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Cardiomyocyte generation using stem cells and directly reprogrammed cells

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1. ABSTRACT

Cardiomyocytes are terminally differentiated cells with limited regenerative capacity in the adult heart, making cell replacement therapy an attractive option to repair injured hearts. Embryonic stem (ES) cells and induced pluripotent stem (iPS) cells are pluripotent and capable of infinite expansion *in vitro*, implicating them as ideal cell types for cell replacement therapy. During the past several years, significant advances in iPS cell generation technology, cardiac differentiation, and cell purification protocols were achieved for the development of stem cell-based heart therapies. The discovery of iPS cells has also sparked the novel idea of direct conversion of mature cell types into another cell type without passing through a pluripotent stem cell state. Functional cardiomyocytes could therefore be directly reprogrammed from differentiated somatic cells by transduction of the three cardiac transcription factors, Gata4, Mef2c, and Tbx5. Herein, we review the recent research achievements and discuss future challenges in stem cell-based cardiac generation and direct cardiac reprogramming technology for heart regeneration.

2. INTRODUCTION

Heart disease remains a major cause of mortality and morbidity worldwide, and current treatments offer no prospect of cure. Adult hearts have little regenerative ability, and malfunction or significant loss of cardiomyocytes due to disease is potentially lethal (1-4). Heart transplantation is an established therapy for heart failure, but is limited due to the number of donor organs available (5). There is, therefore, a pressing need to develop novel therapeutic strategies for lethal heart disease. Cell replacement therapy is an attractive option for myocardial repair and significant advances in regenerative research were achieved during the past decades (6). In particular, advances in stem cell research have generated tremendous excitement surrounding the possibility of using stem cells to repair damaged hearts (7-9).

Several types of stem cells have been used to regenerate functional cardiomyocytes in damaged myocardium, including cardiac stem/progenitor cells, bone marrow-derived mesenchymal stem cells, and hematopoietic stem cells (10-11). Some stem cell therapies

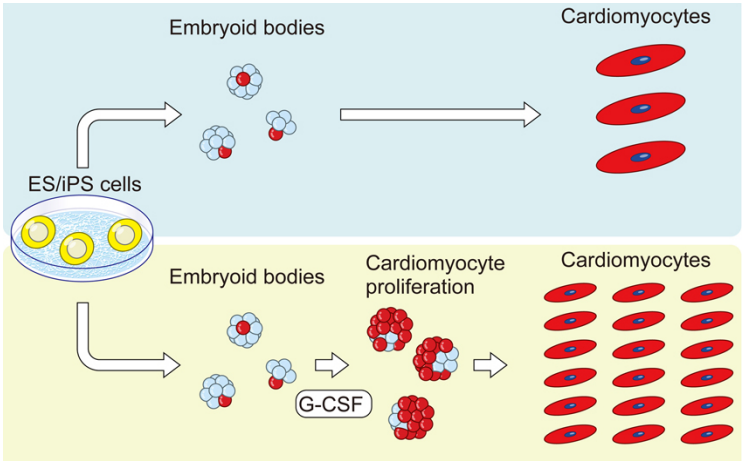

Figure 1. G-CSF promotes cardiomyocyte proliferation and increases cell number in ES/iPS cell-derived cardiomyocytes. The upper panel represents the control treatment and the lower panel represents the G-CSF treatment.

demonstrated beneficial effects on cardiac function, although the mechanisms underlying this improvement are ambiguous because the efficiency of cardiac differentiation from graft cells is unclear1. Embryonic stem (ES) cells are undifferentiated, pluripotent cells, isolated from the inner cell mass of preimplantation blastocyst-stage embryos (12-14). Upon differentiation, ES cells can give rise to cells of all three embryonic germ layers and their derivatives, including cardiomyocytes (15-16). Although ES cells might be ethically and legally problematic (17-18), the recent development of induced pluripotent stem (iPS) cell generation may overcome such issues. The discovery of iPS cells has also sparked a new idea - conversion of mature cell types directly into another cell type without first becoming a stem cell (19-23). This article reviews recent research achievements in stem cell-based cardiac regeneration and direct cardiac reprogramming technology.

