MANIPULATING PROGLUCAGON PROCESSING IN THE PANCREATIC ALPHA-CELL FOR THE TREATMENT OF DIABETES

by

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ABSTRACT

Glucagon-like peptide-1 (GLP-1) has received much attention as a novel diabetes therapeutic due to its pleotropic blood glucose-lowering effects, including enhancement of glucose-stimulated insulin secretion, inhibition of gastric emptying and glucagon secretion, and promotion of β-cell survival and proliferation. GLP-1 is produced in the intestinal L-cell via processing of the proglucagon precursor by prohormone convertase (PC) 1/3. Proglucagon is also expressed in the pancreatic α-cell; however, in this tissue PC2 is typically expressed instead of PC1/3, resulting in differential cleavage of proglucagon to yield glucagon as the major product. We hypothesized that expression of PC1/3 in the α-cell would induce GLP-1 production in this tissue, and that this intervention would improve islet function and survival.

Initial studies in α-cell lines demonstrate that adenoviral delivery of PC1/3 to α-cells increases GLP-1 production. By encapsulating and transplanting either PC1/3- or PC2-expressing α-cells, the following studies show that while PC2-expressing α-cells increase fasting blood glucose and impair glucose tolerance, PC1/3-expressing α-cells decrease fasting blood glucose and dramatically improve glucose tolerance in normal mice and in mouse models of diabetes. We further show that transplantation of PC1/3-expressing α-cells prevents streptozotocin (STZ)-induced hyperglycemia. We also found that PC1/3-expressing α-cells also improve cold-induced thermogenesis in db/db mice, demonstrating a previously unappreciated effect of one or more of the PC1/3-derived proglucagon products. Studies in isolated mouse islets demonstrate that adenoviral delivery of PC1/3 to isolated islets increases islet GLP-1 secretion and improves glucose-stimulated insulin secretion and islet survival. Experiments with diabetic mice show that these GLP-1-producing islets are better able to restore normoglycemia in recipient mice following islet transplantation.

Taken together, these studies demonstrate that the α-cell can be induced to process proglucagon into PC1/3-derived products, and that this shift redirects the α-cell from a hyperglycemia-promoting fate to a blood glucose-lowering one. This research opens up avenues for further investigating the therapeutic potential of inducing islet GLP-1 production in isolated human islets and in vivo in diabetes patients, and may represent a novel way to intervene in the progressive loss of β-cells that characterizes diabetes.
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<tr>
<td>ARG</td>
<td>arginine</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>AUC</td>
<td>area under the curve</td>
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<tr>
<td>BAT</td>
<td>brown adipose tissue</td>
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<tr>
<td>BCA</td>
<td>bicinchoninic acid</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
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<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
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<tr>
<td>CMV</td>
<td>cytomegalovirus</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
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<tr>
<td>CPE</td>
<td>cytopathic effects</td>
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<tr>
<td>CRE</td>
<td>cAMP response element</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DPPIV</td>
<td>dipeptidyl peptidase IV</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
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<tr>
<td>FACS</td>
<td>fluorescent-activated cell sorting</td>
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<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GIP</td>
<td>glucose-dependent insulinotropic polypeptide</td>
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<tr>
<td>GLP-1</td>
<td>glucagon-like peptide-1</td>
</tr>
<tr>
<td>GLP-1R</td>
<td>glucagon-like peptide-1 receptor</td>
</tr>
<tr>
<td>GLP-2</td>
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</tr>
<tr>
<td>GLP-2R</td>
<td>glucagon-like peptide-2 receptor</td>
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<tr>
<td>GRPP</td>
<td>glicentin-related pancreatic polypeptide</td>
</tr>
<tr>
<td>GSIS</td>
<td>glucose-stimulated insulin secretion</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
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<tr>
<td>HD</td>
<td>high dose</td>
</tr>
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<td>HG-DMEM</td>
<td>high glucose DMEM</td>
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<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>ICC</td>
<td>immunocytochemistry</td>
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<tr>
<td>i.c.v.</td>
<td>intracerebroventricular</td>
</tr>
<tr>
<td>IE</td>
<td>islet equivalent</td>
</tr>
<tr>
<td>IFNγ</td>
<td>interferon-γ</td>
</tr>
<tr>
<td>IL-1β</td>
<td>interleukin-1β</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>i.v.</td>
<td>intravenous</td>
</tr>
<tr>
<td>IP-1</td>
<td>intervening peptide-1</td>
</tr>
<tr>
<td>IP-2</td>
<td>intervening peptide-2</td>
</tr>
<tr>
<td>IPGTT</td>
<td>intraperitoneal glucose tolerance test</td>
</tr>
<tr>
<td>ITT</td>
<td>insulin tolerance test</td>
</tr>
<tr>
<td>KRBB</td>
<td>Krebs’ Ringer bicarbonate buffer</td>
</tr>
<tr>
<td>LD</td>
<td>low dose</td>
</tr>
<tr>
<td>MEM</td>
<td>modified Eagle’s medium</td>
</tr>
<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
</tr>
<tr>
<td>MOPS</td>
<td>morpholinepropanesulfonic acid</td>
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<tr>
<td>MPGF</td>
<td>major proglucagon fragment</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>NOD</td>
<td>non-obese diabetic</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>Nx</td>
<td>nephrectomy</td>
</tr>
<tr>
<td>OGTT</td>
<td>oral glucose tolerance test</td>
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<td>OXM</td>
<td>oxyntomodulin</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>prohormone convertase</td>
</tr>
<tr>
<td>PC1/3</td>
<td>prohormone convertase 1/3</td>
</tr>
<tr>
<td>PC2</td>
<td>prohormone convertase 2</td>
</tr>
<tr>
<td>PCNA</td>
<td>proliferating cellular nuclear antigen</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PFU</td>
<td>plaque-forming units</td>
</tr>
<tr>
<td>PGDP</td>
<td>proglucagon-derived peptide</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKB</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>PTX</td>
<td>pancreatectomy</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene difluoride</td>
</tr>
<tr>
<td>PYY</td>
<td>peptide YY</td>
</tr>
<tr>
<td>rcf</td>
<td>relative centrifugal force</td>
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<tr>
<td>RPG</td>
<td>rat proglucagon promoter</td>
</tr>
<tr>
<td>RIA</td>
<td>radioimmunoassay</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>STZ</td>
<td>streptozotocin</td>
</tr>
<tr>
<td>T1D</td>
<td>type 1 diabetes</td>
</tr>
<tr>
<td>T2D</td>
<td>type 2 diabetes</td>
</tr>
<tr>
<td>TAM</td>
<td>tamoxifen</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumour necrosis factor α</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris(Hydroxymethyl)aminomethane-buffered saline</td>
</tr>
<tr>
<td>TBST</td>
<td>TBS + 0.1% Tween</td>
</tr>
<tr>
<td>WAT</td>
<td>white adipose tissue</td>
</tr>
<tr>
<td>Xgal</td>
<td>X-galactosidase</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

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INTRODUCTION

Healthy humans have a remarkable ability to maintain blood glucose levels within a fairly narrow range despite wide variation in the types and amounts of food ingested, changing levels of physical activity, and longer-term alterations in metabolic requirements. This ability to defend blood glucose levels is critically important since left untreated, hypoglycemia can result in loss of consciousness and even death, while chronic hyperglycemia damages the fine nerve endings and microvasculature, which can lead to cardiovascular disease, stroke, renal failure, and loss of vision. Insulin, a hormone produced by the pancreatic β-cell, bears the primary responsibility for lowering blood glucose levels and does so by facilitating glucose uptake from the circulation to peripheral metabolic tissues (e.g. muscle and adipose tissue). Glucagon, a hormone released from the pancreatic α-cells, serves a key counter-regulatory role in opposing insulin action by increasing hepatic glucose output.

NORMAL ISLET STRUCTURE AND FUNCTION

α-cells and β-cells are organized within structures called the islets of Langerhans along with somatostatin-secreting δ-cells and much less numerous pancreatic polypeptide-secreting PP cells and ghrelin-secreting ε-cells (1, 2). These clusters of endocrine cells are found scattered throughout the pancreas and make up only ~1% of total pancreatic mass, the remainder consisting of exocrine tissue, which secretes digestive enzymes, as well as microvasculature, nerves, and ducts (3). In the healthy adult rodent islet, β-cells make up the majority of the islet cells and tend to be located in the centre core of the islet, with α- and δ-cells situated on the islet periphery (1, 3), though islets located in the dorsal pancreas seem to have more non-β-cells than those of ventral origin (4). In humans and non-human primates, non-β-cells make up a greater number of total islet cells, and are dispersed throughout the islet rather than being largely limited to the islet periphery as in rodent islets (5, 6). The reasons for these species-specific differences in islet architecture remain unclear, though it is possible that different paracrine interactions and/or vascular connections between islet cells exist amongst species. The loss of normal islet architecture in both rodents (7-9) and humans (10-13) in disease states such as diabetes suggests that maintenance of cellular organization within the islet is important, though how islet architecture might contribute to normal islet function remains to be determined.
Islets are highly vascularized and innervated to allow for rapid titration of pancreatic hormone release to match changing metabolic needs. Blood flow has been proposed to proceed from the $\beta$-cell to the $\alpha$-cell to the $\delta$-cell, though this remains controversial (3, 14) and has recently been challenged with respect to the human islet (6). Nevertheless, such an arrangement is consistent with the finding that anterograde but not retrograde perfusion of anti-insulin antibodies increases glucagon secretion (15-17).

**Diabetes and the Current Treatment Paradigm**

Diabetes mellitus is a metabolic disorder characterized by defective production, secretion, and/or action of insulin. It is estimated that, with new cases of diabetes developing at a rate of 7 million per year, the total number of individuals with diabetes will be $\sim$380 million by 2025. Despite vast global spending on the prevention and treatment of diabetes (~US$232 billion in 2007), it remains the 4th leading cause of disease-related death, with $>3.8$ million fatalities annually (18). For many patients, diabetes not only reduces life expectancy and quality of life, but also presents significant financial pressure in the form of treatment costs and lost income when the disease interferes with work. In addition to the burden this places on patients and their families, diabetes threatens to place worldwide economic strain on governments and health insurance providers due to direct treatment costs and lost productivity arising from diabetes-related illness, disability, and premature death. There is therefore significant incentive to better understand the pathology of this disease and to apply effective prevention and treatment regimens.

The most common form of diabetes is type 2 diabetes (T2D), in which peripheral insulin resistance develops. Compensatory changes, including increased $\beta$-cell size/number and enhanced insulin secretion, are initially made in order to maintain normal blood glucose levels. However, if these mechanisms fail such that insulin is no longer secreted at levels sufficient to maintain blood glucose levels within an appropriate range, T2D develops (19). T2D is associated with obesity and accounts for the majority of cases of diabetes. Current therapies include diet/exercise interventions; insulin secretagogues, including the sulfonylureas; $\alpha$-glucosidase inhibitors, which decrease carbohydrate breakdown in the gut; thiazolidinediones and related drugs, which sensitize peripheral tissues to insulin; metformin and related drugs, which decrease gluconeogenesis from the liver; and in late stages of disease, insulin (19, 20).
However, none of these drugs reverse the onset of diabetes—disease progression is delayed at best (21).

In contrast to T2D, type 1 diabetes (T1D) affects a much smaller number of people and develops when β-cells undergo autoimmune destruction, resulting in a lack of endogenous insulin. Patients must therefore depend upon multiple daily injections of exogenous insulin to maintain good control of blood glucose levels. However, it is impossible to match the sensitive, dynamic insulin secretion profile achieved by the healthy β-cell with injections. Moreover, injections carry the risk of inducing potentially fatal hypoglycemia and do not prevent the development of pathologies secondary to diabetes (22, 23). A true cure for T1D will thus involve replacing lost β-cell function by organ transplantation, stimulating β-cell regeneration \textit{in vivo}, inducing differentiation of progenitor cells to insulin-producing cells for transplantation, stimulating insulin production by “surrogate” cells, or reversing autoimmune attack (24). Whole pancreas transplant has been used to treat diabetes and results in moderate blood glucose control, but is a difficult and risky surgical intervention. Transplantation of whole islets, isolated from cadaveric donors, represents a simpler and safer means of transplanting surrogate insulin-producing cells to T1D patients (25), but is not without its own shortcomings. Transplant recipients require lifelong immunosuppression (to prevent graft rejection and also recurrence of autoimmune β-cell destruction), and in many cases islet function and survival remains insufficient to maintain insulin independence over the long-term (26, 27). Perhaps most importantly, however, there is a significant supply and demand imbalance—current organ donation and transplant practices yield enough islets for perhaps 1000 transplants (<0.1% of T1D patients) per year in the U.S. (24). Many patients require islets from 2-3 cadaveric donors to achieve insulin independence. This is thought to relate to poor islet survival in the immediate post-transplant period, perhaps due to glucotoxicity (28), hypoxia (29) and limited graft vascularization (30), and/or cytotoxic and anti-proliferative effects of commonly used immunosuppressants (31-33). Therefore, because of the increasing incidence of and costs associated with both major forms of diabetes, and due to the inadequacy of currently available treatments, there is great interest in developing better treatments for these diseases.
PROGLUCAGON AND THE PROGLUCAGON-DERIVED PEPTIDES (PGDPs)

History and discovery of the PGDPs and the incretin concept

It has long been recognized that the secretory activity of the endocrine pancreas can be modulated by gastrointestinal-derived hormones (34-37). The development of an insulin radioimmunoassay (38) facilitated quantification of this phenomenon, and it became evident that there is a much greater insulin secretory response to oral glucose compared to intravenous glucose (39, 40). The mediators of this effect were termed “incretins”, a term first proposed by Labarre in 1932 (41), the criteria for inclusion in this category being that these mediators must be released in response to ingested nutrients and further, that they must stimulate insulin secretion from pancreatic β-cells (41, 42). The gut-derived hormone gastric inhibitory polypeptide (GIP) was named for its ability to inhibit gastric acid secretion (43), but the acronym GIP was given the alternate designation glucose-dependent insulinotropic polypeptide following reports of this namesake novel activity in the perfused rat pancreas and in vivo in dogs and humans (44-47). The N-terminally truncated forms of glucagon-like peptide-1 (GLP-1) were later recognized to also have potent insulinotropic activity in the perfused rat pancreas (48) and in humans administered doses approximating postprandial plasma levels (49). Today, GIP and GLP-1 are widely considered to be incretin hormones and together are estimated to be responsible for 50% of postprandial insulin secretion (50, 51). Several studies have investigated the relative physiological contribution of the incretin hormones in potentiating insulin release. While GLP-1 stimulates insulin secretion at lower concentrations than GIP, plasma levels of GIP appear to be several-fold greater (49, 52, 53). Indeed, it has been suggested that GLP-1’s primary physiological role may lie not in potentiating insulin release but rather in suppressing glucagon release and in slowing gastric emptying (54). Nevertheless, immunoneutralization and receptor agonist studies have confirmed the importance of both GIP and GLP-1 in blood glucose homeostasis (55-59).

Glucagon-like activity was first identified in bovine pancreatic extracts (60), and later established as glucagon, a product of islet α-cells. However, following the development of assays for glucagon-like immunoreactivity (61), it became clear that glucagon-like peptides were also present in specific areas of the intestine and the brain. The cloning of the proglucagon cDNA (62-68) and the isolation and sequencing of the intestinally derived glucagon-like peptides led to the identification and localization of the various PGDPs. Amongst the PGDPs identified, the most extensively studied are glucagon, GLP-1, and glucagon-like peptide-2 (GLP-2).
The glucagon superfamily

The PGDPs belong to the larger glucagon superfamily of peptide hormones, which also includes pituitary adenylate cyclase-activating polypeptide, glucagon, growth hormone-releasing factor, vasoactive intestinal polypeptide, secretin, and GIP (51, 69). Despite diverse and sometimes opposing regulatory functions, the glucagon superfamily members bear significant similarity in terms of gene organization and peptide sequence (see Fig. 1). The proglucagon gene encodes GLP-1, GLP-2, glucagon, oxyntomodulin, glicentin, glicentin-related pancreatic polypeptide (GRPP), and several intervening peptides. Of these, three bioactive peptides—glucagon, GLP-1, and GLP-2—have well-defined effects on nutrient homeostasis, while the function of the others remains largely unknown, though a role for oxyntomodulin in regulation of energy expenditure and food intake is emerging (70-72).

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>GLUCAGON</td>
<td>HSQGFTSDYSKLDSSRAQDFVQWLMT</td>
</tr>
<tr>
<td>GIP</td>
<td>YAEFTFSYSDKTHQODFVYNLLAQKGKKNDWKHNITQ</td>
</tr>
<tr>
<td>GLP-17-37</td>
<td>HAEGFTSDVSYLEGQAKFSIAWLVRKGR</td>
</tr>
<tr>
<td>GLP-17-36NH2</td>
<td>HAEGFTSDVSYLEGQAKFSIAWLVKGRNH2</td>
</tr>
<tr>
<td>EXENDIN-4</td>
<td>HGEGFTSDLJMQEVEAVRLFIEMWLKLNGGPPSSAPPPSH2</td>
</tr>
<tr>
<td>GLP-2</td>
<td>HADGSFSDEMNTILDNLAARDPFINWLQOTKITD</td>
</tr>
</tbody>
</table>

Figure 1: Sequence alignment of several glucagon superfamily members

Amino acid sequences of several members of the glucagon superfamily are shown. Standard one-letter amino acid code has been used to indicate amino acid sequence, and residues identical to those in glucagon are underlined and bold.

In addition to significant homology amongst the PGDPs, their sequences are themselves highly conserved amongst different species; for example, GLP-1’s amino acid sequence is identical amongst all mammalian species examined thus far (51). This high degree of conservation is indicative of the importance of these peptides as key mediators of nutrient homeostasis and metabolic function.

Synthesis of proglucagon

The proglucagon gene is expressed in the pancreatic α-cells, the intestinal L-cells, and in the neurons of the caudal nucleus tractus solitarius and the hypothalamus. Gene expression is controlled by the proglucagon gene promoter, in which several key transcriptional elements have been identified, largely via studies using immortalized glucagon-expressing cell lines (51). The G1 element is required for pancreatic α-cell-specific expression, while the G2, G3, G4, and G5
elements are enhancers for α-cell-specific expression (73-75). The presence of a cAMP response element (CRE) is consistent with the observation that GLP-1 and GLP-2 production is cAMP-dependent (76). The intestinal specific element is required for intestinal L-cell-specific proglucagon expression (77). A number of transcription factors, including Pax-6 (78), Pax-4 (79), Foxa1 (80), Cdx-2/3 (81, 82), c-Maf (83), Brn-4 (84), and Isl-1 (85), have been shown to interact with the various gene regulatory elements in the proglucagon promoter.

The prohormone convertases and processing of proglucagon

While in lower vertebrates alternative exon splicing is responsible for generating the various PGDPs (64), in mammals it is differential post-translational processing which results in the production of PGDPs from the proglucagon precursor (51, 86). This processing is carried out by prohormone convertases (PCs) 1/3 and 2, which belong to the larger family of subtilisin-like PCs named thus due to homology of their catalytic domains to that of the bacterial serine protease subtilisin (87). Members of this family share specificity for dibasic sequences in substrates (typically Lys-Arg or Arg-Arg), and can be broadly classified into two subtypes: those expressed ubiquitously, including furin and PACE4, which are involved in processing of proteins in the constitutive secretory pathway; and those with more limited tissue distribution, including PC1/3, PC2, PC4, PC5/6, and PC7, which are involved in processing in the regulated secretory pathway (87). Members of this family contain an N-terminal signal peptide and proregion, a catalytic region, a P domain, and a C-terminal domain (87).

The PCs themselves are synthesized as propeptides that become fully activated as they move through the secretory pathway. The proregion of PC1/3 is rapidly cleaved by autocatalysis in the endoplasmic reticulum (ER) but remains associated with the propeptide until reaching the acidic, calcium-rich distal Golgi, when the proregion is released (88). An additional autocatalytic cleavage occurs in the C-terminal domain in the maturing secretory granules to yield fully bioactive PC1/3 (89, 90). Compared to PC1/3 and other PC family members, PC2 is retained in its inactive precursor form for a longer period of time owing to its slow folding time in the ER and its interaction with the neuroendocrine protein 7B2 (91), which seems to stabilize proPC2 in a form amenable to transport from the ER to the Golgi (92). From the trans-Golgi, sorting of both PC1/3 and PC2 to the secretory granules of the regulated pathway occurs via an α-helical domain in the C-terminal region (93, 94). This region, when fused to a protein normally destined for the constitutive pathway, is sufficient to redirect proteins to the regulated
secretory pathway by facilitating interaction with the membrane of the nascent secretory granules (95). In this way, PC1/3 and PC2 mature and are targeted to the dense core granules of the regulated secretory pathway along with their substrates; this facilitates the generation and exocytosis of mature, fully bioactive peptides.

The glucagon-like peptides, along with oxyntomodulin and glicentin, are liberated from the proglucagon precursor via PC1/3-mediated processing in the brain and enteroendocrine L-cells (96-98), located in high concentration in the distal intestine (Fig. 2). The role of PC1/3 in the processing of proglucagon to release GLP-1 is highlighted by the PC1/3 knockout mouse, in

Figure 2: Processing of proglucagon by PC1/3 or PC2
(A) Proglucagon contains multiple mono-/di-basic sites (indicated in standard three-letter amino acid code, along with their position in the proglucagon molecule) which are cleaved by PC2 or PC1/3. Some sites are specific for cleavage by PC2 (Lys-Arg at position 62-63) or PC1/3 (Arg at position 77, Arg-Arg at positions 109-10 and 124-125) while others can be cleaved by either enzyme (Lys-Arg at positions 31-32 and 70-71). (B) Processing of proglucagon by PC2 at positions 31-32, 62-63, and 70-71 yields GRPP, glucagon, IP1, and the major proglucagon fragment (99-103). (C) Processing of proglucagon by PC1/3 at positions 70-71, 109-110, and 124-125 yields glicentin, GLP-1_{1-37} or GLP-1_{1-36NH2}, IP2 and GLP-2 (96-98, 104-107). Glicentin and GLP-1 can be further cleaved by PC1/3 at positions 31-32 and 77 respectively to yield oxyntomodulin and GLP-1_{7-37} or GLP-1_{7-36NH2}. GLP-1_{1-37} and GLP-1_{7-37} are converted into GLP-1_{1-36NH2} and GLP-1_{7-36NH2} respectively by the activity of the enzyme peptidylglycine α-amidating monooxygenase (108, 109).
which GLP-1 and GLP-2 are undetectable in plasma, while processing of glucagon remains intact (104-107). Interestingly, a human patient with PC1/3 deficiency has been reported to have measurable circulating levels of immunoreactive GLP-1 despite an inactivating mutation in the PC1/3 gene (110, 111). This suggests that in humans an alternate PC may be able to compensate at least in part for lack of PC1/3 activity. The most likely candidate is PC5/6, given its broad expression pattern in the intestine (112, 113) and that in an in vitro system, it is capable of cleaving proglucagon at many of the sites normally cleaved by PC1/3, the arginine residue at position 77 being a notable exception (99).

In contrast to the L-cells, in the pancreatic α-cells, the expression of the alternate enzyme PC2 results in a different cleavage pattern for proglucagon (see Fig. 2) which results in glucagon and the major proglucagon fragment (MPGF) as the major products yielded (100, 101). The absolute requirement for PC2 in the liberation of glucagon is highlighted by the PC2−/− mouse, which demonstrates a severe defect in the processing of proglucagon to glucagon and gross compensatory hyperplasia and hypertrophy of pancreatic α-cells (102, 103). Studies in an in vitro system have confirmed that processing of proglucagon at the dibasic residues 62 and 63 to release glucagon seems to be exclusive to PC2, with no other (known) PCs capable of processing at this site (99).

**Physiological and Pharmacological Effects of the PGDPs**

**Secretion and metabolism of the PGDPs**

In addition to regulation at the level of expression, the glucagon-like peptides are also subject to regulation at the secretory level. The diffuse distribution of L-cells throughout the mucosa has complicated study of the mechanisms controlling the release of PGDPs from the L-cells, although the development of primary intestinal cell cultures and tumor-derived cell lines has been helpful in this area. It is clear that regulation is complex and integrated, involving both neural and hormonal inputs in addition to nutritional ones.

The precise mechanism by which the presence of ingested nutrients in the upper intestinal tract is coupled to secretion of PGDPs from the distal gut is not completely understood. The primary stimulus for release of hormones from the L-cell into the bloodstream is ingestion of carbohydrates or fats, while systemic glucose administration does not enhance plasma levels of intestinal PGDPs (114, 115). It appears that secretion of GLP-1 and GLP-2 is stimulated to a greater degree by long-chain, monounsaturated fatty acids than by short-chain, polyunsaturated,
or saturated fatty acids (116). Ingestion of protein-containing meals also enhances secretion; however, proteins or amino acids alone do not seem to stimulate GLP-1 release (114). GLP-1 secretion may involve the sodium/glucose co-transporter, since sugars which make use of this transporter (e.g. glucose, galactose) are potent stimulators of secretion, while non-transportable sugars are not (117). It is unlikely that direct nutrient contact with L-cells is a requirement for secretion of the glucagon-like peptides since their levels increase within 10 minutes of nutrient ingestion (114) and the majority of nutrients are absorbed prior to reaching L-cells of the distal GI tract (118). Nevertheless, it has recently been reported that the L-cell expresses α-gustducin, a G protein that couples activation of taste receptors to downstream intracellular signalling cascades, and that α-gustducin null mice have deficient postprandial GLP-1 secretion (119). This suggests that taste receptors may function as a direct ‘glucose sensor’ in the L-cell, although it is likely that there are other hormonal and neural mediators regulating hormone release from the L-cell. For example, GIP, released from the upper intestinal tract K-cells, may potentiate GLP-1 and GLP-2 release from the more distal L-cells via direct and/or indirect means (120, 121). However, there is a lack of clear evidence elucidating these pathways in humans. An alternate possibility is that a population of K/L cells may exist in the mid-small intestine, resulting in the simultaneous secretion of GIP, GLP-1, and GLP-2 (122, 123).

