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Harnessing gut cells for functional insulin production: Strategies and challenges

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ABSTRACT

Reprogrammed glucose-responsive, insulin + cells ("β-like") exhibit the potential to bypass the hurdles of exogenous insulin delivery in treating diabetes mellitus. Current cell-based therapies-transcription factor regulation, biomolecule-mediated enteric signaling, and transgenics - have demonstrated the promise of reprogramming either mature or progenitor gut cells into surrogate "β-like" cells. However, there are predominant challenges impeding the use of gut "β-like" cells as clinical replacements for insulin therapy. Reprogrammed "β-like" gut cells, even those of enteroendocrine origin, mostly do not exhibit glucose – potentiated insulin secretion. Despite the exceptionally low conversion rate of gut cells into surrogate "β-like" cells, the therapeutic quantity of gut "β-like" cells needed for normoglycemia has not even been established. There is also a lingering uncertainty regarding the functionality and bioavailability of gut derived insulin. Herein, we review the strategies, challenges, and opportunities in the generation of functional, reprogrammed "β-like" cells.

1. Introduction

Recent innovations in minimally invasive, transdermal delivery techniques makes insulin delivery painless and relatively accurate, leading to better compliance and saving diabetics from catastrophic macro and microvascular dysglycemic complications.[1–5] However, the large molecular weight of insulin limits the efficiency of these innovations by restricting transdermal permeation, bioavailability, and sustained release.[2,6,7] Furthermore, these novel technologies are expensive, less user-friendly, and come with technical difficulties.[4,8–10] More aggressive treatments, like islet transplantation, possess the potential to restore euglycemia in both Type 1 and advanced Type 2 diabetic patients.[11,12] However, durable donor islets are rare and strongly immunoreactive, rendering patients to a long-term dependency on immunosuppressants to prevent donor islet rejection.[13,14] Recently, Pomposelli et al. suggested that transplanting islets as conglomerated units of pre-vascularized, islet-kidney grafts limits islet apoptosis and provides a physical barrier against host immune responses.[15] Yet, continuous graft loss still renders transplanted islets to eventual host immune assualt.[15] Thus, endogenously engineering host cells into glucose-responsive "β-like" cells exemplifies a more permissive approach in overcoming the limitations of exogenous insulin delivery, potentially curing diabetes.

The gastrointestinal (GI) tract serves as a reservoir for accessible enteroendocrine and stem cells that can be coaxed, through transcriptional control and enteric signaling, into self-renewable "β-like" cells to produce endogenous insulin in a reliable, nutrient-responsive manner.[16–19] Gut stem cells can preserve genomic alterations to allow for therapies to be tailored to patient needs with time, establishing endurance of the desired gene therapy outcome.[20–24] GI tract stem cells exhibit high levels of indefinite turnover and regeneration which counter cell population decline.[16,25–27] In the case of type 1 diabetes, this high turnover could allow for their continued replenishment despite a possible autoimmune attack.[28] Collectively, these properties make the gut an attractive option for creating populations of glucose-responsive insulin-producing cells.

Proof-of-principle studies have shown significant potential in attaining functional, reprogrammed "β-like" intestinal tract cells. However, there are distinct challenges and unanswered questions regarding the employment of these cells as clinical interventions for insulin replacement therapy. Since pancreatic insulin secretion encompasses complex molecular and cellular processes in addition to insulin gene expression, reprogrammed "β-like" cells should not only be able to make insulin, but also have workable receptors for ambient glucose detection,

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cell stimulation, and insulin release from docked secretory granules.[29–32] Yet, attained reprogrammed "β-like" cells do not always temporally release insulin in response to varying glucose concentrations. Considering that the typical length of an adult gastrointestinal (GI) tract stretches into an enormous surface area of ~250–400 m^2 with ~10^{14} cells, it becomes realistic to suppose that a fraction of these gut cells can be reprogrammed into a comprehensive source of insulin to treat diabetes without interfering with normal gut function.[33–36] Regrettably, regardless of the approach adopted, the conversion rate of "β-like" intestinal cells tends to be very low.[36,37] Moreover, the sufficient, therapeutic number of "β-like" intestinal cells needed to reverse diabetes and escape consequent immune responses has not been established.[38–41] Only few studies have discussed or shown that reprogrammed gut "β-like" cells, exhibit the necessary genes for the proteases (Prohormone convertases - PC1/3, PC2, and carboxypeptidase E/H), or at least induce an enteric microenvironment with conditions, necessary for bioactive maturation of secreted insulin. This paper evaluates the various strategic pathways of attaining functional insulin from host gut cells by highlighting the challenges, uncertainties, and possible innovative routes for achieving functional gut "β-like" cell.

