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REVIEW

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Pancreatic β-cell replacement: advances in protocols used for differentiation of pancreatic progenitors to β-like cells

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Abstract
Insulin-producing cells derived from *in vitro* differentiation of stem cells and non-stem cells by using different factors can spare the need for genetic manipulation and provide a cure for diabetes. In this context, pancreatic progenitors differentiating to β-like cells garner increasing attention as β-cell replacement source. This kind of cell therapy has the potential to cure diabetes, but is still on its way of being clinically useful. The primary restriction for *in vitro* production of mature and functional β-cells is developing a physiologically relevant *in vitro* culture system which can mimic *in vivo* pathways of islet development. In order to achieve this target, different approaches have been attempted for the differentiation of pancreatic stem/progenitor cells to β-like cells. Here, we will review some of the state-of-the-art protocols for the differentiation of pancreatic progenitors and differentiated pancreatic cells into β-like cells with a focus on pancreatic duct cells. *(Folia Histochemica et Cytobiologica 2019, Vol. 57, No. 3, 101–115)*

Key words: β-cell replacement; transdifferentiation; pancreatic duct cells; acinar cells; centroacinar cells; endocrine cells; mesenchymal stem cells; β-like cells

Abbreviations:

3D — three-dimensional; Akt — protein kinase 1; ALDH1 — aldehyde dehydrogenase 1; ALK3 — activin-like kinase 3; ARIP — adult rat pancreatic ductal epithelial cell line; Arx — aristaless-related homeobox; Ascl1b — achaete-scute homolog 1b; BLCs — beta-like cells; BMP7 — bone morphogenetic protein 7; B-PMSCs — bovine pancreatic MSCs; BrdU — bromodeoxyuridine; CACs — centroacinar cells; CK19 — cytokeratin 19; CNF — ciliary neurotrophic factor; Dnmt1 — DNA methyltransferase 1; DT — diphtheria toxin; DTZ — dithizone; E12.5 — embryonic day 12.5; eBCs — enriched β-clusters; EGF — epidermal growth factor; EGFP — enhanced green fluorescent protein; EGF-R — EGF receptor; EMT — epithelial-to-mesenchymal transition; Ex-4 — exendin-4; FACS — fluorescence-activated cell sorting; FGF — fibroblast growth factor; Fstl3 — follistatin-like 3; GABA — gamma-aminobutyric acid; GFP — green fluorescent protein; GH — growth hormone; GLP-1 — glucagon-like peptide-1; HA — hyaluronic acid; HDAC — histone deacetylase; HDACi — histone deacetylase inhibitor; Hes-1 — Hairy/Enhancer of split-1; HGF — hepatocyte growth factor; hNEPT — human non-endocrine pancreatic tissue; Hnf6 — hepatocyte nuclear factor 6; ICCs — islet-like cell clusters; IDX-1 — islet duodenal homeobox-1; INS — insulin; IPCs — insulin-producing cells; KGF — keratinocyte growth factor; KO — knockout; LIF — leukemia inhibiting factor; MafA/B — musculoaponeurotic fibrosarcoma oncogene A/B; MSCs — mesenchymal stem cells; NeuroD — neurogenic differentiation; Ngn3 — neurogenin 3; Nkx6.1 — NK6 homeobox 1; NPPCs — nestin-positive progenitor cells; NOD — non-obese diabetic;

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NOD-SCID — non-obese diabetic-severe combined immunodeficiency; p16INK4a — tumor suppressor gene; PANC-1 — human pancreatic cancer cell line; PAX4 — paired box 4; PDEC — pancreatic duct epithelial cell; PDL — partial duct ligation; PDSCs — pancreatic duct stem cells; Pdx1 — pancreatic and duodenal homeobox 1; PI-MSCs — pancreatic islet-derived mesenchymal stem cells; PLGA — poly-lactic co-glycolic acid; PP — pancreatic polypeptide; PRL — prolactin; Px — pancreatectomy; RSPO1 — R-spondin-1; SCs — stem cells; siRNA — small interfering RNA; Sox9 — sex determining region Y box 9; STAT3 — signal transducer and activator of transcription 3; STZ — streptozotocin; Tcf2 — transcription factor 2; TF — transcription factor; T3 — tri-iodothyronine; TGF — transforming growth factor; YFP — yellow fluorescent protein.

Introduction

In recent decades, the occurrence of diabetes has increased globally, with over 425 million diabetic people living worldwide whose number is expected to be 629 million in 2045 [1]. Diabetes is a form of metabolic disorder characterized by dysfunction and loss of β-cell mass resulting in chronic hyperglycemia [2, 3]. Regarding the treatment of diabetes, exogenous insulin can only control blood glucose levels without amending the devastating consequences of diabetes. Islet transplantation also has potential and is a clinical method for restoring normoglycemia, however, not without problems related to donor shortage and immune rejection for which long term immune suppression therapy is necessary [4]. Alternatively, a stem-cells-based β-cell replacement can resolve most disease-related problems of diabetic patients [5, 6].

The pancreas is composed of exocrine (acinar, centroacinar and ductal cells) and endocrine compartments (islet of Langerhans) [7, 8]. Regarding the regeneration, most of the adult tissues are composed of the differentiated cells making them dependent on stem cells for repair and regeneration [9]. However, some tissues like adult pancreas lack the existence of true stem cells, undifferentiated cells which are dedicated for providing an unlimited supply of freshly differentiated cells when these are lost, discarded, or needed in greater numbers, and rely on the unipotent and/or multipotent facultative progenitors [10]. The facultative progenitors are differentiated cells responsible for particular functions, but they retain the ability to de-differentiate [11], proliferate and eventually redifferentiate towards another cell type [12] for repairing the tissue after injury [13].The plasticity of differentiated pancreatic cells enables them to serve as a facultative progenitor in a tissue which lacks true stem cells [14, 15].

