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Review article

In vivo reprogramming for heart regeneration: A glance at efficiency, environmental impacts, challenges and future directions

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ABSTRACT

Replacing dying or diseased cells of a tissue with new ones that are converted from patient's own cells is an attractive strategy in regenerative medicine. *In vivo* reprogramming is a novel strategy that can circumvent the hurdles of autologous/allogeneic cell injection therapies. Interestingly, studies have demonstrated that direct injection of cardiac transcription factors or specific miRNAs into the infarct border zone of murine hearts following myocardial infarction converts resident cardiac fibroblasts into functional cardiomyocytes. Moreover, *in vivo* cardiac reprogramming not only drives cardiac tissue regeneration, but also improves cardiac function and survival rate after myocardial infarction. Thanks to the influence of cardiac microenvironment and the same developmental origin, cardiac fibroblasts seem to be more amenable to reprogramming toward cardiomyocyte fate than other cell sources (e.g. skin fibroblasts). Thus, reprogramming of cardiac fibroblasts to functional induced cardiomyocytes in the cardiac environment holds great promises for induced regeneration and potential clinical purposes. Application of small molecules in future studies may represent a major advancement in this arena and pharmacological reprogramming would convey reprogramming technology to the translational medicine paradigm. This study reviews accomplishments in the field of *in vitro* and *in vivo* mouse cardiac reprogramming and then deals with strategies for the enhancement of the efficiency and quality of the process. Furthermore, it discusses challenges ahead and provides suggestions for future research. Human cardiac reprogramming is also addressed as a foundation for possible application of *in vivo* cardiac reprogramming for human heart regeneration in the future.

Contents

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1. Introduction

According to the World Health Organization, cardiovascular diseases (CVDs) are the number one cause of death worldwide and coronary artery disease accounted for >40% of CVD deaths in 2012 [1]. Current therapies mostly rely on the restoration of blood flow to the damaged area which cannot restore pump function following injury. Moreover, the shortage of donors for heart transplantation is the most unfavorable scenario for the end-stage patients [2].

A huge number of cardiomyocytes are necrotized and lost following myocardial infarction (MI). The human heart has very limited ability to regenerate lost cardiomyocytes and responds to injury by replacement of the infarcted area by activation of fibroblasts and formation of scar tissue. This response cannot regenerate cardiomyocytes and often impairs heart function [2]. Neonatal mammalian heart has the ability to regenerate following injury. However, this ability is lost in adult hearts [3]. Biological efforts to address this issue have led to the investigation of cellular and molecular approaches to restore heart function. These approaches include transplantation of stem/progenitor cells [4] or their lineage-restricted derivatives [5–7] and activation of endogenous cardiac progenitor cells [8].

Transplantation of cells from various sources into the heart of patients has been used as a regenerative cardiac therapy in clinical trials [4,9–12]. Having said that, culture of autologous cells from adult tissues takes a long time, which hampers future scaling-up of this treatment as well as its application in acute MI settings [9,11,13–16]. Although cell injection is a hope, there are challenges ahead, including delivery, retention of the cells, their integration with endogenous cells, rejection and cellular maturation.

Direct reprogramming of somatic cells into cardiomyocyte-like cells both *in vitro* and *in vivo* [17–20] is a new hope to restore heart function and induce regeneration. Indeed, the endogenous reparatory mechanism of the heart can be redirected and promoted into a regenerative process counting on the large population of cardiac fibroblasts [21,22], which can be regarded as a potential cell source for reprogramming toward cardiomyocyte fate and induced regeneration [23]. Several elegant efforts have been done to induce heart regeneration by direct reprogramming of cardiac fibroblasts of the infarcted area into induced cardiomyocytes (iCMs). Interestingly, findings revealed that *in situ* transdifferentiation of cardiac fibroblasts into induced cardiomyocyte-like cells (iCMs) results in functional improvements in mouse models of MI [18–20]. Translation of this new experimental technology into the clinic is a promising strategy for human cardiac tissue regeneration.

The current study specifically reviews what is known about *in vivo* or *in situ* direct cardiac reprogramming/transdifferentiation, its applicability, challenges ahead and future directions. It also deals with methods that can be applied for enhancement of reprogramming efficiency and quality. Furthermore, human *in vitro* cardiac reprogramming is discussed as a foundation for a possible future therapy in human heart regeneration.

2. *In vitro* cardiomyocyte reprogramming

Three decades ago, the Weintraub laboratory [24,25] revealed that ectopic expression of transcription factor (TF) MyoD is sufficient to convert mouse fibroblasts into stable myoblasts [25]. These findings were the first reports of direct cellular reprogramming by the forced expression of lineage-specific transcription factors (TFs). Then, in 1996, Murry and colleagues showed that high-dose *MyoD* adenovirus (10^{10} pfu) can induce myogenin and embryonic MHC expression (embryonic myofiber phenotype) in a few cells of cardiac granulation tissue *in vivo* [26]. They used MyoD (myogenic reprogramming factor [25]) for induction of regeneration in a model of cardiac injury [26]. 20 years later, after the advent of induced pluripotent stem cell (iPSC) technology in 2006 [27], direct reprogramming re-attracted attention as a hope for the production of a variety of cell types. To date, laboratories worldwide reported combinations of TFs capable of engineering cell fate. As a result, different cell types have been produced directly from terminally differentiated somatic cells.

To date, plenty of efforts have been accomplished to identify cardiomyocyte master regulators whose overexpression is capable of induction of cardiomyocyte transdifferentiation in fibroblasts (Table 1). In 2010, Srivastava's group [17] identified a set of three TFs, Gata4, Mef2c, and Tbx5 (GMT), which reprogrammed mouse postnatal cardiac and dermal fibroblasts into functional iCMs *in vitro*. A year after that, Ding and colleagues reported conversion of mouse fibroblasts into cardiomyocytes using a different approach named cell-activation and signaling-directed (CASD) lineage conversion [28,29]. Indeed, in their new approach, they utilized transient expression of pluripotency factors (Oct4, Sox2, Klf4 and c-Myc; OSKM) to induce a plastic state and then lineage-specific signals (small molecule Janus kinase (JAK) inhibitor) to direct reprogramming toward cardiomyocyte fate. Then, Song et al. added Hand2 to the GMT cocktail and showed that GHMT converts adult murine fibroblasts into beating iCMs *in vitro* more efficiently than GMT [20] (Table 1).

Surprisingly, despite significant overexpression of GMT factors, Chen et al. [30] failed to achieve the results of Ieda et al. [17] and reported that the method induced an inefficient and incomplete reprogramming. In their setting, GMT could not induce molecular and electrophysiological phenotypes of mature cardiomyocytes and no beating cardiomyocyte was observed. Thus, further optimizations seemed necessary to increase the efficiency of GMT cardiac reprogramming and also its reliability. Subsequently, certain efforts were made to improve the reliability and robustness of cardiac reprogramming.

Using a new TF screening approach, Protze et al. [31] identified the combination of Tbx5, Mef2c, and Myocd more efficient than GMT in upregulation of a broader spectrum of cardiac genes. This set of cardiac reprogramming factors transduced mouse fibroblasts into cardiomyocytes with expression of cardiac contractile proteins, cardiac-like sodium and potassium currents and action potentials; however, they did not beat. After that, Christoforou et al. demonstrated that addition of either MYOCD and SRF alone or in

conjunction with Mesp1 and SMARCD3 significantly enhances the cardio-inducing effect of GMT [32]. Although the reprogrammed cells developed the capacity to cycle intracellular Ca^{2+}, no significant membrane hyperpolarization or spontaneous contractile activity were detected.

Quantifying calcium activity as a stringent functional measure of success, Addis et al. [33] indicated that GMT plus Hand2 and Nkx2.5 (HNGMT) reprograms mouse fibroblasts into cardiomyocytes >50-fold more efficiently than GMT alone. Moreover, this combination induced robust calcium oscillation and increased the prevalence of spontaneously beating iCMs that persisted for weeks after inactivation of reprogramming factors.

Regarding the facilitation of transcriptional activity of reprogramming factors by fusion of the MyoD transactivation domain, Hirai et al. fused the MyoD domain to GMT and Hand2, and introduced these genes in different combinations into mouse fibroblasts. Interestingly, transduction of the chimeric Mef2c with the wild-types of the three others generated far larger contracting clusters of iCMs, faster, and 15-fold more efficient than the combination of the four wild-type genes [34].

