REVIEW

Direct Reprogramming into Desired Cell Types by Defined Factors

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In the field of developmental biology, the concept that cells, once terminally differentiated, are fixed in their cell fate was long believed to be true. However, Dr. Gurdon and colleagues challenged this fundamental doctrine and demonstrated that cellular reprogramming and cell fate conversion are possible by somatic nuclear transfer and cell fusion. The Weintraub laboratory discovered in the 1980s that a single transcription factor, MyoD, can convert fibroblasts into skeletal muscle cells, and subsequent studies also demonstrated that several transcription factors are lineage converting factors within the blood cell lineage. In 2006, Takahashi and Yamanaka discovered that transduction of the four stem cell-specific transcription factors Oct4, Sox2, Klf4, and c-Myc can reprogram mouse fibroblast cells into a pluripotent state. In 2007, they demonstrated that the same four factors similarly reprogram human somatic cells into pluripotent stem cells. These discoveries by Dr. Yamanaka and colleagues fundamentally changed research in the fields of disease modeling and regenerative medicine and also inspired the next stage of cellular reprogramming, i.e., the generation of desired cell types without reverting to stem cells by overexpression of lineage-specific transcription factors. Recent studies demonstrated that a diverse range of cell types, such as pancreatic β cells, neurons, neural progenitors, cardiomyocytes, and hepatocytes, can be directly induced from somatic cells by combinations of specific factors. In this article, I review the pioneering works of cellular reprogramming and discuss the recent progress and future perspectives of direct reprogramming technology.
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Introduction

In the 1960s, John Gurdon and colleagues demonstrated that the nucleus of a differentiated frog cell could be reprogrammed back to that of a totipotent cell when transferred into an enucleated egg. Such cells can then give rise to a whole new frog,[1,2] and this research lead to the success of clone generation in mammals by somatic nuclear transfer. The birth of Dolly the sheep was reported in 1997, and many other species of cloned mammals were subsequently reported.[3–11] Moreover, identification of *MyoD*, a master gene for skeletal myocytes, demonstrated that cell fates can be changed simply through the overexpression of specific transcription factors.[12–18] These studies suggested that cell fates are more plastic than previously expected and that dormant gene expression pro-

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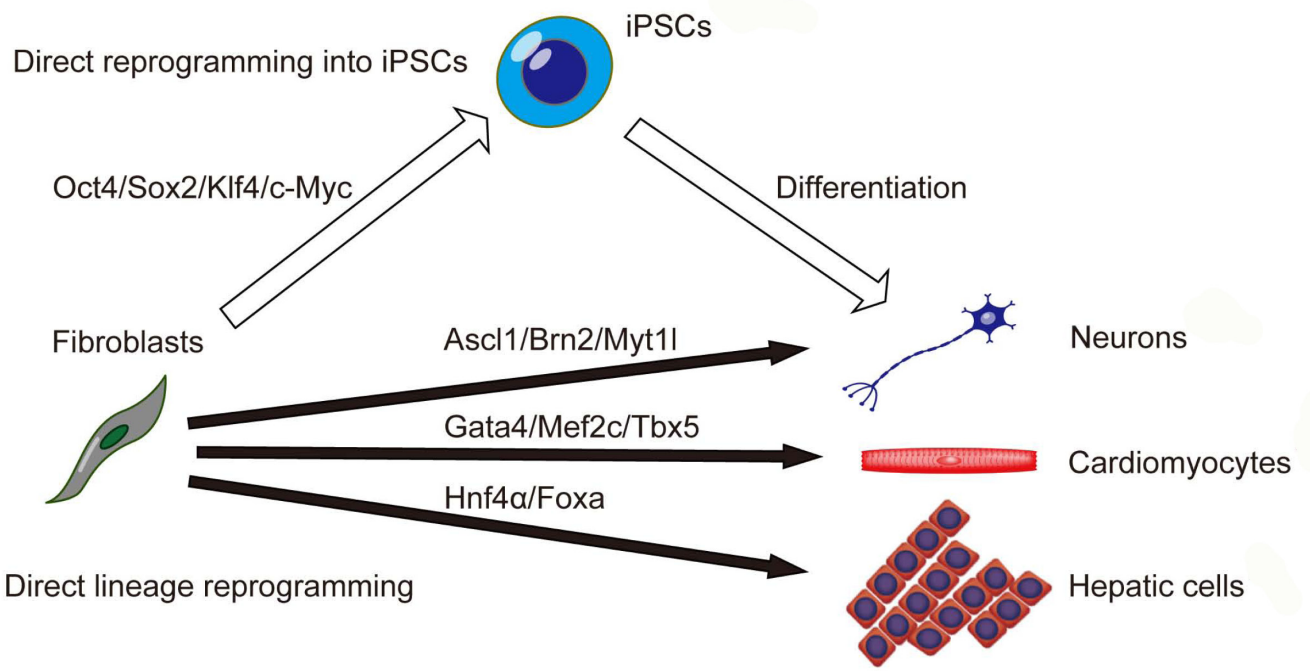

