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Research Article

# Direct Reprogramming: A New Strategy for the Treatment of Diabetes

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**Abstract**

Type 1 and 2 diabetes is characterized by a deficiency in β-cell mass, which cannot be reversed with existing therapeutic strategies. Therefore, restoration of the endogenous insulin-producing cell mass holds great promise for curing diabetes in the future. Although cellular differentiation and lineage commitment have previously been considered as irreversible processes, recent studies have indicated that differentiated adult cells can be reprogrammed to pluripotency and, in some cases, directly into alternate committed lineages. Direct reprogramming means that one type of terminally differentiated cell is directly converted into another without the process of dedifferentiation and redifferentiation by skipping the state of induced pluripotent stem cell. The direct reprogramming of a patient's own somatic cells into pancreatic β-cells skips the phase of stem cell induction, thereby eliminating the risk of neoplasia formation. In this paper, we briefly review studies on the direct reprogramming of endoderm-derived somatic cell types into functional β-like cells and its primary application in diabetic animal models. We also analyze the efficiency and function of the induction process and the existing problems for translating direct reprogramming strategy into the clinical setting.

## ABBREVIATIONS

**IPCs:** insulin-producing cells; **ESCs:** Embryonic stem cells; **iPSCs:** The induced pluripotent stem cells; **STZ:** streptozotocin; **HDAD:** adenovirus-helper dependent adenovirus; **GBCs**: gall bladder cells; **IHBECs:** Intrahepatic biliary epithelial cells

## INTRODUCTION

According to the World Health Organization, 346 million people worldwide, or roughly the combined populations of the United States and Canada, have diabetes [1]. All type I diabetes and severe type II diabetes patients need routine insulin injection, and ultimately, a pancreas or islet-cell transplantation would be required. Due to the shortage of donor pancreas or islet cells, many islet transplantation surgeries could not be performed, and most of the patients undoubtedly suffer from the serious complications with the disease progression. Thus, exploring a new pancreas or islet source is urgently needed for treating advanced diabetes.

To date, regenerative medicine is rapidly progressing, especially in the field of cellular reprogramming or lineage reprogramming. The induced pluripotent stem cells (iPSCs) technology developed by two distinguished scientists has recently been awarded the Nobel Prize. Embryonic stem cells (ESCs) or induced pluripotent stem cells, which are believed to be pluripotent and self-renewable, were widely used to regain cell development in culture to produce insulin-producing cells (IPCs), which could restore normal blood glucose metabolism [2,3]. However, the major hurdle in clinical application of ESCs and iPSCs is the risk of tumor formation. Direct reprogramming has been considered as a new approach for the generation of desired cell types, which is derived from one somatic cell type using combination of lineage-specific transcription factors and other modulators [4]. Direct reprogramming of patient's own somatic cells into pancreatic β-cells skips the stem cell induction phase, thereby eliminating immune rejection and the risk of neoplasia formation [5]. Previous studies indicated that the differentiated somatic cells proximal to the pancreas endocrine cells might be an ideal starting material for pancreatic direct reprogramming. Hepatocytes [6], gall bladder cells [7], pancreas ductal cells [8], and endocrine α cells [9] have been successfully transformed into IPCs. Recently, pig dermal fibroblast has been successfully reprogrammed to pancreatic IPCs by using 5-aza-CR, a DNA methyltransferase inhibitor, and a three step induction methods developed for the mouse ESCs. Cell differentiation peaked on day 36, and could last for 102 days. The reprogramming efficiency

reached 38.1 ±9.2%. Remarkably, the induction process did not involve any transcription factors and micro RNAs [10]. Here, we will briefly review recent progress in direct reprogramming of mature somatic cells to IPCs, analyze the efficiency of induction process and potential in reversing hyperglycemia of diabetes, and discuss major problems of translating the reprogrammed IPCs into clinical settings.

## Direct reprogramming and its features

Direct reprogramming, also referred to as direct lineage conversion or transdifferentiation, is a process of directly converting one somatic cell to another differentiated adult cell type mostly by overexpression of a transcription factor cocktail. By forced expression of one or a small number of key transcription factors, direct reprogramming results in a substantial phenotype switch between two distinct cell types [11-13]. Cells generated by the process that does not pass through a pluripotent state are probably not tumorigenic, and may serve as an alternative for cell replacement therapy. Direct reprogramming skips the complex steps of iPS generating process, directly produces patient specific reprogrammed cells, and eliminates the possibility of immune rejection and as well as ethical issues.

