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STATE-OF-THE-ART REVIEW

Fibroblast Reprogramming in Cardiac Repair

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HIGHLIGHTS

- **Direct cardiac reprogramming, which directly converts fibroblasts into cardiomyocytes, holds great promise in cardiac repair post injury.**
- **This review will discuss recent progress of fibroblast reprogramming in cardiac repair, including different types and states of reprogrammed fibroblasts, optimization of reprogramming protocols, in vitro mechanistic exploration, and in vivo translation efforts.**
- **Further studies will be needed to address some challenges still remaining, such as multi-omics study of the reprogramming process, the maturation of induced cardiomyocytes, and optimization of the in vivo delivery system.**

SUMMARY

Cardiovascular disease is one of the major causes of death worldwide. Limited proliferative capacity of adult mammalian cardiomyocytes has prompted researchers to exploit regenerative therapy after myocardial injury, such as myocardial infarction, to attenuate heart dysfunction caused by such injury. Direct cardiac reprogramming is a recently emerged promising approach to repair damaged myocardium by directly converting resident cardiac fibroblasts into cardiomyocyte-like cells. The achievement of in vivo direct reprogramming of fibroblasts has been shown, by multiple laboratories independently, to improve cardiac function and mitigate fibrosis post-myocardial infarction, which holds great potential for clinical application. There have been numerous pieces of valuable work in both basic and translational research to enhance our understanding and continued refinement of direct cardiac reprogramming in recent years. However, there remain many challenges to overcome before we can truly take advantage of this technique to treat patients with ischemic cardiac diseases. Here, we review recent progress of fibroblast reprogramming in cardiac repair, including the optimization of several reprogramming strategies, mechanistic exploration, and translational efforts, and we make recommendations for future research to further understand and translate direct cardiac reprogramming from bench to bedside. Challenges relating to these efforts will also be discussed. (J Am Coll Cardiol Basic Trans Science 2024;9:145–160) © 2024 The Authors. Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Cardiovascular disease (CVD), which is the most common noncommunicable disease accounting for up to 18 million deaths worldwide each year, poses a heavy burden on public health and well-being throughout the world.[1] This major prevalence of CVD is partially attributable to the fact that adult mammalian cardiomyocytes are terminally differentiated cells with little or no

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ABBREVIATIONS AND ACRONYMS

AAV = adeno-associated virus

ATAC-seq = assay for transposase-accessible chromatin using sequencing

CF = cardiac fibroblast

CVD = cardiovascular disease

iCM = induced cardiomyocyte

iCPC = induced cardiac progenitor cell

MEF = mouse embryonic fibroblast

MGT = Mef2c, Gata4, and Tbx5

MI = myocardial infarction

TTF = tail tip fibroblast

proliferative capacity, which renders the heart incapable of clinically relevant regeneration after injury.[2,3] As such, cardiac muscle lost to injury such as myocardial infarction (MI), one of the most common and severe CVDs, is often replaced by noncontractile scar tissue. This reparative mechanism, however, fails in the long-term to restore normal cardiac function, and often results in heart failure or even cardiac death.[4] To address the fundamental problem of irreversible loss of cardiomyocytes following MI, a number of experimental strategies—namely, transplantation of pluripotent stem cells,[5] stimulation of endogenous cardiomyocyte proliferation,[6] and direct reprogramming of resident fibroblasts into cardiomyocytes[7,8]—have been developed to restore functional myocardium.

Direct reprogramming, which directly converts one somatic cell type into another without entering the pluripotent state, has been achieved for a battery of cell types in different tissues and organs (brain, liver, heart, pancreas islet, and so on).[9,10] Cardiac fibroblasts, which account for a large proportion of nonmyocytes in the mammalian hearts,[11,12] are activated and massively proliferative after MI,[13,14] making them the ideal targets for direct reprogramming. If such conversion of fibroblasts into functional induced cardiomyocytes (iCMs) in the infarcted heart could be achieved in patients, it would not only regenerate damaged myocardium but also mitigate cardiac fibrosis, leading to reduced scar size and improved cardiac function. In this review, we highlight the recent advances in method optimizations, mechanistic studies, and translational efforts in pushing this approach closer to clinical application.

HISTORY AND DEVELOPMENT OF iCM REPROGRAMMING

In 1987, the finding that overexpression of MYOD, a transcription factor highly expressed in skeletal muscle cells, could convert mouse embryonic fibroblasts (MEFs) into myoblasts inspired a generation of research into altering somatic cell identity and a race to add new cell types to the direct reprogramming repertoire.[15] Direct conversion of fibroblasts to cardiomyocyte-like cells, identified by flow cytometry for an αMHC-GFP reporter as well as cardiac troponin T (cTnT), was first accomplished in vitro in 2010. After an extensive screening of the different combinations of 14 cardiac transcriptional factors, Ieda et al[16] showed that ectopic expression of Mef2c, Gata4, and Tbx5 (MGT) was sufficient to convert neonatal mouse cardiac fibroblasts (CFs) into iCMs. The group was also able to show that a small proportion of iCMs exhibited calcium transients, spontaneous beating, and action potentials, suggestive of functional similarity to native cardiomyocytes. In the intervening years, techniques for assessment of cardiac reprogramming efficacy have remained fairly stable. Generally speaking, several experiments must be done to measure the successful conversion from fibroblasts to iCMs. On a molecular level, reprogramming is assessed by quantitative real-time polymerase chain reaction to detect relative expression of cardiomyocyte- and fibroblast-specific genes, flow cytometry and immunofluorescent staining of cardiomyocyte markers (mainly cTnT and αActinin), and evaluating organization of sarcomere proteins.[17-19] On a functional level, assays such as calcium flux imaging, counting beating loci, measuring action potentials, and testing response to pharmacological agents are performed to assess functional maturity of iCMs.[16,20-22] Still, although iCMs may exhibit many of the phenotypes of native cardiomyocytes, genetic difference still exists between iCMs and endogenous CMs as revealed by microarray data[16] and transcriptome sequencing.[23] This is perhaps not surprising, but the differences bear further research. Two years after the publication of MGT reprogramming, in a mouse model of MI, a direct conversion of resident cardiac fibroblasts into iCMs was shown to be accomplished in vivo by local delivery of MGT.[10] Importantly, isolated iCMs from the reprogrammed heart exhibited ventricular cardiomyocyte-like action potentials, beat upon electrical stimulation, and showed evidence of electrical coupling. Song et al[20] independently demonstrated that MGT was able to induce iCM reprogramming both in vitro and in vivo, and the addition of a fourth transcription factor Hand2 improved the reprogramming efficiency, with an ∼4-fold higher percentage of generated iCMs (9.2%) than MGT-induced iCMs (2.5%), determined by flow cytometry analysis of αMHC-GFP and cTnT. Subsequently, successful conversion of mouse fibroblasts into iCMs has also been achieved by forced expression of a combination of 4 cardiac-enriched microRNAs: miR-1, 133, 208, 499 (miR Combo) both in vitro and in vivo.[24,25] Interestingly, however, among the muscle-related microRNAs, miR-133 was found to be the only microRNA capable of enhancing the efficiency of MGT-mediated iCM reprogramming.[26] According to flow cytometry analysis of cTnT or αActinin, miR-133 could sharply increase the ratio of $cTnT^+$ (∼4-fold) or $\alpha Actinin^+$ cells (∼5-fold). Additionally, more calcium transient positive cells

and more beating cells (both around 6-fold) were observed in miR-133 plus MGT group, which suggests that miR-133 enhances generation of functional iCMs. In addition to an miR combo, a small molecule cocktail (CRFVPTZ), including inhibitors for epigenetic silencers and TGFβ, WNT, and GSK3 signaling, also generated spontaneously beating cardiomyocyte-like cells from MEFs.[27] Further, in 2016, Cao et al[22] identified a combination of 9 small molecules that could efficiently reprogram human fibroblasts into iCMs. Mechanistically, they found that chemical cocktail treatment resulted in more open-chromatin conformation at genomic loci encoding key cardiac developmental genes. The generation of iCMs with pharmacological reagents, as in Cao et al,[22] provides a means for in vivo direct cardiac reprogramming without the introduction of exogenous genetic material. Through these studies, approaches to induce iCM formation have been established by various cocktails and molecules (summarized in **Tables 1 and 2**), allowing for detailed mechanistic investigation and bringing clinical application closer and closer to viable.