For the maintenance of β-cell mass, pancreatic β-cells can duplicate themselves [16], but this proliferation is not enough to withhold the excessive loss of β-cells in stress conditions. Recently, Domínguez-Bendala *et al.* reported that in the adult murine and human pancreas, when normal turnover of β-cells is required, β-cells are formed by the replication of existing β-cells or can also be formed by de-differentiated α-cells through an intermediate cell stage called 'virgin β-cell' [17]. The existence of 'virgin β-cell', which serves as an intermediate stage for transdifferentiation of α-cells to β-cells, as a neogenic niche at the periphery of pancreatic islets of mice was confirmed by Meulen *et al*. through single-cell transcriptome analysis [18]. It has been suggested by studies in mice that during metabolic stress, β-cell de-differentiation, and then redifferentiation mechanisms regenerate the islets [19]. However, when the pancreas is damaged excessively then ductal cell progenitors' regenerate islets [17]. The *in vivo* and *in vitro* studies have shown that pancreatic duct epithelial cells (PDECs), acinar cells, and centroacinar cells were the source for neogenesis of β-like cells (BLCs) [20–22]. The main characteristics of BLCs involve the ability to synthesize insulin; however, in most cases, the secretion of insulin stimulated by glucose and other agents is usually lower than in beta cells isolated from pancreatic islets. The *in vitro* studies on endocrine cells showed that replication of β-cells and transdifferentiation of α and δ cells are possible sources of insulin producing β-like cells [23–25]. Additionally, the pancreatic islet-derived mesenchymal stem cells also show some potential for *in vitro* development of β-like cells [26]. Added to their role as a β-cell replacement, *in vitro* derived BLCs can be used as a model to study diabetes pathology [27] and can also provide a consistent and uniform supply for the screening of pharmaceutical drugs for improving β-cell function and survival [28, 29]. However, for the success of these *in vitro* stem cell culture approaches imitation of *in vivo* islet milieu is required.

In this review, we summarized different protocols used for the differentiation of pancreatic progenitors to β-like cells. Furthermore, the available cell sources within the pancreas were also assessed for their potential as the progenitors of β-cells.

Pancreatic exocrine cells as a source of β-cell/β-like cells

The exocrine part of the pancreas is more than 95% in rodents and the pancreas in humans also have more than 95% to 98% of the exocrine part [7, 30]. It includes the acinar cells — secreting digestive enzymes, centroacinar cells — secreting bicarbonate

ions and mucins, and pancreatic ducts — transferring these secretions to the duodenum [7]. All types of exocrine cells in the pancreas have been used *in vitro* for expansion into β-cells in different studies which will be discussed shortly.

Pancreatic ductal epithelial cell's induction to β-cell/β-like cells

Lessons from classical animal models of β-cells regeneration

These models include mainly partial duct ligation (PDL) of the pancreatic duct, partial pancreatectomy (Px) and targeted β-cells damage by diphtheria toxin (DT) and streptozotocin (STZ). The PDL method used by Xu *et al.* [31] and Inada *et al.* [32] in adult mouse showed the presence of multipotent islet progenitors in ductal linings. Injury-induced by PDL caused the β-cell mass expansion. This β-cell mass expansion needed the activation of Nuerogenin3 (Ngn3) gene expression which they believed to be the response for the inflammatory damage and loss of acinar cells due to injury [31, 32]. Inada *et al.* used carbonic anhydrase II-Cre-lineage tracing to explore the source of new β-cells [32]. Pancreatectomy model of 90% Px in adult rats by Li *et al.* [33] illustrates that mature epithelial duct cells can be dedifferentiated and become facultative progenitors which then differentiate to both exocrine and endocrine cell types. During dedifferentiation resulting from pancreatectomy, the duct cells lost Hnf6, a ductal differentiation marker [34, 35], and expressed Pdx1, Tcf2, and Sox9 transcription factors, markers for embryonic pancreatic epithelium [36–38]. These pancreatic epithelial cells acted as progenitors and differentiated to $Ngn3^+$ cells. Differentiation and maturation of $Ngn3^+$ progenitor cells led in the growing pancreas to the formation of $MafA^+$, $insulin^+$ cells [33]. In another murine model, the 60% pancreatectomy was used for finding the source of β-cell regeneration. The $Sox9^+$ viral lineage tracing confirmed that in young, but not adult, mice and humans intra-islet pancreatic ducts are the source of β-cell regeneration [39]. In an adult mouse model, diphtheria toxin (DT) was used to ablate β-cells to show that epithelial cells within the pancreatic ducts contribute to the regeneration of endocrine and acinar cells. The results of lineage tracing in DT-induced ablated β-cell mouse model showed that β-cells were regenerated from pancreatic ductal cells [40]. In another study, a model of streptozotocin-induced diabetic mice was used. The *in vivo* differentiation of oligopotent progenitors, which can differentiate into pancreatic ductal and endocrine cell types including β-cells, was investigated and it was confirmed that after STZ-induced diabetes new β-cells were formed through differentiation of oligopotent progenitors [41].

We have learned from these models that pancreas has the facultative multipotent progenitors, which are activated by a specific type of injury to the pancreas. Pancreatic ductal epithelial cells, particularly, are believed to be these multipotent progenitors which can give rise to either endocrine or exocrine cells depending on the severity of injury to the pancreas. However, the source of β-cell regeneration under physiological conditions is not confirmed hitherto.

In vitro β-cell expansion using pancreatic duct cells

Pancreatic duct epithelial cells can dedifferentiate when there is stress or injury to the pancreas and can re-differentiate to endocrine or exocrine cells depending on the severity of the injury. As described in classical models of differentiation, in the pancreas of adult mice, pancreatic duct epithelial cells (PDECs) can contribute to the regeneration of endocrine and exocrine cell types. The regenerative pathways and cell types that contribute to this process depend upon the severity of injury [40]. Therefore, most researchers have used pancreatic duct cells in their studies to find a potential cell therapy for diabetes. Here, we summarize some protocols used in different studies for the differentiation of PDECs to islet cells or, particularly, insulin-secreting β-like cells.

$Sox9^+$ ductal cells in the adult pancreas can be differentiated to β-cells *in vitro,* but this way of *in vivo* conversion remained controversial until the Zhang group reported first *in vivo* study to resolve this controversy [42]. Using lineage-tracing, Zhang *et al.* proved that genetically labeled $Sox9^+$ adult rat pancreatic ductal cells could be induced to insulin-secreting cells *in vivo*. They used hyperglycemia in combination with long-term, low-dose epidermal growth factor (EGF) infusion as synergistic stimulants for the induction of duct cells to insulin-secreting β-cells. This treatment resulted in normoglycemia in non-autoimmune diabetic mice [42]. Recently, Shaotang *et al*. found the multipotency of adult rat pancreatic ductal epithelial cells by inducing their differentiation into pancreatic islets, nerve cells, adipose cells, and osteoblasts [43]. The development of these target cells was made possible by respective culture protocols for directing the differentiation of PDECs [43]. Furthermore, Qadir *et al.* confirmed the existence of pancreatic progenitors in human exocrine pancreas and ductal epithelial cells were confirmed as pancreatic progenitors [8]. The human exocrine pancreas is a reservoir of multipotent cells expressing PDX1, an important transcription factor expressed by pancreatic endocrine progenitors [44] and ALK3,

and BMP receptor 1A which is associated with the regeneration of tissues [45], mostly found in major pancreatic ducts. Qadir *et al.* also showed that BMP7, which binds to ALK3 could stimulate pancreatic progenitor cells proliferation [8].

