Strategies for Heart Regeneration
Approaches Ranging from Induced Pluripotent Stem Cells to Direct Cardiac Reprogramming

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SUMMARY

Cardiovascular disease remains a leading cause of death for which current therapeutic regimens are limited. Following myocardial injury, endogenous cardiac fibroblasts, which account for more than half of the cells in the heart, proliferate and synthesize extracellular matrix, leading to fibrosis and heart failure. As terminally differentiated cardiomyocytes have little regenerative capacity following injury, development of cardiac regenerative therapy is highly desired. Embryonic stem (ES) and induced pluripotent stem (iPS) cells are promising tools for regenerative medicine; however, these stem cells demonstrate variable cardiac differentiation efficiency and tumorigenicity, which should be solved for clinical applications. Up until the last decade, it was an established theory that cardiomyocytes could only be produced from fibroblasts mediating through stem cells. However, in 2010, we reported for the first time a novel method of the direct reprogramming of fibroblasts into cardiomyocytes, demonstrating various reprogramming pathways exist. This review summarizes the latest trends in stem cell and regenerative research, touching upon iPS cells, partial reprogramming strategy, and direct cardiac reprogramming. Specifically, we examine the many recent advances in both in vitro and in vivo direct cardiac reprogramming, and explore the application of these methods to cardiovascular regenerative medicine. (Int Heart J 2015; 56: 1-5)

Key words: Cardiomyocytes, Transcription factors, microRNAs, Regeneration, Induced cardiomyocytes, Direct reprogramming, iPS cells

Heart disease is a leading cause of death in the developed countries for which current therapeutic regimens are limited. Heart disease, including heart failure, is usually treated with medical therapy, mechanical device implantation, and surgical intervention. When a patient exhibits extremely low cardiac function, a heart transplant is typically required; however, donor shortage is a major problem for heart transplantation (both in Japan and throughout the world). Thus, cardiac regenerative medicine is an attractive alternative therapy to transplantation. For the last two decades, embryonic stem (ES) cells have been used in the field of regenerative medicine, given their self-replication competence and cardiac differentiation ability; however, human ES cells are accompanied by ethical and legal concerns, as well as the threat of immunologic refusal. To solve these problems, Yamanaka and colleagues developed induced pluripotent stem (iPS) cells, which were created by introducing four stem cell-specific transcription factors (Oct3/4, Sox2, c-Myc, and Klf4; OSKM) into human dermal fibroblasts. Prior to our work, it was an established theory that the reprogramming and subsequent differentiation of fibroblasts into cardiomyocytes required an iPS cell intermediate; however, our research introduced the new concept that a direct reprogramming pathway existed for the production of cardiomyocytes from fibroblasts—one that did not involve iPS cells. Here, we summarize current knowledge about cardiac reprogramming, and detail the induction of cardiomyocytes in vivo. Furthermore, we discuss future applications of cardiac reprogramming in regenerative medicine.
Three Pathways to Generate New Cardiomyocytes

Currently, three methods are used to generate cardiomyocytes from fibroblasts (Figure 1): 1) Full reprogramming of fibroblasts into iPS cells and subsequent cardiac differentiation, 2) Partial reprogramming of fibroblasts to cardiac progenitor cells and subsequent differentiation, and 3) Direct reprogramming of fibroblasts into cardiomyocytes.

Cardiomyocytes generated from any of these three pathways can be transplanted into an infarcted or failing heart. The direct reprogramming approach is particularly attractive, as cardiac-reprogramming transcription factors could be introduced directly into a heart, bypassing the need for the engraftation of iCMs. In this section, we review the data on these three cardiac regeneration strategies, and summarize the advantages of each technique. 3)

Full reprogramming of fibroblasts into iPS cells and subsequent cardiac differentiation: Currently, the major strategy to form cardiomyocytes requires the full reprogramming of fibroblasts into iPS cells, and their subsequent differentiation. In other words, this strategy requires the complete initialization to undifferentiated cells (eg, iPS cells) from fibroblasts, and differentiation from iPS cells to cardiomyocytes. 4) Mouse and human iPS cells were established by Takahashi and Yamanaka in 2006 and 2007, respectively. 5) In both instances, iPS cells were made from fibroblasts by transducing these cells with genes encoding four transcription factors (OSKM) using retroviruses. iPS cells have brought about a major revolution in regenerative medicine, 6) and iPS cells having a differentiation ability similar to ES cells can be exposed to cardiac differentiation protocols perfected in ES cells. Following the initial establishment of human iPS cells, functional analyses of iPS cell-derived cardiomyocytes were performed, demonstrating that they are embryonic or immature cardiomyocytes rather than adult type cardiomyocytes. 5,6) Cardiomyocytes derived from human iPS cells were used for a disease modeling, 7) and many laboratories have established various disease models using iPS cells. More recently, Chong, et al reported that directed cardiac differentiation from PSCs, which mimic the developmental signals, generated cardiomyocytes efficiently, and that transplantation of hES cell-derived cardiomyocytes can remuscularize substantial amounts of the infarcted monkey heart, although they induced ventricular arrhythmic complications. 8)

