Special Issue: Direct Reprogramming

Mini Review

Direct reprogramming into cardiomyocytes

Kazutaka Miyamoto[1,2)] and Masaki Ieda[1,2,3,*)]

[1)]Department of Clinical and Molecular Cardiovascular Research, Keio University School of Medicine, Tokyo, Japan

[2)]Department of Cardiology, Keio University School of Medicine, Tokyo, Japan

[3)]JST, CREST, Tokyo, Japan

Adult cardiomyocytes have little regenerative capacity following injury, and damaged myocardium heals via fibroblast proliferation and scar formation, leading to cardiac remodeling and heart failure. We and other reported that functional cardiomyocytes can be directly generated from fibroblasts using several combinations of cardiac-specific defined factors. Mouse fibroblasts can be directly converted into cardiomyocyte-like cells by overexpression of cardiac-specific transcription factors, Gata4, Mef2c, and Tbx5 (GMT), GMT plus Hand2 (GHMT), or Mef2c, Myocd, and Tbx5 *in vitro*. More recently, we and others reported that human fibroblasts can be reprogrammed into differentiated cardiomyocyte-like cells by overexpressing GMT plus Myocd and Mesp1 or Gata4, Hand2, Tbx5, Myocd, miR-1, and miR-133. We found that miR-133 promoted cardiac reprogramming by directly suppressing Snai1, a master gene of fibroblasts, and silencing fibroblast signature. *In vivo* cardiac reprogramming by GMT or GHMT also converted endogenous CFs into cardiomyocyte-like cells *in situ*, and improved cardiac function after acute myocardial infarction in mouse. These studies demonstrate that direct cardiac reprogramming technology may be a potential approach that could regenerate diseased hearts. The present article reviews the recent studies in cardiac reprogramming, and discusses the hopes and challenges of direct cardiac reprogramming towards regenerative therapy.

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*Correspondence should be addressed to:
Masaki Ieda, M.D., Ph.D., Department of Clinical and Molecular Cardiovascular Research; Department of Cardiology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. Phone: +81-3-5843-6702, Fax: +81-3-5363-3875, E-mail: mieda@z8.keio.jp

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Introduction

Heart disease is a major cause of adult and childhood mortality worldwide. Because postnatal cardiomyocytes have little or no regenerative capacity after injury, damaged hearts heal by scar formation rather than by cardiac muscle regeneration, potentially leading to cardiac remodeling and ultimately resulting in heart failure. Although current medical therapies can support damaged hearts, we cannot repair damaged cardiac tissues. Heart transplantation is an effective therapy for heart failure, but this opportunity is dependent on the number of donor organ available. Thus, successful and efficient cardiac regenerative therapies are desirable. The adult heart is composed of cardiomyocytes and other cell types, including cardiac fibroblasts (CFs)[1]. The CFs exist in cardiac tissue, up to 50% of all cells in the adult heart, and supply a supporting structure in the developing heart. However, once adult heart is injured, CFs proliferate and discharge extracellular matrix and growth factors, leading to scar formation and fibrosis[2-4]. Thus, given the abundance of fibroblasts in the heart, the strategy which reprograms resident cardiac fibroblasts into functional cardiomyocytes *in situ* may regenertate injured hearts and improve cardiac function.