3. EFFICIENT CARDIOMYOCYTE INDUCTION FROM EMBRYONIC STEM CELLS

ES cells possess the ability to remain undifferentiated and propagate indefinitely *in vitro* or differentiate into all three embryonic germ layers (ectoderm, mesoderm, and endoderm) and their derivatives, including cardiomyocytes (24-25). Embryoid bodies, which are aggregates of ES cells, have a spontaneous propensity for cardiac differentiation, resembling stem cell development *in utero*. Numerous developmental biology studies have elucidated the step-wise stages of cardiac differentiation, from mesoderm to cardiac progenitor cells and finally to terminally differentiated cardiomyocytes (26-30). In addition to cell-autonomous differentiation of cardiac cells, paracrine factors from the surrounding microenvironment influence and support reproducible cardiogenesis (31).

The process of cardiogenesis in mice provides the framework to understand stem-cell-derived cardiogeneis *in vitro*, and so far the most successful differentiation approaches with ES cells are those that recapitulate the regulatory pathways in the early embryo (26-30, 32). Using cues from developmental biology, significant advances have been made in cardiac differentiation from mouse and human ES cells. Stage-specific induction of ES cells with exogenous factors, activin A, bone morphogenetic protein-4, basic fibroblast growth factor, vascular endothelial growth factor, and Dickkopf-1 results in the generation of cardiac progenitor cells, marked by the expression of vascular endothelial growth factor receptor-2 (KDR/Flk-1) and platelet-derived growth factor receptor (PDGFR)-alpha. The KDR/PDGFR-alpha double-positive mouse and human cardiac progenitor cells can efficiently differentiate into cardiomyocytes *in vitro* and further modifications of this protocol may enable the induction of a large enough number of cardiomyocytes for regenerative purposes (27, 32). Willems and colleagues developed a human ES cell-based high-throughput screening assay to identify small molecules that drive cardiogenic differentiation from mesodermal cells (33). Using this assay, they found that Wnt inhibition was sufficient to drive human ES cell-derived mesoderm to a cardiac fate in the absence of other signaling modulators. Importantly, all of the tested small molecules, which target different cellular components of the pathway and are structurally diverse Wnt inhibitors, showed higher cardiogenic potential than the natural Wnt inhibitor DKK1.

We investigated key molecules that promote cardiomyocyte proliferation in early embryos and cardiac cells derived from stem cells at the later developmental stages (30, 34). In developing embryos, cardiomyocytes abundantly expressed G-CSF receptor at embryonic day (E) 9.5 and intrauterine G-CSF administration strongly promoted the proliferation of embryonic cardiomyocytes. Moreover, G-CSF receptor knockout mice exhibited fetal death in 50% of mice due to myocardial wall thinning. Based on these *in vivo* findings, we applied G-CSF to ES and iPS cell-derived cardiomyocytes and found that G-CSF dramatically increased the number of stem cell-derived cardiomyocytes (Figure 1). These results indicated that G-CSF directly regulates cardiomyocyte proliferation *in vitro*

and *in vivo*, and that G-CSF can be used to expand cardiac cell number (34).

As stem cell-derived cardiac cells mature *in vitro*, specialized heart muscle cells become evident with characteristic ion channel sets of the ventricular, atrial, and pacemaker cell types. The developmental changes in ES cell-derived cardiomyocytes correlate with the length of time *in vitro*, in which pacemaker cells appear early and working myocardial cells (atrial and ventricular myocytes) appear late, although in general a heterogeneous population of all three cardiac cells is seen in culture. One of the remaining challenges is to produce stem cell-derived cardiomyocytes that are functional, but immature in the calcium handling and electrophysiological properties that might be arrhythmogenic in cell transplantation (35). Indeed, the derivation of specific cardiac cell types and terminally differentiated cardiomyocytes that are not arrhythmogenic is an area of tremendous interest for future research.

