



miR-204 Controls Glucagon-Like Peptide 1 Receptor Expression and Agonist Function

SeongHo Jo, Junqin Chen, Guanlan Xu, Truman B. Grayson, Lance A. Thielen, and Anath Shalev

Diabetes 2018;67:256–264 | <https://doi.org/10.2337/db17-0506>

Glucagon-like peptide 1 receptor (GLP1R) agonists are widely used to treat diabetes. However, their function is dependent on adequate GLP1R expression, which is downregulated in diabetes. GLP1R is highly expressed on pancreatic β -cells, and activation by endogenous incretin or GLP1R agonists increases cAMP generation, which stimulates glucose-induced β -cell insulin secretion and helps maintain glucose homeostasis. We now have discovered that the highly β -cell-enriched microRNA, miR-204, directly targets the 3' UTR of GLP1R and thereby downregulates its expression in the β -cell-derived rat INS-1 cell line and primary mouse and human islets. Furthermore, *in vivo* deletion of miR-204 promoted islet GLP1R expression and enhanced responsiveness to GLP1R agonists, resulting in improved glucose tolerance, cAMP production, and insulin secretion as well as protection against diabetes. Since we recently identified thioredoxin-interacting protein (TXNIP) as an upstream regulator of miR-204, we also assessed whether *in vivo* deletion of TXNIP could mimic that of miR-204. Indeed, it also enhanced islet GLP1R expression and GLP1R agonist-induced insulin secretion and glucose tolerance. Thus, the present studies show for the first time that GLP1R is under the control of a microRNA, miR-204, and uncover a previously unappreciated link between TXNIP and incretin action.

Small, noncoding RNAs or microRNAs (miRNAs) have emerged as powerful regulators of gene expression, including that of pancreatic β -cells (1–3). Especially miR-204 has been shown to be highly enriched in β -cells as opposed to α -cells (2,4–6), suggesting that it may play a particularly important role in β -cell biology. Indeed, we recently found that miR-204 targets the insulin transcription factor MafA and thereby downregulates insulin production (4). Moreover, we found

that miR-204 also targets PERK and thereby regulates the unfolded protein response and β -cell apoptosis (7). However, considering the fact that miRNAs can, by imperfect base pairing of their seed sequence, bind to the 3' UTR of a multitude of mRNAs, leading to destabilization or translational inhibition of these target genes (8), we searched for additional putative genes that might be regulated by miR-204.

Intriguingly, using miRWalk as well as microrna.org target prediction software, we identified the glucagon-like peptide 1 receptor (GLP1R) as a potential target of miR-204. Conversely, when using these software programs to predict putative miRNAs that may target the GLP1R 3' UTR, miR-204 again emerged as the most promising miRNA (all other predicted miRNAs were either not expressed in β -cells or had much shorter seed lengths, making actual targeting unlikely). The GLP1R is a G protein-coupled seven-transmembrane receptor highly expressed on pancreatic β -cells that plays a critical role in conferring the effects of GLP-1, the major incretin produced in intestinal L cells and pancreatic islet α -cells (9,10). GLP-1 is secreted in response to food intake, binds to the GLP1R, and leads to increased generation of cAMP, which stimulates glucose-induced β -cell insulin secretion and helps maintain glucose homeostasis (11). However, GLP1R not only mediates the effects of endogenously produced GLP-1, it is also responsible for the action of a large class of incretin mimetic diabetes drugs widely used in the treatment of type 2 diabetes (12), including injectable GLP-1 mimetics and analogs (e.g., exenatide and liraglutide) and oral dipeptidyl peptidase-4 (DPP-4) inhibitors that block GLP-1 degradation (e.g., sitagliptin and linagliptin).

β -Cell expression of GLP1R has been reported to be downregulated in type 2 diabetes (13), in response to glucose (14,15)

Comprehensive Diabetes Center and Department of Medicine, Division of Endocrinology, Diabetes, and Metabolism, University of Alabama at Birmingham, Birmingham, AL

Corresponding author: Anath Shalev, shalev@uab.edu.

Received 28 April 2017 and accepted 30 October 2017.

This article contains Supplementary Data online at <http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db17-0506/-/DC1>.