Suarez-Pinzon *et al.* studied the effect of the combination of epidermal growth factor (EGF) and gastrin on inducing β-cells from pancreatic duct stem cells, both in human and mice [46]. Treatment with EGF and gastrin for two weeks in non-obese diabetic (NOD) mice with autoimmune diabetes (NOD-SCID) resulted in normoglycemia as the β-cell mass was expanded from 15 to 47% of normal cell mass. Immunosuppressive therapy was not done as the combination of EGF and gastrin reduced insulitis, showing that this treatment could also stop autoimmune destruction of β-cells in NOD-SCID mice [46]. On the other hand, the glucagon-like peptide-1 analog, exendin-4, reversed hyperglycemia in diabetic NOD mice; though, this required synchronized immunosuppressive treatment with anti-lymphocyte serum [47]. In their next experiment, Suarez-Pinzon *et al.* showed that β-cell mass in adult human pancreatic islets was increased after combination therapy with EGF and gastrin both *in vitro* and *in vivo*, and this was as a result of the induction of β-cell neogenesis from pancreatic duct cells [48]. In both studies by Suarez-Pinzon*et al.*, the mechanism which initiated the neogenesis of β-cells from pancreatic duct cells was not elucidated. However, the authors suggested that EGF caused an increase in the proliferation of CK19-positive duct cells while gastrin induced the expression of Pdx1 and differentiation of $Pdx1^+$ cells to insulin-positive β-like cells [48]. Likewise, the importance of EGF was reported that the perturbation of EGF-R-mediated signaling resulted in delayed β-cell development in EGF-R deficient $(-/-)$ mice [49]. These studies showing regeneration of β-cell mass through the use of EGF and gastrin combination, not only reveal the role of these peptides in the differentiation of pancreatic progenitors to β-cells in the damaged pancreas but also support the concept that pancreatic ducts contain the progenitor cells which can give rise to β-cells through epithelial-to-mesenchymal (EMT) cell transition [50]. The EMT requires some inducing stimuli, provision of which can cause *in vivo* regeneration of β-cells and also can cause the *in vitro* production of insulin-producing cells (IPCs) from pancreatic ductal epithelial cells.

Hui *et al.* investigated the effect of glucagon-like peptide-1 (GLP-1) on inducing differentiation of pancreatic ductal epithelial cells into insulin-secreting cells [51]. To test the effect of GLP-1, rat (ARIP) and human (PANC-1) cell lines, both derived from the pancreatic ductal epithelium, were used. Using fluorescence-activated cell sorting (FACS) analysis, they showed that GLP-1 induced the differentiation of ARIP cells into insulin-synthesising cells, although it did not affected the phenotype of PANC-1 cells. The expression of Glut2, insulin, and glucokinase transcripts was increased respectively in ARIC cells after GLP-1 treatment. GLP-1 was found to be associated with cause of this differentiation of ARIP cells by inducing the expression of β-cell differentiation marker, islet duodenal homeobox-1 (IDX-1). Albeit, the transfection of IDX-1 gene into PANC-1 cells made them responsive to GLP-1 treatment. The effect of GLP-1 on differentiation was confirmed by using exendin-9, a GLP-1 receptor antagonist, which inhibited the expression of β-cell-specific genes from ARIP and PANC-1 cell line pancreatic epithelial cells [51]. In an attempt to improve the efficiency of GLP-1-induced differentiation, Li *et al.* used sodium butyrate ($C_4H_7NaO_2$), histone deacetylase inhibitor (HDACi), along with GLP-1 to differentiate the FACS-sorted nestin-EGFP-positive progenitor cells (NPPCs) of transgenic (nestin and EGFP) mice into insulin-producing cells *in vitro* [52]. The treatment of purified nestin-EGFP-positive cells with a combination of sodium butyrate and HDACs resulted in increased levels of transcripts which encoded for pancreatic development factors and insulin. The population of insulin-secreting cells and volume, almost 65 ng when exposed to 25 mM glucose, of insulin secretion, both increased as a consequence. The addition of sodium butyrate resulted in de-condensation of chromatin whereas GLP-1 caused the increased expression of Pdx1 which by binding to the insulin gene promoted its transcription [52]. Additional evidence showed that HDACs treatment promoted $Ngn3^+$ proendocrine lineage, obtained from rat embryo, differentiation to an increased pool of endocrine progenitors [53]. Treatment with trichostatin A and sodium butyrate, inhibitors of both class I and II HDACs enhanced the mass of the β-cell as assessed by quantification after immunohistological staining against insulin. These results also suggest that HDACs are one of the critical key factors in endo- and exocrine pancreatic differentiation [53].

Another study conducted by Chen *et al.* reported the protocol to expand and differentiate rat PDECs into insulin-secreting islet-like cell clusters in a dynamic three-dimensional (3D) cell culture system [54]. They cultured cells for 14 days in serum-free culture media supplemented with nicotinamide, keratinocyte growth factor (KGF), and β-fibroblast growth factor (β-FGF) which resulted in the formation of islet-like cell clusters.

These islet-like cellular clusters were positive for insulin detection in the extracellular fluid and cytoplasm after 14 days of differentiation. However, the obtained insulin-secreting cells were not as functional like human islet cells [54]. In a recent study, Tan *et al.* used fibroblast-coated Poly-lactic acid-co-glycolic acid (PLGA) diaphragm to form a biological membrane [20]. They cultured nestin-positive pancreatic stem cells isolated from Wistar rats and, using two-step induction method, pancreatic stem cells were induced into insulin-secreting cells. A two-step induction method was used: in the first step, pancreatic stem cells were cultured in a media supplemented with bFGF and nicotinamide. After the detection of islet-like cell mass in culture media, activin-A, β-catenin, and exendin-4 (Ex-4) were used as supplements. The results showed that the number of nuclei was higher in induced cells and cells in this group were aggregated. The amount of insulin secreted upon stimulation was also significantly higher in the induced group as compared to the normal group but was not the same as of islet β-cells [20].

A summary of these and other factors used in different studies by various researchers for the differentiation of pancreatic duct stem cells into insulin-secreting BLCs is given in Table 1.

