

# Engineering the gut for insulin replacement to treat diabetes

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## ABSTRACT

The gut epithelium's large surface area, its direct exposure to ingested nutrients, its vast stem cell population and its immunotolerogenic environment make it an excellent candidate for therapeutic cells to treat diabetes. Thus, several attempts have been made to coax immature gut cells to differentiate into insulin-producing cells by altering the expression patterns of specific transcription factors. Furthermore, because of similarities in enteroendocrine and pancreatic endocrine cell differentiation pathways, other approaches have used genetically engineered enteroendocrine cells to produce insulin in addition to their endogenous secreted hormones. Several studies support the utility of both of these approaches for the treatment of diabetes. Converting a patient's own gut cells into meal-regulated insulin factories in a safe and immunotolerogenic environment is an attractive approach to treat and potentially cure diabetes. Here, we review work on these approaches and indicate where we feel further advancements are required.

## INTRODUCTION

Most patients with type 1 diabetes are dependent on daily insulin injections for survival. Despite the life-saving impact of insulin in patients with this disease, insulin delivery regimens are far from optimal. Diabetic patients require intensive disease management, as they deal with a wide range of complications resulting from inaccurate insulin dosing, from the potentially deadly consequences of hypoglycemic episodes, to chronic complications, such as neuropathy and nephropathy as a result of prolonged hyperglycemia. The wide fluctuations in glucose levels observed throughout the day in the typical patient with diabetes stem from the difficulties of attempting to mimic the tightly regulated and nutrient-responsive insulin secretion seen in normal physiology, using bolus insulin injections.

To circumvent the challenges and shortcomings of insulin injections, numerous strategies are being explored that could provide a more physiological insulin delivery to treat diabetes. Islet transplantation is a promising approach as a replacement for insulin therapy that is already in clinical use and can successfully restore tight glucose control without the need for insulin injections<sup>1,2</sup>. However, the limited supply of donor

pancreases, the requirement for immunosuppressive drugs and the failure of transplanted islets in most patients over the first 5 years have significantly limited the wide application of this approach in patients with diabetes. Hence, to overcome these limitations, there is tremendous need to find alternate strategies to restore the natural rhythm of insulin production seen in normal physiology. Recent advances using differentiated human embryonic stem cells are promising, and have opened a brand new avenue for the treatment of diabetes<sup>3,4</sup>. The feasibility, side-effects, and efficiency of this approach need to be determined and are already under investigation, with clinical trials recently initiated by ViaCyte, Inc., San Diego, CA, USA. In parallel, the gut is considered a potential source of stem cells to differentiate into endogenous insulin-secreting cells. The gut contains one of the largest populations of stem cells in the body, potentially making it a replenishable source of insulin-producing cells. The gut also contains enteroendocrine cells that express molecules involved in glucose responsiveness and processing of prohormones. Furthermore, as the pancreas and the gut both originate from the endoderm and have many differentiation pathways in common, this opens the possibility of reprogramming a patient's own gut cells to produce insulin without the need for immunosuppressants. Here, we review advances in utilizing and engineering gut cells to treat diabetes.