4. GENERATION OF IPS CELLS AND IPS CELL-DERIVED CARDIOMYOCYTES

ES cell-derived cardiomyocytes are an attractive cell source for cardiac repair, but immune rejection and ethical concerns remain problematic for clinical application (17). Accordingly, Dr Yamanaka and colleagues recently opened a new paradigm in regenerative research that changed the stem cell research field dramatically (36-37). They discovered that four ES cell-specific transcription factors (Oct4, Sox2, Klf4, and c-Myc) can reprogram mouse and human fibroblasts into ES cell-like cells, the so-called iPS cells (36-37). Importantly, many other laboratories reproducibly generated iPS cells after the initial reports (38-43). In order to use iPS cells as efficient research tools and ultimately translate this technology into clinical applications, suitable techniques of factor delivery are crucial. Initially, iPS cells were made from somatic cells by retroviral or lentiviral transduction of the required transcription factors for gene integration into the host genome (36-37, 44-49). Strategies to derive iPS cells free of transgenic sequences were aimed at circumventing the potentially harmful effects of insertional mutagenesis, and the first integration-free iPS cells were generated using adenoviral vectors and with plasmids (50-52). These experiments provided the proof of principle that transient expression of the four reprogramming factors is sufficient to induce pluripotency in somatic cells. However, reprogramming efficiencies with these methods were much lower (0.001%) than those with integrating vectors (0.1-1%). We successfully generated integration-free iPS cells from human activated T cells (in peripheral blood) using a temperature-sensitive mutated Sendai virus that encodes human Oct4, Sox2, Klf4, and c-Myc (53). Sendai virus vector is a minus-strand RNA virus that is not integrated into the host genome and is not pathogenic in human (54). The generation of human iPS cells by this method is easy, efficient (0.1%), and safe, achieved within a month (Figure 2). More recently, successful reprogramming has been achieved without using viral or plasmid vectors, when iPS cells were generated from fibroblasts by delivering the reprogramming factors as purified recombinant proteins and modified RNA (55-57).

As ES cells give rise to cardiac tissue *in vitro*, iPS cells have also demonstrated a capacity for cardiac differentiation. Using methodology established for ES-cell-derived cardiogenesis, iPS cells can be differentiated into cardiomyocytes through mesoderm lineages and cardiac progenitors (32, 58-59). Generation of patient-specific iPS cells might also represent a novel platform for understanding mechanisms of heart disease (60-63). Patient-specific iPS cell-derived cardiomyocytes could also be useful in regenerative medicine by avoiding ethical and legal concerns. Moreover, iPS cells can offer the possibility of understanding the mechanisms and finding new therapeutic interventions for genetic diseases by generating patient-specific cardiac cells (62, 64).

However, there are several concerns arising because iPS cells and ES cells are similar but not identical. For example, Jiang *et al.* reported electrophysiological differences between human iPS cells and ES cells (70). While the ion channel expressions in human iPS cells largely resemble those in ES cells, there are specific differences in their properties and biological roles. A better understanding of the basic biology and further investigation of iPS cell-derived cardiomyocytes may facilitate their future clinical application (65).

5. ES AND IPS CELL-BASED CARDIAC REGENERATION

Heart disease is a serious problem in developed countries, as cardiomyocytes are terminally differentiated cells and myocardial regeneration is very limited. Stem cell-derived cardiomyocytes are a potentially promising cell source for cardiac repair and several studies have been conducted using animal models (66-69). Laflamme *et al.* reported an improvement in cardiac function in immunodeficient rats 4 weeks after coronary ligation by injecting human ES cell-derived cardiomyocytes (66). In contrast, van Laake *et al.* reported improvements in mouse heart function at 1 month, but not at 3 months after myocardial infarction with the injection of ES cell-derived cardiomyocytes into the hearts (72-73). Grafted cardiomyocytes are easily washed out from transplanted hearts and the majority of cells disappear several days after the direct injection (66, 70). Thus, to enhance the effect of stem cell-based cardiac regeneration, it is critical to improve the survival and attachment of grafted cells. To this end, administering prosurvival factors with the grafted cells may improve cell survival (66, 71). We reported that aggregate formation of stem cell-derived cardiomyocytes through homophilic cell-cell adhesion improved their survival in the immunodeficient mouse heart (72) (Figure 3). We confirmed the expression of basic fibroblast growth factor, epidermal growth factor, platelet-derived growth factor-beta dimer, and endothelin-1 and their receptors in these aggregates. Application of these growth factors in culture strongly enhanced the growth of cardiomyocyte aggregates, suggesting that both autocrine and paracrine stimulation promotes the survival and growth of grafted

