β-cell replacement sources for type 1 diabetes: a focus on pancreatic ductal cells

Elisa Corritore, Yong-Syu Lee, Etienne M. Sokal and Philippe A. Lysy

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***Abstract*:** Thorough research on the capacity of human islet transplantation to cure type 1 diabetes led to the achievement of 3- to 5-year-long insulin independence in nearly half of transplanted patients. Yet, translation of this technique to clinical routine is limited by organ shortage and the need for long-term immunosuppression, restricting its use to adults with unstable disease. The production of new *bona fide* β cells *in vitro* was thus investigated and finally achieved with human pluripotent stem cells (PSCs). Besides ethical concerns about the use of human embryos, studies are now evaluating the possibility of circumventing the spontaneous tumor formation associated with transplantation of PSCs. These issues fueled the search for cell candidates for β-cell engineering with safe profiles for clinical translation. *In vivo* studies revealed the regeneration capacity of the exocrine pancreas after injury that depends at least partially on facultative progenitors in the ductal compartment. These stimulated subpopulations of pancreatic ductal cells (PDCs) underwent β-cell transdifferentiation through reactivation of embryonic signaling pathways. *In vitro* models for expansion and differentiation of purified PDCs toward insulin-producing cells were described using cocktails of growth factors, extracellular-matrix proteins and transcription factor overexpression. In this review, we will describe the latest findings in pancreatic β-cell mass regeneration due to adult ductal progenitor cells. We will further describe recent advances in human PDC transdifferentiation to insulin-producing cells with potential for clinical translational studies.

Keywords: β cells, diabetes, differentiation, duct cells, insulin, progenitors

Introduction

Diabetes is a metabolic disorder occurring when the pancreas does not produce enough insulin or when the body does not properly use the released insulin. The common effect is a chronic hyperglycemia that leads to a long-term risk of micro- and macroangiopathy. The worldwide incidence of diabetes is steadily increasing and the World Health Organization estimated that about 552 million people will be affected by the disease by 2030 [Ding *et al.* 2013].

Type 1 diabetes (T1D) affects 5–10% of all patients with diabetes and usually develops in children and young adults. This disease is characterized by progressive destruction of pancreatic insulin-producing β cells provoked by a B- and T-lymphocyte-dependent autoimmune assault [Atkinson *et al.* 2011]. Although the origins of the causative autoimmune reactions are still uncertain [Ludvigsson, 2013], several studies have shown correlations between T1D onset and environmental factors such as enteroviral infections [Krogvold *et al.* 2015], early infant nutrition [Mayer-Davis *et al.* 2013], or vitamin D deficiency [Mathieu, 2015]. The genetic association between human leucocyte antigen (HLA) and the onset of T1D has also been confirmed by many studies since the 1970s. About 50% of the risk for developing T1D is explained by the polymorphism of more than 50 different HLA loci [Lysy, 2014]. Most of these genes are involved in immune regulation and were associated with the risk of developing other autoimmune disorders such as celiac disease, systemic lupus erythematosus and multiple sclerosis [Noble, 2015]. Current treatments for T1D are primarily focused on insulin supplementation that improves glucose

Correspondence to:
Philippe A. Lysy, MD, PhD
Institut de Recherche Expérimentale et Clinique, Pediatric Research Laboratory, Université Catholique de Louvain, Brussels, Belgium
philippe.lysy@uclouvain.be

Elisa Corritore, PhD student
Yong-Syu Lee, PhD student
Etienne M. Sokal, MD, PhD
Institut de Recherche Expérimentale et Clinique, Pediatric Research Laboratory, Université Catholique de Louvain, Brussels, Belgium

homeostasis but fails to achieve treatment targets for many patients [Lind *et al.* 2014].

Pancreas and islet transplantation

In this context, the replacement of functional β cells would be the only cure for patients with T1D, as demonstrated by the accumulated experience in whole pancreas [Niederhaus, 2015] and human islet transplantation [Shapiro and Lakey, 2000; Bellin *et al.* 2012; Barton *et al.* 2012]. Currently, more than 13,000 patients with diabetes mellitus have benefited from a successful pancreas (i.e. pancreas alone, or pancreas-after-kidney) transplantation [Kerr *et al.* 2015] and graft survival improved by up to 81.5% 1 year after organ transplant [Kaufman, 2015]. This procedure is often proposed to patients with severe renal failure requiring concomitant kidney transplant [Johannesson *et al.* 2015]. Despite its curative potential, pancreas transplantation remains a difficult procedure with significant morbidity and mortality (22% mortality rate 10 years after transplant), and with limitations associated to organ shortage [Kandaswamy *et al.* 2016].

Human islet isolation was developed to provide patients with a minimally invasive cell-replacement protocol, and functionality of transplanted islets was greatly improved in the last decade [Bruni *et al.* 2014]. A review by Barton and colleagues from the Collaborative Islet Transplant (CIT) Registry showed insulin independence during 3 years after human-islet transplantation in about 44% of patients [Barton *et al.* 2012]. Recently, Brennan and colleagues showed functional islet engraftment and glucose tolerance 54 months after transplantation under the Edmonton protocol, in patients followed up for 12 years and treated with tacrolimus and sirolimus or mycophenolate mofetil [Brennan *et al.* 2016]. The development of a new immunosuppressive regimen that combined sirolimus and tacrolimus with classical drugs such as daclizumab and etanercept in addition to granulocyte-colony stimulating (G-CSF) and exenatide showed prolonged graft function in 70% of the patients for about 12 years [Inverardi, 2015]. Previously, Long and colleagues showed the efficiency of rapamycin combined with interleukin-2 (IL-2) as treatment for autoimmune diabetes [Long *et al.* 2012]. Indeed, nine diabetic patients in a phase I clinical trial were treated with this cocktail (administration of rapamycin for 3 months and IL-2 for 1 month) and showed effective augmentation of Treg cells. However, transient β-cell dysfunction, decreased levels of C-peptide and negative effects on β-cell regeneration represented the greatest limitations of this combinatorial therapy.

