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Pancreatic Stem Cells: Unresolved Business

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1. Introduction

Diabetes mellitus has recently manifested a global trend in increased prevalence and is now a major public health problem around the world including in developing countries, such as China and India. It currently affects approximately 200 million people, and this number is likely to increase to 400 million by 2030 (Lock and Tzanakakis, 2007). Approximately 10% of these cases will have type 1 diabetes mellitus (T1D), caused by absolute deficiency of insulin-producing β cells. Therefore, a cellular therapy is the best prospect for a cure of T1D, provided autoimmunity to β cells can be controlled and there is a sufficient supply of insulin-secreting β cells for transplantation. However, the poor availability of donor islets has severely restricted the broad clinical use of islet transplantation. The lack of sufficient donor islets is why much attention has recently been paid to stem cells as a renewable source of β cells.

The term 'stem cell' was initially used in embryology in the late 19th century in the context of the origin of the blood system and gametes (Ramalho-Santos and Willenbring, 2007). Stem cells are undifferentiated cells that are capable of both self-renewal and giving rise to specialized functional cells. Depending on the developmental stages of their origin, stem cells can be divided into embryonic stem cells (derived from the inner cell mass of pre-implanted embryos) (Evans and Kaufman, 1981; Martin, 1981); epiblast stem cells (derived from post-implanted epiblast-stage embryos) (Brons et al, 2007; Tesar et al, 2007); germline-derived stem cells (derived from embryonic gonadial ridges or postnatal testes) (Shamblott et al, 1998; Kanatsu-Shinohara et al, 2004; Guan et al, 2006); induced pluripotent stem cells (from foetal or adult cells) (Takahashi and Yamanaka, 2006; Aoi et al, 2008; Hanna et al, 2008; Park et al, 2008) or adult stem cells (derived from postnatal tissues). Adult stem cells are a rare population in specific tissues but show powerful potential for regeneration. They can be further divided based on their tissue origin into a number of categories such as haematopoietic stem cells, neuronal stem cells, skin stem cells as well as mesenchymal stem cells. Unlike other tissue-specific stem cells, pancreatic stem cells (PSC) were proposed only relatively recently (Ramiya et al, 2000). However, despite intense research, the presence and origin of PSC are hotly debated. In order to understand the role and potential of PSC, better knowledge of pancreas development and function is required. In this review, we will particularly discuss several types of β-cell regeneration in physiological and pathophysiological conditions, and explore the mechanisms of regeneration of β cells. We hope that this will give readers a taste of this controversial but important area of research.

2. Pancreas development and physiology

2.1 Embryology

The pancreas is an organ derived from endoderm. The endoderm is one of the three primitive germ layers formed during the early embryonic stage known as gastrulation. Taking the mouse as an example, the pancreas originates from the thickened endodermal epithelium along the dorsal and ventral surfaces of the posterior foregut. These thickenings can be identified histologically at embryonic day (E) 9.0-9.5 (Pictet et al, 1972). Subsequently, these epithelia evaginate into the surrounding mesoderm-derived mesenchymal tissue and form dorsal and ventral pancreatic buds. These buds continue to expand, branch and fuse as a result of gut rotation that brings the buds together. The fused developing pancreas continues to proliferate, differentiate and, ultimately, develop into the mature pancreas. The adult pancreas consists of digestive enzyme-secreting exocrine tissue, digestive enzyme-transporting ductal tissue and hormone-producing tissue located in the islets of Langerhans.
In humans, the dorsal bud can be detected as early as 26 days postcoitum (dpc, an equivalent stage to E9.5 mouse embryos), but insulin-positive cells are not visible until 52 dpc, approximately 2 weeks later than the equivalent stage seen in mice. The appearance of human insulin-positive cells precedes that of glucagon-positive cells at 8-10 weeks of development (Piper et al, 2004). All islet cells are detectable at the end of the first trimester in humans (Piper et al, 2004), but at later stages in mice (Herrera et al, 1991). These data indicate a human-mouse temporal difference in lineage development (Richardson et al, 1997), and this is supported by differences in gene expression patterns during developmental and disease processes in these two species (Fougerousse et al, 2000). More reviews of human pancreas development can be found elsewhere (De Krijger et al, 1992; Lukinius et al, 1992; Polak et al, 2000).

2.2 Pancreatic progenitors

In the thickened DE epithelium along the dorsal and ventral surfaces of the posterior foregut at E9.0-9.5, there are a group of cells express a parahox homeobox transcription factor (TF) gene termed *Pdx1* (pancreas and duodenum TF 1). This is essential for pancreas expansion but not for pancreas initiation in early development and β-cell function in adults (Ohneda et al, 2000; Gu et al, 2002). The specification and differentiation of the various lineages in the pancreas along with transcriptional regulation has been extensively studied and reviewed (Wilson et al, 2003; Jensen, 2004; Servitja and Ferrer, 2004; Habener et al, 2005; Murtaugh, 2007; Best et al, 2008) and is briefly summarised in Figure 1.
Genetic lineage tracing experiment demonstrated that Pdx1-expressing ($Pdx1^+$) cells are multipotent pancreatic progenitors because they give rise to exocrine, endocrine and duct tissues in the pancreas (Gu et al, 2002). These cells are located at the tip of the branching pancreatic tree marked by $Pdx1^+Ptf1a^+$(pancreas transcription factor 1a) $Cpa1^+$(carboxypeptidase 1) (Zhou et al, 2007). To provide cues whether these cells are proliferative, transcriptome profiling analysis was performed showing that when DE cells commit to $Pdx1^+$ pancreatic progenitors there are at least 28 out of 69 (40.6%) cell-cycle and cell-proliferation genes up-regulated (Figure 2) (Jiang et al, 2010).
Surprisingly, direct evidence of proliferation and self-renewal of these $Pdx1^+Ptf1a^+Cpa1^+$ cells has not been produced. However, indirect evidence shows that these $Pdx1^+$ cells may take up bromodeoxyuridine (BrdU), a thymidine analogue that may be incorporated into DNA during S-phase of the cell cycle, indicative of proliferation (Seymour et al, 2007). Unfortunately, due to the lack of a specific marker, this ability of purified $Pdx1^+$ cells has not

been examined *in vitro* either. Development of technologies that allow the demonstration of Pdx1$^+$ cells to self-renew *in vitro* and to specify all pancreatic lineages will be not only important for pancreas developmental biology, but also crucial for a future ESC-derived cellular therapy for T1D. To serve this purpose, there are two ways to obtain purified Pdx1$^+$ cells: from embryos *in vivo* or from directed ESC culture *in vitro*.