The mechanism underlying direct reprogramming has not been fully understood, but it is recognized that epigenetic modulations by ectopic expression of principal transcription factors or signaling pathway modulators have important functions in the direct cell conversion process [5,14]. In contrast to iPS induction, epigenetic memory of the donor cells is not simply removed but the epigenetic features of the target cell type are actively induced, resulting in a direct conversion of the two cell states without physiological intermediates [5]. From assessments of the cell-type-specific histone methylation profiles of alpha, beta and exocrine cells, numerous genes of differentiated alpha cells were found to have bivalent histone modifications, marked by activating the trimethylation of the fourth amino acid (lysine) Histone H3 (H3K4me3) and repressing H3K27me3 histone alteration [15]. A great percentage of these genes were found to be β-cell signature genes involved in transcriptional regulation. These findings have indicated that manipulation of histone methylation signature of human pancreatic islets could explain the possible mechanism of successful alpha to beta cell direct reprogramming.

Different transcription factors cocktails have been screened for successful direct reprogramming of somatic cells. These include Asc11, Brn2, and Myth11 for direct reprogramming of neurons; MyoD for skeletal muscles cells; CATA-1 for eosinophils and platelet; CEBP-αand CEBP-β for macrophages; Cata4, Tbx5, and Baf60c for cardiomyocytes; and Pdx1, Ngn3, and MafA for pancreatic β-cells. Gene delivery system for these transcription factors is initiated from adenovirus and retrovirus methods. The efficiency of this strategy is high, but its integration to the host genome could activate or silence certain genes and therefore change the host phenotype. For safety considerations, other non-integrating gene delivery vectors and methods have been recently reported, including recombinant adenovirus related virus [16], human artificial chromosomes [17], plasmid vectors induced by doxycycline [18], small molecules [19], cellular signal peptides [20], and miRNAs [21]. The next step is to apply the cocktail with the least amount of transcription factors using an efficient and safe transfection technology into the clinic. The clinical application of direct reprogramming is possibly through two ways. One is generating potential target cells in the culture system, and then transplanting to patients after proper screening. The other is to induce direct lineage conversion inside the patients' body.

Although cellular and molecular mechanisms underlying direct reprogramming are still unclear, scientists have already successfully converted different somatic cell types to pancreatic endocrine β-cells in both in vitro and in vivo settings (Table 1).

## Conversion from non β-cells within the pancreas to IPCs

Many studies have demonstrated cell fate conversion of two distinct cell types via direct reprogramming process. Cells within the pancreas share the most similar epigenetic features and microenvironment. Therefore, generating functional β-like cells within pancreas would be feasible and more easily induced.

The adult pancreas consists of both endocrine and exocrine parts, through which they achieve the important functions of food digestion and blood glucose regulation. The exocrine pancreas is composed of acinar cells and ductal cells, which accounts for more than 90% of the entire pancreas, whereas the endocrine pancreas is about 2%.

The first in vivo reprogramming experiments by overexpression of transcription factors within pancreas, being abundant and having a high degree of similarity to the endocrine pancreas, proved that the reprogrammed β-like cells came from acinar cells [13]. Studies by Zhou *et al.* demonstrated that by overexpression of three transcription factors, differentiated pancreatic acinar cells in adult mice could be directly reprogrammed to be β-like cells. They found that transient expression of Pdx1, Ngn3, and MafA by adenovirus could efficiently and stably convert 20% pancreatic acinar cells to β-like cells. The conversion of acinar cells to β-like cells was achieved by a direct reprogramming process, because the absence of Sox9 and Hnf6 expression during the reprogramming process indicated no intermediacy of a progenitor state. The in vivo reprogramming manipulation could rescue the hyperglycemia in a streptozomicin-induced type I diabetes mouse model, demonstrating the successful replacement effect of reprogrammed β-like cells [13]. Another study provided an example of in vitro induction of β-like cells by overexpression of three classic transcription factors in established exocrine cell lines-AR42j-B13 cells [22]. Akinci et al. demonstrated that the strategy could work in culture conditions and transplanting the reprogrammed β-like cells to an immunodeficient diabetic mouse model could reverse hyperglycemia, but not in a regulatory manner.