Fig. 1 Generation of desired cell types via induced pluripotent stem cells (iPSCs) and by direct lineage reprogramming. Transduction of Oct4, Sox2, Klf4, and c-Myc converts fibroblasts into iPSCs. Terminally differentiated cells, including neurons, cardiomyocytes, and hepatocytes, can be generated from iPSCs. Alternatively, neurons, cardiomyocytes, and hepatocytes can be directly induced from fibroblasts by overexpression of lineage-specific transcription factors.

grams can be activated in terminally differentiated cells by defined factors.[2–4,19] This pioneering research led to the landmark discovery by Takahashi and Yamanaka in 2006 that reprogramming mouse fibroblasts into pluripotent stem cells is possible by transduction of four stem cell-specific transcription factors. Sir John Gurdon and Dr. Shinya Yamanaka received the 2012 Nobel Prize in Physiology or Medicine as a result of these achievements.

The generation of induced pluripotent stem cells (iPSCs) by Yamanaka factors has revolutionized the field of regenerative medicine and has triggered studies on cell therapy, disease modeling, and personalized medicine.[20–30] Moreover, the discovery of iPSCs has delivered a new approach for the generation of desired cell types called lineage reprogramming, which is the direct conversion of one adult cell type into another using combinations of lineage-specific transcription factors or microRNAs without the cell passing through the pluripotent stem cell state (**Fig. 1**). Recent studies have demonstrated that direct lineage reprogramming can yield a diverse range of medically relevant cell types, such as pancreatic β cells, neurons, cardiomyocytes, and hepatocytes.[31–35]Here, I will give an overview of the history and recent progress of cellular reprogramming and its potential future applications in regenerative medicine.

MyoD Converts Fibroblasts into Skeletal Myocytes

MyoD is a classic example of a master gene for cell differentiation in the sense that transduction of this gene is sufficient to activate the whole genetic program of muscle differentiation in non-muscle cells.[36,37] In 1979, Taylor and Jones found that treatment of the mouse embryonic fibroblast cell line with 5-azacytidine (5-azaC) induced differentiation of these cells into skeletal muscle cells.[38,39] This observation led to the hypothesis that DNA demethylation and the resulting activation of unidentified genes were responsible for the conversion to muscle cells.[40] A subtractive hybridization experiment comparing untreated and 5-azaC-treated fibroblasts led to the identification of MyoD.[12] When overexpressed in primary fibroblasts or in a wide variety of other cell types, including pigment, nerve, fat, and liver cells, MyoD can convert these cells to skeletal muscle cells.[15,16] These findings provided the first evidence that a single gene, acting as a master switch, can initiate a complex program of differentiation.

