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# Pancreatic β-cell regeneration as a possible therapy for diabetes

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## Abstract

Diabetes is the result of having inadequate supply of functional insulin-producing β cells. Two possible approaches for replenishing the β cells are: 1) replacement by transplanting cadaveric islets or β cells derived from human embryonic stem (hESC)/induced pluripotent stem (iPSC) cells and 2) induction of endogenous regeneration. This review focuses on endogenous regeneration, which can follow two pathways: enhanced replication of existing β cells and formation of new β cells from cells not expressing insulin, either by conversion from a differentiated cell type (transdifferentiation) or differentiation from progenitors (neogenesis). Exciting progress on both pathways suggest that regeneration may have therapeutic promise.

## eTOC BLURB

Aguayo-Mazzucato and Bonner-Weir review the current approaches toward inducing endogenous β-cell regeneration. The authors highlight exciting progress in our understanding and enhancement of regenerative pathways (proliferation, transdifferentiation and neogenesis) which may hold therapeutic promise in alleviating some of the disease burden by overcoming the inadequate β-cell supply in diabetes.

## INTRODUCTION

Diabetes, both type 1 and type 2, is a disease characterized by an absolute or relative deficiency of β cells (Weir et al., 1990). Therefore, replenishing the lost functional or absolute β cell mass is a strategy that can alleviate some of the burdens of the disease. There are two general approaches to replenish β cells: 1) replacement therapy by transplanting cadaveric islets or β cells derived from human embryonic stem (hESC)/induced pluripotent stem (iPSC) cells and 2) induction of endogenous regeneration. The latter approach is the topic of this review.

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Replacement therapy using cadaveric islets has been shown as a proof of principle (Shapiro et al., 2000) to reverse diabetes in 88% of patients at 1 year and 71% at 2 years (Hering et al., 2016) with 10% maintaining insulin independence at 5 years but 80% with detectable C-peptide at the same time point (Ryan et al., 2005). However, the availability of healthy islets is insufficient for widespread application of this. The recent breakthroughs in deriving glucose responsive β-like cells from human pluripotent stem cells (Korytnikov and Nostro, 2016; Pagliuca et al., 2014; Rezania et al., 2014; Russ et al., 2015) have given encouragement for β cell replacement therapy. However, besides the need to gain fully functional insulin responses, this approach has other major challenges in becoming a therapy, including recurrent autoimmune attacks in type 1 diabetes, the inherent risks of placing foreign tissue in the body and potential tumor formation from not fully differentiated cells. Encapsulation of cell aggregates within immunobarriers, either microcapsules or macrocapsules, is the main strategy to protect the transplanted cells as well as containing potentially undifferentiated cell that can later be removed. However, encapsulation brings substantial issues of insufficient oxygen/nutrient access, foreign body response and impaired insulin kinetics (O'Sullivan et al., 2011; Weir, 2013). Even so, some clinical trials with macroencapsulation of hESC-derived cells have started (Clinicaltrials.gov NCT02939118; NCT02239354).

Therefore, there is a continued interest in understanding the processes of endogenous expansion of β cells in order to regenerate their endogenous mass. β cell mass is defined as the total weight of β cells within a pancreas and is determined by the balance between death (apoptosis/necrosis) and birth (replication of existing cells and neogenesis/transdifferentiation) of β cells as well as individual cell volume (atrophy/hypertrophy). The endocrine pancreas is a slow turnover tissue with relatively long lifespan (rodents 2–3 months)(Finegood et al., 1995). The renewal capacity of the pancreatic β cell pool is lower than tissues with well-characterized adult stem cell niches such as blood, skin and gut. Even so, the low frequency of both proliferation and apoptosis in the adult allows sustained β cell mass expansion over the first 7 months in rats (Montanya et al., 2000). Many rodent studies, often using genetically modified mice, have examined pathways involved in development or postnatal growth. In this review, we will combine studies on neogenesis and transdifferentiation. Even though these terms are often used differently, they both represent new β cells derived from a cell not expressing insulin. Neogenesis is usually considered as newly formed β cells by differentiation from stem/progenitor cells; these progenitors may have arisen from dedifferentiated duct cells (Bonner-Weir et al., 2004). On the other hand, transdifferentiation has been defined (Shen et al., 2003) as the direct conversion of a terminally differentiated cell type into another cell type-β cells in this case.

Islet regeneration has mainly been studied in rodent models and only more recently isolated human islet, acinar or duct cells are being used *in vitro*. While the concept of islet regeneration by any means other than replication of preexisting cells suffered disfavor for about a decade, neogenesis/transdifferentiation has regained favor. It is likely that both proliferation and new formation of β cells are mechanisms of islet regeneration, with different emphasis depending on the injury and on the species. We will discuss the state of our knowledge of β cell/islet regeneration in rodents and humans with highlights of some exciting new studies.

## CLASSIC ISLET REGENERATION MODELS

The classic rodent models of pancreatic regeneration, partial pancreatectomy (Bonner-Weir et al., 1993) and partial duct ligation (Wang et al., 1995), have both increased β-cell replication and neogenesis reported. A third model becoming a classic model of regeneration is that of diphtheria toxin-targeted cell ablation.