© 2017 by the American Diabetes Association. Readers may use this article as long as the work is properly cited, the use is educational and not for profit, and the work is not altered. More information is available at <http://www.diabetesjournals.org/content/license>.

and by its own ligand, GLP-1 (16), whereas upregulation by metformin (17,18) and 5-amino-4-imidazolecarboxamide riboside (AICAR) (18) and stabilization by *N*-glycosylation (19) has been suggested. However, the factors regulating GLP1R expression are still not fully understood. Taken together with the important role that GLP1R plays in β -cell biology, glucose homeostasis, and diabetes treatment, this makes the possibility that β -cell GLP1R expression might be under the control of an miRNA even more intriguing. The present studies were therefore aimed at investigating this possibility.

RESEARCH DESIGN AND METHODS

Tissue Culture

HEK293 cells and native INS-1 insulinoma cells were grown as previously described (20,21). Mouse pancreatic islets were isolated by collagenase digestion as detailed previously (22). Human islets were obtained from the Integrated Islet Distribution Program, and islets from the same donor were always used as control, and islets from at least three different donors were used per experiment.

Animal Studies

All mouse studies were approved by the University of Alabama at Birmingham (UAB) Animal Care and Use Committee and conform to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. The miR-204 knockout mouse (204KO) was generated by the UAB Transgenic & Genetically Engineered Models (TGEMs) facility using blastocyst injection of C57BL/6 embryonic stem cells with deletion of miR-204 (N01299P1_C_239W_B9/H11; NorCOMM, Saskatoon, Saskatchewan, Canada). The β -cell-specific thioredoxin-interacting protein (TXNIP) knockout mice (bTKO) and their controls have been described previously (23). Diabetes was induced in control and 204KO mice by multiple low-dose streptozotocin (STZ) injections (40 mg/kg body weight, freshly prepared in 0.1 mmol/L sodium citrate at pH 4.5) and intraperitoneally administered daily for five consecutive days as described previously (23). GLP1R signaling was inhibited using daily intraperitoneal injections of the GLP1R antagonist exendin(9–39) (Ex-9; 0.6 mg/kg body weight) (Bachem, Torrance, CA) as described previously (24). For glucose tolerance tests, mice received glucose (1.5 g/kg) by intraperitoneal injection after a 16-h fast (9). To test the effect of Ex-4 on insulin secretion and glucose metabolism, Ex-4 (10 nmol/kg) (CHI Scientific, Maynard, MA) or vehicle (PBS) was administered via intraperitoneal injection 30 min prior to the glucose injection, as described previously (9). Blood glucose was measured at –30, 0, 15, 30, 60, 90, and 120 min. The glucose area under the curve (AUC) was calculated from 0 to 120 min using the trapezoidal rule. Serum samples harvested at –30 and 10 min were used for insulin measurement with the Mouse Ultrasensitive Insulin ELISA kit (ALPCO, Salem, NH). For ex vivo islet insulin secretion in response to Ex-4, isolated islets (three per tube, four tubes per mouse and condition) were incubated in 16.7 mmol/L glucose Krebs-Ringer buffer with or without 100 nmol/L

Ex-4 at 37°C for 45 min, and the supernatant was used for insulin measurements by ELISA.

cAMP Measurements

After overnight recovery at 37°C in RPMI containing 5 mmol/L glucose, isolated mouse islets (20 per tube) were transferred into Krebs-Ringer buffer containing 1.67 mmol/L glucose for 1 h followed by 15 min at 16.7 mmol/L glucose with or without 10 nmol/L Ex-4. To terminate the reaction, cold ethanol was added to the tubes at a final concentration of 65%. Intracellular cAMP levels were measured using the cAMP Direct Immunoassay Kit (Abcam, Cambridge, MA) and according to the manufacturer's instructions.

