In vivo reprogramming of murine cardiac fibroblasts into induced cardiomyocytes

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The reprogramming of adult cells into pluripotent cells or directly into alternative adult cell types holds great promise for regenerative medicine. We reported previously that cardiac fibroblasts, which represent 50% of the cells in the mammalian heart, can be directly reprogrammed to adult cardiomyocyte-like cells in vitro by the addition of Gata4, Mef2c and Tbx5 (GMT). Here we use genetic lineage tracing to show that resident non-myocytes in the murine heart can be reprogrammed into cardiomyocyte-like cells in vivo by local delivery of GMT after coronary ligation. Induced cardiomyocytes became binucleate, assembled sarcomeres and had cardiomyocyte-like gene expression. Analysis of single cells revealed ventricular cardiomyocyte-like action potentials, beating upon electrical stimulation, and evidence of electrical coupling. In vivo delivery of GMT decreased infarct size and modestly attenuated cardiac dysfunction up to 3 months after coronary ligation. Delivery of the pro-angiogenic and fibroblast-activating peptide, thymosin β4, along with GMT, resulted in further improvements in scar area and cardiac function. These findings demonstrate that cardiac fibroblasts can be reprogrammed into cardiomyocyte-like cells in their native environment for potential regenerative purposes.

Heart failure affects over 14 million people worldwide and is a leading cause of death in adults and in children. Because postnatal cardiomyocytes (CMs) have little or no regenerative capacity, therapies are limited at present. The introduction of exogenous stem-cell-derived CMs holds promise, but also challenges, including delivery, integration, rejection and cellular maturation1–3. Reprogramming adult CMs holds promise, but also challenges, including delivery, integration, rejection and cellular maturation1–3. Reprogramming adult fibroblasts into induced pluripotent stem cells (iPSCs) that are similar to embryonic stem cells addresses some issues4–6, but others, including efficient directed differentiation into CMs and effective delivery, remain.

A new generation of reprogramming technology involves trans-differentiating one adult somatic cell type directly into another7–11. We reported direct reprogramming of fibroblasts into CM-like cells in vitro by expressing three transcription factors: Gata4, Mef2c and Tbx5 (GMT). As observed in reprogramming to iPSCs, the percentage of fibroblast cells fully reprogrammed to beating CMs in vitro was small, but far more were partially reprogrammed, much like pre-iPSCs that can become fully pluripotent with additional stimuli12. We posited that cardiac fibroblasts may reprogram more fully in vivo in their native environment, which might promote survival, maturation, and coupling with neighbouring cells. If so, the vast pool of cardiac fibroblasts in the heart could serve as an endogenous source of new CMs for regenerative therapy.

Retroviral delivery of GMT in vivo

We used a retroviral system to express GMT, and/or dsRed as a marker, in the hearts of 2-month-old male mice by direct intramyocardial injection. After 2 days, transverse sections of the injected area were prepared and co-stained for dsRed, α-actinin (a CM marker) and vimentin (enriched in fibroblasts). No markers are uniquely specific for cardiac fibroblasts, but fibroblasts are known to express vimentin and the surface markers Thy1 and DDR2 (ref. 13). At baseline, it was difficult to detect α-actinin- or vimentin-positive cells that also expressed dsRed, suggesting minimal viral uptake, and consistent with the observation that retroviruses only infect actively dividing cells14.

Fibroblasts are embryologically distinct from CMs in their origin15, and following myocardial infarction (MI) become activated, migrate to the injury site, and proliferate16,17. We induced cardiac injury by coronary artery ligation and injected dsRed retrovirus into the myocardium bordering the infarct zone. Whereas cells co-expressing dsRed and α-actinin were still undetectable, many vimentin-positive cells were also positive for dsRed (Supplementary Fig. 1). By fluorescence-activated cell sorting (FACS), over 4% of cells (98,238 ± 5,523) from the left ventricle of injected hearts were dsRed+ Thy1+ 2 days after injury, suggesting successful delivery of virus into cardiac fibroblasts and possibly other non-myocytes upon injury (Fig. 1a, b). By quantitative polymerase chain reaction (qPCR), dsRed+ Thy1+-sorted cells expressed about 60-fold more GMT than dsRed− Thy1+ cells, and 6–8-fold more than endogenous CMs (Fig. 1c). A similar number of dsRed+ Thy1− cells represented other non-myocyte cell types. Endothelial cells (PECAM+) and some perivascular cells (NG2+; also known as Cspg4) were also transduced by the retrovirus, but haematopoietic (CD34+) and pericardial (WT1+) cells were not (Supplementary Fig. 1).

