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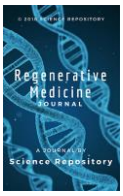

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Research Article

Advances in direct reprogramming and its future clinical application using a protein-based cell engineering system

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ABSTRACT

Direct reprogramming is a promising technology in regenerative medicine. However, there is no report on the clinical applications of cells prepared by direct reprogramming. In the current review, we describe direct reprogramming methods of somatic cells to hepatocytes, pancreatic β-cells, cardiomyocytes, and endothelial cells. Next, we discuss current issues that should be clarified for their future clinical applications. As the most critical issue, it is necessary to establish a vector-free system for cellular engineering, because most studies on direct reprogramming have been performed using viral vectors or plasmid DNA. We recently developed a protein-based cell engineering system, in which a newly identified cell penetrating peptide (NTP) was combined with an artificial transcription factor system (NTP-ATF). By using NTP-ATF, endogenous gene expression can be induced by exogenous recombinant proteins. Here, we briefly introduce the NTP-ATF system and discuss its future applications by combining chemical compounds that are competent for the induction of differentiation. We also propose that the NTP-ATF system can be utilized for expansion of somatic cells, which is another issue for cell therapy using somatic cells.

Introduction

Since Takahashi and Yamanaka first developed induced pluripotent stem cells (iPSCs) from somatic cells using four transcription factors, iPSC technology has been used in many fields, including disease modeling, drug screening, and cell-based therapy [1]. However, further studies are still required to expand the clinical applications of iPSCs, because the establishment of iPSCs from patients and safety assessment of the cells require long periods of time, and immunosuppressive therapy is required when allogeneic iPSCs are clinically utilized.

In contrast, several lines of evidence indicate that by combined forced expression of lineage-specific transcription factors, somatic cells can be manipulated into various tissue cells that include neurons, hepatocytes, pancreatic β-cells, cardiomyocytes, and endothelial cells [2-4]. Although achieving efficiency and safety with direct reprogramming is challenging, direct reprogramming from patients' somatic cells to tissue-specific cells is attractive because additional treatment for overcoming immunological problems is not required. In this review, we focus on direct reprogramming from somatic fibroblasts into hepatocytes, pancreatic β-cells, cardiomyocytes, and endothelial cells (Table 1). Finally, we address current problems associated with direct reprogramming and discuss how our recently developed reprogramming method using a protein-based approach would be suitable for the manipulation of somatic cells.

Direct reprogramming into hepatocytes

Hepatocyte transplantation is the most effective treatment for a malfunctioning liver caused by chronic liver disease. Two research

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groups used direct reprogramming to reprogram mouse fibroblasts into functional induced hepatocyte-like (iHep) cells and screened several transcription factors related to liver function [5, 6]. Huang et al. generated iHep cells by forced expression of GATA4, HNF1a and FOXA3, whereas Sekiya et al. performed direct reprogramming of iHep cells by forced expression of HNF4a and FOXA1. Moreover, these iHep cells express several hepatic genes and acquired hepatocyte functions, including cytochrome P450 enzyme activity or albumin-producing activity. The iHep cells also rescued mice lacking fumarylacetoacetate hydrolase (Fah-/-), a common mouse model of liver failure.

Two research groups generated expandable iHep cells from fibroblasts [7, 8]. Du et al. used HNF1a, HNF4a, HNF6, ATF5, PROX1, CEBPA, c-MYC, and p53 shRNA. Huang et al. forced the expression of HNF1a, HNF4a, FOXA3, and SV40 large T antigen. These expandable iHep cells were useful for the large-scale production of hepatocytes.