Partial pancreatectomy (Px), consisting of the removal of 60–90% of the pancreas in adult rats and 50–80% in adult mice, has demonstrated the substantial regenerative capacity of the adult pancreas with whole new lobes being formed and enhanced proliferation of pre-existing β and acinar cells (Bonner-Weir et al., 1993; Bonner-Weir et al., 1983; Brockenbrough et al., 1988; Li et al., 2010; Peshavaria et al., 2006). We showed that after partial pancreatectomy in adult rats, pancreatic-duct cells undergo a reproducible dedifferentiation and expansion forming transient areas composed of proliferating ductules; these foci of regeneration subsequently recapitulate aspects of embryonic pancreatic differentiation to form new pancreatic lobes with new islets (Bonner-Weir et al., 1993; Li et al., 2010). Recent data has shown that by 1 year of age there was a loss of this plasticity of the pancreatic ducts resulting in less neogenesis (Tellez et al., 2016). In these older rats, without the neogenesis, the β cell mass did not increase after Px even though β cell replication and apoptosis frequency did not differ with that of 1 month old and individual cell size had similar increases. At least two groups using 60% Px in adult mice also reported both neogenesis and enhanced replication (Ackermann Misfeldt et al., 2008; Peshavaria et al., 2006) while others reported only enhanced replication (Dor et al., 2004; Lee et al., 2006; Teta et al., 2007). Using genetically modified mice and this model, Ackermann Misfeldt et al. showed that the regulation of β cell proliferation by the transcription factor FoxM1 differed between β cells formed by neogenesis, which did not need the transcription factor to proliferate, and the mature adult ones that depended on FoxM1 to divide. This difference in the dependency of a transcription factor had been previously reported between embryonic and adult β cells (Zhang et al., 2006). In summary, partial pancreatectomy provides a model to study both neogenesis and replication in rodents.

Partial duct ligation involves the surgical ligation of the pancreatic duct at the level of the pylorus, resulting in an obstruction of drainage of exocrine secretions from the distal tail region and subsequent loss of acinar cells by death and dedifferentiation. This model has been used for several decades to study mechanisms involved in β cell formation (Edstrom, 1971a, b; Hultquist et al., 1979; Lardon et al., 2004; Rooman et al., 2002; Wang et al., 1995). Two distinct features of this model are 1) that regeneration is limited to the pancreatic portion distal to the ligation allowing an internal control and 2) there is no change in glucose homeostasis. In a comprehensive study in rats Wang et al. found a doubling of β cell population that could not be accounted for by a low BrdU incorporation. Finding an increased population of small clusters or singlet insulin positive cells suggested to them that neogenesis also occurred. One of the first studies (Xu et al., 2008) to use this model in mice showed expansion of β cell mass from endogenous multipotent progenitor cells that depended partly on activation of neurogenin 3 (*Ngn3)*, a transcription factor that is necessary for pancreatic endocrine cell development. They identified and sorted GFP-expressing cells from the ligated portion of the pancreas of Ngn3:GFP mice and showed that when

transplanted into *Ngn3*$^{null}$ pancreas *in vitro* these cells could become islet cells including insulin-expressing β cells. With knockdown of *Ngn3* using an intraductal injection of lentivirus, they showed that the activation of *Ngn3* in cells within the ductal complexes was involved. However, subsequently considerable controversy has arisen over this model with most other groups finding no increase in β–cells. The question of whether the PDL-induced changes in tissue composition might skew the results was raised (see (Kopp et al., 2011; Van de Casteele et al., 2014). A rigorous study using pancreatic acinar-specific transcription factor 1a (*Ptf1a*) promoter-driven lineage tracing (Pan et al., 2013) provided evidence of a small contribution of β cells from acinar cells; there was a rapid dedifferentiation of acinar cells to a ductal phenotype and then endocrine differentiation over several weeks. Hyperglycemia due to partial ablation of the β cells by streptozocin accelerated this process.

The diphtheria toxin-targeted cell ablation was originally developed by introducing the diphtheria toxin A chain as a transgene into mice such that only cells that transcribed the specific promoter of choice would be killed(Herrera et al., 1994). An inducible version directing the toxin to the insulin-expressing cells was used to show the regenerative capacity of 20–30% residual β cells and that the immunosuppressive drugs used in islet transplantation blocked the recovery (Nir et al., 2007). Another inducible variation, first used in hepatocytes (Saito et al., 2001) and then in islet cells by Herrera's group (Thorel et al., 2010), has targeted transgene expression of the human heparin-binding epidermal growth factor-like precursor (HB-EGF), which acts as the diphtheria toxin receptor (DTR). Primates are 1000 fold more sensitive to diphtheria toxin (DT) than mice so the human receptor as transgene confers sensitivity; expressing cells are only ablated when DT is injected. The studies of the Herrera group are described below in the Transdifferentiation section. Using crosses of ROSA26 LSL:DTR mice with either *Elastase* (acinar-specific) or pancreatic and duodenal homeobox 1 (*Pdx1*)(global pancreas)-driven expression of Cre and injections of DT, Criscimanna et al. (Criscimanna et al., 2011) showed that the severity and type of pancreatic injury determined the regenerative mechanism. In the PDX$^{cre}$ROSA$^{DTR}$ mice in which both the acinar and islet tissue were ablated, both regenerated from the pancreatic duct cells (which for an unexplained reason had not been ablated) but in Ela$^{cre}$ROSA$^{DTR}$ in which only the acinar cells were ablated, only new acinar regenerated from the ductal cells. Overall, the diphtheria toxin β-cell ablation model may induce less inflammation than the partial pancreatectomy and PDL and therefore may have less confounding effects introduced by edema or inflammation.

