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(RE)GENERATING HUMAN BETA CELLS: STATUS, PITFALLS, AND PERSPECTIVES

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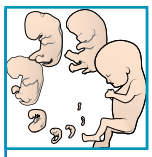

Baeyens L, Lemper M, Staels W, De Groef S, De Leu N, Heremans Y, German MS, Heimberg H. (Re)generating Human Beta Cells: Status, Pitfalls, and Perspectives. *Physiol Rev* 98: 1143–1167, 2018. Published May 2, 2018; doi:10.1152/physrev.00034.2016.—Diabetes mellitus results from disturbed glucose homeostasis due to an absolute (type 1) or relative (type 2) deficiency of insulin, a peptide hormone almost exclusively produced by the beta cells of the endocrine pancreas in a tightly regulated manner. Current therapy only delays disease progression through insulin injection and/or oral medications that increase insulin secretion or sensitivity, decrease hepatic glucose production, or promote glucosuria. These drugs have turned diabetes into a chronic disease as they do not solve the underlying beta cell defects or entirely prevent the long-term complications of hyperglycemia. Beta cell replacement through islet transplantation is a more physiological therapeutic alternative but is severely hampered by donor shortage and immune rejection. A curative strategy should combine newer approaches to immunomodulation with beta cell replacement. Success of this approach depends on the development of practical methods for generating beta cells, either in vitro or in situ through beta cell replication or beta cell differentiation. This review provides an overview of human beta cell generation.

I. KEY POINTS

- Current knowledge on human endocrine pancreas development, gained from scarce histopathology samples, indicates some analogy with rodents.
- Human beta cells are long-lived with limited expansion potential. Nevertheless, the adult human beta cell mass retains plasticity to adapt to changing metabolic needs.
- High-content screening has identified several novel adult beta cell mitogens. However, a lack of cell specificity warrants caution for clinical translation.
- Breakthroughs in directed differentiation of human embryonic stem/induced pluripotent stem cells create hope for cell therapy in diabetes. Overcoming the risk for teratoma formation and immune rejection will determine its true clinical potential.
- Multiple approaches, including transdifferentiation of adult human non-beta cells, harbor potential for clinical translation as an alternative to stem cell-based therapy. A particularly attractive strategy would be in vivo reprogramming of human acinar cells.

II. INTRODUCTION

Diabetes mellitus is a chronic metabolic disorder characterized by elevated blood glucose levels and increased risk of micro- and macrovascular complications. Currently 415 million people have diabetes globally, and that number is expected to rise to 642 million by 2040 (116a). Several subtypes of diabetes can be clinically distinguished, including polygenic/multifactorial, monogenic, and secondary forms (165). The two most common forms of diabetes, type 1 (T1DM) and type 2 (T2DM) diabetes, are polygenic and multifactorial. A large number of rarer monogenic and secondary varieties together explain roughly 10% of diabetes (79, 246). Gestational diabetes [GDM, prevalence of

around 10% of pregnancies (19, 138)], defined as maternal diabetes commencing during pregnancy, represents a transient form of polygenic and multifactorial diabetes caused by situational, relative insulin deficiency.

T1DM is characterized by an absolute shortage of circulating insulin due to progressive autoimmune destruction of the insulin-producing pancreatic beta cells (3). In contrast, T2DM results from a relative shortage of circulating insulin due to *1*) failure of the beta cell mass to adapt to rising metabolic demands, most often caused by obesity-related peripheral insulin resistance; and *2*) increased beta cell death and dysfunction (41, 109, 183, 278). Reduced insulin signaling (whether from insulin resistance or beta cell failure), results in decreased tissue uptake of glucose and increased glucose release by the liver and kidney. The resulting chronic hyperglycemia in turn damages blood vessels and nerves, culminating in multiorgan damage (83, 108).

Current pharmacological therapy for diabetes consists of exogenous insulin injections and/or oral glucose-lowering medications. None of these therapies directly addresses the underlying beta cell deficit in most patients with diabetes. Beta cell replacement therapies could effectively restore and protect the functional beta cell mass. Transplantation of cadaveric human donor islets represents one approach to beta cell replacement.

The landmark Edmonton protocol overcame prior poor success rates by using improved islet isolation techniques, fresh islet preparations, and a steroid-free immunosuppressive regimen to significantly improve transplantation outcomes (220, 233, 234). This study pioneered a series of reproducibility trials and improved protocols worldwide, leading to insulin-independence durations of at least 5 yr in 50% of engrafted patients (20). Nevertheless, shortage of donor islets severely limits more widespread clinical use and has lowered the goal from achieving insulin independence to reducing glycemic variability and elimination of hypoglycemia unawareness (107).

Therefore, the need for alternative supply of human islet cells is obvious. Insulin-producing cells can be generated by replication of preexisting beta cells and/or by (trans) differentiation of non-beta cells (38, 125). Since rodent models remain the most prevalent and accessible tool for preclinical research, insights obtained from rodent pancreas development have guided the design of human beta cell generation protocols. Although some analogy exists between human and rodent pancreas development and function, notable interspecies differences remain. Understanding the pathways that control human beta cell generation will be critical to devising a successful beta cell therapy for diabetes.

III. HUMAN PANCREAS DEVELOPMENT AND POSTNATAL HOMEOSTASIS

A. Human Pancreas Development

1. Specification and early differentiation of multipotent pancreatic progenitor cells

Similar to rodent, the human embryonic pancreas develops from the primitive gut tube early in development. The timeline of human development is defined according to the Carnegie system (Carnegie Stage, CS), based on age estimates until 60 days post conception (dpc) when recognizable human features become apparent and nomenclature is switched from embryo to fetus (185). The human embryonic pancreas evolves as one dorsal and two ventral buds from the primitive foregut at 30–33 dpc (CS12–13) (118, 119, 198). After regression of the left ventral bud, the right ventral bud fuses with the dorsal bud around 58 dpc (CS22–23) (169, 198, 200) **(FIGURE 1)**.

Due to limited access to relevant early developmental human pancreatic tissue, insights into the earliest developmental stages of gut tube regionalization and pancreas specification rely almost solely on model organisms. Prior to budding (25–27 dpc, CS10) and similar to pancreas development in chick (104, 136) and mouse (135), the notochord directly contacts the dorsal prepancreatic endoderm, leading to regional repression of sonic hedgehog (SHH) (118). This notochord-endoderm interaction is interrupted upon fusion of the paired dorsal aortas around 29–31 dpc (CS11) **(FIGURE 1)**. In contrast to mouse where the developmental transcription factor pancreatic and duodenal homeobox 1 (*PDX1*) can be detected at the earliest stage of pancreas development (E8.0) when endoderm is still in contact with the notochord (85, 124, 186, 251), PDX1 does not appear in human pancreas until 29–31 dpc (CS12) when the notochord-endoderm interaction is disrupted (118, 240).

At 30–33 dpc (CS13), the stratified epithelium of the developing pancreatic expresses the same early pancreatic transcription factors–*PDX1*, pancreas specific transcription factor 1A (*PTF1A*), forkhead box A2 (*FOXA2*), GATA binding protein 4 (*GATA4*), Nirenberg and Kim homeobox factor 6.1 (*NKX6.1*), and sex determining region Y-box 9 (*SOX9*)–that define the multipotent pancreatic progenitor cells (MPPCs) that give rise to all pancreatic cell lineages in the developing murine pancreas **(FIGURE 2)** (57, 118, 160, 198). In analogy to murine development, mesenchyme-derived fibroblast growth factor (FGF)7 and FGF10 amplify the MPPC pool by stimulating proliferation of $PDX1^+$ $cytokeratin^+$ MPPCs, resulting in substantial organ growth between 33 and 45 dpc (CS14–18) **(FIGURE 1)** (25, 27, 282). Expression of genes involved in WNT signaling including numerous noncanonical WNT regulators (45) increases in

