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1. Introduction

Type 1 diabetes mellitus is a classical autoimmune disease that results from immune-mediated destruction of pancreatic β-cells, primarily by T cells specific for β-cell antigens, leading to an absolute loss of insulin production (Gillard and Mathieu et al., 2011; van Belle et al. 2011). While the disease can become clinically apparent at any age, it commonly starts during childhood, and may appear later in adulthood in approximately 30-40% of affected individuals (Fändrich and Ungefroren, 2010; Knip, 1997). A combination of a genetic predisposition and autoimmune processes contribute to its development resulting in the gradual destruction of the insulin-producing β-cells. Daily insulin delivery by injection or pump to manage hyperglycemia by no means represents a cure, often resulting in hypoglycemic episodes (Leitão et al., 2008; Noguchi, 2009). Current strategies to prevent or reverse diabetes are broadly based on the concepts of β-cell regeneration, replacement or protection from T-cell-mediated autoimmune destruction. While transplantation of both, whole pancreas as well as islets of Langerhans, is able to restore endocrine function and glucose homeostasis, islet transplantation offers added advantage in terms of being minimally invasive, preventing the incidence of severe hypoglycemic episodes and significantly lowering hypoglycemic unawareness, thereby improving the quality of life of the transplant recipients (Bretzel et al., 2007; Langer, 2010; Noguchi et al., 2009). Furthermore, manipulation of islets in vitro, prior to transplantation, provides an opportunity for the development of various therapeutic manipulations aimed at achieving better transplant outcomes. However, despite islet transplantation being an excellent “proof of principle” for β-cell replacement therapy, its wide-spread applicability is limited by the scarcity of donor organs resulting in inadequate number of islets available for transplant as well as the harmful side-effects of immunosuppressive therapy (Huang et al., 2008). To overcome these limitations, generating a large quantity of β-cells that would allow transplantation of
sufficient β-cell mass to achieve normoglycemia, along with restoration of immunologic
tolerance, represents a highly attractive alternative. To this end, proposed mechanisms for islet
regeneration consist of replication of pre-existing β-cells, neogenesis from ductal and non β-
cell progenitors, transdifferentiation of fully differentiated acinar cells and directed-
differentiation of stem cells (putative β-cell progenitors/pancreatic stem cells; embryonic,
mesenchymal, hematopoietic and umbilical cord blood-derived stem cells etc). While several
studies demonstrate these mechanisms in mice, proving the occurrence of these phenomena in
humans is hard to achieve. In this review, we will focus on various approaches to obtain an
expandable mass of functional, insulin-secreting β-cells, emphasizing the major cell candidates
for β-cell regeneration, the specific factors and stimuli involved in β-cell differentiation and
expansion and alternate strategies that may enhance the effective β-cell mass and function. The
basic science driving these discoveries and the obstacles that hinder clinical translation of these
avenues will be highlighted from the perspective of islet transplantation.

2. Endocrine pancreas plasticity in physiological conditions

Although the formation of new islets in adults has primarily been demonstrated in response
to pancreatic injury (eg. pancreatic duct ligation (PDL), β-cell ablation, partial pancreactomy
etc.) and metabolic stress, there is ample evidence that β-cell replication from existing cells
occurs throughout adulthood (Brennand et al, 2007; Dor et al., 2004; Levetan, 2010; Nir et al.,
2007; Teta et al., 2007). This has been observed in several physiologic situations including
amongst others, late pregnancy and obesity (Bernard-Kargar and Ktorza, 2001). In both
these cases, increases in β-cell mass have been observed in response to insulin resistance and
contribute to insulin oversecretion. Several studies have demonstrated a doubling of β-cell
mass by the end of pregnancy that decreased progressively after parturition, a good
illustration of the plasticity of the endocrine pancreas (Karnik et al., 2007; Rieck & Kaestner,
2010; Scaglia et al., 1995). As in pregnancy, euglycemia is maintained in obesity by increased
insulin secretion, due not only to enhanced individual β-cell activity but also to β-cell
growth. This increase in β-cell mass approximates 50% in obese, non-diabetic humans and
seems consigned to β-cells, with α, δ and PP cell mass remaining unchanged (Bernard-Kargar & Ktorza, 2001). In addition to β-cell expansion based on self-duplication, there is
evidence indicating the contribution of stem cell differentiation towards pancreatic β-cell
maintenance (Bonner-Weir et al.,2004, 2006,2010). The mechanism of islet regeneration
remains controversial, making identification of β-cell progenitors and the in-depth
understanding of the underlying mechanisms that trigger β-cell regeneration and expansion
absolutely critical in order to apply this strategy in a clinical scenario.

2.1 β-cell progenitors within the pancreas

The exocrine tissue of the pancreas consists of acinar cells that secrete digestive enzymes
into a branched ductal network that drains into the gastrointestinal tract. The endocrine cells
consist of α, β, δ, ε, and PP cells that are grouped into islets of Langerhans and secrete
insulin and other polypeptide hormones into the bloodstream. Numerous studies propose
that in addition to replication of pre-existing β-cells, new β-cells can be produced from
differentiated adult cells by interconversions amongst different pancreatic cell
compartments as well as neogenesis from ductal and circulating progenitors and putative
pancreatic stem cells (Bonner-Weir et al., 2004,2006; Gao et al., 2003, 2005; Granger and
Present Accomplishments and Future Prospects of Cell-Based Therapies for Type 1 Diabetes Mellitus

Kushner, 2009; Juhl et al., 2010; Kikugawa et al., 2009; Pittenger et al., 2009). However, while these strategies demonstrate the generation of insulin-producing cells, the major challenge encountered is the inability to generate sufficient amounts of ‘glucose-responsive’ β-cells to normalize hyperglycemia. True mature β-cells are defined by the ability to store large amounts of insulin and secrete it in a regulated manner in response to glucose challenge. This inability to mature into ‘glucose responsive’ β-cells may indicate an underlying inability to dedifferentiate completely to a progenitor state or to efficiently redifferentiate/transdifferentiate into β-cells. Therefore, while these studies outline the therapeutic potential of various regenerative strategies for β-cell expansion, they also highlight the importance of elucidating the underlying mechanisms required to form islets that perform similar to primary islets used in clinical applications.

2.1.1 Can β-cells be derived from the exocrine pancreas - the plasticity of the pancreatic acinar cell.

The exocrine tissue consisting of acinar and ductal cells, comprises approximately 95% of the adult pancreas and shares a common progenitor with endocrine cells, namely, pancreatic and duodenal homeobox 1 (Pdx1)-expressing cells (Gu et al, 2002). Several studies indicate the capability of acinar cells to transdifferentiate into insulin positive cells (IPCs) either directly (Minami et al., 2005; Minami and Seino S, 2008) or via a ductal intermediate (Means et al., 2005), usually accompanied by a corresponding increase in functional β-cell mass. However, other studies using cultured, genetically marked, murine acinar cells indicate that these cells are only able to rapidly transdifferentiate into a amylase-negative, keratin 19- and mucin-antigens positive ductal phenotype and not into functional β-cells (Blaine et al., 2010). Using a mouse model that develops hyperplastic ducts containing IPCs in response to the transforming growth factor (TGF-α), Blaine et al. performed genetic lineage tracing experiments and demonstrated that hyperplastic ductal cells arose largely from acinar cells that transdifferentiated into ductal cells, while IPCs adjacent to acinar-derived ductal cells arose from pre-existing IPCs, suggesting that islet endocrine cells can intercalate into hyperplastic ducts as they develop. Thus, the apparent pancreatic plasticity resulted from both, the ability of acinar cells to transdifferentiate and of endocrine cells to reorganize in association with duct structures. Enthusiasm was further curbed by a Cre/loxP-based lineage tracing study suggesting that transdifferentiation of acinar to β-cells was unlikely a part of the normal β-cell turnover, even after injury such as pancreatectomy, ductal ligation, or pancreatitis (Desai et al., 2007). A recent time-specific lineage tracing study indicated that in mice containing genetically marked ductal and acinar cells carrying the mucin gene Muc1, the Muc1 positive cells only gave rise to β-cells and other islet cells in utero (Kopinke & Murtaugh, 2010). From birth onwards, Muc1 lineage-labeled cells were confined to the exocrine compartment with no detectable contribution to islet cells. In contrast, other studies showed that differentiated exocrine cells were capable of reverting to a partially dedifferentiated state with the capacity to transdifferentiate into different phenotypes, including ductal cells (Rooman et al., 2000), hepatocyte-like cells (Lardon et al., 2004) and IPCs (Baeyens et al., 2005, 2008). Treatment of rat exocrine pancreatic cells in vitro with epidermal growth factor (EGF) and leukaemia inhibitory factor (LIF) resulted in an 11-fold increase of the β-cell mass in a 3 day culture period with the newly-formed cells secreting insulin in response to glucose, containing insulin immunoreactive secretory granules, immunoreactive for C-peptide-I, Pdx-1 and Glucose transporter-2 (Glut-2) and able to
restore normoglycemia upon transplantation into alloxan-diabetic mice. Interestingly, the cells were immunoreactive for amylase and cytokeratin-20, characteristics of exocrine cells. Similar results were obtained using adult mouse pancreatic acinar cells cultured in suspension in the presence of EGF and nicotinamide. Analysis using a Cre/loxP-based direct cell lineage tracing system indicated that newly made cells originated from amylase/elastase-expressing pancreatic acinar cells and had insulin-containing secretory granules (Minami et al., 2005). Insulin secretion was stimulated by glucose, sulfonylurea, and carbachol; potentiation by glucagon-like peptide-1 (GLP-1) was also observed. In addition, enzymatic dissociation of pancreatic acini itself lead to activation of EGF signaling and inhibition of EGF receptor kinase blocked transdifferentiation, suggesting that activation of EGF signaling was required for transdifferentiation of pancreatic acinar cells into insulin-secreting cells with secretory properties similar to native pancreatic β-cells (Minami & Seino, 2008). Using a different approach, Melton and coworkers demonstrated that a specific combination of transcription factors Neurogenin3 (Ngn3), Pdx1 and MafA could reprogram differentiated, pancreatic exocrine cells in adult mice into cells that were indistinguishable from endogenous islet β-cells in size, shape and ultrastructure and expressed genes essential for β-cell function (Zhou et al., 2008). These cells increased by 20% after the triple adenoviral transduction and were capable of ameliorating hyperglycemia by remodeling local vasculature and secreting insulin, providing proof that 1) exocrine cells can assume an insulin-secreting phenotype and 2) that cellular reprogramming of exocrine pancreatic tissue using defined factors in an adult organ was possible without reverting to a pluripotent stem cell state or involving activation of the cell cycle. The drawback of this study however, was that these cells did not form islets nor became incorporated into them. Also, the use of viruses raises concerns about insertional mutagenesis and tumor initiation. A more clinically suitable study through induction of acinar-to-islet transdifferentiation was the expansion of adult, human islet tissue via a duct epithelial-like intermediate using a combination of gastrin, hepatocyte growth factor (HGF), and the Reg family member INGAP, islet neogenesis gene associated protein (Lipsett et al., 2007). A 6-fold increase in total dithizone (DTZ) positivity and approximately 15-fold increase in neoislets compared with untreated control tissue cultures was observed. However, these cells were not as efficient as native islets in the production of insulin and the success of this process depended heavily on the reorganization of the extracellular matrix begging definition of the role of extracellular scaffolding in increasing the output of islet-like cells or their insulin-secreting function(Okuno et al., 2007). Thus, transdifferentiation into insulin-secreting cells with low insulin expression levels and secretory response, absence of transcription factors essential for β-cell development and the inability to fully mature remain serious concerns.

