Despite great advances of modern medical therapy, cardiac disease remains a leading cause of death worldwide. As adult cardiomyocytes have little regenerative capacity, extensive myocardial injury such as myocardial infarction leads to scar formation, cardiac remodeling, and heart failure, which are associated with high mortality. Heart transplantation is an established therapy for severe heart failure, but remains plagued with several issues, including a limited number of donor organs, chronic immunosuppressant therapy, and the subsequent development of graft vasculopathy. Thus, advances in cardiac regenerative therapy would provide an avenue to overcome these hindrances.

Currently, there are several potential strategies for heart regeneration. One such approach is to generate new cardiomyocytes from endogenous cell sources in situ. This includes differentiation of resident cardiac progenitor cells into cardiomyocytes and renewal of pre-existing adult cardiomyocytes in damaged hearts. Alternatively, new cardiomyocytes could be generated from fibroblasts by direct reprogramming without reverting a stem cell state by overexpressing lineage-specific factors. Indeed, direct reprogramming has proven sufficient in yielding a diverse range of cell types from fibroblasts, including neurons, cardiomyocytes, endothelial cells, hematopoietic stem/progenitor cells, and hepatocytes. These studies revealed that somatic cells are more plastic than anticipated, and that transcription factors, microRNAs, epigenetic factors, secreted molecules, as well as the cellular microenvironment are all important for cell fate specification. With respect to the field of cardiology, the cardiac reprogramming presents as a novel method to regenerate damaged myocardium by directly converting endogenous cardiac fibroblasts into induced cardiomyocyte-like cells in situ. The first in vivo cardiac reprogramming reports were promising to repair infarcted hearts; however, the low induction efficiency of fully reprogrammed, functional induced cardiomyocyte-like cells has become a major challenge and hampered our understanding of the reprogramming process. Nevertheless, recent studies have identified several critical factors that may affect the efficiency and quality of cardiac induction and have provided new insights into the mechanisms of cardiac reprogramming. Here, we review the progress in direct reprogramming research and discuss the perspectives and challenges of this nascent technology in basic biology and clinical applications.
also be supplied from exogenous sources by cell transplantation, in which embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) might be most promising candidates to remuscularize failing hearts.6–10 However, several issues remain to be resolved before using iPSC-derived cardiomyocytes in the clinic, such as the heterogeneous and relatively immature phenotype of cardiomyocytes generated from stem cells, the possibility of teratoma formation by iPSC contamination, and the poor survival of transplanted cells in the injured heart.11,12

The discovery of iPSCs in 2006 by Takahashi and Yamanaka13–15 inspired a new approach that generates specific cell types without needing to transition through a stem cell state by introducing combinations of lineage-specific factors, called direct reprogramming. Recent studies demonstrate that direct reprogramming has the potential to yield a diverse range of cell types from fibroblasts, including neurons, cardiomyocytes, endothelial cells, hematopoietic stem/progenitor cells, and hepatocytes (Figure 1).16–22 These findings indicate that cell fate plasticity is much wider than previously anticipated, and that direct reprogramming may offer a new system to study the mechanisms underlying cell fate decisions during development, which is currently a major focus of investigation in basic biology.

We found that a cocktail of 3 cardiac-specific transcription factors, Gata4, Mef2c, and Tbx5 (GMT), was the minimum needed to reprogram mouse cardiac fibroblasts (CFs) into induced cardiomyocyte-like cells (iCMs) directly in vitro.21 Subsequently, Srivastava and Olson laboratories independently reported that endogenous CFs could be directly reprogrammed into iCMs to improve the cardiac function of infarcted mouse hearts by gene delivery of GMT with or without Hand2.23,24 Later reports also demonstrated that human fibroblasts could be also reprogrammed into iCMs with various combinations of cardiac transcription factors in the presence or absence of microRNAs (miRNAs), albeit at a lower efficiency compared with that observed in mice.25–27 These results suggest that direct reprogramming technology may hold immense potential for heart regeneration through the generation of new myocytes from fibroblasts and improve cardiac function after myocardial injury. However, the reprogramming efficiency of fully reprogrammed iCMs was low, and reproducibility of the cardiac induction with this nascent technology has been controversial.28–30 Moreover, the mechanisms of cardiac reprogramming and the properties of newly generated iCMs were obscure. Since our discovery of cardiac reprogramming, multiple groups have improved the iCM reprogramming efficiency, which could help answer to some of these important questions. Furthermore, lessons from direct reprogramming studies in other fields may facilitate our understanding of the cardiac reprogramming process. Here, we first briefly review the progress in other direct reprogramming fields, and then discuss the recent advances and challenges of cardiac reprogramming for basic biology and its future in clinical applications.

Reprogramming Into Pluripotency by Oct4, Sox2, Klf4, and c-Myc

Although the prototype of direct reprogramming or transdifferentiation was described many years ago by the demonstration of MyoD, a master gene of skeletal myocyte,30 the field of direct reprogramming started after the epoch making discovery of iPSCs. Two pioneering studies mark the advent of this developing field. Gurdon31,32 first demonstrated pluripotent reprogramming by somatic cell nuclear transfer in the 1960s, and recently Takahashi and Yamanaka15 achieved a breakthrough in this field by overexpressing 4 ESC-enriched transcription factors, Oct4, Sox2, Klf4, and c-Myc (also known as the Yamanaka factors), in mouse fibroblasts to induce a pluripotent state. Many laboratories including Yamanaka group have since improved iPSC generation techniques to show that mouse iPSCs share all defining features with bona fide mouse ESCs, including the expression of pluripotency markers, DNA methylation patterns, and the ability to generate chimeric mice and all-iPSC mice, the latter of which is the most stringent criteria for pluripotent stem cells.33–40 Subsequently, iPSCs have been successfully derived from several different species, including humans, rats, and rhesus monkeys, either by expression of the 4 Yamanaka factors or combinations of other factors.41–46 Similarly, iPSCs have been derived from other differentiated cell populations, such as keratinocytes, neural cells, stomach cells, liver cells, and terminally differentiated lymphocytes, demonstrating the universality of induced pluripotency.47–50 Although the initial induction efficiency of mouse iPSCs and human iPSCs under the original conditions was 0.1% and 0.01%, respectively, this has been greatly improved with the modification of transcription factors, miRNAs, small molecules, and epigenetic modifiers.52–56 One study even claimed 100% efficiency of reprogramming in mouse and human, which they designated deterministic reprogramming.56 Recently, extensive efforts have been directed toward safe cell therapies using iPSCs, which include the appropriate selection of safe and high-quality iPSC lines, generation of integration-free iPSCs with episomal vectors, Sendai viruses, and chemical compounds, and efficient differentiation and purification methods of differentiated cells from iPSCs.57–59 First-in-man clinical trial using iPSC-derived cells was undertaken by Takahashi and colleagues57 in 2014, in which the autologous iPSC-derived retinal pigment epithelium cell sheets were
transplanted in the patient with exudative age-related macular degeneration. Thus, the progress of iPSC field has grown enormously in past years, although this will not be discussed in detail because of the space limitation.