Several circulating forms of each of the glucagon-like peptides have been identified. GLP-21-33, GLP-17-36 amide, and GLP-17-37 are generally understood to be the most highly bioactive forms of the glucagon-like peptides, while GLP-23-33, GLP-19-36 amide, and GLP-19-37 are less active early breakdown products resulting from cleavage by the ubiquitous serine protease dipeptidyl peptidase IV (DPPIV; (124, 125)). GLP-17-36amide and GLP-17-37 have been shown to be equipotent in terms of their insulinotropic activity (126), although in humans the majority of GLP-1 secreted from the gut is GLP-17-36amide (127). DPPIV activity results in a very short circulating half life (~1-2 min) for both GLP-17-36amide and GLP-17-37 (124, 128, 129). The role of DPPIV in GLP-1 clearance is highlighted by the enhanced circulating bioactive GLP-1 levels seen both in DPPIV-deficient animals and in animals administered DPPIV-resistant GLP-1 analogs (130). It is clear that the kidney also plays a role in clearance of both GLP-1 and GLP-2, based on increased circulating immunoreactive peptide levels in patients with renal failure and in nephrectomized animals (131, 132). Thus activity of GLP-1 and GLP-2 is controlled not only at the level of secretion, but also via clearance mechanisms which function as a fast ‘metabolic brake’ to allow for minute-to-minute control over circulating levels of these hormones.
Physiological actions of GLP-1, GLP-2 and oxyntomodulin

As a result of binding its receptor (GLP-1R) in various tissues, GLP-1 elicits effects including inhibition of glucagon secretion, gastric emptying, and food intake, and induction of satiety (51, 133). Perhaps its best-documented effect is its stimulation of insulin synthesis and secretion (48, 49, 134). A critical difference between GLP-1 and the sulfonylurea class of drugs is the glucose-dependence of GLP-1’s insulinotropism—it effects insulin secretion only when glucose concentrations exceed ~4.5 mM (135, 136), ensuring that insulin is not inappropriately released under low glucose conditions. GLP-1 can induce glucose responsiveness in otherwise unresponsive β-cells (136), and it enhances transcription of specific genes involved in glucose sensing and metabolism (for example, Glut-2, glucokinase, and hexokinase-I), thereby enhancing the long-term function of β-cells (137). Furthermore, GLP-1 is a potent inhibitor of glucagon secretion (138, 139). Importantly, this effect is observed only when blood glucose levels exceed normal fasting levels (140), preventing GLP-1-induced inhibition of glucagon secretion under low glucose conditions, which would perpetuate hypoglycemia. The mechanism of GLP-1’s glucagonostatic effect has not been fully elucidated, but is likely mediated indirectly through increased insulin and somatostatin secretion (141), both of which inhibit glucagon secretion, and/or via direct activation of the GLP-1R on the α-cell (142). GLP-1 is also a counterregulatory hormone opposing the actions of leptin, which suppresses insulin synthesis and secretion (51). The counterregulatory nature of GLP-1 and leptin is evident when either hormone is disrupted; the GLP-1R−/− mouse has increased leptin sensitivity (143), while mice with mutated leptin receptors have enhanced sensitivity to GLP-1 (144).

In addition to its effects on hormone secretion, GLP-1 has been shown to act as a trophic factor for β-cells, with evidence that this is the result of induction of neogenesis (145, 146) and proliferation (147), and inhibition of apoptosis and necrosis (148-150). Short-term treatment with a GLP-1R agonist increases β-cell regeneration in response to partial pancreatectomy in rodents (151, 152), enhances human islet survival during culture (153), promotes recovery from treatment with a β-cell toxin in neonatal rats (145), and, when administered along with an immunosuppressant, induces remission from diabetes in 88% of non-obese diabetic (NOD) mice (154). The importance of GLP-1 signalling in the normal pancreas is highlighted by the GLP-1R−/− mouse, which exhibits abnormal islet topography and mild glucose intolerance as well as impaired protection and recovery from β-cell insult (155). The proliferative and reparative properties of GLP-1 may thus provide one means of maintaining a functional mass of β-cells,
even under conditions of injury. However, the doses used to promote β-cell growth in rodents (154, 156, 157) are typically much higher than can be tolerated in humans (158). Therefore it may not be possible to harness the β-cell growth-promoting actions of GLP-1R agonists when administered by peripheral injection.

GLP-1, GLP-2, and oxyntomodulin are all thought to have a role in regulating food and water intake. Intracerebroventricular (ICV) administration of GLP-1 inhibits food intake in rats, and a recent study reported that gastric distension activates medullary GLP-1- and GLP-2-containing neurons in rats (159). Oxyntomodulin and GLP-2 also suppress food intake in rodents (160-162), and disruption of GLP-1R signalling by genetic or pharmacological means results in enhanced inhibition of food intake by GLP-2 (163). This suggests that at least in rodents, GLP-1 and GLP-2 may work in concert to regulate food intake. A role for GLP-1 (164, 165) and oxyntomodulin (166), but not GLP-2 (164, 165), in decreasing food intake has been described in humans. This has led to the investigation of oxyntomodulin as a treatment for obesity in humans, as will be further discussed later.

In addition to stimulating the activity of central neurons involved in satiety, GLP-1 has a longer-term role in neuroprotection and cognition in rodents, and has effects on the cardiovascular system. GLP-1 promotes survival of hippocampal neurons following treatment with apoptosis-inducing agents including kainate and glutamate (167, 168). It also enhances learning, memory, and neuroprotection in rodents, while the learning deficits evident in GLP-1R knockout mice are corrected by restoration of central GLP-1R expression (168, 169). It has therefore been proposed that GLP-1R agonism may have therapeutic potential for the treatment of neurodegenerative disease (170). GLP-1R agonists also increase heart rate and blood pressure, although these effects are more pronounced in rodents (171) than in humans (172, 173). The importance of endogenous GLP-1 in cardiac structure and function is highlighted by the finding that GLP-1R knockout mice have abnormal cardiac contractility and altered myocardial structure (174). GLP-1 infusion also improves left ventricular function in patients with a myocardial infarct or heart failure, and is associated with better recovery from angioplasty (175, 176). Interestingly, cardiovascular function in dogs is improved after administration of the truncated peptide GLP-1-9-36NH₂, suggesting that an alternate receptor for GLP-1-9-36NH₂ may mediate the cardiovascular effects of native GLP-1 (177). Taken together, these studies suggest that GLP-1 and/or its truncated metabolites may be therapeutically useful in the field of cardiovascular medicine,
GLP-1, GLP-2, and oxyntomodulin also have effects on the gastrointestinal system. For some time it has been supposed that there exists in humans an ‘ileal brake’ which inhibits gastric emptying. By slowing the delivery of gastric chyme into the small intestine, this ‘brake’ ensures that the intestine receives chyme in amounts that it is capable of neutralizing, breaking down, and ultimately, absorbing nutrients from. GLP-1 and GLP-2 are thought to mediate the ileal brake effect by inhibiting gastric acid secretion and gastric motility, perhaps via indirect stimulation of central satiety centers (117, 178), and oxyntomodulin also inhibits gastric acid secretion and gastric emptying (179-181). Indeed it has been argued that, at least in healthy humans, the primary effect of GLP-1 may be delayed gastric emptying rather than promotion of insulin secretion (54). However, others have shown that administration of the GLP-1R antagonist exendin\textsubscript{9-39} increases postprandial insulin secretion in healthy humans, suggesting that endogenous GLP-1 plays an essential role in maintaining normal glucose tolerance (182). Nevertheless, GLP-1, GLP-2, and oxyntomodulin all exert potent effects on gastric motility.

GLP-2 seems to have important effects beyond inhibition of gastric emptying in the gastrointestinal tract. It is well-established that circulating GLP-2 levels increase significantly following bowel resection, and that this is associated with enhanced proliferation and decreased apoptosis of enterocytes (130). Exogenous GLP-2 promotes crypt cell proliferation, increases crypt depth and villus height, and thereby increases mucosal tissue mass and surface area in rodents (183-185). Exogenous GLP-2 also protects crypt cells and enterocytes against experimentally-induced apoptosis (186, 187), and has been demonstrated to have anti-resorptive effects on bone in post-menopausal women (188). The intestinal PGDPs thus have a remarkably diverse array of effects on multiple tissues and systems.

**Role of islet GLP-1 in development and adaptation**

While in the normal adult pancreas \(\alpha\)-cells produce little GLP-1 owing to their predominant expression of PC2 rather than PC1/3, it seems that PC1/3 expression is activated in \(\alpha\)-cells under certain conditions, resulting in GLP-1 production. In mice, the earliest pancreatic progenitor cells co-express proglucagon, islet amyloid polypeptide (restricted to \(\beta\)-cells in the adult mouse), PC2, and PC1/3, suggesting that these cells may produce GLP-1. It is possible that the GLP-1 produced by these cells may play a role during normal pancreatic development (189). In addition, numerous models of pancreatic injury are characterized by \(\alpha\)-cell hyperplasia and islet GLP-1 production. Treatment of neonatal rats with streptozotocin (STZ) induces the
appearance of islet cells coexpressing multiple islet hormones, including GLP-1, and α-cell hyperplasia, but this latter effect is absent when exendin9-39, a GLP-1R antagonist, is administered after STZ (190, 191). STZ treatment of adult rats increases expression of PC1/3 and PC2 in glucagon-immunoreactive cells in islets, and this is correlated with increased levels of bioactive GLP-1 in islet extracts and plasma (152). Further support for the role of GLP-1 in stimulating islet regrowth comes from the finding that GLP-1R−/− mice exhibit impaired recovery from partial pancreatectomy (151). Disruption of glucagon receptor expression in rodents has also been associated with increased pancreatic and serum levels of GLP-1, α-cell hyperplasia/hypertrophy, and enhanced islet, but not gut, preproglucagon mRNA levels (192, 193). Taken together, these studies confirm that islets have the capacity to produce GLP-1, and further, suggest that the liberation of GLP-1 in α-cells by the expression of PC1/3 may be a natural strategy employed by islets to promote islet formation and function under specific conditions.

Mechanisms of action of the PGDPs

The PGDPs bind receptors that have been identified as members of the seven transmembrane spanning G protein-coupled family of receptors, which signal largely via the activation of adenylylate cyclase. Adenylylate cyclase catalyzes an increase in intracellular cAMP, which can have varied effects depending on the cell type in question. cAMP-dependent effects may include changes in intracellular calcium levels and ion channel activity, activation of transcription factors and subsequent changes in gene transcription levels, and exocytosis of stored hormones. In β-cells, for instance, GLP-1-induced enhancement of cAMP levels results in closure of ATP-sensitive K⁺ channels, which depolarizes the cell and, in turn, activates voltage-dependent Ca²⁺ channels to allow Ca²⁺ influx (194, 195). GLP-1 binding may also activate Ca²⁺ release from intracellular Ca²⁺ stores (196). The net increase in intracellular Ca²⁺ levels is the key stimulus for insulin release, coupling GLP-1 release to enhanced insulin secretion. In addition, GLP-1 regulates insulin gene transcription (and transcription of a host of other genes critical to normal β-cell function) via a PKA-dependent mechanism (197).

The GLP-1R has a strong binding affinity for GLP-1, while binding other ligands from the glucagon superfamily with lower affinity or not at all (198, 199). Exendin-4, a peptide isolated from the lizard Heloderma suspectum which bears 53% amino acid sequence homology to GLP-1, is a strong GLP-1R agonist, while the truncated form (exendin9-39) is a potent receptor antagonist which blocks GLP-1 binding (200). Oxyntomodulin is a known ligand for the GLP-
and it is through this receptor that its effects on food intake are mediated (72), although it also has GLP-1R-independent effects (201) mediated via an alternate receptor which remains to be identified. The broad expression pattern of the GLP-1R, which has been identified in brain, lung, pancreatic, gastrointestinal, heart, and kidney tissues, reflects GLP-1’s wide-ranging effects exerted in different systems.

In contrast to the GLP-1 receptor, much less is known about the GLP-2R. Study of the GLP-2R has been complicated by the lack of tumor-derived cell lines and enriched populations of enteroendocrine cells expressing the GLP-2R, and by the lack of available GLP-2R agonists and antagonists. GLP-2R expression has been localized to a distinct subset of intestinal enteroendocrine cells (expressing GIP, serotonin, PYY, chromogranin, or GLP-1) and not to intestinal epithelial cells (117). This receptor distribution suggests that while some of GLP-2’s effects may be directly exerted on the GLP-2R-bearing enteroendocrine cells, many effects may be mediated via an autocrine, paracrine, or endocrine factor released from enteroendocrine cells which acts on intestinal epithelial cells. Indeed, GLP-2’s pro-proliferative effects appear to involve GLP-2R-expressing enteric neurons which activate crypt cell proliferation (155, 202, 203). The GLP-2R has also been localized to the brain (204, 205), suggesting that GLP-2 may have central effects which are, as of yet, unidentified, and more recently to the pancreatic α-cell (206).

The therapeutic potential of GLP-2, oxyntomodulin, and GLP-1

Based on their potent biological activities, all three of the PGDPs arising from the gut are either under investigation or in usage as therapeutic agents. GLP-2 may have therapeutic utility in treating intestinal disorders including small bowel resection, colitis, drug-induced intestinal injury, inflammatory bowel syndrome, and others (155). Both native GLP-2 and a DPPIV-resistant GLP-2 analog are currently in clinical trials for the treatment of short-bowel syndrome, where treatment has thus far been shown to enhance weight gain and intestinal nutrient absorption in subjects (207, 208).

The potent effect of exogenous oxyntomodulin on food intake in rats (160, 161) has led to recent clinical studies investigating the potential of this hormone as a weight loss agent. In one study, i.v. infusion of oxyntomodulin for 90 min decreased ad libitum feeding and hunger sensation in humans, without nausea or food aversion (166). Longer-term, thrice daily subcutaneous dosing has also been found to be effective, resulting in one study in a 0.5 kg weight loss per week in patients compared to placebo-treated controls (209). Weight loss
resulting from oxyntomodulin administration is thought to be the combined result of reduced food intake and increased energy expenditure (71). Therefore, oxyntomodulin may be a promising target for the treatment of obesity in humans.

Compared to GLP-2 and oxyntomodulin, to date GLP-1 has been the subject of much more intense clinical interest and development. The glucose-dependent insulinotropic and proliferative effects of GLP-1 make it an ideal candidate to enhance β-cell function and survival in diabetes patients without the risk of hypoglycemia. However, as discussed previously, GLP-1 is rapidly metabolized by DPPIV, resulting in a short in vivo half-life and limiting the effectiveness of exogenous GLP-1. Thus several alternate strategies for exploiting the beneficial effects of GLP-1 for diabetes therapy are under investigation.

One line of development has been aimed at developing DPPIV inhibitors to enhance endogenous active GLP-1 levels. The first in this class of drugs to receive clinical approval was sitagliptin (Januvia®), which in a single dose achieves 80% inhibition of DPPIV and a ~2-fold increase in circulating bioactive GLP-1 and GIP levels in humans (210). Administration of a DPPIV inhibitor for 10 d lowered glycated hemoglobin and fasting blood glucose levels in T2D patients (211). Longer-term DPPIV inhibition delivered in combination with metformin therapy for 24 wk decreased fasting blood glucose and postprandial glycemic excursion (212). However, DPPIV has a long and diverse list of substrates which it activates or inactivates, including at least 9 chemokines and many hormones and neuropeptides, among them prolactin, growth hormone-releasing hormone, PYY, and substance P (213). Whether long-term DPPIV inhibition produces meaningful changes in the bioactive levels of many of these substrates remains unclear and will require further study.

An alternative option has been to develop long-acting, DPPIV-resistant GLP-1 mimetics. One example is exendin-4, a peptide originally isolated from the venom of Heloderma suspectum that bears 53% amino acid sequence homology to GLP-1 (see Fig. 1) but is not subject to DPPIV-mediated degradation because it has a glycine residue in the penultimate position (214). Indeed, the first GLP-1 mimetic to receive FDA approval was Byetta®, a synthetic form of exendin-4, for the treatment of T2D as an adjunctive therapy for patients already treated with metformin and/or sulfonylureas. In most T2D patients twice daily injection of Byetta® improves fasting and post-prandial blood glucose, enhances insulin secretion, reduces glucagon secretion, delays gastric emptying, decreases food intake, and causes weight loss (215-217). In one recent study, a single bedtime injection of a long-acting GLP-1 derivative (t1/2~10 h) enhanced the
insulin secretory response, decreased basal and post-prandial glucose levels, and inhibited gastric emptying in human subjects with T2D (218). Very long-acting GLP-1R agonists are also being developed by conjugating GLP-1 mimetics to albumin derivatives to increase their plasma half-life (219-221). Whether any of these strategies impact β-cell mass will require further study. Moreover, many patients have reported dose-related adverse gastrointestinal events (e.g. cramping, nausea, and diarrhea) related to delayed gastric emptying, and use of Byetta® in conjunction with sulfonylureas may increase incidence of hypoglycemia (218, 222, 223).

One drawback of the GLP-1R agonists currently in clinical usage or undergoing clinical trials is that they must be injected, which may present problems with patient compliance. However, the first nonpeptidic GLP-1R agonists have recently been reported (224, 225). Thus far these agents have only been tested in rodents, and further clinical study will be required to assess their pharmacokinetics and efficacy in humans. Nevertheless, the identification of these agents suggests that one day orally available GLP-1R agonists, along with injectable GLP-1R agonists and DPPIV inhibitors, might become an important new class of drugs targeting GLP-1R signalling for diabetes therapy.
THESIS INVESTIGATION

The PC1/3-derived proglucagon product GLP-1 has received much attention as a novel diabetes therapeutic given its range of blood glucose-lowering effects. The GLP-1 analog Byetta® is presently in use as an injectable treatment for T2D, but while the currently used dosing regimens successfully increase glucose-dependent insulin secretion, these doses are 50-100 times lower than the minimum doses necessary to induce β-cell proliferation and survival in rodents. Moreover, the high incidence of nausea and cramping reported even at these low doses suggest that it may be impossible to take advantage of the full potential of GLP-1 on β-cell survival and proliferation using peripheral injection. Given the convenient location of α-cells near to the β-cells, the overall objective of this research was to examine whether we could harness the full potential of GLP-1 and other PC1/3-derived proglucagon products by upregulating PC1/3 expression in the α-cell.

The first major goal was to induce PC1/3 expression in an α-cell line and to characterize PC1/3-expressing versus PC2-expressing α-cell lines. Further, we sought to compare and contrast the metabolic effects of encapsulated α-cells expressing either PC1/3 or PC2 when transplanted into mice. A second line of investigation was aimed at elucidating the metabolic effects of encapsulated PC1/3-expressing α-cells in a rodent model of T2D, which led us to note previously unappreciated effects of one or more of the PC1/3-derived PGDPs on thermoregulation. Given the challenges associated with poor islet survival and function following islet transplantation in recipients with T1D, a third major goal was to examine whether GLP-1 production from α-cells could be induced in whole, isolated islets, and whether this intervention might improve islet function and survival. Taken together, these studies demonstrate that the α-cell can indeed be induced to process proglucagon into PC1/3-derived products, and that this shift redirects the α-cell from a hyperglycemia-promoting fate to a blood glucose-lowering one. This research opens up avenues for further investigating the therapeutic potential of inducing islet GLP-1 production in isolated human islets and in vivo in diabetes patients, and may represent a novel way to intervene in the progressive loss of β-cells that characterizes both major forms of diabetes.
MATERIALS AND METHODS

Tissue culture media, antibiotics, fetal bovine serum (FBS), X-galactosidase (X-gal), live/dead assay kit (Molecular Probes L-3224), TRIzol®, and Alexa Fluor-conjugated secondary antibodies used for immunocytochemistry were obtained from Invitrogen Canada (Burlington, ON). Collagenase, bovine serum albumin (BSA), glutaraldehyde, protease inhibitor cocktail (#8340), Cytodex I microcarrier beads, and goat anti-rabbit Cy3 and mouse anti-glucagon antisera were from Sigma-Aldrich Canada (Oakville, ON). Sensitive Insulin RIA, Active GLP-1 ELISA, Total GLP-1 RIA, and Glucagon RIA kits, as well as guinea pig anti-insulin antibody and DPPIV inhibitor were from Linco Research Inc. (St. Charles, MO). Glucagon, GLP-2, and human insulin ELISA and oxyntomodulin RIA kits were from Alpco Diagnostics (Salem, NH). Western blotting reagents were from GE Healthcare (Buckinghamshire, United Kingdom), and the Bradford and BCA kits for protein quantification were from Bio-Rad (Hercules, CA) and Pierce (Rockford, IL) respectively. Imaging and quantification was performed using an Axiovert 200 microscope (Carl Zeiss, Toronto, ON) connected to a digital camera (Retiga 2000R, QImaging, Burnaby, BC) controlled with Openlab 5.0 software (Improvision, Lexington, MA).

ANIMALS

All experiments were approved by the UBC Animal Care Committee. Male mice, age 8-10 weeks, were used except where noted. CD-1 mice were from the UBC Animal Care Facility; C57BL/6 (stock #000664), db/db mice and wildtype control C57BLKS/J mice (BKS.Cg-m +/- Lepr<sup>db</sup>/J, stock #000642 and stock #000662 respectively; received at 4 wk age), and diet-induced obese C57BL/6 mice (fed a 60% kcal from fat diet from 6 wk age and obtained at 16 wk age) were obtained from Jackson Laboratories (Bar Harbor, ME). Mice were maintained on a standard 12 h light/dark cycle at an environmental temperature of 18-22°C and received a standard chow diet (#5015 from Lab Diet Inc.; St. Louis, MO; contains 19.8% kcal from protein, 54.9% kcal from carbohydrates, and 25.3% kcal from fat, 60% of which is soy- or lecithin-derived and 40.0% of which is from lard) except for diet-induced obese mice, which were maintained on diet D12492 from Research Diets Inc. (New Brunswick, NJ; contains 20.0% kcal from protein, 20.0% kcal from carbohydrate, and 60% kcal from fat, comprised of 9.3% soybean oil and 90.7% lard). Blood glucose and body weight were monitored 2-3 times weekly following a 4 h a.m. fast. Blood glucose monitoring and survival blood sampling was carried out on
restrained, unanaesthetized mice via the tail vein or the saphenous vein. Blood samples were collected via heparinized 70 µL microcapillary tubes, promptly expelled into microcentrifuge tubes, and centrifuged at 7000 rpm for 9 min at 4°C. Plasma was drawn off to a fresh set of tubes and stored at -20°C until assay.

**Streptozotocin (STZ) treatment of C57BL/6 mice**

For studies involving transplantation of encapsulated αTC-1 or αTCΔPC2 cells, STZ (Sigma; St. Louis, MO) was freshly prepared in citrate buffer (40 mM; pH 4.5) and delivered by intraperitoneal injection (50 mg/kg) for 5 consecutive days beginning 8 d post-transplant in C57BL/6 mice. For islet transplantation studies, recipient mice were treated with a single intraperitoneal dose (200 mg/kg) of STZ 5 d prior to transplantation, and diabetes was confirmed by blood glucose readings >20 mM on at least 2 consecutive days prior to transplant. For all studies, STZ was injected within 15 min of preparation, and we routinely achieved >80% incidence of diabetes in STZ-treated mice.

**Glucose and insulin tolerance tests**

Except where noted, glucose tolerance tests were performed by oral gavage (OGTT) or intraperitoneal (IPGTT) delivery of glucose (2 g/kg) to recipient mice following an overnight fast. Blood glucose was monitored for 2 h after glucose delivery and insulin was measured using an ultrasensitive mouse insulin ELISA (Alpco Diagnostics, Salem, NH). Intraperitoneal insulin tolerance tests (ITTs) were carried out in transplant recipients following a 4 h a.m. fast, using synthetic human insulin (Novo Nordisk, Toronto, ON; 2U/kg) and monitoring blood glucose levels for 2 h post-injection.

**GLP-1R^{-/-} mice**

GLP-1R^{-/-} mice (226) were provided by Dr. D. Drucker (University of Toronto) and were maintained on a C57BL/6 background. We initially attempted to genotype these mice using PCR primers GLP-1R F1 and GLP-1R R1, which are directed at a 424 bp region of intron 5, and primers that amplify 280 bp of the NeoR insert. When these GLP-1R primers proved ineffective, we changed our strategy to use primers GLP-1R F2 and GLP-1R R2, which are directed at a 437 bp region of intron 6, the region disrupted in mice bearing a knockout allele (227), and the same NeoR primers. Primer sequences are as follows:

GLP-1R F2 5’ TACACAATGGGGAGCCCTA 3’
GLP-1R R2  5’ AAGTCATGGGATGTGTCTGGA 3’
Neo\(^R\) forward  5’ CTTGGGTGGAGAGGCTATTC 3’
Neo\(^R\) reverse  5’ AGGTGAGATGACAGGAGATC 3’

PCR was carried out as follows using standard reaction mix (3 µL template plus 1 µM of each GLP-1R primer and 0.75 µM of each Neo\(^R\) primer, 1 U of Taq polymerase, 200 µM dNTPs, 1x PCR buffer, and 1.5 mM MgCl\(_2\)): 2 min at 94°C; 30 cycles of 94°C for 1 min, 61°C for 1 min, and 72°C for 1 min; and 72°C for 10 min. Wildtype mice had a single 437 bp product, GLP-1R\(^{-/-}\) mice had a single 280 bp product, and GLP-1R\(^{+/-}\) mice had both products.