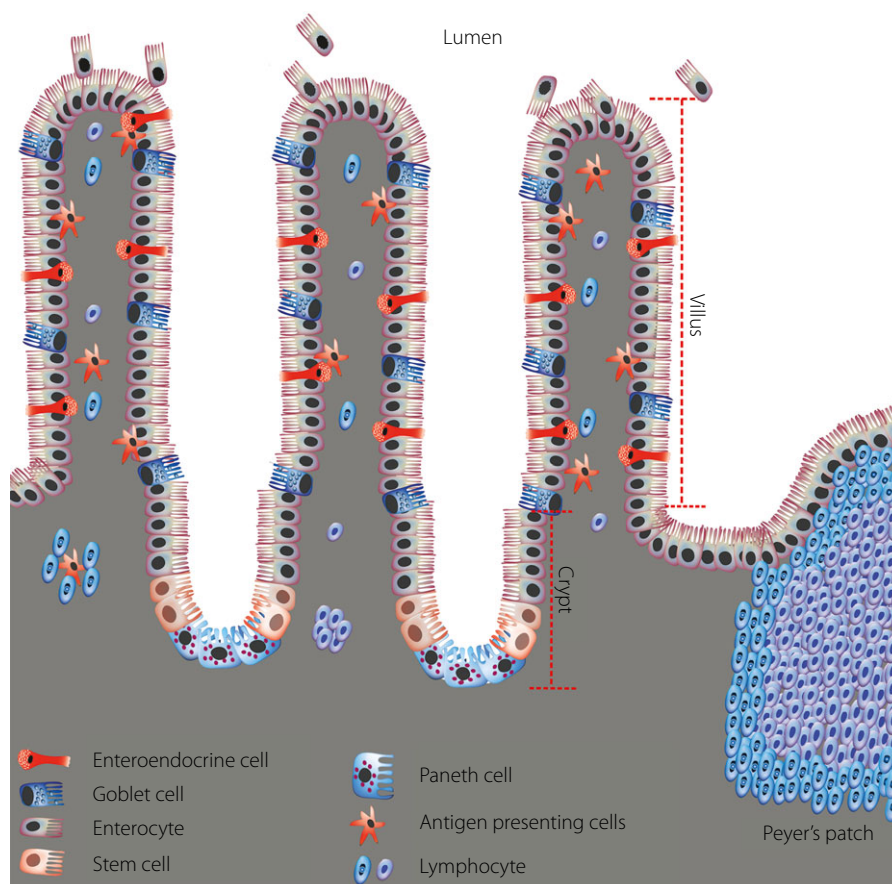
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## GUT AS A UNIQUE CANDIDATE SITE FOR INSULIN REPLACEMENT

The gut epithelium has a well-defined architecture with a high rate of cell renewal. Its epithelial layer is folded for maximal surface area in order to facilitate the efficient absorption of nutrients, with a surface area over 2,000 square feet and a length of approximately 6 m<sup>5</sup>. Epithelial cells migrate from the crypts of Lieberkühn to the villi, where they are differentiated into one of three different cell lineages: goblet cells, enterocytes and enteroendocrine cells (Figure 1). The fourth type of differentiated cell, the Paneth cell, remains at the base of the crypt and releases antimicrobial molecules into the lumen. It is believed that cells in the epithelial layer show minimal differen-

tiation, and turn over every 3–5 days by undergoing apoptosis or shedding from the tip of the villus into the gut lumen<sup>6</sup>. This amounts to an estimated turnover of approximately 100 billion cells in the adult human intestine every day<sup>6</sup>. The gut mucosa has the remarkable ability to maintain gut cell types and numbers, in addition to the ability to adjust the cell type and number to adapt to changes in diet composition. More importantly, gut stem cells can regenerate gut epithelium after acute damage, and it has been shown that an entire crypt can be regenerated from a single surviving stem cell<sup>7</sup>. The gut stem cells also have genome-protective mechanisms; that is, the ability to retain old error-free deoxyribonucleic acid template strands in times of mitosis. If any replication errors do occur in daughter cells,



**Figure 1** | Cytoarchitecture of the intestinal epithelium. The intestinal epithelial cells constitute a single cell layer separating the intestinal lumen from the underlying lamina propria (grey region). The diagram illustrates the finger-like structures of intestinal epithelium, the villi, which project into the lumen of the intestine. The epithelium at the base of the villi invaginates downward to form tube-shaped in-folds called crypts of Lieberkühn, the sites for production of new epithelial cells. The stem cell clusters lie near the base of the crypt just above the Paneth cell cluster. Transitory progenitor cells derived from stem cells migrate bidirectionally along the crypt–villus axis, with the large majority moving upward. These transitory cells divide rapidly, with an average cell-cycle time of approximately 12 h. The upward moving progenitor cells ultimately differentiate into three of the four lineages of functional epithelial cells (goblet, enteroendocrine and absorptive enterocyte cells). The time required for migration of these cells to the villus tip takes approximately 3–5 days, after which the cells are shed into the intestinal lumen. Progenitor cells that are destined to become Paneth cells migrate to the base of the crypt. The lamina propria, which lies under the epithelial layer, contains large numbers of immune cells, including lymphocytes and antigen presenting cells. The intestinal epithelial cells play an important role in the gut immune response by delivering samples of foreign antigens from the lumen of the intestine to the underlying lymphoid tissue, which include the lymphoid nodules known as Peyer's patches.

