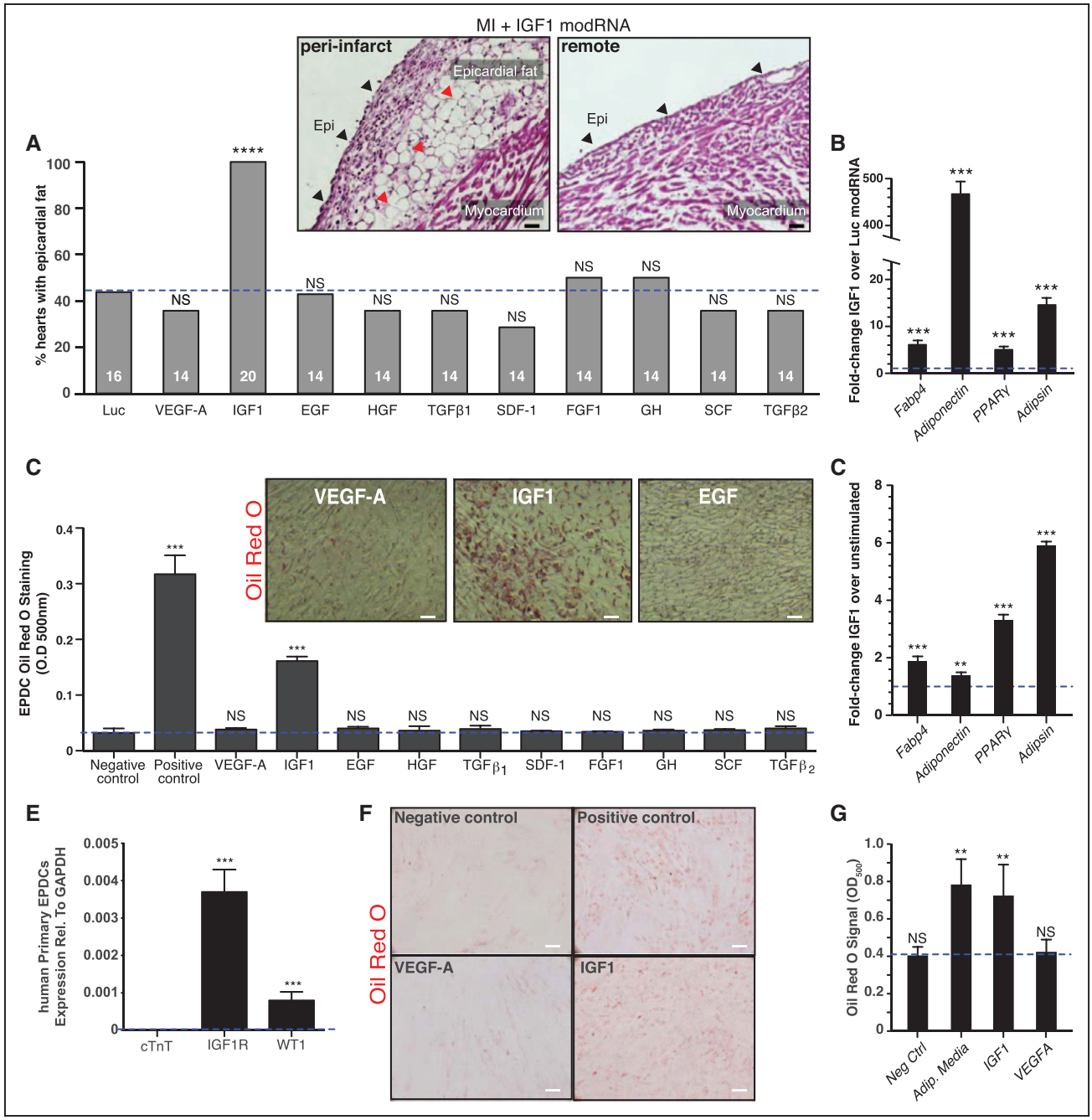

Figure 3. Insulin-like growth factor 1 (IGF1) stimulates myocardial infarction (MI)-induced formation of epicardial adipose tissue.

Mice underwent MI operation and concurrent injection of indicated paracrine factor-modified RNA (modRNA). **A,** Percentage of hearts with grossly visible epicardial fat after MI and injection of indicated paracrine modRNAs. Numbers inside bars indicate sample size (*****P*<0.0001; N.S., not significant; Fisher exact test). Inset shows H&E-stained sections from peri-infarct and remote regions of IGF1 modRNA-injected heart. Red arrowheads indicate adipocytes within the expanded epicardial layer. Black arrowheads delineate the epicardial surface of the heart. **B,** IGF1 modRNA increased adipogenic marker gene expression 10 days after MI. Gene expression in left ventricular (LV) myocardium was measured by quantitative reverse transcription polymerase chain reaction (qRTPCR) and expressed as fold change in IGF1 modRNA compared with Luc treatment (n=3, ****P*<0.001, *t* test). **C,** Adipogenic effect of indicated paracrine factors on primary cultured murine EPDCs. epicardium-derived cells (EPDCs) after MI were cultured in the presence of the indicated paracrine factors (100 μg/mL). The negative control was PBS, and the positive control was adipogenic media (R&D Systems). After 28 days, the cultures were stained with Oil red O to detect lipid accumulation (representative images shown in inset). Lipid accumulation was quantitated by extracting Oil red O from stained cells and measuring the OD_{500}. Dashed line indicates background OD_{500} observed in the negative control (n=3, ****P*<0.001, *t* test). **D,** IGF1 increased adipogenic marker gene expression in cultured EPDCs. After 10 days of (*Continued*)

muscle and endothelial lineages (online-only Data Supplement Figure V). Together these data suggest that IGF1 regulates EPDC fate decisions after MI, directing them toward the adipocyte lineage.

Our finding that IGF1 stimulates adipogenic differentiation suggested that the IGF1 receptor signaling axis is required for MI-induced formation of EAT from EPDCs. To test this hypothesis, we designed at strategy to inhibit IGF1 receptor signaling in EPDCs with either a dominant-negative IGF1 receptor mutant[27] (IGF1R-DN) or a dominant-negative IRS1 mutant[28] (IRS1-DN). To validate that these dominant-negative proteins block IGF1R phosphorylation, we transfected cultured EPDCs cells with IGF1R-DN, IRS1-DN, or Luc (negative control) and stimulated the cells with IGF1 protein. Western blotting of cell extracts 24 hours later showed that both dominant-negative proteins inhibited IGF1R phosphorylation compared with Luc control (Figure 5A). Furthermore, IGF1-stimulated adipogenic differentiation of cultured EPDCs, measured by Oil red O staining, was inhibited 4-fold by the dominant-negative mutants (Figure 5B and online-only Data Supplement Figure VIA). This inhibitor effect was further confirmed by quantitative reverse transcription PCR measurement of adipogenic lineage marker expression in the EPDC cultures (online-only Data Supplement Figure VIB).

To study the requirement of IGF1 receptor signaling in EPDCs for their differentiation toward adipocytes in response to IGF1 modRNA and MI, we delivered the dominant-negative IGF1R antagonists to epicardial cells with modRNA gel. With $Wt1^{CreERT2}$; $Rosa26^{Tomato}$ lineage-tracing mice, we followed the fate of transduced Wt1LCs after MI and IGF1 modRNA treatment. IGF1R-DN or IRS1-DN modRNA gel treatment reduced the frequency of EAT after MI by ≈50% (Figure 5C), reduced the fraction of adipocytes that arise from Wt1LCs (Figure 5D), and decreased Wt1LC differentiation to preadipocytes and adipocytes (Figure 5E). We confirmed these results with modRNA gel to both Cre label cells derived from epicardium and to concurrently express IGF1R-DN or IRS1-DN. As in the $Wt1^{CreERT2}$ lineage-tracing model, inhibition of IGF1R signaling by either dominant-negative mutant in the Cre gel lineage-tracing model also reduced adipogenesis of Cre-marked cells in the context of IGF1 modRNA plus MI (Figure 5F–H). Indeed, the effect of the dominant-negative IGF1R inhibitors was slightly more pronounced in the Cre gel lineage-tracing model most likely because most cells that take up 1 modRNA when cotransfected with multiple modRNAs will concurrently take up all of the modRNAs (online-only Data Supplement Figure VII). This effect would make lineage-traced and dominant-negative inhibitor expressing cells coincide more precisely in the modRNA cotransfection experiment (Figure 5F–H) compared with separate $Wt1^{CreERT2}$ labeling and modRNA inhibitor gel expression (Figure 5C–E). Together both lineage-tracing studies indicate that in the heart after MI, exogenous IGF1 signals act on EPDCs by IGF1R to stimulate their adipogenic differentiation.

To determine whether the IGF1 signaling axis is required for MI-induced adipogenic differentiation of EPDCs in the absence of exogenously added IGF1 stimulation, we first asked whether MI activates the IGF1-IGF1R signaling axis in EPDCs. Immunostaining of peri-infarct myocardium 1 day after MI showed that IGF1 is upregulated in epicardial cells (Figure 6A–B). This finding was further validated by quantitative reverse transcription PCR of IGF1 transcripts in peri-infarct tissue, which confirmed marked IGF1 upregulation (Figure 6C). Immunostaining for activated IGF1R (IGF1R phosphorylated on tyrosine 1161) likewise demonstrated its strong upregulation in EPDCs after MI (Figure 6D). We investigated hypoxia as a potential trigger for IGF1R activation in EPDCs by culturing EPDCs in normoxic or hypoxic conditions in the presence or absence of exogenous IGF1 (online-only Data Supplement Figure VIII). In the absence of exogenous IGF1, hypoxia had little effect on total or activated IGF1R. However, the addition of exogenous IGF1 in combination with hypoxia increased IGF1R activation, even in the face of decreased total IGF1R. These data indicate that hypoxia strengthens IGF1R activation in EPDCs, suggesting a potential mechanism for the observed interaction between IGF1 and MI in inducing adipogenesis.

Next, we induced MI without the addition of IGF1 modRNA and used modRNA gel to both Cre label EPDCs and express IGF1R-DN or IRS1-DN, thereby selectively inhibiting IGF1R signaling in CGLCs, including EPDCs. IGF1R inhibition reduced the fraction of hearts with grossly evident EAT (Figure 6E) and reduced the expression of adipogenic markers (Figure 6F). Evaluation of the fate of CGLCs by confocal imaging showed that infrequent Perilipin A^+ adipocytes expressed the lineage tracer in hearts after MI with intact IGF1R signaling (Luc modRNA gel treatment; arrows, Figure 6G, top panel). Inhibition of IGF1R signaling in CGLCs with either IGF1R-DN or IRS1-DN abolished their differentiation into EAT (Figure 6G, bottom 2 panels). This finding was confirmed by quantitative analysis of imaging data, which showed that the fraction of adipocytes derived from CGLCs (Figure 6H)

Figure 3 Continued. culture with or without IGF1, gene expression was measured by qRTPCR and displayed as a ratio of expression in IGF1 stimulated to unstimulated cells. Dotted line indicates no change in expression (ratio=1; n=3, ** $P<0.01$, *** $P<0.001$, *t* test). **E,** Expression of Wt1 and IGFR1 relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in human primary EPDCs. Gene expression was measured by qRTPCR. **F** and **G,** IGF1 stimulated adipogenesis in human EPDCs. Negative control, standard media; positive control, adiopogenic media. Fixed cells were stained with Oil red O. Oil red O signal was quantitated as described for panel (**C**).

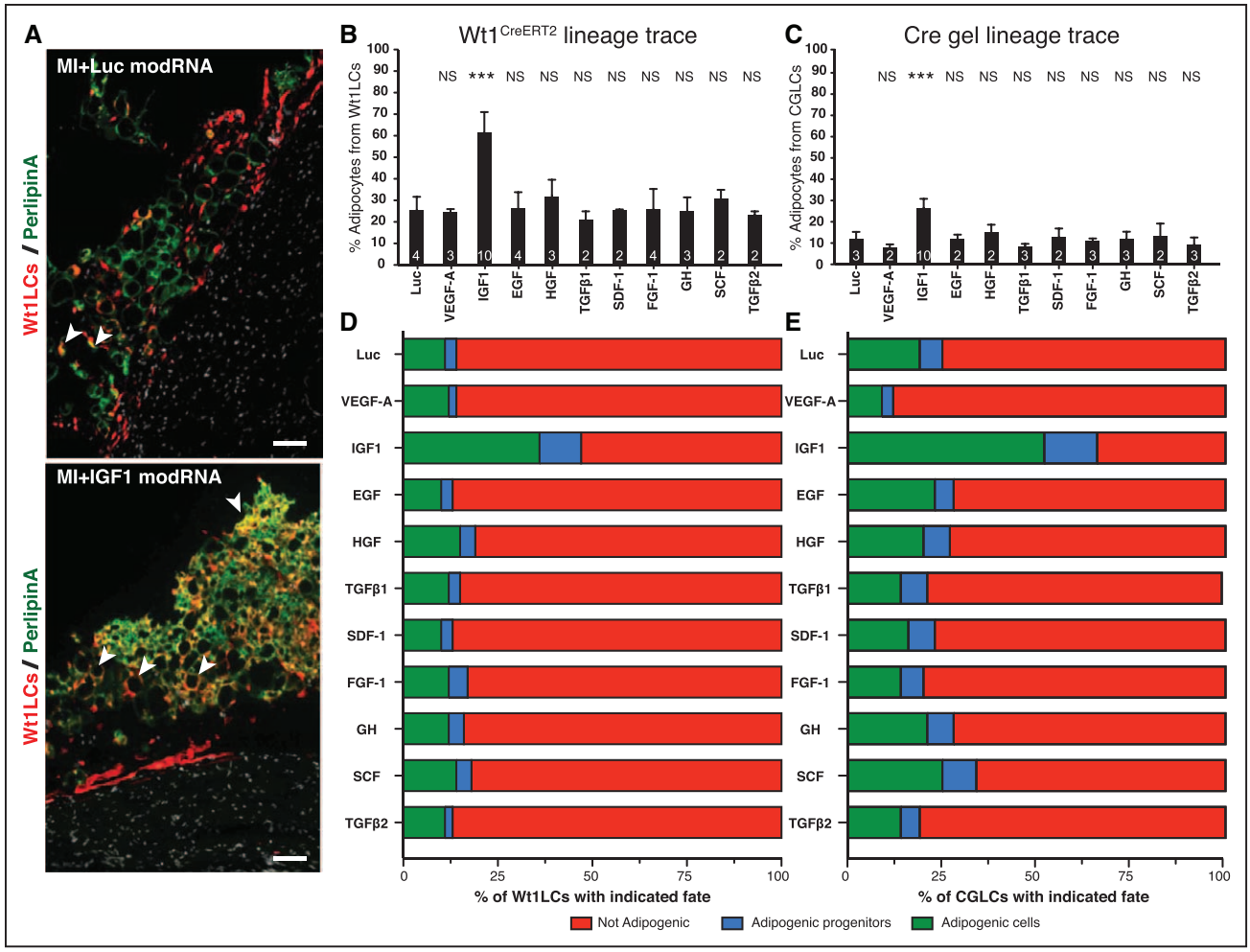

Figure 4. Insulin-like growth factor 1 (IGF1) modified RNA (modRNA) stimulated adipogenic differentiation of epicardial progenitors.

Epicardium-derived cells (EPDCs) were genetically labeled by red fluorescent protein by either treating $Wt1^{CreERT2/+}$; $Rosa26^{Tomato/+}$ mice with tamoxifen or treating $Rosa26^{Tomato/+}$ mice with Cre modRNA gel. Myocardial infarction (MI) was then induced and the fate of EPDCs was determined 4 weeks after MI by confocal imaging of immunostained sections. **A,** Representative confocal images of $Wt1^{CreERT2/+}$ lineage tracing. Arrowheads indicate examples of adipocytes coexpressing EPDC lineage tracer. **B** and **C,** The percentage of mature adipocytes ($CD24^{-}$ Perilipin A^{+}) that coexpressed the EPDC lineage tracer was quantified (*** $P<0.001$, *t* test). **D** and **E,** Quantification of the percentage of EDPCs that coexpressed markers of adipogenic progenitors ($CD24^{+}$ Perilipin A^{-}) and mature adipocytes ($CD24^{-}$ Perilipin A^{+}). Numbers in bars in (**B** and **C**) indicate sample sizes for (**B** through **E**).

and the fraction of labeled CGLCs that expressed adipocyte markers (Figure 6I) were strongly decreased by IGF1R inhibition. Because EPDCs are the predominant constituent of CGLCs, together these data suggest that IGF1R signaling is activated in EPDCs after MI and is required for MI-induced EPDC differentiation into adipocytes.

