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Modified mRNA directs the fate of heart progenitor cells and induces vascular regeneration after myocardial infarction

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Author contributions

L.Z. (lzangi@enders.tch.harvard.edu) worked in the Chien, Rossi, and Pu labs, and designed and carried out most of the experiments, analyzed most of the data, and wrote the manuscript. K.O.L in the Chien lab designed and performed experiments and analyzed the qRT-PCR and immunostaining data, and wrote the manuscript. Her contribution is similar in significance to the contributions of L.Z. A.V.G performed and analyzed the *Wt1*-related experiments. Q.M and R.G. carried out myocardial infarction experiments. W.E, carried out plasmid preparation. L.M.P, performed blinded analysis of imaging data and wrote the manuscript. D.S performed and analyzed skeletal muscle in vivo transfection. H.X performed isolation of neonatal mouse cardiomyocytes. M.T performed and analyzed in vitro transfection of mouse adult myotubes. B.S, carried out and analyzed the MRI experiment. M.N, D.M.B, R.A.L, A.J.W, designed experiments, analyzed data, and revised the manuscript. D.J.R. (Derrick.Rossi@childrens.harvard.edu) designed in vitro cardiomyocyte experiments and revised the manuscript. K.R.C. (kchien@harvard.edu; kenneth.chien@ki.se) conceived the initial project and experimental studies, and with W.T.P. (wpu@enders.tch.harvard.edu) designed further experiments, analyzed data, and wrote the manuscript.

Disclosures

K.R.C. and D.J.R. are co-founders of Moderna Therapeutics, a Cambridge, MA, company that is developing therapeutics based on modified-mRNA. K.R.C. is an advisor to Astrazeneca, which has an interest in cardiovascular therapeutic applications of modRNA..

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Abstract

In a cell-free approach to regenerative therapeutics, transient application of paracrine factors *in vivo* could be used to alter the behavior and fate of progenitor cells to achieve sustained clinical benefits. Here we show that intramyocardial injection of synthetic modified RNA (modRNA) encoding human vascular endothelial growth factor-A (VEGF-A) resulted in the expansion and directed differentiation of endogenous heart progenitors in a murine myocardial infarction model. VEGF-A modRNA markedly improved heart function and enhanced long-term survival of recipients. This improvement was in part due to mobilization of epicardial progenitor cells and redirection of their differentiation toward cardiovascular cell types. Direct in vivo comparison with DNA vectors, and temporal control with VEGF inhibitors, documented the markedly increased efficacy of pulse-like delivery of VEGF-A. Our results suggest that modRNA is a versatile approach for expressing paracrine factors as cell fate switches to control progenitor cell fate and thereby enhance long term organ repair.

Myocardial infarction in humans causes the death of billions of cardiomyocytes. The heart's limited capacity to regenerate these lost cardiomyocytes leads to compromised cardiac function and high morbidity and mortality. As a result, there has been intense interest in developing treatments to reduce or reverse myocardial injury. A number of strategies have been proposed for regenerative cardiovascular therapeutics, including transplantation of *ex-vivo* expanded cells, delivery of therapeutic genes on naked DNA plasmids or viral vectors, and administration of recombinant proteins. Thus far, these approaches have had mixed results. Cell-based therapies have shown limited long-term engraftment and low efficacy. Gene-based methods have suffered from poor control of dosage and duration, low gene transfer efficiency, risk of genomic integration and associated tumorigenesis, and anti-viral immune responses. Recombinant proteins have suffered from fleeting tissue half-lives, poor targeting to specific organs, and complications due to systemic release. modRNA, in which replacement of one or more nucleotides by modified nucleotides, represents a potential alternative therapeutic platform. Previous work showed that modRNA mediates highly efficient, transient protein expression *in vitro* and *in vivo* without eliciting an innate immune response[1-6]. We therefore hypothesized that modRNA might provide an effective means to control the spatial and temporal delivery of gene products to enhance tissue repair or regeneration after injury.

Paracrine factors play key roles in regulating progenitor cell activity in heart development, and recent studies have likewise implicated paracrine factors in promoting cardiac repair and regeneration after MI in experimental model systems[7-9]. In part, paracrine factors promote heart regeneration by stimulating cardiomyogenic activity of poorly defined endogenous heart progenitors[7, 8, 10]. Given that native paracrine signals are often transient and precisely regulated in time and space, we further hypothesized that the pulse-like expression profile of

modRNA might be well suited to delivering paracrine factor signals that modulate heart progenitor activity and thereby promote heart repair or regeneration.

To test these hypotheses, we studied the kinetics and efficacy of modRNA-mediated gene transfer in a murine myocardial infarction model. Previous studies have shown that epicardial heart progenitors are activated within 48 hours after murine myocardial infarction and amplify in a thickened epicardial layer at the surface of the heart. However, these cells are not mobilized to enter the myocardium and differentiate towards cardiovascular lineages as they do in the fetal heart[11-13], but rather remain on the heart surface and differentiate largely into fibroblasts and myofibroblasts. For initial proof-of-concept experiments, we studied modRNA encoding VEGF-A, as our recent in vitro studies identified a new function for VEGF-A as a cell fate switch for multipotent *Islet1*$^{+}$ (*Isl1*$^{+}$) human heart progenitors, driving them away from a cardiac muscle fate and towards a vascular endothelial cells[14]. Here, we asked if in vivo delivery of VEGF-A modRNA given at the time of epicardial cell activation following MI would stimulate epicardial progenitor mobilization or modulate their differentiation. We found that modRNA mediates "pulse-like" expression of VEGF-A and is superior to plasmid DNA in reducing infarct size, enhancing myocardial perfusion, and improving survival. In part, this effect was due to a previously unknown effect of VEGF-A on epicardial progenitors: VEGF-A modRNA amplified these progenitors, mobilized their migration into the myocardium, and redirected their differentiation towards cardiovascular lineages. These results indicate that modRNA gene transfer drives *in vivo* heart progenitor cell fate to enhance cardiac repair.

Results

"Pulse-like" kinetics of modRNA gene delivery to heart and skeletal muscle

We evaluated the suitability of modRNA for gene transfer to heart and skeletal muscle—tissues that have been historically difficult to transfect. Notably, modRNA transfected primary fetal human, neonatal mouse, and adult rat cardiomyocytes or adult mouse skeletal myotubes with high efficiency (89%, 72%, 68%, and 100%, respectively; **Supplementary Fig. 1**) and minimal toxicity (~80% cell survival, comparable to transfection vehicle control). This high efficiency represents a 10- to 40-fold increase compared to typical transfection efficiencies attained using non-viral DNA mediated transfection[15, 16]. modRNA likewise mediated efficient protein production in cardiac cells in vivo. Direct, single, intramyocardial (IM) injection of luciferase (Luc) modRNA yielded robust bioluminescent signal indicative of dose- and time-dependent luciferase protein expression localized to the injection site (**Fig. 1a-c**). Luc expression was immediately expressed and reached high level of expression after only 3 hours, peaked at 18 hours, and returned to baseline at 144-150 hours (**Fig. 1c**). These kinetics differed significantly from luciferase DNA, which peaked at 72 hours and retained high signal for more than 10 days post injection.

To examine cardiac cell types transduced by modRNA, we injected Cre recombinase DNA plasmid or modRNA into Rosa26-lox-stop-lox-LacZ (R26^{fsLacZ}) mouse hearts, followed by X-gal staining for Cre-activated β-galactosidase (blue stain, **Fig. 1d-j**). Whereas DNA plasmid transfection resulted in infrequent cell transfection (**Fig. 1e-g**), modRNA transfected the vast majority of cells in the injection region, including endothelial cells

(~90%), cardiomyocytes (~80%), and smooth muscle cells (~90%) (**Fig. 1h-j**). A single injection of modRNA into the cardiac apex efficiently transfected a substantial area of myocardium (~25% of the left ventricle), a level of transfection that was at least 10 times greater than that achieved by injection of naked plasmid DNA (**Fig. 1k**). Similarly efficient in vivo modRNA transfection was observed in skeletal muscle (**Supplementary Fig. 2**).

VEGF-A modRNA improves myocardial outcome after myocardial infarction

The "pulse-like" kinetics of modRNA gene delivery led us to hypothesize that modRNA might be an effective means to deliver paracrine factors that could alter progenitor cell fate decisions and thereby durably modify organ injury responses. The fetal and post-natal mammalian heart contains a diverse set of endogenous cardiovascular progenitors[7, 9, 11, 13, 17-23], but native expansion, mobilization, and differentiation of these progenitors in vivo has been difficult, and is inadequate to restore myocardial function after injury[24]. We hypothesized that modRNA activation of paracrine signaling pathways responsible for regulation of endogenous heart progenitors might enhance myocardial outcome after myocardial infarction.

We elected to focus on VEGF-A, a potent angiogenic factor that we recently reported acts as a cell fate switch on cultured human cardiac progenitors[14]. Based on its angiogenic activity, VEGF-A has been proposed as a therapeutic agent to improve myocardial outcome after ischemic injury (see **Supplementary Appendix** and **Supplementary Tables 1-2**, and references therein). Previous human and animal studies of VEGF-A using naked DNA plasmids, recombinant proteins, and engineered viruses showed limited efficacy after myocardial infarction, but these negative results may have been due to suboptimal delivery and/or anti-viral immune responses[16, 25-27]. Temporal and spatial control of VEGF-A expression is likely to be critical for its therapeutic efficacy, as two independent groups showed that prolonged exposure of normal muscle to VEGF-A caused excessive vascular permeability[28-30]. Furthermore, the potential activity of VEGF-A on endogenous adult cardiac progenitors has not been studied in vivo or in the context of myocardial injury. We therefore set out to determine whether VEGF-A modRNA has beneficial activity in myocardial infarction, and we benchmarked its performance in comparison to traditional, non-viral, VEGF-A delivery by plasmid DNA. First, we examined the kinetic profile of VEGF-A expression after VEGF-A modRNA administration in vivo and in vitro. Previous work established that cardiac VEGF-A ectopic expression does not elevate VEGF-A protein levels in serum[31]. However, in vitro using cardiac cells transfected with VEGF-A modRNA we were able to show that VEGF-A modRNA-transfected cardiac cells translated and secreted VEGF-A protein with pulse-like kinetics that peaked rapidly and declined to basal levels by 2-3 days (**Fig. 1l**). In contrast, VEGF-A DNA plasmid (DNA) transfection induced a much broader secretion profile that peaked at a lower level at 3 days and gradually declined, with protein levels remaining above baseline at day 10.