DIFFERENT TYPES AND STATES OF FIBROBLASTS FOR iCM REPROGRAMMING

As the starting cells for direct reprogramming, the origin and age of the fibroblasts considerably influence the conversion efficiency and quality of generated iCMs. CFs are the major noncardiomyocyte cell type in the heart and have been the choice of starting cell type for the original and a series of subsequent reprogramming work.[47] Importantly for translational potential, CFs become proliferative and activated at the injury site upon MI, meaning targeting these transforming CFs to generate iCMs could simultaneously regenerate damaged myocardium and reduce the fibrotic scar.[13] For basic research, however, fibroblasts of different origins have been used. MEFs gained considerable popularity as the starting fibroblast type, in part because they are widely used in induced pluripotent stem cell (iPSC) reprogramming[48] as well as direct reprogramming into other lineages such as hepatocyte and neuron.[49,50] Nevertheless, results from within the same laboratory consistently suggest that the efficiency of reprogramming MEFs to iCMs (~0.1% cTnT$^+$ cells and ~4.5% αMHC-GFP$^+$ cells in Zhou et al[17]) is considerably lower than that of generating iCMs from neonatal CFs (~9.3% cTnT$^+$ cells and ~10.7% αMHC-GFP$^+$ cells), but still higher than adult CFs (~3.8% αMHC-GFP$^+$ cells), as shown by parallel experiments under the same culture and reprogramming conditions.[17,19] However, some conclusions that came from CF-based experiments could not be repeated in MEFs. For example, Jayawardena et al[24] showed that miR Combo was able to induce iCM reprogramming of neonatal CFs in 2012, whereas in 2015, Muraoka et al[26] found that miR Combo could not convert αMHC-GFP MEFs into iCMs as revealed by flow cytometry. Although these results await further investigation and validation from additional laboratories, the seemingly discrepant results could be biologically interesting, suggesting differences in cell plasticity even among fibroblasts from different origins. Tail tip fibroblasts (TTFs), more accessible than CFs and MEFs in mice and rats, have also been used in reprogramming research, albeit giving rise to the reported lowest efficiency, possibly because of further epigenetic barriers in this type of fibroblast.[17,19,27] Zhou et al[17] detected fewer than 1% of cells in both neonatal and adult TTFs reprogrammed with MGT that were αMHC-GFP$^+$, compared with 4% to 10% positive cells generated from neonatal and adult CFs, MEFs, and endothelial cells. For human cardiac reprogramming, H9 human ESC-derived fibroblasts and human foreskin fibroblasts have been widely utilized as starting cells.[21,51,52] Distinct from mouse iCM reprogramming, overexpression of MGT alone, without additional transcription factors or microRNAs, cannot directly convert human fibroblasts into functional iCMs. This topic will be discussed in detail in the following text, but it highlights species differences in reprogramming capacity. Although the neonatal CFs from postnatal day 1 mice are commonly used for in vitro iCM reprogramming, adult or aged CFs have been rarely studied because of the complexity of isolation,[53,54] challenges in culturing them without changing cellular features,[55,56] and difficulty in viral infection or protein delivery. Nonetheless, in broad terms for in vitro cardiac reprogramming efficiency, neonatal CFs are superior to other types of fibroblasts, including adult CFs, TTFs, and MEFs, demonstrating the importance of both histological origin and cell age or maturity for the plasticity required for direct reprogramming. Yet, with the rapid progress made in the cardiac reprogramming and aging fields in recent years,[57,58] direct reprogramming of CFs from the aged heart may be achievable with high efficiency in the near future.

Even within 1 specific type of fibroblast, cell heterogeneity inevitably affects reprogramming outcomes. Liu et al[59] utilized single-cell RNA sequencing to analyze global transcriptome changes at the early stages during reprogramming of murine CFs to iCMs. They examined the cellular composition of the isolated starting CFs and identified 5 subpopulations

TABLE 1 Different Approaches for iCM Reprogramming In Vitro

First Author	Year	Fibroblast type	Reprogramming Cocktail	Cell Function Assay	Other Techniques
Ieda et al[16]	2010	Neonatal CFs and TTFs	G/M/T	Ca^{2+} Flux, beating, action potential	Microarray analysis
Song et al[20]	2012	Adult TTFs and CFs	G/M/H/T	Ca^{2+} Flux, beating, action potential	Microarray analysis
Jayawardena et al[24]	2012	Neonatal CFs	miR-1/133a/208/499	Ca^{2+} Flux, beating, action potential	None
Wang et al[28]	2014	MEFs	Oct4, SB431542, Parnate, forskolin, CHIR99021,	Ca^{2+} Flux, action potential	Pharmacological tests
Wang et al[29]	2015	Neonatal CFs	MGT (polycistronic)	Ca^{2+} Flux, beating	None
Zhou et al[30]	2015	TTFs, MEFs, and CFs	G/H/M/T+Akt1	Ca^{2+} Flux, beating	RNA-seq
Zhao et al[31]	2015	MEFs and adult CFs	G/M/T or G/H/M/T plus Y-27632 and A83-01	Ca^{2+} Flux, beating	RNA-seq
Yamakawa et al[32]	2015	MEFs	G/M/T+FGF10+VEGF	Ca^{2+} Flux, beating	Microarray analysis
Fu et al[27]	2016	MEFs	Chemical inhibitor CRFVPTZ	Ca^{2+} Flux, beating, action potential	Microarray analysis
Mohamed et al[33]	2017	Neonatal CFs	MGT plus SB431542 and XAV939	Ca^{2+} Flux, beating,	RNA-seq
Muraoka et al[34]	2019	Neonatal and adult TTFs	GMT or GHMT plus Diclofenac	Ca^{2+} Flux, beating	Microarray analysis
Garry et al[18]	2021	TTFs, MEFs, and CFs	AGHMT+PHF7	Ca^{2+} Flux, beating	RNA-seq, ChIP, ATAC-seq
Kim et al[35]	2022	MEFs	Extracellular vesicles during mESC differentiation	Ca^{2+} Flux, action potential	Microarray analysis
Wang et al[36]	2022	MEFs, neonatal CFs	Mef2c/Ascl1	Ca^{2+} Flux, beating, action potential	RNA-seq, scRNA-seq, scATAC-seq, ChIP-seq

