The Enigma of β-Cell Regeneration in the Adult Pancreas: Self-Renewal Versus Neogenesis

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1. Introduction

The pancreas is constituted by two distinctly different tissues: the exocrine component, i.e., pancreatic acinar cells that secrete digestive enzymes; and the endocrine component, the islets of Langerhans, constituted by hormone secreting cells. In the islet, the α-cells produce glucagon; the β-cells, insulin; the δ-cells, somatostatin; γ-cells, pancreatic polypeptide (Figure 1). Diabetes is caused either by an absolute (type 1) or relative (type 2) defect of insulin-producing β-cells in the pancreas. Therefore, regardless of the different pathogenesis, diabetes is the perfect candidate for cell replacement therapy. Currently the two available alternatives for β-cell replacement therapy are whole pancreas or isolated islet transplantation (Shapiro et al., 2000). However, these approaches are severely limited by a shortage of human organ donors and the need of lifelong immunosuppressive therapy. In the absence of other clearly suitable and renewable sources of surrogate β-cells, an alternative strategy to exogenous cell replacement therapy might be fostering endogenous β-cell regeneration. Therefore, knowledge of the mechanisms regulating β-cell plasticity in both embryonic and adult life, as well as in pathological conditions, is of particular interest.

During pancreatic development, β-cells derive from a population of endocrine precursors arising from the pancreatic epithelium (Gittes, 2009). Activation of cell-specific transcription factors guides the initially multipotent progenitors and determines their differentiation into mature β-cells. The final size of the endocrine pancreas is limited by the size of the progenitor cell pool in the developing pancreatic bud (Stanger et al., 2007). After birth, most β-cells are considered quiescent, however, it has been shown that the β-cell mass can adaptively expand under some physiologic or pathologic circumstances, such as pregnancy and obesity, both in mammals and rodents (Bernard-Kargar & Ktorza, 2001).

In mouse models there is also evidence that the pancreas preserves the ability to regenerate its β-cell mass in response to several non-physiological injuries, such as selective chemical destruction or surgical excision (Trucco, 2005; Thorel, 2010). Furthermore, in non-obese diabetic mice (NOD) it has been shown that recovery of sufficient endogenous insulin

production is possible via combination of strategies involving reversal of the autoimmune attack (Zorina et al., 2003; Kodama et al., 2003; Suri et al., 2006; Chong et al., 2006; Nishio et al., 2006).

In humans it is still debated whether this recovery is possible and, if so, to what extent it is feasible. Spontaneous recovery of β-cell function has been reported in patients with recent onset type 1 diabetes, suggesting that β-cells can regenerate despite underlying autoimmunity (Karges et al., 2004, 2006; Meier et al., 2006a; Butler et al., 2007). Additionally, the observation that people with long-standing type 1 diabetes still possess β-cells despite their destruction by the enduring autoimmunity and glucotoxicity, suggests that new β-cell formation might occur throughout life [Meier et al., 2005]. However, it remains largely unclear through which molecular and cellular mechanisms it occurs.
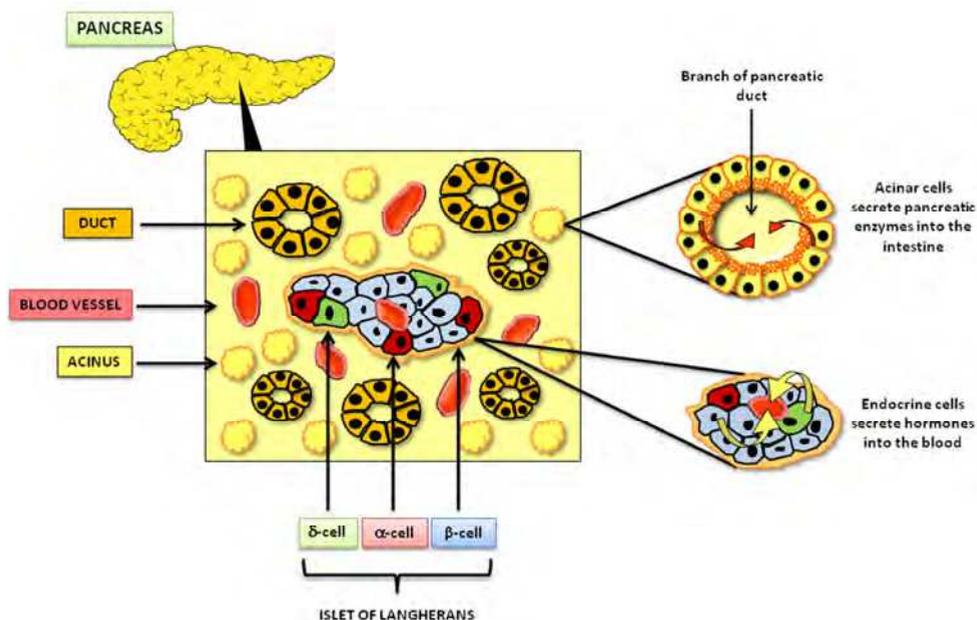

Fig. 1. Cells of the pancreas

The pancreas houses two different tissues. Its bulk is comprised of exocrine tissue, which is made up of acinar cells. These cells secrete pancreatic enzymes delivered to the intestine to facilitate the digestion of food. Scattered throughout the exocrine tissue are many thousands of clusters of endocrine cells known as islets of Langerhans. Within the islet, α-cells produce glucagon; β-cells, insulin; δ-cells, somatostatin; and γ-cells, pancreatic polypeptide — all of which are delivered into the blood stream.

2. Can the endocrine pancreas regenerate? Models of artificially induced diabetes

To address the question whether the pancreas possesses the ability to regenerate, several models have been used to artificially reduce the β-cell mass in order to stimulate a

pancreatic response. Most of these models allow exploring the plasticity of the pancreas in the absence of concurrent autoimmunity, which might prevent or block any attempt of β-cell restoration.

2.1 Partial pancreatectomy

Partial pancreatectomy (>90% removal) was shown to induce limited re-growth of the remnant organ in rats (Pearson et al., 1977). In comparison to liver regeneration second to partial hepatectomy, subtotal pancreatectomy is followed only by a limited regenerative growth that is proportional to the size of the excision. In addition, regeneration is mostly related to the exocrine tissue, while the endocrine part was shown to rescue, at its best, only approximately 30% of its initial mass (De Leon et al., 2003). The extent of the surgical intervention seems to be important, and could explain discrepancies in some reports describing absent (Dor et al., 2004) or vigorous pancreatic regeneration [Bonner-Weir et al., 1993] after 70% and 90% organ resection, respectively. In addition, hyperglycemia, which is present only in the latter case, could act as co-stimulator.

