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Mini Review

Direct cardiac reprogramming by defined factors

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The potency of specific transcription factors as cell fate determinants was first demonstrated by the discovery of MyoD, a master gene for skeletal muscle differentiation, and by the subsequent identification of several genes as lineage-converting factors within the blood cell lineage. These pioneer works led to the landmark study by Dr. Yamanaka and colleagues that is reprogramming of somatic cells into a pluripotent state by transduction of the four stem cell-specific transcription factors, Oct4, Sox2, Klf4, and c-Myc. This study fundamentally altered the approach to regenerative medicine and also inspired a new strategy to generate desired cell types by introducing combinations of lineage-specific transcription factors. In fact, it has been demonstrated that a diverse range of cell types, such as pancreatic β-cells, neurons, chondrocytes, and hepatocytes, can be induced from differentiated somatic cells using lineage specific-reprogramming factors. We and other reported that functional cardiomyocytes can be generated directly from fibroblasts using several combinations of cardiac-enriched factors *in vitro* and *in vivo*. The present article reviews the pioneering and recent studies in cellular reprogramming, and discusses the perspectives and challenges of direct cardiac reprogramming in regenerative therapy.

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Introduction

Developmental biologists have long recognized that differentiated cells maintain their state for years and rarely switch to a new differentiation state. Indeed, once cells reach a stable position by a progressive or sequential differentiation from the pluripotent state, some become terminally differentiated and undergo no further cell division. However, experiments performed several decades ago showed that dormant gene expression programs could be induced in differentiated cells by cell fusion or nuclear transfer to produce a different cell type[1)]. Moreover, lineage conversions have been accomplished simply through the introduction of defined transcription factors[2)]. While cell-type conversion phenomena are scientifically intriguing, the

usefulness of this technology for regenerative medicine remained largely neglected until the generation of induced pluripotent stem cells (iPSCs) by Takahashi and Yamanaka[3, 4].

The discovery of iPSCs recently brought a new approach in regenerative technology which is the direct generation of specific cell types from mature cells using a combination of lineage-specific factors that bypasses the need to go through a stem cell state. Recent studies have demonstrated that direct lineage reprogramming yields a diverse range of medically relevant cell types, including pancreatic β-cells, neurons, chondrocytes, and hepatocytes[5-11]. Recently, we and others showed that somatic cells could be reprogrammed directly into cardiomyocyte-like cells without first becoming stem/progenitor cells[12-16]. We found that a combination of three cardiac-specific transcription factors, namely *Gata4, Mef2c*, and *Tbx5*, directly induced cardiomyocyte-like cells from mouse fibroblasts *in vitro*[12]. Direct gene transfer of the reprogramming factors *in vivo* converts resident cardiac fibroblasts into functional cardiomyocytes in mouse injured hearts, improving cardiac function[16-18]. More recently, we and others reported that human fibroblasts could be reprogrammed into differentiated cardiomyocytes by using either Gata4, Mef2c,Tbx5, Myocd, and Mesp1 or Gata4, Hand2, Tbx5, Myocd, miR-1, and miR-133, and into a cardiac progenitor-like state by using Ets2 and Mesp1[19-21]. Here I will review the pioneering works of cell fate conversion and discuss recent studies of direct cardiac reprogramming using defined factors.

Transcription Factors Induce Cell Fate Conversion

In the 1960s, John Gurdon and colleagues demonstrated that the nucleus of a differentiated frog cell, when transferred into an enucleated egg, could be reprogrammed back to the totipotency of a zygote and then give rise to a whole new frog[1]. Following this work, the potency of specific transcription factors as cell fate determinants was first demonstrated by the discovery of MyoD. Davis et al. found that *MyoD*, a crucial transcription factor in muscle formation and differentiation in vivo, converted fibroblast cell lines into stable myoblasts[2, 22, 23]. After the discovery of *MyoD*, conversion of one cell type into another was predominantly demonstrated for hematopoietic cells, which are an ideal cell type for lineage conversion experiments because the cellular lineages are well defined[24-28].

Takahashi and Yamanaka achieved a breakthrough in this field by overexpressing four embryonic stem cell (ESC)-specific transcription factors in fibroblasts to induce a pluripotent state, resulting in the so-called iPS cells. Using retroviral vectors, they expressed 24 candidate genes and selected for reprogrammed cells by incorporating neomycin resistance and β-galactosidase reporter genes into Fbx15, a gene specifically expressed in pluripotent stem cells. The combination of 24 factors activated Fbx15 and induced the formation of drug-resistant colonies with characteristic ESC morphology. Successive selection rounds to eliminate individual dispensable factors led to the minimally required core set of four genes, comprising Oct4, Sox2, Klf4, and c-Myc and referred to as the Yamanaka factors[3]. Many laboratories have since improved iPSC generation techniques to show that iPSCs share all defining features with ESCs, including expression of pluripotency markers, reactivation of both X chromosomes, and the ability to generate chimeric mice.

