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Discordance between GLP-1R gene and protein expression in mouse pancreatic islet cells

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Abstract

The insulinotropic actions of glucagon-like peptide 1 receptor (GLP-1R) in β-cells have made it a useful target to manage type 2 diabetes. Metabolic stress reduces β-cell sensitivity to GLP-1, yet the underlying mechanisms are unknown. We hypothesized that *Glp1r* expression is heterogeneous among β-cells and that metabolic stress decreases the number of GLP-1R positive β-cells. Here, analyses of publicly-available single-cell RNA-Seq sequencing (scRNASeq) data from mouse and human β-cells indicated that significant populations of β-cells do not express the *Glp1r* gene, supporting heterogeneous GLP-1R expression. To check these results, we used complementary approaches employing FACS coupled with quantitative RT-PCR, a validated GLP-1R antibody, and flow cytometry to quantify GLP-1R promoter activity, gene expression, and protein expression in mouse α-, β-, and δ-cells. Experiments with *Glp1r* reporter mice and a validated GLP-1R antibody indicated that >90% of the β-cells are GLP-1R positive, contradicting the findings with the scRNASeq data. α-cells did not express *Glp1r* mRNA and δ-cells expressed *Glp1r* mRNA but not protein. We also examined the expression patterns of GLP-1R in mouse models of metabolic stress. Multiparous female mice had significantly decreased β-cell *Glp1r* expression, but no reduction in GLP-1R protein levels or GLP-1R-mediated insulin secretion. These findings suggest caution in interpreting the results of scRNASeq for low abundance transcripts such as the incretin receptors and indicate that GLP-1R is widely expressed in β-cells, absent in α-cells, and expressed at the mRNA, but not protein, level in δ-cells.

Introduction

The incretin hormones glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP) are secreted from the gastrointestinal tract following a meal and augment insulin secretion, an effect that is blunted in individuals with type 2 diabetes (T2D) (1). Agonists of the GLP-1 receptor (GLP-1R) have been useful therapeutics for people with T2D (2), and recent iterations of drug development have coupled GLP-1R agonism in single peptides that also stimulate the GIP receptor (GIPR) (3). While the GLP-1R and GIPR have distinct expression across various tissues (4), both receptors were originally identified in β-cells where they mediate the principle action of incretins, insulin secretion. Studies in humans suggest additivity of GIP and GLP-1 on glucose-stimulated insulin secretion (5) and GIPR/GLP-1R dual agonists have greater glucose lowering potency than GLP-1R agonists in mice and humans (6, 7). However, it is not clear whether these effects are due to joint stimulation of β-cells expressing both incretin receptors, or if populations of β-cells have specific expression of one or the other incretin receptors. Heterogeneity of incretin receptor expression among β-cells has not yet been rigorously tested, but this possibility has significant implications for physiology and therapeutics.

Estimates of incretin receptor expression across islet populations have been approached mainly with histologic (8–10) and single-cell gene expression techniques. Immunostaining approaches have been limited by the lack of specific GLP-1R antibodies to label functional receptors (11), and the inherent lack of precision in distinguishing among individual cells on histologic specimens. Several studies have compared the transcriptional profiles of islet α-, β-, and δ-cells in healthy and T2D subjects to gain insight into normal and pathologic physiology. Application of single-cell RNA sequencing (scRNAseq) has demonstrated greater variability among islet cell types than previously appreciated, in addition to supporting distinct gene expression in the development of diabetes (12–14). This approach has the potential for understanding specific physiology, such as the incretin effect, and important clinical applications, such as the mechanism of action of multireceptor agonists.

Previous work has demonstrated that diabetes or metabolic stress decreases *Glp1r* expression in lysates from rodent islets (8), or cell lines (15). These preclinical findings are compatible with the reduced incretin effect among persons with T2D (16), and the blunted response of diabetic subjects to exogenous infusion of GLP-1 (17). However, to date the contribution of GLP-1R activity to abnormal β-cell function has been limited to the analysis of RNA expression, and it remains unclear if the levels of GLP-1R protein are reduced in dysglycemic states, and if so, whether this occurs in all β-cells. Although a validated GLP-1R antibody has recently been developed (9), detailed comparisons of *Glp1r* expression and GLP-1R protein content in individual islet cells have not been made.

In this paper, we describe a series of experiments to test the hypothesis that GLP-1R expression is heterogeneous in β-cells. The work starts with analysis of human and mouse single-cell RNA sequencing data to document the expression of the incretin receptor genes in islets cells. These data suggested significant heterogeneity in incretin receptor expression, which we then expanded upon with studies measuring *Glp1r* RNA expression and GLP-1R protein presence on individual islet α-, β-, and δ-cells. Finally, the role of reduced β-cell *Glp1r* transcription in metabolic stress was tested in the context of GLP-1R protein and activity measurements.

Results

Single cell RNAseq data suggest incretin receptor expression is heterogeneous in β-cells

To test whether incretin receptors were heterogeneously expressed in β-cells, transcriptomes from publicly-available human (12, 13, 18–21) and mouse (22) scRNAseq datasets were analyzed to determine the expression patterns of *GLP1R/Glp1r* and *GIPR/Gipr*. The expression of incretin receptors in single β-cells was calculated (Table 1) and plotted to visualize the extent of co-expression (Fig 1). In human β-cells, *GLP1R/GIPR* co-expression was variable across datasets, ranging from no co-expression (19) to up to ~27% of β-cells expressing both receptors (13). All human data sets demonstrated a significant number of β-cells that did not express either incretin receptor (range: 7-92%; Table 1). Fewer datasets were available for mouse β-cells; however, available data (22) suggest that while ~56% of β-cells had both incretin receptors, 25% expressed *Glp1r* only, 9% expressed *Gipr* only, and 9% of β-cells did not express either. Across species and platforms, scRNAseq suggests considerable heterogeneity in the expression of incretin receptors in β-cells, with a surprising number of β-cells expressing either only a single receptor or none at all.

The Glp1r promoter is active in both β- and δ-cells, but not α-cells

To test the hypothesis that β-cell *Glp1r* expression is heterogeneous, *Glp1r* reporter mice were generated by crossing *Glp1r-Cre* mice (23) with *mTmG reporter* (24) mice (*Glp1r:mTmG*). This reporter model is a constitutive reporter and GFP^+ (Cre^+) cells reflect those that express *Glp1r* promoter activity at any stage of development. Islet cells from *Glp1r:mTmG* mice were isolated, dispersed, and separated by FACS into GFP^+ (Cre^+) and tdTomato (Tom^+, Cre^-) populations (Fig 2A). Quantification of the distinct cell populations demonstrated that 78% of islets cells were GFP^+ and 20% were Tom^+ (Fig 2B). A small percentage (2%) of cells were GFP^+/Tom^+, but given their low abundance these were not analyzed further. Only GFP^+ cells expressed *Glp1r* mRNA, validating the model (Fig 2C). To identify the types of cells constituting GFP^+ and Tom^+ populations, expression of genes specific to α-, β-, and δ-cells were measured by qPCR and expressed relative to whole islet levels. Glucagon (*Gcg*), defining α-cells, was highly enriched in Tom^+ cells and nearly absent in the GFP^+ cells (Fig 2D). Conversely, insulin II (*Ins2*) and somatostatin (*Sst*), markers of β- and δ-cells respectively, were enriched in GFP^+ cells and low/absent in Tom^+ cells (Fig 2E,F). These findings suggest that *Glp1r* expression coincides with markers for β- and δ-cells, but not α-cells in mouse islets, which aligns with other reports (25). Moreover, *Ins2* expression was nearly undetectable in Tom^+ cells, suggesting the number of potential *Glp1r* negative β-cells in mouse islets is very low. While it is possible that an *Ins2* signal is diluted by the abundance of α-cells in the Tom^+ population, the data from mouse scRNAseq data set (22) (Table 1) would suggest that ~18% of β-cells are *Glp1r* negative. This percentage of cells should comprise enough of the Tom$^+$ population to produce an *Ins2* signal, which was not the case here. This indicates that the number of *Glp1r* negative β-cells is substantially lower than 18%, a result incompatible with the hypothesis of heterogeneous *Glp1r* expression in β-cells generated by the scRNAseq data.