Besides transcription factors, other key developmental regulators could also trigger the reprogramming process within the pancreas [23]. Furuya et al. [24] found that adenovirus vectors-mediated gene expression of thyroid receptor α induced the conversion of pancreatic acinar cells into insulin producing cells in a streptozotocin (STZ)-treated diabetic model. Ectopic expression of TRα gene could induce the expression of three

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**Table 1**: Generating pancreatic β-like cells from different endoderm cell types by direct reprogramming strategy: current status.

| Cell source | | Race | *In vivo* | *In vitro* | Factors | Transduction methods | Efficiency | Glucose sensitivity | References |
|---|---|---|---|---|---|---|---|---|---|
| exocrine cells | duct epithelial cells | rodents | + | — | Ngn3, Myt1, δ-notch signaling modulator | adenovirus | low, ~6% of the estimated pancreatic reprogramming path completed | N/A | Nathalie Swales [8] |
| | acinar cells | rodents | + | + | TRα | adenovirus vector | 70% transduction efficiency; ~35% c-peptide positive reprogrammed cells | no | Fumihiko Furuya [24] |
| | acinar cells | rodents | + | — | Pdx1, Ngn3,MafA | adenovirus induction | high, > 20% | yes | Zhou [13]; Akinci [22] |
| endocrine cells | alpha cells, | rodents | + | — | pancreatic duct ligation and alloxan | N/A | ~10% of the normal β-cell mass | no | Thorel [30]; Bramswig [15]; Chung [29] |
| liver cells | human liver tissue | human | — | + | Exendin 4, Pdx1 | recombinant adenovirus | 10% insulin positive cells | no | Aviv [51]; |
| | hepatocytes | human | — | + | Pdx1, Ngn3 | Nucleofection | 50% transduction efficiency | yes, partly | Motoyama [48] |
| | hepatocytes | rodents | + | — | Pdx 1 | recombinant adenovirus | 1% insulin contents in liver than that in normal pancreas; plasma insulin contents is 3 fold higher than control | N/A | Ferber [43] |
| | hepatic oval cells | rodents | + | — | Ngn3, Bcellulin | helper-dependant adenoviral vectors (HDAd) | insulin content/mg total protein was ~20% that of the non-diabetic pancreas | yes | Yechoor[46] |
| | hepatocytes | rodents | + | — | Pdx1, Ngn3, MafA | hydrodynamic gene delivery | N/A | no | Cim [6] |
| | hepatocytes | rodents | + | — | Pdx1, Ngn3, MafA | recombinant adenovirus | This insulin content of about 23% of normal islets | yes | Banga[52] |
| gall bladder cells | gall bladder epithelial cells | rodents | — | + | Hes1, Pdx1 | recombinant adenovirus | ~40%–50% transduction efficiency; ~0.1% insulin contents of normal islets | yes | Coad [57] |
| | primary mouse gall bladder epithelial cells | rodents | — | + | Ngn3, Pdx1, and MafA; addition of retinoic acid and inhibition of Notch signaling | E1-deleted adenovirus (serotype 5) | 50% transduction efficiency; 20.5% of total GBCs positive for insulin | no | Hickey [7] |
| | primary intrahepatic biliary epithelial cells | rodents | — | + | Pdx1; NeuroD; Pdx1/vp16 | recombinant adenoviruses | 30-50% transduction efficiency; 6% of the transduced cultures was c-peptide positive | no | Nagaya [58] |

key transcription factors-Pdx1, Ngn3, and MafA- and insulin production inside the pancreas. The reprogramming effect of TRα is attributed to its role in the activation of PI3K signaling pathway.

Duct cells within the pancreas have long been considered as pancreatic progenitor cells because of their critical function during pancreas development and their capability to differentiate into multiple pancreatic cell types under certain circumstances, in vitro and in vivo [8,25,26].

Within the pancreas, besides exocrine cells and β-cells, the most abundant cells are α cells, which are considered as the more suitable starting cell types for β-cells neogenesis. Pancreatic α cells share a more common developmental process with β-cells during organogenesis of endocrine pancreas, and possibly acquire the most similar epigenetic characteristics, which in turn allow for an easier and more efficient direct conversion. A recent study by Bramswig et al. found that differentiated α cells exhibited more epigenetic plasticity in the genes for β-cell development, specifically by activating H3K4me3 and repressing H3K27me3 histone modulations [15], which suggested that α cells may function as progenitor cells in physiological and/or pathological situations. Treating cultured pancreatic islets with the unspecific histone methyltransferase inhibitor Adox could generate glucagon and insulin colocalization to a small group of cells, but the coexpression of PDX1 and glucagon was at a higher frequency within the cells. The results are consistent with the previous knowledge that α cells and glucagon-secreting cells along the ductal line could be considered as pancreatic progenitor cells, which give rise to the β-cell regeneration during β-cell loss in injury or disease. Another advantage of α cell reprogramming is the native microenvironment or niche. These cells, residing in the same microenvironment with β-cells, would be more easily reprogrammed and remain more stable when compared to less

related cells [27] after the reprogramming process.

