Chapter 12

Stem Cell Therapies for Type I Diabetes

Jesus Ciriza and Jennifer O. Manilay

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/47803

1. Introduction

Diabetes mellitus is a chronic metabolic disease that results in high levels of glucose in the blood. Normally, glucose is transported into cells from the blood for energy, and this transport is initiated in response to the hormone, insulin. In diabetic patients, cellular glucose uptake is defective, in part due to the inability of cells to respond to insulin, or the inability of the body to produce the insulin itself. According to the International Diabetes Foundation, 366 million people worldwide had been diagnosed with diabetes in 2011, and this number is continuing to increase in every country (http://www.idf.org/). It is an especially challenging health problem, as treatments to both control hyperglycemia as well as the debilitating side effects of diabetes, such as injury to the blood vessels and nerves, must be addressed. Amongst the three types of diabetes (Type I - autoimmune, gestational and Type II – adult onset), Type II diabetes is the most prevalent, in which hyperglycemia is uncontrolled due to the body's inability to produce enough insulin or due to the body's inability to respond appropriately to lower the blood glucose level. In contrast, in patients with Type I diabetes (TID), insulin-producing beta (β) cells are destroyed by the immune system. Studies of TID by many groups have provided extensive insight to the fields of immunological tolerance and pancreatic developmental biology. In this chapter, we will briefly describe TID, provide a synopsis of pancreatic β cell development in mice as compared to humans, review of some of the medical treatments currently available for TID, and discuss current studies that have explored the use of stem cells as alternative possible therapies for TID.

2. Type I diabetes in humans and mice

2.1. TID in humans

A fully comprehensive comparison of TID in humans and mice has recently been published, so we will only highlight certain areas (van Belle, Coppieters et al. 2011). TID is a multigenic disease, and several candidates have been discovered that implicate disruptions in the

normal process of negative selection of autoreactive T cells during thymic development, genetic association with specific major histocompatibility complex (MHC) genes in the human leukocyte antigen (HLA) locus, as well as dysregulation of mature T lymphocyte responses. For example, patients with mutations in the *FoxP3* or *Aire* genes, which are important for the development and function of regulatory T cells (T_{regs}) and expression of "self-antigens" by medullary thymic epithelial cells, often present with autoimmune diabetes, as well as other autoimmune disorders (Michels and Gottlieb 2010). On the other end of the spectrum, the inability to downregulate or prevent mature T cell responses due to mutations in cytokine receptor expression (Garg, Tyler et al. 2012) or low surface expression of CTLA-4 (Haseda, Imagawa et al. 2011) can also result in sustained T cell cytotoxicity or lack of T_{reg} response towards diabetes-related antigens. Environmental insults, such as viral infections, molecular mimicry between some viral proteins and diabetes-related autoantigens, changes in gut flora and the intestinal microenvironment, have also been linked with the onset of TID in humans.

2.2. TID in mice

The identification of the mechanisms underlying the autoimmunity of TID in humans has been facilitated by the use of rodent models of the disease (Van Belle, Taylor et al. 2009; von Herrath and Nepom 2009). Despite the fact that TID is a multigenic disease with heterogeneous etiologies, there is overall consensus that TID is caused by the destruction of pancreatic β cells by sets of immune cells that recognize many potential autoantigens (such as insulin, glutamate decarboxylase (GAD), islet antigens, and heat shock proteins-60 and -70) and that the disease can be transferred by the transplantation of autoreactive T lymphocytes that are specific for these antigens (Mallone, Brezar et al. 2011). Several spontaneous, induced and transgenic mouse models currently exist, which have provided important insights into the genetics, cellular mechanisms and immunological aspects of TID. However, the non-obese diabetic (NOD) mouse strain remains the primary animal strain for studies of TID. The utility of the NOD mouse and comparison of the TID in the NOD mouse with human TID have been extensively reviewed and debated, and we direct the reader to these excellent reviews for further reference (Anderson and Bluestone 2005; Roep and Peakman 2011). The NOD mouse strain, when housed in specific-pathogen free conditions, displays the onset of diabetes after 12 weeks of age in both sexes. Insulitis is observed as mononuclear infiltration in the islets before full blown diabetes is evident, and this cellular infiltrate is comprised of primary T lymphocytes, but other lymphoid and myeloid cells are also present. The insulitis results in the destruction of the pancreatic islets and the insulin-producing β cells. The NOD mouse also displays defects in functional macrophages and natural killer cells, NKT cells and regulatory T cells and complement. The NOD mouse, therefore, has allowed for the investigation of numerous immune mechanisms that contribute to the development of TID. The peripheral neuropathy that accompanies TID in humans is also evident in the NOD mouse, which has allowed for analysis and management of the side effects of TID (Anderson and Bluestone 2005; Roep and Peakman 2011). Similar to human diabetes, the MHC of the NOD mouse contains several loci that are linked to

increased susceptibility to TID. The NOD mouse background has also been used to derive the NOD/*scid*/$\gamma_c^{-/-}$ (NSG) immunocompromised strain, into which human cells can be transplanted (Shultz, Saito et al. 2010).

Many studies in mice have focused on mechanisms to control the aberrant autoimmune response in TID, as well as strategies to replace dysfunctional β cells, using simple insulin replacement or rescue/regeneration of the β cells. Despite the similarities between TID in humans and in the NOD mouse, clear differences have been noted. TID in the NOD mouse appears to be a more aggressive disease compared than that in humans, so the NOD mouse may be more beneficial for studies of therapies for long-term TID than for discovery of pre-susceptibility markers of TID. β cells in the NOD mouse may also be more proliferative and could possibly regenerate better than other strains (Sherry, Kushner et al. 2006), and evidence that pancreatic development is altered in the NOD mouse also exists (Homo-Delarche 2001). Therefore, in addition to its clear autoimmune etiology, TID may also result from defects in pancreatic development.

2.3. Pancreatic development in the mouse and human

The adult pancreas organ is comprised of many cell types, including exocrine and endocrine cells, and is highly vascularized. Although TID is a disease that occurs in the fully formed pancreas, strategies to treat or cure TID via β cell regeneration or β cell differentiation from embryonic stem cells (ESCs) or induced pluripotent cells (iPSCs) have relied on the current knowledge of pancreatic development during the fetal period. Here, we briefly review the development of pancreas in the mouse and human, and then move to a description of development of β cells only.

In the mouse, pancreas formation occurs in three phases (Jorgenson, Ahnfelt-Ronne et al. 2007). In the first phase, pancreatic formation from the endodermal gut tube begins at embryonic day (e) 8.5. Patterning of the gut tube in the pancreatic region is reliant on retinoic acid (RA) signaling from the mesoderm. The dorsal and ventral pancreas buds form from the pancreatic epithelium in the primitive gut tube. Recently, the critical role of the Wnt signaling in the pancreatic mesenchyme in the differentiation of pancreatic epithelial cells was shown *in vivo* (Landsman, Nijagal et al. 2011). Eventually, the ventral pancreas rotates toward the dorsal pancreas, and they fuse into one organ by e12.5. At this stage, the pancreas organ undergoes significant cell proliferation and branching. In the second phase (e13.5), the cells within the immature pancreas begin to differentiate into exocrine cells that are required for the production of digestive enzymes, and endocrine cells that secrete hormones into the blood, such as glucagon, insulin, somatostatin, pancreatic polypeptide and ghrelin. Each of these hormones is produced by a distinct endocrine cell type (with insulin produced by the β cells) (**Figure 1**). The third phase of pancreatic development occurs after birth, and is associated with the ability of the pancreas to respond to dietary intake and maintain glucose homeostasis.

Islet development in humans occurs at a slower rate compared to what is observed in mice, but the reasons for this are not understood. The size of the human islet is comparable to that

of the mouse, but in the mouse, the mean area ratio of β cells is higher than observed in the human (Kim, Miller et al. 2009). Fetal antigen-1 is a protein expressed exclusively in human endodermal tissue and pancreatic epithelial cells before islet formation (Tornehave, Jansen et al. 1993). Islets can be located in the human fetus as early as 12-13 weeks post coitus (Piper, Brickwood et al. 2004), although functional β cells that are insulin-positive can be detected as early as 8 weeks.

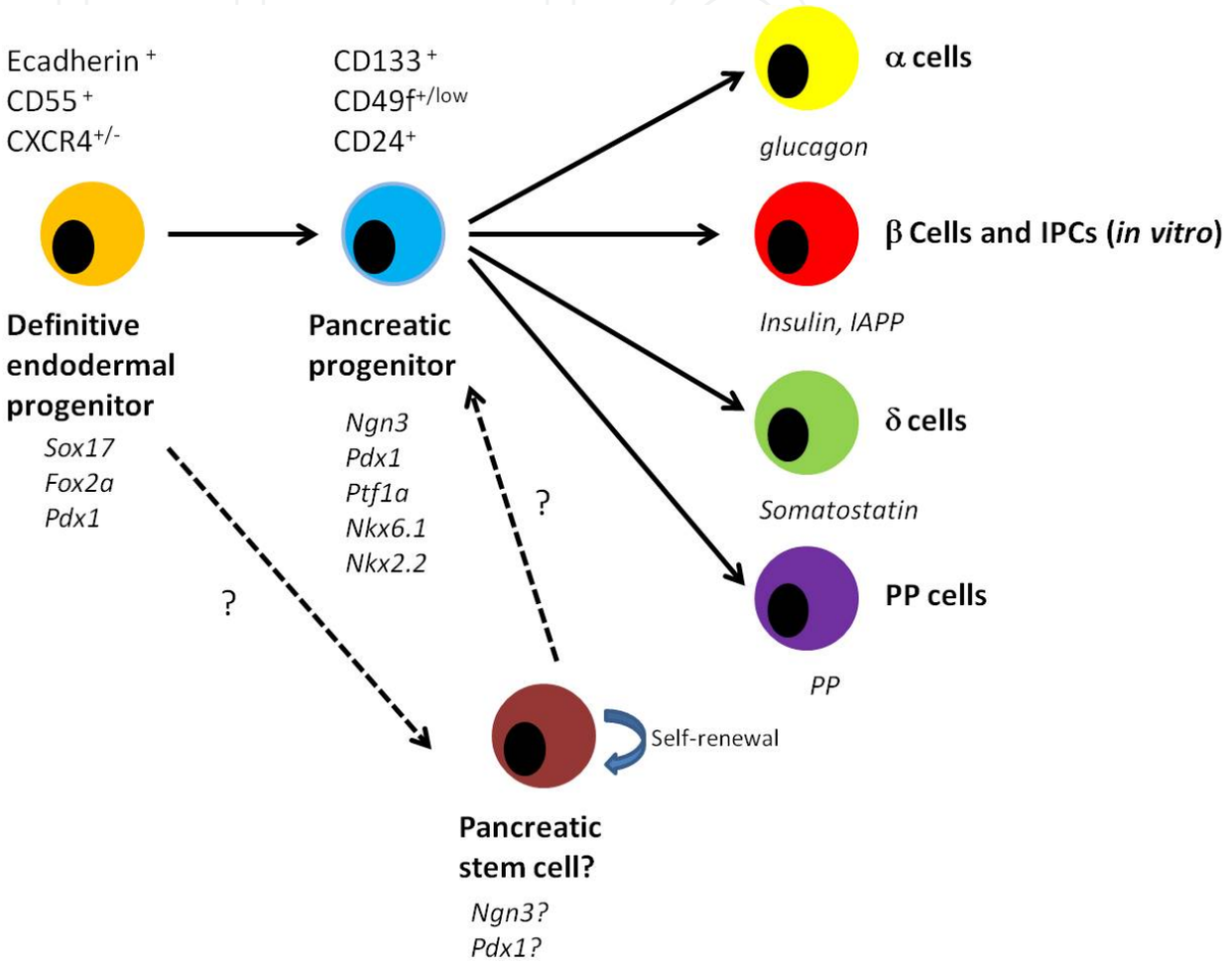

Figure 1. *An overview of pancreatic endocrine cell development*. The development of pancreatic endocrine cells starts with an early endocrine progenitor and ends with mature hormone-producing islet cell types. Cell surface markers (noted above the cell types) and gene expression (in italics) in distinct pancreatic cell lineages are shown. The existence of the self-renewing pancreatic stem cell is still controversial.

An overview of pancreatic endocrine cell development is shown in **Figure 1**. **Definitive endodermal progenitor cells** can differentiate into pancreatic progenitor cells, which have been identified by the expression of two critical transcriptional factors: *Pdx1* and *neurogenin 3* (*Ngn3*). Experiments with conditional *Pdx1* transgenic reporter mice (inducible Cre-ER™-LoxP system to mark the progeny of cells that express *Pdx1*) demonstrated that *Pdx1*⁺ cells give rise to all three types of pancreatic tissue: exocrine, endocrine and duct (Gu, Dubauskaite et al. 2002). In line with this, *Pdx1* deficiency in mouse and humans leads to a

complete pancreas agenesis at birth (Ahlgren, Jonsson et al. 1996). *Pdx1* is also a critical transcriptional activator of insulin and somatostatin in adult islet cells (Ohlsson, Thor et al. 1991; Leonard, Peers et al. 1993). Similar to *Pdx1*, *Ngn3*$^+$ cells give rise to all islet lineage cells during embryogenesis (Gradwohl, Dierich et al. 2000), with peak Ngn3 expression at 15.5 days post-coitus. Although some have reported that *Ngn3* is undetectable in adult pancreas (Gradwohl, Dierich et al. 2000; Johansson, Dursun et al. 2007), *Ngn3*$^+$ cells in duct-ligated adult pancreas were detected and observed to differentiate into islet cells (Gu, Dubauskaite et al. 2002; Xu, D'Hoker et al. 2008). Beta cells in the human, like in the mouse, are derived from a heterogeneous population of **pancreatic progenitor cells** (Scharfmann, Xiao et al. 2008). Whether or not the pancreatic progenitors are derived from a self-renewing **pancreatic stem cell** is still under debate (Jiang and Morahan 2011), and is discussed further in Section 3.1.

For β cell development, the essential role of three key transcription factors, Pax4, Nkx6.1, Nkx2.2, in the mouse and their relationships to the human β cell development is clear. Pax4 (paired box 4) is required for the commitment to the pancreatic lineages (Wang, Elghazi et al. 2004), as well as for the regulation of β cell mass size, proliferation and survival in mice (Brun and Gauthier 2008), and mutations in the *Pax4* gene have been linked to susceptibility to both Type I and Type II diabetes in humans (Yokoi, Kanamori et al. 2006; Chavali, Mahajan et al. 2011). In addition, both human and mice insulinoma cells overexpress Pax4 in comparison to mature β cells, suggesting that Pax4 may also play a role in β cell proliferation and resistance to apoptosis. *Nkx2.2* is expressed early during embryonic pancreatic development (Doyle, Loomis et al. 2007), whereas *Nkx6.1* is expressed later and restricted to the β cells. *Nkx2.2* and *Nkx6.1* knockout mice die in the neonatal period due to the lack of IPCs (Sussel, Kalamaras et al. 1998). Nkx2.2 has both activator and repressor functions; the repressor function is important for regulation of β cell function postnatally (Doyle and Sussel 2007). Nkx6.1 functions exclusively in β cells and is required for β cell development and specification, control of insulin secretion after glucose challenge, and can also induce human and rat β cell proliferation (Schisler, Fueger et al. 2008). However, *in vivo* overexpression of Nkx6.1 does not have any effect on β cells in the mouse (Schaffer, Yang et al. 2011).