Glp1r is highly expressed in β-cells enriched from wild-type mouse islets

To support the results of the *Glp1r:mTmG* model, a complementary approach was used to assess *Glp1r* expression in wild-type islet cells. First, enriched populations of islet cells were separated by FACS (Fig 3A) based on endogenous FADPH-based fluorescence and side scatter (26, 27). Using this approach, the distribution of cells collected from islets of 17 mice was 22% α-cells, 65% β-cells, and 13% δ-cells (Fig 3B). These percentages align with those obtained by the *Glp1r:mTmG* model (Fig 2B) as well as with previous studies estimating islet cell composition in fixed tissue (28). The enriched populations of cells from 8 of the mice were validated with qPCR to determine expression of *Gcg*, *Ins2*, and *Sst* (Fig 3C-E). *Gcg* and *Sst* expression were virtually exclusive to the α- and δ-cell populations, respectively. *Ins2* was most robustly expressed in β-cells. Since islet cells express relatively high levels of the genes for hormones that are not secreted (*e.g.*, *Ins2* in α- and δ-cells (25)), lower-expressing genes that are more specific for α-cells (Somatostatin receptor 2, *Sstr2*) and β-cells (Solute carrier family 2, member 2, *Slc2a2*; Galanin receptor 1, *Galr*)(25) were also measured to confirm fidelity of the enriched populations. Expression of *Sstr2* identified the α-cell population as highly enriched, as did the measures of S*lc2a2* and *Gal1r* for β-cells (Fig S1A-C). *Glp1r* expression was highest in the enriched β-cell population, with lower, but detectable, measures in α- and δ-cells (Fig 3F). All three endocrine populations have been reported to express the *Glp1r* at variable levels using a bulk RNAseq approach (28). These findings align with those reports and with the robust expression of *Glp1r* in β- and δ-cells in the *Glp1r:mTmG* mice. However, the detectable expression of *Glp1r* in enriched α-cells of WT mice contrasts with the lack of promoter activity in α-cells from *Glp1r:mTmG* mice.

GLP-1R protein is detectable in nearly all β-cells

The estimates of *Glp1r* expression patterns in β-cells assessed by scRNAseq data sets did not align with those produced by with qPCR in FACS separated islet cells. This discrepancy may reflect methodological shortcomings of scRNAseq when targeting genes expressed at low abundance (29). To rectify the discordant findings and provide an important linkage between *Glp1r* transcription and translation, an assay to measure protein expression of GLP-1R on individual, live islet cells was developed using antibody Glp1R0017 (9) conjugated to an allophycocyanin (APC) fluorophore. All experiments used the IgG-APC control to set gating parameters (GLP-1R$^-$ and GLP-1R$^+$) and serve as an index of non-specific binding (NSB). Dispersed islet cells were treated with GLP1R-APC or IgG-APC and staining determined in enriched β-cells from control and *Glp1r*$^{\beta cell-/-}$ mice. β-cells from wild-type mice had >90% GLP-1R$^+$ staining, while staining in the cells from *Glp1r*$^{\beta cell-/-}$ mice was comparable to the IgG control (Fig 4A). As a complementary approach, GLP1R-APC binding was also tested in *Glp1r:mTmG* mice to compare *Glp1r* promoter activity with protein expression. Over 90% of GFP$^+$ cells stained GLP-1R$^+$ and virtually all (>97%) of Tom$^+$ cells did not stain with the antibody (GLP-1R$^-$, Fig 4B). These staining characteristics were consistent with qPCR measures of *Glp1r* expression in these discrete cell populations (Fig 4C). Collectively, these data demonstrate specificity of GLP1R-APC for GLP-1R protein and its suitability for flow cytometry applications. Moreover, there appears to be a strong overall concordance of *Glp1r* promoter activity and protein expression. However, a small population of cells (<10%) were GLP-1R$^-$ despite being GFP$^+$.

To characterize the population of cells from *Glp1r:mTmG* islets that were GFP$^+$ but had no GLP-1R immunostaining, gene expression was measured by qPCR in each cell population. Consistent with the findings shown in Fig 2D, *Gcg* expression was enriched in Tom$^+$ cells (Fig

5A), indicating this population of cells are α-cells (no *Glp1r* promoter activity; no immunodetection of GLP-1R). *Ins2* was highest in the GFP^+ cells that stained GLP-$1R^+$ (Fig 5B), consistent with this population being β-cells (high *Glp1r* promoter activity; high immunodetection of GLP-1R). Interestingly, *Sst* was highly enriched in GFP^+ cells that were GLP-$1R^-$ (Fig 5C), indicating that these are δ-cells. Thus, FACS separation was able to generate β-cells with robust staining of GLP-1R, but did not yield GLP-$1R^+$ δ-cells.

In parallel experiments, islets from WT mice were evaluated for GLP1R-APC staining in enriched α-, β-, and δ-cell populations (Fig 6). In α-cells, the IgG-APC control produced a positive signal in 7.1% of the cells (an index of background staining), while the GLP1R-APC produced a positive signal in 16.8% of the cells, a difference of only borderline statistical significance (p=0.07), compatible with, but not definitive for, a small population of GLP-$1R^+$ α-cells. In contrast, the vast majority of β-cells (89.7%) stained positive for GLP-1R-APC (Fig 6B). Finally, in the δ-cell population, 7.4% of the cells stained positive for GLP1R-APC versus 2.9% with IgG (Fig 6C, $p < 0.05$). Thus, analysis of GLP-1R positive cells in enriched populations from WT islets align with the findings from the *Glp1r:mTmG* islets; GLP-1R-APC robustly stains the majority of β-cells, but only produces a signal in small minorities of α- or δ-cells.

Finally, the different populations of islets cells were characterized by gene expression based on FACS separation (Fig 3A) and GLP-1R-APC staining (Fig 6). In addition to α-cells (GLP-$1R^-$), β-cells (GLP-$1R^+$), and δ-cells (GLP-$1R^-$), a group of GLP-$1R^-$ cells characterized as β-cells by autofluorescence were collected and analyzed. Similar to the flow cytometry analysis (Fig 6), FACS sorting of islets cells produced a GLP-1R-APC signal in the majority of β-cells (Fig S2B, E), but only a small proportion of α- and δ-cells (Fig S2A-C, D, F). Given the low abundance of GLP-$1R^+$ α- and δ-cells, there was not enough cellular material to get consistent, sufficient amounts of RNA for qPCR in these populations. *Gcg*, *Ins2*, and *Sst* were highest in the α-, β- and δ-cell populations, respectively (Fig S2G-J). Both GLP-$1R^+$ and GLP-$1R^-$ β-cell populations expressed similar levels of *Ins2* (Fig S2H). Interestingly, *Glp1r* expression was robust in both β-cell populations, but not in α- or δ-cells (Fig S2J). Small, but detectable increases in *Gcg* and *Sst* were observed in GLP-$1R^-$ β-cells, suggesting either small numbers of α- or δ-cells contaminating this gating or that a subset of GLP-$1R^-$ β-cells are multihormonal expressing cells. Taken together, the FACS and flow cytometry assays of wild type islet cells demonstrate minimal GLP-1R staining in enriched α- and δ-cell populations, but nearly uniform staining in enriched β-cells.