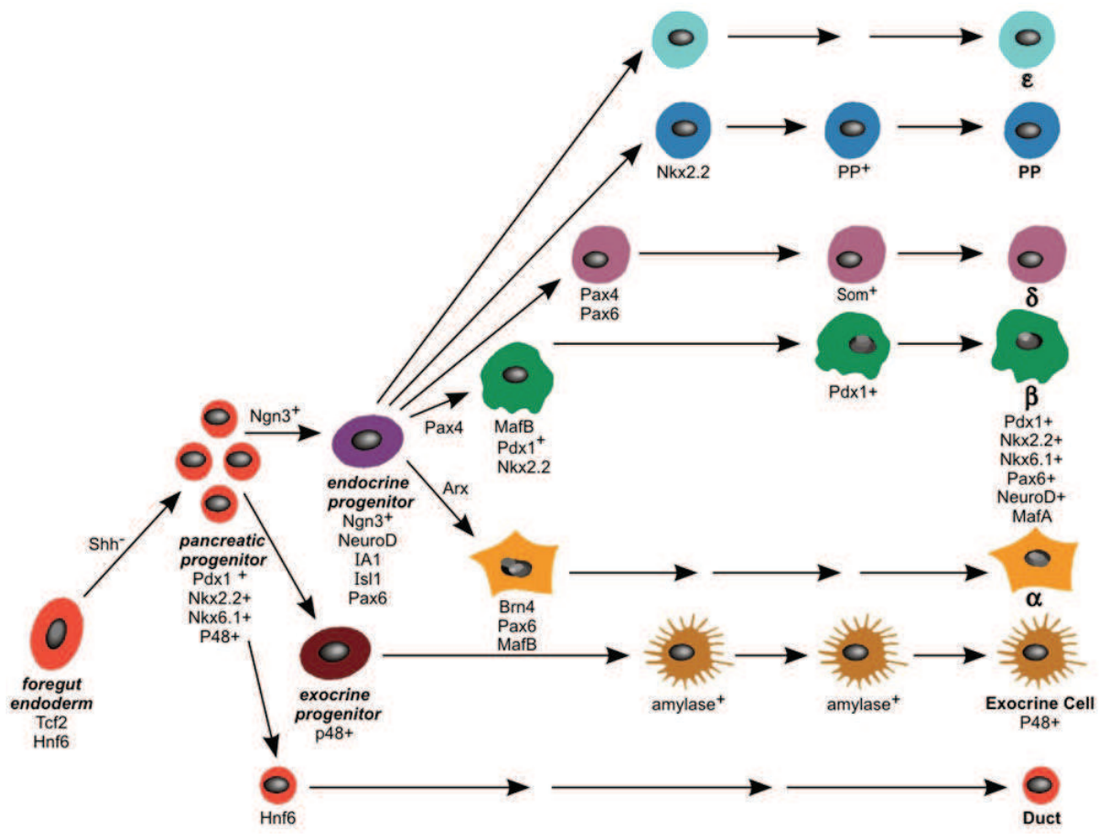

Fig. 1. Lineage development in the pancreas showing role of defined transcription factors. Progenitors within a defined domain of foregut endoderm express *Tcf*2 (*T cell factor 2*, also known as *hepatocyte nuclear factor 1b*, *Hnf1b)* and *Hnf6* (also known as *Onecut 1*). Suppression of *sonic hedgehog* (*Shh*) signalling leads to the development of pancreatic progenitor cells, which are marked by expression of a number of transcription factors, especially Pdx1 (pancreas and duodenum transcription factor 1, also known as Ipf1), pancreas transcription factor 1a (Ptf1a), Nkx2.2 (Nk family homeobox factor 2.2), Nkx61 and Hb9 [also known as motor neuron and pancreas homeobox 1 (Mnx1)]. The Sry-related HMG box transcription factor 9 (Sox9) and suppression of the Notch signalling activates an *neurogenin-3* (*Ngn3*) which allows these pancreatic progenitors to commit to precursors of the endocrine islet lineages; these endocrine progenitors also express *NeuroD* (*neural differentiation 1*), *IA1* (*insulinoma associated 1*), *Isl1* (*Islet 1*) and *Pax6* (*paired box factor 6*). The endocrine progenitors then may differentiate into five types of islet cells [α, β, δ (somatostatin), PP (pancreatic polypeptide) and ε (ghrelin)]. For example, a group of *MafB*- (musculoaponeurotic fibrosarcoma oncogene family protein B), *Pdx1*-, *Pax4*- and *Nkx2.2*-expressing cells will give rise to mature insulin-secreting β cells, whereas cells that express *Brn4* (the brain-specific POU-box factor) and *Pax6* are destined to become glucagon-secreting α cells.