Early reports showed spontaneous conversion from α cells to functional insulin secreting β-cells in extreme injury or almost complete ablation of β-cell mass [28-30], implying the feasibility of α to β-cell conversion. Ectopic expression of transcription factors Pax4, α cells display a conversion to insulin-secreting cell phenotype and could ameliorate hyperglycemia in STZ -induced diabetic models [31]. Most studies on the conversion of α cell to β-cells are performed in extreme cases or in cases of β-cells removal. Some groups demonstrated that other methods could also elicit this conversion in normal diabetic models. Fomina et al. used several small molecule drugs, inhibitors of RSK kinase family [32], and CDK2 [33], inducing α cells to β-cells transdifferentiation in culture. The β-like cells gained partial features of actual β-cells.

## Generating functional β-like cells from outside of pancreas

The major reasons behind the liver being a possible source of functional surrogate β-cells are as follows. First, liver and pancreas are developmentally related, because both organs are derived from appendages of the upper primitive foregut endoderm. Both adult liver and pancreas cells have similar epigenetic memory of their common embryonic origin. The existence of potential β-cell precursors in the adult liver is of obvious medical interest [34,35].Second, both tissues have many characteristics in common, including responsiveness to glucose, and expression of several common transcription factors, such as HNF1, CEPB/β, and E47 [36,37]. Finally, unlike mature pancreatic β-cells, liver regenerates efficiently mainly by proliferation of mature hepatocytes. A transit compartment of liver progenitor cells has been found among hepatic oval cells in mice [38], which appear to be the main group responsible for liver cell regeneration, especially when hepatocyte proliferation is blocked or delayed [38]. In addition, liver is known for having a large amount of functional redundancy [39]. Notably, insulin and glucagon-producing cells are mainly located around the hepatic central veins, possibly allowing direct hormone release into the bloodstream, without affecting normal hepatic function.

Studies by Ferber et al. showed that ectopic expression of Pdx1 by recombinant adenovirus *in vivo* could induce substantial insulin production in hepatic tissues, in which the transient expression of Pdx1 induced the endogenous gene expression of mouse insulin 1, insulin 2, and prohormone convertase 1/3. Both insulin 1 and insulin 2 were secreted to blood and processed to mature phenotype. In STZ-induced diabetic mice, hepatic immunoreative insulin induced by Pdx1 could ameliorate hyperglycemia [40], which provided first evidence that ectopic expression of key pancreatic transcription factors in liver could induce a small group of liver cells shifting to a β-cell phenotype. In 2003, the same group further found that transient expression of Pdx-1 in liver could induce the expression of numerous pancreatic endocrine and exocrine genes. After ectopic expression of Pdx1, endogenous Pdx1 was up regulated possibly by auto-induction, which may explain the genuine “liver to pancreas” reprogramming. The hepatic insulin secretion induced by Pdx-1 recombinant adenovirus is functional that can prevent STZ-induced hyperglycemia in Balb/c mice for 8 months [39]. In 2004, Yang et al. over expressed Pdx1-VP16 driven by lentiviral vectors in a hepatic oval cells line (WB-1), and successfully reprogrammed these cells into functional insulin producing cells [41]. VP16 is the activation domain from the Herpes simplex virus, and fusing it with Pdx1 could turn Pdx1 into a hyperactive state [42]. It has become clear that high glucose is an essential condition for successfully reprogramming liver to β-cells lineage in vitro, while in a diabetic mouse, endogenous hyperglycemia might facilitate the direct reprogramming process.