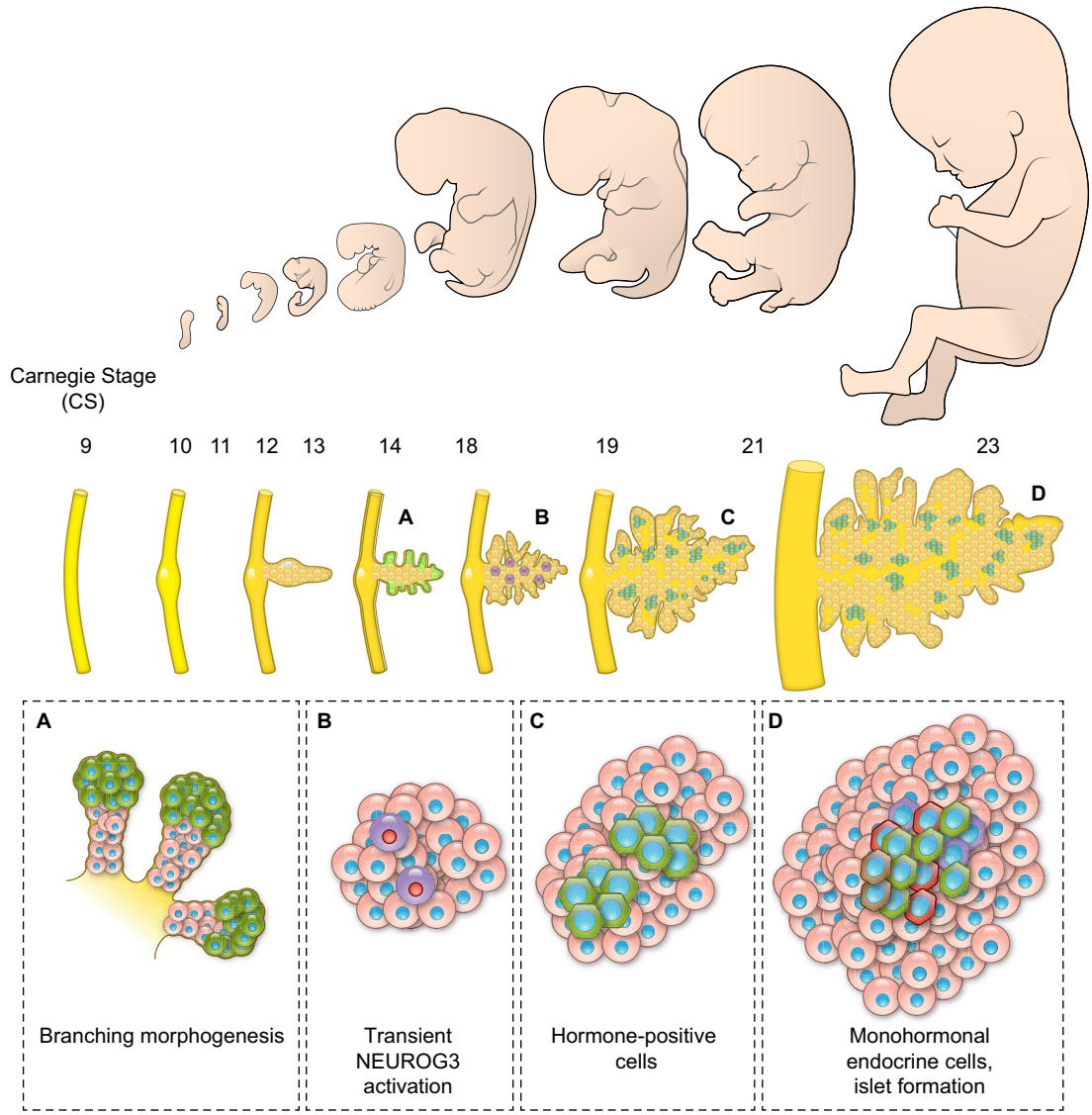

FIGURE 1. Schematic representation of human pancreas development. *Top panel*: human pancreas development is shown in relation to the Carnegie classification system (CS; numbers 9 to 23). Embryos are drawn proportional to the UNSW Human Embryo Resource. *Middle panel*: pancreas morphogenesis is not depicted proportionally but is intended to frame the key developmental steps (*bottom panel*) in the development of the organ. The human embryonic pancreas evolves as one dorsal and two ventral buds from the primitive foregut (yellow tube) at CS12–13. Prior to budding, the dorsal prepancreatic endoderm is in contact with the notochord (red line). After regression of the left ventral bud, the right ventral bud fuses with the dorsal bud around CS22–23. *Bottom panel*: key developmental steps include branching morphogenesis (green, tip cells; orange, trunk cells) (*A*), the first appearance of NEUROG3$^+$ cells (pink) (*B*), formation of the initial hormone$^+$ cells (green) (*C*), and establishment of lineage-committed monohormonal endocrine cells (green, red, brown, purple) and formation of the islet cytoarchitecture (*D*).

MPPCs during 37–45 dpc (CS16–18), before significant acinar and endocrine cell differentiation. From 37 to 40 dpc (CS16) onwards, active growth and branching results in a lobular epithelium (118, 200). Starting around 45–47 dpc (CS19), the growing epithelial branches begin to organize into distinct trunk and tip domains **(FIGURE 1)** (118). The progenitor cells in the tip domain give rise to acinar cells while the trunk progenitors yield ductal and endocrine cells. At 14 wk post conception (wpc), tip-trunk segregation is complete and trunk cells lose *GATA4* expression while tip cells cease *SOX9* expression (118).

Notably, the succession of appearance of endocrine cells in the early developing human pancreas differs from mouse. Rather than the glucagon$^+$ that appear first in the mouse, insulin$^+$ cells appear first in the developing human pancreas, around 49–52 dpc (CS21) **(FIGURE 1)**, 3 wk after budding, followed by glucagon$^+$ and somatostatin$^+$ cells at 8.5 wpc and by pancreatic polypeptide$^+$ and ghrelin$^+$ cells at 9 wpc (118, 160, 198, 214). At mid-gestation (14–16 wpc), a near 1:1 ratio between insulin$^+$ and glucagon$^+$ cells is observed that increases with time to approximate 1.5:1 postnatally (120). While 20–40% of the early endocrine

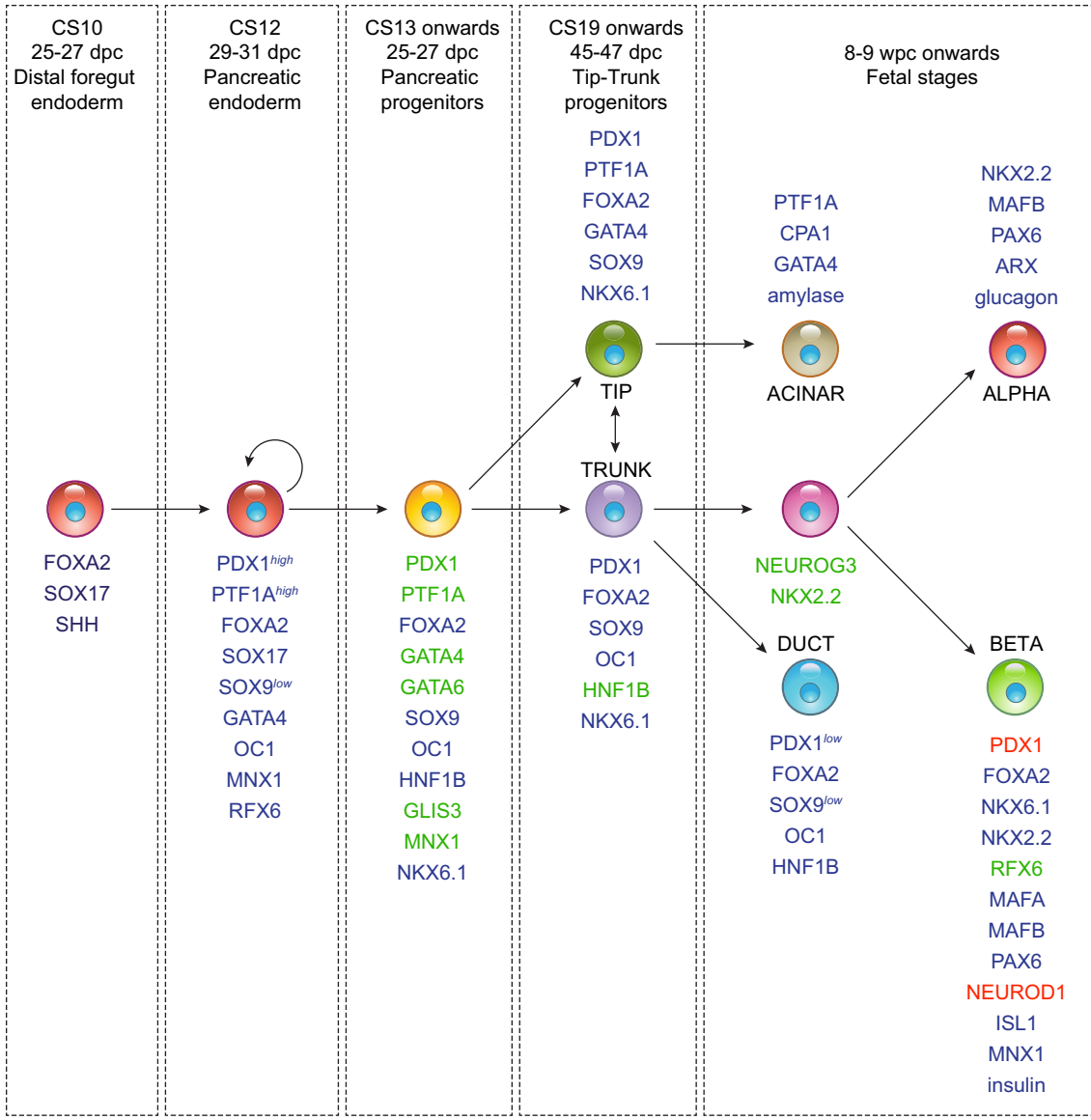

FIGURE 2. Overview of transcription factor expression during human beta cell development. Schematic overview of the signaling cascade governing human pancreas development including currently known transcription factors responsible for cell type fate determination. Transcription factors depicted in green are associated with the development of monogenic diabetes; in red are the transcription factors known to cause MODY.

cells coexpressed insulin and glucagon between 9 and 16 wpc, this fraction declines by 21 wpc such that the vast majority of fetal endocrine cells is monohormonal (120, 214). Notably, ghrelin does not colocalize with insulin or glucagon, suggesting a separate ontology of epsilon cells (214). While clusters of fetal beta cells are present by 10 wpc (118), true islet-like structures only appear from 12 wpc onward (198), reaching a maximum average size at 14 wpc (169).

Rodent studies have demonstrated the importance of the vasculature in pancreatic endocrine development (70, 146, 287; reviewed in Ref. 272). In human pancreas, endothelial cells localize near small beta cell clusters at 10 wpc (198) and, by 14 wpc, vascular structures invade the fetal islets (169, 198). Beyond this observation, more work is needed to fully understand the role of endothelial cell differentiation and blood vessel formation in the developing human pancreas.