Next, we evaluated the toxicity and immunogenicity of VEGF-A modRNA in vitro and in vivo. VEGF-A modRNA induced greater VEGF-A protein secretion than VEGF-A mRNA (**Supplementary Fig. 3a**). Unlike mRNA, modRNA did not cause apoptosis or upregulation of RIG-1, INF- α, or INF- β, hallmarks of the innate immune response (**Supplementary Fig.**

3b-c). VEGF-A modRNA likewise exhibited minimal immunogenicity when delivered to skeletal muscle in vivo (**Supplementary Fig. 3c**). In addition, DNA plasmid delivery in vivo into cardiac muscle upregulated INF- β and RIG-1. These results are consistent with prior studies that demonstrated low modRNA toxicity and immunogenicity[1-6] and immunogenicity of DNA plasmid[32, 33]. Together, these data show that modRNA is an efficient and non-toxic approach for transient, highly efficient, and localized gene delivery to heart and skeletal muscle.

To assess the efficacy of VEGF-A modRNA in the murine myocardial infarction model, we administered it at the time of coronary artery ligation by direct intramyocardial injection into the ischemic region. VEGF-A modRNA stimulated formation of systemically perfused vessels in the area of injection (**Fig. 2a**). Both VEGF-A modRNA and VEGF-A DNA reduced infarct size and apoptotic cell frequency, and increased capillary density at 1 and 4 weeks after myocardial infarction (**Fig. 2b**, $P \leq 0.05$; **Supplementary Fig. 4**). These beneficial effects required VEGF-A signaling through its canonical receptor KDR (also known as VEGFR2), because they were **[AU: OK?OK]** blocked by either SU5614 or PTK787, specific small-molecule inhibitors of KDR (**Fig. 2c**; $P \leq 0.05$).

Although both VEGF-A modRNA and DNA increased myocardial capillary density and reduced infarct size and cell death, the vessels formed by these treatments were functionally different. In VEGF-A DNA–treated hearts, vessels showed excessive permeability as demonstrated by extravasation of 70 kd dextran beads(**Fig. 2d-f**). In contrast, vessels in VEGF-A modRNA–treated hearts did not show this abnormal vascular permeability (**Fig. 2d-f**). The difference in vascular permeability was readily apparent on inspection of hearts: VEGF-A modRNA–treated hearts were similar in shape to control hearts, whereas VEGF-A DNA-treated hearts displayed obvious edema (**Fig. 2g**). This difference in vessel function was likely due to prolonged exposure to VEGF-A with DNA-mediated gene transfer, as increased vascular permeability is a known consequence of lengthy VEGF-A expression[34-36]. To further evaluate the importance of expression kinetics on outcome, we administered Avastin, a neutralizing antibody specific for transfected human VEGF-A starting at 3 days post-myocardial infarction. Avastin did not detectably affect VEGF-A modRNA activity in increasing vessel density (**Fig. 2d-g**) but blocked vessel induction by VEGF-A DNA, consistent with the differing expression kinetics of each modality. On the other hand, Avastin reduced the abnormal vascular permeability and edema induced by VEGF-A DNA. These data indicate that the rapid, brief pulse of VEGF-A delivered by modRNA stimulated growth of functional vessels, whereas the more prolonged VEGF-A expression delivered by DNA stimulated formation of leaky vessels.

We next evaluated the effect of VEGF-A modRNA and DNA on short-term survival after myocardial infarction (**Fig. 3a**). Although VEGF-A DNA augmented vessel number, it caused increased mortality compared to vehicle controls (P=0.02 vs. vehicle control), consistent with earlier reports[30] This detrimental effect was likely due to increased vascular permeability and cardiac edema from prolonged VEGF-A exposure, because Avastin blockade of human VEGF-A beginning on post-myocardial infarction day 3 restored mortality to control levels (P=0.04 in presence vs. absence of Avastin). Unlike VEGF-A DNA, VEGF-A modRNA did not have an adverse effect on short-term survival, and

consistent with VEGF-A modRNA expression kinetics this was not significantly changed by Avastin given on post-myocardial infarction day 3 (**Fig. 3a**). Together, these data point to the importance of VEGF-A expression kinetics mediated by different gene expression systems in determining their biological effect (**Suppl. Table 3**).

To assess the long-term effect of VEGF-A modRNA on outcome after myocardial infarction, we continued to monitor the survival of control mice and mice treated with VEGF-A modRNA to one year after myocardial infarction. VEGF-A DNA–treated mice were not included in this long-term study because of their poor survival in the initial month following MI. In the control group, the 1-month survival was ~60%, similar to other reported studies[37-39]. Survival was significantly higher in the VEGF-A modRNA group than controls at this late end point (**Fig. 3b**; *P*=0.04; n=14). The beneficial effect of VEGF-A modRNA on survival was reflected in its effect on cardiac function, as determined by cardiac magnetic resonance imaging (MRI) measurement of cardiac ejection fraction (EF; **Fig. 3c-d**). At day 1 following myocardial infarction, EF was reduced to a similar extent in control and VEGF-A modRNA groups, indicating equivalent severity of myocardial injury (**Fig. 3d**). At 21 days after myocardial infarction, EF was better preserved in the VEGF-A modRNA group (**Fig. 3d**; *P*=0.001, n=4; Supplementary Movies 1-3). Consistent with declining heart function in controls compared to VEGF-A modRNA treatment, heart rate increased between days 1 and 21 after myocardial infarction in controls, and this effect was blocked by VEGF-A modRNA (**Supplementary Fig. 5**). Other MRI indices were not significantly different between groups (**Supplementary Fig. 5**). MRI assessment at later time points was not performed because excess mortality in the control group would have introduced strong survivor bias. Collectively these data indicate that VEGF-A modRNA has sustained beneficial effects on myocardial outcome and on long-term survival after myocardial infarction.

VEGF-A modRNA activates epicardial cardiac progenitor cells via KDR

To further investigate mechanisms that underlie the beneficial activity of VEGF-A modRNA, we measured the expression of known cardiovascular progenitor and differentiated cell lineage markers in peri-infarct tissue of control and VEGF-A modRNA-treated hearts. qRT-PCR analysis demonstrated upregulation of the cardiomyocyte marker *Tnnt2* and the endothelial cell markers *Pecam1* and *Kdr* (**Fig. 4a** and **Supplementary Fig. 6**). Among cardiac progenitor markers, Wilm's tumor gene 1 (*Wt1*) was highly upregulated by VEGF-A modRNA compared to control treatment, whereas other heart progenitor markers, such as *Isl1*[17-21] and *Nkx2-5,* were not substantially changed (**Fig. 4a** and **Supplementary Fig. 6**). In the heart, *Wt1* is a marker of an epicardial progenitor population that has an important role in heart injury responses[9]. Upregulation of *Wt1* was confined to the heart, and was not observed in other compartments including those that might be sources of blood-borne cells (**Supplementary Fig. 7**). *Wt1* upregulation by VEGF-A modRNA suggested that the VEGF-A pulse affected the epicardial progenitor population. We confirmed a marked increase in $WT1^+$ cells in the peri-infarct region by immunohistochemistry (**Fig. 4b**). Importantly, this amplification of $WT1^+$ cells by VEGF-A modRNA required activation of epicardial cells by injury, as it was not observed in VEGF-A modRNA-treated, sham-operated hearts (**Fig. 4b**). KDR inhibition by either SU5614 or

PTK787 blocked the effect of VEGF-A modRNA (**Fig. 4c**), indicating that its action requires canonical VEGF-A signaling through the KDR receptor. These results were confirmed in an unbiased and independent FACS-based approach that took advantage of the $Wt1^{GFPCre}$ mouse line, in which a GFP-Cre fusion protein is knocked into the endogenous *Wt1* locus[13]. Consistent with quantitation of $WT1^+$ cells by immunohistochemistry, FACS-based quantitation of the number of $WT1^+$ cells by GFP signal showed that myocardial infarction alone substantially expanded this cell population (**Fig. 4d**), as we reported previously[9]. VEGF-A modRNA, but not Luc modRNA, further strongly amplified this population by ~4-fold. Again, this effect was blocked by KDR inhibitors SU5614 or PTK787.

To determine whether VEGF-A acts directly on $WT1^+$ epicardial progenitors to drive their amplification, we measured the abundance of KDR receptor on these cells by FACS (**Fig. 4e**). In sham-operated heart, $WT1^+$ cells expressed low levels of the KDR receptor (mean KDR fluorescence intensity ~2-fold above isotype control background). $WT1^+$ cell expression of the KDR receptor increased significantly (~6-fold) after myocardial infarction, and VEGF-A modRNA treatment further upregulated KDR expression by 5.5-fold, indicating a positive feedback response in which VEGF-A reinforces KDR expression, as previously noted in endothelial cells[40]. Upregulation of KDR after myocardial infarction coincides with general activation of numerous epicardial genes with injury[9] and likely accounts for the requirement of myocardial infarction to enable $WT1^+$ epicardial cells to respond to VEGF-A modRNA (**Fig. 4b**). To further determine whether VEGF-A acts directly on post-myocardial infarction $WT1^+$ epicardial cells, we FACS purified these cells and measured their proliferation in response to recombinant VEGF-A (**Fig. 4f**). VEGF-A increased proliferation of cultured $WT1^+$ epicardial cells, and KDR inhibition powerfully blocked their proliferation. These data indicate that VEGF-A acts directly through KDR on activated, post-myocardial infarction $WT1^+$ epicardial progenitors.