2.1.2 The feasibility of β-cell regeneration through neogenesis from pancreatic ductal cells

Morphological observations of islet-ductal complexes comprising IPCs within or near adult pancreatic ducts in instances such as pregnancy or obesity, damage or disease, supported by various lineage-tracing studies in both human and rodent pancreata, suggest that differentiated pancreatic ductal cells act as in vivo progenitors for pancreatic β-cells (Bonner-Weir et al., 2008; Inada et al., 2008; Suarez-Pinzon et al., 2005; Xu et al., 2008, Xia et al. 2009). This is particularly impressive in the pancreata of individuals with a clinical diagnosis of chronic pancreatitis or with asymptomatic pancreatic fibrosis wherein immunophenotypical
characterization of duct-associated islet-like cells revealed positivity for both ductal and endocrine markers (Gianani et al., 2006, 2011). The presence of insulin- and cytokeratin-positive cells has also been reported in transplanted pancreata of individuals with recurrence of β-cell autoimmunity (Martin-Pagola et al., 2008). Transient expression of the Ngn3 marks progenitor cells in the pancreas as they differentiate into islet cells. Using lineage-tracing and pancreatic ductal ligation in adult mice, Xu et al. revealed the appearance of facultative Ngn3+ Cytokeratin19+ progenitors in the ductal lining of the regenerating portion but not in the non-injured pancreas during the robust β-cell expansion that followed pancreatic duct ligation (PDL), suggesting that endogeneous duct cells represented an obvious target for therapeutic regeneration of β-cells in diabetes (Xu et al., 2008). Concerns raised about using Ngn3 expression as a marker of endocrine progenitors and neogenesis were allayed in a very recent study that developed a transgenic mouse line that could monitor Ngn-3 expression, and thus islet cell genesis, by surrogate markers that secreted alkaline phosphatase (SeAP) and enhanced green florescent protein (EGFP) (Shimajiri et al., 2011). They demonstrated that in transgenic embryos, cells expressing EGFP lined the pancreatic ducts while SeAP was readily detectable in embryos, in the media of cultured embryonic pancreases and in the serum of adult animals. Duct ligation in adult mice caused an increase in circulating SeAP levels. A genetic lineage tracing study using a human carbonic anhydrase II (CA2) promoter fragment to express Cre and CreER in differentiated duct cells showed that a significant proportion of adult pancreatic ductal cells retain the potential to differentiate into other pancreatic cell types including β-cells. Impressively, two weeks after duct ligation, 42% of islets and 24% of β-cells expressed the lineage marker (Inada et al., 2008). In fact, dedifferentiation of ductal epithelial cells is often associated with the epithelial-mesenchymal transition (EMT) (Fanjul et al., 2010). An interesting study by Seaberg et al reported the identification of multipotent precursor cells from the adult mouse pancreatic islet and ductal populations (Seaberg et al., 2004) that proliferated in vitro to form clonal colonies coexpressing neural and pancreatic precursor markers. Upon differentiation, individual clonal colonies produced distinct populations of neurons and glial cells, pancreatic endocrine β-, α- and δ-cells and pancreatic exocrine and stellate cells. The newly generated β-like cells demonstrated glucose-dependent Ca(2+) responsiveness and insulin release, representing a previously unidentified, intrinsic, pancreatic precursor population that is a promising candidate for cell-based therapeutic strategies. Taken together, these data show that the ductal epithelium in human as well as rodent pancreata is capable of producing IPCs, however whether these cells are mature β-cells with the full complement of molecules necessary for maintaining optimal glucose homeostasis remains to be seen. Despite these advances, the ductal origin hypothesis of pancreatic regeneration is under attack (Kushner et al., 2010). While some reports suggest that β-cell replication rather than neogenesis is the main source of regenerating islets in the murine pancreas (Dor et al., 2004), others using genetic labeling to fate map embryonic and adult pancreatic duct cells have demonstrated that pancreatic ductal epithelial cells do not contribute to endocrine or acinar cells postnataally (Solar et al., 2009). The latter study demonstrated that as the embryonic Hnf1β+ epithelium gradually acquires the anatomical configuration of the pancreatic ductal network, it sequentially sets aside cells for the acinar and endocrine lineages and finally takes on a differentiated pancreatic duct phenotype. The embryonic ducts give rise to both differentiated endocrine and ductal cells but once the pancreatic duct epithelium acquires a differentiated phenotype, it does not contribute
significantly to new β-cells that are formed in diverse physiological or regenerative settings. However, certain caveats have not been addressed in this study; amongst them being low labeling efficiency during embryonic development and variable expression of Hnf1β in ductal progenitors with the cells that do become marked, representing a population of duct cells with a higher activity of Hnf1β transcription or greater accessibility to Cre recombinase. Thus, taking into account possible low levels of leakiness of the Cre-lox system over time with the strong insulin promoter, the small proportion of the islets counted, limited (only 30%) labeling of the β-cells and the lack of examination of the new lobes after pancreatectomy, neogenesis in the adult cannot be ruled out by negative data of a lack of dilution of labeled β-cells (Kushner et al., 2010). The observed discrepancies warrant rigorous investigation with definitive experiments, using lineage tracing studies with additional markers that label either ducts or other candidate precursor cells to tease out the underlying mechanisms. Until then, however, the identification of differentiated pancreatic duct epithelium represents an expandable source of facultative progenitor cells for generating β-cells with major implications for replenishment therapy for diabetes.

2.1.3 Replication of pre-existing β-cells: contribution to β-cell growth and regeneration

Following stimuli such as pregnancy, obesity, glucose infusion, manipulating growth-hormone expression, toxigene-mediated β-cell ablation and partial pancreatectomy, self-renewal by β-cell replication has emerged as a dominant mechanism for homeostatic maintenance of β-cell mass postnatally. This was elegantly demonstrated in a study by Melton and coworkers wherein, using lineage-tracing highly specific for β-cells (double transgenic mice bearing a tamoxifen-dependent Cre-recombinase construct under the control of a rat insulin promoter together with a reporter Z/AP gene) they showed that pre-existing terminally differentiated β-cells, rather than pluripotent stem cells, were the major source of new β-cells retaining significant proliferative capacity in vivo during adult life, even after pancreatectomy (Dor et al., 2004). This conclusion was supported by the observation that forced cell-cycle arrest of β-cells by transgenic expression of the cyclin-dependent kinase inhibitor p27 blocked β-cell proliferation, severely restricting postnatal but not embryonic β-cell mass, indicating that non β-cells were unable to compensate (Nir & Dor, 2005). Deletion of cyclin-dependent kinase 4 (Cdk4) showed similar results that could be rescued by β-cell-specific expression of Cdk4, suggesting that the underlying proliferative defect in Cdk4-null mice lay in β-cells (Martin et al., 2003; Rane et al., 1999, Uchida et al., 2005). A recent in vivo pulse-chase labeling assay investigating the replication dynamics of adult mouse β-cells indicated that replicated β-cells were able to re-enter the cell division cycle shortly after mitosis and regained their normal proliferative potential after a short quiescence period of several days (Salpeter et al., 2010). Metabolic demand was a key determinant of cell cycle re-entry as quiescence period was lengthened with advanced age, but shortened during injury-driven β-cell regeneration and following treatment with a pharmacological activator of glucokinase. Their results implicated glucose control of cyclin D2 expression in regulating the capacity of β-cells to re-enter the cell cycle post-mitosis. A novel DNA analog-based lineage-tracing technique to detect multiple rounds of cell division in vivo indicated that β-cells rather than specialized progenitors contributed to adult β-cells, slowed by a replication refractory period that prevented β-cells from immediately redividing (Teta et al., 2007). Also, label-retaining experiments, clonal analysis and sequential thymidine analogue labeling indicated that all β-cells replicated homogeneously,
contribute equally to islet growth and maintenance in healthy adult mice as well as during periods of increased replication such as pregnancy, neonatal growth and following pancreatectomy (Brennand et al., 2007, Teta et al., 2007). By counting accumulated mitotic figures following colchicine treatment (Bonner-weir et al., 1989), 5-bromo-2’-deoxyuridine (BrdU) incorporation (Montana et al., 1994), tritiated thymidine incorporation (Kaung, 1994) or expression of the cell cycle marker Ki67 (Meier et al., 2008), it has been shown that β-cell replication rates in rodents and human beings in vivo are a function of age, highest during late embryonic development and the neonatal period and declining significantly throughout adulthood (Brennand & Melton, 2009). However, hyperglycemia and hyperinsulinemia can induce a robust increase in β-cell replication even in older mice. Unfortunately human β-cell replication is difficult to demonstrate compared to murine pancreas (Butler et al., 2003, 2010; Menge et al., 2008). Although there have been reports of human β-cell proliferation in the presence of HGF (Kayali et al., 2007) or by overexpression of Cdk6 (Fiaschi-Taesch et al., 2010), the results were questionable, since insulin expression was lost after 5 doublings in the former study and the investigators in the latter study did not measure β-cell mass or number thereby raising doubts whether their approach did induce proliferation of β-cells. While β-cell replication offers an attractive mechanism for postnatal homeostatic maintenance of β-cells, the complete absence of β-cells in patients with type 1 diabetes and a declining ability to replicate with age are questions that need to be addressed.