ATAC-seq = assay for transposase-accessible chromatin using sequencing; CF = cardiac fibroblast; ChIP-seq = Chromatin Immunoprecipitation sequencing; iCM = induced cardiomyocyte; MEF = mouse embryonic fibroblast; MGT = Mef2c = Gata4 = and Tbx5; TTF = tail tip fibroblast.

with expression of distinct nonmyocyte lineage markers. They further discovered that suppression of nonmyocyte lineage genes during reprogramming occurs differently in the 5 subpopulations, possibly caused by epigenetic memories. Namely, suppression of endothelial and epicardial genes occurred rapidly while suppression of fibroblast and myofibroblast/smooth muscle genes occurred only with final conversion to iCMs, marking a sharp contrast with reprogramming to pluripotency.[59] Later, Wang et al[60] mapped the epigenetic landscapes and characterized the transcriptomic profiles of nonmyocytes from adult murine hearts through single-cell dual omics (RNA+ATAC) analyses. They revealed extensive heterogeneity of CFs through unbiased subclustering and functional annotation. This study identified 3 subtypes of CFs with distinct functional states related to response to stimuli, cytoskeleton organization, or immune regulation. Enrichment of murine CFs for in vitro cardiac reprogramming is often accomplished by sorting cells for Thy1 positivity, but whether the $Thy1^+$ population is enriched for any of these functional states is not currently known. In fact, little work has been done to identify differences in reprogramming capacity for different CF subtypes. In 2022, Zhang et al[61] used genetic lineage tracing strategies to show that most iCMs generated from CFs in vitro do not pass through a transient myofibroblast state. Indeed, they found that development of sarcomeric structures and characteristic myofibroblast cytoskeletal structures were mutually exclusive. This finding is an important step, but understanding and, ultimately, manipulating specific subtypes of CFs may facilitate further optimization of in vivo direct cardiac reprogramming.

OPTIMIZATION OF iCM COCKTAILS AND PLATFORMS

The original low conversion rate from fibroblasts to iCMs has prompted efforts to optimize the reprogramming cocktail and platform (Table 1). To address the question of whether separate delivery of reprogramming factors into fibroblasts (M+G+T) could lead to low reprogramming efficiency because of a suboptimal stoichiometry of factor expression, Wang et al[29] expressed G, M, T in a single, polycistronic vector with all 6 possible permutations. This study showed that changing the order of factors in a polycistronic construct, and hence the protein expression levels, had a dramatic impact on the efficiency and quality of iCM reprogramming. MGT, with Mef2c in the first place (expressed in the highest level) and Tbx5 in the last position (expressed in the lowest

TABLE 2 In Vivo Investigations of iCM Reprogramming

First Author	Year	Delivery Method	Reprogramming Cocktail	Lineage Tracing Strategy	Other Techniques
Qian et al[10]	2012	Intramyocardial injection of retrovirus	M/G/T	Fsp1-Cre/lacZ mice Periostin-Cre/LacZ mice αMHC-Cre/EYFP mice	Electron microscope, echocardiography, CMR
Song et al[20]	2012	Intramyocardial injection of retrovirus	G/M/H/T	Periostin-Cre/LacZ mice Tcf21-iCre/tdTomato mice	Echocardiography, CMR
Inagawa et al[37]	2012	Intramyocardial injection of retrovirus and GFP lentivirus	G/M/T	αMHC-GFP TG mice (Virus tracing)	None
Mathison et al[38]	2014	Intramyocardial injection of adenovirus (Rat)	GMT (polycistronic)	None	Echocardiography
Ma et al[39]	2015	Intramyocardial injection of retrovirus	MGT (polycistronic)	Tcf21-iCre/LacZ mice	Echocardiography
Jayawardena et al[25]	2015	Intramyocardial injection of lentivirus	miR Combo	Fsp1-Cre/tdTomato mice	Echocardiography
Miyamoto et al[19]	2018	Intramyocardial injection of Sendai virus	GMT	Tcf21-iCre/tdTomato mice	Echocardiography
Mohamed et al[33]	2018	Intramyocardial injection of retrovirus, intraperitoneally injection of inhibitor	GMT plus SB431542 and XAV939	Periostin-Cre/YFP mice	Echocardiography, CMR
Yoo et al[40]	2018	Intramyocardial injection of AAV	GMT+Tβ4	None	Echocardiography
Chang et al[41]	2019	Intramyocardial injection of nanoparticles	pMX-GMT (plasmid)	None	Echocardiography
Wang et al[42]	2020	Intramyocardial injection of retrovirus in Beclin1$^{+/-}$ mice	MGT	None	Echocardiography
Kang et al[43]	2020	Intramyocardial injection of AAV-1	miR Combo	Fsp1-Cre/tdTomato mice	None
Wang et al[44]	2021	Intravenous injection of biomimetic nanoparticles	miR Combo	Fsp1-Cre/tdTomato mice	Echocardiography
Kaur et al[45]	2021	Intramyocardial injection of modRNA	GMTHA+DnTGFb+DnWnt8 (modRNA)	Tnnt-mTmG mice	Echocardiography, scRNA-seq
Kim et al[35]	2022	Intramyocardial injection of extracellular vesicles	Extracellular vesicles from mESC differentiation into CM	None	Echocardiography
Tani et al[46]	2023	Tamoxifen induction of endogenous genes	M/G/T/H	Tcf21-iCre/Tomato mice, Tcf21-iCre/mTmG mice	Echocardiography

AAV = adeno-associated virus; CMR = cardiac magnetic resonance imaging; other abbreviations as in Table 1.

level), was identified as the ideal combination that resulted in a better conversion of both neonatal CFs and TTFs into iCMs (~5%-8% cTnT$^+$ cells vs ~0.5%-3% with M+G+T). Quantitative real-time polymerase chain reaction results suggested much higher mRNA expression level of cardiomyocyte marker genes in MGT-mediated reprogramming than other groups. This enhancement of cardiac reprogramming by the polycistronic MGT was further confirmed in an adult mouse model of MI where lineage tracing through β-galactosidase (Gal) activity showed that MGT led to an ~2-fold increase in α-Actinin$^+$β-Gal$^+$ iCMs per ventricular section compared with M+G+T.[39] Furthermore, Liu et al[62] systematically compared the impact of currently available 2A peptides (P2A, T2A, and E2A) in a polycistronic construct on protein expression and, thus, iCM conversion. This study reinforced the importance of gene order (their highest reprogramming efficiency occurred with their tdTomato reporter in second position in their quadcistronic vector, MtdTomGT vs other permutations) and showed that 2A order was less important to efficiency than using different 2A peptides to separate each gene locus (ie, PTE2A and TPE2A both outperformed 3P2A in their quadcistronic vectors).