2. Modification of expression patterns of endocrine transcription factors sufficiently establishes morphogenesis of reprogrammed "β-like" gut cells

Over the last twenty years, most studies have focused on subsets of transcription factors associated with insulin transcription and release via the ectopic expression of either one, or some combination of the PMN factors (PDX1, MAFA and NGN3), the consistently proven transcription factor triad critical to insulin transcription and the development, maturation, and function of pancreatic β-cells[42–44] (see (a) in Figure). Even though PMN-mediated experiments have successfully demonstrated the induction and secretion of insulin from gut cells in vivo, the ability of these reprogrammed cells to alter the magnitude of insulin secretion in correspondence with glucose concentrations remains a challenge in the field. It seems the ectopic expression of pancreatic transcription factors is not potent enough to establish abundant populations of robust β-like gut cells due to transient delivery inefficiencies, tissue damage from inappropriate dosages of reprogramming hormones or constructs, and native immune attack.[45–47]

Immature rat intestinal cells (IEC-6)expressed insulin when made to overexpress PDX1 along with Insulin Gene Enhancer Protein (ISL1) or when exposed to betacellulin, an epithelial growth factor known to induce ISL1 expression and pancreatic β-cell differentiation.[48–50] However, after transplantation in mice, PDX-1^+ IEC-6 cells failed to secrete insulin in response to increasing glucose and secretagogue concentrations.[48,49] Koizumi et al. showed insulin expression in Streptozotocin (STZ)-induced diabetic mice by injecting into the mice ileum a recombinant, replication-deficient adenovirus carrying the PDX1 gene.[51] The group did not determine the temporal glucose responsivity of converted cells.[51] STZ-induced diabetic mice, after being fed with engineered recombinant adenovirus expressing MAFA under control of the musculoaponeurotic fibrosarcoma oncogene homolog A gene (Ad-MAFA), produced sufficient ileal insulin to ameliorate hyperglycemia.[52] However, insulin levels did not increase after an oral glucose tolerance test in the Ad-MAFA-treated STZ – induced diabetic rats, stipulating that Ad-MAFA-dependent insulin secretion was not regulated or amplified by glucose.[52] The group showed that intestinal epithelial cells, positive for insulin, expressed both PC1 and PC3, but the group did not assess the levels of mature insulin produced.[52] Matsuoka et al. showed that insulin was expressed in mouse IEC-6 cells only when MAFA was co-transduced with PDX1 and BETA2.[53] Unfortunately, the group did not assess insulin secretion in response to glucose.[53]

Chen et al., through the collective expression of all three PMN transcription factors, acquired clusters of crypt-residing, insulin + intestinal cells in mice treated with doxycycline, but insulin secretion was only observed in response to high (15 mM), but not low (3 mM) concentrations of glucose.[54] Lee et al. successfully reprogrammed murine intestinal cells to produce insulin through adenoviral delivery of PMN transcription factors but did not discuss how sensitive and responsive these cells were to glucose.[46] Two groups, utilizing the overexpression of PMN factors, efficiently harnessed mice NGN3-marked gut cells, notably at different locations along the GI tract, into glucose-responsive "β-like" cells at seemingly high frequencies. The intestinal "neo-islets' 'obtained by Chen et al. were observed to be converted at a remarkable rate of 50%, but these unstable cells disappeared after the deinduction of PMN factors.[54] Ariyachet et al. reported that about 42% of highly regenerative, monohormonal antral stomach NKX 6.1 and PC2+ cells in mice were positive for insulin and could reverse hyperglycemia in a glucose-responsive manner.[55] The group suggested the harvesting and coalescing of these cells into transplantable "mini-organs'' for the treatment of diabetes - an interesting alternative to attaining numerous gut "β-like" cells.[55] The Stanger group showed that their crypt-residing "neo-islet" in doxycycline-treated mice, similarly to pancreatic beta-cells, possess ultra-structural components with the ability to process preproinsulin, with the release of C-peptide in its mature form, but the group did not discuss the expression of proteases responsible for insulin maturation.[54]

