Direct Reprogramming of Fibroblasts into Cardiomyocytes for Cardiac Regenerative Medicine

Ji-Dong Fu, PhD; Deepak Srivastava, MD

Cardiac fibroblasts play critical roles in maintaining normal cardiac function and in cardiac remodeling during pathological conditions such as myocardial infarction (MI). Adult cardiomyocytes (CMs) have little to no regenerative capacity; damaged CMs in the heart after MI are replaced by cardiac fibroblasts that become activated and transform into myofibroblasts, which preserves the structural integrity. Unfortunately, this process typically causes fibrosis and reduces cardiac function. Directly reprogramming adult cardiac fibroblasts into induced CM-like cells (iCMs) holds great promise for restoring heart function. Direct cardiac reprogramming also provides a new research model to investigate which transcription factors and microRNAs control the molecular network that guides cardiac cell fate. We review the approaches and characterization of in vitro and in vivo reprogrammed iCMs from different laboratories, and outline the future directions needed to translate this new approach into a practical therapy for damaged hearts. (Circ J 2015; 79: 245–254)

Key Words: Cardiomyocytes; Epigenetic reprogramming; Heart failure; Regenerative medicine

Heart disease remains the leading cause of death worldwide, despite improved treatments that have decreased death rates. According to the annual report from the American Heart Association, coronary artery disease caused nearly 915,000 new or recurrent coronary events in 2010. Because cardiomyocytes (CMs) rarely regenerate in the adult heart, survivors of myocardial infarction (MI) typically develop chronic heart failure. Unfortunately, endstage heart failure can only be addressed by heart transplantation, which is limited by the number of donor organs available. Alternative solutions include cellular therapy that replaces lost CMs either by transplanting CMs or inducing new CMs in situ at the areas affected by the infarction.

After several decades, researchers discovered that adult somatic cells, which were thought to be fixed in their cell fate after terminal differentiation, could be converted into other types of cells through epigenetic reprogramming. With this technology, for example, somatic cells, such as fibroblasts, can be dedifferentiated into pluripotent stem cells by nuclear transfer or with defined transcription factors. Direct reprogramming of fibroblasts into the chief functional cells of different organs, including CMs, neurons, hepatocytes, hematopoietic cells, and endothelial cells, has been accomplished and holds great promise for regenerative medicine. In particular, cardiac fibroblasts comprise over half of the cells in the adult heart, and thus may provide a large pool of cells from which to generate new CMs through epigenetic reprogramming. In 2010, we reported that mouse cardiac and dermal fibroblasts could be directly reprogrammed into induced CM-like cells (iCMs) in vitro by a combination of 3 developmental cardiac transcription factors, Gata4, Mef2c, and Tbx5 (GMT). Since then, other laboratories around the world have reported success in reprogramming mouse fibroblasts into iCMs with similar cocktails of reprogramming factors. Several reviews highlight the potential and challenges of this new avenue for cardiac regenerative medicine. In this review, we consider the approaches and characterization of in vitro (Table 1) and in vivo (Table 2) reprogrammed iCMs reported from different laboratories. We hope that this up-to-date discussion will help outline the major challenges of this new approach, and summarize future efforts needed to translate it toward a practical therapy for damaged hearts.

Molecular Networks Regulating Cardiac Cell Fate

In vertebrate embryos, the heart is the first functional organ to form. Analyses of genetic lineage tracing found that 2 distinct mesodermal heart fields with a common origin, first heart field (FHF) and second heart field (SHF), contribute cells to the developing heart. Cells from these fields coalesce along the ventral midline to first form a primitive heart tube, and then...
### Table 1. Published Studies of In Vitro Direct Transdifferentiation of Fibroblasts into Cardiac-Like Cells

