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PATHOGENESIS OF TYPE 1 DIABETES (AG ZIEGLER, SECTION EDITOR)

Understanding Pancreas Development for β-Cell Repair and Replacement Therapies

Aurelia Raducanu · Heiko Lickert

Abstract The lack or dysfunction of insulin-producing β-cells is the cause of all forms of diabetes. In vitro generation of β-cells from pluripotent stem cells for cell-replacement therapy or triggering endogenous mechanisms of β-cell repair have great potential in the field of regenerative medicine. Both approaches rely on a thorough understanding of β-cell development and homeostasis. Here, we briefly summarize the current knowledge of β-cell differentiation during pancreas development in the mouse. Furthermore, we describe how this knowledge is translated to instruct differentiation of both mouse and human pluripotent stem cells towards the β-cell lineage. Finally, we shortly summarize the current efforts to identify stem or progenitor cells in the adult pancreatic organ and to harness the endogenous regenerative potential. Understanding development and regeneration of β-cells already led to identification of molecular targets for therapy and informed on pathomechanisms of diabetes and in the future might lead to β-cell repair and replacement therapies.

Keywords Development · Regeneration · Differentiation · Pancreas · β-cell · Embryonic stem cells · Progenitor cells · Signaling · Transcription factors · Diabetes

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Introduction

Diabetes mellitus is responsible for the death of 4.6 million people every year. There are currently 346 million people with diabetes in the world and the number of patients is expected to double by 2030 according to World Health Organization (WHO). In light of these predictions, the United Nations has declared diabetes a global threat.

Diabetic patients fail to maintain blood glucose homeostasis due to insufficient use or a lack of insulin. Type-1 diabetes (T1D) is an auto-immune disease where pancreatic β-cells are destroyed by cytotoxic T-cells. It accounts for 5 %–10 % of all clinical cases and occurs mostly in children. Type-2 diabetes (T2D) is the most common form and is characterized by β-cell dysfunction. It manifests as a metabolic disorder characterized by peripheral insulin resistance and glucose intolerance. The reduced β-cell mass in T2D was not recognized for long time due to lack of human studies. Recently, autopsies of T2D patients have revealed a decrease in β-cell mass between 40 % and 60 % [1, 2]. Rare congenital monogenic forms of diabetes are caused by mutations in genes critical for β-cell development and function [3]. Consequently, dysfunction or loss of β-cell mass is the cause for all forms of diabetes and there is great interest in developing β-cell-replacement therapies. Transplantation of the entire pancreas or isolated islets has been shown to be effective in treatment of diabetes but the shortage of donors and immune rejection of the grafts limit this approach [4, 5]. Current strategies in regenerative medicine are to identify the cellular, genetic, and biochemical pathways governing the generation of insulin-producing β-cells from various cell sources.

In this review, we will introduce the major principles of pancreas development and β-cell differentiation and discuss the status of current experimental approaches in β-cell replacement therapy. There are other excellent reviews on pancreas development and regeneration, which are complementary and which we recommend to the interested reader [6–9].

Pancreas Development in the Mouse Embryo

Our understanding of pancreas development and β-cell lineage differentiation has significantly increased in the last 20 years through the use of mouse genetics. However, the picture is not yet complete. Pancreas development and β-cell differentiation can be divided into 4 major events: (1) formation of the endoderm germ layer from which the pancreas arises, (2) specification of the pancreas primordium, endocrine-lineage specification, (3) islet formation, and (4) β-cell maturation [7, 10, 11].

Upon fertilization, the zygote undergoes a series of rapid divisions, giving rise to the 32-64 cell-stage blastocyst [12]. Cells towards the blastocoel cavity form the inner cell mass (ICM) that will give rise to the embryo proper and its associated tissues: the yolk sac, allantois, and amnion. Embryonic stem cells (ESCs) can be generated from the ICM of human and mouse blastocysts [13–16]. ESCs are defined by their pluripotency and self-renewal activity. Thus, they can be maintained indefinitely and differentiate into all cell types of the mammalian body in culture and in mouse chimeras. Mouse ESCs (mESCs) are in the ground state of pluripotency characterized by expression of key transcription factors such as Oct4, Nanog and Sox2 [17]. Due to their developmental potential mESCs and human ESCs (hESCs) are currently the major tools for studying differentiation in vitro to allow cell-replacement therapy [18].