Recently, a new approach to generate specific cell types by using combinations of specific transcription factors has emerged[7]. Based on the knowledge of the transcriptional circuits that specify cardiac lineage and differentiation of cardiomyocytes during embryogenesis, different combinations of cardiac transcription factors have been inspected for their ability to activate cardiac program in CFs. We initially found that mouse fibroblasts can be directly converted into cardiomyocyte-like cells by overexpressing cardiac-specigic transcription factors, Gata4, Mef2c, and Tbx5(GMT) in the fibroblasts[7]. Following our report, other groups also succeeded in direct cardiac reprogramming from mouse fibroblasts by expressing cardiac transcription factors-either (a) GMT plus Hand2 (GHMT) or (b) Mef2c, Myocd, and Tbx5 (MMT)-or by using cardiac-enriched microRNAs (miRNAs)[5, 6, 8]. More recently, we and others reported that human fibroblasts can be reprogrammed into differentiated cardiomyocytes by using GMT plus Myocd and Mesp1 or Gata4, Hand2, Tbx5, Myocd, miR-1, and miR-133[19-21]. *In vivo* cardiac reprogramming by GMT or GHMT also reprogrammed endogenous CFs into cardiomyocyte-like cells and improved cardiac function after myocardial infarction[8, 17, 18]. We developed a polycistronic vector of GMT to transduce the three factors equally and found this system superior to injection of the three separate vectors for *in vivo* reprogramming[9]. These studies together suggested that direct cardiac reprogramming technology may be a promising new therapeutic approach for regenerative medicine that could reduce fibrotic tissues and improve cardiac function in diseased hearts. Here, we review recent studies of direct cardiac reprogramming and discuss the hopes and challenges of using this new technology in regenerative medicine (Fig.1).

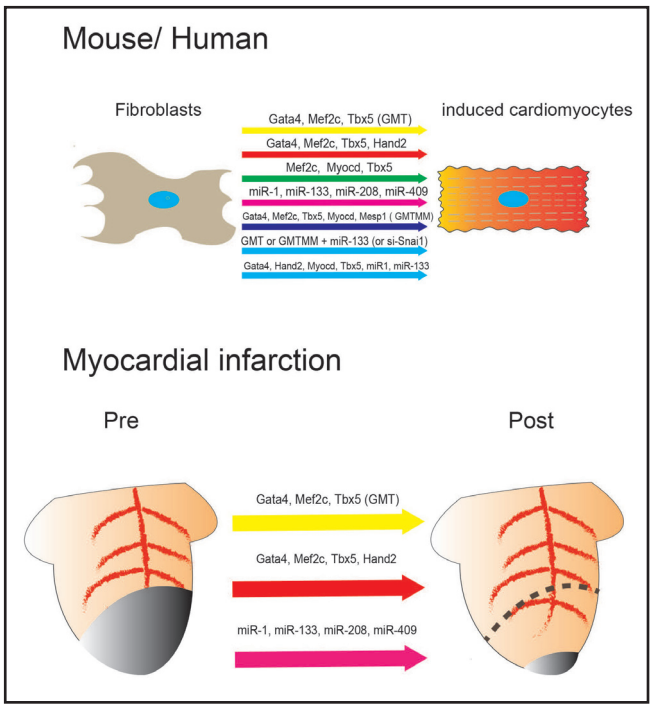

Fig.1 Mouse and human fibroblasts can be directly converted into cardiomyocyte-like cells by over-expressing multiple cocktails of cardiac-specific transcription factors and miRNAs *in vitro* and *in vivo*.

Direct Reprogramming of Mouse Cardiac Fibroblasts into Cardiomyocytes by Transcription Factors

The ability to convert one mature cell type into another by overexpressing a transcription factor was first reported in 1987[10-11]. Davis et al.[10] found that *MyoD*, which encodes a transcription factor crucial for skeletal muscle formation and differentiation, converted fibroblasts into skeletal muscle cells in culture. In contrast, Evans et al. showed that a dominant muscle fate was not the case for heterokaryons of fibroblasts and cardiomyocytes, and thus the transdifferentiation of fibroblasts into cardiomyocytes

seemed to be a challenging endeavor. In spite of the substantial efforts to identify novel cardiac genes that are important for cardiogenesis, a reprogramming gene into cardiomyocytes, a cardiac equivalent of MyoD, has not yet to be identified[12-15]. In 2006, Drs. Takahashi & Yamanaka[16] reported that differentiated somatic cells could be reprogrammed into iPSCs, which are very similar to embryonic stem cells, by transduction of four stem cell-enriched transcription factors: Oct4, Sox2, Klf4, and c-Myc. Since this discovery of iPSC generation, researchers recognized that combinations of multiple transcription factors rather than single factors might be effecitve to convert fibroblasts into another cell type.