<table>
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<tr>
<th>Reprogramming cocktail</th>
<th>Organism</th>
<th>Starting fibroblasts</th>
<th>Cardiac phenotype characterization</th>
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<tr>
<td>Gata4, Mef2c, Tbx5</td>
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<td>Thy1</td>
<td>+</td>
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<tr>
<td>Adult ventricular CM transcriptome</td>
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<td>Thy1</td>
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<tr>
<td>miR1, miR133, miR208, miR499, Jrf</td>
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<td>Thy1</td>
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<td>+</td>
<td>Jayawardena et al, 2012</td>
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<td>+</td>
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<td>+</td>
<td>+</td>
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<td>Protze et al, 2012</td>
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<tr>
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<td>+</td>
<td>+</td>
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<td>Christoforou et al, 2013</td>
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<tr>
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<td>+</td>
<td>+</td>
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<tr>
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<tr>
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<td>Thyl</td>
<td>+</td>
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<tr>
<td>GATA4, MEF2C, TBX5, ESRRG, MESP1, ZFPM2, Myocardin</td>
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<td>Thy1</td>
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<td>Rare</td>
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<tr>
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<tr>
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<td>Mouse</td>
<td>+</td>
<td>+</td>
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</tbody>
</table>

CM, cardiomyocyte; iCM, induced CM-like cell; miR, microRNA.
Published Studies of In Vivo Direct Transdifferentiation into Cardiac-Like Cells

<table>
<thead>
<tr>
<th>Reference</th>
<th>Organism</th>
<th>Cell characterization</th>
<th>Transcription factors targeted</th>
<th>Other abbreviations as in Table 1</th>
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<td>Actc1, Myl2, Myl7, Tnnt2, Myh6, Gata4, Mef2c, Tbx5</td>
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<td>+, +, &gt;50% beating</td>
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<td>Mouse</td>
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<td>Actc1, Myl2, Myl7, Tnnt2, Myh6, Gata4, Mef2c, Tbx5</td>
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<td>+, +, +, +</td>
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<tr>
<td>Jayawardena et al, 2019</td>
<td>Mouse</td>
<td>Actc1, Myl2, Myl7, Tnnt2, Myh6, Gata4, Mef2c, Tbx5</td>
<td>+, +, &gt;50% beating</td>
<td>+, +, +, +</td>
</tr>
</tbody>
</table>

Cardiac Fibroblasts in the Normal and Remodeling Heart

Fibroblasts are mesenchymal cells that produce many extracellular matrix components in organs. Fibroblasts show heterogeneity based on morphology, glycogen pools, collagen production, cell surface markers, and global gene expression profiles. Although the percentage of fibroblasts among the total cells in the heart varies between species, a large population of fibroblasts is quiescent and abundantly distributed in the interstitial and perivascular matrix in the normal heart. Cardiac fibroblasts are highly heterogeneous with distinct origins. Domain receptor (DDR)-2, Thy1, and fibroblast-specific protein-1 (Fsp1) have been used to identify cardiac fibroblasts in some studies; however, cell-specific markers that truly define fibroblasts have not been identified, which challenges attempts to define and compare cardiac fibroblasts in the different studies.

Cardiac fibroblasts synthesize extracellular matrix to provide a 3D network for myocytes and other cells of the heart; they also regulate the biological and electrophysiological response of CMs during physiological and pathological development. In embryonic mouse hearts, cardiac fibroblasts induce proliferation of CMs via paracrine signals of fibronectin, continuously migrate in a temporally and spatially specific manner, and ultimately form a functional heart with 4-chamber structure. The morphological development of the heart has been summarized in several reviews. During development, numerous signaling and transcriptional cascades regulate cell fate decisions in the FHF and SHF, including bone morphogenetic protein (Bmp), sonic hedgehog, fibroblast growth factor, Wnt, and Notch. Among those signaling networks (Figure 1A), the Gata-, Mef2-, Hand-, Nkx- and T-box family of transcription factors control expression of cardiac genes and direct the specification and differentiation of cardiac myocytes. These transcription factors have been frequently included in studies of directly converting fibroblasts into cardiac cells (Figure 1B).