2.4. Current therapies for TID

A fully mature, functional β cell produces and appropriately secretes the mature form of insulin (i.e. proteolytic processing of pro-insulin to form C-peptide is evident), in response to glucose to maintain normal glycemic levels in the blood. The current treatment for TID is long-term insulin replacement therapy that is delivered via injection, insulin pens or pumps, and has been quite successful for control of hyperglycemia. However, insulin replacement therapy is not considered a cure for TID, as patients receiving insulin long-term still manifest abnormalities in metabolism, as measured by above-normal levels of glycated hemoglobin (HbA1c). Furthermore, for children, the daily requirement for insulin replacement can limit their day-to-day activities, and TID-related complications, such as vascular disease, retinopathy and neuropathy, still persist even with insulin therapy.

Pancreatic transplantation has been performed for TID patients with success, but the main limitations of this strategy are the low number of suitable donors required for transplant and the decision if the risks of surgery and transplantation outweigh the benefits (Jahansouz, Kumer et al. 2011). Furthermore, even with insulin replacement therapy or pancreatic transplantation, autoreactive immune cells that can attack and destroy any residual β cells in the patient can still remain. For this reason, therapies that modulate the immune response to β cells and β cell antigens also exist, and their safety and efficacy have been or are currently being tested in clinical trials (Bluestone, Herold et al. 2010; Waldron-Lynch and Herold 2011). GAD65 specific-antigen-based immunotherapy with alum adjuvant demonstrated preservation of C-peptide fasting levels in younger new-onset patients four years after treatment (Ludvigsson, Hjorth et al. 2011), but this was not supported in the larger Phase III trial (Ludvigsson, Krisky et al. 2012). Humanized anti-CD3 antibody (in which human Fc immunoglobulin domains are engineered to mouse CD3-binding portions of the antibody) therapy given in a single dose reduced the decline of insulin production and the amount of exogenous insulin required in the patients (Herold, Gitelman et al. 2009). Interestingly, the anti-CD3 antibodies reduced the numbers of circulating T lymphocytes, but whether or not autoreactive T cells are specifically depleted is unclear. There is evidence that the anti-CD3 antibody acts as a weak TCR agonist and stimulates the production of regulatory T cells (T_{regs}) in humans. B lymphocytes in TID have been postulated to act as antigen-presenting cells and also to produce autoantibodies that can result in β cell destruction (Bluestone, Herold et al. 2010). Anti-CD20 antibodies target and deplete B lymphocytes, and lead to a preservation of β cell function in patients, as measured by C-peptide production and reduction of HbA1c levels compared to controls. However, the level of autoantibodies in these TID patients was not determined, so the mechanism by which anti-CD20 therapy works in TID patients is still uncertain. As we will explain further below, these immune modulation therapies may also be important for the future success of β cell regeneration and transplantation.

3. Experimental alternative treatments for TID: Stem cells

Pancreas transplantation has succeeded as a replacement for insulin-secreting β cells for TID patients, but this relies on a limited source of cadaveric pancreatic tissue (Danovitch, Cohen et al. 2005). Early studies demonstrated that isolated human islets did not proliferate in suspension culture, and that adherent islet cells showed limited replication of β cells (Nielsen, Brunstedt et al. 1979; Nielsen, Galsgaard et al. 2001). Therefore, alternative sources of IPCs are needed, and stem cells have been suggested as this source. Stem cells are undifferentiated cells that are capable of self-renewal and differentiation into any cell type. They can be classified depending on their origin. Embryonic stem cells (ESCs) are derived from the inner cell mass of implanted embryos (Evans and Kaufman 1981), induced pluripotent stem cells (iPSCs) are adult or fetal cells that have been "reprogrammed" to an ESC-like state (Takahashi and Yamanaka 2006), adult stem cells are isolated from adult tissues (Becker, Mc et al. 1963) and germline-derived stem cells are generated from embryonic or adult gonads (Shamblott, Axelman et al. 1998). In this section, we will discuss

the state-of-the-art technologies in which the different types of stem cells are generated and differentiated and their possible clinical applications as cellular replacement therapies for TID.

3.1. Tissue-specific pancreatic stem cells

The existence of pancreatic stem cells was proposed in the last decade (Ramiya, Maraist et al. 2000), and is still a topic of debate (Dor, Brown et al. 2004; Smukler, Arntfield et al. 2011) (**Figure 1**). When defining a "stem cell" by function, the standard tests are to examine the ability of a putative stem cell to **self-renew,** often using proliferation assays; and **differentiate**, measured by changes in function, cell surface marker and/or gene expression.

Evidence from fetal and adult pancreas biology in physiological as well as pathological conditions support the hypothesis of the existence of **pancreatic adult stem cells** based on proliferation assays. For example, pancreas from pregnant rats and mice displayed an uptake of bromodeoxyuridine (BrdU) during pregnancy, suggesting the proliferation of islets (Parsons, Brelje et al. 1992; Karnik, Chen et al. 2007). This proliferation was stimulated by prolactin (Nielsen, Svensson et al. 1999; Rieck and Kaestner) that reduced expression of *multiple endocrine neoplasia type 1* (*MEN1*), a tumor suppressor (Karnik, Chen et al. 2007), and enhanced expression of *tryptophan hydroxylase 1* (*Tph1*), which allows for incorporation of BrdU in the cell (Kim, Toyofuku et al. 2010). In addition, expression of Ki-67 (a marker associated with proliferation) and β-cell mass increase has been described in the pancreas of obese mice, perhaps as a response to insulin resistance (Butler, Janson et al. 2003; Butler, Janson et al. 2003). Other authors have shown that mature β cells are capable of self-replication (**Figure 2**), and that this is the mechanism by which new beta cells in the adult are generated. Young diabetic rats are able to regenerate β cells after streptozotocin (STZ)-induced β cell destruction, but this capacity for regeneration declines rapidly during the first days of life (Wang, Bouwens et al. 1996). β cell regeneration is also detected after pancreatectomies, but the regeneration response is different between species, with a higher rate of regeneration in rodents than in larger mammals (Stagner and Samols 1991; Bonner-Weir and Sharma 2002; Robertson, Lanz et al. 2002; Matveyenko, Veldhuis et al. 2006). More recently, human islet precursor cells were identified from IPCs that underwent an epithelial-to-mesenchymal transition, demonstrating that, *in vitro*, human IPCs from the islets of cadaveric pancreas donors could spontaneously produce highly proliferative "de-differentiated" fibroblast-like cells that could then possibly be expanded and later re-induced into insulin-producing, β-like cells (Gershengorn, Hardikar et al. 2004; Russ, Bar et al. 2008; Russ, Sintov et al. 2011).

As mentioned previously, *Pdx1* and *Ngn3* are two transcription factors which are essential for endocrine cell differentiation during pancreas development, and have been targets in the search of pancreatic stem cells. However, it has not been demonstrated if $Pdx1^+$ cells can self-renew (Jiang and Morahan 2011), a requirement for stem cell definition. $Ngn3^+$ cells from duct-ligated adult pancreas can develop into islet cells (Xu, D'Hoker et al. 2008), satisfying the stem cell differentiation requirement. However, *Ngn3* expression in the embryo inhibits

proliferation by inducing *cyclin-dependent kinase inhibitor 1a* (*Cdkn1a*) (Miyatsuka, Kosaka et al. 2011), resulting in limited mitotic potential of *Ngn3*$^{+}$ cells (Gradwohl, Dierich et al. 2000; Johansson, Dursun et al. 2007), which is evidence against self-renewal.

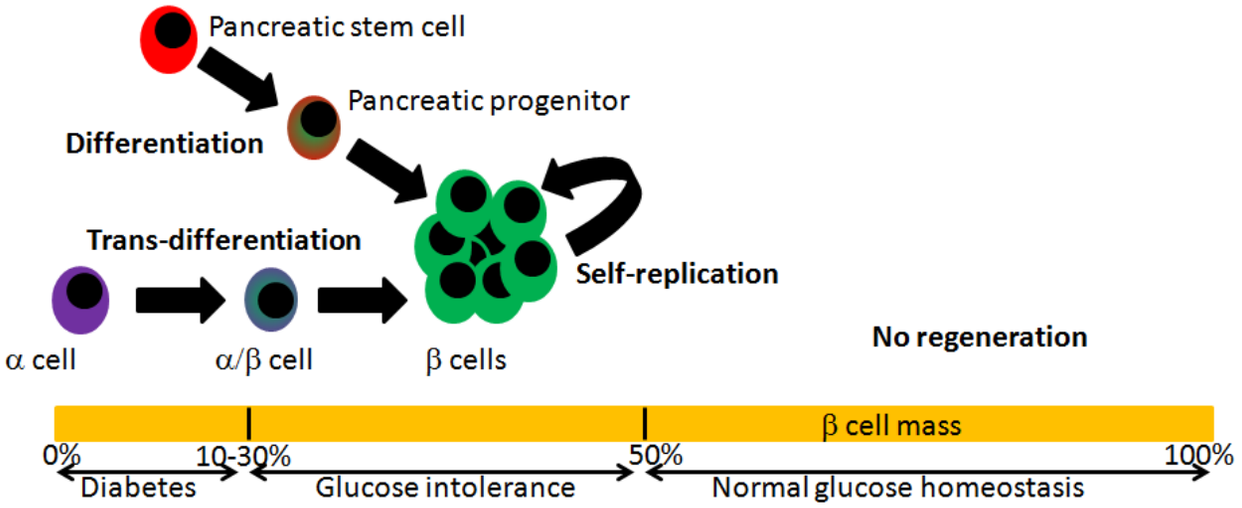

Figure 2. *Different mechanisms for β cell regeneration based on β cell mass*. Differentiation from pancreatic stem cells and transdifferentiation from β cells would occur when less than 30% of the normal β cell mass is present, whereas self-replication of residual β cells would occur if 30-50% of the normal β cell mass is present.

To test whether β cells can self-replicate in the adult, transgenic mice in which pre-existing β cells were specifically labeled were utilized (Dor, Brown et al. 2004). In the absence of pancreatic injury, these labeled cells were not replaced by new IPCs, arguing against the existence of an adult pancreatic stem cell. However, the labeled IPCs were able to uptake BrdU after pancreatectomy, indicating that adult β cells contain regenerative ability (Dor, Brown et al. 2004). A different model in which apoptosis was induced specifically in β cells using a diphtheria toxin receptor (DTR) transgenic mouse model showed β cell recovery, restoration to euglycemia, and that the surviving β cells were involved in their own regeneration (Nir, Melton et al. 2007). These studies assumed that insulin expression was limited exclusively to cells, but it has been shown that in certain circumstances, other cells and tissues can express insulin (Kojima, Fujimiya et al. 2004). In support of this, rare multipotent stem cells which express insulin have been described in both embryonic and adult mouse and human pancreas that are distinct from mature β cells (Smukler, Arntfield et al. 2011). Importantly, these cells were shown to self-renew, expressed *Pdx1*, *Nkx6.1*, *Ngn3* and *insulin*, and differentiate into IPCs that could alleviate diabetes in mice. Therefore, it appears that adult pancreatic stem cells do indeed exist, and the assumption that insulin is not expressed in β cell progenitors is incorrect. These findings can now be used to model additional cellular alternatives for β cell replacement in TID.

Questions regarding the existence and location of pancreatic stem cells and progenitors outside of the pancreas are still an area of investigation. Hepatic and pancreatic cells are derived from a common endodermal progenitor during embryogenesis, and this has led to the hypothesis that cells from fetal liver tissue could be used as an alternative β cell source.

Pancreatic duct cells and liver cells can be induced to express certain β cell gene products in culture (Heremans, Van De Casteele et al. 2002; Sapir, Shternhall et al. 2005). In support of this, $CD45^-$ $Ter119^-$ $c\text{-}kit^-$ hepatic stem cells were isolated from mouse fetal liver and differentiated into IPCs in vitro. These differentiated cells were then transplanted into drug-induced diabetic mice, which showed a significant reduction of their blood glucose levels (Feng, Du et al. 2005). Bone marrow has also been proposed as another possible source of IPCs. For example, human fetal bone marrow-derived $CD45^-$ Glycophorin A^- mesodermal progenitor cells generated insulin secreting islet-like clusters when injected into human fetal pancreatic tissues that were implanted in STZ-treated NOD/severe combined immunodeficiency (SCID) mice (Ai, Todorov et al. 2007). In the mouse, progeny of $Mac\text{-}1^-$ Sca^+ bone marrow cells cultured with cytokines such as IL-3, IL-6, IL-11 and stem cell factor (SCF) migrate into the pancreas islets and have been reported to convert into $CD45^-$ $CD34^+$ insulin-expressing cells (Luo, Luo et al. 2009). The mechanisms involved in the conversion of these bone marrow cells into IPCs were not investigated.

3.2. Transdifferentiated cells as heterologous sources of β cells

Transdifferentiation is defined as a process by which a "terminally" differentiated cell changes into another type of differentiated cell. Transdifferentiation of pancreatic α cells into β cells has been suggested as a source of β cell replacement for TID. Transdifferentiation of α cells into β cells has been observed *in vivo* (Thorel, Nepote et al. 2010). Mice in which more than 99% of β cells were ablated eventually recovered β cell mass, due to transdifferentiated α cells. The rates of recovery from α cells ranged between 32 to 81% among individuals. These data have led to a model in which several β cell regenerative mechanisms can occur, but each mechanism is used under different pathological conditions (**Figure 2**). For example, in this model, pancreatic stem cells and progenitors, as well as transdifferentiated cells, naturally replace β cells when β cell mass is extremely depleted (less than 30% of normal), but β cell mass is regenerated by self-replication of β cells when the loss is 30 - 50% of normal (Thorel, Nepote et al. 2010). Interestingly, during transdifferentiation from the α to β phenotype, cells that express both insulin and glucagon can be temporally detected (Thorel, Nepote et al. 2010). These "bi-hormonal" cells have been described during pancreas development by immunohistochemistry or single cell gene expression analysis (Chiang and Melton 2003). Their existence is controversial and more studies are required before they can be accepted to be true endocrine progenitor cells, such as clear isolation of the population and definitive evidence that they can give rise to α or β cells *in vitro* or *in vivo* (Alpert, Hanahan et al. 1988; Teitelman, Alpert et al. 1993; Herrera, Huarte et al. 1994; Herrera 2000; Jensen, Heller et al. 2000).

One important question that has not yet been answered pertains to the location of the cells that can undergo transdifferentiation into β cells *in vivo*. Two major pancreas compartments contain cells with the potential of generating new β cells via transdifferentiation *in vivo*: ductal epithelium and acinar tissue. Islet-like clusters of human ductal epithelium cells reverse diabetes after transplantation (Bonner-Weir, Taneja et al. 2000; Ramiya, Maraist et al. 2000). After pancreatic duct ligation, ductal cells that express *Ngn3* (a marker of pancreatic

progenitors, see Section 2.3*)* differentiate into β cells if transplanted into fetal pancreas (Xu, D'Hoker et al. 2008). Experiments using ductal cell lineage tracing with the carbonic anydrase II (*CA2*, an enzyme specific for ductal cells) promoter showed *CA2* expression in islet cells under normal conditions or after pancreatic duct ligation, adding more evidence of ductal epithelial cells' ability to differentiate into islet cells. Some authors have suggested that these pancreatic ductal cells are mesenchymal stem cells (MSC) (Seeberger, Dufour et al. 2006). Whether these MSC differentiate directly into pancreatic stem cells or transdifferentiate from epithelial cells to pancreatic stem cells still remains unclear (Bonner-Weir, Inada et al. 2008; Inada, Nienaber et al. 2008). The exocrine tissue, in particular acinar cells, could provide a new alternative β cell replacement source for the treatment of diabetes. Acinar cells are usually discarded after islet purification from donated pancreas for transplantation, but could be reprogrammed *in vivo* to β cells by introducing a combination of the transcription factors *Ngn3*, *Pdx1* and *Mafa* by means of adenovirus. These transcription factors are required only transiently and they are not detectable after two months of transfection (Zhou, Martin et al. 2008). Epithelial cells that were purified from non-endocrine islet cells were co-transplanted with fetal pancreatic cells and differentiated into endocrine cells (Hao, Tyrberg et al. 2006). Moreover, cells which express *amylase/elastase*, specific markers of acinar cells, differentiated into insulin expressing cells, as measured by cell tracing (Minami, Okuno et al. 2005).

