Incretin action in the pancreas: Potential promise, possible perils, and pathological pitfalls

Running title: Incretin biology and the pancreas

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Glucagon-like peptide-1 (GLP-1) and glucose-dependent insulino-tropic polypeptide (GIP) are incretin hormones that control secretion of insulin, glucagon and somatostatin to facilitate glucose disposal. The actions of incretin hormones are terminated via enzymatic cleavage by dipeptidyl peptidase-4 (DPP-4), and through renal clearance. GLP-1 and GIP promote β-cell proliferation and β-cell survival in rodent β-cells. DPP-4 inhibitors expand β-cell and reduce α-cell mass and inhibit glucagon secretion in preclinical studies however whether incretin-based therapies sustain functional β-cell mass in human diabetic subjects remains unclear. GLP-1 and GIP exert their actions predominantly through unique G protein coupled receptors expressed on β-cells and other pancreatic cell types. Accurate localization of incretin receptor expression in pancreatic ductal or acinar cells in normal or diabetic human pancreas is challenging as antisera employed for detection of the GLP-1R are often neither sufficiently sensitive nor specific to yield reliable data. We review recent advances and controversies in incretin hormone action in the pancreas and contrast established mechanisms with areas of uncertainty. Furthermore, we highlight methodological challenges and pitfalls and outline key areas requiring additional scientific investigation.
Introduction

Incretins are gut-derived circulating peptide hormones that potentiate glucose-dependent insulin secretion following meal ingestion. Glucose-dependent insulinotropic polypeptide (GIP), and glucagon-like peptide-1 (GLP-1), are the major incretin hormones. The insulinotropic actions of endogenously secreted GLP-1 and GIP are transient, as both peptides are rapidly cleared by the kidney, and inactivated by cleavage at the N-terminus by a ubiquitous exopeptidase, dipeptidyl peptidase-4 (DPP-4). Potentiation of incretin action underlines two therapeutic classes of glucose-lowering agents, the GLP-1R agonists, and the DPP-4 inhibitors (1). Original concepts of GIP and GLP-1 biology focused primarily on islet β-cells have been expanded to include actions on other cell types within and outside the pancreas (2; 3). There is now considerable interest in understanding how potentiation of incretin action controls multiple facets of pancreatic biology, encompassing regulation of glucose sensing, hormone secretion, cell proliferation, differentiation, and survival. Recent studies have suggested that incretin therapies promote pancreatic inflammation, and aberrant cell proliferation within the endocrine and exocrine pancreas (4; 5), however substantial technical and methodological issues limit the generalizability of these findings. This Perspective evaluates the science supporting existing dogma, and discusses new concepts, controversies, and uncertainties in the biology of incretin action in the pancreas.

Localization of incretin receptor expression in the pancreas

Several dozen commercial antisera are available for detection of GLP-1 and GIP receptor expression by immunohistochemical techniques and Western blotting, and Real Time PCR is widely used to quantify incretin receptor gene expression in pancreatic exocrine and endocrine
compartments. Most antisera used to detect GLP-1R expression (by immunohistochemistry or Western blot analysis) are neither sensitive nor specific (6; 7). Important control experiments (absorption of the antibody with a peptide epitope, demonstration that the antibody recognizes only a single protein, and fails to generate a signal in cells that do not express a full length receptor mRNA transcript or in tissues from *Glp1r*−/− mice) are usually absent. Furthermore, multiple studies describe GLP-1 receptor protein expression in cells or tissues that do not express a full length *Glp1r* mRNA. The widespread use of tightly cropped bands in Western blot analysis precludes accurate assessment of whether a putative band/protein detected by Western blotting is the correct size, the only GLP-1R-immunoreactive protein visualized, or one of several unrelated immunoreactive proteins detected by the same antisera.

Scientists interested in incretin hormone receptor expression face the challenging task of assessing how much, if any, of the data published with these antisera is correct. For example, immunoreactive GLP-1R protein expression or *Glp1r* mRNA transcripts have been detected throughout the heart and ventricle, however we and others determined that cardiac *Glp1r* expression was restricted to the atria, and absent from the ventricles in mice (8) and rats (9). How do the limitations of available reagents impact our understanding of incretin action in the pancreas? The putative localization of incretin receptor expression in the exocrine pancreas provides an instructive example. Abundant immunohistochemical GLP-1 receptor expression in ductal and acinar cells was reported in rodent and human pancreas, in papillary thyroid cancer and pancreatic adenocarcinoma (10; 11). Characterization of multiple GLP-1R antisera, including one of the reagents used in these studies, Abcam39072 (11), revealed major problems with sensitivity and specificity. These antisera detected multiple spurious bands in Western blot
analyses of fibroblasts that do not express the GLP-1R and in cellular extracts from Glp1r-/- mice (6). We now extend these analyses to detection of the human GLP-1R. Western blot analysis using fibroblasts transfected with the human GLP-1R cDNA shows that Abcam39072 does not detect the human GLP-1R (Figure 1). A second antiserum distributed by Novus Biologicals (1940002), recognizes the human GLP-1R protein (Figure 1) however this antiserum also detected multiple spurious bands/proteins in control cells that do not express the Glp1r (Figure 1). Similar problems with sensitivity and specificity of GLP-1R antisera have been described by others (7). Hence, the majority of published studies employing multiple GLP-1R antisera must be discounted until the experimental data is independently verified with validated highly sensitive and specific antisera.

