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# Understanding and Preventing Hypoglycemia in Diabetes

## Abstract Book

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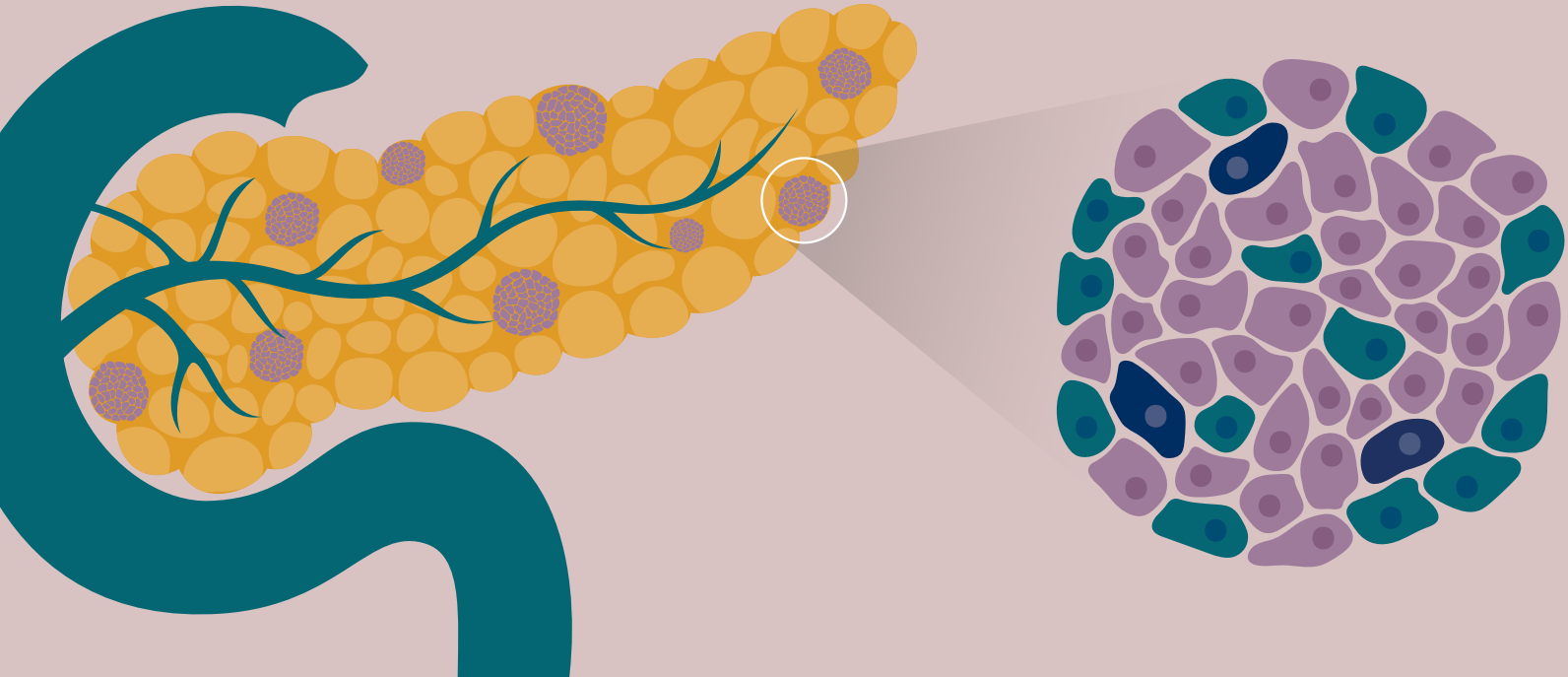

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## 1. Screening human islets to identify small molecules enhancing glucagon secretion under hypoglycemia

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**Background:** In individuals with type 1 diabetes (T1D), α cell function is disrupted, resulting in decreased glucagon secretion during episodes of hypoglycemia. This impairment occurs prior to a reduction in the secretion of epinephrine, a crucial stimulus for α cells, produced by adrenal chromaffin cells. Recurrent episodes of hypoglycemia, which are associated with the progressive loss of β cells in T1D, can result in compromised sympathoadrenal responses and an increased risk of developing hypoglycemia unawareness syndrome, a condition that can be life-threatening.

**Methods:** To explore potential compounds that could activate signaling pathways in human α cells and enhance glucagon secretion during hypoglycemia, we conducted a screen of 1027 FDA-approved compounds from ion channel and GPCR targeted compound libraries (APExBIO) using our primary human pseudoislet system. We optimized our screening protocol to facilitate the formation of pseudoislets in a 96-well format, employing state-of-the-art liquid-handling platforms for the addition of compounds and the analysis of glucagon secretion from individual pseudoislets, with the inclusion of carefully selected internal standards.

**Results:** Through this screen, we identified several promising compounds, referred to as 'hit' compounds, that targeted both ion channels and GPCRs. These compounds demonstrated an increase in glucagon secretion above the levels observed in positive controls, which included 1.7 mM glucose + 20 mM arginine. Notably, over 50 compounds targeting GPCRs, such as histamine, dopamine, 5-HT, opioid, adrenergic, and muscarinic receptors, and ion channels including potassium and sodium channel, elicited robust glucagon responses across multiple human islet donors (N=3-4). Further validation of these hit compounds was conducted in a refined screen providing within donor repeat measurements of compound efficacy. Moreover, we utilized our single-cell and bulk RNA-seq datasets to assess the specificity of targets and to aid in the selection of compounds. This involved evaluating changes in target expression observed in α cells of individuals with T1D. Our primary human pseudoislet system is an optimal platform for this screen, as it maintains the three-dimensional arrangement of islet cells and the islet microenvironment. Additionally, it allows for the generation of α cell-enriched pseudoislets that closely resemble conditions seen in T1D, providing an ideal model system for further validation of identified compounds.

