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Review

# Cardiac regeneration with pluripotent stem cell-derived cardiomyocytes and direct cardiac reprogramming

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ABSTRACT

Cardiovascular disease is the leading cause of death globally. Cardiomyocytes (CMs) have poor regenerative capacity, and pharmacological therapies have limited efficacy in severe heart failure. Currently, there are several promising strategies for cardiac regeneration. The most promising approach to remuscularize failing hearts is cell transplantation therapy using newly generated CMs from exogenous sources, such as pluripotent stem cells. Alternatively, approaches to generate new CMs from endogenous cell sources *in situ* may also repair the injured heart and improve cardiac function. Direct cardiac reprogramming has emerged as a novel therapeutic approach to regenerate injured hearts by directly converting endogenous cardiac fibroblasts into CM-like cells. Through cell transplantation and direct cardiac reprogramming, new CMs can be generated and scar tissue reduced to improve cardiac function; therefore, cardiac regeneration may serve as a powerful strategy for treatment of severe heart failure. While substantial progress has been made in these two strategies for cardiac regeneration over the past several years, challenges remain for clinical translation. This review provide an overview of previous reports and current challenges in this field.



**Contents**



## 1. Introduction

Cardiovascular diseases are the leading cause of death and disability worldwide, despite decades of development of therapeutic approaches for treating such diseases. As adult cardiomyocytes (CMs) have little regenerative capacity, the injured heart follows a progression to severe heart failure that results in the death of CMs, which are replaced with fibrotic scar tissue [1,2].

*Abbreviations:* BMP, bone morphogenic protein; CFs, cardiac fibroblasts; CMs, cardiomyocytes; CPCs, cardiac progenitor cells; ESCs, embryonic stem cells; GHMT, GMT plus Hand2; GMT, Gata4; Mef2c, and Tbx5; iCMs, induced cardiomyocyte-like cells; iPSCs, induced pluripotent stem cells; MI, myocardial infarction; miRs, microRNAs; PSCs, pluripotent stem cells; SeV-GMT, Sendai virus vector expressing GMT.

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Pharmacological approaches have been the most validated clinical strategy for treating heart failure. Although these medical therapies have decreased heart failure mortality, they demonstrate that neither CM regeneration nor replacement of lost CMs occurs in these patients. A promising approach to renew the injured heart is heart transplantation; however, the availability of donor organs is very limited. Therefore, cardiac regeneration has attracted attention as a novel therapeutic approach for patients with heart failure.

CMs are considered to be in a terminally differentiated state, and their regenerative capacity is insufficient to completely regenerate lost myocardium [3,4]. Initial regenerative approaches based on transplanting noncardiac cells and cardiac-derived cells attempted to replace this myocardial tissue. Although these cell-based clinical trials using bone marrow-derived cells, mesenchymal stem cells, and cardiac progenitor cells (CPCs) showed subtle effects on cardiac recovery, mainly due to paracrine signaling, these methods have encountered problems related to safety and low engraftment rates [5,6].

Another regenerative approach is using CMs derived from allogeneic pluripotent stem cells (PSCs), such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). CMs have been generated *in vitro* from PSCs using directed differentiation, which recapitulates the developmental process in embryos and represents a valuable platform to study the mechanisms of cell-fate specification [7,8]. Although tremendous progress has been made in this field, PSC-based therapies have engendered some concerns that must be overcome before their practical use in patients, including low engraftment rate and potential risk of tumorigenesis [7]. To overcome the major issues arising from the use of PSCs, alternative approaches for heart regeneration have targeted resident cardiac fibroblasts (CFs). Direct cardiac reprogramming is a new approach for heart repair, in which resident CFs are converted to induced cardiomyocyte-like cells (iCMs), without reverting to PSCs, by transduction of cardiac-specific factors [9]. In this review, the advances in cardiac regeneration are summarized, and the discussion is limited to these two promising approaches for heart repair.

## 2. PSC-based therapies for cardiac repair

### 2.1. Directed cardiac differentiation from PSCs

Many approaches to induce cardiac differentiation from PSCs have been investigated. Early approaches for CM generation used a spontaneous differentiation protocol, resulting in the creation of embryoid bodies with media containing serum; however, the efficiency was ~10% [10]. Since then, several research groups have developed ways to improve the efficiency of CM differentiation. Current approaches, so-called directed cardiac differentiation, have employed various protocols, each aimed at recapitulating the early differentiation stages that take place in the embryo to generate CMs [11].