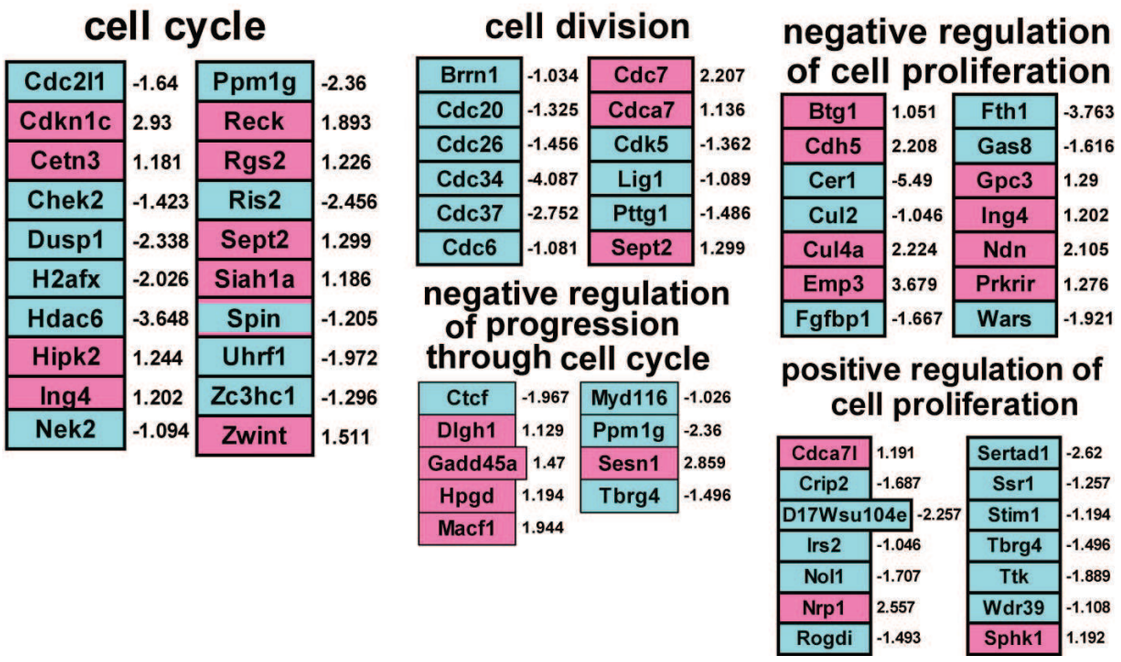

Fig. 2. Dynamic patterns of genes in Pdx1$^+$ cells that regulate cell cycle and cell proliferation. Microarray datasets were generated from dissected definitive endodermal (DE) cells and purified Pdx1$^+$ pancreas progenitors (Gu et al, 2004), followed by GenMapp analysis. Each box identifies a gene. The colour of each box in red or blue represents up- or down-regulation of the gene compared to its expression in DE. The number on the right-hand side is up or down (-) regulation of gene expression in the Log_2 scale. [more detail see (Jiang et al, 2010)].

In humans, numerous PDX1$^+$ cells can be easily detected in the pancreas between 8 and 21 weeks of age (Lyttle et al, 2008; Jeon et al, 2009). The number of PDX1$^+$ cells colocalized with insulin or somatostain is progressively increasing during this period of development (Lyttle et al, 2008). Unfortunately again, these studies did not provide data to show whether the PDX1$^+$ cells are generated by self-renewal themselves or commitment from their progenitors.

2.3 Islet progenitors

At around E9.5 in mice, a small group of cells in the thickened DE epithelium begin to express the basic helix-loop-helix TF neurogenin 3 (Ngn3) (Gradwohl et al, 2000; Gu et al, 2002; Xu et al, 2008). Accumulating evidence indicates that Ngn3-expressing (Ngn3$^+$) cells in the pancreas are islet progenitors that give rise to all islet lineage cells because: (1) in *Ngn3* knockout mice, islet cells do not develop (Gradwohl et al, 2000); (2) gene lineage tracing shows that Ngn3$^+$ cells give rise to all pancreatic endocrine cells (Gu et al, 2002); (3) in adult pancreas, Ngn3$^+$ cells can be activated by partial duct ligation and (4) after injection into a foetal pancreas *in vitro*, purified Ngn3$^+$ cells differentiate into all islet cell types (Xu et al, 2008). Whereas mouse *Ngn3* mRNA expression peaks around E15.5 (equivalent to weeks 7-8 in humans), human *NGN3* expression was low prior to 9 weeks, but from 9 weeks onward, its expression increased sharply and remained high untill 17 weeks (Jeon et al, 2009). Although a few studies showed that Ngn3$^+$ cells were proliferative (Jensen et al, 2000;

Oliver-Krasinski et al, 2009), the recent genetic clonal analyses of mosaic analysis with double marker (MADM) demonstrated that Ngn3+ cells are quiescent and these cells give rise to a single islet cell type (Desgraz and Herrera, 2009). Consistent with this, a recent seminal study shows that the expression of Ngn3 inhibits proliferation by inducing *cyclin-dependent kinase inhibitor 1a* (*Cdkn1a*) (Miyatsuka et al, 2011). Our recent study demonstrate that in addition to induction of *Cdkn1a*, numerous genes involved in cell-cycle and cell-proliferation are down-regulated (95/127 or 74.8%) (Figure 3) (Jiang et al, 2010). Even though there is no literature reporting whether Ngn3+ cells proliferate *in vitro*, it is not likely that Ngn3+ cells are the so-called PSC.