2.1.4 Intra-Islet cells: role of α and δ cells in β-cell regeneration
Several studies using the streptozotocin (STZ)-induced diabetic model have indicated the presence of intra-islet precursor cells with the potential to differentiate into neo islets/pancreatic β-cells upon appropriate stimulation (Banerjee & Bhonde, 2003; Guz et al., 2001; Kodama et al., 2005). While some studies indicate that administration of betacellulin improved STZ-induced hyperglycemia by promoting neoformation of β-cells mainly from somatostatin-positive islet cells (Li et al., 2003), others suggested that differentiation of multipotent nestin-positive stem cells isolated from adult pancreatic islets resulted in pancreatic endocrine, exocrine, and hepatic phenotypes ex vivo (Zulewski et al., 2001). By conditional and ectopic expression of Pax4 using different cell-specific promoters, Collombat et al have demonstrated that Pax4 forces endocrine precursor cells as well as mature α-cells to adopt a β-cell destiny (Collombat et al., 2009). Upon Pax4 ectopic expression, the resulting glucagon deficiency provoked a compensatory and continuous glucagon-positive cell neogenesis requiring the re-expression of the proendocrine gene Ngn3, with the newly-formed α-cells acquiring a β-cell phenotype. Thus, ectopic expression of Pax4 in α-cells caused a cycle of neogenesis and redifferentiation capable of restoring a functional β-mass and restoring normoglycemia in animals that had been chemically depleted of β-cells. In an elegant experiment using alloxan damage to eliminate all pre-existing β-cells followed by PDL to stimulate β-cell neogenesis, Fred Levine and coworkers demonstrated β-cell neogenesis from α-cells (Chung et al., 2010a, 2010b). They showed that virtually all β-cells that appeared after treatment were neogenic, mostly arising from adult α-cells within 14 days. At one week, 58% of IPCs coexpressed glucagon and β-cell-specific transcription factors such as Pdx1 and Nkx6.1. By two weeks, these MafB-expressing immature IPCs demonstrated a predominant mature phenotype defined by MafA expression and lack of glucagon. Furthermore, in this PDL plus alloxan model, in response to environmental cues, α-cells could a) replicate first, forming a potential reservoir of β-cell
progenitors; b) could directly differentiate into β-cells without intervening replication; or c) replicate first and then convert to β-cells. β-cell regeneration from α-cells was also demonstrated using a transgenic model of diphtheria-toxin-induced, acute, selective, near-total β-cell ablation (Thorel et al., 2010). Administration of insulin resulted in β-cell mass augmentation with time and lineage-tracing to label the glucagon-producing α-cells before β-cell ablation tracked large fractions of regenerated β-cells as derived from α-cells. Transdifferentiation of glucagon-expressing cells into insulin-expressing cells on menin inactivation, without the necessity of Pdx1, MafA, Pax4, and Ngn3 expression, has also been demonstrated albeit in a scenario relating to islet tumorigenesis (Lu et al., 2010). The lack of Ngn3, Pdx1, and MafA expression in these cells makes them more similar to the immature MafB+Ins+ cells that have been documented during development, however, further investigation will be required to clarify the similarities and the differences. Taken together, these results demonstrate that adult α-cells can serve as in vivo source for generating β-cells. There are only a few published records of this phenomenon in human pancreata. Gianani et al. reported the presence of insulin–glucagon double-positive cells in fibrotic pancreata and speculated that in conditions like chronic pancreatitis and pancreatic fibrosis, α-cells increased through neogenesis from ductal cells followed by transdifferentiation into insulin-producing cells (Gianani et al., 2006, 2011). This theory is supported by experiments demonstrating insulin–glucagon double-positive cells intermixed with a severe insulitic islet infiltrate in the pancreata of a subset of NOD mice that remain non-diabetic despite the total loss of insulin-producing cells. Since α-cells appear to be capable of resisting immune-mediated destruction in autoimmune diabetes, with residual α-cells persisting long after the onset of disease, this scenario is particularly attractive in terms of potential therapy. Other studies indicating the presence of intra-islet β-cell precursors include the development of a tissue culture platform wherein isolated adult human pancreatic islets formed proliferative duct-like structures expressing ductal and progenitor markers. Short-term treatment with INGAP induced these structures to reform islet-like structures that resembled freshly isolated islets with respect to the frequency and distribution of the four endocrine cell types, islet gene expression and hormone production, insulin content, and glucose-responsive insulin secretion (Hanley & Rosenberg, 2009). Similar results were obtained by dedifferentiation of fresh human islets into a duct cell phenotype with further redifferentiation into β-cells in appropriate conditions in vivo (Gao et al., 2005). While the plasticity of adult human islets has significant implications for islet regeneration, until the maximum extent of neogenesis achieved using intra-islet cells can be determined, significant investment in developing regenerative strategies based on these cells should be reconsidered.

3. Stem cells strategies for β-cell regeneration

Obtaining a large source of β-cells for cellular therapy is a major challenge in the treatment of diabetes. While efforts thus far are based on deriving maximal utilization of all the unwanted tissue from the donor organ, the insignificant yield of differentiated β-cells, diminished function and insignificant amounts of insulin secreted both in vitro and in vivo, make it necessary to seek alternative approaches. Regeneration of β-cells from a self renewing, expandable stock of stem/progenitor cells via processes that include dedifferentiation, proliferation, tissue-specific directed differentiation and genetic
reprogramming offer an attractive alternate source towards achieving insulin independency in diabetic patients. Based on their origins, stems cells from blastocyst-stage embryos can be classified as pluripotent embryonic stem cells (ESCs), whilst those obtained from niches of mature adult tissues and bone marrow as adult multipotent stem cells. The tissue-specific differentiation of ESCs, pancreas-derived multipotent progenitor/stem cells, extra-pancreatic adult stem cells (bone-marrow (BM) derived stem cells, neural progenitor cells, umbilical cord blood (UCB)-derived stem cells, etc.) or induced pluripotent stem cells (iPSCs) established from adult differentiated cells, into unprecedented quantities of cells with an insulin expressing phenotype in vitro has tremendous potential in γ-cells replacement therapy(Aguayo-Mazzucato & Bonner-Weir, 2010; Furth et al., 2009; Guo et al., 2009; Santana et al., 2006; Sordi et al., 2008; Stanley et al., 2008; Tang 2004). Additionally, the immunosuppressive, anti-inflammatory properties of stem cells shared among ESCs and several types of non-haematopoietic stem cells (HSCs) such as BM-derived mesenchymal stem cells (BM-MSCs) and UCB-derived stem cells are an added advantage. Better yet is the ability of iPSCs to generate an unlimited supply of clinically compliant, functional γ-cells derived from a patient’s own somatic cells, and therefore not subject to allograft rejection, providing a framework for a solution to the cited limits of islet transplantation, tissue supply and chronic immunosuppression.

3.1 Potential stem/progenitor cell sources for regeneration of insulin-producing γ-cells

3.1.1 Regeneration of γ-cells from embryonic stem cells

ESCs are pluripotent with high self-renewal potential and a limitless capacity of proliferation. There are several elegant strategies to induce γ-cell generation from ESCs, based on sequential exposure of human ESCs to epigenetic signals that mimic in-vivo pancreatic development, beginning with the formation of definite endoderm, followed by pancreatic endoderm, endocrine tissue and finally γ-cell maturation (Aguayo-Mazzucato & Bonner-Weir, 2010). These differentiated γ-cells display architecture consistent with mature islets, contain islet hormones, respond successfully to glucose challenge in glucose-tolerance tests (GTT) and reverse hyperglycemia in diabetic mice. While several growth factors, such as activin A, fibroblast growth factor 10 (FGF10) and retinoic acid (RA) drive differentiation of hESCs into cells expressing Pdx1, recent efforts have focused on the identification of efficient, less expensive, small molecule inducers capable of controlling differentiation by modulating signal transduction pathways, gene expression or metabolism (Borowiak et al., 2009). For eg. induce definitive endoderm (IDE) 1 and 2 have been shown to induce the formation of large amounts of definitive endoderm expressing multiple endodermal markers from mouse and human ESCs with a 70–80% efficiency, higher than Activin A or Nodal. Further development of these endodermal cells into pancreatic progenitors in vitro has been shown to occur in response to FGF10, RA, inhibitors of hedgehog signaling and more recently small molecule indolactam V (Aguayo-Mazzucato & Bonner-Weir, 2010; Chen et al., 2009). While these hESC-derived γ-cells resemble human islets, in that they are capable of synthesizing insulin, glucagon, somatostatin, pancreatic polypeptide and ghrelin (D’Amour et al., 2006; Kroon et al., 2008) and prolong graft survival, the lack of glucose-stimulated insulin secretion (GSIS) is one of the main drawbacks. In an elegant study utilizing a five-stage hESC differentiation protocol, Kroon et al assessed the competence of hESC-derived pancreatic endoderm to produce functional endocrine cells in vivo. Direct
engraftment of hESC derived pancreatic endoderm from the penultimate fourth stage of the five-step differentiation protocol into immunodeficient mice generated ‘glucose-responsive’ endocrine cells with maximal insulin secretion which developed during a 3 month period post implant. These glucose-responsive cells had insulin secretory properties similar in kinetics and magnitude to ~ 3000 adult human islets similarly engrafted, expressed critical β-cell transcription factors, demonstrated appropriate processing of proinsulin and the presence of mature endocrine secretory granules. 92% of the mice receiving hESC-derived pancreatic endoderm implants achieved insulin levels sufficient to fully protect against STZ-induced hyperglycemia, providing definitive and compelling evidence that hESCs are competent to generate glucose-responsive, insulin-secreting cells and serve as a renewable source of mature functional islets for diabetes cell-replacement therapies. This study also demonstrates the contribution of in vivo factors for the final stages of maturation and the need to identify these factors for efficient in vitro differentiation in order to produce sufficient amounts of functional β-cells.

3.1.2 Islet-derived stem/precursor cells as a source for insulin-producing β-cell regeneration

While ESCs have tremendous potential in tissue engineering, their use is hampered by ethical, legal and scientific considerations. On the contrary, non-embryonic adult stem cells are multipotent and can be derived from several sources including bone marrow, umbilical cord tissue, amniotic fluid, fat tissue, skin, retina and central nervous system (Chhabra et al., 2009). The existence of putative pancreatic stem cells that express low amounts of insulin mRNA in vitro with clonogenic potential and multipotency has been described previously (Bonner-Weir & Weir, 2005; Seaberg et al., 2004). Suzuki et al also demonstrated the existence of a possible pancreatic stem/progenitor cell candidate that expressed HGF c-Met receptor, did not express hematopoietic and vascular endothelial antigens such as CD45, TER119, c-Kit, and Flk-1, formed clonal colonies in vitro, differentiated into multiple pancreatic lineage cells and expanded with self-renewing cell divisions in culture (Suzuki et al., 2004). Following transplantation, these cells differentiated into pancreatic endocrine and acinar cells in vivo. A recent study reported the isolation, culture and functional characterization of islet-derived stem/precursor cells from purified human islet preparations (Carlotti et al., 2011). The cultured stem/precursor cells did not express the genes for endocrine hormones and when transferred to serum-free medium, aggregated to form clusters expressing insulin, glucagon, and somatostatin genes. They were not of hematopoietic, endothelial, or of ductal origin, expressed MSC markers CD105, CD90, CD73, CD44, CD29, and CD13 as well as nestin and vimentin and pericyte markers CD146, NG2, αSMA and PDGF-Rβ. Immuno-flowcytometry and confocal microscopic analyses revealed 2.0±0.8% of CD105/CD90 double-positive cells resided within the human islets, supporting the presence of a distinct MSC-like stem cell population in isolated human islets.