VEGF-A modRNA induced $WT1^+$ epicardial progenitor differentiation into cardiovascular cell types *in vitro*

During heart development, $WT1^+$ epicardial progenitors undergo an epithelial to mesenchymal transition. The resulting epicardium-derived cells (EPDCs) migrate into the myocardium and predominantly differentiate into fibroblasts and smooth muscle cells. Infrequently, EPDCs contribute to the endothelial lineage[13, 41], and they have also been found to differentiate into cardiomyocytes[11, 23, 41]. VEGF-A modRNA increased capillary density and upregulated endothelial markers in peri-infarct tissue (**Fig. 2b**). We hypothesized that VEGF-A might alter the fate of the $WT1^+$ epicardial progenitors and enhance their endothelial differentiation. qRT-PCR analysis of FACS-purified post-myocardial infarction $WT1^+$ epicardial progenitors indicated that VEGF-A modRNA strongly upregulated expression of endothelial markers *Pecam1* and *Kdr* (**Fig. 5a**). VEGF-A stimulation of endothelial differentiation of epicardial progenitors was further demonstrated by culturing FACS-purified post-myocardial infarction $Wt1^+$ cells for 7 days in the presence or absence of recombinant VEGF-A. Analysis of marker genes by qRT-PCR revealed that VEGF-A stimulation strongly upregulated endothelial markers *VE-Cadherin*, *Pecam1*, and *Kdr* (**Supplementary Fig. 8a**). This result was corroborated by FACS analysis, which

showed that VEGF-A treatment markedly increased the frequency of KDR^{+}/VE-cadherin^{+} endothelial cells (68% vs. 14%; **Supplementary Fig. 8b**), and by immunofluorescence imaging, which demonstrated co-expression of VE-cadherin and GFP (expressed from Wt1^{GFPCre}) in cultures stimulated by VEGF-A (**Supplementary Fig. 8c**).

We further tested the hypothesis that VEGF-A influences WT1^{+} epicardial progenitor cell lineage decisions using an in vitro clonal assay. FACS-purified post-myocardial infarction WT1^{+} epicardial cells were individually plated in 96 well dishes, clonally expanded, and assessed for differentiation into the major cardiac lineages in the presence of absence of recombinant VEGFA by qRT-PCR (**Fig. 5b**). This assay demonstrated the multipotency of WT1^{+} epicardial progenitors at the clonal level (**Fig. 5c**). VEGF-A treatment for 7 days increased the fraction of clones that were positive for the endothelial marker *VE-Cadherin* from 12% to 52% (**Fig. 5c**). To further demonstrate that this was a clonal event rather than polyclonal contamination of endothelial cells, we subcloned a clone that was initially *VE-Cadherin* negative in the absence of VEGF-A and repeated the clonal differentiation assay. VEGF-A again strongly stimulated differentiation towards an endothelial fate (24% vs. 0%, **Fig. 5d**). These clonal assays confirm the multipotency of WT1^{+} epicardial progenitor cells, and demonstrate at the clonal level that VEGF-A biases epicardial progenitor fate decisions towards the endothelial lineage.

In summary, these studies identify epicardial progenitors as a novel target for VEGF as a vasculogenic cell fate switch, in addition to its already well known effect on promoting the proliferation of already differentiated endothelial cells

VEGF-A modRNA induced WT1^{+} epicardial cell differentiation into endovascular cell types *in vivo*

To directly track the fate of EPDCs to different lineages following myocardial infarction, we performed genetic lineage tracing using WT1$^{CreERT2/+}$ mice, in which tamoxifen-activated CreERT2 was expressed from the endogenous *Wt1* locus[9]. Treatment of adult mice with tamoxifen triggered recombination of the R26^{mTmG} reporter[42] in *Wt1*-expressing cells, irreversibly labeling them with membrane-localized GFP and simultaneously inactivating expression of membrane-localized Tomato fluorescent protein. We defined these cardiac GFP^{+} cells, consisting of *Wt1*-expressing cells and their descendants, as EPDCs[9]. After allowing clearance of tamoxifen for one week, myocardial infarction was performed with injection of VEGF-A or Luc (control gene) modRNA into the peri-infarct zone. After 7 days, the fate of Wt1^{CreERT2}-labeled EPDCs was evaluated by FACS, qRTPCR, and immunofluorescent imaging (Fig. **6a-e** and **Supplementary Fig. 9**). FACS demonstrated that VEGF-A modRNA increased the number of EPDCs 4-fold (**Fig. 6b**). The fraction of PECAM1+ cells was 26% greater in VEGF-A modRNA versus Luc modRNA hearts (44% vs. 18%). Therefore we estimate that ~23% (6%/26%) of the VEGF-A stimulated increase in PECAM1^{+} cells arose from EPDCs. This result was supported by qRT-PCR of FACS-purified EPDCs, which showed that VEGF-A modRNA increased expression of *Pecam1* and *Kdr* (**Fig. 6c**). This assay also revealed that EPDCs in the VEGF-A modRNA group upregulated the cardiomyocyte marker *Tnnt2*, suggesting that VEGF-A enhanced EPDC differentiation towards the cardiomyocyte lineage.

Confocal analysis of immunostained sections further substantiated these conclusions. In controls without injury or with myocardial infarction and vehicle treatment, EPDCs stayed on the epicardial surface of the heart[9] (**Supplementary Fig 9**). In contrast, myocardial infarction and VEGF-A modRNA mobilized EPDCs so that they migrated into the myocardium (**Supplementary Fig. 9**) and increased differentiation towards the endothelial lineage (58% with VEGF-A modRNA vs. 16% with Luc modRNA; **Fig. 6d-e**). We also detected EPDCs that co-expressed the cardiomyocyte marker TNNI3 in VEGF-A modRNA hearts, but not in controls (5% vs. 0%, respectively; **Fig. 6d-e**). This result was also unlikely due to VEGF-A-induced upregulation of *Wt1* (and therefore the CreERT2 lineage tracer) in cardiomyocytes, since qRT-PCR indicated that VEGF-A had no effect on adult cardiomyocyte *Wt1* expression (**Supplementary Fig. 10**). Furthermore, tamoxifen labeling was performed prior to either myocardial infarction or VEGF-A modRNA treatment, and the level of tamoxifen-independent Cre activity in myocardial infarction and VEGF-A modRNA treated hearts was trivial (0.005% of cardiomyocytes and 0.003% of endothelial cells were GFP^+).

To confirm our results using an independent system that did not critically depend on *Wt1*-driven marker alleles, we used a Cre modRNA-containing biocompatible gel to selectively label and trace the fate of epicardium-derived cells in the adult heart (**Fig. 6f**). When applied to $R26^{mTmG}$ hearts, Cre modRNA gel selectively labeled cells in the epicardial layer (**Fig. 6g**). We tested the kinetics of gene transfer via modRNA gel by applying Luc modRNA gel onto hearts of CFW mice (**Supplementary Fig. 11a-b**). Luciferase bioluminescence was detected at near peak levels by 3 hours, peaked at 24 hours, and was no longer detectable at 72 hours. Similarly, Cre modRNA gel expressed Cre protein in cells confined to the epicardial layer two days after gel application, but Cre protein was no longer detectable at 14 days (**Supplementary Fig. 11c**).

Based on these data, we developed a Cre modRNA gel-based "prelabeling" strategy (**Fig. 6f**) that minimized the possibility of non-epicardial labeling in the complex environment induced by the myocardial infarction. We applied Cre modRNA gel to the heart to label epicardial cells, waited two weeks for decay of Cre activity, and then performed LAD ligation and concurrent myocardial injection of VEGF-A or Luc modRNA into the infarct region. One week later, we assessed the fate of Cre modRNA-labeled EPDCs by confocal analysis of immunostained cryosections. Consistent with the $Wt1^{CreERT2}$ labeling result, EPDCs remained in the epicardial layer in controls with sham operation or myocardial infarction plus Luc modRNA. In contrast, myocardial infarction plus VEGF-A modRNA mobilized EPDCs from the epicardial layer and allowed their migration into the myocardium (**Fig. 6g** and **Supplementary Fig. 12**). EPDCs co-expressed smooth muscle, endothelial, and cardiomyocyte lineage markers (**Fig. 6h** and **Supplementary Fig. 12**). Quantitation of the percentage of EPDCs expressing each lineage marker showed that VEGF-A modRNA directed EPDCs towards an endothelial fate (48% vs 9%), and led to a small but reproducible subset of EPDCs co-expressing cardiomyocyte markers (**Fig. 6g-i**). Collectively, the $Wt1^{CreERT2}$ and Cre modRNA gel fate mapping experiments demonstrate through two independent approaches that VEGF-A modRNA alters EPDC fate in the

postnatal heart, driving their differentiation into endothelial cells and potentially to cardiomyocytes.

Discussion

Our study advances an approach to solid-organ repair and regeneration in which delivery of appropriate signal(s) at the right time and place modifies endogenous progenitor cell activity and thereby promotes longstanding therapeutic benefits. We show that modRNA is an effective, robust approach to implement this approach. modRNA avoids several of the apparent problems that have arisen with conventional cardiac gene therapy vectors[15, 43], including lack of genomic integration, persistence of expression, immunogenicity, difficulty in scalability and production, need for life long monitoring for tumorigenesis and other adverse clinical outcomes, and the potential for vector escape into the systemic circulation and long-term expression elsewhere in the body. For these reasons, modRNA has enormous translational potential.