The heart is derived from cardiac mesoderm and is the first functional organ to be formed in embryos. The cardiac mesoderm arises from the primitive streak and initially becomes specified as CPCs that then differentiate into cardiovascular lineages [12,13]. Previous studies have revealed that temporal activation and inhibition of various combinations of heart development-related signaling molecules, including bone morphogenic protein (BMP), Nodal/Activin, and Wnt, induced CMs from PSCs [14–16]. A recent study demonstrated that PSCs could be exclusively differentiated to a desired lineage by providing the positive signals to induce a given fate while repressing inhibitory signals that induce the unwanted fate [17]. As a result, the efficiency of differentiation into CMs has greatly improved with the use of defined conditions, yielding high purity and sufficient numbers [18–20]. Despite recent success in directed differentiation from PSCs, the molecular mechanisms for cardiovascular induction and lineage diversification remain elusive.

Intriguingly, direct regulation of key developmental genes by overexpression of T-box transcription factors resulted in cardiac mesoderm specification in PSC differentiation [21–24]. Recently, we demonstrated that Tbx6 is critical for PSC differentiation into mesoderm and cardiovascular lineages [25]. In contrast to well-known cardiac development regulator genes that were induced in previous studies, Tbx6 has been regarded as a marker of paraxial mesoderm and implicated mainly in somite development, in which the axial skeleton, skeletal muscle, and dermis are formed [26–28]. To identify new key regulators, we adopted direct reprogramming-based screening, which can be simpler and faster than generating multiple PSC lines expressing a series of candidate genes. We transduced each candidate factor into fibroblasts and analyzed the induction of *Mesp1*, a marker of nascent mesoderm. Only Tbx6 strongly induced *Mesp1* mRNA expression, and *Tbx6*-transduced cells expressed nascent mesoderm genes and specific surface markers. Indeed, single-cell RNA sequencing revealed that Tbx6 expressed nascent mesoderm co-expressed genes related to cardiac mesoderm and CPCs in mouse embryos. Finally, we demonstrated that Tbx6 induces nascent mesoderm from PSCs and determines cardiovascular and somite lineage specification via its temporal overexpression in the absence of exogenous cytokines. Although these results suggested that Tbx6 plays critical roles in cardiac mesoderm specification and cardiogenesis, Tbx6 mutant mice exhibit relatively mild cardiovascular defects, and the heart does not fail to develop [26]. This may be due to redundancy with other T-box genes, such as *Mesp1*, *T*, and *Eomes*, which are also expressed in cardiac mesoderm. Consistent with this, the expression of these mRNAs as well as cardiac differentiation were suppressed but not ablated in *Tbx6* knockout PSCs. These results indicated that Tbx6 is important but not required for mesoderm formation and cardiovascular differentiation in mammals. Thus, steady progress has been made in cardiac differentiation from PSC over the past several years.

### 2.2. Cardiac regeneration using PSC-derived CMs

In recent years, numerous attempts have been made to achieve cardiac regeneration using PSC-derived CMs, and this field has advanced tremendously. Cardiac repair using PSC-derived CMs is therefore expected to become useful as a treatment for severe heart failure. However, PSC-based therapies require further refinement before their practical use in patients, due to the low engraftment rate and challenges with elimination of residual PSCs and immature PSC-derived CMs [7]. Tissue engineering has improved the low engraftment rate of transplanted cells by, for instance, providing scaffolds, such as hydrogels or cell sheets; in addition, a pro-survival cocktail has been shown to improve the survival of the transplanted cells through antiapoptotic effects [29–31]. Shimizu et al. demonstrated that layered rat neonatal CM sheets produce thick, cell-dense myocardium, with microvessel network formation observed just after implantation in rat dorsal subcutaneous tissues [32]. Moreover, Kawamura et al. showed that transplantation of human iPSC-derived CM sheets improved cardiac function in a porcine myocardial infarction (MI) model [30]. Another approach to overcome the low engraftment rates was proposed by Hattori et al. [33], who showed that purified ESC-derived CM aggregates exhibited improved survival and grew in immunodeficient mouse heart through homophilic cell-cell adhesion and autoparacrine signaling. Thus, engineering of PSC-derived CMs into forms such as cell sheets and aggregates is critical for improvement of transplanted cell survival through interactions with adjacent cells.