To sum up, till now we have learned that pancreatic ducts have the progenitors residing in their epithelium which can be differentiated *in situ* and *ex vivo* into BLCs by inducing their differentiation with a combination of different factors.

Acinar to β-cell neogenesis

Acinar cells are differentiated cells with a specialized function but have the plasticity to dedifferentiate towards duct-like endocrine progenitor cell phenotype which further differentiates towards β-cells [13, 55]. In pathological terms, this is referred to as metaplasia which is important for tissue repair after the injury. Owing to the abundance of their number and their ability to give rise to β-cells, the acinar cells are also a reliable candidate for β-cell regeneration and replacement. The transformation capability of acinar cells is mainly influenced by the type and extent of the pancreatic injury as confirmed by the lineage-tracing studies [40, 56].

Baeyens *et al.* showed that administration of EGF and ciliary neurotrophic factor (CNF) to adult STZ-induced diabetic mice stimulated the conversion of acinar cells to BLCs. The neo-β-like cells were glucose-responsive and restored normoglycemia. This conversion was dependent on the expression of Ngn3 which in turn was mediated by the Stat3 signaling [57]. The overexpression of three transcription factors (TFs), MafA, Pdx1, and Ngn3 in the acinar cells of transgenic mice *in vivo* resulted in acinar to β-cell reprogramming [58]. The overexpression of these three TFs caused the reprogramming-induced inflammation, which resulted in ductal cell metaplasia of acinar cells. The metaplasia of acinar to ductal cell phenotype resulted in the conversion of ductal cell phenotype to neo-β-cells. The neo-β-cells reversed diabetes in these mice and these results suggest that the expression of β-cell-specific transcription factors are the key to the transition of acinar cells to β-cells [58]. However, the viral vector system for the overexpression of TFs used in this study makes this approach less feasible for clinical application.

The *in vitro* culture of acinar cells, isolated from adult Wistar rats, in the presence of EGF and leukaemia inhibiting factor (LIF) inhibited the Notch1 signaling resulting in neogenesis of β-like cells [59]. The inhibition of Notch1 signaling caused the dedifferentiation of adult rat acinar cells to a state in which they expressed endocrine progenitor transcription factor, Ngn3, and afterwards differentiated with β-like cells. However, the newly formed β-cells were immature as compared to islet β-cells but they became mature phenotypically and resembled islet β-cells when transplanted at the ectopic site, under the kidney capsule of nude mice [59]. The presence of tri-iodothyronine (T3) receptors in murine embryonic pancreas laid the foundation for *ex vivo* culture of acinar cells in the presence of T3 to develop β-like cells [60]. Culturing of embryonic day 12.5 (E12.5) mouse embryonic pancreas tissue with T3 induced ductal phenotype at the expense of acinar tissue. Furthermore, T3 induced the endocrine fate in murine E12.5 pancreatic explant culture and also in the mouse acinar cell line 266-6. The T3-mediated conversion of acinar to β-cells was dependent upon the Akt signaling pathway and the T3-mediated effects were abrogated by Akt inhibitors [60]. These results confer that acinar cells can be converted to β-like cells by the addition of T3 in culture media. Dagmar Klein *et al.* [21] showed that exposure of adult human non-endocrine pancreatic tissue (hNEPT) to BMP-7 resulted in the conversion of the Pdx1-positive ductal cells to insulin-expressing cells. BMP-7 exposure instigated the ectopic expression of endocrine transcription factors in the exocrine cells which triggered this conversion. The use of *in vitro* lineage tracing confirmed that insulin-secreting cells were derivatives of mature ductal cells [21].

Summarily, the transformation of acinar to β-cell/ /β-like cells is possible as evidenced in many studies reported. This transformation was not direct from acinar to β-cell, instead, it was mediated by $Ngn3^+$ endocrine precursor cell phenotype. Thus, it may be

Table 1. Factors which caused the differentiation of pancreatic duct epithelial cells to β-cell/β-like cells [20, 39, 42, 46, 48, 51, 52, 54, 112–119]

Model and protocol used	Main obtained results	Reference
***In vitro* studies**		
Rat (ARIP*) and human (PANC-1) pancreatic ductal epithelial cells treated with GLP-1	ARIP cells were differentiated to insulin synthesizing cells while PANC-1 cells phenotype was not affected	51
Human islet cells containing endocrine and duct cells cultured for 4 weeks in media with EGF and gastrin	The number of CK19 positive duct cells expressing Pdx1, insulin, and C-peptide was significantly increased (+678%) with increased β-like cells (+118%). Thus, CK19 positive pancreatic ductal cells were a source of new β-cells	48
FACS-sorted PDECs from adult mouse cultured with bFGF	bFGF caused the differentiation of these cells to insulin-secreting cells	112
Murine FACS-sorted NPPCs exposed to GLP-1 and sodium butyrate	The NPPCs were differentiated to insulin-producing cells	52
Culture of human pancreatic duct-rich populations with Ex-4, nicotinamide, KGF, Pdx-1 and NeuroD proteins	After two weeks of culture, human pancreatic progenitors were differentiated to insulin-producing cells	113
Pancreatic ductal fragments from mouse pancreas grown in RSPO1-based 3D cultures	The fragments developed to organoids able to differentiate into ductal as well as endocrine cells upon transplantation under kidney capsule of immunodeficient mice	114
Progenitors from human and mouse embryonic pancreas stimulated by the Wnt agonist RSPO1, FGF10 and/or EGF in 3D cultures	Pancreatic progenitors were expanded to duct-like structures in the presence of EGF and were differentiated to cyst-like structures containing insulin, glucagon, and somatostatin-positive cells in the absence but not presence of EGF	115
Rat PDSCs were cultured in a 3D cell culture system in media supplemented with nicotinamide, FGF, and KGF	Rat PDSCs were differentiated to islet-like cell clusters which expressed insulin as detected by DTZ staining	54
Adult mouse pancreatic progenitor-like cells were cultured in media supplemented with insulin, transferrin, selenium, glucose, and nicotinamide	After two weeks of culture small dense cell clusters were formed and insulin presence in these clusters was confirmed by DTZ staining	116
Rat NPPCs cultured in PLGA membranes in media supplemented with bFGF, nicotinamide, activin-A, β-catenin, and exendin-4	Nestin-positive cells were differentiated mainly to insulin-secreting cells but the amount of insulin released was lower than in primary β-cell cultures	20
***In vivo* studies**		
Human fetal pancreatic cells were transplanted to rats treated with KGF	The ductal cells proliferated and differentiated to β-cells resulting in increased β-cell mass	117
NOD diabetic mice treated with EGF and gastrin	After 2 weeks of treatment, normoglycemia was achieved and β-cell mass was expanded from 15% to 47% of normal cell mass as a result of neogenesis from pancreatic ductal networks	46
The human betacellulin-adenoviral vector was retrogradely injected to mice pancreatic duct	The betacellulin gene transduction resulted in the formation of insulin-positive cells in the duct linings or associated islet-like cell clusters	118
STZ-diabetic rats were treated with GLP-1/ /exendin-4	The number of small cell clusters in pancreatic ducts was increased which improved glucose tolerance in diabetic rats	119
The partly pancreatectomized transgenic mice overexpressing TGF-β used to find the source of β-cells	Intraislet pancreatic ductal networks were present in transgenic but not in wild type mice and lineage tracing confirmed that neo-β-cells were derivatives of duct cells	39
In non-autoimmune diabetic C57BL/6 mice hyperglycemia was induced by alloxan and mice were treated for 56 days with low dose EGF and gastrin	Lineage tracing showed that $Sox9^+$ cells differentiated into insulin-producing β-cells which normalized blood glucose levels	42

