

Advances in β cell replacement and regeneration strategies for treating diabetes

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In the past decade, new approaches have been explored that are aimed at restoring functional β cell mass as a treatment strategy for diabetes. The two most intensely pursued strategies are β cell replacement through conversion of other cell types and β cell regeneration by enhancement of β cell replication. The approach closest to clinical implementation is the replacement of β cells with human pluripotent stem cell-derived (hPSC-derived) cells, which are currently under investigation in a clinical trial to assess their safety in humans. In addition, there has been success in reprogramming developmentally related cell types into β cells. Reprogramming approaches could find therapeutic applications by inducing β cell conversion in vivo or by reprogramming cells ex vivo followed by implantation. Finally, recent studies have revealed novel pharmacologic targets for stimulating β cell replication. Manipulating these targets or the pathways they regulate could be a strategy for promoting the expansion of residual β cells in diabetic patients. Here, we provide an overview of progress made toward β cell replacement and regeneration and discuss promises and challenges for clinical implementation of these strategies.

Introduction

Diabetes mellitus is a chronic disease affecting an estimated 422 million people worldwide in 2014 (1). Characterized by elevated blood sugar levels, diabetes occurs in two major forms, type 1 (T1D) and type 2 diabetes (T2D). T1D results from autoimmune destruction of the insulin-producing β cells in the pancreas, while T2D is characterized by insulin resistance and inadequate insulin secretion by the β cells. Recent studies suggest that β cell dysfunction occurs early in T2D and precedes the reduction in β cell mass observed later during disease progression (2). Because both types of diabetes eventually lead to β cell loss, research has focused on developing β cell replacement strategies to compensate for insulin deficiency. Islet transplantation has proven to be a successful therapy (3), but its clinical application is limited because of the shortage of donor cadaveric islets and the requirement for lifelong immune suppression. In the past decade, there have been intense efforts to identify alternative sources of β cells. β cell replacement strategies based on the in vitro differentiation of human pluripotent stem cells (hPSCs) toward insulin-producing cells have led to an ongoing human clinical trial (Figure 1). In addition, there have been exciting advances in in vivo regeneration approaches aimed at replenishing β cell mass either by converting related cell types into β cells, or by promoting the expansion of residual β cells in diabetic patients (Figure 2). In this Review, we focus on the recent progress toward clinically relevant therapeutic approaches for regenerating β cells.

β cell replacement by implantation of hPSC-derived cells

In the past decade, protocols have been developed that allow for the generation of pancreatic cells from hPSCs (4–7). These mul-

tistep protocols, which are based on developmental paradigms, use sequential stimulation or inhibition of key signaling pathways through small molecules and growth factors to differentiate hPSCs toward β cells. Early protocols support the in vitro differentiation of hPSCs up to the pancreatic progenitor cell stage (4, 6). Sixteen weeks after implantation of these progenitors into mice, they spontaneously differentiate into islet-like structures that contain β and non- β islet cell types (4, 5). When endogenous mouse β cells are ablated after in vivo differentiation of the hPSC-derived progenitor cell grafts, the mice are protected from developing diabetes. These findings in mice have provided the basis for the ongoing human phase I/II trial for patients with T1D (ViaCyte Inc. clinical trials identifier: NCT02239354). There is, however, a risk of immature cells having tumorigenic potential, and teratoma-like lesions have been observed around grafts after pancreatic progenitor cell engraftment into mice (4). To mitigate this risk, as well as to protect the implanted cells from alloimmune and autoimmune attack, in the current clinical trial hPSC-derived progenitors are placed in an encapsulation device (Figure 1). The employed macroencapsulation device holds large numbers of cells and is made of a semipermeable membrane that allows diffusion of oxygen, nutrients, and hormones while also creating a barrier against immune cells. In mice, encapsulated pancreatic progenitor cells are able to mature into functional β cells capable of reversing hyperglycemia (8–10). Microencapsulation technology, which uses hydrogel polymers to create a semipermeable sphere around cell aggregates, has also been explored for implanting cells without immune suppression. While earlier microencapsulation materials evoked a foreign body response that led to device failure (11, 12), recent improvements in the hydrogel composition mitigate this response (13). hPSC-derived pancreatic cells encapsulated in these new microencapsulation devices survive and function for at least 6 months after implantation into immunocompetent mice (14). Both types of encapsulation methods could provide immune protection to hPSC-

