Circulation Research
JOURNAL OF THE AMERICAN HEART ASSOCIATION

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Circ Res. 2012;111:5-8
doi: 10.1161/CIRCRESAHA.112.271452
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 0009-7330. Online ISSN: 1524-4571

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Cellular reprogramming achieved by somatic nuclear transfer or cell fusion has long been recognized.[1] The potency of specific transcription factors as cell fate determinants was first demonstrated by the discovery of MyoD, a master regulator for skeletal muscle differentiation, and by the subsequent identification of several genes as lineage-converting transcription factors in blood cells.[2,3] These pioneering works led to the landmark study by the Yamanaka laboratory that demonstrated the generation of induced pluripotent stem cells from fibroblasts by transducing four stem cell-enriched transcription factors, Oct4, Sox2, Klf4, and c-Myc.[4,5] Numerous subsequent improvements in techniques and additional factors have increased the efficiency and robustness of the technology, and such enhancements continue, as do analyses of the similarities and differences of induced pluripotent stem cells to embryonic stem cells. Increasingly efficient differentiation protocols now permit us to make significant quantities of many individual cell types from induced pluripotent stem cells.

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More recently, a "next generation" of cellular reprogramming has involved the direct conversion of one adult cell type into another by combinations of lineage-specific transcription factors or miRNAs, without passing through a pluripotent stem cell state. Direct lineage reprogramming, also known as transdifferentiation, can yield a diverse range of medically relevant cell types, including pancreatic β-cell-like, neuron-like, neural stem cell–like, cardiomyocyte-like, and hepatocyte-like cells from somatic cells.[6–10] In some cases, the resulting cellular phenotype has been more mature and adult-like than the corresponding pluripotent stem cell–derived phenotype, likely reflecting the lack of an embryonic intermediate during the reprogramming process.

A limitation of direct reprogramming is that cells appear to quickly exit the cell cycle as they adopt a unique fate. Thus, the utility of reprogramming in vitro is limited given the inability to generate larger numbers of cells for potential therapeutic or investigative use. However, leveraging the in vitro system as a screening tool to identify combinations of factors that could more fully reprogram cells in an in vivo setting to stimulate organ regeneration could be powerful. A greater efficiency of cellular reprogramming in the in vivo microenvironment, likely involving spatial cues, extracellular matrix proteins, and tensile forces, among other factors, was highlighted by work from Melton and colleagues.[6] In their work, a combination of transcription factors enriched in pancreatic β-cells could convert pancreatic exocrine cells into insulin-secreting endocrine-like cells in vivo, but not fibroblasts in vitro.[6]

In 2010, we reported the in vitro reprogramming of fibroblasts to cardiomyocyte-like cells by various combinations of core cardiac developmental transcription factors. We found a minimum cocktail of Gata4, Mef2c, and Tbx5 (GMT) was sufficient to broadly reset gene transcription toward a cardiomyocyte-like state.[7] Although global transcriptome changes were observed in pooled cells that activated cardiac reporter gene expression (approximately 15% of cells), only approximately 0.5% of these became fully reprogrammed with the ability to contract; we termed these cells induced cardiomyocytes. This efficiency was similar to that achieved by the original set of induced pluripotent stem cell reprogramming factors; however, unlike induced pluripotent stem cells, induced cardiomyocytes cannot be expanded in vitro. More recently, 2 other groups have reported the ability to reprogram cardiac fibroblasts in vitro into cardiomyocyte-like cells with GMT, while indicating improved reprogramming with either GMT plus addition of Hand2,[11] or by replacement of Gata4 with Myocardin.[12] A third group described in vitro reprogramming into cardiomyocyte-like cells with a combination of cardiac-enriched miRNAs and a small molecule inhibitor of Jak.[13] Combinations of transcription factors, miRNAs, and small molecules are currently being tried by many to identify the preferred combination of stimuli for cardiac reprogramming, as should be expected for any new technology.

Successful reprogramming of fibroblast cells to cardiomyocyte-like cells eluded the field for several decades, so it is no surprise that the conditions conducive to reprogramming are difficult to engineer. The approaches described to date require significant optimization of myriad experimental details, with many pitfalls that may lead to failure of reprogramming. Highly standardized conditions that make the process more efficient and more easily transferable among different laboratories will undoubtedly be developed, thereby facilitating successful entry into the field with greater ease. The difficulty for some laboratories to successfully achieve cardiac reprogramming is highlighted in the article by Chen et al,[14] which is published in this issue of *Circulation Research*.

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(*Circ Res*. 2012;111:5-8.)