2.2 Pancreatic duct ligation

Pancreatic duct ligation (PDL) was also used to determine obstruction and consequently local inflammation and stimulation of pancreatic regeneration. During the first week post-ligation an increase in the β-cell number and the presence of intermediate ductal/endocrine (Wang et al., 1995) or acinar/endocrine phenotypes (Bertelli & Bendayan, 1997; Inada et al., 2008) has been observed. Additional stimulation of the expansion of the β-cell mass following PDL can be achieved by gastrin infusion (Rooman et al., 2002).

2.3 Wrapping of the pancreas with cellophane

Wrapping the pancreas with cellophane has also been used to induce islet neogenesis from ducts, and it has been reported to reverse streptozotocin-induced diabetes in hamsters (Rosenberg et al., 1996).

2.4 Selective β-cell destruction

Selective β-cell destruction can be obtained by chemical ablation with streptozotocin or alloxan, alone and in combination with pancreatectomy (Finegood et al., 1999; Wang et al., 1996; Rood et al., 2006). Streptozotocin (STZ) is a drug that leads to cell death by DNA alkylation, while alloxan is a generator of oxygen free radicals causing extensive DNA damage. Adult mice rendered diabetic with a high dose of STZ or alloxan are unable to recover endogenous β-cell function (Szkudelski, 2001). Interestingly, β-cell neogenesis can be stimulated in STZ-diabetic newborn rats by administration of the hormone glucagon-like peptide-1 (GLP-1), resulting in improved glucose homeostasis persisting at adult age (Tourrel et al., 2001). In another murine experimental model of alloxan-induced beta-cell destruction, treatment with gastrin and epidermal growth factor (EGF) was found to restore glycemic control and 30–40% of the normal beta-cell mass within 7 days (Rooman & Bouwens, 2004). Combination of the same growth factors proved to be effective also in facilitating islet β-cell neogeneisis in NOD mice with autoimmune diabetes (Suarez-Pinzon et al., 2005). In addition, rescue of endogenous islet function was shown in STZ-diabetic mice after removal of the kidney bearing syngeneic islets, which temporarily maintained mice normoglicemic (Yin et al., 2006), thus indicating that glucose control might be relevant

to facilitate the regenerative process. On the other hand, the possibility that recovery of the endogenous β-cell function may occur independently of glucose control exists, i.e., by the mediation of cytokines, which may activate residual β-cell proliferation or progenitor cell differentiation. To note, in the study by Yin et al., a facilitating role was exerted by the presence of the spleen, which probably plays an indirect role as modulator of the inflammatory process in the pancreas, thereby stimulating recovery of the STZ-damaged islets. STZ seems to trigger a pancreatic regenerative response also in non-human primate models, although it does not lead to a substantial endogenous β-cell recovery in absence of additional stimuli, like the failure of exogenous islet transplantation in the liver (Bottino et al., 2009).

Method	Target Cells	Potential Mechanism for Regeneration
Pancreatectomy	Endocrine Exocrine	Replication of pre-existing β-cells Reactivation of embryonic program
Pancreatic duct ligation	Endocrine Exocrine	Regeneration through Ngn3+ precursors Regeneration through Ca-II and Sox-9+ progenitors
Streptozotocin	β-cell	β-cell neogenesis from ductal cells
Alloxan	β-cell	β-cell neogenesis from ductal cells

CA-II: carbonic anhydrase II

Table 1. Summary of the models used to investigate pancreatic regeneration.

3. Lineage tracing techniques

Lineage tracing techniques have been widely used to investigate both the ontogeny of pancreatic cell fates during mouse embryogenesis as well as the identification of progenitor cells *in vivo* during regeneration (see Figure 2 and 3 for further explanations).

In lineage analysis, specific cells are labeled or marked so that their progeny can be identified later during development. In the pancreas, lineage analysis has been used to recognize not only the progenitor cells giving rise to mature endocrine and exocrine cells, but also the stage at which each set of progenitors is restricted to a particular cell fate. Lineage tracing is also useful to label and isolate marked cells in order to study their gene expression profile and *in vitro* differentiation.

In pancreatic lineage analysis, cells can be labeled using distinct approaches. A physical label - such as dye or a replication-incompetent retrovirus - can be directly injected into embryos to label cells within a tissue. The tissue is allowed to mature *in vivo* or in culture, and the cell types that become labeled reveal the lineage of the starting cells. However, since this method marks cells indiscriminately, in most tissues it cannot be used to label specific sub-populations. A more reliable approach is to genetically mark progenitor cells using endogenous gene expression patterns. This method selectively labels cells that express a particular gene, thus revealing the fate of their progeny. In most cases, a tissue specific promoter (for example Pdx-1) driving Cre recombinase is used to irreversibly tag cells. Other options include the use of a transgene driven by a specific promoter within different cell types, and lineage ablation, either using gene-inactivation mutants (knockout) or transgenic expression of cellular toxins. All these approaches have been used to follow pancreatic cell lineages (reviewed in Gu et al. 2003).

3.1 Cre/LoxP system

Cells can be irreversibly marked using the Cre/LoxP system, thus permitting detection of progeny cells that no longer express the gene of interest. This system uses two transgenic mouse lines, the "reporter" and the "deletor" (Figure 2).

The reporter line uses promoter 1 (Pro.1, black rectangle) to drive reporter gene expression (green rectangle). Upstream of the reporter gene coding region is a STOP cassette made of three repeats of a polyadenilation signal (red rectangle). Flanking the blocking signal are two LoxP sites (blue arrows). Promoter 1 can be tissue specific or ubiquitous. In the deletor line, another tissue specific promoter (Pro. 2, black rectangle) is used to drive the expression of *Cre* recombinase (yellow rectangle). When the two mouse lines are crossed, CRE is expressed in the cells in which promoter 2 is active, thus deleting the blocking signal. This results in the expression of the reporter gene in cells that also express promoter 1.

Fig. 2. Design plan for direct cell lineage analysis.