2. Proliferation of MPPCs and early endocrine cells

Reported cell proliferation rates in pancreas range widely, at least partially due to the use of different proliferation markers (191). As the human fetal pancreas grows, proliferation of the epithelial cells drops significantly, with high-

est rates seen around the expanding periphery of the organ while endocrine differentiation from progenitor cells occurs more centrally (119, 198). Both exocrine and endocrine cell replication rates decrease between 8 and 34 wpc (169). However, a transient burst of proliferation occurs neonatally in differentiated duct and beta cells (95).

As noted above, mesenchymal signals, especially Fgf7 (74) and Fgf10 (25), drive the expansion of the MPPCs in rodents. Human pancreatic mesenchyme also expresses both growth factors, their levels parallel MPPC expansion (22), and they activate the proliferation of human pancreatic epithelial cells in vitro (282). Together these data suggest a similar role for FGF7 and FGF10 in both human and rodent MMPC growth.

A careful analysis of the transcriptional regulatory program in human MPPCs revealed a role for noncanonical WNT regulatory genes, similar to developing mouse pancreas, and the Hippo signaling effector TEAD1 and its coactivator YAP in human MPPC expansion, mediated in part through the transcription factor SOX9 (45), which regulates MPPC growth in mouse and human (119, 197, 232). In addition, the WNT agonist R-spondin1 (RSPO1), FGF10 and epidermal growth factor (EGF) enhance human MPPC expansion in long-term cultures (27). Although Notch and retinoic acid signaling also increase mouse MPPC proliferation (reviewed in Refs. 126, 192), their role in human pancreas development is unknown. Taken together, despite evidence for the involvement of specific extracellular signals in human MMPC expansion, a comprehensive picture is still lacking.

3. Monogenic diabetes and transcription factor hierarchy in human pancreas development

The spatiotemporal expression pattern of transcription factors in the developing human pancreas taken together with the discovery of monogenic forms of diabetes caused by mutations in many of the same transcription factors confirm their importance in human pancreatic development (reviewed in Ref. 57). The hierarchy of transcription factors guiding human pancreas development has been partially disclosed by genetic studies of permanent neonatal diabetes (PNDM). Genes that cause pancreas agenesis and therefore likely contribute to early pancreas specification in human include *PDX1* (251), *PTF1A* (230, 276), and *GATA6* (49, 63, 147). Defects in endocrine cell development and manifestation of PNDM or MODY are linked to mutations in *GATA4* (236), *SOX9* (197), *RFX6* (242), *GLIS3* (66, 231), *MNX1* (28, 81), *NEUROD1* (216), and *NKX2.2* (81). In addition, heterozygous loss-of-function mutations of PDX1 and *HNF1b* are associated with MODY4 (250) and MODY5 (69, 286), respectively.

NEUROG3, a basic helix-loop-helix transcription factor that acts as the initiating pro-endocrine transcription factor in rodents (7, 94, 229), is transiently expressed in scattered epithelial cells during human pancreas development around 8 wpc, concomitant with the appearance of the first hormone-expressing cells **(FIGURE 2)** (118). NEUROG3 expression in the developing pancreas peaks between 10 and 14 wpc, declines after 18 wpc on (57, 118, 222), and disappears around 35–41 wpc (222). This wave of NEUROG3 expression overlaps with the appearance of differentiated endocrine cells in the pancreas (120, 222, 225). In addition, NEUROG3$^+$/PDX1$^+$, NEUROG3$^+$/INS$^+$, and NEUROG3$^+$/GLUC$^+$ cells can be detected briefly during these early stages of development (44, 160). However, in contrast to the biphasic Neurog3 expression pattern that parallels the first and second transition of endocrine differentiation in murine pancreatic development (271), only a single phase of NEUROG3 expression is observed in the developing human pancreas (271). Interestingly, the lack of an early phase of NEUROG3 expression and of a separate primary transition of endocrine differentiation may result from differences in the vascular signals: in human development, the paired dorsal aortas do not have early contact with the dorsal endoderm as they do in mouse embryos, underscoring the potential importance of vascular-derived signals in development of the human endocrine pancreas (118).

Based on the hierarchy of transcription factors during human pancreas development, the success of endocrine (trans) differentiation protocols can be gauged as follows (118): *1*) foregut endoderm should express FOXA2 and SOX17 (dorsal endoderm) or SHH (ventral endoderm); *2*) endoderm fated to become pancreas should express PDX1, FOXA2, GATA4, NKX6.1, and SOX9; *3*) trunk progenitors that give rise to ductal and endocrine cells should express PDX1, FOXA2, SOX9, and NKX6.1; and *4*) transient expression of NEUROG3 to initiate endocrine differentiation should correlate with loss of SOX9 and with gain of NKX2.2 and ISL1 to define fetal beta cells (118). Additional transcription factors such as PAX6, RFX6, PTF1A, HNF1B, GATA6, ONECUT1, and MNX1 should be added to this checklist since clinical case reports have demonstrated their necessity for human islet development (81).

4. A ductal niche for MPPCs

Because the MPPCs form the branching, ductlike epithelium where NEUROG3+ cells and differentiated endocrine cells first appear in the developing human pancreas, duct cells have been proposed as endocrine progenitors (32). In addition, during early fetal human development, all pancreatic epithelial cells express the ductal marker keratin 19 (KRT19) while some single endocrine cells and small islet cell clusters express both duct and endocrine markers (32, 33, 169). Many transcription factors that are confined to the postnatal ductal compartment are also expressed in MPPCs during development. Furthermore, lineage tracings using "duct-specific" promoters during early mouse pan-

creas development suggest a ductal origin of endocrine cells (86, 140, 141, 244). However, lineage tracing past the MPPC stage, when terminal differentiation is established, argues against a duct cell origin of new endocrine cells after that time (86, 140, 141, 244). Moreover, the proximity of two cells types cannot distinguish an important anatomical niche from an actual lineage relationship in which one cell type differentiates into another. Location alone is never sufficient; formal proof should be obtained from carefully designed lineage tracing experiments.

B. Postnatal Human Beta Cell Homeostasis

In humans, the adult beta cell mass roughly constitutes 2% of the total pancreas mass, corresponding to 1–2 g of tissue (278). Under normal conditions, beta cell turnover is tightly regulated. Beta cell expansion in human mainly occurs during the perinatal period, i.e., 2 mo before and after birth, when ~3% of the insulin$^+$ cells express Ki67, a marker of active cell cycling (131, 168, 169). This expansion decreases rapidly with age such that, by adulthood and under normal physiological conditions, human beta cells are mostly quiescent with replication rates below 0.1% (194). Consistent with a very low rate of replacement, human beta cells have a long half-life (54) and rarely undergo cell death (41, 168).

Notably, rates of adult beta cell proliferation are substantially lower in humans than in rodents. This difference in replicative potential between human and rodent beta cells might be explained by species-specific replicative aging: in humans, telomere shortening limits replication and leads to senescence (279). This mechanism is considered a critical anti-tumor defense, selected under evolutionary pressure to compensate for the much larger human body and its longer lifespan (237).

However, as in rodents, human beta cells can respond to increased metabolic demands, such as obesity (101) or pregnancy (40, 212), by numeric or functional compensation. In humans, the source of new beta cells in settings of increased metabolic demand or pancreatic damage remains controversial (91), but in mice, after birth, lineage tracing indicates that in most circumstances new beta cells come from the proliferation of preexisting beta cells (68, 264, 282).

In general, nutrients, especially glucose, activate signaling pathways that can mediate beta cell growth (288). Hypoglycemia leads to beta cell atrophy, whereas persistent hyperglycemia provokes hypertrophy and hyperplasia (129, 162). Beta cells are uniquely designed to detect even small changes in extracellular glucose concentrations. For instance, in mice, a 4-day-long infusion of 50% glucose results in mild hyperglycemia, hyperinsulinemia, and increased beta cell replication (3), the latter correlating with increased levels and nuclear localization of cyclin D2 in beta cells (3). Glucose metabolism also shortens the refractory period of beta cells, primarily by increasing the abundance of cyclin D2 postmitosis (223). Notably, aging negatively correlates with the mitogenic effects of glucose on the beta cell in vitro, possibly due to decreased PDX1 expression (161). A mitogenic effect of glucose on human beta cells has been observed both in vitro and after transplantation under the kidney capsule of immune-deficient mice (154, 174, 248).

Glucose phosphorylation by glucokinase and subsequent ATP-dependent closure of potassium (K_{ATP}) channels and membrane depolarization are crucial for glucose-mediated beta cell replication (201). Haploinsufficiency for glucokinase in mouse beta cells blunts beta cell hyperplasia following high-fat diet (261). In humans, a glucokinase-activating mutation was associated with abnormally large islets and increased beta cell proliferation in pancreatic tissue resected from a 3 yr old (130), and treatment with a glucokinase-activating drug induced the proliferation of human beta cells via protein kinase C (PKC)-ζ (145). The glucose-sensing transcription factor carbohydrate response element-binding protein (ChREBP) also plays a role in glucose-stimulated expression of cell cycle regulatory genes and beta cell proliferation (174, 293).

The increase in insulin production and secretion induced by glucose catabolism also contributes to beta cell expansion through the induction of compensatory endoplasmic reticulum signals (235), or possibly through autocrine stimulation of the insulin receptor itself (11). Glucose catabolism also activates insulin receptor substrate 2 (IRS-2) via the Ca^{2+}/calcineurin/NFAT pathway (64) and regulates mTOR and cyclin D2 via activation of PKC-ζ to promote beta cell replication independent of the insulin receptor (145, 248).