*The abbreviations are explained at the beginning of the paper

concluded that first acinar cells dedifferentiate back to progenitor phenotype and then redifferentiate to insulin-secreting cells.

Centroacinar cells as a source of β-cells

Centroacinar cells (CACs) are a type of the exocrine pancreas cell and are present at the centre of acini in

juxtaposition to the terminal pancreatic ducts. CACs have a unique cell morphology and express the endocrine differentiation marker Sox9 [61–64]. Beer *et al.* reported that centroacinar cells (CACs) are a type of ductal pancreatic cell which possesses the progenitor properties and replaces β-cells in zebrafish [65].

The centroacinar cells in zebrafish led to the secondary islet formation after inhibition of Notch signaling [66]. The Nkx6.1-transgenic zebrafish showed fluorescent markers for endocrine progenitor cell phenotype under the Notch-inhibition. The Notch-responsive cells were centroacinar cells, which showed a pancreatic endocrine progenitor phenotype and led to the regeneration of endocrine cells after Notch-inhibition [66]. In order to further assess the ability of centroacinar cells for endocrine pancreas regeneration in adult zebrafish, Delaspre *et al.* established the transcriptome of adult CACs. Then, through the use of gene ontology and *in situ* hybridization, they found that CACs were enriched in pancreatic progenitor markers. Moreover, new β-cells arose from CACs after β-cell ablation or partial pancreatectomy as confirmed by the lineage tracing [22]. Another group also used the β-cell ablation model of zebrafish to show the progenitor properties of CACs. Ghaye *et al.* [67] developed the transgenic lines of Nkx6.1 and Ascl1b-positive cells expressing GFP. In the near complete β-cell ablation model of adult zebrafish, the Nkx6.1 cells were traced as progenitors of pancreatic tissue while Ascl1b-positive cells only gave rise to endocrine cells. These Nkx6.1 cells were present at the end to a ductal tree and were responsive to Notch signaling showing their centroacinar phenotype [67]. The findings of both of these studies suggest that adult CACs are similar to larval CACs and retain the ability for β-cell neogenesis. Thus, it is safe to say that CACs are the progenitors of endocrine cells, especially β-cells in larval as well as adult zebrafish.

For the probing of the progenitor capabilities of centroacinar cells in mammals Rovira *et al.* used the mouse as a model animal in their study [68]. They found that centroacinar and terminal ductal cells showed high levels of ALDH1 enzymatic activity which was used for FACS-based isolation of these cells. The transcription analysis of FACS-isolated cells revealed that they were not the differentiated cells but rather were enriched for markers associated with pancreatic endocrine progenitor population. Moreover, the culturing of CACs in suspension culture resulted in pancreatosphere formation which displayed the pancreatic endocrine and exocrine cell differentiation capacity along with glucose-responsive insulin secretion. For pancreatosphere formation assays on CACs, cells were grown for 5–7 days in cultures

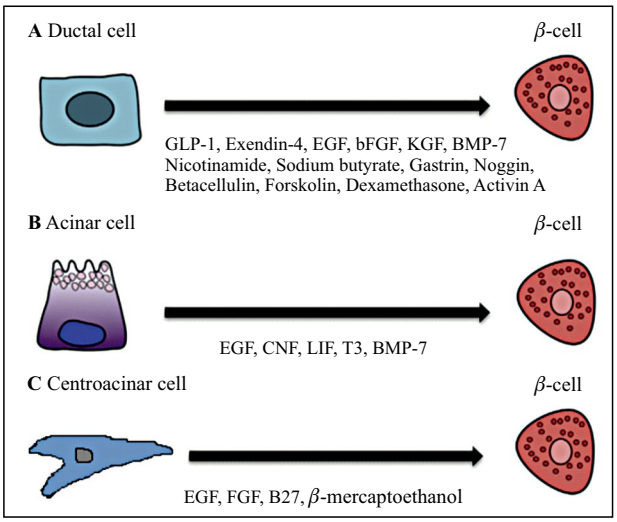

Figure 1. Some of the factors used for in vitro differentiation of pancreatic ductal (A), acinar (B) and centroacinar cells (C). The ductal and acinar cells do not directly transit to β-cell phenotype but instead first attain a progenitor stage and then redifferentiate to insulin-secreting cells.

enriched with EGF, FGF-2, B-27 cell culture supplement, β-mercaptoethanol, nonessential amino acid, and LIF. Furthermore, the CACs and terminal ductal cells also showed intense expansion under chronic epithelial injury conditions [68]. These findings support the reported zebrafish studies and suggest that CACs cells are certainly adept of progenitor's task to provide the upkeep of tissue homeostasis in the adult mouse pancreas.

In summary, so far, we have well understood that centroacinar cells are a type of progenitor cells in the pancreas with the ability of pancreatic exocrine and endocrine tissue regeneration according to the conditions. The findings of the studies mentioned above also support the β-cell neogenesis pathway for the regeneration of β-cell mass. These CACs cells can also be sorted *in vitro* for the expansion of islets or only β-cells to provide a source of β-cells replacement.

Figure 1 shows different factors used for *in vitro* differentiation of pancreatic ductal, acinar and centroacinar cells to β-cell.