Similar concerns relate to interpretation of some experiments using regular PCR (polymerase chain reaction) or Real Time PCR to detect incretin receptor gene expression. Real Time PCR detects Glp1r mRNA transcripts by generating an amplicon of less than 100 base pairs (b.p.) and regular PCR frequently employs primer pairs that generate Glp1r PCR products several hundred b.p. in length, both far smaller than the entire full length GLP-1R open reading frame. However, cells may generate noncoding mRNA transcripts detectable by regular or Real Time PCR. Analysis of Gipr expression revealed ~ 64 possible Gipr mRNA splice variants in RNA from human adipose tissue, only two of which were predicted to contain an open reading frame sufficient to give rise to a fully functional, membrane-spanning GIPR protein (12). Whether one or more of these variant Gipr RNA transcripts encodes a truncated GIPR protein that might exhibit dominant negative signaling activity, as described in mouse beta cells (13), requires further investigation. Furthermore, using a polyclonal antiserum, an immunoreactive GIPR
protein was detected in human skeletal muscle (12), a tissue not previously reported to express full length \textit{Gipr} mRNA transcripts (14). Despite reports describing detection of a) partial \textit{Glp1r} mRNA transcripts by PCR, or b) immunoreactive GLP-1R proteins by Western blotting or immunohistochemistry, in murine liver, macrophages, or ventricular cardiomyocytes (2), we could not detect full length \textit{Glp1r} mRNA transcripts in the same cells and tissues (6; 8).

Given the considerable limitations of commonly used reagents and techniques, how should we interpret available data reporting localization of GLP-1R expression in the endocrine and exocrine pancreas? The difficulty in isolating pure ductal, acinar, or islet cell RNA free from contamination with other cell types renders use of such cell fractions suboptimal for analysis of cell-specific gene expression. Some groups have localized GLP-1R expression in islet $\alpha$-cells (15), however, analysis of \textit{Glp1r} mRNA transcripts in RNA from purified murine FACS-sorted $\alpha$-cells and $\beta$-cells failed to detect \textit{Glp1r} mRNA transcripts in $\alpha$-cells (Furuyama, K., and Herrera, P., 2013, personal communication). Similarly, \textit{Glp1r} and \textit{Gcgr} mRNA transcripts were not detected in rat and mouse $\alpha$-cells, respectively, by \textit{in situ} hybridization (16; 17). Although \textit{Gipr} mRNA transcripts were detected in rodent $\alpha$ cells (18), less information is available regarding \textit{Glp1r} or \textit{Gipr} expression in human $\alpha$-cells. GLP-1R activation stimulates islet somatostatin secretion, however whether some, most or few somatostatin-producing $\delta$-cells express the GLP-1R has not been established. Cell surface DPP-4 expression has been identified on murine $\alpha$-cells, $\beta$-cells, and even more strongly on ductal cells (19), however whether DPP-4 activity locally regulates bioactive incretin activity within these pancreatic cell types has not been determined.
Glp1r mRNA transcripts have been detected in pancreatic ductal cell lines, and in human pancreatic adenocarcinoma cell lines (20). However the GLP-1R agonist exendin-4 failed to stimulate growth or enhance cell survival in 5 different human pancreatic cancer cell lines that express an endogenous Glp1r mRNA transcript. Whether Glp1r mRNA transcripts are expressed in non-immortalized pancreatic ductal or acinar cells remains uncertain. Tornehave and colleagues were unable to demonstrate Glp1r mRNA transcripts in pancreatic duct cells from mice and rats by in situ hybridization, despite detection of an immunoreactive protein in ducts using a GLP-1R antibody subsequently shown to exhibit suboptimal specificity (6; 16).

