Pharmacological conversion of gut epithelial cells into insulin-producing cells lowers glycemia in diabetic animals

Wen Du, …, Sandro Belvedere, Domenico Accili


Graphical abstract

Find the latest version:

https://jci.me/162720/pdf
Pharmacological conversion of gut epithelial cells into insulin-producing cells lowers glycemia in diabetic animals

Wen Du¹,*, Junqiang Wang², Taiyi Kuo¹,³, Liheng Wang¹, Wendy M. McKimpson¹, Jinsook Son¹, Hitoshi Watanabe¹, Takumi Kitamoto¹, YunKyoung Lee⁴, Remi J. Creusot¹, Lloyd E. Ratner⁵, Kasi McCune⁵, Ya-Wen Chen⁶,⁷,⁸, Brendan H. Grubbs⁹, Matthew E. Thornton⁹, Jason Fan¹⁰, Nishat Sultana¹, Bryan Diaz¹, Iyshwarya Balasubramanian¹¹, Nan Gao¹¹, Sandro Belvedere⁴, Domenico Accili¹

¹Department of Medicine and Naomi Berrie Diabetes Center, and
²Systems Biology Institute, Vagelos College of Physicians and Surgeons, Columbia University, New York, NY, 10032
³Department of Neurobiology, Physiology, & Behavior, College of Biological Sciences, University of California, Davis, CA, 95616
⁴Forkhead BioTherapeutics Corp., New York, NY, 10032
⁵Department of Surgery, Columbia University Medical Center, New York, NY 10032
⁶Department of Otolaryngology, ⁷Department of Cell, Developmental, and Regenerative Biology and ⁸Black Family Stem Cell Institute, Icahn School of Medicine at Mount Sinai, New York, NY, 10029
⁹Department of Obstetrics and Gynecology, Keck School of Medicine, University of Southern California, Los Angeles, CA, 90033
¹⁰Bascom Palmer Eye Institute, Department of Ophthalmology, Miami, FL, 33136
¹¹Department of Biological Sciences, Rutgers University, Newark, NJ, 07102

*Indicates correspondence authorship.

Short title: Pharmacological conversion of gut epithelial cells into insulin-producing cells

Key words: Cell conversion; Diabetes; Intestinal epithelial lineage; Intestinal organoids; Insulin

Address correspondence to: Domenico Accili or Wen Du, Department of Medicine and Naomi Berrie Diabetes Center, Vagelos College of Physicians and Surgeons, Columbia University, 1150 ST Nicholas AVE, R234, New York, NY, 10032. Phone: 01. 212.851.5333;

Email: da230@cumc.columbia.edu (DA) and wd2294@cumc.columbia.edu (WD)
1 Conflict-of-interest statement

2 DA was a founder, director, and chair of the advisory board of Forkhead Biotherapeutics. Y.L. and S.B.

3 performed this work as employees of Forkhead Biotherapeutics.
Abstract

As a highly regenerative organ, the intestine is a promising source for cellular reprogramming to replace lost pancreatic β-cells in diabetes. Gut enterochromaffin cells can be converted to insulin-producing cells by FoxO1 ablation, but their numbers are limited. In this study we report that insulin-immunoreactive cells with Paneth/goblet cell features are present in human fetal intestine. Accordingly, lineage tracing experiments show that, upon genetic or pharmacologic FoxO1 ablation the Paneth/goblet lineage can also undergo conversion to the insulin lineage. We designed a screening platform in gut organoids to accurately quantitate β-like cell reprogramming and fine-tune a combination treatment to increase the efficiency of the conversion process in mice and human adult intestinal organoids. We identified a triple blockade of FOXO1, Notch, and TGFβ that, when tested in insulin-deficient STZ or NOD diabetic animals resulted in near-normalization of glucose levels, associated with the generation of intestinal insulin-producing cells. The findings illustrate a therapeutic approach to replace insulin treatment in diabetes.
Introduction

The Center for Disease Control (CDC) estimates that 1.6 million adults and 283,000 children and adolescents have type 1 diabetes in the US alone (https://www.cdc.gov/diabetes/data/statistics-report/diagnosed-diabetes.html). Insulin replacement is a life-saving treatment, but it is not a cure and poses a significant burden on patients and their families. Restoration of endogenous insulin production to cure T1D remains a topic of intense interest (1). Two alternative approaches have been proposed: transplantation and regeneration.

As early as the 1970s, isolated cadaveric pancreatic islets have been transplanted into T1D recipients to treat diabetes (2). Recent developments in stem cell technologies enabled human embryonic stem cells (hESCs)- or induced pluripotent stem cells (iPSCs)-derived islet replacement (3-5). Mature glucose-responsive β-like cells that are functionally equivalent to cadaveric islets can be obtained by different protocols (6-8). Two studies reporting interim data from ongoing first-in-human iPSCs-based transplants showed restoration of meal-induced C-peptide response for up to 1 year in one patient after implantation of iPSCs-derived islet cells, providing proof-of-concept for this approach (9, 10).

Another potential solution is to convert cell types developmentally related to pancreatic β-cells into functional insulin-secreting cells in vivo. However, despite a wealth of reports illustrating conversion of different cell types into β-like cells, poor reproducibility has plagued this area, as well as the intrinsic difficulty of targeting this process pharmacologically (11, 12). Although most studies focused on conversion of different pancreatic cell types, there are reports of trans-differentiation from organs developmentally related to the pancreas, such as liver (13, 14), stomach (15), and intestine (16).