In an attempt to identify cardiac reprogramming factors, we first developed a screening system in which the induction of cardiac genes in fibroblasts could be analyzed quantitatively by reporter-based fluorescence-activated cell sorting (FACS). We generated αMHC-GFP [α myosin heavy chain) promoter-driven enhanced green fluorescent protein (GFP)] transgenic mice, in which cells with activated cardiac programs express the GFP. We isolated Thy1$^+$/αMHC-GFP- CFs from αMHC-GFP neonatal mouse hearts by FACS and transduced 14 candidate cardiogenic factors into the fibroblasts using retroviruses. One week after the transduction, 1-2% of the cells expressed αMHC-GFP, suggesting that the 14 candidates included reprogramming factors. Serial removal of indivisual factors demonstrated that a minimum cocktail of the three factors, Gata4, Mef2c, and Tbx5 (GMT), could induce αMHC-GFP activation in 15-20% of CFs, which we designated induced cardiomyocytes (iCMs). However, the vast majority of iCMs were only partially reprogrammed, with approximately 5% of the starting populations expressing cardiac troponin T (cTnT) after 1 week of GMT transduction, and of these, only a few could contract spontaneously after 4-5 weeks in culture, suggesting that heterogeneous populations composed the iCMs. The efficiency of inducing fully reprogrammed functional cardiomyocytes from CFs by GMT *in vitro* was similar to that achieved in iPSC generation (0.01-0.1%). Despite the heterogeneity of our iCM populations produced as above, a small subset of the cells gradually matured over time to express multiple cardiac proteins, including sarcomeric α-actinin, cTnT, and atrial natriuretic peptide, and to form well-defined striated structures. Global gene expression analyses revealed the expression of a broad range of cardiac genes, with silencing of the fibroblast program in FACS-sorted iCMs. The histone and DNA epigenetic marks in some cardiac-specific promoters in the iCMs were similar to those in neonatal cardiomyocytes but were different from those in the original CFs. We also demonstrated the route of cardiac induction by using lineage tracing. Using Isl1-Cre–YFP (yellow fluorescent protein) and Mesp1-Cre–YFP mice, obtained by crossing Isl1-Cre or Mesp1-Cre mice with R26R-EYFP mice, we found neither *isl1* nor *Mesp1* gene activation, a marker of cardiac progenitor cells, during cardiac reprogramming. These results suggested that the fibroblasts were reprogrammed directly into differentiated cardiomyocytes without passing through a progenitor cell state by GMT transduction.

To analyze whether other types of terminally differentiated cells could also be reprogrammed directly into cardiomyocytes by GMT, we used mouse tail-tip fibroblasts (TTFs). By transduction of retroviral GMT into TTFs, we produced ~15% of αMHC-GFP$^+$ cells, but the efficiency of cTnT$^+$ cells in TTF-derived iCMs was half of that in CF-derived iCMs. Expression of the repressive histone mark, H3K27me3, in cardiac gene promoters was also decreased in the TTF-derived iCMs than original TTFs, but remained high compared with that in CF-derived iCMs and neonatal cardiomyocytes. The TTF-derived iCMs exhibited spontaneous intracellular calcium transients, a functional characteristic of cardiomyocytes, but they did not beat spontaneously, suggesting that TTFs may be more resistant to cardiac reprogramming by GMT[7].

Following our initial publication of direct cardiac reprogramming, several other groups also reported the same phenomenon. Song et al.[8] sought to investigate the optimal combination of the cardiac transcription factors to reprogramm adult TTFs into functional cardiomyocytes. They also used αMHC-GFP reporter mouse, and screened six cardiac transcription factors (Gata4, Hand2, Mef2c, Mesp1, Nkx2.5, and Tbx5) as candidate factors for cardiac reprogramming. According to their results, adding a bHLH (basic loop-helix-loop) transcription factor, Hand2, to GMT (GHMT) led adult CFs and TTFs to functional cardiomyocyte-like cells more efficiently than did any other combinations, including GMT. GHMT induced approximately 9% of starting populations to express both αMHC-GFP and cTnT cardiac genes, which was higher than those with GMT. Microarray and quantitative RT-PCR analyses demonstrated that a broad range of cardiac specific genes was upregulated and fibroblast genes, including *Fsp1* (fibroblast-specific protein1), were synchronously downregulated in the fibroblasts transduced with GHMT.