Plasmid Construction, Transfection, and Luciferase Assays

The wild-type (WT) human and rat GLP1R 3' UTR regions containing miR-204 binding sites were amplified from genomic DNA by using the primers listed in Supplementary Table 1. To generate the GLP1R 3' UTRs with mutated miR-204 binding site, mutations were introduced by PCR and primers listed in Supplementary Table 1. PCR products were subcloned into the *Spe*I and *Pme*I sites of the pMIR-REPORT luciferase vector (Thermo Fisher Scientific), yielding WT GLP1R (GLP1R-WT) and mutant GLP1R-M 3' UTR luciferase reporter plasmids. All plasmids were confirmed by sequencing. For luciferase assays, HEK293 cells were plated in 12-well plates and grown overnight to ~60% confluency. Cells were transfected with GLP1R-WT or GLP1R-M together with hsa-miR-204 mirVana miRNA mimic or negative control oligos (Thermo Fisher Scientific) using DharmaFECT Duo transfection reagent (GE Dharmacon, Lafayette, CO). To control for transfection efficiency, cells were cotransfected with pRL-TK (Promega, Fitchburg, WI) control plasmid expressing renilla luciferase, and 48 h after transfection, firefly as well as renilla luciferase activity was determined using the Dual Luciferase Assay Kit (Promega). To overexpress or knock down miR-204, INS-1 cells were plated in six-well plates and grown overnight to ~60% confluency. Human islets or mouse islets were gently dispersed by incubation for 5–8 min in 200 μ L of 0.05% trypsin-EDTA (Thermo Fisher Scientific) at 37°C, washed, and resuspended in culture medium as described previously (4). Cells were transfected with hsa-miR-204-5p mirVana miRNA mimic (Thermo Fisher Scientific) or miRIDIAN miRNA has-miR-204-5p inhibitor (GE Dharmacon) or corresponding control oligos at a final concentration of 25 nmol/L using the DharmaFECT1 transfection reagent (GE Dharmacon).

Quantitative Real-time RT-PCR

RNA isolation and quantitative real-time RT-PCR (qRT-PCR) were performed as described previously (4,23) using primers listed in Supplementary Table 1, and gene and miRNA expression results were corrected for 18S and U6 run as internal standards, respectively.

Immunoblotting

Protein extracts were prepared as described previously (22). Protein concentrations were measured by Pierce BCA Protein Assay (Thermo Fisher Scientific), and equal amounts of

protein were loaded. The following antibodies were used: rabbit anti-GLP1R IgG (sc-66911; Santa Cruz Biotechnology, Dallas, TX), mouse anti-actin IgG (ab3280; Abcam), goat anti-rabbit IgG (sc-2004; Santa Cruz Biotechnology), and goat anti-mouse IgG (sc-2005; Santa Cruz Biotechnology), and bands were visualized by ECL Plus (GE Healthcare, Little Chalfont, U.K.) and quantified by ImageQuant TL software (GE Healthcare).

Statistical Analysis

Student *t* tests were used to calculate the significance of a difference between two groups. For data sets of more than two groups, we performed one-way ANOVA calculations.

RESULTS

miR-204 Targets the GLP1R 3' UTR

To address the question of whether GLP1R may indeed be targeted by miR-204, we performed a detailed analysis of the 3' UTR of the GLP1R, which revealed two potential miR-204 binding sites in humans (Fig. 1A) and one in rodents (Fig. 1B). We therefore generated a WT human GLP1R 3' UTR luciferase reporter construct as well as three additional mutant constructs disrupting the first, second, or both potential miR-204 seed sequence binding sites. Cotransfection experiments revealed that miR-204 significantly decreased luciferase levels through the WT GLP1R 3'

UTR, and mutations of the first or second site each reduced this effect and the double mutation completely blunted it (Fig. 1C). Similar results were also obtained with the rat GLP1R 3' UTR (Fig. 1D) and mouse GLP1R 3' UTR, confirming that GLP1R is truly a direct target of miR-204 and contains conserved miR-204 seed sequence binding sites (Supplementary Fig. 1).

miR-204 Regulates GLP1R Expression in Rat INS-1 Cells and Primary Mouse and Human Islets

Next, we analyzed the effects of miR-204 on endogenous GLP1R in primary human and mouse islets and in rat INS-1 cells. Consistent with the results of our 3' UTR luciferase assays, we found that miR-204 overexpression led to a decrease in GLP1R mRNA (Fig. 2A–C) and protein expression (Fig. 2D and Supplementary Fig. 2), whereas knockdown of miR-204 with anti-miR-204 resulted in a significant increase in GLP1R expression (Fig. 2E). These results demonstrate that miR-204 indeed regulates endogenous GLP1R and that this effect is not restricted to a cell line or a particular species.