The ability of MyoD to convert somatic cells into skeletal muscle cells suggested that it might have a central role in myogenesis, and subsequent studies sought to determine its biological roles and molecular mechanisms in muscle development.[41] The MyoD protein contains a basic helix-loop-helix (bHLH) motif that is common

to a large family of transcription factors.[41–43] In addition to MyoD, the highly related proteins Myf5, Mrf4, and myogenin are also expressed in skeletal muscle, and each sequentially and synergistically plays a crucial role in muscle cell specification and differentiation during development *in vivo*.[44–46] These observations led to the idea that lineage-associated transcription factors that determine cellular identity during development can change cell fate when ectopically expressed in certain heterologous cells. Following the discovery of MyoD, conversion of one cell type into another was demonstrated within hematopoietic cell lineages by overexpression of transcription factors, including Gata1 and C/EBPα, or deletion of Pax5.[13,14,47–53]

Oct4, Sox2, Klf4, and c-Myc Reprogram Fibroblasts into Pluripotent Stem Cells

In 2006, Takahashi and Yamanaka achieved a breakthrough by demonstrating that the overexpression of four embryonic stem cell (ESC)-specific transcription factors, Oct4, Sox2, Klf4, and c-Myc, can convert fibroblasts into pluripotent stem cells, or iPSCs.[54] 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.[55] The combination of 24 factors activated *Fbx15* and induced the formation of drug-resistant colonies with characteristic ESC morphology. Successive rounds of elimination of individual dispensable factors led to identification of the minimally required core set of four genes: Oct4, Sox2, Klf4, and c-Myc, now known as Yamanaka factors. Following this finding, many laboratories reproduced their results, improved iPSC generation techniques, and demonstrated that murine iPSCs share all their features with naive mouse ESCs, including expression of pluripotency markers, reactivation of both X chromosomes, and the ability to generate chimeric mice.[56–63]

iPSCs can be derived from a number of different species, including humans, rats, and rhesus monkeys, by expression of the four Yamanaka factors.[64–68] Similarly, iPSCs have been derived from other somatic cell populations, such as keratinocytes, neural cells, stomach and liver cells, and melanocytes, as well as from terminally differentiated blood cells, demonstrating the universality of induced pluripotency by these factors.[69–73] Importantly, reprogramming can be induced not only by Oct4, Sox2, Klf4, and c-Myc but also by alternative combinations that employ Nanog, Lin28, ESRRB, NR5A2, and other genes that establish the core transcriptional circuitry of stem cells.[65,74–76]

Reprogramming of Pancreatic Exocrine Cells into Endocrine β cells *in Vivo*

The generation of iPSCs sparked a new idea: the conversion of mature cell types directly into another cell type without passing through a stem cell state by combination of several lineage-specific factors.[31,33,35,77,78] Zhou *et al.* reported that adenoviral gene transfer of a combination of three transcription factors, Ngn3, Pdx1, and Mafa, can efficiently reprogram pancreatic exocrine cells into functional β cells in mice.[35] The three pancreatic reprogramming factors, Ngn3, Pdx1, and Mafa, are known to be important in the embryonic development of pancreas and β cells.[79] The induced β cells were indistinguishable from endogenous islet β cells in structure and they expressed genes essential for β cell function. Importantly, induced β cells can ameliorate hyperglycemia in type 1 diabetic mice. Although the new β cells were not reorganized into islet structures (and this may limit their effectiveness), this study provides the first evidence of cellular reprogramming *in vivo* by defined factors.

Reprogramming of Mouse and Human Fibroblasts into Neural Cells

It is conceivable that cell type conversion within the same lineage might be easy, but conversion to other lineages may be challenging. Vierbuchen *et al.* reported that neuronal lineage-specific transcription factors Ascl1, Brn2, and Myt1l efficiently convert mouse dermal fibroblasts into functional neurons *in vitro*.[33] These induced neuronal (iN) cells expressed multiple neuron-specific proteins, generated action potentials, and formed functional synapses. The same group also reported that when combined with the bHLH transcription factor NeuroD1, these three factors could also convert human fibroblasts into iN cells.[80] Human iN cells also showed typical neuronal morphologies and expressed multiple neuronal markers, even after downregulation of the exogenous transcription factors. Subsequently, it was reported that a combination of other transcription factors, Ascl1, Nurr1, and Lmx1a, was able to generate functional dopaminergic neurons from mouse and human fibroblasts without reverting to a progenitor cell stage.[81] This study also showed that dopaminergic neuronal cells can be generated from cells from patients with Parkinson's disease by the same factors. These studies first revealed that lineage conversions are not restricted to within the same lineage or germ layer, since fibroblasts are mesodermal in origin and neurons are derived from ectoderm. Recently, Wernig's group also reported that terminally differentiated hepatocytes, derived from endoderm, can be converted into iN cells by overexpression of Ascl1, Brn2, and Myt1l.[82]