To obviate the adverse effects of Matrigel-coated tissue culture polystyrene (TCPS) on cardiomyocyte transdifferentiation, Smith et al. [35] improved the CASD lineage conversion protocol of Efe et al. [28] via replacing Matrigel-coated TCPS by custom-engineered materials (poly(ethylene glycol) hydrogels). Their substrate improved reprogramming efficiency and yielded iCMs twice as much as the originally described substrate.

In 2014, Ding and colleagues improved their CASD approach and achieved successful reprogramming of mouse embryonic fibroblasts (MEFs) and tail tip fibroblasts (TTFs) into iCMs using a single factor (Oct4) and a chemical cocktail comprised of CHIR99021 (Wnt activator), SB431542 (TGFβ inhibitor), Parnate, and Forskolin (SCPF). These iCMs were spontaneously contracting and passed through a cardiac progenitor stage without experiencing a transient pluripotency state [36]. This finding reveals the feasibility of using small molecules to accurately specify cardiac cell fate.

To enhance the efficiency of the conversion, Ifkovits et al. [37], meanwhile, showed that the small molecule SB432542 (a TGF-β inhibitor) can increase the efficiency of HNGMT-mediated reprogramming of MEFs and adult cardiac fibroblasts (ACFs) by 5-fold. Their findings regarding the fact that TGF-β is a barrier of cardiac reprogramming then were confirmed by others [38,39].

On the role of miRNAs in the enhancement of cardiac reprogramming, Muraoka et al. indicated that addition of miR-133a to GMT (GMT/miR-133a) improves cardiac reprogramming from mouse fibroblasts [40]. In detail, miR-133a improved GMT transdifferentiation by silencing fibroblast program (i.e. repression of Snai1, a master regulator of epithelial-to-mesenchymal transition), as an important roadblock of cardiac reprogramming [40]. MiR-133 overexpression with GMT not only produced 7-fold more contracting iCMs from MEFs but also shortened duration of the process from 30 to 10 days, compared to GMT alone. Although not at the level detected with miR-133 overexpression, knockdown of Snai1 repressed fibroblast signatures and induced more beating iCMs with GMT transduction, almost reiterating the effects of miR-133 overexpression.

To avoid the use of viral vectors, miRNAs are appropriate alternatives, which can be chemically synthesized and delivered. Moreover, several miRNAs can be packed into the same delivery vector to increase the reprogramming efficiency and homogeneity [41,42]. In this respect, Jayawardena et al. showed that a single transient transfection of miRNAs 1, 133, 208, and 499 converts mouse cardiac fibroblasts to functional iCMs *in vitro* [42]. Moreover, treatment with JAK inhibitor I significantly improved not only the efficiency of reprogramming (up to 10 fold) but also the quality of converted cells. These miRNA reprogrammed iCMs exhibited molecular and cellular functional characteristics of cardiomyocytes, including cardiomyocyte specific gene expression, sarcomeric organization, calcium oscillations, and spontaneous beating [42].

Surprisingly, in an attempt to repeat chemical production of iPSCs [43], Fu et al. found some beating cardiomyocytes in their culture [44]. Then, they developed a two-stage reprogramming strategy to transdifferentiate mouse fibroblasts into spontaneously beating iCMs using only chemical cocktails. Interestingly, their chemical method for production of chemical iCMs passed through a cardiac precursor-like stage, and both atrial-like and ventricular-like cells were found in the culture. This can be considered as a safer approach for production of iCMs by circumventing the use of viral-derived factors and consequently safety concerns for potential clinical applications.

Together, these findings reveal applicability of direct cardiac reprogramming in culture dish using various approaches that suggests ideas for drug screening, cell therapy and *in vivo* reprogramming.

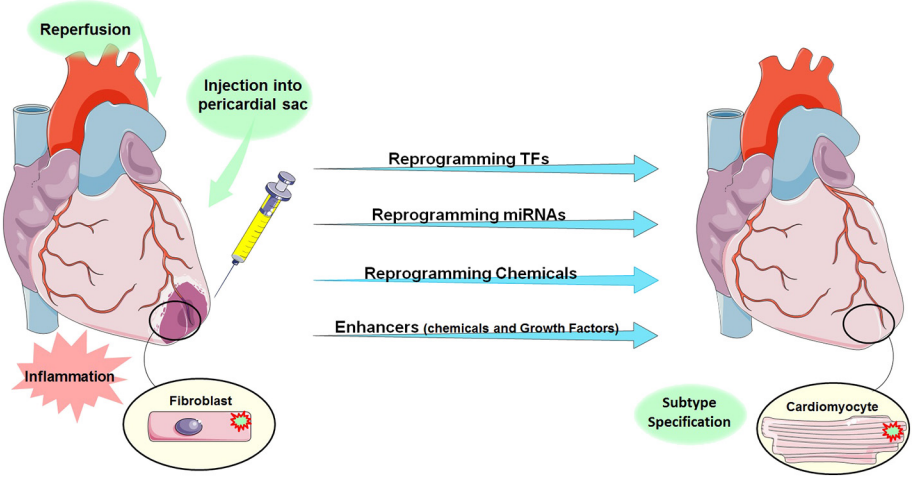

Fig. 1. A schematic representation of *in vivo* reprogramming of cardiac fibroblasts into cardiomyocytes. The figure illustrates different approaches that have been accomplished or can be implemented in the future to enhance cardiac regeneration by *in vivo* cardiac reprogramming. Reprogramming can be completed by transcription factors (TFs) or miRNAs. Moreover, *in vitro* chemical-only cardiac reprogramming is considered as a safer and more convenient strategy for heart regeneration without the risk of destruction by immune system responses. Furthermore, specific small molecules, growth factors and actions (e.g. reperfusion) can be used to enhance the efficiency and quality of reprogramming. However, subtype specification is another issue that is needed to be addressed to have a fully functional cell type that can integrate efficiently with surrounding tissue. Suggestively, reprogramming agents might be injected into the pericardial sac to have a less invasive operation in the future. (Green filled red outlined star inside cells shows cell tracer.)

3. *In vivo* cardiac reprogramming

In 1996, Murry et al. revealed that injection of *MyoD* into cryoinjured rat hearts can convert cardiac fibroblasts into skeletal muscle cells [26]. Indeed, they aimed at induction of heart regeneration by skeletal muscle master regulator MyoD. This was the first effort that was devoted to *in vivo* cardiac reprogramming. Then, in an attempt to investigate the role of cardiac TFs during cardiac development, Reiter et al. revealed that microinjection of *gata5* mRNA into zebrafish embryos induces ectopic expression of certain myocardial regulatory genes (*nkx2.5*, *gata4*, and *gata6*) and can generate ectopic regions of rhythmically contracting tissue in the head and tail [45]. In 2008, David et al. showed that ectopic expression of *MesP1* induces formation of ectopic heart tissue in *Xenopus laevis* [46]. They injected plasmids encoding *MesP1* into the animal pole (one blastomere) of two-cell embryos of *Xenopus laevis*. Interestingly, this ectopic expression of *MesP1* led to the generation of ectopic beating cardiomyocytes in various parts of developed tadpoles [46]. A year after this, Takeuchi and Bruneau [47] reported that ectopic expression of *Gata4*, *Tbx5*, and chromatin remodeling factor *Baf60c* in noncardiogenic mouse mesoderm induces spontaneously beating cardiomyocytes in 50% of transfected embryos (Table 2). In this transdifferentiation paradigm, Gata4 initiated the cardiac program, Tbx5 was found essential for full differentiation into beating cardiomyocytes, and Baf60c potentiated the function of Gata4 and Tbx5, partly by permitting binding of Gata4 to cardiac loci [47].

After the promise of *in vitro* cardiomyocyte reprogramming in 2010 [17], four independent groups, in 2012, revealed feasibility of *in vivo* cardiac reprogramming in mouse MI models [18–20,42,48] (Table 2) (Fig. 1). To elaborate, regarding their finding of *in vitro* reprogramming of mouse fibroblasts toward cardiomyocyte fate [17], Srivastava and colleagues indicated that local delivery of GMT encoding retroviruses into the peri-infarct areas of the mouse myocardium after coronary ligation converts non-myocytes into iCMs *in vivo* [19]. Using transgenic mice, lineage tracing studies revealed that non-myocytes, mostly fibroblasts, are the origin of reprogrammed cells. Different assays showed that iCMs were functional with ventricular cardiomyocyte-like action potentials and electrically coupled with neighboring endogenous cardiomyocytes indicating electrical maturation. Moreover, MRI and echocardiography assays indicated that *in vivo* GMT reprogramming of the infarcted mouse myocardium can reduce scar formation, increase vascular density in the border zone and improve heart function 3 months after MI (Supplementary information). Interestingly, they observed that *in vivo* reprogrammed cardiomyocytes were more mature than *in vitro* reprogrammed ones.