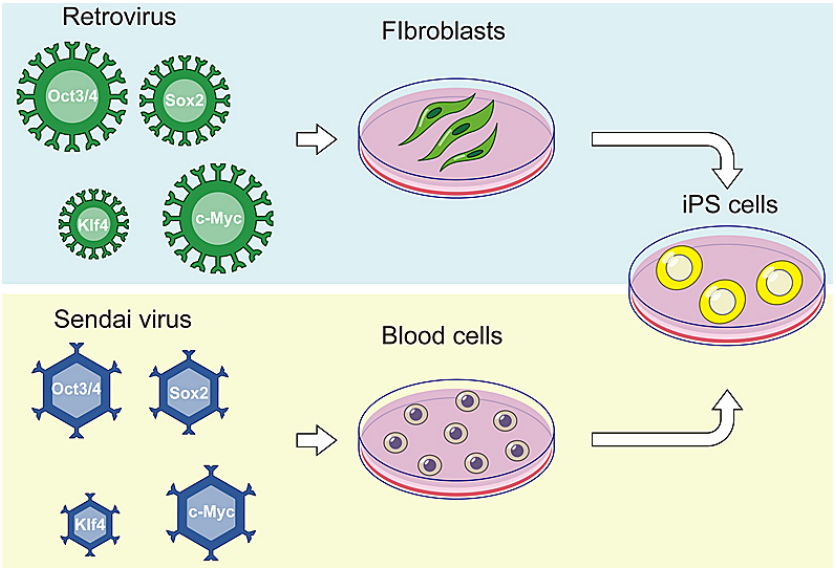

Figure 2. The upper panel illustrates an original iPS cell generation method that uses retroviral vectors for iPS generation from fibroblasts. The lower panel demonstrates that integration-free vectors, such as Sendai viruses, reprogram blood cells into iPS cells. This new method might be more efficient, easier, and safer.

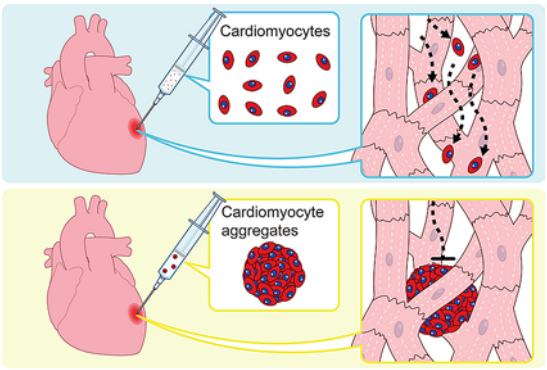

Figure 3. The upper panel illustrates the direct injection of single cardiomyocytes into hearts. Cardiomyocytes are easily washed out and not retained in the heart. The lower panel shows that cardiomyocyte aggregates survive and attach better than those delivered by single cell injection.

cardiomyocytes. Okano *et al.* reported that myocardial cell sheets generated in temperature-sensitive culture dishes were successfully transplanted into murine hearts (77-78). However, they could not observe functional benefits, as the triple-layered cardiomyocyte sheets might not be sufficient for functional recovery in the damaged hearts. Further modifications and appropriate environmental factors such as blood supply and support cells might be necessary to consider for successful cell therapy in the future (73).