Risk of arrhythmia is another hurdle to clinical application, because ventricular tachycardia may occur after intramyocardial injection of PSC-derived CMs [34,35]. Potential mechanisms of this arrhythmogenesis remain undefined; one cause may be the inability of immature CMs to electromechanically integrate with surrounding CMs [36]. Many studies have focused on generating a pure population of more mature cardiac cells, and these improvements may lead to an improved engraftment rate and reduced tumorigenesis and arrhythmia [20,33]. A recent study demonstrated that purified allogenic iPSC-derived CMs transplanted in the infarcted macaque heart improved cardiac function. Although transient non-lethal ventricular tachycardia was observed after transplantation, peaking at day 14, the transplanted CMs survived for 12 weeks without any tumor formation [35]. There has been enormous progress in this field; the first human clinical trial using iPSC-derived CMs, in which allogeneic iPSC-derived CM sheets will be transplanted into a patient with ischemic cardiomyopathy, is being planned by Japanese groups. Thus, PSC-based cardiac regeneration may be a potential approach for treatment of severe heart failure.

## 3. Direct reprogramming of fibroblasts into CMs by defined factors

### *3.1. Development of reprogramming cocktails and overcoming molecular hurdles*

In 2006, Takahashi and Yamanaka demonstrated that overexpression of four transcription factors—Oct4, Sox2, Klf4, and c-Myc—converted fibroblasts into a pluripotent state [37]. The epoch-making discovery of iPSCs has enabled the generation of desired cell types without passing through a stem cell stage by introducing combinations of multiple lineage-specific factors, in a process called direct reprogramming. CFs, constituting ~10% of total cardiac cells, are the most abundant cells in the heart. Given that CFs are activated and increased by replication of the resident CFs in injured heart tissue, CFs would be the potential starting cells in direct cardiac reprogramming [38–40]. Converting CFs to CMs is a promising approach for heart regeneration. Applying the concept of iPSC reprogramming, in 2010, we found that the combination of three transcription factors, Gata4, Mef2c, and Tbx5 (G, M, and T; GMT), could directly convert CFs into iCMs [9]. The iCMs had CM characteristics, such as well-organized sarcomeric structures, global gene expression profiles, action potentials, and spontaneous contractions. Subsequently, many researchers demonstrated that the addition or modifications of reprogramming factors, such as other transcription factors and microRNAs (miRs), may promote reprogramming efficiency and maturation. Song et al. demonstrated that GMT plus Hand2 (GHMT) resulted in more efficient reprogramming of CFs into beating iCMs as compared to GMT alone [41,42]. Jayawardena et al. introduced a combination of muscle-specific miRs (miR-1, miR- 133, miR-208, and miR-499) into neonatal CFs, resulting in functional beating iCMs [43,44]. Although many attempts resulted in the identification of multiple reprogramming cocktails to improve reprogramming efficiency, only a small minority of cells became spontaneously beating iCMs, suggesting the existence of numerous molecular hurdles on the road to successful reprogramming.

Recent advances in direct cardiac reprogramming have provided new insights into mechanisms that maintain original fibroblast cell identity programs and have enabled complete reprogramming. These results demonstrate that optimization of reprogramming cocktails and modification of signaling pathways, epigenetics, and inflammation represent potential strategies for addressing challenges in successful cardiac reprogramming.

Fibroblast signatures were the first identified hurdle to efficient and successful cardiac reprogramming. Muraoka et al. demonstrated that the addition of miR-133 to GMT promoted cardiac reprogramming efficiency and maturation [45]. This approach generated 7-fold more beating iCMs compared to GMT treatment alone and also shortened the time required to induce beating iCMs as compared to GMT. MiRs are short non-coding RNAs that regulate gene expression post-transcriptionally and play critical roles in embryonic cardiac development and specification [46,47]. Mechanistically, miRs bind to the 3′-untranslated region of their target mRNAs and repress protein production by destabilizing the mRNA and translational silencing. In cardiac reprogramming, miR-133 directly targeted *Snai1*, a master regulator of the epithelial-to-mesenchymal transition [45]. *Snai1* knockdown suppressed fibroblast genes and upregulated cardiac gene expression, while *Snai1* overexpression maintained fibroblast gene expression and inhibited the generation of beating iCMs. These results indicated that the residual fibroblast signature is a hurdle to complete cardiac reprogramming. Consistent with this, inhibition of fibroblast-related signaling pathways, such as transforming growth factor-β (TGFβ) and WNT, enhanced cardiac reprogramming efficiency and maturation through silencing of fibroblast signatures [48,49].