Taken together, the studies cited in this section demonstrate that there are many possible pluripotent and even terminally differentiated cells *in vivo* that could be used to derive new IPCs that can alleviate hyperglycemia in diabetic mouse models. However, the alternative source that been most exhaustively researched is the totipotent **embryonic stem cell** (ESC). In the next section, we review studies of ESC differentiation into IPCs.

3.3. Differentiation of embryonic stem cells into insulin producing cells

The use of ESCs for cell replacement therapies has great promise, due to the potentially unlimited self-renewal of ESC in their undifferentiated state, and the potential of ESC to differentiate into any type of cellular derivatives. Many groups have used diverse methodologies to differentiate human or murine ESCs into IPCs for β cell replacement in diabetes (**Figure 3**). The most common obstacles amongst the different protocols are the inability of ESC-derived IPCs (ESC-IPCs) to secrete insulin after glucose-stimulation, the risk of teratoma formation due to undifferentiated ESC that remain in the ESC-IPC culture, and immune rejection of the ESC-IPC after transplantation *in vivo*. Moreover, murine and human ESC lines do not behave similarly when they are differentiated into IPCs, which has led to species-distinct protocols (**Figure 3**).

The efficiency of ESC-IPC differentiation *in vitro* between murine and human ESC cells is clearly different. One reason for this appears to be the manner by which the endodermal germ layer is initiated in ESC culture. The embryoid body (EB) is a three-dimensional cluster of ESC that induces the differentiation of the endoderm, mesoderm, and ectoderm germ layers. In contrast to the EB method, ESCs can also differentiate in two-dimensional

monolayers. For ESC-IPC differentiation, murine ESCs have been observed to differentiate very efficiently to endodermal cells via EB formation, with approximately 50% of the cells expressing *Foxa2*. In contrast, production of definitive endoderm cells in human EBs is less efficient, ranging from 5% to 19% of cells expressing *Foxa2* or *Sox17* (Shim, Kim et al. 2007). Instead, when cultured in monolayers, human ESCs show an efficiency of definitive endoderm differentiation over 80%, as measured by co-expression of *Foxa2* and *Sox17* (D'Amour, Agulnick et al. 2005; McLean, D'Amour et al. 2007), but murine ESCs are not efficiently differentiated into endoderm with this method (Kim, Hoffman et al. 2010). Consequently, most methods described for murine ESCs utilize EB formation, whereas human ESC methods use monolayers. Finding a common consensus protocol is one future challenge to the improvement of ESC-IPC differentiation and function. A summary of some murine and human IPC differentiation studies that includes a comparison of the IPC functional tests performed is presented in **Table 1 and Table 2,** respectively.

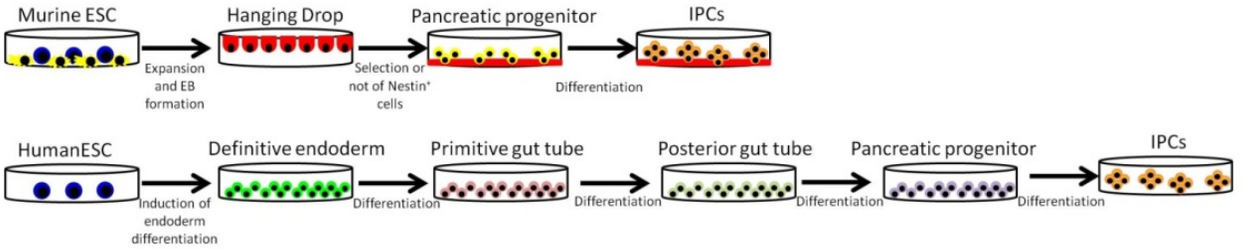

Figure 3. *Comparison of murine versus human ESC differentiation protocols to insulin-producing cells*. The schematic shows the differences in culture methods to produce IPCs from murine ESC (top) and human ESC (bottom). Please see the main text for more details.

ES cell lines used	Insulin mRNA	c-peptide protein expression	*In vitro* glucose response	Rescue of diabetic mice	Differentiation protocol reference
R1	Not done	Not done	Yes	Yes	(Soria, Roche et al. 2000)
B5, E14.1 and ESF122	Yes	Yes (controversial)	Yes	Failed (Fujikawa, Oh et al. 2005)	(Lumelsky, Blondel et al. 2001)
JM1, ROSA, and ESF122	Yes	Yes	Yes	Yes	(Hori, Rulifson et al. 2002)
R1 and ESF122	Yes	Yes	Yes	Yes with *Pax4* transfection	(Blyszczuk, Asbrand et al. 2004)
D3	Yes	Not done	Not done	Not done	(McKiernan, O'Driscoll et al. 2007)
D3	Yes	Not done	Yes	Not done	(Wang and Ye 2009)
B6	Yes	Yes	Yes	Not done	(Kim, Hoffman et al. 2010)
EB3	Yes	Yes	Yes	Not done	(Miyazaki, Yamato et al. 2004)
R1	Yes	Yes	Yes	Not done	(Banerjee, Sharma et al.)

Table 1. Murine ESC-IPC differentiation protocols

ES cell lines used	Insulin mRNA	c-peptide protein expression	*In vitro* glucose response	Rescue of diabetic mice	Differentiation protocol reference
CyT25, CyT49 CyT203	Yes	Yes	Yes	Yes	(D'Amour, Bang et al. 2006; Kroon, Martinson et al. 2008)
H1and H9	Yes	Yes	Yes	Yes	(Jiang, Shi et al. 2007)
H1, H7 and H9	Yes	Yes	Yes	Not done	(Jiang, Au et al. 2007)
Hes3	Yes	Yes	Yes	No	(Phillips, Hentze et al. 2007)

Table 2. Human ESC-IPC differentiation protocols

The first successful approach differentiating ESCs into functional IPCs utilized murine ESCs that were transfected with a neomycin selection gene under the control of the human *insulin* promoter (Soria, Roche et al. 2000). IPCs from EBs were selected with neomycin and nicotinamide, and no teratoma formation was described after transplantation. These ESC-IPCs normalized blood glycemia in diabetic mice during the first 4 weeks after transplant; however, 40% of the mice became hyperglycemic 12 weeks after implantation, suggesting that the ESC-IPCs function or lifespan was diminished (Soria, Roche et al. 2000). Since this initial report, numerous other approaches for differentiating ES cells into IPCs have been described, but three protocols by Lumelsky et al., Hori et al., and Blyszczuk et al. appear to be the most referenced (Lumelsky, Blondel et al. 2001; Hori, Rulifson et al. 2002; Blyszczuk, Asbrand et al. 2004). Non-transgenic ESCs were differentiated into IPCs by means of a five-step protocol: 1) expansion of ES cells, 2) formation of EBs, 3) selection of *nestin*-expressing cells with a serum-free medium containing insulin, transferrin, selenium and fibronectin (ITSFn), 4) expansion of pancreatic endocrine progenitor cells with a N2 medium containing B27 and the mitogenic basic fibroblast growth factor (bFGF) and 5) induction of differentiation of IPCs by withdrawal of bFGF in presence of nicotinamide (Lumelsky, Blondel et al. 2001). ESC-IPCs expressed insulin-1 and insulin-2 (murine isoforms) as detected by immunohistochemistry and PCR, and secreted insulin after glucose challenge (Lumelsky, Blondel et al. 2001). However, two years later, ESC-IPCs produced by this method became controversial, as it was reported that they were incapable of *de novo* insulin synthesis, and instead acquired insulin from the culture medium (Rajagopal, Anderson et al. 2003). This controversy currently is still unresolved, as some groups suggest that the insulin released by the ESC-IPCs is a mixture of insulin produced *de novo,* as well as insulin uptake from the medium (Paek, Morgan et al. 2005), and other groups state that the insulin expression by ESC-IPCs is an artifact due to apoptotic or necrotic cells (Hansson, Tonning et al. 2004).

To settle this controversy, the detection of C-peptide, a by-product of endogenous insulin synthesis, will be needed in order to prove that ESC-IPCs produce insulin *de novo*. ESC-IPCs produced with the Lumelsky protocol have been shown to express C-peptide, but they

failed to alleviate diabetes in mice, due to teratoma formation (indicating that not all of the ESCs were completely differentiated) (Fujikawa, Oh et al. 2005). Hori et al. provided an alternative methodology, using the selection of *nestin*$^+$ cells, a marker for mitotically active cells. Nestin is a neuronal marker, and it is not surprising that the Lumelsky and the Hori protocols would also generate neuronal cells when differentiating ES cells into IPCs (Lumelsky, Blondel et al. 2001; Hori, Rulifson et al. 2002). Moreover, Hori et al. added LY294002, an inhibitor of phosphoinositide 3-kinase, an essential intracellular signaling regulator, to the culture media at the IPC induction step (Hori, Rulifson et al. 2002). ESC-IPCs produced via the Hori protocol were smaller, with a reduced cytoplasmic volume compared to pancreatic β cells, probably due to the ability of LY294002 to reduce cell size (Vanhaesebroeck, Leevers et al. 2001). Compared to the Lumelsky protocol, IPCs produced by the Hori protocol were able to release more insulin and rescue glycemic blood levels after 3 weeks of transplantation in diabetic mice (Hori, Rulifson et al. 2002). However, these results could not been reproduced by other authors (Boyd, Wu et al. 2008).

Blyszczuk et al. described a third ESC-IPC differentiation protocol (Blyszczuk, Asbrand et al. 2004). This protocol consisted of three steps: 1) formation of EBs, 2) spontaneous differentiation of EBs into cells of ectodermal, mesodermal and endodermal lineages by transferring EBs into gelatin-coated plates, and 3) induction of differentiation into IPCs by culturing the gelatin-plated EB cell suspension with culture media containing progesterone, laminin, putrescine, selenium, nicotinamide, insulin, transferrin and B27 (Blyszczuk, Asbrand et al. 2004; Schroeder, Kania et al. 2006; Schroeder, Rolletschek et al. 2006). ESC-IPCs generated by the Blyszczuk protocol released insulin *in vitro* after glucose challenge, but they did not succeed in rescuing diabetic mice *in vivo*. The generation of ESC-IPC via the activation of differentiation genes such as *Pax4* was also studied. When *Pax4*-transduced ESCs differentiated into IPCs and transplanted to diabetic mice, blood glucose levels were reduced for at least 4 weeks. Similar reduced blood glucose levels were detected in diabetic mice transplanted with IPCs transfected with *Pax4* by nucleofection (Lin, Kao et al. 2007). However, mice treated either with unmodified or *Pax4*-modified ESC-IPCs developed teratomas (Blyszczuk, Asbrand et al. 2004). Exhaustive microarray studies have compared gene expression in ESC-IPC derived from wild type and *Pax4*$^+$ ESC at different stages of *in vitro* differentiation in order to attempt understand the mechanisms and factors required to produce fully functional ESC-IPC. The authors conclude that murine ESC-derived pancreatic cells are unable to complete pancreatic differentiation *in vitro*, as they appear to be blocked at the embryonic/fetal stage of development, as deduced by their gene expression profiles (Rolletschek, Schroeder et al. 2010). To date, murine ESC-IPCs are only able to acquire full functional β cell characteristics *in vivo*, but not *in vitro,* so the challenge to identify the missing factors to induce complete maturation of ESC-IPC *in vitro* remains.

Solving the ESC-IPC differentiation problem is complicated, as individual ESC lines do not behave similarly before and after differentiation, which also makes direct cross-comparison of the published ESC-IPC differentiation protocols difficult (Osafune, Caron et al. 2008). The three ESC-IPC differentiation protocols described above were compared using three different ESC cell lines, with the ES122 ESC line providing the best results (Boyd, Wu et al.

2008). When the ES122 ESC line was differentiated using the Lumelsky, Hori or Blyszczuk protocols, the Blyszczuk protocol proved to be superior: higher insulin-1 and insulin-2 gene and protein expression, greater increase in insulin secretion after glucose challenge *in vitro* and longer duration of normoglycemic blood level in diabetic mice after transplantation were achieved. However, consistent with previous reports, IPCs formed teratomas after three weeks of transplantation (Boyd, Wu et al. 2008). Therefore, in order for ESC-IPCs to be used in the clinic, it is clear that additional measures must included in the ESC-IPC protocols before transplantation, such as: the depletion of undifferentiated cells, using markers like stage-specific embryonic antigen 1 (SSEA-1) in order to avoid teratoma formation, methods to efficiently scale-up cell numbers before transplantation in order to reach similar blood glycemia levels as with healthy β cells, and/or the identification and addition of factors that can induce ESC-IPC to more mature, functional status when cultured *in vitro*.

More differentiation protocols to generate ESC-IPCs from murine ESCs have been described, but all of the known assays to test function of ESC-IPC have not been performed (**Table 1**). A comparative analysis in which the murine ESC D3 line was differentiated through the formation of EBs in the presence of retinoic acid showed how the subsequent exposure to sodium butyrate produced IPCs that expressed insulin-1 and insulin-2. However, C-peptide was not expressed and *in vitro* glucose response assays or transplantation in diabetic mice were not performed (McKiernan, O'Driscoll et al. 2007). Another approach, using a three-dimensional (3D) system with a Type I rat tail collagen solution, generated IPCs from the murine ESC D3 line that secreted more insulin after *in vitro* glucose challenge than IPCs generated in a two-dimensional (2D) system (Wang and Ye 2009); however, no *in vivo* functional analysis of these IPCs was performed. Comparing the 3D system with the differentiation protocols described by Blyszczuk et al., Lumelsky et al. or Hori et al. using the same cell line would be interesting. In another protocol that omitted EB formation, ESC-IPCs were generated by following a four step protocol: 1) treatment of ES cells with all-*trans*-retinoic acid (ATRA) and fibroblast growth factor (bFGF), 2) withdrawal of ATRA, 3) induction of formation of cell cluster by growth in presence of N2 and B27 supplements and 4) culture of clusters in dibutyryl cAMP and nicotinamide (Kim, Hoffman et al. 2010). These ESC-IPCs expressed insulin and responded to glucose challenge, but transplantation into diabetic mice was not performed, so the functionality of these cells *in vivo* is unknown. As transgenesis of ESCs with *Pax4* seemed to influence ESC-IPC differentiation, other groups have attempted to control the expression of pancreatic genes during ESC-IPC differentiation. For instance, regulation of *Pdx1* expression via the "Tet-off" system generated IPCs that increased *insulin-2* and *Ngn3* expression and secreted more insulin after glucose challenge *in vitro* (Miyazaki, Yamato et al. 2004). The co-culture of ESCs with other cell types has also successfully generated IPCs that express insulin and C-peptide, and respond to glucose. Banerjee et al. used 3 co-culture steps: 1) induction toward endoderm-like cells by co-culturing with primary hepatocytes, 2) inhibition of Sonic hedgehog by culturing in presence of KAAD-cyclopamine on Matrigel and retinoid induction, and 3) maturation to islet cells by co-culture with endothelial cells in medium containing nicotinamide, insulin, selenium, transferring and B27 (Banerjee, Sharma et al.). These studies

are all examples of the diverse approaches that have been explored to produce ESC-IPCs; however, none of the ESC-IPCs generated by these protocols have shown regulation of blood glucose levels after transplantation, as has been shown by Lumelsky et al., Hori et al. and Blyszczuk et al.