Similarly, Song et al. demonstrated that injection of GMT plus *Hand2* (GHMT), as the most optimal combination of cardiomyocyte reprogramming factors, converts non-cardiomyocytes into functional cardiomyocytes *in vivo* [20]. They displayed that *in vivo* iCMs that comprised about 6% of the cardiomyocytes of the peri-infarct area were functional, with Ca^{2+} transients and action potentials. They also

Table 1
Selected reports of mouse direct cardiac reprogramming.

Starting cell source	Reprogramming factors	Vector type	Enhancer	Ref
Adult cardiac fibroblasts (CFs) & tail-tip fibroblasts (TTFs)	Gata4, Mef2c & Tbx5 (GMT)	Individual pMXs Retroviral Vector or inducible Lentiviruses		[17]
Mouse embryonic fibroblasts (MEFs)	Oct4, Sox2, Klf4 & c-Myc (OSKM)	Individual pMXs retroviral vector	JAK inhibitor I & BMP4	[28]
CFs & TTFs	GMT plus Hand2	Individual pBabe Retroviruses		[20]
CFs & TTFs	GMT	Individual inducible Lentiviruses		[30]
MEFs & neonatal CFs	Tbx5, Mef2c, and Myocd	Individual Lentiviruses		[31]
Adult CFs from the infarcted area	GMT	Individual pMXs Retroviral Vector		[18]
CFs	miR-1, miR-133, miR-208 & miR-499	Introduction of synthetic mimics of mature miRNAs with Dharmafect1	JAK inhibitor 1	[42]
MEFs & adult CFs	GMT plus Hand2 & Nkx2.5 (HNGMT)	Individual inducible Lentiviruses		[33]
MEFs	GMT plus Mesp1, Myocd, Smarcd3 & SRF	Individual inducible Lentiviruses	Valproic acid and JAK inhibitor	[32]
Secondary or reprogrammable doxycycline-inducible transgenic MEFs & TTFs	Doxycycline-inducible OSKM	Transgenic cell line system	JAK inhibitor I & BMP4 Culture on engineered poly(ethylene glycol) hydrogels	[35]
MEFs & neonatal TTFs	Chimeric Mef2c (fused with the MyoD domain) plus wild type of Gata4, Hand2, and Tbx5	Individual pMXs-IRES-Puro Retroviral Vectors	Chimeric Mef2c	[34]
MEFs& Adult CFs	HNGMT	Individual inducible Lentiviruses	TGFβ inhibitor, SB431542	[37]
MEFs & Adult TTFs	Oct4	Inducible Retroviral Vector	SB431542, CHIR99021 (Wnt activator), plus parnate, and forskolin (SCPF)	[36]
MEFs & adult CFs	GMT plus miR-133	1. Individual pMXs Retroviral Vectors for GMT 2. Synthetic mimic of mature miR-133	JAK inhibitor I did not increase efficiency	[40]
Adult CFs, Neonatal & Adult TTFs	MGT: Mef2c-P2A-Gata4-T2A-Tbx5	Splice-ordered Single Polycistronic pMXs Retroviral Vector.		[50]
MEFs & Neonatal TTFs	CHIR99021, RepSox, Forskolin, VPA, Parnate, TTNPB, DZnep		1. Rolipram Enhancers for TTFs: Neuregulin 1 & granulocyte-colony stimulating factor	[44]
MEFs & Adult TTFs	Gata4, Mef2c, Tbx5 (Hand2)	Individual pMXs Retroviral Vectors	FGF2, FGF10, & VEGF	[64]
MEFs, Adult TTFs & Adult CFs	Gata4, Mef2c, Tbx5, Hand2 (GHMT), miR-1, miR-133	1. Individual Retroviral Vectors 2. Adeno-Associated Viral Vectors (inefficient)	A83–01 (inhibitor of TGF-β1), Y-27632 (inhibitor of ROCK)	[38]
MEFs, CFs & TTFs	Gata4, Mef2c, Tbx5, Hand2 (GHMT)	Individual pBabe Retroviruses	Akt1	[79]
Adult and Neonatal CFs, Adult and Neonatal TTFs, MEFs, $CD31^+$ Endothelial Cells	1. MGT 2. MT: Mef2c-Tbx5	Splice-ordered Single Polycistronic pMXs Retroviral Vector.	Bmi-1 knockdown	[83]
Neonatal CFs	GMT	1. Individual pMXs Retroviral Vectors. 2. Polycistronic pMXs Retroviral Vector.	1. Day 1 SB431542 2. Day 2 XAV939 (WNT inhibitor)	[39]

were coupled with surrounding endogenous or other reprogrammed myocytes. Moreover, GHMT reprogramming decreased scar size, increased muscle tissue, and improved contractile function of the infarcted mouse hearts at 12 weeks after MI (Supplementary information). In stark contrast, however, functional improvement was delayed and less complete using GMT without *Hand2*.

Confirming these works, another independent study by Inagawa et al. showed that injection of a polycistronic vector encoding GMT into the infarcted hearts converts resident non-myocytes into iCMs [18]. Although they demonstrated cardiomyocyte characteristics of reprogrammed cells by immunofluorescence and gene expression analysis, they did not evaluate functional recovery of infarcted mouse hearts after GMT delivery.

During the years after the reintroduction of *in vivo* cardiac reprogramming, multiple studies have reported certain modifications to demonstrate applicability and effectiveness of this regenerative approach. These works will be discussed in different parts of this review.

3.1. In vivo cardiac reprogramming using miRNAs

As an alternative to TF mediated reprogramming, Jayawardena et al. showed that cardiac TFs can be replaced by microRNAs [41,42] (Table 2). They demonstrated that a combination of four microRNAs (i.e. miR-1, -133, -208, and -499; miR combo) in the presence of JAK inhibitor JI1 converts neonatal mouse fibroblasts into iCMs *in vitro*. Moreover, they revealed that injection of lentiviruses encoding miR combo into the ischemic mouse myocardium converts cardiac fibroblasts into functional cardiomyocytes *in vivo*. In this study, they only investigated cellular and molecular characteristics of the reprogrammed cells [42]. In a recent study, they presented that *in vivo* iCMs acquired morphological, physiological, and functional properties of adult cardiomyocytes, including expression of cardiac myocyte markers, sarcomeric organization, excitation-contraction coupling, and action potentials [41]. Moreover, serial echocardiography by measuring left ventricular (LV) contractile function and fractional shortening revealed gradual improvement of cardiac function 6 weeks after MI/injection with an enhanced effect after three months [41]. What is more, miR combo

Table 2
Selected reports of *in vivo* cardiac reprogramming.