Reprogramming into induced cardiomyocytes

To determine whether new cardiomyocytes could be created in vivo from cells other than post-mitotic CMs, we used lineage-tracing experiments to track the origin of putative induced cardiomyocytes (iCMs). To label cells genetically, we used a mouse transgenic line that expresses Cre recombinase under the promoter of the fibroblast-enriched gene, periostin18,19. When intercrossed with the R26R-lacZ reporter line20, in which β-galactosidase is activated only in periostin-Cre-expressing cells and their progeny (Fig. 1d–f), we found β-galactosidase activity in many, but not all, cardiac fibroblasts and

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with well-formed sarcomeres and shapes similar to β-galactosidase--myocytes, suggesting that they were descendants of cells that once expressed periostin (Fig. 1f). Similar results were obtained using transgenic mice in which Cre recombinase was under the control of the fibroblast-specific protein 1 (Fsp1; also known as S100a4) promoter to label the non-myocyte population (Fig. 1g and Supplementary Fig. 2a–f). Thus, analogous to induction of cardiac fibroblasts into skeletal muscle in vivo upon introduction of the skeletal muscle master regulator, MyoD, GMT appeared to induce the formation of cardiomyocytes in vivo. We determined if endothelial and circulating haematopoietic cells marked by Tie2-Cre:R26R-lacZ transgenic mice could be reprogrammed to express sarcomeric markers, but found no evidence for such an event (Supplementary Fig. 2g–i).

We formally tested whether retroviral introduction of GMT into non-myocytes could promote cell fusion events in the heart, thereby generating α-actinin--β-galactosidase+ cells. We 'pulse-labelled' endogenous CMs in transgenic mice with Cre under inducible control of the α-MHC (also known as Myb6) promoter (α-MHC-MerCreMer) crossed with R26R-EYFP mice (Supplementary Fig. 3). Subsequently, hearts were injured and infected retrovirally with GMT and dsRed to mark infected dividing cells. After 4 weeks, we detected no YFP-- cells co-labelled with dsRed in the GMTdsRed- or dsRed-infected hearts (Fig. 2a, b and Supplementary Fig. 4a). Because pulse labelling marks only ~80% of endogenous CMs in the uninjured heart and ~60% in the infarct border zone, we quantified the percentage of YFP-- pulse-labelled endogenous CMs at the border area. GMT introduction resulted in a reduced percentage of YFP-- endogenous CMs compared to total CMs, indicating that the CMs in this region were refreshed by new iCMs (Fig. 2c). These findings suggest that it is unlikely that cell fusion makes a major contribution to the α-actinin--β-galactosidase+ cell population, although a minor contribution cannot be ruled out.

If α-actinin--β-galactosidase+ cells instead resulted from cellular reprogramming, one might detect progressive stages of reprogramming over time, as cell fusion would yield mature cells soon after fusion without intermediate stages. We therefore analysed heart sections 1, 2, 3 and 4 weeks after injury and GMT injection, and classified cells into four groups based on increasing α-actinin expression and organization into sarcomeres. The number of α-actinin--β-galactosidase+ cells in the infarct area increased temporally, as did the maturity of the cells, with progressive increases in the percentage of cells with well-developed sarcomeres (Supplementary Fig. 5).

To avoid false positives from overlaying cells due to the thickness of the heart sections, we isolated adult CMs at the single-cell level from the infarct/border zone of periostin-Cre:R26R-lacZ reprogrammed hearts 4 weeks after coronary ligation (Supplementary Fig. 6a). In this preparation, non-myocytes were removed, and cells were assayed 2–4 h after primary culture. No CMs isolated from dsRed-infected hearts were β-galactosidase+ by immunostaining (Fig. 2d). Similarly, CMs from periostin-Cre:R26R-EYFP mice were all YFP+, among the thousands of cells visualized, in agreement with the absence of periostin-Cre activity in myocytes after injury. In contrast, 35% of cells in the CM preparation from the border/infarct zone were β-galactosidase+ after GMT injection (Fig. 2e, f and Supplementary Fig. 6b). Among the β-galactosidase+ cells, 98% were also α-actinin+ (Supplementary Fig. 7a–d). Furthermore, in hearts co-injected with GMT and dsRed retrovirus, β-galactosidase+ CMs were also positive for dsRed, indicating retroviral infection and their likely origin from non-post-mitotic CMs (Fig. 2g–k).

