Production of Pancreatic Beta-Cells from Stem Cells

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Abstract: Diabetes remains a major burden. More than 200 million people are affected worldwide, which represents 6% of the population. The success achieved over the last decade with islet transplantation suggests that diabetes can be cured by the replenishment of deficient beta cells. These observations are proof-of-concept and have intensified interest in treating diabetes not only by cell transplantation but also by stem cells. Regeneration of beta cells from stem and progenitor cells is an attractive method to restore islet cell mass. Pancreatic stem/progenitor cells have been identified, and the formation of new beta cells from pancreatic duct, acinar and liver cells is an active area of investigation. Protocols for the *in vitro* differentiation of embryonic stem (ES) cells based on normal developmental processes have generated beta-like cells that produce high levels of insulin, even though at low efficiency and without full responsiveness to extracellular levels of glucose. Induced pluripotent stem (iPS) cells can also yield insulin-producing cells following similar approaches. Some agents including glucagon-like peptide-1/exendin-4 can stimulate the regeneration of beta cells *in vivo* as well as *in vitro*. Overexpression of embryonic transcription factors in stem cells could efficiently induce their differentiation into insulin-expressing cells. Recent progress in the search for new sources of beta cells has opened up several possibilities for the development of new treatments for diabetes.

Keywords: Islets, Pancreatic stem/Progenitor cells, Embryonic stem cells, Induced pluriopotent stem cells, Islet transplantation.

INTRODUCTION

Diabetes mellitus afflicts more than 200 million people worldwide. Type 1 diabetes results from a cell-mediated autoimmune attack against insulin-secreting β cells in the islets of Langerhans of the pancreas. At the time of clinical diagnosis nearly 70% of β cell mass has been destroyed. In contrast, type 2 diabetes is mainly caused by a combination of systemic insulin resistance and inadequate insulin secretion by pancreatic β cells. In later stages of type 2 diabetes, β cell mass is reduced by about 50%, and oral hypoglycemic agents fail in 20-30% of these patients, requiring exogenous insulin to control hyperglycemia. Surgical resection of the pancreas may also cause insulin-dependent diabetes depending on the size of the remaining pancreas. Although the current therapy using exogenous insulin has greatly improved the lives of diabetic patients, especially type 1 diabetic patients, since the 1920s [1], this method is inaccurate and does not completely control the minute-to-minute fluctuations in systemic blood glucose.

The successes achieved over the last few decades by the transplantation of whole pancreas and isolated islets suggest that diabetes can be cured by the replenishment of deficient β cells. Cell replacement therapies such as transplantation of purified islets have emerged as promising alternatives to whole-organ transplantation [2-15]. Islet transplantation carries the advantage of being less invasive and results in fewer complications compared with the traditional pancreas or pancreas-kidney transplantation. Although the first human islet allograft transplant was performed in 1974 [16], the clinical success rates were not outstanding until 2000 [7]. At this time, dramatic improvement was achieved with the Edmonton Protocol [2]. Since the Edmonton protocol was announced, more than 600 type 1 diabetics in more than fifty institutions have undergone islet transplantation to ameliorate their disease. Islet transplantation technology has advanced considerably, including improvements in islet after kidney transplantation, utilization of non-heart-beating donors [4, 5, 13], single-donor islet transplantation [11], and living-donor islet transplantation [3]. These advances were based on revised immunosuppression protocols [8], improved pancreas procurement strategies [12, 17], improved islet isolation methods [4-6, 9], and enhanced islet engraftment [18, 19]. However, islet transplantation efforts have limitations including the short supply of donor pancreata, the paucity of experienced islet isolation teams, side effects of immunosuppressants and poor long-term results [20]. Further improvements are necessary to make islet transplantation a routine and effective clinical treatment. Nevertheless, the results obtained through human pancreatic islet transplantation have spurred the search for new sources of insulin-producing cells [21]. This review summarizes recent studies on the differentiation of stem cells into insulin-producing cells.