Metabolic stress reduces Glp1r expression but not GLP-1R levels or activity

Islets from young mice fed a standard rodent diet produced β-cells with robust and nearly-ubiquitous staining for GLP-1R (Fig 6B, Fig S2B,E). Previous work has shown that hyperglycemia in pancreatectomized rats (8) and cultured MIN6 cells (15) decreases *Glp1r* expression. Moreover, the insulin secretion response to physiological levels of exogenous GLP-1 is decreased in people with T2D (17). These measurements have not been extended to the resolution of individual β-cells. Thus, male mice were fed a 60% HFD for 4-weeks to determine if this metabolic stress reduces *Glp1r* expression in individual β-cells, and whether this translates to differences in the proportion of GLP-$1R^+$ β-cells. Both WT and *Glp1r:mTmG* mice were included to provide independent measures of *Glp1r* activity. High-fat feeding increased body weight and ambient glycemia in both mouse lines (Fig S3A, B), consistent with induction of metabolic stress. However, the expression of *Glp1r* or *Gipr*, which has also been shown to be reduced by hyperglycemia (8), in enriched β-cell populations did not decrease in either mouse model (Fig S3C, D, F, G). Moreover, the number of GLP-1R positive β-cells remained unchanged in both the WT (Fig S3E) and *Glp1r:mTmG* mice (Fig S3E, H). These results do not conform to previous studies, possibly because a period of longer than 4 weeks of high-fat feeding, or more extreme hyperglycemia, is required to reduce *Glp1r* expression.

To induce metabolic stress through an alternative approach, the expression of GLP-1R was compared in multiparous (MP) and nulliparous (NP) female mice. Multiparity is associated with increased adiposity and impaired glucose tolerance (30). Moreover, multiparity has been shown to be a physiological stress that reduces β-cell mass in mice through dedifferentiation of β-cells into an α-cell-like phenotype (31). This model was used to test the hypothesis that a dedifferentiating β-cell could present an *Ins+:Glp1r-* profile and produce a heterogeneous population of β-cells with respect to GLP-1R expression. MP mice were significantly heavier than NP mice (Fig 7A), but had similar ambient glycemia (Fig 7B). *Glp1r* and *Gipr* expression in enriched β-cells was significantly reduced in MP mice (Fig 7C,D). However, the number of GLP-1R positive β-cells in islets from MP mice was similar to NP mice, and the GLP-1R was present in nearly all β-cells (Fig 7E). To test whether reduced *Glp1r* expression translated into reduced GLP-1R activity, insulin secretion from perifused NP and MP islets was measured in response to increasing concentrations of GLP-1. These concentrations were based on our prior studies testing GLP-1-mediated insulin secretion, where the EC_{50} of GLP-1 was 0.03 nM and maximal response was achieved at 1 nM (32). MP mice had elevated glucose-stimulated insulin secretion (Fig 7F), likely reflecting insulin resistance and metabolic stress. However, the response to GLP-1, measured as the relative increase over GSIS, was comparable between NP and MP mice (Fig 7F,G). In contrast, the reduced expression of β-cell *Gipr* in islets from MP mice (Fig 7D) was associated with a reduced insulin secretory response to GIP in perifused islets compared to NP islets (Fig 7H, I). These data demonstrate that decreased *Glp1r* message did not reflect reduction of GLP-1R protein or activity in MP animals, demonstrating robust maintenance of functional receptors despite a physiologic challenge.

Discussion

The incretin axis, long recognized as an important regulator of glucose tolerance, has taken on increased significance as a target of drug development in recent years. Yet the factors that regulate incretin receptor expression in β-cells remain incompletely understood. As one example, the question of whether all β-cells have similar expression and activity of the GLP-1R has been widely assumed but rarely tested. Moreover, the mechanisms that cause decreased GLP-1 sensitivity in T2D remain unexplained. Here, we observed considerable heterogeneity in the expression patterns of *GLP1R/Glp1r* in human and mouse β-cells from scRNAseq datasets. However, further investigation using *Glp1r* promoter activity, gene expression, protein expression, and ultimately GLP-1R function, demonstrates discordance between measures of gene expression and actual receptor content in islet cells. Based on our results it appears that the majority of adult mouse β-cells express GLP-1R on their plasma membrane. Moreover, GLP-1R function is not necessarily reflected by the level of *Glp1r* gene expression. These findings are consistent with a model whereby GLP-1R signaling is a uniform and resilient feature of healthy β-cells.

Single cell RNA sequencing provides tremendous breadth in the analysis of β-cell gene expression, albeit at a cost of potential false negative reads, usually due to limits in the cDNA library preparation or the sequencing depth of that library (29, 33). The findings presented here exemplify one of these limitations. In published datasets of islet scRNAseq (12, 13, 18–22) the majority of human β-cells did not express at least one of the incretin receptors, while the majority of mouse β-cells express considerable heterogeneity in incretin receptor expression and both had a surprising paucity of cells positive for both receptors (Fig 1, Table 1). However, this dichotomous pattern of *Glp1r* expression was not confirmed with more directed experiments that, in fact, support the presence of the receptor on most β-cells. Thus, the results in this paper are consistent with previous cautions that scRNAseq has limited precision for detecting low-expressing transcripts such as *GLP1R/Glp1r* (29, 33).

A key reagent in this line of investigation was an effective and well-validated antibody for the GLP-1R (9). After conjugation to a fluorophore, Glp1R0017 binds specifically to GLP-1R in live, dispersed β-cells as demonstrated by our studies using *Glp1r*$^{\beta cell-/-}$ mice as negative controls. Moreover, GLP1R-APC labeled 97% of islet cells that had *Glp1r* promoter activity (i.e., GFP^{+} cells), and virtually none that did not (Tom^{+}), providing a second, independent measure of its specificity in this application. In the present study, GFP^{+}/GLP-1R^{+} cells had β-cell markers and most of the enriched β-cells bound GLP1R-APC. We interpret these results as demonstrating that nearly all β-cells in an adult mouse express the GLP-1R, a conclusion previously advanced in reports of studies using immunostaining of fixed sections of pancreas from mice (9) and islets from humans (10, 34), as well as gene expression in dispersed islet cells from mice (35). Our findings add to this established literature with a more definitive approach that mitigates some of the methodologic limits of previous work.

In addition to profiling the expression patterns of GLP-1R on β-cells, our approach also enabled the investigation of expression patterns in α- and δ-cells. Recent islet cell transcriptomics datasets also demonstrate very low *Glp1r* in mouse α-cells (25, 36), although others have reported α-cell GLP-1R (37, 38). While a numerically greater number of α-cells bound GLP1R-APC compared to IgG-APC in our flow cytometry experiments (Fig 6), we were unable to acquire enough cells to perform a reliable qPCR analysis of this population (Fig S2). Contamination of the α-cell pool with β-cells is possible in this experiment, as others have noted (29). Due to technical difficulties, a live/dead stain was not used in these assays and since the cells were not sorted, it is not clear whether dying or dead β-cells contaminated α- and δ-cell populations. Regardless, our findings indicate little or no GLP-1R on α-cells, consistent with the data from both the reporter and gene expression studies, and in line with a recent human study that reported <0.5% of α-cells stained with a GLP-1R antibody (10). Thus, the bulk of current evidence suggests that any actions of GLP-1 on α-cell function are likely to be indirect.

Given that most studies do not support significant GLP-1R in α-cells, the consensus to explain GLP-1 action to decrease glucagon release has rested on a paracrine model. The most common explanation involves GLP-1 stimulation of somatostatin release from δ-cells with secondary inhibition of α-cells. For example, treatment of perfused pancreata with pharmacologic antagonists of somatostatin receptors mute the inhibitory effect of GLP-1 on glucagon secretion, implicating a role for δ-cells to mediate this response (39, 40). Likewise, exposure of cultured human islets to the GLP-1R agonist liraglutide reduces drug-induced glucagon secretion, an effect which is abolished by an SSTR2 inhibitor (34). While there is little histologic data available that demonstrates localization of GLP-1R on δ-cells, especially in mouse islets where these cells are relatively infrequent, recent studies in mouse (38) and human (34) islets suggest some colocalization. The observations reported here neither support nor challenge a role of somatostatin to mediate GLP-1 effects on glucagon release. However, they do bring into question whether this is mediated by a direct action of GLP-1 on δ-cells. Despite clear concurrence of *Sst* expression and *Glp1r* promoter activity in *Glp1r:mTmG* mice, GLP1R-APC staining in enriched δ-cells was only slightly greater than that of IgG-APC. Attempts to sort GLP-1R^{+} δ-cells did not provide sufficient material for qPCR analysis. While GLP1R-APC is specific and stains β-cells convincingly, it remains possible that our staining parameters did not allow for the detection of the GLP-1R in δ-cells. Alternatively, it is possible that the *Glp1r* promoter is active in δ-cells during development, but not expressed or translated in the adult mouse. Reconciling the observation that robust *Glp1r* expression in δ-cells does not translate to more than minimal GLP-1R protein levels in isolated δ-cells warrants future investigation.