Direct Reprogramming Into Pancreatic β-Cells, Neural Cells, and Hepatocytes

Taking the concept of pluripotent reprogramming with the Yamanaka factors, we, along with others, demonstrated that combinations of several lineage-specific factors could convert mature cell types directly into a different cell type without needing to first become a stem cell. Zhou et al\textsuperscript{22} showed that adenoviral gene transfer of the transcription factors, Neurogenin 3, Pdx1, and Mafa, could efficiently reprogram pancreatic exocrine cells into functional β-cells in mouse pancreas and ameliorated hyperglycemia in diabetic mice by secreting insulin from newly generated β-cells. This study provided the first evidence of in vivo reprogramming by defined factors. More recently, Baeyens et al\textsuperscript{58} reported that just transient administration of 2 growth factors, epidermal growth factor and ciliary neurotrophic factor, to adult mice efficiently converted terminally differentiated acinar cells to functional β-cells in vivo and reinstated normal glycemic control in chronic hyperglycemia mouse without genetic manipulation. Thus, the generation of pancreatic β-cells by in vivo direct reprogramming may represent a new therapeutic strategy for diabetes mellitus.

Neural reprogramming is one of the most advanced research fields in direct reprogramming. Vierbuchen et al\textsuperscript{20} has shown that mouse dermal fibroblasts (mesodermal cells) can be converted into functional excitatory neurons (ectodermal cells) in vitro using combination of the neuronal lineage-specific transcription factors, Brn2, Ascl1, and Myt1l. These induced neuronal (iN) cells express multiple neuron-specific proteins, generate action potentials, and can form functional synapses, suggesting that direct reprogramming is possible even between different lineages or germ layers. Mechanistically, Ascl1 acts as a pioneer factor by immediately occupying most cognate genomic sites in fibroblasts to open the chromatin structure and allow the recruitment of Brn2 and Myt1l to target sites genome wide.\textsuperscript{59,60} Subsequently, several groups reported on neural reprogramming in human cells using mouse reprogramming factors in combination with other transcription factors and miRNAs.\textsuperscript{61,62} The induction efficiency of human iN cells with Brn2, Ascl1, and Myt1l plus NeuroD1 in the original conditions (2%–4%) was ≈10-fold lower than that
in mouse (20%), while a recent study demonstrated that small molecule–based inhibition of glycogen synthase kinase-3 β and Smad signaling in combination with the transcription factors could convert postnatal human fibroblasts into functional iPSCs with yields up to >200% and neuronal purities up to >80%.62,63 Induction of clinically relevant subtypes of iPSCs has been succeeded with the addition of subtype-specific transcription factors to the neural reprogramming factors. Four factors (Lhx3, Hb9, Is1, and Ngn2) in combination with the Bnn2, Ascl1, and Myt1f factors generated motor neuronal cells from mouse embryonic fibroblasts,64 and Ascl1, Nurr1, and Lmx1a could generate functional dopaminergic neurons from mouse and human fibroblasts, as well as from patients with Parkinson disease.65–67 Ring et al68 reported on the generation of induced neural stem cells from mouse and human fibroblasts through the overexpression of a single factor, Sox2.69 Thus, steady progress has been made in the neural reprogramming field during the past years.

Two groups independently demonstrated that mouse fibroblasts can be directly reprogrammed to functional induced hepatocyte-like (iHep) cells by defined factors.17,19,60–62 Sekiya and Suzuki19 demonstrated the direct induction of functional iHep cells from mouse fibroblasts by transduction of Hnf4α and Foxa1. These iHep cells showed typical epithelial morphology, expressed hepatic genes, and acquired hepatocyte functions. However, using CellNet analyses, Morris et al70 recently demonstrated that the induced cells generated with the method reported by Sekiya and Suzuki were endoderm progenitors rather than differentiated hepatocytes. They found that the induced cells have the capacity to differentiate into either hepatocyte-like cells or intestine-like cells in vitro, depending on the expression levels of Hnf4α and Foxa1 and the combination with other transcription factors. Intriguingly, transplantation of the induced cells into liver and colon led to differentiation of the cells into mature hepatocytes and colonic epithelium, respectively, suggesting that the microenvironment is critical for cell fate determination. The human iHep cells were directly generated with a combination of hepatic fate conversion factors, Hnf1α, Hnf4A, and Hnf6, together with the hepatic maturation factors, Atf5, Prox1, and Cebpa.63,65 The resultant human iHep cells were functionally mature hepatocytes with drug metabolic function, which is difficult to obtain from human PSCs. Thus, ectopic expression of both cell fate determination and functional maturation factors could generate various types of mature cells.