Notably, after 5 weeks of GHMT transduction into adult CFs and TTFs, calcium transients, action potentials, and spontaneous contractions were observed in a subset of iCMs, suggesting that GHMT may be more potent than GMT to overcome the hurdles of cardiac reprogramming in TTFs. This and other reports revealed that transient overexpression of GHMT or GHMT/Nkx2.5 for 10 days was sufficient to convert fibroblasts into fucntional iCMs, indicating that the cardiac reprogramming event is stable[23].

Cardiac Reprogramming Using MicroRNAs

MicroRNAs (miRNAs) have numerous targets related to signaling pathways, transcription factors, and epigenetic regulation, and play important roles in cell fate decisions. MiRNAs suppress the expression of hundreds of genes, primarily through binding to the 3'-untranslated region (UTR) of target mRNAs. Jayawardena et al. reported that a combination of muscle-specific miRNAs (miR-1, 133, 208, 499) alone reprogrammed neonatal mouse CFs into cardiomyocyte-like cells[6]. Using αMHC-CFP (cyan fluorescent protein) transgenic mice as a cardiac reporter, they found that *in vitro* transfection of mature miR-1, miR-133, miR-208, and miR-499 mimics induced 5% of αMHC-CFP+ cells in CFs. They also found that iCMs generated by miRNAs expressed several cardiac specific proteins and had sarcomeric structures, and interestingly, additional treatment with a Janus tyrosine kinase (JAK) inhibitor enhanced reprogramming efficiency and quality, leading to spontaneous cell contractions in 1-2% of the starting cells. Although it was unclear whether miRNAs alone could be sufficient for cardiac induction in other types of fibroblasts, such as TTFs, these results suggest that the miRNAs can reprogram CFs into iCMs and may have advantages for clinical applications in terms of safety, as miRNA mimics are synthetic oligonucleotides.

Reprogramming Human Fibroblasts to Cardiomyocytes by Defined Factors

Toward clinical application, it would be necessary to translate the mouse system into human. Nam et al.[19] found that GHMT, reprogramming factors in mouse fibroblasts, were ineffective in activating cardiac gene expression in human fibroblasts, but addition of Myocd and 2 miRNAs, miR1 and miR133, to GHMT could reprogram up to 20% of human fibroblasts into cardiomyocyte-like cells which expressed cTnT. The iCMs generated from human fibroblasts expressed multiple cardiac specific genes, and had clear sarcomeric structures. In addition, a small subset of the cells derived from adult cardiac fibroblasts started contraction spontaneously after 11weeks in culture. We reported that a different combination of transcritpion factors, Gata4, Mef2c, Tbx5, Myocd, and Mesp1, could reprogram human fibroblasts into cardiac cells that had many properties of human cardiomyocytes[20]. Human iCMs generated from our protocol did not beat spontaneously, but matured to exhibit action potentials and to contract synchronously in coculture with murine cardiomyocytes. Islas et al. reported that human dermal fibroblasts were reprogrammed into cardiac progenitor-like cells by overexpressing Ets2 and Mesp1, followed by activin A and bone morphogenetic protein 2 treatment[21]. Thus, the findings obtained from us and others would proceed the potential of direct reprogramming technology towards clinical applications. However, the efficiency of human cardiac reprogramming is lower than that of mouse cardiac reprogramming. These results suggest that human cells are more resistant to reprogramming, perhaps reflecting epigenetic and genetic blocks that need to be overcome for adequate cell fate conversion. Future studies are needed to thoroughly optimize conditions for human cardiomyocyte generation and maturation, and to characterize the properties of human iCMs.