MicroRNAs (miRs), which are small single-stranded non-coding RNAs that negatively regulate the stability of gene transcripts, also regulate cardiac gene expression. Transcription factors regulate miR expression, and in turn, miRs can modulate the activities of transcription factors through positive and negative feedback loops. One of the major regulators of cardiac lineage determination during heart development is miR-1, Expression of miR-1 in either mouse or human embryonic stem cells (ESCs) causes them to favor the muscle cell fate. In contrast, miR-133 promotes muscle progenitor expansion and prevents terminal differentiation, while another miRNA, miR-499, promotes the ventricular cell fate in human ESC-differentiated CMs and causes cardiac hypertrophy and enlarged hearts in miR-499 transgenic mice. These studies demonstrate that miRs cooperate with transcription factors to form an intertwined network that reinforces specific cell fate decisions and differentiation during cardiac development (Figure 1A).

Molecular and genetic studies using ESCs and animal models have disclosed the transcription factors and miRs that are essential for determining cardiac cell fate. However, we still do not know which transcription factors and/or miRs comprise the molecular network that establishes and maintains the cell fate of CMs and can reestablish a new cardiac cell fate in non-muscle cells that are terminally differentiated, such as cardiac fibroblasts.
suggesting that in the normal heart cardiac fibroblasts could conduct electric signaling between different regions of myocytes that are electrically isolated by connective tissue.

Cardiac fibroblasts also form intracellular electrical coupling and communicate with myocytes through gap junctions, suggesting that in the normal heart cardiac fibroblasts could conduct electric signaling between different regions of myocytes that are electrically isolated by connective tissue.

Cardiac fibroblasts play a critical role in cardiac remodeling during pathological conditions such as MI. During MI,
ischemic injury causes acute sudden death of CMs, which rapidly activates innate immune pathways that trigger a transient but intense inflammatory response. This response produces cytokines, inflammatory chemokines, and growth factors to activate and differentiate cardiac fibroblasts into myofibroblasts, which migrate to the injured region to promote wound healing and scar formation.2 Cardiac fibroblasts could also become pro-inflammatory cells in the early stage of infarct healing, thereby activating the inflammatory and producing cytokines, chemokines, and proteases, such as interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α.5 All these responses are initially critical to clear dead cells from the infarcted area and preserve the structure of the heart; however, they become severe over time and ultimately cause fibrosis and contribute to heart failure. Thus, the ultimate goal is to replace fibroblasts in the scar region with newly regenerated CMs, ideally by directly converting fibroblasts to CMs.

**Direct Cardiac Reprogramming In Vitro**

The possibility of cell fate conversion was recognized from cell fusion experiments in which non-muscle cells transformed into the cell fate of skeletal muscle.53 After this, Davis et al found that this conversion was controlled by a single transcription factor, MyoD,54 which activates muscle genes in several differentiated cell lines.55 Although skeletal and cardiac muscles share similar characteristics, a cardiac equivalent to MyoD that could convert fibroblasts into cardiac cells has been elusive. Interestingly, Kim et al56 reported that a combination of cardiac genes was successfully activated in mouse embryonic fibroblasts that were exposed to the whole-transcriptome of adult ventricular myocytes. In their study, transcriptome-effected CMs (tCMs) displayed CM-like morphologies, generated Ca²⁺ oscillation, and could elicit action potentials; however, beating cells were not observed. These generated tCMs demonstrated that cardiac cell fate is controlled by a combination of factors, similar to that in pluripotent stem cells.4