Table 1: The direct reprogramming strategies

Organ	Cell types	Genes	Transduction strategies	Reference
Hepatocyte	MEFs	GATA4, HNF1a FOXA3	LV	[5]
	MEFs	HNF4a plus FOXA1, FOXA2 or FOXA3	RV	[6]
	HEFs	HNF1a, HNF4a, HNF6 FOXA3, HNF1a, HNF4a	LV	[7]
	HEFs		LV	[8]
	MEFs	GATA4, HNF1a, FOXA3	EV	[44]
	MEFs	HNF4a, FOXA3	Piggy bac	[47]
	HEFs	HNF1a plus two of the FOXA1, FOXA3, HNF4a	Transfection of synthetic modified mRNA	[49]
	HEFs, MEFs	HNF1a	RV plus CC	[51]
	MEFs	FOXA1, FOXA2 or FOXA3	LV plus CC	[52]
Pancreas β -cell	Mouse hepatocyte (*in vivo*)	PDX1	Injection	[11]
	Human adult liver fibroblast cells	PDX1	AV	[12]
	Pig liver (*in vivo*)	PDX1, NGN3, MAFA, PAX4, NEUROD	AV	[13]
	Mouse (*in vivo*)	PDX1, NGN3, MAFA	Tail vain injection	[14]
	Human fibroblast cells	PDX1	Plasmid transfection	[15]
	Adult mouse intrahepatic biliary cell	PDX1, NGN3	AV	[17]
	Intestinal crypt cells	PDX1, NGN3, MAFA	LV	[18]
	Human pancreatic ductal cells	PDX1, NGN3, MAFA, PAX4	AV	[19]
	Human pancreatic ductal cells	PDX1, NGN3, MAFA, PAX6	AV	[20]
	Mouse pancreatic acinar cells (*in vivo*)	PDX1, NGN3, MAFA	AV injection	[21]
	Human adult hepatocyte cells	PDX1, NGN3	Plasmid transfection	[22]
	hiPSCs	miR-375	LV	[26]
	Human ductal cells		CC	[56, 57]
Cardiomyocyte	MEFs	GATA4, MEF2, TBX5	LV	[27]
	Mouse tail-tip fibroblast cells	GATA4, MEF2, TBX5	LV	[28]
	MEFs	GATA4, MEF2, TBX5	RV	[29]
	Adult mouse tail-tip fibroblast cells	GATA4, HAND2 MEF2C, TBX5	RV	[30]
	MEFs	TBX5, MEF2C, MYOCD	LV	[31]
	MEFs	GATA4, HAND2 MEF2C, TBX5, NKX2.2	LV	[32]
	MEFs	GATA4, HAND2, TBX5	RV	[33]

	Mouse cardiac fibroblast cells (*in vivo*)	miR-1, 133, 208, 499	LV	[34]
	MEFs	miR-133a, GATA4, MEF2C, TBX5	LV	[35]
	HEFs	miR-1, 133, GATA4, HAND2, TBX5	RV	[36]
	Mouse tail tip fibroblast cells	OCT4	LV plus CC	[59]
	Mouse cardiac fibroblast cells	GATA4, MEF2C, TBX5	RV plus CC	[63]
	MEFs		CC	[64]
	MEFs	GATA4, MEF2C, TBX5	LV plus CC	[66]
	MEFs		CC	[67]
Endothelial	Human amniotic cells	ETV2, FL1, ERG1	LV	[37]
	Human adult skin fibroblast cells	FOXO1, ER71, KLF2, TAL1, LMO2	LV	[38]
	Human adult skin fibroblast cells	ETV2	LV	[39]
	Human foreskin cells	ETV2	LV	[40]
	Human ductal fibroblast cells	ER71, ETV2	LV	[41]

MEFs: mouse embryonic fibroblast HEFs: human embryonic fibroblast LV: lenti viral vector RV: retro viral vector
AV: adeno viral vector EV: episomal vector CC: chemical compound

Direct reprogramming into pancreatic β-cells

Diabetes mellitus is caused by a deficiency in, or dysfunctional, pancreatic β-cells, and an effective treatment has not yet been established. Transplanting pancreatic β-cells is an option and can be achieved by direct reprogramming. As sources of direct reprogramming, somatic cells including hepatocytes, biliary cells, gastrointestinal cells, pancreatic ductal cells, pancreatic acinar cells, and pancreatic endocrine cells have been utilized [9, 10]. In the first report of hepatocyte reprogramming into pancreatic β-cells, forced expression of PDX1 was achieved in mice [11]. In a similar manner, Sapir et al. reprogrammed human adult liver cells into pancreatic β-cells, which allowed β-cells to produce insulin *in vitro* and ameliorated hyperglycemia after transplantation *in vivo* [12]. Subsequently, Ham et al. and Tang et al. enhanced the direct reprogramming efficiency by PDX1 with pancreatic transcription factors, including NEUROD, NGN3, MAFA, and PAX4 [13, 14]. Horb et al. improved the efficiency of pancreatic β-cells by fusing PDX1 to the transcription activation domain of VP-16 [15, 16]. Nagaya et al. generated pancreatic β-cells from biliary cells by using PDX1 and NGN3 [17]. Chen et al. generated β-cells from gastrointestinal cells by expressing NGN3, PDX1 and MAFA; these induced pancreatic β-cells produced insulin in response to a high concentration of glucose [18]. After direct reprogramming of pancreas-derived cells into pancreatic β-cells, Lima et al. showed that human pancreatic ductal cells could be converted into pancreatic β-cells by forced expression of PDX1, NGN3, MAFA, and PAX4; these induced β-cells expressed high glucagon but little insulin [19]. Interestingly, Lee et al. generated insulin-secreting cells from fluorescence activated cell-sorted adult human pancreatic duct cells by forced expression of PDX1, NGN3, MAFA, and PAX6 *in vitro*, whereas Zhou et al. reprogrammed adult pancreatic acinar cells into β-like cells by introducing PDX1, NGN3 and MAFA *in vivo* [20, 21]. The initial cell type affects the reprogramming efficiency and induced cell functions.