they will be short-lived, as these cells will be discarded a few days later from the villus tip<sup>8</sup>. Also, in gut stem cells, deoxyribonucleic acid repair pathways, which can be error-prone, are not active; therefore, when any deoxyribonucleic acid damage is detected, these cells undergo programmed cell death<sup>7,9</sup>. These features of gut cells are attractive in the context of gene therapy approaches, as the risk and consequences of errors would be minimized compared with other targets.

Another potentially beneficial feature of the gut for insulin replacement strategies is its vast immune system. The gut constitutes the largest source of immune cells in the body<sup>10</sup>, and under normal conditions, suppresses immune reactions against dietary antigens and commensal bacteria, a process called oral tolerance<sup>11</sup>. Exfoliation of intestinal epithelial cells into the gut lumen is also an important defense strategy against pathogens, and it is enhanced during infection to rid the body of infected enterocytes<sup>12–14</sup>. In humans, several reports suggest a link between the gut immune system and type 1 diabetes<sup>15–17</sup>. Interestingly, it has been shown that tolerance generated to an antigen in the mucosa can promote systemic tolerance to the antigen<sup>18</sup>. Exploiting this attribute could have great clinical use in the treatment of autoimmune disorders, such as type 1 diabetes. In support of this, in the non-obese diabetic (NOD) mouse, a model of human type 1 diabetes, oral delivery of insulin to the intestinal mucosa was able to prevent disease when given before the onset of autoimmunity<sup>19–22</sup>. Thus, not only could insulin produced from gut cells provide a surrogate source of insulin, but it could potentially promote immunotolerance toward insulin produced in the pancreas, and thereby reduce autoimmune attack of  $\beta$ -cells.

## CONVERTING GUT CELLS INTO INSULIN-SECRETING CELLS

Several attempts have been made to convert immature gut cells into insulin-secreting cells by taking advantage of similarities between enteroendocrine and pancreatic endocrine cell differentiation pathways. The IEC-6 (rat small intestine epithelioid) cell line<sup>23</sup> was shown to produce and secrete insulin after transfection with the  $\beta$ -cell transcription factor pancreatic and duodenal homeobox protein 1 (PDX1) after treatment with betacellulin (an epidermal growth factor) or after transplantation in diabetic rats<sup>24</sup>. A similar approach showed that overexpression of PDX1 along with islet-1 in IEC-6 cells resulted in insulin production and secretion without the need for betacellulin<sup>25</sup>. In both studies, the transformed IEC-6 cells were able to improve blood glucose levels when transplanted into rats, but they did not display glucose-regulated insulin secretion. Musculoaponeurotic fibrosarcoma oncogene homolog A (MafA), another critical transcription factor in the development of pancreatic  $\beta$ -cells that is particularly important for  $\beta$ -cell maturation and glucose-responsiveness, was overexpressed in rat intestinal cells through oral administration of an adenovirus containing the MafA gene, resulting in the differentiation of intestinal epithelial cells into insulin-producing cells<sup>26</sup>. Mice

with streptozotocin (STZ)-induced diabetes administered with this adenovirus vector had reduced hyperglycemia and increased plasma insulin levels; however, the insulin secretion was not regulated by oral glucose<sup>26</sup>. A recent study reported that the transient intestinal expression of three  $\beta$ -cell transcription factors, Pdx1, MafA and neurogenin 3 (Ngn3), promoted the conversion of intestinal crypt cells into insulin-producing endocrine cells with ultrastructural characteristics of  $\beta$ -cells. The intestinal islet-like clusters were glucose responsive and could reverse STZ-induced diabetes in mice. Interestingly, lineage-tracing strategies showed that some of the insulin-positive cells were the progeny of Ngn3+ endocrine progenitor cells<sup>27</sup>. However, this lineage-tracing study did not fully exclude the possibility that some insulin-positive cells were enteroendocrine cells<sup>27</sup>.