To identify the combination of cardiac reprogramming factors, we generated a transgenic mouse in which enhanced green fluorescent protein (EGFP) was driven by the α myosin heavy chain (αMHC) promoter,57 which allowed us to observe cardiac myocytes by green fluorescence. Because cardiac fibroblasts are heterogeneous, we purified those that were Thy1-positive but αMHC-EGFP-negative by FACS. Then, with retrovirus, we overexpressed 14 transcription factors, which are critical for heart development, in cardiac fibroblasts isolated from neonatal mice. We found that a small percentage of cells became αMHC-EGFP-positive, indicating cell fate conversion.5 After serially deleting individual factors to remove those that were dispensable or inhibitory, we found that a combination of 3 transcription factors—GMT—could convert 15–20% of cardiac fibroblasts into αMHC-EGFP-positive cells, which we called iCMs. iCMs formed sarcomere-like structures and displayed whole-transcriptome expression profiles that shifted significantly toward the profile of CMs. Although most in vitro iCMs were only partially reprogrammed, many of them could generate Ca²⁺ transients, and some started beating spontaneously 4–6 weeks after reprogramming. GMT-induced iCMs gained a CM-like chromatin state of many cardiac-specific genes, such as Ryr2, Tnt2, and Actn2, indicating that they were epigenetically reprogrammed. Using a lineage-tracing strategy in mice (eg, Isl1-Cre-YFP and Mesp1-Cre-YFP), we did not observe activation of cardiac progenitor markers during GMT cardiac reprogramming,5 suggesting that GMT directly converted fibroblasts toward the cardiac cell fate without dedifferentiation back into a progenitor status.

We also transduced GMT retrovirus into mouse tail-tip fibroblasts (TTFs), which converted 15% of TTFs into αMHC-EGFP-positive cells, some of which expressed cardiac troponin T and could generate Ca²⁺ transients. However, it did not reprogram TTFs into beating iCMs. Soon after our study, Song et al58 found that a basic helix-loop-helix transcription factor, Hand2, could help GMT to convert TTFs into functional beating iCMs. Using αMHC-EGFP mice, they investigated the optimal combination of core factors that could reprogram adult fibroblasts into functional CMs by analyzing the potential cardiogenic activity of 6 cardiac transcription factors: Mesp1, Nkx2.5, Hand2 and GMT. They confirmed that GMT is necessary and sufficient to convert cardiac fibroblasts into iCMs, and found that, compared with other combinations, GMT plus Hand2 (GHMT) could more efficiently convert adult cardiac fibroblasts and TTFs into iCMs. GHMT activated a number of cardiac genes and induced a CM-like transcriptome profile in reprogrammed fibroblasts. GHMT-reprogrammed iCMs formed sarcomere-like structures and could generate Ca²⁺ transients. Additionally, a small subset of iCMs reprogrammed from both cardiac fibroblasts and TTFs could beat spontaneously 5 weeks after inducing GHMT,59 indicating that GHMT induced direct cardiac reprogramming in TTFs.

In these 2 studies, investigators used fibroblasts with αMHC-EGFP reporter to screen a better combination of reprogramming factors. These cells are useful and practical for narrowing down the most efficient combination of reprogramming factors by serially removing individual factors; however, this strategy might identify some transcription factors that preferentially activate the αMHC promoter and might not comprise the optimal combination for cardiac cell fate conversion.60 Rather than measuring the activation of a single reporter, Protze et al12 determined the activation of a panel of 5 cardiac genes (Myh6, Myl2, Actc1, Nkx2.5, and Snc5a) in reprogrammed fibroblasts by quantitative polymerase chain reaction (q-PCR). They introduced 120 different triplet-combinations of 10 critical transcription factors into mouse embryonic fibroblasts. They reported that Mef2c, Tbx5, and myocardin comprised an optimal combination that activated more cardiac genes and converted fibroblasts more into myocyte-like cells. These cells expressed cardiac contractile proteins, had cardiac-like potassium and sodium currents, and could elicit action potentials, but failed to contract. Meanwhile, Christoforou et al15 established different criteria to determine the capacity of cellular reprogramming with different combinations of factors. In addition to activating reporters driven by cardiac-specific promoters, they combined the expression of 34 endogenous cardiac-specific genes, including cardiac cytoskeletal proteins, cardiac transcription factors, calcium-handling proteins and CM-enriched ion channel proteins. They introduced different combinations of reprogramming factors into mouse embryonic fibroblasts, and found that myocardin and SRF could enhance cardiac reprogramming of GMT. In these iCMs, they observed Ca²⁺ oscillations, but no spontaneous contraction.