Genetic ablation of FoxO1 in Neurog3+ progenitor cells can convert enteroendocrine cells (EEC) into insulin-producing β-like cells in mice (17). Moreover, FOXO1 inhibition using a dominant-negative
mutant or lentivirus-encoded small hairpin RNA promotes generation of insulin-secreting cells in human iPSC-derived gut organoids (18). The potential therapeutic significance of this work was amplified by recent reports: one identifying β-like cells in the human fetal intestine—and thus implying that conversion restores a fetal cell type (19); and the others showing that previously described small molecule FOXO1 inhibitors can yield insulin-producing cells in vivo and lower glycemia in diabetic mice (20, 21). These findings prompted us to investigate whether other descendents of Neurog3+ progenitors, such as subsets of goblet and Paneth cells have the potential to be converted into insulin-secreting β-like cells. Based on the identification of cells with mixed lineage of insulin and Paneth/goblet features in human fetal intestines, we developed cellular assays to accurately quantitate cell reprogramming and sought to identify a combination treatment to increase the efficiency of the conversion process by leveraging the expansion of the Neurogenin3 and Paneth/goblet lineages. We found that triple blockade of FOXO1, Notch, and TGFβ can bolster conversion and result in a robust glucose-lowering effect in streptozotocin and NOD diabetic animals. Our findings provide a mechanism underlying intestinal cell trans-differentiation into pancreatic β-like cells and expand its potential therapeutic applications.
Results

A subset of fetal insulin-positive intestinal secretory lineage cells

Pancreas and small intestine share a common endodermal origin. Recent studies show that enteroendocrine K/L cells express insulin during fetal, but not post-natal life (19). These data provide a plausible developmental explanation for the observation that FoxO1 deletion in Neurog3+ endocrine progenitors generates gut β-like, insulin-secreting cells in a cell-autonomous manner (17), since FoxO1 is generally activated upon terminal differentiation in a variety of cell types (22, 23). We tested the relationship between FOXO1 expression/activity and insulin immunoreactivity in the human fetal intestine by analyzing whole rolls of small intestine from one 15-week gestational age (GA) subject, two 17-week GA subjects, and one 19-week GA subject. Combined immunohistochemistry and in situ hybridization detected cells co-expressing insulin mRNA and protein in fetal human intestine at 15-17 weeks GA (Fig. 1A, B), but barely in 19 weeks GA (especially in the villus tip pattern). Interestingly, only one third of cells expressing INS-mRNA also expressed the insulin protein, consistent with the possibility that fetal intestinal insulin expression is transient (Fig. 1B), likely restricted to the early second trimester. Immunostaining also revealed co-reactivity with intestinal secretory cell type markers 5HT (enterochromaffin), lysozyme (Paneth), and GLP-1 (EEC K/L cell) (Fig. 1C). Double-positive 5HT/insulin and lysozyme/insulin cells were detected more frequently at the tip of villi in the proximal small intestine, whereas double-positive GLP-1/insulin cells were mainly located in the distal region. No insulin protein and RNA positive cells were found in adult human intestine biopsies (Fig. 1D, Fig. S1A). INS antibody specificity was tested using negative and positive control samples (Fig. S1B). Co-staining with FOXO1 indicated that most insulin-positive cells did not express FOXO1, consistent with the possibility that FoxO1 ablation in rodents or organoids recapitulates a developmental stage in human fetal intestine (Fig. 1E, F).
Separate subsets of Neurog3 lineage yield intestinal β-like cells

The discovery of cells with mixed insulin/Paneth/goblet features is consistent with the notion that different cell types arise from Neurog3⁺ progenitors: EEC, goblet, and Paneth cells (Fig. S4A) (24).

Therefore, we asked whether the latter two subtypes also give rise to gut β-like cells, because this finding would greatly expand the repertoire of target cells for conversion to β-like cells. To answer this question, we used a two-step enrichment procedure of in vivo lineage tracing with Neurog3Cre-FoxO1 /I; Rosa26\textsuperscript{tdTomato} mice to label FoxO1 knockout cells derived from Neurog3 progenitors (NFKO), followed by CD24 immunostaining to distinguish among EEC, goblet, and Paneth cells (Fig. S2) (25). FoxO1 expression was significantly reduced in sorted Neurog3⁺-derived Tomato⁺ cells from NFKO mice, while FoxO3 and FoxO4 were unchanged (Fig. S3). Quantitative flow cytometry analysis (FACS) revealed that NFKO increased Neurog3⁺-derived cells ~1.7 fold, from 1.54% to 2.65% (P<0.0001) (Fig. 2A, B). Single-cell RNAseq showed an expansion of the EEC and goblet/Paneth lineages among Neurog3 daughter cells of NFKO mice (Fig. S4A, B). Interestingly, both subpopulations included insulin-immunoreactive cells (Fig. 2C). CD24 staining allowed us to subdivide Neurog3⁺-derived (Tomato⁺) cells into two distinct populations: CD24\textsuperscript{neg}/Tomato⁺ and CD24⁺/Tomato⁺ (Fig. 2D). CD24⁺ cells included both Paneth and 5HT cells (Fig. S2). QPCR data demonstrated that CD24\textsuperscript{neg}/Tomato⁺ cells from NFKO mice were highly enriched in Ins1 and Ins2 mRNA (500- to 1,000-fold), while CD24⁺/Tomato⁺ cells showed a more limited 10- to 30-fold enrichment (Fig. 2E). Consistently, insulin-immunoreactive cells showed weak or absent CD24 membrane staining (Fig. 2F). Notably, gene-set enrichment analysis (GSEA) of bulk RNAseq data showed increased pancreatic β-cell- and protein secretion-related transcripts in the CD24\textsuperscript{neg} Tomato⁺ population (Fig. 2G, H). The morphology and gene expression profiles of these cells was consistent with a dual origin from EEC and Paneth/goblet cells (Fig. 2C, F). Besides Paneth/goblet lineage markers (Spink4, Defa24, Muc2, Lyz1) and EEC lineage markers (Chga, Gcg, Tph1, Cck, Pyy), we detected quiescent stem cells markers HOPX and Olfm4 in the insulin-immunoreactive population (Fig. S4C, D).
Moreover, CytoTRACE analysis (26) showed that the insulin-immunoreactive population represents a less differentiated cell state compared with insulin-negative cells from NFKO mice (Fig. S4E), supporting the notion that FoxO1 ablation in Neurog3-derived cells brings about a fetal-like stage.