For human ESCs, differentiation into IPCs through monolayers, without EB formation, is the methodology most commonly followed. The protocol by D'Amour *et al*., in which the *in vivo* differentiation steps from the definitive endoderm to endocrine pancreatic islets are mimicked *in vitro,* is currently considered the reference protocol for differentiating human ESC to IPC (D'Amour, Bang et al. 2006; Kroon, Martinson et al. 2008). Endodermal intermediates can be identified at each step: 1) definitive endoderm markers (*Sox17*, *FoxA2* and *Cxcr4*) are detected after the addition of activin A and Wnt3a in a medium with low serum concentration, 2) primitive gut tube markers (*Hnf1b* and *Hnf4a*) are induced by addition of FGF1- and KAAD-cyclopamine , 3) posterior foregut markers (*Pdx1*, *Hnf6* or *Sox9*) are observed by induction with ATRA, KAAD-cyclopamine, FGF10 and B27, 4) pancreatic endoderm and endocrine precursor gene and protein expression (*Nkx6-1*, *Ngn3*, *Pax4* or *Nkx2-2*) can be detected by induction with exendin-4, and 5) hormone-expressing endocrine cell markers (*insulin*, *glucagon*, *somatostatin* or *poly-peptide Y*) are induced by exposure of cells to IGF1 and HGF. Human ESC-IPCs generated via this protocol release C-peptide at levels similar to β cells from islets in response to multiple secretory stimuli, but unfortunately, not to glucose (D'Amour, Bang et al. 2006). Interestingly, cells that co-express insulin and glucagon are observed, indicating that IPCs generated by the D'Amour protocol may still be immature β cell progenitors. A slight modification of the D'Amour protocol generated pancreatic endodermal cells from human ESCs, which protected diabetic immunocompromised SCID-Bg mice from hyperglycemia after transplant (Kroon, Martinson et al. 2008).

Further modifications to the D'Amour protocol have shown some improvements in IPC properties. For example, human ESC-IPC that secrete insulin and respond to glucose were generated by combining activin A and ATRA in a chemically defined medium, and other maturation factors, such as bFGF and nicotinamide in DMEM/F12. ESC-IPCs differentiated by this system were able to rescue 30% of diabetic *nu/nu* mice from hyperglycemia for at least 6 weeks, with no teratoma formation observed for three months post-transplantation (Jiang, Shi et al. 2007). Other modifications, such as increasing the time of suspension cultures and different growth factor combinations have succeeded in producing ESC-IPCs. Those suspension cultures contained ductal, exocrine and endocrine-like cells and secreted insulin in response to glucose challenge, but their therapeutic potential for diabetes was not tested *in vivo* (Jiang, Au et al. 2007).

Human ESC-IPCs have been also generated through the formation of EBs. Human EBs in a 3D matrix were induced to differentiate into definitive endoderm by the presence of activin A and Bmp4, and then into IPCs via the addition of growth factors such as FGF18, EGF, TGF-α, IGF-1, IGF-1 and VEGF. In contrast with other reports, these ESC-IPCs expressed only insulin and no other pancreatic hormones; however, they did not rescue diabetic mice and formed teratomas instead. When *Pdx1* gene expression was quantified among six

human ESC lines following this protocol, different levels of expression were detected (Phillips, Hentze et al. 2007). This result is not surprising, since distinct cell behaviors and differentiation potentials have been observed amongst independent stem cell lines (Osafune, Caron et al. 2008).

Therefore, for human and murine ESC-IPC differentiation, no "standard" protocol currently exists, as the results appear to be dependent on the ESC line utilized. In addition, there is no consensus on the composition of the differentiation culture mediums to guide cells from definitive endoderm to the IPC fate. However, some compounds appear to be key factors for the success of ESC-IPC differentiation. Activin A, a member of the transforming growth factor-beta (TGF-β) family, is involved in cellular proliferation and differentiation, and seems to be essential at the first steps of differentiation of human ESC into definitive endodermal cells (D'Amour, Bang et al. 2006; Kroon, Martinson et al. 2008). Nicotinamide, the amide of vitamin B3, is a well-known inducer of endocrine differentiation (Otonkoski, Beattie et al. 1993), and has been used in the differentiation of human and murine ESCs into IPCs (Jiang, Shi et al. 2007; Boyd, Wu et al. 2008). Epidermal Growth Factor (EGF) and Basic Fibroblast Growth Factor (bFGF) have been also applied in human and murine ESC-IPC differentiation, as both factors induce cell proliferation and differentiation (Lumelsky, Blondel et al. 2001; Jiang, Au et al. 2007). However, EGF, but not bFGF, needs to be removed from the cultures during the last stages of the differentiation protocols in order to allow the formation of islet-like cell aggregates (Cras-Meneur, Elghazi et al. 2001; Hardikar, Marcus-Samuels et al. 2003). Hepatocyte Growth Factor (HGF) is an inducer of cell proliferation that is secreted by mesenchymal cells, and has been used for human ESC-IPC differentiation (Otonkoski, Cirulli et al. 1996; D'Amour, Bang et al. 2006; Phillips, Hentze et al. 2007) as well as murine fetal liver differentiation into IPCs (Feng, Du et al. 2005). Exendin-4 stimulates β cell proliferation (Xu, Stoffers et al. 1999) and it has been used for murine and human ESC-IPC differentiation (Tang, Cao et al. 2004; Wu, Liu et al. 2007; Gabr, Sobh et al. 2008; Gao, Wu et al. 2008; Li, Lam et al.). A combination of insulin, transferrin, selenium and fibronectin (ITSFn) has been a common cocktail in murine ESC-IPC differentiation, although the presence of insulin in the medium brought some controversy about the endogenous insulin production of IPCs (Lumelsky, Blondel et al. 2001; Hori, Rulifson et al. 2002; Blyszczuk, Asbrand et al. 2004; Hansson, Tonning et al. 2004). The concentration of glucose in the culture media can also influence ESC-IPC differentiation and their properties. Low glucose concentrations (5mM) increase the content of insulin in islet-like clusters, whereas higher concentrations (20-30 mM) induce their replication (Bonner-Weir, Deery et al. 1989; Guillemain, Filhoulaud et al. 2007). Importantly, the presence or absence of serum has a clear effect on ESC-IPC differentiation. It is widely accepted by all cell culture professionals that lot-to-lot variation of serum sources can affect the success of *in vitro* cell culture and assays. The most referenced protocols for murine or human ESC-IPC use serum-free medium, as they claim serum inhibits differentiation; however, the specific component in the serum has not been identified (Blyszczuk, Asbrand et al. 2004; Jiang, Au et al. 2007; Jiang, Shi et al. 2007). The inhibitory role of serum is somewhat controversial, as other reports have described how reduced serum concentrations (0-10%) are permissive for murine ESC-IPC differentiation (Vincent and Odorico 2009), yet other authors produced

ESC-IPC successfully with relatively high 15% serum concentrations (Ibii, Shimada et al. 2007). Inherent variability in the behavior of individual ESC lines, in conjunction with these responses in the absence or presence of serum, could explain these results. Studies that identify the molecules released by developing pancreas could help to determine the main factors needed in ESC-IPC differentiation *in vitro* (Vaca, Martin et al. 2006). These studies could also be applied in the development of synthetic molecules, such as indolactam V, IDE1 or IDE2, for easier production and control of murine and human ESC-IPC differentiation (Borowiak, Maehr et al. 2009; Chen, Borowiak et al. 2009).

In summary, despite the progress made to date, investigation must continue to optimize ESC-IPC differentiation before their clinical use is possible. ESC-IPC cultures contain heterogeneous cell populations, and have not been well-identified. Moreover, the ESC-IPCs do not always respond to glucose challenge and do not release insulin, limiting their use as a pancreatic β cell replacement in TID. Identification of novel factors to induce maturation of ESC-IPC phenotypes may help to achieve full functional status. However, perhaps a better strategy would be to identify and transplant the immature pancreatic progenitor cells within the ESC-IPC cultures, as they may mature and respond more appropriately *in vivo*.

3.4. Identifying markers of endoderm and pancreatic progenitor cells in ESC-IPC cultures

ESC-IPCs to date have not recapitulated pancreatic mature β cell phenotypes. Pancreatic progenitors that are generated within the heterogeneous ESC-IPCs may be suitable for cellular replacement therapy in TID. The pancreatic progenitors may not self-renew as stem cells do, but could differentiate into endocrine cells after transplantation, including β cells. The identification of specific cell surface markers on pancreatic progenitors would allow for their isolation and for the transplantation of a homogenous population of ESC-derived pancreatic progenitors. We will discuss some current advances in the identification of pancreatic progenitors *in vivo* that could be applied to the detection and isolation of ESC-derived pancreatic progenitors from ESC-IPC cultures.

3.4.1. Definitive endoderm progenitors

In mice, putative definitive endodermal cells that co-express E-cadherin and Decay Accelerating Factor (DAF1/CD55) cell surface markers also express *Pdx1* (Shiraki, Harada et al. 2010). Discoidin domain receptor tyrosine kinase 1 (DDR1) and delta/notch-like EGF related receptor (DNER) have been also indicated as possible murine pancreatic progenitor surface markers with bioinformatics studies confirmed by immunohistochemistry (Hald, Galbo et al. 2011). Murine ESCs bearing the GFP reporter gene and human IL2Rα (CD25) marker genes in the *goosecoid* (GSc) and *Soc17* loci have allowed for the identification of seven surface markers that are differentially expressed between definitive and visceral endoderm cells: Amn (Amnionless), Sdc4 (Syndecan-4), Pthr1 (Parathyroid hormone receptor 1), Tmprss2 (Transmembrane protease, serine 2), Tm4sf2 (Transmembrane 4 superfamily 2), Cxcr4 and Gpc1 (Glypican 1). Cxcr4, in combination with E-cadherin,

proved to be appropriate extracellular markers for the identification and isolation of definitive endoderm cells (E-cadherin$^+$ Cxcr4$^+$) from unmanipulated ES-derived cells (Yasunaga, Tada et al. 2005). Thus, Cxcr4 permits the isolation of definitive endoderm cells from human ES cells after 5 days of differentiation to nearly 100% purity, eliminating undifferentiated ESCs (D'Amour, Agulnick et al. 2005). However, Cxcr4 expression decreases during development, making the isolation of late definitive endoderm cells difficult. The combination of E-cadherin with CD55 has circumvented this problem by identifying early and late definitive endoderm cells in the embryo, and in ESC-derived cell cultures where GFP is expressed under the promoter of *Pdx1* (Shiraki, Harada et al. 2010). Our group has also observed the presence of E-cadherin$^+$ CD55$^+$ cells in our ESC-IPC cultures (**Figure 4B**). *Pdx1*$^+$Cxcr4$^+$ ESC-derived cells spontaneously undergo differentiation into IPCs *in vivo*, and lead to a sustained correction of hyperglycemia in diabetic mice (Raikwar and Zavazava 2011).

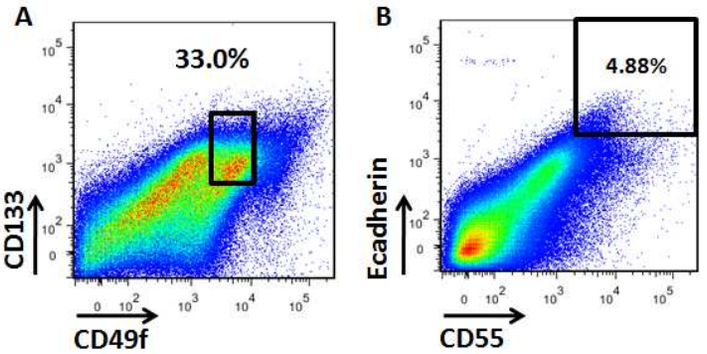

Figure 4. *Identification of putative pancreatic progenitor from murine ESC-IPC cultures.* ESC-IPCs were stained for markers of pancreatic progenitors found in the mouse embryo. A) CD133$^+$CD49f$^{low/+}$ cells. B) E-cadherin$^+$CD55$^+$ cells. The differentiation potential and properties of these in vitro-derived putative pancreatic progenitors requires further study.

3.4.2. Pancreatic progenitors

First isolated using *Ngn3*-fluorescent reporter mice (Gu, Wells et al. 2004; Mellitzer, Martin et al. 2004), *Ngn3*$^+$ cells have been suggested as possible pancreatic progenitors. In later studies, CD133 (prominin 1) was described as an extracellular marker that identified cells which expressed *Ngn3* in fetal and adult pancreas (Oshima, Suzuki et al. 2007; Sugiyama, Rodriguez et al. 2007). Mouse ductal cells with the phenotype CD133$^+$CD34$^-$CD45$^-$Ter119$^-$ CD49f$^{low/+}$ were isolated from fetal and adult murine tissues by flow cytometry. CD133$^+$CD49f$^{low/+}$ cells expressed *Ngn3,* increasing their isolation purity 1.4 fold when co-stained for CD24. Moreover, CD133$^+$CD49f$^{low/+}$ cells expressed *insulin-1*, *glucagon*, *somatostatin* and *pancreatic polypeptide* after culturing them with mitomycin C-treated mouse embryonic fibroblast (MEFs) for 2 days (Sugiyama, Rodriguez et al. 2007). Pancreatic progenitors isolated from neonatal, but not from adult, pancreases were able to differentiate into endocrine cells when cultured *in vitro* with HGF and EGF (Oshima, Suzuki et al. 2007). A newer study confirmed CD133 as well as platelet-derived growth factor receptor β (PDFGRβ) as markers of pancreatic progenitors in murine fetal pancreas (Hori, Fukumoto et

al. 2008). $CD133^+$ $PDFGR\beta^-$ cells were $CD49f^{low/+}$ and expressed *Ngn3* and *Pdx1*. *In vitro* co-culture of $CD133^+$ $PDFGR\beta^-$ cells with MEFs or their *in vivo* transplantation induced the formation of IPCs that expressed C-peptide in 7 days. Moreover, serial transplantation of these $CD133^+$ $PDFGR\beta^-$ cells demonstrated self-renewal properties and suggested they might be true pancreatic stem cells (Hori, Fukumoto et al. 2008). Inclusion of CD24 as a positive marker with CD133 and CD49f during cell isolation increases the yield of $Ngn3^+$ cells after sorting from mouse embryos (Sugiyama, Rodriguez et al. 2007).

Murine *Ngn3*-GFP ESC lines have been differentiated and $Ngn3^+$ cells were detected and isolated from EBs. $Ngn3^+$ cells were then sorted and differentiated *in vitro* into cells that expressed *insulin, glucagon* and *Pdx1*, among other pancreatic markers (Ku, Chai et al. 2007). Inducible *Ngn*3 expression in ESCs enhances expression of glucagon, somatostatin and insulin when they are differentiated into endocrine lineages, which reinforces the idea that *Ngn3* is an intermediate gene during IPCs differentiation (Serafimidis, Rakatzi et al. 2008). However, the description of non-transgenic *Ngn3*-expressing-pancreatic progenitor cells identified by cell surface markers in ESC-differentiation cultures has not been reported in the primary literature. Our group has analyzed the percentage of $CD133^+CD49f^{low/+}$ cells during ESC-IPC differentiation following the Blyszczuk protocol, and we have detected an increase in the number of $CD133^+CD49f^{low/+}$ cells in our cultures (**Figure 4A**). $CD24^+$ has been cited as a marker of murine pancreatic progenitors, and $CD24^+$ cells isolated from human ESC-IPC express *Pdx1,* and express insulin after 1 week *in vitro* (Jiang, Sui et al. 2011). Studies *in vivo* will confirm the differentiation potential of $CD24^+$ cells in ESC-IPC cultures. The CD142 protein is expressed in the human fetal pancreatic epithelium at 8 weeks of gestation, and has been implicated in human ESC-IPC differentiation. Isolation of $CD142^+$ cells allowed for the enrichment of pancreatic endoderm cells, and after transplantation into SCID/Bg mice, $CD142^+$ cells differentiated to all pancreatic cell lineages, including IPCs that responded to glucose stimulation *in vivo* and did not form teratomas (Kelly, Chan et al. 2011). However, these recipients were not diabetic, so future studies are required to demonstrate if they can rescue hyperglycemia in diabetic mice.