One of the keys to paracrine signal therapeutics is to deliver a transient, strong signal at a time and place that coincides with initial activation of an endogenous progenitor pool. As shown in this study, a transient pulse delivered in this manner can achieve long-term benefit through modification of progenitor cell activity and fate. Specifically, we demonstrate that a single intramyocardial injection of VEGF-A modRNA improved myocardial outcome and survival after myocardial infarction. This salutary response was due to improved formation of functional vessels in the peri-infarct region, which is associated with altered activity of epicardial progenitors: pulse-like VEGF-A expression after myocardial infarction amplified the $WT1^+$ epicardial progenitor pool and enhanced their differentiation towards the endothelial lineage (**Fig. 7**), forming a substantial subset of the additional endothelial cells generated under VEGF-A stimulation. This VEGF-A effect on epicardial progenitors is reminiscent of the effect of VEGFA on multipotent *Isl1* heart progenitors that we recently reported[14], and indeed VEGF-A may similarly effect other cardiac progenitor populations. The unique kinetics of modRNA delivery were required to obtain benefit, as it permitted pulse-like VEGF-A delivery at precisely the time that myocardial injury activates epicardial cells from their quiescent state in the normal heart. The transient nature of VEGF-A modRNA delivery was also crucial, as sustained VEGF-A delivery by DNA injection led to adverse effects on vascular function.

EDPC lineage tracing using several different genetic labels has indicated that a subset of EPDCs differentiate into cardiomyocytes under certain conditions in the developing and adult heart[11, 22, 44-46]. Consistent with prior studies, we show that adult EPDCs have little native potential to differentiation towards the cardiomyocyte lineage[9, 13]. Here we show that VEGF-A stimulation increased cardiomyocyte differentiation to consistently detectable, albeit low, levels. We confirmed this result using an independent lineage tracing system, bolstering the evidence that EPDCs differentiate into cardiomyocytes. However, given the pitfalls and limitations of genetic lineage tracing approaches[47, 48], and the lack of cardiomyocyte differentiation in the in vitro clonal assay (which might be attributable to inadequacies of the in vitro culture system), additional studies are needed to further support this conclusion. The number of cardiomyocytes formed by EPDCs was reproducible but low

and likely not sufficient to account for the therapeutic benefit of VEGF-A modRNA. Nevertheless, this finding raises the exciting possibility that additional paracrine signals might be identified that will achieve differentiation of EPDCs to cardiomyocytes at therapeutically meaningful levels.

In summary, we demonstrated that modRNA is an efficient approach for transient, high level, and localized gene transfer into heart and skeletal muscle. Furthermore, we show that the unique pharmacokinetic properties of modRNA make it well suited to implement a therapeutic approach in which transient manipulation of signaling events redirects progenitor cell behavior and fate to achieve a long term, sustained therapeutic benefits, which have thus far been elusive using other therapeutic modalities[10]. This therapeutic approach of pulse paracrine therpeutics and the modRNA gene transfer technology will likely be applicable to repair and regeneration of solid organs in diverse disease contexts.

Methods

Construction of IVT templates and synthesis of modRNA

Production of in vitro transcription (IVT) template constructs and subsequent RNA synthesis have been described previously[3]. All oligonucleotide reagents were synthesized by Integrated DNA Technologies (Coralville). ORFs were amplified by PCR from plasmids encoding GFP, mCherry, firefly luciferase, Cre recombinase, and human VEGF-A (165) (Addgene, see Supplementary Table 4 for ORF sequences). PCR reactions were performed with HiFi Hotstart (KAPA Biosystems) according to the manufacturer's instructions. Splint-mediated ligations were carried out with Ampligase Thermostable DNA Ligase (Epicenter Biotechnologies). UTR ligations were conducted in the presence of 200 nM UTR oligos and 100 nM splint oligos. All intermediate PCR and ligation products were purified with QIAquick spin columns (Qiagen) before further processing. Template PCR amplicons were subcloned with the pcDNA 3.3-TOPO TA cloning kit (Invitrogen). Plasmid inserts were excised by restriction digest and recovered with SizeSelect gels (Invitrogen) before being used to template Poly A tail PCRs. RNA was synthesized with the MEGAscript T7 kit (Ambion), with 1.6 μg of purified tail PCR product to template each 40 μL reaction. A custom ribonucleoside blend was used comprising 3’-O-Me-m7G(5’)ppp(5’)G cap analog (New England Biolabs), adenosine triphosphate and guanosine triphosphate (USB), 5-methylcytidine triphosphate and pseudouridine triphosphate (TriLink Biotechnologies). Final nucleotide concentrations in the reaction mixture were 6 mM for the cap analog, 1.5 mM for guanosine triphosphate, and 7.5 mM for the other nucleotides. RNA was purified with Ambion MEGAclear spin columns and then treated with Antarctic Phosphatase (New England Biolabs) for 30 min at 37°C to remove residual 5’-phosphates. Treated RNA was repurified, quantitated by Nanodrop (Thermo Scientific) and precipitated with 5 M Ammonium Acetate according to the manufacturer's instructions. modRNA was resuspended in 10 mM Tris HCl, 1 mM EDTA at 100 ng/μl for in vitro use or 20-30 μg/μl for in vivo use.

modRNA Transfection

modRNA and RNAiMAX (Invitrogen) transfection agent were each dissolved separately in Opti-MEM (Invitrogen), 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. In vitro transfection was performed by adding the transfection mixture to cells plated in DMEM with 2% FBS and 200ng/ml B18R (eBioscience, San Diego, CA). For in vivo transfection the transfection mixture was injected directly into the cardiac or skeletal muscle of animals.

Epicardial lineage tracing using modRNA gel

The Cre modRNA gel, with a mixture of Cre modRNA (10 μl of modRNA at 20 μg/μl)), lipofectamine (30 μl), and 0.05% polyacrylic acid (10 μl; Sigma), was painted on the surface of the Rosa26 (R26)^{mTmG} hearts 2 weeks before LAD ligation and injection of VEGF-A or Luc modRNA. VEGF-A or Luc modRNA treated hearts were assessed for expression of GFP and different myocardial markers to examine the cell fate of the EPDC derivatives 7 days post myocardial infarction.

Mice

Wt1$^{GFPCre/+}$, Wt1^{CreERT2}, R26^{fsLacZ}, and R26^{mTmG} alleles have been described previously[9, 13, 23, 42, 49]. Genetically engineered mice were in a mixed C57BL6/CFW background and both male and female mice were used. 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), myocardial infarction was induced by ligation of the left anterior descending coronary artery as described below. Hearts were subsequently assessed using a combination of FACS, immunofluorescence and real-time-qPCR (RT-qPCR) analyses for GFP expression and myocardial markers after 7 days. Wt1^{GFPCre} was used for isolating Wt1^{+} progenitor cells by the Wt1-driven GFP marker. The fate of Wt1^{+} epicardial progenitors was determined using adult stage irreversible labeling in the Wt1$^{CreERT2/+}$::R26^{mTmG} model. Mice that were Luc modRNA treated after Tam induction in the presence of myocardial infarction were used as controls. To examine “leaky” CreERT2 activity that might occur under stress in the absence of Tam, mice were treated with sunflower seed oil without Tam prior to myocardial infarction and subsequently underwent myocardial infarction and VEGF-A modRNA or control treatments in parallel with Tam-treated mice. Wild-type were CFW strain (only males). Animals were not randomized, but procedures were performed blinded to genotype and treatment group. All animals that started an experimental protocol and that survived to the measurement point were included. All mice housing and handling were performed in accordance with protocols approved by Institutional Animal Care and Use Committees at Massachusetts General Hospital or Children's Hospital Boston or Harvard University.

Cell Culture

Adult CFW or WT1$^{GFPCre/+}$ hearts were digested using the Neomyt Cardiomyocyte Isolation kit (Cellutron) to achieve a single-cell suspension of adult murine cardiac cells,

according to the manufacturer's instructions. Adult murine cardiac cells were cultured in Mesenchymal Stem Cell Growth Medium (Lonza) containing 10% FBS. Primary cultures of human fetal cardiomyocytes (obtained from Advanced Bioscience Resources, Inc at gestational 20 weeks) were prepared from human fetal ventricles as described before[19]. Briefly, cardiomyocytes were dissociated by means of repeated (6×) enzymatic digestion swith collagenase II solution (Life Technologies) at 37°C. Dissociated cells were pelleted (30 g × 2 minutes) and plated at a density of 1×10^5 cells/cm^2 on 35-mm culture dishes with 2 mL of culture medium (3:1 DMEM: M-199 medium with 5% fetal calf serum and 10% neonatal calf serum). Pre-plating of seeded cells onto 100-mm culture dishes to remove non-cardiomyocytes (for 3 consecutive days) yielded cultures containing approximately 80% cTnT heavy chain-positive cardiac myocytes. Murine neonatal hearts were dissociated to single cells by collagenase II (Sigma) as described previously[9]. Murine neonatal cardiomyocytes were cultured in DMEM containing 5% FBS, 10% horse serum and 1 μg/ml insulin. Rat adult cardiomyocytes were a kind gift from Dr. Ronglih Liao, and isolation and culture methods have been described previously[50]. All cell lines were found negative for mycoplasma contamination.

The secretion of VEGF-A protein was measured using supernatant of murine adult cardiac cells after transfection with VEGF-A modRNA or DNA or RNA using ELISA (R&D systems). Cell transfection efficiency and survival after modRNA transfection was determined as follows: 4 wells were transfected with each different concentration of modRNA GFP (with 0, 0.3,1 or 3 μg per 10^5 cells in a well of a 6 well plate). 16 hours after transfection of cardiac cells, 2 wells from each treatment were trypsinized and stained for trypan blue. The % of intact cells was calculated as the number of trypan blue negative cells per treatment well / number of trypan blue negative cells per well without any treatment *100. To determine transfection efficiency, the two remaining wells were stained for TNNT2 (red) and GFP (green), and double positive cells were measured using ImageJ software.