**CELL CULTURE AND ANALYSIS OF ADENOVIRUS-TRANSUCED CELLS**

**Cell culture**

αTC-1 (clone 9) and HEK293 cells were from the American Type Culture Collection, and InR1-G9 cells and STC-1 cells were kindly provided by Dr. P. Brubaker (University of Toronto) and Dr. D. Drucker (University of Toronto) respectively. αTCΔPC2 cells, kindly provided by Dr. D. F. Steiner (University of Chicago), are an α-cell line derived from mice lacking active PC2, precluding glucagon production (228). HEK293 cells were cultured in Modified Eagle’s Medium (MEM) containing 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin. All other cells were cultured in high-glucose Dulbecco’s Modified Eagle Medium (HG-DMEM) containing 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin. DPPIV inhibitor (10 µL/mL) was included in media when active GLP-1 was to be measured. Cells were maintained at 37°C/5% CO\(_2\) and passaged as necessary using 0.25% trypsin/1 mM EDTA with media refreshment every 2-4 d except where noted.

**Low-glucose culture and static nutrient-stimulated GLP-1 secretion in αTCΔPC2 cells**

αTCΔPC2 cells were cultured in standard high glucose (25 mM) or in parallel in low-glucose (5.6 mM) DMEM for 3 wk, passaging as necessary. Cells (p28) were seeded at 1.2x10\(^6\) cells/well in a 6-well plate and cultured for 2 d until 50% confluent. On the day of the nutrient-stimulated GLP-1 secretion experiment, Kreb’s Ringer Bicarbonate Buffer solution (KRBB; 129 mM NaCl, 4.8 mM KCl, 1.2 mM MgSO\(_4\), 1.2 mM KH\(_2\)PO\(_4\), 2.5 mM CaCl\(_2\), 5 mM NaHCO\(_3\), 10 mM HEPES, and 0.5% BSA) containing different concentrations of glucose or arginine was prepared and equilibrated in suspension culture dishes at 37°C/5% CO\(_2\) for 1 h. Cells were
washed in KRBB containing 5.6 mM glucose (KRBB5.6) and cells were incubated in 1 mL fresh KRBB5.6 for 1 h. Equilibration medium was aspirated off all wells and 700µL fresh KRBB containing the indicated amount of glucose or arginine was added to each well. Plates were incubated for an additional hour before medium was removed, cleared by centrifugation (14000 rpm/1min), and the supernatants transferred to fresh tubes. Samples were stored at -20°C prior to assay for GLP-1 using a GLP-1 (Active) ELISA (Linco Research Inc.; St Charles, MO).

**Propagation of adenoviruses**

AdPC1/3, AdPC2, and Ad5lacZ have been described previously (229, 230) and were propagated by infecting ~70% confluent HEK293 cells in 2 x T75 flasks with adenovirus at ~6x10⁷ PFU (10⁷ PFU•[confluence of HEK293 culture-10%]/10) prepared in 3 mL infection medium (serum- and antibiotic-free MEM). Infection was carried out for 2 h before aspirating the infection medium and replacing it with 10 mL growth medium. Cells were monitored for cytopathic effects (CPE; rounding up and detachment of cells) for 3-6 d post-infection and when >90% of cells were detached, the suspension of cells was collected and centrifuged (5 min; 1500 rcf). The cell pellet was resuspended in 5 mL infection medium and lysed by 3 consecutive freeze/thaw cycles in a dry ice/EtOH bath and 37°C waterbath respectively, with a 30 s vortex between cycles. The lysate was centrifuged (5 min; 1500 rcf) and the supernatant drawn into a 5cc syringe via an 18 G blunt-ended cannula and filtered through a 0.22 µm syringe filter. Filtered supernatant was used to infect 2 x T150 flasks of HEK293 cells for 2 h as above, and following the development of CPE, the harvest procedure was repeated. Filtered supernatant was used to infect 5 x T150 flasks of HEK293 cells. After CPE development, cultures were harvested as before, and following freeze/thawing, the supernatant was filtered through a 0.45 µm bottle-top filter, and purified chromatographically using a commercially available kit according to the manufacturer’s instructions (Adenopure Adenovirus Purification kit; Puresyn; Malvern, PA). Virus stock was dialyzed into a lower salt buffer (2.5% glycerol, 25 mM NaCl, 20 mM Tris, pH 8) using a commercial dialysis kit (66810; Pierce; Rockford, IL) to increase longevity and ability to withstand freeze/thawing, and stored at -80°C until use.

To estimate the titer of the viruses, HEK293 were seeded in a 96-well plate and at ~90% confluence were infected with serial dilutions of adenovirus (10⁰-10¹² dilution). ~6 d post-infection, each well was scored as negative or positive for CPE. Titer was determined as 10^{(x+0.8)}, where x is the sum of wells, infected at 10¹-10¹², which were positive for CPE.
(represented as a fraction of total wells). Titer was averaged for 3 parallel dilutions, each done in duplicate for a total of 6 plates, and typically reached $\sim 10^9$ PFU/mL using this method.

**Transduction of cultured cells with adenovirus**

InR1-G9, αTC-1, αTCAPC2, and/or STC-1 (enteroendocrine) cells were seeded at equal density in 6-well plates and the following day (at $\sim 35\%$ confluence) were transduced with AdPC1/3 or Ad5lacZ at MOI 1, 10 or 100 in a minimal volume of media (1.5 mL for 10 cm plates; 400 µL for 6-well plates) at 37°C/5% CO₂ for 2 h. Mock-transduced cells (MOI 0) were treated with an equal volume of media alone. Cells were washed well with media at the end of the transduction and a complete medium change was performed every 24 h for 3 d thereafter. The 48-72 h medium samples were assayed for glucagon, GLP-1, GLP-2, and/or oxyntomodulin. Three days post-transduction, Ad5lacZ-treated cells were rinsed in PBS-CaCl₂, fixed in 0.2% glutaraldehyde prepared in PBS-CaCl₂ for 5 min at room temperature, exposed to freshly prepared X-gal solution (1 mg/mL X-gal in dimethyl sulfoxide; 5 mM each potassium ferrocyanide and potassium ferricyanide; 2 mM MgCl₂•H₂O; freshly prepared in PBS-CaCl₂) for 2 h at 37°C, and directly imaged using brightfield microscopy.

**Northern blotting and immunoblotting for PC1/3**

Three days after transduction, RNA was isolated using the TRIZOL® method, or cell lysates were prepared. For immunoblotting, lysates were prepared by washing cells in PBS-CaCl₂, and incubating on ice for 30 min in lysis buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 1% Triton X, 20 mM ethylenediamine tetraacetic acid, 100 mM NaF, 10 mM Na₃P₂O₇, 1 mM phenylmethanesulphonylfluoride, 1 mM Na₂VO₄, and 1% protease inhibitor cocktail). Lysates were cleared by centrifugation (15000 rcf for 15 min at 4°C) and protein concentration determined by the Bradford or BCA methods. Equal amounts of protein were electrophoresed on a 10% acrylamide gel and transferred to a 0.2 µm PVDF membrane (Bio-Rad, Hercules, CA). After blocking in 5% milk powder in TBS + 0.1% Tween-20 (TBST), the membrane was incubated with rabbit anti-PC1/3 antiserum (Dr. L. Devi; Mount Sinai School of Medicine; 1:1000 in TBST) and developed using HRP-conjugated secondary antibody (1:5000 in TBST) and enhanced chemiluminescence reagents. For northern blotting, 20 µg RNA was electrophoresed on a 1.5% denaturing agarose gel, transferred to a 0.45 µm nitrocellulose membrane, and UV-crosslinked. $[^{32}P]$-labeled β-actin (Ambion; Austin, TX) and rat proglucagon cDNA probes (provided by Dr. D. Drucker, University of Toronto) were prepared.
by the random-priming method (using the Rediprime II Random Prime Labelling System; Amersham Biosciences; Buckinghamshire, UK). Densitometric analysis was performed using ImageQuant 5.2 (Amersham Biosciences).

**Immunoblotting for GLP-1 using the Phastsystem™**

Recombinant GLP-1\textsubscript{7-36amide} was obtained from American Peptide Company Inc. (#46-1-13; Sunnyvale, CA); protein lysates were prepared from STC-1 or InR1-G9 cells (transduced with AdPC1/3) and protein concentration determined as described above. Lysates were electrophoresed (separation method #6) on a premade high-density sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel using 6 well/4 µL loading combs and the Phastsystem™ (Pharmacia; Uppsala, Sweden) according to the manufacturer’s instructions. Electrophoresed products were transferred to a 0.2 µm PVDF membrane using the Phastsystem™ (separation method #9) and blocked for 30 min with 5% skim milk powder in TBST. Blots were incubated 1 h in primary antibody (Hm 156; 1:1000 in TBST) and, after washing in TBST for 30 min, in HRP-conjugated secondary antisera (Amersham NA934V; 1:4000 in TBST). Blots were developed as above.

**Immunohistochemistry**

Immunohistochemistry was performed on paraffin sections prepared by the UBC Histochemistry Laboratory or by Wax-It Inc. (Vancouver, BC). Sections were dewaxed and rehydrated (3 x 5 min in xylene, 2 x 5 min in 100% EtOH, 5 min in 95% EtOH, 5 min in 70% EtOH, and 5 min in PBS) and antigen retrieval was performed by heating slides in 10 mM citrate buffer (pH 6.0) in 3 x 5 min intervals at microwave power setting 4. Slides were cooled to room temperature, washed well in PBS, and sections were outlined with an hydrophobic pen before blocking in a serum-free commercial agent (X0909; DAKO, Carpenteria, CA) for 20-30 min at room temperature. Slides were incubated in a humidified chamber overnight at 4°C in the indicated primary antibody prepared in a commercial diluent (0809; DAKO). The following morning slides were washed in PBS and incubated in the indicated secondary antibody for 1 h at room temperature. After washing in PBS, slides incubated in fluorescent secondary antisera were mounted with Vectashield (Vector Laboratories; Burlingame, CA). Slides incubated in HRP-conjugated secondary antisera were developed using an ABC Vectastain Elite kit and DAB substrate kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions.
Analysis of GLP-1 antisera using pancreas sections from PC1/3−/− mice

Paraffin-sectioned pancreata from PC1/3−/− mice were obtained from Dr. D. F. Steiner (University of Chicago). PC1/3−/− mice have been previously described and have a complete block in the processing of proglucagon to bioactive GLP-1 (106, 107). Slides were incubated with one of 4 antisera reported to be specific for fully processed, bioactive GLP-1. These antisera were as follows: Alpco Diagnostics #A6104 (1:2500; Salem, NH); Phoenix Pharmaceuticals #H-028-11 (1:400; Burlingame, CA); Hm157 (1:250); and Hm156 (1:250). Hm157 and Hm 156 were kindly provided by Dr. J. Habener (Harvard Medical School). Slides were washed in PBS and incubated in AF594-conjugated secondary antisera (1:800) before mounting and visualization.

Transplantation of Encapsulated Cells

For adenovirus-transduced InR1-G9 cell transplants, passages 10-13 were used. Cells were grown to 30-40% confluence and were transduced with AdPC1/3 or Ad5lacZ at MOI 10 for 2 h at 37°C/5% CO2. ~7.5 million encapsulated, AdPC1/3-transduced cells (transduced at MOI 10) were transplanted in 4 mL volume to the i.p. cavity of 8 week old male CD-1 mice 1 d post-transduction or to 6 wk old male db/db mice 2 d post-transduction. On the day of encapsulation, cells were ~80% confluent. For αTC-1 and αTCΔPC2 cell transplants, cells were grown to ~80% confluence before encapsulation. For all αTC-1 and αTCΔPC2 transplants, 1.4-3 mL of capsules (~4.0x10^7 cells/mouse) were transplanted in <3.5 mL total volume. Experiments were performed using passages 10-14 or 20-29 for αTC-1 and αTCΔPC2 cells respectively.

After trypsinization, pelleted cells were resuspended in PBS without CaCl2 and a cell count was performed using a haematocytometer. Cells were resuspended in a mixture of 1.5% sodium alginate (IE-1010; Inotech Biosystems International; Rockville, MD; molecular weight 475 kDa; G/M ratio 65-75%/25-35%) and morpholinepropanesulfonic acid (MOPS) and transferred to a sterile encapsulator (Inotech Biosystems International). To calculate the volume of capsules that should be transplanted in order to deliver a given number of cells, the desired cell number was divided by the concentration of cells in the alginate/MOPS suspension that was loaded to the encapsulator. Encapsulation was performed according to the manufacturer’s instructions for encapsulation of animal cells using a 250 µm nozzle and encapsulation settings as follows: electrical charge ~1.0 kV, vibration frequency ~1200 Hz, pump speed ~600. Capsules (500-700 µm) were washed in PBS without CaCl2 and loaded to sterile syringes.
attached to 18 G catheters. Recipient mice were anaesthetized using isoflurane, and an incision was made midway between the ventral midline and the animal’s side, ~1.5 cm anterior of the hind leg. Capsules were injected to the intraperitoneal cavity and the musculature and skin were closed with a running suture and a wound clip respectively.

All transplants were completed within 1.5 h of cell encapsulation, and capsules awaiting transplantation were stored in the biosafety cabinet in PBS without CaCl₂ during this time. This was based on preliminary experiments performed by others in our laboratory indicating that longer incubation of encapsulated cells in PBS led to significant cell death. We observed no correlation between the amount of time capsules were stored prior to transplant and loss of cell function in vivo, though cell viability was not directly tested. Sham-operated mice received an equal volume of sterile saline under identical conditions.

**ADENOVIRAL TRANSDUCTION OF MOUSE ISLETS AND HUMAN ISLETS**

**Isolation of mouse islets**

Islets were isolated from male mice (8-12 wk) by injection of collagenase (Type XI; 1000 U/mL) freshly prepared in Hank’s Balanced Salt Solution (HBSS) without CaCl₂ (137 mM NaCl, 5.4 mM KCl, 4.2 mM NaH₂PO₄, 4.1 mM KH₂PO₄, 10 mM HEPES, 1 mM MgCl₂, and 5 mM glucose) through the bile duct to the pancreas. Donor mice were anaesthetized with inhalable isoflurane and a cervical dislocation was performed. An incision was made through the skin and muscle layers from the sternum to the gonads and the peritoneum was exposed using restraint cords fixed to a small rodent surgery board. The diaphragm was cut and, using a saline-soaked cotton-tipped swab, the liver lobes were gently rolled up into the chest cavity to expose the common bile duct. With the aid of a dissecting microscope (Stemi 2000C; Carl Zeiss Canada; Toronto, ON), the point at which the bile duct joins the upper duodenum was located and clamped off with a hemostat. Two and a half mL collagenase was loaded to a 5 cc syringe and a 27 G or 30 G needle bent to a ~45° inside angle was attached. Adjusting the hemostat such that the bile duct and needle lay parallel to one another, the needle (bevel facing up) was inserted into the bile duct just below the branch to the liver. Collagenase was slowly injected through the bile duct to distend the pancreas. Once distended, the pancreas was carefully removed by grasping the upper duodenum with small forceps and making small incisions and/or using downward brushing motions with a pair of scissors to free the pancreas from the intestine and stomach. The spleen was then grasped with forceps and used as a ‘handle’ as the pancreas was
freed from underlying tissue. Finally, after cutting away the spleen and any obvious lymph or adipose tissue, the excised pancreas was transferred to a 50 mL conical vial on and stored on ice while the process was repeated for up to 3 other mice. Total storage time on ice was <1 h, and pancreata were processed in groups of 4, with each pancreas in a separate 50 mL conical vial.

Islets were isolated by standard dextran gradient procedure (231) initially, but we found that using a filtration modification described by Salvalaggio et al. (232) gave us much better quality islets and greater yields. An additional 2.5 mL of collagenase was added to each pancreas-containing vial, and after wrapping the top of each tube in parafilm, tubes were placed horizontally in a shaking 37°C water bath (WB 22; Memmert GmbH; Schwabach, Germany) set at the maximum agitation setting. After 12-14 min digestion, tubes were removed from the waterbath and firmly shaken by hand 5 times each to dissociate pancreatic tissue. Pre-chilled HBSS with 1 mM CaCl₂ was promptly added to a final volume of 40 mL, and tubes were centrifuged at 4°C/200 rcf for 1 min. Supernatants were poured off and discarded and tissue pellets were resuspended in ~5 mL HBSS+CaCl₂ using a 10 mL pipet, being careful not to draw up solution beyond the 3 mL volume mark in the pipet. HBSS + 1 mM CaCl₂ was again added to each tube to a final 40 mL volume and the centrifugation, resuspension, and washing steps were repeated again. After the last centrifugation, tissue pellets were resuspended in 5 mL Hams F10 containing 0.5% BSA, 6.1 mM glucose, 100 U/mL penicillin and 100 µg/mL streptomycin (complete medium). A 100 µm cell strainer (352360; BD Biosciences; San Jose, CA) was placed in a fresh 50 mL conical vial and each vial of digested tissue was applied to a separate strainer in a separate tube, adding 1-2 mL at a time and rinsing the strainer with 5 mL complete medium after each addition to keep tissue from clogging the strainer. The vial containing the digested tissue was washed with ~6 mL complete medium, which was then applied to the strainer. The strainer was removed from the 50 mL conical and inverted over a 6-10 cm suspension culture plate and islets were washed into the dish by washing the strainer with several 5 mL volumes of complete medium. Islets from each pancreas were washed into a separate plate. With the aid of the dissecting microscope, islets were handpicked with a 200 µL pipettor into a fresh dish of complete medium. Handpicking of islets or removal or exocrine tissue was repeated until islets were >90% pure, at which point islets from several mice were sometimes pooled for experiments. Except where noted, islets were cultured overnight in complete medium before any experiments were performed. Where active GLP-1 was to be measured, DPPIV inhibitor was included in the culture media. Islets were counted as islet equivalents (IE), where one 150 µm
islet (roughly measured using a calibrated eyepiece reticle fitted to the dissecting microscope) was considered 1 IE and smaller and larger islets were counted based on this standard.

**Transduction of mouse islets with adenovirus**

One day post-isolation, islets (300 IE/condition) were mock-transduced or transduced with Ad5lacZ or AdPC1/3 at MOI 10 or 100 (assuming 1000 cells/islet) for 1 h at 37°C/5% CO₂ in a minimal volume of media (600 µL in a 6 cm plate or 175 µL in a 24-well plate. After transduction islets were washed well and cultured in complete medium with daily medium changes. Three days post-transduction, Ad5lacZ-transduced islets were fixed in 0.2% glutaraldehyde, exposed to X-galactosidase for 2 h at 37°C and paraffin-embedded. Sections were incubated with mouse anti-glucagon antisera (1:1800) and anti-mouse HRP-conjugated (1:800) secondary antisera. Other islets were transduced as above, cultured for 3 d with a daily complete medium change, and 48-72 h medium samples were assayed for GLP-1 and glucagon. Three days post-transduction, islets were fixed in 4% paraformaldehyde, embedded in paraffin, and sections were immunostained with rabbit anti-PC1/3 antiserum (1:900; Affinity Bioreagents; Golden, Colorado) and goat anti-rabbit-Cy3 antiserum (1:800).

**Culture and transduction of human islets with adenovirus**

Human islets were kindly provided by the Irving K. Barber Islet Isolation Laboratory at Vancouver General Hospital and were cultured in a proprietary final wash medium (99-785-CV; Mediatech Inc.; Herndon, VA) containing 5.5 mM glucose and supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin. Human islets were transduced with AdPC1/3 or Ad5lacZ using the same conditions outlined for mouse islets above and were cultured for 3 d post-transduction with a daily medium change. Media samples corresponding to 48-72 h post-transduction were assayed for GLP-1 content.

**EX VIVO ANALYSIS OF MOUSE ISLETS AND HUMAN ISLETS**

**Static glucose-stimulated insulin secretion (GSIS) in mouse islets**

To assess GSIS in freshly isolated mouse islets, 6 h post-isolation islets were handpicked in 20 IE aliquots to wells of a suspension 24-well plate in KRBB containing 5 mM glucose and equilibrated for 1 h at 37°C/5% CO₂. The plate was gently swirled to collect islets in the centre of the wells, and medium was carefully removed from the edge of the well using a 200 µL pipettor. Fresh KRBB with 5 mM glucose was added dropwise and islets were cultured for an
hour before replacing medium as above with KRBB containing 15 mM glucose. High glucose incubation was carried out for an additional hour before washing islets and culturing in complete medium for 72 h. The static GSIS experiment was repeated 72 h post-isolation. The same procedure was used to compare static GSIS in mock-transduced and AdPC1/3-transduced islets 72 h post-transduction, although in this case 50 IE aliquots were used.

**Static GSIS in human islets**

Seventy-two hours post-infection, human islets (50 IE per well in a 6-well suspension plate) were pre-equilibrated at 37°C/5% CO₂ in KRBB containing 7 mM glucose for 1 h. Media were removed as above and KRBB containing 2 mM glucose was added. After a 30 min incubation, 50 µL medium was removed and replaced with an equal volume of concentrated glucose to bring the final concentration of glucose in the medium to 20 mM. The plate was gently tilted side to side to thoroughly mix concentrated glucose into the medium. Stimulation with high glucose ensued for an additional 30 min, and medium was collected again. Media were assayed using a human insulin ELISA (08-10-1113-01, Alpco; Salem, NH).

**Perfusion of mouse islets**

Initial pilot experiments were performed using normal, untransduced CD-1 or C57BL/6 mouse islets to set up the Endotronics Acu-syst S Perifusion apparatus and confirm this method as a reliable means of measuring GSIS. KRBB was pre-equilibrated to 37°C/5% CO₂ and the perifusion apparatus was preheated to 37°C using a Haake heated water jacket. Cytodex I microcarrier beads were pre-washed in warm KRBB with 3 mM glucose and islets were handpicked into siliconized microcentrifuge tubes (Fisher Scientific; Ottawa, ON). Tubes were transferred, lids open, to a 37°C/5% CO₂ incubator and islets were allowed to gravity-settle while the chambers were assembled. Chamber ends for the large perifusion chambers were assembled with a piece of 700 µm nylon mesh covered by a 0.45 µm round filter paper (Millipore; Billerica, MA) held in place by a rubber O-ring and the lower chamber end was attached to the chamber body. Resuspended Cytodex beads (100 µL) were loaded to the chamber bodies and islets were loaded on top of bead layer in <25 µL volume. After layering 50 µL of Cytodex beads on top of the islets the top chamber end was attached. Media were fed into the chambers via 60 cc syringes fitted with stopcocks, and media flow was controlled by the perifusion pump (setting 20, corresponding to a flow rate of ~0.4 mL/min), and media were collected from the chambers in 5 min fractions collected using a fraction collector. Islets were
equilibrated in the assembled chambers in basal media for 1 h prior to the GSIS experiment. Fractions were stored at -20°C and assayed for insulin using a multi-species sensitive insulin RIA.

Upon confirmation of the perifusion method as a means of assessing GSIS, 100 islets/chamber were loaded to the perifusion apparatus 3 d after transduction with AdPC1/3 (MOI 100). KRBB containing 0.5% BSA and 3 mM or 20 mM glucose was pumped through the chambers following a 1 h preincubation under basal conditions.

**Live/Dead imaging of mouse islets**

Forty-eight hours post-transduction, 12 to 25 AdPC1/3- or Ad5lacZ-transduced islets (MOI 10) were handpicked to wells of a black 96-well plate. Some wells contained H₂O₂ or one or more of IL-1β, TNFα, and IFNγ (R&D Systems; Minneapolis, MN) and islets were cultured for an additional 24 h. Analysis of live and dead cells was performed according to the manufacturer’s instructions using a kit in which ethidium homodimer-1 fluoresces red in cells with damaged membranes, while Calcein-AM is converted to a green fluorescent product in living cells. Images were captured at the central plane of focus on both red and green channels, and merged. Mean red fluorescent intensity was determined by normalizing total internal islet red fluorescence for islet area.

**Transplantation of Mouse Islets**

Islets were isolated from C57BL/6 mice to match the strain of the recipient mice. Following overnight culture, isolated islets were mock-transduced (MOI 0) or transduced with AdPC1/3 at MOI 10 or 100. One to two days post-transduction, islets were hand-picked into aliquots of 150 or 200 islet equivalents (as indicated in figure legends) in a siliconized microcentrifuge tube (Fisher Scientific; Ottawa, ON) and washed in serum-free media. Islets were slowly drawn up into ~13 inches of PE50 tubing (BD427411; Becton Dickinson; Franklin Lakes, NJ) via a 25 G needle attached to a micromanipulator (Mitutoyo; Mississauga, ON) and the tubing was clamped shut with two ligaclips (LS200; Johnson & Johnson; Markham, ON). The micromanipulator and tubing were spun using a modified mechanical spinner (model RZR1; Heidolph Instruments; Schwabach, Germany) for a total of ~30 s at setting 4 to collect the islet pellet at the bottom of the tubing. Recipient mice were anaesthetized with isoflurane in a biosafety cabinet, and a ~1 cm incision was made through the skin and muscle layers covering the left kidney. The kidney was externalized using a saline-wetted cotton-tipped swab and a
superficial hole was made in the kidney capsule with a 30 G needle. A small area was cleared beneath the kidney capsule by inserting a small, round-ended glass probe (hand-pulled from a glass Pasteur pipette) through the nick made in the capsule and rotating the probe from side to side. Islets were ejected from the PE50 tubing and the opening of the pouch was sealed off using a cautery pen (H101; Bovie Medical Corp; St. Petersburg, FL). The muscle layer was closed with a single running suture, and the skin closed with ~3 wound clips (BD427631; Becton Dickinson; Franklin Lakes, NJ) before the animal was allowed to recover from anaesthesia. Two days or twenty-one days after transplantation, survival nephrectomy was performed to remove the islet graft-bearing kidney. Under isoflurane anaesthesia, the incision through which the transplant had been made was re-opened, and the graft-bearing kidney was externalized using the technique outlined above. The renal artery and vein were tied off with a nonabsorbable 5.0 silk suture and the grafted kidney was removed and fixed in 4% PFA before paraffin embedding and sectioning. Blood glucose was monitored for several days after graft excision to confirm recurrence of hyperglycemia.