DISCUSSION

Our work illustrates the power of modRNA paracrine factor libraries to discover novel in vivo functions of these factors as regulators of progenitor cell fate. By enabling the delivery of pulses of a panel of paracrine factor at the relevant time and place in an in vivo disease context, this approach allows rapid identification of signaling pathways that regulate tissue injury responses in vivo. Here, we used a modRNA paracrine factor screen to identify IGF1 as a factor required for EAT formation.

EAT has an intimate anatomic relationship to the coronary vasculature and myocardium. Epidemiological studies demonstrate that EAT is closely related to coronary artery disease, suggesting that paracrine or metabolic signaling between EAT and the coronary vessels and myocardium influence the evolution of atherosclerotic lesions and heart disease.[1–3] Our work shows that IGF1 receptor signaling drives EAT formation by directing progenitor cells into the adipocyte lineage in the context of MI. This function is consistent with the proadipogenic ac-

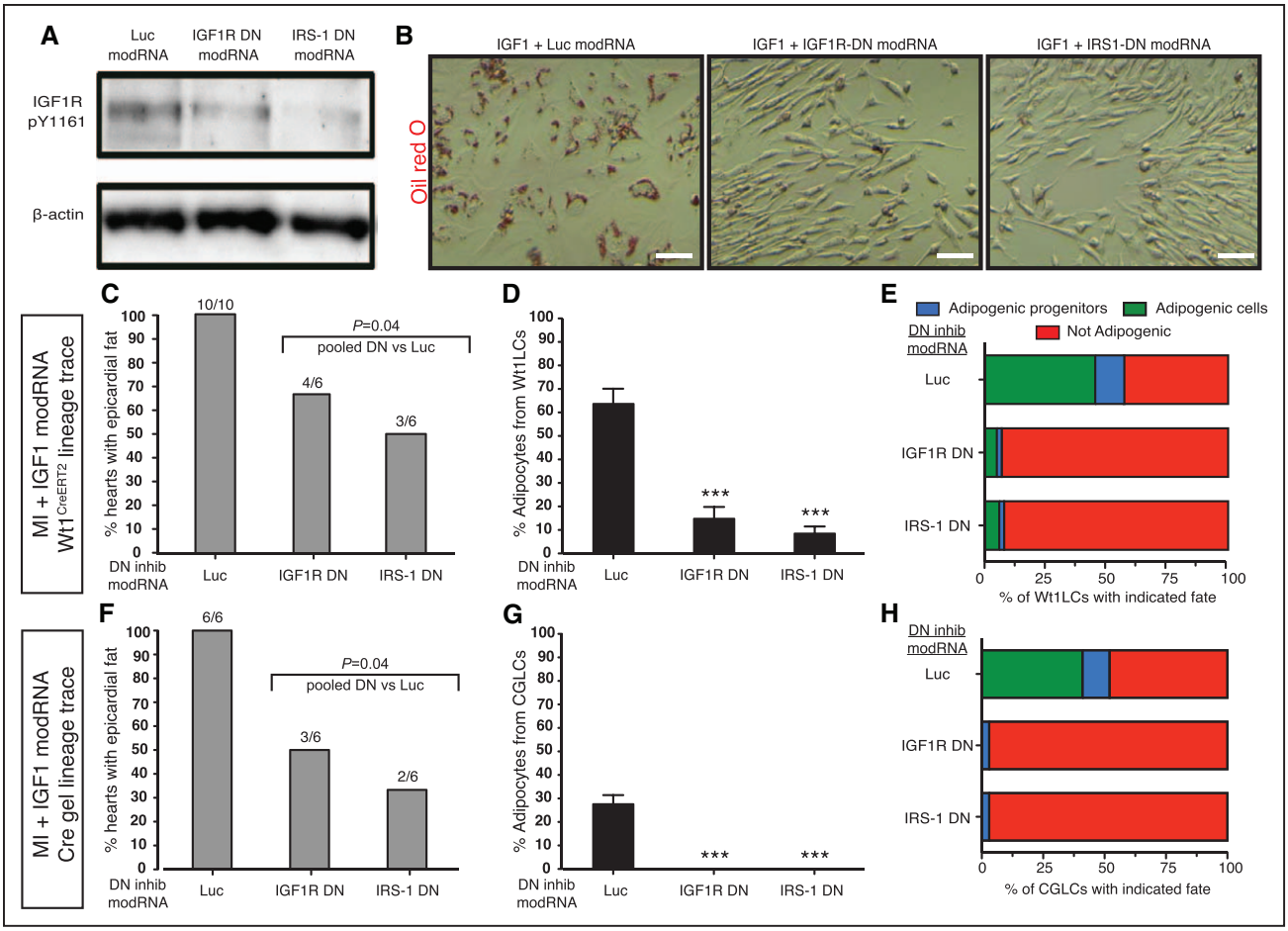

Figure 5. Insulin-like growth factor 1 receptor (IGF1R) signaling is required for epicardium-derived cells (EPDC) differentiation into adipocytes.

A, Primary cultured EPDCs were transfected with Luc (control), IGF1R-DN, or IRS1-DN factor-modified RNAs (modRNAs) and then cultured with IGF1 protein. After 24 hours, cell lysates were immunoblotted for activated IGF1R (IGF1R phosphorylated on tyrosine 1161) and β-actin. **B,** Cultured EPDCs were repeatedly transfected with Luc (control), IGF1R-DN, or IRS1-DN modRNAs and stimulated with IGF1 protein for 28 days. Cells were then stained for lipid accumulation (Oil red O). Representative images are displayed. Quantitation is shown in online-only Data Supplement Figure VI. **C** through E**,** After genetically labeling EPDCs by activating Rosa26^{Tomato} with Wt1^{CreERT2} and tamoxifen pulse, mice underwent MI operation, injection of IGF1 modRNA, and application of gel containing the indicated modRNA. Graphs show the percentage of hearts with grossly visible epicardial fat (**C**; numbers above bars indicate fat-positive and all hearts), the percentage of mature adipocytes (CD24$^-$ Perilipin A$^+$) that coexpress the EPDC lineage tracer (**D**), and the percentage of EPDCs that differentiated into adipogenic progenitors (CD24$^+$ Perilipin A$^-$) or mature adipocytes (**E**). Positive hearts in (**C**) were analyzed in (**D** and **E**). **F** through **H,** Mice underwent MI operation, injection of IGF1 modRNA, and application of gel containing Cre modRNA plus the indicated modRNA. Experimental readouts were as described in (**C** through **E**).

tivity of IGF1 on preadipocytes and mesenchymal stem cells.[29]

The IGF1 pathway has been linked to cardioprotection[30] and enhanced cardiomyocyte differentiation of cardiac progenitor cells.[31] Our results delineate potential negative consequences of IGF1 pathway activation and will need to be considered when evaluating therapeutic interventions based on manipulating the IGF1 pathway. However, because EAT shares common developmental origins with other types of visceral fat,[19] IGF1 may also control expansion of other visceral fat depots. Targeting such signaling pathways or stabilizing mesothelial cells in their epithelial state may be novel approaches to treating the burgeoning problems of ischemic heart disease and obesity.

Consistent with prior studies,[19–21] our work indicated that epicardial progenitors are a source of EAT. This result was supported by 2 independent lineage-tracing strategies, both of which pointed to EPDCs as the IGF1-responsive adipogenic progenitor (Figure 7): IGF1 receptor activation increased their MI-induced differentiation into adipocytes, whereas IGF1 receptor inhibition prevented this lineage conversion. The adipogenic effect of IGF1 was conserved in humans EPDCs. Thus, IGF1

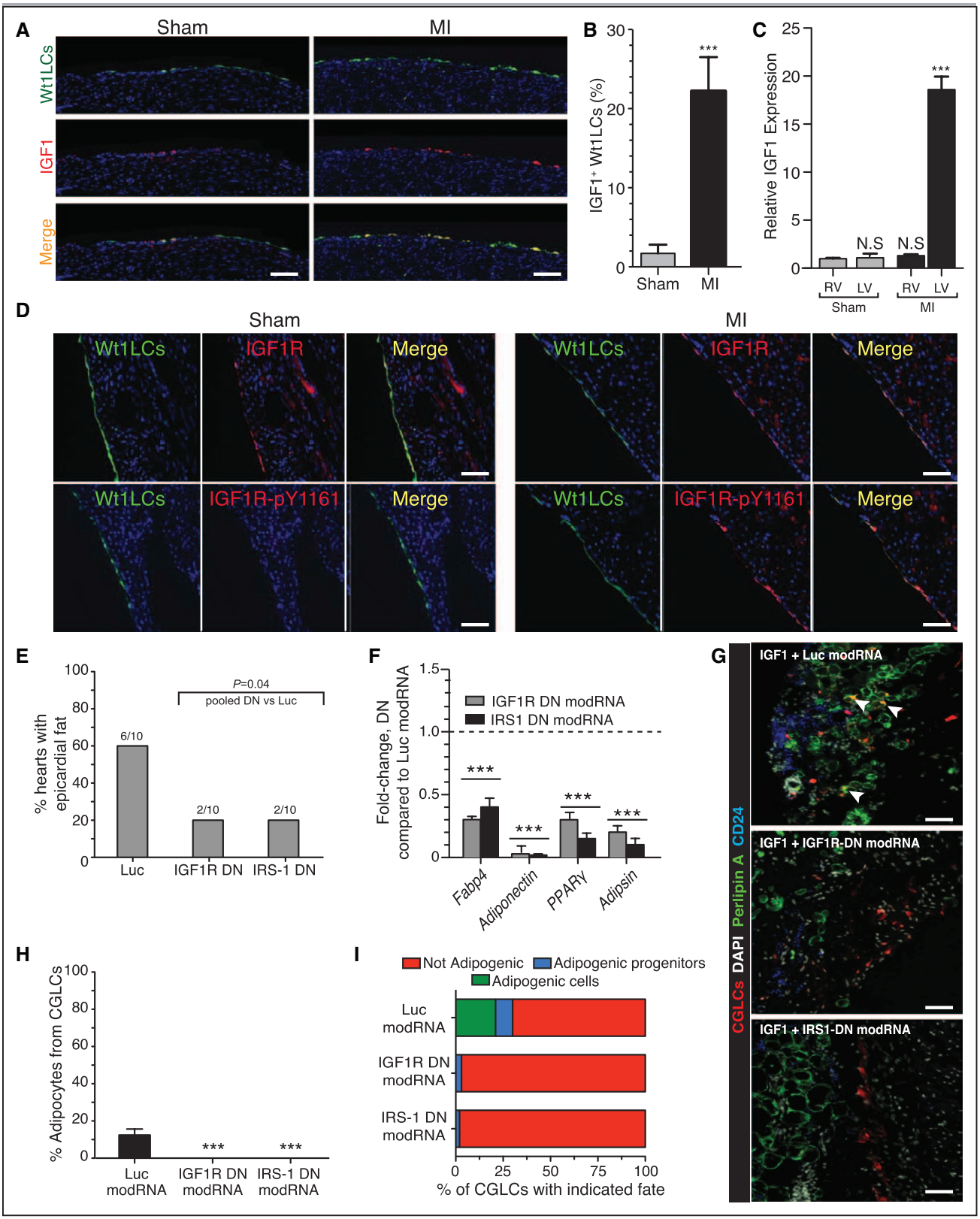

Figure 6. Endogenous insulin-like growth factor 1 receptor (IGF1R) activation is required for myocardial infarction (MI)-induced epicardium-derived cells (EPDC) to fat differentiation.
A through **D,** Wt1$^{CreERT2/+}$ Rosa26^{Tomato} mice were induced with tamoxifen and then underwent sham or MI operation. The next day, hearts were fixed, sectioned, and stained for the Wt1LC genetic marker and IGF1 protein (**A**). The fraction of Wt1LCs coexpressing IGF1 were quantitated (**B**). Peri-infarct LV or remote RV tissue underwent quantitative reverse transcription polymerase chain reaction (qRTPCR) to measure the IGF1 transcript levels 1 day after MI (**C**). Peri-infarct myocardium was stained *(Continued)*

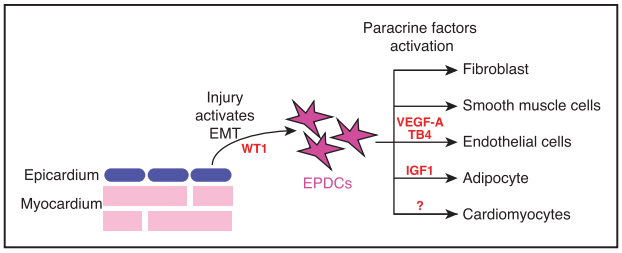

Figure 7. Paracrine factor switches guide the fate of cardiac progenitor cells.
Myocardial infarction (MI) induces epicardial epithelial to mesenchymal transition (EMT) to form epicardium-derived cells (EPDCs), a process that requires WT1. EPDCs have lineage plasticity and endogenously tend to differentiate into fibroblasts, smooth muscle cells, and myofibroblasts. Vascular endothelial growth factor A (VEGFA)[8] and Thymosin B4 (TB4)[32] guide EPDCs to differentiate into endothelial cells,[33] and here we show that insulin-like growth factor 1 (IGF1) guides EPDCs to differentiate into adipocytes. Cardiomyocytes may be an alternative fate of EPDCs,[6,26,34,35] although this notion remains controversial.[36,37]

signaling is a physiological switch that promotes adipogenic differentiation of epicardial progenitor cells.

It is important to note that the IGF1-driven progenitor fate switch appeared to be operative only within a brief time window after MI because IGF1 activation did not stimulate adipogenic differentiation of progenitor cells in the absence of MI, and transient inhibition of IGF1 receptor signaling with modRNA-mediated expression of dominant negative proteins was sufficient to suppress MI-induced differentiation into adipocytes. The requirement for MI in combination with IGF1 to stimulate progenitor differentiation into adipocytes may result from 2 factors. First, our data suggest that IGF1 more strongly activates IGF1R under hypoxic conditions. Second, MI activation of progenitors may be a prerequisite for their differentiation into adipocytes. MI activates epicardial EMT, which transforms epicardial cells from epithelial cells into mesenchymal cells with greater developmental plasticity.[7] Supporting this conclusion, *Wt1* is required for epicardial EMT,[23,24] and MI-induced adipogenesis likewise required *Wt1* (Figure 7).

Infiltration of myocardium by adipocytes is also a hallmark of arrhythmogenic cardiomyopathy, most often caused by mutations in desmosome proteins.[38] Presently, the origin of this adipose tissue is controversial, and our studies suggest the possibility that desmosomal mutations alter the behavior of epicardial progenitors and direct them to migrate into the myocardium and form adipocytes. The epicardium to endocardium direction of adipogenic involvement in this disease would be consistent with this possibility. Lombardi et al[39] showed that adipogenic cells in a mouse model of this disease are marked by Isl1Cre. Although this finding was interpreted as demonstrating a second heart field origin of the adiogenic cells, Isl1Cre also marks epicardial cells,[40] and thus the results are consistent with epicardial contribution to adipogenesis in arrhythmogenic cardiomyopathy.

Delineating lineage relationships between populations with Cre labeling approaches is vulnerable to misinterpretation. In this study, we showed that the IGF1R-responsive adipogenic progenitor population is labeled by both $Wt1^{CreERT2}$ and Cre modRNA. Within the heart, EPDCs are the population that would be labeled by both techniques. However, the MI model distorts normal tissue planes, removes portions of the pericardium, and can cause cardiac adhesions to surrounding tissues. In this setting, noncardiac $Wt1^{+}$ cells from surrounding tissues, such as the mesothelial lining of the chest wall, could contact and infiltrate the heart. Such cells may also be labeled by Cre modRNA gel. The adipocytes in our study had characteristics of EAT, including expression of the brown fat marker UCP1, perfusion by coronary vessels, and covering by epicardium, which excludes mistaking simple pericardial adhesions for EAT. However, we cannot fully exclude the possibility that $Wt1^{+}$ cells lining the surface of other parts of the chest cavity could have infiltrated EAT and differentiated into EAT-like adipocytes.