## PHYSIOLOGICAL MODELS OF COMPENSATORY GROWTH IN RODENTS AND HUMANS: PREGNANCY AND INSULIN RESISTANCE

Pregnancy and high fat diets are not really regeneration models but rather models of physiological compensatory growth that help identify and study relevant pathways that can then be applied in islet regenerative strategies. The concept of compensatory growth to meet increased demand due to obesity, pregnancy or solely insulin resistance has been well studied in rodents and seen mainly as enhanced replication rather than neogenesis. Both models have provided insights into pathways involved with enhanced β cell growth in the adult. Interestingly, comparison of rodent pancreas and autopsied or surgically resected

human pancreas has shown differences in compensatory growth between the species and between physiological compensatory models.

In pregnant rodents, proliferation of preexisting β cells and their enhanced function (Parsons et al., 1992) seem to be the main mechanisms of compensating although at least one lineage tracing study reported neogenesis in addition (Toselli et al., 2014). Signaling through the prolactin receptor by placental lactogen or prolactin was shown stimulate β cell proliferation. The mechanism of this includes the repression of the expression of menin, which as a tumor suppressor blocked β cell replication (Karnik et al., 2007; Kim et al., 2010), and induced serotonin production (Schraenen et al., 2010); (Kim et al., 2010) which in turn stimulated proliferation in an autocrine and/or paracrine manner (Kim et al., 2010). In pregnant humans, while there is a 40% increase in the relative volume of β cells (%β cell/pancreas), there was no observed change in replication nor apoptosis (Butler et al., 2010). However there were increased proportion of small islets, increased number of insulin$^+$ cells with the ducts (1.2±0.02 % vs 0.4±0.1%) and a 3-fold increase in singlet insulin$^+$ cells. Even so, one must be cautious about concluding the expansion was all neogenesis since the lack of measurable increase in replication may be due to the suppression of protein expression of the marker of proliferation Ki67 by autopsy conditions of warm and cold ischemia (Sullivan et al., 2015).

Another model of physiological β cell compensation is insulin resistance or obesity that can result in 30 fold increased β cell mass in mice (Bruning et al., 1997) but only about 30% in humans (Kloppel et al., 1985; Rahier et al., 2008). Moreover, while this compensatory growth is mainly by replication in mice, neogenesis rather than enhanced replication has been suggested in two studies of humans. Using surgical specimens Yoneda et al (Yoneda et al., 2013) reported increased number of single or small clusters of insulin$^+$ cells, bihormone-expressing cells and percentage of insulin$^+$ cells within ducts in patients with impaired glucose tolerance or newly diagnosed T2D compared to non diabetic patients; no difference in proliferation (Ki67$^+$) was seen. Similarly Mezza et al. reported in surgical specimens from insulin-resistant subjects an increased proportion of scattered insulin$^+$ cells and small islets and 3-fold increase of cells co-expressing insulin and the duct marker cytokeratin 19 compared to insulin-sensitive subjects; proliferation as judged by KI67 staining was not detectable (Mezza et al., 2014). A third study (Hanley et al., 2010), using organ donor pancreas, reported overall increased neogenesis (clusters of 3 or less insulin$^+$ cells) with obesity or T2D. Additionally increased PCNA$^+$ insulin cells were found in the non-diabetic obese but decreased PCNA$^+$ cells in obese T2D.

We have learned from these physiological β-cell compensation models that the predominant compensatory mechanism may vary by species and condition, reinforcing the need to look into both strategies as a way to regenerate β-cells as a treatment of diabetes.

## ENHANCED PROLIFERATION AS A STRATEGY

Inducing β cell proliferation is, in theory, a straightforward and effective way to increase absolute β cell mass; however, in human islets it has been quite difficult. Over the years there has been much controversy as to whether adult human β cells could proliferate, but in

2001 (Tyrberg et al., 2001) showed that adult human islets incorporated $H^3$ thymidine when transplanted in mice and this incorporation increased in response to hyperglycemia. In both rodents and human there is a decline in the percentage of replicating β cells after the neonatal period (Gregg et al., 2012; Meier et al., 2008; Scaglia et al., 1997; Teta et al., 2005), but with a low stable frequency well in to adulthood. However, as often ignored but pointed out by Chintinne et al. (Chintinne et al., 2010), the actual pool of replicating β cells is six fold greater in adults than in neonates even with the lower frequency because the overall population is substantially larger.

One of the goals of the field has been to understand what regulated the age-related decrease in replication and strategies to enhance β cell proliferation (Table 1) both in rodents as proof of principle and in humans. In rodents many of the different strategies effective in increasing β cell proliferation have been loss or gain of function studies of different cell cycle molecules. For example, knockout of the nuclear regulator of cyclin-dependent kinase cyclin D2 showed it was necessary to regulate the transition of β cells to a replicative state (Georgia et al., 2010). Consistent with an age-related decrease in the proliferative capacity of β cells there are increases of expression of cell cycle inhibitors. The accumulation of $p16^{Ink4a}$ in cells has been seen as both an effector of senescence and an indicator of cellular senescence in β cells (Krishnamurthy et al., 2006). $P16^{Ink4a}$ blocks replication but its expression is repressed by a histone-lysine N/methyltransferase enzyme encoded by the *enhancer of zeste 2 polycomb repressive complex 2 (Ezh2*) (Zhou et al., 2013). Ezh2 levels decrease with age, in parallel $p16^{Ink4a}$ increases. Replenishing Ezh2 in young mice increased β cell replication, but after 8 months of age this strategy was no longer effective due to an enrichment of a trithorax group protein complex at the Ink4a locus (Zhou et al., 2013). Others showed that the transgenic maintenance of platelet-derived growth factor receptor a (Pdgfra) expression led to sustained Ezh2 expression and β cell replication in 14 month old mice (Chen et al., 2011). Senescent (proliferative-arrested) cells secrete an array of cytokines and chemokines (known as senescence-associated secreted proteins) that can impair proliferation of neighboring cells (Rodier et al., 2009). Senolytic therapies (Palmer et al., 2015) that specifically eliminate senescent cells may allow the remaining β cells to respond to physiological stimuli of proliferation such as pregnancy, hyperglycemia or weight gain.