Endocrine cells giving rise to β-cells

The endocrine pancreas has various endocrine cell types secreting different hormones which are α-cells (glucagon), β-cells (insulin), δ-cells (somatostatin), ε-cells (ghrelin), and PP cells (pancreatic polypeptide) [7]. These hormone-secreting cells are found in condensed structures in the pancreas called islet of

Langerhans. Islet β-cells sense the glucose-in-blood concentration, synthesize and secrete insulin into blood circulation which is responsible for regulating glucose metabolism in the body [69]. The pancreatic islet is likely the dwelling to search for cellular sources of new β-cells because of the common developmental lineage between the different endocrine cells in the islet of Langerhans [70]. The possibilities of islet cells as progenitors will be discussed in this section.

Potential of β-cell replication for its replenishing

The primary mechanism for expansion of β-cell mass during the neonatal period of growth is the proliferation of existing β-cells [71]. Lineage tracing studies confirmed that the replication of preexisting β-cells is the main mechanism accountable for normal β-cell turnover in adult mice [16, 72] and also for regeneration of β-cell mass following β-cell ablation [73, 74]. So, in rodents, the major source for expansion of β-cell mass is the replication of existing β-cells [75]. Though the increase in β-cell mass of neonates occurs by replication of preexisting β-cell, this mechanism disappears after 2 years in humans [71, 76]. Under the physiological conditions in adult humans, the regeneration of β-cells is rare, and so it is not confirmed hitherto from where comes the β-cells in normal conditions [7, 76]. However, the increased regenerative ability of β-cells in conditions of pregnancy [77] and obesity [78] gives us a clue about the replication of existing insulin-producing cells. Nonetheless, replication of existing β-cells and their *in vitro* expansion ability is a well-studied mechanism for restoring β-cell mass of diabetics [10, 79].

Dhawan *et al.* [23] reported that the likely reason for the decreased regenerative capacity of adult β-cells was an accumulation of p16INK4a which caused inhibition of the cell cycle ending up in the limited regenerative capacity of adult endocrine cells. Additionally, they found that TGF-β signaling maintained the p16INK4a through Smad3 which joined in with trithorax to maintain and activate the p16INK4a levels to prevent the replication of β-cells. Thus, the inhibition of TGF-β signaling with different chemical inhibitors resulted in the repression of p16INK4a locus ending up in increased replication of β-cells in adult mice [23]. Recently, Puri *et al.* studied the role of c-Myc in β-cell replication [80]. They used c-Myc transgenic mice and INS-1 cells with c-Myc overexpression (siRNA) which were cultured with L-glutamine, sodium pyruvate and 2-mercaptoethanol. They showed that increased expression of c-Myc resulted in increased proliferation of β-cells but the cells produced were immature. The transcriptome analysis validated the immaturity of new β-cells as the proliferating cellular transcripts were increased and transcripts of the mature β-cells phenotype were decreased which resembled development of islets [80]. Moreover, the genetic deletion of c-Myc resulted in a ~50% decline in the proliferative cell pool during postnatal expansion, which showed the importance of this protein during the replication of β-cells. The authors concluded that the adult β-cells retain a balance between functional and proliferative properties as the proliferation of β-cells leads to functional immaturity [80].

Nielsen *et al*. reviewed the possible factors used for enhancement of β-cell replication in *in vivo* culture. Cytokines such as GLP-1, growth hormone (GH) and prolactin (PRL) were proposed as the tangible factors for the uplifting of *in vitro* β-cell replication. These factors worked best when the cells were dedifferentiated and entered the proliferative phase [81]. *In vitro* expansion of β-cells using adult human islets was successfully attempted by Ouziel-Yahalo *et al*. [82]. The culturing conditions included activin A, betacellulin, and exendin 4 as differentiation-inducing factors. After seven days of culture, the number of β-cells was doubled as measured by BrdU labeling. The dedifferentiation of β-cells resulted in their increased size and redifferentiation to β-cell phenotype, especially in betacellulin supplemented media. However, the functionality of replicated β-cells was not high compared to the primary islet β-cells [82].

In summary, these studies suggest that β-cell replication is a possible mechanism and it involves the dedifferentiation of mature β-cells to cells with less mature phenotype which further proliferate to form new β-cells.

α- to β-cell transdifferentiation

Mature islet cell's plasticity has remained a puzzle until lineage tracing models in mouse [83] and zebrafish [84] confirmed the transdifferentiation ability of α-cells by the transition to β-cells given the conditions of severe β-cell loss [83, 84]. As we already know, adult β-cells have a long lifespan, and they replicate rarely, though they have the self-duplication ability [16, 72]. However, this self-duplication cannot counterbalance the excessive loss of β-cells and for compensating the excessive loss of β-cells, mature glucagon secreting α-cells have been traced to differentiate to β-cells [85]. This kind of cellular transition is possible because α- and β-cells are formed from the same multipotent progenitors during pancreatic islet development [70, 86].

Lineage tracing in diphtheria-toxin induced total β-cell ablation mouse model showed that α-cells were the source of β-cell regeneration [83]. In another

in vivo model of β-cell regeneration, Chung *et al.* used partial duct ligation and alloxan for ablation of existing β-cells [85]. After two weeks it was found that neogenic β-cells came from α-cells. The authors documented that in this model α-cells were not directly converted to β-cells, but instead, they passed through an intermediate stage in which they express the markers for both α-and β-cells and secrete both glucagon and insulin after one week of β-cell ablation [85]. However, after two weeks of PDL plus alloxan combination, the glucagon secretion was minute, and the cells resembled β-cells in phenotype and the expression of MafA was also high [85]. In another study, zebrafish was used as a model for lineage tracing of β-cell regeneration using near total ablation of existing β-cells [84]. The expression of glucagon was increased after the injury suggesting its role in α- to β-cell transdifferentiation. These results, in favor of previous studies, showed that α-cells serve as a reservoir pool for the β-cell regeneration [84] and imply the plasticity of the α-cells to be converted into β-cells [70].

Andrzejewski *et al.* reported the role of activin signaling in α-to β-cell phenotype transition. They treated α-and β-cell lines and also sorted mouse islet cells with activin [24]. In αTC1-6 α-cell line, the expression of α-cell genes, Aristaless-related homeobox (Arx), glucagon, and MafB was suppressed following the treatment with activin A or B. In INS-1E β-cell line, the expression of Pax4 and insulin was increased following the activin A treatment [24]. Furthermore, the exposure to activin A suppressed the expression of α-cell genes and enhanced the expression of β-cell genes in sorted primary islet cells. These results suggest that activin signaling destabilized the α-cell phenotype whereas promoted a β-cell fate in cultured cells [24]. To further verify the hypothesis of activin mediated α- to β-cell transdifferentiation, Brown *et al.* developed Fstl3 knockout mouse labeled with Gluc-Cre/yellow fluorescent protein (YFP) for lineage tracing [87]. Follistatin-like 3 (Fstl3) is the antagonist of activin, and its inactivation resulted in the expansion of β-cell mass and improved glucose homeostasis [88]. The number of Ins^+/YFP^+ cells was significantly increased in Fstl3 KO mice compared with wild type mice. The treatment of isolated islets with activin resulted in a significantly increased number of YFP^+/ /Ins^+ cells [87]. These pieces of evidence suggested that transdifferentiation of α-cells to β-cells was influenced by activin signaling and contributed substantially to β-cell mass [87].

