



Published in final edited form as:

Circ Res. 2013 September 13; 113(7): . doi:10.1161/CIRCRESAHA.112.300625.

Direct Cardiac Reprogramming: From Developmental Biology to Cardiac Regeneration

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Abstract

Heart disease affects millions worldwide and is a progressive condition involving loss of cardiomyocytes. The human heart has limited endogenous regenerative capacity and is thus an important target for novel regenerative medicine approaches. While cell-based regenerative therapies hold promise, cellular reprogramming of endogenous cardiac fibroblasts, which represent more than half of the cells in the mammalian heart, may be an attractive alternative strategy for regenerating cardiac muscle. Recent advances leveraging years of developmental biology point to the feasibility of generating *de novo* cardiomyocyte-like cells from terminally differentiated non-myocytes in the heart in situ after ischemic damage. Here, we review the progress in cardiac reprogramming methods and consider the opportunities and challenges that lie ahead in refining this technology for regenerative medicine.

Keywords

Cellular reprogramming; cardiomyocyte; fibroblasts; heart disease

Cardiomyocyte death is associated with diverse forms of heart disease, which is the leading cause of morbidity and mortality in industrialized countries. Myocardial infarctions (MI) are a major public health problem and affect more than 1 million Americans yearly. A tremendous loss of cardiomyocytes through apoptosis and/or necrosis underlies both acute and chronic MI. To compensate for the death of cardiomyocytes in the injured area, scar tissue is formed by activated fibroblasts. In addition, heart diseases related to pressure overload, such as hypertension or valvular disease, involve the death of cardiac muscle cells but the progression is slow and usually occurs over a long period of time. Human aging is associated with an irreversible loss of cardiomyocytes, which may account for the increased vulnerability of aged hearts to various risk factors¹.

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DISCLOSURES

D.S. serves on the Scientific Advisory Board of iPierian, Inc. and RegeneRx Biopharmaceuticals, Inc.

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Unlike amphibian and fish hearts², the human heart has limited regenerative potential. Although postnatal vertebrate cardiomyocytes undergo some degree of cell renewal, as suggested by transgenic mouse experiments³⁻⁵ and a more recent human radiocarbon dating study⁶, the rate of cardiomyocyte renewal is very low. A genetic fate map study in mice indicated that the endogenous regenerative capacity of the adult heart, albeit limited, largely comes from the differentiation of cardiac progenitor cells rather than replacement by existing cardiomyocytes through cell division^{7, 8}. A major challenge moving forward is to identify such cardiac progenitor pools in the adult heart and promote their expansion and differentiation potential *in vivo*. In contrast, Porrello et al. reported the neonatal mouse heart has a remarkable regenerative capacity within the first 7 days of life, similar to that of the zebrafish heart⁹. Genetic fate mapping suggests that myocyte proliferation is the main mechanism for this regeneration⁹, which is reminiscent of what has been found in the adult zebrafish heart^{10, 11}. Furthermore, in a recent report using stable isotope labeling with genetic fate-mapping in combination with multi-isotope imaging mass spectrometry, the authors concluded that pre-existing CMs are the dominant source of myocyte renewal in the adult mouse heart with or without injury⁵. Cardiomyocyte proliferation has been reported to be enhanced by overexpressing cyclinD2^{12, 13} or administering factors, such as Periostin¹⁴, fibroblast growth factor-1 (FGF-1)¹⁵ or neuregulin 1 (NRG1)¹⁶ in mouse models of MI. Most recently, addition of miR-199a and miR-590 also stimulated cardiomyocyte proliferation *in vitro* and *in vivo*¹⁷. Thus, there is increasing evidence that adult mammalian cardiomyocytes have the capacity to proliferate, but whether approaches to enhance this feature are sufficient to compensate for the functional loss of damaged myocardium awaits confirmation.