Deletion of miR-204 Enhances Islet GLP1R Expression and Function and Promotes Insulin Secretion and Glucose Control In Vivo in Mice

To study the in vivo role of miR-204, we used 204KO. Mice appeared healthy overall and had normal body weight,

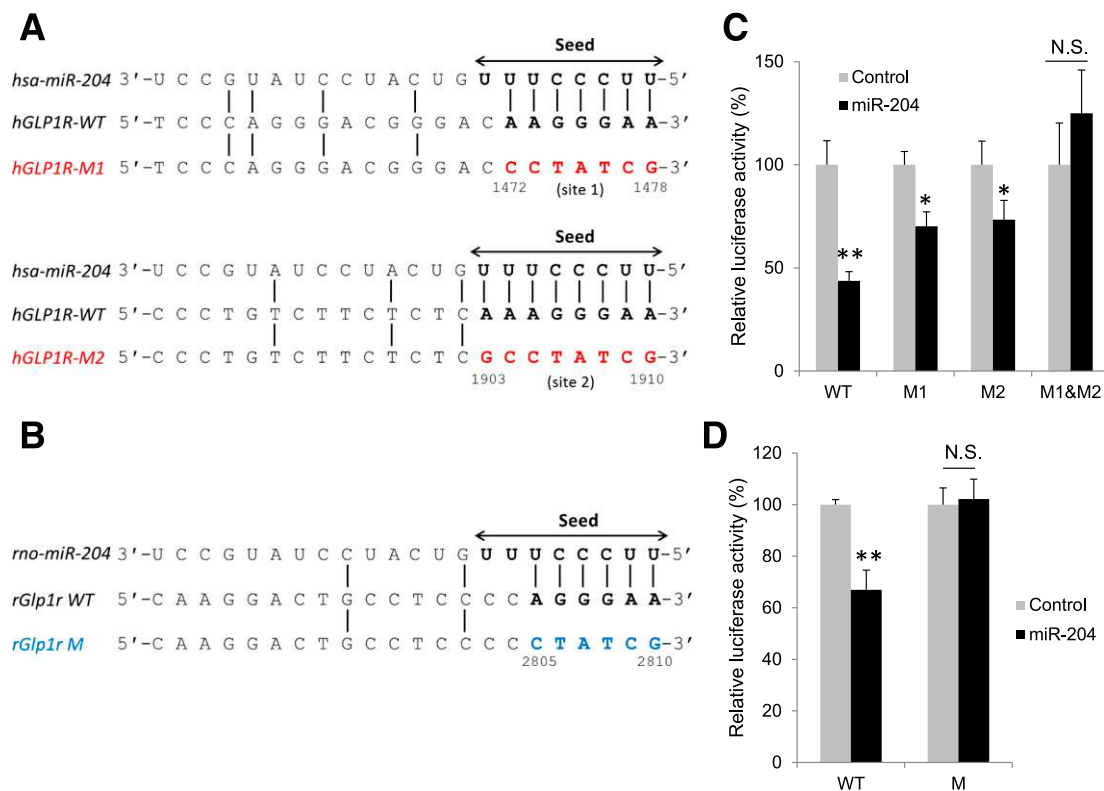


Figure 1—miR-204 targets the GLP1R 3' UTR. Alignment of the miR-204 seed sequence (arrow) with target sequences (bold) identified in the WT GLP1R 3' UTR of human (*hGLP1R-WT*, site 1 and site 2) (A) or rat (*rGlp1r WT*) (B); mutated (M) target sequences are shown in red or blue, respectively. HEK293 cells were cotransfected with WT or mutant 3' UTR luciferase reporter plasmids and with miR-204 mimic or negative control, and miR-204-directed repression of the luciferase reporter gene bearing human (C) or rat (D) WT or mutant 3' UTR segments was assessed 48 h after transfection. Bars represent means \pm SEM of at least three independent experiments. **P* < 0.05; ***P* < 0.01.