**Lineage tracing identifies a dual source of β-like cells following FoxO1 ablation**

The presence of two distinct cells subtypes with β-like features raised the possibility that other intestinal cell types can be converted to insulin-immunoreactive cells by FoxO1 ablation. To critically test this hypothesis, we assessed generation of β-like cells by tracing the 5HT lineage using primary organoids from Tph1Cre<sup>ERT2/+; Rosa26<sup>tdTomato</sup> mice to identify EEC-derived β-like cells, and the goblet/Paneth lineage using Lyz1Cre<sup>Er/+; Rosa26<sup>tdTomato</sup> organoids. After inducing Tph1 reporter-dependent gene activation with 4-OH-TAM, we enriched organoids in EEC by incubating them in medium containing inhibitors of Notch, WNT, and MEK (DAPT, IWP2, and PD0325901, respectively) (27). To induce conversion into β-like cells, we added the chemical FOXO1 inhibitor AS1708727 (AS) (28, 29). QPCR analysis showed significant increases of Ins1, Ins2, and Tph1 mRNA after incubation in EEC medium with or without AS (Fig. 3A). We found ~11% 5HT cells by immunostaining and flow cytometry (Fig. 3B, C). Treatment with AS increased the percentage of 5HT cells 1.5-fold (Fig. 3C). Pulse-chase labelling also showed that after 4-OH-TAM treatment, insulin-positive cells colocalized with newly generated 5HT cells (Tomato<sup>+</sup> cells) regardless of whether organoids had been subjected to the EEC differentiation protocol (Fig. 3D, Fig. S5). Induction of 5HT-positive cells was also evident in EEC-enriched human gut organoids (hGO) (Fig. 3E, F). In sorted 5HT-positive cells from hGOs, insulin mRNA was induced ~ six-fold by incubation in EEC medium, and 30-fold by addition of another FOXO1 inhibitor, FBT10 (Fig. 3G) (30, 31). These data are consistent with the hypothesis that FOXO1 inhibition facilitates conversion of 5HT cells into β-like cells.

Next, we performed similar experiments in organoids derived from Lyz1Cre<sup>Er/+; Rosa26<sup>tdTomato</sup> mice to label goblet/Paneth cells, followed by induction of these two interrelated lineages (Fig. 3H). We
optimized chemical induction of the Paneth/goblet lineage by different combinations of the GSK3β inhibitor Chir99021, Notch inhibitor DAPT, and TGFβ inhibitor Repsox (32, 33). QPCR data showed that single Notch inhibition enriched all secretory cell markers, such as Neurog3, Tph1, Lyz1 and Muc2; this effect was strengthened by the TGFβ inhibitor. In contrast, addition of the GSK3β inhibitor increased Neurog3 and Lyz1 but decreased Tph1 and Muc2, consistent with the possibility that this combination promotes an earlier stage of EEC differentiation. Single treatment with either TGFβ or GSK3β inhibitor had no effect (Fig. 3H-K). The triple combination of Notch, TGFβ, and GSK3β inhibitors resulted in a 10-fold induction of Ins1 and Ins2 mRNA along with EEC progenitor markers, indicating a trend toward the β-cell-like phenotype. The effect of the triple blockade was amplified by adding the FOXO1 inhibitor AS, with both Ins1 and Ins2 mRNA expression increasing by 20- and 14-fold, respectively (Fig. 3L). Adding AS also strengthen the effect of the dual Notch/TGFβ or Notch/GSK3β blockade (Fig. 3L). To confirm the origin of β-like cells, we performed immunohistochemistry and found that insulin colocalized with LYZ1-tomato cells, indicating that β-like cells can also arise from Paneth/goblet cells (Fig. 3M).

**Newly developed FOXO1 inhibitors increase β-like cell conversion**

Based on these data, we sought to increase the efficiency of generating β-like cells using a modified secretory cell conversion protocol combined with FOXO1 inhibition in mouse intestinal organoids (34) (Fig. 4A). TGFβ inhibition resulted in strong induction of Ins1 and Ins2; the latter was further increased ~ twofold by the FOXO1 inhibitor, AS. Notch inhibition had a stronger effect on Ins1 than on Ins2, and addition of AS increased both. Addition of the Notch inhibitor to the TGFβ blockade, either in the presence or absence of AS, had no effect, indicating that Notch is epistatic to TGFβ in the pathway leading to β-like cell conversion (Fig. 4B, Fig. S6). Measurements of insulin content in organoid extracts are consistent with the mRNA findings and show that FOXO1 inhibition increased the amount of insulin recovered after single TGFβ or dual Notch/TGFβ blockade (Fig. 4C).
Next, we set up quantitative assays with cultured gut organoids derived from mice bearing a Rip-Cre; Rosa26tdTomato reporter allele to evaluate the efficiency of β-like cell conversion. In this assay, we used FACS to separate and quantitate cells in which Ins2 expression had been activated, as indicated by the Tomato reporter, from non-insulin-expressing epithelial cells. In a typical experiment, about 14% of cultured organoid cells demonstrated onset of red fluorescence following combined TGFβ/FOXO1 inhibition (Fig. 4D). Using this screening platform, we validated several new novel FOXO1 inhibitors (FBT) based on their potency in reporter promoter assays (20). We selected two compounds, FBT10 and FBT374, that outperformed AS in conversion frequency to β-like cells and Ins2 expression (Fig. 4G, H). Using Tomato+ (β-like) cells isolated by FACS, we compared expression of β-cell-specific genes between converted INS2-Tom+ β-like cells vs. INS2-Tom−cells. RNAseq showed that organoid-derived insulin-immunoreactive cells express pancreatic β-cell markers, including Nkx6.2, MafA, Pcsk2, and Abcc8 (Fig. 4F), while also retaining some intestinal epithelial markers (Fig. S7). GSEA of KEGG pathway revealed that Ins2-Tom+ cells were highly enriched in genes related to biological processes in Maturity-Onset Diabetes of the Young and Type 2 diabetes (Fig. 4E). Thus, intestinal β-like-cells obtained by FOXO1 inhibition share a common molecular signature with islet β-cells.