Another strategy to repair an injured heart is to supply new cardiomyocytes differentiated from multipotent cardiovascular progenitor cells (CPCs) or pluripotent stem cells, including embryonic stem (ES) cells and induced pluripotent stem (iPS) cells (reviewed extensively elsewhere¹⁸⁻²¹). Cardiomyocytes derived from pluripotent stem cells are a promising source of cells due to recent advances in efficiency of human cardiomyocyte differentiation. However, the poor survival, low maturational efficiency, and limited functional integration of engrafted cells are hurdles that must still be overcome. Introduction of adult progenitor cells in animal models has positive effects after MI, but the cells do not appear to persist, pointing to a potential paracrine effect (reviewed in^{22, 23}). Clinical trials are underway to evaluate efficacy, with mixed results to date²⁴.

Here, we will review a newly emerging strategy for cardiac regeneration that involves reprogramming the vast pool of non-myocytes in the heart into new cardiomyocytes²⁵. This approach builds on recent cellular programming knowledge²⁶ and leverages over 15 years of advances in cardiac developmental biology²⁰ to induce a cardiomyocyte-like fate in the adult heart. Cardiac reprogramming can be induced by a discrete set of factors but appears to have critical epigenetic blocks *in vitro* which are significantly overcome upon reprogramming of cells in their native environment *in vivo*^{25, 27-30}. We will review how advances in cellular reprogramming and developmental biology led to our more recent work in direct cardiac reprogramming and will consider the future potential of this rapidly evolving approach to cardiac regeneration.

Cellular reprogramming

For decades, the concept in the field of developmental biology had been that cells, once terminally differentiated, were relatively fixed in their cell fate. This dogma was first challenged in the 1960s by the observation that a somatic cell can obtain totipotency through nuclear transfer into an enucleated frog egg³¹. Cloning of Dolly the sheep in 1997 by nuclear transfer revealed that the mammalian egg also had similar ability in reprogramming

an adult nucleus to the embryonic state³². In the late 80s and 90s, further evidence suggested that cell fate conversion can take place by a more direct route. Blau and colleagues demonstrated that fusion of skeletal muscle cells with fibroblasts resulted in heterokaryons that induced conversion of fibroblasts into a skeletal muscle-like phenotype³³. Studies from the Weintraub lab subsequently demonstrated that fibroblasts could be converted into skeletal muscle cells *in vitro* with forced expression of the skeletal muscle “master regulator” gene MyoD, which encodes a basic helix-loop-helix domain-containing transcription factor^{34, 35}. Despite the race to identify individual transcription factors that could function to guide cell fate similar to MyoD for other lineages, including cardiomyocytes, the MyoD paradigm appeared to be an exception, rather than the rule. Meanwhile, studies in model organisms showed that forced expression of individual master regulatory transcription factors containing homeobox domains could induce formation of complex body structures, which is best exemplified by the induction of ectopic eye formation on the legs of *Drosophila* by overexpressing *eyeless*, the fly orthologue of Pax6³⁶. The concept of cell fate conversion bypassing normal developmental lineage progression therefore emerged, but was still largely neglected with respect to the application of such concepts in regenerative medicine.

Takahashi and Yamanaka's milestone publication in 2006 demonstrating the creation of induced pluripotent stem cells (iPSCs)³⁷ ushered in a new era of utilizing cellular reprogramming in regenerative medicine³⁸⁻⁴⁰. Co-expression of four transcription factors (Oct4, Sox2, Klf4, c-Myc) was sufficient to reprogram fibroblasts to pluripotent stem cells that had the potential to develop into viable animals^{41, 42}. Abundant evidence has demonstrated that iPSCs can be differentiated efficiently into multiple cell types that someday could be used for regenerative therapies, disease modeling and drug discovery. A key lesson from iPSC reprogramming was the recognition that a combinatorial code involving a discrete number of regulatory factors could be sufficient to induce cell fate change^{43, 44}.