These homeostatic mechanisms for balancing beta cell mass with metabolic demands suggest that human beta cell replication can be triggered even when aging and maturation have forced them into a dormant quiescent state. If we can better understand and control these pathways, we may develop clinically useful tools for controlled beta cell expansion.

1. Postnatal human beta cell neogenesis

Despite indirect evidence, neogenesis of beta cells in postnatal humans has never been directly demonstrated. The indirect evidence consists of histological observations of single insulin$^+$ cells dispersed over the pancreatic parenchyma (31), of cells coexpressing insulin and the duct marker, and of insulin$^+$ cells within ducts (29). However, as noted before, conclusions based on histology alone can be misleading and do not provide definitive proof.

Beta cell neogenesis from a non-beta cell also requires further steps of maturation to yield a normally functioning

adult beta cell that can correctly regulate insulin secretion in response to glucose and other physiological signals. This maturation process is still poorly understood. Relative to mature beta cells, FACS-purified immature human beta cells ($PDX1^+/INS^{low}$) are enriched for transcripts encoding proteins characteristic of beta cell development, cell cycling, apoptosis, and other islet cell types. Thus the heterogeneous expression pattern of INS and PDX1, as described previously in vitro (255), also occurs in vivo (256). This subset of $PDX1^+/INS^{low}$ beta cells also contain C-peptide and GLUT2 (42). More extensive knowledge of human beta cell maturation will further refine strategies for in vitro generation of fully functional beta cells for the treatment of diabetes mellitus.

2. Postnatal human beta cell replication

Given the technical hurdles to studying the human pancreas in vivo, available data on human beta cell mass and turnover are very scarce. Most studies are limited to analysis of post mortem tissue samples or to in vitro study of cells isolated from cadaveric pancreases. Human pancreas samples are inherently variable, mandating the analysis of a sufficient number of, preferably healthy, donors to draw valid conclusions (115, 131, 168, 215). Furthermore, ante mortem events such as prolonged life support and post mortem handling of the tissues can alter assessment of beta cell proliferation as assessed by Ki67 staining (115, 254).

Notwithstanding these obstacles, we know that the highest rates of human beta cell replication occur during infancy (0–3 yr), thereafter declining from youth to adulthood (131, 168, 275), with the peak in beta cell mass per kilogram body weight preceding the increase in insulin demand that occurs during puberty (177). The increase in beta cell mass during infancy mainly results from an increase in the number of beta cells per islet rather than the number of islets (168). A study using thymidine analogs administered 8 days to 4 yr before death found that beta cell labeling could be detected only in the youngest patients (18–20 yr old) and was absent from subjects over 30 yr of age (194), confirming that the human beta cell mass is largely established by the age of 20 (55).

Similar to other cell populations, the beta cell mass is not only determined by hyperplasia but also balanced by cell death through apoptosis, a process collectively referred to as "remodeling" (90). The frequency of beta cell apoptosis in pancreases obtained from normal human fetuses and infants is low during weeks 17–32 of gestation, increases perinatally, and declines again after 6 mo of age, adding to the relative increase in beta cell abundance at early childhood.

The limited proliferative capacity of adult human beta cells implies they have an extended lifespan. Cellular longevity can be deduced from intracellular lipofuscin body content. Lipofuscin bodies are multivesicular storage organelles from the lysosomal system, and their accumulation in a cell is an indication of cell aging. An age-related increase in the fraction of lipofuscin body-positive beta cells has been observed in human (89% in 1 yr olds, 95% in 5 yr olds, 97% in 20 yr olds, and 98% after age 50), again supporting the conclusion that the human beta cell mass is largely established by the age of 20 (54, 55).

Despite the low rates of beta cell replication in adult humans, some research efforts have focused on the stimulation of beta cell replication as a means of beta cell replacement. Although a number of growth factors have been proposed to drive beta cell replication in vivo, few, if any, have been unequivocally validated as effective human beta cell mitogens. In mice, beta cell proliferation and mass expansion can be stimulated by infusion with the insulin receptor antagonist S961. Initially, these effects were attributed to a liver-derived mRNA encoding angiopoietin-like 8 (ANGPTL8, renamed "betatrophin") (122, 283). However, S961 failed to induce a similar proliferative response in human beta cells engrafted into mice (77), and elevated plasma concentrations of betatrophin were found in patients with longstanding T1DM, suggesting that betatrophin alone may not protect or expand human beta cell (76). Subsequently, repeated studies could not confirm the betatrophin effects in rodents that could not attribute the effects of the insulin receptor antagonist to betatrophin (60, 98, 284, 285). The effects of S961, however, were reproducible in rodents, and other studies suggest that the liver secretes the protease inhibitor SerpinB1 in response to insulin resistance and obesity in mouse, zebrafish, and human (73). SerpinB1 acts as an inhibitor of elastase and modulates phosphorylation of several factors related to the beta cell cycle and survival, including p-GSK3, p-MAPK3, and p-PRKAR2B (73).

As it stands, the list of mouse beta cell mitogens that fail to induce a comparable human beta cell proliferative response is growing and, among others, includes growth hormone, prolactin, insulin-like growth factor I (IGF-I), placental lactogen, betacellulin, hepatocyte growth factor, serotonin, gastrin, gamma-aminobutyric acid, osteocalcin, and adenosine kinase inhibitor (24, 33, 249). Nevertheless, the search for effective human beta cell mitogens to restore an adequate beta cell mass remains important, given the presence of residual $insulin^+$ cells in most diabetic patients, even in those with longstanding T1DM (167). Importantly, studies that evaluate cell proliferation should examine the presence of mitotic figures and positivity for the G_2 to M transition marker phosphohistone H3 to prove that thymidine analog or Ki67-positive cells indeed are driven towards a proliferative pathway, rather than toward a polyploid state that is relatively frequent in normal human pancreas (72). Obviously, any clinical application of human beta cell mitogens will require concurrent inhibition of the original

cause of beta cell loss, and must be monitored for aberrant cell growth and tumorigenesis.

C. Pregnancy-Associated Changes in Human Beta Cell Mass

Proper fetal growth relies on a constant and adequate flow of nutrients from the mother to the fetus. The largest portion of the energy required by the growing fetus comes from glucose diffusion across the placenta (17, 18, 103). During early pregnancy, a passive glucose gradient is maintained by the fetal beta cells through high basal insulin secretion (10, 78). However, as the fetus grows, the fraction of maternal glucose diverted to the fetus grows. To guarantee a sufficiently high maternal glucose concentration to maintain this glucose gradient in the later part of pregnancy, hormones secreted by the placenta induce maternal insulin resistance (144, 184). To prevent excessive nutrient flows to the fetus after meals, the maternal peripheral insulin resistance is balanced by an increase in maternal beta cell mass in both rodents and humans (1, 40, 262, 265, 268, 280). In pregnant rodents, the maternal beta cell mass expansion occurs mainly, if not exclusively, by beta cell replication (1, 262, 265, 280). The dominant mechanism in human, be it replication, neogenesis, or a combination of both, remains unclear.

In rodents, lactogenic hormones from the pituitary and placenta trigger beta cell replication as demonstrated by the necessity of an intact prolactin receptor (PRLR) (5, 84, 93, 113, 206) and by the effects of prolactin and placental lactogen in vitro (35–37, 245). PRLR signaling induces key genes that control serotonin production in the maternal beta cells (133, 148, 213, 228). In addition, a beta cell-specific switch in serotonin receptor expression occurs during rodent pregnancy: expression of the $G\alpha_q$-linked HTR2B receptor increases while expression of the $G\alpha_i$-linked HTR1D receptor (133) falls. These changes result in increased beta cell replication driven by the HTR2B receptor. At the end of pregnancy and post partum, HTR2B expression is downregulated and HTR1D expression reinstated, causing a drop in beta cell replication and increased beta cell apoptosis. In addition, serotonin signaling causes enhanced insulin secretion by mildly depolarizing the cells through upregulation of the ionotropic HTR3A receptor (187). During rodent pregnancy, PRLR-induced JAK2/STAT5 signaling impacts many other signaling pathway components that may contribute to beta cell adaptation, including insulin receptor substrate 1 (IRS1), insulin receptor substrate 2 (IRS2), phosphatidylinositol 3-kinase (PI3K), AKT, p70S6K, mTOR FOXM1, HNF4a, FOXD3, and menin (5, 6, 97, 114, 128, 199, 289, 291, 292).

The recent, rapid, and independent evolution of the placenta and the lactogenic hormones and their receptors makes direct comparisons among the placental mammals difficult (53, 99). In addition, compared with rodent, human beta cells are much more resistant to mitogenic stimuli and rarely divide (275). However, we do know that, as in rodents, beta cell mass increases in pregnant women, based on two autopsy studies. The first reported a 1.4-fold increase in human beta cell mass during pregnancy, did not detect an increase in beta cell proliferation, and concluded that new beta cells were generated by neogenesis (40). However, as noted above, heterogeneity among pathological samples may complicate correct interpretation of histological observations. Also, a temporal peak in human beta cell replication during pregnancy is easily missed if sampling does not span the entire period of pregnancy. The second study only assessed maternal pancreases from the latter half of and detected a 2.4-fold increase in human beta cell mass (268).

It remains to be determined whether the increase in beta cells during human pregnancy results from increases in proliferation or in neogenesis, or whether any of the pathways that contribute to murine maternal beta cell mass adaptation are active in human pregnancy. Treatment of human islet cells in vitro with prolactin, placental lactogen, or growth hormone increases glucose-stimulated insulin secretion (36), but beta cell proliferation is unaffected (50, 193). Overexpression of murine instead of human STAT5 in human beta cells bypasses this restraint on human beta cell proliferation, leading to the hypothesis that human beta cells have inherent defects in prolactin signaling (50).