To test the plasticity of GLP-1R gene and protein expression, multiparity was used as a means to induce metabolic stress (30), based on the models others have reported to identify islet cell plasticity and β-to-α-cell transitions (31, 41). In rodents, and to a lesser extent, humans (42, 43), pregnancy induces transcriptional changes often associated with increased β-cell mass (44, 45), which return to pre-pregnancy levels shortly after parturition (46, 47). Mice lacking GLP-1R fail to increase β-cell mass during pregnancy (48), suggesting an important role for GLP-1R signaling during gestation. The MP model had a second advantage as a model that may increase the population of islet cells that others have described as co-expressing *Gcg*, *Ins*, and *Glp1r* (35, 49). Importantly, the MP model induced a decrease in β-cell *Glp1r* that was similar in magnitude to other studies (8, 15). However, the decreased levels of *Glp1r* expression did not translate into altered expression of GLP-1R protein on the plasma membrane in live β-cells. Moreover, the action of GLP-1R to stimulate insulin secretion in MP mice remained intact, in line with studies finding similar GLP-1 sensitivity in lean and obese humans (50). These data indicate that RNA expression is not sufficient to determine changes in GLP-1R protein/activity.

Taken together with the other findings reported in this paper, the results from the MP mice suggest nearly universal expression of GLP-1R and continued GLP-1R function in this distinct setting of metabolic stress. However, this model does support potential dynamic regulation of *Gipr*. Most previous work indicates that the GIPR is expressed in α-, β-, and δ-cells, in contrast to what we show here for the GLP-1R. Although our general approach to studying GLP-1R on islet cells is applicable to the GIPR we have not yet found a suitable antibody for labeling and cell sorting. While we have shown here that gene expression does not necessarily reflect protein expression, it should be noted that MP mice had decreased sensitivity to exogenous GIP (Fig 6), suggesting potential differences in incretin receptor regulation following metabolic stress. These divergent effects on incretin action have been reported in mice (48) and humans (51). Given universal expression of GLP-1R in β-cells and interest in co-agonists for both receptors to treat T2D (3, 52) and the different responses of these receptors to ligand (53), understanding the interplay between GLP-1R and GIPR is of interest.

There are some caveats to consider with the data presented in this paper. First, while our scRNAseq data were similar between mouse and human, our subsequent studies were limited to mouse islets. Thus, it remains to be determined whether the distribution of GLP-1R among islet cells described here would differ in human specimens. While others have observed similar staining patterns in whole islet sections (10, 34), this question has not been addressed at the level of individual islet cells. Second, despite demonstrations of Glp1R0017 specificity provided here and in a prior paper (9), this validation has been mostly with β-cells and we cannot exclude the possibility that our findings in δ-cells is due to different antibody binding in the two cell types or differences in the cellular localization of GLP-1R. Western blotting may allow for detecting intracellular GLP-1R, but we did not validated Glp1R0017 for this application. Third, our measures of GLP-1R heterogeneity among islet cells used a dichotomous definition, e.g. present or absent. We cannot determine from our results the variation of receptor expression across the population of β-cells in a mouse islet. Finally, we used MP mice due to their decreased *Glp1r* expression and general metabolic stress; however, we did not control for age compared to NP mice or for important reproductive factors such as estrus cycle or time since last pregnancy. Our studies do not address whether age contributes to the discrepancies we've observed between human and mouse beta cell findings. These factors could also have implications for incretin receptor modulation due to changes in β-cell proliferation and apoptosis (45).

In summary, the findings reported here demonstrate that the majority of β-cells express the GLP-1R, refuting the hypothesis suggested by scRNAseq analyses that a significant percentage of these cells are receptor negative. We also show that WT, adult α-cells neither express *Glp1r* nor contain GLP-1R in the plasma membrane to any significant extent. Mouse δ-cells express the *Glp1r*, at least at some time during development, but have limited membrane receptors in adulthood. The discrepancy between gene expression and amount of active receptors is also seen in MP mice, which downregulate *Glp1r* but maintain normal membrane receptor content and intact responsiveness to GLP-1. Overall, our results support a model whereby direct signaling of GLP-1 is limited to β-cells in adult mouse islets and not modified during metabolic stress.

Experimental procedures

Single-cell RNA sequencing data analysis

Single-cell RNA sequencing data were obtained from seven published studies (12, 13, 18–22) (Table 1). The datasets were all analyzed in Seurat v3 (54), namely, expression was normalized by log normalization method. Pancreatic islet cell identities were taken from the results of each study, and β-cells were extracted for GPCR co-expression analysis.

GPCR expression in human and mouse β-cells

GPCR gene annotation was obtained from HUGO Gene Nomenclature Committee. GPCRs detected in more than 10 β-cells were included in the analysis. Pairwise co-expression pattern was examined for all the detected GPCRs in β-cells. The normalized expression of *GLP1R* (*Glp1r* in mouse) and *GIPR* (*Gipr* in mouse) was used to illustrate co-expression patterns in the human and mouse β-cells. Each dataset has specific expression units according to its single-cell sequencing platform.

Animals

C57Bl/6 mice (WT) were maintained through our internal breeding colony. *Glp1r:mTmG* mice were generated by crossing *Glp1r-ires-cre* (23) with *Gt(ROSA)26Sor*$^{tm4(ACTB\text{-}tdTomato,\text{-}EGFP)Luo/J}$ reporter mice (24) and maintained on a mixed background. These mice express endogenous, membrane-bound tdTomato fluorescence. Following cre-mediated recombination, tdTomato is excised and GFP is transcribed and expressed on the plasma membrane (24). To test GLP-1R antibody specificity, *Glp1r:Gcgr*$^{\beta\text{-cell-/-}}$ were produced by breeding *Glpr1*$^{fl/fl}$*Gcgr*$^{fl/fl}$ mice with MIPcreERT (*MIP-Cre*) mice to generate inducible, β-cell specific knockouts. Control (*Glpr1*$^{fl/fl}$*Gcgr*$^{fl/fl}$*:MIP-Cre*$^{+/+}$) and knockout mice (*Glpr1*$^{fl/fl}$*Gcgr*$^{fl/fl}$*:MIP-Cre*$^{Cre/+}$) were treated with tamoxifen by oral gavage at 6 weeks of age and mice were used at least 4 weeks after treatment as previously reported (32). High-fat diet fed mice received 60% high-fat diet (HFD, Research Diets D12492) for 4 weeks. Multiparous (≥4 pregnancies) female mice were maintained on standard breeder chow (Lab Diet 5058). All other mice received standard rodent chow (Lab Diet 5053). Animal experiments were conducted in accordance with Duke IACUC guidelines.

Islet isolation

Islets were isolated by inflating the pancreas with collagenase type V (0.8 mg/ml) in HBSS injected retrograde through the pancreatic duct. Digestion occurred at 37°C and was stopped with application of ice-cold RMPI (2 mM L-glutamine, 0.25% BSA). Islets were separated from pancreatic tissue using a histopaque gradient and allowed to recover in RMPI (11.1 mM glucose, 10% FBS, 1% pen/strep) overnight before experiments were performed.

Islet dispersion

After overnight recovery, 70-100 islets were collected from each mouse and rinsed once in PBS before incubation with Accutase (Sigma, A6964) for 12-15 min at 37°C with intermittent vortexing. Digestion was stopped with addition of cold RPMI and dispersed islet cells were centrifuged for 3 min, 350 xg, at 4°C. RPMI was aspirated and islets were washed with sorting buffer (RPMI 1640 without phenol red (11835030), 11.1 mM glucose, 1% FBS, 1% pen/strep, 2 uM HEPES, 10 U/ml DNAse). Islets not receiving antibody staining were washed again in sorting buffer before FACS; islets receiving antibody staining were processed as described below.