Most β-galactosidase+ cells were large, rod-shaped and binucleated, closely resembling endogenous CMs that were β-galactosidase-- from the same preparation. In addition to α-actinin, β-galactosidase+ cells expressed multiple sarcomeric markers, including tropomyosin (Fig. 2m), α-MHC (Fig. 2n), and cardiac troponin T (cTnT; also known as Tnnt2) (Fig. 2o). Half of the cells had nearly normal sarcomeric structures throughout the cell. The full spectrum of reprogrammed cells, classified by quality of sarcomeric structure, is shown in

some endocardial and endothelial cells, as previously reported. Periostin-Cre activity was absent in bone marrow cells (not shown). β-Galactosidase activity was not detected in any cardiomyocytes, even 4 weeks after injury, consistent with thoracic aortic banding studies, confirming that the periostin-Cre mice mark only descendants of the non-myocyte population, even after MI (Fig. 1e) Isolation of single CMs from these hearts confirmed the absence of β-galactosidase activity in over 1,500 CMs per heart from 6 mice.

In contrast, 4 weeks after MI and retroviral delivery of GMT, numerous β-galactosidase+ cells were α-actinin+ in the injured areas,
To determine whether iCMs expressed proteins involved in cell–cell communication similar to endogenous CMs, we examined the expression pattern of N-cadherin, a cell-surface Ca$^{2+}$-dependent adhesion molecule normally found in intercalated disks within the myocardium$^{26}$. We found that over 90% of iCMs expressed N-cadherin, with 60% of cells localizing N-cadherin appropriately at the cell border (Fig. 3a). Similarly, about 90% of iCMs expressed Cx43, the major gap junction protein in the heart that promotes electrical coupling and synchronized contraction of myocytes$^{27}$. Half of the iCMs expressed Cx43 at high levels with good localization at the cell border (Fig. 3a). Similarly, about 90% of iCMs expressed N-cadherin, with 60% of cells localizing N-cadherin appropriately at the cell border (Fig. 3a). Similarly, about 90% of iCMs expressed Cx43, the major gap junction protein in the heart that promotes electrical coupling and synchronized contraction of myocytes$^{27}$.

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By electron microscopy, about half of the cells from periostin-Cre:R26R-Tomato reprogrammed hearts exhibited well-organized sarcomeres and mitochondria (Fig. 2p, q), although the sarcomeres were consistently shorter than endogenous CMs and their Z-bands more diffuse. Other Tomato$^{+}$ cells displayed sarcomeric organization in parts of the cell and variable mitochondria organization (Supplementary Fig. 8). For simplicity, we will refer to the β-galactosidase$^{−}$α-actinin$^{+}$ CM-like cells as in vivo iCMs, based on morphology and sarcomeric structure.

Supplementary Fig. 7. Characterization of single dsRed$^{+}$ YFP$^{−}$ cells derived from the α-MHC-Mer-Cre-Mer-YFP pulse-labelled reprogrammed hearts revealed good sarcomere formation and expression of α-actinin, cTnT and connexin 43 (Cx43; also known as Gja1), like dsRed$^{−}$ YFP$^{+}$ cells (Supplementary Fig. 4b).

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Finally, we assessed the reprogramming of gene expression in iCMs by qPCR, focusing on the messenger RNA levels of 20 genes normally enriched in mature CMs or cardiac fibroblasts. We tested iCMs isolated from multiple independent hearts alongside cardiac fibroblasts and endogenous CMs. mRNA levels in iCMs were similar to CMs (Fig. 2r and Supplementary Fig. 9), including the downregulation of periostin and Fsp1, consistent with the morphological changes described earlier.

In vivo iCMs electrically mature and couple

To determine whether iCMs expressed proteins involved in cell–cell communication similar to endogenous CMs, we examined the expression pattern of N-cadherin, a cell-surface Ca$^{2+}$-dependent adhesion molecule normally found in intercalated disks within the myocardium$^{26}$. We found that over 90% of iCMs expressed N-cadherin, with 60% of cells localizing N-cadherin appropriately at the cell border (Fig. 3a). Similarly, about 90% of iCMs expressed Cx43, the major gap junction protein in the heart that promotes electrical coupling and synchronized contraction of myocytes$^{27}$. Half of the iCMs expressed Cx43 at high levels with good localization relative to endogenous CMs (Fig. 3b), and in 4% of these cells, the Cx43 localization pattern was almost indistinguishable from endogenous CMs or reprogrammed CMs, as identified by the periostin-

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calcium releases and cell shortening in iCMs were comparable to endogenous CMs (Fig. 3h). The distribution of action potential durations (APDs) was bimodal in iCMs and CMs, suggesting that reprogrammed cells were incorporated near the epicardial (short APD<sub>90</sub> action potential duration at 90% of repolarization)) and endocardial (long APD<sub>90</sub>) sides of ventricular tissue (Supplementary Fig. 10).