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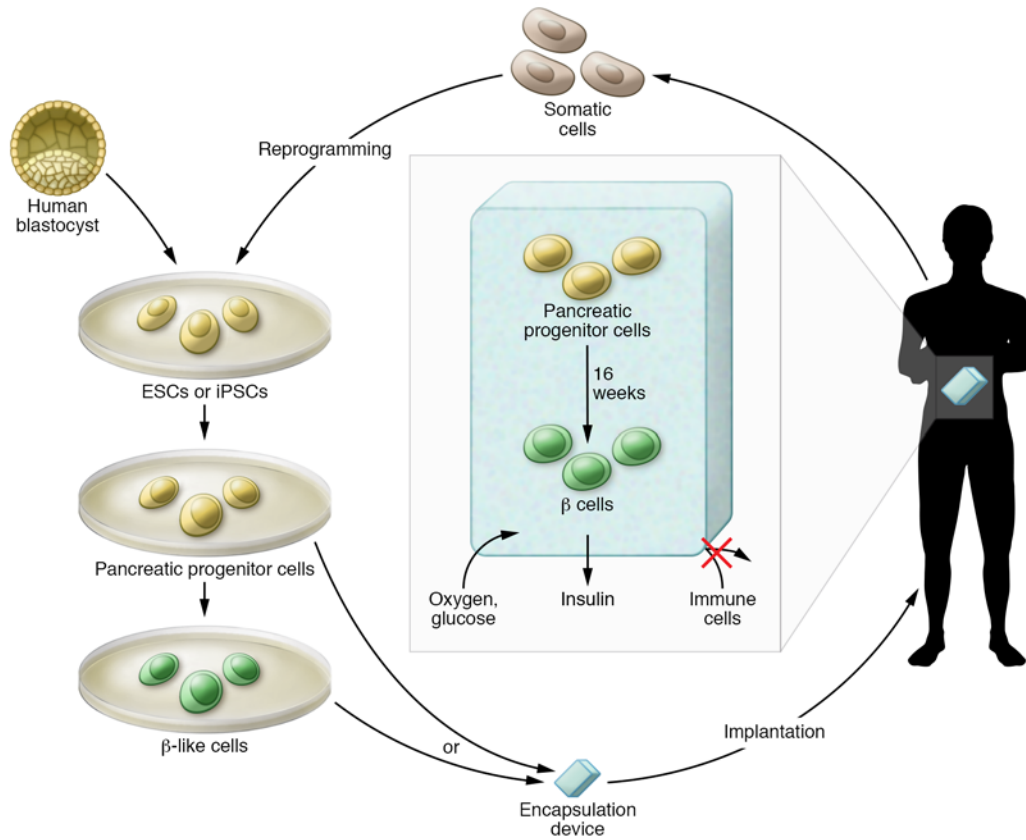


Figure 1. β cell replacement from human pluripotent stem cell sources. Currently pursued approaches include implantation of in vitro-generated pancreatic progenitor cells or β -like cells. In vitro-produced pancreatic progenitor cells differentiate into β cells within 16 weeks after implantation. Cell delivery in an encapsulation device prevents immune cells from contacting implanted cells derived from human embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs), while allowing free exchange of nutrients and hormones, including oxygen, glucose, and insulin. Precursors to β cells are depicted in yellow and insulin-producing cells in green.

derived cells in patients without the need for immunosuppression. Whether encapsulated pancreatic progenitor cells will differentiate into functional β cells in humans, as seen in mice, is still unclear and will emerge from ViaCyte’s ongoing trial.

Recent in vitro differentiation protocols can now also produce β -like cells from hPSCs, including from T1D patient-derived human induced pluripotent stem cells (hiPSCs) (7, 15, 16). Compared with earlier differentiation protocols, a critical feature of the recent improvements was to delay the induction of endocrine cell differentiation until the pancreatic progenitor cell program is fully established. This was accomplished by first suppressing expression of the proendocrine transcription factor neurogenin 3 (NGN-3) by addition of vitamin C, and then transiently inducing NGN-3 at subsequent stages with thyroid hormone and specific inhibitors of the TGF- β signaling pathway. These alterations to the protocol promoted the formation of insulin-positive cells that express a full spectrum of β cell-specific transcription factors (15). However, even with these improvements, in vitro-produced β -like cells still exhibit an insufficient insulin secretory response to a glucose challenge (7, 15). Upon implantation, these β -like cells require a shorter in vivo maturation period than pancreatic progenitor cells until acquiring the capacity to reverse hyperglycemia in mice (7, 15). It is still unclear whether engrafting β -like cells will prove advantageous to the engraftment of progenitor cells. Although β -like cells