**Cold Tolerance Testing and Body Temperature Analysis**

While under isoflurane anaesthesia for cell transplantation, some mice were implanted subcutaneously with sterile temperature transponders (IPTT-300; Bio Medic Data Systems; Seaford, DE) through a ~3 mm incision in the intrascapular region. Blunt-ended scissors were used to separate the skin from underlying muscle and a transponder was implanted longitudinally in this subcutaneous space, parallel but to one side of the spine so as not to interfere with the animal's movement or handling. The incision was closed with a single suture or wound clip before the animal was allowed to recover from anaesthesia. In the case of GLP-1R\(^{-}\) and \(ob/ob\) mice, transponders were transplanted using this procedure 2 d prior to cold tolerance testing. Twenty-four hour body temperature profiles were assessed using a hand-held Pocket Scanner (DAS-5007; Bio Medic Data Systems; Seaford, DE) to read the temperature transponders in singly housed mice. Food intake was assessed at the same time by measuring food mass in the hopper before and after the test period. For cold tolerance testing, conscious mice were singly housed in cages without bedding for the duration of the experiment. Mice were housed at 4°C and body temperature was measured every 15 min for 2 hr using the Pocket Scanner. Any mouse that lost \(\geq7°C\) from basal body temperature was returned to ambient temperature, warmed using a heating pad, and excluded from the study.
Body composition analysis

Measurements were performed with a Bruker Biospec 70/30 7 Tesla MRI scanner (Bruker Biospin; Ettlingen, Germany). NMR signal from the body was acquired with a quadrature volume RF coil tuned to 300 MHz. The “free” water component corresponding to body fluids, \textit{e.g.} urine and CSF) was typically <5% of the total signal. The ratio of lean/fat tissue, expressed as weight/weight, was calculated from the NMR data as described elsewhere (233).

Plasma and tissue analysis of recipient mice

Plasma and tissue collection

At the indicated time, mice were anaesthetized with isoflurane and a cardiac puncture was performed to collect plasma prior to cervical dislocation. Pancreas, jejunum (8-10 cm distal to the pylorus), intrascapular brown adipose tissue (BAT), and/or epididymal white adipose tissue (WAT) were removed, rinsed in PBS, fixed in 4% paraformaldehyde and paraffin-sectioned. In some studies intrascapular BAT and epididymal WAT depots were collected, rinsed in PBS, and weighed, and portions were flash frozen in liquid nitrogen and stored at -80°C until analysis. Plasma analytes were measured using total GLP-1 RIA, GLP-2 ELISA, and/or glucagon ELISA kits.

Brown and white adipose tissue analysis

BAT and epididymal WAT was stained with hematoxylin and eosin and imaged using brightfield microscopy. Lipid droplet size was measured using the inbuilt ‘magic wand’ algorithm with the tolerance level set at 30. Other sections were stained with a rabbit uncoupling protein 1 (UCP-1) antibody (10983; 1:1000; Abcam; Cambridge, MA) and HRP-conjugated secondary antisera.

UCP-1 protein measurement

Intrascapular BAT was ground using a mortar and pestle pre-chilled at -80°C. Ground tissue was transferred to a pre-chilled microcentrifuge tube and 100 µL of lysis buffer, containing 50 mM HEPES, 150 mM NaCl, 10 mM EDTA, 10 mM NaH₂PO₄, 100 mM NaF, with 1 mM PMSF, 2 mM Na₃VO₄, 0.4% Triton-X, and 1% protease inhibitor cocktail added immediately before use. Samples were vortexed and incubated on ice for 30 min, then centrifuged at 15000 g for 5 min at 4°C. The protein-containing fraction was removed to a fresh
set of tubes and protein concentrations were determined using the BCA method. Equal amounts of protein were electrophoresed on a 10% acrylamide gel using SDS-PAGE and transferred to a 0.2 μm PVDF membrane (Bio-Rad). After blocking the membrane was incubated with rabbit anti-UCP-1 antibody (1:4000; (234)) overnight at 4°C. The membrane was incubated with alkaline phosphatase-linked polyclonal secondary antibody (1:5000; GE Healthcare, Buckinghamshire, UK) and developed using an enhanced chemiluminescence kit (GE Healthcare). The membrane was washed, re-blocked and immunoblotted with an alkaline phosphatase linked-β-actin antibody and UCP-1 expression was normalized for β-actin levels.

**Analysis of jejunal crypt cell proliferation**

Jejunal crypt cell proliferation was assessed using a method described elsewhere (235). Sections were stained for proliferating cell nuclear antigen (PCNA) and for ≥6 upright, intact crypts per mouse, the first 20 cells extending upward from the crypt base (position 1) were scored as PCNA-negative or PCNA-positive. Crypt plus villus height was determined by measuring from the crypt base to the tip of the villus in 4-10 upright, intact villi in hematoxylin-stained sections for each mouse.

**Analysis of the pancreas in recipients of encapsulated αTC-1 or αTCΔPC2 cells**

Pancreas sections were stained using guinea pig anti-insulin (1:900; Linco Research; St. Charles, MO), rabbit anti-glucagon (1:1800; Sigma; St. Louis, MO), mouse anti-PCNA (1:200; BD Biosciences; San Jose, CA), and secondary antisera conjugated to Alexa Fluor 488 or 594 (1:800). Total insulin-/glucagon-positive area, as well as the number and average size of insulin-/glucagon-positive clusters were determined in one section per mouse and normalized for sectional pancreatic area. The proportion of α- and β-cells per islet was determined by counting nuclei of all glucagon- and insulin-positive cells of ≥5 randomly selected islets for each mouse. α-cells within 2 cells of the islet edge were considered ‘peripheral’, and otherwise were considered ‘central’. Some sections were co-stained for insulin and PCNA, and the number of insulin-positive/PCNA-positive cells was quantified in ≥7 randomly selected islets per animal.

**Analysis of islet grafts from islet transplant recipients**

Islet grafts were retrieved 2 d or 21 d post-transplant, fixed in 4% paraformaldehyde and paraffin-sectioned. Sections were incubated with rabbit anti-Pdx1 (1:1000), guinea pig anti-insulin (1:900), mouse anti-glucagon (1:1800), and/or rabbit anti-PC1/3 (1:1000) antisera, and
secondary antisera conjugated to Alexa Fluor 488 or 594 (1:800). Some sections were co-stained for insulin and Pdx1, and the intensity of nuclear Pdx1 staining was quantified in 10 randomly selected insulin-positive cells from each of 4 non-overlapping fields of view for each graft. Intensity of insulin staining and the relative proportions of α-/β-cells were determined in sections costained for insulin and glucagon. Intensity of insulin staining was determined in ≥7 nonoverlapping fields of view for each graft. The proportion of α- and β-cells was determined by counting nuclei of all glucagon- and insulin-positive cells in 3 to 8 nonoverlapping fields of view for each graft. Intensity measurements were made on images taken at identical, nonsaturating exposure using an inbuilt algorithm that measures total pixel intensity in an area defined using the ‘lasso’ tool with the tolerance set at 5.

**Data Analysis**

Data analysis was carried out using Prism 4.0 (GraphPad, San Diego, CA). Data are presented as mean ± S.E.M. and were analyzed using the student’s t-test or analysis of variance (ANOVA) followed by a Bonferroni post hoc test as appropriate. Statistical significant was set at 5% and the number of experiments performed are shown in figure legends. *P<0.05, **P<0.01, ***P<0.001.
RESULTS

CHARACTERIZATION AND TRANSPLANTATION OF α-CELL LINES TRANSDUCED WITH Ad5lacZ OR AdPC1/3

Transduction of tumour-derived α-cells with AdPC1/3

Under normal circumstances, the adult α-cell normally expresses PC2 but not PC1/3 (Fig. 3A). We initially tested viral transduction conditions using the reporter virus Ad5lacZ, and found that transduction of InR1-G9 α-cells induced significant and dose-dependent expression of the reporter gene β-galactosidase (Fig. 3B). Using a replication incompetent adenoviral vector carrying the CMV promoter-linked PC1/3 cDNA expression construct, we achieved a dose-dependent increase in expression of the 82 kDa and the bioactive 64 kDa form of PC1/3 in the α-cell lines InR1-G9 and αTC-1 (Fig. 3C, D). PC1/3 overexpression induced significant accumulation of GLP-1 in the culture medium of InR1-G9 (0.58±0.29 nM at MOI 0 versus 4.8±0.64 nM at MOI 10; \( P<0.001 \)) and αTC-1 cells (2.0±0.55 nM at MOI 0 versus 53.0±18.3 nM at MOI 100; \( P<0.001 \)), while transduction with the reporter adenovirus Ad5lacZ, bearing a β-galactosidase construct under the control of a CMV promoter, had no impact on GLP-1 secretion (Fig. 3F, G). Treatment with either vector at MOI 100 decreased glucagon secretion in InR1-G9 cells, but not αTC-1 cells (Fig. 3H, I), and InR1-G9 cells transduced with either vector had decreased proglucagon mRNA levels (Fig. 3E; \( P<0.05 \) versus untransduced control). This decreased glucagon gene transcription and secretion is likely indicative of nonspecific viral toxicity, which was also evident in the reduced cell confluence observed when InR1-G9 cells were transduced at MOI 100 (Fig. 3B).

We sought to confirm GLP-1 expression in AdPC1/3-transduced α-cells via immunoblotting or immunocytochemistry. Preliminary experiments using standard Tris-glycine or Tris-tricine SDS-PAGE and immunoblotting were unsuccessful (data not shown), probably due to technical challenges associated with the small size of the peptide (e.g. diffusing out of the gel or membrane). We altered our approach to use pre-made high-density SDS-PAGE gels designed specifically for low molecular weight peptides. Though we were able to detect recombinant GLP-1 using this method, GLP-1 was not detectable in lysates from AdPC1/3-transduced α-cells (Fig. 4A, B). This likely reflects the limited sample volume we were able to load using this system.
Figure 3: Strategy for inducing GLP-1 production in pancreatic α-cells
(A) Adult pancreatic α-cells liberate glucagon from proglucagon via PC2 activity. We hypothesized that adenoviral delivery of PC1/3 (AdPC1/3) to pancreatic α-cells would liberate GLP-1 from the major proglucagon fragment (MPGF). GRPP, glicentin-related pancreatic polypeptide; IP-1 and IP-2, intervening peptides. (B) X-galactosidase staining in InR1-G9 cells infected with Ad5lacZ at different multiplicities of infection (MOI). Scale bar=100 µm. (C, D) Western blot for PC1/3 expression in control gut (STC-1) cells, InR1-G9 (C), and αTC-1 (D) cells 72 h post-transduction with AdPC1/3 at MOI 0, 10 or 100 (representative of ≥3 experiments). (E) Northern blot for proglucagon (PG) mRNA levels in InR1-G9 cells transduced with AdPC1/3 or Ad5lacZ (representative of ≥3 experiments). Data are normalized for β-actin and expressed relative to mock-transduced control (Ctl); *P<0.05 versus control using t-test. (F-I) GLP-1 and glucagon (GLGN) secretion by InR1-G9 (F, H) and αTC-1 (G, I) cells after treatment with AdPC1/3 or Ad5lacZ. *P<0.05, **P<0.01 and ***P<0.001 versus Ad5lacZ; n≥3.
Before performing immunocytochemistry for GLP-1 in AdPC1/3-transduced α-cells, we first sought to confirm the specificity of several of the available GLP-1 antisera claiming to be specific for fully processed, bioactive GLP-1\textsubscript{7-36amide} or GLP-1\textsubscript{7-37}. We rationalized that since PC1/3 is an absolute requirement for release of bioactive GLP-1 from α-cells (105, 236), in PC1/3\textsuperscript{−/−} mice there should be no ligand for antisera truly specific for fully processed GLP-1. As ileal sections from PC1/3\textsuperscript{−/−} mice were unavailable, we performed our analysis in pancreas sections. All 4 antisera tested bound an antigen both in PC1/3\textsuperscript{+/+} mice and PC1/3\textsuperscript{−/−} mice (Fig. 5).

This indicated that none of the antisera available to us were truly specific for bioactive GLP-1 and we were therefore unable to confirm GLP-1 expression in PC1/3-expressing α-cells by either immunoblotting or immunocytochemistry.
Based on our observation of greater GLP-1 secretion in AdPC1/3-transduced α-cells, we next sought to determine the impact of transplantation of these GLP-1-producing α-cells in normal and pre-diabetic mice. We chose encapsulation as a means of delivering cells without the need for immunosuppression, and were routinely able to obtain capsules of similar shape, size and cell density (Fig. 6A). Transplantation of 8 million encapsulated AdPC1/3-transduced InR1-G9 cells to CD-1 mice had no effect on body weight (Fig. 6B), but increased fasting blood glucose compared to sham-operated mice 3d and 9d post-transplant (Fig. 6C; $P<0.05$ at 3d and 9d post-transplant). An OGTT (1.5 g/kg; performed 29 d post-transplant) revealed no significant change in glucose tolerance compared to sham-operated controls (Fig. 6D, E).

We hypothesized that the absence of improvements observed in fasting or meal-stimulated glucose levels might be due to GLP-1’s glucose dependence and the fact that we transplanted cells into normoglycemic mice. We therefore sought to examine the impact of transplantation of GLP-1-producing α-cells in a mouse model of glucose intolerance, insulin resistance, and T2D—the $db/db$ mouse. Transplantation of 7 million encapsulated AdPC1/3- or

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**Figure 6**: Transplantation of AdPC1/3-transduced InR1-G9 cells to normal CD-1 mice
(A) Representative image of encapsulated α-cells. Scale bar, 800 μm. InR1-G9 cells (p10) were transduced with AdPC1/3 at MOI 10 1 d prior to transplant. ~8 million encapsulated, AdPC1/3-transduced cells were transplanted to the i.p. cavity of 8 week old male CD-1 mice ($n=5$ mice per group). Body weight (B) and blood glucose (C) were monitored following a 4 h a.m. fast. (D) An OGTT (1.5 g/kg) was performed 29 d post-transplant following a 4 h a.m. fast. (E) Area under curve (AUC) for OGTT. *$P<0.05$ compared to sham.
Ad5lacZ-transduced InR1-G9 cells to pre-diabetic db/db mice had no significant impact on body weight or blood glucose (Fig. 7A, B), although in an OGTT (1.5 g/kg; performed 7 d post-transplant), mice that received AdPC1/3- or Ad5lacZ-transduced InR1-G9s had slightly lower blood glucose levels at the 60 min post-gavage timepoint ($P<0.05$ for both transplant groups compared to sham-operated controls), as well as a slight decrease in total glycemic excursion (Fig. 7C, D; $P<0.05$ for Ad5lacZ-transduced InR1-G9s and $P=0.069$ for AdPC1/3-transduced InR1-G9s versus sham). Two weeks after transplantation, we removed and depolymerized capsules from several of the mice that received Ad5lacZ-transduced InR1-G9 cells and cultured the resulting cells overnight. X-galactosidase staining revealed that compared to freshly transduced cells (Fig. 3B), very few retrieved cells had strong β-galactosidase activity at this timepoint (data not shown). Given that AdPC1/3-transduced InR1-G9 cells were not only secreting GLP-1 but also their normal PC2-derived product, glucagon (Fig. 2; Fig. 3I), we also...
suspected that the glucose-raising effects of glucagon might have masked or muted any glucose-lowering effects of GLP-1.

**CHARACTERIZATION AND TRANSPLANTATION OF PC2- OR PC1/3-EXPRESSING α-CELLS**

**In vitro characterization of αTC-1 and αTCΔPC2 cells**

To study the impact of long-term PC1/3 expression in the α-cell in the absence of glucagon, we utilized αTCΔPC2 cells, an α-cell line derived from mice lacking active PC2 (228). Using immunocytochemistry and immunoblotting, we confirmed that αTC-1 cells express PC2 (data not shown) but little or no PC1/3; αTCΔPC2 cells, in contrast, express PC1/3 but not bioactive PC2 (Fig. 8A, B). αTC-1 cells secreted 10-fold more glucagon than αTCΔPC2 cells (Fig. 8C; 113.0 ± 9.6 versus 10.0 ± 1.9 ng/mL; *P*<0.01; *n*≥2). Consistent with minimal PC1/3 expression, αTC-1 cells secreted very little GLP-1 compared to PC1/3-expressing

![Figure 8](image-url)

**Figure 8:** αTC-1 cells express PC2 and produce glucagon, while αTCΔPC2 cells express PC1/3 and produce GLP-1, GLP-2 and oxyntomodulin

(A) In PC2-expressing αTC-1 cells, proglucagon is processed to yield glucagon as the major bioactive product. PC1/3-expressing αTCΔPC2 cells process proglucagon differently resulting in production of GLP-1, GLP-2, and oxyntomodulin. (B) Western blot for PC1/3 in αTC-1 and αTCΔPC2 cell lysates (representative of ≥4 experiments). (C) Glucagon (GLU), GLP-1, GLP-2, and oxyntomodulin (OXM) secretion from αTC-1 and αTCΔPC2 cells (*n*≥2). *P*<0.05, **P*<0.01. IP, intervening peptide; MPGF, major proglucagon fragment; GRPP, glicentin-related polypeptide.
αTCΔPC2 cells (Fig. 8C; 6.6 ± 1.7 ng/mL versus 276.9 ± 96.0 ng/mL; \( P<0.05; n=2 \)). αTC-1 cells also secreted less of the PC1/3-derived proglucagon products GLP-2 and oxyntomodulin than did αTCΔPC2 cells (Fig. 8C; \( P<0.05 \) for GLP-2 and \( P<0.01 \) for oxyntomodulin; \( n=2 \)).

We attempted to increase GLP-1 secretion even further in αTCΔPC2 cells by transducing them with AdPC1/3. Despite an apparent increase in PC1/3 expression in cells transduced at MOI 10 and 100 (particularly the 64 kDa form; Fig. 9A) we found that even transduction at MOI 100 did not further increase GLP-1 secretion (Fig. 9B). This may in part be due to viral toxicity at the higher doses of virus used; indeed, wells transduced at MOI 100 appeared less confluent.

**Figure 9: Characterization of αTCΔPC2 cells**
(A) Western blot showing PC1/3 levels in ‘normal’ α-cells (αTC-1), control enteroendocrine cells (STC-1), and αTCΔPC2 cells, either uninfected (MOI 0) or transduced with AdPC1/3 or AdlacZ, in lysates collected 72 h post-infection. (B) Static GLP-1 secretion from αTCΔPC2 cells, measured from 48 h to 72 h post-transduction with AdlacZ or AdPC1/3 (\( n=2-4 \)). (C) Treatment of αTCΔPC2 cells with AdPC2 (MOI 100) increases glucagon secretion but does not reduce GLP-1 secretion. Media was collected after a static incubation from 48-72 h post-transduction. **\( P<0.01 \) versus MOI 0; \( n=2-4 \). (D) Low-glucose culture decreases secretion from αTCΔPC2 cells and does not restore their glucose sensitivity. Cells were cultured and passaged in either standard high glucose (HG) or low-glucose (LG) DMEM for 3 wk, then stimulated with different concentrations of glucose (5.6 mM-25 mM) or arginine (ARG). *\( P<0.05 \), **\( P<0.01 \) for LG or HG versus 5.6 mM glucose control; #\( P<0.05 \), ##\( P<0.01 \) for HG versus LG (\( n=2 \)).
than mock-transduced wells when media samples were taken for GLP-1 measurement (data not shown). The reason for the drastic upregulation of PC1/3 expression in αTCΔPC2 cells remains unclear; however, we speculated that the high degree of PC1/3 expression in these cells might be at least in part a compensatory effort aimed at counteracting the loss of PC2 bioactivity. We therefore sought to test the impact of reintroducing bioactive PC2 to these cells. AdPC2-transduced αTCΔPC2 cells secreted more glucagon than mock-transduced cells, consistent with reintroduction of bioactive PC2. However, restoration of bioactive PC2 expression and glucagon secretion did not alter PC1/3 expression or GLP-1 secretion (data not shown and Fig. 9C).

In contrast to the native α-cell, which increases secretion of glucagon in response to low circulating glucose concentrations, αTCΔPC2 cells were unresponsive to changes in media glucose concentration, although stimulation with arginine did increase GLP-1 release (data not shown). αTCΔPC2 cells are cultured in 25 mM glucose, and we suspected that long-term high-glucose culture conditions might have desensitized pathways mediating glucose-responsiveness in these cells. We therefore cultured αTCΔPC2 cells in high-glucose DMEM as normal, or in DMEM containing 5.6 mM glucose for 3 wk, passaging them as necessary, and repeated the glucose/arginine stimulation experiment again. Low glucose culture conditions decreased GLP-1 output from αTCΔPC2 cells in response to acute stimulation with several different concentrations of glucose/arginine, and overall did not improve the glucose- or arginine-sensitivity of αTCΔPC2 cells (Fig. 9D).

**Transplantation of PC2- or PC1/3-expressing α-cells to normal mice and GLP-1R−/− mice**

Because αTCΔPC2 cells express PC1/3 but not bioactive PC2, they allowed us to investigate the *in vivo* effects of transplanting α-cells producing only PC1/3-derived proglucagon products. We performed an initial experiment to determine: (a) whether transplantation of αTCΔPC2 cells improves glucose homeostasis in normal mice; (b) if there is a cell dose-dependency to any improvements seen; and (c) how long-lived any effects on glucose homeostasis were. Normal CD-1 mice received either sham surgery or an intraperitoneal transplant of encapsulated αTCΔPC2 cells at either a low dose (~5.7x10^6 cells) or a high dose (4.0x10^7 cells). Neither dose of cells impacted body weight or 4 h fasted blood glucose compared to sham-operated controls (Fig. 10A, B). However, an IPGTT performed 5 d post-transplant revealed a significant improvement in glucose tolerance in mice that received high-
dose, but not low-dose $\alpha$TCΔPC2 cell transplants. This was reflected in lower absolute blood glucose levels (Fig. 10C; $P<0.05$ at 10 min; $P<0.01$ at 20 min and 30 min; $n\geq5$) and in a 61.6%

Figure 10: Transplantation of ~5.7 million or ~40 million encapsulated $\alpha$TCΔPC2 cells to CD-1 mice
Male CD-1 mice (age 9 wks) were transplanted with ~40 million or ~5.7 million cells ($n\geq5$ mice per group). Body weight (A) and blood glucose (B) were measured after a 4 h a.m. fast. IPGTTs (2 g/kg) were performed 5 d (C, D), 25 d (E, F), 37 d (G, H), and 57 d (I, J) post-transplant, following a 5 h (C, D), or an overnight fast (E-J). AUC, area under curve; LD, low dose; HD, high dose. *$P<0.05$, **$P<0.01$, and ***$P<0.001$ for high-dose versus sham.
decrease in total glycemic excursion (Fig. 10D; \(P<0.01; n=5\)). The improvement in glucose tolerance in high-dose \(\alpha\)TC\(\Delta\)PC2 cell recipients was consistently seen in IPGTTs performed 25 d post-transplant (Fig. 10E, \(P<0.001\) at 7 min and 15 min; Fig. 10F, \(P<0.01\)) and 37 d post-transplant (Fig. 10G, \(P<0.05\) at 7 min and 15 min; Fig. 10H, \(P<0.05\)), though the magnitude of the improvement decreased over time and in fact was absent by 57 d post-transplant (Fig. 10I, J).

Having identified a dose of \(\alpha\)TC\(\Delta\)PC2 cells capable of improving glucose tolerance in normal mice, we next sought to confirm that this effect was indeed the result of a PC1/3-derived proglucagon product by directly comparing the impact of transplantation of PC2-expressing (\(\alpha\)TC-1) or PC1/3-expressing (\(\alpha\)TC\(\Delta\)PC2) \(\alpha\)-cells in normal mice. C57BL/6 mice transplanted with encapsulated \(\alpha\)TC-1 cells had slightly increased fasting blood glucose levels during the 14 d following transplant (Fig. 11A; \(P=0.06\) at 8d; \(P=0.054\) at 12 d; \(P<0.05\) at 14d; \(n\geq 7\)), while blood glucose of \(\alpha\)TC\(\Delta\)PC2 recipients was slightly lower than sham-operated mice 2 d post-transplant (8.3 \(\pm\) 0.2 mM versus 9.0 \(\pm\) 0.2 mM respectively; \(P<0.05; n=8\)) but not different thereafter. Body weights were not different amongst the 3 groups (Fig. 11B). Glucose handling was assessed using an IPGTT 7 d post-transplant (Fig. 11C). While sham-operated mice and \(\alpha\)TC-1 recipients behaved similarly, \(\alpha\)TC\(\Delta\)PC2 recipients had a dramatic improvement in glucose tolerance, with lower peak glucose levels (10.0 \(\pm\) 0.2 mM versus 16.7 \(\pm\) 1.9 mM for sham at 15 min; \(P<0.001; n=7\)) and a 41% decrease in area under the curve (Fig. 11D; \(P<0.01; n=7\)).