The establishment of intermediate lineage-restricted progenitor cell lines (similar to the pancreatic progenitor cells) that generate homogeneous IPCs could be useful for scalable IPC production. Several clones were isolated from differentiation protocols into IPCs with the E-RoSH cell line (an endothelial lineage-restricted clonal murine ES cell derived line). The eight clones that were isolated could be frozen and thawed for months, and when cultured, they displayed properties of pancreatic β cells, such as expression of *insulin-1, insulin-2, Pdx1, Nkx2.2, Nkx6.1, Isl1, and Foxa2*, no expression of *Ngn3*, and expression of C-peptide. Moreover, the clones were able to rescue diabetic mice and did not form teratomas, demonstrating the absence of undifferentiated ESC. However, these cells continued proliferating after transplantation and showed an abnormal karyotype, suggesting an insulinoma phenotype, that may be responsible for the hyperinsulinemia and hypoglycemia observed in the animals (Li, Luo et al. 2009).

In summary, isolation and characterization of pancreatic progenitors from embryonic development and ESC-IPCs could provide another possible β cell replacement source for the

treatment of diabetes. These multipotent cells are not teratoma-forming, and can differentiate into IPCs and other hormone expressing cells, and have been shown to reverse hyperglycemic blood levels after transplantation. However, the isolation of pure or enriched pancreatic progenitors using cell-specific surface markers from ESC-IPCs cell differentiation cultures that are able to cure diabetic mice still has not been demonstrated. More studies to assess the efficiency of human ESC-IPC differentiation and optimization of sorting methods to remove any undifferentiated teratoma-forming cells are necessary before clinical translation. Nevertheless, even if fully functional, teratoma-free ESC-IPCs are derived, they must circumvent another possible barrier after transplantation: the host immune system (see Section 3.6). For this reason, induced pluripotent stem cells have been proposed as an autologous alternative to ESCs.

3.5. Differentiation of induced pluripotent stem cells into insulin-producing cells

Induced pluripotent stem cells (iPSCs) are reprogrammed somatic cells that behave like ESCs and can be re-differentiated into all three germ layers and potentially, any terminally differentiated cell type (Takahashi and Yamanaka 2006). The generation of iPSCs was firstly achieved by the viral delivery of reprogramming factors (Takahashi and Yamanaka 2006) in murine cells, and later by non-viral methods (Yu, Hu et al. 2009). Human iPSCs have also been generated (Yu, Hu et al. 2009). iPSC are envisioned as the future of "personalized" medicine since they could be used to generate autologous therapies that will not require pharmacologic immune suppression after transplantation (Okita, Ichisaka et al. 2007). However, the immunogenicity of syngeneic iPSC, which was unexpected by the stem cell biology field, demonstrated that this is not the case (Zhao, Zhang et al. 2011). Moreover, autologous iPSC generated from patients with genetic diseases likely will retain the propensity to develop disease following re-differentiation, unless they are modified (Raya, Rodriguez-Piza et al. 2009). The use of allogeneic iPSC could alleviate this possibility, but would have the disadvantage of possible immune rejection. iPSCs differ from ESCs in gene expression profiles (Chin, Mason et al. 2009), persistence of donor-cell gene expression (Marchetto, Yeo et al. 2009; Ghosh, Wilson et al. 2010), differentiation abilities (Feng, Lu et al. 2010; Hu, Weick et al. 2010) and genetic stability (Mayshar, Ben-David et al. 2010; Laurent, Ulitsky et al. 2011).

Currently, there is no standard iPSC to IPC differentiation protocol, but scientists have followed strategies similar to ESC to IPC differentiation (**Table 3**). Murine skin fibroblasts reprogrammed into iPSCs were differentiated into IPCs that were glucose responsive *in vitro* and normalized hyperglycemia in syngeneic mice with Type I and Type II diabetes. Teratoma formation was prevented by sorting out $SSEA1^+$ IPCs before transplantation, and no immune rejection was detected (Alipio, Liao et al. 2010). Human iPSC to IPC differentiation has been performed using a four-stage protocol: 1) endoderm induction by activin A and wortmannin, 2) pancreatic differentiation with retinoic acid, Noggin and FGF7, 3) expansion of pancreatic endoderm cells with EGF and 4) maturation of IPCs with nicotinamide, bFGF, exendin-4 and BMP4 (Zhang, Jiang et al. 2009), and another method

following a human ESC-IPC protocol (Maehr, Chen et al. 2009; Thatava, Nelson et al. 2010). These IPCs expressed and released C-peptide after glucose challenge *in vitro*. Differentiation of distinct human iPSC lines also produced IPC that expressed C-peptide and insulin, but at lower levels than ESC-IPCs (Zhang, Jiang et al. 2009). Interestingly, iPSC-IPC generated from TID patients also appear to be responsive to glucose challenge *in vitro* (Maehr, Chen et al. 2009). iPSC generated from human β-cells (BiPSCs) appear to be predisposed to re-differentiation into IPCs, as shown by the epigenetics of *Pdx1* and *insulin* promoters. BiPSC express more insulin and C-peptide than ESC-IPCs after transplantation into SCID mice (Bar-Nur, Russ et al. 2011; Russ, Sintov et al. 2011). Human iPSC-IPCs have not yet been transplanted into diabetic humanized mouse models, so their therapeutic potential to normalize blood glucose levels and their safety *in vivo* still requires further investigation.

Source	Insulin mRNA	c-peptide protein expression	*In vitro* glucose response	Rescue of diabetic mice	Differentiation protocol reference
Murine skin fibroblast	Yes	Not done	Yes	Yes	(Alipio, Liao et al. 2010)
Human fibroblast	Yes	Yes	Not done	Not done	(Zhang, Jiang et al. 2009)
Skin human TID adult and healthy neonatal fibroblast	Yes	Yes	Yes	Not done	(Maehr, Chen et al. 2009; Thatava, Nelson et al. 2010)

Table 3. iPS to IPC differentiation protocols

3.6. Addressing the immunogenicity of ESC-IPCs and iPSC-IPCs

Inflammation, due to local tissue damage, is a regular occurrence during transplantation of cells or organs, even in a syngeneic setting (Turvey, Gonzalez-Nicolini et al. 2000; Carvalho-Gaspar, Billing et al. 2005). For instance, cellular infiltrates are evident in rat islet β cell allografts within one week post-transplant, and are completely destroyed within 2 weeks. Depletion of major histocompatibility complex class II (MHC II)-positive islet cells (which presumably are antigen-presenting cells) delays β cell rejection up to 5 weeks, while re-aggregation of islet β cells after islet dissociation and β cell purification promotes allograft survival up to 20 weeks (Pipeleers, Pipeleers-Marichal et al. 1991). Embryonic tissue has not been considered to be highly immunogenic, mainly based on the "immune privileged" status of the fetus during gestation, which protects it from the maternal immune system. However, this immune privileged status is not generally observed outside the womb. For example, transplanted human β cells from fetal pancreas, which have a greater proliferative capacity than adult tissue (Hayek and Beattie 1997; Castaing, Peault et al. 2001), normalize blood glucose levels in diabetic immunoincompetent rodents (Hullett, Falany et al. 1987; Tuch, Osgerby et al. 1988; Tuch and Monk 1991; Castaing, Peault et al. 2001), but are rejected when transplanted into humanized SCID mice (immune reconstituted with human fetal liver and

thymus tissue) (Rouleau, Namikawa et al. 1996). Human pancreas obtained from first trimester fetus are less immunogenic that pancreas from the second trimester of gestation, as indicated by cellular infiltrates that contain high levels of host $CD45^+$ cells. This is consistent with an upregulation of T cell activating molecules, such as MHC II, the chemokine ligand 19 (CCL19), complement component 3 (C3) and tumour necrosis factor superfamily 10 (TNFSF10, also known as TNF-related apoptosis-inducing ligand, TRAIL), in second trimester human fetal pancreas compared to first trimester tissue (Brands, Colvin et al. 2008).

ESCs and iPSCs were also considered to be non-immunogenic, until recently. Murine ESCs do not express MHC I or MHC II, but increase MHC I expression when they differentiate spontaneously (Wu, Boyd et al. 2008). Despite of the lack of MHC I expression, ESCs are not deleted by natural killer (NK) cells (Koch, Geraldes et al. 2008). Interestingly, MHC I-positive murine ESCs are not recognized by cytotoxic $CD8^+$ T cells, even when antigen presenting cells (APCs) are used to prime T cells, suggesting that ES cells have some immune privilege. Nevertheless, this immune privilege is fragile and the addition of $CD4^+$ helper T cells can induce ESC rejection *in vivo* (Wu, Boyd et al. 2008). In immunocompetent mice, ESC allografts contain infiltrates of $CD11b^+$ macrophages, $CD4^+$ and $CD8^+$ T cells as early as 10 days post-transplantation (Wu, Boyd et al. 2008). In line with this, allogeneic EBs are not rejected if the CD4 and CD8 co-receptors are blocked, suggesting that ESC-derived tissues could be tolerated in allogeneic host (Lui, Boyd et al. 2010). In the absence of blocking, rejection of allogeneic EBs is mediated by $CD8^+$ T cells and active $F4/80^+$ MHC II^+ mannose receptor-negative macrophages. IL-10, a cytokine that downregulates immune responses, is expressed in syngeneic and allogeneic EBs grafts that are not rejected (Lui, Boyd et al. 2010). This tolerance is completely eliminated when host $CD4^+$ $FoxP3^+$ regulatory T cells (T_{reg}) are ablated. T_{regs} seem to protect EBs by keeping $F4/80^+$ macrophages in a low activation state by preventing their upregulation of MHC II, as well as by suppressing cytotoxic $CD8^+$ T cell responses by downregulating their expression of granzyme B (Lui, Boyd et al. 2010). It remains to be seen whether or not these mechanisms will apply universally to all ESC-derived tissues.

To date, there have been few descriptions of the immunogenicity of ESC-IPCs, but the existing data indicate that both innate and adaptive arms of the host immune system could be activated after ESC-IPC transplantation. ESC-IPCs can normalize blood glucose levels of diabetic immune compromised mice short-term, but when transplanted into immunocompetent hosts, their functionality is impaired, even under syngeneic conditions (Wu, Boyd et al. 2008). One explanation for the immune rejection of ESC-IPCs is that they express higher levels of MHC I than ES cells, and express higher levels of both MHC I and MHC II after exposure with IFNγ *in vitro*. Incidentally, IFNγ is up-regulated within both syngeneic and allogeneic ESC-IPC grafts three days after transplantation (Boyd and Wood 2009). However, the level of cellular infiltration differs between syngeneic and allogeneic ESC-IPC grafts. For example, $Gr1^+$ leukocytes can be observed in allogeneic, but not syngeneic, ESC-IPC grafts, within the first 24 hours post-transplantation. $CD11b^+$ macrophages can be detected in both syngeneic and allogeneic grafts by three days after transplantation, indicating a general response to local injury (Boyd and Wood 2010). In

addition, early inflammatory molecules that activate macrophages and granulocytes, such as IL-6, Gro-β (CXCL2 or macrophage inflammatory protein 2), Lix (CXCL5 or epithelial-derived neutrophil-activating peptide 78), MIP-1α (CCL3 or macrophage inflammatory protein 1-alpha) and MIP-1β (CCL4 or macrophage inflammatory protein 1-beta, IP-10 (Interferon gamma-induced protein 10 or CXCL10) and Mig (monokine induced by gamma interferon or CXCL9), are induced within both syngeneic and allogeneic ESC-IPC grafts within three days post-transplantation. Of these cytokines, IP-10, and Mig are associated with acute graft rejection (Burns, Wang et al. 2005). The adaptive immune system, as expected, is slower to respond, and infiltrates of reactive $CD8^+$ T cells are observed in allogeneic ESC-IPCs 10 days post-transplantation. In all cases, the immune response to allogeneic ESC-IPC observed was lower than the response to transplanted allogeneic pancreatic islets (Boyd and Wood 2010). These studies suggest that IPCs are suitable for transplantation, but some immune response by macrophages and T cells should be expected, so it is likely that immunosuppressive drugs to prevent graft rejection will have to be administered with ESC-IPC grafts.

Strategies to circumvent the immune system barrier to ESC-IPCs will be needed if they will be used for the treatment of diabetic patients. For instance, a short term immunosuppression approach blocking cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), CD40 ligand (CD40L) or lymphocyte function-associated antigen 1(LFA-1) for 6 days, has shown to allow for the engraftment of murine and human ESCs (Pearl, Lee et al. 2011). Since CTLA4Ig treatment has previously induced long-term survival of xenogeneic pancreatic islet grafts (Lenschow, Zeng et al. 1992; Eventov-Friedman, Tchorsh et al. 2006), it is possible that similar pre-conditioning treatments in recipients will allow for the acceptance of ESC-IPC grafts. An alternative strategy is the co-culture of ESC-IPC with bone marrow-derived mesenchymal stem cells, since co-culture of β cells with bone marrow-derived mesenchymal stem cells induces the expression of protective molecules such as IL6 (in a paradoxically anti-inflammatory role) and TGFβ1, an immunosuppressive cytokine, and anti-apoptotic genes like *Mapkapk2*, *Tnip1* or *Bcl3* (Karaoz, Genc et al. 2010). Another interesting strategy to prevent immune rejection of pancreatic progenitors is their encapsulation in alginate capsules with pores that allow for the passage of nutrients and insulin, but not immune cells (Tuch, Hughes et al. 2011). The use of autologous iPSC, instead of ESCs, was thought to avoid these immune rejection issues, but new evidence has demonstrated that syngeneic iPS-derived tissues can in fact, be rejected, as shown by T cell infiltration in the grafts (Zhao, Zhang et al. 2011). This rejection appears to involve minor histocompatibility antigens. Candidate genes, such as *Hormad1* (Horma Domain-Containing 1) or *Zg16* (zymogen granule protein 16), were shown to be directly involved in the activation of antigen-specific T cells, as shown by IFN-γ release. Based on this evidence, it is clear that the potential of immune rejection of both ESC- and iPSC-derived tissues will be an obstacle to be overcome before they can be applied in the clinic. Moreover, a detailed understanding of the immunogenicity profiles of ESC- and iPSC-derived tissues of any type (i.e. not only IPCs) could provide important information about the type of potential immune response(s) to expect and prevent before transplantation and helping to identify alternative strategies to facilitate their acceptance.

3.7. Hematopoietic stem cell transplantation to induce immune tolerance for type I diabetes

The autoimmune nature of TID provides two main targets in which hematopoietic stem cell transplantation (HSCT) could be used to ameliorate disease: the replacement of autoimmune T cells to prevent the onset of TID, and the induction of immunological tolerance to islet replacements via a previous HSCT or HSC co-transplantation. In both cases, induction of immunological tolerance to donor antigens is required for long-term survival of a transplanted organ. This is more easily achieved with autologous bone marrow transplantation, but can also occur when allogeneic bone marrow donors are used. In allogeneic bone marrow transplantation, induction of central and peripheral tolerance to donor antigens has been accomplished by creating mixed hematopoietic chimerism using non-myeloablative conditioning regimens (Sykes 2007; Sykes 2009). An important component to the induction of this tolerance is the education of donor and host T cells to recognize each other as "self" to avoid graft-versus-host disease (GVHD) and well as host-versus-graft reactions.