Wt1-GFP$^+$ cell isolation and in vitro clonal assays

WT1$^+$ epicardial progenitors were isolated from the heart explants of WT1$^{GFPCre/+}$ mice 7 days after myocardial infarction. Cardiac cells (non-myocytes) were allowed to expand from heart explant cultures. As a control, hearts from uninjured Wt1$^{GFPCre/+}$ or Wt1$^{CreERT2/+}$::R26^{mTmG} mice treated either with vehicle or hVEGF-A modRNA were also analyzed. After 1-2 weeks, cells were FACS sorted (FACS Aria III) for GFP$^+$ cells (WT1$^+$). Single cells or pooled cells of WT1$^+$ epicardial progenitors were plated in a fibronectin-coated (5 ng/ml for 2 hours at 37°C) 96 well plate or 1 well of a 12 well plate, respectively. Cell proliferation of the WT1$^+$ epicardial progenitors was assessed in the presence or absence of VEGF-A (50 ng/ml) or different KDR inhibitors, including SU5614 or PTK787 (10 nM/L) or DMSO control at different time points (4, 8 and 14 days). Media were changed every 3 days. Calibration curve of DMSO and KDR inhibitors indicate that the optimal range (ratio of cell death of vehicle treatment (DMSO control) to KDR inhibitors) of using these inhibitors in vitro is between 4-10 nmol/l. Cells were counted using an automated cell counter (Invitrogen).

For clonal assays, epicardial explants from $Wt1^{GFPCre/+}$ myocardial infarction mice were cultured as described[23]. Explant outgrowths were then dissociated and $WT1^{+}$ epicardial cells were FACS sorted. Single sorted cells were deposited into fibronectin-coated 96-well plates and clonally expanded in the presence or absence of VEGF-A (100 μg/ml) for 7-14 days before examination for their cell fate change in vitro. For FACS analyses, sorted cells were incubated with flurochrome-conjugated primary antibodies at 4°C for 30 minutes followed by 3 washes with PBS/2% FBS and resuspended in Hank's balanced salt solution. Flow cytometric analyses were performed using a BD FACSCanto analyzer. GFP^{+} fibroblasts, cardiomyocytes, smooth muscle and endothelial cells were assessed by immunostaining and treatment-blind cell counts were performed through serial sections using ImageJ software.

Immunodetection methods

Immunostaining was performed on cryosections using standard protocols with the antibodies listed in Supplementary Table 5. Isolectin B4 (Vector Lab) was used to stain endothelial cells in cryosections to determine capillary density. TUNEL (Roche) or Annexin V staining (eBiosciences) was performed to detect apoptosis, according to the manufacturer's instructions. To examine blood vessel leakiness, a mixture of 250 μl Isolectin B4 (0.5 mg/ml, Vector Lab) and 250 μl 70 kD FITC-dextran beads (50 mg/ml, Sigma) was injected into the tail vein 7 days post-myocardial infarction. Hearts were removed for histological analysis 30 minutes after tail vein injection. Quantification of immunostaining in cardiac sections was performed using the ImageJ Software. For each image, color channels (red, blue, and green) were first separated into different images. After separation, the intensity of single-color signals within each image was quantitated by the software. Specific structures in the images (e.g., blue ovals corresponding to DAPI-stained nuclei) were defined by intensity threshold analysis. Definition of discrete structures by the software was further refined by contour and area analysis.

Statistical analyses

Statistical significance was determined paired t-test for the MRI results, Log-rank (Mantel-Cox) test for survival curves or Student's t-test for other experiments, with $P < 0.05$ taken as significant. Values were reported as mean ± standard error of the mean. Two-sided Student's t-test based on assumed normal distributions. Sample sizes were selected for 80% power to detect a biologically meaningful effect given our past experience with intragroup variance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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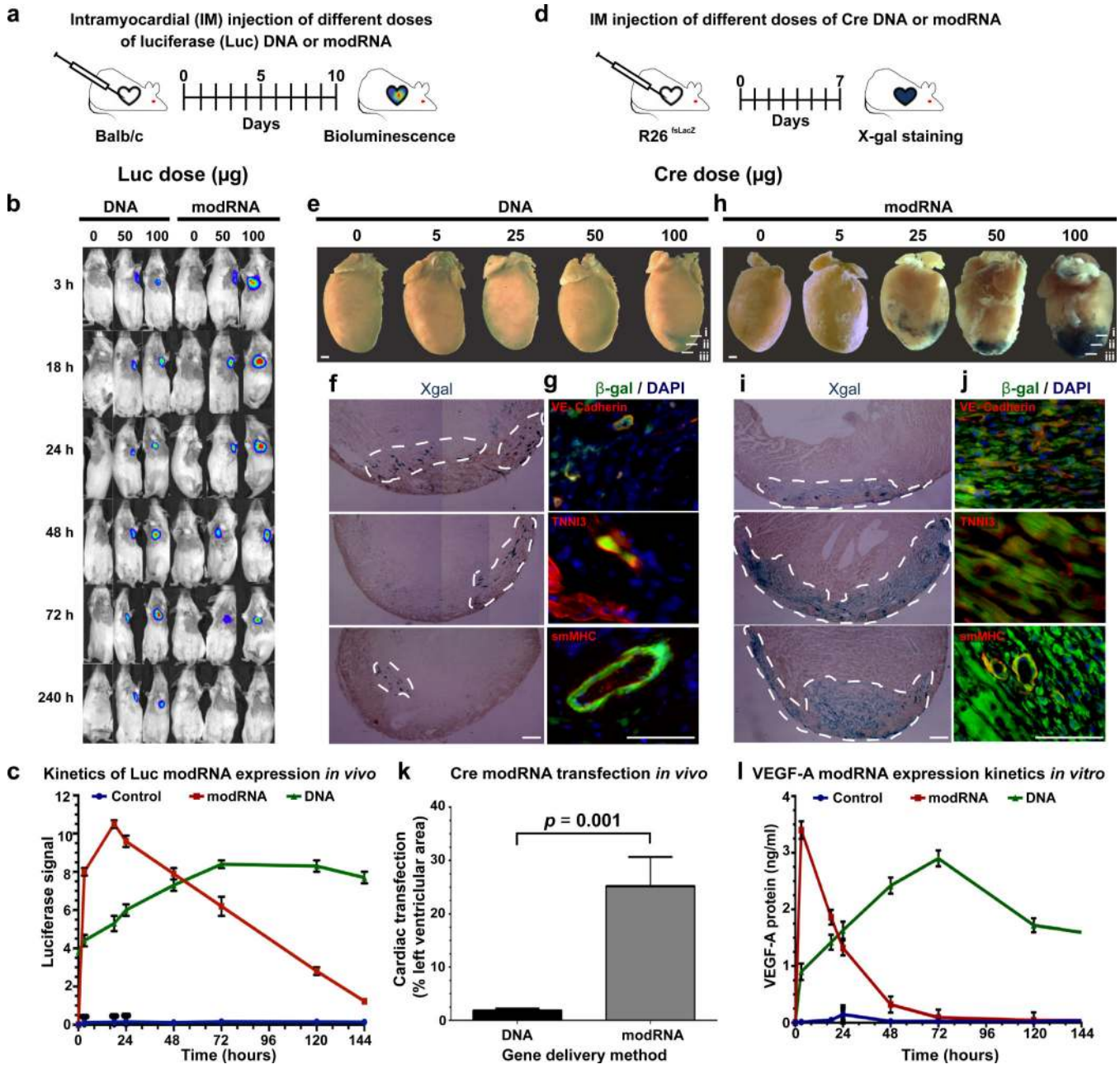

Figure 1. Highly efficient, transient gene transfer in vivo using modRNA

a-b. Luciferase DNA or modRNA was delivered by myocardial injection. Protein expression was assayed by bioluminescence.

c. Time course of luciferase activity after injection of luciferase DNA or modRNA (100 μg) or vehicle only (control) per heart.

d. Cre DNA or modRNA, delivered by myocardial injection, catalyzed cardiac recombination, detected by X-gal staining (blue) for Cre-activated expression of LacZ from $R26^{fsLacZ}$.

e-g. Indicated doses of Cre DNA were injected intramyocardially into $R26^{fsLacZ}$ hearts. DNA plasmid inefficiently transfected cells expressing endothelial, cardiomyocyte, and

smooth muscle lineage markers. Lines labeled i, ii, and iii indicate planes of section in panel f. Bar = 400 μm (e) or 50 μm (f-g).

h-j. Indicated doses of Cre modRNA were injected intramyocardially into $R26^{fsLacZ}$ hearts. modRNA efficiently transfected cells expressing endothelial, cardiomyocyte, and smooth muscle lineage markers. Lines labeled i, ii, and iii indicate planes of section in panel i. Bar = 400 μm (h) or 50 μm (i-j).

k. Summary of transfection efficiency of single 100 μg DNA or modRNA injection into cardiac muscle (left ventricle).

l. Kinetics of VEGFA modRNA expression in cardiac cells cultured in vitro. * For a-l, n=3, Representative of 2 independent experiments.