Figure 11: Transplantation with encapsulated \(\alpha\)TC\(\Delta\)PC2 cells improves glucose tolerance in normal mice (A-C) CD-1 mice were transplanted with 4.0x10^7 \(\alpha\)TC\(\Delta\)PC2 or \(\alpha\)TC-1 cells \((n\geq 6)\). (A) Four-hour a.m. fasted blood glucose. (B) IPGTT (2 g/kg) performed 6 d post-transplant. (C) Area under curve (AUC) for IPGTT. \#\(P<0.05\) (\(\alpha\)TC-1 versus sham); \*\(P<0.05\), \**\(P<0.01\), \***\(P<0.001\) (\(\alpha\)TC\(\Delta\)PC2 versus sham).
Sixteen days post-transplant, CD-1 mice were sacrificed and pancreas tissue collected for histological examination. All groups had similar total and average insulin-positive area and number of insulin-positive areas (Fig. 12A-E). However, αTC-1 recipients had relatively fewer

Figure 12: Analysis of pancreata from mice transplanted with encapsulated αTCΔPC2 or αTC-1 cells
CD-1 mice transplanted with αTCΔPC2 or αTC-1 cells were sacrificed 16 d post-transplant (n≥4). (A) Insulin (green) and glucagon (red) in representative islets from transplant recipients. Scale bar, 100 µm. (B) Total insulin-positive area per section, expressed as % of sectional pancreas area. (C) Number of insulin-positive clusters per section, normalized for sectional pancreas area. (D) Islet number, stratified by islet size, expressed as % of total islets per section. White bars, <2500 µm²; hatched bars, 2501-10000 µm²; black bars, 10001-50000 µm²; striped bars, >50001 µm². (E) Average size of an individual insulin-positive area (µm²). (F) Proportion of α-cells/β-cells, expressed as % of total α- and β-cells per islet. (G) Total glucagon-positive area per section, expressed as % of sectional pancreas area. (H) Number of glucagon-positive clusters per section, normalized for sectional pancreas area. (I) Average size of an individual glucagon-positive area. *P<0.05 (sham versus αTC-1). #P<0.05 (αTCΔPC2 versus αTC-1).
α-cells per islet (Fig. 12F; \( P<0.05; n \geq 4 \)) and a trend towards decreased total glucagon-positive area \((P=0.11)\) owing to decreased average size of glucagon-positive areas (Fig. 12G-I; \( P<0.05 \) for mean glucagon-positive area compared to sham).

To assess the contribution of GLP-1R signalling to the improved glucose tolerance observed after αTCΔPC2 transplant, we acquired GLP-1R\(^{-/-}\) mice from Dr. D. Drucker (University of Toronto). In order to distinguish wildtype, knockout, and heterozygous mice, we sought to develop a PCR-based genotyping protocol, since such a protocol had not previously been reported. Based on the initial description of the knockout GLP-1R allele as having a Neo\(^R\) insert disrupting the region encoding transmembrane domains 1 through 3 of the GLP-1R (part of exon 5 to part of exon 7, and intervening introns; (226)), we designed primers targeted at this area (GLP-1R F1 and R1 and Neo\(^R\) forward and reverse; Fig. 13A, B). However, we found that even in mice previously identified by Dr. Drucker as knockouts, GLP-1R F1 and R1 primers still

**Figure 13:** Development of a PCR-based genotyping protocol for GLP-1R\(^{-/-}\) mice

(A, B) Schematics of protein structure (A) and simplified intron/exon sequences (B) showing the region originally reported to be disrupted in GLP-1R\(^{-/-}\) mice (yellow) and the actual region disrupted in GLP-1R\(^{-/-}\) mice (red). Overlap between these regions is shown in orange. The exon regions encoding transmembrane domains (TM) 1-4 are shown in (B). (C, D) PCR products amplified from known \(-/-, +/-, +/+\) mice using primers GLP-1R F1 and GLP-1R R1 (C) or primers GLP-1R F2 and GLP-1R R2 (D).
amplified the 424 bp wildtype GLP-1R band (Fig. 13C). Three other sets of primers targeted to this region yielded the same result (data not shown). A review of the literature on GLP-1R<sup>−/−</sup> mice yielded one other report in which the targeting region of the Neo<sup>R</sup> insert in the knockout GLP-1R allele was discussed (227). Interestingly, this later report localized the Neo<sup>R</sup> insert to the region encoding transmembrane domains 2 through 4 of the GLP1-R (Fig. 13A, B). We therefore designed an alternate pair of primers (GLP-1R F2 and R2) targeted to intron 6 of the GLP-1R. These primers amplified a 437 bp wildtype GLP-1R band in known wildtype and heterozygous mice, but not in known knockout mice, and used in combination with Neo<sup>R</sup> primers, allowed us to distinguish knockout mice from heterozygotes and wildtypes (Fig. 13D).

Having developed a means of PCR-based genotyping to identify GLP-1R<sup>−/−</sup> and GLP-1R<sup>+/−</sup> mice, we proceeded to transplant 4.0x10<sup>7</sup> encapsulated αTCΔPC2 cells to these mice. αTCΔPC2 cell transplant did not change body weight or 4 h fasted blood glucose in either GLP-1R<sup>+/−</sup> or GLP-1R<sup>−/−</sup> mice (Fig. 14A, B). However, compared to sham-operated controls,

**Figure 14: The ability of encapsulated αTCΔPC2 cells to improve glucose tolerance is largely dependent on GLP-1R signalling**

GLP-1R<sup>+/−</sup> and GLP-1R<sup>−/−</sup> mice were transplanted with 4.0x10<sup>7</sup> αTCΔPC2 cells on day 0 (n=5-10). Blood glucose (A) and body weight (B) were monitored following a 4 h a.m. fast. (C-E) IPGTT (2 g/kg) performed in GLP-1R<sup>+/−</sup> (C) or GLP-1R<sup>−/−</sup> (D) mice 7 d post-transplantation. (E) AUC for IPGTTs. *P<0.05 (αTCΔPC2 versus sham).
GLP-1R\(^{+/+}\) mice receiving \(\alpha\)TC\(\Delta\)PC2 cells had a 33% decrease in peak glucose levels (Fig. 14C; \(P<0.05\) at 7, 15 and 60 min; \(n\geq5\)) and a 25% decrease in AUC in an IPGTT performed 7d post-transplant (Fig. 14E; \(P<0.05\)). In contrast, GLP-1R\(^{-/-}\) mice receiving \(\alpha\)TC\(\Delta\)PC2 cells had a mild decrease in peak glucose (Fig. 14D; \(P<0.05\) at 7 min; \(n\geq5\)) and no change in AUC compared to controls (Fig. 14E).

**ADMINISTRATION OF MULTIPLE LOW-DOSE STZ TO MICE FOLLOWING TRANSPLANTATION OF PC2- OR PC1/3-EXPRESSING \(\alpha\)-CELLS**

Since transplantation of PC1/3-expressing \(\alpha\)TC\(\Delta\)PC2 cells or PC2-expressing \(\alpha\)TC-1 cells resulted in opposite effects on glucose homeostasis in non-diabetic mice, we sought to examine the impact of \(\alpha\)TC\(\Delta\)PC2 or \(\alpha\)TC-1 cells in a mouse model of T1D. C57BL/6 mice received sham surgery or encapsulated \(\alpha\)TC\(\Delta\)PC2 or \(\alpha\)TC-1 cells on day 0 and a low-dose regimen of the \(\beta\)-cell toxin STZ was administered from 8-12 d post-transplant. \(\alpha\)TC\(\Delta\)PC2 recipients exhibited lower fasting blood glucose levels than sham-operated mice even before STZ was administered (Fig. 15A; \(P<0.05\) on d5, \(P<0.01\) on d 8; \(n\geq8\)), and while control mice developed mild diabetes within \(\sim\)10 d of the last dose of STZ, \(\alpha\)TC\(\Delta\)PC2 recipients did not.

![Figure 15](image.png)

**Figure 15: Transplantation of encapsulated \(\alpha\)TC\(\Delta\)PC2 cells prevents hyperglycemia onset in multiple low-dose STZ-treated mice**

C57BL/6 mice received sham surgery or cell transplant on day 0, and daily STZ (50 mg/kg) from days 8-12 inclusive (\(n\geq8\)). Four-hour a.m. fasted blood glucose (A) and body weight (B) of recipients. (C) IPGTT (2 g/kg) performed 7 d post-transplant. Inset, area under curve (AUC). (D) Oral glucose tolerance test (2 g/kg) performed 20 d post-transplant. Inset, area under curve. \(*P<0.05\), \(**P<0.01\), ***\(P<0.001\) compared to sham.
progress to diabetes (Fig. 15A). Body weights of control mice and αTCΔPC2 recipients did not differ (Fig. 15B). An IPGTT was performed 7 d post-transplant (prior to STZ administration), and consistent with our observations in CD-1 mice, αTCΔPC2 recipients exhibited a dramatic improvement in glucose tolerance, with lower blood glucose levels at all timepoints (Fig. 15C; P<0.01 at 0 min, P<0.001 at 7 min, P<0.05 at 15 min and 60 min) and a 55.6% decrease in AUC (Fig. 15C inset; P<0.05). Plasma GLP-1 was measured in blood samples collected just before glucose administration, and while GLP-1 was below the level of detection in control mice (<8.9 pg/mL) αTCΔPC2 recipients had plasma GLP-1 levels of 297.7 ± 36.7 pg/mL (n=6). In an OGTT performed 20 d post-transplant (just prior to STZ-induced diabetes onset), αTCΔPC2 recipients again displayed improved glucose disposal, with lower peak blood glucose values (Fig. 15D; P<0.05 at 7 min, P<0.01 at 15 min, P<0.001 at 120 min) and a 24.9% decrease in AUC (Fig. 15D inset; P<0.05).

In contrast to αTCΔPC2 recipients, αTC-1-transplanted mice had consistently higher blood glucose levels than sham-operated controls following transplant (Fig. 16A; P<0.001 at d3; P<0.01 at d5, d10 and d12; P<0.05 at d14 and d17). An IPGTT was performed 7 d post-transplant, and unlike αTCΔPC2 recipients, αTC-1 recipients exhibited impaired glucose

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**Figure 16:** Transplantation of encapsulated αTC-1 cells does not prevent hyperglycemia in multiple low-dose STZ-treated mice

C57BL/6 mice received sham surgery or cell transplant on day 0, and daily STZ (50 mg/kg) from days 8-12 inclusive (n=8). Four-hour a.m. fasted blood glucose (A) and body weight (B) of recipients. (C) IPGTT (2 g/kg) performed 7 d post-transplant. Inset, area under curve (AUC). (D) Oral glucose tolerance test (2 g/kg) performed 20 d post-transplant. Inset, area under curve. *P<0.05, **P<0.01, ***P<0.001 compared to sham.
tolerance relative to control mice, with higher peak blood glucose levels (Fig. 16C; P<0.01 at 7 min and 15 min) and a 53.0% increase in AUC (Fig. 16C inset; P<0.05). Plasma glucagon was measured in plasma collected just before glucose administration and αTC-1 recipients had 3-fold greater glucagon levels than controls (799.6 ± 60.2 pg/mL versus 246.6 ± 41.2 pg/mL; n=8; P<0.001). In response to STZ treatment, αTC-1 recipients developed hyperglycemia similar to control mice (Fig. 16A). An OGTT was performed 20 d post-transplant, and in contrast to αTCΔPC2 recipients, αTC-1 recipients did not exhibit improved glucose handling (Fig. 16D).

Plasma and histological analysis of STZ-treated αTCΔPC2 or αTC-1 recipient mice

Mice were sacrificed 30 d post-transplant, and plasma and pancreatic tissue were collected for further analysis. αTCΔPC2-treated mice had 3-fold higher plasma GLP-1 levels.

![Figure 17: Analysis of pancreata from STZ-treated mice receiving encapsulated αTCΔPC2 cells](image)

Mice were sacrificed 30 d post-transplant (n≥3). (A) Insulin (green) and glucagon (red) in representative islet from recipients. Scale bar, 100 µm. (B) Proportion of α-cells and β-cells in islets, expressed as percentage of total combined α- and β-cells per islet. (C) Total insulin-positive area, expressed as % of sectional pancreas area. (D) Average area of an individual insulin-positive region. (E) Number of insulin-positive areas per animal, normalized for sectional pancreas area. (F) Insulin (green) and PCNA (red) staining in representative islet from recipients. Scale bar, 50 µm. Arrow identifies a PCNA+ β-cell. (G) Number of PCNA+ β-cells, expressed as a percentage of total insulin-positive cells. *P<0.05 versus sham.
(177.7 ± 56.5 pg/mL versus 59.9 ± 12.0 pg/mL; \( P < 0.05; n \geq 5 \)) and 1.8-fold greater plasma GLP-2 levels than sham-operated controls at this time (1.3 ± 0.2 versus 0.7 ± 0.02 ng/mL; \( P < 0.05; n \geq 8 \)). \( \alpha \)TC-1 recipients had 8.3-fold higher plasma glucagon levels than controls (494.6 ± 164.7 pg/mL versus 59.77 ± 11.37 pg/mL; \( P < 0.05; n = 8 \)).

Histological examination of the pancreas revealed that sham-operated, STZ-treated mice had relatively more \( \alpha \)-cells and fewer \( \beta \)-cells per islet than did \( \alpha \)TC\( \Delta \)PC2-transplanted, STZ-treated mice (Fig. 17A, B). Sham-operated mice exhibited disrupted islet architecture, with more centrally-located \( \alpha \)-cells, while mice receiving \( \alpha \)TC\( \Delta \)PC2 cells were protected against this STZ-induced abnormality (28.0 ± 3.2 % versus 15.8 ± 1.6 % respectively; \( P < 0.01; n \geq 7 \)). \( \alpha \)TC\( \Delta \)PC2 recipients tended to have more insulin-positive pancreatic area (\( P = 0.062 \)) and on average, larger islets, though the number of islets was unchanged (Fig. 17C-E). \( \alpha \)TC\( \Delta \)PC2 recipients also

Figure 18: Analysis of pancreata from STZ-treated mice receiving encapsulated \( \alpha \)TC-1 cells
Mice were sacrificed 30 d post-transplant (\( n \geq 6 \)). (A) Insulin (green) and glucagon (red) in representative islets from recipients. Scale bar, 100 \( \mu \)m. (B) Proportion of \( \alpha \)-cells and \( \beta \)-cells in islets, expressed as % of total combined \( \alpha \)- and \( \beta \)-cells per islet. (C) Total insulin-positive area per animal, expressed as % of sectional pancreas area. (D) Average area of an individual insulin-positive cluster. (E) Number of insulin-positive areas, normalized for sectional pancreas area. (F) Total glucagon-positive area per section, expressed as % of sectional pancreas area. (G) Average size of an individual glucagon-positive area. (H) Number of glucagon-positive areas, normalized for sectional pancreas area. *\( P < 0.05 \), **\( P < 0.01 \) versus sham.
displayed an increased percentage of β-cells that were PCNA-positive (Fig. 17F, G). STZ-treated, αTC-1-transplanted mice also displayed relatively fewer α-cells and more β-cells per islet (Fig. 18A, B; \(P<0.01\)). Sham-operated mice had more centrally-located α-cells than did αTC-1 recipients (28.8 ± 1.8 % versus 16.7 ± 2.4 % of α-cells respectively; \(P<0.01\); \(n\geq7\)). However, while αTC-1 recipients exhibited no change in insulin-positive area, islet size, or islet number relative to sham-operated controls (Fig. 18C-E), they tended to have decreased total glucagon-positive pancreatic area (Fig. 18F; \(P=0.068\)). The average glucagon-positive area per islet was decreased in αTC-1 recipients (Fig. 18G; \(P<0.05\)), though the number of glucagon-positive areas was unchanged (Fig. 18H). β-cell replication as assessed by insulin/PCNA co-staining was not altered in αTC-1 recipients relative to controls (data not shown).

**Figure 19: Jejunal proliferation in STZ-treated mice receiving encapsulated αTCΔPC2 or αTC-1 cells**

Jejunal proliferation was assessed in sections prepared from αTCΔPC2 (A-D) or αTC-1 (E-F) recipients 30 d post-transplantation (\(n=6-8\) mice per group). Representative images are shown for quantification of crypt plus villus height (A; scale bar= 250 μm) and of PCNA staining (B; scale bar=100 μm). Crypt plus villus height was measured from the crypt base to the tip of the villus in duplicate for ≥4 upright, intact villi per animal (C, E). Distribution of PCNA-positive cells in jejunal crypts (D, F). The cell at the midpoint of the crypt base was defined as position 1, and the first 20 cells extending upward from this position were scored as PCNA-negative (0) or PCNA-positive (1). *\(P<0.05\), **\(P<0.01\) compared to respective sham.
We also examined jejunal sections for evidence of possible effects that any αTCΔPC2- or αTC-1-derived products might have had on this tissue; representative images are shown in Fig. 19A, B. Compared to sham-operated controls, αTCΔPC2 recipients displayed increased crypt plus villus height and increased proliferation (i.e. PCNA-positive cells) in the region 16-20 cells up from the crypt base (Fig. 19C, \(P<0.05\); Fig. 19D, \(P<0.05\) for cell positions 17 and 20; \(P<0.01\) for cell position 18). In contrast, αTC-1 recipients displayed no change in crypt plus villus height or crypt cell proliferation compared to their respective controls (Fig. 19E, F).

**The Effect of Transplanted PC2- versus PC1/3-expressing α-cells on Diabetes Development in Mouse Models of T2D**

Characterizing the impact of αTCΔPC2 cell transplant on glucose homeostasis in high-fat fed and db/db mice

Our observation that transplantation of PC1/3-expressing α-cells prevented STZ-induced diabetes led us to investigate their therapeutic efficacy in a mouse model of glucose intolerance. We transplanted \(40 \times 10^6\) encapsulated αTCΔPC2 cells to the i.p. cavity of 16 week old C57BL/6 mice that had been fed a 60% kcal from fat diet from 4 wk of age. Cell transplantation had no effect on body weight (Fig. 20A) and we could not detect any changes in food intake (data not shown).

![Figure 20](image-url)

**Figure 20:** Transplantation of encapsulated αTCΔPC2 cells improves glucose homeostasis in a mouse model of diet-induced obesity
16 wk old male mice fed a 60% fat diet from 4 wk of age received sham surgery or cell transplant on day 0 (\(n=8\) per condition). Body weight (A) and blood glucose (B) were monitored after a 4 h a.m. fast. (C-D) An IPGTT (2 g/kg) was performed 7 d post-transplant. AUC, area under curve. *\(P<0.05\), **\(P<0.01\), ***\(P<0.001\) compared to sham.
However, αTCΔPC2 recipients had lower fasted blood glucose levels than sham-operated controls at two post-transplant timepoints (Fig. 20B; 8.8 ± 0.3 mM versus 10.4 ± 0.5 mM at d4, P<0.05; 9.8 ± 0.3 mM versus 11.0 ± 0.3 mM at d13, P<0.05). An IPGTT performed 7d post-transplant revealed that αTCΔPC2 recipients had better glucose tolerance than controls, as indicated by a more rapid lowering of blood glucose levels (Fig. 20C; P<0.001 at 60 min and P<0.05 at 120 min) and an overall decrease in glycemic excursion (Fig. 20D; P<0.01).

Since αTCΔPC2 cells improved glycemia in the high-fat fed model of glucose intolerance, we hypothesized that transplantation of αTCΔPC2 cells might delay the onset of

Figure 21: Glucose homeostasis in db/db mice receiving αTCΔPC2 cell transplantation
Blood glucose (A) and body weight (B) were measured followed a 4 h morning fast. IPGTTs (2 g/kg) were performed 7 d (C, D) or 28 d (F, G) post-transplant following an overnight fast. AUC, area under curve. For the d 7 IPGTT, plasma was collected at the indicated timepoints and assayed for insulin (E), expressed as % increase compared to basal. (H) An insulin tolerance test (2U/kg) was performed 24 d post-transplant. *P<0.05, **P<0.01, ***P<0.001 using two-tailed student’s t-test (n=5-6 mice per group).
diabetes in the *db/db* mouse, a mouse model of T2D. While αTCΔPC2 cell transplant initially lowered fasting blood glucose levels post-transplant (6.6 ± 0.2 mM versus 7.7 ± 0.3 mM at 2d post-transplant; *P*<0.01), blood glucose levels were not different thereafter and both groups of animals developed diabetes at a similar rate (Fig. 21A). Nevertheless, as we had observed in other mouse models, αTCΔPC2 cells improved glucose tolerance, with cell-treated mice having lower blood glucose levels at each timepoint (Fig. 21C; *P*<0.05 at 0 min, *P*<0.01 at 7 min, 60 min, and 120 min, and *P*<0.001 at 15 min) and a 40% reduction in total glycemic excursion compared to sham-operated controls (Fig. 21D; *P*<0.01). As has been reported previously (237, 238), *db/db* mice were hyperinsulinemic even after an overnight fast and had defective GSIS in the IPGTT. However, αTCΔPC2 cell recipients displayed lower fasting insulin levels (2.13 ± 0.28 µg/L versus 3.47 ± 0.43 µg/L for sham-operated controls; *P*<0.05) and a re-establishment of insulin secretion in response to glucose injection (Fig. 21E; *P*<0.05 at 7 min and 15 min, *P*<0.01 at 60 min). An insulin tolerance test performed 24 d post-transplant revealed no change in insulin sensitivity in αTCΔPC2 cell recipients compared to controls (Fig. 21H). By 28 d post-transplant, the beneficial impact of αTCΔPC2 cells on glucose tolerance was no longer evident (Fig. 21F, G) except for a slight lowering of blood glucose levels at the 15 min timepoint (Fig. 21F; *P*<0.05 at 7 min). To determine whether αTCΔPC2 cell transplant had impacted body composition in *db/db* mice, we performed NMR 17 d post-transplant. Cell transplant recipients were found to have a slight but statistically significant increase in the ratio of lean:lipid body mass (Fig. 22C; *P*<0.05) without any change in overall body mass (data not shown), indicating that some product from the transplanted cells decreased fat mass in these mice.

Mice with genetic defects in the leptin receptor signalling pathway are known to have

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**Figure 22: Cold-induced thermogenesis in *db/db* mice receiving αTCΔPC2 cell transplantation**

29 d post-transplant, mice were housed at 4°C for 2 h and body temperature was monitored using a hand-held transponder (*n*=5-6 mice per group). Body temperature is expressed in absolute values (A) or as a % of basal body temperature (B). (C) Lean-to-lipid body mass ratio was determined by NMR 17 d post-transplant. *P*<0.05, **P*<0.01, ***P*<0.001 using two-tailed student’s *t*-test.
lower body temperature in both the light and dark periods, as well as defective cold-induced thermogenesis, compared to littermate controls (239-241). To assess whether PC1/3-derived proglucagon products can improve body temperature regulation in this model, we implanted indwelling temperature sensors in the interscapular region of db/db mice that had received αTCΔPC2 cell transplant or sham surgery. Cell transplant recipients had a remarkable improvement in their ability to maintain their body temperature during acute housing in a 4°C environment, with higher body temperature at almost every timepoint (Fig. 22A; P<0.05 at 20, 75, 90 and 105 min; P<0.01 at 45, 60 and 120 min).

**Characterizing the impact of αTCΔPC2 cell transplant on body temperature in db/db mice**

To further characterize the beneficial effects of αTCΔPC2 cell transplant on body temperature regulation additional cell transplants were performed in db/db mice and sham-operated wildtype C57BLKS controls. To measure the success of cell transplant, we repeated

![Graphs](image-url)

**Figure 23: Glucose homeostasis in db/db mice receiving αTCΔPC2 cell transplantation**

Blood glucose (A) and body weight (B) were measured followed a 4 h morning fast in db/db mice and wildtype controls. (C-F) An IPGTT (2 g/kg) was performed 7 d post-transplant following an overnight fast. AUC, area under curve. Plasma was collected assayed for insulin, expressed as absolute values (E) and as % increase compared to basal (F). *P<0.05, **P<0.01, ***P<0.001 for db/db sham versus db/db αTCΔPC2 transplant using student’s t-test; n=7-8 mice per group.
some of the metabolic measurements performed in our prior study. As in our previous study, transplantation of αTCΔPC2 cells had no impact on body weight (Fig. 23B) and did not delay diabetes onset in db/db mice, although transplant recipients tended to have lower blood glucose than sham-operated db/db controls (Fig. 23A; $P<0.001$ for entire post-transplant period using paired student’s $t$-test). As we had observed previously, αTCΔPC2 cell transplant improved glucose tolerance in db/db mice, with lower blood glucose levels at several timepoints (Fig. 23C; $P<0.01$ at 7 min and 60 min; $P<0.05$ at 120 min) and a 45% decrease in area under the curve to levels indistinguishable from wildtype (Fig. 23D; $P<0.01$ compared to sham). Cell transplant recipients had higher insulin levels prior to and following glucose injection (Fig. 23E; $P<0.05$ at 7 min, $P<0.001$ at 15 min), suggesting that transplanted cells increased insulin secretion.

We performed an initial cold tolerance test 5 d post-transplant, and found no difference between sham-operated and αTCΔPC2-transplanted db/db mice, though both groups had an impaired ability to maintain their body temperature compared to wildtype controls (Fig. 24A).

![Figure 24: Cold tolerance and diurnal body temperature in db/db mice receiving αTCΔPC2 cell transplantation](image)

Mice were individually housed for 2 h at 4°C 5 d (A) and 26 d (B) post-transplant. (C) Diurnal body temperature was measured in mice housed individually at room temperature. (D) Cold tolerance was assessed in GLP-1R$^{++}$ and GLP-1R$^{-/-}$ mice. *$P<0.05$, **$P<0.01$ for db/db sham versus db/db αTCΔPC2 transplant using student’s $t$-test; $n=7-8$.

However, by 26 d post-transplant, db/db αTCΔPC2 cell recipients were better able to maintain their body temperature than db/db controls (Fig. 24B; $P<0.05$ at 0, 30, 60, 75, and 90 min; $P<0.01$ at 45 min), though they still had lower body temperatures than wildtype controls towards the end of the cold exposure period. We monitored changes in body temperature over a 24 h
period in a cohort of singly housed mice, and found that at all timepoints, both groups of \(db/db\) mice had lower body temperature than wildtype controls. However, \(\alpha\)TCΔPC2 recipients tended to have higher body temperatures than sham-operated \(db/db\) mice, particularly during the dark phase (Fig. 24C; \(P<0.05\) at 2400h; \(P=0.013\) for entire 24 h period using paired student’s \(t\)-test).