**Conclusion:** The outcomes of this study hold promise for the repurposing of FDA-approved drugs for the treatment of hypoglycemia and could potentially expedite their translation into clinical testing, thereby addressing a critical need in the management of T1D.

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## 2. Identification of novel MAFB target genes in endocrine progenitor cells.

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**Background:** Glucose homeostasis is maintained by counteracting actions of insulin and glucagon hormones. The MAFB transcription factor regulates both insulin and glucagon transcription and is critical for alpha and beta cell development. MAFB expression is initially observed in differentiating alpha and beta cells, but also in a fraction of Neurog3$^{+}$ endocrine progenitor cells. This indicates potential roles in endocrine cell fate decisions and islet formation. The aim of this study is to investigate the impact of MafB deficiency on endocrine cell differentiation during fetal development.

**Methods:** We use MafB-GFP knock in animals to investigate the impact of MafB deficiency on endocrine differentiation *in utero*. Single-cell analysis was performed using Chromium Single Cell 3' solution on a 10x Genomics platform, followed by sequencing with a Novaseq sequencer.

**Results:** scRNA-seq analysis showed that loss of MafB mainly affects gene expression in Neurog3+ endocrine progenitor cells, representing the early stages of endocrine cell differentiation. Over 200 differentially expressed genes were identified, with a notable proportion of these genes being associated with transcriptional regulation. Furthermore, scRNA-seq analysis of MAFB deficient endocrine cells derived from human ES cells revealed that most of these transcription factors (TFs) were also reduced in MAFB deficient cells. This observation strongly suggests that MafB plays a pivotal role in driving the differentiation of endocrine progenitor cells toward the alpha-and/or beta-cell lineages in both human and mouse pancreas. In addition, we identified a previously unknown cluster of islet TFs expressed during the development of endocrine cells towards either alpha or beta cell lineages, including AUTS2, ETV1, MEIS2, RUNX1T1, and TOX3. Remarkably, the majority of the newly TFs regulated by MAFB are expressed in human endocrine progenitor cells. Type 2 diabetes risk alleles were identified in the genomic regions of AUTS2 and ETV1 genes suggesting that these genes are critical for human islet function and/or islet mass.

**Conclusions:** Our study highlights the critical role of MafB expression in early endocrine progenitor cells during pancreatic development. We have identified several novel transcription factors, uniquely expressed in endocrine progenitor and alpha or beta progenitor cells in both human and mice. Notably, the presence of type 2 diabetes risk alleles highlights potential molecular mechanisms underlying diabetes susceptibility. These findings significantly advance our understanding of pancreatic development and the genetic basis of diabetes.

**Acknowledgments:** This project is supported by the Diabetes Wellness, EFSD/NovoNordisk, and Påhlsson foundations.

**3. Lactate and ROS-mediated activation of α-cell KATP channels inhibits glucagon secretion by hyperpolarizing the membrane potential and reducing $Ca^{2+}$ entry.**

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Elevations in pancreatic α-cell intracellular $Ca^{2+}$ ($[Ca^{2+}]_i$) stimulate glucagon (GCG) secretion. Because α-cell ATP-sensitive $K^+$ ($K_{ATP}$) channels are largely inhibited under low and high glucose conditions (>99% tonic inhibition), conditions that activate this channel are likely to have a more profound impact on membrane potential and $Ca^{2+}$ handling. However, the mechanisms that activate α-cell $K_{ATP}$ still remain to be elucidated. This this study investigated mechanisms that activate $K_{ATP}$ in α-cells and how they control $Ca^{2+}$ handling and GCG secretion. As lactate activates ATP-sensitive $K^+$ ($K_{ATP}$) channels in cardiomyocytes, lactate may also modulate α-cell $K_{ATP}$. Lactate enters cells through monocarboxylate transporters (MCTs) and is also produced during glycolysis by lactate dehydrogenase A (LDHA), an enzyme expressed in α-cells. Lactate-inhibited mouse (75 ± 25%) and human (47 ± 9%) α-cell $[Ca^{2+}]_i$ fluctuations only under low-glucose conditions (1 mM) but had no effect on β- or δ-cells $[Ca^{2+}]_i$. Glyburide inhibition of $K_{ATP}$ channels restored α-cell $[Ca^{2+}]_i$ fluctuations in the presence of lactate. Lactate transport into α-cells via MCTs hyperpolarized mouse (14 ± 1 mV) and human (12 ± 1 mV) α-cell membrane potential ($V_m$) and activated $K_{ATP}$ channels. Lactate-induced inhibition of α-cell $[Ca^{2+}]_i$ influx resulted in reduced GCG secretion in mouse (62 ± 6%) and human (43 ± 13%) islets. Another activator of pancreatic β-cell $K_{ATP}$ channels is reactive oxygen species (ROS). When α-cell ROS levels were elevated with either $H_2O_2$ or decreasing glutathione levels, $K_{ATP}$ channels were activated in both mouse and human a α-cells. ROS activation of $K_{ATP}$ reduced α-cell $[Ca^{2+}]_i$ influx and inhibited GCG secretion. Thus, ROS control of α-cell $K_{ATP}$ may be a physiological mechanism that tunes α-cell GCG secretion. These data demonstrate for the first time that lactate or ROS production in α-cells results in $K_{ATP}$ activation, $V_m$ hyperpolarization, reduced $[Ca^{2+}]_i$, and inhibition of GCG secretion. Importantly, we find that lactate and ROS also controlled α-cell function in human type1-diabetic-like islets without β-cells. This suggests that conditions that selectively activate $K_{ATP}$ in high glucose could be targeted to restore glucose-inhibition of GCG secretion in T1D.