The iPS cell technology is clearly not yet ready for clinical use, with the main issue being safety; iPS cells, like ES cells, tend to form teratomas, and current differentiation protocols cannot eliminate undifferentiated cells (37, 74-75). Cardiomyocyte-specific gene reporter systems might be successfully applied for the high-grade purification of cardiomyocytes, although this method requires genetic modifications (76-77). We found that a fluorescent dye that labels mitochondria, tetramethylrhodamine methyl ester perchlorate, can selectively mark ES/iPS cell-derived cardiomyocytes (72, 78). Cardiomyocytes could subsequently be enriched (> 99% purity) by fluorescence-activated cell sorting (FACS) using this dye, and purified cardiomyocytes transplanted into testes did not form teratomas. However, as cardiomyocytes are fragile and sensitive to high-pressure passage during FACS, non-genetic methods without FACS are necessary for future clinical applications.

6. DIRECT CONVERSION TO CARDIOMYOCYTES

The discovery of reprogramming of fibroblasts to iPS cells raises the possibility that a somatic cell could be reprogrammed to an alternative differentiated cell fate

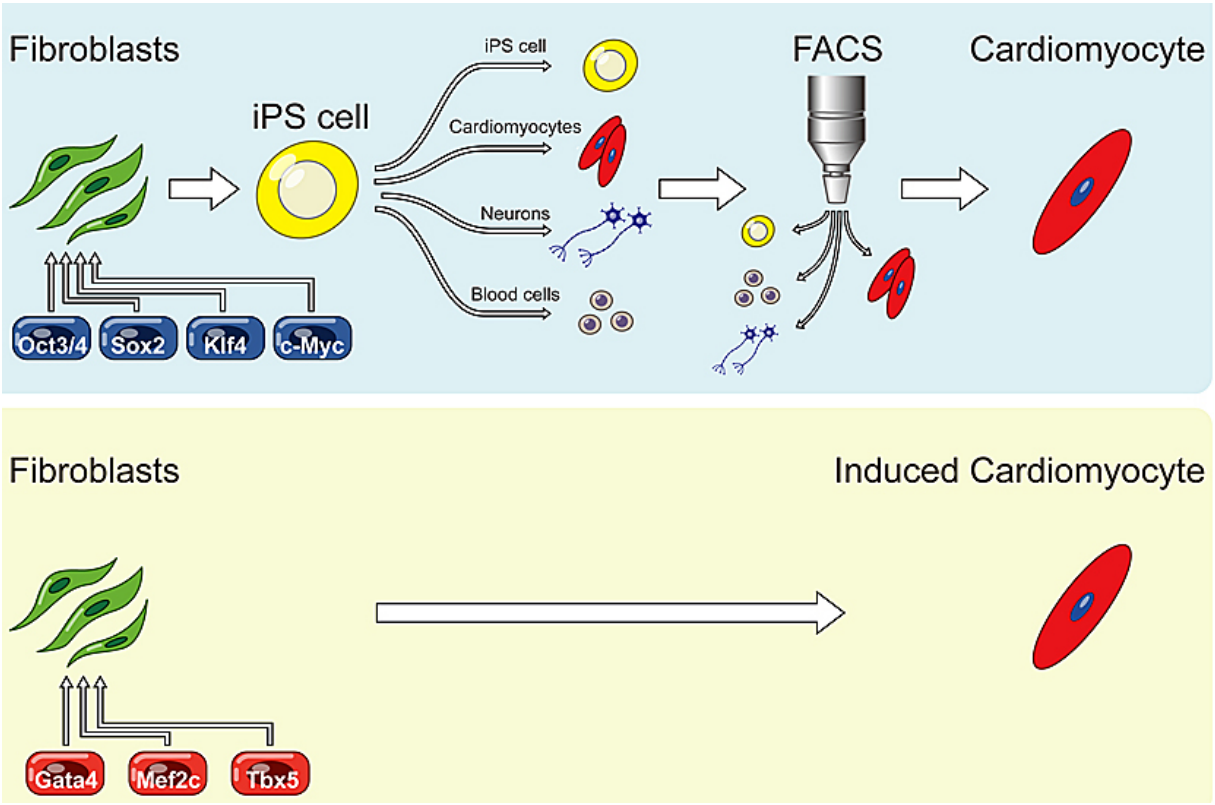

Figure 4. The upper panel represents the derivation of cardiomyocytes through iPS cell generation from fibroblasts in a process requiring several steps. The lower panel demonstrates direct reprogramming of fibroblasts into cardiomyocytes by defined factors such as Gata4, Mef2c, and Tbx5.