To enhance reprogramming efficiency, Lu et al. co-transfected miR-302 with pancreas-specific transcription factors PDX1, NGN3, and MAFA, and successfully reprogrammed human adult hepatocytes into pancreatic islet-like cells that were able to release the hormone in response to glucose *in vitro* [22, 23]. Moreover, it was reported that miR-375 or miR-187 is important for pancreatic β-cell function [24, 25], whereas Lahmy et al. generated pancreatic β-cells from iPSCs using miR-375 [26].

Direct reprogramming into cardiac cells

The heart is an organ with low regenerative capacity, and transplantation of cardiac cells or the heart itself is the only method for treating severe heart failure. Ieda et al. screened 14 factors and determined that GATA4, MEF2c and TBX5 (GMT) are sufficient for cardiac direct reprogramming [27]. Additionally, Chen et al. reported that forced expression of GMT was insufficient to convert adult mouse tail-tip fibroblasts into cardiomyocyte-like cells [28]. To improve reprogramming efficiency, Wang et al. generated induced cardiac myocytes using a polycistronic vector of GMT, by which reprogramming efficiency was enhanced up to 10-fold [29]. Of note, Song et al. and Protze et al. screened 6 and 10 candidate transcription factors, respectively, for reprogramming into cardiac cells and demonstrated direct reprogramming by different combinations of transcription factors (GATA4, HAND2, MEF2c, and TBX5 or MEF2c, TBX5, and MYOCD) from mouse fibroblasts [30, 31]. Finally, Addis et al. optimized the combination of transcription factors using a calcium indicator and found that the combination of HAND2, NKX2.2, GATA4, MEF2c, and TBX5 was the most efficient, having 50-fold more

efficiency than GMT [32]. Hirai et al. fused the MyoD transactivation domain to GATA4, MEF2c, TBX5, and HAND2, and expressed this combination in mouse fibroblasts; this system enhanced reprogramming efficiency 15-fold [33]. Jayawardena et al., Muraoka et al. and Nam et al. reported that the addition of microRNA-1, 133, 208, 499, and 133a to GMT improved cardiac direct reprogramming in mouse embryonic fibroblasts (MEFs), adult mouse cardiac fibroblasts, and human fibroblasts [34-36].

Direct reprogramming into endothelial cells

Ginsberg et al. showed that human midgestation c-Kit (-) lineage-committed amniotic cells (ACs) could be reprogrammed into vascular endothelial cells [37]. They generated mature endothelial cells by forced expression of ETV2 with FL1 or ERG1 in ACs, whereas transient ETV2 expression generated immature endothelial cells. Han et al. showed that adult mouse fibroblasts could be reprogrammed into endothelial cells using five transcription factors: FOXO1, ER71, KLF2, TAL, and LMO2 [38]. Morita et al., Van et al. and Lee et al. recently reported that human fibroblasts could be directly reprogrammed into endothelial cells by forced expressed of ETV2 only; these induced endothelial cells acquired mature vascular endothelial cell functions [39-41]. In particular, Morita et al. showed that the induced endothelial cells improved blood flow recovery in a mouse model of hind limb ischemia.

Current problems associated with direct reprogramming

In recent years, guidelines of clinical trials using iPSCs have been established, and such trials using iPSCs have been performed. In contrast, no clinical trials using cells prepared by direct reprogramming have been reported. However, cell therapy, in which cells are prepared from patients, reprogrammed and injected into the same patients, has a great advantage because it is not necessary to add an immunosuppressive. However, certain problems should be addressed before clinical application of cells that are engineered by direct reprogramming.

First, the efficiency of direct reprogramming is generally low. Many research groups have investigated methods using transcription factors, small molecules, and microRNAs; however, these methods did not improve reprogramming efficiency. Second, differentiated somatic cells cannot proliferate, and large amounts of cells are required at the beginning of the direct reprogramming procedure. Du et al. and Huang et al. generated expandable induced hepatocytes. As one possible approach for expanding somatic cells, forced expression of hTERT and Bmi-1 has been attempted and its effectiveness has been confirmed by several groups [42, 43]. These methods would be suitable for the large-scale production of human cells. Third, most studies of regenerative medicine that include expansion of somatic cells have used a viral vector but obtaining a nontoxic-free system for direct reprogramming is the most critical issue for the clinical application of direct reprogramming.