Despite significant improvements, islet transplantation still remains limited by the shortage of cadaveric donors, the necessity for long-term immunosuppression, restricting its indication for adults with unstable diabetes (e.g. recurrence of severe hypoglycemias, intractable glucose variability, diabetes-related complications), due to the complexity of cell isolation protocols and islet viability after cryopreservation. Although Manning Fox and colleagues recently showed that human islets cryopreserved for 20 years maintained correct purity, functional intracellular Ca^{2+} influx and glucose-stimulated insulin secretion (GSIS), these stored cells had decreased levels of insulin content and lower abilities to reverse diabetes in a mouse model as compared with fresh islets [Manning Fox *et al.* 2015].

Pluripotent stem cells

These difficulties fostered the finding of new cell sources with potential to provide large supplies of insulin-producing cells for clinical settings. Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) represent the most widely investigated candidates for *in vitro* reprogramming purposes because of their theoretically unlimited proliferation and high differentiation potential [Lysy *et al.* 2012]. Over the last 10 years, researchers designed protocols for differentiation of pluripotent stem cells (PSCs) that mimicked pancreas embryogenesis by using cocktails of molecules with specific influences on key intracellular pathways [Blyszczuk *et al.* 2004; Ku *et al.* 2004; Schroeder *et al.* 2006; Schiesser and Wells, 2014]. Combinations of activin A, retinoic acid, transforming growth factor (TGF) β, nicotinamide, bone morphogenetic protein (BMP), keratinocyte growth factor (KGF), cyclopamine, basic fibroblastic growth factor (bFGF), Noggin, wingless-type mouse mammary tumor virus (MMTV) integration-site family-member 3a (Wnt3a), sonic hedgehog pathway antagonist (SANT1) and insulin were used to induce the differentiation of ESCs toward pancreatic endocrine precursors through incubations of 3–4 weeks [Ku *et al.* 2004; Yasunaga *et al.* 2005; D'Amour *et al.* 2006; Kroon *et al.* 2008; Rezania *et al.* 2012]. During differentiation, ESCs showed acquisition of typical gene profiles

of pancreatic stepwise development: expression of *OCT4*, *SOX2*, and *Brachyury* followed by activation of *PDX1*, *NGN3*, *NKX6.1* until final expression of key β-cell markers such as *MAFA*, *PAX4* and *insulin* [D'Amour *et al.* 2006; Jackson *et al.* 2010; Hrvatin *et al.* 2014]. In 2006, D'Amour and colleagues made great advances by obtaining ESC-derived β-cell populations containing up to 12% of insulin$^+$ cells after *in vitro* stepwise protocols [D'Amour *et al.* 2005, 2006]. These cells completed their reprogramming into functional insulin-producing derivatives 3–4 months after transplantation into rodents [D'Amour *et al.* 2006; Kroon *et al.* 2008; Rezania *et al.* 2012]. Yet PSCs lacked essential properties of *bona fide* β cells, including GSIS [Nostro *et al.* 2011; Kelly *et al.* 2011; Bruin *et al.* 2014; Hrvatin *et al.* 2014]. Recently, two groups [Pagliuca *et al.* 2014; Bruin *et al.* 2014] demonstrated the possibility of obtaining functional differentiation of human ESCs *in vitro* with insulin secretion patterns close to human islet cells; yet only the Rezania and Pagliuca teams described the attainment of monohormonal ESC-derived β cells after implantation under the kidney capsule of diabetic mice [Pagliuca *et al.* 2014; Rezania *et al.* 2014]. In these studies, human insulin was detected in the mouse bloodstream with subsequent glycemic normalization within 40 days post transplantation. These data are putting PSCs closer to potential exploitation in the clinical setting.

Tumor formation remains a major concern in the use of ESCs, fetal stem cells and iPSCs for transplantation studies. Unwanted 'off-target' mesodermal formation was reported with human ESCs in transplantation sites [Kroon *et al.* 2008; Rezania *et al.* 2012]. Many groups have tried selection of ESC-derived endocrine cells [Kelly *et al.* 2015; Jiang and Morahan, 2015] or the use of micro- and macroencapsulation devices [Motte *et al.* 2014; Agulnick *et al.* 2015; Song and Roy, 2015] such as the Theracyte^{TM} (Inc. manufactures, Laguna Hills, California) system to circumvent the undesired cell growth, but also to promote angiogenesis of the cells and to protect those from the host immune system [Pepper *et al.* 2015]. PSC-derived functional insulin-producing cells were recently injected into a Theracyte-based device (called Encaptra^{TM}, San Diego, California) and subsequently transplanted subcutaneously [Agulnick *et al.* 2015]. Furthermore, this system permitted the survival of mouse neonatal pancreatic tissue maintaining euglycemia up to 95 days post-transplant in diabetic RIP-LCMV.GP mice [Boettler *et al.* 2015]. Clinical trials in Phase I/II [ClinicalTrials.gov identifiers: NCT02239354 and NCT01996228] are currently assessing long-term tolerability and efficacy of this system, with results eagerly awaited for. Very recently, alginate-based encapsulation was used to intraperitoneally transplant human ESC-derived β-like cells into immunocompetent mice [Vegas *et al.* 2016]. Grafts retained functionality at 174 days of follow up with significant effects on glucose homeostasis that mirrored C-peptide secretion levels. These important results bring additional evidence of the translational capacity of human ESCs.

Principal modes of β-cell regeneration

Whether progenitors or stem cells reside in the adult human pancreas or not is still unknown, but regeneration of pancreatic compartments were described after birth and after injury [Chintinne *et al.* 2010; Li *et al.* 2010; Nakamura *et al.* 2011]. The principal mechanisms investigated to explain β-cell regeneration within the pancreas include: neogenesis (differentiation of islet cells from facultative pancreatic progenitors), replication of pre-existent β cells and transdifferentiation (being the conversion from one cell type to another) [Bonner-Weir *et al.* 2010, 2012].