Addition of MIR-133 Promotes Cardiac Reprogramming by Targeting SNAI-1

As stated above, we and others reported direct cardiac reprogramming in mouse and human fibroblasts. However, induction of functional cardiomyocytes was inefficient, and the molecular mechanisms of direct reprogramming remained undefined. Recently, we demonstrated that addition of miR-133a (miR-133) to Gata4, Mef2c, and Tbx5 (GMT) or GMT plus Mesp1 and Myocd improved cardiac reprogramming in mouse or human fibroblasts by directly repressing Snai1, a master regulator of epithelial-to-mesenchymal transition[22]. MiR-133 overexpression with GMT generated sevenfold more beating iCMs from mouse embryonic fibroblasts and shortened the duration to induce beating cells from 30 to 10 days, compared to GMT alone. Snai1 knockdown suppressed fibroblast genes, upregulated cardiac gene expression, and induced more contracting iCMs with GMT transduction, recapitulating the effects of miR-133 overexpression. In contrast, over-expression of Snai1 in GMT/miR-133-transduced cells maintained fibroblast signatures and inhibited generation

of beating iCMs. MiR-133-mediated Snai1 repression was also critical for cardiac reprogramming in adult mouse and human cardiac fibroblasts. Thus, silencing fibroblast signatures, mediated by miR-133/Snai1, is a key molecular roadblock during cardiac reprogramming. This is a first report determining a molecular mechanism and identifying inhibtory factors during direct cardiac reprogramming.

Reprogramming Endogenous Cardiac Fibloblasts into Cardiomyocytes *in vivo*

We speculated the main cause of low cardiac reprogramming efficiency *in vitro* might be the lack of natural environment for cardiomyocytes in plastic dishes. *In vivo* environment might be better for cardiac differentiation than that *in vitro*. To test the possibility of direct cardiac reprogramming *in vivo*, we and others investigated whether the gene transfer of reprogramming factors into infarcted mouse hearts could convert resident CFs into cardiomyocytes *in situ*[9, 8, 17, 18]. Mice harboring various lineage markers for the non-myocyte population were subjected to left anterior descending (LAD) ligation, causing fibroblasts to proliferate to form a scar. Qian et al.[17] directly injected GMT retroviruses into mouse hearts after myocardial infarction (MI) and showed that approximately 35% of cardiomyocytes in the border/infarct zone were newly generated iCMs derived from resident CFs. Of these iCMs, 50% had well-organized sarcomeric structures and showed the functional characteristics of adult ventricular cardiomyocytes, including cellular contraction, electrophysiological properties, and functional coupling to other cardiomyocytes. The hearts also included partially reprogrammed iCMs, and time course experiments revealed a progressive reprogramming process in which a more complete cardiac phenotype arose over time in *in vivo* iCMs. Significant improvements, including increased cardiac output, ejection fraction, and stroke volume, and decreased scar size, were observed upon GMT treatment. Functional studies revealed that the retroviral GMT gene transfer sustained improvement of cardiac function and reduction of fibrosis until at least 3 months after MI. Song et al.[8] also reported that GHMT retroviral injection into mouse ischemic hearts converted endogenous CFs into functional cardiomyocyte-like cells *in situ*. Using fibroblast-lineage-tracing mice, Song et al. also showed that 2-6% of cardiomyocytes in the border/infarct area were newly generated cardiomyocyte-like cells with clear striations and functional properties similar to those of endogenous ventricular cardiomyocytes. They used αMHC-MerCreMer/Rosa26-LacZ mice, in which endogenous cardiomyocytes could be chased by pulse labeling with tamoxifen, to exclude the possibity of cell fusion events in new cardiomyocyte generation. They demonstrated that the ejection fraction was increased by two fold in GHMT-treated mice compared with control, and that the scar size was reduced by 50% at 12 weeks after myocardial infarction.