Recently, Addis et al14 evaluated functional outcomes that assess the yields of cardiac reprogramming from several combinations of transcription factors. In their study, they visualized the induction of calcium oscillations in reprogrammed cells with a transgenic calcium reporter, GCaMP5, driven by a cardiac-specific gene promoter. They found that a combination of 5 cardiac transcription factors, GMT, Hand2, and Nkx2.5
(GMTHN), more efficiently reprogrammed mouse embryonic fibroblasts. They also found that GCaMP5 helps track the location of rare beating iCMs that represent fully reprogrammed cells. With the same method, Ifkovits et al. found that a small-molecule inhibitor of TGF-β, SB432542, increased reprogramming efficiency via GMTHN up to nearly 5-fold and generated more beating iCMs in mouse embryonic fibroblasts.

In addition to transcription factors, directly converting fibroblasts into iCMs was stimulated with miRs, which interactively direct cardiac cell fate decisions during heart development. Jayawardena et al.11 reported that a combination of 4 miRs – miR-1, miR-133, miR-208, and miR-499 (abundantly expressed in CMs and directly regulating their development and functions53,37,38,59,69) – could convert cardiac fibroblasts from neonatal mice into CM-like cells in vitro. Specifically, by transfecting mimics of these miRs in vitro, they induced expression of cardiac genes in cardiac fibroblasts, and reported that a JAK inhibitor could enhance reprogramming efficiency and sporadically induce spontaneous beating in iCMs.11 Additionally, Muraoka et al.13 found that adding miR-133a to GMT generated 7-fold more beating iCMs from mouse embryonic fibroblasts, compared with GMT alone. They also found that miR-133 could directly repress Snail to silence fibroblast signatures, a key molecular roadblock during cardiac reprogramming.

A number of varying criteria have been applied to evaluate the efficiency of cardiac reprogramming in different laboratories. Some studies evaluated the activation of cardiac-specific genes in reprogrammed iCMs by Q-PCR and/or histological immunostaining, which has lower stringency than methods such as global transcriptome analysis, CM-specific epigenetic marks, Ca2⁺ handling, electrophysiological properties, and cellular contraction.25 Nevertheless, the summary of findings (listed in Table 1) suggests that endogenous cardiac fibroblasts could become a viable cell source to generate new iCMs as a replacement of damaged CMs in the heart. Indeed, investigations aiming to identify the most ideal combination of reprogramming factors are also developing new approaches to study and validate the molecular networks that can establish cardiac cell fate.

Direct Cardiac Reprogramming In Vivo

The ultimate goal in generating new iCMs is to improve the systolic function of the damaged heart and restore its normal structure and function. Therefore, we tested direct cardiac reprogramming in vivo with the hypothesis that the heart’s native microenvironment would promote direct reprogramming of fibroblasts to CMs. Takeuchi and Bruneau62 found that the combination of Gata4, Tbx5, and Balf60c could convert non-cardiogenic embryonic mesoderm tissue into functional cardiac myocytes that beat in mouse embryos, but not in vitro. We expected that direct cardiac reprogramming would be enhanced in the native heart and could improve the function of the damaged heart.