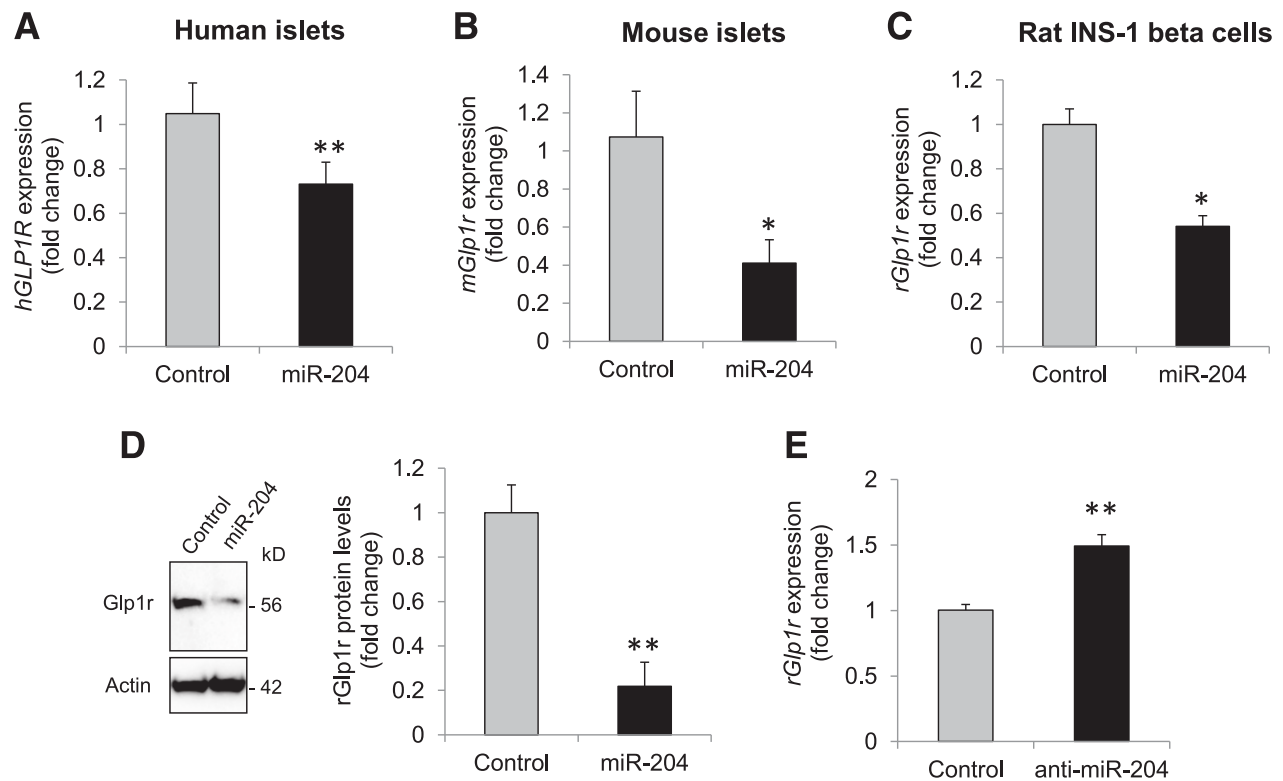


Figure 2—miR-204 regulates GLP1R expression in primary human and mouse islets and in rat INS-1 cells. Primary human islets (A), mouse islets (B), and rat INS-1 cells (C) were transfected with miR-204 mimic or negative control, and 72 h later, GLP1R mRNA expression was assessed by qRT-PCR. D: GLP1R protein was measured by immunoblotting and quantified by ImageQuant in INS-1 cells 72 h after transfection with miR-204 mimic or negative control. E: INS-1 cells were transfected with miR-204 inhibitor or inhibitor negative control, and GLP1R mRNA expression was assessed 48 h later. All data are shown as the mean \pm SEM of at least three independent experiments. * $P < 0.05$; ** $P < 0.01$.