Our observation that \(\alpha\)TCΔPC2 cells improved body temperature regulation in \(db/db\) mice led us to hypothesize that one or more of the PC1/3-derived proglucagon products might have a role in body temperature regulation. We reasoned that if GLP-1 were involved in

![Graphs and images]

**Figure 25: BAT and WAT morphology in \(db/db\) mice receiving \(\alpha\)TCΔPC2 cell transplantation**

Intrascapular BAT and epididymal WAT were collected 34 d post-transplant. (A, B) WAT and BAT mass as a % of body weight. (C, D) Representative hematoxylin- and eosin-stained images of WAT and BAT. (scale bar=120 \(\mu\)m). (E,F) Western blot showing UCP-1 and \(\beta\)-actin levels. Blots are representative of 3-5 experiments. UCP-1 protein expression is expressed as pixel intensity and is normalized for blot background and for \(\beta\)-actin expression. *** and ###, \(P<0.001\) versus wildtype; #, \(P<0.05\) versus wildtype; \(n\geq 4\) per group.
maintenance of body temperature, then mice with ablation of the GLP-1R might have an impaired ability to thermoregulate in a cold environment. However, a 2 h cold tolerance test revealed no difference in body temperature between GLP-1R\textsuperscript{−/−} and GLP-1R\textsuperscript{+/+} mice, and both groups were better able to thermoregulate than ob/ob control mice (Fig. 24D).

\textit{db/db} and wildtype mice were sacrificed 34 d post-transplant and tissues were collected for analysis. As expected, \textit{db/db} mice had greater epididymal WAT mass (Fig. 25A; P<0.001 for both groups versus wildtype) and intrascapular BAT mass (Fig. 25B; P<0.001 for sham and P<0.05 for \textit{αTCΔPC2} recipients versus wildtype) than did wildtype controls, though the mass of the fat depots did not differ between sham-operated and cell-transplanted \textit{db/db}s. Though both groups of \textit{db/db} mice had larger adipocytes in the WAT depot and larger lipid droplets in the adipocytes in the BAT depot compared to wildtype controls, we were not able to detect any difference between the groups of \textit{db/db} mice (Fig. 25C, D and data not shown). Using western blot analysis, we could not detect any difference in UCP-1 protein levels in the BAT depot of sham- versus \textit{αTCΔPC2}-treated \textit{db/db} mice (Fig. 25 E, F).

**TREATMENT OF ISOLATED MOUSE AND HUMAN ISLETS WITH ADENOVIRUS TO INDUCE α-CELL PC1/3 EXPRESSION**

**Transduction of isolated mouse islets with AdPC1/3**

As α-cells are concentrated in the periphery of mouse islets, we next developed conditions to achieve gene transfer to this region using Ad5lacZ. Treatment of isolated islets with Ad5lacZ at MOI 10 resulted in a pattern of patchy, weak transduction in the α-cell-rich islet periphery. Treatment at MOI 100, however, resulted in uniform transduction of virtually the entire islet periphery but not the β-cells that lie in the core of the islets, except in some very small islets and islet fragments (Fig. 26A). Costaining of X-galactosidase-stained islets for glucagon revealed that the virus-transduced region was primarily localized to glucagon-positive areas (Fig. 26A, B).

Immunostaining showed that using the same gene transfer conditions with AdPC1/3, we were successful in introducing PC1/3 to cells in the islet periphery, increasing the levels of PC1/3 immunoreactivity in the periphery to exceed those in the islet β-cell core, which expresses this enzyme endogenously (Fig. 26C). Consistent with our results in InR1-G9 and \textit{αTC}-1 cells (Fig. 3E, F), increased peripheral PC1/3 expression in islets significantly increased GLP-1
release, by a magnitude of up to 29 times (Fig. 26D; 84.3±7.9 pM at MOI 100 versus 2.9±0.9 pM for control islets; \( P < 0.001 \)).

![Image](image_url)

**Figure 26:** Optimization of adenoviral transduction conditions to target the mouse islet periphery and induce islet GLP-1 secretion  
Ad5lacZ was used to optimize transduction conditions such that the islet periphery was transduced in isolated islets. Transduced cells appear blue; glucagon-positive cells appear brown. Scale bar=250 µm (A) or 100 µm (B, C). (C) PC1/3 immunoreactivity in mouse islets 72 h after treatment with AdPC1/3. (D) GLP-1 secretion by mouse islets 72 h after treatment with AdPC1/3. Data were analyzed using one-way ANOVA; ***\( P < 0.001 \) versus MOI 0; \( n = 3 \).

Assessment of glucose-stimulated insulin secretion (GSIS) and survival of AdPC1/3-transduced mouse islets

Our next goal was to test the impact of increased islet GLP-1 production on islet function. Initial studies showed that we were able to successfully culture mouse islets for at least 72 h without loss of GSIS, and confirmed our protocol for assessing static GSIS in isolated mouse islets (Fig. 27A; \( P < 0.001 \) for freshly isolated islets and \( P = 0.08 \) for 72 h culture). However,
Figure 27: Assessment of mouse islet function using static glucose-stimulated insulin secretion

(A) Static glucose-stimulated insulin secretion in mouse islets just after isolation and again 72 h later. ***P<0.001 versus low glucose control using student’s t-test; n=2-3 per condition. (B) Static glucose-stimulated insulin secretion assay cannot distinguish between the function of uninfected (control) islets and AdPC1/3-transduced islets. Islets were infected with AdPC1/3 at MOI 100 48 h prior to the GSIS experiment. The same GSIS procedure as outlined in (A) was used except using 50 islets per well. **P<0.01 versus low-glucose control using student’s t-test; n=4-6 per condition.

Despite robust expression of the known insulin secretogogue GLP-1 (Fig. 26D), AdPC1/3-transduced islets showed no improvement in static GSIS compared to mock-transduced controls using the same static GSIS protocol (Fig. 27B). We also sought to assess GSIS in AdPC1/3-transduced islets using perifusion. Mock- or AdPC1/3-transduced islets were challenged with high (20 mM) glucose and glucose-stimulated insulin output was assessed by insulin RIA in 5 min perifusate fractions. AdPC1/3-transduced islets (MOI 100) had a greater insulin response to glucose stimulation than mock-transduced islets, with greater peak insulin levels (Fig. 28A; 4.76 ± 1.15 ng/mL versus 2.63 ± 0.55 ng/mL at 40 min; P<0.05) and a 57% increase in total insulin output (Fig. 28B; 88.2 ± 20.69 ng/mL versus 56.27 ± 17.0 ng/mL•min; P<0.01).

Figure 28: Assessment of mouse islet function using perifusion

(A) AdPC1/3 transduction enhances glucose-stimulated insulin secretion in isolated mouse islets. Perifusion was performed 72 h post-transduction with AdPC1/3 (MOI 100). Area under curve (B) was determined for entire curve normalized for baseline. Data were analyzed using a paired two-tailed student’s t-test; n=4 with 2 independent replicates per experiment.

We also wanted to examine whether transduction with AdPC1/3 offered islets protection against cell death, since the PC1/3-derived proglucagon product GLP-1 protects β-cells against
necrotic and apoptotic cell death (148-150). To this end, we treated islets with H$_2$O$_2$ to induce oxidative stress, or with one or more of the pro-inflammatory cytokines interferon $\gamma$ (IFN$\gamma$), tumor necrosis factor $\alpha$ (TNF$\alpha$), and interleukin-1$\beta$ (IL-1$\beta$). After a 24 h treatment period, a live/dead assay was performed to visualize dead and dying cells within whole islets. There was no detectable difference in the amount of live cells in islets that received virus and/or cytokine or H$_2$O$_2$ treatment compared to untreated islets (data not shown and Fig. 29C). Initial tests showed that all of the compounds tested increased islet cell death compared to uninsulted control islets (Fig. 29A). We selected 50 $\mu$M H$_2$O$_2$ for further study in islets transduced with Ad5lacZ or AdPC1/3. Interestingly, there was a trend towards increased cell death in virus-transduced islets

![Figure 29: Quantification of islet cell death using the live/dead assay](image)

(A) Mouse islets were treated with various combinations and doses of cytokines or H$_2$O$_2$ for 24 h prior to live/dead assay. Quantification of cell death in islets is reported as average red fluorescence intensity normalized for islet area. Data were collected from $\geq$7 individual islets and analysis was performed using two-tailed student’s t-test to compare each condition to uninsulted islet control (*$P<0.05$, **$P<0.01$, ***$P<0.001$ versus uninsulted). (B) AdPC1/3 transduction does not protect mouse islets from cell death induced by a 24 h culture with 50 $\mu$M H$_2$O$_2$. (C-D) AdPC1/3-transduced islets are protected from cell death induced by 24 h IL-1$\beta$ treatment. (C) Merged image of live (green) and dead (red) cells in whole mouse islets treated with virus (MOI 10) and with or without IL-1$\beta$ (50 U/mL). Scale bar=200 $\mu$m. (D) Quantification of cell death in IL-1$\beta$-treated islets, reported as mean red fluorescent intensity, normalized for islet area. Data were collected from 6-15 individual islets for two independent experiments and analysis was performed using t-test. *$P<0.05$, **$P<0.01$ versus no IL-1$\beta$ control.
(both AdPC1/3 and Ad5lacZ), though this trend did not reach statistical significance (Fig. 29B,D). This is probably due to non-specific toxicity associated with adenoviral transduction. We were unable, however, to detect any protective effect of AdPC1/3 transduction against H$_2$O$_2$-induced cell death (Fig. 29B), perhaps because GLP-1 was unable to overcome such a harsh death-inducing agent. We thus chose a less severe treatment using 50 U/mL IL-1β for further testing in AdPC1/3-transduced islets. Treatment with IL-1β induced a significant increase in cell death in control and Ad5lacZ-transduced islets (Fig. 29C, D; $P<0.01$ for IL-1β-treated versus untreated control islets; $P<0.05$ for IL-1β-treated versus untreated Ad5lacZ islets). Remarkably, however, AdPC1/3-transduced islets showed no increase in cell death upon exposure to IL-1β and thus appeared to be protected against IL-1β-induced cell death (Fig. 29C, D).

**Transplantation of AdPC1/3-transduced mouse islets**

Having established that AdPC1/3-transduced islets have enhanced function and survival *in vitro*, we next tested the performance of these GLP-1-producing islets in a marginal mass model of islet transplantation in mice. We reasoned that GLP-1 production in AdPC1/3-transduced islets might render a marginal mass of islets (that is, one that would normally be insufficient to correct STZ-induced hyperglycemia) able to restore normoglycemia.

Recipient C57BL/6 mice were treated with STZ 5 d prior to transplant; this induced weight loss (Fig. 30A, C) and rapid development of fasting hyperglycemia (Fig. 30B, D). As transduction of islets with a high dose of virus *in vitro* was associated with a slight increase in cell death (Fig. 29D), we opted to test a lower dose of virus (MOI 10) in initial *in vivo* studies. Based on previous work reporting the minimal mass of syngeneic islets necessary to reverse STZ-induced diabetes in mice as 150-200 IE (242, 243), we transplanted 150 IE, either mock-transduced or transduced with AdPC1/3 at MOI 10, beneath the kidney capsule of diabetic recipient mice. This islet dose was insufficient to reverse hyperglycemia in all recipients, even when islets had been transduced with AdPC1/3 to induce GLP-1 production (Fig. 30B). We therefore tested a higher islet dose for transplantation (200 IE) along with a higher MOI for AdPC1/3 transduction to increase GLP-1 production further. At this islet dose, islets transduced at MOI 0 and MOI 100 were unable to restore normoglycemia, while islets transduced at MOI 10 restored normal fasting blood glucose levels within the first week after transplant (Fig. 30D; $P<0.05$ for MOI 10 recipients versus controls at d2, 3, 4, 6 and 10). Recipients of islets
Figure 30: Preliminary islet transplantation experiments in mice

Mice were treated with 200 mg/kg STZ 5 d prior to transplantation. Islets were infected with AdPC1/3 (or mock-infected) at MOI 10 or 100 for 1 h. Diabetic recipient mice received transplantation of 150 IE (A-B; n=6 per condition) or 200 IE (C-F; n=4-5 per condition), and 4 h a.m. fasted body weight (A, C) and blood glucose (B, D) were measured. 150 IE was not sufficient to restore normoglycemia in any animal. (E-F) An IPGTT (2 g/kg) was performed in recipients of 200 IE 4 d post-transplant. AUC, area under curve. *P<0.05, **P<0.01 for AdPC1/3 MOI 10 versus control using two-tailed student’s t-test.

transduced with AdPC1/3 at MOI 10, but not MOI 100, had lower blood glucose levels at most timepoints in an IPGTT performed 4 d post-transplant (Fig. 30E; P<0.05 at 0, 20 and 30 min; P<0.01 at 60 min), although integrated response to glucose was not altered (Fig. 30F). We therefore chose a dose of 200 IE transduced with AdPC1/3 at MOI 10 for further study.

Following induction of diabetes with STZ, mice received a subcapsular transplant of 200 non-transduced control islets or AdPC1/3-transduced islets. Mock-transduced control islets were slow to restore normoglycemia in recipient mice, or incapable of ameliorating hyperglycemia at all. In marked contrast, however, transplantation of AdPC1/3-transduced islets resulted in a prompt return to normoglycemia in all recipients (Fig. 31A, B). When recipient mice were challenged with an IPGTT 5 d post-transplant, mice that had received AdPC1/3-treated islets
Figure 31: AdPC1/3 transduction improves islet transplantation outcomes in STZ-treated mice
STZ-treated diabetic mice received subcapsular transplant of mock- (control) or AdPC1/3-transduced (MOI 10) islets on day 0 and nephrectomy (Nx) on Day 21 (n=.
(A) Blood glucose levels in transplant recipients. (B) Blood glucose normalization curves for transplant recipients, expressed as % of animals normalized. The day of normalization was considered the second of ≥2 consecutive days where blood glucose was <15 mM. (C) IPGTT (2 g/kg) performed 5 d post-transplantation. *P<0.05, **P<0.01 versus control.

demonstrated lower blood glucose levels at all time points (Fig. 31C; P<0.05 at 0, 10, 20 and 30 min) although AUC was unchanged (data not shown). There was no effect of AdPC1/3 treatment of islets on the body weight of recipient animals (not shown). There also did not appear to be any deleterious, non-specific effects of virus transduction on the long-term function of islet grafts (or any such effects were masked by the benefits of GLP-1 production), since all recipient mice maintained normoglycemia for the duration of the study. Blood glucose levels rapidly returned to pre-transplant levels in all animals upon removal of the islet graft-bearing kidney 2 d (not shown) or 21 d post-transplant (Fig. 31A).

Histological examination was performed on grafts retrieved from mice. Both α- and β-cells were easily identified by robust glucagon and insulin immunofluoresence (Fig. 32A), though PC1/3 expression was not detectable in α-cells of grafts harvested 21 d post-transplant (not shown). There was a trend towards more intense insulin staining in β-cells of AdPC1/3-
transduced grafts, but this did not reach statistical significance (Fig. 32C). Islet grafts harvested 2 d post-transplantation exhibited weaker insulin staining and β-cell degranulation (not shown). However, a subset of α-cells was clearly PC1/3- and glucagon-positive at this time point (white arrowhead, Fig. 32B). There was no difference in the proportion of α-cells or β-cells in control versus AdPC1/3-transduced islet grafts at either 2 d or 21 d post-transplant (Fig. 32D). Since GLP-1’s effects on β-cells include enhanced expression and activity of the transcription factor Pdx1 (137), we performed double immunofluorescence for Pdx1 and insulin in islet grafts. We could not detect significant cytoplasmic Pdx1 staining in either control or AdPC1/3-transduced grafts (Fig. 32E). However, nuclear Pdx1 staining was more intense in β-cells in AdPC1/3-transduced grafts compared to controls (Fig. 32F), though this effect was significant only for the grafts harvested 21 d after transplant.
AdPC1/3 transduction of human islets

Having observed that treatment of mouse islets with AdPC1/3 induces GLP-1 production and enhances islet function and survival in vitro and in vivo, we next wanted to extend these studies to human islets. Using the same viral transduction conditions as in mouse islets, we were unable to induce any significant increase in GLP-1 secretion from human islets transduced at MOI 10 or MOI 100 (Fig. 33A). We were also unable to detect any difference in static GSIS in AdPC1/3-transduced human islets compared to sham-operated controls (Fig. 33B). In the absence of any effect of AdPC1/3 treatment on GLP-1 levels, we did not test GSIS using the perifusion protocol.

Figure 33: Treatment of human islets with AdPC1/3
(A-B) The conditions under which AdPC1/3 delivery to mouse islets increases islet GLP-1 production neither induces GLP-1 production nor enhances GSIS in human islets. (A) Active GLP-1 content was measured in human islet media after a 24 h static culture carried out 48-72 h post-transduction. n=2-4 per condition. (B) Static GSIS in human islets 72 h post-infection with AdPC1/3. *P<0.05, **P<0.01 versus 2 mM glucose; n=2-4 per condition.
DISCUSSION

Based on its pleiotropic effects on insulin secretion, gastric emptying, and food intake, GLP-1 has recently been the subject of intense clinical study as a novel therapy for diabetes. While GLP-1R agonists and DPPIV inhibitors are available for clinical usage, they require frequent administration. Since GLP-1’s insulinotropic action is glucose-dependent and therefore should not induce hypoglycemia, one proposed alternative has been to use gene therapy as a means of achieving long-term, continuous GLP-1 delivery. Indeed, intramuscular delivery of a GLP-1 or exendin-4 construct via electroporation has been reported to decrease blood glucose levels, improve GSIS, and increase β-cell mass in the multiple low-dose STZ model of T1D and to improve glucose homeostasis in db/db mice (244, 245). Others have shown that induction of ectopic GLP-1 expression using systemically delivered adenoviral or plasmid-based constructs enhances GSIS and peripheral glucose uptake and decreases hepatic glucose output in ob/ob mice (246) and improves glucose tolerance and decreases food intake and weight gain in db/db mice and Zucker Diabetic Fatty rats (247). However, all of the gene therapy approaches reported thus far have utilized constitutive, non-specific expression systems. The consequences and long-term efficacy of chronic GLP-1 expression in a non-endocrine cell are unclear, and such non-specific vectors would face significant regulatory hurdles before they could be tested clinically.

With this in mind, we sought an alternative site for ectopic GLP-1 production. Others have proposed the β-cell itself as a potential site for GLP-1 production. Indeed, transduction of insulinoma cells with a GLP-1 minigene increases insulin gene transcription and secretion and is protective against the toxic effects of immunosuppressive drugs (248, 249), and overexpression of a DPPIV-resistant form of GLP-1 improves GSIS in a β-cell line (250). However, these findings have yet to be extended to primary β-cells, and whether long-term expression of a non-native protein in the β-cell might have untoward effects on cell viability and function remains an open question, particularly given the sensitivity of the β-cell to ER stress (251).

We therefore looked to the α-cell, which possesses several key characteristics that may be advantageous for GLP-1 production. First, the native α-cell is located in close proximity to β-cells—the very cells whose function and survival diabetes therapies seek to promote. We hypothesized that GLP-1 produced locally within islet α-cells might reach neighbouring β-cells via vascular and/or interstitial connections. Secondly, the α-cell is an endocrine cell already equipped with the transcriptional and secretory machinery necessary for efficient production of a
secreted protein. Finally, it is also already equipped with the proglucagon precursor from which GLP-1 is derived and as has been discussed, there is evidence that during development and under specific types of postnatal stress, the α-cell itself turns on GLP-1 expression.

In keeping with its key counteregulatory role, secretion of glucagon from the α-cell is increased in response to low glucose and in healthy humans, decreased in response to high blood glucose levels (252-254). The α-cell might seem a counterintuitive site for GLP-1 production if the objective were solely to exploit GLP-1’s insulinotropic effect to treat high blood glucose levels. However, since significant time is spent in the postabsorptive state over a 24 h period, such an arrangement may be beneficial for harnessing GLP-1’s long-term, pro-survival effects on the β-cell. Furthermore, α-cell secretion is active at a basal level even under high glucose conditions (255, 256). Low levels of insulin have been shown to promote survival of primary mouse and human β-cells (257); we reasoned that perhaps small amounts of GLP-1 released from the α-cell under postabsorptive conditions might have a similar effect.

Therefore, the goal of the current work was to investigate whether the pancreatic α-cell, in which proglucagon is normally processed to yield glucagon (Fig. 2), could be manipulated to liberate the alternate hormone GLP-1. We further sought to assess whether this intervention would be therapeutically useful in the context of diabetes. While most gene therapy strategies involve direct delivery of the therapeutic product of interest to the diseased or deficient tissue, our strategy employs a unique approach wherein delivery of a processing enzyme allows the liberation of the product of interest from a precursor already present, but not normally processed, in the target tissue.

Prevailing dogma has been that PC1/3 is not produced, or at best is produced in very small amounts, in healthy postnatal islet α-cells, precluding GLP-1 production (258, 259). However, we could detect GLP-1 production in InR1-G9 and αTC-1 cells and GLP-1 was also secreted at low levels in normal, non-transduced islets. This is consistent with several reports demonstrating that islet α-cells do produce small amounts of GLP-1 which may act as a paracrine stimulus on β-cells (260, 261). Nevertheless, initial studies in InR1-G9 and αTC-1 cells showed that adenoviral PC1/3 delivery significantly increased GLP-1 secretion from these cells, suggesting that under normal conditions, low-level PC1/3 expression is the key factor limiting GLP-1 production from α-cells. Interestingly, compared to AdPC1/3-treated InR1-G9 cells, AdPC1/3-treated αTC-1 cells exhibited much lower levels of the 82 kDa precursor and proportionally greater levels of the fully active 64 kDa form of PC1/3, as well as much higher
levels of GLP-1 secretion (Fig. 3). This observation may indicate that αTC-1 cells, derived from a mouse glucagonoma (262), more efficiently process PC1/3 in the secretory pathway than InR1-G9 cells, which were sub-cloned from a hamster insulinoma line (263).

One previous report by Dhanvantari et al. examined the effect of coexpression of PC1/3 and PC2 in α-cells by stably transfecting InR1-G9 cells with a PC1/3 construct (264). This resulted in increased processing of proglucagon to yield glicentin, oxyntomodulin, and GLP-2, but interestingly, not GLP-17-36amide (264). This apparent dissimilarity with the current studies may reflect that we were able to achieve higher levels of fully processed, bioactive PC1/3 in InR1-G9 and αTC-1 cells using adenoviral infection, while PC1/3 maturation was incomplete and/or insufficient in transfected InR1-G9 cells. We did not investigate the order in which PC1/3 and PC2 might cleave proglucagon in α-cells expressing both of these enzymes. However, the processing of proopiomelanocortin in pituitary melanotropes (265) and of proinsulin in pancreatic β-cells (266) is known to proceed in temporal order with PC1/3 preferentially acting first, followed by PC2-mediated precursor cleavage, perhaps because PC1/3 moves through and matures in the secretory pathway more rapidly than PC2, and/or because initial cleavage by PC1/3 opens up the molecule to allow PC2 binding. It is possible that this type of temporal processing first by PC1/3 and then by PC2 also occurs in α-cells coexpressing these processing enzymes. Interestingly, Dey et al. reported that cleavage at the interdomain site dibasic site at residues 70-71 of proglucagon (releasing glicentin and the major proglucagon fragment) preferentially occurs prior to any further processing, suggesting that this initial cleavage may relieve steric hindrance within the molecule and thereby allow further processing to occur (99).

Since PC1/3 and PC2 can both cleave the interdomain site of proglucagon (99), in AdPC1/3-transduced α-cells both enzymes may be able to perform this initial cleavage, releasing the N-terminal glicentin fragment for further processing by PC2 and the C-terminal major proglucagon fragment for further processing by PC1/3 (see Fig. 2).

Transplantation of alginate-encapsulated α-cells

Alginate encapsulation provides a means of protecting transplanted cells from host immune attack and has been used to achieve long-term maintenance of transplanted islets in models of diabetes (267-269). We used cell encapsulation to allow us to examine the long-term delivery of PC1/3-derived PGDPs in mice without the need for immunosuppression or use of an immunocompromised animal model. Capsules remained interspersed throughout the
intraperitoneal cavity of transplant recipients, though beyond ~3-4 weeks post-transplant capsules occasionally hardened and clumped together. This may be due to fibrotic overgrowth of capsules, as has been reported elsewhere (270-272), perhaps due to an inflammatory reaction mounted by the host immune system to the capsules. Indeed, when we removed capsules at the time of sacrifice of the recipients, we could often see cells adhering to the outside of the capsules when observed under the microscope (not shown). However, the inflammatory response to alginate capsules is thought to be primarily a reaction to poly-L-lysine, and not alginate itself (270, 272), and our encapsulation method did not involve coating the capsules with poly-L-lysine.

One of the drawbacks of alginate encapsulation as a means of cell therapy is that, unlike subcapsular transplantation of cells to the kidney, it is difficult to remove transplanted cells and monitor recipients for a reversal of symptoms. While it is theoretically possible to remove capsules by intraperitoneal lavage, in pilot studies we found this too difficult and invasive a procedure for routine survival surgery. One additional limitation of encapsulated cells is that, unlike cells transplanted beneath the kidney capsule, they do not develop direct vascular connections and thus tend not to be sensitive to changes in blood glucose levels (269, 273). However, the ease of the encapsulation and transplantation procedure combined with the ability to transplant a large volume of cells without the need for immunosuppression led us to select the encapsulation method for cell delivery.

Our observation that transplantation of AdPC1/3-transduced InR1-G9 cells had no appreciable effect on glucose handling in normal or db/db mice led us to suspect that in this model, the PC2 product glucagon might be counterbalancing the glucose-lowering effects of the PC1/3 product GLP-1. This was particularly concerning given the relatively short-term expression of transgenes imparted by adenoviral vectors. Indeed, we observed that by 2 wk post-transplant, very few Ad5lacZ-transduced InR1-G9 cells retrieved from transplanted capsules were still expressing adeno-virally-delivered β-galactosidase. We therefore altered our strategy to transplant α-cells expressing either PC2 (αTC-1) or PC1/3 (αTCAPC2) but not both (Fig. 8). As has been reported previously (100, 264), we observed that αTC-1 cells express PC2 but not PC1/3 and secrete glucagon but not GLP-1. In contrast, αTCAPC2 cells, which lack bioactive PC2 (228), expressed abundant levels of PC1/3 and thus secreted high levels of GLP-1 but very little glucagon. The upregulation of PC1/3 in the absence of PC2 seems to be specific to this cell line and not a more general feature of loss of function of PC2 in α-cells, since there is no
apparent induction of PC1/3 expression in the α-cells of PC2\textsuperscript{+/-} mice (102, 274), and antisense-mediated reduction of PC2 expression in α-cell lines has been reported to have no effect on PC1/3 levels (264, 275). Robust PC1/3 expression in αTCΔPC2 cells may reflect developmental arrest induced by transformation at a stage when PC2 and PC1/3 are normally coexpressed in the developing α-cell (189, 228, 274). High glucose culture conditions may have further promoted PC1/3 expression in αTCΔPC2 cells, since hyperglycemia has been shown to increase PC1/3 expression in rodent α-cells (152).