A series of papers from the Dor lab support the concept that glucose metabolism is more important than cell cycle components in regulating β cell proliferation and can overcome cell cycle inhibitors. Cyclin D2, shown to be high in quiescent β cells, did not differ in β cells of mice between 1 and 6 months old. Yet it became downregulated in replicating cells, and regulation of its mRNA was glucose-dependent (Salpeter et al., 2010; Salpeter et al., 2011). Moreover, glucose metabolism (glycolytic flux) within the β cell was shown to be the main regulator of their compensatory growth, with the rate-limiting enzyme of glycolysis glucokinase being a potential target for inducing proliferation (Porat et al., 2011). In parabiosis and transplantation experiments examining replication in islets of old (8 month) and young (1 month) mice, they found β cell replication in old islets increased in a young environment, without changes in levels of cell cycle inhibitors *p16, p18, p27* or *p21* mRNA (Salpeter et al., 2013). Interestingly, they noted that the blood glucose levels were lower in their old mice and speculated that in the young environment, glucose might be driving the

replication. This ability to induce compensatory growth in old mice is consistent with their experiments in which old mice (1–2y) showed 2–3 fold increase in β cell proliferation using either inducible DTA ablation of β cells or a small molecule glucokinase activator (Stolovich-Rain et al., 2012).

However, the translation of these findings into human cells is complicated by the fact that there are some differences in cell cycle proteins in human islets compared to rodent as laid out in the “roadmap” of the cell cycle molecules in human islets by Stewart’s group (Fiaschi-Taesch et al., 2013). One difference seems to be the cytoplasmic location of G1/S cell cycle molecules in human β cells *in vitro*. With adenoviral overexpression, Cdk6 plus cyclin D3 became nuclear and drove replication; however, in parallel, the cell cycle inhibitors p16, p21 and p27 also underwent cytoplasmic-to-nuclear localization (Fiaschi-Taesch et al., 2013). There is a concern that a substantial proportion of the β cells that entered the cell cycle showed accumulation of double-stranded DNA damage, which may result in apoptosis and not in expansion of functional β cells (Rieck et al., 2012). Such lack of completion of cell cycle was also seen with mitogenic push from overexpression of hepatocyte nuclear factor 4 (*Hnf4)* (Rieck et al., 2012). Similarly, long-term over activation of glucokinase or hyperglycemia in T2D was associated with DNA strand breaks, DNA damage response and activation of tumor suppressor protein p53 leading to growth arrest and apoptosis (Tornovsky-Babeay et al., 2014).

Even so, work proceeds on trying to enhance human β-cell proliferation. The most exciting results come from the use of high throughput screens to identify new pathways and compounds that can drive human β cell replication. Adenosine kinase inhibitors promoted β cell replication in mice, rats and pigs without affecting proliferation of α, PP, fibroblasts, exocrine or hepatic cells but, unfortunately the initial report found no effect on isolated human islets (Annes et al., 2012). Since then others have shown 5-iodotubercidin (Dirice et al., 2016) and harmine (Wang et al., 2015), both of which target tyrosine-regulated kinases DYRK1A and NFAT, stimulated human β proliferation. Harmine also stimulated α cell proliferation (Wang et al., 2016). Targeting the same pathway, aminopyrazine compounds stimulated β cell proliferation in dispersed adult human and mouse islets by inhibiting DYRK1a and glycogen synthase kinase 3 β (GSK3β) (Shen et al., 2015). Silencing of CDKN2C/p18 or CDKN1a/p21, both cell cycle inhibitors, was able to drive proliferation of human β cells (Robitaille et al., 2016). Using the LIRKO mouse in which the ablation of the insulin receptor in hepatocytes led to massive β cell hyperplasia, proteinase inhibitor SerpinB1 was identified as a hepatocyte-secreted protein that enhanced β-cell proliferation of human, mice and zebrafish (El Ouaamari et al., 2016). All of these represent potential β cell proliferation strategies but a major potential issue is whether other cell types within the body also respond with enhanced replication; unfortunately, most studies have not examined this question of specificity.

## TRANSDIFFERENTIATION/NEOGENESIS AS A STRATEGY

A different, and complementary, strategy is to replenish the β cell mass by differentiation of new β cells. There is not convincing evidence of a true adult pancreatic stem cell, even though embryonically multipotent progenitors give rise to ductal, endocrine and exocrine

lineages. While considerable work supports new β cell formation either from progenitors or facultative progenitors within the pancreas or by transdifferentiation of differentiated pancreatic cells into functional β cells, the presence of facultative progenitors after birth is not universally accepted. However, the concept of potential plasticity of the different pancreatic cell types has become somewhat accepted. The main questions studied are which cell type is involved, what factors trigger the process and whether these findings translate to human. The cell types suggested are pancreatic duct cells (or some particular cell within the ducts), centroacinar cells, acinar cells and islet cells particularly glucagon-expressing α, and somatostatin-expressing δ cells (Figure 1). With the recent publications of single cell RNAseq data from human islet preparations, there is an increasing awareness of heterogeneities within a particular cell type (Baron et al., 2016; Dorrell et al., 2016) as well as potentially intermediate stages (Segerstolpe et al., 2016; Xin et al., 2016). Whether such intermediates are artifacts of the technique or show transition states remains to be determined. It may well be that only some of these phenotypes have potential plasticity. This methodology of single cell analysis should be useful in furthering our understanding of β cell regeneration.