**4. Real-time detection of single-islet somatostatin release uncovers excessive secretion in type 2 diabetes**

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**Background:** Somatostatin released from pancreatic δ-cells is an important paracrine regulator of insulin and glucagon secretion, but the mechanisms regulating somatostatin release are not well understood and it is not clear if somatostatin secretion is altered in diabetes. To facilitate studies of δ-cell function we aimed to develop a tool for real-time detection of somatostatin secretion from single islets.

**Methods:** Reporter cells responding to somatostatin with increases of the cytoplasmic $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) were generated by co-expressing the somatostatin receptor SSTR2, the G-protein Gα15 and the $Ca^{2+}$ sensor R-GECO1 in HeLa cells.

**Results:** Somatostatin induced dose-dependent $[Ca^{2+}]_i$ increases in reporter cells with half-maximal and maximal effects at 1.6±0.4 and ~30 nM, respectively. In the presence of mouse islets, the reporter cells showed $[Ca^{2+}]_i$ elevations that were inhibited by the SSTR2 antagonist CYN154806. Depolarization of the islets with 30 mM $K^+$, the $K_{ATP}$ channel inhibitor tolbutamide, or by increasing the glucose concentration from 3 to 11 mM evoked concurrent elevations of islet and reporter cell $[Ca^{2+}]_i$. Islets exposed to glucagon, GLP-1 and ghrelin also triggered reporter cell $[Ca^{2+}]_i$ responses, whereas islets treated with insulin, glutamate, GABA and urocortin-3 lacked effects. Similar results were obtained with human islets from healthy donors. In contrast, islets from type 2 diabetic donors induced less reporter cell activity at 3 mM glucose, but more pronounced $[Ca^{2+}]_i$ responses at 11 mM and after $K^+$ depolarization although a lower number of δ-cells were identified by immunostaining.

**Conclusion:** We have developed a semi-quantitative reporter cell assay that enables real-time detection of somatostatin release from single islets. Our results indicate that type 2 diabetes is associated with excessive secretion of somatostatin, which has implications for paracrine regulation of insulin and glucagon secretion.

**5. Ghrelin and LEAP2 affect insulin and glucagon secretion in mouse but not in human islets, and their effects in mouse islets are mediated by somatostatin**

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**Background:** Ghrelin, which activates the growth hormone secretagogue receptor 1a (GHS-R1a), stimulates appetite ("hunger hormone") and impairs glucose homeostasis. Conversely, liver-expressed antimicrobial peptide-2 (LEAP2), which acts as an endogenous antagonist and inverse agonist of GHS-R1a, counteracts the effects of ghrelin and improves glucose homeostasis. The mechanisms of control of insulin (INS) and glucagon (GCG) secretion by ghrelin and LEAP2 are debated or poorly understood. Since δ-cells strongly express GHS-R1a, ghrelin and LEAP2 are expected to affect somatostatin (SST) secretion. This study aims to explore the effects of ghrelin and LEAP2 on pancreatic hormone secretion in mice and humans, to test the efficacy of two other GHSR inverse agonists, $LEAP2_{(38-47)}$ (a fragment of LEAP2 upregulated in Roux-en-Y gastric bypass) and PF-5190457 (PF), and to determine whether their effects are mediated directly or indirectly via SST.

**Methods:** The experiments were performed on islets from control ($Sst^{+/+}$) and SST knockout ($Sst^{-/-}$) mice (both strains on C57BL/6N background), and human islets (IIDP). Islets were incubated for 1h in a medium containing a 6 mM amino acid mixture (2 mM alanine, 2 mM arginine and 2 mM glutamine) and various glucose concentrations (1 mM (G1), 7 mM (G7), or 15 mM (G15)), ghrelin (acyl-ghrelin) or LEAP2 (both tested at 0.1, 1, 10 or 100 nM), LEAP2(38-47) or PF (both tested at 0.1, 1, 10, 100 nM or 1 μM).

**Results:** As expected, glucose stimulated INS and SST secretion, and inhibited GCG release from $Sst^{+/+}$ mouse and human islets. $Sst^{-/-}$ islets showed similar INS secretion but higher GCG secretion compared to $Sst^{+/+}$ islets. In $Sst^{+/+}$ islets, ghrelin dose-dependently inhibited GCG release in G1 (by 35% to 50% with 0.1 nM and 100 nM, respectively; $P<0.01$), whereas only 100 nM ghrelin inhibited INS secretion in G15 (by 20%; $P<0.05$). LEAP2, at 100 nM only, increased GCG secretion in G1 (by 45%; $P<0.01$), and increased INS secretion in G15 (by 19%; $P<0.05$). [Ghrelin] $\geq$1 nM and [LEAP2] $\geq$10 nM stimulated and inhibited ($P<0.01$), respectively, SST secretion from $Sst^{+/+}$ islets. Neither ghrelin nor LEAP2 affected INS or GCG secretion from $Sst^{-/-}$ islets demonstrating that the effects they exerted in $Sst^{+/+}$ islets were mediated by SST. $LEAP2_{(38-47)}$ and PF failed to stimulate GCG and INS release from mouse $Sst^{+/+}$ islets. In human islets, neither ghrelin nor LEAP2 affected pancreatic hormone secretion, except for a 60% stimulation of SST release by 100 nM ghrelin in G15 ($P<0.01$).