***Circulation Research* is available at http://circres.ahajournals.org**
DOI: 10.1161/CIRCRESAHA.112.271452

Table. Comparing Methods for Cardiac Reprogramming

	Ieda et al (GMT)		Song et al (GMHT)	Chen et al (GMT)
Vector	Retrovirus	Dox-on Lentivurus	Retrovirus	Dox-on Lentivirus
Transgenic mouse	α-MHC-GFP	α-MHC-GFP	α-MHC-GFP	α-MHC-Cre Nkx2.5-Cre cTnT-Cre
Cell type	Neonatal/adult CF Neonatal TTF	Neonatal TTF	Adult TTF/CF	Adult TTF/CF
Transduction efficiency	>95% (TTF/CF)	80% (TTF) 40% (CF)	ND	ND
GMT expression	6–8 fold greater than cardiomyocytes		ND	18-fold to 1000-fold more than fibroblasts
Toxicity	No	Yes	No	ND
Cardiac induction	After 1 wk by FACS (α-MHC-GFP)		After 1 wk by FACS (α-MHC-GFP)	After 3 wk by FACS
αMHC-GFP	15%	0.5–1%	15–18%	0% (α-MHC-Cre reporter)
cTnT	5%	ND	9%	35% (cTnT-Cre reporter)
Gene expression analyses				
Cell type	αMHC-GFP$^+$ cells	ND	CF transduced with GHMT	Tbx5-expressing cells
Array data	Similar to neonatal CMs	ND	Cardiac gene upregulation	Similar to TTF/CF
Immunohistochemistry	α-Actinin$^+$ cTnT$^+$ ANP$^+$	α-Actinin$^+$	α-Actinin$^+$ cTnT$^+$ cTnI$^+$	ND
Function	Action potential Cell contraction Ca^{2+} transient	ND	Action potential Cell contraction Ca^{2+} transient	Ca^{2+} channel–mediated depolarization
Cell transplantation	α-MHC-GFP$^+$ α-Actinin$^+$	ND	ND	Cell death

ANP indicates atrial natriuretic peptide; CF, cardiac fibroblast; CM, cardiomyocyte; cTnT, cardiac troponin T; FACS, fluorescence-activated cell sorting; GFP, green fluorescent protein; GMHT, Gata4/Mef2c/Hand2/Tbx5; GMT, Gata4/Mef2c/Tbx5; MHC, myosin heavy chain; ND, not determined; TTF, tail-tip fibroblast.

Ieda et al used retroviral or Dox-inducible lentiviral vectors to express GMT but performed most experiments and in-depth analyses in neonatal CFs with retroviruses.[7] Song et al[11] used retroviral vectors for GMHT overexpression in adult CFs or TTFs. Chen et al used adult CFs/TTFs with lentiviral vectors expressing GMT.[14]

Chen et al report their difficulty in expressing levels of Gata4, Mef2c, and Tbx5 sufficiently high to promote robust reprogramming and correspondingly observe only a few changes toward a cardiomyocyte-like phenotype and a minimal shift in the overall transcriptome of the overall population of transduced cells in vitro.

It is worthwhile to consider the technical differences between the reprogramming achieved by Ieda et al[7] and Song et al,[11] compared with the less successful results described by Chen et al,[14] with the objective of clarifying aspects of reprogramming that remain challenging and that should be addressed by future research (Table). First, the starting cell type and the condition of the cells are pivotal for successful reprogramming. Chen et al utilized 3- to 6-week-old tail-tip fibroblasts (TTFs) or cardiac fibroblasts for their studies. In our experience, neonatal cardiac fibroblasts were more amenable to reprogramming than TTFs, with a greater percentage of α-myosin heavy chain (α-MHC)-GFP$^+$ cells expressing cardiac troponin T and more complete alteration of epigenetic marks at specific loci. We did not see any TTF that were more fully reprogrammed with the ability to contract. Similarly, Protze et al[12] had more success with GMT in neonatal cardiac fibroblasts compared with TTFs, although Song et al report the emergence of some beating cells from TTF with GMT plus addition of Hand2 after prolonged periods in culture for more than 1 month. In addition to the source of fibroblasts, the health and senescent state of primary fibroblasts had major effects on reprogramming. We found α-MHC-GFP induction by GMT was best in fresh primary fibroblasts without passage, and the efficiency progressively decreased by approximately 50% with each passage of primary fibroblasts.

Second, high expression levels and proper stoichiometry of reprogramming factors are necessary for success. In our experience, cells reprogrammed to activate the α-MHC-GFP reporter using retroviral vectors had six-fold to eight-fold more expression of all three factors than neonatal cardiomyocytes, which was much greater than fibroblasts; fibroblast cells that were infected by the viral vectors but failed to reprogram had significantly lower levels of expression. Chen et al utilized lentiviral vectors but never achieved high levels of expression of all three reprogramming genes in the same cell population. In their experience, Mef2c levels were only 10-fold greater in transduced TTFs than in nontransduced cardiac fibroblasts; Gata4 levels were only eight-fold greater in transduced cardiac fibroblasts than fibroblasts. One would

not expect significant reprogramming with the levels of expression achieved by Chen et al because greater levels of each factor would be required.