Our approach to test the efficiency of *in vivo* cardiac reprogramming differed from those of the two other groups. They used mainly fibroblast-lineage-tracing mice to demonstrate cardiac conversion from endogenous CFs, whereas we co-transduced GMT retroviruses and reporter genes (such as GFP) to determine cardiac conversion from nonmyocytes. We used retroviruses, as the viruses infect only proliferating cells, which are mainly CFs in an infarcted myocardium. Also, different from their reports, we used immunosuppressive mice to promote the survival of the virally transduced cells. We found that GMT gene transfer induced mostly immature α-actinin$^+$ cells *in vivo*, with a conversion rate of approximately 1% in the transduced cells. To improve this transduction, we then generated a polycistronic retrovirus expressing GMT at near equimolar levels from the same promoter by using self-cleaving 2A peptides. Gene transfer of this polycistronic GMT retrovirus induced morphologically more mature cardiomyocyte-like cells in fibrotic tissues than those generated by injecting the three separate vectors. Thus, polycistronic vectors may be a valuable tool for *in vivo* reprogramming strategy[9].

Conclusions

Despite excitement and huge potential of direct reprogrammng technology, many important questions and issues remain (Table 1). Recently, we demonstrated that Snai1 is a key molecular roadblock during cardiac reprogramming, but other moelucules important for cardiac reprogramming remain unclear. We speculate secreted proteins, electrical and mechanical stimulation, and cell-to-cell contact may promote cardiac reprogramming, accoding to the co-culture experiments in human iCMs. The safety issue associated with viral delivery of reprogramming factors may be another concern for clinical applications. As a major limitation in the previous studies is the use of potentially harmful genome-integrating retroviruses, future study investigating whether fibroblasts can be reprogrammed into functional iCMs without viral integration is critical. Non-integrating viral vectors expressing cardiac

Table 1 Summary of published reports of direct cardiac reprogramming in mouse and human fibroblasts by various defined factors

in vitro or in vivo	Reprogramming factors	Species	References	Cardiac induction (α-MHC)	Cardiac induction (cTnT)	Gene expression	Function
in vitro and in vivo	Gata4, Mef2c, Tbx5	Mouse	7, 9, 17	15% (in vitro)	5% (in vitro)	Myh6 Actc1 Actn2 Nppa	Action potential Cell contraction Ca^{2+} transient
in vitro and in vivo	Gata4, Mef2c, Tbx5, Hand2	Mouse	8	15-18% (in vitro)	9% (in vitro)	Myl1 Myl2 Myl4 Myl6 Nppa	Action potential Cell contraction Ca^{2+} transient
in vitro	Mef2c, Myocd, Tbx5	Mouse	5	2.2%	2.5%	Myh6 Myl2 Actc1 Scn5a	Action potential
in vitro and in vivo	miR-1, miR-133, miR-208, miR-409 and JAK inhibitor	Mouse	6	1,1-5.3% (in vitro)		Mef2c Tnni3 Myh6	Cell contraction Ca^{2+} transient
in vitro	Gata4, Mef2c, Tbx5, Myocd, Mesp1	Human	20		5.9%	Actc1 Myh6 Myl2 Scn5	Action potential Cell contraction (co-culture) ca^{2+} transient
in vitro	Mesp1, Ets2	Human	21			Myl2 Myh6 Myh7	ca^{2+} transient
in vitro	Gata4, Hand2, Myocd, Tbx5, miR-1, miR-133	Human	19		13.9%	Actc1 Myh6 Myl7 Tnnt2	Cell contraction Ca^{2+} transient
in vitro	Gata4, Mef2c, Tbx5, miR-133 Gata4, Mef2c, Tbx5, Myocd, Mesp1, miR-133	Mouse Human	22	17-34%	5-13% (Mouse) 18-28% (Human)	Myh6 Actn2 Ttn Nppa	Cell contraction (Mouse) Ca^{2+} transient (Mouse)
in vitro	Gata4, Mef2c, Tbx5	Mouse	24	0%	35%	cTnT αMHC βMHC	Ca^{2+}channel-mediated depolarization

reprogramming factors, cardiogenic small molecules, and synthetic oligonucleotides might be attractive options in clinical applications. Moreover, all *in vivo* studies reported so far were performed in acute MI mouse models, and it remains to be determined whether *in vivo* reprogramming can be applied to the context of chronic heart failure. Further studies are required to address these questions, and optimization of cardiac reprogramming in human fibroblasts and demonstration of the therapeutic efficacy and safety of this approach in larger animals will be needed to advance this technology.

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Conflict of interests

None

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