Direct reprogramming by integration-free vectors: As one approach to avoid alternations in the genome structure by a vector system, integration-free vectors have been used. Kim et al. reported that MEFs could be converted into iHep cells by transfection of oriP/Epstein-Barr nuclear antigen-1-based episomal vectors containing GATA4, HNF1a and FOXA3 [44-46]. Katayama et al. generated iHep cells using Piggy bac, a type of transposon system, whereas Simeonov et al. demonstrated direct reprogramming using a synthetic modified mRNA of HNF1a plus any of the following two factors: FOXA1, FOXA3, or HNF4a [47-49].

Direct reprogramming by chemical compounds: For non-integrated approaches, small molecules have also been used for direct reprogramming of somatic cells into certain cell types [50]. Lim et al. and Guo et al. showed that small molecules could reprogram somatic cells [51, 52]. Lim et al. reported that mouse fibroblasts could be converted into iHep cells by forced expression of a single transcription factor, Hnf1a, in combination with two small molecules, A-83-01 and CHRI99021; these small molecules inhibit TGF-β and GSK-3 β signaling [53, 54]. Guo et al. generated iHep cells from MEFs using one transcription factor (FOXA1, 2, or 3) plus a chemical cocktail (CHIR99021, RepSox, Forskolin, VPA, TTNPB, DZnep) [55]. To generate pancreatic β-cells, Yuan et al. reported that BRD7552 induces PDX1, whereas Lefebvre et al. showed that the DNA methyltransferase inhibitor 5-aza-dC increased the activity of NGN3 [56, 57]. In direct cardiac reprogramming, Wang et al. demonstrated that mouse fibroblasts were reprogrammed into cardiomyocyte-like cells by the induction of OCT4 and four small molecules (SB431542, CHIR99021, parnate, and Forskolin) [58-61]. Moharmed et al. and Fu et al. reported that reprogramming mouse fibroblasts into cardiomyocytes could be performed by using the small molecules SB431542, XAV939 or CRFVPTZ [62-64, 55]. Ifkovits et al. and Zhou et al. improved reprogramming efficiency by inhibiting TGF-β or Akt signaling [65, 66].

Vector-free system: It is preferable to develop a simple, designable and safe system by which both direct programming and cell expansion can be performed. We recently identified a potent cell-penetrating peptide, NTP (nuclear trafficking peptide), and developed a protein-based artificial transcription system (ATF) [67]. NTP-tagged ATF is composed of NTP, TALE (transcription activator-like effector), which was developed as a second generation of the genome-editing tool, and the transcription activator domain, VP64. By using TALE targeting the proximal promoter of the miRNA-302/367 cluster gene, we successfully established iPSCs (NTP-iPSCs). Of note, we observed no apparent chromosome aberrations in NTP-iPSCs and chimeric mice were obtained. Additionally, NTP-ATF has a wide safety range: its effective dose is approximately 0.25 nM, whereas its toxic dose, which impaired cellular growth as judged by the MTT assay, is approximately 100 nM. As a next step to prove its usefulness, we are currently attempting to convert human fibroblasts into hepatocytes. Because TALE is a designable DNA binding protein and is applicable to any gene of interest, it is plausible that NTP-ATF, which targets hTERT and Bmi-1, will make it possible to expand somatic cells.

Conclusion and Future Perspectives

Recent progress in regenerative medicine has proven that cells of interest can be manipulated from fibroblasts or somatic stem cells by forced expression of lineage-specific genes. However, it is critically important to develop a vector-free system of cell manipulation, and to expand somatic cells into a sufficient number of cells for cell therapy. Although using chemical compounds is an attractive approach, some transcription factors are not sufficiently expressed. Moreover, sustained treatment of small compounds could cause chromosomal aberrations, and more studies are required to identify suitable combinations of chemical

cocktails for direct reprogramming. It is possible that the NTP-ATF system could be combined with chemical compounds because the NTP-ATF system is applicable to any gene of interest. It would be worthwhile to carry out direct reprogramming based on chemical compounds, with support of the NTP-ATF system, for inducing miRNA expression of a gene responsible for the terminal differentiation of the cells of interest but not being fully activated by chemical compounds. Additionally, the NTP-ATF system could be used to expand somatic stem cells for which no chemical compounds are currently available.

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