In addition to varying the stoichiometry of MGT expression, additional transcriptional factors, growth factors, and microRNAs have been included in the MGT cocktail to further enhance cardiac reprogramming efficiency. The helix-loop-helix transcription factor Hand2 was shown to promote direct cardiac reprogramming of most of the previously mentioned fibroblast cell types. Compared with MGT, the combination of Gata4, Hand2, Mef2c, and Tbx5 (GHMT) was able to induce a higher ratio of iCMs to CFs (~5%-20% αMHC-GFP$^+$cTnT$^+$ cells with GHMT compared with ~1.5%-6% with only MGT) and more organized sarcomeres in parallel experiments both in vitro and in vivo.[20] In a screen of a library of 192 kinases, Zhou et al[30] found that addition of Akt1, a serine/threonine protein kinase, drastically increased the efficiency of GHMT-mediated reprogramming from fibroblasts into

iCMs. A nearly 20-fold increase was observed in relative mRNA expression of Myh6 in GHMT with Akt1 over GHMT only. The results also showed that Akt1 enhances generation of iCMs with Ca^{2+} flux (~3× more cells per field showing Ca^{2+} transients) and beating cells, which indicated that more functional iCMs were induced with the addition of Akt1. Furthermore, growth factors, including vascular endothelial growth factor alone or in combination with fibroblast growth factors, were found to promote cardiac reprogramming with a drastic increase in the number of functional, beating iCMs.[32,63] MiR-133, one of the microRNAs in miR Combo, was found to enhance MGT-based reprogramming by suppressing fibroblast gene signature.[26]

In recent years, small molecule inhibitors, which hold translational potential, have gained popularity. In 2015, Zhao et al[31] found that TGF-β or ROCK signaling inhibitors dramatically enhanced the ability of reprogramming transcription factors to convert mouse fetal or adult fibroblasts into functional iCMs by suppression of fibrotic events.[31] A high-throughput chemical screen of 5,500 compounds revealed that the combination of the TGF-β pathway inhibitor SB431542 and the WNT inhibitor XAV939 increased MGT-based reprogramming efficiency by 8-fold in vitro and in vivo.[33] A similar small molecule screening effort led to the identification of diclofenac, a nonsteroidal anti-inflammatory drug, as an enhancer of MGT or GHMT-based cardiac reprogramming, which suggests that suppression of at least some proinflammatory signals can improve cardiac reprogramming.[34]

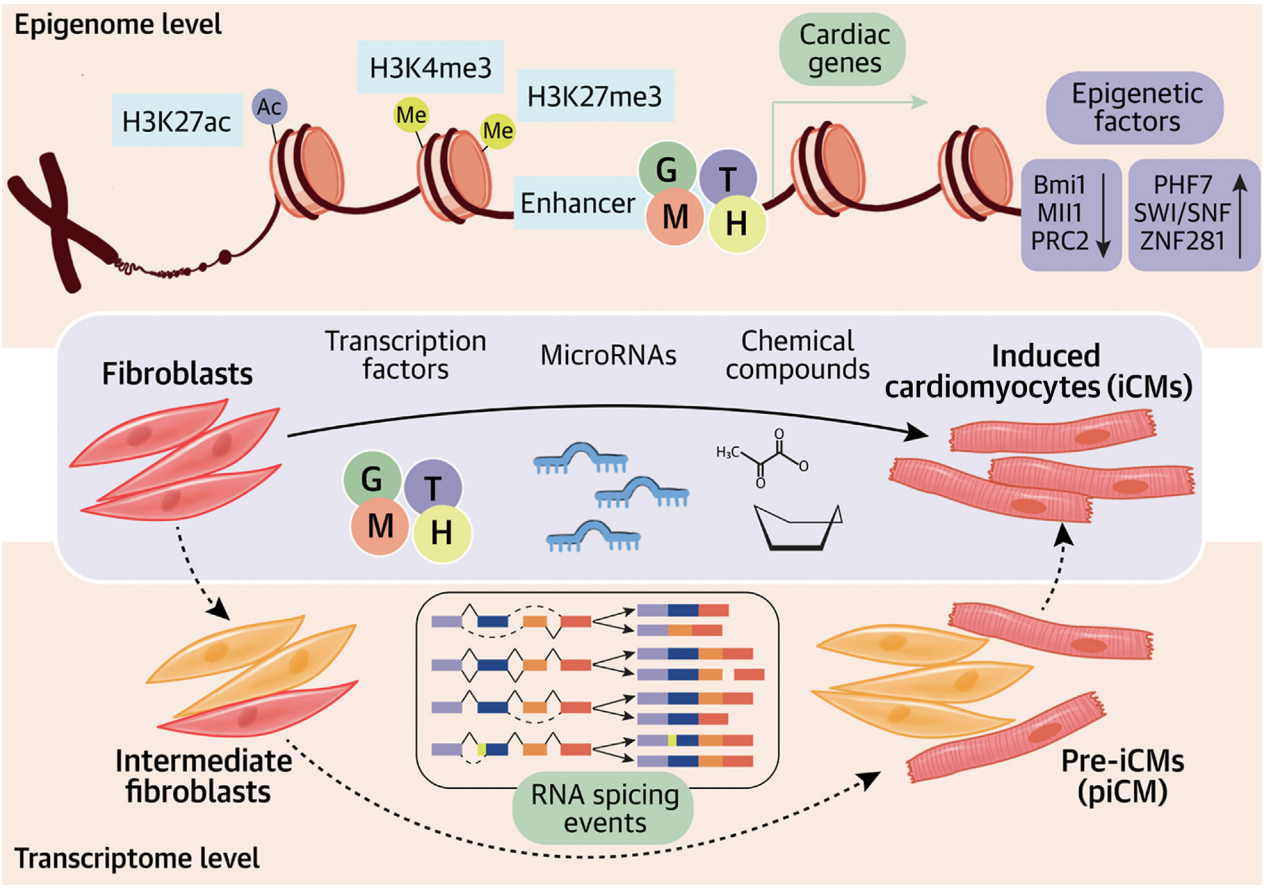
Taken together, these findings demonstrate that there is still room for optimization of reprogramming cocktails toward the clinical translation of cardiac reprogramming. This includes exploring novel reprogramming pathways outside of those seen during development of native cardiomyocytes. For example, Wang et al,[36] who recently explored the cross-lineage reprogramming potential of a neural lineage-specific transcriptional factor, Ascl1, to generate induced CMs, neurons, and hepatocytes, found that overexpression of Ascl1 activated a set of cardiac genes alongside the well-established activation of neuron-related genes. Together with Ascl1, Mef2c, the classical cardiac transcriptional factor, could drive Ascl1 binding away from neuronal loci to further activate cardiac program, which results in iCM reprogramming with high efficiency and maturity. Moreover, their working model of Ascl1 and Mef2c's cooperation in inducing transcriptional and epigenetic changes that ultimately generate iCMs differs significantly from that of MGT or GHMT-based reprogramming. This cross-lineage capacity of Ascl1 opens the door to the tantalizing possibility of looking outside the factors canonically associated with cardiomyocyte development when optimizing cardiac reprogramming cocktails and may shed light on common mechanisms of direct reprogramming more broadly.