Inhibition of Notch and TGFβ in FoxO1-deficient mice increases Neurog3+ and β-like cells

Next, we tested whether triple blockade of Notch, TGFβ and FOXO1 can induce cell conversion in vivo. To this end, we combined genetic FoxO1 ablation in Neurog3Cre-FoxO1f/f; Rosa26tdTomato mice with pharmacological treatment with Repsox and a different Notch inhibitor, the γ-secretase inhibitor, DBZ. We injected DBZ for the first 2 days, followed by 5 days of Repsox oral dosing (21, 35) (Fig. 5A). Immunohistochemistry and quantitative FACS showed that DBZ increased Neurog3-derived cells by ~2.5-fold, and the DBZ/Repsox combination by 7-fold, to account for ~15% of all live duodenal epithelial cells. Repsox had no effect by itself (Fig. 5B-C, Fig. S8A). The treatment had similar effects on the
percentage of 5HT cells, consistent with data in cultured organoids (S8B-C). These data show that triple
inhibition expanded the EEC lineage, a necessary condition for β-like conversion of a subset of cells.

To test whether these treatments resulted in the formation of functional gut β-like cells, we
rendered the animals diabetic with STZ and measured the effects of the various interventions on fasting
glucose, glucose tolerance, plasma insulin, and generation of insulin-immunoreactive intestinal cells (Fig.
5D). FoxO1 ablation resulted in lower fasting glycemia and improved GTT after STZ administration,
consistent with prior observations (17). After a 6-day course of dual inhibition with Repsox and DBZ in
FoxO1 knockout mice plasma insulin levels increased following 4-hr-fast or 1-hr-refeeding (Fig. 5E, F). In
oral glucose tolerance tests, this treatment yielded the largest improvement compared with vehicle or
any single treatment, or dual Repsox and DBZ inhibition in WT controls (Fig. 5G-I). Dual inhibition
increased 5HT and goblet cell population while decreasing the Paneth cell marker lysozyme (Fig S9).
Immunohistochemistry revealed abundant insulin-immunoreactive cells within intestinal crypts, and
colocalization with S10T and lysozyme/MUC2 (Fig. 5J) as well as pancreatic β cells markers, PC2, MAFA ,
and SUR1 (Fig S12), suggesting that these cells undergo conversion to β-like cells. Moreover, the various
treatments had no effects on residual endocrine pancreas β-cells (Fig. S10A, B), total pancreatic insulin
content (Fig. S10C) and proliferating β-cells as assessed by Ki-67 staining (Fig. S10D). DBZ alone or
combined with Repsox did similarly enhance circulating GLP1 levels (Fig. S11A, B), as well as the number
of GLP1- and GIP-expressing cells in the small intestine (Fig. S11C-F). As these treatments had no effect
on plasma insulin and pancreatic insulin content was anyway negligible, the increased plasma insulin
and lower glycemia seen Repsox/DBZ-treated FoxO1 knockout mice should be attributed to the
induction of intestinal insulin-positive cells rather than expansion of other EEC lineages. Furthermore,
we performed glucose- and KCl-induced insulin secretion assays from gut isolated from Repsox/DBZ-
treated FoxO1 knockouts and found that these intestinal β-like cells release insulin in response to
secretagogues (Fig 5K, L). Taken together, these data indicate that combined FOXO1, Notch, and TGFβ
Du et al, Pharmacological conversion of gut epithelial cells into insulin-producing cells

inhibition increases the efficiency of cell conversion in vivo and is associated with a commensurate glucose-lowering effect in diabetic animals.

**Triple combination therapy lower blood glucose and induces gut β-like cells in NOD mice**

To evaluate the translational value of this triple combination therapy in an autoimmune model of diabetes, we used Repsox and the gamma secretase inhibitor PF-03084014, currently in Phase II for the treatment of different forms of cancer (36), in combination with the chemical FOXO1 inhibitor, FBT10 (20, 21). 5 days of oral administration with FBT10, PF, and Repsox only slightly decreased body weight (Fig 6B), with a significant increase of plasma insulin and GLP1 levels (Fig 6C, D). Triple combination therapy decreased 4h fasting blood glucose levels by 400mg/dl, and nearly normalized OGTT compared with vehicle-treated controls (Fig 6E, F). Immunohistochemistry confirmed the presence of β-like cells in the intestine of the treatment group, partly co-immunoreactive with 5HT or lysozyme/MUC2, but not in the vehicle group (Fig. 6G and Fig. S13A). There were no differences in residual islet β-cells, and both groups showed evidence of islet immune cell infiltration (Fig. S13B).

**Combination treatment of human gut organoids induces insulin+ cells**

We finally determined the effects of triple chemical blockade of Notch, TGFβ and FOXO1 using primary human duodenal organoids (Fig. 7A). QPCR analysis indicated a remarkable induction of insulin and CD49a, a membrane marker of human stem cell-derived β-cells, by the triple combination treatment (7)(Fig. 7B). Measurements of insulin content and C-peptide immunohistochemistry confirmed these findings (Fig. 7C, D). β-like cell generated from human gut organoids showed insulin secretion. Interestingly, similar to ES-derived β-like cells, they failed to clearly respond to high glucose or KCl (Fig 7E-G). This likely reflects an immature stage due to the short course of the differentiation experiment.
Discussion

Pancreas and intestine share a common developmental origin, and their endocrine compartments share a common progenitor as well as several terminally differentiated cell types, such as SST- and Ghrelin-producing cells. Other cells, for example α- and K/L cells, give rise to alternatively spliced products of the same pre-proglucagon gene in the two organs (37). Insulin-producing cells are however restricted to the pancreas. We were therefore quite surprised when, a decade ago, we observed that genetic ablation of FoxO1 in endocrine progenitors resulted in the generation of intestinal cells with highly differentiated β-like cell features normally only found in pancreatic islets. Three subsequent pieces of evidence clarified this finding. First, Stanger and Zhou independently replicated these findings using a forced expression approach with NEUROG3, PDX1, and MAFA (15, 16), confirming the potential of the gut to undergo this conversion. Next, we showed that FOXO1 inhibition appeared to reprogram enterochromaffin 5HT cells into β-like cells in human gut organoids (18). And the Melton laboratory reported that enterochromaffin-like cells are a “byproduct” of stem cell differentiation into β-cells, including expression of genes related to 5HT biosynthesis. The similarities between these two cell populations suggest that there is a relationship between enterochromaffin and β-cell fates (7). This, in turn, dovetails with the notion that pancreatic β-cells synthesize 5HT (38). Finally, the recent description of bona fide insulin-producing cells in the fetal human gut suggests that FOXO1 ablation is arresting Neurog3 progenitor differentiation at a fetal-like stage (19), providing a plausible underpinning as well as unifying mechanism for these disparate observations.