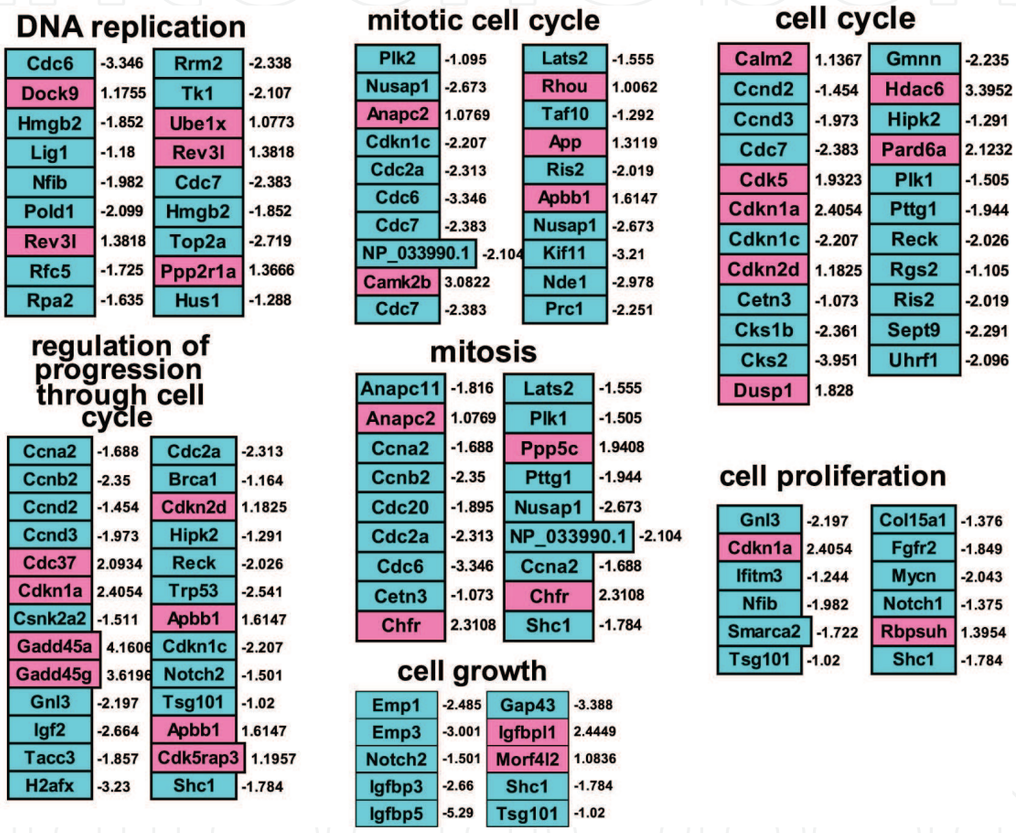

Fig. 3. Dynamic patterns of genes in Ngn3+ cells that regulate cell cycle and cell proliferation. Microarray datasets were generated from purified Pdx1+ pancreas progenitors and Ngn3+ islet progenitors (Gu et al, 2004), followed by GenMapp analysis. Each box identifies a gene. The colour of each box in red or blue represents up- or down-regulation of the gene compared to Pdx1+ cells. The number on the right-hand side is up or down (-) regulation of gene expression in the Log_2 scale. [more detail see (Jiang et al, 2010)].

2.4 Physiology

The islets are composed mainly of α, β, δ, ε and PP cells (Figure 1) that secrete glucagon, insulin, somatostatin, ghrelin and pancreatic polypeptide respectively (Jorgensen et al, 2007). These hormones are generally responsible for the regulation of glucose homeostasis. For this function, there is a set of fine tuned paracrine interactions among these endocrine

cells which is summarized in Figure 4. In adult humans, there are 2,000–3,000 β cells/islet of Langerhans, with approximately 1 million islets scattered throughout the pancreas (Stefan et al, 1982). The β cells sense the fluctuation of blood glucose levels and secrete insulin in a manner dependent on the glucose concentration. Insulin regulates circulating blood glucose concentrations through its actions on peripheral tissues, such as to inhibit hepatic glucose release and stimulate glucose uptake and storage by skeletal muscle and adipocyte tissue.

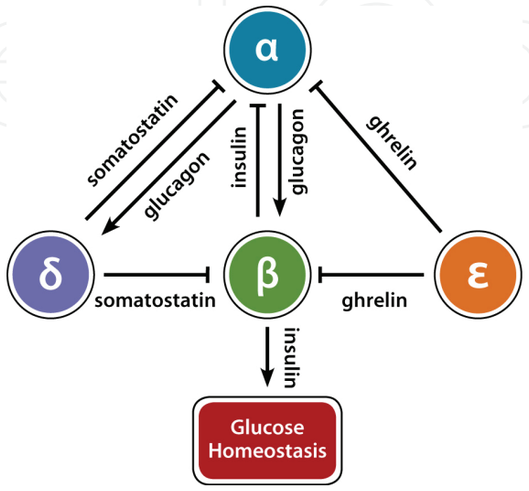

Fig. 4. Paracrine interactions between islet cells.
Insulin secreted by β cells acts as a prime hormone of glucose homeostasis and inhibits glucagon secretion by α cells. Whereas glucagon activates insulin and somatostatin secretion, somatostatin secreted by δ cells and ghrelin by ε cells inhibit insulin secretion.

3. Regeneration of β cells occurs physiologically and pathophysiologically

β cells are indeed observed to be regenerated at least during pregnancy, partial pancreatectomy and obesity. These observations lead to the birth of the PSC concept (Bonner-Weir and Sharma, 2002). The existence of PSC is also inferred from the continued function of islets after transplantation (Ryan et al, 2002; Ryan et al, 2005). Since there is no convincing evidence of contribution of haematopoietic stem cells to islet cells (Wagers et al, 2002) nor that β cells are long-lived (Bonner-Weir, 2000), the continued function of transplanted islets suggests that PSC reside inside the islets and/or the functional β cells are capable of self-renewal.

3.1 Regeneration of β cells during pregnancy

To cope with physiological demand, pancreatic β cells do regenerate during pregnancy in humans and experimental animals. For example, the uptake of BrdU increases 3-fold at E10 and 10-fold at E14 in islets of pregnant rats (Parsons et al, 1992), providing indirect evidence that proliferation of islet cells contributes significantly to the increase of islet volume (Hellman, 1960; Van Assche, 1974). However, there only is a 2-fold increase in islet volume and a 3-fold increase in BrdU labelling at E15.5 maternal mouse islets (Karnik et al, 2007). This discrepancy may reflect a species difference in regeneration capacity or a difference in sensitivity of detection methods. During human pregnancy, both an increase in volume of

maternal islets and hyperplasia of 'β' cells have also been observed (Van Assche et al, 1978), but direct evidence of proliferation in these islets is still lacking. In rodents and humans, the proliferation of β cells during pregnancy may be stimulated by prolactin and placental lactogens (Nielsen et al, 1999).

Recently, genetic studies provided molecular insights into how β-cell proliferation occurs during pregnancy. The pregnancy hormone prolactin suppresses the transcriptional co-activator menin, encoded by the gene *multiple endocrine neoplasia type 1 (MEN1)*, resulting in β-cell proliferation. This is demonstrated by experiments in which a short infusion of prolactin is sufficient to reduce menin expression and stimulate proliferation of mouse islet cells (Karnik et al, 2007). In addition, the pregnancy hormones prolactin and placental lactogen also induce expression of *tryptophan hydroxylase 1* (*Tph1*), encoding the enzyme Tph1 essential for serotonin production, which induces BrdU incorporation into isolated islet cells (Kim et al, 2010). However, it is still unknown whether proliferation comes from functional β cells and/or from PSC within islets. Future research should be directed to recapitulate this effect *in vitro* with purified subpopulations of islet cells, from which novel stimuli and molecular pathways may be identified. Defining these pathways may establish a platform on which novel strategies can be developed for a cure of T1D.