### Direct Reprogramming of Mouse Fibroblasts Into Cardiomyocytes by GMT

Although embryonic mesoderm can be induced to generate cardiomyocytes, no master regulator of cardiac differentiation has been identified.60,77,78 We hypothesized that a combination of key developmental cardiac transcription factors is required to directly convert fibroblasts into cardiomyocytes. In an attempt to identify cardiac reprogramming factors, we first developed a screening system in which the induction of cardiac genes could be analyzed quantitatively by reporter-based fluorescence-activated cell sorting. We generated α-myosin heavy chain promoter–driven enhanced green fluorescent protein (α-MHC-GFP) transgenic mice, in which cells with activated cardiac programs express GFP. After retroviral expression of 14 candidate genes in neonatal CFs, we detected cells with cardiac morphologies and expression of cardiac markers, suggesting that such a conversion may indeed be possible. Serial reduction of individual factors revealed that the 3 cardiac transcription factors, GMT, were minimally required to induce iCMs.21 Transduction with GMT in CFs activates a cardiac reporter, α-MHC-GFP, in ∼15% to 20% of the cells, and cardiac protein expression in 5% of the fibroblasts after 1 week; however, few cells became spontaneously beating iCMs after 4 weeks (only 0.01%–0.1% of the starting fibroblasts), suggesting that the vast majority of the α-MHC-GFP-cells were partially reprogrammed iCMs (Figure 2). The efficiency of generation of beating iCMs by GMT was similar to that in iPSC generation, with the original conditions. Despite the heterogeneity of the iCM populations, produced as above, global gene expression analyses revealed the expression of a broad range of cardiac genes, with silencing of the fibroblast program in

### Direct Reprogramming Into Hematopoietic Stem/Progenitor and Endothelial Cell Lineages

The hematopoietic stem-like cells capable of multilineage long-term engraftment were successfully generated from committed lymphoid and myeloid progenitors and myeloid effector cells by transient ectopic expression of 6 transcription factors, Runx1t1, Hlf, Lmo2, Prdm5, Pbx1, and Zif37, which are selectively expressed in hematopoietic stem/progenitor cells.17 The successful generation of engraftable hematopoietic stem cells required in vivo bone marrow environment, a supportive niche for hematopoietic stem/progenitor cells, emphasizing the importance of microenvironment for cell fate conversion. Another approach to generate hematopoietic stem/progenitor cells is the induction of hematopoietic progenitors mediated through an endothelial cell intermediate, mimicking the developmental process in embryos in which definitive hematopoiesis emerges via an endothelial-to-hematopoietic transition. Batta et al76 demonstrated that mouse fibroblasts can be efficiently reprogrammed to multilineage hematopoietic progenitors by the ectopic expression of the transcription factors Erg, Gata2, Lmo2, Runx1c, and Scl. Generation of hematopoietic cells was preceded by the appearance of hemogenic endothelium, recapitulating the normal embryonic program of blood development. These results suggest that a simple combination of transcription factors is sufficient to induce a complex, dynamic, and multistep developmental program in vitro and that direct reprogramming may provide a platform to dissect the molecular mechanisms of cell fate determination during development.

Han et al15 recently reported that adult mouse fibroblasts could be directly reprogrammed into functional differentiated endothelial cells, using a cocktail of 5 transcription factors, Foxo1, Er71, Kh2, Tal1, and Lmo2. The newly generated induced endothelial cells functionally behaved like mature endothelial cells in that they released nitric oxide on stimulation with acetylcholine or vascular endothelial growth factor. Transplantation of induced endothelial cells enhanced angiogenesis and limb perfusion in a murine model of hindlimb ischemia, demonstrating the therapeutic potential for regenerative purposes.
fluorescence-activated cell sorting–purified α-MHC-GFP+ iCMs. The histone and DNA epigenetic marks in several cardiac gene promoters in the iCMs were similar to those in neonatal cardiomyocytes but were different from those in the original CFs. GMT transduction also induced cardiac gene expression and spontaneous intracellular calcium transients in tail-tip fibroblasts (TTFs), but the TTF-derived iCMs did not beat spontaneously. The lineage-tracing experiments using Isl1-Cre-yellow fluorescent protein and Mesp1-Cre-yellow fluorescent protein mice demonstrated that neither Isl1 nor Mesp1 gene was activated during cardiac reprogramming, suggesting that fibroblasts were converted directly into cardiomyocytes without passing through a progenitor cell state. Our study was the first to represent the global gene expression profiles and epigenetic status of directly induced cells and demonstrates the route of reprogramming unambiguously using several reporter mice.

Although the achievement of direct cardiac reprogramming is exciting in the cardiac research, the induction efficiency of iCMs remained variable depending on the protocol. Chen et al28 reported that the lentiviral expression of GMT did not efficiently induce cardiac reprogramming in adult mouse CFs and TTFs. The GMT-transduced fibroblasts expressed several cardiac genes and exhibited voltage-dependent calcium currents, but spontaneous action potentials and cellular contractions were not observed, suggesting the cells were in incomplete reprogramming. The culture conditions for fibroblasts and iCMs, the starting cell types used for reprogramming, and titers of viruses expressing the reprogramming factors may be critical for successful cardiac reprogramming, as they are for the induction of iPSCs.79–81 The expression of GMT by lentiviruses might be different from that by retroviruses, and the starting fibroblasts used in our study were mainly neonatal CFs, while they used adult CFs and TTFs, which were more resistant to reprogramming.29

The low and variable reprogramming efficiency by GMT may be because of the stoichiometry of reprogramming factors in the cells transduced with separate vectors.80,82 To address this, Wang et al83 recently generated all possible polycistronic vectors of GMT and demonstrated that the stoichiometry of reprogramming factors is indeed critical for cardiac reprogramming. They found that the 2 polycistronic vectors, MGT and MTG, which expressed a high level of Mef2c and low levels of Gata4 and Tbx5, enhanced cardiac reprogramming, whereas the other vectors reduced reprogramming efficiency (Figure 2). This need for precise stoichiometry of factors for successful cardiac reprogramming demonstrated that subtle differences in experimental conditions may result in failure of cardiac reprogramming. It is conceivable that the inefficient reprogramming reported by Chen et al may have been caused by higher Gata4 and Tbx5 expression and lower Mef2c expression in their transduced cells. Although the findings by Wang et al provided important insights into the variable reprogramming efficiency reported by different laboratories, they did not compare the cardiac gene expression in the iCMs generated by the polycistronic vectors with those in neonatal cardiomyocytes or adult cardiomyocytes. Further investigation is needed to determine the similarity of the MGT-induced iCMs to bona fide cardiomyocytes.