could reverse hyperglycemia more immediately, their high oxygen demand might also render β cells particularly prone to death early after implantation. While this may be mitigated by encapsulation in devices with an integrated oxygen reservoir (17), transplantation of progenitor cells offers distinct advantages. For one, the lower oxygen demand of progenitor cells could ensure improved graft survival in the first 8 weeks following implantation, during which the encapsulation device becomes vascularized. Moreover, the other endocrine cell types that develop alongside β cells in progenitor grafts could aid in optimizing blood glucose control in patients with diabetes. Paracrine signaling that occurs within islets is important for adequate regulation of hormone secretion. For example, insulin inhibits the release of glucagon from α cells, glucagon stimulates insulin release, and somatostatin from islet δ cells inhibits the release of both insulin and glucagon (18). These paracrine signaling mechanisms between endocrine cell types act as a feedback mechanism and help maintain glucose homeostasis by preventing excessive fluctuations in pancreatic hormone levels.

β cell replacement by transdifferentiation

Transdifferentiation, the direct conversion of one differentiated cell type into another, could provide an alternative to hPSCs as a source of new β cells. A large body of work suggests that developmentally related cells, including other pancreatic cell types and

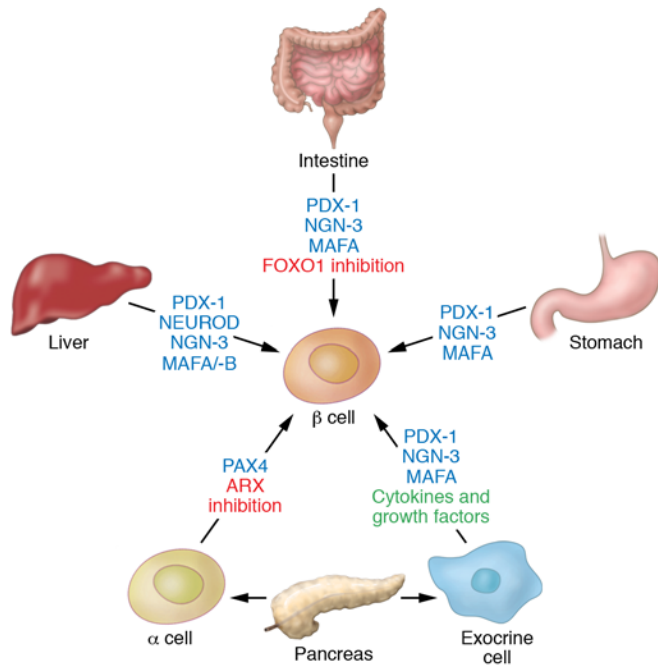


Figure 2. Reprogramming approaches for generating replacement β cells. Cells and organs of similar developmental origin to that of pancreatic β cells, such as liver, stomach, intestine, or other pancreatic cell types, can be converted into β cells by reprogramming with transcription factors or in some instances by exposure to cytokines and growth factors.

cells of the liver and gastrointestinal tract, have the greatest potential to convert into β cells (refs. 19–41 and Figure 2).

Transdifferentiation of pancreatic cells. There is significant plasticity among the different endocrine cell types of the pancreas. In particular, α and δ cells have been shown to spontaneously convert into β cells after near-total β cell ablation in mice (26, 27, 38). Whether spontaneous transdifferentiation of non- β islet cells also occurs in humans in response to extreme β cell loss is still unknown. In patients with T1D, β cell regeneration through transdifferentiation or other mechanisms could be masked by autoimmune destruction of newly generated β cells.

Reprogramming of α cells to β cells can also occur through manipulation of a single transcription factor, either by inactivation of ARX, which is important for specifying α cell fate, or by ectopic expression of PAX4, an essential regulator of β cell development (refs. 19, 28, 29, 40, and Figure 2). Because a single gene is sufficient to induce α -to- β -cell transdifferentiation, it may be possible to identify small molecules that mimic this effect. Recent studies have identified compounds that can promote α -to- β -cell reprogramming, at least in cultured cells. Using a high-content screen for small-molecule inducers of insulin expression, Schreiber, Wagner, and colleagues identified two kinase inhibitors as compounds that upregulate insulin expression in cultured α cells (31, 32). These inhibitors, which target ribosomal S6 kinase (RSK) and cyclin-dependent kinase-2 (CDK2), were shown to upregulate multiple β cell markers, including PAX4, in a dose-dependent manner. Although these compounds have only been tested *in vitro*, the studies suggest that pharmacologic agents could promote pancreatic cell plasticity without the need for genetic modification. Screens

to identify chemical inducers of PAX4 or inhibitors of ARX may reveal compounds that promote α -to- β -cell transdifferentiation and reverse diabetes.