Transcriptome analysis of human pancreatic endocrine and exocrine cells detected glucagon receptor (Gcgr) expression in ductal cells, however Glp1r expression was not reported (21). Despite immunohistochemical depiction of robust GLP-1R immunopositivity in human pancreatic cancer cells (22), we have been unable to find evidence that Glp1r mRNA transcripts are overexpressed in these tumors using transcriptome analysis of publicly available databases (oncomine.com version 4.4.3, and Genome Expression Omnibus (GEO) http://www.ncbi.nlm.nih.gov/geo/). Similarly, Korner and colleagues were unable to detect GLP-1 binding sites in pancreatic adenocarcinomas using in situ ligand binding and autoradiography (23). New studies employing individual endocrine or acinar cells purified by FACS analysis, or isolation of single pancreatic cells using laser capture microdissection, followed by the use of validated antisera and/or PCR analysis using primers that span the full length Glp1r open reading frame should refine our understanding of the direct cellular targets of GLP-1 action in the pancreas.

Incretin-mediated control of islet hormone secretion
The increasing realization that β-cells exhibit considerable functional heterogeneity begs the question of whether there is a gradient of incretin receptor expression and action in different β-cells and whether these putative gradients vary across species amongst islets of different size and location. Although the insulin-stimulating properties of GLP-1R agonists are preserved in experimental models of diabetes and human subjects with T2DM, the actions of GIP on the diabetic β-cell are attenuated, likely due to downregulation of Gipr expression and/or attenuation of signaling pathways coupling GIPR activation to insulin secretion (2). The loss of GIP action in the diabetic pancreas is reversible in animal and human studies. Reduction of glycemia with phlorizin restores islet GIPR expression and insulin secretion in response to GIP in diabetic rats (24; 25), whereas treatment of human subjects with type 2 diabetes (T2D) with insulin for 4 weeks to reduce levels of glycated hemoglobin to ~7% significantly improves the insulin secretory response to exogenous GIP (26).

GLP-1 and GIP exhibit different actions on islet α-cells. GLP-1R agonists (and DPP-4 inhibitors) inhibit glucagon secretion in normoglycemic and diabetic animals and humans (27), most likely via GLP-1R-dependent stimulation of islet somatostatin secretion. Somatostatin in turn inhibits glucagon secretion through SSTR2 expression on α-cells (28). Conversely, GIP stimulates glucagon secretion in humans under conditions of hyperglycemia (29; 30), however whether these actions reflect direct activation of α-cell GIP receptors (29) remains unclear. Intriguingly, rodent and human α-cells express immunoreactive and bioactive GIP, hence an intraislet paracrine or autocrine GIP axis, with locally-produced GIP acting through α-cell GIP receptors cannot be excluded (31).

*Pancreatic incretin receptor signaling, cell proliferation and apoptosis*
**Expansion of β-cell mass**

Multiple preclinical studies demonstrate proliferative and anti-apoptotic actions of GLP-1, leading to expansion of β-cell mass (32). Early experiments promoted the concept that GLP-1R agonists stimulated neogenesis of β-cells via activation of a ductal cell GLP-1R (2; 32). However, the contribution of β-cell neogenesis from ductal precursors to generation of new β-cells in adult mice has been elegantly disputed (33). Anti-apoptotic actions of GLP-1R agonists have been demonstrated in rodent and human islets (2; 32) and in preclinical studies of transplanted human islet cells. More disappointing are results of clinical studies assessing whether GLP-1R agonists preserve β-cell function in subjects with type 1 (T1D) or type 2 diabetes (T2D). There is little evidence that prolonged therapy with GLP-1R agonists modifies the progressive decline in β-cell function, an indirect surrogate of β-cell mass, independent of changes in weight loss, in subjects with T2D (34). Similarly, treatment of C-peptide-positive subjects with long standing T1D with exenatide, with or without immunosuppression (daclizumab), for 6-9 months, did not enhance β-cell function or suppress meal-stimulated glucagon levels (35). The available evidence from randomized controlled trials does not support the contention that therapy with exenatide or liraglutide produces a sustained or progressive improvement in β-cell function in subjects with T1D following islet transplantation.

Why have we not seen clinical evidence for expansion of functional β-cell mass in diabetic subjects treated with GLP-1R agonists or DPP-4 inhibitors? The majority of positive preclinical experiments were carried out in younger animals (2), whereas older rodent β-cells exhibit a substantially diminished or absent proliferative response to multiple regenerative stimuli, including GLP-1R agonists (36; 37). The diminution in β-cell replicative capacity in response to
GLP-1R agonists has been attributed to loss of cell cycle regulating proteins such as Skp2 (that controls p27) and sustained expression of p16Ink4a in older rodent and human β-cells (38). Human β-cells appear much less responsive to proliferative agents such as GLP-1 compared to rodent β-cells (39) and β-cell replication is substantially diminished in older human subjects (40). Hence more work is required to understand whether an older diabetic human β-cell retains a meaningful capacity to proliferate, resist cell death, or retain a functional differentiated state, in response to GLP-1R agonists.