**In vivo GMT improves cardiac function**

Because in vivo reprogrammed iCMs had contractile potential and electrically coupled with viable endogenous CMs (and other iCMs), we asked whether converting endogenous non-myocytes into new myocytes translates into partial restoration of heart function after MI. All studies were performed in a blinded fashion, including the retroviral injections, and were decoded only after completion of the measurements. By Evans blue/triphenyltetrazolium chloride (TTC) double staining, the area at risk (AAR) and the infarct size were similar in GMT- or dsRed-injected mice 48 h after coronary ligation (Supplementary Fig. 11a). Three months after MI, cardiac function was examined by magnetic resonance imaging (MRI). The fraction of blood ejected with each ventricular contraction (ejection fraction), the volume of blood ejected (stroke volume), and the total cardiac output were significantly improved in GMT-infected mice, particularly the stroke volume and cardiac output, possibly due to cardiac enlargement (Supplementary Fig. 11b–d). To determine the time course of these improvements, other mice underwent serial high-resolution two-dimensional echocardiography 1 day before MI, and 3 days, 1, 4, 8 and 12 weeks after MI (Supplementary Fig.11b–d). All mice showed a comparable reduction in left ventricular function after coronary artery ligation (Supplementary Fig. 11c). Although different imaging approaches yield different absolute value norms, the overarching trends observed by echocardiography were similar to our MRI findings, in that functional improvements for all parameters were statistically significant 8 and 12 weeks after injection (Supplementary Fig. 11c).

We next performed qPCR to monitor the expression levels of atrial natriuretic factor, brain natriuretic peptide and tenascin C in injured and control hearts. We found that MI led to the upregulation of all three peptides, but this upregulation was attenuated in GMT-injected infarcted hearts (Fig. 4b). Expression levels of collagen genes, which were increased in dsRed-infected MI hearts, were also partially restored by injecting GMT (Fig. 4c). Furthermore, the scar area calculated from 16 sections at four levels of the heart was significantly smaller 8 weeks after MI in the GMT-treated group. To determine if the muscle cells in the scar area were reprogrammed iCMs, we repeated the experiments in peristin-Cre:R26R-LacZ transgenic mice. α-Actinin<sup>−</sup> cells in the scar area were also β-galactosidase positive, suggesting that they were newly born iCMs of non-myocyte origin (Fig. 4e). Vascular density was significantly increased in the border zone of reprogrammed hearts at 8 weeks (Supplementary Fig. 12). Electrocardiographic (ECG) studies (telemetry) over a 24-h period did not indicate evidence for more arrhythmias in GMT- versus dsRed-injected control mice, and no mice suffered sudden death (not shown).

**Thymosin β4 enhances effects of GMT in vivo**

We hypothesized that infecting more Thy1<sup>+</sup> cells would enhance functional improvement. Thymosin β4, a 43-amino-acid G-actin monomer-binding protein, promotes cell migration<sup>28,29</sup>, cardiac cell survival<sup>28,30</sup> and activates epicardial cells to become more proliferative and yield more cardiac fibroblasts and endothelial cells<sup>31,32</sup>. It also improves cardiac function and decreases scar size after MI<sup>28</sup>. To test cardiac fibroblast migration, we used a cardiac explant migration assay<sup>28</sup>. The average time for fibroblasts to migrate from adult heart explants was 3 weeks; however, thymosin β4 treatment led to equivalent fibroblast migration within 2 weeks and within only 3 days in...
the total GMT-infected cell population remaining at ~12% (Supplementary Figs 13a and 14b).

Injecting thymosin β4 immediately after ligation improved cardiac function, as previously reported. 28-30 Co-injecting thymosin β4 and GMT further improved ejection fraction and cardiac output 8 weeks after infarction (Supplementary Figs 13f and 14d, e). Furthermore, co-injecting thymosin β4 and GMT caused less scarring than injecting either alone (Supplementary Fig. 13g), despite similar areas at risk and initial infarct sizes (Supplementary Fig. 14c).

Discussion
We show that upon cardiac injury, resident cardiac non-myoocytes—primarily fibroblasts—can be converted into CM-like cells in vivo following local delivery of GMT by retroviral-mediated gene transfer. In vivo cardiac reprogramming occurred with similar initial efficiency as observed in vitro (10–15%). However, in vivo iCMs were more fully reprogrammed and more closely resembled endogenous CMs than their cultured counterparts. This may result from factors within the native microenvironment—including extracellular matrix, secreted proteins, and tissue stiffness—that further enhance reprogramming. Improved cardiac function may be explained by the diversion of a small percentage of fibroblasts into new CM-like cells, suggesting functional integration of these muscle cells. Although non-myoocytes convert to iCMs to help regenerate the damaged heart, alteration of fibroblast behaviour by GMT may contribute to the effects on scar formation and cardiac function. Although it is difficult to separate the relative contributions of new muscle formation and other non-cell-autonomous effects, non-myoocyte reprogramming in the heart appears to be beneficial for cardiac function. Optimizing gene delivery to more cells would probably enhance functional benefits.