While reprogramming of α cells has proven to restore functional β cell mass and cure diabetes in animal models (19, 28, 29, 40), it also results in a severe depletion of α cells and a corresponding decrease in the production of glucagon (28, 42). Depletion of α cells in mice does not have overt effects on health or lifespan (42), and even a small number of α cells (2% of total population) is sufficient to maintain glucagon signaling (43). Thus, converting α cells into β cells could be a viable approach to treat diabetes; however, it is currently not known what effect α cell depletion will have in humans. It may be that glucagon deficiency due to α cell loss puts patients at risk for hypoglycemia. While the α cell response is important in recovery from insulin-induced hypoglycemia in humans, epinephrine can compensate for insufficient glucagon secretion (44). Therefore, an intact epinephrine response to hypoglycemia is an important prerequisite when considering α -to- β -cell reprogramming in patients.

Owing to their abundance, pancreatic exocrine cells, which comprise acinar and ductal cells, have been explored as an alternative population for reprogramming. In contrast to α cells, acinar cells harbor extensive repressive histone modifications at the promoters of endocrine genes, which might constitute a barrier for endocrine reprogramming (24). Indeed, the *in vivo* conversion of acinar cells requires the expression of not only one but three transcription factors (PDX-1, NGN-3, and MAFA) that are important for β cell development (ref. 41 and Figure 2). More relevant for possible clinical translation, there is also evidence that exocrine cells can be converted into β -like cells without genetic manipulation through *in vivo* cytokine/growth factor treatment (21, 35, 45). When β cells are destroyed by injection of a β cell toxin, systemic administration of EGF and CNTF induces conversion of acinar cells into β cells and restores β cell mass and normoglycemia (21). It has been observed that the success of exocrine cell reprogramming may depend on the glycemic environment. A recent study showed that hyperglycemia can inhibit *in vivo* reprogramming of acinar cells by PDX-1, NGN-3, and MAFA (46). A better understanding of how glucose levels influence cell plasticity and the response to reprogramming factors will be critical to assess the strategy's potential for future clinical application. One important consideration is that manipulations promoting transdifferentiation could also have undesired effects. For example, loss of the tumor suppressor menin 1 in α cells triggers their transdifferentiation into insulin-expressing cells, but also the development of insulinomas from reprogrammed cells (47). Therefore, before these therapies are implemented, extensive studies are necessary to fully evaluate the state of maturity and stability of reprogrammed cells.

Transdifferentiation of liver and gastrointestinal cells. Reprogramming-based strategies to generate β cells from somatic cells are not limited to cells of the pancreas, but extend to other gut tube-derived organs, such as the liver, intestine, and stomach. In the past decade, most studies have focused on the liver as a potential source of β cells. Adenoviral delivery of pancreatic transcription factors, including PDX-1 (22, 30) and/or NEUROD (33), NGN-3 (37, 39), and more recently MAFA/MAFB (34), has been shown to induce emergence of insulin-producing cells in the liver (Figure