A series of elegant studies from the Accili group showed that Ngn3-Cre-driven knockout of the transcription factor, forkhead box O1 (FOXO1), in mice caused Ngn3+ progenitor cells in the gut to differentiate into cells that released insulin in response to glucose<sup>28</sup>. This FOXO1 ablation increased the expression of  $\beta$ -cell transcription factors including Pdx1, Ngn3, MafA and NKX6.1, as well as prohormone convertase (PC) 2. Notably, although STZ resulted in initial loss of both pancreatic  $\beta$ -cells and intestinal insulin-producing cells, the latter rapidly regenerated in the gut and reversed hyperglycemia, showing that the intestinal insulin in these animals was bioactive and the insulin-producing cells were replenishable<sup>28</sup>. The same group reported that in human gut organoids, both short hairpin ribonucleic acid-mediated inhibition of FOXO1 and transduction with an adenovirus expressing a dominant negative mutant FOXO1, promoted the generation of insulin-positive cells that released C-peptide in response to glucose<sup>29</sup>. When the adenovirus-transduced organoids were transplanted into mice, they maintained their epithelial structure and insulin-immunoreactivity; however, limitations in the amount of transplantable tissue prevented the achievement of detectable circulating human c-peptide levels<sup>29</sup>. Although these approaches are encouraging, a practical method of genetic manipulation of gut epithelium in a safe manner will be required to develop clinically relevant therapeutic strategies.

In addition to transcription factors, there is evidence that the peptide glucagon-like peptide-1<sub>(1–37)</sub> (GLP-1<sub>(1–37)</sub>), derived from the preproglucagon gene that is expressed in intestinal L cells, pancreatic  $\alpha$ -cells and discrete brainstem neurons, can also promote insulin production in intestinal epithelial cells. This was shown in cultures of fetal intestines and was only observed with the full-length peptide, GLP-1<sub>(1–37)</sub>, but not the truncated forms that are typically secreted from L cells; that is, GLP-1<sub>(7–36)</sub> and GLP-1<sub>(7–37)</sub><sup>30</sup>. The GLP-1<sub>(1–37)</sub> treated cells showed glucose-responsiveness in culture, formed islet-like structures when transplanted into the intraperitoneal space of mice and were capable of reversing diabetes<sup>30</sup>. The authors also reported that intraperitoneal injection of GLP-1<sub>(1–37)</sub> in pregnant mice promoted differentiation of neonatal intestinal epithelial progeni-

tors into insulin-producing cells. To overcome the limitations as a result of the short biological half-life of GLP-1 plus difficulties with delivery of a bioactive compound to the luminal side of the gut, a recent study used human commensal bacteria engineered to secrete GLP-1<sub>(1-37)</sub><sup>31</sup>. These bacteria were able to convert rat and human intestinal epithelial cells into insulin-secreting cells that expressed the  $\beta$ -cell markers, PDX1, MafA and forkhead box protein A2<sup>32</sup>. Diabetic rats fed the GLP-1<sub>(1-37)</sub>-secreting bacteria daily for 50 days showed higher circulating insulin levels and had improved glucose tolerance compared to rats fed wild-type bacteria. It remains to be determined whether such an approach would be effective in humans.