CD-1 mice that received transplants of glucagon-producing αTC-1 cells displayed increased fasting blood glucose levels. On the other hand, mice—including normal CD-1 and C57BL/6 models, and db/db and high-fat diet-fed models of T2D—that received GLP-1-producing αTCΔPC2 cells exhibited greatly improved glucose tolerance. Thus by changing the processing enzyme present in α-cells from PC2 to PC1/3, transplanted cells were converted from hyperglycemia-promoting to causing a robust improvement in glucose homeostasis, both in normal mice and in glucose intolerant models. The mechanism driving αTCΔPC2-mediated improvements in glucose handling is likely multi-faceted. In db/db mice, but not other models, αTCΔPC2 cell transplant increased the insulin response to i.p. glucose injection. We were unable to measure plasma glucagon levels with confidence given that all of the currently available assays cross-react with proglucagon and/or its other products, so it is possible that GLP-1 decreased plasma glucagon levels and thereby improved glucose homeostasis. This is consistent with other studies reporting suppression of plasma glucagon in response to chronic treatment with GLP-1R agonists in rodents and humans (276). It is possible that GLP-1 and/or oxyntomodulin increased energy expenditure and/or decreased gastric emptying, but we did not assess either of these parameters. Most likely pro-proliferative and/or anti-apoptotic effects of GLP-1 on the β-cell also contributed to the observed phenotype. The pro-proliferative effects of GLP-1 appear to involve activation of PKB-dependent insulin receptor substrate 2 signalling (277), as well as transactivation of the epidermal growth factor receptor and downstream phosphoinositide-3 kinase- and/or PKB-dependent pathways (147, 278). There is also evidence that the anti-apoptotic effects of GLP-1R agonism involve upregulation of IRS-2 expression and phosphorylation-dependent nuclear exclusion of FoxO1, a negative regulator of β-cell growth (279, 280).
Since at the time of sacrifice mice receiving \(\alpha TC-1\) or \(\alpha TC\Delta PC2\) cells still had elevated plasma levels of glucagon and GLP-1, respectively, transplanted cells likely remained viable even 30 d post-transplantation. This is consistent with previous studies showing long-term survival and function of encapsulated cells (267). It has been reported that encapsulated insulinoma cells proliferate \textit{ex vivo} (281); however, we are unaware of studies quantifying proliferation of tumour-derived endocrine cells in capsules retrieved from recipient mice. It remains possible that encapsulated \(\alpha TC-1\) and \(\alpha TC\Delta PC2\) cells proliferated \textit{in vivo} in the transplanted capsules, although we did not directly quantify this and the cell density in capsules retrieved from recipients did not appear grossly different than at the time of transplant (not shown).

Transplantation of \(\alpha TC\Delta PC2\) cells to GLP-1R\textsuperscript{−/−} mice revealed that the improvement in glucose tolerance observed in normal mice was primarily due to GLP-1R signalling (Fig. 14). Given that GLP-1R signalling has been implicated in \(\beta\)-cell survival (51, 148, 282), this finding led us to compare the effect of \(\alpha TC-1\) and \(\alpha TC\Delta PC2\) transplantation in a mouse model of diabetes. We chose STZ, a glucosamine-nitrosourea compound, as a diabetes-inducing agent. STZ is thought to be taken up into the \(\beta\)-cell via the GLUT2 glucose transporter, which recognizes a glucose moiety on the molecule; once inside the \(\beta\)-cell it causes DNA damage and generates reactive oxygen species which ultimately cause cell death (283, 284). In the low-dose STZ model, sham-operated mice developed hyperglycemia 1-2 weeks after STZ administration, while \(\alpha TC\Delta PC2\) recipients were protected against diabetes development and had improved glucose tolerance. In contrast, \(\alpha TC-1\) recipients had impaired glucose tolerance relative to sham-operated mice, and following STZ administration, developed hyperglycemia along with their respective controls. Histological examination of the pancreas revealed that \(\alpha TC\Delta PC2\) recipients had greater insulin-positive area than controls, while \(\alpha TC-1\) recipients showed no change compared to controls. Interestingly, sham-operated mice displayed the disrupted islet architecture typical of the multiple low-dose STZ model (9), while \(\alpha TC\Delta PC2\) recipients did not (Fig. 17). The relative increase in the number of \(\beta\)-cells per islet, and the increases in islet area and \(\beta\)-cell replication observed in \(\alpha TC\Delta PC2\)-treated mice suggest that a PC1/3-derived PGDP stimulated \(\beta\)-cell proliferation. GLP-1 is the most likely candidate given its known trophic effects on \(\beta\)-cell mass (51, 282, 285). It is possible that GLP-1 produced by the transplanted cells might also have protected islets against STZ-induced apoptosis in the \(\alpha TC\Delta PC2\) recipients,
consistent with GLP-1’s known pro-survival effect on β-cells (51, 148, 282). Further supporting the concept that these effects are mediated by a PC1/3-derived PGDP is the observation that transplanted PC2-expressing αTC-1 cells afforded no protection against STZ-induced hyperglycemia and did not induce greater insulin-positive area or β-cell proliferation (Fig. 18).

Consistent with our observations in CD-1 mice transplanted with αTC-1 cells, αTC-1-transplanted C57BL/6 mice displayed increased fasting blood glucose levels even before STZ treatment (Fig. 16). αTC-1 transplant in this model also caused impaired glucose tolerance. It is unclear how transplanted αTC-1 cells impaired glucose tolerance in C57BL/6 but not CD-1 mice, but it is possible that different genetic backgrounds have different capacities to compensate for hyperglucagonemia, or that the viability of transplanted cells differed between studies. We could not measure any differences in plasma insulin levels of either CD-1 or C57BL/6 mice receiving αTC-1 transplants compared to respective controls (not shown). This is consistent with a previous study in which mice transplanted with a subcutaneous glucagonoma were not hyperinsulinemic (286). However, compensation for chronic hyperglucagonemia may have occurred by other means, including decreased food intake and/or glucagon receptor downregulation. In both CD-1 and C57BL/6 models, chronic stimulation of hepatic glucose output by the excessive glucagon coming from the transplanted αTC-1 cells may have led to the observed perturbations in blood glucose levels. Consistent with the notion that ectopic glucagon decreased the demand for pancreatic glucagon, αTC-1 recipients exhibited α-cell hypoplasia in the endogenous pancreas (Figs. 12, 18). This observation supports previous studies in which α-cell atrophy resulted secondary to hyperglucagonemia induced by transplantation of a glucagonoma (286-288) or by glucagon minipump (289). This may have occurred via induction of α-cell apoptosis, and although we did not directly test this hypothesis, others have shown this to be the mechanism by which pancreatic α-cell hyperplasia is reversed following provision of exogenous glucagon (289). Notably, α-cell hypoplasia was absent in CD-1 mice receiving αTCΔPC2 transplants, providing further evidence that this was a compensatory adaptation specific to the PC2 product glucagon.

Disorganization of islet architecture and a relative increase in number of α-cells have been widely reported in models of β-cell damage or dysfunction (9, 290-293). It remains unclear whether these architectural changes reflect true α-cell hyperplasia or are an artifact of decreased islet size and the inward collapse of islets as β-cells are destroyed. It is also unknown whether
this putative change in α-cell mass is secondary to hyperglycemia or is directly due to the loss of an intra-islet β-cell-derived signal. However, in the current study, αTC-1 recipients were protected against STZ-induced disorganization of islet architecture and α-cell hyperplasia even in the face of hyperglycemia. Our studies therefore suggest that hyperglycemia itself is not sufficient to induce islet disorganization and α-cell hyperplasia.

In summary, our cell transplantation studies demonstrate that simply switching the PC profile in proglucagon-expressing cells can render them either glucose raising (PC2) or glucose lowering (PC1/3). Transplantation of GLP-1-producing αTCΔPC2 cells prevented STZ-induced hyperglycemia, which supports the growing body of evidence suggesting that long-term activation of the GLP-1R in mice promotes β-cell survival and proliferation (148, 245, 294). This work provides additional evidence that modulation of proglucagon processing in the α-cell promotes the release of a beneficial profile of PGDPs and could be useful for diabetes therapy. Moreover, αTCΔPC2 cells provide a model system for studying alternate proglucagon processing and examining the impact of continuous delivery of PC1/3-derived proglucagon products.

**Effects of PC1/3-derived PGDPs in db/db mice**

Similar to our results in normal mice, αTCΔPC2 cells improved glucose tolerance and modestly reduced fasting blood glucose in both cohorts of db/db mice we tested (Fig. 21, 23). However, while in the first db/db study αTCΔPC2 cells reduced fasting insulin levels and restored some degree of GSIS, in the second study cell transplant recipients were actually hyperinsulinemic compared to sham-operated controls at this same timepoint. The different response to αTCΔPC2 cell transplant may relate to the slightly different glycemic status of db/db mice in the two studies: in both sham-operated and cell-transplanted db/db mice, fasting glucose levels were approximately doubled in the second study compared to the first study (Fig. 21C; Fig. 23B). In both studies, however, αTCΔPC2 cell transplant was unable to delay the onset of overt diabetes in db/db mice.

Leptin, a hormone secreted by adipocytes, normally acts via the long form of the leptin receptor (ObRb) to indicate the status of adipose tissue stores to the central nervous system. In db/db mice, however, the ablation of ObRb and loss of this signalling axis renders these mice in a perceived state of negative energy balance, resulting in massive hyperphagia and obesity, decreased energy expenditure and metabolic rate, and suppression of energy-costly activities
such as reproduction and growth (295-298). It is likely that αTCΔPC2 cell transplant was simply unable to overcome this extreme, hyperglycemia-promoting ‘energy conservation’ phenotype in db/db mice for more than a short period of time. Interestingly, despite the known anorectic effects of GLP-1 (299, 300) and oxyntomodulin (72, 161), we did not observe any changes in food intake or body weight in db/db mice receiving αTCΔPC2 cell transplant compared to sham surgery. It is possible that our methods were simply not sensitive enough to observe changes in food intake, or that the pathways mediating these effects might have been desensitized due to chronic receptor stimulation. It is also possible that the perceived state of chronic negative energy balance of db/db mice was simply too powerful to overcome. This is consistent with previous work in which long-term administration of a GLP-1R agonist failed to significantly impact body weight or food intake in ob/ob mice (301).

Loss of leptin receptor signalling in mice also leads to abnormal thermoregulation and decreased capacity for adaptive thermogenesis (240, 302). This is thought to relate to decreased activation of leptin-sensitive sympathetic nerves innervating brown adipose tissue (303-306). In our studies mice were housed at ambient temperature below thermoneutrality—that is, below the temperature at which basal metabolism generates sufficient heat to maintain body temperature (∼28°C for mice). While acute exposure to a sub-thermoneutral temperature mainly activates shivering as a mechanism for maintaining body temperature, longer-term exposure activates non-shivering thermogenesis (or adaptive thermogenesis) to enable long-term adaptation of the animal to this temperature. The key mediator of this thermogenic adaptation is thought to be uncoupling protein 1 (UCP-1), which is expressed almost exclusively in brown adipose tissue and which uncouples cellular respiration from ATP synthesis such that heat is generated (307-310).

In accordance with previous observations (240), we observed that compared to wildtype controls, sham-operated db/db mice displayed a diurnal rhythm in body temperature that mimicked the amplitude and phase of wildtype mice, although at all timepoints their body temperature was below that of the wildtypes. Unexpectedly, we also observed that short-term exposure to 4°C unmasked an improvement in the thermogenic ability of db/db mice bearing an αTCΔPC2 cell transplant, although this was evident only after several weeks of exposure to transplanted αTCΔPC2 cells. This is consistent with the idea that increases in adaptive thermogenic capacity develop over the course of weeks, rather than days (307).
Delivery of exogenous leptin to the leptin-deficient \textit{ob/ob} mouse increases sympathetic activity and UCP-1 expression in brown adipocytes, resulting in increased body temperature and improved ability to withstand cold (241, 311, 312). We hypothesized that a product of the \textit{αTCΔPC2} cells might have induced a leptin-independent upregulation of UCP-1 levels in brown adipocytes. We were not able to detect any differences in UCP-1 protein levels between sham-operated and cell-treated \textit{db/db} mice. However, we also did not detect any difference in UCP-1 protein levels between wildtype and \textit{db/db} mice, despite previous work indicating that \textit{db/db} mice have lower UCP-1 levels than wildtype mice (307, 313). In the absence of this positive control, we cannot yet eliminate the possibility that \textit{αTCΔPC2} cell transplant might have increased UCP-1 protein levels and will need to repeat the experiment to further study this.

The mechanism driving the \textit{αTCΔPC2} cell-induced increases in body temperature of \textit{db/db} mice therefore remains unclear, as does the specific peptide(s) mediating this effect. It is intriguing to note that for some time, glucagon has been proposed to have thermogenic properties, although only when administered at \textasciitilde 1000x circulating levels (305). A more recent report found no thermogenic effect of purified glucagon and suggested that the earlier observations may have arisen from contamination of the glucagon preparation with another PGDP (314). We did not test whether transplantation of PC2-expressing \textit{α}-cells impacted cold tolerance and therefore, from our experiments we cannot exclude the possibility that glucagon or another PC2-derived proglucagon product might also have effects on cold thermogenesis. There is some evidence that GLP-1 and oxyntomodulin can modulate core body temperature, but results from various studies have been somewhat contradictory. Several studies in rats have reported that GLP-1 administered ICV or intravenously increases body temperature (315, 316), while in the Japanese quail GLP-1, but not GLP-2, has been reported to decrease body temperature when administered ICV or intravenously (315, 317). Treatment of rats with ICV oxyntomodulin has also been found to increase core body temperature, perhaps as a result of increased energy expenditure (318). However, a role for these peptides in cold-induced thermogenesis has not, to our knowledge, been described.

In our studies, one possibility is that GLP-1 arising from the transplanted cells improved thermogenesis in the \textit{db/db} mice. This notion is consistent with recent studies showing that peripherally-delivered GLP-1 can activate autonomic control centres in the CNS and thereby influence peripheral sympathetic functions such as blood pressure and heart rate (171, 319). However, we found that GLP-1R\textsuperscript{−/−} mice do not have a defective ability to thermoregulate in the
cold, suggesting that GLP-1R signalling is not essential for cold thermogenesis. This does not preclude any involvement of GLP-1R signalling in body temperature regulation, since effects of GLP-1R ablation on temperature regulation may simply be masked by an upregulation of other redundant mechanisms controlling this key metabolic parameter. Whether other PC1/3-derived PGDPs might contribute to the observed effects also remains possible, and further studies using continuous infusion of a single peptide would be required to assess the contribution of each of the PGDPs individually. Our studies with αTCΔPC2 cells in db/db mice thus indicate that one or more of the PC1/3-derived PGDPs may have a previously unappreciated effect on thermogenesis and body temperature regulation.

**GLP-1 production from within the mouse islet**

As in the α-cell lines, we were able to significantly increase GLP-1 output from native islet α-cells by transducing them with AdPC1/3. While the PC1/3 gene was likely transferred to a small number of peripheral β-cells in addition to α-cells, we do not anticipate adverse effects of such inadvertent delivery, since β-cells endogenously express PC1/3. Indeed, AdPC1/3-transduced islets had enhanced glucose-induced insulin secretion and were protected from IL-1β-mediated cell death. This is consistent with known anti-apoptotic actions of GLP-1. For example, GLP-1R agonists increase the survival and function of isolated rat β-cells following the induction of ER stress, at least in part by protein kinase A-dependent increases in the expression of ATF4 and other transcription factors involved in the stress response (320). Interestingly, our pilot transplantation studies showed that mouse islets transduced at the highest dose of AdPC1/3 tested did not perform as well as those transduced at a lower dose, despite producing greater levels of GLP-1. This was likely due to nonspecific viral toxicity as has been reported elsewhere (321), and we therefore used a lower viral dose for further study. In STZ-treated diabetic mice, islets transduced at this lower dose of AdPC1/3 were better able to restore good glucose control than normal islets, with fasting blood glucose and glucose tolerance approximately equivalent to those of normal mice.

How might delivery of PC1/3 to islet α-cells lead to greater transplantation success in diabetic mice? We do not anticipate that GLP-1 derived from AdPC1/3-transduced islets had any appreciable impact on circulating GLP-1 levels, and indeed, we could not detect any changes in plasma GLP-1 levels in recipients of AdPC1/3-transduced islets compared to untransduced islets (data not shown). Rather, we propose that α-cell-derived GLP-1 may interact with its
receptor on the β-cell surface by diffusion through the interstitial space. As the microcirculation of the native and transplanted islet remains controversial (14), it is also possible that GLP-1 reaches β-cells via direct vascular connections. The insulinotropic effect of GLP-1 could have helped restore normoglycemia sooner in transplant recipients and thereby relieve the glucotoxicity associated with prolonged exposure of islet grafts to high glucose levels (28). GLP-1 is known to activate insulin gene transcription (197), and though we did not directly measure insulin content, there was a trend towards increased insulin immunofluorescence in AdPC1/3-transduced islet grafts. However, because secretion from α-cells is increased at low glucose levels, the improved transplantation outcomes observed may primarily be due to promotion of β-cell survival and function via continuous exposure of islets to local GLP-1 under postabsorptive conditions. This is supported by our observation that AdPC1/3-transduced islets had less cell death than control islets in vitro. GLP-1’s effects on β-cell survival and proliferation are mediated through several pathways; however, activation of the β-cell transcription factor Pdx1 appears to be critical to GLP-1R-dependent promotion of β-cell growth and survival, since GLP-1R agonism cannot stimulate proliferation or inhibit apoptosis in mice specifically lacking Pdx1 in the β-cell (322). Indeed we also observed greater nuclear Pdx1 staining in the β-cells of AdPC1/3-transduced grafts. Although we did not directly assess β-cell apoptosis in islet grafts, this observation suggests that islet-derived GLP-1 led to increased Pdx1 expression in engrafted β-cells, and via inhibition of apoptosis and/or promotion of β-cell function, improved islet transplant outcomes. Our observation that there was no increase in the ratio of β-cells to α-cells in AdPC1/3-transduced islet grafts compared to control grafts argues against any significant effect of islet-derived GLP-1 on β-cell proliferation, although we did not directly test this parameter.

Potential contribution of other PC1/3-derived PGDPs

In addition to GLP-1, it is likely that αTCΔPC2 cells and AdPC1/3-transduced islets also produce oxyntomodulin and GLP-2, since these are also products of PC1/3 cleavage and are cosecreted along with GLP-1 from the enteroendocrine L-cell (51, 96, 282). The observation that αTCΔPC2 transplant improved glucose tolerance in wildtype, but not GLP-1R−/− mice suggests that GLP-1 coming from the transplanted cells is the primary PC1/3-derived proglucagon product that mediates this effect. However, since GLP-1R−/− mice receiving αTCΔPC2 cells had lower blood glucose than controls 15 min after glucose delivery, some other product of
αTCΔPC2 cells may also have beneficial effects on glucose handling. Oxyntomodulin has been reported to stimulate insulin secretion (179, 323) and thus is a potential candidate contributing to glucose-lowering effects, although such actions might also be mediated in part through the GLP-1R (72). Further studies involving continuous administration of either GLP-1 or oxyntomodulin, perhaps in combination with the GLP-1R antagonist exendin9-39, would be required to establish the relative contribution of these two peptides, and in the case of oxyntomodulin, whether any effects observed are mediated solely through the GLP-1R.

GLP-2 could also theoretically have contributed to the phenotype observed in αTCΔPC2 recipients and in AdPC1/3-transduced islet recipients. Until recently, GLP-2’s primary actions were thought to be exerted in the gut as a growth factor for crypt cells and enterocytes (186, 324). Though it has previously been reported that GLP-2R mRNA is not detectable in whole pancreas (205), more recently GLP-2R mRNA has been identified in isolated islets and GLP-2R immunoreactivity localized to α-cells but not other islet cells (206). Exogenous GLP-2 is glucagonotropic in humans (325) and in the perfused rat pancreas, where it can override the glucagonostatic effects of GLP-1 when the two are co-perfused (206). However, since GLP-2 does not have an insulinotropic effect (206, 326) and since the GLP-2 receptor has not been localized to the β-cell (205, 206), we consider it unlikely that GLP-2 acted directly on the β-cell to contribute significantly to the phenotype of αTCΔPC2 recipient mice or AdPC1/3-transduced islet recipients. We did, however, observe effects consistent with the known proliferative effects of GLP-2 on intestinal enterocytes (282, 324, 327). STZ-treated αTCΔPC2 recipients had increased plasma GLP-2 levels relative to controls and hyperplasia of the intestinal epithelium (Fig. 19). Notably, these effects were absent in mice that received αTC-1 cells, which lack the capacity for GLP-2 production. Our results are in agreement with a recent study in which injection of a GLP-2 analog resulted in greater proliferation in the “rapidly proliferating transit zone” defined by cells 11-20 of the jejunal crypts (235).

One of the challenges we consistently faced throughout these studies was that of identifying antisera and assay kits that were specific for the various fully processed, bioactive PGDPs without significant crossreactivity with proglucagon, processing intermediates, or other PGDPs. Previous studies have reported the presence of GLP-1 in the islets of normal (261) or diabetic (152) rats based at least in part on immunocytochemistry using “GLP-1” antisera. Our testing of the various antisera reported to be specific for bioactive GLP-1 in PC1/3+ mice revealed that many of the currently available antisera interact with peptides other than fully
processed GLP-1—most likely proglucagon and/or the major proglucagon fragment. This highlights the importance of using multiple methods and not immunocytochemistry alone to identify true GLP-1\textsubscript{7-36NH\textsubscript{2}} and/or GLP-1\textsubscript{7-37}. That many of the currently available assay kits for glucagon, GLP-1, GLP-2, and oxyntomodulin cross-react with multiple PGDPs further underlines the need for more specific antisera for studying these peptides. In our own studies, we did not use high performance liquid chromatography or mass spectrometry methods to confirm the identity of GLP-1 being released from PC1/3-expressing $\alpha$-cells as bioactive GLP-1\textsubscript{7-36NH\textsubscript{2}} or GLP-1\textsubscript{7-37}. However, our studies in GLP-1R\textsuperscript{−/−} mice indicated that GLP-1R signalling is necessary in order to observe glucose-lowering effects of GLP-1-producing $\alpha$-cells \textit{in vivo}.

Along with the ample evidence of the known biological actions of GLP-1 we observed in several additional \textit{in vivo} models of PC1/3-expressing $\alpha$-cells, this suggests that both islet $\alpha$-cells and $\alpha$-cell lines expressing PC1/3 do, in fact, produce biologically active GLP-1.

**Endogenous islet GLP-1 production**

It is intriguing to note that PC1/3 expression is activated in $\alpha$-cells under certain conditions and therefore, that our strategy of inducing islet GLP-1 expression may be recapitulating a naturally occurring phenomenon. The notion that GLP-1 promotes islet formation and/or function is supported by the observation that islets from GLP-1R\textsuperscript{−/−} mice have altered islet topography (31), increased sensitivity to damage by STZ (17) and impaired recovery from partial pancreatectomy (20). Others have suggested that the GLP-1 mediating such effects might arise from the islet itself (260, 261). Indeed, Masur \textit{et al} showed that conditioned media collected from isolated rat islets activated the GLP-1R in an \textit{in vitro} bioassay system involving COS-7 cells expressing the GLP-1R (261). This effect was inhibited by including exendin\textsubscript{9-39} in the culture media (261). As noted previously, during mouse embryogenesis, the earliest pancreatic progenitor cells co-express proglucagon, islet amyloid polypeptide, PC2, and PC1/3, suggesting that these cells may produce GLP-1 for some period of time (27). In addition, several models of pancreatic injury have been associated with islet GLP-1 production. Treatment of neonatal rats with STZ induces the appearance of islet cells co-expressing glucagon and GLP-1, and increases circulating glucagon and pancreatic GLP-1 content (28-30). STZ treatment of adult rats increases expression of PC1/3 and PC2 in glucagon-immunoreactive cells in islets, and this is correlated with increased GLP-1 levels in islet extracts and plasma (21). Disruption of glucagon receptor expression in rodents has also been associated with increased pancreatic and
serum levels of GLP-1, α-cell hyperplasia/hypertrophy, and enhanced islet, but not gut, preproglucagon mRNA levels (192, 193). Many models of β-cell injury also report α-cell hyperplasia (9, 152, 190, 290-292, 328, 329), though it is possible that this may be an artifact of decreased islet size and the inward collapse of islets as the β-cell core is destroyed.

Nevertheless, it is clear that islets have the capacity to produce GLP-1 and may use this strategy under specific conditions to promote islet formation and function. GLP-1-producing cells could theoretically arise from three different sources: from progenitors that have been stimulated by islet injury to begin the expansion and differentiation processes necessary to generate new endocrine cells; from pre-existing β-cells in which PC1/3 is expressed as normal, but in which proglucagon gene expression has also been activated; or from pre-existing α-cells in which the normal phenotype has been altered such that PC1/3 is expressed abundantly. Since islet GLP-1 production is upregulated in rodents following treatment with STZ (152), which destroys β-cells (330), scenario two seems unlikely. Moreover, it has been shown that β-cell regrowth following damage results from replication of surviving β-cells (331, 332). It seems reasonable to suppose that α-cells might possess a similar proliferative capacity, but this has not been studied. We therefore favour the third scenario and propose further experiments to test the hypothesis that adult α-cells proliferate following pancreatic injury and give rise to GLP-1-producing cells under these circumstances. Further knowledge of the source and identity of GLP-1-producing cells appearing after β-cell injury may allow us to begin to understand the function and fate of such cells in the damaged islet.