One unexplained feature of these findings was that other secretory lineage cells also arise from NEUROG3 progenitors (24). In this regard, it is noteworthy that the non-canonical WNT/planar cell polarity pathway, which controls islet β-cell functional heterogeneity, primes intestinal stem cells toward the EEC or Paneth lineages (39). In this work, we provide direct lineage tracing evidence that
secretory cells in the non-endocrine (Paneth and goblet) fate can also be converted to intestinal β-like cells. In addition, expanding on a recent communication (19), we show that in human fetal intestine of 15- to 17-week gestational age, insulin-immunoreactive cells also colocalize with goblet/Paneth lineage markers but exclude active FOXO1, lending further support to the notion that FOXO1-inactive cells can be converted to β-like cells. These findings address the question of which type of cell can be converted into insulin-immunoreactive β-like cells, extending previous observations (17, 18, 21).

The data above raised the possibility that leveraging additional signaling pathways can modulate the conversion process in synergy with FOXO1. TGFβ, WNT, FGF, Notch, BMP, and FOXO1, along with relevant receptors and signaling pathways, are involved in pancreatic and intestinal tissue patterning (40, 41). FOXO1 and Notch signaling interact in determining intestinal stem cell differentiation into Paneth/goblet (42) and EEC lineages (19, 21, 43). Thus, we combined genetic FoxO1 knockout with pharmacological Notch inhibition (DBZ) to show that dual Notch/FOXO1 inhibition expands the Neurog3+ progenitor pool and its secretory lineage cell descendants. Moreover, adding the TGFβ inhibitor Repsox further increased the Neurog3+ lineage, indicating a synergistic effect on endocrine induction, as observed during the derivation of β-like cells from stem cells (4). Interestingly, TGFβ inhibition in the combination treatment decreased expression of the Paneth cell marker lysozyme, but increases the EC marker, 5HT, and the goblet marker, MUC2, indicating that the two pathways affect sub-lineage specification. Insulin expression levels in reprogrammed gut organoids are lower than islets, suggesting that β-cell maturation is incomplete, similar to ES-derived β-like cells.

Numerous studies tracking the fate of Paneth, goblet, tuft, and EECs have shown that lineage-committed cells are capable of dedifferentiating into multipotent ISCs during gut regeneration (44-46). Dedifferentiated cells can adopt an alternate cell fate upon injury or perturbation of the intestinal epithelium. The molecular mechanisms driving cellular reprogramming remain to be elucidated. Our
findings strengthen the notion that FOXO1 participates in intestinal secretory lineage trans-
differentiation, similar to its role in pancreatic β-cells (47, 48). Single cell RNAseq of FoxO1-ablated cells
also shows the reemergence of HOXP- and OLFM4-positive cells along with β-like cells, suggesting that
committed secretory cells can revert to a stem- or fetal-like stage as a path to differentiate into β-like
cells. This process too bears similarities with the role of FoxO1 in pancreatic islets (17).

Combination treatment had a glucose-lowering effect in mice, adding to an emerging body of
evidence that pharmacological FOXO1 inhibition is a viable option for β-cell replacement. Although our
focus was to probe the mechanistic underpinning of the conversion, the potential use of combination
treatment as an alternative to insulin injections or cell transplant should be considered. Most modern
treatments leverage detailed knowledge of signaling pathways to target disease processes as diverse as
different types of cancer or immune disorders with combination approaches.

In summary, we characterized insulin-secreting β-like cells using genetic and pharmacologic
models of signaling perturbations. Based on this insight, we developed a robust combination treatment
to generate β-like gut cells in mice and cultured human enteroids. The discovery of similar cells in the
human fetal intestine (19) raises the question of whether these manipulations restore a fetal-like cell
type. In addition to providing developmental and mechanistic insight into this process, our findings
expand potential therapeutic options for insulin replacement.
Du et al, Pharmacological conversion of gut epithelial cells into insulin-producing cells

1 **Materials and Methods:**

2 Please refer to the Supplementary Material for comprehensive details.

3 **Animal studies**

4 Mouse strain information is shown in Table S1. A single high-dose injection of STZ (170 mg/kg, Sigma) was administrated intraperitoneally to induce diabetes in 6- to 8-week-old male NFKO and littermate male FoxO1/−/− (WT) mice. Mice that were not hyperglycemic within one week were excluded from further study. Blood glucose were monitored a least twice per week of 11-12 week old NOD female mice, the treatment begin immediately after blood glucose level consistently above 250 mg/dl.

5 For in vivo drug treatment, STZ mice were injected intraperitoneally with 25mg/kg DBZ q.d. for 2 days and/or gavaged with 10mg/kg Repsox q.d. for 5 to 7 days. For NOD mice in vivo drug treatment, FBT10, PF-03084014 and Repsox were dosed orally twice daily at 50 mg/kg/dose, 150 mg/kg/dose and 10mg/kg/dose, respectively. In the fasting-refeeding study, mice were fasted for 4 hours followed by 1 hour refeed. In oral GTT, mice were fasted for 4 hours followed by gavaging of 2 g/kg of D-glucose (Sigma). Blood glucose was measured at 0, 15, 30, 60 and 120 min. Blood was collected from tail vein with DPP4 inhibitor and plasma insulin or GLP-1 were measured by insulin ELISA kit (Mercodia) or GLP-1 ELISA Kit (Crystal Chem).