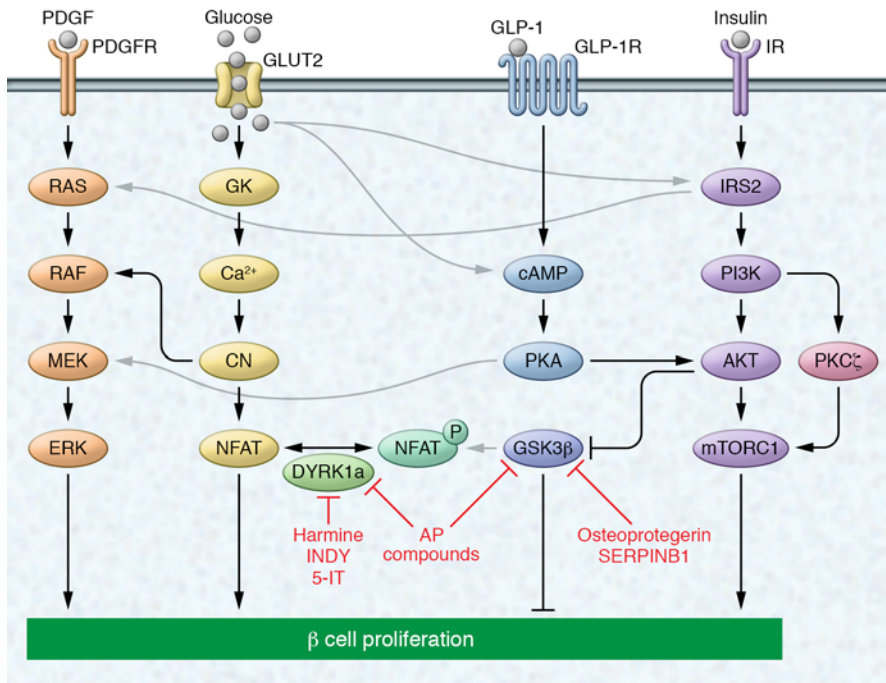


Figure 3. Select signaling pathways known to regulate β cell proliferation. Growth factors (PDGF), nutrients (glucose), and hormones (GLP-1 and insulin) have been shown to induce β cell proliferation by activating both canonical and noncanonical mitogenic pathways. Activation of their respective receptors induces signaling via the ERK (also called MAPK) signaling pathway either directly (in the case of PDGF) or via crosstalk mechanisms. Glucose, GLP-1, and insulin have been shown to activate mitogenic signaling via the PI3K/AKT/mTOR pathway. Glucose signals through the calcineurin/NFAT and ERK pathways, while signaling via GLP-1 induces cAMP synthesis to induce proliferation. Small molecules/compounds have been identified that target proteins in these signaling pathways, inhibiting either DYRK1a or GSK3 β , both of which phosphorylate NFAT, sequestering it in the cytoplasm. Black and gray arrows indicate canonical and noncanonical signaling, respectively. The depicted pathways regulating β cell proliferation are mainly based on evidence in rodents and do not represent a comprehensive overview of all published work. Compounds shown to induce human β cell replication are indicated in red. AP, aminopyrazine; 5-IT, 5-iodotubercidin; Ca²⁺, calcium; CN, calcineurin.

2). While these insulin-producing cells were to some extent able to correct hyperglycemia in diabetic mice, it is less clear whether these cells are glucose-responsive and thus function as true β cells.

Studies have demonstrated that simultaneous transgenic expression of PDX-1, NGN-3, and MAFA can promote rapid conversion of gastrointestinal endocrine cells in the antral stomach and intestine into insulin-expressing cells in vivo (refs. 20, 25, and Figure 2). These reprogrammed cells exhibit ultrastructural features of β cells, are glucose-responsive, and are able to ameliorate hyperglycemia in diabetic mice. Similarly, ablation of the transcription factor FOXO1 in endocrine progenitors of the intestine results in the generation of insulin-expressing cells that are able to reverse hyperglycemia in mice (36). This finding has been reproduced in human gut organoids (23), suggesting that FOXO1 inhibition in gut organoids could offer a source of insulin-producing cells to treat human diabetes. As human gastrointestinal tissue is readily accessible by noninvasive techniques, ex vivo reprogramming of gastrointestinal cells could be a source of autologous β cells for implantation in patients with diabetes.

Recent studies in an animal model of T1D indicate that insulin-producing reprogrammed liver cells are susceptible to autoimmune attack (37). Therefore, to prevent autoimmune destruction

of β cells produced by reprogramming, immunosuppressive therapy will likely be necessary to achieve lasting normoglycemia in T1D patients. Alternatively, ex vivo reprogrammed cells could be implanted in an encapsulation device. Despite remaining hurdles, the progress made in converting other cell types into β cells is remarkable and suggests avenues for clinical translation.

β cell replacement by promotion of β cell expansion

Another, and perhaps the therapeutically most tangible, method for inducing β cell regeneration in vivo is to target pathways that regulate β cell proliferation (Figure 3). The predominant mechanism for adapting β cell mass to increased metabolic demand, as observed during pregnancy or obesity, is through modulation of β cell replication (48, 49). Thus there has been considerable interest in understanding the mechanisms that regulate replication of β cells with the goal of discovering new therapeutic targets to promote their regeneration. This approach could benefit patients with T2D as well as T1D, as residual β cells are frequently found even decades after the onset of T1D (50, 51).

Ageing and β cell replication. Replication of β cells is the predominant mechanism that ensures the rapid expansion of β cell mass early in life; however, the regenerative capacity of β cells rapidly declines with advancing age (52–56). This age-dependent decline in β cell proliferation is regulated

by p16^{ink4a}, a cyclin-dependent kinase inhibitor encoded by the *Cdkn2a* gene (57). Multiple age-dependently regulated factors, including p38MAPK and PcG/trithorax group proteins, have been shown to epigenetically modify the *Cdkn2a* locus and repress p16^{ink4a} expression (58–62). In young β cells, PDGF receptor signaling increases the abundance of the PcG/trithorax group protein EZH2, thereby repressing p16^{ink4a} expression. However, in aged β cells, decline of PDGF receptor expression leads to p16^{ink4a} derepression and β cell cycle arrest (58).