3.1.3 Mesenchymal stem cells: stopping immune destruction and promoting β-cell regeneration

Whole bone marrow contains a mixture of multiple types of stem cells, including BM-HSCs, BM-MSCs, endothelial progenitor cells (EPCs), multipotent adult progenitor cells and side population (SP) cells. BM-MSCs are plastic-adherent cells, typically expressing surface markers such as CD90, CD73, CD105, CD44 and CD29. They lack hematopoietic lineage
markers such as CD34, CD45, CD14 and HLA-DR and can differentiate into cells of connective tissue lineages, including bone, fat, cartilage and muscle (Volarevik et al., 2011). These multipotent cells can be isolated and expanded with high efficiency in culture, are non-immunogenic and display immunosuppressive properties, for eg. inhibiting the proliferation and function of major immune cell populations, including T cells, B cells and natural killer (NK) cells as well as modulating the activities of dendritic cells (DCs) and inducing regulatory T cells (Tregs) both \textit{in vivo} and \textit{in vitro} (Shi et al., 2011). They also express a number of growth factors (GF) such as vascular endothelial growth factor (VEGF), HGF and insulin-like growth factor-1 (IGF-1), secrete tolerogenic cytokines as well as display significant anti-inflammatory and anti-apoptotic features (Uccelli et al., 2011). Thus, non-immunogenic BM-MSCs can enhance repair and regeneration, not only by repopulating damaged tissue, but also by reducing inflammation. These cells also do not possess cell surface human leukocyte antigen (HLA) or major histocompatibility complex (MHC) class II molecules, thereby allowing transplantation across MHC barriers. The possible therapeutic effect of BM-MSCs in type 1 diabetes was suggested by their capacity to generate insulin-producing cells and to abrogate immune injury (Chen et al., 2004; Sun et al., 2007; Tang et al., 2004; Volarevic et al., 2011; Xie et al., 2009). These insulin producing cells expressed multiple genes related to the development or function of pancreatic β-cells including high expression of pdx1, insulin, and glucagon and were able to release insulin in a glucose-dependent manner that led to amelioration of diabetic conditions in STZ-treated nude mice. Bouzama et al. also showed that administration of \textit{in vitro}-expanded syngeneic BM-MSCs in STZ-induced diabetic rats promoted Pdx1 and insulin expression in the islets, altered T cell cytokine pattern toward IL-10/IL-13 production, preserved CD4(+)/CD8(+) Foxp3(+) Tregs in the periphery and induced sustained normoglycemia, thereby establishing a tissue microenvironment that supported β-cell activation/survival in the pancreas (Boumaza et al., 2009). While an increase in pancreatic islets and β-cells producing mouse insulin was observed with intracardiac infusion of human MSCs into STZ-induced diabetic NOD/scid mice, nearly 3% of the infused human MSCs engrafted into the pancreas and 11% in the kidney, the two organs sustaining most damage, effectively demonstrating the homing and tissue repair potential of MSCs (Lee et al., 2006). A recent study (Y. Zhang et al., 2010) demonstrated the effective differentiation of human first-trimester fetal BM-MSCs under a novel four-step induction procedure into functional pancreatic islet-like cell clusters (ILCs) that contained 62±14% IPCs, expressed a broad gene profile related to pancreatic islet β-cell development, and released high levels of insulin (2.245±0.222 pmol/100 clusters per 30 min) and C-peptide (2.200 ± 0.468 pmol/100 clusters per 30 min) in response to glucose challenge \textit{in vitro}. The pancreatic ILCs normalized the blood glucose level of diabetic model mice for at least 9 weeks when xenografted and examination of the grafts indicated that the transplanted cells survived in recipients and produced human insulin and C-peptide \textit{in situ}. Most studies suggest the requirement of \textit{in vivo} hyperglycemia or transplantation into a diabetic model for BM-MSCs to differentiate into IPCs able to produce and release insulin in a glucose-dependent manner and normalize hyperglycemia (Chen et al., 2004; Karnieli et al., 2007; Tang et al., 2004). An added benefit is that MSC and islet co-transplantation has been shown to improve islet graft function and survival in diabetic rats by promoting graft revascularization, suppressing inflammation and maintaining islet organization and morphology (Figliuzzi et al., 2009; Ito et al., 2010; Rackham et al., 2011). In fact, engraftment might not be a necessary prerequisite for human MSCs to enhance tissue repair as MSCs
embolized in the lung were shown to improve myocardial infarction by secreting anti-inflammatory factors (Lee et al., 2009). Due to these findings, MSCs offer an attractive avenue of research in diabetic stem cell therapy and may have the potential to enhance β-cell repair mechanisms even if the infused cells do not contribute significantly to the β-cell pool. However, in order to efficiently use MSCs therapeutically, problems of poor engraftment, limited differentiation under in vivo conditions and possible malignant transformation needs to be overcome.

### 3.1.4 Umbilical cord blood (UCB)-derived stem cells: Potential therapeutic tool in β-cell regeneration

Human UCB-derived stem cells represent a readily available source of MSCs and blood stem cells, both of which are potential sources of IPCs. hUCB-derived mononuclear cells (MNCs) possess MSC-like characteristics (expression of CD44, CD90 and CD105) (McGuckin et al., 2005) and have the ability to differentiate into mature adipocytes, osteoblasts, chondrocytes, skeletal myocytes, cardiomyocytes, neurons, and endothelial cells (Fan et al., 2011; Kogler et al., 2004; Parekh et al., 2009; Yoshida et al., 2005). In vitro differentiation of UCB-derived stem cells into insulin- and C-peptide-producing cells has been demonstrated using different approaches (Koblas et al., 2009; Gao et al., 2008). While some differentiated Oct-4 and SSEA-4 expressing hUCB cells by a protocol consisting of nicotinamide and extracellular matrix proteins (ECMs) laminin and fibronectin (Szymczak et al., 2010), others used RA, nicotinamide, exendin-4 and extracellular matrix proteins to differentiate hUCB-MSCs (Gao et al, 2008). In fact recently, ‘cryopreserved’ human UCB-derived MSCs were induced to differentiate into ILCs expressing nestin, Pdx-1, Ngn3, IIs-1, Pax6, Pax4, Nkx2.2, Nkx6.1, Glut-2 and insulin genes (Phuc et al., 2011). Unfortunately, despite differentiation into insulin producing cells, these cells seemed immature in terms of glucose sensing and/or secretory machinery required for glucose stimulated insulin secretion (GSIS) (D’Amour et al., 2006). In vivo differentiation of hUCB cells into β-cells has also been demonstrated following transplantation into STZ-induced diabetic immunocompromised (Kogler et al., 2004; Parekh et al., 2009; Zhao et al., 2006) or NOD mice (Koblas et al.,2009; Yoshida et al.,2005), though at a low rate. Detailed characterization of freshly isolated hUCB-MNCs indicate the ability to maintain as islet-progenitor cells expressing pdx1 and GLP1R for at least 5 passages of in vitro expansion. Transplantation of these cells into immune-incompetent mice resulted in their differentiation into insulin producing cells, more frequently during partial pancreatectomy, that were immature in terms of GSIS response (Parekh et al., 2009). Thus, it appears that the diabetic status, wherein a demand for neogenesis of insulin producing cells is induced in response to the decreased β-cells mass, results in a higher rate of compensatory hUCB cell differentiation (Yoshida et al., 2005). Kadam et al reported islet neogenesis from constitutively nestin-expressing human umbilical cord matrix stem cells (hUC-MSCs) (Kadam & Bhonde, 2010). These hUC-MSCs expressed CD29, CD44, CD73, CD90, CD105, smooth muscle actin, nestin, vimentin, proliferation marker Ki67 and embryonic markers Oct4, SSEA4, exhibited high proliferating capacity for an extended period and when subjected to a cocktail of specific differentiating factors, differentiated into fat, cartilage, bone, neurons and ILCs. These ILCs stained positive for DTZ, expressed human C-peptide, insulin and glucagon and demonstrated abundance of Pdx-1, Ngn3, insulin, glucagon and somatostatin transcripts. Transplantation into diabetic mice restored normoglycemia and exhibited normal glucose tolerance test, demonstrating
the potential of constitutively-expressing nestin-positive progenitors from hUC as a novel source for islet neogenesis and their usage in cell replacement therapy for diabetes. Interestingly, treatment with CD4(+)CD62L(+) Tregs that were modulated by hUCB derived stem cells was able to simultaneously overcome autoimmunity via systemic and local immunomodulations as well as the shortage of insulin producing cells via stimulation of β-cell regeneration (Zhao et al., 2010). A number of studies indicate the use of these cells for autologous transplantation in type 1 diabetes subjects. An initial trial with promising results of autologous cord blood has been attempted for diabetes reversal in patients with new onset diabetes who had banked cord blood (Haller et al., 2008). However, although the potential of UCB derived stem cells in the future of T1D interventional therapies is immense, the reality remains that multiple therapeutic avenues need to be combined in order to achieve the dream of permanently reversing/preventing T1D.

3.1.5 Generation of insulin producing β-cells from induced pluripotent stem cells

Efforts to create pluripotent stem cells (iPSCs) that are molecularly and functionally similar to ES cells by reprogramming somatic cells have tremendous therapeutic potential. Fibroblasts, B lymphocytes, liver, stomach epithelial cells, UCBs, human fetal and newborn epithelia etc. have all been dedifferentiated by the stable genomic integration and overexpression of various combinations of defined transcription factors that participate in determining pluripotency in cells, for instance, oct3/4, sox2, klf4, c-myc or oct3/4, sox2, nanog, lin28 (Hochdedlinger & Plath, 2009). However, the use of oncogenes (c-myc, klf4) and retroviral and lentiviral vectors have raised concerns about the risk of potential tumorigenicity. Various novel alternatives directed towards making reprogrammed cells safer and more practical for therapeutic use have been explored. For e.g. iPSCs generated by viral integration of only three transcription factors, Oct4/Sox2/Klf4 have exhibited reduced tumorigenicity in chimeras and progeny mice (Nakagawa et al., 2008), albeit with substantially lower reprogramming efficiency. Interestingly, the addition of two factors p53 siRNA and UTF1 enhanced the efficiency of iPSC generation upto 100-fold (Zhao et al., 2008). Furthermore, a combination of two small molecules, BIX-01294 (a G9a histone methyltransferase inhibitor) and BayK8644 (a L-channel calcium agonist) was able to compensate for viral transduction of Sox2, enabling reprogramming of Oct4/Klf4-transduced mouse embryonic fibroblasts, which do not endogenously express the factors essential for reprogramming (Shi et al., 2008). Similarly, valproic acid (VPA), a histone deacetylase inhibitor has been shown to enable reprogramming of primary human fibroblasts with only two factors, Oct4 and Sox2, without the need for the oncogenes c-Myc or Klf4 (Huangfu et al., 2008). In fact, the generation of iPSCs from hUCBs with only Oct4 and Sox2 has also been demonstrated recently (Giorgetti et al., 2010). These two factor-induced human iPSCs resemble human ES cells in pluripotency, global gene expression profiles and epigenetic states, highlighting an important new trend in this field, the replacement of viral-mediated gene transfer with drug therapies such as small molecule gene inducers. However, finding small molecules that induce specified gene targets is not always possible. Alternatively, it is possible to engineer cell-penetrating protein therapies wherein a protein of interest can directly be transferred into the target cell. This approach has been applied in a study that reported the generation of protein-induced pluripotent stem cells (piPSCs) from murine embryonic fibroblasts using recombinant cell-penetrating reprogramming proteins (Zhou et al., 2009). These piPSCs were capable of long-term self-
renewal and were pluripotent in vitro and in vivo. Addition of a transportation tag consisting of 11 linked copies of arginine to four proteins c-Myc, Klf4, Oct4 and Sox2 has also been shown to facilitate translocation across cell and nuclear membranes (Baker, 2009) with addition of valproic acid further boosting reprogramming rates. In fact, when proteins were administered four times over six days at 36-hour intervals, cells over 30 passages were morphologically indistinguishable from ES cells and expressed similar markers. Mixing in these cells with normal mouse embryos and allowing development in a surrogate mother resulted in the reprogrammed cells contributing to the germlayers in 13.5-day-old embryos. Interestingly, UCB-derived iPSCs have also been generated by lentiviral overexpression of oct4, sox2, nanog and lin28 with reprogramming efficiency similar to that of keratinocytes and fibroblasts. Towards development of a reliable protocol for induction of mature insulin-producing cells from iPSCs, a highly efficient strategy involving a) the use of activin A and wortmannin to induce definitive endoderm formation followed by b) priming with FGF10, KAAD-cyclopamine and c) addition of RA, NOGGIN and FGF7 to induce pancreatic specialization and d) EGF to regulate progenitor expansion has been demonstrated (Zhang et al., 2009). Using this stepwise induction strategy, most human iPSC lines could be induced into Pdx1-positive progenitor cells and further differentiated into cells expressing islet cell specific marker genes including Pdx1, MafA, Glut2 and insulin. Various marker genes were expressed at different induction stages: Sox17 on day 4, Pdx1 on day 8, Sox9 on day 13, amylase, Pdx1 and insulin on day 20. Additionally, co-expression of Pdx1 and C-peptide further confirmed insulin-producing cells suggesting that human iPSCs could be efficiently differentiated into pancreatic lineage cells. In a very recent study, a similar stepwise induction protocol was utilized with the exception that the pancreatogenic cocktail was enriched with Indolactam V/GLP-1 (Thatava et al., 2011). Under feeder-free conditions, fate specification of human iPSCs was initiated with activin A and Wnt3a that triggered engagement into definitive endoderm, followed by priming with FGF10 and KAAD-cyclopamine. Addition of RA, boosted by the pancreatic endoderm inducer indolactam V (ILV), yielded pancreatic progenitors expressing Pdx1, Ngn3 and NeuroD1 markers. Further differentiation under IGF-1, HGF and N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) was enhanced by GLP-1 to generate islet-like cells that expressed pancreas-specific markers including insulin and glucagon. Sustained expression of Pdx and functional responsiveness to glucose challenge, secreting up to 230pM of C-peptide, was observed in progeny. Thus, a proficient means for lineage specification of human iPSCs into functional glucose-responsive hormone-producing progeny has further refined the development of a personalized platform for islet-like cell generation. Whilst these studies generate proof of concept that iPSCs may be an appropriate source for the generation of clinically compliant, immunologically quiescent, therapeutic β-cells from an autologous, non-embryonic origin, progress towards clinical application is hampered by a number of factors. Determining the somatic cell candidate for dedifferentiation depends on factors such as relative availability and ease of isolation, definitive identification and characterization markers, ex vivo expansion potential as well as safety upon transplantation. For future consideration, extensive characterization of in vitro differentiated iPSC will be critical and must be investigated. While the use of small molecule gene inducers and cell-penetrating protein therapies address the issues of poor efficiency and slow kinetics of the reprogramming process by viral transfection of the nuclear factors, these methodologies require substantial improvement before they could be used in clinical applications.
Similarly, the use of non-viral gene delivery vectors that can obviate safety concerns regarding the risk of mutagenesis, terratoma and tumor formation that prevail in the use of oncogenes and viral transduction need to be stringently addressed. Last but not least, studies thus far have demonstrated that the induction of the insulin-secreting phenotype is still incomplete with differentiated cells making multiple hormones or inadequate amounts of insulin or lacking response to glucose challenge to be clinically useful. Extensive, in-depth investigation into the underlying mechanisms involved is therefore of critical importance. Thus, while iPSCs derived from autologous somatic cells represent an exciting and important avenue to replenish islet supply while simultaneously obviating immune concerns such as rejection and chronic immunosuppression, until the accompanying risks are completely obviated, other avenues of β-cell replacement need to be judiciously explored.