To track pre-existing CMs and newly generated iCMs that were reprogrammed from cardiac fibroblasts, we performed our experiments in transgenic mouse strains in which non-myocytes, predominately cardiac fibroblasts, were genetically labeled with β-galactosidase (β-gal) or a fluorescent protein that is controlled by a promoter of genes associated with cardiac fibroblasts, such as peristin or Fsp1.63 We delivered GMT into mouse hearts after acute MI via retroviruses. These viruses can only infect actively dividing cells,64 and thus, could deliver the reprogramming factors into non-myocytes (mostly fibroblasts), but not into CMs that exit the cell cycle after differentiation.65 At 4 weeks after introducing GMT, we found numerous β-gal+/α-actinin+ cells within the scar area of the mouse hearts,26 indicating newly born iCMs reprogrammed from cardiac fibroblasts. To rule out the possibility that these β-gal+/α-actinin+ cells arose from cell fusion between cardiac fibroblasts and damaged CMs, we used another transgenic mouse line, aMHC-merCremer:R26r-eYFP, in which CMs could be irreversibly pulse-labeled with yellow fluorescent protein via injecting tamoxifen. After acute MI, we delivered retroviruses containing GMT and dsRed to label infected cells; cells expressing both dsRed and YFP would indicate fusion between virus-reprogrammed cardiac fibroblasts and YFP+ CMs. Importantly, we did not observe YFP+/dsRed− CMs, which confirmed that iCMs were reprogrammed from cardiac fibroblasts by GMT in vivo. We found that more than 50% of in vivo-derived iCMs closely resembled endogenous ventricular CMs, had a rod shape, were binucleate, assembled sarcomeres, generated Ca2⁺ transients, and elicited ventricular-like action potentials and beating activity.63 By microarray analysis, in vivo mouse GMT-iCMs showed similar global gene expression profiles with mouse adult CMs, such that they were clustered as 1 type of cell (Figure 2A).16 The iCMs reprogrammed in vivo were electrically coupled with endogenous CMs, and no arrhythmias were observed in mice that received GMT reprogramming factors. Most importantly, introducing GMT in vivo reduced scar size and cardiac dysfunction up to 12 weeks after coronary ligation.63 Similarly, Inagawa et al.66 successfully reprogrammed cardiac fibroblasts into iCMs in vivo by introducing GMT into the hearts of immunosuppressed mice using a single-poly cystronic retrovirus, which contains GMT with self-cleaving 2A peptides.

Using a similar method to trace the genetic lineage, Song et al.13 found that introducing GMT and Hand2 in vivo could directly convert cardiac fibroblasts into iCMs. By tracing lineages using Fsp1-Cre-, inducible Tcf21-iCre and aMHC-merCremer mouse lines, they demonstrated that the origin of iCMs was likely cardiac fibroblasts. They also found that these in vivo reprogrammed iCMs were rod-shaped, formed sarcomere structures, and generated Ca2⁺ transients and action potentials, similar to endogenous CMs. They observed that introducing reprogramming factors reduced scar size and improved heart function in a mouse model of acute MI.

Jayawardena et al.67 found that directly administering lentivirus containing miR-1, -133, -208, and -499 into FSP1-Cre/tdTomeo mouse infarcted hearts converted resident cardiac fibroblasts into tdTomato+ CM-like cells in situ. The tdTomato+ iCMs displayed adult CMs morphology and function. They found that introducing these 4 miRs decreased scar size and improved the function of mouse hearts with acute MI.

In addition, direct cardiac reprogramming could be combined with other therapeutic factors to further restore the function of damaged hearts. In our in vivo studies, we found that adding the proangiogenic and fibroblast-activating factor thymosin β4,65,66 which independently promotes cardiac repair,69 enhanced the beneficial effects of GMT. Mathison et al.9 replicated in vivo cardiac reprogramming in rat hearts, and found that GMT reduced fibrosis by half and significantly improved the ejection fraction in rat hearts with MI. They also found that administering vascular endothelial growth factor (VEGF) with GMT further improved the ejection fraction four-fold greater than GMT alone.