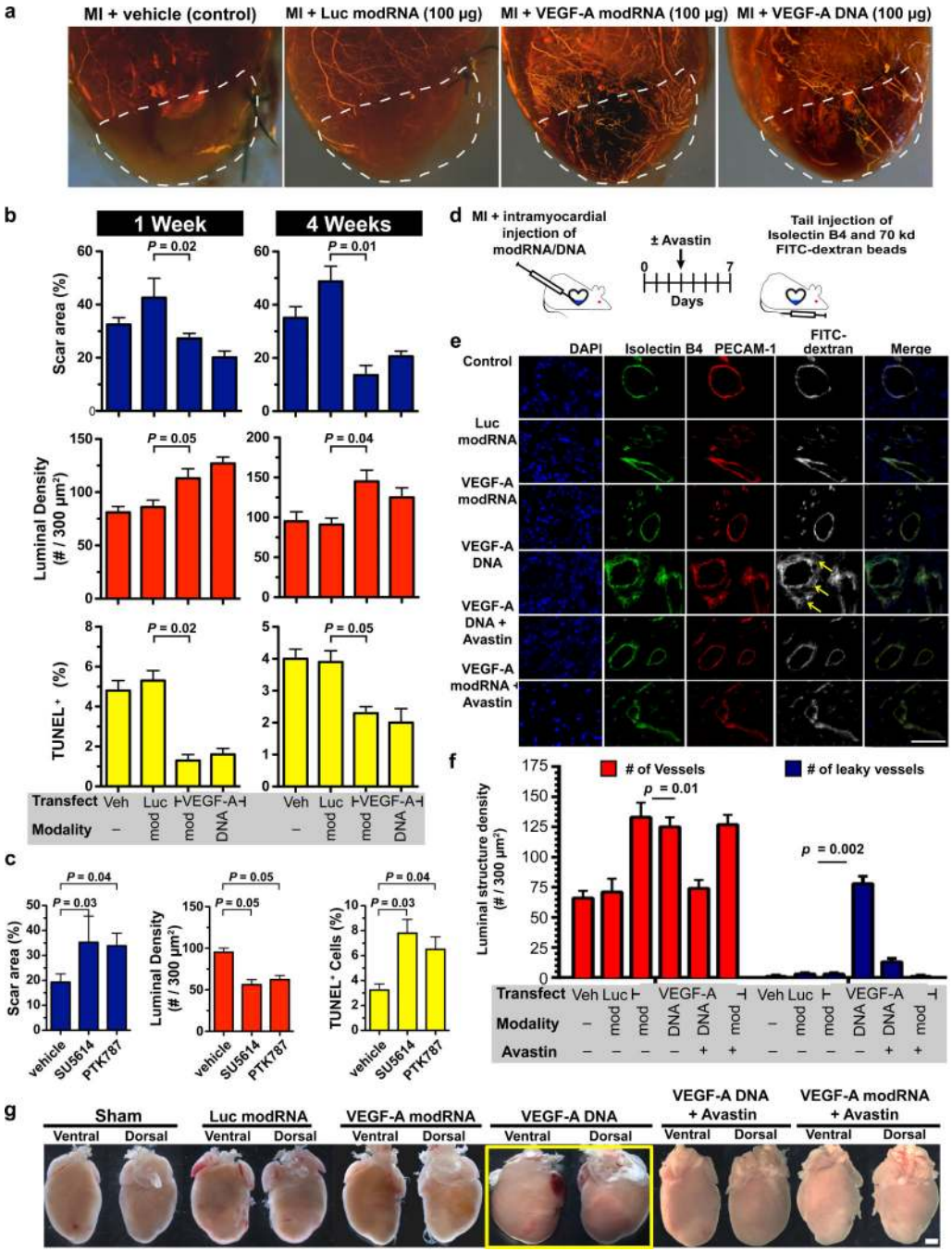

Figure 2. VEGF-A modRNA enhanced formation of functional, non-leaky vessels

a. VEGF-A modRNA, injected into the infarct region at the time experimental myocardial infarction (myocardial infarction), increased vascular density in the peri-infarct region. Seven days after myocardial infarction, the vascular plexus was highlighted by Microfil followed by imaging of cleared hearts. The indicated treatments were injected within the region demarcated by the dashed lines.

b. VEGF-A modRNA reduced scar area and TUNEL$^+$ cells and increased capillary density at 1 week and 4 weeks after myocardial infarction. Capillary density and TUNEL$^+$ fraction

were measured in infarct border zone (left ventricle). Masson's trichrome was used to evaluate scar area (see also Supplementary Figure 4). n=3.

c. Beneficial activity of VEGF-A modRNA required KDR signaling. Mice were treated with myocardial infarction and VEGF-A modRNA. KDR inhibitors SU5614 or PTK787, administered from one day before myocardial infarction to tissue collection at 7 days after myocardial infarction, blocked beneficial effect of VEGF-A modRNA.

d. Experimental design to assess functional angiogenesis. VEGF-A modRNA was injected into the myocardium at the time of LAD ligation. After one week, isolectin B4 and FITC-dextran beads (70 kDa) were injected into the tail vein to assess connection to the systemic vasculature and vascular permeability, respectively.

e. Vessels formed under the influence of VEGF-A DNA, but not VEGF-A modRNA, or VEGF-A DNA with Avastin, neutralizing VEGF-A antibodies (given IP twice a week) were permeable to FITC-dextran (yellow arrows). Scale bar = 50 μm.

f. Density of luminal structures and leaky vessels quantification for different treatments.

g. Macroscopic myocardial edema in VEGF-A DNA (yellow frame), but not VEGF-A modRNA, treated hearts. Scale bar = 5 mm.

*****For a-g, n=3, Representative of 2 independent experiments.

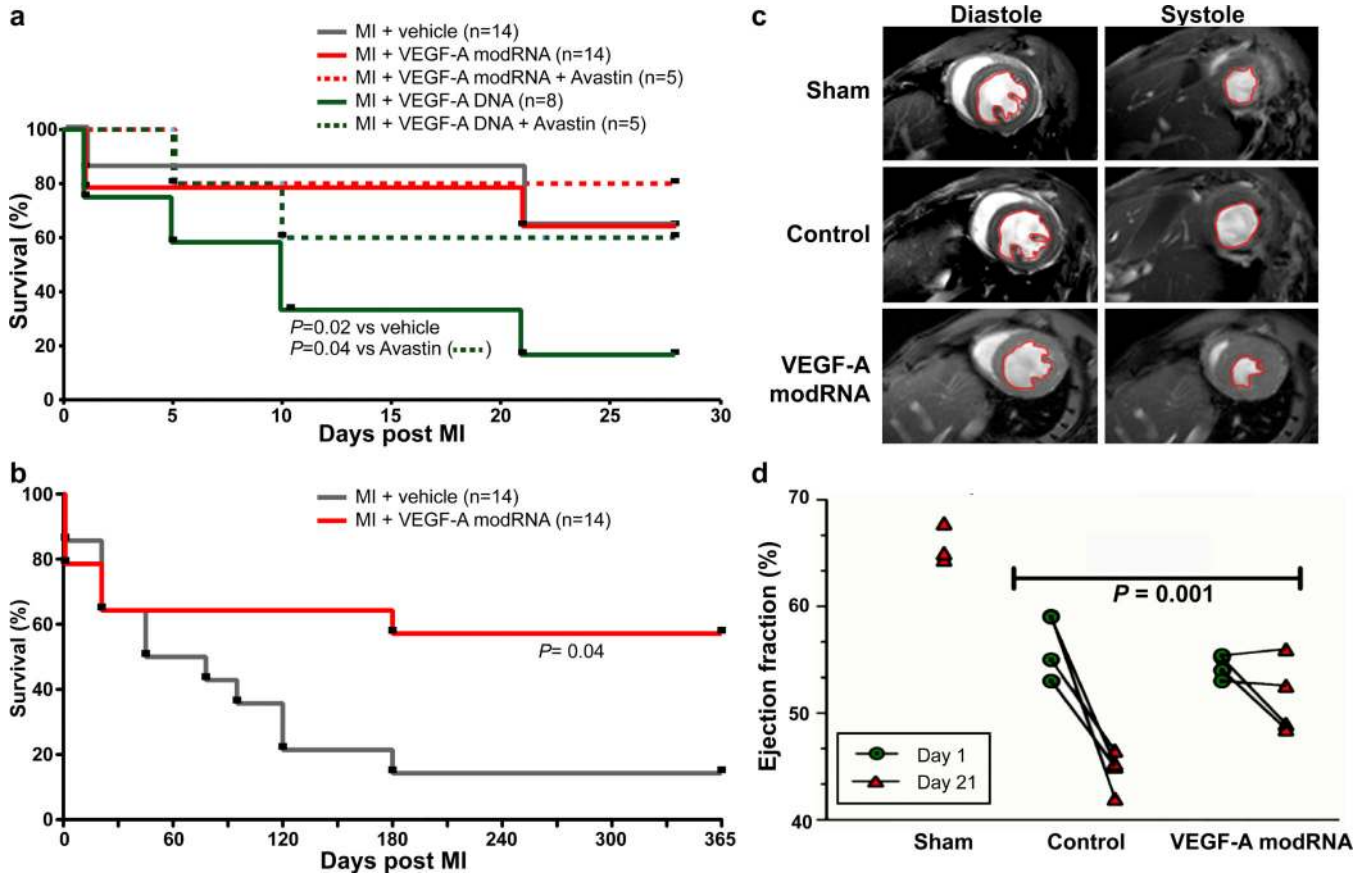

Figure 3. VEGF-A modRNA improved outcome in a murine myocardial infarction model

a. Short term survival curve after myocardial infarction and the indicated treatments. VEGF-A modRNA, DNA, or vehicle were injected into the infarct region at the time of LAD ligation. Avastin was injected twice weekly starting on post-myocardial infarction day 3. P-values were calculated using the Mantel-Cox log-rank test.

b. Long term survival curve after myocardial infarction and VEGF-A modRNA or control treatments. VEGF-A modRNA improved survival at one year compared to control treatment. P-value was calculated as in (a).

c. MRI assessment of left ventricular systolic function. Images show left ventricular chamber (outlined in red) in diastole and systole.

d. Left ventricular systolic function (ejection fraction) was better preserved 21 days after LAD ligation in the VEGF-A modRNA group compared to control. P-value was calculated using paired-test. Sham control, n=3, control or VEGF-A modRNA group, n=5.