Following these studies, other pancreatic development-related transcription factors that induce insulin secreting cells in liver, such as neurogenic differentiation-1 (NeuroD1), Neurogenin 3 (Ngn3), and MafA, have also been reported and confirmed [43-45]. Kojima et al. used a modified adenovirus-helper dependent adenovirus (HDAD) to deliver NeuroD, a basic helix-loop-helix transcription factor downstream of Pdx1, together with betacellulin, a β-cell stimulating hormone, to STZ-treated diabetic mice [44]. They found that in a diabetic liver, the regimen could efficiently induce islet neogenesis, the maturity of which is highly close to the normal islets. The insulin-producing cells formed in a typical islet structure, are sensitive to glucose and sulfonylurea, which possess insulin containing granules in the cytoplasm, similar to the secretory granules in normal pancreatic β-cell. Treated mice in the study were healthy and normoglycemic for a long period (more than 120 days). In contrast, Pdx1 in different doses could elicit insulin production and ameliorate hyperglycemia for a short time, however, the high dose Pdx1 treatment could lead to lethal hepatitis [44]. Yechoor *et al*. demonstrated that Ngn3, together with Btc, can induce endocrine pancreas organogenesis by HDAD vectors in the liver of diabetic mice [43]. The regimen resulted in two waves of insulin secretion, and could reverse hyperglycemia and restore normal glucose-responsive insulin production for a longer period. These cells gave rise to neo-islets containing β cell-like cells, producing insulin in a glucose-responsive manner and completely reversing the diabetes. Their data showed that the neo-islet acquired the function of islet cells, produced the four major islet hormone-producing cell types, and expressed a complete islet specific transcription program (NeuroD1, Pax6, Nkx2.2, Nkx 6.1). The combination of transcription factors Ngn3, NeuroD, and MafA, loaded in a polycistronic adenovirus (Ad) and adenovirus-associated virus (AAV) hybrid virus vector, were infused to diabetic mouse liver. The regimen induced insulin production in liver and reversed hyperglycemia [46]. Afterwards, other important pancreatic transcription factors, such as extendin4 and Nkx6.1 have been reported to promote and enhance the Pdx1-induced direct reprogramming of liver cells to insulin producing cells [47,48].

Recent studies reported that a subgroup of liver cells expressing Sox9 was successfully reprogrammed in vivo into functional insulin secreting duct-like cells, which could maintain normal blood glucose in STZ diabetic mice in the long term [49]. Banga et al. used a polycistronic adenoviral vector expressing the combination of Pdx1, Ngn3, and MafA, where aroud 23% of liver cells could be converted into insulin-secreting cells in a glucose-sensitive manner. It was evident that these insulin-secreting cells did not originate from hepatocytes, because no albumin expression was found in the insulin positive cells. In addition,

only sox9 could be found through the entire induction period, indicating that small bile duct cells or hepatoblast like progenitor cells (hepatic oval cells) might be the true origin [50]. Thus the combination of these three genes could irreversibly elicit a liver-to-β-cells reprogramming, more efficiently than any single factor only.

Several studies [43,49] reported that insulin-producing cells generated by ectopic expression of pancreatic transcription factors were found in the periportal vein region, and expressed ductal cell markers, or showed heptic oval cells morphology and characteristics. It was suggested that the liver to β-cells reprogramming was not a process of transdifferentiation but transdetermination [43], reprogramming occurring in the progenitor cells and not in mature somatic cells. Nonetheless, Ferber et al. demonstrated that liver-derived reprogrammed insulin-producing cells through the overexpression of pancreatic transcription factor mainly came from albumin positive cells, appeared to be mature hepatocytes, and also through expressing other adult hepatic cells maker proteins. Of these certain group cells, EpCAM and other progenitor cell markers were hardly detected [51,52]. Accordingly, the reprogrammed insulin-producing cells are mainly found in the proximity to the central vein, where the adult, terminally differentiated liver cells reside. However, this study suggested that the in vitro operation of the liver cells induces epithelial to mesenchymal transition, through which the cultured liver cells acquire developmental plasticity [52], which was performed in an in vitro culture system. Nevertheless, mature hepatocytes are capable of being directed to become insulin-secreting β-like cells. Further studies are needed to investigate the origin of reprogrammed pancreatic β-like cells, which is meaningful in increasing the efficiency of liver to β-cells reprogramming strategy.