Outside of the specific reprogramming cocktail, the microenvironment has long been speculated to be a key determinant of cardiac reprogramming efficiency and quality. This is illustrated by the fact that iCMs generated in vivo are much more mature than those generated in vitro. Indeed, isolated ventricular iCMs from in vivo reprogramming hearts were mainly rod-shaped and binucleated, closely resembling endogenous CMs, and evinced similar electrophysiological properties. In contrast, iCMs generated in vitro showed a more naïve morphology,[10,20,25] suggesting that the native microenvironment in vivo may promote the induction and maturation of iCMs. To mimic the in vivo mechanical microenvironment for cardiac reprogramming, Li et al[64] engineered a 3-dimensional hydrogel culture system and found that the 3-dimensional fibrin-based hydrogel environment significantly boosted miR combo-mediated cardiac reprogramming via an MMP-dependent mechanism. Similarly, culturing the MGT infected fibroblasts on micro-grooved substrate enhanced the yield of iCMs through the regulation of Mkl1 activity.[65] In 2020, Kurotsu et al[66] developed a Matrigel-based hydrogel culture system to determine the roles of matrix stiffness and mechano-transduction on cardiac reprogramming. They found that a soft matrix comparable in stiffness (~8 kPa) to native myocardium promoted the efficiency and quality of cardiac reprogramming through inhibition of YAP/TAZ.[66] These studies make the impact of microenvironment on reprogramming in vitro clear. It remains to be investigated, however, whether altering microenvironment after MI could promote in vivo direct reprogramming. This is particularly important because damaged myocardium is significantly different in its cellular environment and, relatedly, its cellular composition and function when compared with the healthy myocardium.[67]

MECHANISTIC EXPLORATION OF iCM REPROGRAMMING

Since the initial report of MGT-mediated cardiac reprogramming, studying the underlying mechanisms by which fibroblasts are directly converted into iCMs has become a quite significant and interesting topic, due in no small part to the lack of such a

CENTRAL ILLUSTRATION **Mechanistic Illustration of Fibroblasts Reprogramming Into Induced Cardiomyocytes**



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iCM = induced cardiomyocyte.

conversion observed in nature. A complete decoding of this reprogramming process will further facilitate the improvement of reprogramming efficiency and maturity of iCMs, easing translation to the clinic.

Efforts have been made to characterize the earliest stages of fate conversion through traditional profiling approaches (**Central Illustration**) such as a quantitative mass spectrometry-based proteomic approach, which showed rapid down-regulation and rebound of translation factors as well as up-regulation of ECM proteins and down-regulation of chromatin- and nucleic acid-binding proteins within 72 hours of infection with the MGT retrovirus,[68] and comparing the gene expression profiles of iCMs to more established iPSC-derived cardiomyocytes, which showed that iCMs tend to develop more mature epigenetic and energetic phenotypes than iPSC-derived cardiomyocytes.[23] However, it was not until the emergence of single-cell RNA sequencing (scRNA-seq) that high-resolution molecular characterization of the reprogramming process became possible using transcriptome profiles combined with mathematical modeling at the single-cell level. In 2017, Liu et al[59] performed scRNA-seq on CFs 3 days postinfection of retroviral MGT. They first identified molecularly distinct subpopulations of cells during reprogramming: fibroblast, intermediate fibroblast, pre-iCM, and iCM, which indicated the asynchronous nature of the reprogramming process. They then constructed a pseudo-time trajectory to illustrate the most likely reprogramming route from fibroblasts through intermediates to iCMs. Moreover, the heterogeneity of starting fibroblasts was also presented by dividing them into several subgroups with distinct expression

of nonmyocyte lineage markers, which were suppressed with varied dynamics over the course of reprogramming with epicardial and endothelial markers suppressed before fibroblast and myofibroblast/smooth muscle markers. Interestingly, molecular events related to RNA splicing were found to be enriched in the process of iCM induction. Ptbp1, one of the splicing factors, was recognized as the barrier to cardiac reprogramming. Knockdown of Ptbp1 effectively promoted the conversion of fibroblasts into iCMs. The specific roles of RNA splicing, splicing factors, and RNA binding proteins in direct cardiac reprogramming will need to be elucidated in future research.

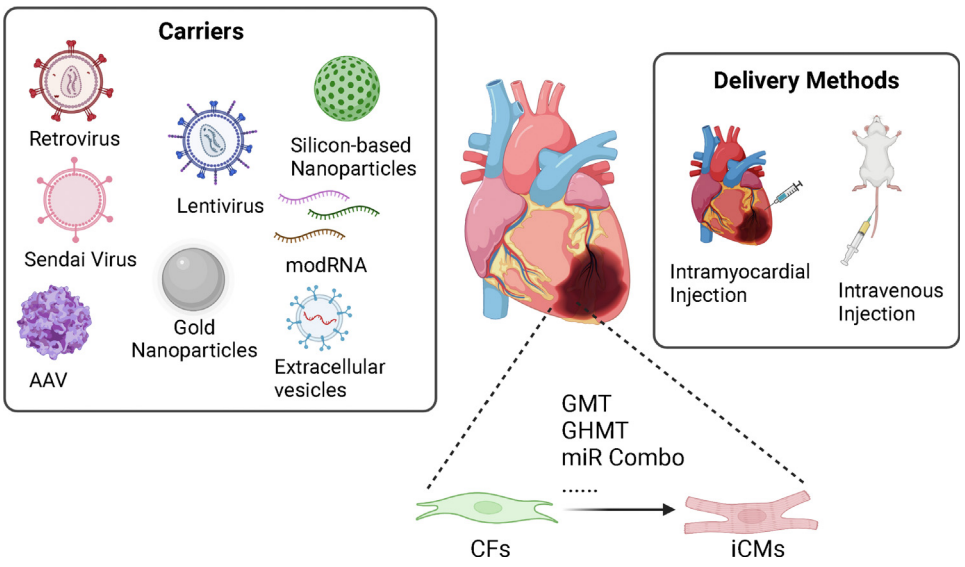
This study is not only the first to utilize scRNA-seq to reconstruct cardiac reprogramming process, but is also a piece of pioneering work that leveraged an interdisciplinary approach to combine single-cell omics, supported by mathematical modeling, with molecular, cellular, and biochemical characterizations to uncover new biology of reprogramming (**Central Illustration**). Following this line of investigation, in 2019 there were 2 studies that both revealed the genome targets of and interactome involving the 3 original iCM reprogramming factors.[69,70] Both groups found that Gata4, Mef2c, and Tbx5 functioned synergistically and cooperatively to guide cardiac reprogramming and suppress fibroblast identity. Stone et al[69] combined single-cell RNA-seq with Assay for Transposase-Accessible Chromatin using sequencing (ATAC-seq), Chromatin Immunoprecipitation sequencing and machine learning to elaborate the mechanisms by which transcription factors act to initiate cardiac reprogramming. They were able to show that the 3 transcription factors facilitate and limit each other's ability to bind to DNA at specific loci, leading to context-specific regulation of cardiac reprogramming. Hashimoto et al,[70] meanwhile, performed genome-wide analyses of cardiogenic transcription factor binding and enhancer dynamics during iCM reprogramming. The enhancer landscape changes resemble the patterns of enhancer activation during embryonic cardiomyogenesis. They further constructed the regulatory network of iCM reprogramming and found that repressing the EGFR pathway helped the augmentation of iCM fate conversion. More recently, Wang et al[71] performed single-cell ATAC-seq on early stages of cardiac reprogramming and unveiled the networks of transcription factors involved in the early (within 3 days) shift of chromatin accessibility. They then integrated their scATAC-seq data with scRNAseq and showed a global rewiring of the cis-regulatory interactions at cardiac genes during reprogramming.[71] Interestingly, both Stone et al[69] and Wang et al[71] identified Smad3 as a barrier to reprogramming initiation, but the latter further showed that Smad3 could facilitate reprogramming at later time points. This suggests a more complicated role for TGFβ signaling in reprogramming than just as profibrotic barrier. Continuing to improve our understanding of cardiac reprogramming mechanisms will allow researchers in this field to identify new barriers and facilitators at each step in the process and design reprogramming cocktails and conditions that further increase reprogramming efficiency and maturity of iCMs. In the long-term, these improvements will be invaluable for clinical translation of cardiac reprogramming, ensuring that clinicians will be targeting the right cells in the right way at the right time. However, the road to application of these techniques in patients is long and requires extensive studies in living animals, so we now turn to recent advances in cardiac reprogramming techniques in vivo.