The first transgenic mouse uses a promoter (promoter 1), which can be tissue specific or ubiquitous, to drive the expression of a reporter gene, such as LacZ or green fluorescent protein (GFP). The second mouse carries a transgene that uses a different tissue specific promoter (promoter 2) to drive the expression of Cre recombinase. In the absence of the Cre deletor transgene, the expression of the reporter protein is prevented by a STOP cassette (multiple repeats of a poly-adenylation signal) upstream of the reporter coding sequence. However, in the presence of Cre recombinase, two LoxP sites flanking the blocking sequence permit this block to be removed. Thus, in double transgenic animals, the reporter gene will be expressed in cells following the excision event, thereby labeling all progeny derived from those precursors that express the deletor transgene.

A fine-tuning of the Cre/LoxP system can be achieved with the CRE-ER™ recombinase, which is a fusion between the catalytic domain of the CRE recombinase and the ligand-binding domain of a modified estrogen receptor. The CRE-ER™ protein requires an artificial ligand, tamoxifen, to catalyze LoxP mediated recombination. Because tamoxifen is active within mouse embryos for less than 48 h, cells expressing Cre-ER™ at a specific developmental stage can be selectively labeled by administration of tamoxifen during that stage. After tamoxifen treatment, the conventional CRE recombinase activates the reporter transgene expression as soon as CRE protein is generated, and labeled cells accumulate in any lineage where Cre has been expressed. This type of recombinase can be used to follow selectively the progeny of cells born at defined developmental stages, including postnatal growth and during regeneration.

3.2 Lineage analysis based on simple transgenes

A simpler transgenic approach drives expression of a reporter gene, such as LacZ or green fluorescent protein (GFP), under the promoter of interest. The drawback of this approach is that any progeny of these cells, which cease expression of the chosen protein cannot be followed. In addition, since this method marks cells from the first time the promoter is activated, and as these cells accumulate during development, it becomes impossible to distinguish new members of the population. Thus, one cannot distinguish the progeny of cells born during embryogenesis from those born in adults.

3.3 Lineage analysis based on cell ablation

Another method to investigate lineage relationship is cell ablation. This can be accomplished by specific gene inactivation mutations (knockout), such as in the *Pdx1* knockout mouse, which has no mature pancreatic cells. Alternatively, a tissue specific promoter can be used to drive the expression of a cellular toxin, such as the Diphtheria Toxin A (DTA) subunit. In these transgenic animals, the DTA subunit will kill those cells whose progenitors express that specific transgene. A similar approach is represented by the Diphtheria Toxin Receptor (DTR)-mediated conditional cell ablation model. DTR is a membrane-anchored form of the heparin-binding EGF-like growth factor (HB-EGF precursor). The human and simian HB-EGF precursors bind DT and function as toxin receptors, whereas HB-EGF from mice and rats do not bind the toxin and therefore remain insensitive to DT. Thus, transgenic expression of the simian or human DTR in mice can render cells DT-sensitive. Recently, a mouse strain was generated (iDTR), in which the gene encoding DTR has been introduced into the *ROSA26* locus (R26^{DTR}), but its expression is dependent on the Cre-mediated removal of a transcriptional STOP cassette. Therefore, only Cre-expressing cells and their progeny will undergo Cre-recombinase activity and subsequently will transcribe DTR. Although viable and normally functioning, these cells are rapidly killed upon DT administration (Figure 3) (Buch et al. 2003).
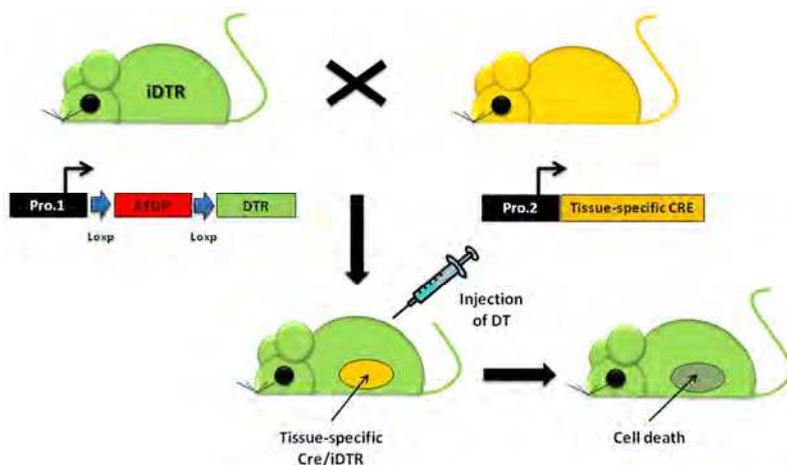
The STOP cassette, which prohibits DTR expression, is removed by crossing the iDTR strain to a tissue-specific Cre-expressing mouse strain. Consecutive expression of the DTR renders the respective tissues sensitive to cell death induced by injection of diphtheria toxin. Filled rectangles, *loxP* sites; arrows, transcriptional activity; open ovals, promoter.

Fig. 3. Design plan of the inducible DTR mouse strain (iDTR).

4. Evidence of pancreatic endocrine progenitors/stem cells in the pancreas

Several cells in the pancreas have been described as potential sources of β-cell renewal.