Gene delivery system	Method of delivery	Reprogramming factors	Substrate (starting) cells	Product cells	Animal model	Ref
50 pg of gata5 mRNA	Microinjection	Gata5 mRNA	1–4 cell stage embryos	Rhythmically Contracting Myocardial Tissue in the Head and the tail	Zebrafish developing embryos at 25 h after fertilization	[45]
MesP1 plasmid DNA (100 pg)	Injection into animal pole of two-cell embryo (one blastomere)	MesP1	Various regions of developed tadpoles: trunk region and dorsal part of ablated eye stage 45 Xenopus tadpole.	Ectopic Beating Cardiomyocytes	Two-cell *Xenopus laevis* embryos	[46]
Transient transfection by Expression constructs	Embryos were injected posteriorly under the visceral endoderm.	Gata4, Tbx5 and Baf60c	Mesoderm (non-cardiogenic posterior mesoderm and the extraembryonic mesoderm of the amnion)	Beating cardiomyocytes	Mouse embryonic day (E) 6.5–7.5	[47]
Polycistronic pMXs retroviral vectors	Injection into peri-infarct of mouse heart	Gata4, Mef2c, and Tbx5	Cardiac Fibroblasts	Cardiomyocyte-like cells	1. wild-type (WT) ICR mouse 2. immunosuppressed nude mouse. 3. α-myosin heavy chain (αMHC)-GFP transgenic mouse	[18]
Retrovirus	Injection into peri-infarct of mouse heart	GATA4, HAND2, MEF2C and TBX5	Cardiac Fibroblasts	Cardiomyocytes	Fsp1-cre/Rosa26-LacZ mice And Tcf21$^{iCre/+}$/R26R^{tdT} mice	[20]
Retrovirus	Injection into peri-infarct	Gata4, Mef2c, Tbx5	Cardiac Fibroblasts	Cardiomyocytes-like cells	Periostin-Cre:R26R-lacZ or Fsp1-Cre:R26R-lacZ mice	[19]
Lentivirus Adenovirus (VEGF)	Injection into peri-infarct	(GMT) + VEGF	Cardiac Non-myocytes	Cardiomyocytes	Fisher 344 rats	[48]
Lentiviraus	One injection at the time of injury, at 2 sites 2 mm below site of ligation	miRNAs 1, 133, 208, and 499	Cardiac Fibroblasts	Cardiomyocytes	1. Fsp1-Cre/*td*Tomato mice 2. αMHC-CFP/Fsp1-Cre/*td*TOMATO transgenic mice	[42]
Lentiviruses	Individual lentiviruses were injected once at the time of injury, at 2 sites 2 mm below the site of ligation	miRNAs 1, 133, 208, or 499	Cardiac Fibroblasts	Cardiomyocytes	Fsp1-Cre/*td*Tomato mice	[41]
Adenovirus	local injection in cardiac granulation tissue (wound) using a 27-gauge needle	MyoD	Cardiac Fibroblasts	Skeletal Muscle	Male Sprague-Dawley rats	[26]
Single polycistronic retrovirus	One injection into the boundary between the infarct zone and border zone.	MGT	Cardiac Fibroblasts	Cardiomyocytes	Periostin-Cre:R26RlacZ mice	[51]
1. Adenovirus for VEGF 2. Single polycistronic retrovirus for GMT	1. Injection of adenovirus encoding 3 major VEGF isoforms 3 weeks before GMT injection 2. Injection of singlet lentivirus encoding G, M, or T or a GMT "triplet" polycistronic lentivirus vector	1. VEGF 2. Gata4, Mef2c, Tbx5	Cardiac Fibroblasts	Cardiomyocytes	Fischer 344 rats	[49]
polycystronic retrovirus	1. Local injection of polycystronic GMT retrovirus 2. Intraperitoneal injection of SB431542 and XAV939 every day for 2 weeks after MI and intramyocardial injection of GMT retrovirus	1. GMT 2. SB431542 and XAV939	Cardiac Fibroblasts	Cardiomyocytes	Periostin-Cre:R26R-YFP mice	[39]

significantly decreased fibrosis one month post injury. As a comparison, both TFs [19,20] and miRNAs [41] improved cardiac function in the same time frame.

Although JI1 was not injected together with miR combo *in vivo*, addition of small-molecule enhancers to reprogramming cocktail may improve the *in vivo* conversion process (Fig. 1). Collectively, miRNA delivery offers a novel strategy for efficient cardiac reprogramming both *in vitro* and *in vivo*.

3.2. In vivo cardiac reprogramming using single polycistronic retrovirus

The use of separate vectors may cause heterogeneous and imperfect delivery of all genes into the cells. To introduce all genes into the cells and to homogenously overexpress all genes at sufficient levels, Inagawa et al. [18] used single polycistronic retroviral vectors expressing GMT using "self-cleaving" 2A peptides to induce full cardiac reprogramming *in vitro* and *in vivo*. The induced cells by this system expressed functional cardiac markers, including sarcomeric α-actinin and cardiac troponin T. Similarly, Mathison et al. used a single-promoter polycistronic vector and 2A self-cleaving peptides to homogenously express exogenous GMT [49] in infarcted rat hearts, this strategy improved the efficiency of iCM generation by 2-fold and also enhanced ventricular function.

As a comparison, Qian et al. [19] reported that 10% to 15% of GMT infected cells were converted into cardiomyocytes using single retroviruses, which is >10-fold higher than the efficiency reported by Inagawa et al. [18]. Although Inagawa et al. used a single promoter polycistronic retrovirus, differences between the reprogramming efficiency of two studies may originate from the different experimental settings, different mouse strains, transgene expression levels, and viral titers that were used in two studies (Table 2).

Recently, Qian and colleagues revealed that stoichiometry of G, M, T protein expression influences the efficiency and quality of cardiomyocyte direct reprogramming *in vitro* [50]. Although the exact underlying mechanisms remain unclear, they found that the polycistronic vector encoding MGT that expresses a relatively high expression of M and low expressions of G and T induces *in vitro* cardiomyocyte reprogramming more efficient (10-fold) than other combinations [50]. Interestingly, in another study, they reported that a single polycistronic retrovirus encoding MGT improves the efficiency of *in vivo* cardiac reprogramming and enhances the improvement of ventricular contractile function in comparison with the separate G, M, and T (G/M/T) delivery. Moreover, single-triplet MGT reduced scar size more than the pooled separate G/M/T viruses during *in vivo* direct cardiac reprogramming [51].

Collectively, one of the optimizations to improve efficiency, flexibility, and consistency of *in vivo* cardiac reprogramming could be the use of a single vector encoding reprogramming genes and results suggest that polycistronic systems can be valuable tools for this purpose [51,52].

3.3. Chemical enhancement of in vivo cardiac reprogramming

Recently, based on their *in vitro* findings, Srivastava and colleagues used chemical inhibitors of TGF-β and WNT signaling to improve the efficiency of *in vivo* GMT reprogramming [39]. They injected SB431542 and XAV939 (WNT inhibitor) intraperitoneally every day for 2 weeks following MI and intramyocardial injection of GMT (GMTc). They indicated that combinatorial chemical inhibition of TGF-β and WNT improves GMT-mediated *in vivo* cardiac reprogramming and cardiac function after MI in terms of quality, quantity, and speed. Compared to GMT alone, different assays showed improvement of heart structure and function as early as 1 week after MI and also its persistence over 12 weeks. GMTc further decreased scar size and thick bands of myocytes were observed throughout the infarct area in comparison to threads of myocytes in GMT alone treated hearts. Moreover, compared to GMT iCMs, *in vivo* GMTc iCMs were more similar to adult control cardiomyocytes concerning functionality and upregulation of cardiac genes. Furthermore, lineage-tracing revealed that remuscularization around the infarct area was due to the conversion of cardiac fibroblasts into newly formed iCMs.

This finding demonstrates the importance of identification and inhibition of barriers to cardiac transdifferentiation and also the feasibility of application of small molecules as enhancers that can improve efficiency and quality of *in vivo* cardiac reprogramming. However, despite encouraging evidence for chemical enhancement of *in vivo* cardiac reprogramming, more research is needed to achieve a robust chemical-only approach and the greatest outcome.

4. The impact of cardiac microenvironment on direct cardiac reprogramming

To regenerate the injured myocardium using *in vivo* reprogramming technology, a possibility is that cardiac microenvironment may specifically have a positive impact on the robustness of the process rather than environments of other tissues or *in vitro* conditions. There are several lines of evidence that confirm this notion. For example, dermal fibroblasts transfected with miRNAs showed a less mature cardiomyocyte phenotype *in vitro* than cardiac fibroblasts [42]. This represents the importance of the origin of the starting cell types that may determine their propensity for direct reprogramming and also the significance of using cardiac own cells to induce regeneration. In an experiment to evaluate the propensity of skin fibroblasts toward cardiac fate *in vivo*, Inagawa et al. injected GMT into the mouse skin. Interestingly, no cardiac transdifferentiation occurred *in vivo*. Suggestively, differences in skin and cardiac microenvironments, tissue permeability, and the resistant epigenetic state of dermal fibroblasts may account for the resistance of skin cells against cardiac transdifferentiation [18]. Indeed, the opposition of skin tissue environment against cardiomyocyte reprogramming shows the importance of compatibility between the origin of starting cells and target fate.

To explore the impact of cardiac microenvironment, Qian et al. indicated that reprogramming *in vivo* yields more mature cardiomyocytes with more similarity to their endogenous counterparts than the *in vitro* setting. Additionally, Song et al. stated that the efficiency of cardiac reprogramming *in vivo* in GHMT-treated hearts seems more than that of *in vitro* [20]. Regarding the favorable effect of the cardiac microenvironment, Ma et al. reported that although a single polycistronic MGT retrovirus induces cardiomyocyte reprogramming more efficient than the separate G/M/T vectors *in vivo*, both G/M/T and MGT result in the generation of iCMs with the same quality. This can be due to the important effect of native microenvironment on the maturation of iCMs *in vivo*. Thus, it seems that native microenvironment has a more significant role than the type of vector in improving the quality of the reprogrammed cells [51].