<table>
<thead>
<tr>
<th>Direct cardiac reprogramming by Gata4, Mef2c, and Tbx5</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time course</td>
<td>0  1-2 weeks  4 weeks</td>
</tr>
<tr>
<td>Efficiency of reprogramming</td>
<td>–  5-20%  0.01-0.1%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Property</th>
<th>Express fibroblast genes</th>
<th>Express αMHC-cTnT and other cardiac genes</th>
<th>Beat spontaneously</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Retroviral expression of Gata4,Mef2c, and Tbx5</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Addition or modification of reprogramming factors</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Lentiviral expression of Gata4, Mef2c, and Tbx5</td>
<td>↓</td>
</tr>
<tr>
<td>2. Stochiometry of Gata4,Mef2c, and Tbx5 (High Mef2c expression)</td>
<td>↑</td>
</tr>
<tr>
<td>3. Hand2*</td>
<td>↑</td>
</tr>
<tr>
<td>4. Fusion of transactivation domain of MyoD to Mef2c</td>
<td>↑</td>
</tr>
<tr>
<td>5. MIR-133 (Sineo1 suppression)</td>
<td>↑ (Silence fibroblast signature)</td>
</tr>
<tr>
<td>6. Nkx2.5*</td>
<td>ND</td>
</tr>
<tr>
<td>7. TGFβ inhibitor*</td>
<td>ND</td>
</tr>
</tbody>
</table>

Figure 2. Modifications of reprogramming factors alter the efficiency and quality of cardiac reprogramming. Time course and efficiency of cardiac induction from mouse fibroblasts by Gata4, Mef2c, and Tbx5, with the original conditions, are shown (upper). Lower, Several interventions could change the yield of partially reprogrammed induced cardiomyocyte-like cells (iCMs) and functional iCMs from mouse fibroblasts. *The studies were conducted in adult cardiac fibroblasts or tail-tip fibroblasts. The functional improvement by addition of Nkx2.5 and transforming growth factor (TGF)-β inhibitor was measured by spontaneous calcium activity in the iCMs. α-MHC indicates α-myosin heavy chain; and cTnT, cardiac troponin T.
Direct Reprogramming Into Cardiomyocytes by Different Factor Combinations

Addition or modifications of transcription factors, miRNAs, and small molecules may promote the cardiac reprogramming efficiency, as shown in the other reprogramming fields (Figure 2). Song et al.83 sought to investigate the optimal combination of the cardiac transcription factors to reprogram adult CFs and TTFs into functional iCMs. They also used α-MHC-GFP reporter mouse and screened 6 cardiac transcription factors (Gata4, Hand2, Mef2c, Tbx5 [GHMT], Mesp1, and Nkx2.5) as candidate factors. Addition of Hand2 to GMT led adult CFs and TTFs to be reprogrammed into functional iCMs more efficiently than did any other combinations, including GMT. GHMT induced ≈9% of starting populations to express both α-MHC-GFP and cardiac troponin T (cTnT) cardiac genes after 1 week, which was higher than those induced with GMT (3%), suggesting that Hand2 promoted early stage of reprogramming process. Notably, after 5 weeks of GHMT transduction into adult CFs and TTFs, the functional properties, including 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 barriers for cardiac reprogramming in TTFs. More recently, Nam et al.84 analyzed the iCM properties induced with GHMT in more detail. Although 10% to 30% of the GHMT-transduced fibroblasts expressed cTnT, the iCMs revealed remarkable degree of sarcomeric structural diversity and only a small portion of the cells (=1% of fibroblasts) formed well-organized sarcomere structure that correlated with spontaneous beating activity (=0.16%). Moreover, they found that GHMT generated all 3 types of immature form of myocytes, atrial, ventricular, and pacemaker cells, at roughly equal proportions, determined by multiplex immunostaining and electrophysiology. Thus, Hand2 promoted cardiac reprogramming at least in part by increasing the cardiac marker-expressing cells at early stage, and the GHMT-iCMs consisted of heterogeneous populations including all 3 cardiac subtypes.

Increasing the transactivation activity of transcription factors may also promote cardiac reprogramming. To address this, Hirai et al.85 fused a powerful transactivation domain of MyoD to GHMT and transduced these genes in various combinations into mouse embryonic fibroblasts. Transduction of the chimeric Mef2c with the wild-type forms of the other 3 genes generated more cardiac protein-expressing cells and beating clusters of iCMs with an efficiency of 3.5%, which was 15-fold greater than the wild-type gene combinations. Intriguingly, transduction of other combinations of chimeric genes reduced the number of beating iCMs, suggesting that increasing the transcriptional activity in only Mef2c is critical for successful cardiac reprogramming, which is consistent with the results by Wang et al.83 using polycistronic vectors.

MiRNAs have numerous targets related to signaling pathways, transcription factors, and epigenetic regulation and play important roles in cell fate specifications and embryonic development. Jayawardena et al.86 introduced a combination of miRNAs (miR-1, miR-133, miR-208, and miR-499) into neonatal CFs to succeed in creating functional iCMs. As miRNAs are not incorporated into host chromosome by transient expression, miRNA-mediated cardiac reprogramming may be safer applications in clinic. This article also revealed that culture conditions are vital to enhance the reprogramming efficiency and quality, as expression of α-MHC-cyan fluorescent protein increased ≈10-fold, and spontaneous cell contractions reached to 1% to 2% of the starting cells by addition of a JAK inhibitor to the culture media.

A new approach to identify optimal combinations of reprogramming factors to generate functional iCMs was proposed by Addis et al.87 They used a reporter system in which the calcium indicator GCAMP was driven by the cTnT promoter for the screening of functional iCMs.88 They found that addition of Nkx2.5 to GHMT resulted in the most efficient combinations for cardiac reprogramming and reported that the fibroblasts were stably reprogrammed into calcium transient+ iCMs after 15-day exposure to GHMT with Nkx2.5 using doxycycline-inducible lentiviral system. More recently, the same group used this reporter system to identify small molecules sufficient to enhance cardiac reprogramming and demonstrated that the transforming growth factor β (TGFβ) inhibitor, SB431542, was capable of increasing the conversion of both mouse embryonic fibroblasts and adult CFs into calcium transient+ iCMs up to 5-fold.89 Inhibition of TGFβ signaling at early in the reprogramming process led to the greatest increase in the conversion of fibroblasts to iCMs.