PANCREATIC STEM/PROGENITOR CELLS, PANCREATIC DUCT CELLS

During embryonic development, cells in the pancreatic anlage migrate from the ducts while differentiating to form

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clusters that will eventually become islets [22], so the postnatal pancreatic duct may harbor islet precursor/stem cells. Islet neogenesis, the budding of new islets from pancreatic stem/progenitor cells located in or near ducts, has long been assumed to be an active process in the postnatal pancreas. Several *in vitro* studies have shown that insulin-producing cells can be generated from adult pancreatic ductal tissues [23-30]. It was shown that human adult ductal tissues cultured with Matrigel formed islet-like buds and differentiated into endocrine cells [26, 27]. Moreover, ductal cells purified from dispersed islet-depleted human pancreatic tissue using CA19-9 antibody differentiated into insulin-producing cells [31]. Some insulin-positive cells co-expressed duct markers (CK19 and CA19-9) and heat shock protein (HSP) 27, a marker of non-islet cells, suggesting the transition from ductal cells [31]. Other groups showed the isolation of multipotent pancreatic progenitors from both neonatal and adult pancreata using fluorescent activated cell sorting [29, 30]. By combining flow cytometry and clonal analysis, they showed that a possible pancreatic stem/progenitor cell candidate that resides in the developing and adult mouse pancreas expresses the receptor for the hepatocyte growth factor (HGF) c-Met, but does not express hematopoietic and vascular endothelial antigens such as CD45, TER119, c-Kit, and Flk-1. Moreover, they identified a newly specific marker for ductal cells, CD133 [30]. The cells purified with CD133 (+), CD34 (-), CD45 (-), Ter199 (-) could not only differentiate into pancreatic endocrine and acinar cells but also expressed multiple markers of non-pancreatic organs including the liver, stomach, and intestine. The expanded colonies also expressed the duct marker, CK-19, and nestin. Furthermore, to directly test whether ductal cells serve as pancreatic progenitors after birth and give rise to new islets, Inada *et al*. generated transgenic mice expressing human carbonic anhydrase II (CAII) promoter: Cre recombinase or inducible CreER to cross with ROSA26 loxP-Stop-loxP LacZ reporter mice [32]. CAII-expressing cells within the pancreas act as progenitors that give rise to both new islets and acini normally after birth and after injury (ductal ligation). This identification of a differentiated pancreatic cell type as an *in vivo* progenitor of all differentiated pancreatic cell types has implications for a potential expandable source of new islets for replenishment therapy for diabetes [32]. Such interesting results suggest the possibility of multipotent progenitors in adult pancreatic ducts.

We and others have shown isolation of mouse pancreatic stem cells are capable of self-renewal and multipotency [33, 34]. We established mouse pancreatic stem cells from the duct rich population after islet isolation [34]. After purification on a density gradient, the cells, which were contained in the density range of 1.062–1.11 g/mL, were inoculated into 96-well plates in limiting dilution. From over two hundred clones, fifteen clones were able to be cultured for over three months. The HN#13 cells, which had the highest expression of insulin mRNA after induction, expressed PDX-1 transcription factor, glucagon-like peptide-1 (GLP-1) receptor, and cytokeratin-19, (duct-like cells). These cells continue to divide actively beyond the population doubling level (PDL) of 300. Exendin-4 treatment and transduction of PDX-1 and NeuroD proteins by protein transduction technology in HN#13 cells induced insulin and pancreas-related gene expression. Yamamoto *et al*. showed a method for isolating pancreatic ductal epithelial cells from adult mice by stimulation of cAMP signaling [33]. Pancreatic ductal cells were grown in medium containing cholera toxin or 8-bromocyclic adenosine monophosphate, which is known to be an intracellular cAMP generator and then cloned by limiting dilution. The isolated clonal cells were maintained for more than a year under the medium condition. The cells expressed high levels of CK and PDX-1 and were differentiated into insulin- and somatostatin-producing cells by adenovirus-mediated expression of ngn3. Furthermore, albumin production was induced by dexamethasone or by long-term culture in serum-containing medium. These cells are able to partially differentiate into pancreatic endocrine cells and hepatocyte-like cells and are therefore considered to have the characteristics of endodermal progenitor cells. These two articles suggest the possibility of multipotent stem cells in or near adult pancreatic ducts.