4.1 Pancreatic ductal progenitor cells

During pancreatic organogenesis, stem cells within the duct pancreatic epithelium give rise to both the endocrine and acinar cells (Gittes, 2009). Therefore it seems reasonable to think that the regeneration process could start within the ductal compartment, recapitulating embryonic and fetal development. In addition, there are similarities between islet regeneration and embryonic pancreas development at the gene expression level. Evidence of adult duct cells harboring stem cells capable of differentiating into β-cells was reported both *in vivo* and *in vitro* (Dudek et al., 1991; Ramiya et al., 2000; Bonner-Weir et al., 2000, 2008; Gao et al., 2003). In 2000 Ramiya et al. claimed that long-term cultivation of pancreatic ductal epithelial cells isolated from pre-diabetic, adult, non-obese diabetic mice contained nestin-positive stem cells able to differentiate into islets of Langerhans (Ramiya et al., 2000). These "surrogate" islets responded *in vitro* to glucose challenge, and reversed insulin-dependent diabetes after being implanted into diabetic NOD mice. Similar observations were reported using more defined culture conditions in which isolated human pancreatic duct preparations led to formation and propagation of human islet-like structures (Bonner-Weir et al., 2000, 2008; Gao et al., 2003). Ogata et al. also derived a similar subset of islet-like insulin secreting cells from pancreatic ducts of neonatal rats (Ogata et al., 2004). After incubation with activin A and betacellulin, cells showed tolbutamide- and glucose-responsive insulin secretion. Transplantation of these pseudo-islets in STZ-diabetic NOD mice improved blood glucose levels. Hao et al. confirmed the existence of endocrine stem or progenitor cells within the epithelial compartment of the adult human pancreas, by isolating stem cells from the non-endocrine fraction after islet separation of adult human pancreas digests (Hao et al., 2006). Following elimination of the contaminating mesenchymal cells, the highly purified population of non-endocrine pancreatic epithelial cells (NEPECs) was transplanted under the kidney capsule of immunodeficient (SCID) mice. Although NEPECs produced only low amounts of insulin, when co-transplanted with fetal pancreatic cells, they were capable of endocrine differentiation. No evidence of β-cell replication or cell fusion was observed. To directly test whether ductal cells serve as pancreatic progenitors after birth and give rise to new islets, a transgenic mouse expressing human carbonic anhydrase II (CAII) promoter was generated. This study showed that CAII-expressing cells within the pancreas act as progenitors that give rise to both new islets and acini normally after birth and after injury (PDL) (Inada et al., 2008).

Additional evidence of the existence of endocrine precursor cells within the ductal compartment come from the detection of the nuclear transcription factor Neurogenin-3 (Ngn3) in the ducts during regeneration after STZ. Ngn3 is a basic helix–loop–helix transcription factor, which is able to commit pancreatic cells to an endocrine cell fate (Schwitzgebel et al., 2000). Lack of Ngn3 leads to an absence of islets (Gradwhol et al., 2000); its ectopic expression determines premature over-commission towards the endocrine lineage (Apelqvist et al., 1999). Presence of Ngn3 is very convincing evidence that pancreatic regeneration starts from pancreatic progenitors and mimics the same pathway followed during normal development (Figure 4). By using an inducible Cre-ERTM-LoxP system to
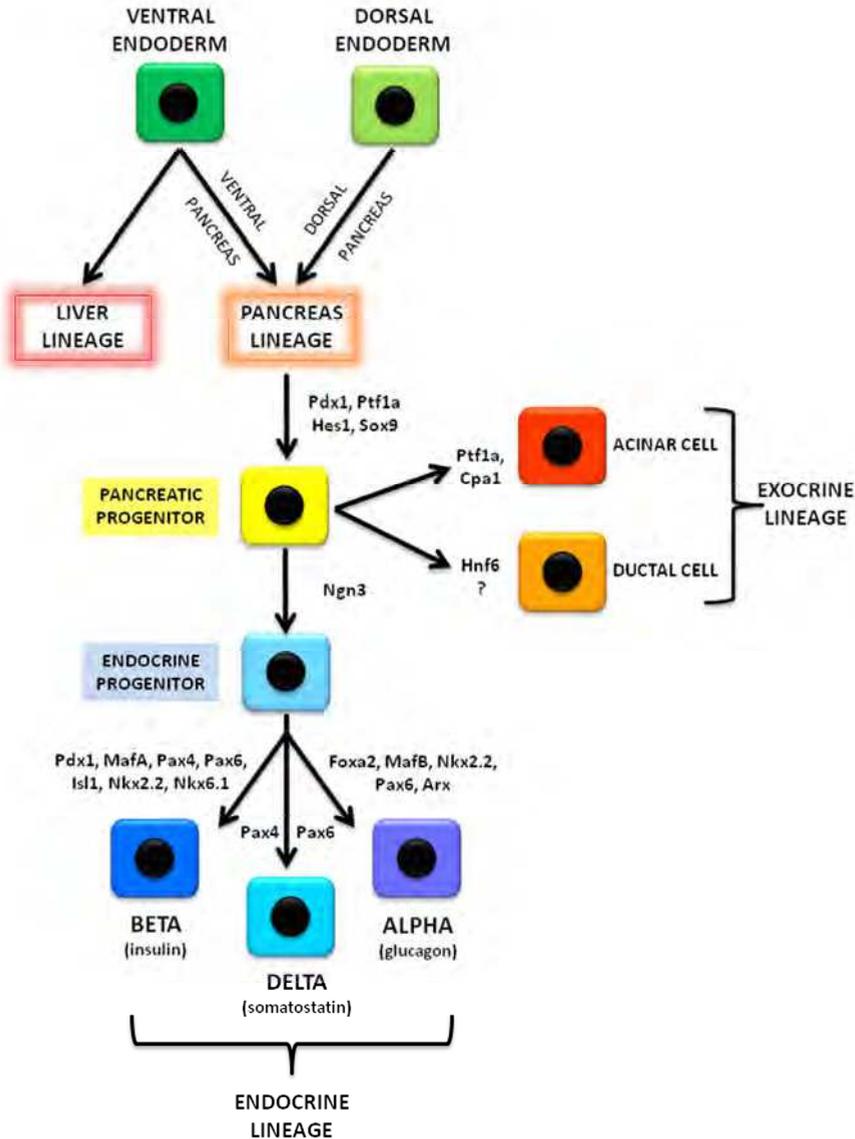
Fig. 4. Regulatory nuclear transcription factors controlling cell type lineages during embryonic pancreas development.

mark the progeny of cells expressing either Ngn3 or Pdx1 at different stages of development, Gu et al. showed that endocrine/exocrine and ductal lineages are separated before E12.5 (Gu et al., 2002). Authors demonstrate that, while cells expressing Pdx1 give rise to all three types of pancreatic tissue (exocrine, endocrine and duct), only the subset Pdx 1/Ngn3+ cells are islet progenitors. The duct cells that do not contain progeny of

Ngn3^{+} cells presumably give rise to the adult duct system and account for the heterogeneity in developmental potential among 'duct-like structures'. Kodama et al. also suggested that in STZ-treated mice, regeneration occurs mainly from intra-islet Ngn3^{+} progenitor cells rather than from ductal precursors (Kodama et al., 2005). Recently, Xu et al. showed that PDL in the pancreatic tail resulted in some β-cell proliferation and, more strikingly, in a large upregulation of Ngn3 gene expression (Xu et al., 2008). Ngn3 positive cells were found to be closely associated with ducts, and possibly cells of the ductal lineage themselves. After isolation, these cells could give rise to all islet cell types, including glucose responsive β-cells, both *in situ* and when cultured in Ngn3^{-/-} embryonic pancreas explants. In addition, White et al. utilized a system based on Ngn3–enhanced green fluorescent protein knock-in mouse model to isolate endocrine progenitor cells from embryonic pancreata to generate an ample gene expression profile of these progenitors and their immediate descendants (White et al., 2008). On the other hand, a recent publication reported low level expression of Ngn3 in adult endocrine cells, raising concerns about using Ngn3 expression as a marker of endocrine progenitors and neogenesis in the adult pancreas (Wang et al., 2009). Furthermore, another study indicates that although PDL leads to Ngn3 expression in a sub-population of cells within the ducts, it does not induce appropriate cues to allow for completion of the entire β-cell neogenesis program (Kopp et al., 2011).