Despite the difficulty of identifying MyoD-like factors for direct cellular reprogramming from one terminally differentiated adult somatic cell directly into another without taking a “detour” back to pluripotency, the iPSC experience raised the possibility of a combination of regulators that could together induce cell fate change, or transdifferentiation. This reprogramming strategy was first demonstrated by Zhou *et al.*, who directly converted exocrine pancreatic cells into insulin-producing endocrine cells in the mouse pancreas with the transcription factors, Ngn3, Pdx and Maf⁴⁵. Similarly, we showed that cardiac and dermal fibroblasts could be reprogrammed into cardiomyocyte-like cells by a combination of three transcription factors, Gata4, Mef2c, and Tbx^{25, 27}. The direct reprogramming of adult fibroblasts to neuronal-like cells was also achieved *in vitro* with forced expression of a combination of transcription factors^{46, 47} or microRNAs (miRNAs)⁴⁸, and similar observations were made for conversion to the hepatocyte lineage^{49, 50}. Unlike direct reprogramming with multiple factors, Szabo *et al.* showed that combining expression of only Oct4 with administration of select cytokines can reprogram human fibroblasts into hematopoietic progenitors⁵¹, while transient expression of iPSC reprogramming factors, followed by Jak/Stat inhibition, resulted in emergence of cardiomyocytes⁵². The advances in cardiac reprogramming are considered in more detail below.

Discovery of transcription factor-based direct cardiac reprogramming

Over the last 20 years, developmental biology studies have revealed complex and intertwined networks of signaling pathways, transcription factors and miRNAs that regulate formation and function of the heart^{20, 53, 54}. The networks are self-reinforcing with layers of positive and negative feedback loops. Transcription factors often function in common

complexes, and human mutations that disrupt their interaction can lead to similar forms of heart malformations, as seen with mutations in GATA4 and TBX5⁵⁵. In 2009, Takeuchi and Bruneau demonstrated that overexpression of Gata4, Tbx5 and the interacting chromatin remodeling protein, Baf60c, converts noncardiogenic mesoderm into beating cardiomyocytes in the embryo by a mechanism involving the induction of Nkx2-5 by Gata4 and Baf60c⁵⁶.

The adult heart has many cell types within the organ that are normally non-cardiogenic. The vast majority of these are cardiac fibroblasts, which comprise over 50% of cells in the heart and are derived from an extracardiac structure known as the proepicardial organ⁵⁷. Fibroblasts play an important structural and paracrine role supporting the neighboring myocytes⁵⁸. Upon injury, cardiac fibroblasts are activated and migrate to the site of injury to create scar tissue that replaces dead myocardium. Due to the abundance of resident cardiac fibroblasts, the ability to reprogram such cells *in situ* would represent a powerful approach for regenerating myocardium.

We leveraged the abundant knowledge of cardiac developmental biology to attempt reprogramming of adult somatic cells into cardiomyocyte-like cells²⁵. Despite the desire to ultimately reprogram *in vivo*, we established an *in vitro* assay to discover the minimal cocktail of factors that could convert non-cardiomyocytes in the heart into a more cardiomyocyte-like phenotype. We generated transgenic mice containing the EGFP reporter downstream of the cardiomyocyte-specific α MHC (Myh6) promoter⁵⁹ and isolated the EGFP-negative non-myocyte population, which consisted largely of fibroblasts. Upon retroviral introduction of 14 transcription factors, and a pool of miRNAs, neonatal α MHC-EGFP⁻ cells activated the expression of α MHC (Myh6) and the α MHC-EGFP reporter, which allowed quantitative analysis by fluorescence activated cell sorting (FACS). The miRNAs in this setting were dispensable. Through serial deletion of one transcription factor at a time, we narrowed the required reprogramming factors to three: Gata4, Mef2c and Tbx5 (GMT). The GMT cocktail was sufficient to induce GFP expression in about 15–20% of the cells, which we termed induced cardiomyocytes (iCMs). However, the vast majority of cells were partially reprogrammed, with 5% of the total infected cell population expressing additional cardiac markers, such as cTnT, and assembling sarcomeric structures; furthermore, only ~0.5% of the α MHC-EGFP⁺/cTnT⁺ cells were capable of beating.