**Conclusion:** In mouse islets, ghrelin exhibits dose- and glucose-dependent inhibitory effects on GCG and INS secretion, while LEAP2 stimulates GCG secretion in G1 and INS secretion in G15. Notably, ghrelin stimulates SST secretion, whereas LEAP2 inhibits it. The effects of ghrelin and LEAP2 on INS and GCG secretion are indirect, mediated by SST, which is compatible with the high expression of GHS-R1a in δ-cells. $LEAP2_{(38-47)}$ and PF do not reproduce the inverse agonistic effect of LEAP2. In human islets, neither ghrelin nor LEAP2 affect INS or GCG secretion. This suggests that modulation of pancreatic hormone secretion by both peptides contributes less to glucose homeostasis in humans than in mice.

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## 6. Glucose modulates cAMP in α-cells by a mechanism that is mainly intrinsic and PDE-dependent

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**Background:** The mechanisms by which glucose controls glucagon release are poorly understood. Recent studies have suggested that a drop of the glucose concentration stimulates glucagon secretion by increasing [cAMP] in α-cells ($[cAMP]_\alpha$). However, whether glucose controls $[cAMP]_\alpha$ by a direct action on α-cells or indirectly via β- or δ-cells is disputed. Moreover, the underlying mechanisms are unclear and were addressed in the present study.

**Methods:** Changes in $[cAMP]_\alpha$ were monitored in live-cell imaging using islets from GCG-iCre-cAMPER mice expressing a cAMP sensor (cAMPER) specifically in α-cells. Glucagon secretion was measured in perifusion and incubation experiments. All media contained a 2.1 mM mixture of amino acids present at physiological concentrations (in mM: 0.4 alanine, 0.5 glutamine, 0.2 lysine, 0.25 glycine, 0.15 leucine, 0.25 valine, 0.15 threonine, 0.1 serine, and 0.1 arginine).

**Results:** Lowering the [glucose] from 7mM (G7) to 1mM (G1) increased [cAMP] in α-cells within islets. Most of this effect is independent of somatostatin (SST) and insulin since it persisted in the presence of SST-14 (1μM), H6056 (1μM) + CYN154008 (0.3μM) which are antagonists of the two main SST receptors expressed by α-cells (SSTR2/3), insulin (1μM), or diazoxide (250 μM) which inhibits insulin and SST secretion. However, SST secreted by δ-cells also contributes to the control of $[cAMP]_\alpha$ by glucose since exogenous SST-14 decreased $[cAMP]_\alpha$ more potently at G1 than at G7, probably because, in the latter situation, endogenous SST secreted by δ-cells in response to G7 has already decreased $[cAMP]_\alpha$. Exogenous insulin (1μM) only slightly decreased $[cAMP]_\alpha$ (more at G1 than at G7), suggesting that it contributes very little to the effect of glucose on $[cAMP]_\alpha$. That G1 also increased $[cAMP]_\alpha$ in dispersed α-cells confirms a direct action on α-cells. We evaluated the involvement of adenylate cyclases (ACs) and phosphodiesterases (PDEs) in the effect of glucose on $[cAMP]_\alpha$. Activation of ACs with forskolin (FSK, 2,5μM) or blockade of PDEs with IBMX (100μM) elevated $[cAMP]_\alpha$ in G7. IBMX, but not FSK, prevented the increase in $[cAMP]_\alpha$ induced by a subsequent drop of [glucose] to G1, suggesting that glucose controls $[cAMP]_\alpha$ by modulating PDEs. The rise in $[cAMP]_\alpha$ induced by G1 was partially prevented by 8MM-IBMX (PDE1 inhibitor, 100μM), but not by cilostamide (PDE3b inhibitor, 250nM) or rolipram (PDE4 inhibitor, 400nM) used alone or in combination. This suggests that PDE1 inhibition is involved in the effect of G1 on $[cAMP]_\alpha$. We next evaluated the role of the cAMP effectors, PKA and EPAC2, in the control of glucagon secretion by glucose. Application of RP-8-CTP-cAMPs (PKA inhibitor, 100μM), or ESI-05 (EPAC2 inhibitor, 25μM) in G1 did not affect glucagon secretion. However, these inhibitors significantly inhibited glucagon secretion (~50%) at G1 when used in combination. Similarly, activating both PKA and EPAC2 with 100μM SS20 in G7 reproduced the effect of G1 on glucagon secretion. Surprisingly, glucagon secretion was frequently not correlated to $[cAMP]_\alpha$, even when $[Ca^{2+}]_c$ was not kept at basal levels.

**Conclusion:** We demonstrate that glucose primarily lowers $[cAMP]_\alpha$ by an intrinsic mechanism involving the activation of PDE. The effects of $[cAMP]_\alpha$ on glucagon secretion are mediated by the combined action of PKA and EPAC2. The primordial role of cAMP in the control of glucagon release is however tempered by observations of poor correlation between both parameters.

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## 7. Restoring glucagon secretion in type 1 diabetes: from physiological insight to therapeutic applications

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Increased glucagon release from alpha cells is the main defense against hypoglycemia. In Type 1 Diabetes (T1D), the glucagon response to a lowering in glycemia is severely reduced, thus increasing the risk of life-threatening hypoglycemia. As β-cell mass diminishes in T1D, there is a substantial loss of paracrine signals that supresss glucagon secretion under hyperglycemic conditions. This not only elevates basal glucagon levels, further promoting hyperglycemia, but also desensitizes autocrine signaling pathways. We hypothesize that loss of inhibitory signals also plays a crucial role in the impaired responsiveness of α-cells to hypoglycemic conditions. If so, reactivating paracrine and autocrine signaling pathways pharmacologically could reverse alpha cell glucose blindness and restore glucagon secretion.