3.2 Identifying stem cells with insulin-generating capability and tracking differentiated β-cells - the necessity for cellular markers.

Nestin, which is detected at low levels in human pancreatic tissue in vitro, has long since been debated as a cellular marker for ESC differentiation into IPCs (Baharvand et al., 2006). The existence of multipotential nestin-positive cells with unusually extended proliferative capacity that might participate in neogenesis of islet endocrine cells has been demonstrated in rat and human pancreatic islets and ducts (Zulewski et al., 2001). Upon confluence, these cells differentiated into cells that expressed liver and exocrine pancreas markers such as α-fetoprotein and pancreatic amylase, and displayed a ductal/endocrine phenotype with expression of CK19, neural-specific cell adhesion molecule, insulin, glucagon, and the pancreas/duodenum specific homeodomain transcription factor, IDX-1. While others dispute that nestin-positive cells residing in the pancreas are a potential source for regenerating or expanding islets (Gao et al., 2003), multipotential stem cells with β-cell generative capacity may be identified by this marker. Very recently, Kadam et al. isolated and characterized hUC-MSCs that constitutively expressed nestin and differentiated into functional ILCs capable of restoring normoglycemia in experimental diabetic mice (Kadam & Bhonde 2010). An effective multistep protocol to efficiently induce pancreatic ductal and β-cell differentiation in vitro from multipotent, nestin-positive rat bone-marrow stem cells in a serum-free system has recently been described (Milanesi et al., 2011). In this procedure, trichostatin A, a regulator of chromatin remodeling and 5-aza 2’ deoxycytidine, an inhibitor of DNA methylase were used to enhance the induction and differentiation toward pancreatic lineage, followed by all-trans RA to promote pancreatic differentiation. Important transcription factor genes, such as Pdx1, Ngn3, and Pax6 were sequentially induced following the in vivo development timeline. Lastly, nicotinamide, was used to induce expression of islet and ducal-specific markers. The differentiated cells displayed glucose-responsive insulin secretion and expressed Glut2, indicating the clinical relevance of ‘nestin-positive’ bone-marrow stem cells. In further characterization of candidate nestin-positive islet progenitors (NIPs), Lechner et al. proposed that some islet cell progenitors possess characteristics of both neural origin, exemplified by nestin, as well as of bone-marrow progenitors, exemplified by the so-called side-positive (SP) cell phenotype (Lechner et al., 2002). This phenotype is characterized by the ability to exclude the vital dye Hoechst through the action of the ATP-binding cassette transporter ABCG2 (BCRP1). The presence of this molecular marker potentially allows for purification of these cells thereby providing an
efficient progenitor cell for islet differentiation. These studies prompted investigation into the possibility that pancreatic SP cells possessed β-cell regenerative potential (Zhang et al., 2005). SP cells were shown to be capable of insulin, glucagon, Glut2 and Pdx1 expression induced by a variety of growth factors, concomitant with a decreased expression of progenitor cell markers such as nestin and Ngn3. These differentiated cells also released insulin in response to high glucose concentrations, though in notably lesser amounts when compared to primary β-cells, calling for continued investigation into standardizing the the conditions required for inducing complete differentiation of SP cells into fully functional β-cells.

As necessary as it is to have markers that identify progenitor cells amenable to the islet pathway, it is of equal importance to have markers that can rapidly identify the small number of successfully differentiated cells. With an aim to improve the efficiency of analyzing and sorting β-like insulin-producing cells from undifferentiated cells, Fukazawa et al. designed a novel β-cell specific and glucose-responsive artificial promoter system designated pGL3.hINS-363 3x (Fukazawa et al., 2006). This system exhibited significant luciferase activity not only in insulin-producing MIN6 m9 cells but also in isolated human islets. The pGL3.hINS-363 3x construct showed no activity in non-insulin producing cells in low glucose conditions (2mM glucose) but demonstrated significant activity with β-cell specificity in high glucose conditions (16mM glucose). Furthermore, pGL3.hINS-363 3x showed significant promoter activity in differentiated AR42J cells that could produce insulin after activin A and betacellulin treatment. Detection of small numbers of newly formed β-cells by this approach could significantly enhance the production of differentiated, insulin-positive, β-cells for transplantation. Growing evidence suggests that microRNAs (miRNA) play an important role in insulin production, secretion and action and that diabetes changes miRNA expression profiles in many tissues (Baroukh et al., 2007; Correa-Medina et al., 2009; Joglekar et al., 2007, 2009; Li et al., 2009; Poy et al., 2004, 2009; Tang et al., 2009). miRNAs are a novel group of highly conserved, endogenous, 22–23 nucleotide non-coding RNAs that regulate biological functions very precisely by negatively modulating the gene expression either by promoting mRNA degradation or through translational repression of proteins. Very recently, Chen et al. have described an approach wherein they isolated miRNAs from human embryonic stem cell (hES-T3 cells) -derived pancreatic ILCs (T3Pi) that expressed insulin, glucagon and somatostatin and showed that these miRNAs negatively regulated the expression of protein-coding mRNAs (Chen et al., 2011; Sebastiani et al., 2011). T3pi clusters showed very high expression of miRNAs, miR-186, miR-199a and miR-339, that downregulated the expression of LIN28, PRDM1, CALB1, GCNT2, RBM47, PLEKHH1, RBPMS2 and PAK6 indicating that these miRNAs and their target genes very likely played important regulatory roles in the development of pancreas and/or differentiation of islet cells. Using this approach, differentiated T3Pi cells with high miRNA expression can be identified and miRNAs may possibly even be manipulated to increase the proportion of β-cells and insulin synthesis for cellular therapy in type I diabetics. Another recent study indicates that miR-24, miR-26,miR-182 or miR-148 act as positive regulators of insulin transcription in cultured β-cells or in isolated primary islets by reducing the expression of insulin transcriptional repressors (Melkman-Zehavi et al., 2011). MiRNAs may also provide a new class of biomarkers for diabetes and progress in the development and use of synthetic miRNAs such as antagonirs (Krutzfeldt et al., 2005) to silence miRNAs such as miR-375 in case of diabetes may provide a novel therapeutic tool for the treatment of diabetes and other diseases in the
future. Furthermore, mechanistic studies on the role of miRNAs in the modulation of the immune system may help identify potential therapeutic targets to ameliorate responses to islet transplantation.

4. Role of transcription factors, growth factors and other cellular targets in enhancing β-cell differentiation and proliferation