## DUCTS

The pancreatic ductal tree serves as the conduit for the digestive enzymes to get from acinar cells to the duodenum, but we had been proponents that, in addition, its epithelium itself serves as a pool for progenitors for both islet and acinar tissues after birth and into adulthood (Bonner-Weir et al., 2016; Bonner-Weir et al., 2004; Inada et al., 2008). Our studies with the partial pancreatectomy rat model (Bonner-Weir et al., 1993; Li et al., 2010; Sharma et al., 1999) support the idea that there is a massive dedifferentiation of the ductal epithelium of the largest ducts (common pancreatic duct) and proliferation across these ducts giving rise to new lobes rapidly; within 1 week after surgery these new lobes were difficult to distinguish from the old ones. The treatment with gastrin in the 95% pancreatectomy induced further duct dedifferentiation and expression of *Ngn3* and the β cell specific transcription factor *Nkx6.1*, followed by increased population of β cells (Tellez and Montanya, 2014). The papers on PDL from the Heimberg lab (mentioned above) also indicated that pancreatic ducts where the source of the cells with activated *Ngn3*. *In vitro* expansion of human ductal tissue and its subsequent differentiation to islet cells was observed after 3–4 weeks culture, when there was a significant increase in insulin as well as formation of functional islet-like structures (Bonner-Weir et al., 2000; Gao et al., 2005). In addition to replication in grafts of human isolated islet preparations under the kidney capsule of mice, duct cells proliferated and had induction of *Nkx6.1* expression (Tyrberg et al., 2001). Similarly, insulin-positive cells expressing duct markers were found in grafts of purified human duct cells (Yatoh et al., 2007).

Several groups have identified markers that potentially identify progenitor populations within the ductal tree (Table 2). The most widely accepted marker is CD133 expressed by the progenitor populations from mouse or human pancreas (Gomez et al., 2015; Jin et al., 2016; Lee et al., 2013; Sugiyama et al., 2007b). Yet, others identified $CD133^{+}$ cells in adult human duct cells as characterized by their expression of cytokeratin 19 (CK19) and carbonic anhydrase 2 (CAR2) (Lee et al., 2013). However, the expression of NGN3 was found in

CD133$^{high}$ CD49f$^{high}$ cells from fetal mice and human pancreas (Sugiyama et al., 2007b) and as CD133$^{+}$ cells from adult human, being 2 % of acinar and duct cells in surgical biopsies and 10% in organ donor pancreas (Gomez et al., 2015). Another subpopulation of cells, those expressing double cortin-like kinase 1 (Dclk1), has also been suggested in mouse and human to be a quiescent but long-lived pancreatic progenitor (Westphalen et al., 2016). These cells were found in both ducts and acini, responded to injury and may give rise to pancreatic cancer, but their role in endocrine pancreas regeneration has not been clarified. The recent identification by single cell RNAseq of at least 4 different gene expression profiles of human duct cells (Baron et al., 2016) does not clarify whether there is a separate progenitor population within the ductal tree or just a generalized potential plasticity.

Centroacinar cells (CAC) are a unique cell type at the junction of the terminal ducts and the acini. In zebrafish, they have been shown to contribute to pancreatic regeneration (Delaspre et al., 2015). In human pancreas they have strong CD133 expression (Immervoll et al., 2011). In mice, isolated aldehyde dehydrogenase isoform 1 (ALDH1)$^{+}$ CACs/terminal ductal cells were shown to generate endocrine cells *in vitro* in either pancreatospheres or dorsal bud explants (Rovira et al., 2010). However the lack of transcription factor *Hes1* in these ALDH1$^{+}$ cells has led to questions of whether they were actually CAC cells (Delaspre et al., 2015) since in the adult pancreas *Hes1* expression is expected to persists in this cell population.

The use of Cre-lox based lineage tracing did not resolve the question of whether pancreatic ducts contain a pool of β-cell progenitors. Such experiments using duct transcription factors *hepatocyte nuclear factor-1-β* (*Hnf1β)* (Solar et al., 2009) or *SRY-Box 9* (*Sox9)* (Kopp et al., 2011) as promoters found no labeling of either islets or acinar cells after birth nor after pancreatic duct ligation in contrast to studies using the *carbonic anhydrase 2* promoter (Inada et al., 2008), the inducible insulin promoter (Nakamura et al., 2011; Zhang et al., 2016) and the *Sox9* promoter (Zhang et al., 2016). Using *Sox9* promoter line of mice from the Sander lab but with a founder with higher recombination efficiency, this latter study found that moderate hyperglycemia was needed to drive SOX9$^{+}$ ductal cells to β cells and that a long-term low dose of gastrin/epidermal growth factor enhanced the neogenesis. One remaining issue with these discrepant lineage-tracing experiments is that those that do not find neogenesis also did not find any postnatal ductal contribution to the acinar population (Kopp et al., 2011; Solar et al., 2009). This lack seems unexplained but the massive growth of the pancreas in the neonatal period, particularly between 2 and 4 weeks when there is a 5-fold increase in pancreatic weight in mice, could not be solely from replication of preexisting acinar cells or their change in cell volume as these would not generate new lobes. Both the ductal branching system that continues to add pancreatic lobes to the growing pancreas after birth (Bonner-Weir et al., 2016) and the labeled pancreatic lobes at 4 weeks age seen with CAII lineage tracing (Inada et al., 2008) support the postnatal pancreas expansion from expanding and differentiating ductal cells. Further evidence that ducts can serve as β-cell progenitors in the adult mouse comes from a series of papers from Collombat in which mice ectopically expressing *paired box 4* (*Pax4)* in glucagon-expressing cells could repeatedly recover from toxin-induced diabetes via duct epithelial cells continuously forming new α cells that then converted to β cells (Al-Hasani et al., 2013; Collombat et al., 2009; Courtney et al., 2013; Pfeifer et al., 2013). The ductal origin of the new glucagon-

expressing cells was lineage-traced using the *Hfn1β*Cre$^{ER}$ driver (Al-Hasani et al., 2013). These experiments are discussed further under islet transdifferentiation.