To test this hypothesis, we conducted experiments using isolated islets and pancreatic tissue slices from both non-diabetic organ donors and donors with T1D. Using functional recordings, we determined α-cell responses to (a) changes in glycemia, (b) agonists, antagonists, and positive allosteric modulators of autocrine and paracrine signaling pathways, and (c) reference stimuli such as adrenaline and KCl depolarization. Additionally, we performed in vivo studies using mouse models with defective glucose counterregulation to determine whether α-cell responses to hypoglycemia could be restored.

Our findings revealed that, unlike β-cells, α-cells did not faithfully track glucose concentrations. α-cell responses to low glucose were transient, contrasting with the sustained responses of β-cells to high glucose. Remarkably, exposing islets for 15 minutes to high glucose to activate inhibitory paracrine signaling fully restored glucagon secretion to low glucose. Our in vitro results in human tissue slices/islets from donors with T1D not only reproduced important features seen in vivo but also revealed mechanistic defects related to autocrine and paracrine inputs. Importantly, reestablishing these inputs pharmacologically rescued glucagon secretion in response to a lowering in glucose concentration as well as to hypoglycemia in the nonobese diabetic (NOD) mouse model. Several of these signaling molecules are used for other medical indications and could be repurposed to develop therapies aimed at preventing hypoglycemia in T1D. Our findings highlight that elucidating the fundamental mechanisms that control α-cell function in the healthy physiological state is still work in progress. A detailed understanding of basic α-cell physiology will provide the foundation for restoring adequate glucagon secretion in T1D.

**8. Insulin-independent regulation of type 1 diabetes via brown adipocyte-secreted proteins and the novel glucagon regulator nidogen-2**

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**Background:** Type 1 diabetes (T1D) is defined by autoimmune-mediated destruction of the insulin-producing pancreatic β-cells and exacerbated by the aberrant hypersecretion of glucagon by α-cells. Current therapeutic strategies for T1D focus on insulin replacement through subcutaneous administration of the hormone or islet/pancreas transplantation. Brown adipose tissue (BAT) has gained attention in metabolic research due to its endocrine functions and role in metabolic regulation. Beyond its well-documented capacity for thermogenesis, BAT acts as a secretory organ, modulating metabolic processes through autocrine, paracrine, and endocrine pathways. Subcutaneous embryonic BAT transplantation leads to insulin-independent recovery of euglycemia, accompanied by elevation of IGF-1 and suppression of glucagon secretion. We aimed to determine whether this transplantation effect was due to BAT secretions, and to identify the secreted factors that mediate the positive effects of transplantation.

**Methods:** We utilized a differentiated BAT cell line to generate substantial quantities of BAT secretions for further purification by molecular weight spin columns, size exclusion, anion exchange and reverse phase chromatography. We assessed the resulting molecular fractions for glucagon inhibition and insulin receptor activation using isolated human and mouse islets, dispersed islet cells, and human cell lines. Promising fractions were further evaluation *in vivo* using the NOD mouse as a T1D model.

**Results:** We showed that a large molecular weight (>100 kDa) BAT-secreted protein fraction, but not exosomes, inhibits glucagon secretion from islets and dispersed islet cells ($p < 0.001$). This fraction not only inhibits glucagon, but it also enhances glucose uptake in adipose tissue, skeletal muscle, and liver through an insulin receptor-dependent pathway. Further, we demonstrate the therapeutic potential of a secreted protein fraction from embryonic brown adipose tissue (BAT), independent of insulin by showing that giving seven daily injections of this fraction suppresses hyperglucagonemia and restores euglycemia in diabetic NOD mice ($p < 0.001$). We observed this reversal of hyperglycemia in 90% of the animals injected with this purified fraction (27 or 30 treated animals), and this effect lasts up to 8 weeks following cessation of the injections. The >100 kDa fraction also promotes *in vivo* white adipocyte differentiation and browning, maintains healthy BAT, and enhances glucose uptake in adipose tissue, skeletal muscle, and liver. Further purification of this fraction by chromatography allowed us to identify nidogen-2 as a critical BAT-secreted protein that reverses hyperglycemia in NOD mice, inhibits glucagon secretion from pancreatic α-cells, and mimics other actions of the entire secreted fraction. These findings confirm that BAT transplants affect physiology and demonstrate that BAT-secreted peptides represent a novel therapeutic approach to diabetes management. Furthermore, our research reveals a novel signaling role for nidogen-2, beyond its traditional classification as an extracellular matrix protein.

## 9. Impact of gap junction electrical coupling between pancreatic delta and beta cells on hormone secretion and glucose homeostasis