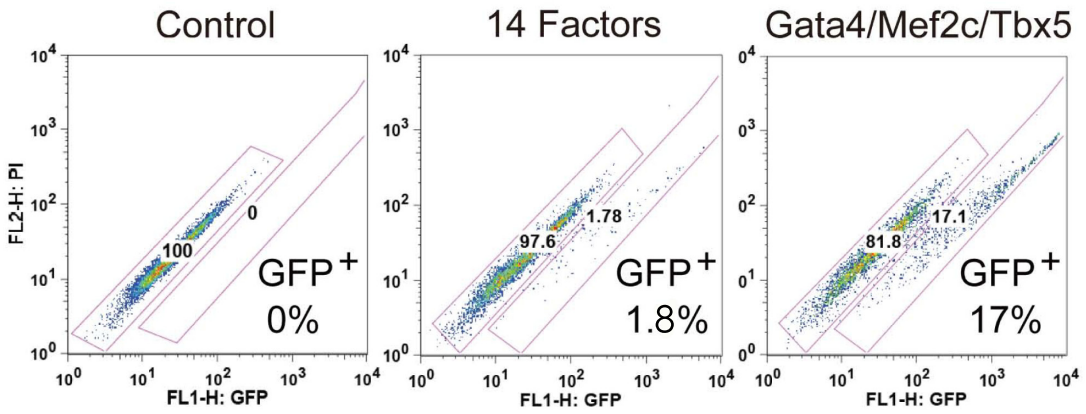

Fig. 2 Screening for cardiac reprogramming factors by fluorescence-activated cell sorting (FACS).
FACS analyses for α-MHC-GFP$^+$ cell (cardiac reporter) induction from fibroblasts. Transduction of 14 factors into fibroblasts induced 1.8% of GFP$^+$ cells, and serial removal revealed that the combination of Gata4, Mef2c, and Tbx5 induced 17% of GFP$^+$ cells 1 week after transduction.[34]

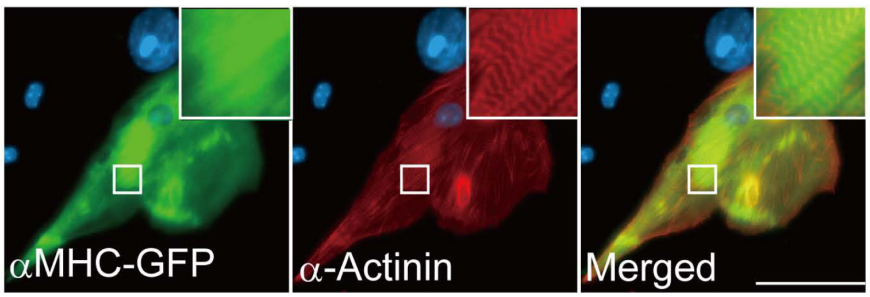

Fig. 3 Induced cardiomyocytes expressed cardiac genes *in vitro*.
Immunofluorescent staining for α-MHC-GFP, α-actinin, and DAPI (nuclei staining) in the Gata4, Mef2c, and Tbx5-transduced fibroblast cells. Induced cardiomyocytes expressed cardiac genes and exhibited sarcomeric organization.[34] Bar 100μm.