Pancreatic development includes the generation of endoderm/gut endothelium, pancreatic differentiation, endocrine specification, and ultimately β-cell differentiation. Therefore, in order to efficiently promote β-cell differentiation and expansion, it is of vital importance to understand the factors that contribute to islet development. A number of signaling molecules and transcriptional regulators including Wnt, TGF-β, FGF, Notch and Hedgehog control various aspects of pancreas and endocrine cell development, proliferation and differentiation (Evans-Molina et al., 2009; Jun et al., 2010; Mishra et al., 2010). During development, it is the definitive endoderm that gives rise to the pancreas and genes such as Wnt/β-catenin, Nodal, GATA4/6, FoxA, Sox17 and Mix amongst others participate in its formation. Pdx1 represents a marker of all pancreatic lineages (endocrine, exocrine and ductal), mediating β-cell function, growth, and proliferation and its inactivation prevents both islet and acinar cell differentiation. Pdx1 regulation is mediated by the IRS2/Akt/FoxO1 pathway, maintaining the ability of β-cells to proliferate and function properly. The notch signaling pathway in turn regulates the expansion and differentiation of the pancreatic progenitor cells by the expression of the ‘pro-endocrine’ gene, Ngn3. Transcription factors such as Pdx-1, Isl1, Ngn-3, Nkx2.2, Nkx6.1, NeuroD, Hlxβ9, Pax-4, MafA and Pax-6 all participate in islet differentiation with Ngn-3 acting as a key transcription factor required for islet cell development. Amongst these transcription factors, Pdx-1 is the most extensively utilized factor, driving both β-cell neogenesis as well as transdifferentiation of pancreatic and extra-pancreatic cells into insulin-producing β-cells. Various factors such as pregnancy, diabetogenic stimuli, growth factors such as HGF, a combination of epidermal growth factor (EGF) and gastrin, clonophylline, betacellulin, GLP-1 or its long-lasting homolog exendin-4, members of the regenerating protein family such as Reg protein and INGAP can stimulate replication and proliferation of β-cells (Jun et al., 2010; Mishra et al., 2010). Herein, we describe a few of the candidates that are used to attain maximal β-cell expansion by targeting pathways that augment mature β-cell proliferation, inhibit apoptosis, or simultaneously target both. Betacellulin is a member of the EGF family, and is known to increase the rate of native β-cell self-duplication and enhance Pdx1-positive progenitor differentiation. It also induces proliferation and differentiation of insulinoma cells as well as converts an exocrine pancreatic cell line (AR42J) into an insulin-expressing phenotype when combined with activin A. This combined treatment potentiates β-cell self-duplication, ductal cell proliferation and δ-cell transdifferentiation into new β-cells (Li et al., 2004). Similarly, activin promotes regeneration and differentiation of newly formed β-cells and together with growth differentiation factors participates in endocrine and exocrine lineage specification (Mishra et al., 2010). The incretin GLP-1 is produced from gut endocrine cells and has been shown to stimulate β-cell proliferation and neogenesis, inhibit β-cell apoptosis and have a profound effect on stimulating the release of insulin and suppression of glucagon from the pancreas. Rapid degradation by dipeptidyl peptidase IV (DPPIV) is responsible for its short biological half life. To overcome this,
exenatide/Exendin-4 (AC2993), a long acting GLP-1 receptor (GLP-1R) agonist and other such stable GLP-1 analogs that are resistant to degradation (iraglutide) and inhibitors of DPPIV (sitagliptin, vildagliptin) are being investigated for treatment of diabetes (Geelhoed-Duijvestijn, 2007). Injections of GLP-1 or exendin-4, have been shown to increase the β-cell mass and improve hyperglycemia. Combination of GLP-1 and gastrin or EGF with gastrin restores β-cell mass and inhibits autoimmune destruction of islet cells (Suarez-Pinzon et al., 2005, 2008). Administration of GLP-1 and gastrin restores normoglycemia by expanding β-cell mass and by downregulating immune response in autoimmune diabetic NOD mice. Diabetic immunodeficient NODscid mice recipients treated with this combination therapy together with transplantation of human islets demonstrated expanded β-cell mass largely derived from cytokeratin 19-positive pancreatic duct cells. Several growth factors play important roles in β-cell differentiation, proliferation and islet survival. For instance, HGF acts by targeting the protein kinase B/Akt involved in an intracellular signaling cascade linked to improving islet cell survival and function. It also upregulates Glut-2, glucokinase, and insulin gene expression in β-cells and has been shown to increase longitudinal and functional graft survival. Keratinocyte growth factor promotes differentiation of IPCs and VEGF regulates insulin gene expression and β-cell proliferation through laminin and maintains adult islet function. IGF1 expression in β-cells of transgenic mice regenerates the endocrine pancreas during type 1 diabetes. Though the IGF-I-mediated mechanism(s) of restoring β-cell mass is not fully understood, early studies indicate that IGF-I modulates cell cycle proteins and increases replication of pre-existing β-cells after damage (Agudo et al., 2008). The Reg gene family belongs to the calcium-dependent lectin (C-type lectin) gene superfamily and is divided into subclasses based on the primary structures of the encoded proteins. Several Reg family genes, such as Reg2, -3α, and -3β, are upregulated to induce β-cell regeneration, growth and protection and also during the progression of autoimmune diabetes. INGAP, one of the Reg family gene products has been used in numerous studies to facilitate β-cell differentiation and expansion. In addition, heparan sulfate, by regulating ligand-receptor interactions, plays an important role in embryonic development and regulation of postnatal islet maturation. Dicer1 (a ribonuclease required by the RNA interference and miRNA pathways to produce the active small RNA component that represses gene expression) maintains the adult pancreas and regulates the differentiation of endocrine precursor cells. Interestingly, Dicer1-deficient β-cells mostly retain their identity including the expression of typical β-cell markers involved in glucose sensing and insulin transcription but show an increase in repressors of insulin transcription (Melkman-Zehavi et al., 2011). Similarly miRNAs reinforce insulin expression by reducing the expression of insulin transcriptional repressors. Before utilizing these factors as treatment options, safety concerns regarding the risk of cancerous transformation and participation in other cellular processes should be investigated in depth.

β-cell proliferation is not only affected by factors that induce expansion and differentiation, but is also tightly regulated by factors that impede/inhibit proliferation. Modulation of these factors therefore represents another alternative to enhance β-cell proliferation. For instance deletion of p27Kip1, a key cell cycle inhibitor, increased the frequency of cellular replication and proliferation during β-cell development and in the early neonatal period (Rachdi et al., 2006). Improved glucose tolerance and hyperinsulinemia associated with increased islet mass and proliferation was observed in p27-deficient mice, while induction of p27 expression resulted in severe glucose intolerance.
and reduced β-cell mass. Additionally, p27(-/-) mice showed decreased susceptibility to
develop STZ-induced diabetes compared to controls that displayed elevated blood glucose
levels (Georgia & Bhushan, 2006). In mice that developed STZ-induced diabetes, β-cells
retained the ability to reenter the cell cycle at a far greater frequency in the p27(-/-) mice
than in wild-type littermates. These studies establish the role of p27 in maintaining the
quiescent state of newly differentiated β-cells generated during embryogenesis and indicate
p27 as a key regulator in the establishment of β-cell mass and thus as an important target in
regenerative therapies for diabetes. The success of this therapy however would depend on
the ability of the cell to reactivate p27 to stop uncontrolled proliferation when metabolic
demands for insulin are met. Apart from the risk of tumorigenesis, another drawback is the
dedifferentiation and loss of insulin secretion commonly observed with repeated cell
division of β-cells. Another key regulator of β-cell replication and expansion is glycogen
synthase kinase-3 (GSK3), known to negatively regulate insulin-mediated glycogen
synthesis and glucose homeostasis (Rayasam et al 2009). Islet β-cell growth is controlled by
endogenous GSK-3β activity via feedback inhibition of the insulin receptor/P3K/Akt
signalling pathway (Liu et al., 2010). In fact, glucose regulates steady-state levels of Pdx1 via
the reciprocal actions of GSK3 and AKT kinases. Glucose-stimulated activation of AKT and
inhibition of GSK3 decreased Pdx1 phosphorylation and delayed Pdx1 degradation
demonstrating the important role of AKT-GSK3 axis in glucose modulation of Pdx1 stability
(Humphrey et al., 2010). Similarly, direct pharmacologic inhibition of AKT destabilized,
while inhibition of GSK3 increased Pdx1 protein stability. Increased expression and activity
of GSK3 has been reported in type II diabetics and obese animal models and consequently,
inhibitors of GSK3 have been demonstrated to have anti-diabetic effects in vitro and in
animal models. However, the use of GSK3 inhibitors poses a challenge as achieving
selectivity of an over achieving kinase involved in various pathways with multiple
substrates may lead to side effects and toxicity.

5. Prolonging long term survival, growth and function of β-cells in vitro

Despite the promising results of β-cell regenerative strategies, maintaining functional islet
cells in long-term cultures with persistent insulin-secreting capabilities has proven
challenging. The efficacy of various cell culture medium supplements in prolonging the
survival and function of newly generated β-cells and enhancing their survival in long-term
cultures for optimal expansion has been investigated. For e.g. human islets were cultured in
vitro for more than a year during which time, using multilabeling immunohistochemical and
immunoelectron microscopic analyses with islet cell markers (antibodies to hormones,
neuron-specific enolase, chromogranin A) and ductal cell markers (cytokeratins 7 and 19,
carbonic anhydrase II, DU-PAN2, CA19-9, and MUC1), islet cells gradually
transdifferentiated into ductal, acinar, and undifferentiated cells, considered pancreatic
precursor (stem) cells (Schmied et al., 2001). Although endocrine cells remained detectable
at day 60, hormone secretion ceased after day 28. Eventually all endocrine and exocrine cells
were replaced by undifferentiated cells that expressed neuron-specific enolase,
chromogranin A, laminin, vimentin, cytokeratin 7 and 19, α-1-antitrypsin, TGF-α, and EGF-
receptor. While this study successfully demonstrated the possibility of culturing human
islets for long periods, it brings into focus the formidable challenge of overcoming the loss of
insulin-secreting capabilities and transdifferentiation of β-cells. The efficacy of supplementing culture medium with growth hormone (GH) and prolactin (PRL) as well as co-culturing islets with fibroblasts on stimulating long term cell proliferation as well as maintenance of insulin-synthesizing and -secreting capacity has also been investigated (Gartner et al., 2006). The supportive role of FGF2 in maintaining functional β-cells in culture has been previously demonstrated (Hardikar et al, 2003). FGF2, acting as a paracrine chemoattractant, stimulates clustering of human islet-derived precursor cells, leading to islet-like cell aggregate formation necessary for the early stages of islet cell differentiation. An impressive protocol that combined bFGF, leukemia inhibitory factor (LIF), and bone morphogenetic protein-4 (BMP-4) proved that islet progenitor-like cells can be derived from islet-enriched fractions under serum-free defined culture conditions and induced to stably express high levels of Pdx1 and Notch pathway-associated genes, characteristic of embryonic pancreatic progenitor cells, for more than 6 months and maintain endodermal and pancreatic phenotypes. Unfortunately, insulin expression remained minimal. Based on the hypothesis that loss of the trophic support provided by surrounding non-endocrine pancreatic cell populations underlies the decline in β-cell mass and insulin secretory function observed in human islets following isolation and culture, the effect of co-culturing islets with ductal epithelial cells on islet structural integrity, β-cell mass and insulin secretory capacity was investigated (Murray et al., 2009). Ten days following isolation, the results showed that co-culturing islets with ductal epithelial cells led to preserved islet morphology and sustained β-cell function, with the presence of ductal epithelial cells beneficial for maintenance of β-cell mass. While reinforcing the possibility of maintaining islets in long-term cultures under appropriate cell culture conditions, the study highlights the necessity for further characterization of regulatory influences in order to realize the promise of its therapeutic potential. The importance of a three-dimensional (3D) environment incorporating extracellular matrix (ECM) components in providing favorable conditions to preserve human islets in long-term culture has also been demonstrated (Daoud et al., 2010). The loss of the ECM basement membrane during isolation contributes to eventual apoptosis in vitro. While collagen I/IV and fibronectin induce adhesion, fibronectin is the only ECM protein capable of maintaining islet structural integrity and insulin content distribution in cultures. Although in this study islet phenotype was eventually lost, insulin gene expression was highest in islets cultured on collagen I and IV. Insulin release peaked with fibronectin along with a decrease in SUR1 expression, while glucose metabolism along with Glut2 and GCK expression was highest on collagen I and IV surfaces. A very recent study consisting of long-term and highly uniform human islet culture within a micro-fabricated scaffold with collagen 1 gel supplemented with ECM components fibronectin and collagen IV with controlled pore structures, displayed an insulin release profile similar to freshly isolated islets, yielding a stimulation index of approximately 1.8 (Daoud et al., 2011). Gene expression was markedly increased for all pancreatic genes, giving approximately 50-fold elevation of insulin gene expression with respect to suspension culture. The level of pancreatic hormones was also highly elevated. These findings provide the groundwork for, 1) establishing a modified 3D construct to culture pancreatic islets, and 2) for understanding the underlying mechanisms of islet interactions with its surroundings, in order to provide a platform for long-term maintenance and preservation of human pancreatic islets in vitro.
6. Hepatocytes and Xenogeneic islets - prospects and barriers as alternative sources