**Control of α-cell mass**

Multiple studies demonstrate that GLP-1R agonists and DPP-4 inhibitors inhibit glucagon secretion (2; 27). Surprisingly, hyperplasia of glucagon-producing α-cells was described in pancreata from diabetic human subjects who received sitagliptin (n=7) or exenatide (n=1) for at least one year, leading to speculation that exposure to DPP-4 inhibitors and/or GLP-1R agonists promotes α-cell hyperplasia via a reduction in glucagon secretion (5). Ki67+ proliferating α-cells were not detected in these pancreata, hence putative mechanisms linking incretin action to expansion of α-cell mass remain unknown. Remarkably, the diabetic controls and incretin-treated subjects were substantially mismatched in regards to age, duration of diabetes, sex, age of diabetes onset, medication profile, and history of ketoacidosis, precluding any meaningful interpretation of the data. Furthermore, these observations are contradicted by extensive preclinical studies in rodents and non-human primates that failed to detect α-cell hyperplasia, despite systemic multiples of exposures to GLP-1R agonists or DPP-4 inhibitors much greater than that achieved in human subjects (41-44). As the majority (7/8) of human pancreata studied were from subjects taking sitagliptin (5) we reviewed preclinical studies reporting changes in α-
cell numbers in preclinical studies with DPP-4 inhibitors (Supplementary Table 1). One of twenty studies described an increase in \( \alpha \)-cells, 6 studies reported no change in \( \alpha \)-cells, and 13 papers described a reduction in \( \alpha \)-cell number and/or decreased \( \alpha \)-cell proliferation. Hence a substantial body of independent scientific experimentation (Supplementary Table 1) taken together with extensive preclinical data spanning thousands of mice, rats and monkeys (41-44), consistently reports \( \alpha \)-cell findings diametrically opposite to those reported in a small human autopsy pancreas study (5).

Scientists reporting \( \alpha \)-cell hyperplasia in pancreata from subjects treated with sitagliptin or exenatide envisioned a pathway linking GLP-1-mediated reduction of glucagon secretion to expansion of \( \alpha \)-cell mass, independent of changes in \( \alpha \)-cell proliferation (5). Complete genetic attenuation of \( Gcgr \) expression in all tissues, or extinction of glucagon receptor signaling in the liver leads to compensatory expansion of \( \alpha \)-cell mass in an attempt to restore glucagon action, achieved via mechanisms linked to increased \( \alpha \)-cell proliferation (45; 46) (Figure 2). However, the robust expansion of \( \alpha \)-cell mass secondary to elimination of \( Gcgr \) signaling is independent of GLP-1R signaling (47; 48). Furthermore, heterozygous \( Gcgr^{+/ -} \) mice do not exhibit \( \alpha \)-cell hyperplasia (14) and less than complete blockade of the \( Gcgr \) using a \( Gcgr \) antagonist administered to high fat fed mice for 82 days did not result in \( \alpha \)-cell hyperplasia (49). Complete elimination of glucagon production also leads to \( \alpha \)-cell hyperplasia (50), however DPP-4 inhibitors or GLP-1R agonists generally produce a 20-50% reduction in plasma glucagon levels (27; 51), a scenario that has never been shown to trigger \( \alpha \)-cell hyperplasia. Hence, a large amount of independent experimentation refutes the existence of a speculative pathway (5).
linking partial reduction of glucagon secretion to expansion of α-cell mass and neuroendocrine tumor formation independent of changes in α-cell proliferation.

Acinar and ductal cells

Notwithstanding the uncertainty about whether rodent or human acinar and ductal cells express a functional GLP-1R, older rodent pancreatic ductal cells retain the capacity to proliferate following GLP-1R activation. Indeed, a 3-fold increase in ductal proliferation was observed after a 7 day course of exendin-4 in three 7 month old mice (38). Nevertheless, the hypothesis that sustained GLP-1R signaling and/or inhibition of Gcgr signaling (which also increases levels of GLP-1) will promote exocrine cell proliferation leading to expansion of exocrine mass (5) in non-sensitized preclinical models has not been independently validated. Treatment of transgenic mice expressing an activated K-ras oncogene, with exendin-4 for 12 weeks increased the expression of low grade pancreatic intraepithelial neoplasia and enhanced ductal cell proliferation, however acinar cell proliferation was not reported (10). The assertion (5) that Gcgr−/− mice or humans with a Gcgr null mutation exhibit enhanced exocrine proliferation is not supported by the published data (14; 52; 53) cited by the same authors (5). Although Gcgr−/− mice exhibit pancreatic enlargement, increased acinar or ductal cell proliferation has not been detected by multiple independent groups that have studied these animals (45; 46; 48; 52).