Chakravarthy *et al.* deciphered another possible mechanism for the conversion of α-cells to β-cells in adult mice [89]. The inactivation of two regulators of α-cell functionality, Arx and DNA methyltransferase 1 (Dnmt1), resulted in the conversion of α-cells to β-cells. This conversion took three months, and the newly formed β-cells were similar to native β-cells when checked for gene expression using RNA-sequence. The neogenic β-cells also secreted insulin upon glucose stimulation [89]. In 2017, two remarkable reports showed the role of gamma-aminobutyric acid (GABA), a neurotransmitter found in the nervous system and also in the endocrine pancreas [90], in α-to β-cell transition [91, 92]. In the first report Ben-Othman *et al.* reported that GABA induces the α-cell-mediated β-cell neogenesis *in vivo* in mice after three months of treatment with GABA. The newly produced β-cells were functional and could replace the native β-cells. When newly transplanted human islets were treated with GABA, they showed an increased number of β-cells at the cost of α-cells [91]. In a similar report, Li *et al.* [92] tested artemisinin, an antimalarial drug which enhances GABA signaling by binding to $GABA_A$ receptors, for β-cell neogenesis from α-cells. Artemisinin repressed the master regulator of glucagon-secreting α-cells, Arx, by causing its displacement to the cytoplasm. Thus, the α-cells lost their identity and were transdifferentiated to β-cells in zebrafish, rodents and primary human pancreatic islets [92]. However, a recent study by Ackermann *et al.* contradicted the role of both GABA and antimalarial drug in the α- to β-cells conversion [93]. They treated mice for three months with artesunate and GABA and showed by cell-specific genetic lineage tracing that no α- to β-cells transdifferentiation happened and insulin secretion was also not stimulated [93]. In another recent report, Shin *et al.* used rhesus monkey to translate the rodent β-cell regeneration model [94]. They used STZ for β-cell ablation followed by porcine islet transplantation to maintain normoglycemia, but in both conditions, there was no increase in β-cell number or serum C-peptide level. They also checked the *in vivo* and *in vitro* transdifferentiation ability of α- to β-cells by GABA treatment, and yet again no β-cell regeneration was found [94]. Eizirik and Gurzov [95] proposed four main reasons for the contrary results of the reported papers [91–94] and first of them was the use of different experimental models: zebrafish, rodents and primary human pancreatic islands [92], a single mice model [93], mice model that was confirmed in human and rat [91], rhesus monkey [94]. Secondly, Ackerman *et al.* used tamoxifen-induced lineage-tracing for following the adult α- to β-cell transition [93] whereas Ben-Othman *et al.* used Glucagon-Cre mice which can also detect the β-cell neogenesis from a transient and putative cell state expressing glucagon [91]. Thirdly, the GABA used in those experiments was not made for purpose of *in*

vivo use and finally, the diet and housing conditions were different in those laboratories which could have affected the murine microbiome and ultimately drug metabolism and systemic mouse metabolism [95].

Thus, we come to know that under the severe loss of β-cells due to metabolic stress or injury, the transition of α-cells to β-cells becomes stimulated. The regulation of this conversion is possible due to the genetic programming which involves the repression of α-cell genes and activation of β-cell genes. Therefore, different factors which can repress the α-cell signature genes and/or enhance the β-cell signature genes in α-cells can be used for designing a fruitful protocol for *in vitro* generation of β-like cells from α-cells.

δ-cells transdifferentiation to β-cells

Delta cells are found in the islets of Langerhans and they are responsible for somatostatin secretion [96]. The attempts to find the source of new β-cells and location of dedicated or facultative progenitors in adult mammals led to the studies for δ- to β-cell transdifferentiation following the models of α- to β-cell transdifferentiation [97]. Chera *et al.* administered diphtheria toxin (DT) to postnatal two week-old mice and adult mice for ablation of β-cells [25]. The administration of DT resulted in 99% ablation of β-cells; however, afterwards mice regenerated β-cells from other islet cells. The lineage tracing revealed that in the adult mice the induction of post-DT β-cell regeneration was by transdifferentiation of α-cells while in mouse pups the δ-cells were dedifferentiated and reprogrammed to β-cells. The dedifferentiated cells showed fewer somatostatin transcripts, and high insulin transcripts and no polyhormonal cells were found [25]. Druelle *et al.* generated and characterized Cre-LoxP transgenic mice that express *Pax4* specifically in somatostatin-expressing δ-cells cells [97]. They demonstrated that the ectopic expression of *Pax4* in δ cells is sufficient to induce their conversion into functional β-like cells that can partly reverse chemically-induced diabetes and induce β-like cell hyperplasia [97].

Although more work is needed for validation of δ-cells as the source of *in vivo* β-cell expansion, these results favor the plasticity of islet cells for their transdifferentiation into β-cells.

The summary of some factors used for the differentiation of pancreatic endocrine cells to β-like cells is presented in Figure 2.