THE EFFICIENCY AND EFFECTIVENESS OF IN VIVO CARDIAC REPROGRAMMING

Alongside the substantial progress made in several aspects of in vitro cardiac reprogramming, significant efforts have been made to study in vivo iCM generation in various mouse and rat models (**Table 2**). The first in vivo experiments in cardiac reprogramming date to the very first published iCM study in 2010 from Ieda et al.[16] The researchers successfully transplanted GMT-transduced CFs into immunosuppressed NOD-SCID mouse hearts. Although no quantification data regarding to the ratio of iCMs induced from injected fibroblasts was presented, they were able to show that a subset of transplanted cells expressed αActinin in patterns suggestive of sarcomeric organization. In 2012, scientists from the same group went a step further and performed in vivo reprogramming of resident CFs into iCMs in a mouse model of MI.[10] Retrovirus was chosen as the GMT-vector in this project for its capacity to infect only cell-cycle active cells, which in the postinjury mouse heart are primarily fibroblasts populating the injured area.

Later, Qian et al[10] adopted a lineage tracing strategy to prove the successful in vivo conversion of fibroblasts into iCMs, as opposed to promoting proliferation of native CMs, by delivering GMT retroviruses to 2 fibroblast-specific tracing mouse lines (Fsp1-Cre and Periostin-Cre), which has been generally regarded as the "gold standard" for fibroblast lineage tracing. They observed the gradual maturation of iCMs (stained with both β-Gal and αActinin) by

FIGURE 1 **Optimization of In Vivo Direct Cardiac Reprogramming**



Optimization of in vivo cardiac reprogramming includes carriers of transcription factors and the delivery methods. Virus-based carriers (Retrovirus, lentivirus, Sendai virus, and adeno-associated virus [AAV]) and nonviral carriers (Gold nanoparticles, silicon-based nanoparticles, and extracellular vesicles) are reported to mediate in vivo reprogramming. Although it has been proved that intravenous injection of sequential targeted nonviral carriers could realize miR Combo-based reprogramming,[44] nearly all of the other studies are achieved by intramyocardial infarction.

immunostaining cryosections from hearts harvested at 1 and 4 weeks of reprogramming. They showed that ~1% to 2% of left ventricle CMs were reprogrammed from fibroblasts. They then showed improved cardiac function in GMT-infected mice 3 months post-MI, with 10% improvement in the absolute value of ejection fraction revealed by both echocardiography and cardiac magnetic resonance imaging.[10] This detailed investigative research established a valuable template for later in vivo studies. Nearly simultaneously, another group reported similar results of iCM reprogramming in vivo by retroviral delivery of GHMT to hearts post-MI. They also used 2 strains of lineage tracing mice (Fsp1-Cre and Tcf21-iCre) and found that nearly 6% of the cardiomyocytes found in the injured area were β-Gal$^+$, ie, reprogrammed from resident fibroblasts. Again, like Qian et al,[10] they showed improved cardiac function by echocardiograph and cardiac magnetic resonance imaging.[20] Subsequent publications followed these fibroblast-specific tracing strategies to support their in vivo reprogramming results, including one focused on testing the optimal polycistronic MGT vector[39] and another that demonstrated the effect of some chemical inhibitors on cardiac reprogramming efficiency both in vitro and in vivo.[33] Apart from genetic strategies of lineage tracing, virus tracing strategies by simultaneous injection of GMT-encoding virus and fluorescent protein-encoding virus together have also been used to demonstrate in vivo reprogramming, although these strategies tend to be less reliable because of the low likelihood that CFs will uptake 2 viruses at once. Nonetheless, Inagawa et al[37] used a virus tracing strategy in 2012 to show that ~3% of retrovirus-infected cells became iCMs.

To develop more clinically relevant delivery systems, some groups began to investigate other (particularly nonintegrating) viral vectors or even nonviral vectors for cardiac reprogramming (**Figure 1**). Because of the smaller size of miRNAs compared with protein-encoding genes, miR-Combo represents a promising strategy for clinically relevant vectors with strict limits on cargo size. In 2015, Jayawardena et al[25] provided evidence for mature iCM generation and cardiac function improvement in (lentiviral) miR Combo-induced cardiac reprogramming in the Fsp1-Cre mouse inducing tdTomato expression in resident fibroblasts. They found high infection efficiency of resident CFs by lentivirus injection and a roughly 3-fold increase of tdTomato$^+$ CMs relative to the negmiR control group, suggestive of some degree of real conversion rather than fusion of native CMs and

fibroblasts. Nonintegrating adenoviruses have also been reported to mediate in vivo cardiac reprogramming in a rat injury model. Mathison et al[38] showed that "triplet" polycistronic adenoviral vectors were more effective than "singlet" vectors in enhancing ventricular function; however, they showed no evidence for efficient delivery to resident CFs and, thus, for successful iCM reprogramming. Other groups have taken advantage of adeno-associated viruses (AAVs) for more specific delivery of factors to cardiac fibroblasts. AAV-DJ[40] and AAV-1[43] showed high transduction efficiency (up to 80% and ~35%, respectively, in CFs with the latter showing high fibroblast tropism) and high reprogramming efficiency in successfully transduced cells (~32% and ~20% $cTnT^+$, respectively). The group using AAV-DJ also showed reduced fibrotic area post-MI, but it is unclear whether this is primarily caused by reprogramming, inhibition of fibrosis, or increased angiogenesis because of their addition of thymosin β4 to the GMT cocktail. More comprehensive research of cardiac fibroblast tropism in AAV serotypes, including chimeric AAVs, is needed to better make use of this promising viral vector. Sendai virus, another nonintegrating virus, proved to induce more efficient cardiac reprogramming than traditional retroviral vectors, as shown by genetic lineage tracing and virus tracing strategies. A major advantage of Sendai viruses is their ability to replicate in the cytoplasm, leading to higher transgene expression at a lower viral load than other viral vectors. Miyamoto et al[19] used the Tcf21-iCre mouse to show that ~1.5% of resident fibroblasts were successfully reprogrammed to cTnT-expressing iCMs (a nearly 3-fold increase over parallel retroviral MGT-induced iCMs) with nearly 20% of those exhibiting mature sarcomere structure (whereas none of the retroviral iCMs showed well-organized sarcomeres). Recently, various groups have explored the capacity of nonviral vectors to deliver reprogramming factors in vivo, including nanoparticles,[44,41] extracellular vesicles,[35] and modified messenger RNA (modRNA).[45] Although all of the groups here cited reported improved left ventricular function, none performed strict verification of in vivo iCM generation using lineage tracing or expression of reporter genes, leaving open the possibility of function improvement through other mechanisms. Indeed, the study using modRNA to deliver reprogramming factors found robust improvement in angiogenesis (both in infarct area and in hindlimb injury area) over control subjects, which may explain some or all of the improvement in ventricular function they observed post-MI. The specific strategies in these studies will be discussed in more detail in the following text, but we introduce the concept of nonviral vectors here to highlight the work that is still needed to validate and optimize various delivery systems in preparation for clinical translation of cardiac reprogramming.