As part of the extrahepatic biliary tissue, gall bladder cells (GBCs) are another possible source of cells for direct conversion to insulin-secreting β-cells. GBCs share a common developmental path with the ventral pancreas during embryogenesis [53]. The specification of these two lineages depends on a series of genetic and epigenetic regulating factors and the extracellular regulating signal pathways, especially the Notch signal modulator Hes1. Coad et al. had successfully induced GBCs into partial insulin expression cells by inhibition of Hes1 in vitro [54]. The induced GBCs were obviously distinguishable from the actual β-cells, implying that GBCs are a possible source of transplantable reprogrammed β-like cells. Inspired by the study, Hickey et al. investigated the extent of GBC derived β-like cells reprogramming and the in vivo functionality of these cells [7]. They employed the classic TFs combination of Ngn3, Pdx1, and MafA, and also added retinoic acid and an inhibitor of notch signaling pathway to the culture condition. Cultured GBCs were rapidly converted towards the β-cell fate. However, the phenotype of these reprogrammed cells was polyhormonal and not responsive to glucose, which is due to incomplete reprogramming into mature β-cells. Nonetheless, cultured GBCs were more prone to be reprogrammed than mouse fibroblast through the same reprogramming regimen. After the transplantation into STZ diabetic mouse, these cells could survive and become insulin positive in vivo for 15 wks, but exhibit no reversal effect of hyperglycemia. The expendable GBCs could be converted by a combination of TFs and auxiliary factors to insulin producing cells, making it possible to obtain enough transplantable functional β-like cells in vitro.

Intrahepatic biliary epithelial cells (IHBECs) have been successfully purified and expanded ex vivo, which could elicit the phenotype of insulin-producing cells upon introduction of key developmental transcription factors. The transcription factors Pdx-1, NeuroD, and Pdx-1/VP16 could induce insulin secretion of IHBEC-derived reprogrammed cells [55]. Although the separation and expansion method for IHBECs are still very time consuming and cumbersome, the study was the first to demonstrate that IHBECs are other candidates for in vitro generation of insulin-producing cells for transplantation into diabetic patients.

## DISCUSSION AND CONCLUSION

Before this strategy becomes translated to clinical application, we need to contemporarily consider the potency and safety of these reprogrammed IPCs. For successful reconstruction of β-cells in diabetic patients, the cells for transplant or those induced in situ needs to be fully functional with enough quantity [44]. By analyzing different pancreatic β-like cells generated by direct reprogramming, the gap between induced cells and endogenous β-cells still exists. Of the current methodologies for lineage reprogramming, transcription factors that play a crucial role in pancreatic β-cells development and maturation have been extensively employed, including Pdx1, Ngn3, NeuroD1, Pax4, MafA, Nkx6.1, and Nkx2.2. However, the reprogramming efficiency reported by different groups greatly differs. The efficiencies were low in most reprogramming experiments (refer to Table 1), indicating that induced cells were not sufficient for successful glucose control. This may partially because the master regulator factors or networks, such as MyoD1 in skeletal myocyte reprogramming or OSKM in iPSCs induction, have not been fully defined in pancreatic endocrine development. Another limitation is the immaturity of reprogrammed pancreatic β-like cells. Only a few studies reported that the final generated β-like cells formed in typical β-cell morphology both in size, shape, and ultrastructure, and also normally function in insulin secretion and glucose regulation [13]. These cells failed to form a normal islet structure, only exhibiting single or in small clusters. Other induced β-like cells only showed an insulin gene expression phenotype, not in β-cell morphology, and not in insulin secretion in a regulated manners [6,24]. Further studies are needed to determine better transcription factor combinations and improve culture conditions such as hypoxia and chemical material addition. The proliferation ability of reprogrammed cells is poor because of their somatic cell origin; thus, the quantity of the cells for transplantation is less to surrogate diseased or damaged host cells. Another reprogramming strategy can solve the proliferation problem because it initially converts somatic cells to progenitor cells and then direct the progenitor cells to target cell types [56,57].

The rationale behind current direct reprogramming technique is that specific transcription factors display a lineage instructive role whereas the reprogrammed process possibly mimics the physiological cell fate transition [58]. Recently, a group from Stanford University revealed a hierarchical mechanism in direct lineage reprogramming of fibroblast into induced neuronal cells. Transcription factors Ascl1, Brn2, and Myt1l were found to

work in a sequential order during the reprogramming process. Ascl1 showed an "on target" pioneer function by occupying most homogeneous genomics sites in the fibroblast, with the other two factors not directly interacting with fibroblast chromatin [59]. This pioneer factor model possibly explains the phenomenon that most direct lineage reprogramming did not require active cell division [5]. It also implies that sequential introduction of transcription factors can improve the reprogramming efficiency. Similarly, as demonstrated in haematopoietic system, the order expression of C/EBPα and GATA2 in a progenitor cell state could possibly generate two distinct cell types. The sequential order and the time point of the key transcription factors can determine the cell fate [4,60]. During pancreas endocrine genesis, the time point of Ngn3 expression could result in a completely different cell type generation. Early Ngn3-expressing cells develop exclusively to α-cells, whereas the later phase expression of Ngn3 gives rise successively to β-, PP-, and δ-cells [61,62] . Ferber et al found that the order of transcription factors did not affect the reprogramming efficiency, but it could increase the number of mature reprogrammed β like cells [63].