Improvement upon thymosin β4 addition is in agreement with the notion that increasing the delivery of GMT to more cells could enhance cardiac repair. Pre-treating hearts with thymosin β4 several days before injury resulted in a small population of epicardial-derived cells that could behave as myocyte precursors, but not if thymosin β4 was given at the time of injury. Nevertheless, transduction of GMT into these progenitors, or other rare progenitors yet to be identified, might promote their differentiation into cardiomyocytes. Because thymosin β4 is also pro-angiogenic,31,32 the cooperation between GMT and thymosin β4 may be multifaceted and will be interesting to explore.

The ability to regenerate adult heart tissue from endogenous cells is a promising approach to treating cardiac disease that may face fewer obstacles to clinical translation than other approaches. Improving the delivery of reprogramming factors, using small molecules and epigenetic modulators, and conducting trials in large animals will be important to refine the technology and assess its safety and efficacy, particularly regarding arrhythmias.

METHODS SUMMARY
Retroviruses. Retroviruses were generated as described using pMXs retroviral vectors containing coding regions of Gata4, Mef2c, Tbx5, and dsRed. Ultra-high titre virus (>1×10^9 plaque-forming units (p.f.u.) per ml) was obtained by ultracentrifugation.

Animals, surgery, echocardiography and electrocardiography. Periostin-Cre:R26R-lacZ and Fsp1-Cre:R26R-lacZ mice were obtained by crossing periostin-Cre mice (31, 32) and R26R-lacZ mice. Periostin-Cre:R26R-EYFP or α-MHC-MerCreMer24 mice and R26R-EYFP mice were obtained by crossing periostin-Cre or α-MHC-MerCreMer24 mice and R26R-EYFP mice. Periostin-Cre:R26R-Tomato mice were obtained by crossing periostin-Cre mice and R26R-Tomato mice. Surgeries and subsequent analyses were performed blinded to genotype and intervention. MI was induced by permanent ligation of the left anterior descending artery (LAD) as described. A pool of concentrated virus (GMT or GMTR) was mixed, and 10 μl of mixed virus plus 10 μl of PBS or 40 ng μl^-1 thymosin β4 were injected along the boundary between the infarct and border zones. Mouse echocardiography and surface electrocardiography were performed as described. All mouse work was done with the approval of the University of California, San Francisco (UCSF) animal care oversight committee.
Immunohistochemistry, immunocytochemistry and electron microscopy. Immunohistochemistry, immunocytochemistry and electron microscopy were performed as described\textsuperscript{1,2}. Scar size was determined by Masson-Trichrome staining\textsuperscript{3,4}. The AAR and myocardial infarct size were determined by Evans blue/TTX labelling\textsuperscript{4}.

Cardiomyocyte isolation, patch-clamp, and fibroblast migration assays. Adult cardiomyocytes were isolated as described with minor modifications\textsuperscript{5}. Single-cell patch-clamp recordings were performed as described\textsuperscript{6}. Migration assays were performed according to published protocols\textsuperscript{7,8}.

FACS and quantitative RT–PCR. Dissociated cardiac cells were stained with APC-conjugated anti-Thy1 antibody (Biociences). Stained cells were sorted by FACSARia2 (BD) and RNA extracted in TRizol (Invitrogen). qPCR was performed using ABI 7900HT (TaqMan, Applied Biosystems).

Statistics. Differences between groups were examined for statistical significance using unpaired Student’s $t$-tests or ANOVA. $P < 0.05$ was regarded as significant.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Author Contributions L.Q., designed, supervised and performed the experiments. Y.H. performed all surgeries, echoes and ECGs, and contributed to tissue sectioning and sample preparation. C.I.S. performed all cellular electrophysiology experiments. A.F. quantified scar size and induced CMs and helped with mouse colony maintenance. V.V. helped with isolation of adult CMs and implantation of transmitters. S.J.C. provided periostin-Cre::Rosa26-lacZ mice and supplemental data. J.-D.F. provided initial reagents and technical knowledge and helpful discussion. D.S. designed and supervised the work. L.Q. and D.S. wrote the manuscript.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare competing financial interests: details accompany the full-text HTML version of the paper at www.nature.com/nature. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to D.S. (dsrivastava@gladstone.ucsf.edu).
METHODS

Retrovirus generation, concentration, and titration. Retroviruses were generated as described. To generate virus, pMXs retroviral vectors containing the coding regions of Gata4, Me2c, Tbx5 and delix were transfected into Plat-E cells using FuGene 6 (Roche). Forty-eight hours after transfection, virus-containing supernatants were collected and concentrated by standard ultracentrifugation. Retroviral titration was performed using the Retro-X qRT–PCR Titration Kit (Clontech), as per the manufacturer’s protocols. Ultra-large titre virus (>1×10^9 plaque-forming units (p.f.u.) per ml) was resuspended in PBS. After verification of high transduction efficiency in cell culture (>95%), a large number of small stock aliquots (10 µl) were made and frozen at −80°C to ensure consistency among experiments. After one freeze–thaw cycle, titrations were repeated to ensure that active virus was maintained at the desired 1×10^10 p.f.u. concentration for in vivo injection.