Pre-existing chromatin states represent another challenge to the reprogramming process [50]. Reprogramming factors must be able to engage genes that are developmentally silenced and inappropriate for expression in fibroblasts. The epigenetic state controls accessibility of transcription factors. Analyses of the epigenetic status of cardiac-specific gene promoters revealed that iCMs were epigenetically converted to CMs during direct cardiac reprogramming [9,51]. It has been shown that inactive chromatin marks increased at fibroblast promoters and decreased at cardiac promoters, while activated chromatin marks were enriched at cardiac promoters. Recently, Zhou et al. identified Bmi1, a polycomb complex protein, as a significant epigenetic barrier to cardiac reprogramming [52]. Among epigenetic modifiers, Bmi1 regulated key cardiogenic genes through direct binding at these loci in fibroblasts, and Bmi1 inhibition promoted an open chromatin status. Thus, these observations indicate that transcription factors could efficiently drive cardiac induction when the epigenetic status was permissive.

Recently, we revealed that age-related inflammation acts as a third hurdle to direct cardiac reprogramming [53]. A high-throughput screening system revealed that diclofenac sodium, a non-steroidal anti-inflammatory drug, enhanced cardiac reprogramming efficiency and maturation in combination with GMT or GHMT in adult fibroblasts. Although adult fibroblasts are less efficient for reprogramming compared with embryonic fibroblasts, the molecular mechanisms of obstacles associated with aging remain elusive. Intriguingly, COX-2 was strongly expressed in adult fibroblasts compared with embryonic fibroblasts in an age-dependent manner. Diclofenac enforced cardiac reprogramming by inhibiting COX-2, PGE2/EP4, cyclic AMP/protein kinase A, and interleukin 1β signaling through silencing inflammatory and fibroblast programs, which were activated in aged fibroblasts. Zhou et al. identified Znf281 as an inducer of cardiac reprogramming from an unbiased screen of human transcription factors. They found that it suppressed the expression of inflammatory genes and modulated cardiac gene expression by interacting with the transcription factor GATA4 [54]. Overall, numerous aspects of the molecular mechanisms underlying reprogramming remain unaddressed, and significant achievements and improvements are needed to further progress the novel technology.

### 3.2. In vivo cardiac repair and regeneration

The major goal of cardiac reprogramming is to provide newly generated CMs from endogenous CFs *in situ*. In contrast, in PSC-based cardiac regeneration, CMs generated *in vitro* are transplanted into the injured heart, followed by *in vivo* delivery of the reprogramming factors directly into the heart, which convert resident CFs. *In vivo* direct reprogramming has been reported with the retroviral or lentiviral delivery of reprogramming cocktails (GMT, GHMT, and miR combo) after acute MI, with improved cardiac function and reduced fibrosis observed in the injured murine heart [41,44,55,56]. A lineage-tracing system showed that these newly generated iCMs were derived from resident CFs and not from cell fusion with endogenous CMs. Intriguingly, *in vivo* iCMs more closely resemble endogenous CMs than those produced *in vitro* and form gap junctions with surrounding CMs [41,57]. Consistent with this observation, physiologically relevant culture conditions *in vitro* promote cardiac reprogramming. To mimic environmental stimuli *in vivo*, Sia et al. demonstrated that micro-grooved substrate induced mature functional iCMs, and Li et al. showed that 3D culture using fibrin hydrogel enhances reprogramming efficiency [58,59]. These results suggested that the *in vivo* environment, including the extracellular matrix, secreted proteins, electromechanical stimulation, and tissue stiffness, may promote direct cardiac reprogramming [60].

Although *in vivo* reprogramming improves cardiac function and reduces fibrosis after MI, the conventional method using retroviral and lentiviral vectors leads to insertional mutagenesis. The use of these vectors is associated with a risk of random genomic integration of virally overexpressed reprogramming factors. For clinical applications, development of a safe delivery method will be required. Recently, we developed a polycistronic Sendai virus vector expressing GMT (SeV-GMT) and demonstrated *in vivo* direct reprogramming [61]. This unique, non-segmented, negative-stranded RNA viral vector remains exclusively in the cytoplasm and does not integrate into the host genome. In addition, SeV-GMT improved cardiac function and fibrosis in mice after acute MI compared to conventional retroviral GMT. Although further investigation is needed, *in vivo* cardiac reprogramming with SeV represents a potential future treatment for heart disease. Despite the progress of *in vivo* direct cardiac reprogramming, there are several roadblocks we must overcome prior to clinical trials. First, experiments in chronic heart failure models are required. Although *in vivo* cardiac reprogramming resulted in improvement of cardiac function and fibrosis, all *in vivo* studies were performed in the acute MI model [41,49,55,56,61]. It remains unknown whether *in vivo* reprogramming could translate effectively to chronic heart failure, for which regenerative medicine is in high demand. Moreover, a system for gene delivery to resident CFs during the chronic phase of heart injury remains elusive. Regardless of the advancements in