To identify the exact identity of *in situ* reprogrammed cardiomyocytes, Cahan et al., using a network biology-based computational platform (CellNet), showed that cardiac niche provides selective/inductive environmental signals favorable to the prosperous establishment of heart gene regulatory networks (GRNs) in the iCMs [53]. Indeed, cardiac tissue microenvironment can complete silencing of the native fibroblast GRN, significantly increase cardiac classification scores and close the fate of iCMs to cardiac GRN status. Building on the CellNet analysis of the *in situ* generated iCMs, *in vivo* niches provide an enhanced environment for direct conversion of resident starting cells to the target cell type [53,54].

In addition, supporting the positive role of the cardiac milieu, it has been presented that injected induced cardiac progenitor cells (iCPCs) into the infarcted mouse myocardium spontaneously differentiate into the three cardiovascular lineages within the adult heart without the need for exogenous cardiogenic signals [55,56]. Furthermore, it has been recently demonstrated that the neonatal rat heart confers maturity to transplanted human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) [57,58]. Interestingly, the infarcted adult

heart induced more cardiomyocyte maturation and hypertrophy than the neonatal heart [58]. This proves that *in vivo* environment of the infarcted ventricle can provide inductive signals specifically for three-lineage cardiovascular differentiation of iCPCs and maturation of injected cardiomyocytes.

The fact that *in vivo* cardiac reprogramming results in a higher efficiency and iCM quality than that of *in vitro* conditions demonstrates that cardiac tissue has a favorable microenvironment for the generation and maturation of newly reprogrammed cardiomyocytes. These positive effects may stem from the cardiac local microenvironment containing extracellular matrix, secreted proteins, and growth factors. Moreover, tissue stiffness, persistent contractility, surrounding contractile cells and other neighboring cell types may make the heart tissue more permissive and favorable to reprogramming than *in vitro* conditions [19,20,51]. Identification of such powerful inducers in cardiac microenvironment could provide new insights into the mechanisms of cardiac reprogramming. These findings are fundamental to potential future clinical applications of *in vivo* cardiac reprogramming.

5. Strategies to improve cardiac transdifferentiation

Generally, efficiency of the current methods of *in vitro* cardiac transdifferentiation is low in both human and mouse cells. Although the efficiency of *in vivo* reprogramming methods was reported higher than that of *in vitro*, it is still lower than expectations for human regeneration. In contrast to investigations reported successful cardiac reprogramming, even though with a low efficiency, Chen et al. reported that they could not achieve a successful cardiac reprogramming using GMT *in vitro* [30]. Moreover, differences between efficiencies of cardiac transdifferentiation of different donor cells can arise from the specific epigenetic state of each cell type and active barriers. Indeed, identification and removal of genetic and epigenetic barriers to cardiomyocyte reprogramming can improve the efficiency and quality of the process and also facilitate robust establishment of cardiac fate [16].

Two strategies can be employed to increase the efficiency of cardiac reprogramming, removal of barriers and/or administration of enhancing factors. Indeed, along with TFs, other enhancing factors (e.g. cytokines and small molecules) can also be added to reprogramming cocktail to increase the efficiency of *in vivo* cardiac reprogramming (Fig. 1). This section deals with several methods for improvement of the efficiency of cardiac reprogramming *in vitro* and *in vivo*.

5.1. Angiogenic factors

Cardiomyocytes are not the only required cell type for heart regeneration. To achieve a robust regeneration in infarcted myocardium, blood circulation is also needed to supplement the area with nutrients and oxygen, representing a need for angiogenesis. This part covers methods that can stimulate angiogenesis.

5.1.1. Thymosin β4

Thymosin β4 is a pro-angiogenic factor which has positive effects on cell migration and cardiac cell survival. This fibroblast-activating peptide also activates proliferation of epicardial cells [19,59]. It also can stimulate coronary vasculogenesis and angiogenesis in heart ischemic areas [59,60] and can prime differentiation of epicardial-derived progenitors into cardiomyocytes [61]. Thus, in promoting cardiac repair, multiple effects have been reported for thymosin β4, including angiogenesis, cell proliferation and cell survival [62]. Based on the hypothesis that infection of more fibroblasts could enhance reprogramming and functional improvement, Qian et al. co-injected thymosin β4 together with GMT into the infarcted hearts [19]. This treatment further reduced scar size and improved cardiac function compared with GMT alone. In this experiment, thymosin β4 did not increase the ratio of iCM reprogramming to total number of GMT-infected cells, remaining at 12%, while, the number of infected cells doubled upon co-injection. Indeed, the beneficial effect of thymosin β4 on improving cardiac function is associated with the delivery of GMT to more starting cells [19]. In this regard, Srivastava and colleagues indicated that fibroblast activator, thymosin β4, promotes cardiac repair and enhances the efficiency of GMT reprogramming by activation of fibroblast proliferation and increasing the number of donor fibroblasts [19,62,63].

5.1.2. Vascular endothelial growth factor

Based on the fact that ischemia adversely affects survival and function of native cardiomyocytes, exogenous transplanted cells and also newly generated iCMs, Mathison et al. hypothesized that formation of new blood vessels or vascularization in the scar tissue may be an important factor that can positively affect the *in situ* cellular reprogramming process [48]. They performed adenovirus-mediated gene delivery of all three major isoforms of vascular endothelial growth factor (*VEGF*), three weeks prior to administration of lentiviruses encoding GMT around the infarct zone of heart in rat MI models. Interestingly, they indicated that this treatment reduces the extent of myocardial fibrosis by half and improves post-infarct myocardial function (4-fold) in contrast to that of GMT or *VEGF* administration alone after 4 weeks. The presumed mechanism of action appears to be pre-neovascularization and reprogramming of non-myocytes into iCMs [48]. Furthermore, Yamakawa et al. indicated that VEGF together with FGF10 and FGF2 promotes cardiac reprogramming under defined serum-free conditions and increases spontaneously contracting iCMs *in vitro* by 100-fold. These growth factors also eliminated the need for Gata4 in induction of cardiac fate [64]. Accordingly, preconditioning the infarcted heart with the pro-angiogenic factor VEGF or supplementation of GMT with VEGF can be considered as a strategy to enhance the efficiency of GMT-mediated *in vivo* reprogramming, reduce scar size and improve myocardial function.

5.1.3. Relaxin and small-molecule compound 8

It has been revealed that relaxin hormone induces expression of *VEGF* and *bFGF* in normal human endometrial (NHE) cells [65] and THP-1 cells (monocyte/macrophage cell line) to induce proliferation and angiogenesis, however, does not directly act on endothelial cells [66]. Interestingly, findings have revealed that systemic administration of relaxin in rats causes an increase in *VEGF* and *bFGF* expression in inflammatory cells of ischemic wound sites and also leads to the formation of new blood vessels [66]. This could be employed as a strategy for induction of targeted revascularization remotely and systematically.

Xiao and colleagues recently reported the identification of a novel series of small-molecule agonists for relaxin/insulin-like family peptide receptor 1 (RXFP1), that non-competitively act with the relaxin hormone to activate this receptor [67]. Moreover, they demonstrated high *in vitro* plasma stability of the small-molecule 'compound 8', an agonist of RXFP1, even after 2 h of exposure. In contrast to the low metabolic stability of the recombinant hormone, compound 8 exhibited a long half-life in plasma and hearts of mice after a single intraperitoneal administration without any abnormal clinical behaviors or acute toxicity. Furthermore, compound 8 significantly activates transcription of *VEGF*, a relaxin target gene, in THP1 cells [67].

According to the beneficial actions of relaxin and its recombinant form, serelaxin, in the cardiovascular system [68,69], compound 8 could replace them as a potent, highly selective, orally bioavailable and easy to synthesize angiogenic small-molecule with a long half-life [67]. In this respect, local administration of compound 8 may improve revascularization at the site of injury and also the efficiency of cardiac reprogramming. Therefore, concerning advantages of small molecules [70,71] and the role of compound 8 in the induction of VEGF secretion at the site of injury, it could be applied to increase the efficiency of *in situ* cardiac reprogramming by stimulation of angiogenesis. Direct effects of relaxin and/or compound 8 on endogenous and iCMs and also infarcted heart remain to be uncovered *in vitro* and *in vivo*.