The design of experimental models, in vivo or in vitro, that mimic pregnancy-mediated human beta cell adaptation will help to unravel the molecular mechanisms underlying human beta cell expansion and function. The use of serum from pregnant women at various time points throughout pregnancy or the use of placental cell types as signaling source may be a first step in better understanding the effects of pregnancy on human beta cells.

D. Obesity-Associated Changes in Human Beta Cell Mass

Ample studies in rodent models have demonstrated beta cell adaptation and increases in beta cell mass in response to increased adiposity and insulin resistance (8, 61, 294). Lineage tracing has demonstrated that the new beta cells generated in these settings come from the proliferation of preexisting beta cells (264, 282).

Human beta cell mass also correlates with adiposity: nondiabetic, obese humans have ~50% more beta cell mass relative to lean nondiabetic individuals based on autopsy studies (204, 221). This increase apparently depends on genetic ethnicity, since surgically resected pancreases from obese Japanese patients have almost no increase in beta cell mass compared with lean Japanese patients (116). Adipos-

ity-driven beta cell mass expansion is not due to cellular hypertrophy (221), but just as with gestational increases in beta cell mass, the source of the increased numbers of beta cells in obese humans remains uncertain in the absence of lineage tracing. Some evidence suggests that the additional beta cells come from neogenesis (41, 101), while other evidence supports a role for proliferation in the generation of these new beta cells (61).

Several mechanisms have been implicated in obesity-induced beta cell adaptation and replication. As discussed earlier, glucose can promote beta cell proliferation through its catabolism in beta cells (145, 248). However, in nondiabetic obese individuals with normal glucose levels, increases in beta cell proliferation cannot be attributed to hyperglycemia.

To prevent hyperglycemia, however, beta cells must produce and secrete more insulin to compensate for the increasing insulin resistance that occurs as central adiposity increases. In mice, beta cell-specific knockout of the insulin receptor disrupted beta cell replication in response to a high-fat diet, supporting the conclusion that insulin signaling plays a role in compensatory beta cell replication during obesity and insulin resistance (186). Insulin and insulin-like growth factors (IGFs) mediate their proliferative actions via IRS2-PI3K/AKT axis. Downstream of AKT [also known as protein kinase B (PKB)], phosphorylation of $p27^{Kip1}$ and of the antiproliferative transcription factor FOXO1 promotes nuclear exclusion of the latter, thereby allowing beta cell replication (137).

In addition, the obesity-induced increase in insulin synthesis and processing in high-fat diet or *db*/*db* mice induces endoplasmic reticulum (ER) stress and the adaptive unfolded protein response (UPR), which promotes beta cell proliferation via activating transcription factor 6 (ATF6). The adaptive UPR also elevates thymidine-analog labeling in human beta cells upon exposure to high glucose in vitro (235).

In addition to glucose, SerpinB1, and insulin, other obesity-induced, extrapancreatic beta cell mitogens have been proposed, including growth hormone (GH) (111), incretins (GLP-1 and GIP) (270), WNTs (96, 142, 164, 207, 227), and hepatocyte growth factor (HGF) (4, 8, 173).

Finally, microRNAs (miRNAs) also regulate the compensatory beta cell expansion in response to obesity in mice (182). Beta cell-specific disruption of Dicer-1, a key processing enzyme required for the synthesis of all miRNA, caused glucose intolerance due to a significant decrease in pancreas insulin content. A more specific knockdown of miR-24, miR-26, miR-182, and miR-148 in isolated murine islets enhanced insulin expression, at least in part, by repressing insulin gene transcriptional repressors (172). Other miRNAs, including miR-375 (202) and miR338–3p (117), play a direct role in compensatory beta cell proliferation by regulating a cluster of genes controlling cell cycle and growth. Additional evidence for the role of miRNAs in adaptive beta cell proliferation comes from the reduced compensatory beta cell expansion in the absence of Argonaut 2 (Ago2), which facilitates interactions of miRNAs with their target mRNAs (260).

Taken together, it is becoming increasingly clear that beta cells integrate various extra- and intracellular signals to adjust their mass in response to the increased insulin demand of obesity.

IV. STRATEGIES FOR GENERATING AN ABUNDANT SUPPLY OF TRANSPLANTABLE HUMAN BETA CELLS

A. In Vitro Beta Cell Replication

Transplantation of in vitro expanded human beta cells could offer a logical approach to beta cell replacement in people with diabetes. However, as discussed above, human beta cells are terminally differentiated and long-lived cells that become established in the first few years of life followed by very low proliferation rates in adult humans (54, 194). The age-dependent decline in replicative potential represents a major obstacle to therapeutic beta cell expansion since currently the principal source of human beta cells is adult cadaveric donors.

In contrast to rodent beta cells, human beta cells have been proven difficult to maintain, let alone expand in vitro (71, 193). In addition, often when replication of human beta cells has been achieved, glucose-stimulated secretion from the proliferated cells has declined (217, 226). For example, while glucose is clearly mitogenic for rodent beta cells, it exerts very limited effects on human beta cell cycling, depending on donor age, duration of glucose exposure, and glucose concentration (161).

The low rates of basal and glucose-stimulated proliferation in human beta cells have led to efforts to identify other and better human beta cell mitogens (267). Among candidate human beta cell growth factors, those signaling through IRS2 have attracted particular interest. Even though the efforts to understand the involvement of IRS2 signaling by IGF-I mainly focused on rodent beta cells (158, 203, 211, 257), in vitro propagation of human beta cells will most likely depend on the identification of growth factors that maintain activation of such a key signal transducer. However, mitogens in this pathway may not display much beta cell specificity.

Recent advances in high-throughput screening with large libraries of compounds have identified a number of factors capable of stimulating significant human beta cell proliferation (239, 274) **(TABLE 1)**. One such molecule, WS6, can stimulate proliferation of human beta cells in a dose-responsive manner (239). Although its mechanism of action is largely unknown, involvement of Erb3 binding protein (EBP1) and of the IκB kinase IKKε has been suggested (239). In additional screens, small molecule inhibitors of the DYRK1A kinase were identified as potent inductors of human beta cell proliferation (67, 238, 274). Two of these, harmine and INDY, inhibit DYRK1A by inserting into its ATP-binding pocket. The pro-proliferative effects of the DYRK1A inhibitors have been linked to the calmodulin-calcineurin-cMyc pathway.

It is clear, however, that these molecules are not beta cell specific but rather trigger cell cycle activation in most cell types, warranting extreme caution and emphasizing the need of beta cell specific delivery of these mitogens. Cell specificity can be enhanced by using combinations of drugs. For example, combining a selective inhibitor of the endocrine tumor suppressor menin combined with a GLP1 receptor agonist (exendin-4) yields potent beta cell-specific mitogenic activity (47).

These reports are encouraging and may lead to strategies to expand human beta cells in vitro or even in vivo. Small molecule screening also identified compounds with activity similar to SerpinB1, the protease inhibitor with beta cell mitogenic activity induced in the liver in response to insulin resistance and obesity (73). Osteoprotegerin (OPG) is yet another factor whose concentration increases in models of beta cell expansion, including obesity and pregnancy. In response to lactogens, OPG induces beta cell proliferation without dedifferentiation, by modulating proliferative pathways in rodent and human islets (139). OPG inhibits glycogen synthase kinase-3 (GSK3), stimulates cAMP response element-binding protein (CREB), and inhibits the RANKL/RANK pathway that acts as a brake on beta cell proliferation (139). Other recently identified human beta cell mitogens were recently reviewed by Shirakawa et al. (241).

Despite the availability of numerous rodent beta cell lines, the first functional human beta cell line (EndoC-βH1) was generated only recently by introducing immortalizing transgenes in human fetal pancreatic tissue (205). This cell line has been further optimized through Cre-mediated excision of the immortalizing transgenes, resulting in a phenotype more similar to genuine human beta cells (22, 226). Despite the potential to generate an unlimited supply of transplantable human beta cells with this or similar lines, the use of lentiviral vectors for immortalization, combined with the potential for incomplete excision of the immortalizing oncogenes in every cell, prevents the direct application of EndoC cells to human therapy, although they remain a uniquely useful tool for drug development and preclinical studies of cell replacement therapy in diabetes.

In contrast to murine beta cells (12, 178, 277), epithelial-to-mesenchymal transition (EMT) of adult human beta cells generates proliferative fibroblast-like cells that, upon serum removal and addition of growth factors, appear to redifferentiate into insulin-producing isletlike structures that can replace beta cell function in hyperglycemic mice (15, 92, 149, 189). The origin, identity, and ultimate fate of these cells have provoked vigorous debate. Eventually the beta cell origin of the proliferating cells was confirmed by genetic lineage tracing (217, 219). Reexpression of insulin and other beta cell-specific genes occurs only in the beta cell-derived mesenchymal cells, demonstrating that these cells arise by redifferentation rather than by de novo differentiation from non-beta cells. Inhibition of NOTCH (15) and Wnt signaling (153) synergistically promote endocrine redifferentiation. Although these cells can further mature in vivo into functional beta-like cells (15), the reproducibility of this approach has been questioned (132). In addition, detailed characterization of the expansion capacity of this approach and the fidelity of the beta cell characteristics of the resulting cells has not been fully established. Also, it remains unknown whether EMT of human beta cells occurs in vivo and, if so, whether it could be therapeutically exploited.