Histological analyses of the pancreas have been carried out after extensive chronic treatment of thousands of mice and rats and dozens of monkeys using high doses of GLP-1R agonists for up to two years. None of the studies, involving multiple doses of structurally distinct GLP-1R agonists has reported expansion of the ductal or exocrine compartments in rodents or non-human primates (41; 42). Similarly, the DPP-4 inhibitors vildagliptin or sitagliptin administered to
hundreds of mice and rats continuously for two years (43; 44) at doses producing high multiples of systemic drug exposure did not result in acinar, ductal or endocrine cell neoplasia. Although data from toxicology studies in diabetic animals is limited, a 3 month treatment regimen of exenatide twice daily at doses of 6, 40 and 250 µg/kg/day produced no changes in pancreatic exocrine structure or ductal proliferation (54). Similarly no proliferative effects of exenatide or liraglutide were detected in the exocrine pancreas of diabetic ZDF rats after 13 weeks of drug administration (55). Sitagliptin was administered for 3 months in monkeys, 12 months in dogs, and 24 months in mice and rats, at doses producing levels of exposure considerably higher than that achieved clinically, with no evidence of pancreatic abnormalities detected on gross or histological analysis of the pancreas; however precise details on the actual analyses carried out in these toxicology studies have not yet been published (44). As each pharmaceutical sponsor of a DPP-4 inhibitor or GLP-1R agonist is required to carry out carcinogenicity studies of 2 years duration in 2 species, there have now been thousands of animals exposed to DPP-4 inhibitors and GLP-1R agonists, in addition to the studies reported above. However, reports of ductal or acinar proliferation or pancreatic adenocarcinoma in preclinical studies have not yet been forthcoming, either in the form of toxicology reports submitted as part of New Drug Applications to regulatory authorities, or as published manuscripts.

Nevertheless, GLP-1R agonists increase the weight of the pancreas in some preclinical studies, most notably in young rodents (10; 56), through incompletely understood mechanisms. Selective restoration of hGLP-1R expression under the control of the Pdx1 promoter in β-cells and ducts normalized glucose homeostasis in Glp1r−/− mice, but was not sufficient to mediate an increase in pancreatic weight in response to exogenous exendin-4 (57). Hence, although insulin secretion is not sufficient for the increase in pancreatic mass observed secondary to GLP-1R activation,
further studies are required to elucidate the precise cell types and mechanisms linking GLP-1R activation to changes in pancreatic weight.

**GLP-1R signaling, DPP-4 inhibition, and pancreatic inflammation**

The glucose reduction achieved with DPP-4 inhibitors requires intact GLP-1R and GIPR signaling (58; 59), however non-glucoregulatory actions may be mediated by other substrates, including SDF-1α (3; 60). There is little data linking non-enzymatic signaling of DPP-4 to specific actions in the endocrine or exocrine pancreas. The widespread expression of GLP-1 receptors on multiple immune cell populations (61), together with the expression and activity of DPP-4( CD26) in the immune system, provides a logical basis for exploring whether GLP-1R agonists and/or DPP-4 inhibitors modulate immune function. The majority of actions ascribed to DPP-4 in immune cells are attributable to non-enzymatic actions of the enzyme; hence DPP-4 signaling in immune cells proceeds independent of its catalytic enzyme activity (62). Accordingly, partial inhibition of the catalytic activity of DPP-4 using highly selective DPP-4 inhibitors would not be predicted to perturb immune function (60). Indeed T-cell dependent immune responses are preserved in Dpp4−/− mice and in mice treated with a highly selective DPP-4 inhibitor (63). Preclinical studies linking incretin action to enhanced pancreatic inflammation include the observation that one of 8 human islet amyloid polypeptide (hIAPP) transgenic rats treated with sitagliptin for 12 weeks developed focal pancreatic inflammation (64).

In an attempt to reproduce abnormalities reported in the exocrine pancreas of hIAPP transgenic rats treated with sitagliptin for 12 weeks (64), Aston-Mourney and colleagues treated high fat fed non-hyperglycemic hIAPP transgenic mice for 12 months with sitagliptin, or metformin alone, or
in combination (65). In contrast to findings observed in transgenic hIAPP rats (64), islet amyloid deposition, ductal cell proliferation, and pancreatic mass were not increased by sitagliptin in hIAPP transgenic mice, however β-cell mass was increased, consistent with the known actions of sitagliptin in mice (2). Furthermore, sitagliptin treatment was not associated with pancreatic inflammation, necrosis, metaplasia, neoplasia, or periductal fibrosis; pancreatic mass was increased in mice treated with metformin but not in mice treated with sitagliptin (65).