The potential for the pancreatic organ to recover the endocrine function after injury has been also investigated in non-human primates, where a ductal involvement was observed in STZ-diabetic monkeys that recovered endogenous β-cell function following pig islet transplantation in the liver (Bottino et al., 2009).

Further evidence that hormone-positive cells arise from the ducts comes from the comparison of 16 donor pancreas specimens and pancreas biopsies from 8 simultaneous pancreas/kidney transplantations (Martin-Pagoda et al., 2008). While in the donor pancreas the frequency of insulin^{+} duct cells was low (0.45%), in five pancreatic transplants with recurrent autoimmunity, 57.5% of the duct cells expressed insulin protein. If new islets were generated from pre-existing ductal tissue, transient co-expression of hormones and residual duct markers could be expected. Indeed, this has been demonstrated in grafts of purified human duct cells (Yatoh et al., 2007).

4.2 Pancreatic non-ductal progenitor cells

Besides ductal progenitors, other groups proposed that pancreatic stem cells may also reside within the islets or in the acinar compartment.

4.2.1 Acinar cells

Acinar cells, which represent the main portion of pancreatic tissue, have been shown to transdifferentiate into islet cells *in vivo* and *in vitro*, through the generation of duct cells as an intermediate step (Lardon et al., 2004; Baeyens et al., 2005; Rooman et al., 2000; Lipsett et al., 2007). Lineage tracing has been used *in vitro* to further strengthen the conclusion that endocrine cells can be generated from exocrine cells via transdifferentiation [Minami et al., 2005]. Earlier *in vivo* lineage tracing experiments in mice showed that acinar cells scarcely contribute to generate new β-cells and duct cells (Desai et al., 2007). However, more recent studies by Collombat et al. demonstrated that upon expression of Pax4, adult α-cells can transdifferentiate to β-cells (Collombat et al., 2009). The ectopic expression of Pax4 forces

endocrine precursor cells as well as mature α-cells, to adopt a β-cell fate. In addition, since α-cells were constantly recruited and converted to β-cells, the resulting glucagon deficiency provoked a compensatory and continuous glucagon+ cell neogenesis through Ngn3+ precursors. On the other hand, Arx misexpression in β-cells, using either an Ins[Cre] or in adult β-cells using an inducible Pdx1[CreERT] system reduced insulin-expressing cells and increased alpha and PP-positive cells (Collombat et al., 2007).

Alpha-to-Beta-cell transdifferentiation was also recently described in a transgenic model of diphtheria-toxin-induced acute selective near-total β-cell ablation (Thorel et al., 2010). Lineage-tracing to label the glucagon-producing α-cells before β-cell ablation, tracked large fractions of regenerated β-cells as deriving from α-cells, revealing a previously unknown flexibility in the functioning of the pancreas in relation to hormone secretion, with the potential for exploiting it to cure diabetes.

Generation of β-cells from α-cells has also been shown with a unique model that combines PDL with alloxan-mediated β-cells destruction (Chung et al., 2010). In this model, large numbers of β-cells were generated primarily from α-cells by two mechanisms: the first involved extensive α-cell proliferation, which provided a large pool of precursors that, in turn, would become β-cells via asymmetric division; the second demonstrated that β-cells could form directly from α-cells via transdifferentiation. This latter mechanism was put forward by the finding of intermediate cells co-expressing α- and β-cell-specific markers. Double-positive cells were detectable in the first week after injury, but their number gradually declined and by the second week some converted into mature β-cells, as shown by loss of glucagon and new expression of MafA, a β-cell-specific transcriptional activator.

4.2.2 Nestin-positive cells

Nestin-positive cells have been identified within adult rat islets as being capable of differentiating into insulin-positive cells *in vitro* (Zulewski et al., 2001). Nestin is an intermediate filament protein expressed by the neural lineage, which, according to some groups can be found in the pancreas (Edlund, 2002), in contrast to others that could not find its expression during development of the human pancreatic epithelium (Piper et al., 2002). More recent lineage-tracing experiments showed that nestin-positive cells contribute to the vasculature as well as acinar lineages but not to the endocrine lineage (Treutelaar et al., 2003; Esni et al., 2004; Delacour et al., 2004).

4.2.3 Proliferative human islet precursor cells (hIPCs)

Proliferative human islet precursor cells (hIPCs) were obtained *in vitro* from preparations of adult human islets after extensive *in vitro* proliferation (Gershengorn et al., 2004). Authors believed that these cells, showing a mesenchymal phenotype, derived from insulin-expressing cells undergoing epithelial-to-mesenchymal transition (EMT). hIPCs could be re-differentiated into insulin-expressing islet-like cell aggregates (ICAs) and secreted insulin when transplanted under the kidney capsule of immunodeficient mice. However, many criticisms were advanced from other groups, claiming that, at least in mouse pancreatic cultures, islet-derived fibroblast-like cells are not generated via EMT from pancreatic β-cells (Chase et al., 2007; Atouf et al., 2007). Later, Gershengorn et al. further confirmed the basic differences between human and mouse cultures, and claimed that hIPCs are a special kind of pancreatic mesenchymal stromal cells (Morton et al., 2007). More recently, using a lineage-tracing *in vitro* technique, Russ et al. found evidence for massive proliferation of

cells derived from human β-cells. Nevertheless, it appears that induction of significant replication *in vitro* results in dedifferentiation. (Russ et al, 2008).