As development proceeds, the ICM of the blastocyst gives rise to the epiblast cells of the gastrula-stage embryo [19–21]. Epiblast cells are organized into a single layer epithelium and become primed to give rise to the 3 embryonic germ layers: ectoderm, mesoderm, and endoderm. In a narrow time window around gastrulation, epiblast stem cells (EpiSCs) can be derived from the epiblast [22, 23]. The EpiSCs can maintain their pluripotency in the presence of fibroblast growth factors (FGFs) and activinA, comparable to human ES cells, and they can differentiate into all lineages in vitro [22–24]. On the molecular level EpiSCs are more similar to hESCs than to mESCs. They express the core pluripotency factors Oct4, Sox2 and, Nanog, but they differ from mESCs, since they also express epiblast-specific markers, such as Sox2, T and Foxa2 [17]. Thus EpiSCs offer an attractive alternative source of mouse pluripotent stem cells for comparison with hESCs in differentiation culture.

Differentiation of the definitive endoderm during gastrulation represents the first step towards pancreas development [25–27]. Definitive endoderm cells exit the pluripotent epiblast in the anterior region of the primitive streak and intercalate between and disperse the visceral endoderm, which will contribute to yolk sac formation [28, 29]. After gastrulation, the endoderm epithelial layer undergoes a series of morphogenetic events, such as fore- and hindgut invagination to become organized into the primitive gut tube. Antero-posterior (AP) patterning regionalizes the sheet-like epithelial layer and primitive gut tube into foregut (precursor to the thymus, lung liver, stomach, and pancreas), midgut (prospective small intestine) and hindgut (the future colon) [30–33]. The molecular control of endoderm formation and patterning is highly conserved during evolution and involves Retinoic Acid (RA), sonic Hedgehog (Shh), nodal/TGFβ, Wnt/β-catenin, FGF, and bone morphogenic protein (BMP) signals, which culminate in the activation of endoderm-specific transcription factors and molecular programs [25, 32, 34].

Pancreatic primordium formation depends especially on neighboring tissue interactions. Around embryonic day (E8.0), first 2 small lateral areas and afterwards a single dorsal domain in the foregut endoderm are specified by mesoderm-derived signals such as FGFs, BMPs, activin, and retinoic acid (RA) to become pancreatic tissue [6, 9, 35]. Signals from the notochord mediated by activin-βB and FGF2 block the expression of *Shh* in the dorsal pancreatic epithelium making it competent to express pancreatic genes such as *Pdx1* [36]. Mice lacking *Pdx1* display pancreatic agenesis demonstrating the crucial role of Pdx1 as a key regulator of pancreas development [37–39]. Prior to and essential for *Pdx1* expression are the more widely expressed genes: *Hlxb9* (*Hb9*), *Hhex* (*Hex*), *Onecut1* (*HNF6*), *Tcf2*/*HNF1β* and *Foxa1* and *2* (*HNF3α* and β) [7, 40]. While initially Shh inhibition is required for pancreas primordium formation later Hh signaling is necessary for pancreas epithelial growth and regulation of the insulin gene in mature β-cells [41, 42]. As development proceeds, the notochord is replaced by the aorta and VEGF secreted by the neighboring aortic endothelium sustains *Pdx1* expression and induces *Ptf1a* expression [43]. Ventral pancreas development is less well studied but seems to originate from a common progenitor with the liver in the ventral foregut [44]. Lateral plate mesoderm signals such as BMP, RA, or activin specifies the presumptive ventral pancreatic endoderm next to the liver domain [45]. FGFs and BMPs released by the cardiac and septum transversum mesoderm promote a liver fate and through a morphogenetic migratory process, the cells that escape these signals will become committed towards a pancreatic fate [44, 46]. Ventral pancreas induction seems to be independent of endothelial signals [47]. Although a different specification program acts in the development of the ventral bud the final destination in the differentiation program is likely identical to the dorsal bud.