We applied the isolation methods to human pancreatic tissue. However, the human cells from the duct-rich population were unable to divide after 30 days under any culture conditions, although the cells were able to differentiate into insulin-producing cells [35]. There are some possibilities for these differences. One possibility is that the human pancreatic stem cells were unable to maintain an undifferentiated state under any culture conditions in our study. Indeed, the culture conditions of embryonic stem cells are different between human and mouse. If so, we need to evaluate other culture conditions. Another possibility is that pancreas preservation and/or isolation stress affected the survival of human pancreatic stem cells because human islet isolation is likely more stressful for cells than mouse islet isolation. A third possibility is that there are no stem cells in the human pancreas, while there are some stem cells in the mouse pancreas. A forth possibility is that there are no stem cells in both human and mouse pancreata and that our established cells from mouse pancreata are spontaneously "immortalized" cells. However, our established cells were maintained only in a specific culture condition, they do not have tumorigenic properties, and do have a normal chromosome, suggesting that the cells are likely to be mouse pancreatic stem cells.

The Edmonton group attempted to define a correlation between graft cellular composition and long-term transplant success using eighty-three transplanted human islet grafts [36]. A significant positive correlation was observed between the number of ductal-epithelial cells transplanted and long-term metabolic success as assessed by an intravenous glucose tolerance test at approximately two years post-transplantation, while no significant correlation was observed between the total islet equivalents and long-term metabolic success. The data showed that the presence of ductal cells in clinical islet transplantation may improve the long-term metabolic outcome.

TRANSDIFFERENTIATION OF ACINAR CELLS TO β CELLS

The transdifferentiation of acinar cells to islets has also been championed [37-39]. Minami *et al*. showed that pancreatic acinar cells could transdifferentiate into insulin-secreting cells with secretory properties similar to those of

native pancreatic β cells [39]. The frequency of insulin-positive cells was only 0.01% in the initial preparation and increased to approximately 5% under culture conditions. Analysis by the Cre/loxP-based direct cell lineage tracing system indicates that these newly made cells originate from amylase/elastase-expressing pancreatic acinar cells. Melton's group showed *in vivo* reprogramming of adult pancreatic exocrine cells to β cells [40]. Using a strategy of re-expressing key developmental regulators *in vivo*, they identified a specific combination of three transcription factors (Ngn3, Pdx1, and MafA) that reprograms differentiated pancreatic exocrine cells in adult mice into cells that closely resemble β cells. The induced β cells were indistinguishable from endogenous islet β cells in size, shape and ultrastructure and expressed genes essential for β cell function. Moreover, the induced cells were able to ameliorate hyperglycaemia by remodeling local vasculature and secreting insulin. These data provide an example of cellular reprogramming using defined factors in an adult organ and suggest a general paradigm for directing cell reprogramming without reversion to a pluripotent stem cell state. Bouwens' group found evidence for acinar-to-islet conversion under the form of transitional cells co-expressing amylase and insulin, suggesting that fully differentiated exocrine pancreatic cells retain the capacity to undergo important phenotypic switches [37]. However, using alloxan diabetic mice treated *in vivo* with epidermal growth factor (EGF) and gastrin, the same group concluded that the observed normalization of blood glucose and increased islet mass resulted from increased neogenesis from ducts [41]. This conclusion was based on the findings of transitional cells expressing both CK and insulin and increased ductal proliferation without increased β cell proliferation.