How might PC1/3 expression be activated in the α-cell? The current lack of knowledge about the PC1/3 promoter presents an obstacle to understanding how PC1/3 expression is activated in α-cells under certain conditions. One hint may come from a study in which αTC-1 cells were transfected with a construct bearing a human PC1/3 promoter-luciferase reporter construct (333). Transfected cells cultured in high glucose had nearly double the luciferase activity of those cultured under basal glucose conditions, suggesting that high glucose itself might activate PC1/3 promoter activity. The proximal PC1/3 promoter has previously been found to contain two cAMP response elements (CREs) that are essential for cAMP-mediated activation of PC1/3 expression (334, 335). Interestingly, the ability of αTC-1 cells to activate PC1/3 expression in response to high glucose culture was dependent on the presence of these CRE elements, since cells transfected with a PC1/3 promoter-luciferase construct lacking these
elements failed to respond to high glucose culture. Diabetic ob/ob mice (336) and STZ-treated diabetic rats (152) also have increased pancreatic PC1/3 expression levels, suggesting that similar mechanisms could be at work in vivo. PC2 and PC1/3 expression are co-regulated with proinsulin expression in β-cell lines (337, 338) and in isolated rodent islets (339) during chronic exposure to high glucose. These effects are mediated via CRE activation in the PC1/3 and PC2 promoters, and are thought to be a means of increasing insulin secretory capacity. Elevated glucose has also been found to regulate PC1/3 and PC2 biosynthesis at the translational level in a β-cell line (338), and expression of PC1/3 and PC2 in the β-cell is also sensitive to free fatty acid concentrations (340). Interestingly, hypothalamic PC1/3 and PC2 expression are inhibited by fasting and stimulated during feeding, an effect possibly mediated by leptin (341). Thus in other tissues and in αTC-1 cells, PC1/3 biosynthesis seems to be regulated by nutritional status. It is possible that similar mechanisms might be activated in the α-cell during chronic exposure to high glucose, although this will require direct study. Indeed, it is possible that our own studies showing that long-term low-glucose culture of αTCΔPC2 cells decreases GLP-1 secretion may be an indirect indication that low extracellular glucose levels decrease PC1/3 expression, although we have yet to directly investigate this possibility.

The bioactivity of PC1/3 is also regulated within the regulated secretory pathway. Full maturation of PC1/3 is dependent on the acidic pH (342) and high calcium concentrations (343) found in the trans-Golgi. Furthermore, in 2000 several groups identified the peptide precursor proSAAS as an endogenous inhibitor of PC1/3 (344-346). proSAAS mRNA has been detected in human islets (347), and intriguingly, appeared to be highly expressed in the α-cell-rich islet periphery in one immunohistochemical study of rat pancreas (348). It is therefore possible that inhibition of proSAAS expression and/or activity in α-cells may represent an additional way to upregulate PC1/3 bioactivity in these cells. Thus, with a better understanding of the transcriptional, translational, and post-translational regulation of PC1/3, we may be able to increase PC1/3 expression and/or alter the milieu within the dense core granules to promote PC1/3 activity in the α-cells.

**Future directions—α-cell mass dynamics and α-cells as a source of GLP-1-producing cells**

In the current work we observed a significant reduction in the proportion of islet α-cells in normal mice 2 wk following transplantation of encapsulated αTC-1 cells, and a relative increase in the number of α-cells in STZ-treated mice. The dynamic ability of α-cell mass to
change is clearly evident from animal models in which glucagon production or function is impaired. Ablation of PC2 in mice results in a severe defect in glucagon production and is associated with marked α-cell hyperplasia (35-37), although this effect is reversible by administration of glucagon by mini-osmotic pump (38). Disruption of glucagon receptor expression in rodents is associated with α-cell hyperplasia/hypertrophy (39, 40). In addition to our findings of apparent increased α-cell mass following STZ, many others have reported that α-cell hyperplasia accompanies β-cell injury (21, 28, 41-46), and there is also some evidence that the islet fraction of α-cells is increased in non-obese humans with T2D (47). Therefore, although less studied relative to the β-cells, it appears as though α-cells have the ability to change dynamically in both function and mass. Indeed, α-cell hyperplasia may contribute to the hyperglucagonemia typically associated with diabetes. However, a systematic quantification of α-cell mass has not been performed and it thus remains possible that apparent α-cell hyperplasia is in part an artifact of decreased islet size and the inward collapse of islets as β-cells are destroyed. The current work therefore supports the necessity of a series of experiments that will allow us to quantify α-cell mass in a rodent model of β-cell damage.

To address the question “Does α-cell number increase after β-cell injury?” we are generating a novel line of mice (Fig. 34A) by crossing Z/RED mice (Jackson Laboratories; 005438), which express a βgeo (lacZ/neoR) construct flanked by loxP sites and upstream of a DsRed gene (349), with RPGCre mice, which bear a constitutively active Cre recombinase construct downstream of 1.3 kb of the rat proglucagon promoter (350). These mice will be crossed with MIPGFP mice (Jackson Laboratories; 005282), which express a modified green fluorescent protein (GFP) downstream of a mouse insulin promoter (MIP) construct (351), to provide dual fluorescent markers for simultaneously tracking both α-cell and β-cell mass (Fig. 34B).

RPGCre;Z/RED;MIPGFP mice (male, 8 wk old) will receive 5 d, low-dose STZ/vehicle treatment, and will be sacrificed 0, 10, 20, or 30 d after the last STZ/vehicle dose. Pancreas sections collected at each time point from both STZ- and vehicle-treated groups will be analyzed by ICC for glucagon, insulin, PC1/3 and PCNA expression. In other mice, the number of α- and β-cells per pancreas will be determined by dispersing isolated islets into a single cell suspension which will be analyzed by fluorescence-activated cell sorting (FACS). This will allow us to
enumerate α-cells and β-cells in the pancreas prior to and following STZ treatment, to determine whether true α-cell hyperplasia does, in fact, accompany β-cell injury during this period.

Figure 34: Generation of RPGCre;Z/RED;MIPGFP mice and anticipated post-STZ islet cell dynamics

(A) The Z/RED mouse expresses a βgeo construct flanked by loxP sites and upstream of the DsRed gene. If Cre is present the βgeo construct will be excised, allowing expression of DsRed. Z/RED mice will be crossed with RPGCre mice in which Cre expression is driven by a 1.3 kb α-cell specific fragment of the proglucagon promoter to generate mice bearing the Z/RED transgene and the -1.3RPGCre transgene (RPGCre;Z/RED). (B) These mice will be crossed with MIPGFP mice, which bear a gene encoding a modified green fluorescent protein (GFP) under the control of the mouse insulin promoter (MIP), to generate RPGCre;Z/RED;MIPGFP mice. In these mice α-cells will fluoresce red, while β-cells will fluoresce green. (C) We expect that following STZ treatment, β-cell number (green line) will drop significantly and then rise again as regeneration occurs. We expect that α-cell number (red line) will increase after STZ treatment, peaking by ~3 wk post STZ.

Purified DsRed+ cells will be collected and extracted for determination of immunoreactive GLP-1 levels by ELISA, with results normalized for α-cell number. In STZ-treated mice we anticipate detecting a significant reduction in β-cell number, followed by a recovery as islet
regeneration occurs (Fig. 34C). Based on our experiments in STZ-treated C57BL/6 mice, we also anticipate that there will be an expansion in α-cell numbers, peaking ~20 d post-STZ administration. In vehicle-treated mice, most cells will be GFP-/DsRed- (e.g. exocrine tissue), but some cells should be GFP+ (i.e. β-cells), and others DsRed+ (i.e. α-cells). This will give a baseline number of α- and β-cells per pancreas in the absence of islet injury that can be compared to the α-/β-cell populations present following administration of STZ. In STZ-treated mice we expect to observe a population of cells expressing both GFP and DsRed that are glucagon- and insulin-positive by ICC. If sufficient numbers exist, future studies will be designed to compare the gene and protein profile of these cells to pure GFP+ or DsRed+ cells obtained by FACS. We anticipate that ELISA of FACS-sorted DsRed+ cell extracts from STZ-mice will reveal a significant increase in immunoreactive GLP-1 compared to vehicle, and that PC1/3 and glucagon will co-localize in some fraction of α-cells in whole pancreas sections.

We have completed the first round of breeding to create transgenic RPGCre;Z/RED mice and have devised a PCR-based genotyping protocol to differentiate double transgenics from single- and non-transgenics (Fig. 35A). Initial ICC experiments performed on pancreas sections from double transgenics indicate that DsRed expression is limited to α-cells and is never observed in other islet or exocrine cells (Fig. 35B). This supports the α-cell specificity of the -1.3kb rat proglucagon promoter in the islet. However, these initial studies indicate that the RPGCre transgene may label only a subset of pancreatic α-cells which may be insufficient for tracking α-cell dynamics. We are now attempting to increase labelling efficiency by breeding reporter mice bearing the RPGCre transgene on both alleles. This approach has also been taken by another group which recently characterized RPGCre-ROSA26EYFP transgenic mice (352).

An additional line of experimentation arising from the current work is necessary to address the question of whether GLP-1 producing cells arising in the islet are α-cell derived. Our approach will mimic that of Dor and colleagues, who reported that replication of existing β-cells is the primary source of new β-cells in adulthood (331). The tamoxifen (TAM)-inducible CreER™ construct (353) has been obtained from A. McMahon (Harvard University) and modified such that CreER™ lies downstream of 2.4 kb of the rat proglucagon promoter (RPG; (354)). We are in the process of generating transgenic RPGCreER™; Z/RED mice in which CreER™ will be expressed only in cells where the proglucagon promoter is active, and even in such cells, only activated upon TAM treatment (Fig. 36A). Ultimately we will cross these mice
Figure 35: Development of a genotyping protocol and initial characterization of RPGCre;Z/RED mice

(A) Gel-electrophoresed PCR products amplified from double transgenic, single transgenic, and transgene-negative mice. (B) DsRed and glucagon staining in pancreata of double-transgenic and transgene-negative mice. Scale bar, 100 µm. See Appendix I for supplemental methods.

with MIPGFP mice to generate RPGCreER™;Z/RED;MIPGFP mice which will express GFP constitutively in insulin-expressing cells, and following TAM treatment, DsRed in proglucagon-producing cells and their progeny. These mice will provide a model to assess whether DsRed+ cells (i.e. proglucagon-expressing cells or their progeny) ever give rise to GLP-1-producing cells and whether insulin-producing cells arise from proglucagon-expressing cells.

To confirm the function of the RPGCreER™ construct prior to using it to generate transgenic mice, we carried out initial double-transfection studies in vitro using this construct and a reporter plasmid (pCall2DsRedMST, kindly provided by Dr. A. Nagy, University of Toronto). pCall2DsRedMST bears a β-galactosidase construct and a stop site upstream of a loxP-flanked DsRed construct, all under the control of a constitutive promoter. We reasoned that cells co-transfected with these plasmids should be positive for β-galactosidase but not DsRed expression prior to TAM treatment. We expected that treatment of cotransfected α-cells with TAM would result in translocation of the CreER™ protein to the nucleus, where it would mediate excision of the βgeo construct, allowing for the activation of DsRed expression. In non-
Figure 36: Generation of RPGCreER™;Z/RED mice and confirmation of the RPGCreER™ construct

(A) RPGCreER™ mice will be crossed with Z/RED mice to generate mice bearing both transgenes; these double-transgenics will be crossed with MIPGFP mice to create triple-transgenic mice (not shown). Upon treatment of RPGCreER™;Z/RED mice with TAM, Cre-mediated excision of the βgeo construct will occur only in cells in which the proglucagon promoter is active, activating expression of DsRed only in α-cells in the islet. (B, C) αTC-1 cells were cotransfected with a RPGCreER™ plasmid and a pCall2DsRedMST plasmid, which is similar to the Z/RED construct shown in (A). Cells were treated with vehicle or TAM. Cells were fixed and stained with X-galactosidase (B), or live cells were imaged directly for DsRed expression (C). See Appendix I for supplementary methods.

α-cells, however, we expected that TAM treatment would be without effect, since in non-α-cells the proglucagon promoter driving expression of the CreER™ protein should be inactive. As predicted, some co-transfected 3T3-L1 cells were positive for β-galactosidase expression, while DsRed expression was never observed in these cells, irrespective of whether they were TAM-treated or not (not shown). Some αTC-1 cells co-transfected with these two plasmids also acquired β-galactosidase expression (Fig. 36B), and a few α-cells were DsRed+, indicating some ‘leakiness’ in the TAM-inducibility of the CreER™ construct (Fig. 36C). However, upon TAM treatment the number of cotransfected αTC-1 cells expressing DsRed increased (Fig. 36C), indicating that in these cells TAM induced nuclear localization of CreER™. We did not observe
a loss of β-galactosidase expression concomitant with activation of DsRed expression. This may have been because the pCall2DsRedMST plasmid copy number was >1/cell and excision of the βgeo construct was only partial, and/or because we did not allow enough time for previously synthesized β-galactosidase to have been degraded within the cell. Having confirmed activity of the RPGCreER™ construct in vitro, we are now in the process of generating mice bearing this transgene.

Once we have generated RPGCreER™;Z/RED;MIPGFP mice, we will initially establish an appropriate TAM dose by administering 8 mg daily via intraperitoneal injection for 5 d, as reported previously (331). TAM will render proglucagon-expressing cells and their progeny DsRed+, although labeling efficiency is likely to be <100%. Mice will be sacrificed 2 d after the last TAM dose and pancreatic tissue will be processed for ICC to assess labeling efficiency (i.e. % α-cells labeled DsRed+) using anti-glucagon and anti-DsRed antibodies. The TAM dose will be adjusted accordingly if labeling efficiency is <50% or if toxicity is observed. The specificity of the labeling will be verified by carefully examining pancreatic sections for DsRed+ cells that are not immunoreactive for glucagon. The extent of DsRed+ labeling in control mice will define the ‘leakiness’ of the transgenic reporter system.

Upon establishing a TAM dose labeling >50% of α-cells, RPGCreER™;Z/RED;MIPGFP mice will be used to determine whether putative α-cell expansion in response to multiple low-dose STZ or partial pancreatectomy results from α-cell proliferation. Reporter mice will be TAM-treated and subjected to 5 d low-dose STZ (or vehicle) or 50% partial pancreatectomy 2 d after the last TAM dose (Fig. 37A). TAM treatment will label α-cells prior to STZ treatment, and α-cells (and their progeny) and β-cells will be enumerated following a chase period to allow for α-cell hyperplasia. Pancreata will be dispersed for FACS (followed by extraction of the sorted DsRed+ fraction to determine GLP-1 content by ELISA). Other pancreata will be processed for immunostaining (for glucagon, insulin and DsRed to determine whether α-cell-derived cells give rise to cells coexpressing insulin and glucagon; or for PC1/3 and glucagon, to quantify the proportion of glucagon-producing cells that also produce GLP-1). The extent of dilution of α-cells positive for DsRed will indicate the likelihood that new α-cells arise from pre-existing proglucagon-expressing cells (no dilution), as we expect, or non-proglucagon-expressing cells (dilution; Fig. 37B).
Figure 37: Experimental outline & possible FACS results
(A) RPGCreER;Z/RED;MIPGFP mice will be given a TAM "pulse" (day -5 to -1) followed by 5-day low-dose STZ (red; day 1 to 5) or partial pancreatectomy (PTX; blue; day 1) to induce islet injury. A "chase" period will follow to allow for cell differentiation/proliferation in response to the injury. TAM treatment will label α-cells prior to STZ or PTX and their progeny. (B) Possible FACS results following STZ. FACS analysis of islets from vehicle-treated mice will provide a baseline number of β-cells (GFP+) and α-cell-derived cells (DsRed+; left panel). GFP+ cell number should decrease in FACS-analyzed islets from STZ-treated mice. If α-cells replicate after β-cell injury, DsRed+ cell number will increase (middle panel). If non-α-cell precursors replace α-cells over time, GFP+ cells will decrease relative to vehicle-treated control, but DsRed+ cell number will also decrease as cells labeled at the time of TAM pulse are ‘diluted out’ by a non-α-cell source (right panel). Some cells may be GFP+/DsRed+ at the post-STZ timepoints; these are depicted in yellow and are represent cells in which the insulin promoter is active and that are derived from cells in which the proglucagon promoter was active at the time of TAM pulse.

Taken together, these studies will quantitatively establish whether α-cell number increases in response to β-cell injury and if so, whether this is due to α-cell proliferation or differentiation of some other cell type to generate glucagon-positive cells. Further, by using a lineage tracing approach, we will determine whether α-cells can give rise to insulin-expressing cells or GLP-1-expressing cells in response to β-cell injury. With a better understanding of the upregulation of islet GLP-1 production following β-cell injury, we may be able to design superior ways to mimic this phenomenon for regenerative purposes in the treatment of diabetes.

Future directions—GLP-1 production in human islets
A key follow-up to the current work will be to assess the feasibility and impact of inducing GLP-1 production in human islets. Islet transplantation improves the lives of subjects
with T1D, but unfortunately this approach typically requires islets from multiple donors and supplementation with exogenous insulin (27). Preventing failure of transplanted islets is thus important not only to improve transplantation outcomes, but also to reduce the number of islets required per recipient and thereby ease the burden on the limited supply of tissue available. In humans, α-cells are found scattered throughout the islet, while in rodents they are generally limited to the islet periphery. However, the greater proportion of α-cells in human islets compared to mouse islets (5, 6) means that the absolute number of α-cells available for transduction in the islet periphery may be similar in rodents and humans. We were unsuccessful in using AdPC1/3 to induce GLP-1 production in human islets. However, with the aid of α-cell-specific gene delivery vectors and/or further optimization of transduction conditions for human islets (for example, by adjusting viral MOI and/or transduction time), inducing local GLP-1 production within islets as described here may yet offer a novel approach to improve the outcome of islet transplantation in humans. It remains an intriguing possibility that this strategy could also be exploited in vivo to intervene in the loss of a functional mass of β-cells which characterizes diabetes. This hypothesis can now be tested since techniques have been developed to deliver genes to islets in vivo (355).
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APPENDIX I: SUPPLEMENTARY METHODS FOR FIGURES 35-36

Animals

Hemizygous Z/RED (1) and RPGCre (2) breeders were obtained commercially and crossbred to generate hemizygous double transgenic mice. Neither single- nor double-transgenics displayed any gross abnormalities in size or growth, although mice tended to have smaller than average litters.

PCR-based genotyping was carried out as follows using standard reaction mix (3 µL template plus 1 µM each of GluCre-F, GluCre-R, oIMR3846, and oIMR3847 primers, plus 0.5 U of Taq polymerase, 200 µM dNTPs, 1x PCR buffer, and 1.5 mM MgCl₂ in 25 µL total volume): 3 min at 94°C; 40 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s; and 72°C for 5 min. Primer sequences are as follows:

- GluCre-F 5’ GAGAAATTTATATTGTACCG 3’
- GluCre-R 5’ ATGGTGATACAAGGAC 3’
- oIMR3846 5’ CCCCGTAATGCAGAAGAA 3’
- oIMR3847 5’ GGTGATGTCCAGCTTGGAGT 3’

PCR products were electrophoresed on a 1.5% agarose gel and imaged using SYBR-Safe. RPGCre<sup>TG+</sup>;Z/RED<sup>TG−</sup> mice had a 208 bp product, RPGCre<sup>TG−</sup>;Z/RED<sup>TG+</sup> mice had a 300 bp product, RPGCre<sup>TG+</sup>;Z/RED<sup>TG+</sup> mice had both products, and RPGCre<sup>TG−</sup>;Z/RED<sup>TG−</sup> mice had neither product. An extraneous ~470 bp product of unknown identity is amplified for all mice and serves as an internal control to ensure successful amplification using PCR.

Immunocytochemistry

Pancreata were collected from 8 wk old male RPGCre<sup>TG+</sup>;Z/RED<sup>TG+</sup> and RPGCre<sup>TG−</sup>;Z/RED<sup>TG−</sup> mice (2 per genotype). ICC was carried out on pancreas sections as outlined in the ‘Materials and Methods’ section using primary rabbit anti-DsRed (AB3216; Millipore; Billerica, MA; 1:300) and mouse anti-glucagon (Sigma-Aldrich; Oakville, ON; 1:900) antisera and Alexa Fluor-conjugated secondary antisera (Invitrogen; Burlington, ON; 1:800).

Generation of the 2.4RPGCreER™ construct

To construct the 2.4RPGCreER™ vector, 2.35 kb of the rat proglucagon promoter was excised from the pGluLUC vector (3) provided to us by Dr. D. Drucker (University of Toronto) with a BamHI/XhoI digest. The promoter and poly linker regions in the pShuttle vector
(Clontech; Mountain View, CA) were modified by Dr. R. Baker in our laboratory. The resulting vector (pScore) was digested with \textit{Bgl} II and \textit{Sal} I and the gel purified 2.35 kb proglucagon promoter and the linearized pScore vector were ligated to form the pScore:rGCGpro plasmid. The \textit{pGEM:rGIP-CreER™} plasmid, based on a \textit{pGEM-T} plasmid backbone (Promega; Madison, WI), was digested with \textit{ApaI/NotI} to linearize the CreER™ open reading frame. The pScore:rGCGpro vector was linearized with \textit{ApaI/NotI} and the 2150 bp CreER™ segment was gel-purified and ligated into this vector to create the pScore:rGCG-CreER™ (-2.4RPGCreER™) plasmid. This vector was sent to the BC Cancer Agency Genetic Modelling Core, where it was linearized with PI-Scele/CIeul, gel-purified and used to create transgenic founder mice.

\textit{In vitro} testing of the -2.4RPGCreER™ construct

αTC-1 and 3T3L1 cells were maintained in HG-DMEM containing 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin (complete medium). To test the fidelity of the -2.4RPGCreER™ plasmid, αTC-1 (p22) and 3T3L1 (p9) cells were seeded at 2.0x10\(^5\) cells/well in a 24-well plate. The \textit{pCall2DsRedMST} plasmid, which bears a loxP-flanked β-galactosidase construct and stop codon upstream of a DsRed construct (1), was obtained from Dr. A. Nagy (University of Toronto). The following day cells were ~35% confluent, and were co-transfected with a total of 0.8 µg of plasmid DNA (0.4 µg each of -2.4RPGCreER™ and \textit{pCall2DsRedMST}). Transfection was carried out with Lipofectamine (Invitrogen; Burlington, ON) in a 400 µL volume according to the manufacturer’s instructions for ~6 h. Transfection media were removed and replaced with fresh complete media containing 20% FBS, and cells were cultured overnight. 24 h after the beginning of the transfection period, media on all cells was changed and replaced with media containing 1 µM TAM (T5648; Sigma; Oakville, ON; prepared as a 20mg/mL stock solution in 100% EtOH and diluted in complete media just before use) or vehicle (100% EtOH diluted in media). After 48 h TAM treatment live cells were analyzed for DsRed expression using fluorescent microscopy (Axiovert 200; Carl Zeiss Canada; Toronto, ON). Immediately following, cells were fixed in 0.2% glutaraldehyde, stained for X-galactosidase using the method outlined in the ‘Materials and Methods’ section, and imaged using brightfield microscopy.

References


APPENDIX 2: LIST OF PUBLICATIONS


APPENDIX 3: BIOSAFETY AND ANIMAL CARE CERTIFICATES

The University of British Columbia

Biohazard Approval Certificate

PROTOCOL NUMBER: H03-0049

INVESTIGATOR OR COURSE DIRECTOR: Kieffer, Timothy

DEPARTMENT: Physiology

PROJECT OR COURSE TITLE: GLP-1 Gene Therapy for Diabetes

APPROVAL DATE: February 14, 2003

APPROVED CONTAINMENT LEVEL: 2

FUNDING AGENCY: Canadian Diabetes Association

The Principal Investigator/Course Director is responsible for ensuring that all research or course work involving biological hazards is conducted in accordance with the Health Canada, Laboratory Biosafety Guidelines, (2nd Edition 1996). Copies of the Guidelines (1996) are available through the Biosafety Office, Department of Health, Safety and Environment, Room 50 - 2975 Westbrook Mall, UBC, Vancouver, BC, V6T 1Z1, 822-7586, Fax: 822-6880.

Approval of the UBC Biohazards Committee by one of:
Chair, Biosafety Committee
Manager, Biosafety Ethics
Director, Office of Research Services

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required.

A copy of this certificate must be displayed in your facility.

Research Ethics, Office of Research Services
110, 2386 East Mall, Vancouver, V6T 1Z3
Phone: 604-827-5111  FAX: 604-827-5117
ANIMAL CARE CERTIFICATE

PROTOCOL NUMBER: A02-0307

INVESTIGATOR OR COURSE DIRECTOR: Kieffer, T.J.

DEPARTMENT: Physiology

PROJECT OR COURSE TITLE: GLP-1 Gene Therapy for Diabetes

ANIMALS: Mice 1360  Rats 320

START DATE: 03-07-01  APPROVAL DATE: 04-08-03

FUNDING AGENCY: Canadian Diabetes Association

The Animal Care Committee has examined and approved the use of animals for the above experimental project or teaching course, and have been given an assurance that the animals involved will be cared for in accordance with the principles contained in Care of Experimental Animals - A Guide for Canada, published by the Canadian Council on Animal Care.

[Signature]

Approval of the UBC Committee on Animal Care by one of:
Dr. W.K. Milsom, Chair
Dr. J. Love, Director, Animal Care Centre
Ms. L. Macdonald, Manager, Animal Care Committee

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.