HSCT has been used to induce immune tolerance to TID antigens. Gene therapy approaches in which transplantation of syngeneic HSCs that were engineered to express pro-insulin under the MHC-II promoter transferred protection against insulitis and the development of spontaneous autoimmune diabetes (Steptoe, Ritchie et al. 2003; Chan, Clements et al. 2006). Transplant of fully allogeneic purified adult hematopoietic stem cells into lethally irradiated NOD mice (Beilhack, Scheffold et al. 2003) and the induction of mixed allogeneic hematopoietic chimerism using a non-myeloablative conditioning regimen cured diabetes in NOD mice and established clear immunological tolerance to both host and donor MHC as well as to insulin (Nikolic, Takeuchi et al. 2004). Recently, it has been shown that mixed chimerism across MHC mismatches is superior than mixed chimerism amongst MHC matched hematopoietic cells for prevention of insulitis in TID (Racine, Wang et al. 2011). Moreover, mesenchymal stem cells (MSC) co-transplanted with hematopoietic stem cells without islets prevented the onset of TID in allogeneic recipients, and co-transplantation of MSC with allogeneic hematopoietic stem cells and islets have been used to prevent the onset of TID in rodent models (Itakura, Asari et al. 2007; Asari, Itakura et al. 2011).

The results of the first human clinical trial of the use of autologous HSC transplant to treat early-stage TID patients were excellent (Voltarelli, Couri et al. 2007). Importantly, in these studies TID was alleviated using hematopoietic grafts alone (i.e. without co-transplantation of islets). However, patients with late-stage TID can only currently be cured by islet transplantation protocols which require islets from multiple matched donors, exceeding donor availability (Shapiro, Ricordi et al. 2006), or other insulin producing cell sources (such as ESC-IPC and iPS-IPC), which will also require induction of immune tolerance. Combined islet and HSC allotransplantation using an 'Edmonton-like' immunosuppression, (i.e. without ablative conditioning) in which high doses of donor HSCs ($4.3 \pm 1.9 \times 10^6$ HSCs/kg) were transplanted after 5 and 11days of islet transplantation, has not resulted in stable hematopoietic chimerism or graft tolerance after 1 year post-transplantation (Mineo, Ricordi

et al. 2008). Whether prior transplantation of HSCs could improve the engraftment of later islet transplantation is not clear, and is not easily tested in the clinic.

The induction of immunological tolerance to ESC-derived tissues is an active and emerging area of investigation. Recent studies have provided proof-of-principle that the induction of immunological tolerance to ESC-derived tissues can be achieved, but the mechanisms of tolerance induction to ESC-derived hematopoietic-progenitors (ES-HP), as well as the ability of ES-HP themselves to induce immunological tolerance across allogeneic barriers, are still not well understood (reviewed in (Thompson and Manilay 2011)). Transplantation of HoxB4-transduced ES-HP resulted in mixed hematopoietic chimersm and the induction of specific transplantation tolerance to allogeneic cardiac grafts (Bonde and Zavazava 2006; Bonde, Chan et al. 2008). It appears that HoxB4-ES-HP-derived T cells are actively "tolerized" to donor antigens in the thymus, and that if not, they can cause lethal GVHD (Kim, Stultz et al. 2011). In line with this, recipients of allogeneic ES-HP lacked evidence of GVHD (Burt, Verda et al. 2004; Bonde and Zavazava 2006; Bonde, Chan et al. 2008). Finally, another study has shown that non-HoxB4 transduced ES-HP can prevent onset of TID in mice (Verda, Kim et al. 2008), but the method of ES-HP production in this study is quite different from the other more recent protocols. Therefore, it appears that ES-HP are similar to bone-marrow derived hematopoietic progenitors, and that they could be used to induce tolerance of allogeneic tissues that are derived from the same ESC line, or that share MHC antigens. The same theory could apply to hematopoietic progenitors that are derived from iPSC (Niwa, Umeda et al. 2009).

4. Conclusion

The replacement of β cells is a promising therapy for diabetic patients, but the process depends on the discovery and detailed study of sources other than cadaveric pancreas. Pancreatic stem cells would be an ideal source for β cell replacement, since they are committed to differentiate into pancreatic cells. However, their cellular marker phenotype, as well as their anatomic location, has not yet been identified. If these questions are resolved, the conditions for their expansion and differentiation will require further optimization, such as emulating the properties of the pancreatic stem cell niche, improving their expansion, and perfecting the composition of culture media to induce their differentiation into endocrine cells. In addition, as pancreatic stem cells will likely be isolated from healthy patients, the immune system barrier to allogeneic transplants will also need to be addressed.

Another alternative to pancreatic stem cells is the differentiation of IPCs from ESC or iPSC, but this first requires the development and acceptance of a standard methodology to generate them. IPCs differentiated by this standard methodology will have to be completely mature and functional (i.e. respond appropriately when blood glucose level are altered, and have absolutely no teratoma-forming potential). The generation of a well-defined homogenous population of transplantable pancreatic progenitors that can be identified with extracellular markers would be an alternative to IPCs. Although they are not completely

mature, pancreatic progenitors could perhaps differentiate more readily into mature endocrine cells in response to the signals in their *in vivo* microenvironment. Studies to demonstrate that pancreatic progenitors can be isolated and differentiated from human ESCs, and can normalize blood glucose levels in humanized diabetic animal models still have not been reported. Perhaps a more complete comprehension of normal pancreas development can shed light into the processes that lead to the complete maturation of β-cells *in vivo*, which can be translated to differentiation protocols from ESC and iPSC. iPSC that are reprogrammed from human β cells can be more efficiently differentiated into IPCs than ESCs, and would avoid the ethical issues related to the use of ESCs. However, optimization of non-viral strategies for reprogramming iPS cells would be preferable before clinical translation (Yu, Hu et al. 2009). Finally, independently of the type of cell transplanted (ESC-IPC, iPSC-IPC, or pancreatic progenitors), the immunogenicity of these cells will have to be considered and determined to predict the potential and type of immune response to block before and after transplantation. The data collected from such analyses will aid in the establishment of strategies that can promote immune tolerance of these IPC grafts.

Author details

Jesus Ciriza
Department of Molecular and Cell Biology, School of Natural Sciences, University of California, Merced, Merced, CA, USA

Jennifer O. Manilay*
Department of Molecular and Cell Biology and Quantitative and Systems Biology Graduate Group, School of Natural Sciences, University of California, Merced, Merced, CA, USA

Acknowledgement

This manuscript is supported by funding from the University of California and the California Institute for Regenerative Medicine (RN1-00554-1).

5. References

Ahlgren, U., J. Jonsson, et al. (1996). "The morphogenesis of the pancreatic mesenchyme is uncoupled from that of the pancreatic epithelium in IPF1/PDX1-deficient mice." *Development* 122(5): 1409-16.

Ai, C., I. Todorov, et al. (2007). "Human marrow-derived mesodermal progenitor cells generate insulin-secreting islet-like clusters in vivo." *Stem Cells Dev* 16(5): 757-70.

Alipio, Z., W. Liao, et al. (2010). "Reversal of hyperglycemia in diabetic mouse models using induced-pluripotent stem (iPS)-derived pancreatic beta-like cells." *Proc Natl Acad Sci U S A* 107(30): 13426-31.

* Corresponding Author

Alpert, S., D. Hanahan, et al. (1988). "Hybrid insulin genes reveal a developmental lineage for pancreatic endocrine cells and imply a relationship with neurons." *Cell* 53(2): 295-308.

Anderson, M. S. and J. A. Bluestone (2005). "The NOD mouse: a model of immune dysregulation." *Annu Rev Immunol* 23: 447-85.

Asari, S., S. Itakura, et al. (2011). "Mesenchymal stem cells facilitate mixed hematopoietic chimerism induction and prevent onset of diabetes in nonobese diabetic mice." *Pancreas* 40(6): 846-54.

Banerjee, I., N. Sharma, et al. (2010). "Impact of co-culture on pancreatic differentiation of embryonic stem cells." *J Tissue Eng Regen Med* 5(4): 313-23.

Bar-Nur, O., H. A. Russ, et al. (2011). "Epigenetic memory and preferential lineage-specific differentiation in induced pluripotent stem cells derived from human pancreatic islet beta cells." *Cell Stem Cell* 9(1): 17-23.

Becker, A. J., C. E. Mc, et al. (1963). "Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells." *Nature* 197: 452-4.

Beilhack, G. F., Y. C. Scheffold, et al. (2003). "Purified allogeneic hematopoietic stem cell transplantation blocks diabetes pathogenesis in NOD mice." *Diabetes* 52(1): 59-68.

Bluestone, J. A., K. Herold, et al. (2010). "Genetics, pathogenesis and clinical interventions in type 1 diabetes." *Nature* 464(7293): 1293-300.

Blyszczuk, P., C. Asbrand, et al. (2004). "Embryonic stem cells differentiate into insulin-producing cells without selection of nestin-expressing cells." *Int J Dev Biol* 48(10): 1095-104.

Bonde, S., K. M. Chan, et al. (2008). "ES-cell derived hematopoietic cells induce transplantation tolerance." *PLoS One* 3(9): e3212.

Bonde, S. and N. Zavazava (2006). "Immunogenicity and engraftment of mouse embryonic stem cells in allogeneic recipients." *Stem Cells* 24(10): 2192-201.

Bonner-Weir, S., D. Deery, et al. (1989). "Compensatory growth of pancreatic beta-cells in adult rats after short-term glucose infusion." *Diabetes* 38(1): 49-53.

Bonner-Weir, S., A. Inada, et al. (2008). "Transdifferentiation of pancreatic ductal cells to endocrine beta-cells." *Biochem Soc Trans* 36(Pt 3): 353-6.

Bonner-Weir, S. and A. Sharma (2002). "Pancreatic stem cells." *J Pathol* 197(4): 519-26.

Bonner-Weir, S., M. Taneja, et al. (2000). "In vitro cultivation of human islets from expanded ductal tissue." *Proc Natl Acad Sci U S A* 97(14): 7999-8004.

Borowiak, M., R. Maehr, et al. (2009). "Small molecules efficiently direct endodermal differentiation of mouse and human embryonic stem cells." *Cell Stem Cell* 4(4): 348-58.

Boyd, A. S. and K. J. Wood (2009). "Variation in MHC expression between undifferentiated mouse ES cells and ES cell-derived insulin-producing cell clusters." *Transplantation* 87(9): 1300-4.

Boyd, A. S. and K. J. Wood (2010). "Characteristics of the early immune response following transplantation of mouse ES cell derived insulin-producing cell clusters." *PLoS One* 5(6): e10965.

Boyd, A. S., D. C. Wu, et al. (2008). "A comparison of protocols used to generate insulin-producing cell clusters from mouse embryonic stem cells." *Stem Cells* 26(5): 1128-37.

Brands, K., E. Colvin, et al. (2008). "Reduced immunogenicity of first-trimester human fetal pancreas." *Diabetes* 57(3): 627-34.

Brun, T. and B. R. Gauthier (2008). "A focus on the role of Pax4 in mature pancreatic islet beta-cell expansion and survival in health and disease." *J Mol Endocrinol* 40(2): 37-45.

Burns, W. R., Y. Wang, et al. (2005). "Recruitment of CXCR3+ and CCR5+ T cells and production of interferon-gamma-inducible chemokines in rejecting human arteries." *Am J Transplant* 5(6): 1226-36.

Burt, R. K., L. Verda, et al. (2004). "Embryonic stem cells as an alternate marrow donor source: engraftment without graft-versus-host disease." *J Exp Med* 199(7): 895-904.

Butler, A. E., J. Janson, et al. (2003). "Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes." *Diabetes* 52(1): 102-10.

Butler, A. E., J. Janson, et al. (2003). "Increased beta-cell apoptosis prevents adaptive increase in beta-cell mass in mouse model of type 2 diabetes: evidence for role of islet amyloid formation rather than direct action of amyloid." *Diabetes* 52(9): 2304-14.

Carvalho-Gaspar, M., J. S. Billing, et al. (2005). "Chemokine gene expression during allograft rejection: comparison of two quantitative PCR techniques." *J Immunol Methods* 301(1-2): 41-52.

Castaing, M., B. Peault, et al. (2001). "Blood glucose normalization upon transplantation of human embryonic pancreas into beta-cell-deficient SCID mice." *Diabetologia* 44(11): 2066-76.

Chan, J., W. Clements, et al. (2006). "Transplantation of bone marrow genetically engineered to express proinsulin II protects against autoimmune insulitis in NOD mice." *J Gene Med* 8(11): 1281-90.

Chavali, S., A. Mahajan, et al. (2011). "Association of variants in genes involved in pancreatic beta-cell development and function with type 2 diabetes in North Indians." *J Hum Genet* 56(10): 695-700.

Chen, S., M. Borowiak, et al. (2009). "A small molecule that directs differentiation of human ESCs into the pancreatic lineage." *Nat Chem Biol* 5(4): 258-65.

Chiang, M. K. and D. A. Melton (2003). "Single-cell transcript analysis of pancreas development." *Dev Cell* 4(3): 383-93.

Chin, M. H., M. J. Mason, et al. (2009). "Induced pluripotent stem cells and embryonic stem cells are distinguished by gene expression signatures." *Cell Stem Cell* 5(1): 111-23.

Cras-Meneur, C., L. Elghazi, et al. (2001). "Epidermal growth factor increases undifferentiated pancreatic embryonic cells in vitro: a balance between proliferation and differentiation." *Diabetes* 50(7): 1571-9.

D'Amour, K. A., A. D. Agulnick, et al. (2005). "Efficient differentiation of human embryonic stem cells to definitive endoderm." *Nat Biotechnol* 23(12): 1534-41.

D'Amour, K. A., A. G. Bang, et al. (2006). "Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells." *Nat Biotechnol* 24(11): 1392-401.

Danovitch, G. M., D. J. Cohen, et al. (2005). "Current status of kidney and pancreas transplantation in the United States, 1994-2003." *Am J Transplant* 5(4 Pt 2): 904-15.

Dor, Y., J. Brown, et al. (2004). "Adult pancreatic beta-cells are formed by self-duplication rather than stem-cell differentiation." *Nature* 429(6987): 41-6.

Doyle, M. J., Z. L. Loomis, et al. (2007). "Nkx2.2-repressor activity is sufficient to specify alpha-cells and a small number of beta-cells in the pancreatic islet." *Development* 134(3): 515-23.

Doyle, M. J. and L. Sussel (2007). "Nkx2.2 regulates beta-cell function in the mature islet." *Diabetes* 56(8): 1999-2007.

Evans, M. J. and M. H. Kaufman (1981). "Establishment in culture of pluripotential cells from mouse embryos." *Nature* 292(5819): 154-6.

Eventov-Friedman, S., D. Tchorsh, et al. (2006). "Embryonic pig pancreatic tissue transplantation for the treatment of diabetes." *PLoS Med* 3(7): e215.

Fabricius, D., S. Bonde, et al. (2005). "Induction of stable mixed chimerism by embryonic stem cells requires functional Fas/FasL engagement." *Transplantation* 79(9): 1040-4.

Feng, Q., S. J. Lu, et al. (2010). "Hemangioblastic derivatives from human induced pluripotent stem cells exhibit limited expansion and early senescence." *Stem Cells* 28(4): 704-12.

Feng, R. Q., L. Y. Du, et al. (2005). "In vitro cultivation and differentiation of fetal liver stem cells from mice." *Cell Res* 15(5): 401-5.