GLP-1R antibody conjugation and staining

Glp1R0017 (9) and control hIgG1 (MedImmune) were conjugated to an APC fluorophore with a commercially-available kit (Abcam, ab201807) per the manufacturer's instructions. Briefly, antibodies were mixed with APC modifier reagent and incubated overnight, in the dark, at room temperature with APC. Then, quenching reagent and sodium azide (0.05% final concentration) was added to yield a concentration of 1.25 mg/ml of Glp1R00017-APC (GLP1R-APC) and hIgG1-APC (IgG-APC). Conjugated antibodies were stored at 4ºC for up to 2 months. For FACS experiments, dispersed islet cells were incubated with GLP1R-APC or IgG-APC control antibodies (10 ug/ml) and Hoechst 33342 (10 uM) for 90 minutes rocking at 4ºC in the dark. For flow cytometry experiments, dispersed islet cells were incubated only with GLP1R-APC or IgG-APC control antibodies (10 ug/ml) for 30 minutes rocking at 4ºC in the dark. Following antibody incubation, cells were spun down and washed 3x with sorting buffer before FACS or flow cytometry analysis.

Flow cytometry

Dispersed islet cells were transported on ice and filtered through 30 um mesh prior to fluorescence-assisted cell sorting (FACS) using a Beckman-Coulter MoFlo Astrios or analyzed using Attune NxT Analyzer (Thermo Fisher A24863). Forward and side scatter (SSC) were used to separate single cells from debris and doublets. For FACS, live islet cells (Hoechst$^-$) from *Glp1r:mTmG* mice were separated into GFP$^+$ and tdTomato$^+$, and by auto-fluorescence and side-scatter, into α-, β-, and δ-cell populations for wild type mice (26, 27) into Trizol. For flow analyzer experiments, wild type islets were gated similarly, but without Hoechst stain due to technical limitations of our instrument. Islets treated with antibody were sorted based on APC staining, with IgG-APC$^+$ cells used to set the negative gating control (GLP-1R$^-$) and fluorescence greater than this was classified as GLP-1R$^+$. Where possible, cell percentages reported in this manuscript are calculated from FACS sorts (i.e. absolute number of cells sorted to Trizol). Post-sort analysis of FACS files in FlowJo (v.10.6.2) is used to present flow cytometry plots and in instances where cells numbers were not collected or cell numbers were not reported. Analysis methods are described in the legend of each figure.

RNA extraction, DNA synthesis, RT-PCR

Whole islets and sorted cells were collected into Trizol for RNA extraction and cDNA was synthesized from 100 ng RNA (Thermo Fisher cat #4368814). qPCR was run using Taqman reagents and primers (Table S1). Data were analyzed by calculating ΔΔCT and each gene of interest was normalized to cyclophilin A. Data are shown as fold change relative to whole islet lysates in control animals.

Islet perifusion

For islet perifusion, 75 islets were handpicked and loaded into 0.275 ml chambers containing KRPH (140 mM NaCl, 4.7 mM KCl, 1.5 mM $CaCl_2$, 1 mM NaH_2PO_4, 1 mM $MgSO_4$, 5 mM HEPES, 2 mM $NaHCO_3$, 1% fatty acid-free BSA) in 2.7 mM glucose. Prior to all experiments, KRPH with 2.7 mM glucose was perifused at a rate of 200 µl/min for 48 min to equilibrate using the BioRep Perifusion system. Following equilibration, experimental conditions were applied and perifusate was collected each minute. GLP-1 (Bachem, cat#4030663) and GIP (Phoenix Pharmaceuticals, cat#027-27) were reconstituted according to manufacturer's instructions and diluted in KRPH prior to experiment. Perifusate insulin concentrations were measured with AlphaLISA (Perkin Elmer).

Statistics

Data in figures are presented as mean ± SEM and data in the text is presented as mean ± SD. Analysis was done using GraphPad Prism (v. 8.3). Pairwise comparisons are stated throughout the text and mice were compared within genetic background. Differences between >2 groups were compared by 1-way ANOVA (with mouse repeated) or mixed effect model where values were missing. Post-hoc tests used Tukey's multiple comparisons test to determine significance. Differences between cell populations within the same mouse were compared by paired *t*-test and differences between two groups were compared with unpaired *t*-test. Incretin responses were normalized to glucose-stimulated insulin secretion and a 2-way ANOVA was run to test for the effect of parity, incretin dose, and parity*dose interaction term. Sidak's multiple comparisons test was used to compare responses between groups within dose. Tests are described in the legend of each figure.

Data Availability: The datasets generated during and analyzed during the current study are available from the corresponding author upon reasonable request.

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Table 1.

Study	Data source	Platform	Species	β-cell #	*GLP1R*$^{+}$ *GIPR*$^{-}$	*GLP1R*$^{-}$ *GIPR*$^{+}$	*GLP1R*$^{+}$ *GIPR*$^{+}$	*GLP1R*$^{-}$ *GIP R*$^{-}$
Baron et al. (18)	GSE84133*	inDrop	Human	2507	187 (7.5%)	216 (8.6%)	30 (1.2%)	2074 (82.7%)
Lawlor et al. (12)	GSE86469*	Fluidigm C1	Human	258	8 (3.1%)	184 (71.3%)	48 (18.6%)	18 (7.0%)
Grun et al. (19)	GSE81076*	CEL-Seq	Human	161	24 (14.9%)	1 (0.6%)	0 (0%)	136 (84.5%)
Muraro et al. (20)	GSE85241*	CEL-Seq2	Human	445	132 (29.7%)	9 (2.0%)	6 (1.3%)	298 (67.0%)
Segerstolpe et al. (13)	E-MTAB-5061*	Smart-Seq2	Human	308	100 (32.5%)	22 (7.1%)	83 (26.9%)	103 (33.4%)
Xin et al. (21)	GSE114297	10x Genomics	Human	7361	510 (6.9%)	98 (1.3%)	10 (0.1%)	6743 (91.6%)
Tabula Muris (22)	Tabula muris github	Smart-Seq2	Mouse	449	115 (25.6%)	42 (9.4%)	254 (56.6%)	38 (8.5%)

Table 1: *GLP1R/Glp1r* and *GIPR/Gipr* expression in β-cells from published scRNAseq data sets. Number of β-cells (% of total) shown for β-cell expressing either, both, or neither incretin receptor. *=The dataset was provided by *SeuratData*.