To more globally define age-associated changes in the β cell epigenome, Kaestner and colleagues carried out a genome-wide analysis of β cells from young and old mice and found that the proliferative decline of β cells correlated with increased de novo promoter methylation and decreased expression of cell cycle regulators (63). This suggests that manipulation of epigenetic regulators could reverse β cell senescence and promote regeneration. Interestingly, this group also observed upregulation of genes involved in β cell function and improved insulin secretory function with age (63). While these results contradict early studies showing a decline of β cell function with age (64–67), they align with more recent work demonstrating sustained or improved β cell secretory function in older animals (68, 69). Because β cell proliferation and β

cell function are tightly linked (70), additional studies are needed to determine whether β cell proliferation can be safely increased without compromising function.

β cell replication in pregnancy. During pregnancy, β cell mass expands in order to adapt the organism to increasing insulin demand (71–74). Multiple factors, including lactogens, serotonin, and components of the EGFR signaling pathway, have been shown to increase β cell replication in pregnant rodents (71, 75–78). There is, however, controversy as to whether adaptive β cell proliferation during pregnancy occurs to the same extent in humans (72). Moreover, conflicting reports have been published regarding the conservation of molecular pathways regulating β cell mass expansion between pregnant rodents and humans. While one study found β cell proliferation to be induced by prolactin and placental lactogen in two human islet samples (75), more recently, Stewart and colleagues were unable to induce human β cell proliferation with prolactin in six independent human islet samples (79). This could be explained, at least in part, by the lack of prolactin receptor expression on human β cells (79). While prolactin receptor agonists may not be effective for stimulating human β cell proliferation, downstream signaling pathways may be conserved and could provide insight into therapeutic targets. In support of this idea, Vasavada and colleagues found that treatment of human islets with recombinant osteoprotegerin, a lactogen target, induced human β cell proliferation (80). Importantly, the FDA-approved osteoporosis drug denosumab mimicked the activity of osteoprotegerin and enhanced human β cell replication in vitro and after engraftment of human islets into mice. The pro-proliferative effect of denosumab suggests that there is potential for repurposing this drug for the treatment of diabetes.

With the goal of discovering novel targets for enhancing β cell proliferation, Ahnfelt-Rønne and colleagues took a proteomic approach to identify proteins that change in abundance during pregnancy in mice (81). The analysis not only confirmed regulation of targets previously shown to be controlled at the mRNA level (77, 82–84), but also identified proteins not previously associated with pregnancy-induced β cell expansion. Two examples are stathmin 1 and nuclear chloride ion channel 1, which have known roles in the regulation of cell proliferation and are being evaluated as drug targets in cancer (85–88). While follow-up studies will be necessary, this study highlights the importance of global approaches to identify novel molecular targets for enhancing β cell proliferation.

β cell replication in hyperglycemia and insulin resistance. Apart from pregnancy, β cell proliferation is also regulated by diet and changes in metabolic state. A recent study suggests that nutritional cues have immediate effects on the capacity of β cells to mount a regenerative response. When mice were prematurely weaned from fat-rich milk to carbohydrate-rich chow, the potential of β cells for compensatory proliferation increased (89). Although it remains to be studied whether a similar mechanism operates during adulthood, this finding suggests that diet composition could have effects on β cell mass. Metabolic regulation of β cell proliferation is also evident during a state of insulin resistance, which is known to trigger compensatory β cell proliferation. This has been demonstrated in multiple rodent models of diabetes, including *ob/ob* mice (90), *db/db* mice (91), and Zucker fatty rats (92), and in high-fat-diet feeding (93). Increased β cell mass is also observed

in hyperinsulinemic humans with obesity and insulin resistance (94–96); however, whether proliferation is increased is less clear.

Glucose and insulin have been identified as inducers of β cell replication (Figure 3). Multiple studies have demonstrated increased proliferation of rodent and human β cells following glucose infusion (97–103). Glucose metabolism is required for β cell proliferation, as lack of glucokinase, a key enzyme in glycolysis that converts glucose to glucose-6-phosphate, decreases β cell proliferation whereas treatment with a small-molecule glucokinase activator stimulates β cell proliferation (104). However, the pro-proliferative effect of glucose is only observed in the short term, while sustained exposure of β cells to high glucose can cause glucotoxicity, resulting in DNA damage and apoptosis, as also seen in β cells from T2D patients (105). Therefore, there is a need to better understand where the mitogenic and DNA damage pathways diverge before the glucose-induced mitogenic pathway can be considered for therapeutic intervention.