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Pancreatic delta cells regulate glucose homeostasis by secreting somatostatin, which inhibits glucagon and insulin secretion from alpha and beta cells, respectively. While delta cell activity is influenced by paracrine interactions of insulin and urocortin3 from beta cells, research suggests that gap junction coupling between delta and beta cells may also play a role in this regulation. Connexin-36 (Cx36) gap junctions between beta cells coordinate their cellular responses to suppress spontaneous electrical activity at low glucose and facilitate synchronized and pulsatile insulin secretion at high glucose. However, the existence of gap junction coupling between delta and beta cells, and its potential impact on somatostatin release, remains to be determined. Previous RNA-seq data indicate that GJD2, the gene encoding Cx36, is downregulated in both beta and delta cells from individuals with Type 1 Diabetes (T1D) or those with multiple islet-autoantibodies. While it is known that disruption of gap junctions in beta cells leads to dysregulated insulin secretion and impaired glucose tolerance, the role of gap junctions in delta cells, particularly their function and disruption in the context of diabetes, is poorly understood. Global Cx36 knockout (KO) and somatostatin (sst)-cre Cx36 KO mouse models were generated to evaluate the effect of loss of gap junction coupling broadly in the pancreatic islet and precisely in delta cells. A sst-cre TdTomato mouse model was developed and validated by immunofluorescence to further study delta cell dynamics. Gap junction permeability was assessed by Fluorescence Recovery after Photobleaching (FRAP). Calcium imaging and hormone secretion assays were performed to assess the impact of gap junction coupling on cellular function. FRAP experiments demonstrated cationic dye diffusion between beta and delta cells, indicating Cx36 gap junction coupling, with no difference between delta and non-delta cells. In contrast, alpha cells showed no fluorescent recovery, indicating a lack of Cx36 gap junction coupling. Calcium imaging revealed that a subset of delta cells exhibited synchronous activity with beta cells, further supporting electrical coupling between these cell types. Islets from Cx36 KO mice showed altered glucagon ($p = 0.018$) and somatostatin ($p = 0.06$) secretion in response to low and high glucose compared to controls. During an intraperitoneal insulin tolerance test, Cx36 KO mice showed similar glucose levels to controls at 0-60 minutes, but differences at 90 minutes post-insulin delivery ($p < 0.05$), suggesting an altered counterregulatory response. Glucose tolerance tests in sst-cre Cx36 +/- mice revealed differences in blood glucose levels compared to littermate controls, consistent with impaired inhibition of insulin secretion by somatostatin. These results suggest that delta cells are gap junction-coupled to beta cells, and these connections are functionally significant for normal hormone secretion. Downregulation of GJD2 in T1D suggests that impaired delta cell gap junction coupling may contribute to altered glucose homeostasis and diabetes pathogenesis. Further research is needed to understand the precise mechanisms by which delta cell gap junction coupling influences islet hormone release.

**10. Human pseudoislet system reveals cell-to-cell contact and hypoglycemia as mechanisms contributing to α cell dysfunction in type 1 diabetes (T1D)**

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**Background:** In T1D, autoimmune destruction of islet β cells leads to hyperglycemia and altered islet composition. Further, individuals with T1D also show impaired glucagon responses to hypoglycemia, which presents a major challenge for insulin therapy. Previous data from our group showed that while remaining β cells in islets from T1D donors have nearly normal function, α cells have impaired glucagon secretion and altered gene expression. The reason(s) for these intrinsic α cell changes are unknown but may include loss of α-to-β cell contact, chronic hyperglycemia, and/or repeated hypoglycemic events.

**Methods:** To test these hypotheses, we utilized cell sorting of human islets from donors without diabetes to create α cell-enriched ("T1D-like") pseudoislets. Pseudoislets were transduced with GCaMP6f and assessed *in vitro* by live-cell imaging and microperifusion, allowing for synchronous capture of intracellular $Ca^{2+}$ ($[Ca^{2+}]_i$) signal and glucagon secretion. To understand the impact of chronic hyperglycemia, we transplanted pseudoislets into *Nod-SCID-IL2Rγ$^{null}$; RIP-Diphtheria Toxin (DT) Receptor* mice made diabetic by DT-mediated depletion of endogenous β cells. To mimic repeat hypoglycemia, we subjected T1D-like pseudoislets to multiple low glucose (MLG) exposures *in vitro*, using those remaining in basal glucose as control.

**Results:** Our initial results suggest hyperresponsiveness of T1D-like pseudoislets to both 16.7 mM glucose (G 16.7; control vs T1D-like AUC: -0.04 ± 0.01 vs. -0.19 ± 0.09% glucagon content; p=0.28) and 2 mM glucose plus epinephrine (G 2 + Epi; 0.09 ± 0.005 vs. 0.32 ± 0.10% glucagon content; p=0.25, n= 2 donors) compared to those with an equal ratio of β cells. This was accompanied by altered α cell $[Ca^{2+}]_i$ signaling in T1D-like pseudoislets in response to both G 16.7 (AUC: 0.57 ± 0.51 vs. 0.08 ± 0.05; p= 0.47) and G 2 + Epi (0.18 ± 0.18 vs. 0.09 ± 0.08; p=0.41). Six weeks post-transplantation, α cells in T1D-like pseudoislets expressed the β cell-enriched transcription factor NKX6.1, recapitulating previous findings from primary T1D islets (control vs T1D-like: 1.3 ± 1.3% α cells vs 48.2 ± 17.7%; n= 2-3 donors). Similar findings were seen in transplants exposed to one month of hyperglycemia (composition effect: p=0.01). Following MLG, T1D-like pseudoislets had reduced static glucagon secretion in response to both G 1.7 (control vs MLG: 1.51 ± 0.18 vs 0.79 ± 0.13% glucagon content; p=0.01, n=4 donors) and G 16.7 (0.79 ± 0.13 vs 0.39 ± 0.08% glucagon content; p=0.02). Additionally, they also show reduced dynamic glucagon secretion in response to G2 + Epi (control vs. MLG: 0.08 ± 0.007 vs. 0.03 ± 0.017% glucagon content; p=0.09, n=3 donors) which was accompanied by reduced $[Ca^{2+}]_i$ signaling during this period (0.30 ± 0.20 vs. 0.02 ± 0.02 p=0.28).

**Conclusion:** These data suggest that loss of β cell contact and repeated exposure to low glucose alter α cell function. Further, chronic hyperglycemia may lead to changes in α cell identity state after β cell loss.