blood glucose, and serum insulin levels as well as normal insulin sensitivity (Supplementary Fig. 3). Analysis of their islets confirmed effective knockout of miR-204 (Fig. 3A). Interestingly, islets of 204KO mice also had clearly higher expression levels of GLP1R (Fig. 3B) compared with control WT littermates, consistent with our in vitro findings (Fig. 2). Moreover, glucose tolerance tests revealed that whereas under basal conditions blood glucose levels were not significantly different in 204KO mice as compared with controls (Fig. 3C), 204KO mice were more responsive to the effects of the GLP1R agonist exendin-4 (Ex-4) and exhibited lower blood glucose levels (Fig. 3C) and glucose AUC in response to Ex-4 (Fig. 3D). In addition, analysis of serum insulin levels demonstrated that Ex-4-induced insulin secretion was significantly increased in 204KO mice (Fig. 3E), again consistent with the higher expression level of GLP1R observed in the islets of these mice. Similarly, Ex-4-induced insulin secretion was also significantly increased ex vivo in isolated 204KO islets as compared with control islets (Fig. 3F), whereas insulin content remained unchanged (Supplementary Fig. 4A). Since GLP-1 signals via cAMP (25), we also assessed Ex-4-induced cAMP production and found that 204KO islets produced significantly more cAMP in response to Ex-4 as compared with control islets (Fig. 3F), further demonstrating that the increase in GLP1R expression in response to miR-204 deletion translates into increased

function and an increase in islet GLP1R signaling. Of note, similar findings have been observed in GLP1R transgenic mice (9). Since GLP-1 has been shown to inhibit α -cell glucagon secretion (26), we also assessed whether miR-204 deletion had any influence on this effect. However, while Ex-4 led to a dramatic decrease in serum glucagon as expected, it did so in both 204KO and control mice, and no difference was observed between the groups (Supplementary Fig. 4B). Similarly, miR-204 deletion did not affect ex vivo islet glucagon secretion from isolated islets (Supplementary Fig. 4C). The fact that miR-204 knockout had no effect on α -cell glucagon secretion is in alignment with the pre-existing low/absent expression of miR-204 in WT α -cells (2,4–6). Also, based on these results, it is very unlikely that any of the observed effects of miR-204 deletion on glucose homeostasis were due to altered glucagon levels.

204KO Mice Exhibit Improved Glucose Control and Increased Serum Insulin Levels in the Context of Diabetes

Increased GLP1R signaling has been shown to improve glucose homeostasis in various mouse models of diabetes, including diabetes induced by multiple low-dose STZ injections (27). We therefore hypothesized that miR-204 deletion and the associated increase in GLP1R might also have beneficial effects in the context of STZ-induced diabetes. Indeed, we found that 204KO mice were protected against