These studies (summarized in Table 2) clearly demonstrate that in vivo reprogramming more efficiently yields higher-
Direct Cardiac Reprogramming for Heart Repair

Establishing the technology of cardiac reprogramming of human cells was a necessary step toward considering clinical application. However, neither GMT nor GHMT, which reprogrammed iCMs from mouse fibroblasts, were able to reprogram human fibroblasts into iCMs in vitro; however, inclusion of additional reprogramming factors resulted in successful reprogramming. Nam et al. found that GHT, without MEF2C, but with another transcription factor, myocardin, and 2 muscle-specific miRNAs, miR-1 and miR-133, could reprogram human fibroblasts into iCMs. These reprogrammed iCMs expressed multiple cardiac genes, developed sarcomere-like structures, and generated Ca\(^{2+}\) transients with a small subset of the cells exhibiting spontaneous contractility after 11 weeks in culture. Wada et al. reported that GMT with MESP1 and myocardin could activate cardiac gene expression in human neonatal and adult cardiac fibroblasts. They did not find spontaneously beating human iCMs, but they did observe that iCMs contracted synchronously in coculture with murine neonatal CMs.

In our study, pairing GMT with ESRGG and MESP1 induced global expression of cardiac genes and shifted the phenotype of human fibroblasts toward the CM-like state; adding 2 transcription factors, myocardin and ZFPM2 further enhanced this cardiac reprogramming in human fibroblasts. Reprogrammed human iCMs were epigenetically stable and formed sarcomere structures, and some could generate Ca\(^{2+}\) transients and action potentials. By comparing whole-transcriptome expression of 4- and 12-week iCMs, we found that reprogramming human cells takes longer than for mouse cells because of their progressive repression of fibroblast genes. However, we did not find spontaneously beating human iCMs in vitro, even 16-weeks after reprogramming. Nonetheless, our analysis of orthologous gene expression indicated that at the global gene expression level, human iCMs were reprogrammed at a level similar to mouse iCMs reprogrammed by GMT in vitro (Figure 1A).

Did the global transcriptome shifting and morphological and functional changes in human iCMs depend on ongoing expression of the reprogramming factors? To answer this question, we introduced reprogramming factors into human fibroblasts with a doxycycline (Dox)-inducible retroviral system for 2 weeks, and then shut off their expression by withdrawing Dox. We found that the percentage of reprogrammed iCMs in culture decreased during the first 2 weeks after Dox withdrawal, but remained stable between 3 and 5 weeks. Considering that non-reprogrammed fibroblasts continued to divide but reprogrammed cells did not, our observation that the percentage of iCM remained relatively unchanged indicates that more than 20% of iCMs were epigenetically and stably reprogrammed and could maintain...
a cardiac cell fate without relying on exogenous genes.

**Current Challenges and Future Directions**

The studies listed in Table 1 and Table 2 indicate that forced expression of 3 or 4 transcription factors can directly convert mouse cardiac fibroblasts into CM-like cells in vitro, although most of these iCMs were partially reprogrammed. These same factors generated more mature CM-like cells in the native heart, improved heart function and reduced scar size in the mouse heart post-MI (Figure 2B). Similarly, expression of 5 to 7 reprogramming factors converted human fibroblasts into non-perfect iCMs in vitro. By comparing in vitro vs. in vivo iCMs in mice, and considering the similarities between human and mouse iCMs in vitro, we speculate that the cocktails recently identified for human fibroblasts may be sufficient to reprogram adult CM-like cells that are fully functional in the in vivo environment, such as in the pig or non-human primate heart, as is the case in mice. We need to determine which combination of reprogramming factors sufficiently and efficiently generates iCMs in vivo, and evaluate their therapeutic efficacy and safety in larger animals.

We also need to develop a more efficient method for in vitro reprogramming that can be replicated more easily between laboratories. With the current protocols (Table 1), most fibroblasts were converted into partially reprogrammed cells in vitro, and only few of these iCMs were fully reprogrammed into beating cells; however, other studies report that different combinations of transcription factors could activate many cardiac genes in fibroblasts, but they failed to find beating iCMs reprogrammed in vitro. For example, Chen et al. delivered GMT into adult mouse cardiac fibroblasts and TTFs via lentivirus and found that GMT-infected TTFs expressed some cardiac genes, including Tnnt2, Serca2a, Tbx20, Gata6, Gja1, and Mybpc, and 21.8% GMT-infected cells displayed some cardiac electrophysiology activities. However, they did not find EGFP+ cells among either aMHC-Cre/Rosa26mTmG or Nkx2.5-Cre/Rosa26mTmG fibroblasts. Their study highlighted the significance of understanding the mechanism involved in efficiently converting fibroblasts into CM-like cells. Very recently, Wang et al. found that, rather than random overexpression of reprogramming factors, a stoichiometric expression of GMT protein really influences the efficiency and quality of iCM reprogramming.