Reprogramming adult mammalian cells is an attractive approach for generating β-cells for replacement therapy. The common embryonic origin of liver and pancreas, the similarity of glucose-sensing systems, the large group of mutually expressed, specific transcription factors and the high level of developmental plasticity exhibited by adult human liver cells suggest that liver stem cells/hepatocytes are a potential source of pancreatic progenitor tissue. For instance, persistent expression of the Pdx1 or its super-active form Pdx1-VP16 fusion protein in hepatic cells reprograms these cells into pancreatic β-cell precursors under condition of hyperglycemia or hepatic regeneration (Yang et al., 2006). Also, most hepatocytes of Ad-pdx-1-infected mice demonstrated positivity for Pdx-1 expression but expressed insulin and somatostatin only in STZ-treated or in STZ-treated plus partial hepatectomy mice vs. nontreated mice. A corresponding amelioration of hyperglycemia and along with expression of other β-cell markers like Glut2 glucokinase was also observed (Kim et al., 2007). Similarly ex vivo lentiviral-mediated Pdx1 expression in isolated adult liver cells resulted in expression of insulin in the transduced cells at both mRNA and protein levels, dependent on the presence of glucose and sulfonylurea (Fodor et al., 2007). Expression of β-cell genes, including those encoding solute carrier family 2 (facilitated glucose transporter), member 2 (Slc2a2), glucokinase (Gck), ATP-binding cassette, sub-family C (CFTR/MRP), member 8 (Abcc8), the potassium inwardly-rectifying channel, subfamily J, member 11 (Kcnj11) and proprotein convertase subtilisin/kexin type 1 (Pcsk1) were also observed. The PDX1-transduced hepatocytes expressed several pancreatic transcription factors related to early pancreatic endocrine development (endogenous Pdx1, NeuroD1, and Nkx6.1) as well as the late-stage pancreatic transcription factors (Pax4, Pax6, and MafA). Transplantation of these transdifferentiated liver cells in STZ-induced diabetic scid mice resulted in improvement in hyperglycemia. This capacity of Pdx-1 to activate the pancreatic lineage in mature liver has been independently confirmed in numerous studies (Imai et al., 2005, Horb et al., 2003, Li et al., 2005; Miyatsu et al., 2003; Zalzman et al., 2005). Also the effects of additional pancreatic transcription factors alone or combined with Pdx-1 expression on this process have been analyzed (Sapir et al., 2005; Kaneto et al., 2005a, 2005b; Kojima et al., 2003; Meivar-Levy et al., 2006). For instance, a recent study indicates the dominant role of Nkx6.1, expressed in mature β-cells, in altering the hepatic developmental fate along the pancreatic lineage and function (Gefen-Halevi et al., 2010; Wang, 2007). Nkx6.1 activated immature pancreatic markers such as Ngn-3 and Isl-1 but not pancreatic hormones gene expression in human liver cells, suggesting a potential role for Nkx6.1 in promoting Pdx-1 reprogrammed cells maturation along the β-cell-like lineage. Complementation of Nkx6.1 by ectopic Pdx-1 expression substantially and specifically promoted insulin expression while addition of glucose augmented processed hormone secretion without increasing the number of reprogrammed cells. Other studies demonstrate that combinations of overexpressed MafA with NeuroD and Ngn3 or NeuroD and betacellulin in diabetic mice markedly induced insulin gene transcription and ameliorated glucose tolerance without producing hepatitis (Kaneto et al., 2005a, 2005b; Kojima et al., 2003). Transient transfection of human hepatoma cells (Li et al., 2005) or hepatic WB cells (Tang et al., 2006) with super-active version Pdx1-VP16 resulted in transdifferentiation into pancreatic cells, characterized by induction of both pancreatic exocrine cells (by detection of amylase protein) and endocrine cells (by detecting insulin, glucagon and somatostatin proteins) and suppression of the hepatic phenotype.
Transdifferentiated β-cells were responsive to physiological stimuli, exhibiting 1) functional β-cell markers including prohormone convertase 1/3 (PC1/3), insulin C-peptide and GLP-1R, 2) increased insulin mRNA expression after treatment of cells with GLP-1 and betacellulin and c) elevated insulin secretion after glucose challenge. Thus, reprogramming with multiple pancreatic transcription factors using first generation, non-toxic, transiently-expressed adenoviral vectors along with appropriate conditions (hyperglycemia and/or partial hepatectomy) can reprogram liver stem/adult liver cells into functional insulin-producing cells. The use of adult human liver cells for generating functional insulin-producing tissue may pave the way to autologous transplantation, circumventing both the shortage in tissue availability, the need for immunosuppressive therapy and the ethical issues associated with ESC therapy. Additionally the ability of the liver to regenerate itself after surgical removal of a portion to use for transdifferentiation without significant harm to the patient is an added bonus.

Xenotransplantation using porcine cells, tissues, or organs may offer a potential solution for the shortage of allogeneic human organs (Denner et al., 2009). However, prior to their clinical use the hurdles of immunologic rejection and risk of transmission of porcine pathogens needs to be overcome. Immunologic rejection in pig to primate xenotransplants consists of hyperacute rejection, acute humoral xenograft rejection (AHXR), acute cellular rejection and chronic rejection (Elliott et al., 2011). Hyperacute rejection results from the activation of the complement cascade that converts graft endothelial cells from an anticoagulant to a procoagulant phenotype. Apart from old world primates and man, all animals possess a cell surface antigen containing the epitope – Galα1–3Galβ1–4GlcNAc-R (‘α-gal’) to which humans have complement fixing antibodies that cause immediate rejection of animal cells when transplanted into humans. This can be prevented by reducing or inhibiting functional ‘α-gal’ activity using knock-out or knock-in procedures involving human alpha-1, 2-Ft or GnT-111 gene expression as well as by depletion of anti-pig antibodies or complement from serum. Adult porcine islets do not express Gal, reducing the antibody-mediated response to them after transplantation. However, fetal and neonatal islets do, and therefore the use GT knockout (α1,3-galactosyltransferase gene-knockout [GTKO]) pigs as the as the sources of all islets (fetal, neonatal and adult) is likely to be advantageous. Similarly, genetically-engineered pigs expressing thrombomodulin, tissue factor (TF) pathway inhibitor, CD39 or other mechanisms that prevent the coagulation dysfunction; or expressing human complement-regulatory proteins, CD46 [membrane cofactor protein] or CD55 [human decayacceleratingfactor] or CD59 or all three in pig islets (Ekser & Cooper, 2010); or expressing anticoagulant and antiplatelet molecules within the graft, may afford some protection (Cowan & d’Apice, 2008). Recombinant antithrombin III may also ameliorate both early graft damage and the development of systemic coagulation disorders in pig-to-human xenotransplantation. This strategy, in parallel with physical methods such as encasing islets in a protective layer, holds promise for reducing the thrombogenicity of pig islet xenografts. Ideally, genetically engineered GTKO/CD46 pigs whose organs and cells are protected from the coagulation dysregulation that include modifications that prevent tissue factor activity on the graft and as well as activation of recipient platelets to express TF and initiate consumptive coagulopathy are required. AHXR or delayed xenograft rejection results from vascular endothelial cell activation and injury caused by the complement and cellular components of the innate immune system. There is increasing evidence that primate neutrophils, natural killer (NK) cells and macrophages
play a role in AHXR, particularly seen following the development of a T-cell dependent elicited antibody response. Administration of CD39, heme oxygenase, thrombomodulin and TF pathway inhibitor have been used in its treatment (Cowan & d’Apice, 2008). Acute cellular rejection involving T- and B-cell infiltration of the graft, T-cell activation and a T-cell-dependent elicited antibody response is believed to be stronger following xenotransplantation than the alloresponse. However, potent pharmacologic agents can largely prevent acute cellular rejection, which is therefore typically not observed with intense immunosuppressive drug regimens. Ekser et al. have obtained promising results using an immunosuppressive regimen consisting of induction therapy with antithymocyte globulin (ATG), and maintenance with an anti-CD154 monoclonal antibody and Mycophenolate mofetil (MMF) (Ekser & Cooper, 2010). A clinically acceptable regimen of ATG, CTLA4-Ig and MMF has also been found to be particularly effective in preventing T-cell activation in the xenotransplantation setting. Chronic rejection symptoms in the form of chronic vasculopathy has been observed in pig to primate graft that survived for more than a few weeks, similar to the chronic rejection seen in long-surviving allografts. The risk of development of xenozoonosis in the recipient of a pig graft (particularly with regard to porcine endogenous retroviruses (PERV), is of concern. However, the stable long-term expression of anti-PERV siRNAs has been shown to be effective in knocking down PERV expression in cells. Breeding of designated pathogen-free pigs can prevent transmission of most porcine microbes (Dieckhoff et al., 2008; Ramsoondar et al., 2009). Another hurdle includes sensitization to pig antigens (e.g., swine leukocyte antigens), resulting in an increase in antibodies to HLA. Fortunately current evidence indicates that antibodies that develop after exposure to a pig xenograft are not crossreactive against HLA, and so would not be detrimental to a subsequent allograft (Cooper et al., 2004). However this does not preclude patients with a high level of HLA-reactive antibodies who may still be at greater risk of rejecting a pig xenograft. Clinical attempts to treat type 1 diabetes with implanted porcine islets are underway and showing promising early results. Immune-suppression, sertoli cell co-transplantation and intraperitoneal implantation of micro-encapsulated neonatal islets have been tried in type 1 diabetic humans with some clinical benefit reported from the latter two. In a very recent study by Elliot et al using the microencapsulation technique, transitory insulin independence of several months duration was observed (Elliot et al., 2011). Interestingly, the treatment appeared to significantly decrease severe hypoglycemic episodes and reduce/abolish hypoglycemic unawareness episodes, even in the absence of insulin independence. Evidence of xenosis in the xenotransplants recipients though diligently sought could not be found, given the credentials of the designated pathogen free source herd used. Virus safety in xenotransplantation is a fast-developing field, and new findings may contribute to improved outcomes.