Two reports described non-diabetic rats treated with exenatide that developed pancreatic damage and inflammation (66; 67). Notably, in both experiments, exenatide-treated rats experienced profound weight loss of 25-30%, however no pair-fed controls were included in these analyses, and mechanisms linking GLP-1R activation to increased pancreatic inflammation were not identified (66; 67). Rapid profound weight loss is frequently associated with a catabolic state, whereas more modest and gradual weight loss, particularly in the setting of pre-existing obesity, is generally associated with reduced tissue and systemic markers of inflammation.

Increased pancreatic inflammation has not been detected in multiple preclinical studies examining chronic effects (up to 2 years) of high dose administration of GLP-1R agonists or DPP-4 inhibitors in non-diabetic rodents or non-human primates (41-44). For example, treatment of diabetic rats with supratherapeutic doses of exenatide or liraglutide for 13 weeks was not associated with histological or biochemical evidence of pancreatic inflammation (54; 55). Moreover, administration of GLP-1R agonists prior to or following the induction of experimental pancreatitis did not enhance pancreatic inflammation in normal or diabetic rats and mice (68; 69); unexpectedly GLP-1R agonists induced an anti-inflammatory gene expression profile in the high fat fed insulin resistant murine pancreas (68).
**Incretin-based therapies and inflammatory markers in humans**

Small increases in plasma levels of amylase and lipase have been reported in diabetic subjects treated with the DPP-4 inhibitors alogliptin and sitagliptin (70), and a separate observational study of diabetic subjects treated with sitagliptin, saxagliptin, or exenatide reported that 35.6% of subjects exhibited increases in plasma levels of amylase and/or lipase, with levels of lipase increasing to a relatively greater extent (71); notably, elevated amylase and lipase levels were also observed, albeit less frequently, in diabetic control subjects not receiving a DPP-4 inhibitor or GLP-1R agonist. Whether the increase in amylase and lipase reflects subclinical pancreatic inflammation, or dysregulated synthesis, secretion, or clearance of these enzymes, requires further study. Administration of GLP-1R agonists or DPP-4 inhibitors is associated with suppression of inflammation (72), however many of these experiments do not control for concomitant reduction in glucose or body weight, which may also indirectly dampen inflammation. Exenatide administered twice daily for 12 weeks in subjects with T2D reduced circulating markers of inflammation in circulating mononuclear cells independent of changes in body weight (73); a single acute 5 ug exenatide injection significantly and rapidly reduced levels of reactive oxygen species, nuclear factor-κB binding activity, and expression of tumor necrosis factor α , interleukin-1β , JNK-1, TLR-4 and SOCS-3 mRNA transcripts in RNA isolated from circulating mononuclear cells (73). Similarly, administration of sitagliptin 100 mg once daily for 12 weeks to 12 subjects with T2D reduced expression of proinflammatory markers in circulating mononuclear cells, whereas acute administration of 100 mg sitagliptin to fasting diabetic subjects significantly reduced mononuclear cell expression of TLR-2, IKKb, CCR-2, CD-26 mRNA transcripts and decreased nuclear factor-κB binding activity (74). Hence the available data indicates that GLP-1R agonists and DPP-4 inhibitors independently exert anti-inflammatory
actions in tissues such as the exocrine and endocrine pancreas, as well as in circulating blood
cells from diabetic subjects, although the mechanisms mediating these actions remain poorly
understood.

**Summary and perspective**

The potential promise of incretin-based therapies has been partially realized, in that we can now
implement anti-diabetic regimens associated with lower rates of hypoglycemia and weight gain.
Although the first actions of GLP-1 on pancreatic islet cells were described more than 25 years
ago, we still have much to learn about how GLP-1R signaling regulates β-cell function. For
example, the molecular mechanisms underlying glucose-sensitive GLP-1R signaling have
remained elusive. The precise cellular localization of the GLP-1R in islet and exocrine cell types
requires more careful study, not only in animals, but also in pancreata from human subjects, over
a broad range of ages, with and without pre-existing diabetes or diseases of the pancreas.