Fujikawa, T., S. H. Oh, et al. (2005). "Teratoma formation leads to failure of treatment for type I diabetes using embryonic stem cell-derived insulin-producing cells." *Am J Pathol* 166(6): 1781-91.

Gabr, M. M., M. M. Sobh, et al. (2008). "Transplantation of insulin-producing clusters derived from adult bone marrow stem cells to treat diabetes in rats." *Exp Clin Transplant* 6(3): 236-43.

Gao, F., D. Q. Wu, et al. (2008). "Extracellular matrix gel is necessary for in vitro cultivation of insulin producing cells from human umbilical cord blood derived mesenchymal stem cells." *Chin Med J (Engl)* 121(9): 811-8.

Garg, G., J. R. Tyler, et al. (2012). "Type 1 Diabetes-Associated IL2RA Variation Lowers IL-2 Signaling and Contributes to Diminished CD4+CD25+ Regulatory T Cell Function." *J Immunol*.

Gershengorn, M. C., A. A. Hardikar, et al. (2004). "Epithelial-to-mesenchymal transition generates proliferative human islet precursor cells." *Science* 306(5705): 2261-4.

Ghosh, Z., K. D. Wilson, et al. (2010). "Persistent donor cell gene expression among human induced pluripotent stem cells contributes to differences with human embryonic stem cells." *PLoS One* 5(2): e8975.

Gradwohl, G., A. Dierich, et al. (2000). "neurogenin3 is required for the development of the four endocrine cell lineages of the pancreas." *Proc Natl Acad Sci U S A* 97(4): 1607-11.

Gu, G., J. Dubauskaite, et al. (2002). "Direct evidence for the pancreatic lineage: NGN3+ cells are islet progenitors and are distinct from duct progenitors." *Development* 129(10): 2447-57.

Gu, G., J. M. Wells, et al. (2004). "Global expression analysis of gene regulatory pathways during endocrine pancreatic development." *Development* 131(1): 165-79.

Guillemain, G., G. Filhoulaud, et al. (2007). "Glucose is necessary for embryonic pancreatic endocrine cell differentiation." *J Biol Chem* 282(20): 15228-37.

Hald, J., T. Galbo, et al. (2011). "Pancreatic islet and progenitor cell surface markers with cell sorting potential." *Diabetologia* 55(1): 154-65.

Hansson, M., A. Tonning, et al. (2004). "Artifactual insulin release from differentiated embryonic stem cells." *Diabetes* 53(10): 2603-9.

Hao, E., B. Tyrberg, et al. (2006). "Beta-cell differentiation from nonendocrine epithelial cells of the adult human pancreas." *Nat Med* 12(3): 310-6.

Hardikar, A. A., B. Marcus-Samuels, et al. (2003). "Human pancreatic precursor cells secrete FGF2 to stimulate clustering into hormone-expressing islet-like cell aggregates." *Proc Natl Acad Sci U S A* 100(12): 7117-22.

Haseda, F., A. Imagawa, et al. (2011). "Low CTLA-4 expression in CD4+ helper T-cells in patients with fulminant type 1 diabetes." *Immunol Lett* 139(1-2): 80-6.

Hayek, A. and G. M. Beattie (1997). "Experimental transplantation of human fetal and adult pancreatic islets." *J Clin Endocrinol Metab* 82(8): 2471-5.

Heremans, Y., M. Van De Casteele, et al. (2002). "Recapitulation of embryonic neuroendocrine differentiation in adult human pancreatic duct cells expressing neurogenin 3." *J Cell Biol* 159(2): 303-12.

Herold, K. C., S. Gitelman, et al. (2009). "Treatment of patients with new onset Type 1 diabetes with a single course of anti-CD3 mAb Teplizumab preserves insulin production for up to 5 years." *Clin Immunol* 132(2): 166-73.

Herrera, P. L. (2000). "Adult insulin- and glucagon-producing cells differentiate from two independent cell lineages." *Development* 127(11): 2317-22.

Herrera, P. L., J. Huarte, et al. (1994). "Ablation of islet endocrine cells by targeted expression of hormone-promoter-driven toxigenes." *Proc Natl Acad Sci U S A* 91(26): 12999-3003.

Homo-Delarche, F. (2001). "Is pancreas development abnormal in the non-obese diabetic mouse, a spontaneous model of type I diabetes?" *Braz J Med Biol Res* 34(4): 437-47.

Hori, Y., M. Fukumoto, et al. (2008). "Enrichment of putative pancreatic progenitor cells from mice by sorting for prominin1 (CD133) and platelet-derived growth factor receptor beta." *Stem Cells* 26(11): 2912-20.

Hori, Y., I. C. Rulifson, et al. (2002). "Growth inhibitors promote differentiation of insulin-producing tissue from embryonic stem cells." *Proc Natl Acad Sci U S A* 99(25): 16105-10.

Hu, B. Y., J. P. Weick, et al. (2010). "Neural differentiation of human induced pluripotent stem cells follows developmental principles but with variable potency." *Proc Natl Acad Sci U S A* 107(9): 4335-40.

Hullett, D. A., J. L. Falany, et al. (1987). "Human fetal pancreas--a potential source for transplantation." *Transplantation* 43(1): 18-22.

Ibii, T., H. Shimada, et al. (2007). "Possibility of insulin-producing cells derived from mouse embryonic stem cells for diabetes treatment." *J Biosci Bioeng* 103(2): 140-6.

Inada, A., C. Nienaber, et al. (2008). "Carbonic anhydrase II-positive pancreatic cells are progenitors for both endocrine and exocrine pancreas after birth." *Proc Natl Acad Sci U S A* 105(50): 19915-9.

Itakura, S., S. Asari, et al. (2007). "Mesenchymal stem cells facilitate the induction of mixed hematopoietic chimerism and islet allograft tolerance without GVHD in the rat." *Am J Transplant* 7(2): 336-46.

Jahansouz, C., S. C. Kumer, et al. (2011). "Evolution of beta-Cell Replacement Therapy in Diabetes Mellitus: Pancreas Transplantation." *Diabetes Technol Ther* 13(3): 395-418.

Jensen, J., R. S. Heller, et al. (2000). "Independent development of pancreatic alpha- and beta-cells from neurogenin3-expressing precursors: a role for the notch pathway in repression of premature differentiation." *Diabetes* 49(2): 163-76.

Jiang, F. X. and G. Morahan (2011). "Pancreatic Stem Cells: From Possible to Probable." *Stem Cell Rev*.

Jiang, J., M. Au, et al. (2007). "Generation of insulin-producing islet-like clusters from human embryonic stem cells." *Stem Cells* 25(8): 1940-53.

Jiang, W., Y. Shi, et al. (2007). "In vitro derivation of functional insulin-producing cells from human embryonic stem cells." *Cell Res* 17(4): 333-44.

Jiang, W., X. Sui, et al. (2011). "CD24: a novel surface marker for PDX1-positive pancreatic progenitors derived from human embryonic stem cells." *Stem Cells* 29(4): 609-17.

Johansson, K. A., U. Dursun, et al. (2007). "Temporal control of neurogenin3 activity in pancreas progenitors reveals competence windows for the generation of different endocrine cell types." *Dev Cell* 12(3): 457-65.

Jorgenson, M. C., J. Ahnfelt-Ronne, et al. (2007). "An illustrated review of early pancreas development in the mouse." *Endocrine Reviews* 28(6): 685-705.

Karaoz, E., Z. S. Genc, et al. (2010). "Protection of rat pancreatic islet function and viability by coculture with rat bone marrow-derived mesenchymal stem cells." *Cell Death Dis* 1: e36.

Karnik, S. K., H. Chen, et al. (2007). "Menin controls growth of pancreatic beta-cells in pregnant mice and promotes gestational diabetes mellitus." *Science* 318(5851): 806-9.

Kelly, O. G., M. Y. Chan, et al. (2011). "Cell-surface markers for the isolation of pancreatic cell types derived from human embryonic stem cells." *Nat Biotechnol* 29(8): 750-6.

Kim, A., K. Miller, et al. (2009). "Islet architecture: A comparative study." *Islets* 1(2): 129-36.

Kim, E. M., R. Stultz, et al. (2011). "Embryonic stem cell-derived T cells induce lethal graft-versus-host disease and reject allogenic skin grafts upon thymic selection." *Am J Transplant* 12(3): 600-9.

Kim, H., Y. Toyofuku, et al. (2010). "Serotonin regulates pancreatic beta cell mass during pregnancy." *Nat Med* 16(7): 804-8.

Kim, P. T., B. G. Hoffman, et al. (2010). "Differentiation of mouse embryonic stem cells into endoderm without embryoid body formation." *PLoS One* 5(11): e14146.

Koch, C. A., P. Geraldes, et al. (2008). "Immunosuppression by embryonic stem cells." *Stem Cells* 26(1): 89-98.

Kojima, H., M. Fujimiya, et al. (2004). "Extrapancreatic insulin-producing cells in multiple organs in diabetes." *Proc Natl Acad Sci U S A* 101(8): 2458-63.

Kroon, E., L. A. Martinson, et al. (2008). "Pancreatic endoderm derived from human embryonic stem cells generates glucose-responsive insulin-secreting cells in vivo." *Nat Biotechnol* 26(4): 443-52.

Ku, H. T., J. Chai, et al. (2007). "Insulin-expressing colonies developed from murine embryonic stem cell-derived progenitors." *Diabetes* 56(4): 921-9.

Landsman, L., A. Nijagal, et al. (2011). "Pancreatic mesenchyme regulates epithelial organogenesis throughout development." *PLoS Biol* 9(9): e1001143.

Laurent, L. C., I. Ulitsky, et al. (2011). "Dynamic changes in the copy number of pluripotency and cell proliferation genes in human ESCs and iPSCs during reprogramming and time in culture." *Cell Stem Cell* 8(1): 106-18.

Lenschow, D. J., Y. Zeng, et al. (1992). "Long-term survival of xenogeneic pancreatic islet grafts induced by CTLA4lg." *Science* 257(5071): 789-92.

Leonard, J., B. Peers, et al. (1993). "Characterization of somatostatin transactivating factor-1, a novel homeobox factor that stimulates somatostatin expression in pancreatic islet cells." *Mol Endocrinol* 7(10): 1275-83.

Li, G., R. Luo, et al. (2009). "Generating mESC-derived insulin-producing cell lines through an intermediate lineage-restricted progenitor line." *Stem Cell Res* 2(1): 41-55.

Li, H., A. Lam, et al. (2010). "High dosage of Exendin-4 increased early insulin secretion in differentiated beta cells from mouse embryonic stem cells." *Acta Pharmacol Sin* 31(5): 570-7.

Lin, H. T., C. L. Kao, et al. (2007). "Enhancement of insulin-producing cell differentiation from embryonic stem cells using pax4-nucleofection method." *World J Gastroenterol* 13(11): 1672-9.

Ludvigsson, J., M. Hjorth, et al. (2011). "Extended evaluation of the safety and efficacy of GAD treatment of children and adolescents with recent-onset type 1 diabetes: a randomised controlled trial." *Diabetologia* 54(3): 634-40.

Ludvigsson, J., D. Krisky, et al. (2012). "GAD65 antigen therapy in recently diagnosed type 1 diabetes mellitus." *N Engl J Med* 366(5): 433-42.

Lui, K. O., A. S. Boyd, et al. (2010). "A role for regulatory T cells in acceptance of ESC-derived tissues transplanted across an major histocompatibility complex barrier." *Stem Cells* 28(10): 1905-14.

Lumelsky, N., O. Blondel, et al. (2001). "Differentiation of embryonic stem cells to insulin-secreting structures similar to pancreatic islets." *Science* 292(5520): 1389-94.

Luo, L., J. Z. Luo, et al. (2009). "Cytokines inducing bone marrow SCA+ cells migration into pancreatic islet and conversion into insulin-positive cells in vivo." *PLoS One* 4(2): e4504.

Maehr, R., S. Chen, et al. (2009). "Generation of pluripotent stem cells from patients with type 1 diabetes." *Proc Natl Acad Sci U S A* 106(37): 15768-73.

Mallone, R., V. Brezar, et al. (2011). "T cell recognition of autoantigens in human type 1 diabetes: clinical perspectives." *Clin Dev Immunol* 2011: 513210.

Marchetto, M. C., G. W. Yeo, et al. (2009). "Transcriptional signature and memory retention of human-induced pluripotent stem cells." *PLoS One* 4(9): e7076.

Matveyenko, A. V., J. D. Veldhuis, et al. (2006). "Mechanisms of impaired fasting glucose and glucose intolerance induced by an approximate 50% pancreatectomy." *Diabetes* 55(8): 2347-56.

Mayshar, Y., U. Ben-David, et al. (2010). "Identification and classification of chromosomal aberrations in human induced pluripotent stem cells." *Cell Stem Cell* 7(4): 521-31.

McKiernan, E., L. O'Driscoll, et al. (2007). "Directed differentiation of mouse embryonic stem cells into pancreatic-like or neuronal- and glial-like phenotypes." *Tissue Eng* 13(10): 2419-30.

McLean, A. B., K. A. D'Amour, et al. (2007). "Activin a efficiently specifies definitive endoderm from human embryonic stem cells only when phosphatidylinositol 3-kinase signaling is suppressed." *Stem Cells* 25(1): 29-38.

Mellitzer, G., M. Martin, et al. (2004). "Pancreatic islet progenitor cells in neurogenin 3-yellow fluorescent protein knock-add-on mice." *Mol Endocrinol* 18(11): 2765-76.

Michels, A. W. and P. A. Gottlieb (2010). "Autoimmune polyglandular syndromes." *Nat Rev Endocrinol* 6(5): 270-7.

Minami, K., M. Okuno, et al. (2005). "Lineage tracing and characterization of insulin-secreting cells generated from adult pancreatic acinar cells." *Proc Natl Acad Sci U S A* 102(42): 15116-21.

Mineo, D., C. Ricordi, et al. (2008). "Combined islet and hematopoietic stem cell allotransplantation: a clinical pilot trial to induce chimerism and graft tolerance." *Am J Transplant* 8(6): 1262-74.

Miyatsuka, T., Y. Kosaka, et al. (2011). "Neurogenin3 inhibits proliferation in endocrine progenitors by inducing Cdkn1a." *Proc Natl Acad Sci U S A* 108(1): 185-90.

Miyazaki, S., E. Yamato, et al. (2004). "Regulated expression of pdx-1 promotes in vitro differentiation of insulin-producing cells from embryonic stem cells." *Diabetes* 53(4): 1030-7.

Nielsen, J. H., J. Brunstedt, et al. (1979). "Preservation of beta cell function in adult human pancreatic islets for several months in vitro." *Diabetologia* 16(2): 97-100.

Nielsen, J. H., E. D. Galsgaard, et al. (2001). "Regulation of beta-cell mass by hormones and growth factors." *Diabetes* 50 Suppl 1: S25-9.

Nielsen, J. H., C. Svensson, et al. (1999). "Beta cell proliferation and growth factors." *J Mol Med (Berl)* 77(1): 62-6.

Nikolic, B., Y. Takeuchi, et al. (2004). "Mixed hematopoietic chimerism allows cure of autoimmune diabetes through allogeneic tolerance and reversal of autoimmunity." *Diabetes* 53(2): 376-83.

Nir, T., D. A. Melton, et al. (2007). "Recovery from diabetes in mice by beta cell regeneration." *J Clin Invest* 117(9): 2553-61.

Niwa, A., K. Umeda, et al. (2009). "Orderly hematopoietic development of induced pluripotent stem cells via Flk-1(+) hemoangiogenic progenitors." *J Cell Physiol* 221(2): 367-77.