Neogenesis

Neogenesis was largely considered the main process responsible for embryonic development, however, its involvement in the adult pancreas is still under investigation [Bonner-Weir *et al.* 2012]. Numerous studies describe the existence of a reservoir of progenitor cells within the human exocrine pancreas. The involvement of PDCs as a niche of committed progenitors in the adult pancreas will be discussed below.

Acinar cells represent the major population in the human pancreas; therefore particular attention was paid to their role as progenitors [Houbracken *et al.* 2010; Mfopou and Bouwens, 2013; Baeyens *et al.* 2014; Pin *et al.* 2015]. The conversion of acinar into endocrine fates was previously reported by lineage-tracing studies, showing formation of ectopic insulin+ cells derived from acinar cells after suppression of *Ptf1a* in zebra fish [Hesselson *et al.* 2011]. Controversy regarding the *in vivo* plasticity of adult acinar was generated by a study showing the lack of acinar-to-β

transdifferentiation following 70% pancreatectomy (Px), pancreatic duct (PDL), and cerulein-induced pancreatitis in mice [Desai *et al.* 2007]. In 2008, Zhou and colleagues showed the direct reprogramming of exocrine cells into β cells after *in-vivo* overexpression of PDX1, NGN3, and MAFA [Zhou *et al.* 2008]. Adenoviral vectors containing the sequences of the three transcription factors (TFs) (and green fluorescent (GFP) as reporter) were transduced in exocrine cells of *Rag1*$^{-/-}$ diabetic mice. Only 10 days after infection, new insulin^{+}/GFP^{+} cells expressing the hallmarks of *bona fide* β cells were observed. Streptozotocin (STZ)-treated animals showed significant decrease of hyperglycemia without complete disease reversal. These results were confirmed by Cavelti-Weder and colleagues, who showed extensive periods (up to 13 months) of euglycemia in two of the TF-transduced diabetic animals [Cavelti-Weder *et al.* 2015]. Similarly, a cytokine-based protocol [containing epidermal growth factor (EGF) and ciliary neurotrophic factor (CNTF)] was developed to circumvent the use of adenoviruses for acinar-to-β transdifferentiation [Bonfanti *et al.* 2015]. In this study, alloxan-induced diabetic mice received EGF and CNTF *via* intraperitoneal osmotic pumps that allowed 65% of animals to increase their levels of serum insulin and normalize their glycemia within 5 days. Acinar cells were detected to be at the origin of the new β cells *via* lineage-tracing experiments. Together, these works provide evidence for a major potential of acinar cells for designing *in situ* transdifferentiation protocols.

Replication

The other mechanism thought to be at play in endogenous replenishment of pancreatic tissues is replication of pre-existing β cells. β-cell proliferation in both human and murine models is normally controlled by activation of proteins that regulate the cell cycle [Vetere *et al.* 2014]. For example, it has been shown that p16, p27, cyclins D1, D2, and D3 have a central role in pancreas regeneration controlling the replicative potential of the β-cell mass. Many efforts were made to identify compounds that could stimulate β-cell proliferation. An increase of about 50% of the β-cell mass and parallel decrease of hyperglycemia in mice was observed after 6 weeks of treatment with WS6 molecule without affecting their differentiation or viability [Shen *et al.* 2013; Boerner *et al.* 2015]. High-throughput screening of more than 850 compounds led to the identification of other molecules such as 5-iodotubercidin and ABT-702, capable of stimulating β-cell expansion in rodent and porcine islets [Annes *et al.* 2012]. Intraperitoneal injections of these adenosine kinase inhibitors in wild-type mice promoted β-cell turnover within 24 hours, as demonstrated by the increase of PDX1^{+}/insulin^{+} cells coexpressing Ki-67 while no replication was found in the exocrine compartment.

Although the replication potential of rodent β cells was observed, very little evidence of β-cell proliferation was provided in the human pancreas [Meier *et al.* 2008]. In this context, Russ and colleagues, in 2011, described an *in-vitro* system to dedifferentiate, expand and redifferentiate human β cells with the aim of obtaining a large supply of functional insulin-producing cells [Russ *et al.* 2011]. The dedifferentiating β cells acquired mesenchymal markers, suggesting epithelial-mesenchymal transition (EMT). Furthermore, the maintenance of epigenetic memories allowed fast redifferentiation of β-cell-derived cells into new insulin^{+} cells, using a combination of soluble factors that included glucose, nicotinamide, exendin-4, and activin A. About 40% of C-peptide^{+} cells were observed in these EMT populations after 8 days of incubation with these molecules. When EMT-derived β-like cells generated from Notch inhibition protocols were transplanted under the kidney capsule of STZ-treated NOD-SCID mice, Efrat's group observed a decrease of blood glucose levels and the detection of human C-peptide [Bar *et al.* 2012]. Collectively, these findings proposed a new system for expansion and generation of a source of functional β cells starting from primary cultures of adult human islets.

Transdifferentiation

The third mechanism evaluated to explain β-cell mass replenishment is the transdifferentiation of pancreatic cells into β cells. Using a transgenic mouse model, Collombat and colleagues, in 2009, described the conversion of glucagon^{+} cells into functional β cells after overexpression of PAX4 [Collombat *et al.* 2009] or selective inhibition of aristaless related homeobox (ARX) in α cells [Courtney *et al.* 2013]. In both studies, *in vivo* overexpression of PAX4 or misexpression of ARX was sufficient to generate new functional β cells capable to revert STZ-induced diabetes. In parallel, it was observed that the lack of glucagon^{+} cells triggered an endocrine-specification

program occurring after re-expression of NGN3$^+$ in 'progenitors' residing in the duct epithelium. This shift of endocrine phenotypes was also observed after diphtheria toxin-induced α-cell death [Thorel *et al.* 2010]. Other groups reported the *in vitro* conversion from α to β cell under specific conditions [Fomina-Yadlin *et al.* 2010; Yang *et al.* 2011; Zhang *et al.* 2015], but at rather low yields. Also, the question remains as to whether these phenomena could take place in humans and how this potential could be translated into pharmacological protocols [Courtney *et al.* 2011; Napolitano *et al.* 2015; Vieira *et al.* 2013; Collombat *et al.* 2010].