TRANSDIFFERENTIATION OF LIVER/INTESTINAL CELLS TO β CELLS

Another approach to the generation of β cells is the differentiation of stem/progenitor cells in other organs derived from endoderm. The pancreas, liver, and gastrointestinal tract are all derived from the anterior endoderm and some articles show that their stem/ progenitor cells can differentiate into insulin-producing cells [42-48]. *In vivo* transduction of mice with an adenovirus expressing PDX-1 [42, 43], both betacellulin and BETA2/NeuroD [45], or a modified form of PDX-1 carrying the VP16 transcriptional activation domain [47], or MafA together with PDX-1 and NeuroD [48] markedly increases insulin biosynthesis and induces various pancreas-related factors in the liver. Furthermore, in streptozotocin-induced diabetic mice, overexpression of these factors in the liver ameliorated glucose tolerance. These data suggest that liver cells may have potential utility in the treatment of diabetes. Other groups showed which cells in liver can trans-differentiate into insulin-producing cells. One group showed that highly purified adult rat hepatic oval cells, which are capable of differentiation to hepatocytes and bile duct epithelium, can trans-differentiate into pancreatic endocrine hormone-producing cells when cultured in a high glucose environment [49]. Nagaya *et al*. recently showed that intrahepatic biliary epithelial cells (IHBECs) could become a new source of insulin-producing cells [50]. IHBECs were transfected with adenoviral-Pdx-1, NeuroD or Pdx-1/VP16. IHBECs expressed some endocrine progenitor genes (Neurog3, NeuroD, Nkx6.1, and Pdx-1 as well as insulin, GLUT2, and prohormone convertase 1 and 2). Transduced cells released insulin (Ad-Pdx-1 0.08+/-0.05, Ad-NeuroD 0.33+/-0.09, Ad-Pdx-1/VP16 0.37+/-0.14 ng/1x10^5 cells after 48 h in culture). These data suggest that oval cells and/or biliary epithelial cells in liver are potential candidates for new sources of insulin-producing cells that can be used for transplantation in diabetes.

PDX-1 and/or Isl-1 induction of immature rat intestinal stem cells (IEC-6) into insulin-producing cells has been reported [51, 52]. It was also shown that GLP-1-(1-37) induces insulin production in developing and, to a lesser extent, adult intestinal epithelial cells *in vitro* and *in vivo*. This process is mediated by the up-regulation of the Notch-related gene, ngn3, and its downstream targets, which are involved in pancreatic endocrine differentiation [53]. These adult intestinal stem cells are also probable candidates for a new therapeutic approach to diabetes mellitus.

EMBRYONIC STEM CELLS

Embryonic stem cells (ES cells) are pluripotent diploid cells, proliferate indefinitely in an undifferentiated state, and can be induced to differentiate into cells of all three germ layers both *in vivo* and *in vitro* [54, 55]. It has been reported that ES cells from mouse [56-63], monkey [64], and human [57, 65] were able to differentiate into insulin-positive cells, a potential source of new β cells. Beginning in 2000, it was reported by many groups that ES cells can differentiate into insulin-producing cells *in vitro* by the selection of ES cells expressing nestin, an intermediate filament protein thought to be a marker of neural stem cells [58-61]. However, this has proven more difficult than expected. Recent reports demonstrated that ES cells give rise to a population of cells that contain insulin, not as a result of biosynthesis but from the uptake of exogenous insulin by cell apoptosis [66-68]. Melton's group took five ES cell lines (both murine and human) and differentiated them into insulin-producing cells. Their results showed that insulin-1 mRNA was not detected and insulin-2 mRNA detection was weak, although 10 to 30% of the cells stain with antibodies to insulin and that fifty-micrometer clusters of insulin-staining cells were produced as previously described [58]. They concluded that most of the insulin-staining cells were apoptotic cells and insulin in the culture medium affected staining [66]. Therefore, the measurement of not only insulin but also C-peptide is needed to prove that insulin has been synthesized. Also, considering that several articles have shown the selection of nestin and that human neural stem cells are capable of producing small amounts of insulin [69], it is possible that the insulin found in ES cell-derived cells arises from aberrant neuronal differentiation. However, these cells have no more than a tiny fraction of the insulin content of normal β cells and exhibit incomplete expression of β cell markers.

The lack of success of these early attempts at differentiating ES cells has focused attention on the fundamentals of normal embryonic development to better understand the early stages of endoderm formation. D'Amour *et al*. have recently developed a five-step protocol for differentiation of human ES cells to endocrine cells capable of synthesizing the pancreatic hormones insulin, glucagon, somatostatin, pancreatic polypeptide and ghrelin [70]. This process mimics