Figure 1

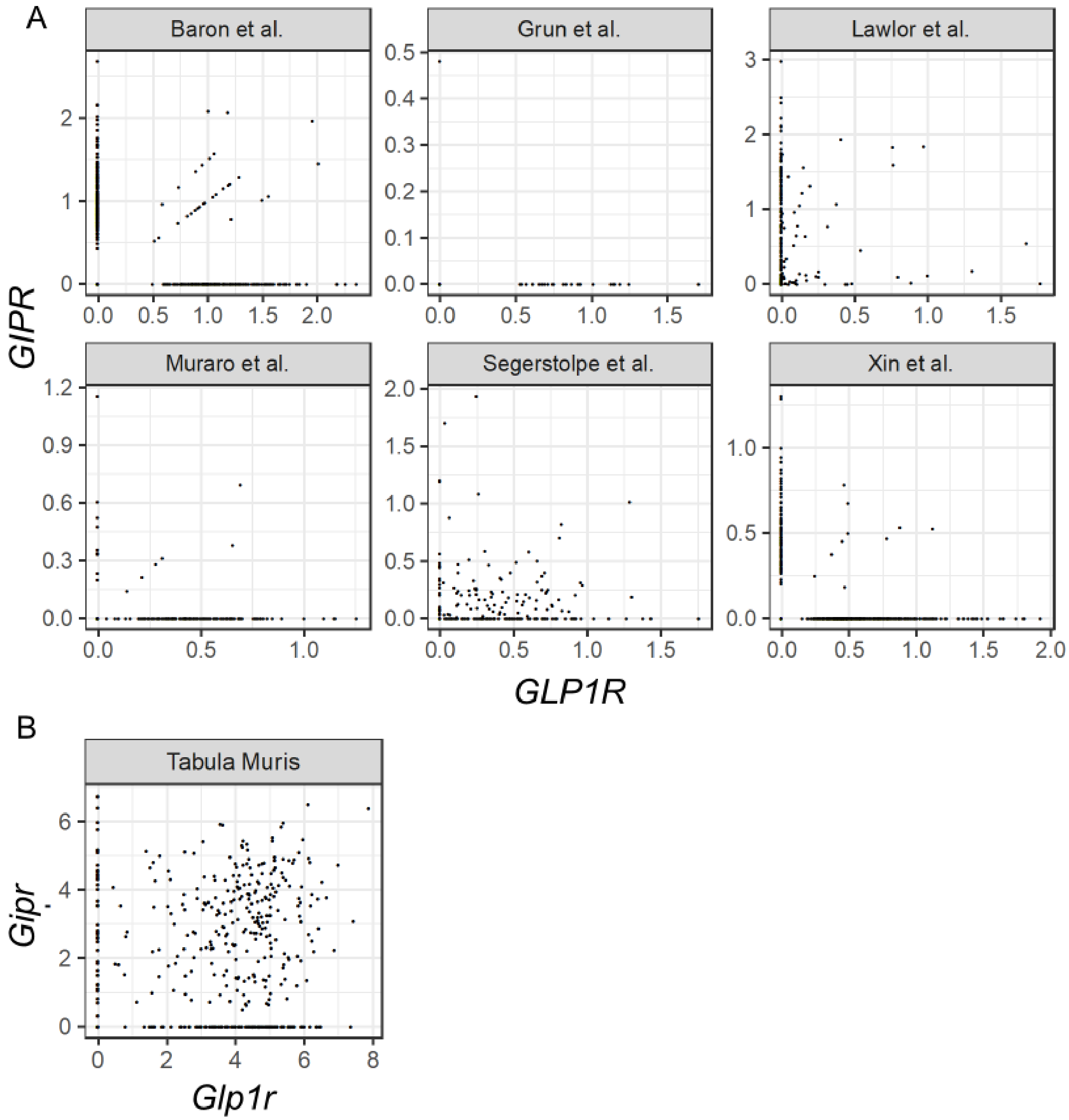


Figure 1. Heterogeneous expression of *GLP1R/Glp1r* and *GIPR/Gipr* in β-cells from published scRNAseq data sets in human (A) and mouse (B). Each circle represents an individual β-cell.

Figure 2

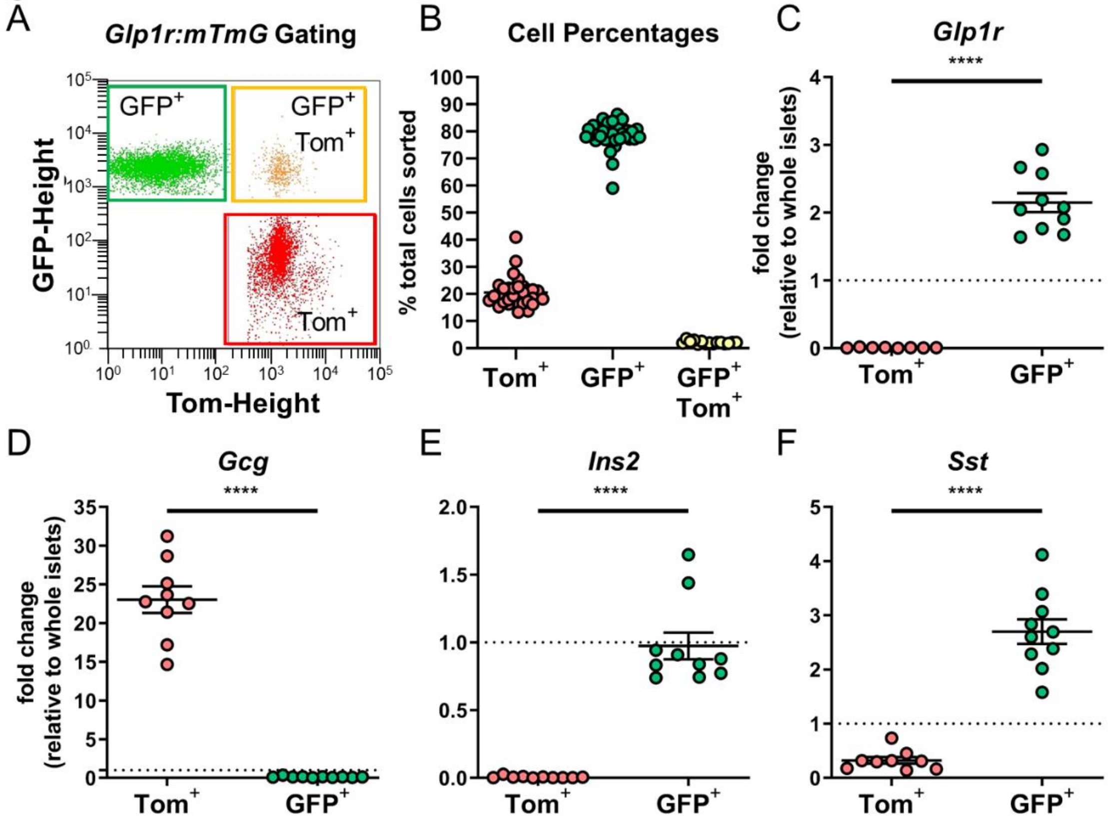


Figure 2. *Glp1r* promoter activity is enriched in β-and δ-cells. A) Gating strategy to separate tdTomato+ (Tom+) and GFP+ islet cells from *Glp1r:mTmG* mice by FACS. B) Quantification of Tom+ (red circles), GFP+ (green circles) and GFP+/Tom+ (yellow circles) cells from individual mice. C-F) qPCR in Tom+ and GFP+ cells for expression of C) *Glp1r*, D) *Gcg*, E) *Ins2*, F) *Sst*. All qPCR data are normalized to gene expression in whole islet lysates. Panels C- F: Comparisons between Tom+ and GFP+ cells were compared by paired *t*-test, *****p*<0.0001. Each circle represents an individual mouse and data are presented as mean ± SEM.