Unrestrained metabolic stress alters the expression of many functionally important and cell type-specific genes in mature beta cells, in a process sometimes broadly labeled "dedifferentiation" or "loss of cell type identity." This process can lead to cells that express typically progenitor- or alpha cell genes and lose glucose-stimulated insulin secretion. These pathological changes may contribute to the beta cell failure in T2DM (123, 258). In this regard, treatment of beta cell dysfunction should aim for reduction of metabolic stress, thereby potentially correcting these pathological beta cell changes and reestablishing metabolic control.

B. In Vitro (Trans)differentiation to Beta Cells

1. Differentiation of embryonic stem and induced pluripotent stem cells to beta cells

Directed differentiation of human embryonic stem (hES) cells and induced pluripotent stem (iPS) cells into glucose-responsive, insulin-producing beta cells represents a promising route for generating an unlimited source of human beta cells for replacement therapy in people with diabetes **(FIGURE 3) (TABLE 1)**. The most successful protocols use a stepwise process that manipulates the cellular micro- and macroenvironment in stages, based on our understanding of pancreatic development gained from developmental

Table 1. *Postnatal sources of new human beta(-like) cells*

Method of Beta Cell Generation	Cell of Origin	Trigger	Mechanism	Reference Nos.
Replication	Beta cell	Glucokinase	Signals via protein kinase C-ζ	130, 145
		Harmine	Calmodulin-calcineurin-cMyc pathway	274
		INDY (harmine analog)	Calmodulin-calcineurin-cMyc pathway	274
		WS6	Involvement of Erb3 binding protein (EBP1) and the IkB kinase IKKe has been suggested	239
		Osteoprotegerin (and denosumab)	Inhibits glycogen synthase kinase-3 (GSK3), stimulates cAMP response element-binding protein (CREB), and inhibits RANKL/RANK pathway	139
		SerpinB1	Activation of proteins in insulin/IGF-I signaling pathway, altered phosphorylation of MAPK, PRKAR2B, and GSK3 subunits	73
Differentiation of ES/iPS cells	ES/iPS	7-stage protocol	Neurog3 induction, thyroid hormone, and a gamma secretase inhibitor addition to induce Nkx6, 1/ins+ cells, and MAFA induction for maturation	208
		Sequential treatment with multiple growth and differentiation factors over a period of 5 wk	Progenitor differentiation by modulating signaling by Wnt, activin, hedgehog, EGF, TGF-β, thyroid hormone, and retinoic acid, as well as γ-secretase inhibition	190
		15- to 21-day protocol to generate monohormonal endocrine cells	Omission of BMP inhibitors at the pancreas progenitor stage, addition of EGF+KGF for rapid Nkx6.1 induction, and BMP+ALK inhibition to synchronize Neurog3 expression	218
Transdifferentiation of adult stem cells	Pancreas-derived multipotent precursors from adult human pancreas	Facultative stem cells within the beta cell population	Spontaneous expansion and differentiation upon isolation and cell culture	243
	Islet-derived mesenchymal stem cells	Mix of appropriate factors under serum-free conditions	Directed differentiation	87
	Human bone marrow-derived MSCs	Exposure to specific growth factors, mimicking diabetic hyperglycemic niche, supplementation of fetal pancreatic extract	Directed differentiation	195
	Human bone marrow-derived MSCs	Pdx1 overexpression, transplantation into hyperglycemic niche	Forced differentiation by transcription factor overexpression	127

Continued

Table 1.—*Continued*

Method of Beta Cell Generation	Cell of Origin	Trigger	Mechanism	Reference Nos.
	Human multipotent adipose tissue-derived stem cells	Multi-step protocol	Directed differentiation	48, 134, 176, 188
	Human multipotent adipose tissue-derived stem cells	PDX1 overexpression	Forced differentiation by transcription factor overexpression	151
Reprogramming of nonpancreatic cell types	Hepatocytes	Ectopic expression of PDX1	Inhibition of liver-gene expression by inhibition of C/EBPβ	23, 170, 171, 181, 224, 290
	Hepatocytes	Overexpression of PDX1 + exendin-4	Exendin-4 induces liver cell proliferation and maturation of transdifferentiated cells	13
	Keratinocytes	Overexpression of PDX1	Forced differentiation by transcription factor overexpression	166
	Fibroblasts	Combination of growth factors, chemical compounds, and nonintegrative episomal reprogramming factors	Directed differentiation	296
Reprogramming of pancreatic cell types	Duct cells	PDX1/NEUROG3/MAFA/PAX4 + epigenetic signaling events	Forced differentiation by transcription factor overexpression	157
		DPP-4i, PPI	Directed differentiation	252, 253
	Acinar cells	MAPK/STAT3 overexpression in an in vitro setting using sequential free-floating and 3D matrix culture	Constitutively active MAPK+STAT3 overexpression	152
	Alpha cells	Nonspecific methyltransferase inhibitor	Potential role for p53	34, 82

Overview of the currently known sources of new human beta(-like) cells in vitro and the mechanisms used to stimulate beta cell generation. See text for definitions.

studies. Nevertheless, this approach remains empirical. The first major breakthrough in the field of directed human stem cell differentiation mimicked the normal developmental program, guiding hES cells through formation of definitive endoderm to pancreatic endoderm and subsequently to endocrine progenitor and beta cell-like stages (62). Early protocols generated cells that often expressed multi-hormones and had poor (62, 196) to moderate (121) glucose-stimulated human insulin C-peptide secretion. Careful analysis of the insulin-expressing cells after in vitro differentiation identified many differences from normal adult beta cells on the levels of function, transcriptome proteome, and epigenome (16). Further maturation to glucose responsive, beta-like cells capable of correcting experimental diabetes was achieved by long-term engraftment in immune-deficient mice (39, 143). In vivo, the polyhormonal cells mainly differentiated to a glucagon-positive phenotype while the progenitors were not immediately able to reverse hyperglycemia and engrafted mice required exogenous insulin until the transplanted cells functionally matured and produced sufficient amounts of insulin (209). Notably, besides characterization of the resulting cells at the transcript, protein, and functional levels, epigenetic profiling may reveal further abnormalities caused by a failure to eliminate polycomb group-mediated repression of endocrine-specific genes (281).

The development of patient-derived iPS cells now allows the generation of autologous cells for transplantation (163), although this approach does not solve the problem of autoimmunity in patients with type 1 diabetes. The availability of genome-wide reference maps of DNA methylation and gene expression of hES and iPS cells facilitates in-depth characterization of different cell lines (26). Genome-wide transcriptional analysis of insulin$^+$ cells derived from three independent human iPS lines

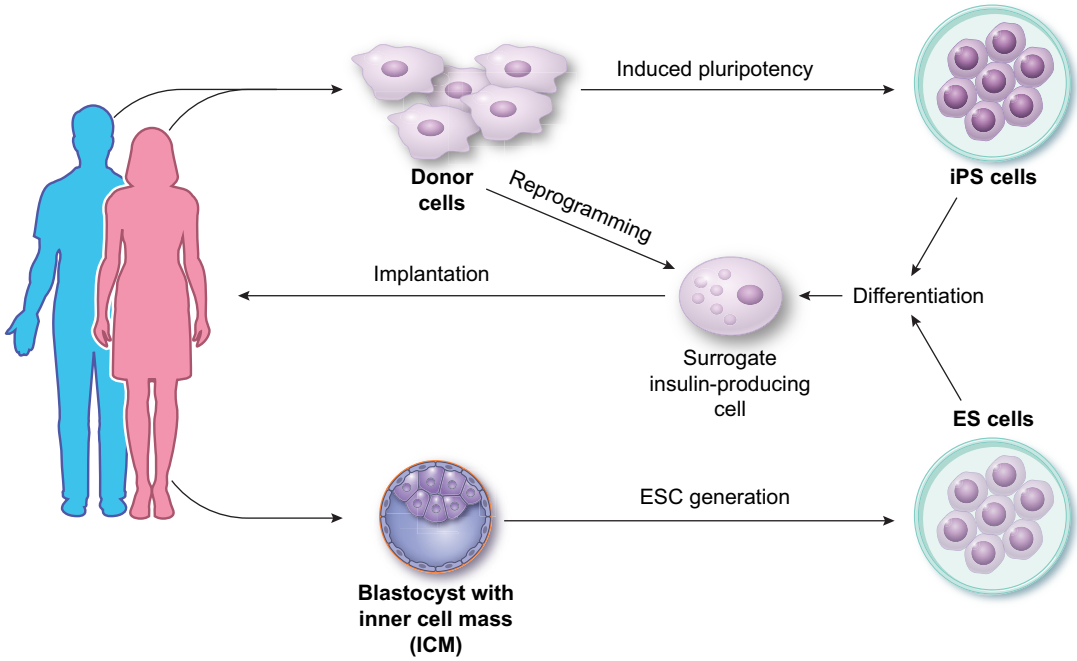

FIGURE 3. Nonpancreatic cell sources for beta cell generation. Illustration of the potential use of nonpancreatic cell types to generate new beta(-like) cells in vitro based on hES/hIPS directed differentiation or postnatal donor cell reprogramming. The cells of origin depicted represent healthy donor cells. Functional beta(-like) cells could be reintroduced to replenish the beta cell pool lost in diabetes patients.

resulted in cells resembling human fetal beta cells rather than adult beta cells. Similar to hES, in vivo maturation was necessary for iPS-derived cells to advance to a more adult beta cell phenotype (112).