5. Evidence of pancreatic progenitors/stem cells outside the pancreas

In addition to pancreatic progenitors, cells from other organs, such as liver, spleen, bone marrow, adipose tissue and limbus have been identified as either new sources of islets or stimulators of islet regeneration.

5.1 Liver stem cells

Pancreas and liver share the same origin from the embryonic endoderm (Zaret, 2000). It has been reported that transdifferentiation of pancreas into liver occurs both *in vitro* and *in vivo* in animal models after a number of experimental treatments (Rao et al., 1986, 1995; Dabeva et al., 1997; Kralowski et al., 1999; Shen et al., 2000). The opposite conversion of liver into pancreas is also possible (Horb et al., 2003). Zalzman et al. were able to immortalize a population of human fetal liver epithelial progenitor cells that, once transfected with the Pdx1 gene, generated a stable population of insulin-producing cells (Zalzman et al., 2003). Intraperitoneal transplantation of these cells into immunodeficient mice led to reversal of diabetes for 80 days. However, Yang et al. showed that expression of Pdx1 in hepatocytes does not result in the formation of functional endocrine pancreas in Pdx1 deficient mice, thus suggesting that Pdx1 is necessary but not sufficient to induce differentiation of the pancreatic tissue (Yang et al., 2002).

5.2 Splenic stem cells

The hypothesis that the spleen may harbor stem cells capable of differentiating into β-cells has also been investigated. Faustman and colleagues initially showed that splenocytes contributed to the reversal of autoimmunity in the NOD mouse model when injected with Freund's complete adjuvant (Ryu et al., 2001). Later they also suggested that splenocytes may directly contribute to islet regeneration by differentiation into β-cells (Kodama et al., 2003). However, these findings proved to be controversial. Indeed several other groups, confirming a partial recovery from the autoimmune attack following splenocyte injections, actually failed to display evidence of a direct contribution of donor cells to β-cell regeneration (Suri et al., 2006; Chong et al., 2006; Nishio et al., 2006). Nonetheless, Yin and colleagues supported a facilitating role of the spleen in the regeneration of endogenous β-cell mass (Yin et al., 2006).

5.3 Mesenchymal stem cells

There are numerous reports suggesting that the bone marrow not only harbors haemopoietic stem cells, which are committed to differentiate into blood cells, but also mesenchymal stem cells (MSCs), capable of differentiation into β-cells (Oh et al., 2004; Moriscot et al., 2005). MSCs were reported to differentiate *in vivo* into glucose-competent pancreatic endocrine cells when transplanted in NOD mice (Ianus et al., 2003). However, following studies resulted in controversial outcomes, suggesting that MSCs do not become *per se* insulin producing cells, rather they take part in islet vascularization, eventually promoting β-cell regeneration (Hess et al., 2003 Chamson-Reig et al., 2010). Transplantation of human MSCs was also shown to induce repair of pancreatic islets and renal glomeruli in immunodeficient mice (NOD/SCID) suffering from STZ-induced diabetes (Lee et al., 2006). The role exerted by bone marrow cells

by quenching autoimmunity allowing therefore functional recovery of residual β-cell mass in the pancreas, has been proven by Zorina et al. (Zorina et al., 2003). In the NOD autoimmune diabetes mouse model" in place of, restoration of endogenous β-cell function to physiologically sufficient levels was achievable after allogeneic bone marrow transplantation. Abrogation of autoimmunity and consequent β-cell mass recovery interestingly occurred even when allogeneic bone marrow cell tranplantation was performed after the clinical onset of diabetes. A recent study has suggested that bone marrow cells might have a role in permitting survival of endogenous β-cells also in humans (Voltarelli et al., 2007). Authors reported insulin independence for up to a year in more than half the cases of a small number of patients with recent-onset type 1 diabetes. Patients were administered high-dose immunosuppressive therapy to kill autoreactive T cell clones followed by autologous non-myeloablative stem cell transplantation. Nonetheless, because autologous bone marrow cell transplantation could not change indefinitely the genetic susceptibility to develop autoimmune diabetes, autoimmunity recurred soon after full immunocompetence was re-established. Therefore, different approaches should be used to obtain durable abrogation of β-cell specific autoimmunity, and allow recovery of insulin production (Giannoukakis et al., 2008).

Collectively these series of reports suggest that bone marrow cells do not give rise directly to new insulin producing cells - however they can indirectly facilitate regeneration of the endocrine pancreas, perhaps by secreting appropriate regenerative factors that still need to be characterized.

Other mesenchymal stem cells have been taken into consideration as a potential source of β-cells. Human and rat multipotent adipose tissue-derived stem cells (ADSCs) have been reported to generate insulin-producing cells after transduction with Pdx1 gene. The surrogate β-cells improved glucose sensitivity when transplanted under the renal capsule of STZ-induced diabetic rats [Lin et al., 2009]. In addition, intraportal infusion of human ADSCs together with bone marrow stem cells could increase endogenous insulin levels reducing exogenous insulin requirements in patients affected by type 1 diabetes (Trivedi et al., 2008).

The human limbus has also been indicated as a source of stem cells. The limbus is a highly specialized region of the eye hosting a well-recognized population of epithelial stem cells, which continuously renew the corneal surface. Additionally, the limbal niche also hosts stromal fibroblast-like stem cells (f-LSCs), with multilineage transdifferentiation potential.

f-LSCs were able to generate functional pancreatic hormone-expressing cells *in vitro* recapitulating pancreatic organogenesis (Criscimanna et al., 2011).