3.2 Regeneration of β cells during obesity

β cells regenerate in response to pathological processes such as obesity. For example, up to 10-fold increase in β-cell mass has been observed in obese rodents, responding to their insulin resistance (Butler et al, 2003a). Double staining of pancreas sections from obese mice and humans can detect insulin-producing cells that express Ki-67, a marker strictly associated with cell proliferation (Butler et al, 2003a; Butler et al, 2003b), indicating that regeneration may occur in the islets. Again, the regeneration capacity seems to be significantly greater in mice than in humans, although the underlying mechanism is unclear yet. Studies of one obese mutant mouse line, termed *Ay*, showed that reduction of menin contributes to adaptive β-cell proliferation (Karnik et al, 2007). Taken together, these data suggest that a similar mechanism for β-cell regeneration may operate in physiological pregnancy and pathophysiological obesity in mice. It would be very interesting to determine whether this mechanism is also at work during human pregnancy or obesity.

3.3 Regeneration of β cells after partial pancreatectomy

Like many other organs in the body, islets do regenerate in response to injury, in this case, pancreatectomy. In rats 4 weeks after 90% pancreatectomy, for example, there is a regeneration to 27% and 45% of sham-operated pancreas and islet mass, respectively (Bonner-Weir and Sharma, 2002). However, there are species differences in regeneration capacity. Even a 50% pancreatectomy in adult dogs would cause impaired fasting glucose in the short term (Matveyenko et al, 2006) and diabetes mellitus in the longer term (Stagner and Samols, 1991). Likewise, a 50% pancreatectomy in adult humans also leads to subsequent obesity and diabetes mellitus (Robertson et al, 2002). These studies again indicate that there is a difference between species in their capacity to mount islet regeneration: this is much more powerful in rodents than in larger mammals. Additional studies are needed to confirm this capacity difference and understand its underlying mechanism. Furthermore, it is less clear to what extent islet regeneration contributes to

maintain β-cell mass in adult humans from existing islet cells and how much is from cells of any other origins. This knowledge is critical for a viable strategy to promote β-cell regeneration both *in vivo* and *in vitro*. Additionally, it is unknown whether the PSC also contribute to this type of regeneration. In the following sections, we will examine several possible mechanisms that may lead to β-cell generation and regeneration.

4. Evidence that β cells are generated from PSC

In addition to the observations of long-term survival of transplanted islets mentioned above, substantial *in vitro* evidence has indicated that "pluripotent PSC" may be present in all three major pancreas compartments, i.e. the ductal epithelium (Cornelius et al, 1997; Ramiya et al, 2000; Suzuki et al, 2002), islets, and acinar tissue (Zulewski et al, 2001; Seaberg et al, 2004). This evidence comes from studies of both rodent and human pancreas. For example, a potential PSC candidate has been purified by flow cytometry in the developing and adult mouse pancreas. These cells are identified by expression of the receptor for hepatocyte growth factor, c-Met, and absence of blood cell surface markers such as CD45, TER119, c-Kit, and Flk-1. These cells can differentiate into multiple pancreatic lineage cells from individual cells *in vitro* and give rise to pancreatic endocrine and acinar cells *in vivo* following transplantation (Suzuki et al, 2004). However, the *in vivo* localization and the molecular characteristics of these c-met expressing cells are largely unknown and clonogenesis at the single cell level has not been established.

4.1 PSC may house in ductal epithelium

Over the last several years, we investigated the differentiation and proliferation of foetal mouse pancreatic cells, believed to be a rich source for potential PSC. We first demonstrated *in vitro* that bone morphogenetic proteins-2, -4, -5 and -6, members of the transforming growth factor β superfamily, can promote the proliferation of pancreatic precursors and the development of pancreatic cystic epithelial colonies containing β cells (Jiang et al, 2002; Jiang and Harrison, 2005a), a process partially recapitulating an *in vivo* developmental stage. In addition, we also found that various isoforms of epidermal growth factors can stimulate colony formation (Jiang and Harrison, 2005b). These data indicate that extracellular signalling molecules, including various families of growth factors, modulate fate changes of pancreas precursor/stem cells. However, currently these colony-forming cells may at most be considered precursors because their self-renewal has not been demonstrated *in vitro* (F.X.J. unpublished observations).

Recently, *in vitro* and *in vivo* experiments have indicated that PSC are localized to the ductal epithelium. Bonner-Weir and colleagues were the first to report that adult human pancreatic ductal epithelial cells can form islet-like clusters and differentiate into insulin-secreting β cells (Bonner-Weir et al, 2000). Ramiya and colleagues reported that transplantation of *in vitro* generated islet-like structures from mouse PSC in the ductal epithelium can reverse diabetes mellitus (Ramiya et al, 2000). Another set of experiments used cultures of "pancreatic ductal cell aggregates" that were left over from pancreas digests after purification of human islets for transplantation. From these cultures, fibroblast-like cells grew out; these have been termed "pancreatic mesenchymal stem cells (pMSC)". These cells can undergo at least 12 passages and express a range of bone marrow-derived MSC markers including CD13, CD29, CD44, CD54, CD105, α_6 integrin subunit (also known as CD49f) and Thy1 (also known as CD90). These pMSC can give rise to cells of at least two germ layer

origins including endoderm-derived cells, but not convincingly pancreatic lineage cells (Seeberger et al, 2006). *In vivo*, the large, small, and centrolobular ducts of the rat pancreas contain foci of cells that express the neural stem cell–specific marker, nestin, but do not express the ductal marker cytokeratin 19 (CK19) (Zulweski et al. 2001), suggestive of "islet progenitor" cells.

However, all the above studies have used mixed cell populations and have failed to demonstrate clonogenesis. Using culture conditions suitable for generating neurospheres ex vivo, mouse pancreatic ductal cells gave rise to neurosphere-like structures that can subsequently be differentiated into several types of islet cells including β cells (Seaberg et al, 2004). The molecular phenotype of progenitor cells for these islet cells remains unknown. On the other hand, after pancreatic duct ligation, numerous $CK19^+$ ductal cells are regenerated and then $Ngn3^+$ cells are observed, transplantation of the latter has further resulted in their differentiation into functional β cells (Xu et al, 2008), suggesting that the regeneration process may resemble that of embryonic pancreas development.