The process of transdifferentiation from duct cells into β cells can be promoted through a number of strategies (Figure 1). Transgenic over-expression of interferon-γ in the β cells (Gu and Sarvetnick, 1993), over-expression of transforming growth factor (TGF)-α in pancreatic ducts (Wang et al., 1993), pancreatic or ductal deletion of *Fbw7* component of the SCF type ubiquitin ligase (Sancho et al., 2014) and *Pax4* ectopic expression in glucagon-positive cells (Collombat et al., 2009) all showed duct-to-β cell progression. Inflammatory cytokines have been shown to stimulate epithelial -to-mesenchymal transition as well as endocrine differentiation program in duct cells through NGN3 activation in a STAT3-dependent manner (Valdez et al., 2016). Transgenic hepatic overexpression of the cytokine TWEAK (TNF-like weak inducer of apoptosis, TNFSF12) promoted pancreatic ductal proliferation and expression of Ngn3 and resulted in focal regions of proliferating ductules and β cells (Wu et al., 2013). *In vitro* culture of human islet-depleted exocrine tissue (both duct and acinar cells) or duct cell lines with BMP7 (Klein et al., 2015), gastrin and epithelial growth factor (EGF) (Suarez-Pinzon et al., 2005), or preadipocyte factor 1 (Pref-1) (Rhee et al., 2016) have had varying success with differentiating cells that when transplanted could reverse diabetes in mice.

On the basis of the above data the authors conclude that at least some, if not most, of the pancreatic duct cells in rodents and in humans can serve postnatally as β-cell progenitors although this process may not be robust

## ACINAR

Acinar cells comprise the most abundant pancreatic cell type and therefore constitute an attractive source of reprogrammable tissue that could generate β cells. Much of the earlier work was on acinar cells *in vitro* and was led by the Brussels groups. After several days of suspension culture, rat exocrine tissue was seen to transdifferentiate/dedifferentiate to ductal cells (Rooman et al., 2000) but if cultured with growth factor EGF and leukemia inhibitory factor, newly formed β cells were seen that could restore normoglycemia in alloxan-diabetic mice (Baeyens et al., 2005). Further work showed that inhibiting notch signaling lead to 30 % of the acinar cells forming β cells (Baeyens et al., 2009). Then a transient cytokine mixture of EGF and ciliary neurotrophic factor (CNTF) that activated Stat3 signaling in mice that led to *in vivo* conversion of acinar-to-β cells could reverse alloxan-induced diabetes (Baeyens et al., 2014). Some of these findings were also found with *in vitro* using human pancreatic tissue (Houbracken et al., 2011), including the activation of STAT3 and MAPK-induced NGN3 expression in transduced cells with some resultant insulin-positive cells (Lemper et al., 2015).

Additional *in vivo* work used modification of transcription factor expression within the acinar compartment. Pioneering work introduced a viral vector driving key developmental transcription factors *Ngn3, Pdx1*, and *Mafa* into the pancreatic parenchyma to reprogram the differentiated exocrine cells in adult mice into β-like cells. These induced β cells were indistinguishable from endogenous islet β cells in size, shape and ultrastructure, and they expressed genes essential for β-cell function and ameliorated hyperglycemia (Zhou et al.,

2008). More recently *Pdx1* expression induced in acinar cells in transgenic mice resulted in reprogramming into endocrine precursor cells that migrated into islets and differentiated into insulin, somatostatin or PP cells; these cells improved STZ-induced diabetes (Miyazaki et al., 2016). As mentioned above, the study on pancreatic duct ligation with *Ptf1a* promoter-driven lineage tracing showed some β cell formation from acinar cells via a dedifferentiation to a ductal phenotype (Pan et al., 2013).

## ISLET CELLS

Pancreatic islets are non-random organized microorgans of several endocrine cell types, with insulin-producing β-cells being the predominant one. Recently there has been surprising evidence using genetically manipulated mice that β cells could come from transdifferentiation of other islet cell types, namely glucagon-producing α-cells and somatostatin-producing δ-cells. The recent identification of a "virgin beta cell subpopulation" that are urocortin $3^{negative}$, $MAFA^{negative}$ and $insulin^{positive}$ subpopulation in the periphery of the islet, adds to the potential list of progenitors that could contribute towards a functional beta cell pool (van der Meulen et al., 2017). Moreover, other studies have shown β cells can lose their phenotype and become dedifferentiated and dysfunctional but could perhaps be redifferentiated.