Elegant studies focusing on the inhibition of the nuclear Forkhead boxO1 (FOXO1) transcription factor have shown some success in achieving glucose-regulated/amplified insulin secretion from reprogrammed gut β-cells (see (b) in figure). During nutrient overload, pancreatic FOXO1 counters PDX1 to hinder β-cell proliferation and insulin secretion, while preserving pancreatic β-cells against acute oxidative stress.[56–59] Under lower nutritive states, FOXO1 promotes β-cell proliferation by inducing Cyclin D and evoking antioxidant mechanisms to prevent β-cell failure, thus sustaining β-cell function.[56–59] A subset of FOXO+ and NGN3+ gut cells have also been shown to determine gastric cell distribution.[60,61] Talchai et al. were able to attain insulin-positive gut cells through somatic Foxo1 ablation in Ngn3-expressing enteroendocrine progenitor cells of adult mice.[62] In an STZ-induced diabetic mouse, the group obtained reprogrammed gut cells that expressed mature pancreatic β-cell markers and secreted bioactive insulin in response to varying glucose and sulfonylurea concentrations.[62] The same group showed that Foxo1 removal from human-derived iPS gut organoids promoted the generation of insulin-positive cells, releasing C-peptide in a dose-dependent manner to concentrations of secretagogues.[63] This group identified a new population of Foxo-expressing cells in the stomach epithelium, of which Foxo1 deletion resulted in a substantial increase in insulin and c-peptide expressing cells with "β-like" gene expression.[64] Unfortunately, only about 4% of the intestinal Ngn3+ cells and about 0.05% of cells human-derived iPS gut organoids, a number that decreased over time, were positive for insulin.[62–64] Recently, three studies from the Accili group showed that the blockade of either a single FoxO1 agent or a triad of Notch-Tgfβ-FoxO1 transcription factors yields immunoreactive "β-like" insulin + gut cells and reverses hyperglycemia in streptozotocin-diabetic insulin-deficient akita mice and cultured human enteroids.[65–67] The synergistic effect of the dual inhibition of FOXO1 and notch signaling increased the number of progenitor enteroendocrine cells by 30%, signifying the possibility of higher conversion frequency.[65]

Unfortunately, FOXO1 ablation in gut cells, despite being an efficient

route for achieving glucose-responsive "β-like" cells, is not without risk. FOXO factors play critical roles in tumor suppression and FOXO1 is mostly deleted, inactivated, or downregulated in some human cancers.[68–71] Thus, any therapeutic approach relying on down-regulating FOXO1 from a patient's gut would have to consider side-effects seriously. Also, FOXO factors have been shown to integrate cues for determining the developmental timing, pool size, and functional features of endocrine progenitor cells, so complete ablation of FOXO1 might unknowingly hinder the functionality and proliferation of host enteroendocrine stem cells.[62,72] Although these setbacks question the safe utilization of FOXO1 ablation, the Accili group has persistently demonstrated that the inhibition of FOXO1 is sufficient to transform gut cells into insulin-producing, glucose-responsive cells.

2.1. Transgenesis shows promise in reprogramming enteroendocrine cells (EECs) into gut "β-like" cells

Enteroendocrine cells, especially that of G -, K-, and L-cells, have sophisticated nutrient/glucose sensing systems and share similar NGN3-precursor differentiation pathway with pancreatic β-cells, hence tightly secrete incretins (gastrin, GIP, and GLP-1) in response to ingested meals with similar kinetics to pancreatic β-cells.[47,73–82]Recently, Egozi et al. made a single-cell atlas of human fetal and neonatal small intestinal cells and found subsets of fetal insulin expressing K and L cells.[83] Unfortunately, compared to the number of adult pancreatic beta cells, the number of fetal K/L INS + cells (9 INS + cells out of 37 fetal K/L cells) were four orders lower in magnitude and ceased with insulin expression with time.[83] The similarity in insulin secretion and incretin secretion kinetics and differentiation pathways between EECs and pancreatic β-cells suggests enteroendocrine cells to be suitable candidates for achieving glucose-regulated insulin secretion. However, glucose-regulated insulin secretion in EECs has seen modest success, and there are compounding uncertainties regarding the most suitable choice of EEC for robust conversion.