Partial reprogramming of fibroblasts to cardiac progenitor cells and subsequent differentiation: The second strategy to generate cardiomyocytes requires the generation of partially reprogrammed cells, including cardiac progenitor cells, which can be achieved in the process of iPSC generation by exposing fibroblasts to OSKM. These cardiac progenitor cells can then be induced to form cardiomyocytes. Efe, et al reported an equivalent to this partial reprogramming method. 9) These researchers reported the successful differentiation of induced cardiomyocytes in fibroblast cultures exposed to OSKM, which were subsequently treated with cardiomyocyte-inducing conditions. Given that fibroblasts in their method may have been partially reprogrammed into cardiac progenitor cells, it is possible that several cardiomyocytes could be derived from a single fibroblast in this procedure. However, it remains to be determined whether this strategy is applicable in human cells.

Direct reprogramming of fibroblasts into cardiomyocytes: Recently, a third strategy has been described to convert fibroblasts into another cell type by introducing a combination of transcription factors into these cells. In 2010, Vierbuchen, et al succeeded in making neuronal-like or induced neuronal cells by introducing three genes, encoding transcription factors (Ascl1, Brn2, and Myt1) necessary for neuronal differentiation, into mouse fibroblasts. 10) This was the first successful report of the direct reprogramming of fibroblasts into a specific cell type (without an iPS cells) using organ-specific transcription factors.

Following the work of Vierbuchen and colleagues, we reported that mouse neonatal cardiac fibroblasts could be converted into cardiomyocyte-like cells or iCMs through the introduction of genes encoding cardiac-specific transcription factors (Gata4, Mef2c, Tbx5: collectively, GMT). 11) More recently, Suzuki, et al reported the direct reprogramming of hepatocyte-like cells or induced hepatocytes from mouse fibroblasts. 12) Direct reprogramming technology converts terminally differentiated fibroblasts into another organ cell type, and does not require the formation of iPS cells. In time, this strategy may provide a safe and novel alternative to heart transplants. In Figure 2, we summarize the three strategies used to derive cardiomyocytes from fibroblasts.

Direct Cardiac Reprogramming in vitro

Generation of mouse iCMs: It has been four years since we discovered that neonatal cardiac fibroblasts could be reprogrammed directly to form iCMs, without going through an intermediate iPSC cell phase. Since then, multiple laboratories have reported the derivation of iCMs using various methods. We and others used cardiac fibroblasts (CFs), tail tip fibroblasts (TTFs), or mouse embryonic fibroblast (MEFs) derived from reporter mice, which express a fluorescent protein when a cardiac-specific promoter—that of α-myosin heavy chain (αMHC) or cardiac troponin T (cTnT)—is activated, as cell sources for the generation of iCMs. To overexpress reprogramming factors in fibroblasts, the researchers used one of two
techniques: 1) genes encoding cardiac-specific transcription factors (Gata4, Mef2c, Tbx5, Hand2, and Myocd etc.) were introduced into cells by viral vectors (retroviruses, lentiviruses, adenoviruses, etc.); or 2) lipofection method was used to transfect cells with cardiac-specific microRNAs (miRs). The reprogramming efficiency can be quantified by counting the number of cells that expressed cardiac reporter or protein (by flow cytometry or FACS) 1−3 weeks after the introduction of reprogramming factors into fibroblasts. As part of a functional analysis, these cells were further evaluated for spontaneous beating, calcium homeostasis, and action potentials. Based on our epoch-making study, Song, et al were able to produce functional iCMs (identified as cTnT+ cells) from adult CFs and TTFs by adding a gene encoding a fourth transcription factor—Hand2—to GMT.\(^\text{12}\) However, Chen, et al showed the difficulty in generating functional cardiomyocytes through GMT, and emphasized the need to examine the reprogramming mechanisms and epigenetic changes induced with this transcription factor cocktail.\(^\text{13}\)