Progenitor cells

Theoretically, pancreatic epithelial cells present valuable properties for β-cell reprogramming since they all arise from the same embryonic precursor; the branching morphogenesis of the pancreas, depending on the ductal system, gives rise to all exocrine and endocrine lineages [Pan and Wright, 2011; Lysy *et al.* 2013] under specific signaling instructions. Pancreas organogenesis is controlled by the embryonic mesoderm that releases molecules such as fibroblastic growth factors (FGFs), retinoic acid, BMPs, Wnt, Sonic Hedgehog, and Notch proteins [Wandzioch and Zaret, 2009; Mfopou *et al.* 2010; Nostro *et al.* 2011; Marquez-Aguirre *et al.* 2015]. Moreover, both exocrine and endocrine compartments are characterized by local expression of specific markers and TFs. For example, Lemaire and colleagues showed that bicaudal C1 (BICC1), a protein normally in duct lining during embryogenesis, is essential for ductal morphogenesis and subsequent progenitor differentiation [Lemaire *et al.* 2015]. Pancreatic sections of BICC1$^{-/-}$ mice showed a 34% decrease of NGN3-expressing endocrine progenitors within the ductal compartment. BICC1 shutdown affected normal tissue formation: the endocrine mass was decreased by 50% and duct cell overproliferation led to cystic dysplasia of the main and interlobular ducts. These results illustrate the possibility to fate-map molecular signals of pancreas morphogenesis.

In vivo studies on pancreatic progenitors

Postnatal *in vivo* experiments performed using several murine pancreatic injury models (e.g. partial Px, cellophane wrapping, duct ligation or intraperitoneal cerulein injection [Rosenberg, 1995; De Breuck *et al.* 2006; Sakaguchi *et al.* 2006; Bonner-Weir *et al.* 2008]) revealed partial tissue regeneration from PDCs (Table 1) expressing PDX1, NGN3, HNF1β, with or without SOX9. The Inada group observed β-cell mass replenishment after PDL in carbonic anhydrase II (CAII)-Cre transgenic mice, which were subsequently mated with the strain ROSA26 loxP-Stop-loxP LacZ (R26R) to trace the progeny of ductal cells [Inada *et al.* 2008]. Two weeks after PDL, mice had 12.1 ± 1.9% of β-galactosidase$^+$ β cells in nonligated portions and 23.6 ± 2.2% in the ligated ones, compared with 5.5 ± 2.0% in control mice. These results suggested, at least partially, the ductal origin of the new β cells. To investigate the role of putative progenitors in pancreas regeneration, Criscimanna and colleagues generated a mouse model of restricted diphtheria-toxin (DT)-receptor expression for induction of global pancreatic damage after toxin injection [Criscimanna *et al.* 2011]. Extensive and selective ablation of elastase$^+$ acinar and PDX1$^+$ endocrine cells were induced in R6DTR/CRE backcrossed animals. Within 3 to 4 weeks after DT injection, highly proliferative ducts contained cells coexpressing amylase, NGN3, insulin, and glucagon. These findings suggested that the global pancreatic damage provoked by DT injection stimulated the differentiation of cells located in the ductal compartment toward acinar and endocrine cells. Recently, lineage-tracing experiments showed duct-to-β-cell conversion after partial Px in normal young developing mice and in adult mice with TGF-β receptor overexpression [El-Gohary *et al.* 2016]. Interestingly, this study showed prolongations of the ductal tree inside surrounding islets, at least in young mice and humans. This feature was not observed in adult mice after injury, but well in TGF-β-receptor-mutant animals, showing the implication of this pathway (implicated in EMT) in endocrine reprogramming of the ducts in adult tissues. These results corroborate previous reports about the requirement for duct-lining cells to enter a state of EMT before endocrine reprogramming can occur (see below) [Pfeifer *et al.* 2013].

The identification of factors with potential to activate β-cell regeneration from progenitors would be a compelling approach to cure diabetes [Piran *et al.* 2014; Riley *et al.* 2015; Dominguez-Bendala *et al.* 2016]. Induction of pancreatic neogenesis in the postnatal pancreas after administration of growth factors *in vivo* was evaluated in murine models using glucagon-like peptide 1 (GLP-1)/

Table 1. Evidence of pancreatic ducts as a source of progenitor cells.

Reference	Research model	Evidence
Suarez-Pinzon *et al.* [2005]	*In vivo*	Treatment with EGF promoted β-cell neogenesis
Xu *et al.* [2006]	*In vivo*	Treatment with exendin-4 facilitated β-cell neogenesis in pancreatic ducts
Tokui *et al.* [2006]	*In vivo*	Betacellulin promoted ductal differentiation towards insulin-expressing cells
Inada *et al.* [2008]	*In vivo*	β-cell mass replenishment occurred from ductal cells 2 weeks post PDL
Bonner-Weir *et al.* [2008]	*In vivo*	Partial pancreatic tissue regeneration occurred from ductal cells
Xu *et al.* [2008]	*In vivo*	Re-expression of NGN3 in ductal lining cells occurred 1 week after PDL
Criscimanna *et al.* [2011]	*In vivo*	Proliferative ducts expressed acinar and endocrine markers 3/4 weeks post-DT injection
Wang *et al.* [2012]	*In vivo*	Administration of gastrin and EGF increased β-cell neogenesis
Pfeifer *et al.* [2013]	*In vivo*	Requirement of EMT for duct-lining cells differentiation into endocrine cells
Courtney *et al.* [2013]	*In vivo*	Continuous activation of $NGN3^+$ cells along the lining duct occurred after ARX inhibition
Téllez *et al.* [2014]	*In vivo*	Appearance of β-cell clusters from ductal epithelium occurred 3 days post 90% Px
Lemaire *et al.* [2015]	*In vivo*	Bicaudal C1 expressed in duct lining cells was essential for progenitor differentiation
Yamaguchi *et al.* [2015]	*In vivo*	Pancreatic ductal glands contained niches of progenitor cells
Carpino *et al.* [2015]	*In vivo*	Pancreatic ductal glands as container of niches for pancreatic precursors
Napolitano *et al.* [2015]	*In vivo*	Continuous activation of $NGN3^+$ cells along the lining duct occurred after PAX4 overexpression
El-Gohary *et al.* [2016]	*In vivo*	Duct-to-β-cell conversion occurred after partial Px in mice overexpressing TGF-β receptor
Zhang *et al.* [2016]	*In vivo*	Conversion of $SOX9^+$ cells into insulin-producing cells occurred after administration of gastrin and EGF
Seaberg *et al.* [2004]	*In vitro*	Progenitor colonies arose from islet and ducts cultured with EGF and FGF2
Rovira *et al.* [2010]	*In vitro*	Centroacinar/terminal ductal cells with progenitor-like phenotype