6 **Human Tissues**

7 Intestinal tissues or endoscopic biopsy were obtained from 8 patients from the Columbia University Irving Medical Center and Presbyterian Hospital and Vanderbilt Clinic. The Columbia University, University of Southern California, and Children’s Hospital Los Angeles Institutional Review Boards have approved all procedures and collection of human fetal tissue samples. All samples were deidentified, and the only clinical information collected was gestational age and additional fetal diagnoses. Intestinal samples ranging in age from 15 to 19 weeks of gestation were received immediately after elective
 terminations and fixed in 4% paraformaldehyde, dehydrated with 30% sucrose, processed for OCT embedding, followed by sectioning and immunostaining.

Chemicals

All small molecule information for intestinal treatment is listed in Table S2. DBZ was from Apexbio Technology; RepSox and PF-03084014 were from Selleck Chemical; FBT10 from ForkheadBio Therapeutics. For STZ mice in vivo treatment, DBZ and Repsox were formulated in 1% DMSO, 0.5% methylcellulose and 0.2% Tween-80 PBS solution, respectively. For NOD mice in vivo treatment, FBT10, PF-03084014 and Repsox was formulated together into N,N-Dimethylacetamide: Solutol HS 15: water= 5:10:85 (v/v/v) solution, pH4-5.

Gut Organoid Cultures

For mouse small intestinal organoids, crypts were isolated using EDTA chelation from the duodenum and cultured as described (49). For human small intestinal organoids, crypts were isolated using EDTA chelation from the duodenum as previously described (50). IntestiCult™ Organoid Growth Medium (Human) or IntestiCult™ Organoid Differentiation Medium (Human) were used for culture or differentiation of human gut organoids (STEMCELL Technologies). Organoids were used prior to passage 3 for optimal efficiency of EEC and β-like cell induction.

Intestinal epithelial cell isolation and sorting

4- to 6-week-old NFKO mice were used to isolate single intestinal cell preparations as described (51). Attached pancreata were removed under a dissection microscope to avoid pancreatic β-cell contamination. Isolated intestinal epithelial cells were stained for 20 min with APC-conjugated anti-CD24 antibody and FITC-conjugated anti-Epcam antibody (Biolegend) prior to sorting using BD Influx.

Flow cytometric analysis of epithelial cells

Single cell suspension was obtained by enzymatic digestion of intestinal mucosa or cultured organoids (51, 52). Suspended cells were first stained with live/dead cell staining kit (Invitrogen), then fixed in BD
Cytofix™ fixation buffer. Cells were washed in permeabilization buffer and followed by intracellular staining before sorting or FACS analysis. When sorted cells were used for RNA isolation, 0.2% RNaseOUT (Invitrogen) was added to the antibody incubation and FACS buffer.

**RNA isolation and quantitative PCR**

Organoids or sorted cells were lysis in 1ml TRIzol (ThermoFisher). RNA was isolated using RNeasy mini kit or RNeasy Micro kit (Qiagen) followed by reverse transcription. RNA isolation from intercellular stained cell sample was as described (53). QPCR was performed with GoTaq® qPCR Master Mix (Promega). Gene expression levels were normalized to Hprt using the $2^{-ΔΔCt}$ method and are presented as relative transcript levels.

**Quantitative measurement of conversion insulin-producing cells with cultured organoids**

Primary gut crypts which derived from a mouse bearing an Rip-Cre;Rosa26tdTomato reporter allele placed in culture, and then induced to undergo cell conversion by applying a protocol based on published patent US20170349884A1. Thereafter, Ins2-expressing cells were analyzed by flow cytometry and collected for further RNA analysis.

**In Situ Hybridization by RNAscope**

RNAscope was performed using the RNAscope® 2.5 HD Detection Reagent RED kit (Advanced Cell Diagnostics) combined with immunofluorescence according to manufacturer’s instructions. A human insulin probe (ACD #313571) was used to detected insulin mRNA.

**Immunohistochemistry**

Swiss rolls of small intestines were prepared from 6- to 8-week-old vehicle- or drug-treated mice and fixed in 4% PFA for 2 hours, followed by dehydration in 30% sucrose in PBS overnight, embedding in Tissue-Tek O.C.T (Sakura), and freezing at -80°C. Mouse and human organoids sections were prepared as described (18). 6µm-thick sections were cut and stained with standard frozen-IHC protocols. The
antibodies used are listed in Table S3. Images were recorded with a confocal laser-scanning microscope (LSM 710, Carl Zeiss) and processed using Image J software (National Institutes of Health).

**Bulk RNA-sequencing and analysis**

RNA-sequencing was performed by the Columbia Genome Center. Poly-A pull-down was used to enrich mRNA from small intestinal epithelial cells sorted from 4 to 6-week-old NFKO or Ins2-tomato⁺ cells from drug-treated organoids. Libraries were constructed and then sequenced using Illumina NovaSeq 6000. Differentially expressed genes were tested using DESeq2. Pathway enrichment was assessed through the pre-ranked version of GSEA (54).

**scRNA-seq and data analysis**

Tomato⁺ cells were isolated and sorted from NFKO mice as described above. Samples’ viabilities above 90% were processed by 10x Genomics 3’ Single Cell Gene Expression microfluidics platform, library preparation and sequencing were performed by the Columbia Genome Center as described (55). The R package Seurat was used to do the clustering analysis and cell type annotation (56) for the raw counts of scRNA-seq data analysis. The differentiation potential of INS⁺ and INS⁻ cells from NFKO mice was predicted using CytoTRACE (26).

**In vitro tissue and organoids insulin secretion assay**

1 cm of duodenum (after removal of pancreas) or cultured human organoids (after removal of medium and matrigel) were preincubated in Krebs buffer (2.6mM glucose) for 1h, then switch to stimulation Krebs buffer (2.6 mM glucose, 16.8 mM glucose or 30mM KCl) for another hour. Supernatant was collected for the insulin ELISA measurement. Secreted insulin was normalized with total tissue protein.

**Data availability**

The bulk RNA-seq and scRNA-seq data have been deposited in the NCBI’s Gene Expression Omnibus (GEO GSE201832, GSE213445 and GSE201776) and are publicly available as of the date of publication.