### TARGETING GUT ENTEROENDOCRINE CELLS

Genetically engineering gut endocrine cells to produce insulin is an intriguing approach for the treatment of diabetes. Although insulin production has been achieved in several tissues, such as muscle and the liver, the difficulty in generating meal-responsive insulin secretion from these surrogate cells limits their potential for insulin replacement therapy. Approaches that target non-endocrine cells and rely on transcriptional control of insulin production, with glucose-responsive promoter elements, will never reproduce the rapid on and off insulin secretion kinetics of  $\beta$ -cells. This issue might be overcome by using gut enteroendocrine cells that already show several features of  $\beta$ -cells. Specifically, the G-, K- and L cells, three types of enteroendocrine cell located in the epithelium of the small intestine producing gastrin, glucose-dependent insulinotropic polypeptide (GIP) and GLP-1, respectively, all carry sophisticated glucose/nutrient-sensing machinery. Insulin production in G cells was achieved in transgenic mice harboring a chimeric gene consisting of a gastrin promoter fused to the human insulin gene<sup>33</sup>. Although the kinetics of insulin secretion from these cells was not discussed<sup>33</sup>, considering that G cells are primarily responsive to protein, but not glucose, we speculate that these cells might not be adequately glucose responsive and therefore less than optimal for development as surrogate  $\beta$ -cells. Conversely, K- and L cells rapidly release GIP and GLP-1, respectively, during meal ingestion or with glucose alone, and serve to enhance insulin secretion from  $\beta$ -cells in a glucose-dependent manner. Indeed, GIP and GLP-1 have similar glucose-induced secretion patterns as insulin<sup>34</sup>, raising the possibility of using these cells as surrogates for  $\beta$ -cells to recapitulate meal-regulated insulin release. Furthermore, enteroendocrine cells contain prohormone convertases that could process proinsulin into mature bioactive insulin and, like  $\beta$ -cells, can store hormone products in secretory vesicles<sup>35-38</sup>.

The ability of L cells to serve as surrogates for  $\beta$ -cells has been studied in various GLP-1-secreting intestinal cell lines. NCI-H716 human intestinal cells were engineered to produce insulin driven by a cytomegalovirus promoter, using a recombinant adeno-associated virus; insulin and GLP-1 were co-localized in the cells and released with similar dynamics<sup>39</sup>. Murine

glucagon gene-similar virus-40 large T-antigen cells transfected with a plasmid containing the human insulin gene driven by a cytomegalovirus promoter were capable of secreting both GLP-1 and mature insulin in response to multiple secretagogues; however, the transfected cells did not increase insulin secretion when stimulated with different concentrations of glucose<sup>36</sup>. Furthermore, transplanted engineered glucagon gene-similar virus-40 large T-antigen cells did not produce enough insulin to ameliorate diabetes in mice<sup>40</sup>. Murine STC-1 cells, which produce a number of gut hormones including GLP-1, were transfected with a plasmid containing the insulin gene driven by a proglucagon promoter. Although the transduced cells produced insulin, little in the way of glucose-responsive insulin secretion was observed<sup>41</sup>. Studies with primary L cells will be required to determine if this cell population can produce insulin in a sufficiently robust glucose-dependent manner to appropriately regulate blood glucose levels.

Compared with GLP-1 secretion levels from L cells, K cells produce higher levels of GIP under both basal and stimulated conditions<sup>42</sup>. Therefore, our group rationalized that K cells might be more suitable surrogates for  $\beta$ -cells. We and others evaluated the feasibility of using these cells both *in vitro*, using a K cell line, and *in vivo*, using various transgenic mouse models<sup>43-45</sup>. Moderately glucose-responsive production of insulin was obtained from GIP-expressing murine GTC-1 cells (a derivative of STC-1 cells) after transfection with a transgene comprised of a rat GIP promoter upstream of human preproinsulin<sup>43</sup>. In another study, STC-1 cells engineered to produce mouse insulin reversed diabetes after transplant under the kidney capsule in diabetic immunodeficient mice<sup>45</sup>. However, by 4 weeks post-transplantation, the mice developed progressive hypoglycemia, possibly as a result of overgrowth of the tumoral STC-1 cells<sup>45</sup>. Another study with STC-1 cells producing human insulin also reversed diabetes in mice by 3 weeks post-transplant, but longer-term tracking was not reported<sup>46</sup>. We generated a regulatable cell-based system using GTC-1 cells in which transcriptional control of insulin was inducible by mifepristone in a dose-dependent manner<sup>47</sup>. When transplanted into mice, these cells were able to ameliorate STZ-induced diabetes. Unfortunately, most transformed cell lines do not match the tightly regulated hormone secretion of enteroendocrine cells. Therefore, it is difficult to extrapolate these findings to engineered native K cells.