ACKNOWLEDGMENTS

The authors thank V. Huff, University of Texas MD Anderson Cancer Center, for the $Wt1^{flox}$ mouse line, and Drs Marie Jose Goumans and Adriana Gittenberger-de Groot at Leiden University Medical Center for providing human EPDCs. The authors thank Aibin He, Alexander von Gise, Sean Stevens, Valentin Fuster, and Roger Hajjar for their contributions to this project. The authors also thank Lauren Drowley and Anna Jonebring for their helpful advice regarding huEPDC cell culture.

Figure 6 Continued. for the Wt1LC genetic marker and IGF1R or activated IGF1R (IGF1R-pY1161; **D**). MI upregulated IGF1 expression and activated IGF1R in EPDCs. **E** through **I,** $Rosa26^{Tomato/+}$ mice underwent MI operation and were treated with modRNA containing Cre and either IGF1R-DN, IRS-1-DN, or Luc (negative control). The percentage of hearts with grossly evident EAT was determined 28 days later (**E**). Numbers above bar indicate fat positive and all hearts. Inhibition of IGF1R signaling significantly decreased the frequency of epicardial adipose tissue (EAT) (P=0.04, Fisher exact test using pooled DN modRNA groups compared with Luc control). LV myocardial expression of adipocyte marker genes in hearts treated with DN modRNAs compared with Luc modRNA was measured by qRTPCR (**F**; dotted line indicates level in Luc control) (n=3, ***P<0.001, t test). Heart sections were imaged with a confocal microscope. Representative images are shown in (**G**). Arrowheads indicate adipocytes coexpressing the Cre gel-activated lineage tracer. Bar = 10 μm. The fraction of perilipin A^{+} adipocytes that coexpressed the Cre gel lineage cells (CGLC) genetic marker (**H**) and the fraction of CGLCs that differentiated into mature adipocytes, adipogenic progenitors, or nonadipocytes (**I**) was quantified (***P<0.001, t test).

SOURCES OF FUNDING

Dr Pu was supported by National Institutes of Health grants R01 HL094683 and U01 HL100401, an Established Investigator Award from the American Heart Association, and charitable contributions from Dr and Mrs Edwin A. Boger. Dr Zangi was supported by a postdoctoral fellowship from the American Heart Association. Dr Chien was supported by the Wallenberg Foundation, the Karolinska Institute-AstraZeneca Integrated Cardio Metabolic Center, and a Distinguished Professorship of the Swedish Research Council. Dr Zangi was also supported by seed package funds from the Cardiovascular Research Center, Icahn School of Medicine at Mt. Sinai, New York.

DISCLOSURES

Dr Chien is Chair of the External Science Panel for AstraZeneca and Co-Founder of Moderna Therapeutics, which have financial interest in modified RNAs. Drs Später and Wang are employees of AstraZeneca.

Dr Zangi designed and performed experiments, analyzed the data, and wrote the manuscript. Drs Oliveira and Ye contributed equally. Drs Oliveira, Ye, and Sultana designed, performed, and analyzed quantitative reverse transcription PCR and immunostaining experiments. Dr Ye also performed animal husbandry and cardiac perfusion. Dr Ma carried out all mouse surgery. Drs Später and Wang performed experiments on human EPDCs. Dr Zhou performed microarray analysis of EPDCs after MI. Dr Chew performed molecular biology experiments, and Dr Abrial performed and analyzed immunostaining data. Dr Pu designed experiments, analyzed data, and wrote the manuscript. Dr Chien contributed to the design of experiments, data review, and manuscript writing.

AFFILIATIONS

From Cardiovascular Research Center, Department of Genetics and Genomic Sciences, and Black Family Stem Cell Institute, Icahn School of Medicine at Mount Sinai, New York (L.Z., N.S., Y.H.); Department of Cardiology, Boston Children's Hospital, MA (L.Z., M.S.O., L.Y.Y., Q.M., W.T.P.); Cardiovascular and Metabolic Diseases Innovative Medicine Biotech Unit, AstraZeneca, Mölndal, Sweden (D.S., Q.-D.W.); The State Key Laboratory of Cell Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (B.Z.); Department of Genetics (W.L.C.), Harvard Stem Cell Institute (W.E., W.T.P.), Harvard Medical School, and Cardiovascular Research Center, Massachusetts General Hospital (M.A.), Harvard Medical School, Boston, MA; and Department of Cell and Molecular Biology and Medicine, Karolinska Institutet, Stockholm, Sweden (K.R.C.).

FOOTNOTES

Received August 31, 2015; accepted October 8, 2016.

The online-only Data Supplement is available with this article at http://circ.ahajournals.org/lookup/suppl/doi:10.1161/CIRCULATIONAHA.116.022064/-/DC1.

Circulation is available at http://circ.ahajournals.org.

REFERENCES

1. Corradi D, Maestri R, Callegari S, Pastori P, Goldoni M, Luong TV, Bordi C. The ventricular epicardial fat is related to the myocardial mass in normal, ischemic and hypertrophic hearts. *Cardiovasc Pathol*. 2004;13:313–316. doi: 10.1016/j.carpath.2004.08.005.
2. Cherian S, Lopaschuk GD, Carvalho E. Cellular cross-talk between epicardial adipose tissue and myocardium in relation to the pathogenesis of cardiovascular disease. *Am J Physiol Endocrinol Metab*. 2012;303:E937–E949. doi: 10.1152/ajpendo.00061.2012.
3. Greif M, Becker A, von Ziegler F, Lebherz C, Lehrke M, Broedl UC, Tittus J, Parhofer K, Becker C, Reiser M, Knez A, Leber AW. Pericardial adipose tissue determined by dual source CT is a risk factor for coronary atherosclerosis. *Arterioscler Thromb Vasc Biol*. 2009;29:781–786. doi: 10.1161/ATVBAHA.108.180653.
4. Wilm B, Ipenberg A, Hastie ND, Burch JB, Bader DM. The serosal mesothelium is a major source of smooth muscle cells of the gut vasculature. *Development*. 2005;132:5317–5328. doi: 10.1242/dev.02141.
5. von Gise A, Pu WT. Endocardial and epicardial epithelial to mesenchymal transitions in heart development and disease. *Circ Res*. 2012;110:1628–1645. doi: 10.1161/CIRCRESAHA.111.259960.
6. Zhou B, Ma Q, Rajagopal S, Wu SM, Domian I, Rivera-Feliciano J, Jiang D, von Gise A, Ikeda S, Chien KR, Pu WT. Epicardial progenitors contribute to the cardiomyocyte lineage in the developing heart. *Nature*. 2008;454:109–113. doi: 10.1038/nature07060.
7. Zhou B, Honor LB, He H, Ma Q, Oh JH, Butterfield C, Lin RZ, Melero-Martin JM, Dolmatova E, Duffy HS, Gise Av, Zhou P, Hu YW, Wang G, Zhang B, Wang L, Hall JL, Moses MA, McGowan FX, Pu WT. Adult mouse epicardium modulates myocardial injury by secreting paracrine factors. *J Clin Invest*. 2011;121:1894–1904. doi: 10.1172/JCI45529.
8. Zangi L, Lui KO, von Gise A, Ma Q, Ebina W, Ptaszek LM, Später D, Xu H, Tabebordbar M, Gorbatov R, Sena B, Nahrendorf M, Briscoe DM, Li RA, Wagers AJ, Rossi DJ, Pu WT, Chien KR. Modified mRNA directs the fate of heart progenitor cells and induces vascular regeneration after myocardial infarction. *Nat Biotechnol*. 2013;31:898–907. doi: 10.1038/nbt.2682.
9. Gao F, Maiti S, Alam N, Zhang Z, Deng JM, Behringer RR, Lécureuil C, Guillou F, Huff V. The Wilms tumor gene, Wt1, is required for Sox9 expression and maintenance of tubular architecture in the developing testis. *Proc Natl Acad Sci U S A*. 2006;103:11987–11992. doi: 10.1073/pnas.0600994103.
10. Sohal DS, Nghiem M, Crackower MA, Witt SA, Kimball TR, Tymitz KM, Penninger JM, Molkentin JD. Temporally regulated and tissue-specific gene manipulations in the adult and embryonic heart using a tamoxifen-inducible Cre protein. *Circ Res*. 2001;89:20–25.
11. Madisen L, Zwingman TA, Sunkin SM, Oh SW, Zariwala HA, Gu H, Ng LL, Palmiter RD, Hawrylycz MJ, Jones AR, Lein ES, Zeng H. A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. *Nat Neurosci*. 2010;13:133–140. doi: 10.1038/nn.2467.
12. Muzumdar MD, Tasic B, Miyamichi K, Li L, Luo L. A global double-fluorescent Cre reporter mouse. *Genesis*. 2007;45:593–605. doi: 10.1002/dvg.20335.
13. Tarnavski O, McMullen JR, Schinke M, Nie Q, Kong S, Izumo S. Mouse cardiac surgery: comprehensive techniques for the generation of mouse models of human diseases and their application for genomic studies. *Physiol Genomics*. 2004;16:349–360. doi: 10.1152/physiolgenomics.00041.2003.
14. Warren L, Manos PD, Ahfeldt T, Loh YH, Li H, Lau F, Ebina W, Mandal PK, Smith ZD, Meissner A, Daley GQ, Brack AS, Collins JJ,

Cowan C, Schlaeger TM, Rossi DJ. Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. *Cell Stem Cell*. 2010;7:618–630. doi: 10.1016/j.stem.2010.08.012.
15. Lui KO, Zangi L, Silva EA, Bu L, Sahara M, Li RA, Mooney DJ, Chien KR. Driving vascular endothelial cell fate of human multipotent Isl1+ heart progenitors with VEGF modified mRNA. *Cell Res*. 2013;23:1172–1186. doi: 10.1038/cr.2013.112.
16. Meirelles Lda S, Nardi NB. Murine marrow-derived mesenchymal stem cell: isolation, *in vitro* expansion, and characterization. *Br J Haematol*. 2003;123:702–711.
17. Anjos-Afonso F, Siapati EK, Bonnet D. *In vivo* contribution of murine mesenchymal stem cells into multiple cell-types under minimal damage conditions. *J Cell Sci*. 2004;117(Pt 23):5655–5664. doi: 10.1242/jcs.01488.
18. Bax NA, van Oorschot AA, Maas S, Braun J, van Tuyn J, de Vries AA, Groot AC, Goumans MJ. *In vitro* epithelial-to-mesenchymal transformation in human adult epicardial cells is regulated by TGFβ-signaling and WT1. *Basic Res Cardiol*. 2011;106:829–847. doi: 10.1007/s00395-011-0181-0.
19. Chau YY, Bandiera R, Serrels A, Martínez-Estrada OM, Qing W, Lee M, Slight J, Thornburn A, Berry R, McHaffie S, Stimson RH, Walker BR, Chapuli RM, Schedl A, Hastie N. Visceral and subcutaneous fat have different origins and evidence supports a mesothelial source. *Nat Cell Biol*. 2014;16:367–375. doi: 10.1038/ncb2922.
20. Liu Q, Huang X, Oh JH, Lin RZ, Duan S, Yu Y, Yang R, Qiu J, Melero-Martin JM, Pu WT, Zhou B. Epicardium-to-fat transition in injured heart. *Cell Res*. 2014;24:1367–1369. doi: 10.1038/cr.2014.125.
21. Yamaguchi Y, Cavallero S, Patterson M, Shen H, Xu J, Kumar SR, Sucov HM. Adipogenesis and epicardial adipose tissue: a novel fate of the epicardium induced by mesenchymal transformation and PPARγ activation. *Proc Natl Acad Sci U S A*. 2015;112:2070–2075. doi: 10.1073/pnas.1417232112.
22. Samulin J, Lien S, Grindflek E, Berget I, Ruyter B, Sundvold H. Depot specific differences during adipogenesis of porcine stromal-vascular cells. *Cell Biol Int*. 2008;32:525–531. doi: 10.1016/j.cellbi.2008.01.001.
23. von Gise A, Zhou B, Honor LB, Ma Q, Petryk A, Pu WT. WT1 regulates epicardial epithelial to mesenchymal transition through β-catenin and retinoic acid signaling pathways. *Dev Biol*. 2011;356:421–431. doi: 10.1016/j.ydbio.2011.05.668.
24. Moore AW, McInnes L, Kreidberg J, Hastie ND, Schedl A. YAC complementation shows a requirement for Wt1 in the development of epicardium, adrenal gland and throughout nephrogenesis. *Development*. 1999;126:1845–1857.
25. Chau YY, Brownstein D, Mjoseng H, Lee WC, Buza-Vidas N, Nerlov C, Jacobsen SE, Perry P, Berry R, Thornburn A, Sexton D, Morton N, Hohenstein P, Freyer E, Samuel K, van't Hof R, Hastie N. Acute multiple organ failure in adult mice deleted for the developmental regulator Wt1. *PLoS Genet*. 2011;7:e1002404. doi: 10.1371/journal.pgen.1002404.
26. Cai CL, Martin JC, Sun Y, Cui L, Wang L, Ouyang K, Yang L, Bu L, Liang X, Zhang X, Stallcup WB, Denton CP, McCulloch A, Chen J, Evans SM. A myocardial lineage derives from Tbx18 epicardial cells. *Nature*. 2008;454:104–108. doi: 10.1038/nature06969.
27. Prager D, Li HL, Asa S, Melmed S. Dominant negative inhibition of tumorigenesis *in vivo* by human insulin-like growth factor I receptor mutant. *Proc Natl Acad Sci U S A*. 1994;91:2181–2185.
28. Tanaka S, Wands JR. A carboxy-terminal truncated insulin receptor substrate-1 dominant negative protein reverses the human hepatocellular carcinoma malignant phenotype. *J Clin Invest*. 1996;98:2100–2108. doi: 10.1172/JCI119016.
29. MacDougald OA, Mandrup S. Adipogenesis: forces that tip the scales. *Trends Endocrinol Metab*. 2002;13:5–11.
30. McMullen JR, Izumo S. Role of the insulin-like growth factor 1 (IGF1)/phosphoinositide-3-kinase (PI3K) pathway mediating physiological cardiac hypertrophy. *Novartis Found Symp*. 2006;274:90–111.
31. Birket MJ, Ribeiro MC, Verkerk AO, Ward D, Leitoguinho AR, den Hartogh SC, Orlova VV, Devalla HD, Schwach V, Bellin M, Passier R, Mummery CL. Expansion and patterning of cardiovascular progenitors derived from human pluripotent stem cells. *Nat Biotechnol*. 2015;33:970–979. doi: 10.1038/nbt.3271.
32. Smart N, Risebro CA, Melville AA, Moses K, Schwartz RJ, Chien KR, Riley PR. Thymosin beta4 induces adult epicardial progenitor mobilization and neovascularization. *Nature*. 2007;445:177–182. doi: 10.1038/nature05383.
33. Tian X, Pu WT, Zhou B. Cellular origin and developmental program of coronary angiogenesis. *Circ Res*. 2015;116:515–530. doi: 10.1161/CIRCRESAHA.116.305097.
34. Smart N, Bollini S, Dubé KN, Vieira JM, Zhou B, Davidson S, Yellon D, Riegler J, Price AN, Lythgoe MF, Pu WT, Riley PR. De novo cardiomyocytes from within the activated adult heart after injury. *Nature*. 2011;474:640–644. doi: 10.1038/nature10188.
35. Zhou B, Pu WT. Genetic Cre-loxP assessment of epicardial cell fate using Wt1-driven Cre alleles. *Circ Res*. 2012;111:e276–e280. doi: 10.1161/CIRCRESAHA.112.275784.
36. Christoffels VM, Grieskamp T, Norden J, Mommersteeg MT, Rudat C, Kispert A. Tbx18 and the fate of epicardial progenitors. *Nature*. 2009;458:E8–E9. doi: 10.1038/nature07916.
37. Rudat C, Kispert A. Wt1 and epicardial fate mapping. *Circ Res*. 2012;111:165–169. doi: 10.1161/CIRCRESAHA.112.273946.
38. Iyer VR, Chin AJ. Arrhythmogenic right ventricular cardiomyopathy/dysplasia (ARVC/D). *Am J Med Genet C Semin Med Genet*. 2013;163C:185–197. doi: 10.1002/ajmg.c.31368.
39. Lombardi R, Dong J, Rodriguez G, Bell A, Leung TK, Schwartz RJ, Willerson JT, Brugada R, Marian AJ. Genetic fate mapping identifies second heart field progenitor cells as a source of adipocytes in arrhythmogenic right ventricular cardiomyopathy. *Circ Res*. 2009;104:1076–1084. doi: 10.1161/CIRCRESAHA.109.196899.
40. Zhou B, von Gise A, Ma Q, Rivera-Feliciano J, Pu WT. Nkx2-5- and Isl1-expressing cardiac progenitors contribute to proepicardium. *Biochem Biophys Res Commun*. 2008;375:450–453. doi: 10.1016/j.bbrc.2008.08.044.