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**11. The glucagon-like peptide-1 receptor antagonist, exendin (9-39) amide, improves the impaired glucagon response to hypoglycemia in type 1 diabetes.**

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**Background:** In type 1 diabetes (T1D), counterregulatory islet α-cell glucagon secretion becomes insufficient, contributing to potentially fatal hypoglycemia. This defect is partly caused by a dysregulated paracrine increase in somatostatin signalling from the neighbouring δ-cells. The incretin hormone glucagon-like peptide-1 (GLP-1) is derived from proglucagon, the precursor of islet glucagon. Normally, GLP-1 is not predominantly secreted by the islet α-cells due to the selective expression of prohormone convertase (PC)-2, an enzyme that processes proglucagon to glucagon. However, recent observations suggest that the activity of α-cell PC-1/3, an enzyme that processes proglucagon to GLP-1 in the gut, may be increased in T1D. GLP-1(7-36) activates the GLP-1 receptor (GLP-1R) expressed by both α- and δ-cells. GLP-1(7-36) inhibits glucagon secretion both directly, by activating the α-cell GLP-1R; and indirectly, by stimulating GLP-1R-induced somatostatin release from the δ-cells. Our aim was to determine the contribution of α-cell derived GLP-1 and its blockade on glucagon secretion during hypoglycemia using the spontaneous, autoimmune non-obese diabetic (NOD) mouse model of T1D and islets from human T1D donors.

**Methods:** Islet hormone secretion was assessed in adult diabetic (blood glucose ≥ 25 mmol/l) and normoglycemic pre-diabetic control (blood glucose 5-8 mmol/l) NOD/ShiLtJ mice by *in situ* pancreas perfusion, as well as from human T1D donor islets by static secretion. Glucagon and total GLP-1 secretion was quantified from the pancreas perfusate by ELISA (Mercodia, Sweden). Islet insulin-positive cells were examined by morphometry. GLP-1R expression was assessed in both diabetic and control islets by fluorescent immunohistochemistry.

**Results:** Both diabetic and pre-diabetic NOD islets showed immune cell infiltration, with NOD diabetic islets exhibiting a marked reduction in insulin-positive area/islet. While hypoglycemia normally increased glucagon secretion from the perfused NOD control pancreas, this response was abolished from the diabetic pancreas (10 mmol/l vs 1 mmol/l glucose: control, 9.17±1.36 vs 21.5±4.32 pg/min, n = 7; diabetic, 4.93±1.52 vs 6.76±1.54 pg/min, n = 3). Total GLP-1 secretion in response to hypoglycemia was 3.7-fold higher from the diabetic pancreas, when compared to the control pancreas (1 mmol/l glucose: control, 3.03±0.19 pg/min, n = 3; diabetic, 11.3±0.51 pg/min, n = 3; ***$p < 0.001$). The GLP-1R antagonist, exendin(9-39) amide (Ex9-39), significantly improved hypoglycemia-induced glucagon secretion from the diabetic NOD pancreas by 2.3-fold (1 mmol/l glucose vs 1 mmol/l glucose + 1 μmol/l Ex9-39: 6.76±1.54 vs 15.98 ± 1.42, n = 3; *$p < 0.05$) and from human T1D islets by 1.9-fold (*$p < 0.05$, n = 2 T1D donors).

**Conclusion:** Autoimmune diabetes in the NOD mouse is associated with an increase in islet-derived GLP-1 secretion. The impaired glucagon response to hypoglycemia from α-cells from both NOD diabetic and human T1D islets is significantly improved by GLP-1R blockade using the GLP-1R antagonist, exendin(9-39) amide. This suggests that intra-islet GLP-1 has a significant role in the defective glucagon secretion seen in T1D and provides a rationale for GLP-1R antagonists as a potential adjunct therapy to insulin for reducing the risk of hypoglycemia in individuals with T1D.

## 12. Additive glucagonotropic and insulinotropic effects of glucose-dependent insulinotropic polypeptide and alanine in fasted healthy men

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**Background:** Glucose-dependent insulinotropic polypeptide (GIP) has bidirectional effects on plasma glucose levels via its insulinotropic and glucagonotropic properties exerted at high and low plasma glucose concentrations, respectively. *In vitro*, GIP has also been shown to potentiate the glucagonotropic and insulinotropic effects of alanine in isolated mouse islets. Here, we evaluated the separate and combined effects of GIP and alanine infusions on plasma glucagon and insulin levels in fasted healthy men.

**Methods:** In this placebo-controlled, randomized, double-blind crossover study, 10 healthy men (mean ± SD; age 22.9 ± 2.8 years, BMI 23.7 ± 2.1 kg/m$^2$) underwent four experimental days involving continuous 90-minute i.v. infusions of GIP (6 pmol/kg/min for 10 minutes and then 4 pmol/kg/minute for 80 minutes), alanine (28 µmol/kg/min), GIP + alanine, and saline (placebo), respectively, after a 10-hour overnight fast. Differences in area under the curve (AUC) were evaluated using Student's *t* test with Benjamini-Hochberg control for false discovery rate.

**Results:** Compared to placebo, both GIP and alanine stimulated glucagon secretion with supraadditive effects during co-infusion eliciting significantly greater placebo-corrected AUC (GIP+Alanine: 1,613 ± 719 (mean ± SD) min × pmol/l) as compared to each of the separate infusions (Alanine: 995 ± 445 min × pmol/l, $p = 0.005$; GIP: 298 ± 226 min × pmol/l, $p < 0.0001$). Likewise, both GIP and alanine increased C-peptide responses compared to placebo, and co-infusion elicited an additive effect evaluated from placebo-corrected AUCs (GIP+Alanine: 25,999 ± 11,859 (mean ± SD) min × pmol/l; Alanine: 16,711 ± 11,359 min × pmol/l, $p = 0.02$; GIP: 9,803 ± 9,553 min × pmol/l, $p = 0.0002$).