Mouse lines. Periostin (Ptn)-Cre;R26R-lacZ mice were obtained by crossing Postn-Cre mice and Rosa26-lacZ mice. Postn-Cre;R26R-YFP mice were obtained by crossing Postn-Cre mice and Rosa26-EYFP mice, and Postn-Cre;R26R-Tomato mice were obtained by crossing Postn-Cre mice and Rosa26-Tomato mice. All transgenic lines for immunohistochemistry and single cell isolation were maintained by crossing C57BL6 mice (Charles River). BALB/C mice (Charles River) were used for all functional studies after permanent ligation of the left anterior descending artery (LAD) and virus injection. Fsp1-Cre, Tie2-Cre, Myh6-MerCreMer mice were obtained from Jackson Labs, and lines were validated before further breeding. Fsp1-Cre, Tie2-Cre and Myh6-MerCreMer-YFP mice were obtained by crossing Fsp1-Cre, Tie2-Cre or Myh6-MerCreMer mice to R26R-lacZ or R26R-EYFP mice. Efficiency of Cre recombination induction for Myh6-MerCreMer-YFP was tested by immunohistochemistry for YFP after injection of various doses of tamoxifen. To pulse label the pre-existing CMs, adult Myh6-MerCreMer-YFP mice (8–12 weeks old) were treated with tamoxifen (Sigma) by intraperitoneal injection once a day for 5 days at a dosage of 20 mg kg⁻¹ day⁻¹. GMT delivery and coronary artery ligation were performed 2 days afterwards.

Mouse MI model and in vivo delivery. The animal protocol for surgery was approved by institutional guidelines (UCSF Institutional Animal Care and Use Committee). All surgeries and subsequent analyses were performed blinded for genotype and intervention. Mice were anesthetized with 2.4% isoflurane/97.6% oxygen and placed in a supine position on a heating pad (37°C). Animals were intubated with a 19 G stump needle and ventilated with room air using a MiniVent Type 845 mouse ventilator (Hugo Sachs Elektronik-Harvard Apparatus; stroke volume, 250 µl; respiratory rate, 120 breaths per minute). MI was induced by permanent ligation of the LAD with a 7-0 prolene suture as described. Sham-operated animals served as surgical controls and were subjected to the same procedures as the experimental animals with the exception that the LAD was not ligated. A pool of concentrated virus (GMT, or GMTR) was mixed, and 10 µl of mixed virus plus 10 µl of PBS or 40 ng µl⁻¹ thymosin β4 was injected into the myocardium through an insulin syringe with an incorporated 29 G needle (BD). Injection with a full dosage was carried out along the boundary of the infarct zone and border zone based on the blanched infarct area after isolation.

Isolation of adult CMs. Adult CM isolation was performed as described with minor modifications. Briefly, adult mice were anesthetized with isoflurane and mechanically ventilated. Hearts were removed and perfused into the aorta, thus all myocardial tissue was stained blue except the left ventricle end-systolic and end-diastolic diameter were measured from the left ventricular M-mode tracing with a sweep speed of 50 mm s⁻¹ at the papillary muscle level for calculating the shortening fraction. B-mode was used for two-dimensional measurements of end-systolic and end-diastolic dimensions.

Mouse echocardiography. Echocardiography was performed by the Vevo 770 High-Resolution Micro-Imaging System (VisualSonics) with a 15-MHz linear array ultrasound transducer. The left ventricle was assessed in both parasternal long-axis and short-axis views at a frame rate of 120 Hz. End-systole or end-diastole was defined as the phase in which the smallest or largest area of the left ventricle, respectively, was observed and used for measurements.

Mouse surface electrocardiography. Mice were anaesthetized with 1.75% isoflurane at a core temperature of 37–38°C. Four needle electrodes (AD Instruments) were placed subcutaneously in standard limb lead configurations. For each mouse, 10–20 s of continuous signals were sampled at 10 kHz in each lead configuration with a PowerLab/4i 30 interface (AD Instruments). Data analysis was performed offline with electrocalipers on averaged beats (ChartPro v5.4, AD Instruments).