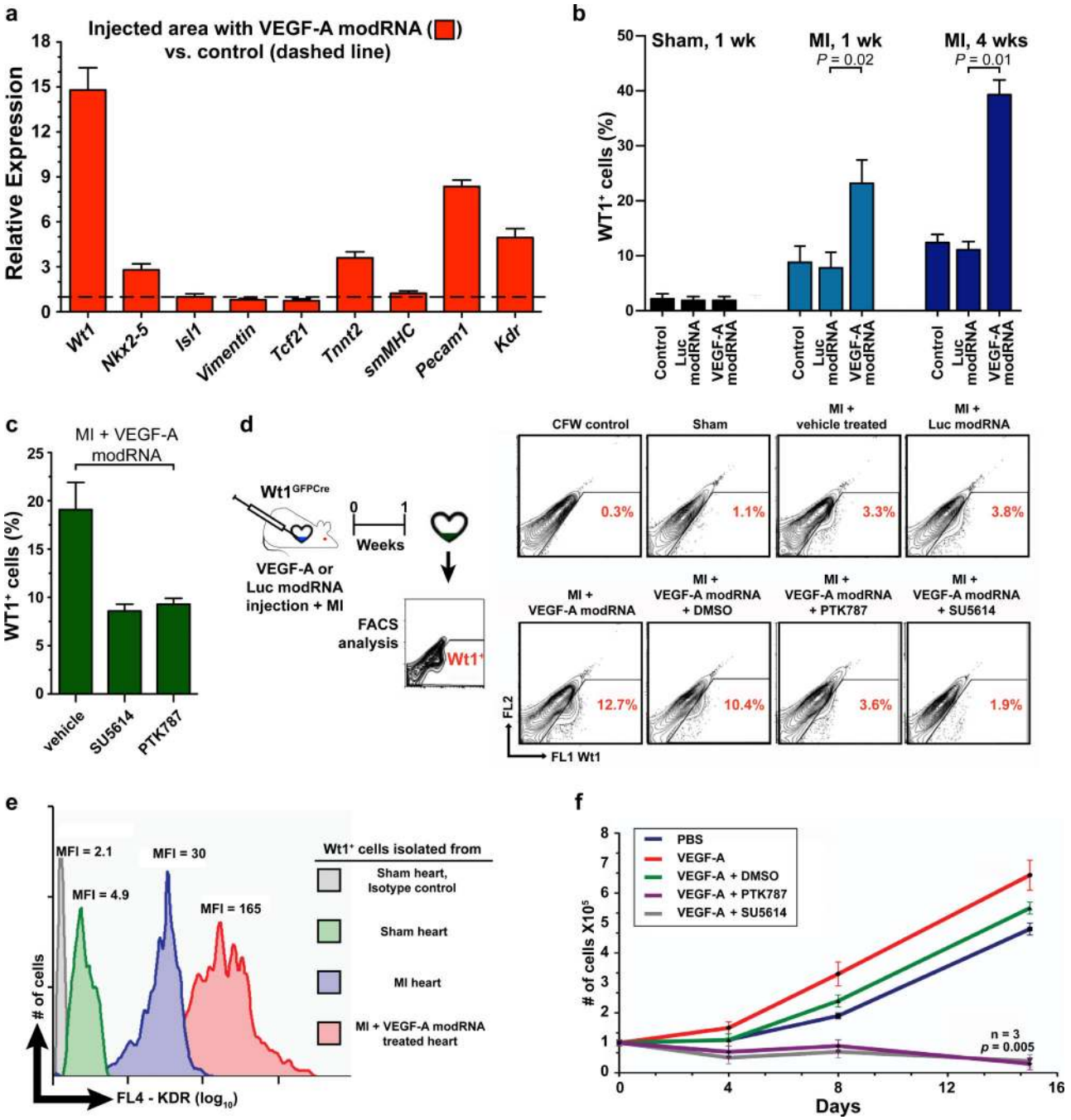

Figure 4. VEGF-A modRNA reduced scar area and apoptosis and increased capillary density and WT1$^+$ cells proliferation after myocardial infarction in a KDR-dependent manner

a. Marker gene analysis showed that VEGF-A modRNA dramatically upregulated *Wt1* expression. qRT-PCR was performed on peri-infarct tissue 3 days post-myocardial infarction. Expression was calculated relative to vehicle-treated heart (dashed line).

b. Quantitation of Wt1$^+$ cells in the infarct border zone (left ventricale) of immunostained heart sections. VEGF-A modRNA increased frequency of WT1$^+$ cells at 1 week and 4 weeks after myocardial infarction but not after sham treatment.

c. Increase in $Wt1^+$ cells in myocardial infarction + VEGF-A modRNA-treated hearts required signaling through KDR. Samples were analyzed as in (b), 1 week after myocardial infarction.

d. FACS-based quantitation of Wt1+ cells after myocardial infarction and control or VEGFA modRNA treatment. $WT1^+$ epicardial progenitors were isolated from dissociated $Wt1^{GFPCre}$ heart by GFP FACS sorting. VEGF-A modRNA treatment increased the frequency of GFP^+ (Wt1-expressing) cells 1 week after myocardial infarction. Red numbers within the region of interest indicate the fraction of cells that were GFP^+ ($WT1^+$).

e. KDR expression on $WT1^+$ epicardial progenitors was measured by FACS. Dissociated $Wt1^{GFPCre/+}$ hearts were stained for KDR, then analyzed by FACS. The histogram shows KDR immunostaining intensity on $WT1^+$ epicardial progenitors (GFP^+). myocardial infarction and VEGF-A modRNA treatment increased KDR mean fluorescence intensity (MFI) on these progenitors.

f. VEGF-A protein increased, and KDR antagonists reduced proliferation of FACS-purified $WT1^+$ epicardial progenitors. Cell number was measured using an automated cell counter at days 4, 8 and 14 of cell culture.

*For a-f, n=3, Representative of 2 independent experiments.

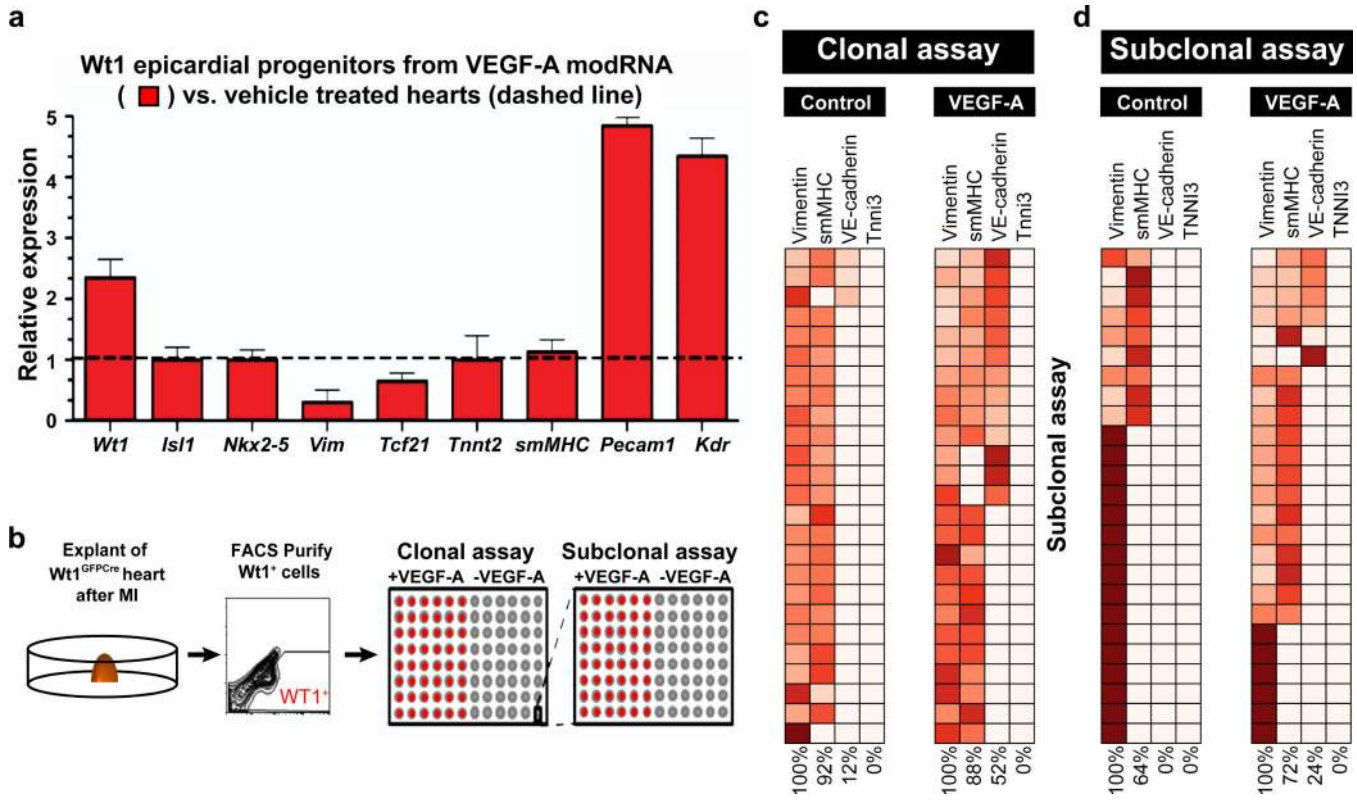

Figure 5. VEGF-A modRNA induced WT1$^+$ epicardial progenitor proliferation and shifted differentiation towards the endothelial lineage

a. VEGF-A modRNA increased endothelial marker gene expression in FACS-purified GFP$^+$ cells after myocardial infarction. Gene expression, determined by qRT-PCR, was calculated relative to GFP$^+$ cells isolated from control-treated, post-myocardial infarction hearts. n=3 Representative of 2 independent experiments.

b. Experimental design of clonal assays to assess VEGF-A modulation of WT1$^+$ epicardial cell fate decisions. Individual FACS-purified cardiac WT1$^+$ cells were deposited in 96 well dishes, clonally expanded, and assessed for differentiation to the indicated lineages by qRT-PCR. One VEGF-A-naïve, VE-Cadherin negative clone was subcloned and the individual subclones were further tested in the clonal differentiation assay.

c. VEGF-A modRNA promoted Wt1$^+$ epicardial progenitor differentiation towards the endothelial lineage. Each row represents an individual clone, and each column indicates the relative qRT-PCR-measured expression of the indicated lineage marker. The precentage of clones with detectable expression of each lineage marker is indicated at the bottom of each column.

d. VEGF-A modRNA effect on Wt1$^+$ epicardial progenitor differentiation was recapitulated in the subclonal assay. This confirmed multipotency of WT1$^+$ epicardial cells, and made polyclonal contamination highly unlikely.