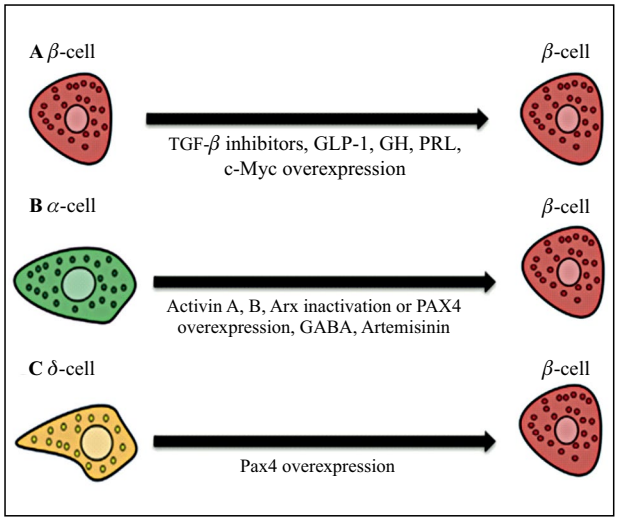

Figure 2. Some factors used for in vitro β-cell replication (A) as well as α-cell (B) and δ-cell (C) transdifferentiation to β-cell

Pancreatic islet-derived mesenchymal stem cells giving rise to β-like cells

Mesenchymal stem cells (MSCs) can differentiate into many target cells depending upon the applied conditions [98–100]. It was shown that transplantation of bone marrow-derived [101] and adipose tissue-derived [102] MSCs enhanced the regeneration of pancreatic islet and decreased the blood glucose levels of diabetic animals [103]. Furthermore, MSCs have shown a decent safety profile in clinical trials with limited risk of tumor formation [104]. The pancreatic islet also has mesenchymal stem cells (PI-MSCs) which play important roles as a source of ECM and during pancreatic islet injury. Here, we will shortly report studies showing the use of these PI-MSCs as a source of insulin-producing cells.

The quest for finding the stem or progenitor cells in the pancreas has led the researchers to search for these cells in different compartments of the pancreas. Zulewski *et al*. showed that rat and human pancreatic islets contain the distinct population of cells positive for neural stem-cell-specific marker nestin [105]. Apart from the islets of Langerhans, pancreatic ducts also contain the nestin-positive cells with the capability of giving rise to β-cells as we have discussed earlier. In *in vitro* culture, the nestin-positive cells have shown boundless proliferation and differentiation abilities as these cells were differentiated to acinar, ductal, and endocrine phenotype cells [105]. These findings suggested that the pancreatic islets have the progenitor cells with the ability to regenerate the islets of Langerhans [105]. Karaoz *et al.* isolated stem cells (SCs) from pancreatic islets and characterized their stem cell properties [106]. They found that the pancreatic islets SCs expressed the markers of embryonic SCs (Oct-4, Sox-2, and Rex-1) and also showed the high proliferation ability coinciding with the

bone marrow MSCs. Furthermore, these pancreatic islets SCs expressed the differentiation markers of adipo-, chondro-, neuro-, myo-, and osteogenic cells [106]. Thus, pancreatic islets contain stem cell with the ability to be expanded *in vivo* for replacement therapy of diabetes [106]. Gong *et al.* found that the CD117-positive cells were very few in normal pancreatic tissue, but their number increased after induction of pancreatitis [107]. In this model of pancreatic inflammation, the CD117-positive cells were only present in the pancreatic islet and participated only in the repair of islets of Langerhans but not in the repair of extra-islet pancreatic tissue. Thus, these findings suggested that the CD117 positive progenitor cells are not the true stem cells, but merely a type of islet progenitor or precursor cells [107].

It has been shown that microenvironment is important for the regulation of stem cells proliferation and differentiation [26]. Use of glycated collagen in culture media resulted in the induction of rat pancreatic islet-derived mesenchymal stem cells differentiation to insulin-secreting cells. PI-MSCs were induced by using glucose, hEGF, nicotinamide, activin-A, exendin-4, hHGF, and pentagastrin [26]. The differentiation efficiency and insulin expression in collagen cultures were higher than in control cultures; however, glucagon was also present in media showing the presence of α-cells [26]. Coskun *et al.* showed that valproic acid, class-I histone deacetylase inhibitor (HDACi) and glucose induced the differentiation of PI-MSCs to insulin-secreting cells with a fair efficiency, however, the newly formed β-cells were non-functional [108].

Gao *et al.* were the first to culture bovine pancreatic MSCs of (B-PMSCs) for differentiation to insulin-producing cells [109]. Growth kinetics of B-PMSCs revealed their decent *in vitro* self-renewal capability. Retinoic acid, HGF, and EGF were used for the induction of stem cells to insulin-secreting clusters. After 21 days of culture, the DTZ staining showed the presence of β-cells which were positive for glucose-stimulated insulin-secretion in culture media [109]. Most recently, the differentiation of MSCs from different sources into IPCs within 3D alginate matrices was performed by Cañibano-Hernández *et al.* [5]. MSCs from different sources were used, but the quantity of insulin released was high in IPCs differentiated from pancreatic islet-derived MSCs. Moreover, the amount of insulin secreted was increased after adding hyaluronic acid (HA) in alginate microcapsules [5].

The β-like cells developed *in vitro* in the presence of cytokines and/or inhibitors mentioned above were less in number and also were not functional enough to serve as the β-cell replacement source. In order to achieve the maturation of *in vitro* produced β-like cells, Nair *et al.* elegantly reported a reliable method for the maturation of *in vitro* generated β-like cells [110]. They recapitulated the culture conditions mimicking *in vivo* pancreatic islet organogenesis and β-cell maturation. In these conditions, the immature β-like cells were isolated and reaggregated in the form of islet-size enriched β-cell clusters (eBCs) — each eBCs comprised by aggregation of 1000 β-like cells mimicking the number of cells present in human islet [110]. After seven days, eBCs showed the properties of mature β-cells. The physiological properties exhibited by eBCs presented dynamic insulin secretion, increased calcium signaling in response to secretagogues, and improved mitochondrial energetics resembling primary human β-cells. Unlike previous studies in which long time was required for insulin secretion [28, 111], within three days of transplantation in mice, eBCs showed glucose-stimulated insulin secretion [110]. However, some dual-hormone cells, the likely progenitors of glucagon-secreting α-cells were also present in the clusters produced.

Conclusions

Attempts to control the diabetic epidemic have resulted in the development of many strategies for the replacement of β-cells. Still, to the best of our knowledge, *in vitro* development of functional islets or insulin-producing cells is not efficient enough to be used as a routine clinical option for curing diabetes due to many problems. These problems include production in low number, immature β-cell production, secretion of low amount of insulin, polyhormonal nature of cells, the impurity of cells, and the high cost of factors used in protocols for *in vitro* differentiation. While developing *in vitro* protocol, tactics like the recapitulation of *in vivo* conditions can warrant the production of an ample number of mature and functional β-cells. However, for mirroring *in vivo* conditions, first, we need to have vibrant knowledge about pancreas development which is instead a convoluted process and not fully understood yet. Though, the use of three-dimensional culture systems and tools like single-cell transcriptome analysis can help us understand the cellular and molecular mechanisms of pancreatic development assisting in the development of viable protocols for *in vitro* production of *bona fide* β-like cells.

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Conflict of interest

The authors hereby declare that they do not have any conflicting interest.

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