These studies did not explore the immediate origin of $Ngn3^+$ cells in adult pancreas and whether the cells that give rise to these $Ngn3^+$ cells possess PSC features. Recently, the use of the *in vivo* genetic tracing Cre-loxP system has generated further knowledge in this regard. In these experiments, Cre expression was directed by the promoter of carbonic anhydrase II, a marker of mature ductal cells, resulting in the excision of the stop cassette (Rosa-loxP-stop-loxP-lacZ) in the transgenic Rosa26 (R26R) mice. Therefore β-galactosidase activity is expressed in the cells that express Cre (ductal cells). At 4 weeks, β-galactosidase is detected in many ducts, patched acinar cells and 35-40% of islet cells (Bonner-Weir et al, 2008). These data provide direct evidence that adult ductal epithelial cells can give rise to islet cells, at least in mice. It is still unknown whether the carbonic anhydrase II-expressing cell population is homogenous or heterogenous, and whether this is a differentiation process from PSC or a transdifferentiation process from mature ductal cells. Anyway, these results should be repeated by independent laboratories and even in other experimental animals.

4.2 PSC may reside in the islets

Accumulated *in vitro* evidence suggests that a subpopulation of islet cells has PSC potential. A well-planned *in vitro* study found that a subset of unspecified cells in mouse islets and ducts named as pancreas-derived multipotent precursor cells give rise to neurosphere-like structures and subsequently could differentiate into islet β-like cells. This was a minor property, as most of these cells developed into neural lineage cells (Seaberg et al, 2004). The phenotype of these potential PSC cells was not defined.

In rat and human islets, a distinct population of $nestin^+$ cells that do not express the hormones insulin, glucagon, somatostatin or pancreatic polypeptide has been identified. When cultured *in vitro*, these cells proliferate extensively (~8 months), give rise to cells that express liver and exocrine pancreas markers, such as α-fetoprotein and pancreatic amylase, and display a ductal/endocrine phenotype with expression of CK19, neural-specific cell adhesion molecule, insulin, glucagon, and PDX1. These $nestin^+$ putative progenitor cells may therefore participate in the neogenesis of islet endocrine cells (Zulewski et al, 2001), mediated at least partially by glucagon-like peptide-1, an incretin hormone derived from processing proglucagon (Abraham et al, 2002). However, recent studies *in vivo* indicate that $Nestin^+$ cells are mostly restricted in non-endodermal-derived cells (Lardon et al, 2002; Selander and Edlund, 2002). In addition, from donated adult human islets, outgrown

fibroblast-like cells do not express hormones, but proliferate readily and give rise *in vitro* to hormone-expressing cell aggregates. However, these aggregates cannot be induced to become typical functional islet cells (Gershengorn et al, 2004). Furthermore, a rigorous genetic-based lineage tracing in mice under the control of *Pdx1* or *rat insulin promoter* (*RIP*) demonstrated that neither PDX1- nor RIP-expressing cells contribute significantly to these fibroblast-like cells *in vitro* (Chase et al, 2007). In contrast, human islet RIP-expressing cells could be dedifferentiated to fibroblast-like cells in which insulin expression was not detectable and proliferate *in vitro* up to 16 population doublings (Russ et al, 2008). Whether this discrepancy is due to species differences remains to be clarified. Because of its critical importance, such tracing experiments should be reproduced by independent research groups. Further investigations are required to resolve the inconsistent results from the current studies.

4.3 PSC may locate in the exocrine tissue

In the clinic, a large population of nonendocrine pancreatic acinar cells would be discarded after purification of islets from donated pancreas for transplantation. The possibility of making use of these cells has attracted significant interest in recent years. After co-transplantation with foetal pancreatic cells under the kidney capsule of immunodeficient mice, these nonendocrine pancreatic epithelial cells have been shown to be capable of endocrine differentiation though without evidence of β-cell replication or cell fusion. These experiments suggest the existence of PSC or progenitor cells within the acinar compartment of the adult human pancreas (Hao et al, 2006). More recently, analysis using the Cre/loxP-based tracing system demonstrated that amylase/elastase-expressing acinar cells can give rise to insulin-positive cells in a suspension culture (Minami et al, 2005). However, because clonal assay of these amylase/elastase-expressing cells and their intermediate steps have not been investigated, this study may simply reveal that mouse and rat pancreatic acinar cells are able to transdifferentiate into surrogate insulin-expressing cells (Baeyens et al, 2005; Minami et al, 2008). This possibility was further supported by a recent study which shows that mouse acinar cells can be directly re-programmed *in vivo* to β-like cells with just three transcription factor genes, namely, *Pdx1*, *Ngn3* and *MafA* (Zhou et al, 2008). On the other hand, similar lineage tracing experiments *in vivo* demonstrate that after 70-80% pancreatectomy, pre-existing mouse pancreatic acinar cells do not contribute to regeneration of islet β cells (Desai et al, 2007). The contradiction of *in vitro* and *in vivo* findings requires further reconciliation.

5. Evidence that islet β cells are capable of self-replication

There are several pieces of strong evidence demonstrating that islet β cells act as functional "stem" cells to reproduce themselves. Using RIP-driven reporter genes to genetically trace the fate of functional insulin-secreting cells, Dor and colleagues (Dor et al, 2004) first revealed that adult mouse pancreatic β cells are duplicated by RIP-expressing cells within the islets, either physiologically or after partial pancreatectomy. This study assumed that all RIP-expressing cells in adult islets are functional β cells and did not exclude the presence of PSC. Similarly, by using a transgenic model, in which the expression of diphtheria toxin was directed by RIP to β cells, diphtheria expression results in apoptosis of 70%-80% of β cells, destruction of islet architecture and, finally, diabetes mellitus. Withdrawal of diphtheria expression led to a significant regeneration of β-cell

mass and a spontaneous normalization of blood glucose levels and islet architecture. Simultaneously, RIP-based lineage tracing analysis indicated that the proliferation of 20-30% surviving 'β' cells played a major role in this regeneration and in recovery of euglycemia (Nir et al, 2007).