Mouse awake electrocardiography. To record awake electrocardiograms in six reprogrammed post-MI mice and six control post-MI mice, transmitters were surgically implanted according to the manufacturer’s instructions (Data Sciences International). After a 3-day recovery period, the electrocardiogram was recorded continuously for 48 h in each mouse. Tracings were analysed off-line and were scored by a blinded investigator for the presence and frequency of arrhythmias.

MRI. MRI was performed on a Varian DirectDrive 7T small-animal scanner. Each mouse was anaesthetized by inhalation of 2% isoflurane/98% oxygen administered via an MR-compatible mobile inhalation anesthesia system (VetEquip). The mice were put in supine position on a homemade heating bed to keep the temperature at 37°C. Two ECG leads were inserted into the right front and left rear leg. ECG waveforms were monitored with a small animal monitoring and gating system (SA instruments). The mouse was then placed into a homemade 1H birdcage coil with an inner diameter of 32 mm. A group of ECG (R–wave rising edge) triggered spin echo scout images were acquired first to define the oblique plane of the short axis. Then a ECG-triggered two-dimensional gradient echo sequence with an echo time of 2.75 ms, repetition time of 200 ms and a flip angle of 45° was used to obtain nine short-axis images at 12 or 13 phases per cardiac cycle. Each scan consisted of 8–9 contiguous slices spanning the left ventricle from apex to base with 1-mm thickness, a matrix size of 128×128, a field of view of 5.6×25.6 mm, and four averages.

Isolation of adult CMs. Adult CM isolation was performed as described with minor modifications. Briefly, adult mice were anaesthetized with isoflurane and mechanically ventilated. Hearts were removed and perfused retrogradely via aortic cannulation with a constant flow of 3 ml min⁻¹ in a Langendorf apparatus. Hearts were perfused at 37°C for 5 min with supplemented Wittenberg Isolation Medium (WIM) containing (in µM): 116 NaCl, 5.4 KCl, 6.7 MgCl₂, 12 glucose, 2 glutamine, 3.5 NaHCO₃, 1.5 KH₂PO₄, 1.0 NaH₂PO₄, 21 HEPES with 1.5 M insulin, essential vitamins (GIBCO), and essential amino acids (GIBCO) (pH 7.4), followed by digestion solution (WIM, supplemented with 0.8 mg ml⁻¹ collagenase II and 10 µM CaCl₂) for 10 min (4 min for paired CMs that were used in cell–cell coupling experiments). Hearts were then removed from the Langendorf apparatus while intact (with tissues loosely connected). Desired areas (that is, border/infarct zone) were then micro-dissected under the microscope, followed by mechanical dissociation, triturating, and resuspension in a low-calcium solution (WIM, supplemented with 5 mg ml⁻¹ BSA, 10 mM tauro, and 150 mM CaCl₂). Cells were then spun at low speed, supernatant was removed, and calcium was gradually reintroduced through a series of washes. For electrophysiology experiments, cells were used on the same day as isolation and until recording were stored at room temperature (21°C) in M199 (Gibco) supplemented with 5 mM creatine, 2 mM i-carnitine, 5 mM tauro and 1.5 mM insulin. For immunohistochemistry, cells were plated onto laminin-coated culture slides, allowed to adhere, and fixed on the day of isolation. For electron microscopy or qPCR, CMs were selected manually by micro-pipette based on the presence of peristin-Cre;R26R-YFP/Tomato signal under the fluorescent microscope right after isolation.