Direct Reprogramming into Cardiomyocytes by Gata4, Mef2c, and Tbx5

Although embryonic mesoderm can be induced to differentiate into cardiomyocytes, no master regulator of cardiac differentiation has been identified, despite much research inspired by the discovery of MyoD.[12,83,84] We hypothesized that no single gene can directly convert fibroblasts into cardiomyocytes, but that a combination of key developmental cardiac genes might achieve direct conversion. We selected 14 genes as candidates for cardiac reprogramming; these candidate genes are specifically expressed in embryonic cardiomyocytes and are critical for cardiac cell fate specification, as demonstrated by knockout studies in mice.[85] Cardiac fibroblasts were isolated from transgenic mice expressing enhanced green fluorescent protein (EGFP) under a cardiac-specific alpha myosin heavy chain (αMHC) promoter, and fibroblast cells that did not express EGFP were used for screening.[86,87] Transduction of all 14 factors into fibroblasts induced 1.8% of GFP$^+$ cells, and serial reduction of individual factors demonstrated that a combination of three factors, Gata4, Mef2c, and Tbx5, were sufficient for GFP$^+$ cell induction (around 15%, **Fig. 2**). We designated these GFP$^+$ cardiomyocyte-like cells induced cardiomyocytes (iCMs).[34] The three cardiac reprogramming factors, Gata4, Mef2c, and Tbx5, are core cardiac transcription factors in early heart development[88–90] and are known to interact with one another, to coactivate cardiac gene expression, and to promote cardiomyocyte differentiation.[91–93]

We determined the molecular features of iCMs by genetic and epigenetic analyses and by cell fate mapping studies. The iCMs expressed several cardiac-specific genes and had sarcomeric structures (**Fig. 3**). Microarray analyses established that the global gene expression profile of iCMs is similar to that of neonatal cardiomyocytes, but different from that of the original fibroblasts. For some cardiac gene promoters, the histone modifications and DNA methylation patterns of iCMs were also similar to cardiomyocytes.[94] A subset of iCMs exhibited intracellular Ca^{2+} transients and contracted spontaneously after

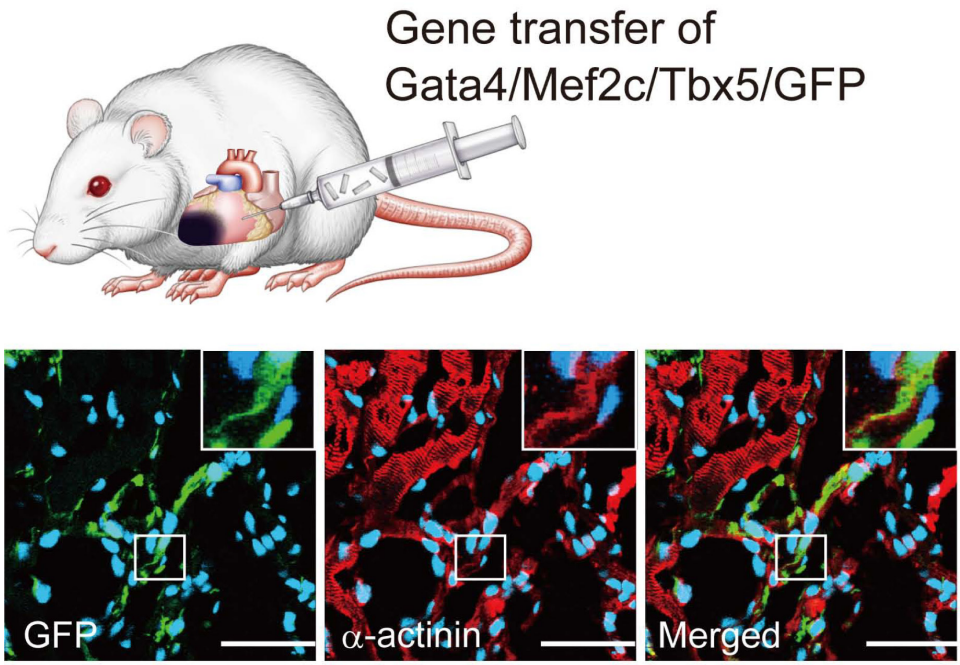

Fig. 4 Direct cardiac reprogramming in infarcted mouse hearts by Gata4, Mef2c, and Tbx5.
Gata4, Mef2c, Tbx5, and GFP were directly injected into the mouse hearts after myocardial infarction. Gata4, Mef2c, Tbx5, and GFP-transduced fibroblasts expressed cardiac gene α-actinin and exhibited sarcomeric structures.[102] Bars 50μm.