in vivo pancreatic organogenesis by directing cells through stages resembling definitive endoderm, gut-tube endoderm, pancreatic endoderm and endocrine precursor-en route to cells that express endocrine hormones. In stage 1, human iPS cells were transitioned through mesendoderm to definitive endoderm using high concentrations of activin A treatment period to 3 days and adding Wnt3a during the first day of activin exposure in the context of low FBS supplementation, as previously reported [71, 72]. As with the protocol, it was shown that the human ES cell-derived definitive endoderm expressed the markers SOX17 and CXCR4, and showed anterior character as indicated by expression of the anterior definitive endoderm markers CER and FOXA2 [70]. In stage 2, activin A was removed, which is essential to allow the transition of definitive endoderm to a stage resembling the primitive gut tube. During stage 2, a considerable upregulation of the gut-tube markers HNF1B and HNF4A at both the mRNA and protein levels was shown in human ES cell-derived definitive endoderm. Simultaneously, expression of the definitive endoderm markers CER and CXCR4 was considerably reduced. Although removal of activin A was sufficient to induce expression of gut-tube markers, it was shown that addition of FGF10 and the hedgehog-signaling inhibitor KAAD-cyclopamine in human ES cell-derived definitive endoderm resulted in a 160-fold increase in insulin mRNA compared with activin removal alone. In stage 3, the gut-tube endoderm was exposed to retinoic acid (RA) together with KAAD-cyclopamine and FGF10. Upon addition of RA, the cells of human ES cell-derived gut-tube endoderm rapidly began to express high levels of PDX1 and HNF6 while maintaining or increasing expression of HNF1B and HNF4A. Expression of this combination of genes is indicative of posterior foregut. During stage 4, the PDX1-expressing posterior foregut endoderm cells were recruited to the pancreatic and endocrine lineages. The cells derived from human ES cells expressed PDX1, NKX6-1, NKX2-2, NGN3, and/or PAX4 after treatment with DAPT and exendin-4. During stage 5, endocrine cells expressing the pancreatic hormones insulin, glucagon, somatostatin, pancreatic polypeptide and ghrelin are produced. The cells derived from human ES cells co-expressed the pan-endocrine markers NEUROD1, ISL1, PAX6 and synaptophysin (encoded by SYP), and cells that expressed insulin also expressed islet amyloid polypeptide (encoded by IAPP) after treatment with exendin-4, IGF-1, and HGF. The human ES cell-derived insulin-expressing cells have an insulin content approaching that of adult islets. Similar to fetal β-cells, they release C-peptide in response to multiple secretory stimuli, but only minimally to glucose *in vitro* [70] or *in vivo* [73]. Although only a few hormone-expressing endocrine cells, which do not respond to glucose, are presenting fetal human pancreatic tissues at 6–9 weeks of age, these tissues have been shown to differentiate into glucose-responsive islets that correct hyperglycemia after implantation in diabetic mice [74, 75]. Cells at stage 4 of the five-stage protocol are shown to be similar to fetal 6- to 9-week pancreatic tissue in that they consist primarily of pancreatic epithelial cells, with few hormone-expressing cells [70, 71]. The same group recently showed that the stage-4 cells developed *in vivo* into endocrine cells that are morphologically and functionally similar to pancreatic islets and protect mice against streptozotocin (STZ)-induced hyperglycemia [76].

Melton's group recently reported small molecules that efficiently direct endodermal differentiation of mouse and human embryonic stem cells [77]. In a screen of 4000 compounds, they identified two cell-permeable small molecules that direct differentiation of ES cells into the endodermal lineage. These compounds induced nearly 80% of ES cells to form definitive endoderm, a higher efficiency than that achieved by Activin A or Nodal, commonly used protein inducers of endoderm. The chemically induced endoderm expressed multiple endodermal markers, was able to participate in normal development when injected into developing embryos, and was able to form pancreatic progenitors. The application of these small molecules to differentiate ES cells into endoderm could achieve a reproducible and efficient production of desired ES cell derivatives.

On the other hand, a significant number of problems remain unsolved in terms of their clinical application, such as the risk of tumorigenicity and immunosuppression after transplantation. The ethical issue is another major obstacle to the clinical use of ES cells.