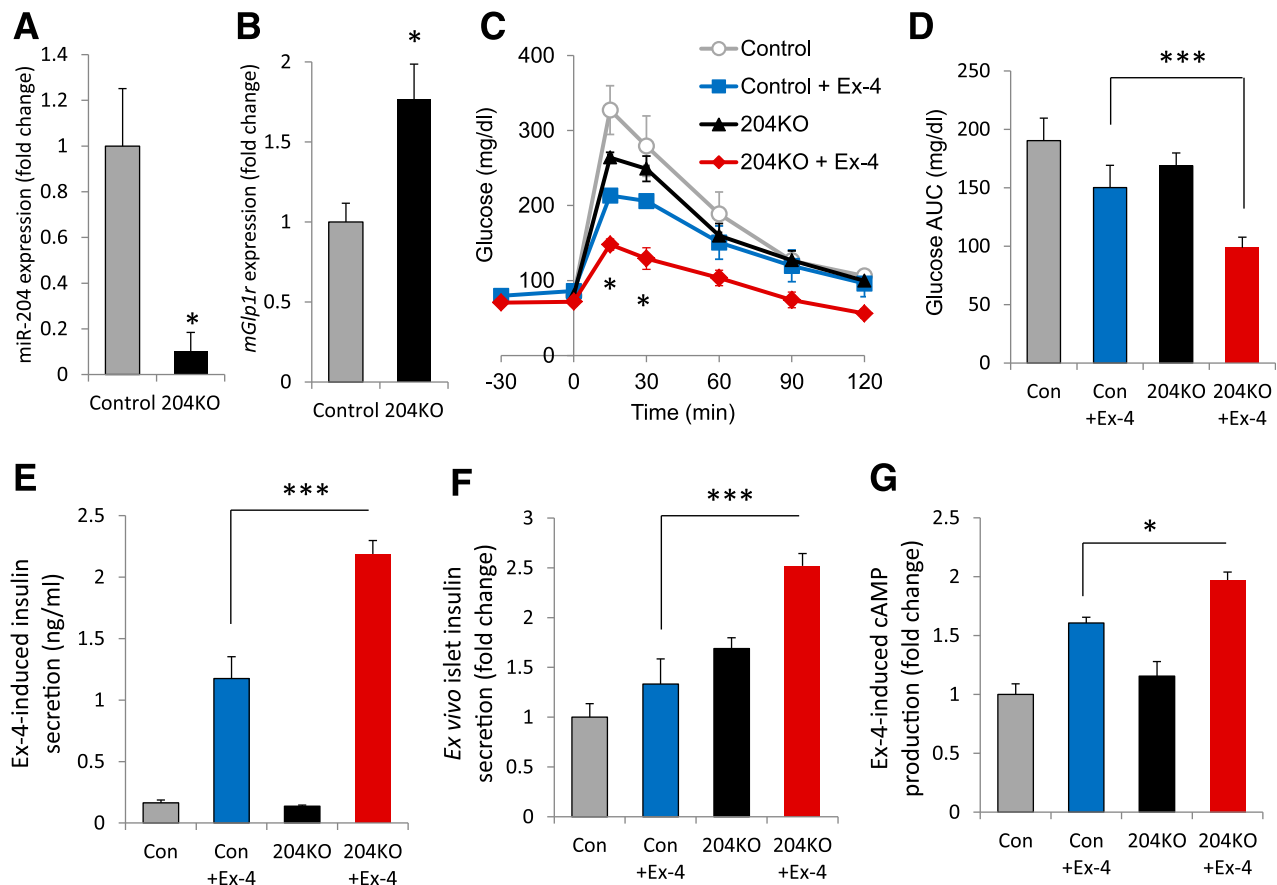


Figure 3—Deletion of miR-204 enhances islet GLP1R expression and promotes Ex-4-induced insulin secretion and glucose control in vivo in mice. miR-204 (A) and GLP1R (B) expression in islets of 204KO mice and control littermates as assessed by qRT-PCR and corrected for U6 and 18S, respectively. C: Glucose tolerance tests with or without injection of Ex-4 (10 nmol/kg i.p. at –30 min) using age- and sex-matched 204KO or control mice. Glucose (1.5 g/kg i.p.) was injected at 0 min, and blood glucose was measured at the designated time points; glucose AUC (D) and Ex-4-induced changes in serum insulin (E). F: Insulin secretion from isolated islets of 204KO or control mice in response to Ex-4. G: Intracellular cAMP production in response to Ex-4 was assessed in isolated islets of 204KO or control mice. All data are shown as the mean \pm SEM; $n = 3$ –6 mice per group. * $P < 0.05$; *** $P < 0.001$.

the hyperglycemia observed in control mice and maintained significantly lower blood glucose (Fig. 4A) and increased insulin levels (Fig. 4B). Moreover, treatment of 204KO mice with the GLP1R antagonist Ex-9 blunted this protective effect and mice developed STZ-induced diabetes similarly to control mice (Fig. 4A), suggesting that the beneficial effects of miR-204 deletion were primarily mediated by GLP1R signaling. Of note, we have previously shown that downregulation of TXNIP, the upstream regulator of miR-204, also improves glucose homeostasis in the context of this and other diabetes models (23).

miR-204 Expression Is Low or Absent in Stomach or Exocrine Pancreas and GLP1R Expression Remains Unaltered in Response to miR-204 Deletion in These Tissues

GLP1R is highly expressed on pancreatic β -cells but also in the exocrine pancreas, stomach, small intestine, and brain (28,29), and inhibition of gastric and small intestinal motor function may even contribute to the lowering of postprandial glycemia in response to GLP-1 agonists (30). However, it may also contribute to some of the dose-dependent side

effects of GLP1R agonists, including delayed gastric emptying and nausea (30,31), as well as to the potentially elevated risk for pancreatitis and pancreatic cancer, an issue that has remained controversial (32–34). In contrast, whereas miR-204 is also highly expressed in pancreatic β -cells (2,4,6), we found no or very low expression in the exocrine pancreas and stomach (Fig. 5A). Consistent with these findings and in contrast to the observed increase in islets (Fig. 3B), GLP1R expression was unaltered in the stomach and exocrine pancreas of global 204KO mice as compared with controls (Fig. 5B and C).