**Statistical analysis**
Data analysis was conducted using Prism 6.0 software (GraphPad) unless otherwise stated. Proper statistical methods were chosen based on data type and distribution. The statistical test and significance level are indicated in the figure legends.

Study approval

All animal studies were approved and overseen by Columbia University Institutional Animal Care and Use Committee (AABG6551). Human small intestine tissue for organoids culture was obtained in accordance with an approved Institutional Review Boards (Columbia University, AAAS9243). University of Southern California, and Children’s Hospital Los Angeles Institutional Review Boards have approved all procedures and collection of human fetal tissue samples (HS-19-00837).
Author contributions

W.D. designed, executed experiments, analyzed results, and wrote the manuscript; J.W. analyzed the sc(Seq data; T.K., W.L., W.M.M., J.S., H.W., T.K., L.Y., R.J.C., N.S., B.D. performed experiments and edited the manuscript; Y.C., B.H.G and M.E.T collected the fetal tissue and perform the human fetal tissue related experiment; L.E.R. and K.M. performed surgeries from which donor samples were obtained; N.G. and L.B. maintain the mouse stain; L.Y. and S.B. provide FBT compounds in this study; D.A. designed experiments, oversaw research and wrote the manuscript.

Acknowledgements

We thank members of the Accili laboratory for helpful discussions and critical data review and T. Kolar and A.M. Flete for technical help. We thank Forkhead BioTherapeutics Inc for providing FBT compounds. We thank Drs. Que (Columbia University) and Merchant (University of Arizona) for donating mice, Kuo (Stanford University) and Clevers (Hubrecht Institute) for cell lines. We thank M. L. Wilson (Department of Preventive Medicine, University of Southern California and Family Planning Associates) for coordinating fetal tissue collection. Mrs. Xu and Sun performed histology, Drs. Lu and Chen flow cytometry. This research was supported by a grant from the JPB Foundation to D.A., R01DK119198 to N.G., 1K01DK121873 to W.M.M and by Core Facilities supported by DK63608 (Columbia Diabetes Center).
References


Jeffrey Michael Karp RSL, Xiaolei Yin. United States; 2017.


Holst JJ. From the Incretin Concept and the Discovery of GLP-1 to Today's Diabetes Therapy. *Front Endocrinol (Lausanne).* 2019;10:260.

Du et al, Pharmacological conversion of gut epithelial cells into insulin-producing cells


Figure legends

Figure 1. INSULIN and FOXO1 expression in human fetal small intestine secretory lineage cells

(A) The representative image (GA=17 weeks) of tile scanning of ¼ fetal proximal intestinal roll section stained with INS(mRNA) in red and INS protein in green, scale bar indicate in the image;

(B) Quantification of INS-protein⁺, INS-mRNA⁺ and double positive cells (n= 3 different donors, GA=15-17 weeks, bar graphs show mean ± SEM);

(C) Insulin (red) and 5HT, Lysozyme or GLP-1 (green) staining in fetal human anterior intestine (GA=17 weeks), colocalization is shown in yellow color, scale bar = 20µm;

(D) Insulin (red) and 5HT, Lysozyme or GLP-1 (green) staining in adult human duodenum, colocalization is shown in yellow color, scale bar = 40µm;

(E) Insulin (green) and FOXO1 (red) staining in fetal human anterior intestine, scale bar = 20µm.

(F) Quantification of FOXO1⁻Insulin⁺ vs. FOXO1⁺ Insulin⁺ cells in fetal human proximal intestine (n= 3 different donors, each point shows averaged counting value from 3-4 different images per donor, bar graphs show mean ± SEM, t-test).
Figure 2. Expanded Neurog3 lineage and β-like cells in gut of Neurog3 FoxO1 KO mice

(A) FACS of isolated Tomato+ cells from either Neurog3Cre+FoxO1+/f; ROSA tdTomato (NFKO) or Neurog3Cre+; ROSA tdTomato (WT) gut epithelial cells. Red gate indicates sorting window for Neurog3-derived Tomato+ cells;

(B) Tomato+ cells frequency assessed by FACS in NFKO and Neurog3Cre (WT) mice (NFKO, n=23; WT, n=16 mice. Bar graphs show mean ± SEM. t-test);

(C) Representative IHC image of two types of Neurog3-derived β-like cells from NFKO mice: Paneth pattern (upper panel) and EEC pattern (lower panel), scale bar = 40µm;

(D) FACS plot CD24 based sorting strategy of dissociated Tomato+ single cells from NFKO small intestinal epithelial cells;

(E) Ins1 and Ins2 mRNA in sorted CD24+Tomato+, CD24neg Tomato+ and Tomatoneg population (n= 4 mice, Mann-Whitney Rank Sum Test);

(F) Representative IHC of Insulin, CD24 and Tomato. Paneth (upper panel) and EEC pattern (lower panel) of CD24 staining in insulin+ cells (green and red channel double colocalization is shown in yellow; green, red, and magenta triple colocalization is shown in white);

(G-H) Enriched hallmark gene sets in CD24neg Tomato+ vs. Tomatoneg population predicted by the GSEA.
Figure 3. Dual source of β-like cells in murine and human gut organoids

(A) QPCR of mouse intestinal organoids following EEC induction (n=3 independent experiments, bar graphs represent means ± SEM, paired t-test);

(B) Tomato staining of Tph1CreERT2 organoids after 4 days in differentiation (Diff) and EEC induction medium following activation of Tomato reporter (n=3 independent experiments, scale bar: 20 µm).