6. Evidence of β-cell regeneration via proliferation of pre-existing β-cells

During the fetal stage, differentiation from precursor cells is the major mechanism by which β-cells are formed, while β-cells replication is enhanced during the perinatal and neonatal period. Lineage-tracing experiments in rodents provided convincing proof to the theory that adult β-cells predominantly arise from other β-cells without significant contributions from underlying stem or progenitor cell populations (Cano et al., 2008). Several studies conducted by the group of Melton and colleagues showed that, after pancreatic resection (Dor et al., 2004), or in a diabetic status induced by transgenic expression of diphtheria toxin (Nir et al., 2007), mouse β-cells possess significant capacity for spontaneous regeneration, sufficient to recover from overt diabetes. Authors claim that failure of β-cell regeneration in both autoimmune and pharmacological models of diabetes is due to confounding factors

disguising the innate regenerative response, such as the persistence of circulating autoreactive T cells. To further sustain this hypothesis, it has been demonstrated how therapeutic protocols intended at blocking autoimmunity in NOD mice (Chatenoud et al., 1994) and in humans with type 1 diabetes (Herold et al., 2002, 2005, Bresson et al., 2006) resulted in partial remission from the disease. Whether this is due to a true regeneration process or just recovery of dysfunctional β-cells is still debated.
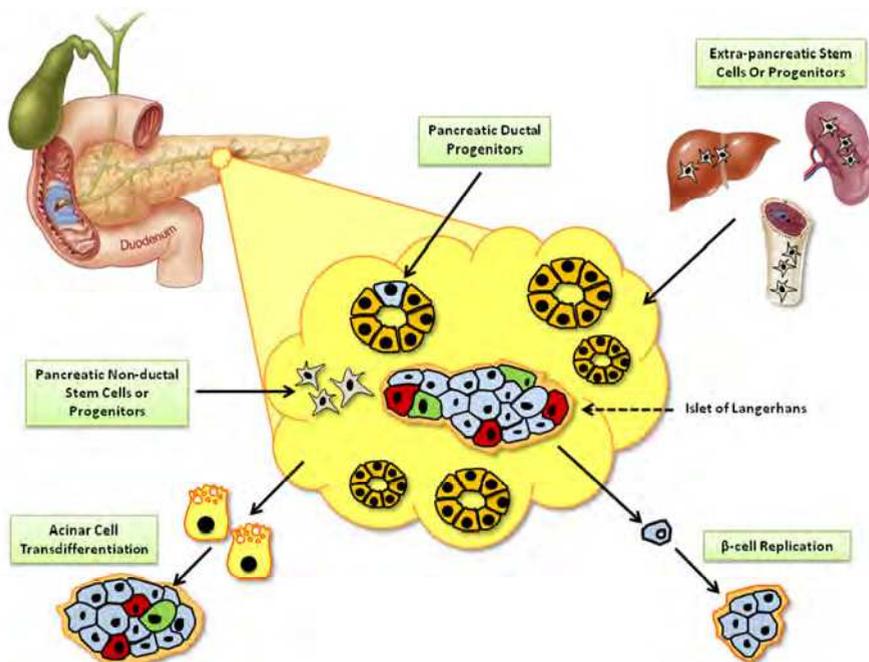
Fig. 5. Schematic illustration of potential cell sources for postnatal ß cell regeneration

Recently, Brennand et al. also demonstrated that in adult mice all β-cells, not just a subpopulation, equally contribute to islet growth and maintenance (Brennand et al., 2007). Two approaches were performed to address this issue. First, evaluation of the replicative potential of the entire β-cell mass was performed by monitoring the disappearance of a fluorescent marker accompanying cell division. Second, clonal analysis of dividing β-cells was completed. Because a uniform loss of label (cell division) across the entire cell population was observed, and all clones were of comparable size, authors conclude that the β-cell pool homogeneously possesses replication ability.

In humans, increased β-cell replication has been documented adjacent to intrapancreatic gastrinomas, suggesting that adult human β-cells can be driven, under specific circumstances, into the cell cycle (Meier et al., 2006b). Support to this remark was also given by another study, which demonstrated that β-cell replication is the primary mechanism responsible for the postnatal expansion of the β-cell mass in a population of young non-diabetic individuals [Meier et al., 2008]. In particular, it was shown that β-cell mass is able to (1) expand by several folds from birth to adulthood, (2) this is accomplished by an increase in number of β-cells per islet with a concomitant expansion in islet size (3) the relative rate

of β-cell growth is higher in infancy and gradually declines thereafter to adulthood with no secondary accelerated growth phase during adolescence, (4) β-cell mass (and presumably growth) is highly variable between individuals, (5) a high rate of β-cell replication is coincident with the major postnatal expansion of β-cell mass. A summary of the theories on β-cell origin is presented in Figure 5.

7. The chronic pancreatitis model

Regenerative responses from the pancreatic tissue can be the result of a surgical or inflammatory injury. It has been described that pancreatic stellate cells, the star shaped cells representing approximately 4% of the resident pancreatic cell pool, are involved in fibrogenesis and pancreas regeneration. In chronic pancreatitis, these cells undergo transformation from quiescent to activated myofibroblast-like cells. Upon activation, which can be triggered by reactive oxygen intermediates, ethanol, Transforming Growth Factor (TGF) α and - β1, they can disclose special features, including the capability to increase the synthesis of collagen, fibronectins, in addition to cytokines. Interestingly TGF-β_1 is expressed by ductal cells in chronic pancreatitis, with a role in development of fibrosis and glandular atrophy (Demois et al. 2002). Recently it has been shown in the adult human pancreas of patients with chronic pancreatitis increased numbers of insulin as well as glucagon -containing cells in the ducts, in addition to other cells containing endocrine and exocrine markers. Such findings were also associated to higher numbers of proliferating cells (Ki67 positive) and Pdx1^{+} cells, suggestive of a "metaplastic" status (Philips et al.,2007).

In our own experience as a reference center for the isolation of human islet cells, we observed peculiar morphological features in the pancreatic sections in patients with chronic pancreatitis. Not only did we find a higher density of islets, probably due to the destruction of the surrounding exocrine tissue (Figure 6), but, interestingly, we found that the intra-islet β-cell area relative to the α-cell area was significantly higher in chronic pancreatitis patients. Co-expression of the epithelial marker CK19, typical of ductal cells, with Ngn3, a transcription factor expressed in endocrine-committed cells during pancreatic development (Figure 7) was also identified.
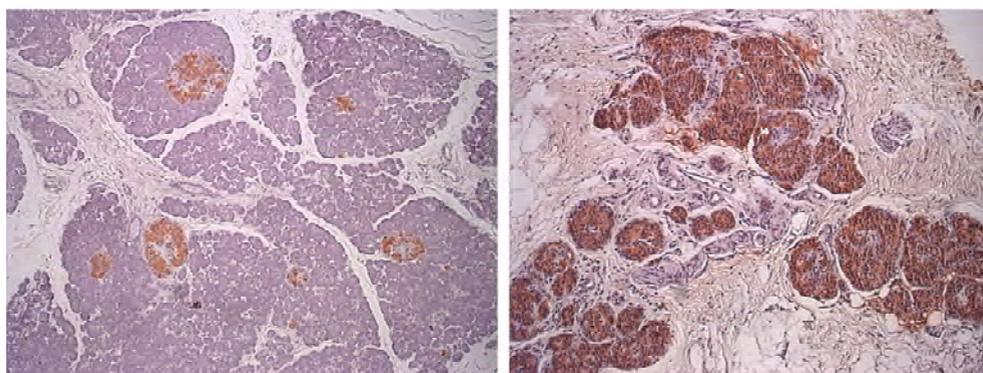

Fig. 6. Histological pancreatic features in chronic pancreatitis.