Gastric G cells in transgenic mice with a chimeric gene consisting of a gastrin promoter fused to the human insulin gene produced insulin, but the kinetics of insulin secretion was not discussed.[84] Aiming to decipher the kinetics of insulin secretion, Lu et al. achieved functional insulin production in G-cells by creating transgenic rats that had the human insulin coding sequence knocked into the mouse gastrin gene.[85] Though the insulin produced was able to ameliorate hyperglycemia in STZ - induced diabetic rats, the group found that gastric G-cells could only secrete insulin when stimulated by peptone, not glucose[85] Given that G-cells are mainly responsive to proteins, researchers speculate that transforming these cells into surrogate *β*-cells might not lead to glucose –potentiated insulin release.[47,86,87] K- cells, derived from the STC-1intestinal cell line and induced to produce NGN3 and NKX6.1, upregulated insulin gene expression but could not secrete insulin to lower glucose after transplantation in diabetic mice.[88] Likewise, GIP-enriched K-cells, GTC-1 cells, were transcriptionally controlled to produce insulin via a dose and time-dependent introduction of mifepristone, not glucose.[89] After transplantation of these mifepristone-treated cells in STZ-induced diabetic mice, the mice were surprisingly observed to be hypoglycemic, and the group attributed this result to the fact the reprogrammed mice K-cells lacked the precise glucose sensitivity of the native human K-cell, releasing insulin even under low glucose conditions for normal physiological functions in humans.[89] Also, these cells lost glucose sensitivity and insulin secretion capacity over time: indicating that the transplanted cells lacked the requisite interactions characteristic of human K-cells.[89]

Dose-dependent, acute insulin and endogenous GLP-1 secretion was observed in human NCI–H716 intestinal cell lines (an enteroendocrine L-cell replica) transduced with a recombinant adeno-associated virus (rAAV) vector carrying the human insulin gene. Insulin was induced only upon stimulation by meat hydrolysate.[90] The same group achieved in vitro insulin secretion after transfecting murine GLUTag L-cell line with a plasmid co-expressing human insulin and neomycin resistance.[91] Nevertheless, insulin secretion was induced at a sub physiological glucose concentration level with no significant insulin secretion changes from cells exposed to escalated glucose concentrations (5 mM vs 20 mM), showing that the secreted insulin was rendered, but not regulated by glucose.[91] Murine GLUTag-INS L-cells also expressed both PC1/3 and PC2; however, it was not demonstrated that the insulin produced was bioactive and mature[91]

After employing western blotting on intestinal lysates to evaluate the immunoreactivity of murine K-cell-derived insulin, Mojibian et al. found that most of the secreted insulin was proinsulin and not mature[92] The scientists speculated this sub-optimal processing of insulin was because the murine K-cell expresses PC1 and PC3, but not PC2.[92] The same group recently further challenged the long-standing theory of insulin processing by showing that PC2 is not required for proinsulin processing into mature insulin in humans.[93] It is reasonable to assert that an immune attack against gut-derived beta-like cells could cause a low observed reprogramming rate. However, the gut has been evidenced to have "immune privilege," denoting that reprogrammed gut, insulin + cells, which do not express β-cell autoantigens, could evade an immune attack.[94,95] In NOD mice, K- cells, upon overexpression of preproinsulin, produced insulin and showed high levels of immunoreactivity and secreted very few inflammatory cytokines, escaping immune attack.[92] In view of this immune advantage, researchers may not need to address immune attack on β-like gut cells; however, more studies are needed to determine if gut-originated insulin + cells can resist immune attack in animal models over time.

During embryonic development, the Paired box 4 (PAX4) factor establishes the endocrine lineage of pancreatic β-cells and EECs, while selectively promoting mature β-cell expansion and resistive survival against apoptosis by oxidative metabolic stress.[96–102] PAX4 has also been evidenced to promote β-cell differentiation of human embryonic stem cells.[103–108] Relying on both their previous results of PAX4 misexpression converting somatostatin-producing δ-cells into "β-like" cells and the high frequency of somatostatin + gut cells, the Collombat group found that misexpressing PAX4 in somatostatin + gut cells was sufficient to produce robust gut "β-like" cells[28,109,110] (see (c) in Figure). The group, from lineage tracing, determined that the somatostatin + gut cells were D-cells.[28] Interestingly, the group attained a high frequency of 65% beta-like cells co-expressing insulin and somatostatin and approx. 25% expressing insulin only.[28] Moreover, the group also saw PC1/3, an enzyme involved in insulin maturation, signifying that the insulin produced was mature.[28] So far, PAX4 misexpression in gut somatostatin D cells have been shown to overcome almost all hurdles of functional insulin secretion of gut beta-like cells and deserves careful attention going forward.