Figure 3

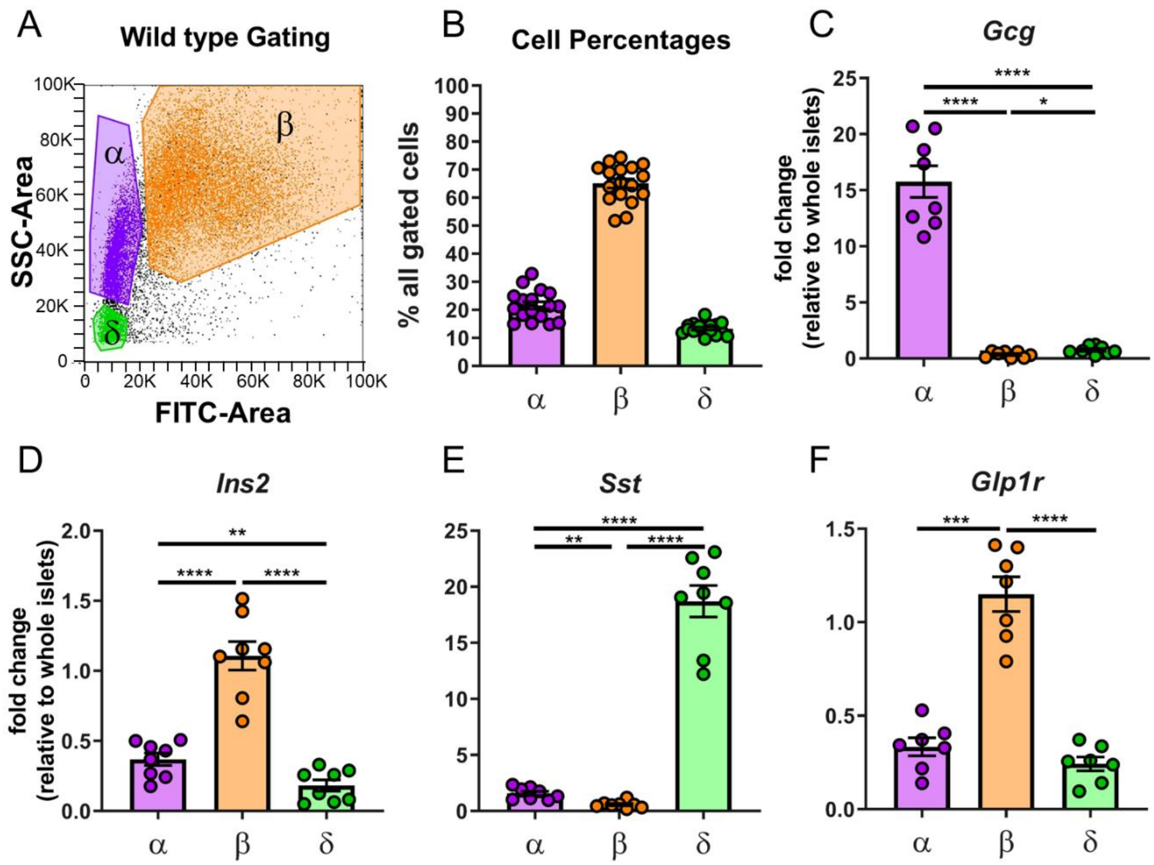
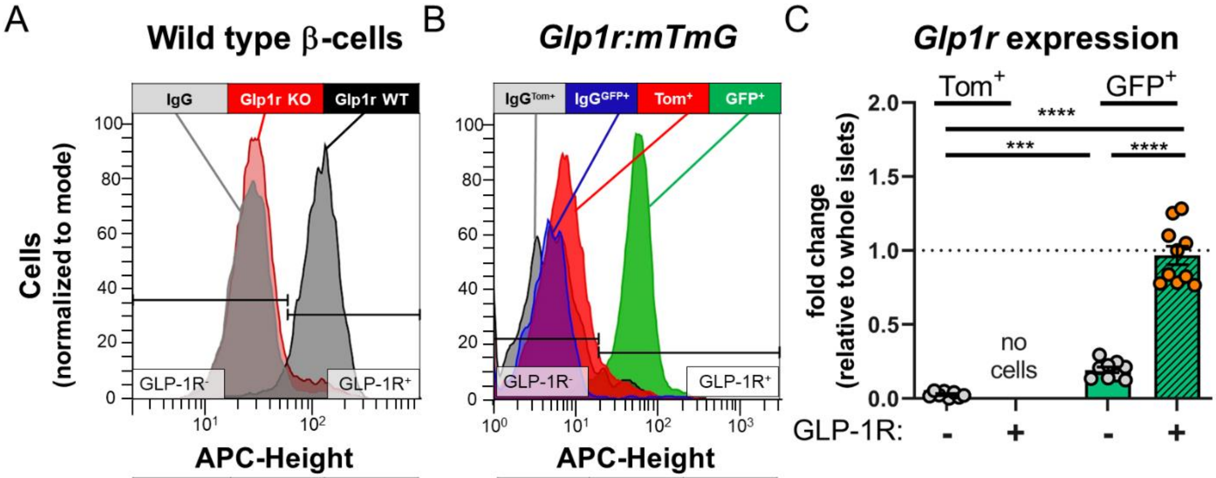


Figure 3. *Glp1r* is highly expressed in enriched populations of β-cells from wild-type mouse islets. A) Gating strategy to separate enriched populations of α-cells (purple), β-cells (orange), and δ-cells (green) from wild type islets by autofluorescence (FITC-Area) and side scatter (SSC-Area). B) Quantification of cells separated by this method. Enriched populations were verified for enrichment of C) *Gcg* in α -cells, D) *Ins2* in β-cells, E) *Sst in* δ-cells. F) *Glp1r* expression in enriched populations. All qPCR data are normalized to gene expression in whole islet lysates. Panel C-F: Populations were compared by 1-way ANOVA with Tukey's multiple comparisons test for pairwise comparisons between α-, β-, δ-cells. Significant differences are denoted by *$p\leq0.05$ **$p<0.01$, ****$p<0.0001$. Each circle represents an individual mouse and data are presented as mean ± SEM.

Figure 4



Pop.	GLP-1R-	GLP-1R+
Glp1r WT	6.4	93.6
Glp1r KO	90.2	9.8
IgG	92.2	7.8

Pop.	GLP-1R-	GLP-1R+
GFP+	6.4	93.6
Tom+	97.5	2.5
IgGGFP+	96	4
IgGTom+	90.5	9.5

Figure 4. GLP1R-APC is specific for GLP-1R protein detection in islet cells. A) Representative histogram of β-cells from Glp1r WT (black, *Glp1r*$^{\beta cell+/+}$, n=1) and Glp1r KO (red, *Glp1r*$^{\beta cell-/-}$, n=3) mice stained with GLP1R-APC; IgG-APC staining control is shown in gray and population statistics below. B) Representative histogram of islet cells from *Glp1r:mTmG* mice (n=10) stained with GLP1R-APC in Tom$^+$ (red) and GFP$^+$ (green) populations. IgG-APC^{Tom+} and IgG-APC^{GFP+} staining controls are shown in gray and blue, respectively with population statistics below. C) *Glp1r* expression in Tom$^+$/GLP1R-APC$^-$, GFP$^+$/GLP1R-APC$^-$, GFP$^+$/GLP1R-APC$^+$ populations from *Glp1r:mTmG* mice incubated with GLP1R-APC. A, B) Histograms are normalized to mode. C) *Glp1r* expression is normalized to whole islet lysates. Populations were compared by mixed effects analysis and Tukey's multiple comparisons test was used for pairwise comparisons. Significant differences are denoted by ***p<0.001, ****p<0.0001. Each circle represents an individual mouse, and data are presented as mean ± SEM.

Figure 5

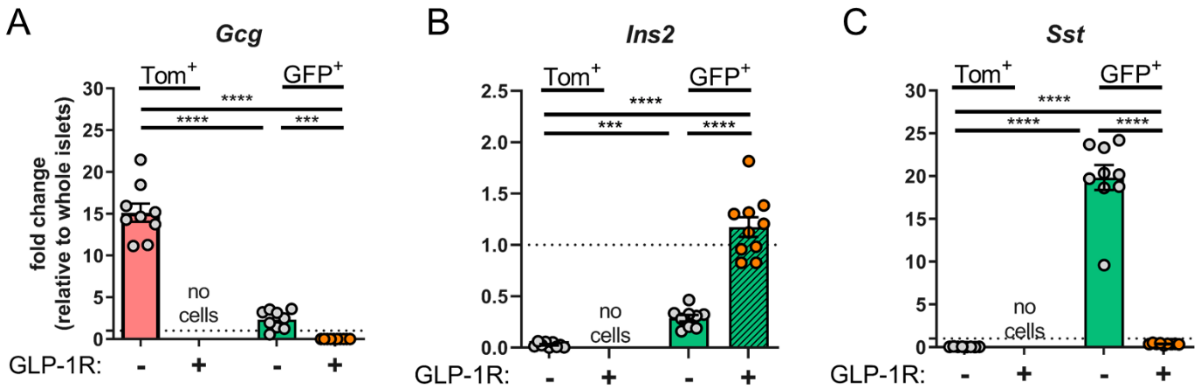
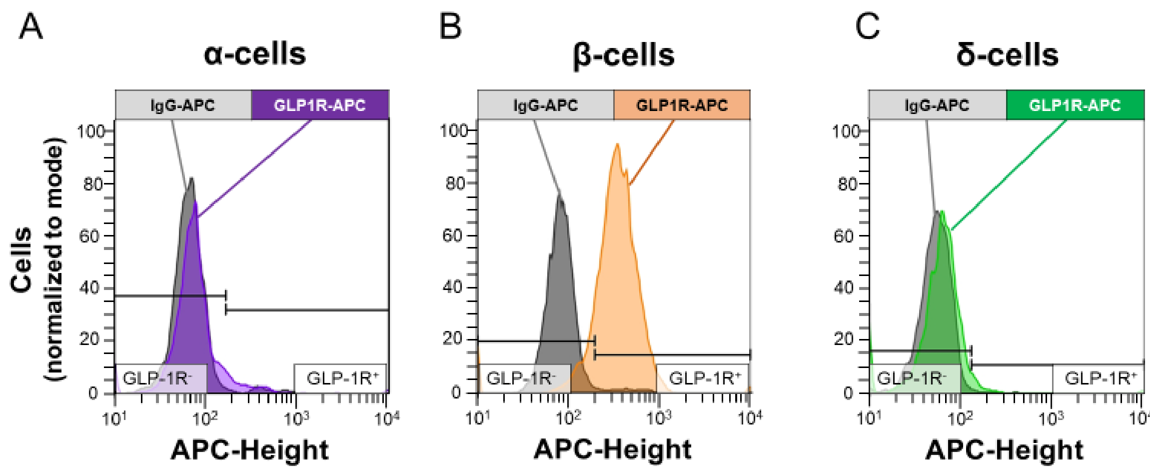