The in vivo studies we have discussed so far have been conducted in animal models of acute myocardial injury, meaning delivery of reprogramming factors was performed at the acute phase, typically within a day of MI. However, most patients who could potentially benefit from generation of iCMs from resident fibroblasts present with old or chronic MI, wherein the injury area has already formed a fibrotic scar with fewer fibroblasts actively dividing. Hoping to address this discrepancy, Tani et al[46] established a Tcf21-iCre inducible transgenic mouse line to simultaneously induce the expression of GHMT and label resident CFs. They then waited to induce the expression of reprogramming factors in the CFs until 4 weeks after MI injury. Mice showed significant improvement in cardiac function after GHMT induction. However, it is unclear whether the improvement is due primarily to reprogramming as such or global inhibition of fibrotic signaling, given that both factor expression after induction and overall reprogramming efficiency were quite low compared with other in vivo studies. Still, these findings make it possible to imagine the possibilities for cardiac reprogramming in chronic models, although the problem of how to successfully deliver reprogramming factors in fibrotic hearts with few actively dividing fibroblasts remains to be addressed.

Much work still remains ahead of clinical application of cardiac reprogramming strategies. Improvements to iCM generation as well as a more comprehensive understanding of the reprogramming process in rodent models are still needed to justify transition to large animals and ultimately nonhuman primates. Lineage tracing demonstrating conclusive cell fate conversion using the various strategies that have been or will be developed, a model of how much the various consequences of reprogramming (iCM generation, reduction of fibrosis, novel angiogenesis, and so on), and evaluation of side effects and other off-target effects and any differences in reprogramming between species and individuals are crucial avenues for future in vivo research.

DIRECT CARDIAC REPROGRAMMING OF HUMAN FIBROBLASTS

Animal studies make it clear that direct conversion of fibroblasts into beating cardiomyocytes holds great promise for improving patient outcomes in clinical

application. However, the classical cocktail of MGT is not sufficient to induce functional human induced cardiomyocytes (hiCMs). To address this issue, several groups sought to develop a viable protocol for efficient hiCM reprogramming. They found that with the supplement of additional transcriptional factors (eg, Hand2, Mesp1, and Myocd) and microRNAs (miR-1 and miR-133) to the classical MGT combination, human fibroblasts can be transdifferentiated into cardiac-like myocytes with sarcomere-like structures and action potentials, although the efficiency remains low relative to direct reprogramming of murine fibroblasts.[21,51,52] Later, alternative methods and protocols, like hMGT+miR-133 only,[72] miR Combo only,[73] and small molecules only,[22] were optimized for human cardiac reprogramming. Understanding the differences between hiCM and murine iCM generation is key for future clinical translation. In 2019, Zhou et al[72] performed scRNA-seq to study the molecular and cellular dynamics of hiCM reprogramming and identified hiCM-specific features when compared with murine iCM. In particular, they identified a "decision" point on the human cardiac reprogramming trajectory where a cell decides to either continue down the reprogramming pathway or enter a "refractory pathway" and regress to its initial fibroblast fate. They also developed a "cell fate index" (CFI) to quantitively assess the progression of reprogramming, with which they demonstrated that hiCM generation progresses significantly more slowly than that of murine iCMs. Common to both species, however, were 2 inflection points in reprogramming velocity demarcating 3 phases: an initial phase of rapid progression down the reprogramming pathway, a rapid deceleration leading to a phase of slower but still steady reprogramming, and a final acceleration marked by rapid acquisition of iCM fate. Additionally, the CFI can be a valuable tool for studying other biological processes involving cell fate transition.

These findings provided insights on the molecular networks governing human cardiac reprogramming, revealing key commonalities and differences between human and murine iCM reprogramming pathways and kinetics. Future work should focus on improving human cardiac reprogramming efficiency, both by addressing the barriers leading into the shift to the slower second phase of reprogramming and by coaxing cells away from the refractory pathway and toward the reprogramming pathway. Additionally, in recent years, the combinatorial use of various bioengineering techniques has created new avenues for improving human cardiac reprogramming, including developing nonviral delivery of plasmid[74] or microRNAs[75] and improving microenvironment by using a cardiac-like extracellular matrix.[76]

TRANSLATIONAL EFFORTS IN iCM REPROGRAMMING

Although the underlying mechanisms of direct cardiac reprogramming are being actively investigated in human and murine cells, translational efforts are simultaneously being made in various model systems to maximize the utility of these strategies in a clinical setting. These efforts include characterizations of iCM subtypes, alternative reprogramming into cardiac progenitor cells (CPCs), utilization of bioengineering techniques, and mRNA-mediated reprogramming.

Optimal heart pumping relies on functionally distinct CMs, including interconnected atrial, ventricular, and pacemaker CMs. Therefore, Nam et al[77] employed a pacemaker-specific Hcn4-GFP reporter mouse and a spectrum of CM subtype-specific markers to investigate possible subtypes of iCMs induced from fibroblasts. They found that there was indeed expression of cardiac subtype-specific marker genes in the just over 1% of the total cell count identified as successfully GHMT-reprogrammed iCMs with well-organized sarcomeres, including all 3 major subtypes (~35% atrial, ~22% ventricular, and ~32% pacemaker with ~11% marked "other"). All subtypes seemed to be immature, and, in particular, no beating Hcn4-GFP positive pacemakers were observed. Some years later, the lead author of this study became the corresponding author in a study that sought to determine whether chamber-specific CMs can be induced during GHMT-induced iCM reprogramming in vivo. Interestingly, they found that a large fraction of iCMs express both atrial and ventricular markers in vitro while in vivo reprogramming exclusively generated ventricular-like iCMs.[78] The latter finding is promising in terms of clinical relevance given that the regions of the heart most commonly damaged by MI are in the left ventricle. However, it will be worthwhile for future research to investigate strategies for specific induction of atrial and pacemaker CMs.

Another major translational effort is to directly reprogram fibroblasts into induced cardiac progenitor cells (iCPCs), because iCPCs may provide an alternative regenerative method to repair a damaged myocardium. CPCs are highly proliferative and are capable of differentiating into various cardiac cell types, including cardiomyocytes, endothelial cells,

and smooth muscle cells.[79,80] Although there is some overlap in strategies, reprogramming fibroblasts into iCPCs requires distinct sets of transcription and epigenetic factors from iCM reprogramming, including Mesp1, Tbx5, Baf60c, Gata4, and so on.[81,82] Pioneers in this field showed that their reprogrammed iCPCs maintained long-term proliferative ability and exhibited multipotency toward all cardiac lineages. More importantly, transplantation of iCPCs into adult murine myocardium improved cardiac repair after MI, which suggests that iCPCs could serve as an attractive resource for cell transplantation into damaged hearts. More recent research on iCPCs has aimed at optimizing induction protocols and validating them in vivo. Leveraging the CRISPR/dCas9 activation system where the endogenous expression of cardiac-specific transcriptional factors can be elevated through the deactivated Cas9 (dCas9) engineered with transcription activation elements, iCPCs were generated from fibroblasts without persistent overexpression of transcription factors.[83,84] Expandable iCPCs could also be reprogrammed from fibroblasts, with either murine or human origin, via treatment of 6 small molecules in xeno-free conditions, a meaningful advance toward clinical translation. Recently, another group revealed that combination of miR-208 (one of the microRNAs in the miR Combo), ascorbic acid, and bone morphogenetic protein 4 could reprogram mouse fibroblasts into the mixture of the previously mentioned 3 types of cells, further forming partial cardiovascular tissues in vitro, which could also be transplanted into the injured murine heart and aid cardiac repair, sharing a similar concept with the utilization of iCPCs.[85]