Successfully achieving cardiac reprogramming requires high expression and proper stoichiometry of reprogramming factors, healthy and non-senescent fibroblasts, and optimal conditions for cell culture. Cardiac fibroblasts are heterogeneous, which raises the question of whether we can find a subpopulation of cardiac fibroblasts that is favorable for direct cardiac reprogramming. In addition, we still do not have a proper culture method to facilitate or maintain the maturation of adult CMs in vitro; while cultured in petri dishes, the rod-shaped adult CMs dedifferentiated into a flattened, spreading morphology to adapt the new environment. Therefore, we need to identify the optimal conditions for cell culture to enhance in vitro cardiac reprogramming. Although culture conditions, such as electric-stimulation, might help facilitate and maintain the functional maturation of iCMs in the late stages (ie, after cardiac cell fate conversion), the process may require some small-molecule compounds and growth factors to overcome epigenetic barriers in the early stages of in vitro reprogramming.

The epigenetic barriers that prevent cardiac reprogramming in vitro remain unknown; however, the in vivo environment of the heart after acute MI appears to overcome these epigenetic blocks. Non-myocyte lineages in the heart, such as endothelial cells, dynamically interact with myocytes and direct CM cell fate decisions during development, and also regulate the contractility functions of adult CMs. We speculate that secreted factors and direct cell-cell interactions, including mechanical and electrical, from myocytes and non-myocytes may work together to improve direct cardiac reprogramming.

In addition, Aurora et al. reported that an immune response was required for heart regeneration in neonatal mice after MI. It is possible that inflammatory cytokines and chemokines produced after acute MI may facilitate direct cardiac reprogramming, because cardiac fibroblasts were activated by inflammatory responses and became myofibroblasts. By understanding how environmental factors overcome epigenetic barriers in vivo, we can combine them with reprogramming factors to activate and reprogram quiescent fibroblasts within the scar of chronic heart failure patients.

Another elusive concept is the molecular mechanism that underlies direct cardiac reprogramming. Transcription factors bind genomic DNA to activate target genes that induce cellular events of cell fate conversion. Hirai et al. fused the MyoD transactivation domain with reprogramming factors, and found this modification could accelerate direct reprogramming and produced more beating iCMs from mouse fibroblasts. What are the DNA targets of those reprogramming factors? How are those transcriptional changes epigenetically stabilized during reprogramming? We need to understand the cascading cellular events, initiated by reprogramming factors individually and combinatorially, during this cardiac cell fate conversion. Our single-cell qRT-PCR results disclosed the variability in reprogramming among individual iCMs reprogrammed with factors expressed at different levels. Therefore, we also need to investigate the molecular mechanism within single cells, which will allow us to directly correlate the expression of critical transcription factors with that of their downstream targets. By combining mechanism assays at whole-population and single-cell levels, which should be mutual and consistent, we can gain a more integrated and comprehensive understanding of how core transcription factors establish a self-reinforcing molecular network that controls cardiac cell fate.

Although many challenges and hurdles remain in this blossoming research field, the high demand for regenerative medicine strategies for the heart emphasizes the significance of these efforts in discovering new therapeutic strategies. Observing the functional benefits of iCMs reprogramming in mouse heart and the promising and similar degree of reprogramming in mouse and human iCMs, we are endeavoring to translate direct cardiac reprogramming for future clinical applications.

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**Disclosures**

The authors report no conflicts.

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