INDUCED PLURIPOTENT STEM CELLS

Initial induced pluripotent stem (iPS) cells have been generated from mouse and human somatic cells by introducing Oct3/4 and Sox2 with either Klf4 and c-Myc or Nanog and Lin28 using retroviruses [78-82]. Mouse and human iPS cells are similar to ES cells in morphology, gene expression, epigenetic status and *in vitro* differentiation. Furthermore, mouse iPS cells give rise to adult chimeras and show competence for germline transmission [83-85]. This technical breakthrough has significant implications for overcoming the ethical issues associated with ES cell derivation from embryos. However, chimeras and progeny mice derived from iPS cells frequently develop tumors, which in some cases may be due to reactivation of the c-Myc oncogene [84]. Recently it has been shown that it is possible to generate iPS cells without retroviral insertion of c-Myc [86, 87], albeit at a lower efficiency. Moreover, it has been reported that valproic acid (VPA), a histone deacetylase inhibitor, enables reprogramming of primary human fibroblasts with only two factors, Oct4 and Sox2, without the need for the c-Myc or Klf4 [88]. The results support the possibility of reprogramming through some chemical means, which would make therapeutic use of reprogrammed cells safer and more practical. Another group showed that adult mouse neural stem cells expressed higher endogenous levels of Sox2 and c-Myc than embryonic stem cells, and that exogenous Oct4 together with either Klf4 or c-Myc was sufficient to generate iPS cells from neural stem cells (NSCs) [89]. The same group also showed that exogenous expression of the germline-specific transcription factor Oct4 was sufficient to generate pluripotent stem cells from adult mouse NSCs [90]. These data suggest that, in inducing pluripotency, the number of reprogramming factors can be reduced when using somatic cells that endogenously express appropriate levels of complementing factors.

However, retroviral integration of the transcription factors may activate or inactivate host genes, resulting in tumorigenecity, as was the case in some patients who underwent gene therapy. Yamanaka's group reported the generation of mouse iPS cells without viral vectors. Repeated trans-

fection of two expression plasmids, one containing the complementary DNAs (cDNAs) of Oct3/4, Sox2, and Klf4 and the other containing the c-Myc cDNA, into mouse embryonic fibroblasts resulted in iPS cells without evidence of plasmid integration [91]. The generation of mouse iPS cells from fibroblasts and liver cells by using non-integrating adenoviruses transiently expressing Oct4, Sox2, Klf4, and c-Myc has also been shown [92]. Moreover, the generation of human iPS cells without genomic integration of exogenous reprogramming factors by plasmids expressing OCT3/4, SOX2, KLF4, c-MYC, NANOG, LIN28, and SV40LT has been reported [93]. More recently, Ding's group reported generation of protein-induced pluripotent stem cells (piPSCs) from murine embryonic fibroblasts using recombinant cell-penetrating reprogramming proteins [94]. A polyarginine (11R) protein transduction domain (PTD) was fused to the C terminus of four reprogramming factors: Oct4, Sox2, Klf4, and c-Myc. They demonstrated that such piPSCs by 4 factors-11R were able to long-term self-renew and were pluripotent *in vitro* and *in vivo*. These four reports provide strong evidence that insertional mutagenesis is not required for *in vitro* reprogramming. The production of iPS cells without integration into host genome addresses a critical safety concern for potential use of iPS cells in regenerative medicine.

Although two papers showed the generation of insulin-secreting islet-like clusters from human iPS cells [95, 96], the efficiency of the method into insulin-producing cells seems low. The method as shown in section of embryonic stem cells may represent a critical step in the development of insulin-producing cells from iPS cells.

CONCLUSION

The most difficult and yet unsolved issue is how to differentiate stem/progenitor cells and acquire fully functional islets. The possibility that pharmacological agents might increase β cell mass is tantalizing because a decrease in β cell mass is the root cause of both types of diabetes. We recently showed that protein transduction technology [23-25, 97-101], as also used for induction to iPS cells, is an effective method for the induction of insulin-producing cells from stem cells. We reported that transduction of PDX-1 and NeuroD proteins induces insulin gene expression [24, 98, 99]. Other groups also showed that transduction of NeuroD *in vivo* or TAT-Ngn3 fused TAT-PTD induced insulin-producing cells [102, 103]. These results also raised the possibility of differentiating stem/progenitor cells into insulin-producing cells by using the appropriate sequence and combination of PTD-fused transcription factors. Further investigations to induce differentiation of stem/progenitor cells into insulin-producing cells will help to establish cell-based therapies in diabetes.

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