Insulin-Like Growth Factor 1 Receptor-Dependent Pathway Drives Epicardial Adipose Tissue Formation After Myocardial Injury

Lior Zangi, Marcela S. Oliveira, Lillian Y. Ye, Qing Ma, Nishat Sultana, Yoav Hadas, Elena Chepurko, Daniela Später, Bin Zhou, Wei Leong Chew, Wataru Ebina, Maryline Abrial, Qing-Dong Wang, William T. Pu and Kenneth R. Chien

Circulation. 2017;135:59-72; originally published online November 1, 2016;
doi: 10.1161/CIRCULATIONAHA.116.022064
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 0009-7322. Online ISSN: 1524-4539

SUPPLEMENTAL MATERIAL

Expanded Methods

Mice. All animal procedures were performed under protocols approved by the Boston Children's Hospital Institutional Care and Use Committee. $Wt1^{CreERT2}$, $Wt1^{GFPCre/+}$, $Wt1^{flox/flox}$, $\alpha MHC^{MerCreMer}$, $R26^{Tomato}$, and $R26^{mTmG}$ alleles have been described previously.[1-5] Genetically engineered mice were in a mixed background. Both male and female mice were used. Wild-type CFW mice were used to measure adipogenic gene expression 10 days after MI. Tamoxifen free base (Tam) was dissolved in sunflower seed oil at 12 mg/ml by sonication. 0.12 mg/g body weight Tam was administered to adult mice twice weekly for 3 weeks to induce CreERT2-mediated recombination. One week after completion of Tam dosing (to allow Tam clearance), the left anterior descending coronary artery was ligated to induce MI.[6] In order to determine the effect of different modRNAs on EPDCs differentiation outcome after MI, paracrine factor modRNAs (100 μg/heart, Luc modRNA serve as control) were injected into the infarct zone myocardium immediately after LAD ligation.[7] Procedures and measurements were performed blinded to genotype and treatment group. All animals that started an experimental protocol and that survived to the measurement point were included.

Construction of IVT templates and synthesis of modRNA. ModRNAs were in vitro transcribed from plasmid templates as described previously [7-9] Briefly, PCR amplified open reading frames(see Supplementary Table 1 for sequences) were subclonedusing the pcDNA 3.3-TOPO TA cloning kit (Life Technologies). Plasmid inserts were excised by restriction digestion, recovered with SizeSelect gels (Life Technologies), and subject to polyA tailing PCR. The MEGAscript T7 kit (Ambion) was used to transcribed 1.6 μg of purified tail PCR productin a 40 μl reaction containing a custom ribonucleoside blend of 3'-O-Me-m7G(5')ppp(5')G cap analog (6 mM, New England Biolabs), guanosine triphosphate (1.5 mM, USB), adenosine triphosphate (7.5 mM, USB), and 5-methylcytidine triphosphate and pseudouridine triphosphate (7.5 mM, TriLink Biotechnologies). RNA was purified with AmbionMEGAclear spin columns and then treated with Antarctic Phosphatase (New England Biolabs) for 30 min at 37ºC. After repurification, RNA was quantitated by Nanodrop (Thermo Scientific) and precipitated with ethanol and Ammonium Acetate. ModRNA was resuspended in 10 mMTrisHCl, 1 mM EDTA at 100 ng/μl for in vitro use or 10-30 μg/μl for in vivo use.

modRNA transfection. modRNA and RNAiMAX (Life Technologies) were separately dissolved in Opti-MEM (Life Technologies), combined, and then incubated for 15 minutes at

room temperature to generate the transfection mixture. 5 or 0.5 μl of RNAiMAX reagent was used for every microgram of modRNA for in vitro or in vivo transfection, respectively. In vitro transfection was performed by adding the transfection mixture to cells plated in DMEM with 2% FBS and 200 ng/ml B18R (eBioscience, San Diego, CA). For in vivo transfection the transfection mixture was injected directly into cardiac muscle.

modRNA gel. modRNA gel, was made by mixing Cre modRNA (10 μl modRNA at 20 μg/μl), Lipofectamine 2000 (30 μl, Life Technologies), and 0.05% polyacrylic acid (10 µl; Sigma) incubated for 15 minutes at room temperature to generate the gel transfection mixture. The gel was painted on the heart surface. For lineage tracing experiments (Figs. 1 and 3), Cre modRNA gel was applied 2 weeks before LAD ligation via a lower thoracotomy site. For combined IGF1R signaling inhibition with dominant negative mutants and Cre lineage tracing (Fig. 4), the modRNAs were premixed, assembled into the modRNA gel, and then applied to the surface of the heart at the time of LAD ligation.

Isolation and culture of EPDCs and mesenchymal stem cells. Mesenchymal stem cells (MSCs) were isolated from adult (6-8 weeks) CFW femurs as described previously.[10] Femurs were manually crushed and bone marrow cells were collected and sieved. Bone marrow cells were plated in 12 well plates (1 x 10^6 cells per well) for 2 days, allowing MSCs to adher. Isolated cells were cultured in StemXvivo Osteogenic/Adipogenic Base media (R&D system) with Penicillin-Streptomycin (1:100). Media was changed every 3-4 days. $WT1^+$ EPDCs were isolated from heart explants of $WT1^{GFPCre/+}$ mice 2 days after MI. Cardiac cells (non-myocytes) were allowed to expand from heart explant cultures. After 2 weeks, GFP^+ cells were isolated by FACS (FACS Aria III) and plated plated in fibronectin-coated (5 ng/ml for 2 hours at 37ºC) wells of a 12 well plate (70,000 cells per well). Culture media and media change was performed as for MSCs.

Human EPDCs were isolated from the atria of patients over the age of 45 who were undergoing heart operations for clinical indications. The EPDCs were isolated and cultured as described.[11] The EPDCs were used at 9 passages or less.

Adipogenic differentiation. For enhancement of adipocyte differentiation in MSCs or EPDCs, culture medium (StemXvivo Osteogenic/Adipogenic Base media) was supplemented with Adipogenic supplemental vial (R&D system, 1:20). Media was changed every 3-4 days. ModRNAs were transfected every 3-4 days during adipogenic differentiation. For detection of oil droplets, cultures were stained with saturated Oil red O solution (Sigma) in 60% isopropanol, 28 days after initiation of adipogenic differentiation, as described previously.[12] To quantitate oil red

O staining, plates were dried and extracted with 1 ml 100% isopropanol. After 10 minutes incubation with gentle shaking, the OD_{500} was recorded. For gene expression measurements, cells were collected after 10 days of treatment and analyzed for expression of adipogenic transcripts by qRTPCR.

Immunostaining. The peri-infarct zone near the apex was fixed in 4% PFA and embedded in OCT. Immunostaining was performed on cryosections using the antibodies listed in Supplementary Table 2. Quantification of immunostaining in cardiac sections was performed using ImageJ Software to analyze individual color channel intensity thresholds, image contours, and areas. Adipogenic differentiation of EPDCs was measured as both the fraction of adipocytes expressing the epicardial lineage tracer and the fraction of EPDCs that expressed adipocyte markers.

Isolectin B4 coronary perfusion was performed by isolating hearts and inserting a perfusion needle into the ascending aorta. Hearts were perfused with PBS, then PBS containing isolectin B4 conjugated to Alexa488 (Life Technologies), then 4% PFA.

RNA isolation and gene expression profiling. The peri-infarct zone near the apex was snap-frozen. Total RNA was isolated using the RNeasy mini kit (Qiagen) and reverse transcribed using Superscript III reverse transcriptase (Life Technologies). Real-time qPCR analyses were performed on a Mastercycler Realplex 4 Sequence Detector (Eppendoff) using SYBR Green (Quantitect SYBR Green PCR Kit, Qiagen). Fold-changes in gene expression were determined by the ∂∂CT method and were presented relative to *Gapdh* internal control. PCR primer sequences are shown in Supplementary Table 3. To characterize GFP^+ progenitors, total RNA was obtained from FACS-sorted GFP^+ cells, isolated after collagenase digestion of $WT1^{GFPCre/+}$ mice 2 days post-MI using a FACSAria III cell sorter.

Microarray gene expression profiling and analysis. $WT1^{GFPCre/+}$ mice underwent LAD ligation, and 7 days later the hearts were dissociated. GFP^+ EPDCs were isolated by GFP FACS. RNA was isolated and used to probe Affymetrix Gene 1.0 ST arrays (n=3). Gene expression values were determined using Affy Power Tools, and mean values were used to define the detectable gene expression threshold. Genes with gene ontology terms “receptor activity” or “plasma membrane” were manually curated to define a set of cell surface receptors.

Western blot. Western blotting was performed to measure phosphorylated IGF1R in EPDCs stimulated with IGF1 protein (PeproTech). Samples containing equal amounts of protein were separated by SDS-PAGE, transferred to nitrocellulose, probed with IGFR (pY-1161)

antibody (Abcam, 1:1000) followed by donkey anti-Rabbit HRP antibody, and visualized by chemo-luminescent detection.

Statistical analyses. Values are reported as mean ± standard error of the mean. Comparisons between groups were made using Welch's 2-tailed *t*-test (continuous variables) or Fisher's exact test (proportions).

References

1. Zhou B, Ma Q, Rajagopal S, Wu SM, Domian I, Rivera-Feliciano J, Jiang D, von Gise A, Ikeda S, Chien KR, and Pu WT. Epicardial progenitors contribute to the cardiomyocyte lineage in the developing heart. Nature. 2008;454:109-13.
2. Gao F, Maiti S, Alam N, Zhang Z, Deng JM, Behringer RR, Lecureuil C, Guillou F, and Huff V. The Wilms tumor gene, Wt1, is required for Sox9 expression and maintenance of tubular architecture in the developing testis. Proc Natl Acad Sci U S A. 2006;103:11987-92.
3. Sohal DS, Nghiem M, Crackower MA, Witt SA, Kimball TR, Tymitz KM, Penninger JM, and Molkentin JD. Temporally regulated and tissue-specific gene manipulations in the adult and embryonic heart using a tamoxifen-inducible Cre protein. Circ Res. 2001;89:20-5.
4. Madisen L, Zwingman TA, Sunkin SM, Oh SW, Zariwala HA, Gu H, Ng LL, Palmiter RD, Hawrylycz MJ, Jones AR, Lein ES, and Zeng H. A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. Nat Neurosci. United States; 2010;13:133-40.
5. Muzumdar MD, Tasic B, Miyamichi K, Li L, and Luo L. A global double-fluorescent Cre reporter mouse. Genesis. 2007;45:593-605.
6. Tarnavski O, McMullen JR, Schinke M, Nie Q, Kong S, and Izumo S. Mouse cardiac surgery: comprehensive techniques for the generation of mouse models of human diseases and their application for genomic studies. Physiol Genomics. 2004;16:349-60.
7. Zangi L, Lui KO, von Gise A, Ma Q, Ebina W, Ptaszek LM, Später D, Xu H, Tabebordbar M, Gorbatov R, Sena B, Nahrendorf M, Briscoe DM, Li RA, Wagers AJ, Rossi DJ, Pu WT, and Chien KR. Modified mRNA directs the fate of heart progenitor cells and induces vascular regeneration after myocardial infarction. Nat Biotechnol. 2013;31:898-907.
8. Warren L, Manos PD, Ahfeldt T, Loh YH, Li H, Lau F, Ebina W, Mandal PK, Smith ZD, Meissner A, Daley GQ, Brack AS, Collins JJ, Cowan C, Schlaeger TM, and Rossi DJ. Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. Cell Stem Cell. United States; 2010;7:618-30.

9. Lui KO, Zangi L, Silva EA, Bu L, Sahara M, Li RA, Mooney DJ, and Chien KR. Driving vascular endothelial cell fate of human multipotent Isl1+ heart progenitors with VEGF modified mRNA. Cell Res. England; 2013;23:1172-86.
10. Meirelles LDS, and Nardi NB. Murine marrow-derived mesenchymal stem cell: isolation, in vitro expansion, and characterization. Br J Haematol. England; 2003;123:702-11.
11. Bax NA, van Oorschot AA, Maas S, Braun J, van Tuyn J, de Vries AA, Groot AC, and Goumans MJ. In vitro epithelial-to-mesenchymal transformation in human adult epicardial cells is regulated by TGFbeta-signaling and WT1. Basic Res Cardiol. 2011;106:829-47.
12. Anjos-Afonso F, Siapati EK, and Bonnet D. In vivo contribution of murine mesenchymal stem cells into multiple cell-types under minimal damage conditions. J Cell Sci. England; 2004;117:5655-64.

Supplementary Tables

Table S1: Open reading frame sequences used for modRNA production for this study.

Name	Sequence
Luciferase	atggccgatgctaagaacattaagaagggccctgctcccttctaccctctggagg atggcaccgctggcgagcagctgcacaaggccatgaagaggtatgccctggtgcc tggcaccattgccttcaccgatgcccacattgaggtggacatcacctatgccgag tacttcgagatgtctgtgcgcctggccgaggccatgaagaggtacggcctgaaca ccaaccaccgcatcgtggtgtgctctgagaactctctgcagttcttcatgccagt gctgggcgccctgttcatcggagtggccgtggcccctgctaacgacatttacaac gagcgcgagctgctgaacagcatgggcatttctcagcctaccgtggtgttcgtgt ctaagaagggcctgcagaagatcctgaacgtgcagaagaagctgcctatcatcca gaagatcatcatcatggactctaagaccgactaccagggcttccagagcatgtac acattcgtgacatctcatctgcctcctggcttcaacgagtacgacttcgtgccag agtctttcgacagggacaaaaccattgccctgatcatgaacagctctgggtctac cggcctgcctaagggcgtggccctgcctcatcgcaccgcctgtgtgcgcttctct cacgcccgcgaccctattttcggcaaccagatcatccccgacaccgctattctga gcgtggtgccattccaccacggcttcggcatgttcaccaccctgggctacctgat ttgcggctttcgggtggtgctgatgtaccgcttcgaggaggagctgttcctgcgc agcctgcaagactacaaaattcagtctgccctgctggtgccaaccctgttcagct tcttcgctaagagcaccctgatcgacaagtacgacctgtctaacctgcacgagat tgcctctggcggcgccccactgtctaaggaggtgggcgaagccgtggccaagcgc tttcatctgccaggcatccgccagggctacggcctgaccgagacaaccagcgcca ttctgattaccccagagggcgacgacaagcctggcgccgtgggcaaggtggtgcc attcttcgaggccaaggtggtggacctggacaccggcaagaccctgggagtgaac cagcgcggcgagctgtgtgtgcgcggccctatgattatgtccggctacgtgaata accctgaggccacaaacgccctgatcgacaaggacggctggctgcactctggcga cattgcctactgggacgaggacgagcacttcttcatcgtggaccgcctgaagtct ctgatcaagtacaagggctaccaggtggccccagccgagctggagtctatcctgc tgcagcaccctaacattttcgacgccggagtggccggcctgcccgacgacgatgc cggcgagctgcctgccgccgtcgtcgtgctggaacacggcaagaccatgaccgag aaggagatcgtggactatgtggccagccaggtgacaaccgccaagaagctgcgcg gcggagtggtgttcgtggacgaggtgcccaagggcctgaccggcaagctggacgc ccgcaagatccgcgagatcctgatcaaggctaagaaaggcggcaagatcgccgtg taa
hVEGF-A_{165}	atgaactttctgctgtcttgggtgcattggagccttgccttgctgctctacctcc accatgccaagtggtcccaggctgcacccatggcagaaggaggagggcagaatca tcacgaagtggtgaagttcatggatgtctatcagcgcagctactgccatccaatc gagaccctggtggacatcttccaggagtaccctgatgagatcgagtacatcttca agccatcctgtgtgcccctgatgcgatgcgggggctgctgcaatgacgagggcct ggagtgtgtgcccactgaggagtccaacatcaccatgcagattatgcggatcaaa cctcaccaaggccagcacataggagagatgagcttcctacagcacaacaaatgtg aatgcagaccaaagaaagatagagcaagacaagaaaatccctgtgggccttgctc agagcggagaaagcatttgtttgtacaagatccgcagacgtgtaaatgttcctgc aaaaacacagactcgcgttgcaaggcgaggcagcttgagttaaacgaacgtactt gcagatgtgacaagccgaggcggtga
IGF1	atgaccgcacctgcaataaagatacacatcatgtcgtcttcacacctcttctacc tggcgctctgcttgctcaccttcaccagctccaccacagctggaccagagaccct