Using the more sophisticated MADM system in mice known as RIP-CreER; $Rosa26^{GR}$/ $Rosa26^{RG}$, each RIP-expressing clone has been demonstrated to consist of 5.1±5.4 or 8.2±6.9 cells after one or two months of chase (Brennand et al, 2007). These RIP-expressing clones have been interpreted as further evidence of regeneration of functional β cells. An additional loss-of-function study following knockout of the Hnf4α (hepatocyte nuclear factor 4α) gene suggested that the β-cell regeneration may involve the Ras/Erk signalling cascade (Gupta et al, 2007) and ultimately be regulated by cycling modulators including cyclin D2 (Georgia and Bhushan, 2004). Taken together, further identification and characterization of the so-called self-replicative or dedifferentiative RIP-expressing cells both *in vivo* and *in vitro* will be urgently needed because they may hold the key for a regenerative therapy for type 1 diabetes mellitus.

Again using thymidine-based lineage tracing, β cells were demonstrated to be produced within an islet by rare self-renewing cells with a slow replication-refractory period (Teta et al, 2007), although the identity of these unique cells and the length of their replication-refractory period remain to be determined. The frequency of these self-renewal cells can be significantly increased after partial pancreatectomy or during pregnancy. Further studies should determine the molecular signature and biological potential of these replicating self-renewal cells. Because of ethical issues, similar studies cannot be performed in human islet tissues, but such investigation should at least be repeated in larger mammals.

Nevertheless, the β-cell population in the adult islets is in fact functionally heterogeneous (Heimberg et al, 1993; Pipeleers et al, 1994; Szabat et al, 2009). By using a dual fluorescence reporter mouse line, a few $Ngn3^+$ cells in the developing pancreas have been observed to coexpress insulin (Hara et al, 2006). In humans a few $NGN3^+$ cells in the foetal pancreas are also observed to coexpress insulin from 10 to 21 weeks of age (Lyttle et al, 2008). Consistent with these studies, the insulin gene expression has been detected from $Pdx1^+$ progenitors through $Ngn3^+$ cells during development to mature islet cells (Jiang et al, 2010) (Figure 5).

An early study indicated that the RIP-expressing cells in the developing pancreas gave rise to other islet cell types in addition to β cells (Alpert et al, 1988). The $CD105^+CD37^+CD90^+$ mesenchymal stromal cells present in adult human islets have been shown to express a low level of insulin mRNA (Davani et al, 2007). After considering all these studies, therefore, lineage tracing studies under the control of other mature β-cell specific transcription factor gene promoters such as Pdx1 or MafA should be performed. Just like the β-cell line Min6 cells (Miyazaki et al, 1990), the glucose-responsive β cells in the islets may indeed duplicate themselves if the Pdx1- or MafA-expressing cells can be shown to be proliferative similar to the RIP-expressing cells.

6. The identity of PSC is inconclusive

Whereas investigation of β-cell duplication as a mechanism of islet regeneration has attracted great attention in recent years, much progress has been made to identify PSC. However, their identity is still not known. This is due, in addition to the knowledge gap that the signals required for late stage differentiation of functional β cells are largely

unknown, at least partially to lack of the following factors: specific cell surface markers to characterize and purify cells that may have PSC potential; a simple, effective and reproducible *in vitro* assay to examine self-renewal and differentiation potential of a purified cell population; *in vivo* functional assays to determine biological function for both experimental animals and humans. Finally, well-agreed general criteria for defining a PSC are required.

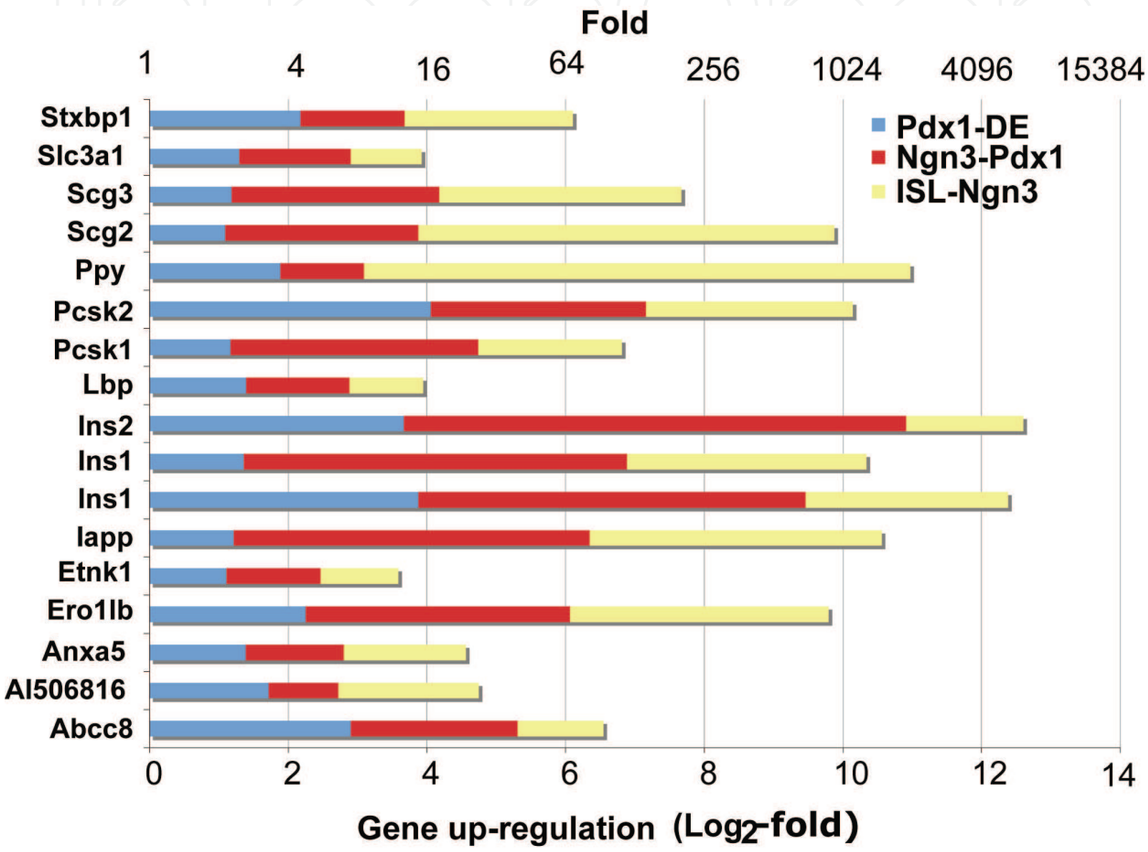

Fig. 5. Insulin genes being continuously up-regulated from pancreas progenitors to mature islet cells.
Microarray datasets were generated from dissected definitive endodermal (DE) cells, purified Pdx1$^+$ pancreas progenitors, Ngn3$^+$ islet progenitors and mature islet cells (ISL) (Gu et al, 2004). Bioinformatics algorithms for time-series datasets were used to calculate expression levels of insulin genes and other genes involved in post-translational modifications of insulin during the stages from Pdx1$^+$ through Ngn3$^+$ islet progenitors to ISL. [more detail see (Jiang et al, 2010)].