Islets of Langerhans (immunostained for insulin in brown/red) of a healthy individual (left) in comparison with those of a patient affected by chronic pancreatitis (right).
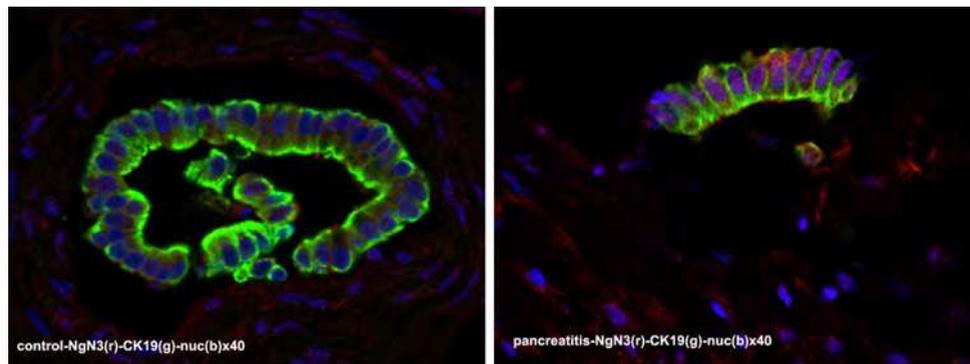
Fig. 7. Peculiar cell phenotypes in chronic pancreatitis.

Co-expression of the epithelial marker CK19 (green) and Ngn3 (red), a transcription factor typically expressed in progenitor cells committed to the endocrine lineage in the pancreas of a patient with chronic pancreatitis (right panel). Control healthy pancreas (left panel).

A number of questions can be asked regarding the process by which damage, and pancreatitis specifically, mobilize these cells. For example, what is the potential role of the immune response to a damage signal in the pancreas in the differentiation of ductal and exocrine cells towards the endocrine lineage? Do immune cells produce soluble factors that facilitate the process of differentiation? What are the characteristics of differentiating non-endocrine and endocrine cells in response to pancreatitis? Can endocrine progenitor cells be better characterized using pancreatitis as an inductive event? Would isolation of such progenitor cells offer a framework for *in vitro* genetic manipulation to further differentiate such cells into defined and fully functional endocrine cells? Would *in vivo* genetic manipulation of the microenvironment exhibiting differentiation processes in response to pancreatitis offer a framework to direct such cells into endocrine lineages? These questions are important in the context of identifying progenitor cells that can serve as the source of endocrine cells, especially insulin-producing β‑cells.

8. Conclusions

In conclusion, caution is required in interpreting studies unraveling the mechanisms involved in the maintenance of the β-cell mass. The extrapolation of information from animal studies can be misleading and not necessarily indicative for regenerative therapy in diabetic patients because mechanisms of regeneration/maintenance of the β-cell mass might be different across species (Hanley et al., 2008). For example, in obese rodents, an increment in β-cell mass is achieved by a massive increase of islet size and β-cell number per islet, consistent with a predominant mechanism of β-cell replication. On the contrary, in obese humans, islets are only modestly increased in size, consistent with minor involvement of β-cell replication (Butler et al., 2003). Also, a 90% pancreatectomy performed in young rodents leads to transient diabetes and regeneration of β-cell mass of ~50% within 2 weeks (Bonner-Weir et al., 1983, 1994), while only a 50% pancreatectomy is sufficient to determine diabetic status in adult humans who afterward became obese too. Certainly, one potential explanation for the reported differences between rodents and humans could be that rodents are more frequently studied at 1–3 months of age, when there is a high capacity for β-cell

replication, whereas human pancreatic tissue is primarily studied in adults. It is therefore of great value to investigate the potential of pancreatic tissue to recover endogenous endocrine function after injury in animal models more similar to humans, such as non-human primates. However, even if the mechanisms regulating the maintenance of the β-cell mass in physiologic conditions are the same in humans, monkeys and rodents, we cannot exclude that during pathological conditions different pathways, species-specific and injury-specific (for type and magnitude), might be activated. For example, although the potential of acinar, duct (epithelial) and mesenchymal cells to differentiate into other cell types has been demonstrated *in vitro*, the mechanisms *in vivo* may be totally different. These discrepancies would not be surprising as cells taken into a foreign environment commonly behave differently than when residing in their natural niche. Another consideration is that, if regeneration occurs by recapitulation of fetal development, adult precursors should respond to fetal inductive signals. Conversely, if regeneration occurs by a different pathway, it is less likely that the process would be influenced by the same signals. The study of the factor(s) secreted by bone marrow precursors may shed some light on these aspects of the regenerative process as well.

In conclusion, the debate between the supporters of neogenesis and self-renewal for maintenance and replacement of β-cells is still open. It is perhaps acceptable to conclude that there is some truth in both proposed hypothesis, and that one does not exclude the other. Understanding the potential contribution of each mechanism will be crucial to find better curative approaches for diabetes.

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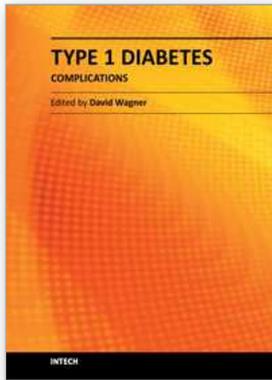
Type 1 Diabetes Complications

Edited by Prof. David Wagner

This book is a compilation of reviews about the complication of Type 1 Diabetes. T1D is a classic autoimmune disease. Genetic factors are clearly determinant but cannot explain the rapid, even overwhelming expanse of this disease. Understanding etiology and pathogenesis of this disease is essential. The complications associated with T1D cover a range of clinical obstacles. A number of experts in the field have covered a range of topics for consideration that are applicable to researcher and clinician alike. This book provides apt descriptions of cutting edge technologies and applications in the ever going search for treatments and cure for diabetes.

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