Several groups have reported the generation entirely in vitro of hES-derived cells with more similarities to mature, functional cells (190, 208) reported optimized protocols for the in vitro generation of mature, functional beta-like cells from hES cells. The first described a seven-stage protocol resulting in the efficient conversion (>50%) of hES cells to monohormonal, phenotypically mature, and glucose responsive beta-like cells, stably expressing NKX6.1, insulin, and MAFA. Although these cells closely resemble adult beta cells, important differences remained. Most notably, the hES-derived beta-like cells showed a blunted and delayed response during dynamic glucose stimulation with normal Ca^{2+} oscillations observed in only a fraction of the insulin^{+} cells. Nonetheless, when transplanted into mice, these cells reversed diabetes within 40 days (208).

The second study reported a scalable three-dimensional culture system using sequential treatment with multiple growth and differentiation factors over a period of 5 wk to transform hES cells into monohormonal, functional beta-like cells. Insulin^{+} cells (33% of final cells) displayed key features of mature human beta cells including normal Ca^{2+} oscillations and glucose responsiveness, expression of mature beta cell markers (PDX1, NKX6.1, insulin, C-peptide, ZnT8), and ultrastructural features reminiscent of adult beta cells. When transplanted into mice, these cells produced detectable circulating human insulin within 2 wk (190).

Recent reports demonstrated that synchronization of NEUROG3 expression following initial induction of PDX1^{+}NKX6.1^{+} MPPCs prevents preemptive endocrine differentiation and generation of dysfunctional polyhormonal cells, allowing greater yields of functional beta-like cells (210, 218). A followup report described the generation of functional beta-like cells by a similar protocol from iPS cells derived from a patient with T1DM (175).

The generation of functional, mature beta-like cells from hES/human iPS cells has moved stem cell-derived beta cells closer to clinical application. The United States Food and Drug Administration approved an Investigational New Drug (IND) application for a phase1/2 safety, tolerability, and efficacy trial of stem cell-derived encapsulated cell replacement therapy in adult patients with T1DM in 2014.

Despite advancements, several concerns regarding the use of pluripotent-stem cell-derived beta cells in patients remain. These concerns include the risk of tumor formation, either from undifferentiated pluripotent cells that might remain among the differentiated beta-like cells or from oncogenic mutations that accumulate during the differentiation process. Also, cells with unregulated insulin secretion could cause hypoglycemia in the recipient.

In addition, transplanted beta-like cells must be protected from allograft rejection (for beta-like cells not derived from the recipient) and autoimmune destruction (in recipients with T1DM) by blocking or modifying the immune response. Clinical application will also require cell culture on an industrial scale, and potentially the generation of an hESC bank based on growth, differentiation, and HLA-type matching (43, 179, 266). Whether patient-derived iPS cells can replace hES cells for cell therapy remains to be determined, but already patient-derived iPS cells are contributing to disease modeling.

2. Differentiation of adult stem cells to beta cells

The end of the last century witnessed much excitement over the possibility that adult stem cells and hematopoietic stem cells (HSCs) might have the capacity to differentiate across tissue lineages and even across germ layer boundaries **(FIGURE 3)**. For example, HSCs were thought to transdifferentiate into lung, gut, skin, muscle, liver, and neurons. In 2003, bone marrow cells transplanted into lethally irradiated mice reportedly generated "glucose-competent" beta cells, but subsequent studies could not replicate these unexpected findings (52, 150, 259). In general, these observations of adult stem cell plasticity were often over-interpreted or could be explained by experimental artifacts (273).

Interestingly, a subsequent study showed that bone marrow-derived stem cells could induce pancreatic regeneration after injection in diabetic mice in a non-cell autonomous manner (110). Rather than transdifferentiating into beta cells, the injected cells acquired an endothelial cell phenotype and stimulated beta cell proliferation (110). Although the exact mechanism remains unclear, this observation fits well with the role of signals from mesoderm-derived tissues in pancreas development and adult beta cell proliferation and function.

Autologous non-myeloablative HSC transplantation led to insulin independence with good glycemic control in a series of patients with newly diagnosed T1DM (58). However, this exciting finding could not be corroborated in patients with T1DM of more than 5 yr duration (75) or when umbilical cord blood was infused intravenously in young children with T1DM (100). This difference in outcomes suggests that the preparatory treatment with cyclophosphamide and rabbit anti-thymocyte globulin altered the disease course in new-onset patients, while the subsequent stem cell infusion had no measurable impact on the disease.

Recent research focus has shifted to the isolation and study of other populations of adult human stem cells besides the well-characterized HSCs. For example, so-called "pancreas-derived multipotent precursors" (PMPs) were isolated from islets of adult human and mouse pancreas, independent of age (243). These PMP cells proliferate and differentiate into multiple differentiated neuronal and pancreatic cell types. The generated beta cells contain about one-third of the amount of insulin of a primary beta cell. After transplantation into mice, these cells produced circulating human C-peptide and ameliorated experimental diabetes. Immunohistochemistry of the graft detected human insulin$^+$ cells (243). Independent confirmation and further characterization of the PMPs and PMP-derived beta cells and their molecular mechanism of differentiation will help determine the utility of this approach to beta cell replacement therapy.

Mesenchymal stem cells (MSCs) isolated from various tissues have also been suggested as a potential source of endocrine progenitors. For example, human islet-derived MSCs develop into isletlike clusters when exposed to a cocktail of differentiation factors under serum-free conditions. After differentiation, expression of glucagon, somatostatin, and transcription factor Islet 1 (ISL1) was noted. Although the resulting cells stained for C-peptide, they did not secrete insulin in a glucose-regulated manner (87).

Human bone marrow-derived MSCs (hBM-MSC) form isletlike aggregates after exposure to a differentiation cocktail. However, significant maturation, secretion of human C-peptide, and normalization of glycemia were only observed after transplantation in mice with lowered beta cell mass after pancreatectomy or following streptozotocin-induced beta cell ablation (195). Similar findings were previously reported using hBM-MSCs ectopically expressing PDX1 (127).

Finally, human multipotent adipose tissue-derived stem cells (hASCs) have been proposed as a readily accessible source of stem cells for beta cell generation (134). Following a multistep differentiation protocol, abdominal fat tissue obtained by liposuction generated isletlike aggregates containing insulin$^+$ cells that secreted insulin in vitro (48, 176, 188). Two to three weeks after transplantation in diabetic mice, these cells could restore near-normoglycemia for up to 8 wk (48). As with hBM-MSCs, overexpression of PDX1 in hASCs enhanced pancreatic differentiation. The resulting cells secreted insulin in response to glucose stimulation in vitro and reduced blood glucose levels in diabetic mice without achieving normoglycemia (151) **(TABLE 1)**.

Despite continued interest in generating beta cells from adult stem cells due to their ready availability and practicality, it remains unclear how similar the derived cells are to normal beta cells at the level of gene expression, stability, or function or how well they compare with beta-like cells derived from hES or human IPS cells.

3. Transdifferentiation of nonpancreatic cells to beta cells

Transdifferentiation of differentiated adult human nonpancreatic cells represents another potential route to new beta

cells **(TABLE 1)**. Because pancreas and liver cells originally derive from bipotent progenitor cells in the gut endoderm during embryonic development (65), transdifferentiation of liver cells became a major focus in the quest for beta cell replacement **(FIGURE 3)**. In the first experiment of its kind, ectopic expression of PDX1 in adult human hepatocytes induced insulin synthesis, processing, storage, and glucose-regulated release. In diabetic mice, these cells reduce blood glucose levels (13, 23, 127, 170, 171, 180, 181, 224, 290). Treatment with GLP1 agonist exendin-4 further enhanced this transdifferentiation to insulin-producing cells (13). The reprogrammed human liver cells expressed pancreas-specific genes, and cell lineage tracing showed that the insulin$^+$ cells originated from genuine hepatocytes. Further study, however, suggested that these insulin$^+$ cells appear only in culture after undergoing EMT and that a similar phenomenon may not occur in vivo (170). More recently, the TALE homeodomain transcription factor *Tgif2* was used to reprogram mouse liver cells to pancreas progenitor-like cells that express *Pdx1*, *Ptf1a*, *Neurod1*, *Pax6*, *MafA*, *Isl1*, and *Insm1*, a potential starting point for generation of beta(-like) cells (46).

Similar to hepatocytes, human keratinocytes ectopically expressing PDX1 also transformed into glucose-responsive beta-like cells (166). The pool of potential nonpancreatic cell sources for beta cell generation was even further enlarged by the derivation, from human fibroblasts, of proliferative endodermal progenitors and, subsequently, pancreatic beta-like cells that exhibit glucose-stimulated insulin secretion in vivo (296). Despite the range of nonpancreatic cell sources (many of which fall beyond the scope of this review), efforts to transdifferentiate nonpancreatic cells towards beta cells have recently dwindled, possibly because these efforts have failed to generate functionally equivalent beta-like cells. Increasing evidence suggests that tissue-specific resident or facultative stem cells may provide a more successful starting cell, and newer protocols using hES/iPS cells are generating cells increasingly similar to normal adult human beta cells.

4. Transdifferentiation of pancreatic cell types to beta cells

Because pancreatic endocrine cells arise during human pancreas development from branching pancreatic endoderm comprised of epidermal MPPCs that share many similarities with adult human pancreatic duct cells, the duct cells are often considered candidates for transdifferentiation towards endocrine cells. Adult human pancreatic duct cells express a network of transcription factors that also play a role in the maintenance of progenitor cell characteristics in embryonic MPPCs (105, 159).