ARX, aristaless related homeobox; DT, diphtheria toxin; EGF, epidermal growth factor; EMT, epithelial-mesenchymal transition; PDL, pancreatic duct ligation; Px, pancreatectomy; TGF, transforming growth factor.

exendin-4, betacellulin, gastrin, INGAP, interferon-γ, soybean trypsin inhibitor and TGF-β. These studies are summarized elsewhere [Bonner-Weir *et al.* 2012]. Intraperitoneal injection of exendin-4 in STZ-treated wild-type rats, repeated for 10 consecutive days, induced a significant reduction of blood glucose for more than 2 weeks [Xu *et al.* 2006]. In another study, adenoviral vectors carrying human betacellulin sequences were injected within ducts in imprinting control region (ICR) mice [Tokui *et al.* 2006]. Only 1 week later, betacellulin and insulin-protein expression were observed in ductal cells, and at 2 and 8 weeks postinfection, numbers of duct-lining cells coexpressing insulin increased [Tokui *et al.* 2006]. Because of potential implications of gastrin in β-cell mass regeneration in rodents [Suarez-Pinzon *et al.* 2008; Suissa *et al.* 2013], a recent study evaluated the role of gastrin in promoting β-cell neogenesis from ductal cells in 90% Px rats [Tellez and Montanya, 2014]. In the gastrin-treated mice, strong proliferation activity was

observed in the ductal epithelium 1 day after Px, with cells acquiring expression of β-cell markers such as PDX1, NGN3, NKX6.1, and NeuroD1. Only 3 days after surgery, β-cell clusters and increased β-cell mass appeared next to the ductal epithelium in gastrin-treated animals. However, blood glucose levels were not improved [Tellez and Montanya, 2014].

Since treatment with EGF was reported to induce β-cell neogenesis from adult ductal cells *in vitro* and *in vivo* [Suarez-Pinzon *et al.* 2005; Wang *et al.* 2012], Zhang and colleagues recently investigated the origin of ductal neogenesis in alloxan-treated diabetic wild-type mice subjected to administration of EGF and gastrin for 56 days [Zhang *et al.* 2016]. Lineage-tracing experiments showed that conversion of $SOX9^+$ PDCs into insulin-producing cells occurred in hyperglycemic conditions and that differentiation significantly increased after long-term administration of low-dose gastrin and EGF (GE). Interestingly, about 75% of animals reversed medium (300–450 mg/dl) but not high hyperglycemia (>450 mg/dl), suggesting a dependency on glucose levels of duct-to-β reprogramming.

Controversy arose about spontaneous postnatal duct-to-β cell conversion after several studies reported the complete absence of endocrine regeneration from ductal lineages in early postnatal mouse models [Solar *et al.* 2009; Furuyama *et al.* 2011; Kopp *et al.* 2011; Pan *et al.* 2013; Rankin *et al.* 2013]. Although β cells appear at day 17.5 in the mouse embryo after activation of $NGN3^+$ cells in the vicinity of the ductal epithelium [Pan and Wright, 2011], Kopinke and Murtaugh could not find evidence of postnatal endocrine specification of PDCs in healthy mice after birth [Kopinke and Murtaugh, 2010]. Using a Cre-lox system within *HNF1β* locus to track embryonic and adult ductal cells after PDL in R26R reporter mice, Solar and colleagues found that the development of endocrine cells was strictly dependent on HNF1β expression in the embryos but not in the adult tissues [Solar *et al.* 2009]. Similar findings were obtained in 2011 with $Sox9CreER^{T2}$ transgenic mouse in which NGN3 expression in PDCs triggered *de novo* formation of endocrine and acinar cells before birth, but not postnatally [Kopp *et al.* 2011]. The same year, a study confirmed the absence of endocrine reprogramming of SOX9-expressing cells in adult mice following partial Px, acute pancreatitis, STZ-induced diabetes, or PDL [Furuyama *et al.* 2011]. In 2013, a study showed the lack of significant changes in β-cell mass and insulin content in adult mice 30 days following PDL [Rankin *et al.* 2013] despite massive expression of NGN3 in the ligated portion. To investigate β-cell regeneration after extreme β-cell loss, Cavelti-Weder and colleagues performed PDL in STZ-treated adult rats transplanted with syngeneic islets [Cavelti-Weder *et al.* 2013]. Although acinar cells showed massive regeneration and self-renewal capacities 10 months after surgery, no evidence of significant β-cell replacement was observed in this model. Only few β cells (about 1.5%) could be observed 5 and 10 months after PDL in ligated portions, with low insulin content (0.39 ± 0.1 μg/pancreas).