Cardiac fibroblast migration assay. The migration assay was performed according to the standard culture protocol as described. Briefly, isolated adult mouse hearts were minced into small pieces less than 1 mm³ in size. The explants were plated on gelatin-coated dishes and cultured in explant medium (IMDM, 20% FBS) until fibroblasts migrated out from minced tissue. The time required for ten heart pieces to have migratory fibroblasts surrounding them were recorded.
FACS analyses and sorting. At 48 h after LAD and viral introduction, hearts were removed and minced into small pieces less than 1 mm³ in size. Blood cells and debris were removed by several washings of PBS. Minced cardiac tissues were digested in an eppendorf tube and shaken with glass beads in enzyme buffer (collagenase/dispace plus DNaseI; Roche) at 37 °C. After passing through a 40-μm cell strainer, dissociated cardiac cells were stained with APC-conjugated anti-Thy1 antibody (eBioscience) for 30 min at room temperature. After washing with PBS twice, stained cells were sorted by FACS (BioBench). The viability duration was measured from the point of maximum depolarizating voltage using pClamp, Microsoft Excel, and Origin (OriginLab) software. Action potential recordings. isolated myocytes were suspended in tissue culture medium and photographed with the Gatan Ultrascan 1000 digital camera (Gatan). The viability of cytosolic Ca²⁺ was examined on a JEOL JEM-1230 transmission electron microscope (JEOL USA). Ultracut S ultramicrotome and counter stained with 0.8% lead citrate. Grids were stained with 2% aqueous uranyl acetate, dehydrated in acetone, infiltrated, and embedded in LX-112 resin (Ladd Research Industries). Samples were ultrathin sectioned on a Reichert Ultracut S ultramicrotome and examined on a Tecnai 12 transmission electron microscope (FEI, USA) to examine the muscle integrity. Field stimulation experiments. Isolated myocytes were loaded with Fluo-4 for 30 min at room temperature before being transferred to the superfusion chamber. The loading solution containing a 1:10 mixture of 5 mM Fluo-4 AM in dry DMSO and PowerloadTM concentrate (Invitrogen) which was diluted 100-fold into extracellular Tyrode’s solution containing suspended myocytes. An additional 20 min was allowed for de-esterification before commencing recordings. Contractions and Ca²⁺ transients were evoked by applying voltage pulses at 0.33 Hz, between platinum wires placed on either side of the cell of interest and connected to a field stimulator (IonOptix, Myopacer). The pulses were of 2 ms duration and set at 150% of the threshold required to elicit twitches. Fluo-4 fluorescence transients were recorded via a standard filter set (#49011 FT, Chroma Technology) in batches of ten to enable signal averaging. Between stimuli, the fluorescence excitation light was blocked by an electromechanical shutter (CS35; Vincent Associates). Resting fluorescence was recorded after cessation of pacing, and background light was obtained after picking up and removing the cell from the field of view with a patch electrode at the end of the experiment. The Ca²⁺ transients were calibrated using the pseudo-ratio method, assuming an in situ dissociation constant of 1.1 μM for Fluo-4. Contractions were optically recorded simultaneously with Ca²⁺ transients by illuminating the cell of interest in red light (λ > 665 nm) and calculated to a CCD camera (IonOptix Myopac). The cell length signals were converted to voltage via a video motion director (VED 205; Crescent Electronics) and contraction amplitudes from different myocytes were normalized by calculating the per cent change in cell length.

Determination of cell–cell coupling. In experiments to assess the interconnectivity between iCMs and CMs, the whole-cell patch-clamp method was used to introduce a gap-junction-permeable (that is, mobile) and an impermeable (that is, immobile) dye into the same cell of interest within a small group of apparently coupled isolated myocytes (n = 5 groups from 5 independent hearts). The mobile dye was calcein (5 mM), and the immobile dye was 1 mM dextran-conjugated Cascade Blue (MW 10.000). The immobile dye was chosen to be well separated spectrally, both from calcine and from tomato, which was used for labelling iCMs (Invitrogen). The dye pair was included in standard intracellular solution (containing 5 mM EGTA) and cytoplasmic loading was allowed to proceed for 2 min, after which the patch electrode was withdrawn from the patched myocyte. The sarcolemma of the cell resealed after pulling off the pipette, aided by high EGTA levels in the filling solution, thereby trapping the dyes in the cytoplasm. Blue fluorescence from the immobile indicator was excited at 365 ± 40 nm, whereas calcine fluorescence was excited at 470 ± 40 nm. Fluorescent images were recorded using IonOptix Myopac via a video frame grabber (#166VCB, Hauppauge) for processing using ImageJ software. The functionality of cell–cell junctions was investigated by imaging the intercellular transmission of Ca²⁺ waves and excitation between myocytes pre-loaded with Fluo-4 AM as detailed above for the field-stimulation studies. Superfusion with 2 mM ouabain for 5–10 min was used to induce intracellular Ca²⁺ overload accompanied by Ca²⁺ wave activity, and videomicroscopy revealed the spatiotemporal relationships of Ca²⁺ waves translocating within and between the individual cells imaged in small, adherent groups (n = 6 groups from 5 independent hearts).

Quantitative RT–PCR. iCMs were manually sorted based on the presence of fluorescent lineage markers. Approximately 100 iCMs were pooled for RNA isolation. Similarly, ~100 endogenous CMs isolated using the standard Langendorf apparatus (see above) and ~500 cardiac fibroblasts using the migration assay (see above) were prepared for RNA isolation. RNA was extracted by the Trizol method (Invitrogen). RT–PCR was performed using the Superscript III first-strand synthesis system (Invitrogen). qPCR was performed using the ABI 7900HT (TaqMan, Applied Biosystems) as per the manufacturer’s protocols. Optimized primers from the Taqman Gene Expression Array were used.

Statistical analyses. Differences between groups were examined for statistical significance using unpaired Student’s t-test or ANOVA. A P-value < 0.05 was regarded as significant. Error bars indicate standard error of the mean (s.e.m.).