In addition to issues on inducing efficiency, safety is another essential aspect requiring careful investigation. An earlier study reported that when using adenoviral vectors encoding pdx1 gene [44], the mice rapidly developed a lethal hepatitis, which was probably caused by trans differentiation of several hepatocytes to an exocrine pancreas phenotype, with the secretion of digestive enzymes, although the cells could also give rise to endocrine β-cells. Another group reported that ectopic expression of Pdx-1 in the liver of transgene mice showed severe liver dysmorphogenesis because of both endocrine and exocrine pancreas differentiation [64]. However, the appearance of Pdx1 in the Ngn3-induced neo-islets did not lead to exocrine enzyme expression [43].

Another possible influencing factor is the gene delivery system itself, the viral vectors. The first-generation adenoviral vectors have been shown to be immunogenic, which could elicit severe liver toxicity. The helper-dependent adenoviral vectors, newly developed from the first generation adenovirus, show reduced immunogenicity upon the removal of adenoviral genes in the vectors, but the adenoviral capsid alone is still immunogenic [65].

Given the risks posed by viral vectors [66], non-viral approaches offer significant advantages. Motoyama et al. reported that nucleofection was a moderately effective way for achieving transient expression of pancreatic transcriptional factors in mature hepatocytes in vitro. They also showed that co-expression of Pdx1 and Ngn3 using a bicistronic expression approach activated the transcription of various islet-related genes, including Ins1 and Ins2 in primary hepatocytes, and that the induced cells acquired the ability to synthesize and secrete insulin. It seems that the use of viral vectors is not indispensable for this approach [45]. The non-viral gene delivery approaches for direct reprogramming have been reported [6]. Cim *et al.* used the hydrodynamic approach to individually deliver genes for rat pdx1, ngn3, and mafA and in combination to livers of normoglycaemic rats. Hydrodynamic gene delivery of multiple transcription factors to rat liver can initiate transdifferentiation to pancreatic β-cells, and could ameliorate hyperglycemia in STZ treated DA rats, but the process is reversible, and probably requires more sustained transcription factor expression. These non viral approaches overcome the immunogenicity and integration risk of a viral system, but the technical challenges in poor targeting and low efficiency still exist. Ultrasound-mediated delivery method is another potential gene delivery tool for somatic cell reprogramming, which has been successfully used in gene transfection of cultured cells and live animals with ideal transfection efficiency [67]. Microbubbles containing target gene vectors could increase gene transfection efficiently with the help of ultrasound. Moreover, it is safer because it is a physical method with good targeting and temporal control [68].

In most studies using different starting tissues and variable induction systems, the reprogrammed cells could secret insulin, and some studies observe reversed hyperglycemia in a diabetic mouse model. However, most of them could not respond to ambient glucose change, because the reprogramming of input cell types is incomplete and the induced cells do not form natural islets structure. Furthermore, the function units inside the pancreas are the islets of Langerhans where insulin producing cells, *i.e.* β-cells, are found in the core and surrounded by glucagon-, somatostatin-, pancreatic polypeptide-, and ghrelin-producing cells. The glucose homeostasis and level of insulin secretion are regulated by various cell types in a cooperative manner. Therefore, further work needs to consider reprogramming of all of the cell types inside the islet, at least the β-cells and α cells. In particular, the reprogrammed cells need to be organized into a normal structure prior to clinical application.

In the future, with a clearer understanding of direct reprogramming and the emergence of new gene delivery methods, the desired surrogate β-cells will be functionally enough for clinical application through the modified direct reprogramming approach. It should be recognized that direct reprogramming is not a natural developmental process; hence the final product cells harvested from this strategy are not true β-cells but are only functionally comparable β-cells, which can be potentially used for the treatment of both type I and advanced type II diabetes mellitus.

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