Existence of stem-cell niches with progenitor potential was recently observed within pancreatic ductal glands (PDGs) [Yamaguchi *et al.* 2015]. PDGs are a newly identified epithelial compartment residing in the mesenchymal structures surrounding pancreatic ducts. Using a mouse model of pancreatitis and BrdU labeling, Yamaguchi and colleagues observed proliferation in PDCs which migrated from the PDGs to the main pancreatic duct 5 days after acute inflammation. Microarrays showed the enrichment of ESC markers, including *SOX2* and *NANOG*, in the PDGs compared with the ductal epithelium. A more extensive anatomical characterization of PDGs and their role as a niche of pancreatic precursors was performed on healthy human pancreata [Carpino *et al.* 2015]. PDGs were identified as heterogeneous populations of $OCT4^-/PDX1^+/SOX9^+$ cells associated with pancreatic ducts and occasionally localized in continuity with islet cluster. A progressive loss of SOX9 expression was detected in PDCs transitioning to the islets; this was interpreted as an indirect demonstration of their commitment to endocrine lineages. Proportion of the duct areas containing PDGs was estimated at 4% in the pancreatic head and in the tail. Few $NGN3^+$ cells were detected in the PDG subpopulation of the main duct but this number increased in interlobular ducts where increased numbers (about 8% of ductal area) of $insulin^+$ or $glucagon^+$ cells were observed. Yet, the function of these PDG-localized insulin-expressing cells remains elusive, in particular, in the context of disease. However, these data suggest the existence of adult-committed progenitors in proximity of intercalated ducts that differ from stem cells and show heterogeneous expression patterns [Yamaguchi *et al.* 2015].

One of the key elements in driving pancreatic progenitors into endocrine lineage is the activation of NGN3$^+$ expression [Desgraz and Herrera, 2009; Desgraz *et al.* 2011]. In a study conducted by Xu and colleagues, who observed an increase of about 40% of the β-cell mass 1 week after PDL in adult mice, re-expression of NGN3 occurred throughout the ductal lining [Xu *et al.* 2008]. Electron microscopy analysis of purified NGN3$^+$ cells showed their structural resemblance to pancreatic progenitor cells in the embryo, whereas their differentiation potential toward functional β cells was reported after microinjection into embryonic NGN3$^{-/-}$ explants. Furthermore, α-to-β cell conversion studies using PAX4 overexpression or ARX inhibition revealed a continuous activation of pancreatic precursors expressing NGN3 that were located along the lining duct [Courtney *et al.* 2011, 2013; Beucher *et al.* 2012; Napolitano *et al.* 2015]. NGN3$^+$ cells differentiated toward α cells to compensate the relative glucagon deficiency. Altogether, these data identified *NGN3* as a pro-endocrine gene involved in a continuous cycle of regeneration from duct-lining cells.

In vitro studies on pancreatic progenitors

Two teams discovered multipotent progenitor cells (PMPs) in the adult mouse and human pancreas with capacities for proliferation and β-cell differentiation [Smukler *et al.* 2011; Razavi *et al.* 2015]. These PMPs were isolated and identified at low frequency (0.02–0.03%) in both nestin$^+$ and nestin$^-$ fractions, obtained following islet and ductal dissociation into single cells. Progenitor colonies arose *in vitro* from islet and ducts cultured at low density with EGF and FGF2 [Seaberg *et al.* 2004]. About 26.3 ± 1.4 % of insulin$^+$ cells deriving from these PMPs were observed in the normal adult pancreas, whereas few cells coexpressing insulin and NGN3 were identified and accounted as possible progenitor populations. The transplantation of PMP spheres under the kidney capsule of diabetic BalbC and NOD-SCID mice significantly improved hyperglycemia and demonstrated GSIS capacities. Immunostaining analysis of the grafts showed clusters of cells coexpressing insulin and glucagon. These studies support the existence within pancreatic ductal tissue of facultative adult progenitors with extensive proliferation and differentiation capacities.

The endocrine differentiation potential of duct-lining cells was also described with centroacinar/terminal ductal (CA/TD) cell [Rovira *et al.* 2010]. CA/TD cells contain high levels of aldehyde dehydrogenase type 1 (ALDH1) enzymatic activity that allows their fluorescence-activated cell sorting (FACS) sorting. These cells show a progenitor-like phenotype characterized by *PTF1a*, *SOX9*, *SCA-1*, *SDF-1*, *c-MET*, and *Nestin* expression. In suspension culture, ALDH1$^+$ cells have self-renewal activity and form 'pancreatospheres' with endocrine and exocrine differentiation capacities. The spheres responded to glucose challenge (0, 5 and 11 mmol/L of glucose) by secreting up to 0.9 ng/ml of C-peptide in culture media. After injection into cultured microdissected embryonic-dorsal-pancreatic buds isolated from E12.5 mouse embryos, 11.7% and 11.6% of ADLH1$^+$ cells expressed glucagon and insulin/C-peptide, respectively. *In vivo*, a massive expansion of CA/TD cells was observed after intraperitoneal injections of mice with low-dose cerulein for 3 consecutive weeks [Strobel *et al.* 2007]. These data suggest the potential application of ADLH1$^+$ cell expansion and transdifferentiation protocols to promote neogenesis *in vitro* or *in vivo*.

In vitro expansion of pancreatic ductal cells

PDCs have other interesting features for β-cell engineering: they represent about 35% of the human pancreatic cell mass and are easily purified; they are resistant to organ enzymatic dissociation and cell isolation procedures, and they attach to plastic. These characteristics offer unique opportunities for testing expansion protocols required to obtain a critical mass of transplantable cells.