5.1.4. Small molecule Trichostatin A

Small molecules that can induce angiogenesis are in great demand to replace angiogenic cytokines. Recently, Palii et al. introduced transcription factor TAL1 as a key mediator of angiogenesis [72]. Indeed, TAL1 and downstream targets are necessary for revascularization function of endothelial colony-forming cells (ECFCs) *in vivo*. Results have demonstrated that trichostatin A (TSA, a histone deacetylase inhibitor) can enhance the migratory ability of ECFCs *in vitro*. It also increased *in vivo* revascularization potential of treated endothelial cells after their injection into the ischemic mouse muscle. As an underling mechanism, TSA exerts its effects by increasing histone acetylation and binding of the p300 at TAL1 target genes (e.g. *CXCR4*, *CDH5*, and *EFNB2*) [72]. Thus, TSA can be considered as a small-molecule that can stimulate revascularization and possibly be beneficial to *in vivo* cardiac reprogramming.

5.1.5. Small molecule Me6TREN

Injection of angiogenic factors is a feasible therapeutic alternative to promote angiogenesis and repair ischemic tissue. Regarding the finding that endothelial progenitor cells (EPCs) from peripheral blood or bone marrow can improve angiogenesis and blood flow recovery in damaged tissues, pharmacological mobilization of these cells can be an efficient therapeutic strategy for ischemic diseases. In this respect, Chen et al. introduced a novel small molecule, Me6TREN (Me6) [73,74], which can efficiently mobilize EPCs into the blood circulation [74]. They showed that a single injection of Me6 induces a long-lasting rise in the number of circulating Flk-1^+/Sca-1^+ EPCs [74]. Indeed, systemic administration of Me6 induced recruitment of autologous EPCs into the ischemic tissues, diminished apoptosis, and augmented the capillary and arteriole density in the ischemic hind limb tissue. This finding suggests Me6 as a potential useful chemical for improvement of *in vivo* cardiac reprogramming acting through enhancing autologous EPC recruitment and promoting angiogenesis.

5.2. Removal of molecular barriers to cardiac reprogramming

In general, to increase the efficiency of a specific type of reprogramming one approach is to remove its molecular barriers. To date, a quantity of barriers of pluripotent reprogramming has been known [54,75,76], while a few barriers of iCM reprogramming have been identified. However, these two different kinds of reprogramming (i.e. pluripotent and cardiogenic) may not completely share similar barriers. Indeed, iCM reprogramming possibly has its own specific barriers. Nevertheless, iCM generation using the CASD lineage conversion, which utilizes iPSC reprogramming factors may share similar roadblocks with pluripotent reprogramming [29]. Accordingly, depending on the strategy that is adopted for cardiomyocyte transdifferentiation (i.e. CASD or cardiomyocyte-TF reprogramming), removal of its own specific barriers can improve the process and increase the efficiency. Multiple efforts have been done to increase the efficiency of TF-mediated direct cardiac reprogramming in MEFs [31,32,37,40,50,64,77–79] although not exceeding 20%. Even more imperfect, the efficiency of cardiac reprogramming of adult mouse fibroblasts was reported <0.1%.

5.2.1. Enhancer and barrier signaling pathways

In 2014, Ifkovits et al. found that the TGF-β pathway is a barrier to cardiac reprogramming and acts at the early stage of the process [80]. To improve reprogramming efficiency, they showed that chemical inhibition of this pathway at the early stage (day−1), simultaneously with exogenous expression of reprogramming factors, gives rise to the greatest increase (5-fold) in iCM yield.

Recently, Song and colleagues also indicated that pro-fibrotic signaling, is a barrier of cardiac reprogramming and strongly antagonizes the process [38]. They showed that concurrent with GHMT-mediated cardiac reprogramming, pro-fibrotic pathways such as TGF-β and Rho-associated kinase (ROCK) pathways are activated during the early stages and inhibit the process. In this respect, they indicated that addition of miR-1 and miR-133 (2m) to GHMT (GHMT2m) improves reprogramming by enhancing cardiac gene expression and inhibiting pro-fibrotic events. Considering beneficial effects of GW788388, an inhibitor of TGF-β type I and II receptor kinases [81], and ROCK inhibitor Y-27632 [82] on restoration of heart function after MI in murine models, chemical inhibition of TGF-β or Rho-associated kinase pathways suppressed pro-fibrotic signaling and consequently enhanced the efficiency of cardiac transdifferentiation of MEFs up to 60% using GHMT2m [38]. Furthermore, this strategy considerably enhanced the kinetics of the process, with spontaneously beating cells emerging in <2 weeks, in contrast to 4 weeks with GHMT alone. In addition to embryonic fibroblasts, this approach improved reprogramming of adult cardiac and dermal fibroblasts into functional cardiomyocytes. This finding is in accordance with the result of Ifkovits et al., who firstly introduced TGF-β signaling as a barrier of mouse cardiac reprogramming [37].

In comparison with other studies, GHMT2m plus A83-01 (TGF-β inhibitor), as the most optimal combination, achieved the highest efficiencies reported to date with induction of ~7000 beating iCMs from 5000 MEFs on day 11, or ~300 beating iCMs from 5000 ACFs/TTFs by 1 month. Although inhibition of either TGF-β signaling or ROCK enhanced the efficiency, results have shown that addition of A83-01 and Y-27632 (ROCK inhibitor) together does not have synergistic effect and cannot increase the reprogramming efficiency more, representing the need for suppression of other barriers to further optimize cardiac transdifferentiation [38].

More recently, Mohamed et al., using a high-throughput chemical screen, identified TGF-β and WNT signaling pathways as barriers to mouse cardiac reprogramming [39]. They achieved an eight-fold increase in cardiac reprogramming when SB431542 and XAV939 were added at day 1 and 2, respectively. This is while they were dispensable after about 1 week of reprogramming. Using these chemicals, GMT-induced reprogramming efficiency (4%) enhanced to ~30% within two weeks and beating cells appeared as early as 1 week, in comparison to 6–8 weeks with GMT alone. Although not completely, the compounds approximated gene expression profile of iCMs to adult mouse ventricular cardiomyocytes. In this setting, combinatorial actions of SB431542 and XAV939 increased not only the quality, but also the quantity and speed of cardiac transdifferentiation *in vitro*. Moreover, this approach improved efficiency of not only mouse embryonic and post-natal fibroblasts, but also human cardiac fibroblasts. In detail, SB431542 downregulated expression of genes involved in fibrotic signal and extra cellular matrix formation, similar to what was reported by Ifkovits et al. [80] and Zhao et al. [38]. Inhibition of WNT signaling also enhanced reprogramming by facilitating GMT chromatin binding at the cardiac gene sites possibly by modulation of chromatin, DNA packaging and nucleosome organization.

To improve the process, meanwhile, Olson and colleagues screened 192 protein kinases and found that Akt/protein kinase B markedly accelerates cardiac reprogramming in mouse embryonic and adult fibroblasts [79] (Table 1). They added Akt1 to their established GHMT factors (AGHMT) and revealed that this treatment improves both quantity and quality of reprogramming. AGHMT improved the efficiency and devoted more maturity to iCMs as observed by spontaneous beating, cellular hypertrophy, and metabolic reprogramming. Interestingly, it was the first report that described polynucleate iCMs generated by direct reprogramming. As an underlying mechanism for enhancing effect of the phosphoinositide 3-kinase (PI3K)/AKT/mTOR pathway on cardiac reprogramming, insulin-like growth factor 1 (IGF1) signals via PI3K to Akt and then downstream signals of Akt (mTORC1 and Foxo3a), which are involved in iCM reprogramming are activated. More recently, this group reported that non-canonical Notch signaling is a barrier of cardiac reprogramming and that its chemical inhibition enhances mouse cardiac GHMT reprogramming by increasing the binding of MEF2C to cardiac gene promoters. Interestingly, combinatorial inhibition of Notch and activation of Akt1 improved the efficiency of the

process up to 70% with 45% of the iCMs representing spontaneous contraction [83].

These findings represent the potential of enhancing chemicals and reprogramming factors as a combinatorial strategy for efficient heart regeneration after MI.