(C) Percentage of Tph1CreERT2-Tomato cells in mouse gut organoids with or without iFOXO1 (AS1842856, AS) treatment (n=3 independent experiments);

(D) Lineage tracing of Tph1CreERT2 4 days after activation of Tomato expression (scale bar: 20 µm);

(E) FACS diagram representing the sorted 5HT+ (pink) population in EEC induced from human gut organoids with or without FBT10 treatment;

(F) Percentage of 5HT+ cells in EEC induced from human gut organoids with or without FBT10 treatment (n=6 independent experiments, bar graphs represent means ± SEM, t-test);

(G) QPCR of Ins and Tph1 in sorted 5HT+ cells with or without FBT10 treatment (n=3 independent experiments);

(H-L) QPCR of different marker genes following treatment with combination of iNotch (DAPT, D), iTGFβ (Repsox, R), iGSK3β (Chir, C); iFOXO1 (AS1842856, AS) (n=3, bar graphs represent means ± SEM, paired t-test);

(M) Lineage tracing of Lyz1CreER 4 days after activation of Tomato expression (scale bar: 20 µm).
Figure 4. Conversion of gut cells into insulin-producing cells by combination treatment

(A) Modified differentiation protocol to induce conversion of INS* cells by the addition of FOXO1 inhibitor to the differentiation medium at different stages. Treatment details explained in Supplementary methods part: Quantitative measurement of conversion insulin-producing cells with cultured organoids. Abbreviations of small molecules indicate in Table S2;

(B) QPCR of Ins1 and Ins2 expression from organoids following treatment with the differentiation cocktail, comprising inhibitors combination of Notch (DAPT, D), and or TGFβ (Repsox, R), followed by the addition of FOXO1 inhibitor (AS1842856, AS) (n=6 independent experiment, bar graphs represent means ± SEM, paired t-test);

(C) Insulin content in organoids following treatment with the differentiation cocktail compared with islets (n=4 independent experiments, bar graphs represent means ± SEM, one-way ANOVA);

(D) FACS diagram presenting the percentage of converted INS2+ cells in ctrl vs. differentiation cocktail-treated organoids (Repsox, R; AS1842856, AS);

(E) Heatmap comparing expression level of typical islet and gut epithelial marker genes in sorted INS2-Tomato+ vs. INS2-Tomato+ from differentiated organoids of INS2-Tomato mice;

(F) Comparative evaluation of the potencies of 2 new FBT compounds and iFOXO1 (AS) to generate Insulin;

(G) Upregulated KEGG pathways in sorted INS2-Tomato+ cells vs. INS2-Tomato+ cells;

(H) Cells by an integrated calculation (Tom Score) of INS2-Tomato intensity, percentage of INS2-Tomato cells, and live-cell percentage, as detected by flow cytometry (n=3 independent experiments, bar graphs represent means ± SEM, paired t-test);

(I) Ins2 relative expression in sorted INS2-Tomato cells (n=4 independent experiments, bar graphs represent means ± SEM, paired t-test).
Figure 5. iNotch and iTGFβ combination therapy generates insulin+ cells in NFKO mice

(A) Experimental design for DBZ and Repsox combination treatment of NFKO mice;

(B) Representative IHC image of Tomato (Red) staining in Neurog3Cre FoxO1+/−;ROSA tdTomato mice following DBZ, Repsox or combination treatment, scale bar: 40μM;

(C) Tomato+ cells frequency measured by FACS in Ctrl, DBZ-, Repsox- or combination-treated Neurog3Cre FoxO1+/−;ROSA tdTomato mice, n=3 mice in each treatment group. Bar graphs represent means ± SEM, two-way ANOVA;

(D) Experimental design for DBZ and Repsox combination treatment in STZ-WT or NFKO mice;

(E) 4-hour fasting plasma insulin levels in STZ-NFKO and STZ-WT mice before and after treatment with the indicated compounds;

(F) 1-hour refed plasma insulin levels in STZ-NFKO and STZ-WT mice before and after treatment with the indicated compounds;

(G) Oral glucose tolerance tests after DBZ, Repsox and dual treatment;

(H) 4 hour fasting glucose level measure before OGTT;

(I) Area under the curve (AUC) of oral glucose tolerance test shown in (G);

(J) Representative IHC image of lysozyme (upper panel, green), MUC2 (middle panel, green) and 5HT (lower panel, green) co-stained with Insulin (red) in combination therapy-treated STZ-NFKO mice, scale bar=40μm, green and red channels colocalization shown in yellow.

Figure 6(E) to (I), n = 6 to 9 mice in each treatment group, bar graphs indicate mean± SEM, two way ANOVA;

(K-L) Glucose or KCl stimulate insulin secretion from duodenum of control or DBZ and Repsox combination treated NFKO mice, n=12 to 25, bar graphs indicate mean± SEM, Mann-Whitney Rank Sum Test.
Figure 6. Triple combination therapy lower blood glucose and induces gut β-like cells in NOD mice

(A) Experimental design for PF-03084014, Repsox and FBT10 triple combination treatment of NOD mice;

(B) Body weight measurement after 5 days combination treatment in Ctrl (vehicle) and Tx (treatment) groups, n=3 mice each group, bar graphs indicate mean± SEM, t-test;

(C-D) Plasma insulin and GLP-1 in Ctrl and Tx groups before and after 5-day treatment;

(E) Oral glucose tolerance tests after 5 days of vehicle or triple combination treatment;

(F) Area under the curve (AUC) of oral glucose tolerance test shown in (E);

(G) Representative IHC of lysozyme (upper panel, green), MUC2 (middle panel, green) and 5HT (lower panel, green) co-stained with Insulin (red) in triple combination therapy-treated NOD mice, scale bar=40µm, green and red channels colocalization shown in yellow.

n=3 mice each group, bar graphs indicate mean± SEM, t-test.
Figure 7. Induction of β-like cells by combination treatment in human gut organoids

(A) Schematic treatment protocol;

(B) qPCR of different marker genes from hGOs treated with the differentiation cocktail, bar graphs represent means ± SEM, paired t-test;

(C) Representative IHC image of C-peptide (green) staining in hGOs treated with differentiation cocktail, scale bar=20 µm;

(D) Insulin content of the differentiation cocktails treated hGOs (n=6 independent experiments, bar graphs represent means ± SEM, one-way ANOVA);

(E-G) Glucose and KCl stimulate Insulin secretion from different cocktails treated hGOs (n=3 independent experiments, 1-3 replicates in each experiment, bar graphs represent means ± SEM, t-test).