	ttgcggggctgagctggtggatgctcttcagttcgtgtgtggaccgaggggctttt tacttcaacaagcccacaggctatggctccagcattcggagggcacctcagacag gcattgtggatgagtgttgcttccggagctgtgatctgaggagactggagatgta ctgtgccccactgaagcctacaaaagcagcccgctctatccgtgcccagcgccac actgacatgcccaagactcagaagtccccgtccctatcgacaaacaagaaaacga agctgcaaaggagaaggaaaggaagtacatttgaagaacacaagtag
EGF	atgcattttgatggaacagactacaaagttctgctcagccggcagatgggaatgg tttttgccttggattatgaccctgtggaaagcaagatatattttgcacagacagc cctgaagtggatagagagggctaatatggatgggtcccagcgagaaagactgatc acagaaggagtagatacgcttgaaggtcttgccctggactggattggccggagaa tctactggacagacagtgggaagtctgttgttggagggagcgatctgagcgggaa gcatcatcgaataatcatccaggagagaatctcgaggccgcgaggaatagctgtg catccaagggccaggagactgttctggacggacgtagggatgtctccacggattg aaagcgcttcccttcaaggttccgaccgggtgctgatagccagctccaatctact ggaacccagtggaatcacgattgactacttaacagacactttgtactggtgtgac accaagaggtctgtgattgaaatggccaatctggatggctccaaacgccgaagac ttatccagaacgacgtaggtcacccccttctctctagccgtgtttgaggatcacct gtgggtctcggattgggctatcccatcggtaataagggtgaacaagaggactggc caaaacagggtacgtcttcaaggcagcatgctgaagccctcgtcactggttgtgg tccatccattggcaaaaccaggtgcagatccctgcttatacaggaatggaggctg tgaacacatctgccaagagagcctgggcacagctcggtgtttgtgtcgtgaaggt tttgtgaaggcctgggatgggaaaatgtgtctccctcaggattatccaatcctgt caggtgaaaatgctgatcttagtaaagaggtgacatcactgagcaactccactca ggctgaagtaccagacgatgatgggacagaatcttccacactagtggctgaaatc atggtgtcaggcatgaactatgaagatgactgtggtcccgggggggtgtggaagcc atgctcgatgcgtttcagacggagagactgctgagtgtcagtgtctgaaagggtt tgccagggatggaaacctgtgttctgatatagatgagtgtgtgctggctagatcg gactgccccagcacctcgtccaggtgcatcaacactgaaggtggctacgtctgca gatgctcagaaggctacgaaggagacgggatctcctgtttcgatattgacgagtg ccagcgggggggcgcacaactgcggtgagaatgccgcctgcaccaacacggaggga ggctacaactgcacctgcgcaggccgcccatcctcgcccggactgagttgccctg actctaccgcaccctctctccttggggaagatggccaccatttggaccgaaatag ttatccaggatgcccatcctcatatgatggatactgcctcaatggtggcgtgtgc atgcatattgaatcactggacagctacacatgcaactgtgttattggctattctg gggatcgatgccagactcgagacctacgatggtgggaactgcgtcatgctggcta cgggcagaagcatgacatcatggtggtggctgtctgcatggtggcactggtcctg ctgctcgtcttggggatgtgggggacttactactacaggactcggaagcagctat caaaccccccaaagaaccccttgtgatgagccaagcggaagtgtgagcagcagcgg acccaacagcagcagcggggcagctgtggcttcttgtccccaaccttggtttgtg gtcctagagaaacaccaagaccccaagaatgggagtctgcctgcggatggtacga atggtgcagtagtagatgctggcctgtctccctccctgcagctcgggtcagtgca tctgacttcatggagacagaagccccacatagatggaatgggcacagggcaaagc tgctggattccaccatcaagtgacagaggaccccaggaaatagagggaaactccc acctaccctcctacagacctgtggggccggagaagctgcattctctccagtcagc taatggatcgtgtcacgaaagggctccagacctgccacggcagacagagccagtt cagtag
HGF	atgatgtgggggaccaaacttctgccggtcctgttgctgcagcatgtcctcctgc acctcctcctgcttcatgtcgccatcccctatgcagaaggacagaagaaaagaag aaatacacttcatgaatttaaaaagtcagcaaaaactactcttaccaaggaagac ccattactgaagattaaaaccaaaaaagtgaactctgcagatgagtgtgccaaca

	ggtgtatcaggaacaggggctttacgttcacttgcaaggccttcgttttttgataa gtcaagaaaacgatgctactggtatcctttcaatagtatgtcaagtggagtgaaa aaagggtttggccatgaatttgacctctatgaaaacaaagactatattagaaact gcatcattggtaaaggaggcagctataaagggacggtatccatcactaagagtgg catcaaatgccagccttggaattccatgatccccccatgaacacagcttttttgcct tcgagctatcgcggtaaagacctacaggaaaactactgtcgaaatcctcgagggg aagaaggggaccctggtgtttcacaagcaatccagaggtacgctacgaagtctg tgacattcctcagtgttcagaagttgaatgcatgacctgcaatggtgaaagctac agaggtcccatggatcacacagaatcaggcaagacttgtcagcgctgggaccagc agacaccacaccggcacaagttcttgccagaaagatatcccgacaagggctttga tgataattattgccgcaatcctgatggcaagccgaggccatggtgctacactctt gaccctgacacccttgggagtattgtgcaattaaaacgtgcgctcacagtgctg tgaatgagactgatgtccctatggaaacaactgaatgcattcaaggccaaggaga aggttacaggggaaccagcaataccatttggaatggaattccctgtcagcgttgg gattcgcagtaccctcacaagcatgatatcactcccgagaacttcaaatgcaagg accttagagaaaattattgccgcaatccagatggggctgaatcaccatggtgttt taccactgacccaaacatccgagttggctactgctctcaaattcccaagtgtgac gtgtcaagtggacaagattgttatcgtggcaatgggaaaaattacatgggcaact tatccaaaacaaggtctggacttacatgttccatgtgggacaagaatatggagga tttacaccgtcatatcttctgggagccagatgctagcaaattgaataagaattac tgccggaatcctgatgatgatgcccatggaccttggtgctacacggggaatcctc ttattccttgggattattgccctatttcccgttgtgaaggagatactacacctac aattgtcaatttggaccatcctgtaatatcctgtgccaaaacaaaacaactgcgg gttgtaaatggcattccaacacaaacaacagtagggtggatggttagtttgaaat acagaaataaacatatctgtggaggatcattgataaaggaaagttgggttcttac tgcaagacaatgttttccagccagaaacaaagacttgaaagactatgaagcttgg cttggcatccacgatgttcatgagagaggcgaggagaagcgcaagcagatcttaa acatttcccagctggtctatggtcctgaaggctcagacttggttttactgaagct tgctcgacctgcaatcctggataactttgtcagtacaattgatttacctagttat ggttgtacaatccctgaaaagaccacttgcagtatttacggctggggctacactg gattgatcaacgcggatggtttattacgagtagctcatctgtatattatggggaa tgagaaatgcagtcagcaccatcaaggcaaggtgactttgaatgagtctgagtta tgtgctggggctgaaaagattggatcaggaccatgtgagggagattatggtggcc cactcatttgtgaacaacacaaaatgagaatggttcttggtgtcattgttcctgg tcgtggatgtgccatcccaaatcgtcctggtatttttgttcgagtagcatattat gcaaaatggatacacaaagtaattttgacatacaagttgtaa
TGFb1	atgccgccctcggggctgcggctactgccgcttctgctcccactcccgtggcttc tagtgctgacgcccgggaggccagccgcgggactctccacctgcaagaccatcga catggagctggtgaaacggaagcgcatcgaagccatccgtggccagatcctgtcc aaactaaggctcgccagtcccccaagccaggggggaggtaccgcccggcccgctgc ccgaggcggtgctcgctttgtacaacagcacccgcgaccgggtggcaggcgagag cgccgacccagagccggagcccgaagcggactactatgctaaagaggtcacccgc gtgctaatggtggaccgcaacaacgccatctatgagaaaaccaaagacatctcac acagtatatatatgttcttcaatacgtcagacattcgggaagcagtgcccgaacc cccattgctgtcccgtgcagagctgcgcttgcagagattaaaatcaagtgtggag caacatgtggaactctaccagaaatatagcaacaattcctggcgttaccttggta accggctgctgacccccactgatacgcctgagtggctgtcttttgacgtcactgg agttgtacggcagtggctgaaccaaggagacggaatacagggctttcgattcagc gctcactgctcttgtgacagcaaagataacaaactccacgtggaaatcaacggga tcagccccaaacgtcggggcgacctgggcaccatccatgacatgaaccggcccctt

	cctgctcctcatggccacccccctggaaagggcccagcacctgcacagctcacgg caccggagagccctggataccaactattgcttcagctccacagagaagaactgct gtgtgcggcagctgtacattgactttaggaaggacctgggttggaagtggatcca cgagcccaagggctaccatgccaacttctgtctgggaccctgcccctatatttgg agcctggacacacagtacagcaaggtccttgccctctacaaccaacacaacccgg gcgcttcggcgtcaccgtgctgcgtgccgcaggctttggagccactgcccatcgt ctactacgtgggtcgcaagcccaaggtggagcagttgtccaacatgattgtgcgc tcctgcaagtgcagctga
SDF1	atggacgccaaggtcgtcgccgtgctggccctggtgctggccgcgctctgcatca gtgacggtaaaccagtcagcctgagctaccgatgcccctgccggttcttcgagag ccacatcgccagagccaacgtcaagcatctgaaaatcctcaacactccaaactgt gcccttcagattgttgcacggctgaagaacaacaacagacaagtgtgcattgacc cgaaattaaagtggatccaagagtacctggagaaagctttaaacaaggggcgcag agaagaaaaagtggggaaaaaagaaaagataggaaaaaagaagcgacagaagaag agaaaggctgcccagaaaaggaaaaactag
FGF1	atggctgaaggggagatcacaaccttcgcagccctgaccgagaggttcaacctgc ctctaggaaactacaaaaagcccaaactgctctactgcagcaacgggggccactt cttgaggatccttcctgatggcaccgtggatgggacaagggacaggagcgaccag cacattcagctgcagctcagtgcggaaagtgcgggcgaagtgtatataaagggta cggagaccggccagtacttggccatggacaccgaagggcttttatacggctcgca gacaccaaatgaggaatgtctgttcctggaaaggctggaagaaaaccattataac acttacacctccaagaagcatgcggagaagaactggtttgtgggcctcaagaaga acgggagctgtaagcgcggtcctcggactcactatggccagaaagccatcttgtt tctgcccctcccggtgtcttctgactag
GH	atggagagattcgcttgcttgtgtgttttcatactggatcaaacattaaacatcc tgagatccaaagactctcggacctcctggctcctgaccgtcagcctgctctgcct gctctggcctcaggaggctagtgcttttcccgccatgcccttgtccagtctgttt tctaatgctgtgctccgagcccagcacctgcaccagctggctgctgacacctaca aagagttcgagcgtgcctacattcccgagggacagcgctattccattcagaatgc ccaggctgctttctgcttctcagagaccatcccggcccccacaggcaaggaggag gcccagcagagaaccgacatggaattgcttcgcttctcgctgctgctcatccagt catggctggggcccgtgcagttcctcagcaggattttcaccaacagcctgatgtt cggcacctcggaccgtgtctatgagaaactgaaggacctggaagagggcatccag gctctgatgcaggagctggaagatggcagcccccgtgttgggcagatcctcaagc aaacctatgacaagtttgacgccaacatgcgcagcgacgacgcgctgctcaaaaa ctatgggctgctctcctgcttcaagaaggacctgcacaaagcggagacctacctg cgggtcatgaagtgtcgccgctttgtggaaagcagctgtgccttctag
SCF	atgaagaagacacaaacttggattatcacttgcatttatcttcaactgctcctat ttaatcctctcgtcaaaactcaggagatctgcaggaatcctgtgactgataatgt aaaagacattacaaaactggtggcgaatcttccaaatgactatatgataaccctc aactatgtcgccgggatggatgttttgcctagtcattgttggttacgagatatgg taacacacttatcagtcagcttgactactcttctggacaagttttcaaatatttc tgaaggcttgagtaattattccatcatagacaaacttgggaaaatagtggatgac ctcgtggcatgtatggaagaaaatgcacctaagaatgtaaaagaatcactgaaga agccagaaactagaaactttactcctgaagaattctttagtattttcaatagatc cattgatgccttcaaggacttcatggtggcatctgacactagtgattgtgtgctc tcttcaacattaggtcctgagaaagattccagagtcagtgtcacaaaaccattta tgttacccccctgttgcagccagttcccttaggaatgacagcagtagcagtaatag gaaagccgcaaagtcccctgaagacccaggcctacaatggacagcaatggcactg ccggctctcatttcgcttgtaattggctttgcttttggagccttatactggaaga