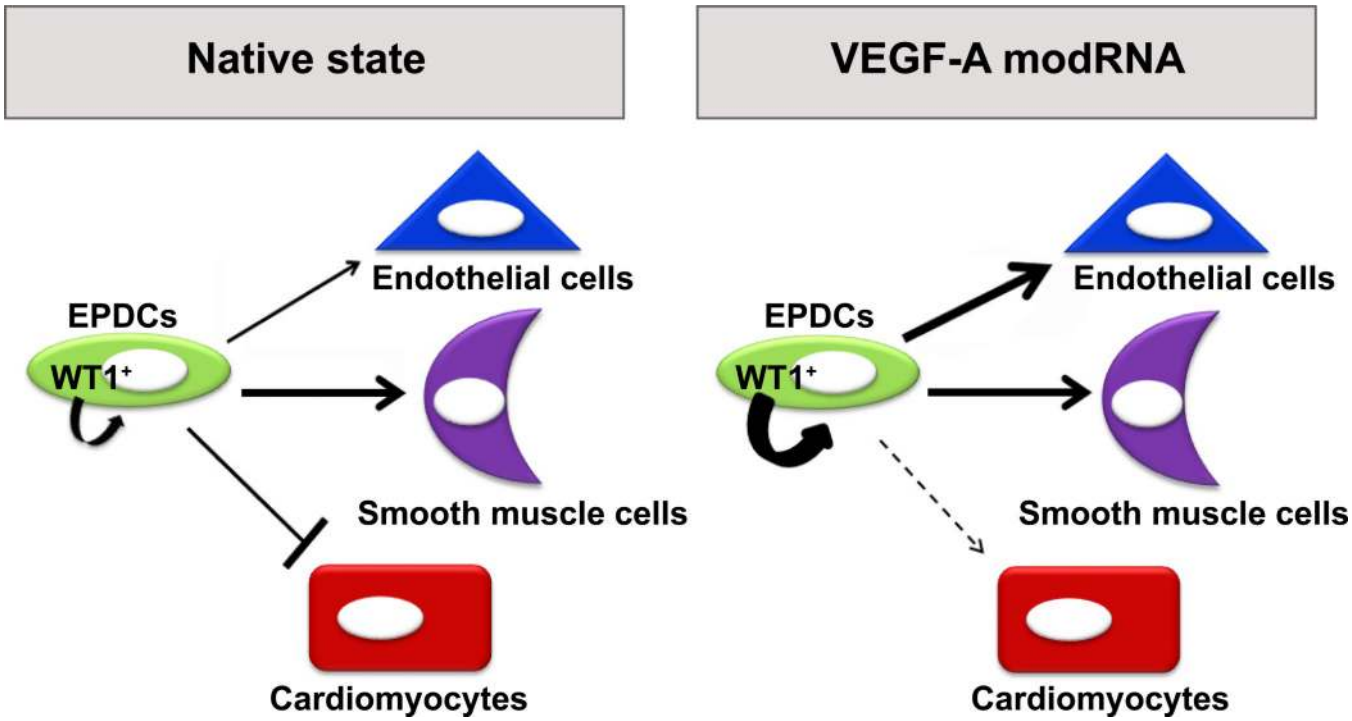

Figure 6. VEGF-A modRNA promoted differentiation of EPDCs towards the cardiovascular lineage in vivo

a. Genetic lineage tracing was used to follow the fate of EPDCs after myocardial infarction with VEGF-A or Luc modRNA treatment. Tamoxifen treatment of $Wt1^{CreERT2/+}$::$R26^{mTmG}$ mice prior to myocardial infarction irreversibly labeled epicardial cells and their descendants with GFP.

b. FACS analysis of dissociated hearts 1 week after myocardial infarction indicated that VEGF-A modRNA increased the frequency of EPDCs expressing the endothelial marker PECAM1 (indicated in red).

c. VEGF-A modRNA increased endothelial and cardiomyocyte marker gene expression in FACS-sorted EPDCs one week after myocardial infarction. Expression of each marker was measured by qRT-PCR and displayed relative to expression in control-treated EPDCs.

d. Immunofluorescent analysis of EPDC fate by $Wt1^{CreERT2}$ genetic lineage tracing. Expression of smooth muscle (smMHC), endothelial (PECAM1), and cardiomyocyte (TNNI3) markers by GFP^+ EPDCs was assessed by immunostaining and confocal microscopy. Bar = 30 μm.

e. Quantitation of d. A minimum of 2000 EPDCs were analyzed in each post-myocardial infarction heart treated with Luc modRNA (n=2) or VEGF-A modRNA (n=5) from 2 independent experiments. The graph shows the percentage of GFP^+ EPDCs that co-expressed the indicated lineage marker.

f. Cre modRNA gel-mediated tracing of epicardial cell fate was used to follow the fate of EPDCs after myocardial infarction with VEGF-A or Luc modRNA treatment. Cre modRNA gel, applied to $R26^{mTmG}$ mice 2 weeks prior to myocardial infarction, irreversibly labeled epicardial cells and their descendants with GFP.

g. Cre modRNA gel selectively labeled epicardial cells with GFP in $R26^{mTmG}$ mice. Note that labeled cells were restricted to the epicardium in controls (Sham or myocardial infarction and Luc modRNA treatment). However in myocardial infarction hearts injected with VEGF-A modRNA, labeled cells were found both in the epicardial layer and within the myocardium, and differentiated into myocytes (yellow asterisk) and non-myocytes (white arrowheads). Bar = 50 μm.

h. Immunofluorescent analysis of EPDC fate with Cre modRNA gel lineage tracing. Bar = 30 μm.

i. Quantitation of h. A minimum of 2000 Cre-gel labeled cells were analyzed in each post-myocardial infarction heart treated with Luc modRNA (n=3) or VEGF-A modRNA (n=4) from 2 independent experiments. The graph shows the percentage of GFP^+ EPDCs that co-expressed the indicated lineage marker.

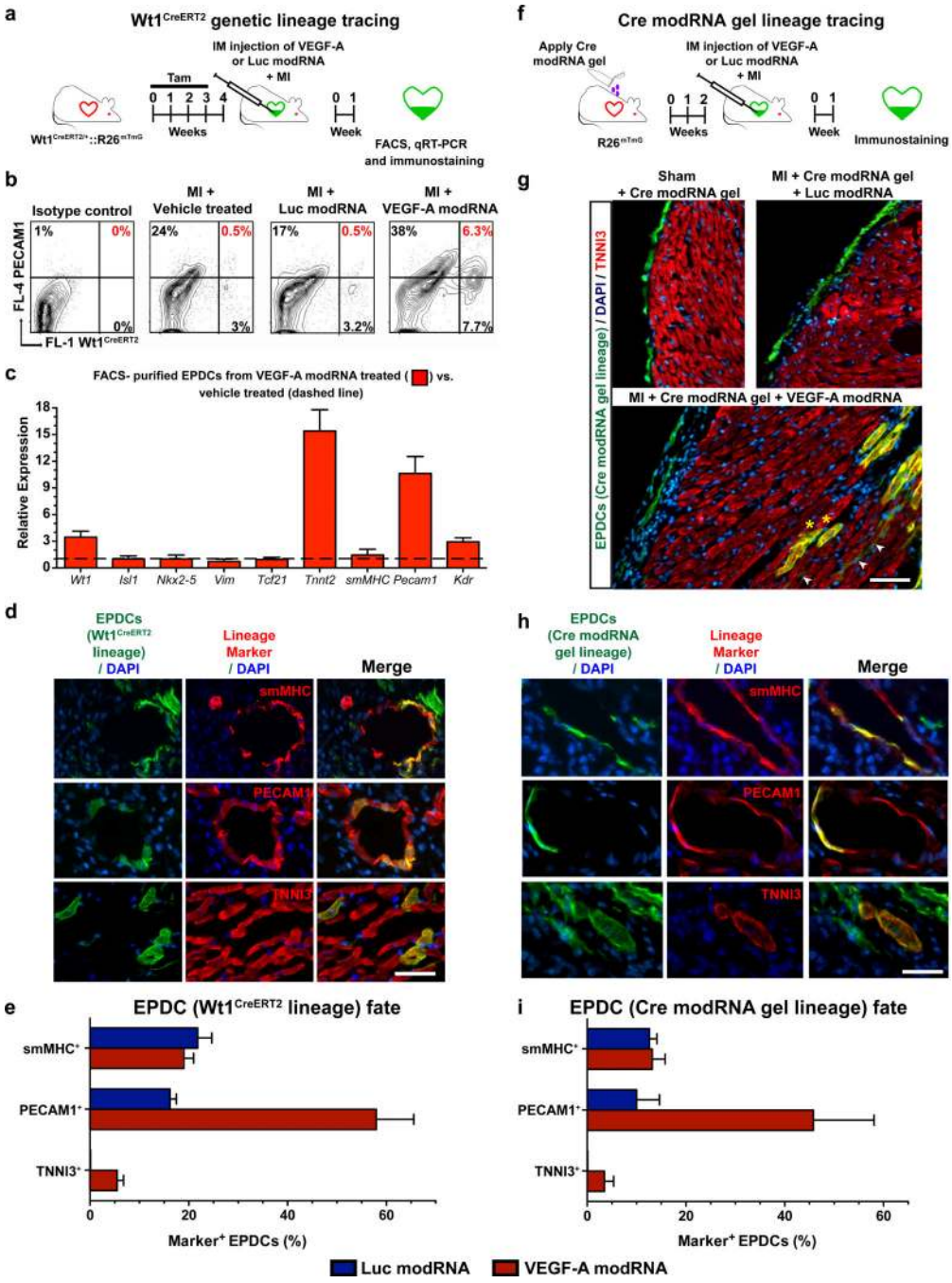

Figure 7. Suggested model for the role of VEGF-A modRNA on EPDCs differentiation in vivo

Schematic summary of results. In the native state, myocardial infarction stimulates amplification of WT1$^+$ EPDCs, which remain confined to the epicardial layer. VEGF-A modRNA in the context of myocardial infarction augments amplification of WT1$^+$ EPDCs, increases their mobilization into the myocardial layer, and enhances their differentiation towards the endothelial lineage.