The first visible anatomical structures of the prospective pancreas appear as multilayered epithelial thickenings surrounded by mesenchymal tissue in the dorsal and ventral domain of the posterior foregut epithelium at E9.0-9.5. Upon gut-tube rotation the 2 pancreatic buds fuse together into a single pancreatic primordium around E11.5 [7, 8]. Removal of mesenchyme prevents epithelial outgrowth of

the dorsal bud [48], a process that depends on Notch activity and mesenchymal FGF10 stimulating the proliferation of pancreatic epithelial progenitors through Fgfr2b [49–51]. Deletion of *Isl1*, a TF expressed early in the dorsal mesenchyme and in the pancreatic epithelium leads to the absence of dorsal mesenchyme and consequently to the lack of dorsal pancreatic bud formation [52].

The stratified epithelial bud contains multipotent pancreatic progenitor cells (MPCs) and a few differentiated immature endocrine cells, which are mainly glucagon+, but transient insulin/glucagon+cells have also been reported [53, 54]. This first wave of differentiation takes place between E9.0-11.5 and is called primary transition. Several TFs start to be expressed at this time downstream of Pdx1. Ptf1a is essential in maintaining the growth of the undifferentiated MPCs [55, 56] and later for exocrine-lineage specification [57]. Sox9 is another TF that promotes survival and proliferation of the MPC progenitor pool in the pancreas [58, 59] and regulates the expression of *Neurogenin3* (*Ngn3*) [60]. Ngn3 is one of the TFs that initiates endocrine-lineage differentiation and mice that lack *Ngn3* are devoid of islets and die shortly after birth due to hyperglycemia [61]. Other TFs downstream of Ngn3 are important for segregation into different lineages. *Nkx2.2*, *Isl1*, *Pax6,* and *NeuroD* are expressed from E9.5 onwards and all are essential for endocrine-lineage formation [7, 40]. Nkx6.1, Nkx6.2, and MafA have also been shown to be involved in β-cell differentiation and maintenance [62–65].

Between E12.5 and 15.5 the pancreatic epithelium experiences massive plexus remodeling that leads to the organization of a branching structure embedded into surrounding mesenchyme together with blood vessels and neuronal cells. Segregation of the epithelium into a proximal trunk and duct region as well as a distal tip region, depends on the distance from the surrounding mesenchyme and Notch signaling [66]. At this time the major 3 lineage decisions into duct, endocrine, and exocrine lineage are being progressively made in the process of so-called secondary transition. MPC initially express a characteristic set of TFs, such as *Pdx1*, *Foxa2*, *Sox9*, *Ptf1a*, *HNF6*, *HNF1β,* and *HNF4α* [7, 9, 40]. After E14.5, the Cpa1+ distal tip cells of the pancreatic epithelial trunk/duct will be committed to the exocrine lineage as shown by the expression of the exocrine promoting TF such as *Ptf1a*, *c-Myc*, and *Mist1* [55, 67–69]. The ducts are the main progenitor pool with bipotent capacities to form duct or endocrine cells [70], but also retain multipotent capacities [71]. This multipotency is illustrated by the concomitant expression of *Pdx1*, *Foxa2*, *Sox9*, *Ptf1a,* and *Nkx6.1*, which are all known to be expressed in early MPCs shown by genetic lineage tracing experiments [7, 40, 72]. Scattered Ngn3+ cells start to leave the duct epithelium through a process that resembles epithelial-mesenchymal transition (EMT) and become differentiated into endocrine precursors [73]. Like previous lineage allocation steps, also the secondary transition process is regulated by mesenchymal-epithelial interactions. Initial studies have shown that removal of the mesenchyme promotes endocrine development at the expense of exocrine differentiation [74–76]. Several signaling molecules have been shown to play a role at this step including FGF, Notch, TGF β and Wnt/ β-catenin, and are essential to regulate epithelial proliferation and differentiation [9]. However, the exact molecular mechanisms that govern the specific endocrine fate allocation in time and space towards a certain hormone-producing cell type are still not fully understood.

At around E18.5 and until the first days after birth, the islets form and morphogenesis takes place. α-Cells encircle clusters of β-cells, migrate away from the ducts and acquire the typical islet ovoid morphology [77]. The β-cells switch from an immature to a mature stage characterized by a specific set of TFs. The MafB+to MafA+switch seems to be important as MafA is required for the expression of genes such as *insulin*, *Pdx1*, *Glut2*, *Nkx6.1*, *Slc30a8,* and *G6pc2*, which are essential for β-cell function and physiology [7, 63].