Possible perils of incretin therapies include the development of complications, including
pancreatitis and cancer. Although some studies combine groups of experimental subjects
exposed to DPP-4 inhibitors and GLP-1R agonists for pooled analyses of adverse events (5; 75),
these two distinct drug classes exhibit multiple fundamental differences in mechanisms of action
(2; 60). Hence it is not scientifically justifiable to pool subjects exposed to DPP-4 inhibitors and
GLP-1R agonists. The hypothesis that activation of GLP-1R signaling might promote increased
cell proliferation and increase the incidence or detection of specific neoplasms is reasonable.
Indeed rats, and to a lesser extent, mice, exhibit C cell hyperplasia and medullary thyroid cancer
after prolonged sustained exposure to GLP-1R agonists (76). Nevertheless, monkeys and humans
exhibit major differences in GLP-1R expression in their thyroid C cells, and calcitonin levels do
not rise into the abnormal range in the vast majority of subjects following prolonged exposure to GLP-1R agonists (77). Studies assessing the pancreata of thousands and mice and rats have not shown dysplasia or tumor formation following treatment with GLP-1R agonists or DPP-4 inhibitors for periods up to 2 years. Furthermore, GLP-1 levels remain substantially elevated for years following many forms of bariatric surgery, yet rates of pancreatitis, medullary thyroid cancer, glucagonomas, or cancer of the pancreas are not increased in this patient population despite more than 10 years of follow-up (78). Hence, the hypothesis that GLP-1R agonists or DPP-4 inhibitors will promote tumor formation (4) is not supported by the available preclinical or clinical data.

Experimental evidence raising the possibility that incretin-based therapy may be associated with a predisposition to develop pancreatitis or pancreatic cancer generates important hypotheses that require testing in mechanistic preclinical studies, and independent validation in large randomized controlled clinical trials. Pathological pitfalls of incretin-based science include the use of non-specific antisera, mismatched cases and controls, the generation of non-validated hypothesis and irreproducible data. As millions of patients with diabetes are being treated with incretin-based therapies, our collective responsibility for carrying out higher quality science has never been greater. Underpowered studies employing poorly validated reagents, or analysis of mismatched cases and controls (5) will have a much greater certainty of not being reproducible, and do not advance our understanding of incretin action in the pancreas. Emerging pharmacovigilance studies, such as the Safety Evaluation of Adverse Reactions in Diabetes (SAFEGUARD) study should shed additional clarity on the risk:benefit ratio of medications used to treat diabetes.
A great deal has been written about incretin action in the pancreas, including statements not substantiated or contradicted by available data. For example, the claim that in *Heloderma suspectum*, “production of exendin-4 causes rapid proliferation of intestinal tissue and a 50% increase in the size of the pancreas” (79) is simply incorrect, and is clearly refuted by the actual experimental data cited (80). The ongoing debate surrounding the mechanisms of action and potential safety of incretin based therapies reminds one of a quotation variably attributed to Daniel Patrick Moynihan, James Schlesinger, or Bernard Baruch. “Everyone is entitled to their own opinions, but they are not entitled to their own facts”. The beauty of science is that it is self-correcting, and provocative experiments and observations that are not highly reproducible are ultimately discarded. Over the next several years, we will learn much more about the potential risks and benefits of incretin-based therapies from large, randomized, ongoing cardiovascular outcome studies, with rigorous independent adjudication of adverse events. Thoughtful scientists await the results of these studies, and ongoing pharmacovigilance studies, with great interest. The results of these trials will be extremely useful for increasing our understanding of incretin action not only in the cardiovascular system but also in the diabetic pancreas.
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Figure Legends

Figure 1  Characterization of the sensitivity and specificity of antisera against the human GLP-1R

BHK cells were transiently transfected with the vector pcDNA3.1 alone (lane 1) or with a human Glp1r cDNA tagged at the C-terminus with GFP cloned into pcDNA3.1 (lane 2). Whole-cell extracts were prepared 48 h after transfection and analyzed by immunoblotting with the indicated commercial GLP-1R antibodies or with a rabbit polyclonal antibody against GFP (Abcam ab6556). Molecular mass standards are indicated on the right. Both the anti-GLP-1R antibody Novus1940002 and the anti-GFP antibody detected similar immunoreactive proteins of ~68 and ~87 kDa, likely representing differently glycosylated species of the GLP-1R-GFP fusion protein.

Figure 2

GLP-1 and DPP-4 inhibitor action in the endocrine pancreas. GLP-1R agonists and DPP-4 inhibitors enhance insulin and reduce glucagon secretion. In preclinical studies, these agents expand β-cell mass and reduce α-cell mass. Genetic mutations that disrupt glucagon receptor signaling or eliminate production of bioactive glucagon result in islet α-cell hyperplasia.
Figure 1
For Peer Review Only

Insulin Secretion
Glucagon Secretion
Somatostatin Secretion

Diabetes

GLP-1R activation
DPP-4 inhibition

Diabetes

Glucagon Secretion
Insulin Secretion
Somatostatin Secretion

β-cells
α-cells
δ-cells

β-cells
α-cells

α-cell hyperplasia

Gcgr^{-/-} mice
Gcgr^{Hep/-} mice
LGsKO mice
Gcg^{+/-} mice
Pcsk2^{+/-} mice
Gcgr human mutation