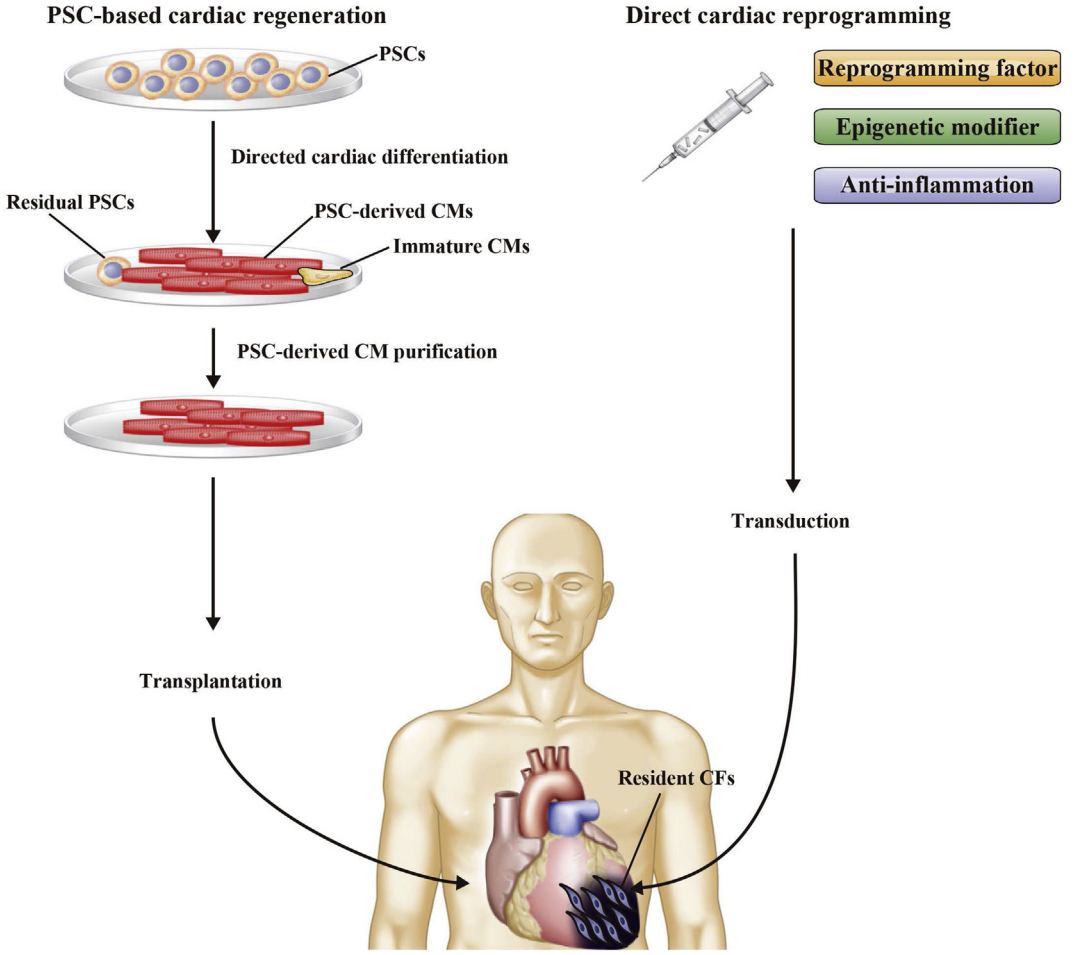


**Fig. 1.** Future regenerative medicine for the treatment of heart disease. As adult CMs have poor regenerative capacity, dead CMs are replaced by fibroblasts, leading to fibrosis, cardiac remodeling, and heart failure, which is associated with high mortality. The cell transplantation-based approach using PSC-derived CMs is shown (left). A direct cardiac reprogramming approach may convert endogenous CFs directly into CMs by defined factors *in situ* (right).

direct reprogramming, before testing these approaches in clinical trials, further studies are warranted to assess the efficacy and safety of these novel approaches for heart regeneration.

## 4. Conclusions

Substantial progress has been made in heart regeneration strategies over the past several years. Transplantation of PSC-derived CMs and direct reprogramming therapy have been suggested as promising and beneficial approaches for heart regeneration (Fig. 1). However, the existing therapeutic approaches for heart failure are unable to completely restore the lost CMs. Since the heart has a limited proliferative and regenerative capacity, effective strategies for cardiac regeneration are needed. Therefore, opportunities for development of such methods are of significant interest.

## Declarations of interest

None.

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