While glucose can increase β cell replication, β cell hyperplasia occurs in *ob/ob* and *db/db* mice prior to the onset of hyperglycemia and is also observed in mouse models of insulin resistance in the absence of hyperglycemia (91, 106–108). These observations suggest that factors other than glucose contribute to β cell mass expansion in the face of insulin resistance. Insulin levels are highly elevated in the insulin-resistant state, and insulin signaling has been shown to account for compensatory β cell growth during insulin resistance. Ablation of the insulin receptor in an insulin-resistant mouse model impaired β cell proliferation and rendered mice prematurely diabetic (109). In contrast, deletion of the IGF receptor had little effect on β cell growth, suggesting that compensatory β cell mass expansion predominantly depends on insulin rather than IGF signaling.

Circulating factors and β cell replication. In recent years, significant effort has been put forth into identifying systemic regulators of β cell proliferation in the context of aging, pregnancy, and metabolic challenge. Circulating factors that are regulated during metabolic adaptation are particularly attractive therapeutic targets, as manipulating their activity might mitigate the risk for inducing tumors in other tissues. Studies have shown that circulating factors from young mice improve regeneration of aged islets. This has been demonstrated in the context of parabiosis experiments, in which a young and an old mouse are surgically joined to develop a shared circulatory system, or after revascularization of an islet graft from aged mice in a young host (68, 110). Likewise, β cell replication increases when islets from metabolically normal mice are grafted under the kidney capsule of insulin-resistant mice (111). While these experiments clearly illustrate the importance of circulating factors in β cell regeneration, the specific factor or factors that account for the effects have remained elusive. Multiple circulating factors, including glucagon-like peptide-1 (GLP-1), secreted by the intestinal L cells (112–114); thyroid hormone (115, 116); the osteoblast-derived hormone osteocalcin (117–120); liver-derived angiopoietin-like protein 8 (ANGPTL8, also known as betatrophin) (121); and recently the liver-secreted protease inhibitor SERPINB1 (122), have been identified as potentially pro-proliferative for β cells, at least in rodents (Figure 3). It is less clear whether these circulating factors can also stimulate human β cell growth. Controversial reports exist regarding effects of GLP-1 analogs on

human β cell growth, with one study finding no effect (123), while another reports stimulation (124). Illustrating the difficulty of controlled studies using primary human cells, subsequent work showed that the age of the islet donor might be a factor contributing to responsiveness of β cells to GLP-1 analogs (125). The original report describing ANGPTL8 as a β cell growth factor received much attention (121). However, further studies on ANGPTL8 have called its role in β cell mass expansion into question. Genetic loss- and gain-of-function experiments with *Angptl8* revealed no effect on β cell mass in insulin-resistant mice (126, 127), showing that ANGPTL8 is not the long-sought-after liver-derived factor that stimulates β cell growth. Consistent with findings by Gromada and colleagues (127), ANGPTL8 also failed to exert a pro-proliferative effect on transplanted human β cells (128). More promising are recent findings on osteocalcin (120) and SERPINB1 (122), which indicate that these hormones could be effective in stimulating human β cell proliferation. Proliferation of β cells was increased after treatment of human islets with decarboxylated osteocalcin or small molecules mimicking SERPINB1 activity both *ex vivo* and after transplantation into mice (122). Preliminary analysis of mice treated with small-molecule mimics of SERPINB1 suggests that the effects on proliferation of extrapancreatic tissues are limited, which raises hope that it might be possible to identify growth-stimulating agents that are selective for β cells.

Intracellular signaling pathways regulating β cell replication.

Many groups have used high-throughput screening methods to discover novel molecules and pathways that could stimulate β cell mass expansion (129–138). Some of these screens have led to the discovery of novel compounds with therapeutic potential. For example, a high-throughput chemical screen recently identified aminopyrazine compounds, harmine, INDY, and 5-iodotubercidin as pro-proliferative in rodent β cell lines, and these compounds were subsequently shown to also augment human β cell proliferation (129, 134, 137, 139). Interestingly, all 4 molecules inhibit the kinase DYRK1A, which blocks nuclear localization of NFAT, a transcription factor that activates expression of cell cycle genes in β cells (refs. 140, 141, and Figure 3). Aminopyrazine compounds have a larger effect on β cell proliferation than harmine, which is explained by the additional inhibition of glycogen synthase kinase-3 β (GSK3 β) by aminopyrazine compounds (137). Like DYRK1A, GSK3 β prevents nuclear localization of NFAT (142) and inhibits β cell proliferation (143–146). Interestingly, osteoprotegerin and SERPINB1 have both been shown to inhibit GSK3 β activity (refs. 80, 122, and Figure 3), suggesting that their effect on β cell proliferation may, at least in part, be mediated through GSK3 β inhibition. A significant hurdle for advancing β cell therapeutics for these pathways is the unclear specificity of many of the small molecules as well as their effects on multiple tissues.