Despite the low percentage of fully reprogrammed cardiomyocytes, genome-wide transcriptome studies of the α MHC-EGFP⁺ cells (~15% of infected cells) showed that the partially reprogrammed population induced a broad cardiac transcriptional program involving thousands of genes and also silenced the fibroblast transcriptome. Moreover, the epigenetic status of iCMs was similar to neonatal endogenous cardiomyocytes at loci examined, and the reprogramming event was stable, not requiring ongoing expression of GMT. Notably, the GMT cocktail also reprogrammed tail-tip dermal fibroblasts, albeit with lower efficiency, suggesting that the presence of a cardiac progenitor pool was not necessary for the presence of iCMs.

Interestingly, the more fully reprogrammed iCMs had action potentials that were most similar to adult ventricular myocytes. This observation was in contrast to the relatively immature electrical activity noted in ES- or iPS-derived cardiomyocytes. Using a Cre-based strategy, we found that iCMs never expressed *Mesp1* or *Isl1*, markers of early cardiac progenitors, during the process of cardiac reprogramming²⁵. This suggested that the reprogramming event represented a direct conversion from one adult cell type to another rather than traversing through a progenitor stage. The rapidity of initial conversion and the more mature electrophysiology observed in iCMs is consistent with this interpretation.

***In vivo* direct cardiac reprogramming**

The relatively poor quality and low efficiency of *in vitro* cardiac reprogramming might be due to the lack of a natural environment for cardiomyocytes on plastic dishes. Because the objective with this strategy was to harness the endogenous cardiac fibroblasts within the organ for regeneration without needing to use cell-based therapy, we attempted to deliver GMT *in vivo* after ischemic injury and convert non-myocytes to cardiomyocyte-like cells. Classic genetic lineage tracing studies with Periostin-Cre, Fsp1-Cre and α MHC-MerCreMer were performed to demonstrate that dividing non-myocytes infected by retroviruses could be converted into iCMs²⁷ over a period of 4 weeks. Careful studies demonstrated that the newly born myocytes arose from *in vivo* conversion rather than leaky Cre expression or cell-cell fusion. Importantly, intermediate stages of reprogramming to iCMs were identified and characterized at varying time points after retroviral infection, further supporting the idea of a progressive reprogramming process.

In vivo-derived iCMs developed many characteristics of endogenous CMs. They were binucleate, assembled sarcomeres and had cardiomyocyte-like gene expression by 4 weeks post-infection with GMT. Furthermore, single-cell analyses by patch clamp technology revealed that 50% of *in vivo*-derived iCMs closely resembled endogenous CMs with beating upon electrical stimulation and ventricular cardiomyocyte-like action potentials. The markedly improved quality of reprogramming *in vivo* compared to *in vitro* might have been due to signals from the microenvironment, exposure to the extracellular matrix, or the influence of mechanical forces while reprogramming. Importantly, we found evidence for electrical coupling of the *in vivo* reprogrammed iCMs with endogenous cardiomyocytes and other iCMs. *In vivo* delivery of GMT intramyocardially decreased scar size and attenuated cardiac dysfunction after coronary ligation, as assessed by MRI and echocardiography. As expected, the cardiomyocytes within the scar area of GMT-treated mice represented newly born iCMs as determined by lineage tracing experiments. The beneficial effects of GMT were enhanced with the addition of the pro-angiogenic and fibroblast-activating factor, Thymosin β 4, which independently promotes cardiac repair^{60, 61}.

In a similar study, Song *et al.*²⁸ replicated the findings of Ieda *et al.*²⁵ with GMT, and demonstrated that the addition of a bHLH domain-containing transcription factor, Hand2, could increase the efficiency of cardiac reprogramming *in vitro*. Hand2 was initially discovered in a search for a cardiac MyoD-like bHLH protein⁶², and is essential for a subset of cardiac progenitors⁶³⁻⁶⁵, but not sufficient to induce the cardiac phenotype. Importantly, Song *et al.* also showed direct conversion from non-myocyte to myocytes *in vivo* by retrovirally transducing fibroblasts with GMT plus Hand2 (GHMT) into hearts after coronary ligation. These *in vivo* induced cardiac-like myocytes were similar to the endogenous cardiomyocytes, based on their gene expression, sarcomere structure and electrophysiological features. Lineage tracing with Fsp1-Cre, an inducible Tcf21-iCre, and α MHC-MerCreMer demonstrated the origin of the induced cardiomyocyte-like cells was likely cardiac fibroblasts. This observation was further supported by the evidence that the genetically pre-labeled old myocyte pool was diluted by these newly born myocyte-like cells. Along with the emergence of new cardiomyocyte-like cells, improved heart function and reduced scar size were observed.