To explore the utility of endogenous K cells for insulin production, and avoid the issues encountered with transformed cell lines, our group generated transgenic mice in which human preproinsulin is coexpressed with GIP in gut K cells, and is secreted into the circulation in a meal-dependent manner<sup>43</sup>. These mice were protected from developing STZ-induced diabetes and maintained normal glucose tolerance in the absence of exogenous insulin<sup>43,44</sup>. To assess whether insulin-producing K cells would be subjected to autoimmune attack similar to that which targets  $\beta$ -cells in type 1 diabetes, we generated transgenic mice expressing murine insulin in K cells in the NOD mouse model

of autoimmune diabetes. These transgenic mice showed high levels of circulating insulin immunoreactivity, but normal insulin sensitivity, bodyweight and glucose tolerance. Notably, diabetes incidence was significantly reduced in these mice. We have also observed that these K cells not only escape immune destruction, but that K cell insulin production appears to reduce the expected assault on pancreatic  $\beta$ -cells in NOD mice, likely through the induction of immune tolerance to insulin<sup>44</sup>.

Our transgenic mice with insulin producing K cells showed high levels of insulin and proinsulin immunoreactivity, yet C-peptide levels were comparable with non-transgenic littermates<sup>44</sup>. Further evaluation of the insulin produced by K cells, using insulin western blotting of intestinal lysates, suggested that the majority of the insulin immunoreactivity was proinsulin, although mature insulin was also produced (unpubl. data). Thus, K cell insulin production can improve glucose regulation in NOD mice, even if only a portion of the proinsulin produced is fully processed into mature insulin. PC1/3 and PC2 are important for efficient processing of proinsulin into mature insulin in mice<sup>48,49</sup>. While mouse  $\beta$ -cells express both PC1/3 and PC2, most K cells express PC1/3, but not PC2<sup>35</sup>. This likely explains the higher proinsulin relative to mature insulin production in our transgenic mice that express insulin in K cells. To circumvent this less than optimal insulin processing, we are currently assessing the impact of modification of the proinsulin gene in our transgene to promote more efficient proinsulin processing by PC1/3 alone in K cells.

## CONCLUSIONS

Patients with type 1 diabetes are dependent on multiple daily injections of insulin to survive. Even when blood glucose levels are closely monitored, and insulin doses are carefully coordinated with meals and physical activity, ideal glucose levels are seldom achievable. Therefore, restoring appropriate endogenous insulin production is a desirable goal. One approach is to utilize a patient's own gut cells to reestablish nutrient-regulated insulin secretion. To exploit the potential of the gut to either reprogram its stem cells into  $\beta$ -cells, or engineer enteroendocrine cells, such as K cells, into insulin-producing cells, advancements in the development of safe and efficient genetic modification approaches are required. The possibility that insulin production by the gut could also promote systemic immune tolerance to insulin is a potentially significant benefit to this tactic. Numerous human gene therapy clinical trials are currently in progress to inform the selection of a clinically acceptable method for gene delivery or gene silencing to the gut cells. However, further investigation and refinements are required, as any clinical approach targeting the intestines for insulin production would need to safeguard against unregulated overexpression of insulin and unintended downregulation of other important cellular products. The burgeoning diabetes population and promising advances in this field warrant continued investments and efforts to explore the utility of engineered gut cells in the treatment of diabetes.

## DISCLOSURE

TJK is a cofounder and shareholder of enGene, Inc. (Montreal, Canada), a biotechnology company developing gene delivery to gastrointestinal mucosal cells for the production of therapeutic proteins. The other authors declare no conflict of interest.

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