5.2.2. The epigenetic regulator Bmi1

Recently, Qian and colleagues found that Bmi1 is a major epigenetic roadblock of the mouse cardiac transdifferentiation and that its inhibition considerably enhances the efficiency of GMT-mediated iCM generation [84]. Interestingly, *Bmi1* depletion allowed iCM reprogramming using *Mef2c* and *Tbx5* alone by de-repression of endogenous *Gata4* and making its exogenous counterpart dispensable. Therefore, identification and removal of specific barriers of iCM reprogramming can not only increase the efficiency of the process, but also decrease the number of required reprogramming factors.

In general, low efficiency of cardiac reprogramming reveals the need for an efficient and reliable method of cardiac reprogramming. Favorable factors and mechanisms inherent to the cardiac microenvironment are the reasons for increased efficiency of *in vivo* transdifferentiation and more mature *in vivo* iCMs. In addition to the abovementioned topics, identifying these favorable factors and also molecular mechanisms of the epigenetic remodeling and cardiac transdifferentiation could assist development of strategies for highly efficient and fast cardiac reprogramming and for *in vitro* (drug toxicity studies and disease modeling) and clinical applications [16]. Furthermore, to improve *in vivo* cardiac reprogramming in terms of quality and quantity a combinatorial strategy should be implemented to achieve the best results (Fig. 1).

6. Challenges and future directions

Besides the exciting developments that have been made in the field of *in vivo* cardiac transdifferentiation, questions have remained to be addressed regarding the translation of this technology into human cardiac regeneration and repair.

6.1. Delivery

Direct injection of GMT retrovirus into the peri-infarct area of a transgenic mouse heart induced expression of αMHC in only 3% of virus-infected cells after one week [18]. Regarding the limited injections and the limited area that each injection covers as well as the outpour of the injected cocktail from contractile myocardium, safer and more efficient methods of delivery with broader coverage are needed to be investigated.

To avoid outpour of the reprogramming cocktail, new methods (e.g. a hydrogel system and a fibrin-based glue approach [85]) are needed to be developed and expanded. Although direct intramyocardial injection following MI allows gene delivery to the desired area, it needs invasive surgery and multiple injections. In this respect, for future regenerative therapies, this method my not be sensible and applicable for some patients who are subjected to angioplasty.

In 1999, Fromes et al. revealed that the injection of an adenovirus vector encoding β-galactosidase into the pericardial sac of adult mice and rats results in a restricted transfection of the pericardial cell layers. To increase the coverage area and the efficiency of transfection, they injected a mixture of collagenase and hyaluronidase with the virus into the pericardial sac. Their results showed a large diffusion of the transgene activity within >40% of the myocardium [86]. Thus, as an alternative technique to intramyocardial injection during open-heart surgery, viruses/inducers could be directly injected into the pericardial space by a closed-chest minimally invasive method [87]. Furthermore, percutaneous transendocardial delivery is another approach that is less invasive than intramyocardial injection [88,89]. These methods could be less harmful for transfer of viruses/inducers into the heart muscle.

In general, as a suggestion, injection of reprogramming factors in combination with agents, which can make epicardium permeable (e.g. proteolytic enzyme) directly into the pericardial space through the chest, under ultrasound guidance, seems to be safer, less invasive and more efficient by delivering genes to more cells.

6.2. Inflammatory conditions

Another drawback, which can reduce the effectiveness of reprogramming treatment is the immune system response to viral vectors encoding reprogramming factors. It has been indicated that the number of fibroblasts infected by GMT retroviruses in infarcted cardiac tissue was reduced during 2 weeks after their injection due to the immune responses of the immunocompetent mouse in comparison with the immunosuppressed mouse. Moreover, treatment with cyclosporine A (an immunosuppressant) could not significantly prevent this reduction, while in nude mouse hearts, retroviral GFP was expressed up to 3 months after injection [18]. This suggests that immune responses contribute to the loss of viral-infected cells in immunocompetent animals. Additionally, intense inflammatory reactions were reported due to injection of a high dose of adenovirus in injured rat heart [26,90,91]. Therefore, immune system response to viral vectors is a substantial complication for induction of reprogramming in human tissues. To translate this technology into the human setting, methods are needed that will not stimulate the immune system (e.g. chemical reprogramming [70,71]). These issues emphasize again on the importance of utilization of small molecules for *in vivo* reprogramming applications. However, more investigations are still required to reveal the merits and demerits of *in vivo* application of viral and chemical reprogramming approaches.

6.3. Blood supply

In human setting, blood flow is returned by administration of a thrombolysis drug (e.g. streptokinase) immediately after MI to dissolve intracoronary thrombi and supply blood into the myocardium. In the next step, angioplasty or coronary artery bypass grafting is applied to open coronary artery narrowing or blockage. The *in vivo* cardiac reprogramming studies used murine models of MI that were created by permanent ligation of the left anterior descending (LAD) coronary artery [18–20]. Then, vectors encoding reprogramming factors immediately were injected into the peri-infarct area after MI. A drawback to this procedure is that induction of regeneration in an infarcted ischemic area seems to be more ineffective than an infarcted one, which is supplemented with blood flow. Required cells for heart regeneration can be generated through *in vivo* reprogramming, but with more efficiency in an environment that is supported with oxygen and nutrients. For instance, Song et al. demonstrated higher density of converted cardiomyocyte in the border zone adjacent to the non-infarct regions thanks to intact vascular structures and higher viral infection in this area [20]. Thus, factor injection in an area without blood supply may not achieve a robust reprogramming and regeneration. Regarding this drawback, injection after reperfusion or induction of blood vessel formation may achieve better results. Several strategies for enhancement of angiogenesis have been discussed in previous sections.

6.4. Cardiac subtype specification

It has been revealed that GMT or GMT/miR-133 mostly generates atrial-type myocytes *in vitro*, while GHMT produced all three types of myocytes [40,92]. Zhao et al. also showed that GHMT/miR-1/miR-133 plus A83–01 produces iCMs that are composed of different subtypes of cardiomyocytes, including ventricular and mostly atrial ones. Moreover, nodal-like action potentials fired by iCMs were representative of early

stage of development of cardiomyocytes [38]. Regarding the different types of cardiac myocytes, including sinoatrial nodal cells, atrial myocytes, and ventricular myocytes, robust protocols are needed to improve cardiac subtype specification and to direct cardiac reprogramming toward the desired mature cell types [52]. It is to be hoped that cardiac environment can differentiate reprogramming products into more specified and mature iCMs.

7. Human cardiac reprogramming

Direct reprogramming technology offers a new regenerative therapy for a damaged human heart, which is a poorly regenerative organ. Regarding the promising results from direct *in vivo* reprogramming of fibroblasts of the infarcted myocardium into functional cardiomyocytes in murine models, this technology could be potentially considered as a regenerative therapy in the future.

To that aim, the first step is to identify appropriate factors and conditions for production of human cardiomyocytes *in vitro*. In this regard, Islas et al., in 2012, reported that forced expression of *ETS2* and *MESP1* in the presence of Activin A and BMP2 converts human fibroblasts into cardiac progenitors [93]. Then, Olson and colleagues showed that cardiac TFs, including *GATA4*, *Hand2*, *T-box5*, and myocardin in combination with miR-1 and miR-133 reprogram neonatal and adult human fibroblasts into cardiomyocytes. After 4–11 weeks, reprogrammed cells acquired cardiomyocyte characteristics [94]. While GMT was found insufficient for human cardiac transdifferentiation, the addition of *Mesp1* and *Myocd* to GMT has been found to induce cardiac gene specific signatures in human fibroblasts *in vitro*. These human iCMs presented action potentials and synchronous contractility in maturation conditions [95]. Similarly, Muraoka et al. demonstrated that GMT plus *Mesp1* and *Myocd* (GMTMM) induces cardiac fate in human fibroblasts and that addition of miR-133a to this combination (GMTMM/miR-133a) significantly increases the efficiency and quality of human iCMs [40] (Table 3).