A third successful example of *in vivo* cardiac reprogramming was reported by Inagawa *et al.*³⁰. The authors used the same GMT cocktail and retroviral delivery method. Different from Qian *et al.* and Song *et al.*, immunosuppressed mice were used in an attempt to promote the survival of viral transduced cells. Reprogramming with these nude mice resulted in more iCMs with well-defined sarcomere structure. Furthermore, in order to improve the transduction of GMT *in vivo*, Inagawa *et al.* generated a polycistronic retrovirus expressing

GMT at near equimolar levels from the same promoter using “self-cleaving” 2A peptides that resulted in a better reprogramming efficiency. Although the global outcome of GMT introduction in this system was not clear, use of a 2A polycistronic vector and immunosuppressed mice further refined the *in vivo* reprogramming technology.

These three studies provide compelling evidence that the abundant non-myocyte pool in the heart, largely composed of fibroblasts, can be transdifferentiated into new cardiomyocyte-like cells *in vivo* after injury, resulting in regeneration of myocardium and improved cardiac function. The functional improvement can be partially explained by new myocytes that increase force generation, but a fundamental alteration in the nature of scar-producing fibroblasts could also account for part of the reduction in scar size and improvement of cardiac output.

Alternative strategies for cardiac reprogramming

The strategy involving serial deletion of one factor at a time can yield potential combinations of factors for reprogramming but, due to the complex interactions between networks, may not necessarily yield the optimal combination. To address this, Protze *et al.*⁶⁶ screened 120 triplet combinations of 10 important developmental cardiac transcription factors expressed by lentiviruses in mouse embryonic fibroblasts (MEFs) for their ability to induce a myocyte-like phenotype. Rather than measuring the activation of a single reporter, they assayed expression of a panel of cardiac genes by qPCR. In this screen, Myocardin, Mef2c and Tbx5 were the optimal combination to convert fibroblasts into myocyte-like cells, determined by gene expression, sarcomere formation and ion channel activity, suggesting multiple combinations may exist for efficient cardiac reprogramming. Protze *et al.* also performed a time-course experiment and showed a progressive process involved in direct cardiac reprogramming in which a more complete cardiac phenotype arose over time.

miRNAs have important roles in cardiomyocyte decisions and are often regulated by the major cardiac transcription factors and, in turn, titrate the dosage of the key transcriptional networks^{54, 67}. In particular, miR-1, the most abundant cardiac miRNA, promotes muscle gene expression and regulates many aspects of cardiac biology⁶⁸⁻⁷¹. Recently, Jayawardena *et al.* showed that a combination of four muscle-specific miRNAs (miR-1, miR-133, miR-208, miR-499) was sufficient to transdifferentiate mouse fibroblasts into cardiomyocyte-like cells *in vitro* and *in vivo*. Remarkably, in the presence of a JAK-1 inhibitor, miR-1 alone was sufficient to reprogram the cardiac fibroblasts²⁹.

While many groups have now successfully reprogrammed fibroblasts to cardiomyocyte-like cells *in vitro*, others have struggled⁷², highlighting the challenges to easily mastering this technique. The use of fresh, non-senescent fibroblasts, high titers of viruses expressing the reprogramming factors, and careful culture conditions are among the variables involved in achieving successful reprogramming⁷³. Future work on standardizing the conditions and improving the conversion efficiency *in vitro* will be necessary to make this a routine procedure in many other laboratories.