7. Future directions for PSC research

We believe arguably that the successful establishment of *in vitro* culture and *in vivo* functional assay systems (Daniel et al, 1989; Dexter, 1989) facilitates the investigation of

haematopoietic stem cells and subsequently leads to the application in clinics as a stem cell therapy. Therefore our future work should reach a consensus that PSC at least satisfy the following criteria: (1) clonogenesis should be demonstrated at the level of single cells sorted by flow cytometry; (2) sorted single cells should self-renew *in vitro*; (3) clonogenic cells should give rise *in vitro* to more than one specialized cell lineage and (4) these cells should differentiate *in vivo* into several functional cell types after transplantation. In the future, the investigation of PSC should be particularly encouraged based on three considerations. First, differentiation of embryonic stem cells into insulin-expressing cells was initially exciting and promising, but safety consideration aside, the generation of functional β cells proves extremely difficult and progress has been far slower than originally expected. PSC and dedifferentiation or transdifferentiation of other cell types in the pancreas may therefore provide an alternative renewable source of surrogate β cells. Second, β-cell duplication theory was mostly established from rodent models. It cannot completely exclude the existence of PSC, and it assumed that all insulin-expressing cells in the adult islets are uniformly functional β cells, but in fact these cells are heterogeneous. Last, as there is a significant difference in regeneration capacity between rodent and human islets, it may be wise not to directly extrapolate regeneration data from rodents to humans. Unlike previous studies that were only performed on *in vivo* or *in vitro* experiments without targeting specific cell types, future PSC work may need to employ integrated approaches, for example, applying cell surface markers to target particular cell populations; examining *in vitro* their potential for self-renewal and clonogenesis, using genetic approaches to dissect molecular mechanisms of their phenotypic changes *in vivo* and finally to examine their lineage contribution and biological function. Performing such integrated research is likely beyond the capacity of most individual laboratories, and therefore requires multi-discipline, multi-laboratory and even multi-national collaborations including the participation of the pharmaceutical sector to generate a therapeutic grade of surrogate β cells for a replacement therapy to diabetes mellitus.

8. Note to add

On the eve of submission, an important study from Dr Derek van der Kooy's laboratory sheds a shiny ray of new light on this inconclusive topic. By using state-of-art genetic lineage tracing techniques, Smukler and colleagues now provide conclusive evidence that the PSC cells (originally called as pancreas-derived multipotent precursor cells) in the adult pancreas were derived from the embryonic pancreatic lineage, but not from the neural crest, as previously supposed. The PSC cells express insulin, along with an array of markers typical of islet progenitors; are distinct from mature functional ells; and give rise to non-beta cells in vivo. In addition to mouse PSC cells, human PSC cells have also been identified. After transplantation to diabetic mice, both mouse and human PSC cells could ameliorate their diabetes mellitus (Smukler et al, 2011).

9. Acknowledgement

The authors are supported by grants from Juvenile Diabetes Research Foundational International (4-2006-1025), the Diabetes Research Foundation of Western Australia, the University of Western Australia, and the Medical Research Foundation of Royal Perth Hospital.

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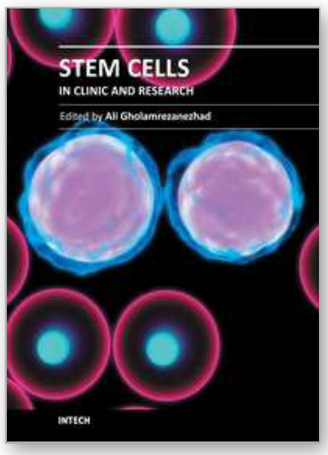

Stem Cells in Clinic and Research
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ISBN 978-953-307-797-0
Hard cover, 804 pages
Publisher InTech
Published online 23, August, 2011
Published in print edition August, 2011

Based on our current understanding of cell biology and strong supporting evidence from previous experiences, different types of human stem cell populations are capable of undergoing differentiation or trans-differentiation into functionally and biologically active cells for use in therapeutic purposes. So far, progress regarding the use of both in vitro and in vivo regenerative medicine models already offers hope for the application of different types of stem cells as a powerful new therapeutic option to treat different diseases that were previously considered to be untreatable. Remarkable achievements in cell biology resulting in the isolation and characterization of various stem cells and progenitor cells has increased the expectation for the development of a new approach to the treatment of genetic and developmental human diseases. Due to the fact that currently stem cells and umbilical cord banks are so strictly defined and available, it seems that this mission is investigationally more practical than in the past. On the other hand, studies performed on stem cells, targeting their conversion into functionally mature tissue, are not necessarily seeking to result in the clinical application of the differentiated cells; In fact, still one of the important goals of these studies is to get acquainted with the natural process of development of mature cells from their immature progenitors during the embryonic period onwards, which can produce valuable results as knowledge of the developmental processes during embryogenesis. For example, the cellular and molecular mechanisms leading to mature and adult cells developmental abnormalities are relatively unknown. This lack of understanding stems from the lack of a good model system to study cell development and differentiation. Hence, the knowledge reached through these studies can prove to be a breakthrough in preventing developmental disorders. Meanwhile, many researchers conduct these studies to understand the molecular and cellular basis of cancer development. The fact that cancer is one of the leading causes of death throughout the world, highlights the importance of these researches in the fields of biology and medicine.

How to reference
In order to correctly reference this scholarly work, feel free to copy and paste the following:

Fang-Xu Jiang and Grant Morahan (2011). Pancreatic Stem Cells: Unresolved Business, Stem Cells in Clinic and Research, Dr. Ali Gholamrezanezhad (Ed.), ISBN: 978-953-307-797-0, InTech, Available from: http://www.intechopen.com/books/stem-cells-in-clinic-and-research/pancreatic-stem-cells-unresolved-business

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