As pointed out previously, nearly all previous studies regarding in vivo cardiac reprogramming take advantage of the retrovirus, which primarily infects cell-cycle active cells—such as the reactivated cardiac fibroblasts in injured hearts—to deliver MGT or other factors to realize direct reprogramming.[10,20,39,33,42] However, intramyocardial injection of integrating viruses into the myocardium has limited clinical relevance, such as potentially carcinogenic genome integration, potential immunogenicity issues, and possible secondary damage. Although Sendai virus has been shown to act as a possible vector to achieve in vivo cardiac reprogramming without genome integration,[19] the high cost might limit its further application. Yet, bioengineering techniques including nanoparticle-based delivery systems,[86-89] extracellular vesicles,[90-92] and microneedle-based patches[93,94] have been developed at an unprecedented pace in recent years and used as vehicles for drug delivery to treat various human diseases.

Recently, bioengineering techniques have begun to be utilized to develop nonviral carrier-based delivery systems for cardiac reprogramming. In 2019, Chang et al[41] showed that cationic gold particles loaded with MGT-expressing plasmid could function as nanocarriers for direct cardiac reprogramming and demonstrated efficient in vitro conversion of fibroblasts into iCMs and improvement of cardiac function in vivo by direct injection of AuNPs into the damaged myocardium post-MI. Approaching from a different angle, Wang et al[44] introduced a biomimetic nonviral system (shown in vitro to convert 16% of isolated fibroblasts to cTnT$^+$ iCMs) to sequentially target CFs of the injury border zone and precisely deliver miR Combo to induce iCM generation from resident cardiac fibroblasts by systemic administration.

Those nanocarriers based on either intramyocardial injection or systemic injection have significant tradeoffs in a clinical setting. Direct intramyocardial injection might increase the reprogramming efficiency while systemic injection is minimally invasive, and thus easier for translation. How to optimize nanoparticle-based delivery systems to balance these pros and cons will be a key question for future research. Extracellular vesicle-guided direct reprogramming of fibroblasts into iCMs was also reported in 2022.[35] Kim et al[35] found that extracellular vesicles derived from 2 stages of embryonic stem cells undergoing cardiac differentiation have the ability to reprogram fibroblasts into functional iCMs with high efficacy. The first stage delivered many miRs related to chromatin remodeling and the second miRs related to cardiac fate. Given the surprisingly high reprogramming efficiency (nearly 60% αMHC-GFP$^+$ cells), the maturity of the generated iCMs, and the undefined nature of these extracellular vesicles, efforts should be made to characterize the underlying mechanisms of this powerful and distinct reprogramming approach. This will improve understanding of cardiac reprogramming writ large and also lead to improvements in generation and maturity of iCMs in strategies that use defined factors.

Most recently, the COVID-19 pandemic has propelled the development and deployment of effective and safe mRNA-based vaccines. In 2021, Kaur et al[45] used a modRNA gene delivery system to deliver a cocktail of reprogramming factors to induce cardiac reprogramming by directly injecting a mixture of 7 modRNAs into the myocardium. This mRNA-based therapy led to efficient, transient expression of reprogramming factors, thus avoiding long-term persistent overexpression of transcription factors, which could result in unwanted side effects compromising cardiac function. Given the widespread use of

modRNAs to vaccinate against SARS-CoV2, this strategy represents a potentially safe and effective avenue for clinical translation. Future research should focus on working out the mechanisms of modRNA-based reprogramming, improving delivery, and promoting the maturity of reprogrammed iCMs.

FUTURE DIRECTIONS AND CHALLENGES

Up to now, although great progress has been made in optimizing reprogramming protocols, elucidating the underlying mechanisms, and improving delivery methods for reprogramming factors, there are still some remaining issues that need to be addressed to advance both basic and clinical development of this promising and interesting cardiac regenerative approach.

To unravel the underlying mechanisms in direct cardiac reprogramming, significant efforts have delineated much of the transcriptional and epigenetic regulation of iCM induction.[59,69,70,72] Through these efforts, epigenetic barriers, such as Bmi1, have been identified, and removing those barriers at early stages of reprogramming significantly enhanced the generation of functional iCMs.[17,95] Although transcriptional and epigenetic regulation are critical for orchestrating cell fate transition and establishment, post-transcriptional regulation at the level of RNA processing, RNA transport, and post-transcriptional modifications and translation, in short, an entire one-third of the central dogma, has been entirely neglected as it concerns cardiac reprogramming.[96-100] It has been reported that post-transcriptional regulation is important in direct reprogramming of other lineages.[101,102] Therefore, delineating the effect of post-transcriptional regulation of direct cardiac reprogramming may lead to discovery of novel mechanisms governing this process.

It is worth mentioning that the function of an iCM is heavily influenced by its maturity level. The iCMs generated in vitro are less mature than those generated in vivo.[10,20,23] The immaturity of iCMs unfortunately hinders detailed functional characterization in vitro, which is crucial to discern the quality of iCMs and their ability to facilitate cardiac repair. Future work is needed to explore additional or improved assays for measuring the maturity level of iCMs in a quantitative yet cost-effective way. Similarly, research guided toward how to improve maturity of iCMs by either genetic manipulation or chemical treatment may offer alternative opportunities for clinical applications and unexpected mechanistic findings.

It will be of great benefit to translate those in vitro findings into clinical applications. In general, in vitro findings of previous research could be reproduced in cardiac reprogramming in mouse hearts, such as intraperitoneal addition of chemical inhibitors[33] and delivering virus in heterogenous knockout mouse.[42] However, experiments on mice are a long distance from the application for human treatment. Therefore, large animal models or human organoid models may be useful to promote the translation process in the future.

Finally, ways to induce reprogrammed cells so far are largely based on overexpression of some transcriptional factors or microRNAs,[10,20,25] which may face delivery hurdles and possible side effects when it comes to clinical translation. First of all, the size of these factors restricts the choice of optimal vectors, such as AAV that has been applied in many clinical trials but only allows a relatively small packaging size.[103] Second, overexpression of cardiogenic transcription factors or microRNAs may cause undesired side effects especially with a persistently high expression level, such as hypertrophy or arrhythmia. Therefore, from the point of view of gene therapy, it might be easier to knock down certain genes rather than overexpressing them. It has been recently shown that knockdown of a single gene, Ptbp1, successfully induced direct reprogramming of fibroblasts into functional neurons in vitro,[104] which can be further achieved in vivo in a model of Parkinson's disease through AAV-mediated delivery,[105] holding great promise for translation in large animals and even humans. Similarly, identification of novel molecular or epigenetic "barriers," knocking down of which is sufficient to induce iCM formation, may open new paths in the field of direct cardiac reprogramming.

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