Challenges and future directions

Despite the excitement and potential of direct cardiac reprogramming technology, several challenges remain, as would be expected for any new technology. All of the work thus far has been performed in rodents. It will be intriguing to determine if human fibroblasts can be similarly reprogrammed into iCMs. Although this might be technically challenging, identification of additional factors or a different combination of factors might allow conversion to occur in human fibroblasts given the success in human neuronal and iPSC reprogramming. Recently, successful reprogramming of human fibroblasts to an early

cardiac progenitor state was reported using the transcription factors, MESP1 and ETS-1⁵². The cardiac progenitors were multipotent and could subsequently differentiate into myocytes with calcium transients. However, direct reprogramming to a human adult-like cardiomyocyte, bypassing the embryonic stage, has not yet been reported. In addition, preclinical trials on large animal models, such as pigs, should be rigorously performed with careful monitoring of potential side effects, especially the risk of arrhythmias. Finally, new non-integrating methods for delivery of reprogramming factors and methods to increase efficiency will be important.

The low conversion rate of fibroblasts to fully reprogrammed iCMs *in vitro* is a major challenge for deciphering the mechanism of reprogramming. The relative inefficiency *in vitro* is not surprising, considering the iPSC reprogramming efficiency rate (0.01–0.1%), which is likely due to major epigenetic barriers that cells cannot easily overcome⁷⁴. Unlike iPSCs, reprogrammed cardiomyocytes rapidly exit the cell cycle, making efficiency a much bigger concern. Understanding how progressive epigenetic and transcriptional changes occur temporally during direct cardiac reprogramming is an important first step towards overcoming these hurdles. As highlighted from two recent genome-wide epigenetic studies of ESC-derived CMs^{75, 76}, temporal alterations in chromatin structure patterns lead to activation of key genes associated with heart development and function at distinctive differentiation stages. Such mechanisms might be informative for cardiac reprogramming. Second, methods aiming to improve delivery efficiency, such as the utilization of a polycistronic vector³⁰ to control for homogenous reprogramming gene expression, nanoparticle techniques for specific and efficient targeting, immunosuppression to promote survival of transduced cells³⁰, and the administration of Thymosin β 4 to mobilize and activate fibroblasts⁶⁰, may increase the final number of reprogrammed iCMs. Third, techniques that enhance the maturation of cardiomyocytes should be used to promote the progression of iCMs from immature to mature stages. These include supplying the cells with cytokines at defined stages, altering the expression pattern of certain ion channel regulators, exposing cells to mechanical forces and providing extracellular matrix and/or endothelial cells. Last, small-molecule and secreted factor screens geared towards increasing iCM numbers and quality should be performed to identify externally administered factors that promote the reprogramming process.

Approaches to improve efficiency *in vitro* will aid in mechanistic understanding of the reprogramming process and may ultimately allow use of direct reprogramming to model human disease. Although current technology does not generate sufficient numbers of fully reprogrammed iCMs for disease modeling studies, improvements in efficiency will be valuable since iCMs appear to achieve electrical maturation that is more similar to ventricular myocytes, particularly when reprogrammed *in vivo*, and this has been difficult to achieve with ESC- or iPSC-derived cardiomyocytes. Generation of sufficient numbers of fully reprogrammed cells *in vitro* would also be valuable for drug toxicity studies and drug screening.

In contrast, current iCM technology is quite efficient for *in vivo* reprogramming. Thus, harnessing the vast endogenous pool of non-cardiomyocytes seems like a viable approach to regenerate heart muscle without cell-based therapy. Although this would require a gene therapy approach ideally using non-integration vectors such as adeno-associated viral (AAV) vectors, a reasonable regulatory path exists for virally mediated gene delivery with scores of FDA-approved trials underway and the recent approval of a gene therapy drug for lipoprotein lipase deficiency in Europe. Future identification of small molecules or secreted proteins that could replace each transcription factor, as has been done for iPSC reprogramming⁷⁷, may allow an alternative to gene therapy. It is possible that *in vivo* reprogramming of cells to regenerate damaged tissue will serve as a new paradigm for many

human diseases, and the lessons learned in the cardiac area will be applicable to strategies to realize this dream in other tissues. While many challenges lie ahead in advancing this nascent technology, the opportunities and the potential benefits are significant, and we are confident the field will continue to push this technology further in the years ahead.