The generation of iPSCs also sparked interest into the new field of converting mature cell types directly into another cell type using a combination of lineage-specific factors. For example, Vierbuchen et al. converted mouse dermal fibroblasts into functional neurons *in vitro* using the neuronal lineage-specific transcription factors *Ascl1, Brn2*, and *Myt1l*[8]. Zhou et al. provided the first evidence of cellular reprogramming *in vivo* using defined factors[9]. They showed that gene transfer of the three transcription factors *Ngn3, Pdx1*, and *Mafa*[29] efficiently reprogrammed pancreatic exocrine cells into functional β-cells in the mouse. Although the three factors were not able to convert fibroblasts into β-cells *in vitro*, the newly generated β-cells *in vivo* were indistinguishable from endogenous islet β-cells in terms of their structure and the gene expression patterns.

Gata4/Mef2c/Tbx5 Directly Reprogram Fibroblasts into Cardiomyocytes *in vitro*

While embryonic mesoderm can be induced to generate cardiomyocytes, no master regulator of cardiac differentiation had been identified that parallels MyoD. We thus hypothesized that a combination of key developmental cardiac genes is required to directly convert fibroblasts into cardiomyocytes. To test this, we selected 14 genes as candidates for cardiac reprogramming; all are specifically expressed in embryonic cardiomyocytes and are critical for

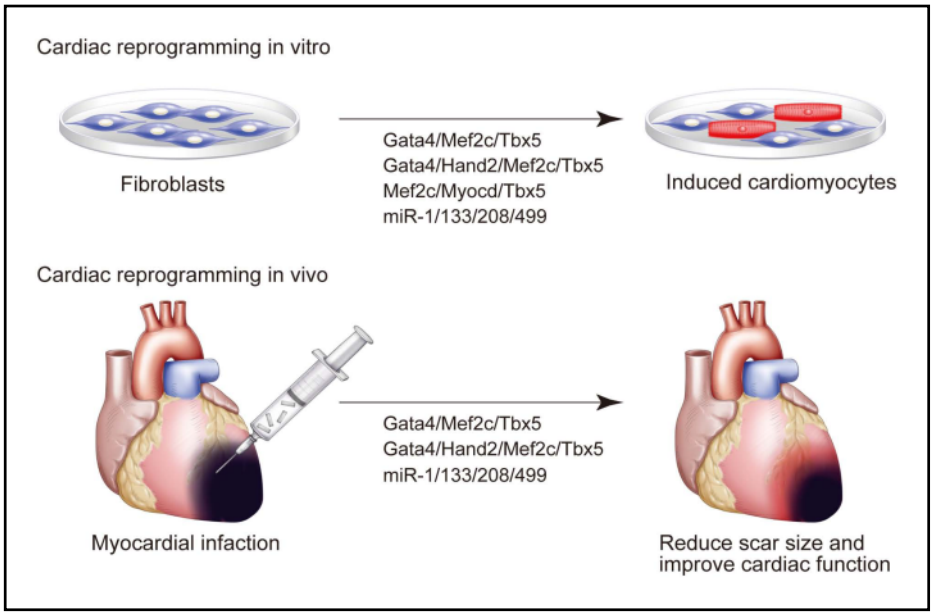

Fig.1 Mouse Cardiac Reprogramming *in vitro* and *in vivo*

Mouse fibroblasts can be converted into cardiomyocyte-like cells using several different combinations of reprogramming factors *in vitro* and *in vivo*.

cardiac cell fate specification as demonstrated by knockout mouse studies. Cardiac fibroblasts were isolated from heart explants of transgenic mice expressing EGFP under a cardiac-specific alpha myosin heavy chain (αMHC) promoter and the fibroblast cells not expressing EGFP were used for screening. Transduction of all 14 factors into fibroblasts induced 1.7% of GFP$^+$ cells, and serial reduction of individual factors demonstrated that a combination of three factors, Gata4, Mef2c, and Tbx5, was sufficient for GFP$^+$ cell induction (around 15%). We designated these GFP$^+$ cardiomyocyte-like cells induced cardiomyocytes (iCMs)[12]. Gata4, Mef2c, and Tbx5 are core cardiac transcription factors in early heart development that interact with each other to coactivate cardiac gene expression and promote cardiomyocyte differentiation.