The signaling pathways that have been identified to act during different steps in β-cell development have been recently used as the basis to differentiate human and mouse ESCs into β-cells in culture. In the next section we will review some of these approaches.

Differentiation of β-Cells From Pluripotent Stem Cells

Pluripotent stem cells are a valuable resource to generate β-cells because they can be indefinitely propagated in culture and they have the capacity to differentiate into any somatic cell type. A stepwise approach that mimics in vivo differentiation processes is a rational way to obtain any kind of differentiated somatic cell type. An initial step toward pancreatic specification is differentiation of definitive endoderm from the epiblast during gastrulation under the control of the TGFβ family members, nodal or activinA. Studies have demonstrated that it is possible to differentiate endoderm from mouse and human ESCs with rather high efficiency [78–80]. When ESCs are cultured under low serum conditions and in the presence of Wnt3a, addition of high concentration of activinA leads to cultures that contain 80 % endodermal cells [78, 80]. An alternative approach is to use small molecules capable of inducing endoderm differentiation, such as the histone-deacetylase inhibitors IDE1 and IDE2 [81]. After definitive endoderm formation, A-P patterning of the primitive gut tube and specification of the pancreatic endoderm has to be mimicked in culture. This

process is highly dependent on the correct timing and dose of Wnt, FGF, and RA [25]. Further inhibition of Shh by FGF10 and activin-β2 in the posterior foregut renders the endoderm progenitor competent for expression of *Pdx1* and downstream genes. In the following step, steering the pancreatic endoderm cells towards the endocrine lineage requires Notch inhibition. Based on the translation of these developmental principles, Novocell (now ViaCyte) developed successfully a 5-step differentiation protocol to generate insulin-producing β-cells [82]. Although this study represents a major breakthrough in obtaining β-like cells in vitro the efficiency is very low—only 7.3 % of the cells were positive for insulin. Moreover, the insulin+cells were not responsive to glucose, and some cells were polyhormonal, a feature specific to naïve endocrine cells generated during primary transition [53]. Recently, up to 25 % insulin-producing β-cells could be differentiated from hESCs by modification of the ViaCyte protocol [83]. The authors fine-tuned several steps in the protocol according to an updated understanding of the signaling involved in pancreas development. This study defined the temporal requirement of TGFβ-family members and canonical Wnt signaling during the differentiation protocol. Moreover, since BMP signaling has been shown to regulate hepatic specification [84], its inhibition was essential in obtaining insulin-producing cells [83]. Although this study shows how the right combination of instructive molecules acts differently at certain times in the developmental program, the outcome was still disappointing, as further studies showed that similar to the ViaCyte protocol, most of the insulin-producing cells were polyhormonal and were not glucose responsive [85, 86]. Importantly, Kroon et al reported in 2008 that naïve β-cell progenitor cells after transplantation into diabetic mice were able to mature to glucose-dependent insulin-secreting cells that normalize blood glucose levels. Although this was a very encouraging result, out of 105 mice treated only a few showed improved glycemic control upon glucose stimulation. Since a mixture of progenitor cells was transplanted, it took almost 3 months for these cells to mature, and currently it is still not clear how functional β-cells can be generated in culture [87]. Thus, employing cell-sorting strategies might improve the effectiveness of transplanted progenitor cells for maturation after transplantation. One study from ViaCyte demonstrated that only pancreatic progenitor cells were able to generate functional β-cells after transplantation, whereas polyhormonal cells generated only glucagon+cells [88•]. Taken together, although currently no functional β-cells can be produced in culture, the fact that progenitor cells can mature to glucose-sensitive insulin-secreting β-cells is very encouraging for future cell-replacement therapies.

Endogenous Mechanisms of Repair

Exploiting the multipotent capacities of progenitors in the pancreatic epithelium and perhaps also in the islets can represent an alternative strategy towards β-cell regeneration.