4 weeks of culture. Lineage-mapping experiments with Mesp1-Cre/R26R-YFP and Isl1-Cre/R26R-YFP reporter mice suggested that the fibroblasts were directly reprogrammed to the differentiated cardiomyocyte fate without reverting to a cardiac mesoderm/progenitor stage.[84,95–97] This study was the first to represent the global gene expression profiles and epigenetic status of directly induced cells and the first to demonstrate cell fate conversion without the cells reverting to stem/progenitor states using reporter mice. Following our report, several other groups also demonstrated cardiac reprogramming from fibroblasts using the same factors and microRNAs.[98–100]

In 2012, we and two other groups reported that gene transfer of Gata4, Mef2c, and Tbx5 with or without Hand2 can convert resident cardiac fibroblasts into cardiomyocyte-like cells in infarcted mouse hearts (**Fig. 4**).[99,101,102] We found that expression of Gata4, Mef2c, and Tbx5 using a polycistronic vector enhanced cardiac maturation *in vivo* compared with injecting the three vectors independently.[102] Srivastava and colleagues and Olson and colleagues demonstrated not only *in vivo* cardiac reprogramming but also improvement of heart function after myocardial infarction by gene transfer of cardiac reprogramming factors.[99,101] These results are striking and may provide a potential new strategy for regenerative medicine. It will be important to understand the molecular mechanisms of cardiac reprogramming utilizing *in vitro* culture models to enhance cardiac induction efficiency *in vivo*.

Induction of Hepatocyte-like Cells by Defined Factors

Overexpression of lineage-specific transcription factors can directly convert terminally differentiated cells into the cells of some other lineages; however, until recently it remained unclear whether transplantation of these induced cells could improve the function of damaged organs. In 2011, two groups demonstrated that induced hepatocyte-like (iHep) cells that were directly generated from fibroblasts could restore damaged hepatic tissues after cell transplantation.[31,32] Huang *et al.* demonstrated the direct induction of proliferative functional iHep cells from mouse tail-tip fibroblasts by transduction of Gata4, Hnf1a, and Foxa3 and inactivation of p19Arf.[31] The iHep cells showed typical epithelial morphology, expressed hepatic genes, and acquired hepatocyte functions. Notably, transplanted iHep cells repopulated the damaged livers in mice and spared almost half of the recipients from death by restoring liver functions. Sekiya *et al.* established that Hnf4a plus Foxa1, Foxa2, or Foxa3 can convert mouse embryonic and adult fibroblasts into iHep cells.[32] Their iHep cells were also proliferative, functional, and rescued damaged hepatic tissues after cell transplantation. These studies provide novel strategies to generate functional hepatocyte-like cells for the purpose of liver engineering and regenerative medicine. However, iHep cell transplantation experiments showed that the rescue was partial and

that the cells were not identical to bona-fide hepatocytes. Further modifications to the process of iHep cell generation might be needed to enable this approach to be applied in clinical settings.

Conclusions

Progress in the field of cellular reprogramming has been extensive and has the potential to change the face of regenerative medicine in the future.[26,34,103,104] Pluripotent reprogramming with directed differentiation is theoretically able to produce all desired cell types, as has been shown in ESC studies.[105,106] A potential advantage of using iPSCs is that pluripotent stem cells have nearly unlimited capacity to proliferate in culture and may provide a large number of desired terminally differentiated cells. In contrast, directly induced cells appear to quickly exit the cell cycle by lineage reprogramming, and the utility of reprogrammed cells *in vitro* might be limited in some instances. Direct induction of progenitor cells, as shown in neural stem/progenitor cell reprogramming, may be an alternative approach to solving this issue.[107,108] It is conceivable that lineage reprogramming techniques might be utilized in *in vivo* reprogramming, i.e., converting endogenous cells directly into desired cell types in situ by gene transfer of defined factors, as has been demonstrated in pancreatic β cell and cardiomyocyte induction.[35,99,101,102] If this is possible, it has several advantages: first, the process is simple and quick; second, the avoidance of reprogramming to pluripotent cells before lineage differentiation might greatly lower the risk of contamination of immature cells; and third, direct injection of defined factors can avoid cell transplantation in which long-term cell survival might be challenging in some organs, such as the heart.[109–112] Detailed analyses of the properties of directly induced cells and understanding of the molecular mechanisms of lineage reprogramming will likely be necessary to advance this technology for future clinical applications.

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