We investigated the genetic and epigenetic status of directly induced cells by microarray and histone and DNA methylation analyses. The iCMs are similar, although not identical, to cardiomyocytes in genetics and epigenetics status. Their global gene expression profile resembles that of neonatal cardiomyocytes, but is different from that of the original cardiac fibroblasts. The histone-modification and DNA-methylation patterns of iCMs were also similar to those of cardiomyocytes, and a subset of iCMs contracted spontaneously after 4 weeks of culture. Lineage-mapping experiments with Mesp1-Cre/R26R-YFP and Isl1 Cre/R26R-YFP reporter mice suggested direct reprogramming of the fibroblasts to the iCM fate without reversion to a cardiac mesoderm/progenitor stage. In these studies, we primarily used neonatal CFs as the cell source for cardiac induction. CFs are present in cardiac tissue along with cardiomyocytes, and account for more than half the total cells in a healthy heart[30-32]. In diseased hearts, fibroblasts proliferate and secrete extracellular matrix and growth factors, leading to fibrosis, myocardial remodeling, and heart failure[33]. Thus, we considered that CFs may be an ideal cell source for newly generated cardiomyocytes, if they can be converted into functional cardiac cells *in vivo*.

Gata4/Mef2c/Tbx5 Directly Convert Resident Cardiac Fibroblasts into Cardiomyocyte-like Cells in Mouse Infarcted Hearts

More recently, we investigated whether direct gene transfer of GMT into the mouse hearts could similarly induce new cardiomyocyte generation from cardiac fibroblasts (Fig.1)[18]. Retrovirus was used as a vector for gene delivery after myocardial infarction in mouse, as this virus infect mainly fibroblasts but not terminally differentiated cardiomyocytes. Injection of GMT retrovirus into the αMHC-GFP transgenic mouse hearts induced expression of GFP, a reporter of cardiomyocytes, in 3% of virus-infected non-myocytes. A mixture of GMT injection into the immunosuppressed nude mouse hearts induced cardiac protein expression in 1% of the transduced fibroblast cells, although few cells showed sarcomeric structures. We next devel-

oped a polycistronic vector expressing GMT separated by 2A "self-cleaving" peptides (3F2A) to improve reprogramming efficiency. Injection of this polycistronic retrovirus vector resulted in generation of induced cardiomyocyte-like cells in fibrotic tissues, which expressed sarcomeric α-actinin, cardiac troponin T, and several cardiac-specific genes. Importantly, more iCMs had well-defined sarcomeric structures by using this system, suggesting 3F2A-iCMs were more mature cardiomyocyte-like cells and that the polycistronic vector can be used for cellular reprogramming *in vivo*. Srivastava and Olson groups independently demonstrated improvement of heart function after myocardial infarction by gene transfer of cardiac reprogramming factors[16, 17]. These results of *in vivo* cardiac reprogramming are striking and may provide a potential new strategy for regenerative medicine.

Following our initial *in vitro* cardiac reprogramming paper, other groups also reported generation of cardiomyocyte-like cells from mouse fibroblasts by transcription factors or microRNAs *in vitro* and *in vivo*. Song et al. reported that adding *Hand2* to GMT converted adult cardiac fibroblasts and tail-tip fibroblasts into functional cardiomyocyte-like cells more efficiently than GMT[16]. Protze et al.[15] found that the combination of *Mef2c, Myocd*, and *Tbx5* upregulated a broader spectrum of cardiac genes compared with other combinations, including GMT. Neonatal cardiac fibroblasts transduced with lentiviral MMT or GMT expressed cardiac contractile proteins, had potassium and sodium currents, and exhibited cardiac-like action potentials. Jayawardena et al. reported that a combination of muscle-specific microRNAs, namely mir-1, 133, 208, and 499, can convert cardiac fibroblasts into functional cardiomyocyte-like cells *in vitro* and *in vivo*[14].

Gata4/Mef2c/Tbx5/Myocd/Mesp1 Reprogram Human Cardiac Fibroblasts into Cardiomyocyte-like Cells *in vitro*

We next analyzed whether human fibroblasts could be directly converted to iCMs by defined factors[21]. We found that GMT was not sufficient for cardiac induction in human cardiac fibroblasts (HCFs), and that addition of Mesp1 and Myocd to GMT upregulated a broader spectrum of cardiac genes more efficiently than GMT. The HCFs and human dermal fibroblasts transduced with GMT, Mesp1, and Myocd (GMTMM) changed the cell morphology to rod-like or polygonal shape, expressed a broad range of cardiac genes and concomitantly suppressed fibroblast genes, and exhibited spontaneous Ca^{2+} oscillations. Moreover, the cells matured to exhibit action potentials and contract synchronously in coculture with murine cardiomyocytes. The EdU assay revealed that the human iCMs generated did not pass through a mitotic stem cell state. Nam et al. reported that a combination of Gata4, Hand2, Tbx5, Myocd, miR-1, and miR-133 induced 13% of adult HCFs to express cardiac troponin T protein and that a small subset of the iCMs exhibited spontaneous contractility after 11 weeks in culture[19].