First, the Herrera lab used their model of near total β cell ablation by injections of diphtheria toxin in RIP:DTR (β-cell expression of diphtheria toxin receptor HB-EGF) transgenic mice. Over 10 months with the first 5 months animals were treated with insulin to maintain blood glucose levels below 20 mM, β cells regenerated to about 10% of mass of the controls (Thorel et al., 2010). All islets were involved, and there were no extrainsular β cells that would suggest neogenesis. Surprisingly, lineage tracing showed when the ablation occurred in adult mice there was α,-to-β cell conversion rather than an expansion of the few residual β cells. Most of the β cells also expressed glucagon. However, in juvenile mice (before puberty) no α,-to-β cell conversion was seen but rather β cells derived from δ cells involving dedifferentiation, proliferation and re-expression of islet developmental genes in a forkhead box protein O1 (FoxO1)-dependent process (Chera et al., 2014). These rigorous experiments were the first to clearly show the plasticity of the pancreatic endocrine cells. The hope of such α- to-β cell conversion occurring in humans has led to studies searching for bihormonal cells in human pancreas. In the several autopsy studies published, only 3–4 % islet cells coexpress both insulin and glucagon were found in T2D pancreas with some aberrant transcription factor expression (Butler et al., 2013) ;(Spijker et al., 2015). In surgical specimens from non diabetic subjects, insulin-resistant subjects had higher frequency of bihormonal cells than in insulin-sensitive subjects (Mezza et al., 2014). Yet these human findings may reflect a dedifferentiation of the β cells rather than their transdifferentiation from another endocrine cell type; dedifferentiation of β cell will be discussed below.

Secondly, a series of papers from Collombat (Collombat et al., 2009);(Al-Hasani et al., 2013) (Courtney et al., 2013) showed that by *in vivo* ectopic expression of *Pax4* or inhibition of transcription factor *Arx*- expression in α cells resulted in their conversion to β cells. Importantly, these neo-generated β-like cells were functional and could reverse chemically-

induced diabetes even after repetitive ablations of the β cells. Moreover, a recent study identified GABA as an inducer of this α-to-β-like cell conversion in mice and in human islet transplanted under the kidney capsule of immuno-incompetent mice (Ben-Othman et al., 2017). The role of GABA had been suggested in expansion of the β cells earlier in a provocative study (Soltani et al., 2011) in which GABA treatment led to improved glucose levels in autoimmune diabetic NOD mice and the reversal of diabetes in a multiple low dose streptozocin diabetes mouse model. A particularly exciting complementary finding was that artemisins, already used for malaria treatment, caused both the translocation of ARX from the nucleus to the cytoplasm, thus inhibiting its function for maintenance of the α cell identity, and the stabilization of gephyrin which enhances GABA receptor signaling (Li et al., 2017). Treatment *in vitro* of human islets with artemisins, led to improved glucose-stimulated insulin release and changes in gene profiles that suggest the α-to-β transdifferentiation seen in mice. These data provide a possible unprecedented β-cell regeneration strategy using a known and approved therapeutic agent; we expect clinical trials will be coming.

A provocative proposal was made that dedifferentiation of β cells was quantitatively more important than actual β cell death/loss in diabetes and that redifferentiation of these β cells might be a reasonable therapy (Talchai et al., 2012). This proposal from the Accili group was based on their findings that β-cell ablation of the transcription factor FoxO1 in mice resulted in chronic hyperglycemia and a reversion of β cells to a progenitor state with a subset adopting an α cell phenotype (Talchai et al., 2012). Yet the term “dedifferentiation” can be more than the loss of hormone protein expression (Weir et al., 2013). In a series of papers in rats we showed that chronic hyperglycemia, even moderate (10–20 mg/dl), resulted in β cell dysfunction and change of phenotype (Jonas et al., 1999; Laybutt et al., 2003; Laybutt et al., 2002). The gene expression changes, including the loss of β cell identity gene expression and the gain of disallowed gene expression, were reversed with normalization of the glycemic levels after 2 weeks of hyperglycemia and partially after 12 weeks. Similarly, after induced transgene expression of a mutated $K_{ATP}$ channel in β cells, hyperglycemia rapidly ensued leading to dedifferentiation and degranulation of the β cells (Brereton et al., 2014; Wang et al., 2014). Treatment with insulin or glibenclamide to maintain normoglycemia prevented the dedifferentiation, and if treatment was started after 4 wks, the β cells recovered function and granulation. Together these data would suggest that the maintenance of the glycemic levels *in vivo* may preserve the functionality of the β cells, but it is not clear yet if there is a limit to the length of time of dedifferentiation of β cells after which reversal is not possible. The Accili group extended this concept to humans with type 2 diabetes (T2D), reporting 16.8% $hormone^{negative}synaptophysin^{positive}$ (ie. dedifferentiated) islet cells in the pancreas of type 2 diabetes subjects, with a large number of these having misexpression of key transcription factors and “no loss of cells with general endocrine features”(Cinti et al., 2016). (Synaptophysin and chromogranin are pan-endocrine makers that have overall equivalent expression in the different islet endocrine cells and can identify an endocrine cell that has lost its hormone expression). They concluded that the 30–50% deficit of β cells seen in T2D (Butler et al., 2003; Rahier et al., 2008; Yoon et al., 2003) was not due to death but dedifferentiation or transdifferentiation of β cells to other islet types. In contrast, the Butlers found that $chromogranin^{positive}hormone^{negative}$ (ie. dedifferentiated)

islet cells could account for no more than 2% of the deficit of β cells in T2D and suggested that instead these hormone$^{negative}$ cells may reflect an attempted regeneration (Butler et al., 2016; Md Moin et al., 2016).

## CONCLUSIONS

There have been great advances in our understanding and enhancement of regenerative strategies of β cells. The major pancreatic cell types (islet, acinar and duct) have been shown to have a degree of plasticity that could lend them to being expanded and differentiated to new β cells. There are recent encouraging findings that suggest endogenous replenishment may be possible in humans. Yet, it is unclear how much enhanced replication or transdifferentiation/neogenesis could be achieved *in vivo* in humans; it is likely that a combination of both processes will be needed to restore normoglycemia. Mechanistic and developmental studies in rodents have laid the foundation for identifying small molecules and drugs already approved for their medical use that could enhance both regenerative pathways. Even so, a major question remains as how the interventions to expand β cell mass could be specifically targeted to β cells.