Ohlsson, H., S. Thor, et al. (1991). "Novel insulin promoter- and enhancer-binding proteins that discriminate between pancreatic alpha- and beta-cells." *Mol Endocrinol* 5(7): 897-904.

Okita, K., T. Ichisaka, et al. (2007). "Generation of germline-competent induced pluripotent stem cells." *Nature* 448(7151): 313-7.

Osafune, K., L. Caron, et al. (2008). "Marked differences in differentiation propensity among human embryonic stem cell lines." *Nat Biotechnol* 26(3): 313-5.

Oshima, Y., A. Suzuki, et al. (2007). "Isolation of mouse pancreatic ductal progenitor cells expressing CD133 and c-Met by flow cytometric cell sorting." *Gastroenterology* 132(2): 720-32.

Otonkoski, T., G. M. Beattie, et al. (1993). "Nicotinamide is a potent inducer of endocrine differentiation in cultured human fetal pancreatic cells." *J Clin Invest* 92(3): 1459-66.

Otonkoski, T., V. Cirulli, et al. (1996). "A role for hepatocyte growth factor/scatter factor in fetal mesenchyme-induced pancreatic beta-cell growth." *Endocrinology* 137(7): 3131-9.

Paek, H. J., J. R. Morgan, et al. (2005). "Sequestration and synthesis: the source of insulin in cell clusters differentiated from murine embryonic stem cells." *Stem Cells* 23(7): 862-7.

Parsons, J. A., T. C. Brelje, et al. (1992). "Adaptation of islets of Langerhans to pregnancy: increased islet cell proliferation and insulin secretion correlates with the onset of placental lactogen secretion." *Endocrinology* 130(3): 1459-66.

Pearl, J. I., A. S. Lee, et al. (2011). "Short-term immunosuppression promotes engraftment of embryonic and induced pluripotent stem cells." *Cell Stem Cell* 8(3): 309-17.

Phillips, B. W., H. Hentze, et al. (2007). "Directed differentiation of human embryonic stem cells into the pancreatic endocrine lineage." *Stem Cells Dev* 16(4): 561-78.

Pipeleers, D. G., M. Pipeleers-Marichal, et al. (1991). "Transplantation of purified islet cells in diabetic rats. II. Immunogenicity of allografted islet beta-cells." *Diabetes* 40(7): 920-30.

Piper, K., S. Brickwood, et al. (2004). "Beta cell differentiation during early human pancreas development." *J Endocrinol* 181(1): 11-23.

Racine, J., M. Wang, et al. (2011). "Induction of mixed chimerism with MHC-mismatched but not matched bone marrow transplants results in thymic deletion of host-type autoreactive T-cells in NOD mice." *Diabetes* 60(2): 555-64.

Raikwar, S. P. and N. Zavazava (2011). "Spontaneous in vivo differentiation of embryonic stem cell-derived pancreatic endoderm-like cells corrects hyperglycemia in diabetic mice." *Transplantation* 91(1): 11-20.

Rajagopal, J., W. J. Anderson, et al. (2003). "Insulin staining of ES cell progeny from insulin uptake." *Science* 299(5605): 363.

Ramiya, V. K., M. Maraist, et al. (2000). "Reversal of insulin-dependent diabetes using islets generated in vitro from pancreatic stem cells." *Nat Med* 6(3): 278-82.

Raya, A., I. Rodriguez-Piza, et al. (2009). "Disease-corrected haematopoietic progenitors from Fanconi anaemia induced pluripotent stem cells." *Nature* 460(7251): 53-9.

Rieck, S. and K. H. Kaestner (2010). "Expansion of beta-cell mass in response to pregnancy." *Trends Endocrinol Metab* 21(3): 151-8.

Robertson, R. P., K. J. Lanz, et al. (2002). "Relationship between diabetes and obesity 9 to 18 years after hemipancreatectomy and transplantation in donors and recipients." *Transplantation* 73(5): 736-41.

Roep, B. O. and M. Peakman (2011). "Diabetogenic T lymphocytes in human Type 1 diabetes." *Curr Opin Immunol* 23(6): 746-53.

Rolletschek, A., I. S. Schroeder, et al. (2010). "Characterization of mouse embryonic stem cell differentiation into the pancreatic lineage in vitro by transcriptional profiling, quantitative RT-PCR and immunocytochemistry." *Int J Dev Biol* 54(1): 41-54.

Rouleau, M., R. Namikawa, et al. (1996). "Antigen-specific cytotoxic T cells mediate human fetal pancreas allograft rejection in SCID-hu mice." *J Immunol* 157(12): 5710-20.

Russ, H. A., Y. Bar, et al. (2008). "In vitro proliferation of cells derived from adult human beta-cells revealed by cell-lineage tracing." *Diabetes* 57(6): 1575-83.

Russ, H. A., E. Sintov, et al. (2011). "Insulin-producing cells generated from dedifferentiated human pancreatic beta cells expanded in vitro." *PLoS One* 6(9): e25566.

Sapir, T., K. Shternhall, et al. (2005). "Cell-replacement therapy for diabetes: Generating functional insulin-producing tissue from adult human liver cells." *Proc Natl Acad Sci U S A* 102(22): 7964-9.

Schaffer, A. E., A. J. Yang, et al. (2011). "Transgenic overexpression of the transcription factor Nkx6.1 in beta-cells of mice does not increase beta-cell proliferation, beta-cell mass, or improve glucose clearance." *Mol Endocrinol* 25(11): 1904-14.

Scharfmann, R., X. Xiao, et al. (2008). "Beta cells within single human islets originate from multiple progenitors." *PLoS One* 3(10): e3559.

Schisler, J. C., P. T. Fueger, et al. (2008). "Stimulation of human and rat islet beta-cell proliferation with retention of function by the homeodomain transcription factor Nkx6.1." *Mol Cell Biol* 28(10): 3465-76.

Schroeder, I. S., G. Kania, et al. (2006). "Insulin-producing cells." *Methods Enzymol* 418: 315-33.

Schroeder, I. S., A. Rolletschek, et al. (2006). "Differentiation of mouse embryonic stem cells to insulin-producing cells." *Nat Protoc* 1(2): 495-507.

Seeberger, K. L., J. M. Dufour, et al. (2006). "Expansion of mesenchymal stem cells from human pancreatic ductal epithelium." *Lab Invest* 86(2): 141-53.

Serafimidis, I., I. Rakatzi, et al. (2008). "Novel effectors of directed and Ngn3-mediated differentiation of mouse embryonic stem cells into endocrine pancreas progenitors." *Stem Cells* 26(1): 3-16.

Shamblott, M. J., J. Axelman, et al. (1998). "Derivation of pluripotent stem cells from cultured human primordial germ cells." *Proc Natl Acad Sci U S A* 95(23): 13726-31.

Shapiro, A. M., C. Ricordi, et al. (2006). "International trial of the Edmonton protocol for islet transplantation." *N Engl J Med* 355(13): 1318-30.

Sherry, N. A., J. A. Kushner, et al. (2006). "Effects of autoimmunity and immune therapy on beta-cell turnover in type 1 diabetes." *Diabetes* 55(12): 3238-45.

Shim, J. H., S. E. Kim, et al. (2007). "Directed differentiation of human embryonic stem cells towards a pancreatic cell fate." *Diabetologia* 50(6): 1228-38.

Shiraki, N., S. Harada, et al. (2010). "Identification of DAF1/CD55, a novel definitive endoderm marker." *Cell Struct Funct* 35(2): 73-80.

Shultz, L. D., Y. Saito, et al. (2010). "Generation of functional human T-cell subsets with HLA-restricted immune responses in HLA class I expressing NOD/SCID/IL2r gamma(null) humanized mice." *Proc Natl Acad Sci U S A* 107(29): 13022-7.

Smukler, S. R., M. E. Arntfield, et al. (2011). "The adult mouse and human pancreas contain rare multipotent stem cells that express insulin." *Cell Stem Cell* 8(3): 281-93.

Soria, B., E. Roche, et al. (2000). "Insulin-secreting cells derived from embryonic stem cells normalize glycemia in streptozotocin-induced diabetic mice." *Diabetes* 49(2): 157-62.

Stagner, J. I. and E. Samols (1991). "Deterioration of islet beta-cell function after hemipancreatectomy in dogs." *Diabetes* 40(11): 1472-9.

Steptoe, R. J., J. M. Ritchie, et al. (2003). "Transfer of hematopoietic stem cells encoding autoantigen prevents autoimmune diabetes." *J Clin Invest* 111(9): 1357-63.

Sugiyama, T., R. T. Rodriguez, et al. (2007). "Conserved markers of fetal pancreatic epithelium permit prospective isolation of islet progenitor cells by FACS." *Proc Natl Acad Sci U S A* 104(1): 175-80.

Sussel, L., J. Kalamaras, et al. (1998). "Mice lacking the homeodomain transcription factor Nkx2.2 have diabetes due to arrested differentiation of pancreatic beta cells." *Development* 125(12): 2213-21.

Sykes, M. (2007). "Mechanisms of tolerance induced via mixed chimerism." *Front Biosci* 12: 2922-34.

Sykes, M. (2009). "Hematopoietic cell transplantation for tolerance induction: animal models to clinical trials." *Transplantation* 87(3): 309-16.

Takahashi, K. and S. Yamanaka (2006). "Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors." *Cell* 126(4): 663-76.

Tang, D. Q., L. Z. Cao, et al. (2004). "In vivo and in vitro characterization of insulin-producing cells obtained from murine bone marrow." *Diabetes* 53(7): 1721-32.

Teitelman, G., S. Alpert, et al. (1993). "Precursor cells of mouse endocrine pancreas coexpress insulin, glucagon and the neuronal proteins tyrosine hydroxylase and neuropeptide Y, but not pancreatic polypeptide." *Development* 118(4): 1031-9.

Thatava, T., T. J. Nelson, et al. (2010). "Indolactam V/GLP-1-mediated differentiation of human iPS cells into glucose-responsive insulin-secreting progeny." *Gene Ther* 18(3): 283-93.

Thompson, H. L. and J. O. Manilay (2011). "Embryonic stem cell-derived hematopoietic stem cells: challenges in development, differentiation, and immunogenicity." *Curr Top Med Chem* 11(13): 1621-37.

Thorel, F., V. Nepote, et al. (2010). "Conversion of adult pancreatic alpha-cells to beta-cells after extreme beta-cell loss." *Nature* 464(7292): 1149-54.

Tornehave, D., P. Jansen, et al. (1993). "Fetal antigen 1 (FA1) in the human pancreas: cell type expression, topological and quantitative variations during development." *Anat Embryol (Berl)* 187(4): 335-41.

Tuch, B. E., T. C. Hughes, et al. (2011). "Encapsulated pancreatic progenitors derived from human embryonic stem cells as a therapy for insulin-dependent diabetes." *Diabetes Metab Res Rev* 27(8): 928-32.

Tuch, B. E. and R. S. Monk (1991). "Regulation of blood glucose to human levels by human fetal pancreatic xenografts." *Transplantation* 51(6): 1156-60.

Tuch, B. E., K. J. Osgerby, et al. (1988). "Normalization of blood glucose levels in nondiabetic nude mice by human fetal pancreas after induction of diabetes." *Transplantation* 46(4): 608-11.

Turvey, S. E., V. Gonzalez-Nicolini, et al. (2000). "Fas ligand-transfected myoblasts and islet cell transplantation." *Transplantation* 69(9): 1972-6.

Vaca, P., F. Martin, et al. (2006). "Induction of differentiation of embryonic stem cells into insulin-secreting cells by fetal soluble factors." *Stem Cells* 24(2): 258-65.

van Belle, T. L., K. T. Coppieters, et al. (2011). "Type 1 diabetes: etiology, immunology, and therapeutic strategies." *Physiol Rev* 91(1): 79-118.

Van Belle, T. L., P. Taylor, et al. (2009). "Mouse Models for Type 1 Diabetes." *Drug Discov Today Dis Models* 6(2): 41-45.

Vanhaesebroeck, B., S. J. Leevers, et al. (2001). "Synthesis and function of 3-phosphorylated inositol lipids." *Annu Rev Biochem* 70: 535-602.

Verda, L., D. A. Kim, et al. (2008). "Hematopoietic mixed chimerism derived from allogeneic embryonic stem cells prevents autoimmune diabetes mellitus in NOD mice." *Stem Cells* 26(2): 381-6.

Vincent, R. K. and J. S. Odorico (2009). "Reduced serum concentration is permissive for increased in vitro endocrine differentiation from murine embryonic stem cells." *Differentiation* 78(1): 24-34.

Voltarelli, J. C., C. E. Couri, et al. (2007). "Autologous nonmyeloablative hematopoietic stem cell transplantation in newly diagnosed type 1 diabetes mellitus." *Jama* 297(14): 1568-76.

von Herrath, M. and G. T. Nepom (2009). "Remodeling rodent models to mimic human type 1 diabetes." *Eur J Immunol* 39(8): 2049-54.

Waldron-Lynch, F. and K. C. Herold (2011). "Immunomodulatory therapy to preserve pancreatic beta-cell function in type 1 diabetes." *Nat Rev Drug Discov* 10(6): 439-52.

Wang, J., L. Elghazi, et al. (2004). "The concerted activities of Pax4 and Nkx2.2 are essential to initiate pancreatic beta-cell differentiation." *Dev Biol* 266(1): 178-89.

Wang, R. N., L. Bouwens, et al. (1996). "Beta-cell growth in adolescent and adult rats treated with streptozotocin during the neonatal period." *Diabetologia* 39(5): 548-57.

Wang, X. and K. Ye (2009). "Three-dimensional differentiation of embryonic stem cells into islet-like insulin-producing clusters." *Tissue Eng Part A* 15(8): 1941-52.

Wu, D. C., A. S. Boyd, et al. (2008). "Embryonic stem cells and their differentiated derivatives have a fragile immune privilege but still represent novel targets of immune attack." *Stem Cells* 26(8): 1939-50.

Wu, X. H., C. P. Liu, et al. (2007). "Reversal of hyperglycemia in diabetic rats by portal vein transplantation of islet-like cells generated from bone marrow mesenchymal stem cells." *World J Gastroenterol* 13(24): 3342-9.

Xu, G., D. A. Stoffers, et al. (1999). "Exendin-4 stimulates both beta-cell replication and neogenesis, resulting in increased beta-cell mass and improved glucose tolerance in diabetic rats." *Diabetes* 48(12): 2270-6.

Xu, X., J. D'Hoker, et al. (2008). "Beta cells can be generated from endogenous progenitors in injured adult mouse pancreas." *Cell* 132(2): 197-207.

Yasunaga, M., S. Tada, et al. (2005). "Induction and monitoring of definitive and visceral endoderm differentiation of mouse ES cells." *Nat Biotechnol* 23(12): 1542-50.

Yokoi, N., M. Kanamori, et al. (2006). "Association studies of variants in the genes involved in pancreatic beta-cell function in type 2 diabetes in Japanese subjects." *Diabetes* 55(8): 2379-86.

Yu, J., K. Hu, et al. (2009). "Human induced pluripotent stem cells free of vector and transgene sequences." *Science* 324(5928): 797-801.

Zhang, D., W. Jiang, et al. (2009). "Highly efficient differentiation of human ES cells and iPS cells into mature pancreatic insulin-producing cells." *Cell Res* 19(4): 429-38.

Zhao, T., Z. N. Zhang, et al. (2011). "Immunogenicity of induced pluripotent stem cells." *Nature* 474(7350): 212-5.

Zhou, B., G. J. Martin, et al. (2008). "Increased phenotypic stability and ethanol tolerance of recombinant Escherichia coli KO11 when immobilized in continuous fluidized bed culture." *Biotechnol Bioeng* 100(4): 627-33.