Although multiple groups promoted cardiac reprogramming by adding transcription factors or small molecules, the molecular mechanisms of direct reprogramming remained undefined. Recently, we demonstrated that addition of miR-133 to GMT promoted cardiac reprogramming via Snai1 repression and silencing the fibroblast gene signature. Cotransduction of miR-133 with GMT increased the number of cells expressing cardiac markers by 9-fold at the early stage of reprogramming, leading to the generation of 7-fold more beating iCMs compared with GMT treatment alone; this treatment also shortened the duration required to induce beating iCMs from 30 to 10 days.80 Mechanistically, miR-133 directly targeted Snai1, a master regulator of epithelial-to-mesenchymal transition, and Snai1 knockdown suppressed fibroblast genes, upregulated cardiac gene expression, and induced more contracting iCMs, recapitulating the effects of miR-133 overexpression. In contrast, overexpression of Snai1 in GMT/miR-133-transduced cells maintained fibroblast signatures and inhibited generation of beating iCMs, suggesting that miR-133–mediated Snai1 repression is critical for cardiac reprogramming. Thus, many laboratories improved the reprogramming efficiency by modifying or adding several factors to GMT,84–86 which in turn may help our understanding of the reprogramming process, as we will discuss later.

Direct Reprogramming Into Cardiomyocytes In Vivo

The most exciting potential of direct cardiac reprogramming lies in the possibility of in vivo use of this new technology, in which a large pool of endogenous CFs could be directly reprogrammed into cardiomyocyte-like cells solely through gene delivery. Moreover, it is conceivable that the in vivo cardiac environment may help the reprogramming process to generate...
high-quality iCMs compared with those in vitro. To test the possibility of direct cardiac reprogramming in vivo, we, along with others, investigated whether the gene transfer of reprogramming factors into infarcted mouse hearts could convert resident CFs into cardiomyocytes in situ.22,24,34,95

Qian et al23 directly injected GMT retrovirus into mouse hearts after coronary ligation and demonstrated in vivo cardiac reprogramming with functional improvement. They used fibroblast lineage-tracing mice (Fsp1-Cre and Periostin-Cre) to demonstrate that iCMs originated from CFs or nonmyocytes and used α-MHC-MerCreMer mice to determine whether the newly generated iCMs arose from cell fusion with pre-existing cardiomyocytes. Using these lineage-tracing approaches, they showed that ≈35% of cardiomyocytes in the border/infarct zone were newly generated iCMs derived from resident CFs rather than cell fusion of fibroblasts with pre-existing cardiomyocytes. Of the in vivo iCMs, 50% had well-organized sarcomeric structures and exhibited functional characteristics of adult ventricular cardiomyocytes, including cellular contraction, electrophysiological properties, and functional coupling to other cardiomyocytes. The hearts also contained intermediate stages of partially reprogrammed iCMs, and time course experiments revealed a progressive reprogramming process in which a more complete cardiac phenotype arose over time. These observations suggested that in vivo reprogramming might yield mature iCMs more efficiently than in vitro conditions. Functional studies with cardiac MRI and echocardiography and histological analyses revealed that retroviral GMT gene transfer significantly improved cardiac function and reduced fibrosis until at least 3 months after myocardial infarction.

Song et al24 also reported that GHMT retroviral injection into mouse infarct hearts converted endogenous CFs into functional iCMs in situ and improved cardiac function after myocardial infarction. They used Fsp1-Cre and inducible Tcf21-iCre mice as fibroblast lineage-tracing mice and found that 2% to 6% of cardiomyocytes in the border/infarct area were newly generated iCMs with clear striations and functional properties similar to endogenous ventricular cardiomyocytes, as determined by electrophysiology, Ca²⁺ transients, and cell contractility. The discrepancy of cardiac induction rate between 2 studies may be because of the difference of reprogramming factors and lineage-tracing mice used in their studies. Song et al also demonstrated that the ejection fraction was increased by 2-fold in GHMT-treated mice compared with controls, and that the scar size was reduced by 50% at 12 weeks after myocardial infarction.

We demonstrated that GMT gene transfer could generate new iCMs in vivo; however, our approach to test the in vivo cardiac reprogramming differed from those used by the Srivastava and Olson groups.44 They used mainly fibroblast lineage-tracing mice to demonstrate in vivo cardiac reprogramming, whereas we took an alternative approach of using cotransduction of retroviral GMT and reporter gene to determine cardiac induction from nonmyocytes. We first confirmed that retroviruses infected only proliferating cells, which are mainly CFs in an infarcted myocardium. In addition, different from that reported in the previous 2 articles, we used immunosuppressed mice to promote the survival of virally transduced cells. We found that separate GMT gene transfer induced mostly immature α-actinin⁺ cells in vivo, with a conversion rate of ≈1% in the transduced cells. To improve the transduction of the 3 factors, we then generated a polycistronic retrovirus expressing GMT from the same promoter using 2A peptides.94,96 Gene transfer of this polycistronic GMT retrovirus induced morphologically more mature iCMs in fibroblastic tissues than those generated by injecting the separate GMT vectors, suggesting that polycistronic systems could be valuable tools for in vivo reprogramming.94,97

Mathison et al95 demonstrated that intramyocardial administration of vascular endothelial growth factors into the infarcted myocardium 3 weeks before GMT gene delivery enhanced functional recovery and reduced scar size in rat hearts after myocardial infarction compared with that observed with the use of GMT or vascular endothelial growth factor alone. These beneficial effects of vascular endothelial growth factor might be because of neovascularization and some other mechanisms, including better survival of the newly generated iCMs in the infarcted hearts.