Additional potentially druggable intracellular regulators of β cell proliferation have been identified through candidate approaches. The literature on intracellular signaling in β cell proliferation has recently been comprehensively reviewed (147–150), and we refer to these reviews for a comprehensive description of all pathways shown to regulate β cell proliferation. From the numerous studies, the MAPK and PI3K/AKT pathways have emerged as critical regulators of β cell proliferation also in humans (Figure 3). The MAPK pathway via ERK1/2 phosphorylation is the key mitogenic

pathway that separates metabolic regulation of β cell function from the regulation of β cell proliferation, as ERK1/2 phosphorylation is not required for glucose-stimulated insulin secretion (151). The MAPK pathway mediates the β cell mitogenic effect of multiple growth factors, hormones, and nutrients, including PDGF, GLP-1, prolactin, insulin, and glucose (58, 152–159). The second major pathway responsible for transducing β cell proliferative signals is the PI3K/AKT/mTOR pathway, which is activated by insulin, GLP-1, and glucose (153, 159–162). AKT activation is an important component that links growth signals to its downstream target mTOR, which coordinates a cell growth response directly through its effect on cell cycle regulators (143, 163). Numerous studies have demonstrated a role of this pathway in promoting β cell proliferation *in vitro* and increasing β cell mass *in vivo* (143, 160, 163–167). Further illustrating its pro-proliferative role, AKT/mTOR signaling is active in pancreatic endocrine tumors (168). Notably, PI3K signaling can induce β cell proliferation not only by activating AKT, but also through AKT-independent PKC ζ , which mediates the proliferative effect of glucose on human β cells (169–172).

It is important to consider that significant crosstalk exists between the signaling pathways. For example, high glucose and GLP-1 levels activate both mTOR and MAPK signaling (153, 158, 160). A recent study nicely illustrates how the balance between different signaling arms determines the β cell response to insulin. Knockdown of PI3K resulted in rerouting of the insulin signal from PI3K-mediated metabolic signaling to ERK-mediated mitogenic signaling, which induced a switch of β cells from highly glucose-responsive to proliferative (173). Extensive feedback inhibition and amplification constitute a further layer of complexity, exemplified by mTOR-mediated negative feedback on insulin signaling via IRS2 (174).

All of these intracellular signals converge to regulate the core G1/S cell cycle machinery (147–150). Successful targeting of β cell proliferation will hinge on the downregulation of cell cycle inhibitors and upregulation of cell cycle activators. The example of aminopyrazine compounds, which target DYRK1A and GSK3 β , illustrates that targeting more than one pathway will likely have a more robust effect on β cell proliferation than targeting one pathway alone. Given the extremely low proliferation rate of human β cells (54), hitting multiple targets might be necessary to produce clinically relevant effects. Furthermore, as regenerative and oncogenic pathways share similar effector proteins, a major challenge will be to enhance β cell proliferation without inducing aberrant growth of β cells or other tissues.

Challenges for therapeutic implementation

Both *in vivo* reprogramming approaches and the induction of β cell proliferation will likely require cell type-specific delivery systems for application in humans. Local delivery of reprogramming or regeneration factors could be achieved by ultrasound destruction of microbubbles carrying plasmid DNA administered into the pancreatic microcirculation (175). Tethering the molecule to the ligand of a β cell-specific receptor could be an alternative approach for delivering molecules directly to the β cell. The efficacy of this approach has recently been demonstrated for GLP-1-estrogen conjugates in β cells (176).

Perhaps the most challenging issue is protecting the newly generated β cells from autoimmune destruction in T1D. Similarly,

the autoimmune and alloimmune responses impose a hurdle for hPSC-based replacement therapies. hiPSC-derived β cells from T1D patients can serve as an autologous source for cell replacement therapy that would obviate the need for systemic immune suppression (16); however, these cells are not protected from autoimmune destruction in T1D patients, and likely would still need to be implanted within an encapsulation device. Recent studies suggest that gene editing strategies can be used to generate hPSCs that are invisible to the immune system and could escape at least allogenic rejection (177). Similar strategies could perhaps also allow the cells to evade autoimmune destruction. Overall, significant progress has been made in the past decade, and the coming decade will show which strategy will hold most promise for translation into clinical therapies.

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