While adult rodent β-cells proliferate after birth and with lower rate at later stages [89, 90], the proliferating capacity of human β-cells dramatically declines after the first years of life [91–93]. Interestingly, β-cell mass can increase upon metabolic demand, such as pregnancy and obesity [1, 94, 95]. The mechanisms underlying the age-dependent decrease in proliferation capacity have not been elucidated till recently. β-Cells of young islets have high levels of Ezh2, a histone methyltransferase from the Polycomb group protein complex, and its activity decreases with age. Ezh2 represses the expression of *p16Ink4a*, which is a cell-cycle inhibitor and senescence gene that has been shown to be highly expressed in β-cells of old islets [96, 97].

Many studies have focused on the cell-cycle regulation in murine and human β-cells [98, 99]. Several growth factors and hormones are known to promote β-cell proliferation in mouse: EGF, IGF1, HGF, human growth hormone, insulin, incretin-like GLP1, as well as metabolites such as glucose, but their role in humans is not being investigated [100]. Drug therapy in T2D is focused on GLP-1 receptor modulation since it was shown that this pathway enhances insulin secretion. GLP-1 analogues with a longer half-life than GLP-1, like exenatide and liraglutide, as well as inhibitors of the GLP1-degrading enzyme DPP4 are currently used as therapeutic agents for T2D [101, 102]. Deciphering the mechanism of β-cell re-entry into the cell cycle can lead to the development of new drugs that can activate the proliferation capacity of senescent β-cells, but these drugs would have to act selectively on islets and not on the ductal epithelium, as pancreatic adenocarcinomas arise from these cells [103].

The main mechanism of β-cell death in diabetes is through apoptosis. Before β-cells undergo apoptosis it seems that they transit to a de-differentiated state [104]. In animal models, hyperglycemia results in regression of the mature β-cell phenotype towards a premature stage when several key genes important for insulin secretion such as *Pdx1*, *Nkx6.1*, *Pax6*, *MafA*, *Glut2* are down-regulated and other progenitor genes, such as *Ngn3*, are up-regulated [104, 105]. Other factors that might induce such a phenotypic shift are hypoxia [106], obesity [107], and inflammation [108]. Thus, exploiting the mechanisms of de-differentiation can be useful in generating drugs able to reverse the immature non-functional phenotype of β-cells.

Stimulating the intrinsic capacity of pancreas to regenerate would offer immense possibility for therapy.

Currently, the existence of progenitor cells with regeneration capacity in adult pancreas is still under intense research. In a partial pancreatectomy model it was shown that β-cell regeneration occurs through duplication of pre-existent β-cells and not from a distinct progenitor population [89, 109]. These findings have been questioned recently by the identification of a rare population of β-cells, which seem to have a progenitor-like character and are able to differentiate into all hormone-producing cells, but surprisingly also into neuronal-like cells [110•]. Further studies are necessary to settle the debates about the existence of an adult pancreatic "stem-like" cell and their respective niche, but if these progenitor cells really exist their potential can be further explored.

Mobilization of progenitor duct cells is another plausible option to regenerate β-cells. In an injury model, which used duct ligation to destroy the distal islets and a reporter gene to trace the carbonic anhydrase II+ductal cells, the regeneration of islets occurred through differentiation of ductal cells as shown by the increased number of reporter-positive cells [111]. Xu et al also used pancreatic duct ligation as an injury model and could trace the Ngn3+ endocrine precursor present in the ducts. Upon injury, these cells were activated in the ducts and could give rise to all endocrine cell types in situ and in embryonic explant cultures [112]. These studies suggest that an intrinsic activation mechanism of ductal progenitors is revived in an injury situation, thus finding small molecules or drugs that can potentially activate such mechanisms in diabetic patients remains a possibility.