Protze, et al introduced 120 combinations of factors into mouse embryonic fibroblasts using a pool of 10 transcription factors in an attempt to induce cardiac differentiation, and confirmed cardiomyocyte properties in treated cells through gene expression analyses. As a result, they showed that the 3F-Myocd combination (Mef2c, Tbx5, and Myocd, in which Myocd was substituted for Gata4) might result in more developed cardiomyocytes.\(^\text{14}\)

In addition, Jayawardena, et al introduced only microRNAs (miR-1, miR-133, miR-208, and miR-499) into neonatal cardiac fibroblasts and succeeded in creating iCMs, distinguishing this report from other research. As microRNAs are not incorporated into host chromosomes by transient expression, microRNA-mediated induction may be safer for applications in humans.\(^\text{15}\) This research also suggested that culture conditions are vital to cardiomyocyte induction, as expression of αMHC-CFP increased nearly tenfold when a JAK inhibitor was added to the culture medium.

Addis, et al found adding Nkx2-5 to GMT and Hand2 was beneficial. Using a transgenic calcium fluorescent reporter driven by a cardiomyocyte-specific gene promoter, they demonstrated that infection with GMT, Hand2, and Nkx2-5 (collectively HNGMT) resulted in the most efficient generation of functional cardiomyocytes.\(^\text{16}\) Christoforou, et al determined that overexpression of the MYOC and SRF transcription fac-

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\(^{12}\) Christoforou, et al determined that overexpression of the MYOC and SRF transcription fac-
tors, alone or in conjunction with Mesp1 and SMARCD3, enhanced the basal cardiac-inducing effects of GMT. Through global gene expression analysis, they demonstrated the significantly greater cardiac-inducing effects of Myocd and Srf.\(^\text{17}\)

Very recently, we demonstrated that miR-133 overexpression paired with GMT generated seven-fold more beating iCMs from mouse embryonic fibroblasts, compared to GMT treatment alone; this treatment also shortened the duration required to induce beating iCMs (from 30 days to 10 days). Furthermore, we found that miR-133-mediated Snai1 repression was critical for cardiac reprogramming in adult mouse (and human cardiac) fibroblasts, and that silencing fibroblast signatures via miR-133/Snai1 was a key molecular roadblock during cardiac reprogramming.\(^\text{20}\) Importantly, this is a first study demonstrating a molecular mechanism underlying cardiac reprogramming by defined factors.

**Generation of human iCMs:** In 2013, three studies including ours applied the concept of direct reprogramming to neonatal and adult human fibroblasts.\(^\text{19,20,21}\) Nam, et al reported that a combination of genes encoding four transcription factors (Gata4, Hand2, Tbx5, and Myocd) and two muscle-specific microRNAs (miR-1 and miR-133) could reprogram up to 20% of human fibroblasts into cTnT+ cells (presumptive cardiomyocytes). Furthermore, a subset of iCMs derived from human cardiac fibroblasts demonstrated spontaneous beating after 11 weeks in culture.\(^\text{19}\) Similarly, Fu, et al reported that a mixture of genes encoding seven transcription factors (Gata4, Mef2c, Tbx5, Mesp1, Myocd, Zfp521, Esrrg) could induce human cardiomyocyte gene expression in treated fibroblasts.\(^\text{20}\) This work also demonstrated that this mixture of reprogramming factors made human iCMs epigenetically stable, and that transforming growth factor-β (TGF-β) signaling improved the efficiency of human iCM reprogramming.\(^\text{20}\) Finally, we found that a combination of genes encoding five transcription factors (GATA4, MEF2C, TBX5, MESP1, and MYOCD) could reprogram human fibroblasts into cardiomyocyte-like cells, which demonstrated action potentials and beating when co-cultured with rat cardiomyocytes.\(^\text{21}\) Islas, et al used two transcription factors (MESP1 and ETS-2) in activin A and BMP2 (bone morphogenetic protein 2)-treated cells to reprogram human dermal fibroblasts into cardiac progenitor-like cells, which could then differentiate into cardiomyocyte-like cells.\(^\text{22}\) Despite these promising results, direct cardiac reprogramming is less efficient in human cells compared to mouse fibroblasts. An optimized combination of appropriate transcription factors and miRNAs for direct human cardiac reprogramming is required, as are inducing conditions for human iCMs.

**Direct Cardiac Reprogramming in vivo**

Perhaps the most exciting potential of cardiac transcription factor-based reprogramming lies in the possibility of using this technology in vivo. For example, cardiac fibroblasts in an infarcted area of a heart could be targeted for cardiogenic reprogramming, resulting in the formation of new cardiomyocytes in situ. In 2012, multiple groups including ours demonstrated the transdifferentiation of fibroblasts into cardiomyocytes in vivo. Olson and Srivastava groups used Cre recombinase driven by fibroblast-specific promoters to trace the cell fate of cardiac fibroblasts and subsequent cardiomyocyte transdifferentiation.