2.2. Incretins possesses the ability to rewire the gut to make glucose-regulated insulin-producing cells through enteric signaling

Incretin hormones potentiate pancreatic insulin release in response to elevated glucose concentrations in the fed state.[31,111–115] Auspiciously, this pancreatic, glucose-dependent insulinotropic effect has been meaningfully exploited to demonstrate insulin secretion through direct interactions between intestinal cells and GLP-1, an incretin that stimulates pancreatic β-cell proliferation and inhibits glucagon

secretion[116–118] (see (d) in Figure). The molecular mechanism responsible for this physiological phenomenon was unclear until recently when studies indicated that GLP-1 analogs and receptor agonists promoted intestinal enteroendocrine cell proliferation – a similar effect on pancreatic beta cells - by allowing for self-adaptation to metabolic stress, hence leading to increased incretin expression against metabolic syndromes, particularly obesity and type 2 diabetes.[119–122]

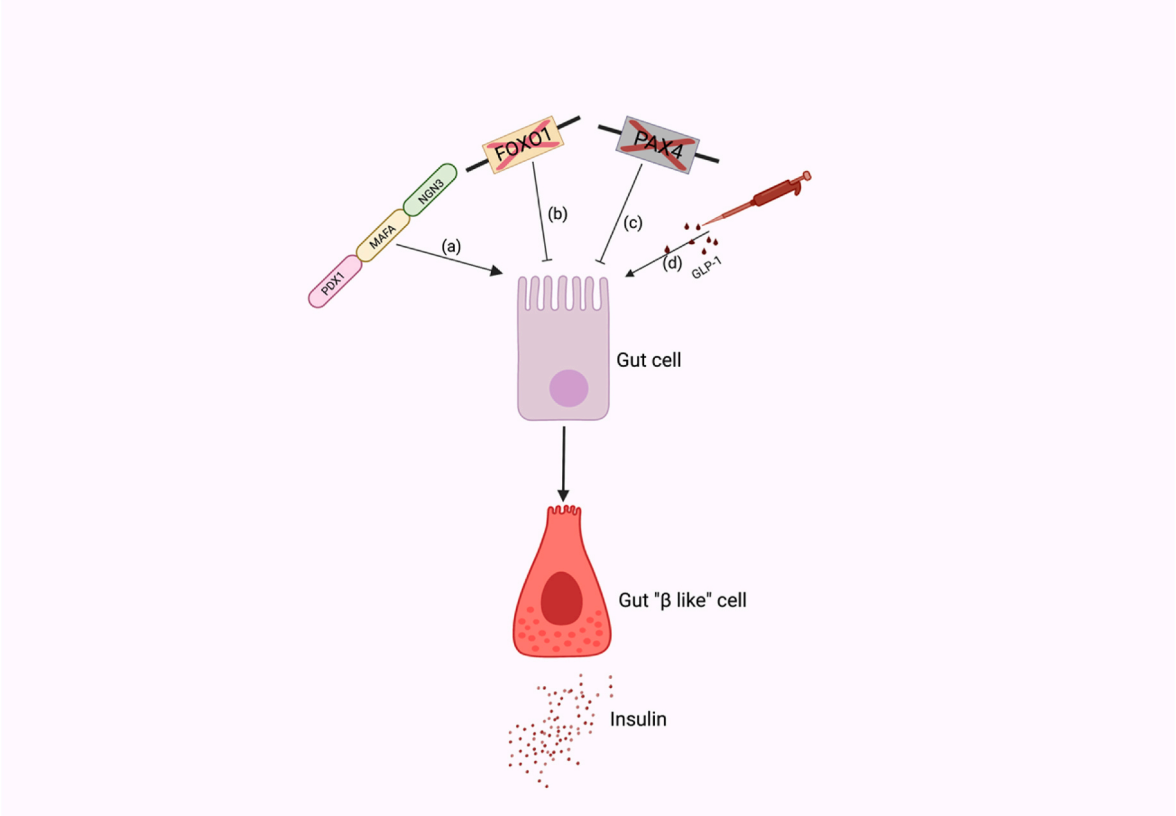
Suzuki et al. first showed that intraperitoneal delivery of the full form of GLP-$1_{(1\text{-}37)}$, by controlling the expression of ngn3 and HNF-6, directly converts both adult and embryonic rat intestinal epithelial cells into glucose-regulated, insulin-secreting cells.[123] Two studies from the March lab have shown that by populating the intestinal villous compartment with engineered bacteria that secrete GLP-$1_{(1\text{-}37)}$ intestinal epithelial cells could be differentiated into glucose-responsive, insulin-producing cells. Human intestinal carcinomas (Caco-2 cells) transformed into insulin-secreting cells, secreting up to 1 $ngml^{-1}$ of insulin when co-cultured with GLP-$1_{(1\text{-}37)}$-secreting *Escherichia coli Nissle 1917,* under the control of the *fliC* promoter.[124] The bacteria exhibited high survival and persistence, augmenting serum insulin to levels needed for steady glycemic control in healthy adults.[124] In the subsequent study, this approach proved successful in vivo, where a 50-day oral administration of GLP-1(1–37)-secreting *Lactobacillus gasseri* reduced but did not fully ameliorate hyperglycemia in STZ-induced diabetic rats.[45] The bacterially-treated rats, relative to wild-type STZ-induced diabetic rats, showed a two-fold increase in serum insulin levels in correspondence with ingested glucose concentrations. In vitro experiments in IEC-6 cells linked this response to HNF-6 expression.[45]