Conversion of α-cells into β-cells in vivo to generate endogenous β-cells could potentially be achievable. Thorel et al used diphtheria-toxin to selectively induce apoptosis in almost 99 % of the β-cell in adult mice. Using lineage-tracing to label glucagon-producing α-cells, it was shown that upon injury the α-cells have a remarkable plasticity and can spontaneously convert into insulin-producing β-cells [113•]. Previously, Zhou et al successfully reprogramed α-cells to β-cells in vivo with a transducing viral vector which expresses *Ngn3*, *Pdx1*, and *MafA*, key TFs required for β-cell maturation [114]. Thus, it can be possible and maybe easier to rather obtain β-cells from other endocrine precursors without recapitulating the whole differentiation program from a pluripotent state. Therefore, developmental relatives of β-cells, the hepatocytes have also been explored in reprogramming attempts. Initially upon overexpression of *Pdx1* [115] and later, of *Pdx1* together with *NeuroD* or *Ngn3* [116] mouse hepatocytes were reprogrammed into cells that express insulin and could restore normal glycemia in diabetic mice. Despite the regenerative capacity of the liver, difficulty of working with hepatocytes, as they do not proliferate in vitro and they de-differentiate in culture, makes this approach limited to in vivo explorations.

Conclusions

Developmental biology during the last decades has provided insights into pancreatic organ formation and β-cell differentiation. Translating developmental principles to culture has allowed differentiation of pluripotent stem cells into β-like cells; however, the efficiencies are low and no functional glucose-responsive insulin-producing β-cell has yet been derived. Improving every single step of β-cell differentiation to endoderm progenitors, endocrine precursors, and mature β-cells has to be achieved. Therefore, more detailed information of the in vivo program should uncover the temporal and spatial cues required for β-cell lineage segregation. Drug screens are on the way to identify small molecules to improve every step of the differentiation protocol. Moreover, isolation of multipotent and lineage restricted progenitor cells from mice is now technically feasible and this will precisely inform about the overall molecular signature that guides progenitor cell differentiation. Isolation of juxtaposed tissues, like the endothelial cells and mesenchymal cells, will provide the external signals for every single step of differentiation. In addition, the identification of surface molecules can deliver markers to build a lineage tree and to enrich specific populations for transplantation. Transplantation paradigms, such as alginate microencapsulation, are being developed for efficient engrafting, optimal blood supply, and protection from the immune system. Infusion of pancreatic islets into the portal vein of the liver has already been demonstrated using the Edmonton protocol, and various preclinical models have shown that cell-replacement therapy can in principle work. Generation of induced pluripotent stem cells (iPSCs) from humans can be used not only as an alternative source of producing human β-like cells but also for modeling diseases in vitro and for pharmacologic screens. These cells alone or in combination with cell-types that are important for disease development (for example T-cell in case of T1D) can be used for further in vitro studies of biochemical mechanisms involved in pathogenic process. Moreover, iPSC can be useful to investigate the mode of action and toxicity of new therapeutic drugs.

Alternatively, triggering endogenous mechanisms to reconstitute functional β-cell mass will require the identification of adult pancreatic progenitor cells that might be able to be mobilized. The field has greatly advanced through the study of injury models in combination with genetic lineage tracing approaches. This has revealed that the pancreas has regenerative capacity, although not at the extent of the liver. Remarkable is that both organs derive from common progenitors, but although the liver can regenerate extensively, the pancreas cannot. Comparing the developmental programs of liver and pancreas progenitor cells might provide insights into the molecular details that allow regeneration.

Moreover, identification of progenitor cells in the ductal epithelium or in the islets using lineage-tracing approaches might enable the mobilization of these cells to become β-cells. Upon metabolic demand, β-cell mass increases mainly via β-cell replication. Thus, stimulating β-cell replication might be one mechanism to regain functional β-cells if they are still present. One complication here might be that stimulation of proliferative mechanisms in the pancreas might lead to carcinomas with the worst prognosis. It is also interesting to note that upon bariatric surgery and extreme calorie restriction T2D patients that where insulin-dependent quickly regain functional β-cell mass to normalize blood glucose levels, even before the effect on body weight loss can be noticed. This demonstrates that β-cells are present, but might not be functional. In this respect, it is interesting that in T2D mouse models, β-cells undergo de-differentiation and one new paradigm could be stimulating the maturation of de-differentiated β-cells. This will also require more detailed analysis of β-cell fate in diabetes models, but might be an alternative strategy to current T2D therapies. Taken together, a combined effort of developmental and stem cell biologists in close cooperation with clinicians might eventually extend the repertoire of immunologic and metabolic therapies and enable regenerative medicine for the treatment of diabetes.

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- Of importance

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AUTHOR QUERIES

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