Third, the reporter system used for screening will affect the results. We generated transgenic mice with the α-MHC promoter driving GFP and screened through many mice before selecting one that had the most reproducibly high expression of GFP in cardiomyocytes with the greatest specificity. Chen et al utilized Cre-dependent reporter systems but saw no activation of the α-MHC-Cre or Nkx2.5-Cre reporters, but there was robust activation of the cardiac troponin T-Cre reporter. Careful validation of the reporter systems and of the percentage of cardiomyocytes expressing the reporter is important, as is monitoring expression of several markers, such as the expression of α-MHC and cardiac troponin T in the same cell, among others, as we had reported.

Finally, for whole transcriptome analysis, meaningful experiments require selection of the subset of cells that have begun to reprogram. In our studies, a reporter system was used to select the fraction of cells that appeared to be shifting in gene expression from the much larger population that received the reprogramming factors but failed to respond. In this setting, we observed a broad resetting of gene expression. Whereas the failure to express high levels of the reprogramming factors by Chen et al likely precluded broad alterations in gene expression, even partial resetting would have been undetected by their approach because all cells that expressed the Tbx5-expressing virus were used for microarray analysis. For reprogramming studies, there is limited value in gene expression analysis without purifying cell types of interest.

The article in this issue[14] of *Circulation Research* highlights the fact that with current technology, direct cardiac reprogramming in vitro is challenging and requires scrupulous attention to a great variety of technical details. Already several groups are improving on the original recipe and, as the field develops, other small molecules, secreted proteins, or miRNAs likely will improve the efficiency in vitro. However, the clinical potential of this technology for cardiac regeneration lies not in the ability to reprogram cells in culture, but rather in harnessing the vast pool of resident cardiac fibroblasts for in situ reprogramming into cardiomyocytes that could integrate with preexisting cardiomyocytes and contribute to force generation of the intact heart. To this end, the recent articles from our group and from the Olson laboratory indicate the plausibility of this approach.[11,15] In these studies, lineage tracing experiments labeling nonmyocytes in the heart demonstrated the emergence of new cardiomyocyte-like cells from the nonmyocyte population on expression of GMT or GMT plus addition of Hand2. Most importantly, both studies suggest that the in vivo microenvironment, likely enriched by secreted signals, components of the extracellular matrix, and mechanical forces, significantly enhances the degree of cardiac reprogramming by GMT or GMT plus addition of Hand2. The percent of reprogrammed cells that contract with electric stimulation increased to approximately 50%.[11] Furthermore, new cardiomyocyte-like cells reprogrammed from fibroblasts resident within the intact heart could electrically couple with preexisting cardiomyocytes. Most importantly, adding the reprogramming factors by a gene therapy approach resulted in improved cardiac function and a decrease in total scar area after myocardial infarction in a murine model. Thus, although it remains important to use a cell culture system to refine cardiac reprogramming technology, it may not be necessary to obtain the optimal type of cardiomyocyte-like cells in culture if the ultimate use of the "recipe" will be in vivo.

To achieve the promise of in vivo reprogramming of tissues to the desired cell types, many challenges remain. In addition to identifying the optimal conditions for generating cardiomyocyte-like cells, efficacy and safety issues will be important to resolve in larger animals. Whereas gene therapy approaches to deliver reprogramming factors may have a viable regulatory path, attempts to replace transcription factors with small molecules and/or secreted proteins would be valuable. Experience from the induced pluripotent stem cell field suggests that at least some reprogramming factors can be substituted with small molecules, and that epigenetic regulators may enhance efficiency. As this young field develops, there will be many challenges to overcome, but the recent reports of successful in vitro and in vivo reprogramming by many groups firmly establish the conceptual advance that nonmyocytes can be transdifferentiated to cardiomyocyte-like cells capable of contractile performance. Future studies in human cells, development of safe and efficient systems for delivery of reprogramming factors into cells of the heart in situ, and understanding the molecular mechanisms involved in direct cardiac reprogramming are necessary to advance this technology for future clinical applications.

Sources of Funding

M.I. was supported by research grants from JST CREST and JSPS. D.S. was supported by grants from National Heart Lung and Blood Institute/National Institutes of Health, the California Institute of Regenerative Medicine (CIRM), the Roddenberry Foundation, the Younger Family Foundation, and the L.K. Whittier Foundation.

Disclosures

D.S. is a scientific cofounder of iPierian and is on the scientific advisory boards of iPierian and RegeneRx Pharmaceuticals.

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