Qian, et al used the periostin and fibroblast-specific protein 1 (FSP-1) promoter Cre transgenic mice, and found that fibroblasts in infarcted hearts were converted into cardiomyocyte-like cells by GMT retroviral gene transfer; global function also developed in treated hearts.\(^\text{23}\) Following the direct injection of GMT retroviruses into infarcted mouse hearts, this work demonstrated that almost 35% of cardiomyocytes in the infarcted area or its border were newly generated iCMs derived from resident cardiac fibroblasts. Furthermore, half of these iCMs had well-organized sarcomeric structures and exhibited functional characteristics of adult ventricular cardiomyocytes, including cellular contraction, electrophysiological properties, and functional coupling to other cardiac cells. These observations suggested that in vivo reprogramming generated functional iCMs more efficiently than in vitro reprogramming.\(^\text{23}\) In contrast to the work of Qian, et al, Song, et al added Hand2 to the GMT cocktail (creating a GHMT cocktail), and utilized FSP1-promoter Cre transgenic and Tcf21-iCre knock-in mice for fibroblast lineage tracing. They reported that GHMT retroviral injection into mouse infarcted hearts converted endogenous cardiac fibroblasts into functional cardiomyocyte-like cells in vivo.\(^\text{17}\) These researchers also demonstrated that approximately 6% of cardiomyocytes in the infarcted area or its border were newly produced cardiomyocyte-like cells with clear striations and functional properties similar to those of endogenous ventricular cardiomyocytes. Twelve weeks after myocardial infarction, Song, et al also demonstrated that the scar zone of injured hearts was reduced by 50%, and the ejection fraction was increased two-fold in GHMT-treated mice compared to controls.\(^\text{12}\)

We generated a polycistronic retrovirus expressing GMT. This polycistronic retrovirus, which expressed GMT at near equimolar levels from the same promoter, was generated using self-cleaving 2A peptides.\(^\text{24}\) We co-injected polycistronic GMT (3F2A) and reporter genes (eg, GFP) to determine cardiac induction from non-myocytes. We found that gene transfer of this polycistronic GMT retrovirus induced more mature cardiomyocyte-like cells (as evidenced by sarcomeric structures) than those generated by the injection of three separate vectors. Mathison, et al injected a mixture of GMT retroviruses and vascular endothelial growth factor (VEGF) into injured myocardial areas in rats. Infarcted areas were reduced in rats treated with VEGF, compared to those only treated with GMT. This reduction of scar in the infarcted area may be due to VEGF-mediated neovascularization or some other unknown mechanisms.\(^\text{25}\) Jayawardena, et al reported that direct injection of lentiviruses containing four microRNAs (miR-1, miR-133, miR-208, and miR-499) into mouse infarcted hearts converted resident cardiac fibroblasts into cardiomyocyte-like cells in vivo. After injection of these microRNAs, approximately 1% of the infarcted area contained new iCMs; however, this work did not report on whether ejection fraction improved after microRNA injection.\(^\text{26}\)

**Conclusions:** Heart is composed of various groups of cells, including the blood vessel endothelial cells, smooth muscle cells, nerve cells, and cardiac fibroblasts. Judging from the absolute number of cells comprising the heart, cardiomyocytes only account for approximately 30% of heart cells, whereas cardiac fibroblasts constitute approximately 50% of this organ. When a large number of cardiomyocytes necrotize by myocardial infarction, the number of cardiac fibroblasts increases in the infarction area. Heart rupture can be prevented by replac-
ing an infarction area with fibrous tissue; however, fibroblasts can result in low cardiac function and potentially fatal arrhythmic focus. Direct reprogramming technology may provide an ideal treatment that could bypass the formation of fibrosis in an infarction region, instead resulting in new cardiomyocyte formation if certain genes are efficiently introduced into cardiac fibroblasts. However, in order for direct reprogramming to be used in clinical applications, the cardiac reprogramming efficiency of this method must be optimized. Currently, the reprogramming efficiency of fibroblasts into mature cardiomyocytes is variable and low. Although there are several reports on the direct reprogramming of human cardiac fibroblasts into cardiomyocytes, further study is required for optimizations. We hope to utilize regenerative medicine-based therapies to treat patients with severe heart failure, potentially employing cardiac muscle cells derived from iPS cells and iCMs.

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