Several studies have shown that adult human duct cells can propagate in vitro as well as generate new isletlike cells **(FIGURE 4)** (30, 89). These conclusions were based on the observation that isletlike structures could bud from three-dimensional ductal cysts (30, 89). Notably, when contaminating endocrine cells were depleted from these cultures, the original findings were no longer reproducible, suggesting that beta cell dedifferentiation rather than neogenesis may explain the former conclusions (88). In a proof-of-concept study, ectopic expression of *Neurog3* in adult human duct cells has shown a capacity for adult duct cells to differentiate into endocrine-like cells (106). Other reports show how cotransplantation with fetal cells (102) or engraftment followed by introduction of gastrin/GLP1 appears to induce beta cell generation from human ducts (252) (253). Importantly, none of these studies included lineage tracing, and in adult mice, lineage tracing with different duct-specific promoters has not supported a duct-related origin of regenerating endocrine cells (86, 140, 141, 244). Working with human cells, lineage tracing is imperative to rule out redifferentiation of residual degranulated or fully dedifferentiated beta cells rather than actual transdifferentiation of exocrine cells.

Human acinar cells were genetically traced and shown to undergo EMT in vitro (156). Upon ectopic expression of NEUROG3, PDX1, MAFA, and PAX4 and a series of (epigenetic) signaling events, the human exocrine cells could give rise to insulin$^+$ cells capable of normalizing blood glucose levels (156), thereby extrapolating the initial findings documented in mice in vivo (295). Of note, in vitro transdifferentiation towards beta-like cells seemed most efficient using freshly isolated tissue under conditions whereby EMT was inhibited. Supplementing this approach with ARX inhibition yielded a monohormonal cell population containing 40% C-peptide$^+$, 4% glucagon$^+$, and $<$2% somatostatin$^+$ cells. The resulting beta-like cells were glucose responsive and expressed insulin protein levels at 15% of adult human islets, similar to hIPS cell-derived beta-like cells. When grafted in hyperglycemic mice, an immediate and prolonged effect on normalization of blood sugar levels was noted (157).

Another approach was based on ectopic expression of activated mitogen-activated protein kinase and STAT3 to induce human acinar-to-beta cell conversion through a NEUROG3$^+$ intermediate (152). The expression of the pro-endocrine factor NEUROG3 was activated in more than half of transduced exocrine cells. Genetic lineage tracing identified human acinar cells as a source of NEUROG3$^+$ and insulin$^+$ cells. However, the number of insulin$^+$ cells in vitro only increased following sequential suspension and three-dimensional Matrigel culture. Long-term engraftment into immune-compromised mice amplified the efficiency of reprogramming to insulin$^+$ cells. Under normoglycemic conditions, the engrafted mice displayed an increase in circulating human C-peptide, starting at around day 90 posttransplantation. Upon chemical destruction of the endogenous rodent

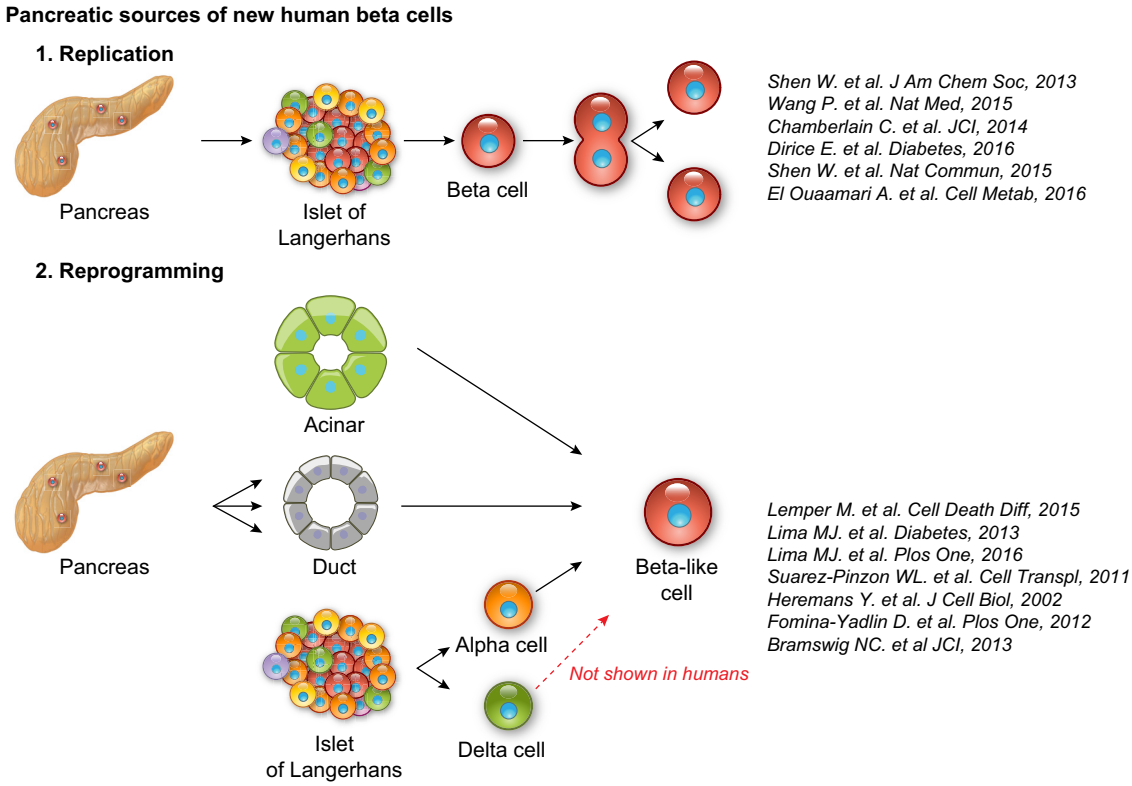

FIGURE 4. Strategies to increase human beta cell numbers in vitro from pancreatic cell types. Schematic overview of the strategies used to generate new beta(-like) cells in vitro using cell types of pancreatic origin. Black arrows represent strategies that have been documented; red arrow depicts a potential strategy that has yet to be shown using human cells.

beta cells, human C-peptide levels further spiked, and the sharp increase in blood glucose observed in controls was attenuated in engrafted mice. Removal of the graft unequivocally identified the human cells as the source of C-peptide and blood glucose control (152).

These data demonstrate that exocrine cells from human pancreas can be transdifferentiated to transplantable insulin-producing cells that acquire functionality **(FIGURE 4)**. Given the large number of exocrine cells in a donor pancreas, this approach presents a novel strategy to expand beta cell therapy in diabetes. Efficient transdifferentiation of human acinar cells would be able to augment the beta cell number by 10-fold or more. Can exocrine cells, shown to be surprisingly plastic in the rodent pancreas, be the key to resolving the shortage in human donor beta cells? Future experiments will need to find the definitive answers. However, acinar cells also force us to be cautious as emerging evidence identifies these cells as potential origin of pancreatic ductal adenocarcinoma (PDAC), one of the most aggressive pancreas malignancies in humans.

Finally, since the endocrine lineages are closely related, it seems likely that, analogous to rodents, non-beta endocrine cells represent another ideal source for generating new beta cells **(FIGURE 4)**. Indeed, similar to rodents, human alpha and beta cells share a close epigenetic relationship making human alpha cells perhaps even more amenable to convert into beta cells compared with other pancreatic cell types (34, 82). Treatment of adult human islets with a nonspecific histone methyltransferase inhibitor generates GCG^+/INS^+ and $GCG^+/PDX1^+$ cells (34). A potential role for p53 in insulin expression by adult human alpha cells was suggested (82). Vice versa, adult human beta cells were recently converted to alpha-like cells in a model of islet cell cluster formation (247). These observations lend evidence to the notion that chromatin-modifying compounds affect plasticity of endocrine cells.

Regarding the potential of pancreatic cell types to serve as a beta cell replacement **(TABLE 1)**, we can conclude that, historically, human duct cells were considered the cell type with the highest probability for harboring transdifferentiation potential. This notion is mainly due to histological studies observing single $insulin^+$ cells as well as whole islets near ducts in the human pancreas and the detection of endocrine progenitors in the lining of embryonic ductules. However, current evidence is still limited to suggest that duct cells have an increased capacity over other cell types to respond to cell transdifferentiation signals. Recently, alpha, delta, or acinar cells (14, 51, 263) have received increasing attention following their successful transdifferentiation in rodent studies. Human alpha cells bear bivalent epigenetic marks, allowing easier transdifferentiation into beta cells.

However, given the relatively small fraction of alpha cells in human islets (9), this approach would generate only a limited amount of transplantable beta cells unless the alpha cell pool would be replenished. This is the case by continuous mobilization of duct-lining progenitor-like cells in mice with ectopic expression of *Pax4* (2, 56) or inactivation of *Arx* (59) selectively in alpha cells. Interestingly, GABA signaling was identified as an inducer of alpha- to beta-like cell conversion (21), and artemisinins were reported to functionally repress Arx via enhancement of $GABA_A$ receptor signaling, also in human cells (155). However, conversion of alpha to mature beta cells through artemisinins has just recently been disproven (269).

V. CONCLUSIONS AND PERSPECTIVES

Given the critical importance of the beta cells in metabolic homeostasis, it is not surprising that the complex forces control the size and function of the beta cell population in the pancreas. Recent evidence suggests that multiple signals orchestrated by the interplay among various organs as well as contributions from autocrine and paracrine signals within the pancreas regulate beta cell mass. Thus it seems possible that new beta cells can be generated via multiple routes, a conclusion that provides optimism that we can develop successful therapies for beta cell regeneration. However, despite important conceptual progress, major practical challenges remain before we can meet the ambitious goal of beta cell (re)generation for people with diabetes.

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GRANTS

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DISCLOSURES

M. S. German owns stock in Viacyte, Inc. The authors declare no other conflicts of interest, financial or otherwise.

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