**Conclusion:** GIP and alanine have additive glucagonotropic and insulinotropic effects in fasted healthy men supporting GIP's glucose stabilising effects during situations with alanine abundance.

## 13. Influence of genetics on variation in continuous glucose monitoring (CGM) features and islet autoantibody status

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**Background**: A one-week CGM home test in healthy relatives of subjects with type 1 diabetes (T1D) demonstrated that the percent time of glucose >180 mg/dL, glucose range (Range), and glucose coefficient of variation (CV) predicts low (no islet autoantibodies (Ab); N=21) *vs*. high (2+ Ab; N=21) immunological risk (TrialNet ancillary study; NCT 02663661). Here, we test whether including genetic information improves CGM-based prediction of 1 Ab versus multiple (2+) Ab, as well as using genome-wide analysis to identify variants associated with CGM features.

**Methods**: Genetic and one-week CGM data (including Time Above (TA) or below (TB) a glucose threshold) were collected in a TrialNet genetic ancillary study from 39 participants (18 with 1 Ab; 21 with 2+ Ab). Following quality control, genetic data from 38 participants were analyzed using a regression framework to predict Ab status using nine CGM features, a 72-SNP Genetic Risk Score (T1D GRS), and genome-wide analysis of CGM features.

**Results**: Analysis of nine CGM features showed that only TA140 (percent time of glucose > 140 mg/dL) was significantly associated (P=0.015) with multiple Ab versus 1 Ab. A higher T1D GRS was strongly associated (P=0.002) with having multiple Ab. Models with CGM features and T1D GRS resulted in only the T1D GRS being significantly associated (P=0.019) with Ab status (higher T1D GRS, 2+ Ab). Genome-wide analysis of the CGM features identified 5 SNPs/genes associated with 7 CGM features: TB54 with rs1009693 (*RBFOX1*, $P=4.3\times10^{-8}$), TB70 with rs10886031 (*SHTN1*, $P=1.9\times10^{-8}$), TA160 with rs17017778 (*COLEC11*, $P=6.9\times10^{-8}$), TA180 with rs7654070 (*TLL1*, $P=3.6\times10^{-8}$), and Range ($P=4.2\times10^{-9}$), CV ($P=6.6\times10^{-8}$), and SD ($P=1.82\times10^{-8}$) with rs7528798 (*COL11A1*).

**Conclusion**: Comparison of 1 Ab (low risk) versus 2+ Ab (high risk) participants is an important consideration for screening and identifying individuals for immune intervention in type 1 diabetes. These results suggest that CGM (TA140) and a T1D GRS can help identify individuals at high risk, and that genetic variation contributes to CGM features.

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## 14. Characterization of pancreas and islet structure and function in longstanding type 1 diabetes

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**Background:** Individuals with longstanding type 1 diabetes (LT1D, >10 years) have higher risk of hypoglycemia, likely attributable to progressive alpha cell dysfunction. However, most studies of T1D human pancreas have focused on donors with recent-onset T1D; our understanding of the LT1D pancreas and islet is limited.

**Methods:** To characterize the features of the LT1D pancreas and understand how these may influence alpha cell function, we studied LT1D pancreata using traditional immunohistochemistry (N = 20 T1D and 20 non-diabetic, ND, donors) and multiplexed fluorescent immunohistochemistry (CODEX, N = 4 ND and 4 LT1D), isolated islets, and generated pancreatic slices. The codex antibody panel included endocrine, acinar, ductal, and extracellular matrix (ECM) markers. Tissue viability and structure of the living pancreas slices were assessed by FDA-PI and H&E staining.

**Results:** By traditional IHC, the LT1D pancreas had greater fibrosis, particularly in a periductal and perivascular distribution, and marked blood vessel wall thickening, compared to ND. On CODEX, LT1D pancreata had greater intra-islet laminin and exocrine collagen-IV area, demonstrating changes in the ECM in both pancreas compartments. LT1D islets had greater pan-cytokeratin, a ductal marker, and more frequent loss of the basement membrane between endocrine and exocrine cells and disruption of the islet-acinar boundary.

Commonly used approaches to isolate islets from LT1D tissues with collagenase resulted in low islet yield and purity. Multiple, alternative islet isolation protocols using collagenase, neutral protease, thermolysin, endopeptidase, elastase, DNAse I, plasmin, hyaluronidase, and clostripan at temperatures ranging from 30-50°C did not improve islet isolation results. The LT1D pancreas was remarkably resistant to enzymatic and mechanical disruption, with notable formation of a coherent matrix that impaired liberation of islets. This outcome was not observed in more than 2000 islet isolations from non-diabetic donors or donors with shorter-duration T1D or T2D. LT1D pancreas slices generated according to published protocols often collapsed or folded during slicing, did not embed well in agarose, and had poor viability. Slices generated using an amended approach, with ductal agarose injection improved cutting, retention of the tissue in the gel, and viability.

**Conclusion:** In LT1D, the endocrine and exocrine compartments have altered morphology, vasculature and ECM, with significant loss of the islet-acinar boundary. These changes likely impact islet function, tissue perfusion, and have implications for drug or targeting vector delivery in vivo. These changes also likely impair the isolation of islets from LT1D donors. The pancreas slice technique with the amended approach may allow functional studies of the LT1D pancreas to better understand alpha cell dysfunction and prevent hypoglycemia in this population.

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