without first becoming a stem cell. If target cells could be obtained directly without passing through the stem-cell stage, the possibility of tumor formation after the cell graft is very low. Zhou *et al.* reported that a combination of transcription factors comprising Neurogenin3, Pdx1, and Mafa could efficiently reprogram pancreatic exocrine cells into functional β-cells in mouse, while Vierbuchen *et al.* reported that neuronal transcription factors, Ascl1, Brn2, and Myt1l, convert dermal fibroblasts into functional neurons (31-32). Although embryonic mesoderm can be induced to differentiate into cardiomyocytes, no "master regulator'' of cardiac differentiation, like MyoD for skeletal muscle, was identified (79-81).

We hypothesized that rather than a single key developmental cardiac gene, a combination of genes might be capable of directly converting cardiac fibroblasts into cardiomyocytes. To determine candidate factors for cardiac reprogramming, we identified genes that are specifically expressed in embryonic cardiomyocytes and critical for cardiogenesis. We developed a novel cell purification system in which embryonic cardiomyocytes and cardiac fibroblasts can be purified using FACS and selected 14 genes as candidates for cardiac reprogramming (31). We developed a screening system using alpha myosin heavy chain (alpha MHC) promoter-driven EGFP-IRES-puromycin transgenic mice (alphaMHC-GFP) in which only mature cardiomyocytes express the green fluorescent protein (GFP) (82-83). Transduction of all 14 factors into fibroblasts induced 1.7% of GFP+ cells, and serial removal of individual factors demonstrated that a combination of three factors (Gata4, Mef2c, and Tbx5) was sufficient for GFP+ cell induction (around 15%). We designated these GFP+ cardiomyocyte-like cells induced cardiomyocytes (iCMs) (Figure 4). The three cardiac reprogramming factors, Gata4, Mef2c, and Tbx5, are core cardiac transcription factors in early heart development and are known to interact with each other, coactivate cardiac gene expression, and promote cardiomyocyte differentiation (84-89).

The iCMs are similar to cardiomyocytes in genetics and epigenetics, although not identical. The global gene expression profile of iCMs is similar to neonatal cardiomyocytes, but different from the original cardiac fibroblasts. The histone modifications and DNA methylation patterns of iCMs were also similar to those in cardiomyocytes (90). In addition, a subset of iCMs exhibited intracellular Ca2+ transient and contracted spontaneously after 4 weeks of culture. A subgroup of reprogrammed cells had physiological properties of bona fide cardiomyocytes, but the others seemed to be only partially reprogrammed. As shown in iPS cell generation, the cardiac reprogramming process is slow and may take time for full reprogramming.

Thus, the efficiency of generating functional cardiomyocytes should be improved by further modifications.

Ding *et al.* showed that transient overexpression of Yamanka 4 factors, Oct4, Sox2, Klf4, and c-Myc, could convert mouse fibroblasts directly into spontaneously contracting cardiomyocytes mediating through cardiac progenitor cells, but not through a pluripotent intermediate (91, 97). Compared to our transdifferentiation protocol, they showed spontaneous contraction beginning after 11 days and a 6-fold higher efficiency yield of cTnT+ cardiomyocytes. This latter result might reflect the generation of mitotically active cardiac precursor cells first rather than terminally differentiated cardiac cells in their protocol. However, transplantation studies are needed to confirm that their fibroblast-derived cardiomyocytes are functionally integrated into host myocardium and that the graft cells do not form teratomas.