Suzuki et al. stated that their achieved GLP-1 $_{(1\text{-}37)}$ -stimulated, glucose-regulated insulin + intestinal cells were in very low frequency in adult rats (Suzuki et al., 2003). The March Lab made a similar observation when only about 0.06% of rat intestinal epithelial cells were able to produce insulin upon daily administration of GLP-$1_{(1\text{-}37)}$ – secreting bacteria (F. F. Duan et al., 2015b). Reasons for this outcome are unknown: however even for the experiments focused on GLP-1 as a reprogramming agent, we speculate that the low reprogramming rates of gut cells are mostly due to the inherent lumenal instability of biomolecules, the ambiguities regarding sufficient, safe biomolecule concentrations adequate to induce reprogramming, and the inadequacies in the biomolecule delivery method.[45] The non-bioactive, non-functional form of GLP-1 molecule, GLP-$1_{(1\text{-}37)}$, has an inherent short half-life due to rapid degradation by the protease DPP-IV thus may not have even reached the crypts to drive intestinal conversion[45,125–131] In all, these promising studies have opened the possibility of exploring incretins as powerful insulin-inducing biomolecules. Future studies should focus on establishing optimal, safe, and precise dosing of incretins for effective attainment of robust gut beta-like cells.

2.3. Graphical summary

Figure. Evidenced strategies for gut cell reprograming into gut 'beta-like' cells.

Descriptive text: Gut cells made to express either one, or some combination of PDX1, MAFA, and NGN3 (PMN factors) have been shown to produce insulin (a).[43,48–51,53–55,132] Gut ablation of FOXO1 has consistently been shown to yield gut "β-like" cells (b).[36,61–67,72] Recently, PAX4 misexpression has been shown to also yield robust gut "β-like" cells in high numbers (c).[28] Incretin delivery (e.g.GLP-1) have also been presented to illicit insulin gut-derived insulin secretion (d).[45,123,124]



3. Conclusion

Reprogramming gut cells into producing endogenous insulin remains a potentially attractive means of managing, and ultimately curing diabetes. Given the evidenced insulinotropic potential of the gut, it is not surprising to witness the several innovative approaches employed to fully manifest this insulinotropic effect. Several studies have succeeded in harnessing gut cells to produce viable insulin, but in very small cell numbers, and at times without robust glucose homeostatic ability. To achieve sturdy insulin + cells through any therapeutic method, we suggest focusing on gaining a complete understanding of all processes, candidate genes, and transcription factors responsible for not only enteric insulin expression and granule secretion, but also for protection of reprogrammed gut "β-like" cells from immune and physiological assault. In-vitro studies should aim at establishing optimal, safe, and precise dosing of gut insulin-inducing biomolecules to ensure maximal conversion. Advanced efforts, including but not limited to encapsulated cell transplantation, for both the delivery and silencing of genes and transcription factors are currently underway. However, alluding to the aforesaid challenges associated with transplantations and humoral protein delivery, formulating a safe oral pill that delivers accurate, yet systemically specific biomolecules and decoys, appears to be an outstanding method for patient ease and compliance. With the high, escalating prevalence of insulin-dependent diabetes, reprogramming gut cells into insulin producing cells remains an innovative, attention worthy approach to meet the exponentially growing demand for safe insulin.

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Declaration of competing interest

All authors disclose that they have no actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work that could inappropriately influence, or be perceived to influence, their work. John C. March is an Editorial Board Member for Biotechnology Notes and was not involved in the editorial review or the decision to publish this article.

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