Human primary PDCs perform one or two rounds of proliferation when plated into classical culture media (i.e. Connaught Medical Research Laboratories (CMRL) or Dulbecco's Modified Eagle Medium (DMEM)) and rapidly enter cell-cycle arrest if not stimulated [Bonner-Weir *et al.* 2000]. To foster expansion of PDCs, three-dimensional (3D) culture systems were developed using human exocrine preparations and extracellular matrices such as agarose or rat-tail collagen [Kerr-Conte *et al.* 1996], or Matrigel [Bonner-Weir *et al.* 2000]. These studies had in common the development of cystic structures with PDCs increasing their proliferation by a factor of 3–7, and by the budding of endocrine cells coexpressing insulin after more than 1 week in culture. In the mouse, a much higher expansion rate (5×10^5 expansion over 11 weeks) of exocrine tissue was observed after its incorporation in

3D semisolid methylcellulose-based cultures using Matrigel [Jin *et al.* 2014]. In this model, authors added roof-plate-specific spondin 1 (RSPO1), a wingless-int (Wnt) signaling activator *via* ligation of LGR4-6 receptors, to the culture media, which greatly enhanced proliferation rates. Huch and colleagues found similar data with Matrigel-embedded mouse pancreatic-duct fragments incubated with RSPO1, EGF, fibroblastic growth factor (FGF) 10 and nicotinamide [Huch *et al.* 2013]. These authors reported the subpassaging of cyst-like organoids at a 1:4–1:5 ratio weekly for over 10 months, without tumoral formation, chromosomal anomalies or aneuploidy.

Many studies described the use of growth factors such as EGF, hepatocyte growth factor (HGF), KGF or nicotinamide to stimulate human PDC proliferation [Bonner-Weir *et al.* 2000; Rescan *et al.* 2005; Hoesli *et al.* 2012] without successfully avoiding rapid senescence and limited growth. Here again, mouse tissue showed a distinct growth pattern, as shown by Oshima and colleagues, with purified $CD133^{+}$c-Met^{+} PDCs cultured in the presence of HGF, EGF, nicotinamide and dexamethasone [Oshima *et al.* 2007]. These FACS-sorted cells were capable of clonal subculture for 3 months, albeit not to the extent required for massive output of reprogrammable cells. It is noteworthy that most successful protocols for mouse PDC expansion failed to trigger replication of their human counterparts. This may be partly explained by the fact that in human cells, telomere shortening restricts replication and leads to senescence, whereas in mice and rats, telomere length is maintained during subculture [Bonner-Weir *et al.* 2010].

As with many other epithelial cells [Khuu *et al.* 2011; Russ *et al.* 2008], PDCs rapidly lose their phenotype after enzymatic disruption of intracellular junctions and plating in adherent culture [Bonner-Weir *et al.* 2000; Todorov *et al.* 2006]. The loss of E-cadherin precedes mesenchymal marker expression, including N-cadherin and Snail1, the master regulator of epithelial-mesenchymal transition (EMT) [Lamouille *et al.* 2014]. The acquisition of mesenchymal features by PDCs was shown initially on unselected human exocrine cultures [Seeberger *et al.* 2009; Fanjul *et al.* 2010], but this was preceded by demonstrations of EMT with human primary islet cultures [Gershengorn *et al.* 2004; Ouziel-Yahalom *et al.* 2006] that were able to redifferentiate into insulin-expressing cells after an expansion phase of over 65,000-fold. Russ and colleagues further exploited these properties, confirming the model by lineage tracing [Russ *et al.* 2008] and produced highly proliferative β-cell derivatives with potential to reverse hyperglycemia in STZ-treated NOD-SCID mice [Russ *et al.* 2011; Bar *et al.* 2012]. Similarly, the natural process of EMT can be fostered in human PDCs to overcome early senescence of primary cultures. Using FACS-sorted CA19-9^{+} PDCs as per the protocol of Yatoh and colleagues [Yatoh *et al.* 2007], we observed the proliferation of purified cultures using endothelial growth media [Corritore *et al.* 2014]. The cells identified as human duct-derived cells (HDDCs) shifted from a cobblestone-like to a spindle-shaped morphology and massively expanded, such that 100×10^{9} cells could be harvested after 1 month of culture. HDDCs went through an E- to N-cadherin switch, while maintaining low levels of *CK19* and *SOX9* expression. Incubation of fresh CA19-9+ cells with TGFβ inhibitor A-83-01 blocked the appearance of HDDCs, which were not capable of clonal expansion under serial dilution experiments. Culture conditions were defined to recapitulate the pancreas embryonic development using small molecules and growth factors. After incubation with a 14-day stepwise protocol, HDDCs acquired β-cell features, including insulin secretion capacities, yet not to the extent of glucose sensitivity. Ongoing work is evaluating the possibility of bringing HDDCs to functionality and diabetes-reversal potential in animal models (Corritore *et al.* 2016).

In vitro reprogramming of pancreatic ductal cells into β cells

Mouse and human studies on growth-factor-based (e.g. EGF, nicotinamide, exendin-4, FGF-7) [Bonner-Weir *et al.* 2000, 2008; Gao *et al.* 2003; Kikugawa *et al.* 2009], spontaneous [Ramiya *et al.* 2000; Yatoh *et al.* 2007], or *in vivo*-induced [Hao *et al.* 2006] differentiation of PDCs demonstrated the potential of these cells to acquire β-cell-like features with GSIS capacities reported in perifusion models [Gao *et al.* 2003].

Because of the difficulties in obtaining critical amounts of epithelial PDCs *in vitro*, many groups investigated the endocrine differentiation potential of ductal-cell lines, such as PANC-1 cells [Zhou *et al.* 2002; Hardikar *et al.* 2003; Zhang *et al.* 2010] derived from a human sample of pancreatic carcinoma. Similarly, human PDCs immortalized by overexpression of E6/E7 genes

from human papillomavirus were developed as a surrogate for normal PDCs because of similar genotypic and phenotypic profiles [Yang *et al.* 1998; Ouyang *et al.* 2000]. Incubation of these human pancreatic ductal epithelial (HPDE) cell lines with islet neogenesis associated protein (INGAP) favored the upregulation of β-cell markers [Assouline-Thomas *et al.* 2015]. After short exposure of a week with INGAP, HPDE cells showed the capacity to secrete insulin in response to glucose, albeit the values observed in basal (5.8 mmol of glucose) and stimulated conditions (25 mmol of glucose) were not statistically significant. Interestingly, an increase of glucagon, somatostatin, and pancreatic polypeptide (PPY) gene expression was also detected at day 7 of the incubation period, suggesting the induction of an endocrine program elicited by INGAP. Nevertheless, the outcome of similar differentiation protocols in unmodified primary cultures is still doubtful.