	agaaacagtcaagtcttacaagggcagttgaaaatatacagattaatgaagagga taatgagataagtatgttgcaacagaaagagagagagtttcaagaggtgtaa
TGFb2	atgcactactgtgtgctgagcacctttttgctcctgcatctggtcccggtggcgc tcagtctgtctacctgcagcaccctcgacatggatcagtttatgcgcaagaggat cgaggccatccgcgggcagatcctgagcaagctgaagctcaccagccccccggaa gactatccggagccggatgaggtccccccggaggtgatttccatctacaacagta ccagggacttactgcaggagaaggcaagccggagggcagccgcctgcgagcgcga gcggagcgacgaggagtactacgccaaggaggtttataaaatcgacatgccgtcc cacctcccctccgaaaatgccatcccgcccactttctacagaccctacttcagaa tcgtccgctttgatgtctcaacaatggagaaaaatgcttcgaatctggtgaaggc agagttcagggtcttccgcttgcaaaaccccaaagccagagtggccgagcagcgg attgaactgtatcagatccttaaatccaaagacttaacatctcccacccagcgct acatcgatagcaaggttgtgaaaaccagagcggagggtgaatggctctccttcga cgtgacagacgctgtgcaggagtggcttcaccacaaagacaggaacctggggttt aaaataagtttacactgcccctgctgtaccttcgtgccgtctaataattacatca tcccgaataaaagcgaagagctcgaggcgagatttgcaggtattgatggcacctc tacatatgccagtggtgatcagaaaactataaagtccactaggaaaaaaaccagt gggaagaccccacatctcctgctaatgttgttgccctcctacagactggagtcac aacagtccagccggcggaagaagcgcgctttggatgctgcctactgctttagaaa tgtgcaggataattgctgccttcgccctctttacattgattttaagagggatctt ggatggaaatggatccatgaacccaaagggtacaatgctaacttctgtgctgggg catgcccatatctatggagttcagacactcaacacaccaaagtcctcagcctgta caacaccataaatcccgaagcttccgcttccccttgctgtgtgtcccaggatctg gaaccactgaccattctctattacattggaaatacgcccaagatcgaacagcttt ccaatatgattgtcaagtcttgtaaatgcagctaa
TB4	atgtctgacaaacccgatatggctgagatcgagaaattcgataagtcgaagttga agaaaacagaaacgcaagagaaaaatcctctgccttcaaaagaaacaattgaaca agagaagcaagctggcgaatcgtaa
IGF1R-DN	atgaagtctggctccggaggagggtccccgacctcgctgtgggggctcctgtttc tctccgccgcgctctcgctctggccgacgagtggagaaatctgcgggccaggcat cgacatccgcaacgactatcagcagctgaagcgcctggagaactgcacggtgatc gagggctacctccacatcctgctcatctccaaggccgaggactaccgcagctacc gcttccccaagctcacggtcattaccgagtacttgctgctgttccgagtggctgg cctcgagagcctcggagacctcttccccaacctcacggtcatccgcggctggaaa ctcttctacaactacgccctggtcatcttcgagatgaccaatctcaaggatattg ggctttacaacctgaggaacattactcgggggccatcaggattgagaaaaatgc tgacctctgttacctctccactgtggactggtccctgatcctggatgcggtgtcc aataactacattgtggggaataagcccccaaaggaatgtggggacctgtgtccag ggaccatggaggagaagccgatgtgtgagaagaccaccatcaacaatgagtacaa ctaccgctgctggaccacaaaccgctgccagaaaatgtgcccaagcacgtgtggg aagcgggcgtgcaccgagaacaatgagtgctgccaccccgagtgcctgggcagct gcagcgcgcctgacaacgacacggcctgtgtagcttgccgccactactactatgc cggtgtctgtgtgcctgcctgcccgcccaacacctacaggtttgagggctggcgc tgtgtggaccgtgacttctgcgccaacatcctcagcgccgagagcagcgactccg aggggtttgtgatccacgacggcgagtgcatgcaggagtgcccctcgggcttcat ccgcaacggcagccagagcatgtactgcatcccttgtgaaggtccttgcccgaag gtctgtgaggaagaaaagaaaacaaagaccattgattctgttacttctgctcaga tgctccaaggatgcaccatcttcaagggcaatttgctcattaacatccgacgggg gaataacattgcttcagagctggagaacttcatggggctcatcgaggtggtgacg ggctacgtgaagatccgccattctcatgccttggtctccttgtccttcctaaaaa

	accttcgcctcatcctaggagaggagcagctagaagggaattactccttctacgt cctcgacaaccagaacttgcagcaactgtgggactgggaccaccgcaacctgacc atcaaagcagggaaaatgtactttgctttcaatcccaaattatgtgtttccgaaa tttaccgcatggaggaagtgacggggactaaagggcgccaaagcaaaggggacat aaacaccaggaacaacggggagagagcctcctgtgaaagtgacgtcctgcatttc acctccaccaccacgtcgaagaatcgcatcatcataacctggcaccggtaccggc cccctgactacagggatctcatcagcttcaccgtttactacaaggaagcaccctt taagaatgtcacagagtatgatgggcaggatgcctgcggctccaacagctggaac atggtggacgtggacctcccgcccaacaaggacgtggagcccggcatcttactac atgggctgaagccctggactcagtacgccgtttacgtcaaggctgtgaccctcac catggtggagaacgaccatatccgtggggccaagagtgagatcttgtacattcgc accaatgcttcagttccttccattcccttggacgttctttcagcatcgaactcct cttctcagttaatcgtgaagtggaaccctccctctctgcccaacggcaacctgag ttactacattgtgcgctggcagcggcagcctcaggacggctacctttaccggcac aattactgctccaaagacaaaatccccatcaggaagtatgccgacggcaccatcg acattgaggaggtcacagagaaccccaagactgaggtgtgtggtggggagaaagg gccttgctgcgcctgccccaaaactgaagccgagaagcaggccgagaaggaggag gctgaataccgcaaagtctttgagaatttcctgcacaactccatcttcgtgccca gacctgaaaggaagcggagagatgtcatgcaagtggccaacaccaccatgtccag ccgaagcaggaacaccacggccgcagacacctacaacatcaccgacccggaagag ctggagacagagtacccttctttgagagcagagtggataacaaggagagaactg tcatttctaaccttcggcctttcacattgtaccgcatcgatatccacagctgcaa ccacgaggctgagaagctgggctgcagcgcctccaacttcgtctttgcaaggact atgcccgcagaaggagcagatgacattcctgggccagtgacctgggagccaaggc ctgaaaactccatcttttaaagtggccggaacctgagaatcccaatggattgat tctaatgtatgaaataaaatacggatcacaagttgaggatcagcgagaatgtgtg tccagacaggaatacaggaagtatggaggggccaagctaaaccggctaaacccgg ggaactacacagcccggattcaggccacatctctctctgggaatgggtcgtggac agatcctgtgttcttctatgtccaggccaaaacaggatatgaaaacttcatccat ctgatcatcgctctgcccgtcgctgtcctgttgatcgtgggagggtaa
IRS1-DN	atggcgagccctccggagagcgatggcttctcggacgtgcgcaaggtgggctacc tgcgcaaacccaagagcatgcacaaacgcttcttcgtactgcgcgcggccagcga ggctgggggcccggcgcgcctcgagtactacgagaacgagaagaagtggcggcac aagtcgagcgcccccaaacgctcgatccccccttgagagctgcttcaacatcaaca agcgggctgactccaagaacaagcacctggtggctctctacacccgggacgagca ctttgccatcgcggcggacagcgaggccgagcaagacagctggtaccaggctctc ctacagctgcacaaccgtgctaagggccaccacgacggagctgcggccctcgggg cgggaggtggtgggggcagctgcagcggcagctccggccttggtgaggctgggga ggacttgagctacggtgacgtgcccccaggacccgcattcaaagaggtctggcaa gtgatcctgaagcccaagggcctgggtcagacaaagaacctgattggtatctacc gcctttgcctgaccagcaagaccatcagcttcgtgaagctgaactcggaggcagc ggccgtggtgctgcagctgatgaacatcaggcgctgtggccactcggaaaacttc ttcttcatcgaggtgggccgttctgccgtgacggggcccggggagttctggatgc aggtggatgactctgtggtggcccagaacatgcacgagaccatcctggaggccat gcgggccatgagtgatgagttccgccctcgcagcaagagccagtcctcgtccaac tgctctaaccccatcagcgtccccctgcgccggcaccatctcaacaatcccccgc ccagccaggtggggctgacccgccgatcacgcactgagagcatcaccgccacctc cccggccagcatggtgggcgggaagccaggctccttccgtgtccgcgcctccagt gacggcgaaggcaccatgtcccgcccagcctcggtggacggcagccctgtgagtc ccagcaccaacagaacccacgcccaccggcatcggggcagcgcccggctgcaccc

	cccgctcaaccacagccgctccatccccatgccggcttcccgctgctcgccttcg gccaccagcccggtcagtctgtcgtccagtagcaccagtggccatggctccacct cggattgtctcttcccacggcgatctagtgcttcggtgtctggttcccccagcga tggcggtttcatctcctcggatgagtatggctccagtccctgcgatttccggagt tccttccgcagtgtcactccggattccctgggccacaccccaccagcccgcggtg aggaggagctaagcaactatatctgcatgggtggcaaggggccctccaccctgac cgcccccaacggtcactacatttgtctcggggtggcaatggccaccgctgcacc ccaggaacaggcttgggcacgagtccagccttggctggggatgaagcagccagtg ctgcagattaa
mCherry	atggtgagcaagggcgaggaggataacatggccatcatcaaggagttcatgcgct tcaaggtgcacatggagggctccgtgaacggccacgagttcgagatcgagggcga gggcgagggccgcccctacgagggcacccagaccgccaagctgaaggtgaccaag ggtggcccctgcccttcgcctgggacatcctgtcccctcagttcatgtacggct ccaaggcctacgtgaagcaccccgccgacatccccgactacttgaagctgtcctt ccccgagggcttcaagtgggagcgcgtgatgaacttcgaggacggcggcgtggtg accgtgacccaggactcctccctgcaggacggcgagttcatctacaaggtgaagc tgcgcggcaccaacttcccctccgacggccccgtaatgcagaagaagaccatggg ctgggaggcctcctccgagcggatgtaccccgaggacggcgccctgaagggcgag atcaagcagaggctgaagctgaaggacggcggccactacgacgctgaggtcaaga ccacctacaaggccaagaagcccgtgcagctgcccggcgcctacaacgtcaacat caagttggacatcacctcccacaacgaggactacaccatcgtggaacagtacgaa cgcgccgagggccgccactccaccggcggcatggacgagctgtacaagtaa
Nuclear GFP	atggtgagcaagggcgaggagctgttcaccggggtggtgcccatcctggtcgagc tggacggcgacgtaaacggccacaagttcagcgtgtccggcgagggcgagggcga tgccacctacggcaagctgaccctgaagttcatctgcaccaccggcaagctgccc gtgccctggcccaccctcgtgaccaccctgacctacggcgtgcagtgcttcagcc gctaccccgaccacatgaagcagcacgacttcttcaagtccgccatgcccgaagg ctacgtccaggagcgcaccatcttcttcaaggacgacggcaactacaagacccgc gccgaggtgaagttcgagggcgacaccctggtgaaccgcatcgagctgaagggca tcgacttcaaggaggacggcaacatcctggggcacaagctggagtacaactacaa cagccacaacgtctatatcatggccgacaagcagaagaacggcatcaaggtgaac ttcaagatccgccacaacatcgaggacggcagcgtgcagctcgccgaccactacc agcagaacaccccatcggcgacggccccgtgctgctgcccgacaaccactacct gagcacccagtccgccctgagcaaagaccccaacgagaagcgcgatcacatggtc ctgctggagttcgtgaccgccgccgggatcactctcggcatggacgagctgtaca agggagatccaaaaaagaagagaaaggtaggcgatccaaaaaagaagagaaaggt aggtgatccaaaaaagaagagaaaggtataa
Cre Recombinase	atgtccaatttactgaccgtacaccaaaatttgcctgcattaccggtcgatgcaa cgagtgatgaggttcgcaagaacctgatggacatgttcagggatcgccaggcgtt ttctgagcatacctggaaaatgcttctgtccgtttgccggtcgtgggcggcatgg tgcaagttgaataaccggaaatggtttcccgcagaacctgaagatgttcgcgatt atcttctatatcttcaggcgcgcggtctggcagtaaaaactatccagcaacattt gggccagctaaacatgcttcatcgtcggtccgggctgccacgaccaagtgacagc aatgctgtttcactggttatgcggcggatccgaaaagaaaacgttgatgccggtg aacgtgcaaaacaggctctagcgttcgaacgcactgatttcgaccaggttcgttc actcatggaaaatagcgatcgctgccaggatatacgtaatctggcatttctgggg attgcttataacaccctgttacgtatagccgaaattgccaggatcagggttaaag atatctcacgtactgacggtgggagaatgttaatccatattggcagaacgaaaac gctggttagcaccgcaggtgtagagaaggcacttagcctgggggtaactaaactg gtcgagcgatggatttccgtctctggtgtagctgatgatccgaataactacctgt

	tttgccgggtcagaaaaaatggtgttgccgcgccatctgccaccagccagctatc aactcgcgccctggaagggatttttgaagcaactcatcgattgatttacggcgct aaggatgactctggtcagagatacctggcctggtctggacacagtgcccgtgtcg gagccgcgcgagatatggcccgcgctggagtttcaataccggagatcatgcaagc tggtggctggaccaatgtaaatattgtcatgaactatatccgtaacctggatagt gaaacaggggcaatggtgcgcctgctagaagatggcgattag

Table S2: Antibodies used in this study

Name	Company	Catalog #
Perilipin A	abcam	ab3526
CD24	abcam	ab25550
VEGFR2	BD biosciences	561993
IGFR1	Sigma Aldrich	SAB4300359
EGFR	R&D systems	AF1280
HGFR	R&D systems	AF527
TGFBR1	Sigma Aldrich	SAB4502958
CXCR4	R&D systems	MAB21651
FGF1R	abcam	ab63601
GHR	R&D systems	AF1360
C-kit	R&D systems	AF1356
TGFR2	abcam	ab61213
IGFR-P (Y1161)	abcam	ab72965
hCD25	Biolegend	302624
CD31	eBioscience	12-0311-83
Myosin smooth muscle	Biotechnologies INC.	BT-562
Cardiac troponin I	abcam	ab47003
Actin	abcam	ab179467
Alexa-488	Life Technology / Invitrogen	A21202, A21208, A11055, A21206
Alexa-555	Life Technology / Invitrogen	A11056, A10036, A10040, SA5-10027
Alexa-647	Life Technology / Invitrogen	A31573, A21472, A31571, S32357, A21083
DAPI	Sigma	D9542

Suppl. Table S3: Primer Sequences for qPCR in this study

Gene	Forward	Reverse
Gapdh	ttgtctcctgcgacttcaac	gtcataccaggaaatgagcttg
Adipsin	tccgcccctgaaccctacaa	taatggtgactaccccgtca
Adiponectin	gctcctgctttggtccctccac	gcccttcagctcctgtcattcc
FABP4	gcgtggaattcgatgaaatca	cccgccatctagggttatga
Pparγ2	tctgggagattctcctgttga	ggtgggccagaatggcatct
Myh6	acggtgaccataaaggagga	tgtcctcgatcttgtcgaac
Tnni3	gaagcaggagatggaacgag	ttaaacttgccacggaggtc
Tnnt2	ctgagacagaggaggccaac	ttctcgaagtgagcctcgat
Isl1	agcaccagcatcctctctgt	tgaagcctatgctgcacttg
Wt1	agacacacaggtgtgaaacca	atgagtcctggtgtgggtct
Tbx18	atggcctccagaatgcgtatg	tgtcccccatcaagcctgtt
Vegfr2	agaacaccaaaagagagaggaacg	gcacacaggcagaaaccagtag
Igf1r	atggagagcgtcccactggacc	ccgggccattctcagccttgtg
Egfr	gtgtgaagaagtgcccccgaaac	aacgaccgccaaagaaaactgacc
Met	gtcaacatgaagtatcagctccc	tgtagtttgtggctccgagat
Tgfbr1	cctgcttctcatcgtgttggt	aggtggcagaaacactgtaatgc
Cxcr4	tcagtggctgacctcctctt	tttcagccagcagtttcctt
Fgfr1	agcgaacaaccctatgagcac	tcgtttggctgggataactcg
Ghr	cgttcccctgaactggagac	cagcttgtcgttggctttcc
c-Kit	ccgggatcagcttattgca	gctacagctctcgcccaagt
Tgfbr2	tatgagcccccgtttggttc	gccaggagctgggaatttct
Csf2rb	taagcagcttcaggactc	cttgtcctctgtcggagag
Csf3r	tcatggccaccagtcgagc	cacgctggagtcccagaag
Erbb4	ggctgctgagttttcaaggatg	gcttcatacgatcatcaccctga
Ngfr	gactaacctaggccacccaa	cagacgtcgtttccagatgt