Islas et al. reported that transient overexpression of Ets2 and Mesp1 could reprogram human dermal fibroblasts into cardiac progenitor-like cells[20]. The induced cardiac progenitor-like cells differentiated into immature cardiomyocytes and exhibited calcium activities. These findings may represent an important initial step toward potential therapeutic applications of the direct reprogramming approach in clinical situations. However, human cardiac reprogramming process is slower and less efficient than mouse reprogramming, much like the induction of human iPSCs and neuronal cells. Future studies are needed to thoroughly optimize conditions for human cardiomyocyte generation and maturation and to characterize the properties of human iCMs. Although further works in larger animals and more efficient protocols of cardiac reprogramming are needed, these reports demonstrate that new direct reprograming strategy might be a potential approach for heart regeneration in the future[34].

Future Perspectives of Direct Cardiac Reprogramming

As discussed above, the cardiac reprogramming field has been extensively progressed and may change the regenerative medicine in the future (Table 1). The directly induced cardiac cells appear to quickly exit the cell cycle following the lineage conversion, and the utility of iCMs *in vitro* might be limited in some instances. Alternatively, direct induction of progenitor cells, as shown in neural stem/progenitor cell reprogramming, may be a good approach to solve this issue[35]. In contrast, introduction of the cardiac reprogramming factors directly into the damaged heart may convert the endogenous fibroblast population, which represents > 50% of cardiac cells, into new functional cardiomyocytes in situ, and may improve cardiac function. This *in vivo* reprogramming approach may have several advantages com-

Table 1 Direct Cardiac Reprogramming in Mouse and Human fibroblasts by Several Combinations of Defined Factors

Reprogramming factors	Mouse				Human		References
	In vitro			In vivo			
	Reporter	Gene	Function		Gene	Function	
Gata4/Mef2c/Tbx5	15%	5%	0.5% of iCMs	○	Not efficient	ND	[12] [17] [18] [21]
Gata4/Hand2/ Mef2c/Tbx5	15%	9%	0.2% of iCLMs	○	Not efficient	ND	[16] [19]
Mef2c/Myocd/Tbx5	2%	12%	0.08% of total	ND	ND	ND	[15]
miR-1/133/208/499	5% 28% (JAKi)	ND	1-2% of total	○	ND	ND	[14]
Gata4/Hand2/Myocd/ Tbx5/miR-1/miR-133	ND	ND	ND	ND	13%	beating+	[19]
Gata4/Mef2c/Tbx5/ Myocd/Mesp1	ND	ND	ND	ND	5%	Co-culture beating+	[21]
Ets2/Mesp1	ND	ND	ND	ND	CPC gene	Ca transient+	[20]

○ indicates successful *in vivo* reprogramming, Reporter indicates αMHC-reporter expression, Gene indicates cardiac troponin T expression, Function indicates percentage of spontaneously beating cells, and ND indicates not determined.

pared with cell transplantation-based regeneration. First, the process is simple and short; second, avoiding the reprogramming of pluripotent cells before cardiac differentiation would greatly lower the risk of tumor formation; and third, direct injection of defined factors obviates the need for cell transplantation, for which long-term cardiac cell survival remains a challenge.

Although direct cardiac induction using defined factors has been demonstrated by several laboratories, the reprogramming efficiency remains low and many pitfalls remain that may lead to reprogramming failure[36, 37]. Future studies will be needed to thoroughly optimize conditions for iCM generation and maturation, and to characterize the properties of iCMs. Given that secreted proteins, electrical and mechanical stimulation, and cell-to-cell contact might promote cardiac differentiation and reprogramming in our human iCM coculture, the in vitro system might represent a valuable platform for screening such key factors. Highly standardized protocols that make the process more efficient and more easily transferable among different laboratories should be developed in the future to push this field forward.

Conclusions

Cellular reprogramming has long been recognized as a possibility, although the impact of cell type conversion by defined factors was most prominently exemplified only recently by the discovery of iPSCs. This landmark finding fundamentally altered approaches to regenerative medicine, and provided a broad strategy to induce desired cell types by introducing lineage-specific factors. Detail analyses of the properties of directly induced cells and understanding the molecular mechanisms of lineage reprogramming might be necessary to advance this nascent technology for future clinical applications.

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Disclosures

None.

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