Figure 5. qPCR of FACS-sorted populations from *Glp1r:mTmG* mice incubated with GLP1R-APC. Sorted populations from Tom$^+$/GLP1R-APC$^-$ (red bar/gray circles), GFP$^+$/GLP1R-APC$^-$ (green bar/gray circles), and GFP$^+$/GLP1R-APC$^+$ (green bar/orange circles) were analyzed for expression of A) *Gcg*, B) *Ins2*, and C) *Sst*. Tom$^+$/GLP1R-APC$^+$ cells were not acquired. All qPCR data are normalized to gene expression in whole islet lysates. Populations were compared by mixed effects analysis and Tukey's multiple comparisons test was used for pairwise comparisons. Significant differences are denoted by ***p<0.001, ****p<0.0001. Each circle represents an individual mouse and data are presented as mean ± SEM.

Figure 6



α-cells	GLP-1R$^-$ (SEM)	GLP-1R$^+$ (SEM)
GLP1R	83.2 (4.5)	16.8 (4.5)
IgG	92.9 (1.0)	7.1 (1.0)

β-cells	GLP-1R$^-$ (SEM)	GLP-1R$^+$ (SEM)
GLP1R	10.3 (1.8)	89.7**** (1.8)
IgG	89.6 (1.4)	10.4 (1.4)

δ-cells	GLP-1R$^-$ (SEM)	GLP-1R$^+$ (SEM)
GLP1R	92.7 (1.7)	7.4* (1.7)
IgG	91.7 (0.6)	2.9 (0.6)

Figure 6. GLP-1R staining is robust in enriched β-cells by flow cytometry. A) GLP1R-APC staining in α-cells (purple) did not reach statistical significance (*p*=0.07). B) β-cells had robust GLP1R-APC staining (orange). C) δ-cells had a slightly greater GLP1R-APC staining (green) than IgG-APC control. Representative histograms of staining in α-, β-, and δ-cells are shown with IgG-APC (gray) displayed for each cell type. Population statistics are reported below histograms as a percentage of enriched cells (SEM) . N=7 experiments. ****p<0.0001; *p<0.05, paired t-test.

Figure 7

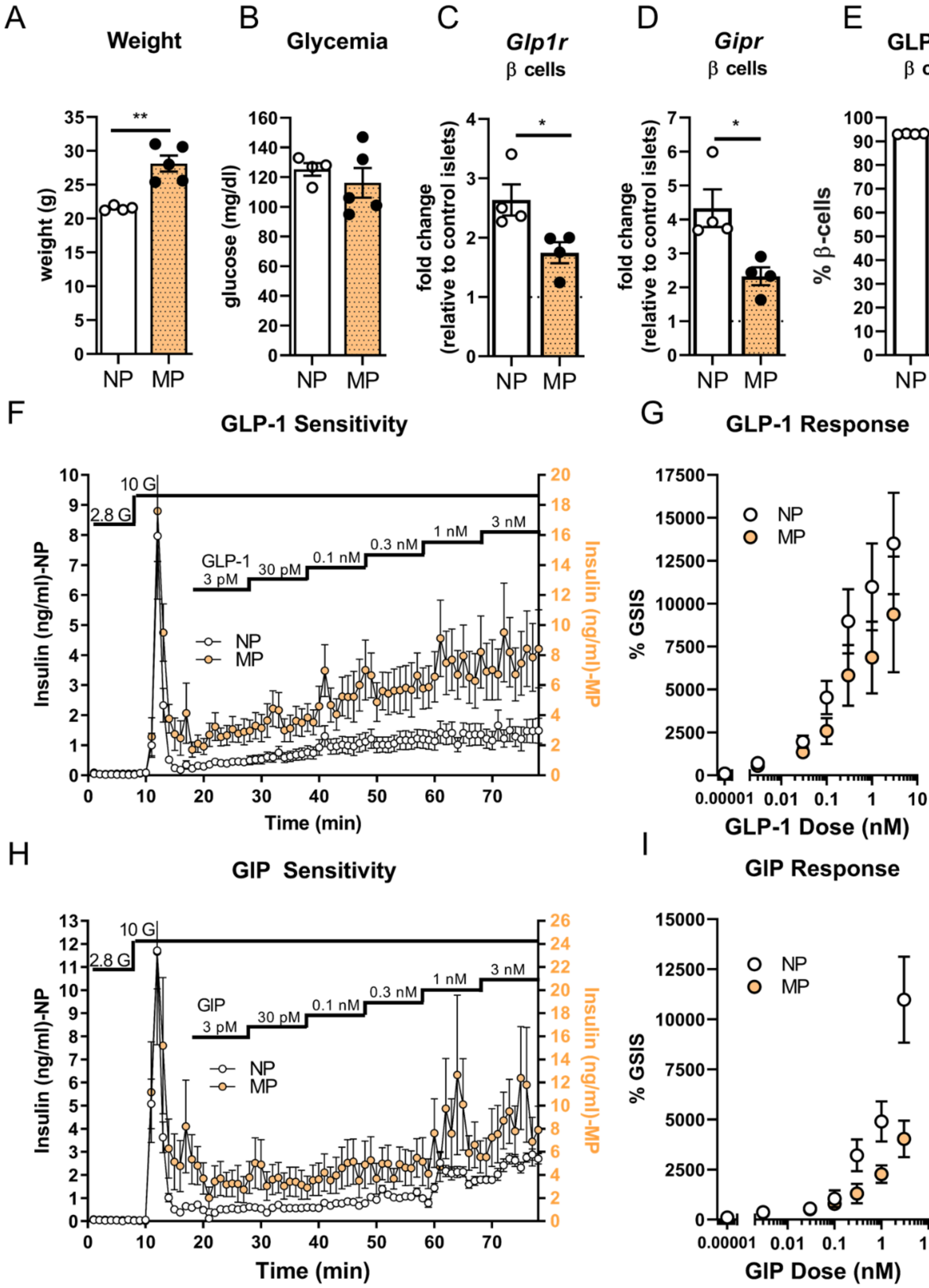


Figure 7. GLP-1R and GIPR expression and function in metabolically-stressed, multiparious female mice. A) Weight and B) blood glucose at sacrifice in nulliparous (NP, white) or multiparous (MP, yellow) mice. C) *Glp1r*, D) *Gipr* expression, and E) GLP1R-APC staining in enriched β-cells from NP and MP mice. F) Insulin secretion from NP and MP islets perifused with increasing concentrations of GLP-1 and G) GLP-1 response normalized to glucose-stimulated insulin secretion (GSIS, N=9 per group). H) Insulin secretion

from NP and MP islets perifused with increasing concentrations of GIP and G) GIP response normalized to GSIS (N=6 per group). *$p<0.05$, *t*-test.

Discordance between GLP-1R gene and protein expression in mouse pancreatic islet cells

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