In parallel with studies using cell lines, the β-cell differentiation capacities of the pancreatic exocrine tissue was also investigated, with leaders in the field being Bouwen's and Baeyens's groups [Baeyens and Bouwens, 2008; Bouwens *et al.* 2013] that accumulated *in vitro* studies both on murine and human tissues. Recent developments in their work showed the potential to induce NGN3 expression in human exocrine cells by lentiviral-induced overexpression of mitogen-activated protein kinase, and signal transducer and activator of transcription 3 [Lemper *et al.* 2015]. The NGN3-expressing cells entered an endocrine program and specified into functional β cells 42 days after transplantation into normoglycemic immunocompromised mice. Although elastase 2A-expressing acinar cells were shown as the parent of insulin$^+$ cells, a state of acinar-to-ductal dedifferentiation was observed before endocrine reprogramming, as described earlier with human cells [Houbracken *et al.* 2011; Staels *et al.* 2015]. This particularity of a ductal intermediate was already suggested *in vitro* by Gomez and colleagues [Gomez *et al.* 2015], who showed the possibility to FACS-sort NGN3$^+$ cells from human exocrine tissue cultures based on their selective coexpression of CD133, expressed on the apical surface of CA19–9+ PDCs. Interestingly, these NGN3$^+$CD133$^+$ duct-lining cells, which resembled endocrine progenitors in their expression profile, had features of both acinar and ductal lineages, confirming the continuum between those two exocrine compartments, at least in culture. Similar duct-lining cells coexpressing NGN3 and duct markers (e.g. osteopontin or HNF1β) were purified from adult mice subjected to pancreas injury [Xu *et al.* 2008] or to genetic modifications to foster α-to-β cell transdifferentiation [Al-Hasani *et al.* 2013]. How these cells participate in the endocrine reprogramming potential of the exocrine compartment is unclear since *in vitro* studies involved *ipso facto* unselected tissues. A recent study by Klein and colleagues using cultures of human islet-depleted exocrine tissue showed that acinar (elastase 3a$^+$) and ductal (CAII$^+$) cells participated poorly to bone morphogenetic 7-induced endocrine reprogramming [Klein *et al.* 2015]. Instead, cells expressing PDX1 at the time of lentiviral-based tagging participated to almost 50% of the C-peptide$^+$ cells retrieved after differentiation protocols. Although these results might be partly influenced by lineage-dependent transduction efficiency, it clearly highlights issues related with the use of unselected exocrine preparations, that is, contamination with endocrine cells (69% of fresh cells expressed PDX1 protein) and natural EMT processes (79% of the insulin-Cre progeny expressed vimentin after 12 days) leading to overestimation of the initial presence of progenitor cells and/or of the contribution of a cellular subtype in the endocrine reprogramming.

Purification of human PDCs unequivocally addresses the potential of the ductal-cell compartment for *in vitro* neogenesis. Several membrane proteins were studied for their duct-specific expression, such as lectin (binding peanut agglutinin [Barresi *et al.* 1993], CA19-9 [Gmyr *et al.* 2004; Yatoh *et al.* 2007], CD133 [Lee *et al.* 2013], or antigens targeted by specific monoclonal antibodies [Dorrell *et al.* 2008; Hald *et al.* 2012]. The Bonner-Weir group was one of the first to show spontaneous differentiation of purified human PDCs after aggregation [Yatoh *et al.* 2007]. In their study, CA19-9$^+$ PDCs had increased capacities to form insulin-producing cells after coculture with pancreatic stromal cells. Likewise, human CD133$^+$ PDCs exclusively expressing duct-specific markers (such as keratin 19) were successfully isolated using flow cytometry [Lee *et al.* 2013]. These cells were submitted to adenoviral-based concomitant overexpression of PDX1, MAFA, NGN3, and PAX6 that triggered β-cell differentiation to levels close to β cells regarding insulin content (up to 15.2 pmol/μg DNA), GSIS and ultrastructure. This study confirmed previous reports of unselected PDCs that

showed reprogramming of PDCs into insulin-producing cells after transduction of NGN3 [Heremans *et al.* 2002] with or without MYT1 [Swales *et al.* 2012]. Similar outcomes were obtained in adult mouse PDCs subjected to reprogramming after incubation with a polycistronic construct carrying NGN3, PDX1, and MAFA TFs [Yamada *et al.* 2015] borrowed from the study by Zhou and colleagues that achieved acinar-to-β transdifferentiation [Cavelti-Weder *et al.* 2015]. Interestingly, Yamada and colleagues [Yamada *et al.* 2015] showed the need to add exendin-4 to culture media for PDCs to acquire GSIS, a feature not observed *in vivo* by other groups [Zhou *et al.* 2008; Cavelti-Weder *et al.* 2015].

Conclusion

Over the last 15 years, strategies to generate insulin-producing cells have been thoroughly investigated. Committed progenitor cells with capacity for renewal of the endocrine compartment after tissue damage were identified in adult pancreata within the ductal epithelium. Re-expression of NGN3 in this subpopulation of pancreatic precursors was identified as an essential step anticipating the regeneration process. *In vivo* experiments performed in adult pancreas showed replenishment of β-cell mass and restoration of glycemic levels in STZ- or alloxan-treated rodents due to conversion of ductal progenitors into insulin-producing cells following treatment with growth factors or small molecules.

In parallel, efforts have been made to exploit PDCs as a safe source of β-like cells for translational studies. Current investigations are focusing on the potential to expand and differentiate PDCs after purification. Recent models for expansion of purified human PDCs were reported, and *in vitro* differentiation protocols achieved functional β-cell phenotypes within a few weeks. Animal studies will help to determine long-term functionality of these systems and the safety of expanded PDCs after transplantation. Once the proof of concept of diabetes reversal with purified human PDCs is unequivocally demonstrated, PDCs might be considered for developing new strategies for β-cell mass regeneration in the human setting.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

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