7. THE HOPE AND HURDLES OF CELL THERAPIES FOR FUTURE CARDIAC REGENERATION

During the past several years, tremendous progress has been achieved in the field of cardiac regeneration (76, 92-94). Efficient and safe stem cell-based cardiac regeneration protocols have been reported by many laboratories. For the process of cardiac regeneration using iPS cell-derived cardiomyocytes, first, the patient's own blood T cells or fibroblasts would be harvested, expanded *ex vivo*, and transduced with Yamanaka's four factors. The Sendai virus can make integration-free iPS cells that might be safer than those derived using retro or lentiviral vectors. Cardiomyocytes are differentiated from iPS cells using the accumulated knowledge from developmental biology and purified by FACS to avoid contamination of undifferentiated cells and other cell types. The iPS cell-derived cardiomyocytes could be transplanted into patient hearts as aggregates or cell sheets by a surgical operation. In this case, the process is straightforward and sufficient numbers of cardiomyocytes might be generated from iPS cells for cardiac repair, because stem cells can expand infinitely. However, there are concerns that stem cell-derived cardiomyocytes are phenotypically young and immature cardiomyocytes compared with adult cardiomyocytes (65, 95-99). It also seems difficult to make stem cell-derived cardiac cells mature using conventional culturing methods, and there are concerns that cell-based therapies might produce electrical heterogeneities and abnormal conduction that could trigger arrhythmias. Continued efforts to produce more mature cardiac cells and successful integration into the recipient myocardium are necessary for clinical success.

The new direct cardiac reprogramming technologies may change the field of cardiac regeneration in the future (91, 100). Yamanaka 4 factor-transduced fibroblasts can be converted into cardiomyocytes, and after cardiac cell selection the cells might be transplanted into damaged hearts. Gata4/Mef2c/Tbx5-transduced cardiac fibroblasts could be transplanted into mouse hearts and be converted into cardiomyocyte-like cells within the heart. Moreover, in the future, cardiomyocytes might be directly generated from endogenous cardiac fibroblasts in the infarct area by injecting the reprogramming factors. Such direct administration into the damaged heart may reprogram the endogenous fibroblast population, which represents more than 50% of the cells, into new cardiomyocytes. This possibility carries several significant advantages: first, the process is simple and short; second, the avoidance of reprogramming to pluripotent cells before cardiac differentiation would greatly lower the risk of tumor formation; third, direct injection of defined factors can avoid cell transplantation in which long term cardiac cell survival is still challenging (66, 70, 77, 101). However, the functional properties of induced cardiomyocytes should be characterized more carefully and it is still unclear whether direct cardiac reprogramming is possible in human cells. Moreover, the efficiency of cardiac reprogramming, particularly generation of bona fide cardiac cells, is still low and needs to be improved by future research. Studies in human cells and understanding of the molecular mechanism of direct cardiac reprogramming are necessary to advance this technology for future clinical applications.

8. CONCLUSIONS

Heart disease is still one of the most life-threatening diseases worldwide. Given the general lack of heart transplantation donors, cell replacement therapy is one of the most promising and exciting research areas to be pursued. It is clear that the discovery of iPS cells has fundamentally altered the approach to regenerative medicine, but the field is still in its infancy. The work of numerous laboratories has led to significant therapeutic and scientific advances in cell therapy to cardiac regenerative medicine. However, many questions and challenges remain. The issues of appropriate cardiac differentiation, risk of tumor formation due to contamination of immature cells, and proper integration into the recipient myocardium need to be improved before clinical applications can be considered. The new direct cardiac reprogramming technology has just emerged and much refinement and characterization of the reprogramming process will be necessary. We hope that cardiac regeneration therapy will become a next-generation strategy for helping heart disease patients in the future.

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Abbreviations: ES: embryonic stem; iPS: induced pluripotent stem; vascular endothelial growth factor receptor-2: KDR/Flk-1; platelet-derived growth factor receptor: PDGFR; fluorescence-activated cell sorting: FACS; green fluorescent protein: GFP; induced cardiomyocytes: iCMs; myosin heavy chain: MHC

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