In parallel, Srivastava and colleagues revealed that GMT plus *ESRRG*, *MESP1*, *MYOCD*, and *ZFPM2* (7F) changes gene expression and phenotype of human fibroblasts into a cardiomyocyte-like state [96]. Their findings displayed that a minimum of five factors (5F; GMT, *ESRRG*, and *MESP1*) are sufficient to generate iCMs. Although *MYOCD* and *ZFPM2* were dispensable, their presence quantitatively and qualitatively improved human cardiac reprogramming, resulting in more mature iCMs. Furthermore, TGF-β1 improved the efficiency of 5F human iCM reprogramming to a level comparable to the 7F reprogramming. This is while TGF-β1 did not enhance 7F reprogramming. Although most of the iCMs were partially reprogrammed without any visible contraction after a long time in culture, approximately 20% of them represented some functional electrophysiological properties [96] (Table 3). This group, more recently, found that TGF-β and WNT signaling pathways are barriers of cardiac transdifferentiation and that chemical inhibitors of these pathways not only improve the quality and efficiency of human cardiac reprogramming but also decrease the number of required transcription factors to 4 factors (4F: GMT plus Myocardin) [39]. Indeed, this treatment made *Mesp1*, *Zfpm2* and *Esrrg* genes dispensable. Besides, addition of SB431542 and XAV939 to 4F (4Fc) or 7F (7Fc) doubled the percentage of reprogrammed human iCMs. Interestingly, 4Fc reprogrammed iCMs acquired calcium transients after just 10 days of reprogramming and exhibited sarcomere formation as early as 3 weeks.

Chemical enhancement of human cardiac reprogramming and reduction of required TFs will expedite the application of direct cardiac reprogramming strategy for the treatment of heart failure.

7.1. Integration free human cardiac reprogramming

Development of strategies to convert human somatic cells into different lineages (e.g. cardiomyocytes) using integration-free approaches will assist this technology to be applicable for future clinical applications. Toward this goal, Islas et al. [93] reported successful *in vitro* reprogramming of human dermal fibroblasts into replicative progenitor cells expressing core cardiac TFs by non-integrating TAT-ETS2 and TAT-MESP1-cell permeant proteins in the presence of activin A, and BMP2 [93]. Then, Li et al. demonstrated that delivery of modified proteins of GHMT using QQ-reagent together with three cytokines (BMP4, activin A, and bFGF) quickly and efficiently converts human adult fibroblasts into induced cardiac progenitor cells (iCPCs) [97]. These cells were multipotent cardiac progenitors and differentiated into three cardiac lineages *in vitro*. Moreover, transplantation of these iCPCs into the infarcted rat hearts decreased fibrosis and improved cardiac function after 4 weeks [97].

In a recent research, Ding and colleagues indicated that a combination of nine chemicals (9C) converts human fibroblasts into functional cardiomyocyte-like cells [98] (Table 3). In this study, fibroblasts were treated with 9C for 6 days and then cultured for 5 days in an optimized cardiac induction medium (CIM) containing cardiogenic molecules. For maturation, the induced cells were treated with human cardiomyocyte-conditioned medium for 20 days. Interestingly, this chemical reprogramming process was consistent with the developmental sequence of cardiogenesis during cardiac differentiation of human

Table 3
Selected reports of *in vitro* human cardiac reprogramming.

Starting cells	Gene delivery system	Reprogramming factors	Product	Ref
Human Neonatal Foreskin, Adult Cardiac and Dermal Fibroblasts	Retrovirus	GATA4, Hand2, T-box5, and myocardin in combination with miR-1 and miR-133	Cardiomyocyte-like cells	[93]
Human Neonatal Foreskin and Adult Cardiac Fibroblasts	Pantropic retrovirus	GMT plus Mesp1 and Myocd	Cardiomyocyte-like cells	[94]
Human Neonatal Dermal Fibroblasts	1. Doxycycline-regulated lentiviral vector system 2. TAT-ETS2 and TAT-MESP1 Proteins	ETS2 and MESP1 in the presence of Activin A and BMP2	Cardiomyocyte-like cells	[92]
Human Adult Cardiac Fibroblasts	Lentiviral vectors	GMT plus Mesp1, Myocd, and miR-133a (GMTMM/miR-133a)	Cardiomyocyte-like cells	[40]
Human Adult Dermal Fibroblasts	Nonviral, QQ-reagent based protein delivery system	GHMT using QQ-reagent together with three cytokines (BMP4, activin A and bFGF)	iCPCs	[96]
human embryonic stem cell derived fibroblasts	Inducible Retroviral Expression System	GMT plus ESRRG, MESP1, MYOCD, and ZFPM2	Cardiomyocyte-like cells	[95]
Human Foreskin Fibroblast and Human Fetal Lung Fibroblasts	Small-molecule	First, 9C for 6 days Next, culture for 5 days in cardiac induction medium (CIM). Finally, human CM–conditioned medium for 20 days	Cardiomyocyte-like cells	[97]
Human adult cardiac fibroblasts	pMXs retroviral vectors	1. Gata4, Mef2c, Tbx5, and Myocardin 2. SB431542 at 24 h post-infection and XAV939 48 h post-infection	Cardiomyocyte-like cells	[39]

pluripotent stem cells. Furthermore, in contrast to cardiomyocytes generated by TF-mediated reprogramming, chemically iCMs were highly reprogrammed and largely homogeneous. To evaluate the functionality, the 9C (6 days) and CIM treated cells (5 days) were transplanted into the infarcted hearts and results showed their maturation and integration into the diseased heart tissue after two weeks. This result shows that 9C induced fibroblasts are compatible with the host environment and can be further matured into cardiomyocytes *in vivo* [98]. Collectively, these findings could raise the hopes for application of an integration-free approach, especially a chemical approach, for *in vivo* reprogramming of heart non-myocytes into cardiomyocytes [29,70,97,98].

Although results of *in vitro* human cardiac reprogramming are promising, its low efficiency and slow kinetics impose major limitations for ultimate applications of this technology in regenerative medicine. Identification of genetic and epigenetic roadblocks of human reprogramming can result in methods for the enhancement of the process and will advance this new technology. Generation of functional human cardiomyocytes *in vivo* using pharmacologic agents is of particular interest. To this aim, more research is needed to enhance the process and close this important technology to potential clinical applications.

Suggestively, regarding the enhanced reprogramming of the mouse cardiac fibroblasts *in vivo*, reprogramming of human cardiac fibroblasts may result in more mature iCMs in *in vivo* condition. To induce regeneration in the human heart, preclinical studies in large animals can evaluate the safety and efficacy of the direct reprogramming technology.

8. Conclusions

Findings that are discussed here illustrate that fibroblasts in murine hearts can be converted into cardiomyocytes and can recover heart function after MI. Remarkably, heart microenvironment has been found to be more favorable to cardiac reprogramming than the *in vitro* conditions. Possibly, extracellular matrix, paracrine agents, contractile forces, electrical currents and other unidentified factors in the native microenvironment may exert their effects to improve maturation of newly reprogrammed iCMs *in vivo* [19,20,51]. As a result, utilizing endogenous cardiac non-myocyte cells as donor cells for *in vivo* cardiac reprogramming is also a promising approach for human heart regeneration. In this regard, although human cardiac reprogramming did not robustly result in fully functional iCMs *in vitro*, it may act better *in vivo*, similar to the mouse *in vivo* cardiac reprogramming [96]. Indeed, *in vivo* reprogramming may face fewer difficulties for its translation to the clinic than the other regenerative approaches such as cell injection. However, potential negative consequences of *in vivo* reprogramming (e.g. arrhythmogenicity) are also needed to be addressed [23]. Thus, despite encouraging results, this approach is still new and needs improvements in safety, delivery routes, types of inducers, efficiency, and kinetics.

Regenerative strategies, including cellular reprogramming and cell and factor-based therapies, can be improved by developing our insights over the genetic and epigenetic-regulatory mechanisms, enabling us to favorable manipulations and utilizations (Fig. 1). Small molecules have properties that make them ideal for induction of reprogramming *in vivo* [29,70,71,75]. Designing chemical-only protocols for induction of cardiac reprogramming can overcome safety-related concerns and allow less variable reprogramming methods. What is more, small molecules not only avoid immune system activation, but also can enhance the efficacy and kinetics of the process. Therefore, thanks to their applicability and convenience, small molecules are valuable alternatives for induction of heart regeneration in an integration-free, safer, non-immunogenic, and more efficient manner. In addition to small molecules, the use of CRISPR technology to substitute reprogramming transgenes by activation of expression of endogenous genes is worthy of investigation and may accelerate clinical translation of *in vivo* reprogramming. Moreover, future studies of *in vivo* cardiac reprogramming in large animal models will be an important step forward to advance its therapeutic applications.

Competing interests

The author declares that there are no conflicts of interest.

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Appendix A. Supplementary data

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