Jayawardena et al98 demonstrated that direct administration of a combination of lentiviral miR-1, miR-133, miR-208, and miR-499 into mouse infarcted hearts converted resident CFs into iCMs and improved cardiac function after myocardial infarction. They used Fsp1-Cre mice for lineage tracing of nonmyocytes and found that 12% of cardiomyocytes in the border/infarct area were newly generated iCMs. The iCMs generated by in vivo reprogramming expressed cardiac myocyte markers, sarcomeric organization, excitation–contraction coupling, and action potentials characteristic of mature ventricular cardiac myocytes. Serial echocardiography revealed that there was a time-delayed and progressive improvement in ventricular function, beginning 1 to 2 months post surgery and was enhanced at 3 months, which was similar to the period reported for reprogramming with transcription factors. For clinical applications, the development of a nonviral delivery method, including chemically synthesized miRNA mimics, may be an attractive therapeutic option, as they do not integrate into the host chromosomes. Together, these results suggest that the abundant pool of endogenous CFs could be a cell source for new cardiomyocytes by direct reprogramming and that this new technology can improve cardiac function and reduce scar size after myocardial infarction. Moreover, the in vivo iCMs are more mature than those in vitro, suggesting that the in vivo environment may improve the quality of cardiac reprogramming.

**Direct Cardiac Reprogramming in Human Fibroblasts**

To advance the direct cardiac reprogramming technology toward clinical applications, it would be necessary to translate the mouse system into human. Nam et al25 found that GHMT reprogramming factors in mouse fibroblasts were ineffective in activating cardiac gene expression in human fibroblasts, and that the additional factor, Myocd, was required for human cardiac gene expression. Furthermore, adding 2 muscle-specific miRNAs, miR-1, and miR-133, further improved the
myocardial conversion of human fibroblasts and eliminated the requirement for Mef2c in cardiac induction from human neonatal foreskin fibroblasts, adult CFs, and adult dermal fibroblasts. The induction efficiency of cTnT-expressing cells from fibroblasts was 10% to 20%, but only a small subset of the cells derived from adult CFs exhibited spontaneous contractility after 11 weeks of culture, suggesting most human iCMs were in a partially reprogrammed state.

We also found that GMT was not sufficient for cardiac reprogramming in human fibroblasts. Thus, we screened 11 additional factors in combination with GMT and found that addition of Mesp1 and Myocd upregulated a broader spectrum of cardiac genes more efficiently in human CFs compared with that observed with GMT alone. Myocd upregulated sarcomeric genes and Mesp1 increased intracellular Ca\(^{2+}\) oscillations, suggesting Myocd and Mesp1 differentially regulate cardiac gene expression. The human CFs and dermal fibroblasts transduced with GMT, Mesp1, and Myocd changed the cell morphology from spindle to rod-like or polygonal shape, expressed multiple cardiac-specific proteins, increased a broad range of cardiac genes and concomitantly suppressed fibroblast genes, and exhibited spontaneous Ca\(^{2+}\) oscillations. Although the human iCMs did not beat spontaneously, the cells matured to exhibit action potentials and contract synchronously in coculture with murine cardiomyocytes. The EdU (5-ethynyl-2-deoxyuridine) assay revealed that the iCMs generated did not pass through a mitotic cell state, suggesting GMT, Mesp1, and Myocd directly reprogram human fibroblasts toward a cardiac fate. More recently, we also found that addition of miR-133 to GMT, Mesp1, and Myocd promoted cardiac reprogramming in human fibroblasts.

The induction of cardiac markers, α-actinin and cTnT, was increased from 2% to 8% to 23% to 27% by addition of miR-133. Mechanistically, miR-133-mediated Sna1 repression was also critical for human cardiac reprogramming, which is in accordance with the results in mouse data.

Fu et al. reported that GMT plus Esrrg, Mesp1, Myocd, and Zfp2 induced global cardiac gene expression and phenotypic shifts to a cardiac state in human fibroblasts derived from ESCs, fetal heart, and neonatal skin. Although most cells were partially reprogrammed, a subset of human iCMs had sarcomere formation, calcium transients, and action potentials. They demonstrated that the epigenetic status of human ESC resembled that of hESC-derived cardiomyocytes in DNA and histone methylation status, and that the iCMs were stably reprogrammed to a cardiac state without the need for continuous expression of reprogramming factors. Furthermore, they found that TGF \(\beta\) signaling was important for cardiac reprogramming and improved the efficiency of human iCM generation.

Islas et al. reported that transient expression of Ets2 and Mesp1, followed by activin A and bone morphogenetic protein 2 treatment, could reprogram human dermal fibroblasts into cardiac progenitor–like cells. The induced cardiac progenitor–like cells expressed several cardiac progenitor markers and differentiated into cardiomyocyte–like cells that expressed cardiac genes, sarcomeric structures, and Ca\(^{2+}\) activities in a prolonged culture. Although these findings of human cardiac reprogramming represent an important step toward potential clinical applications, the process was slower and less efficient than mouse reprogramming.

### Partial Reprogramming With Transient Expression of Pluripotent Factors

Direct reprogramming of somatic cells to a pluripotent state using Yamanaka factors entails extensive genetic and epigenetic resetting. Thus, it is conceivable that Yamanaka factors function to erase cell identity by epigenetic mechanisms and that subsequent exposure to external lineage-specific signals may direct lineage specification and terminal differentiation. Efe et al. showed that transient overexpression of Oct4, Sox2, Klf4, and c-Myc and subsequent exposure to the cardiogenic media, including bone morphogenetic protein 4 and JAK inhibitor, converted mouse fibroblasts into spontaneously contracting cardiomyocytes, via a cardiac progenitor cell state with no pluripotent intermediate. More recently, the same group reported that only a single transcription factor, Oct4, in combination with the small molecule cocktails, consisting of SB431542 (ALK4/5/7 inhibitor), CHIR99021 (GSK3 inhibitor), parnate (LSD1/KDM1 inhibitor), and forskolin (adenylyl cyclase activator), enabled cardiac conversion of mouse fibroblasts without traversing the pluripotent state. A similar approach was also applied to generate neural progenitor cells and blood progenitor cells from mouse fibroblasts.