7. The role of gene therapy in enhancing islet function

While not routinely achieving long-term normoglycemia, islet transplantation does afford substantial benefits in the form of reduced incidences of debilitating hypoglycemic episodes and hypoglycemic unawareness, lower daily insulin requirements, a detectable level of c-peptide, and improved A1c levels. The major limitation of this procedure is the inadequate numbers of donor islets available for transplantation. While we have discussed the various approaches to generate and expand islet β-cells, strategies aimed at increasing the efficiency
of islet function in terms of insulin synthesis and secretion are of equal importance since it would reduce the number of islets required for restoring glucose homeostasis following transplantation. Towards this end, gene therapy has been proposed. A simple conceptual approach to create hyper-functional islets would consist of augmentation of the regulated insulin secretory capacity of an islet graft by insulin gene transfer to the graft before transplantation. Deng et al. demonstrated that Ad-Ins-transduced islets showed superior function in terms of insulin production and secretion as well as GSIS (Deng et al., 2003). The amount of basal insulin secretion and the overall pattern of insulin secretion from the Ad-Ins-transduced islet appeared completely normal and following transplantation, recipients remained normoglycemic for more than 100 days without evidence of deterioration in graft function. Histological examination of the grafts showed normal islet graft morphology and the presence of abundant insulin. Interestingly, although transfer of an exogenous insulin gene under control of a powerful viral promoter forced β-cells to produce more insulin, transplantation of a large number of these islets did not result in recipient hypoglycemia, confirming that Ad-Ins-transduced islets secreted insulin in an appropriately regulated manner. Furthermore, only 25% of the previously needed islet mass was required to reverse diabetes suggesting that four times as many transplants could be performed compared to the unmodified islets. The use of insulin promoter instead of the CMV promoter would have further increased insulin secretion from Ad-Ins-transduced islets in response to changes in blood glucose concentration in a highly regulated manner. Furthermore, the islet gene transfer was carried out using an islet-virus co-culture technique resulting in only partial islet transduction (37% expressing transgene). The authors speculate that Ad-Ins-vector delivery by vascular perfusion of the pancreas, would achieve maximal transgene expression in nearly all islet endocrine cells making it possible to reverse diabetes with islets isolated from only a portion of the perfused pancreas. The consideration of live donors as a source of islets is not unreasonable since laparoscopic-assisted distal pancreatectomy and nephrectomy has been performed successfully in three living donors since December 2007 with minimal peri-operative mortality and acceptable morbidity and the distal pancreas has been used successfully as a segmental vascularised graft (Gruessner et al., 2001; Maruyama et al., 2010; Tan et al., 2005). The uneventful postoperative course and normal functioning of the grafts indicate the technique to be minimally invasive and safe, making the consideration of live related donors for providing islet tissue supply a reasonable solution to the problem of organ shortage until other strategies such as stem-cell derivation of β-cells or xenogeneic sources of islet tissue can be refined. In order to avoid the potential side effects associated with the use of viral vectors, Chen et al. demonstrated targeting of plasmid DNA to the pancreas in vivo using ultrasound-targeted microbubble destruction (UTMD) (Chen et al., 2010). Intravenous microbubbles carrying plasmids were destroyed within the pancreatic microcirculation by ultrasound, achieving local gene expression that was further targeted to β-cells by a modified rat insulin promoter (RIP3.1). Delivery of RIP3.1-NeuroD1 two days after STZ-induced diabetes to rats pretreated with the JNK inhibitor SP600125 successfully blocked β-cell apoptosis and resulted in in vivo islet regeneration, restoration of β-cell mass and normalization of blood glucose level, insulin and C-peptide in rats. The key pancreatic transcription factor Pdx1, possesses a protein transduction domain (PTD) that facilitates its entry into cells (Koya et al., 2008). Reversal of STZ-induced diabetes in mice by cellular transduction with recombinant Pdx1 represents a novel protein transduction domain-based therapy. The potential use of a non-viral, systemic, carrier-based delivery of Fas siRNA gene therapy through intravenous administration using a polymeric carrier, polyethyleneimine
(PEI) offers yet another novel approach (Jeong et al., 2010). Another alternative for obtaining hyper-functional islets lies in hyperinsulinemia. Genetic conditions that include mutations in the potassium channel, the sulfonylurea receptor and the energy-sensing mechanisms of β-cells result in hyperinsulinemia. In diffuse congenital hyperinsulinism of infancy, near-total pancreatectomy (98%) has to be performed as a life-saving procedure in order to achieve glycemic control, demonstrating the extreme potency of these cells when compared to a normal islet (Lovvorn et al., 1999; Pierro et al., 2011). Use of these hyperfunctional islets in islet transplantation, preferably along with normal islet tissue, is an interesting proposition, although the use of diseased tissue would raise several concerns and challenges.

8. Increasing post-transplant islet survival - an immunological approach

From 1999 to 2007, clinical islet transplantation at established centers has resulted in a remarkable reduction in the occurrence of severe hypoglycemia and a success rate of 70% in achievement of insulin independence persisting for 2 years or more in 50% of those achieving insulin independence, or 35% of all islet alone graft recipients. These results are consistent throughout the 8 years of follow-up included in the Collaborative Islet Transplant Registry (Alejandro et al., 2008). Approximately 60% of transplanted islets are lost in the first 10–14 days post-transplantation (Evgenov et al., 2006) most likely due to local hypoxic injury caused by lack of islet vascularity and the deleterious effects of the innate immune response which induces apoptosis, necrosis, coagulation, and complement fixation (Huang et al., 2008). This non-specific inflammatory response results in the production and release of a number of proinflammatory cytokines (e.g. TNF-α, IL-1β, IFN-γ) that further enhance local inflammatory activity, stimulate adaptive immunity and exert deleterious effects on islet β-cell function by inducing apoptosis and cell death (Donath et al., 2008). Islet cells themselves, upon transplantation, possess the ability to receive signals that result in the activation of multiple signaling factors including STAT1, AP-1 and NFκB (Eizirik & Mandrup-Poulsen, 2001). Lisofylline has demonstrated significant ability in down-regulating the systemic inflammatory response by interference with STAT4 signaling resulting in improved islet transplant survival (Yang et al., 2005). Suppression of these pathways has also been attempted through gene therapy approaches (Moore et al., 2006). Currently, islet-directed anti-inflammatory therapy mostly focuses on Toll-like receptor (TLR) signaling pathways within the islets (Huang et al., 2008). Fortifying the β-cell with protection against islet-destructive cytokines represents another avenue to defend against the immune response. For instance, adenoviral transduction of islet cells with a construct expressing IRAp, the interleukin-1 receptor antagonist protein, resulted in improved islet survival and replication caused mainly due to interference in the activation of the IL-1 mediated apoptotic pathway (Tellez et al., 2005). Other strategies that overexpress anti-apoptotic proteins such as the TNF-α inducible transcription factor A20 or bcl-2 demonstrate beneficial effects on islet survival. While immunomodulatory therapies (e.g. monoclonal antibody therapies, CTLA4Ig, anti-thymocyte globulin (ATG), IL-1 receptor antagonist therapy, cellular therapies, etc.) act by either providing immunoregulatory cytokines such as IL-4, IL-10 or TGF-β or by altering the balance between TH1 and TH2 cells, immunosuppressive regimens act by either suppressing the immune response by binding to specific cytoplasmic proteins that inhibit IL-2 secretion and subsequent T cell expansion (Calcineurin inhibitors CNIs; cyclosporine and tacrolimus) or by suppressing IL-2R
signaling thereby inactivating T cells (sirolimus) or by suppressing cell division of lymphocytes (Azathioprine) (Winter & Schatz, 2003). Therefore, combinations of CNIs and steroid-sparing or -free regimens with drugs that demonstrate powerful immunosuppressive/anitinflammatory potency in the absence of nephrotoxicity and diabetogenicity are being investigated. A triple therapy approach that combined rapamycin plus agonist IL-2-related and antagonist-type mutant IL-15-related Ig cytolytic fusion proteins(IL-2.Ig and mutIL-15.Ig) demonstrated a striking ability to reverse diabetes and ameliorate inflammation, mainly due to augmentation of the pro-regulatory effects of IL-2 and inhibition of the proinflammatory mediator IL-15, creating a favorable balance between regulation and inflammation (Koulmanda et al., 2007). Currently, targeted antigen specific/non-specific and antibody-specific immunotherapies that readjust the underlying immunologic imbalance in order to stop or reverse the β-cell-specific autoimmune and inflammatory process within islets and maintain immune tolerance are being combined with islet regeneration therapies in a variety of clinical studies and hold great therapeutic promise for islet transplantation outcomes. These include islet transplantation followed with ATG /alemtuzumab (Campath -1H, monoclonal anti CD52 Ab) / hOKT3 γ induction therapy / anti-CD25 (daclizumab) induction therapy along with a sirolimus-based, prednisone-free maintenance regimen in combination with MMF and low Tacrolimus (Bellin et al., 2008; Gillard et al., 2010; Herold et al. 2005; Magliocca & Knechtle, 2006). Short term ATG treatment in T1DM of recent onset has been shown to contribute to the preservation of residual C-peptide production and to lower insulin requirement following diagnosis. Because Tregs strongly suppress the immune response in syngeneic islet transplantation and improve graft survival and function, several approaches are now emerging to induce/increase host Tregs activity in the transplant setting, including amongst others, systemic TGF-β1 therapy (W. Zhang et al., 2010). Other β-cell therapies currently in Phase II or III stages of development include Otelixizumab (anti-CD3), Teplizumab (anti-CD3), rituximab(anti-CD20), abatacept (CTLA4Ig), DiapPep 277 (heat shock protein) and GAD, Oral Insulin amongst others. Parenteral administration of anti-CD3 mAb for transplantation in humans and for treatment of autoimmune diabetes has been approved. Various combination interventions such as costimulatory blockade with anti-CD4 monoclonal antibodies plus CTLA4Ig and ATG plus CTLA4Ig (Suzuki et al., 2010) as well as combinations of anti-lymphocyte serum (ALS) plus anti-CD3mAb are under investigation. As discussed earlier, GLP-1R agonists like exendin-4 stimulate β-cell proliferation and neogenesis and inhibit β-cell apoptosis while DPPIV inhibitors increase cell insulin content, and therefore are of immense benefit in the above mentioned combination therapies (GLP-1 agonists plus anti-CD3mAB, anti-CD3mAb plus exendin-4, ALS plus exendin-4 etc.) for preserving and expanding β-cell mass following transplantation. Other avenues include encapsulating islets with nanofibre scaffolds or biomatrices synthesized to contain immunosuppressive drugs or drugs that stimulate vasculogenesis/angiogenesis as well as ‘bioartificial pancreas’. Stem cells of embryonic, mesenchymal (prochymal), cord blood and haematopoietic (and some neural stem cell) origin, besides their use for regenerative purposes, also possess potent immunomodulatory functions and have great therapeutic potential in increasing post-transplant survival either alone or in combination with the therapies discussed above. Thus, depending on the time of the therapeutic intervention, various immunological approaches may be employed as monotherapy or in combination with short-term tolerance promoting immunoregulatory drugs or drugs promoting preservation, differentiation, expansion or insulin secretion to increase the functional and
longitudinal survival of islets post-transplantation. However, while assessing the efficacy of these therapies, it is important to keep in mind that both CNIs as well as corticosteroids contribute to an increased risk of developing post-transplant hyperglycemia and to the differential diagnosis of graft rejection (Cantarovich & Vistoli, 2011; Egidi et al., 2005). Conducting large randomized trials to establish guidelines that minimize the adverse effects of immunosuppressive regimens for pancreas transplantation will be useful in achieving long-term, functional graft survival.

9. Conclusion
The possibility of transplanting sufficient quantities of functional, viable islets to induce lifelong euglycemia depends on the successful outcomes of the various strategies discussed herein, namely regeneration of β-cells utilizing every kind cell from the pancreas, stem cells as well as cells from alternate sources, preserving and expanding their number ex vivo, and ensuring their maximal survival after transplantation through protection from hypoxic and immune insults. The possibility of generating a β-cell with a hyperfunctional phenotype would also significantly expand the functional β-cell pool. The evidence presented here indicates that while the challenges are many and accomplishing this task seems formidable, considerable inroads have already been made in each step highlighted in this review, drawing us a step closer to the possibility of widespread applicability of islet transplantation for the reversal of diabetes. Islet transplantation represents a definitive intervention for patients with Type 1 diabetes and with the significant progress achieved in the fields of stem cell therapy, immunomodulation, and gene therapy, the prospect of translating this new found knowledge into clinical applications that promote successful long term outcomes of islet transplantation is looking very promising.

10. References


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Present Accomplishments and Future Prospects of Cell-Based Therapies for Type 1 Diabetes Mellitus


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This book is a compilation of reviews about the pathogenesis of Type 1 Diabetes. T1D is a classic autoimmune disease. Genetic factors are clearly determinant but cannot explain the rapid, even overwhelming expanse of this disease. Understanding etiology and pathogenesis of this disease is essential. A number of experts in the field have covered a range of topics for consideration that are applicable to researcher and clinician alike. This book provides apt descriptions of cutting edge technologies and applications in the ever going search for treatments and cure for diabetes. Areas including T cell development, innate immune responses, imaging of pancreata, potential viral initiators, etc. are considered.

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