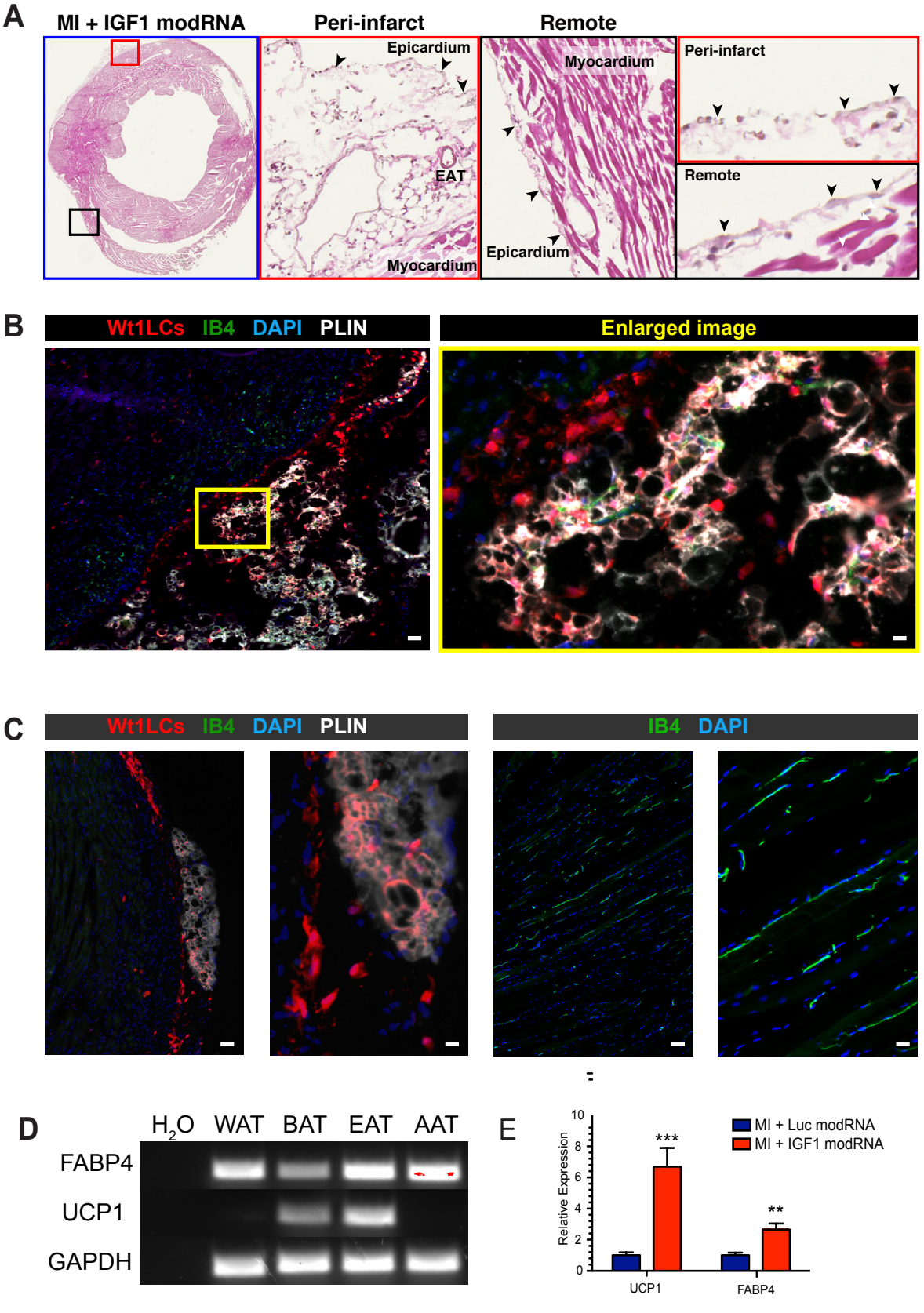

Fig. S1. IGF1 activates Wt1-lineage cell (Wt1LC) differentiation into EAT. A. Wt1LCs were labeled by tamoxifen treatment of $Wt1^{CreERT2/+}$; $Rosa26^{Tomato}$ mice. The mice then underwent MI and IGF1 modRNA injection. The epicardium around the site of injury became thicker and contained EAT, which were not seen remote from the infarct. Arrowheads point to

epicardial cells with flat epithelial morphology on the outer surface of the expanded epicardium, with adipocytes underneath. Immunofluorescent staining showed some adipocytes overlapping Wt1LCs (white arrowheads). **B.** Labeled EAT was perfused from the coronary arteries. After euthansia, the ascending aorta was occluded with a ligature and the right atrium was opened. Isolectin B4 (IB4) was injected into the heart, to perfuse the coronary arteries but not systemic arteries. Blood vessels supplying EAT, containing Wt1LCs, were labeled, as expected for adipose tissue derived from cardiac as opposed to non-cardiac tissue. **C.** IB4 injection into the systemic circulation with exclusion of the coronary circulation did not label vessels in EAT. Mouse was prepared as in B, except that IB4 solution was injected into the aorta distal to the ligature. Skeletal muscle blood vessels were robustly labeled by IB4 (right pair of images), but myocardium and EAT were not (left pair of images). **D.** Expression of brown fat markers in EAT. EAT was collected 28 days after MI for RTPCR. Brown adipose tissue (BAT), white adipose tissue (WAT), and aortic adipose tissue (AAT) controls were obtained from interscapular, inguinal, and peri-aortic fat depots, respectively. **E.** Quantitative RTPCR showed that IGF1 modRNA upregulated both types of fat markers after MI in IGF1 modRNA-treated mice. n=3. **, $P<0.01$. ***, $P<0.001$. t-test. Bar = 500 or 10 μm (A), 100 or 50 μm (B), or 50 or 10 μm (C).

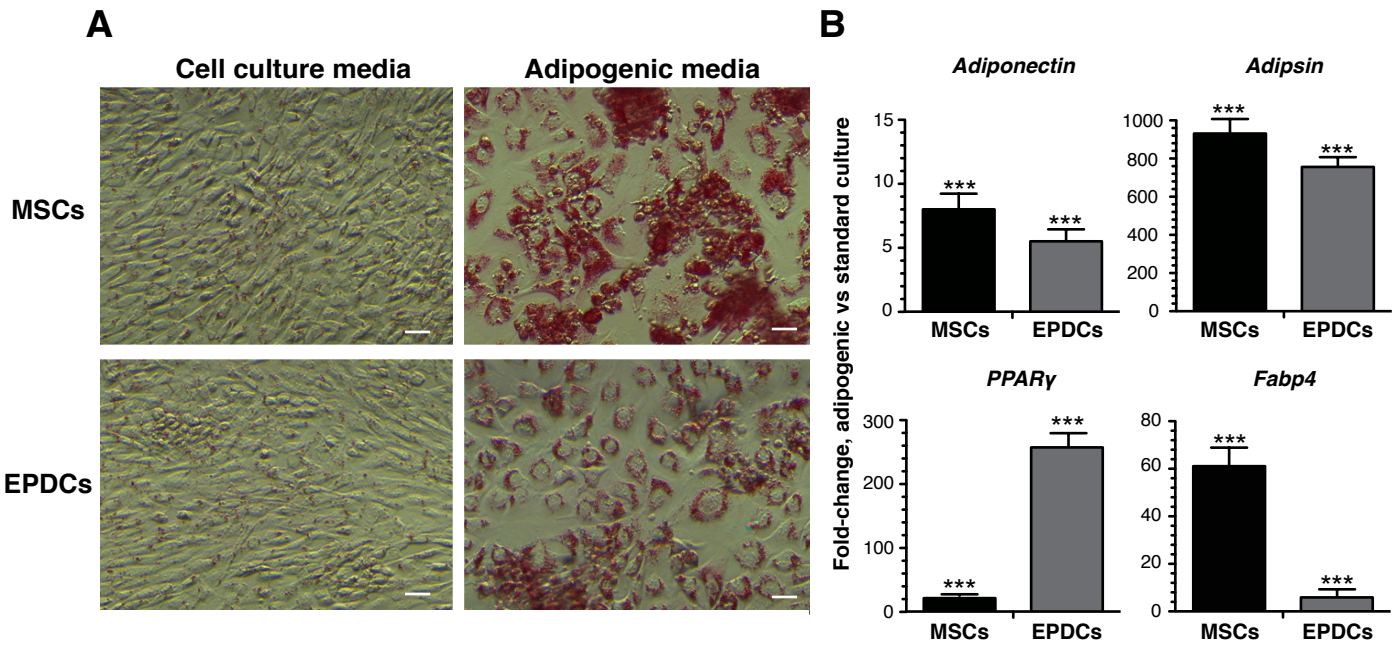

Fig. S2. EPDCs differentiate into adipocytes when cultured in adipogenic media. a. EPDCs were cultured for 28 days in standard or adipogenic media and then stained for lipid accumulation using oil red O. Mesenchymal stem cells (MSCs), a cell type that is known to undergo adipogenic differentiation in the presence of the adipogenic media, were included as a positive control. **b.** Adipogenic media increased expression of adipogenic marker genes. Gene expression was measured by qRTPCR after 10 days of culture in adipogenic or standard media. The fold-change of gene expression in adipogenic compared to standard media is plotted. n=3. ***, P<0.001, *t*-test. bar = 20 μm.

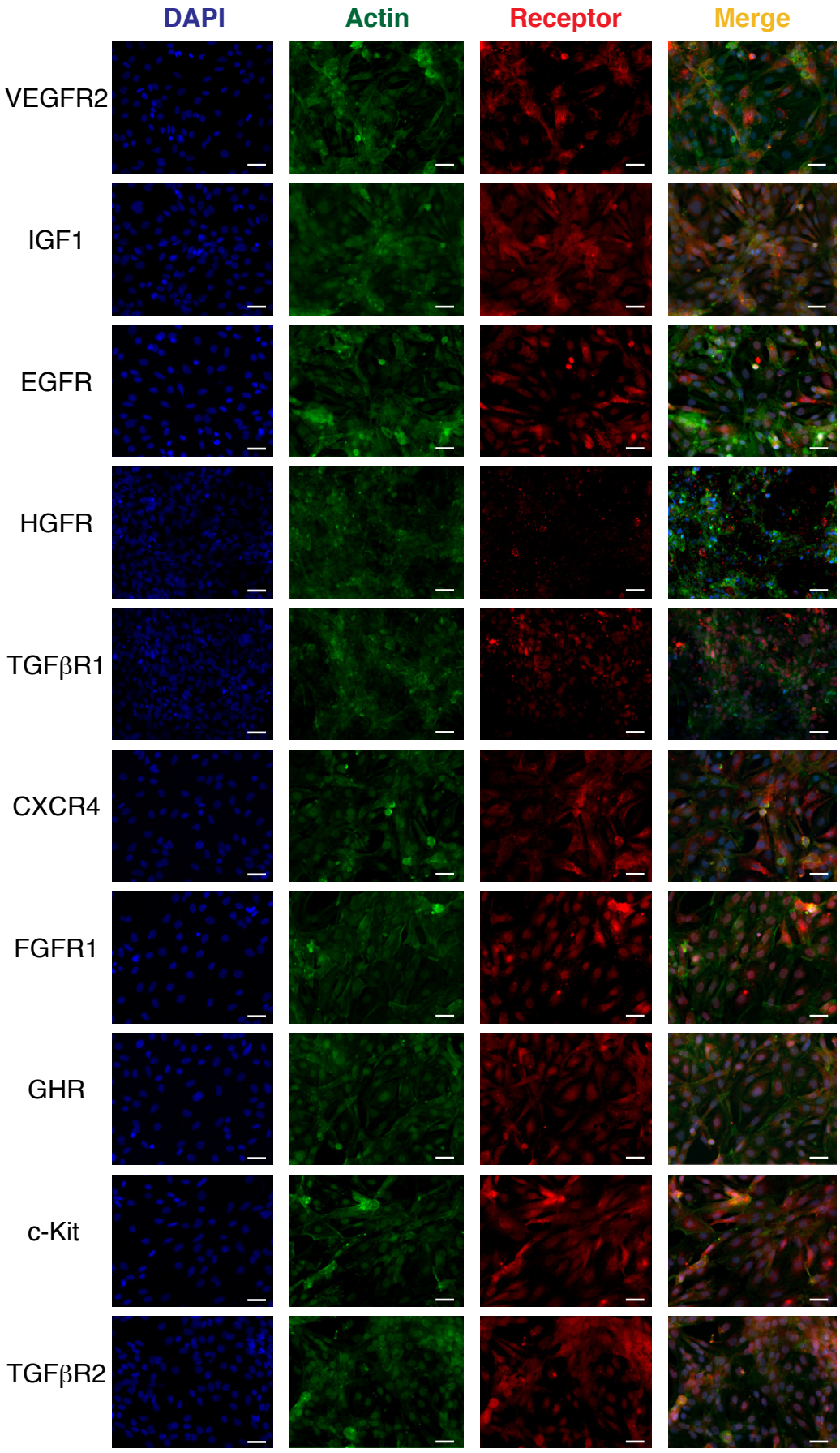

Fig. S3. Cultured EPDCs express 10 candidate paracrine receptors. FACS-sorted EPDCs from $Wt1^{GFPCre}$ mice were immunostained for actin (green) and the indicated paracrine factor receptor (red). bar = 10 mm.

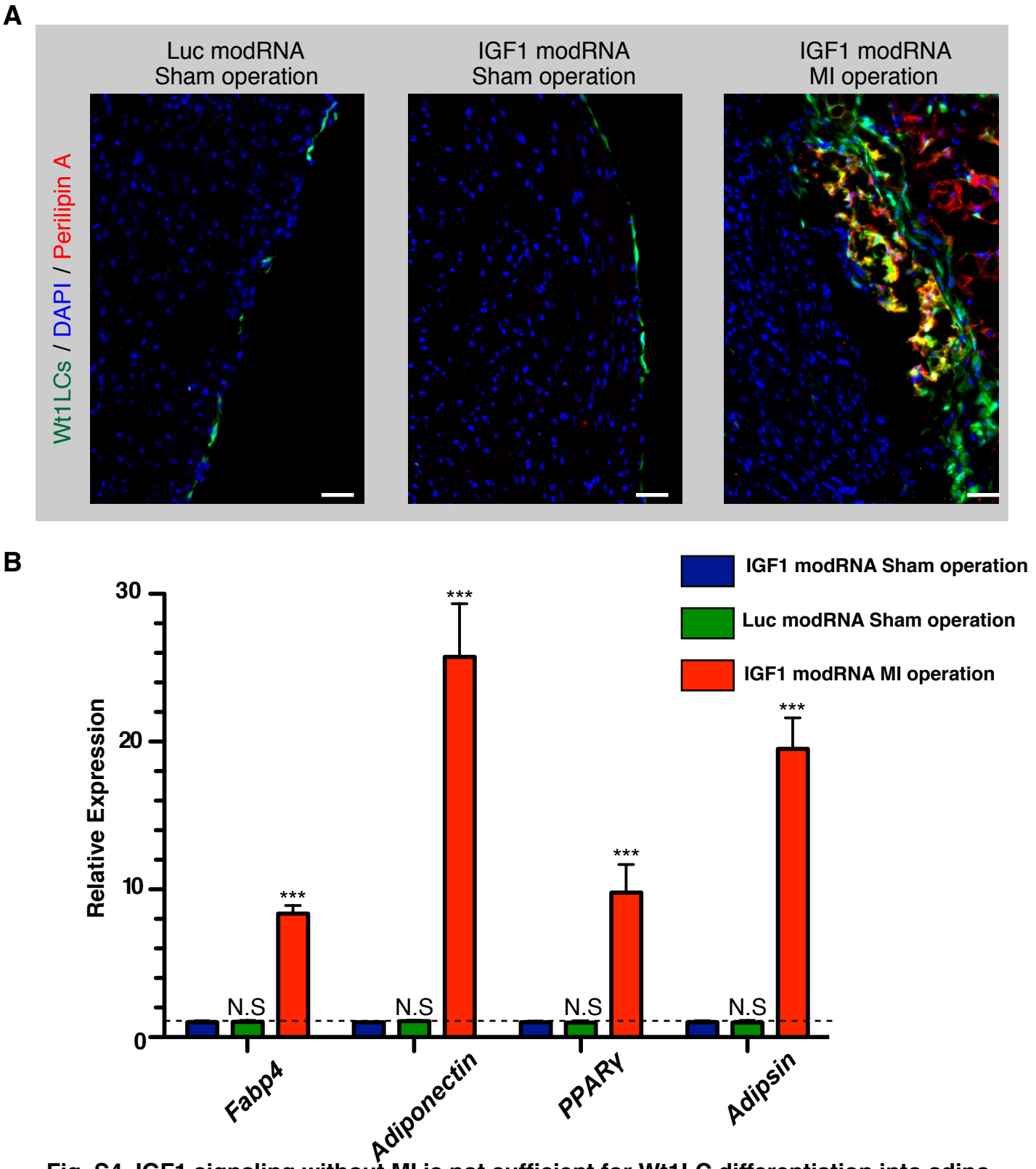

Fig. S4. IGF1 signaling without MI is not sufficient for Wt1LC differentiation into adipocytes. A. Wt1$^{CreERT2/+}$ Rosa26$^{Tomato/+}$ mice were pulse-labeled with tamoxifen. They then underwent either sham or MI operation and injection of the indicated modRNA. After 28 days, heart sections were immunostained and imaged using a confocal microscope. B. Expression of adipogenic markers in perinfarct myocardium was measured using qRTPCR seven days after sham or MI operation and modRNA injection. Relative expression was normalized to IGF1 modRNA-sham operation. Dotted line indicates no change in expression (ratio = 1). NS, not significant. ***, P<0.001. t-test. Bar = 50 μm.