Kim et al. demonstrated that the transient expression of the Yamanaka factors and subsequent exposure to the cell culture conditions favorable for neural precursor cells induced neural progenitor cells from fibroblasts without reverting to iPSCs. To apply the partial reprogramming approach toward heart regeneration, it should be determined whether the cardiomyocyte foci do not form teratoma and functionally integrate into the host myocardium after transplantation, and the same approach could be applied in human cells.

### Direct Cardiac Reprogramming: Challenges and Future Directions

Although the recent progress in cardiac reprogramming is promising, the low reprogramming efficiency of fibroblasts into iCMs in vitro is a major challenge. We first reported that just 3 transcription factors rapidly and efficiently induced fibroblasts to a cardiac fate (activated cardiac genes and suppressed fibroblast genes); however, the reprogramming to generate beating iCMs in vitro was inefficient and slow. Since then, many researchers have demonstrated that modifications of reprogramming factors and culture conditions could improve cardiac induction (Figure 2). Addition of Hand2 to GMT promoted cardiac reprogramming at least in part by increasing the number of cells expressing cardiac markers and proteins at the early stage. Further investigations using a doxycycline-inducible system for transient expression of Hand2 may clarify the role of Hand2 at the late stage of reprogramming. MiR-133 promoted cardiac induction by Sna1 suppression and silencing the fibroblast signature at the early stage. Given that TGF\(\beta\) is an activator of Sna1, it is conceivable that TGF\(\beta\) inhibitor-mediated cardiac reprogramming in mouse fibroblasts, reported by Ifkovits et al., might be partly mediated through suppression of Sna1 and silencing fibroblast signatures. Intriguingly, this process is similar...
to the mesenchymal-to-epithelial transition process mediated through Snail suppression in iPSC generation. In contrast, Fu et al reported that TGFβ signaling promoted human cardiac reprogramming, suggesting that the TGFβ/Snail pathway may affect cardiac reprogramming paradoxically depending on the species and cell lines, as seen in iPSC generation. High transcriotional activity of Mef2c and stoichiometry of transcription factors (high expression of Mef2c and low expression of Gata4 and Tbx5) increased cardiac induction. Although the precise mechanisms remain unclear, this is consistent with the view that precise dosage of transcription factors is critical for proper development and cell fate specification. Addition of Nkx2.5 to GMT or GHMT increased the generation of functional iCMs, determined by the spontaneous calcium activity. In contrast, we, along with others, previously reported that Nkx2.5 reduced the number of cardiac marker–expressing cells, such as α-MHC-GFP, suggesting that Nkx2.5 may increase the proportion of functional iCMs without increasing the partially reprogrammed iCMs. However, it should be determined whether addition of Nkx2.5 indeed increased the number of beating iCMs, which is the more stringent criterion for functional iCMs. Thus, we, along with others, have identified several important molecules that enhance cardiac induction at the early stage of reprogramming; however, the factors critical for the transition of partially reprogrammed iCMs into spontaneously beating iCMs at the late stage remain unclear. As the reprogramming efficiency at early stage may not always correlate with functional reprogramming at late stage, the measurement of cardiac functional outcomes, such as the efficiency of beating iCMs, will identify such molecules. Standardized conditions for efficient and reproducible cardiac induction are needed for the screening, and factors identified in the mouse system might be applied to in vivo reprogramming and human cardiac reprogramming.

Recently, the Daley and Collins groups developed a new biological platform, CellNet, which can compare gene regulatory networks in engineered cell populations with those in vivo counterparts and identify factors that may improve cellular reprogramming. Cahan et al analyzed the genome-wide transcriptional profiles of α-MHC-GFP iCMs generated with GMT, several cell types, and ESC–derived cardiomyocytes using CellNet. They found that the α-MHC-GFP population was exclusively classified as heart, but not other cell types including skeletal muscle and fibroblasts, confirming a global shift in the gene expression profile toward a cardiac fate. They also demonstrated that the α-MHC-GFP cells were less similar to neonatal cardiomyocytes than ESC–derived cardiomyocytes, but this is not surprising, given that the majority of the cells were nonbeating, partially reprogrammed iCMs (Figure 2). Some heart-related genes, including Gata4 and Tbx20, were incompletely activated in the α-MHC-GFP population compared with cardiomyocytes, and addition of these factors might improve cardiac reprogramming. Cahan et al also demonstrated that the in vivo iCMs were similar to bona fide cardiomyocytes and established the heart gene regulatory network more completely than ESC–derived cardiomyocytes. Development of CellNet for single-cell analyses will likely lead to demonstration of the variability of reprogramming among individual iCMs generated with different stoichiometries of reprogramming factors and also identify new targets that enhance the quality of cardiac reprogramming.

As cardiomyocytes consist of several types of myocytes, including sinoatrial nodal cells, atrial myocytes, and ventricular myocytes, it would be important to understand the mechanism of cardiac subtype specification and navigate the cardiac reprogramming toward the desired mature cell types. The beating phenotype in some in vitro iCMs seems to be nonrhythmic, unlike that in postnatal cardiomyocytes or ESC–derived cardiomyocytes that is rhythmic and uniform across the entire cell. Given that murine cardiomyocytes contract irregularly at the early embryonic stages (embryonic days 9.5–11.5), this phenotype may be because of the generation of immature iCMs or partially reprogrammed iCMs, in which the expression of some ion channel genes is not sufficiently high. The detailed characterization of iCM functions, such as contractile force generation and ion channel expression, will reveal the properties and maturation level of iCMs. We demonstrated that iCMs generated in vitro with GMT or GMT/miR-133 were mostly atrial-type myocytes, whereas GHMT induced all 3 types of myocytes in nearly equal proportions. It would be intriguing to determine the phenotypes of myocytes induced with various reprogramming factors, which may reveal key factors for cardiac subtype specification during development. Kapoor et al reported that overexpression of Tbx18, a gene critical for sinoatrial node specification, could convert murine ventricular cardiomyocytes to pacemaker cells in vitro and in vivo. Similarly, forced expression of Tbx3 or activation of Notch signaling in working cardiomyocytes was sufficient to generate conduction cells in vitro and in vivo. Given that GHMT generate all 3 types of immature form of cardiomyocytes from fibroblasts, it is conceivable that addition of these transcription factors or molecules to GHMT may convert fibroblasts directly into the desired cardiac cell types, which has been demonstrated in the iN and iHep cell reprogramming.