There are other major obstacles that need to be overcome in type 1 and type 2 diabetes. Of foremost importance, the autoimmune attack characteristic of type 1 diabetes needs to be addressed so that regenerative strategies can have a long lasting beneficial effect. There have only been a few studies that have tried to replenish β cells in autoimmune diabetic NOD mice. One successful study combined anti-CD3/CD8 conditioning regimen with treatment with gastrin and EGF to reverse diabetes in NOD mice. Both β cell neogenesis and replication were enhanced (Wang et al., 2012; Zhang et al., 2016). For type 2 diabetes, it remains to be determined whether the pancreatic cells are still receptive to growth, expansion and differentiation techniques. If the cells have already become senescent, it is unlikely that they could be activated to regenerate unless the senescence pathway is directly targeted.

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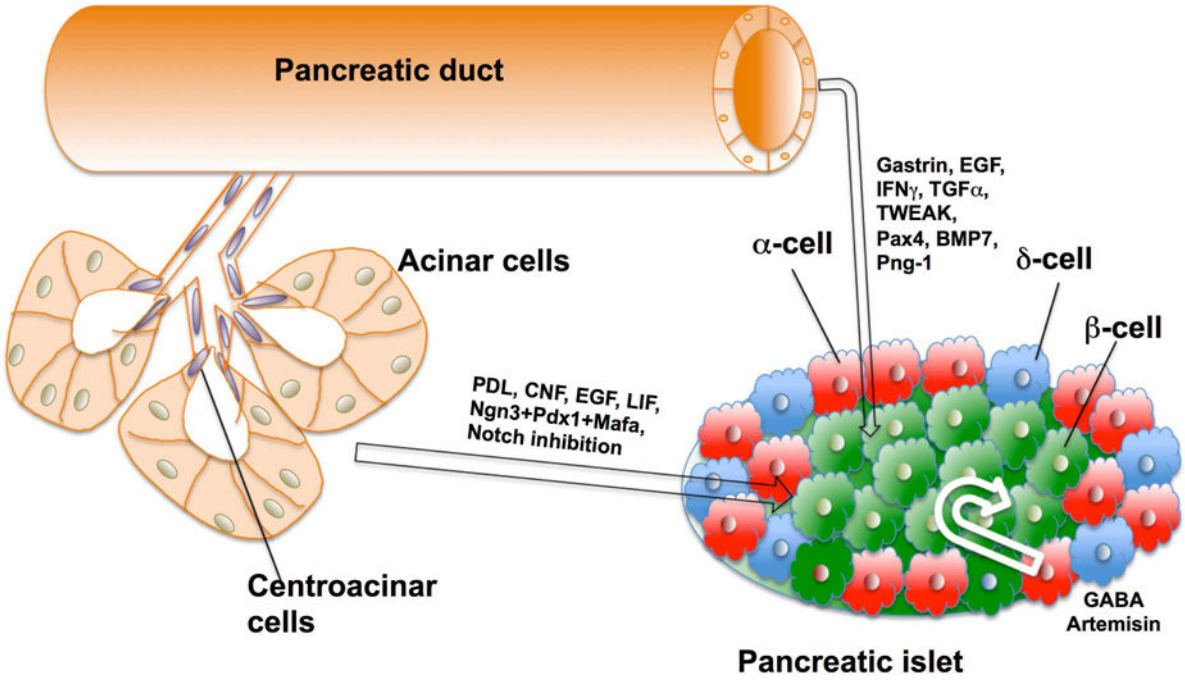


**Figure 1.**

Cell sources within the pancreas that have been shown to differentiate into β-cells. The strategies used to induce this transdifferentiation are indicated within the arrows. For a complete description of these approaches refer to the text.

**Table 1**

Strategies to induce beta cell replication

| | Rodents | Human islets | References |
|---|---|---|---|
| **KO Cyclin D2** | Effective | NA | Georgia et al. 2010 |
| **Increase Ezh2** | Effective | NA | Zhou et al. 2013 |
| **Senolytic therapies** | TBD | TBD | Palmer et al. 2015 |
| **Glycolytic flux and glucokinase activator.** | Effective | Led to DNA damage, growth arrest and apoptosis. | Salpeter et al. 2010, 2011, 2013; Porat et al. 2011; Rieck et al. 2012; Tornovsky-Babeay et al. 2014 |
| **Adenosine kinase inhibitors** | Effective | Not effective | Annes et al. 2012 |
| **Silencing of CDKN2C/p18 or CDKN1a/p21** | TBD | Effective | Robitaille et al. 2016 |
| **Harmine** | Effective | Effective (also induces α-cell proliferation) | Wang P et al. 2015, Wang YJ et al 2016 |
| **Aminopyrazine** | Effective | Effective | Shen et al. 2015 |
| **SerpinB1** | Effective | Effective | El Ouaamari et al. 2016 |

**Table 2**

Markers of ductal progenitors

| Markers | Rodent | Humans | References |
|---|---|---|---|
| **CD133** | ✓ | ✓ | (Gomez et al., 2015; Jin et al., 2016; Lee et al., 2013; Sugiyama et al., 2007a) |
| **CD49f$^{high}$** | ✓ | ✓ | Sugiyama et al., 2007 |
| **Dclk1** | ✓ | ✓ | Westphalen et al., 2016 |
| **ALDH1$^{+}$ CAC** * | ✓ | **TBD** | Rovira et al., 2010 |

* CAC-centroacinar cells