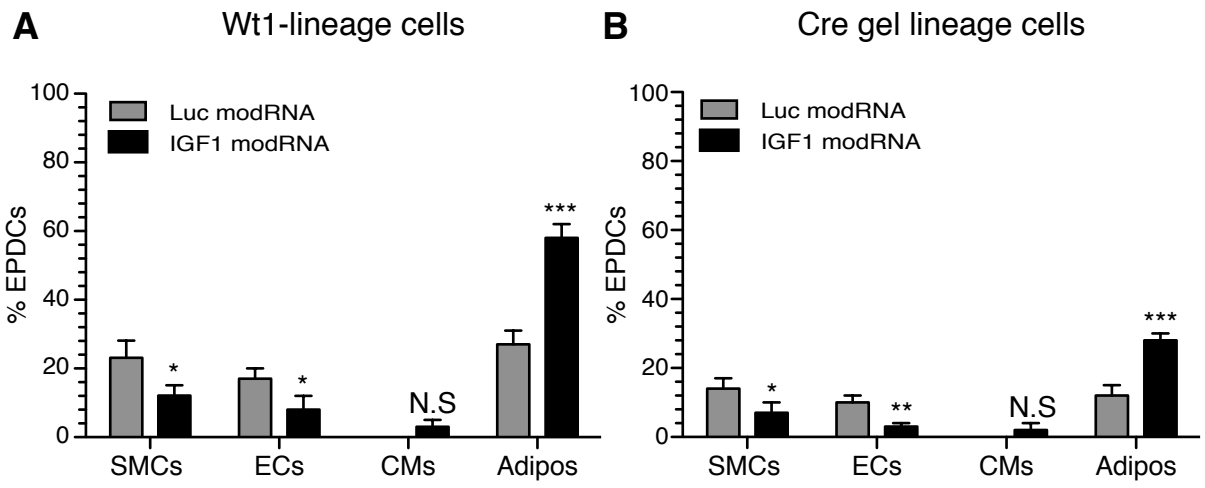

Fig. S5. IGF1 modRNA drives EPDC differentiation into the adipocyte lineage and away from smooth muscle or endothelial lineages. EPDC fate was analyzed using two independent lineage tracing systems. **A.** Immunofluorescent analysis of Wt1-lineage cells. The percentage of Wt1LCs expressing the markers of smooth muscle cells (SMCs), endothelial cells (ECs), cardiomyocytes (CMs) and adipocytes (Adipos) is shown. The markers used were smooth muscle myosin heavy chain (smMHC), PECAM1, troponin I (TNNI3), and perilipin A, respectively. Quantitation was performed on each post-MI heart treated with Luc modRNA (n=4) or IGF1 modRNA (n=4) from 2 independent experiments. The graph shows the percentage of Wt1LCs that co-expressed the indicated lineage marker. **B.** Immunofluorescent analysis of Cre gel lineage cells. Quantification was performed as in A. n=3. Inter-group comparisons were made by t-test. n.s., not significant ($P \geq 0.05$); *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

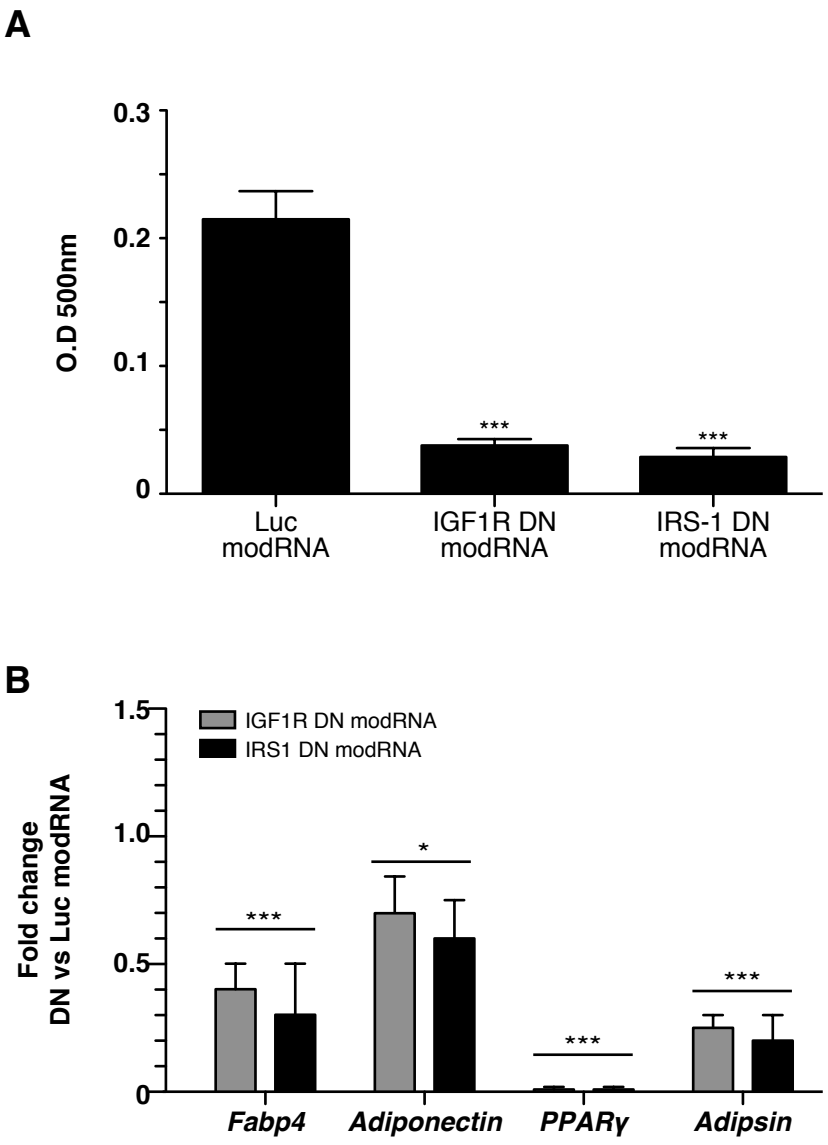

Fig. S6. Blocking IGF1R signaling inhibited EPDC adipogenic differentiation. A. Quantification of effect of IGF1R inhibition on cultured EPDC adipogenic differentiation. EPDCs were cultured in the presence of IGF1 protein and repeatedly transfected with Luc (control), IGF1R-DN, or IRS1-DN modRNAs. After 28 days, cells were stained with oil red O, and lipid accumulation was quantified by extracting oil red O retained by cells and measuring optical density at a wavelength of 500 nm. n=3. ***, P<0.001, *t*-test. **B.** IGF1R-DN and IRS1-DN modRNA transfection decreased expression of adipogenic marker genes in EPDCs. EPDCs were cultured with IGF1 and transfected with Luc modRNA (control) or with either IGF1R-DN or IRS1-DN modRNA. Expression of each marker was measured by qRT-PCR and displayed as its fold-change in cells transfected with DN modRNA compared to Luc modRNA. n=3. *, P<0.05, ***, P<0.001, *t*-test.

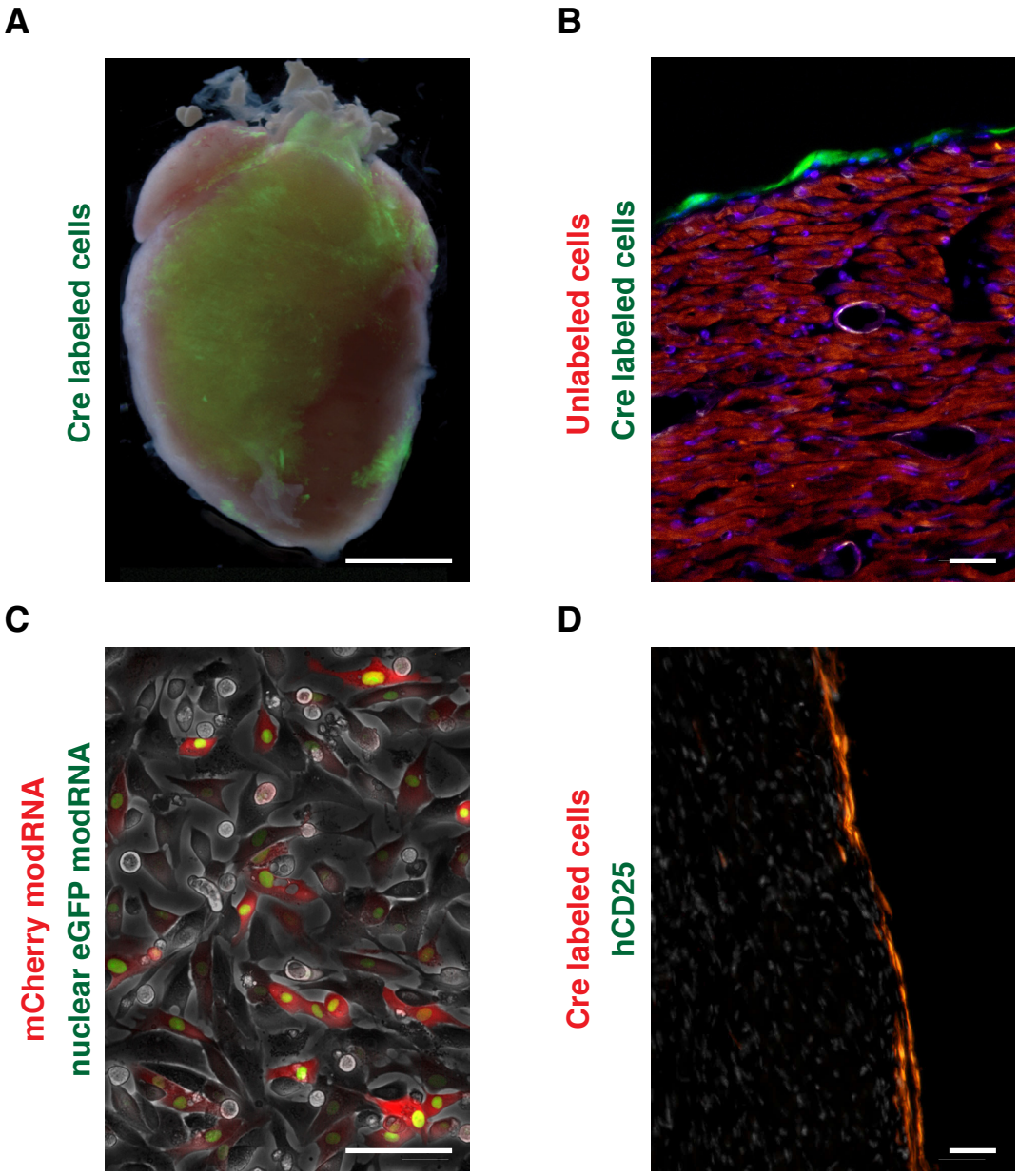

Fig. S7. Gel modRNA as a tool for specific and concurrent gene delivery to EPDCs. A. Hearts of $R26^{mTmG}$ mice (no Cre recombination = red; Cre recombination = green) were treated with Cre modRNA gel. A subset of EPDCs were irreversibly labeled. B. Immunostained cryosections of Cre modRNA gel-treated $R26^{mTmG}$ heart for GFP expression reveals recombination limited to the epicardial layer. C. Co-delivery into EPDCs in vitro of two different modified mRNAs by co-transfection in the same lipid vehicle. Note that the vast majority of cells expressing one modified mRNA also expressed the second mRNA. D. In vivo, mixing two modRNAs (Cre and human CD25) in the same modRNA gel resulted in co-transfection of the two genes into epicardial cells. Scale bar = 1 mm (A) or 20 mm (B-D).

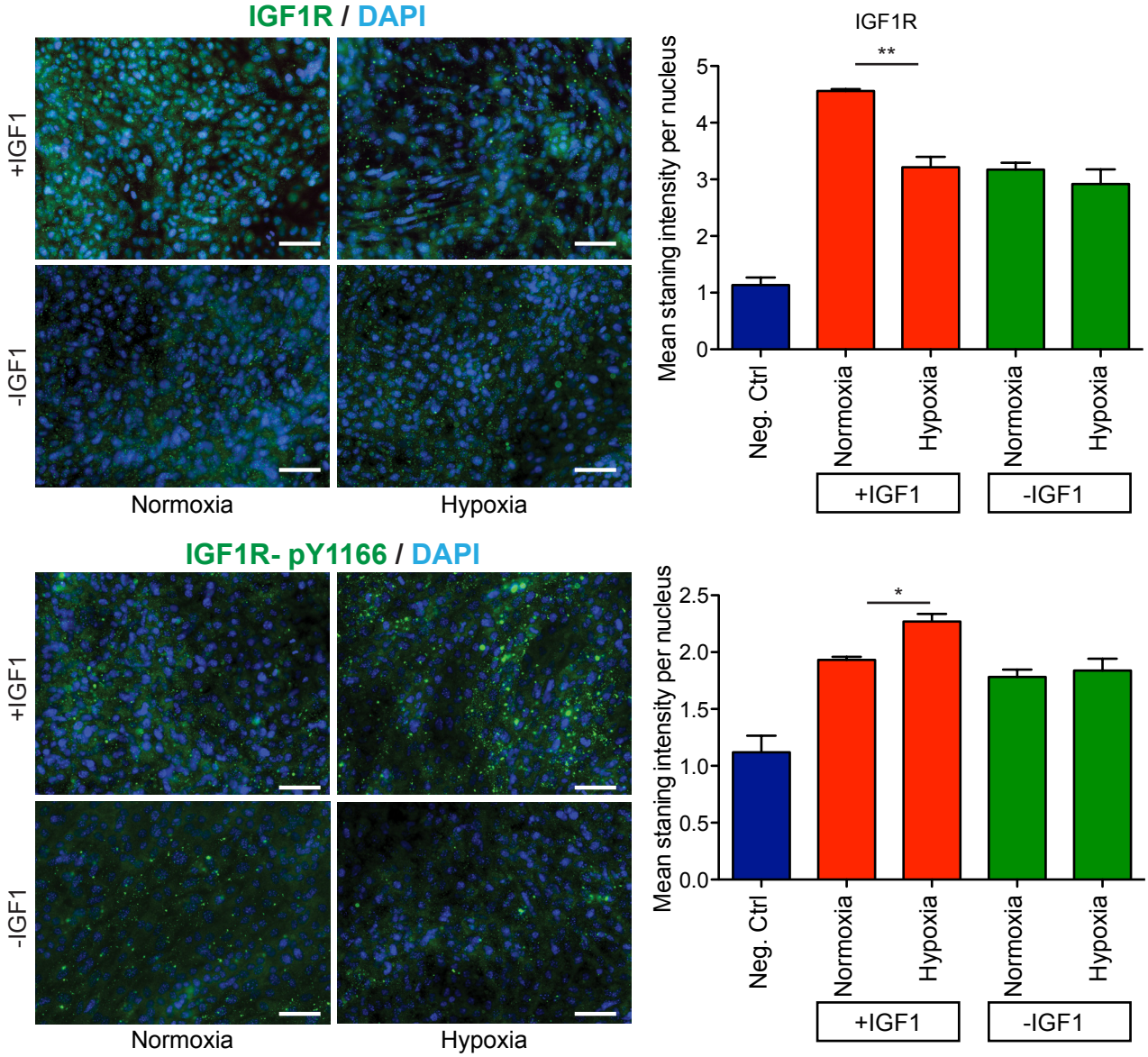

Fig. S8. Hypoxia enhances IGF1R activation by IGF1 in EPDCs. EPDCs were cultured in normoxia or hypoxia (1% O2) with or without IGF1 (100 ng/ml). 24 hours later cells were stained for IGF1R (top) or activated IGF1R (IGF1R-pY1166). Staining was analyzed by fluorescent microscopy to determine the mean staning intensity per nucleus (visualized with DAPI). Negative control, no primary antibody. *, P<0.1, **, P<0.01, *t*-test. bar = 50 µm.