Normal islet
<table>
<thead>
<tr>
<th>Publication</th>
<th>Experimental Model &amp; Exposure</th>
<th>Drug(s)</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ahren (1)</td>
<td>Regular and HFD mice 8 wks</td>
<td>NVP DPP728</td>
<td>No change in islet α cells</td>
</tr>
<tr>
<td>Akarte (2)</td>
<td>Neonatal STZ Rats 8 wks</td>
<td>PKF-275-055 vs. Saline</td>
<td>Reduced number of glucagon+ islet cells</td>
</tr>
<tr>
<td>Busch (3)</td>
<td>Rats &amp; mice 2 year Exposure &gt; 200x</td>
<td>Vildagliptin</td>
<td>No changes in islet α cell number</td>
</tr>
<tr>
<td>Duttaroy (4)</td>
<td>Neonatal rats 19 days</td>
<td>Vildagliptin</td>
<td>No change in islet α cells</td>
</tr>
<tr>
<td>Furuta (5)</td>
<td>HFD-STZ mice 11 wks</td>
<td>DSP-7238</td>
<td>Normalization of α cell topography</td>
</tr>
<tr>
<td>Han (6)</td>
<td>ZDF rats 5 wks</td>
<td>Sitagliptin + metformin</td>
<td>Reduced numbers of islet α cells</td>
</tr>
<tr>
<td>Hou (7)</td>
<td>STZ rats 12 wks</td>
<td>Sitagliptin + mangiferin</td>
<td>Reduced numbers of islet α cells</td>
</tr>
<tr>
<td>Hu (8)</td>
<td>STZ HFD rats 10 wks</td>
<td>Sitagliptin + stem cells</td>
<td>Reduced numbers of islet α cells</td>
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<tr>
<td>Inaba (9)</td>
<td>GK rats 18 weeks</td>
<td>Vildagliptin</td>
<td>Increased α cell proliferation</td>
</tr>
<tr>
<td>Liang (10)</td>
<td>STX mice 2-14 days</td>
<td>Sitagliptin + losartan</td>
<td>Reduced α cell proliferation</td>
</tr>
<tr>
<td>Mu (11)</td>
<td>ICR Mice STZ/HFD 10 wks</td>
<td>Sitagliptin</td>
<td>Reduced numbers of islet α cells</td>
</tr>
<tr>
<td>Mu (12)</td>
<td>ICR mice STZ HFD 11 wks</td>
<td>Des-fluoro sitagliptin vs. SU</td>
<td>Reduced numbers of α cells</td>
</tr>
<tr>
<td>Omar (13)</td>
<td>HFD mice 11 months</td>
<td>Vildagliptin</td>
<td>Reduced numbers of α cells</td>
</tr>
<tr>
<td>Sato (14)</td>
<td>HFD 20 wks IRS2-/- mice</td>
<td>Vildagliptin</td>
<td>No change in islet α cells</td>
</tr>
<tr>
<td>Shimizu (15)</td>
<td>C/EBPB TG mice 24wks</td>
<td>Vildagliptin</td>
<td>No change in islet α cells</td>
</tr>
<tr>
<td>Souza-Mello (16)</td>
<td>HFD mice 6 wks</td>
<td>Sitagliptin + telmisartan</td>
<td>Reduced numbers of α cells</td>
</tr>
<tr>
<td>Tajima (17)</td>
<td>HFD STZ mice 5 wks</td>
<td>TS-021</td>
<td>Reduced α cell; β cell ratio</td>
</tr>
<tr>
<td>Takeda (18)</td>
<td>STZ mice 5 wks</td>
<td>Des-fluoro sitagliptin</td>
<td>Reduced numbers of α cells</td>
</tr>
<tr>
<td>Yeom (19)</td>
<td>Akita/db/db mice 4-6 wks</td>
<td>Sitagliptin</td>
<td>Decreased islet glucagon immunoreactivity</td>
</tr>
<tr>
<td>Zhang (20)</td>
<td>HFD/STZ mice 10 wks</td>
<td>Alogliptin</td>
<td>Reduced numbers of α cells</td>
</tr>
</tbody>
</table>
Supplementary Table 1 Legend

Preclinical studies describing effects of DPP-4 inhibitors on numbers of islet α cells in normal or diabetic animals, exclusive of experiments in NOD mice. Two year toxicology data in rats and mice carried out as part of regulatory requirements for multiple DPP-4 inhibitors was not included in this Table.

References