In contrast to the reprogramming in vitro, the in vivo cardiac reprogramming could generate more mature iCMs and repair infarcted myocardium. Injection of the reprogramming factors directly into the damaged heart may convert the endogenous CF population, which represents >50% of all cardiac cells, into new functional iCMs. This in vivo reprogramming approach may have several advantages over cell transplantation–based therapy. First, the process is simple; second, avoiding the induction of pluripotent cells before cardiac differentiation would greatly lower the risk of tumor formation; and third, direct injection of defined factors obviates the need for cell transplantation, for which long-term cell survival remains challenging. However, many questions and hurdles remain to be overcome to realize the success of this new technology. First, it is not clear how many iCMs would remain in the infarct hearts over a longer term, and to what extent the iCMs would integrate with the native cardiomyocytes and contribute to the observed improvement in cardiac function. Although it is challenging to count the total number of iCMs generated in vivo, it will be important to expect the number of newly generated cardiomyocytes and anticipate its effect on overall cardiac function. Given that the cardiac reprogramming efficiency is
relatively low, the increased ejection fraction and reduced fibrosis after injection of the reprogramming factors into mouse hearts seem to be greater than expected. Some other mechanisms, such as alteration of fibroblast profile, promotion of angiogenesis, and prevention of apoptosis in cardiomyocytes by paracrine factors, may contribute to increased cardiac function. Understanding the mechanism of functional improvement by in vivo reprogramming may identify new targets for cardiac repair. Second, it is possible that the immature iCMs generated in situ may have a propensity to trigger arrhythmias in the damaged heart. There is also a risk of random genomic integration of virally overexpressed transgenes with the current protocols, which may result in adverse effects. Therefore, improving the quality of cardiac reprogramming, optimizing gene delivery systems, and development of nonintegrating vectors or small molecules to replace some or all transcription factors are required to advance this strategy toward future regenerative therapies. Third, the low efficiency of human cardiac reprogramming may raise concerns on whether this approach will be feasible in the near future. Feedback from mouse reprogramming and further modifications of factors and culture conditions will enhance the human cardiac reprogramming. Given that in vivo reprogramming demonstrated functional benefits and higher reprogramming efficiency compared with in vitro reprogramming in mice, it is conceivable that the delivery of human cardiac reprogramming factors into damaged heart may repair cardiac tissues and improve cardiac function. Experiments in larger animals in vitro and in vivo will assess the safety and efficacy of the direct reprogramming approach. Moreover, all in vivo studies thus far were performed in the acute stage of myocardial infarction, and it remains to be determined whether in vivo reprogramming could be applied to chronic heart failure models, in which regenerative medicine is in high demand. Last, it would be intriguing to investigate the molecular mechanisms underlying cardiac reprogramming and cell fate determination. Why are cardiac reprogramming factors different in mice and humans, in contrast to the Yamanaka factors in iPSCs? The genetic and epigenetic networks and time periods required for development differ among species, which may affect the reprogramming efficiency and factors needed for reprogramming. It is conceivable that the iPSC paradigm might be an exception rather than the rule in cell fate conversion, considering that the reprogramming factors in mouse and human are also different in other direct reprogramming fields. Identification of the mechanisms that inhibit human reprogramming by transcriptomic as well as epigenetic analyses will enhance our understanding of the gene regulatory networks during development and advance this new technology toward clinical applications.

Conclusions

Although still in a nascent stage, direct cardiac reprogramming has undergone great advances and attracted considerable attention. From a basic biology standpoint, it may become a new methodology in the developmental and molecular biology. It offers a new way to understand the transcription factor
function and to study the complex interplay among transcriptional regulators, miRNAs, and epigenetic regulators. Moreover, the generation of iCMs represents a novel way to study the mechanisms of cell fate decisions during cardiac development and postmitotic cardiac maturation. From a clinical point of view, direct cardiac reprogramming holds great potential for cardiovascular disease research and treatment, including patient-specific drug screening, cardiac disease modeling, and regenerative purposes. When comparing the ability to generate ex vivo cardiomyocytes through the differentiation of iPSCs versus direct reprogramming, it is clear that the former strategy is far more advanced at this stage. Expandability is obviously a major advantage of iPSCs over postmitotic iCMs, and efficiency of cardiac induction, particularly in humans, is still low in direct reprogramming. Nevertheless, direct reprogramming in vivo possesses several theoretical advantages, which may resolve many of the challenges and issues associated with cell therapies. As the retroviral delivery of reprogramming factors will be a concern in clinical applications, new methods to generate integration-free iCMs would be required. Further studies, including optimization of cardiac reprogramming in human fibroblasts and demonstration of the therapeutic efficacy and safety of in vivo reprogramming approach in chronic heart failure and larger animal models, are needed. As the demand is high for new regenerative therapies, the opportunities and the potential benefits of this direct cardiac reprogramming approach are significant.

Acknowledgments

We are grateful to members of the Ieda laboratory for valuable discussion and contributions.

Sources of Funding

M. Ieda was supported by research grants from Japan Science and Technology Agency (JST) CREST, Japan Society for the Promotion of Science (JSPS), Banyu Life Science, The Uehara Memorial Foundation, Takeda Science Foundation, and SENSHH Medical Research Foundation.

Disclosures

S. Yamanaka is a scientific advisor of IPS Academia Japan without salary. The other authors report no conflicts.

References


Pfisterer U, Kirkeby A, Torper O, Wood J, Neland J, Dufour A, Björklund A, Lindvall O, Jakobsson J, Palmisano M. Direct conversion of...


90. Cirillo LA, Zaret KS. An early developmental transcription factor complex that is more stable on nucleosome core particles than on free DNA. Mol Cell. 1999;4:961–969.


Direct Cardiac Reprogramming: Progress and Challenges in Basic Biology and Clinical Applications
Taketaro Sadahiro, Shinya Yamanaka and Masaki Ieda

Circ Res. 2015;116:1378-1391
doi: 10.1161/CIRCRESAHA.116.305374

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/116/8/1378

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/