Acknowledgments

The authors thank members of the Srivastava lab for valuable discussion and contributions, B. Taylor for assistance with preparation of the manuscript, and G. Howard for editorial assistance.

SOURCES OF FUNDING

D.S. is supported by grants from NHLBI/NIH, the California Institute for Regenerative Medicine, the Younger Family Foundation, the Roddenberry Foundation, and the Whittier Foundation.

Nonstandard Abbreviations

None

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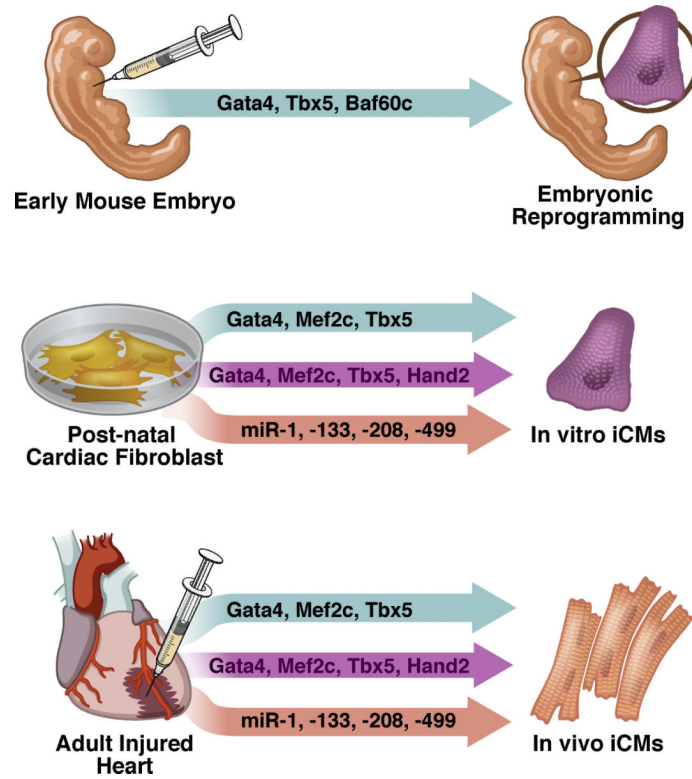


Figure 1. Approaches to direct cardiac reprogramming

Several methods for converting non-myocytes to cardiomyocyte-like cells have been published and are summarized here. In the first example, injection of factors into the non-cardiogenic mesoderm of E7.0 embryos resulted in ectopic beating cells (E8.5 embryos shown for simplicity). In vitro delivery of various cocktails resulted in primarily partially reprogrammed cells, while in vivo delivery yielded more fully reprogrammed cardiomyocyte-like cells.

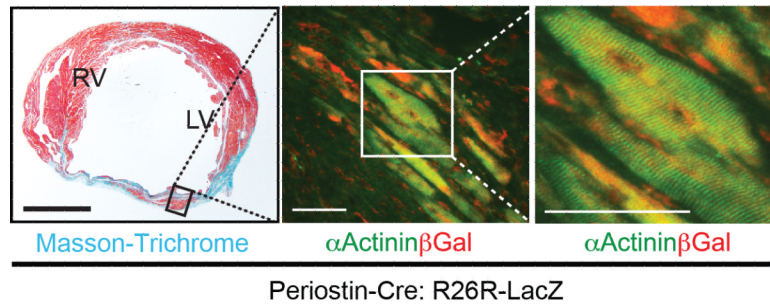


Figure 2. Direct conversion of cardiac fibroblasts into cardiomyocyte-like cells *in vivo*
Masson-Trichrome (left panel) and immunofluorescent staining for α -Actinin and β Gal (right panels) in GMT injected Periostin-Cre:R26R-lacZ mouse heart 4 weeks post-coronary ligation. Scale bars: 500 μ m in the left panel, 50 μ m in the right two panels.