β -Cell-Specific TXNIP Knockout Mice Have Lower miR-204 and Higher GLP1R Expression and Exhibit Enhanced Insulin Secretion and Glucose Control in Response to Ex-4

We initially identified miR-204 as one of the top miRNAs induced by TXNIP (4). Although TXNIP has been known as a ubiquitously expressed cellular redox regulator (35,36), we previously reported that TXNIP is upregulated by glucose and diabetes and plays a major role in the control of pancreatic β -cell mass and that TXNIP deletion or inhibition

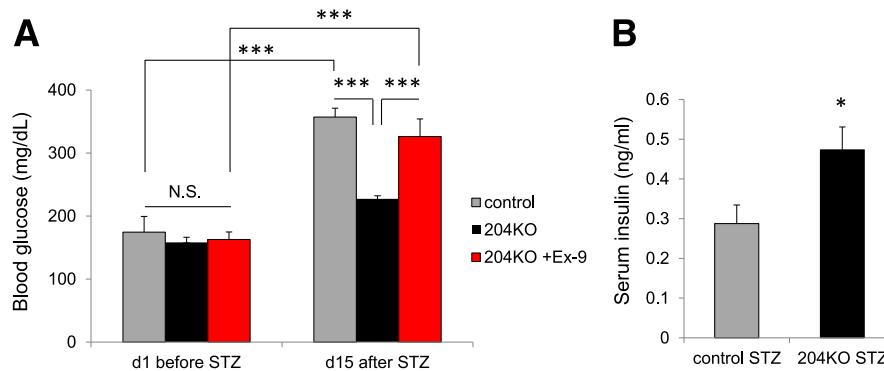


Figure 4—204KO mice exhibit improved glucose control and increased serum insulin levels in the context of diabetes. To induce diabetes, male 204KO and control mice received multiple low-dose STZ injections with or without the GLP1R antagonist Ex-9. **A**: The blood glucose on day 1 prior to STZ and on day 15 after the start of the STZ injections is shown. **B**: Comparison of serum insulin levels on day 15. Bars represent means \pm SEM; $n = 3$ –6 mice per group. * $P < 0.05$; *** $P \leq 0.001$.

protects against type 1 and type 2 diabetes (21–23,37–39). More recently, we also showed that TXNIP upregulates miR-204, whereas miR-204 expression is decreased in response to TXNIP deletion (4). Given this role of TXNIP as an upstream regulator of miR-204, we next investigated whether TXNIP deletion could mimic the effects of miR-204 deletion. Taking advantage of our bTKO mice (23), we assessed whether TXNIP deletion could upregulate GLP1R expression. We found that miR-204 expression was significantly downregulated in bTKO islets (Fig. 6A), and this was associated with a clear increase in GLP1R expression (Fig. 6B). Again glucose tolerance tests showed no difference between the different mouse lines at baseline but revealed significantly lower blood glucose levels in response to Ex-4 in the bTKO mice as compared with control mice (Fig. 6C). Consistent with these findings, the glucose AUC was also significantly lower in bTKO mice as compared with controls (Fig. 6D), whereas Ex-4-induced insulin secretion was upregulated (Fig. 6E). Moreover, consistent with the findings in 204KO islets, Ex-4-induced cAMP production was also increased in bTKO islets as compared with control islets (Fig. 6F). These results suggest that inhibition of

miR-204 or of its upstream regulator TXNIP can modulate islet GLP1R expression and function and with that the responsiveness to Ex-4 administration.

DISCUSSION

Taken together, our work provides the first demonstration that GLP1R is under the control of an miRNA. Moreover, our findings in rat INS-1 cells and primary mouse and human islets as well as in vivo in miR-204- and TXNIP-deficient mouse models reveal that TXNIP-induced miR-204 regulates islet GLP1R expression and that lack of miR-204 or TXNIP promotes the β -cell response to GLP1R agonists, enhances insulin secretion and glucose homeostasis, and has protective effects in the context of diabetes.

The sequence of miR-204 is well conserved between human, rat, and mouse, and it initially was just described to be expressed in kidney, retina, and insulinomas (40–42), but it has now been identified as the most highly enriched miRNA in human β -cells as opposed to α -cells (2,4–6), suggesting that miR-204 may play a particularly important role in β -cell biology. In fact, our current discovery of miR-204 downregulating islet GLP1R, which is critical for

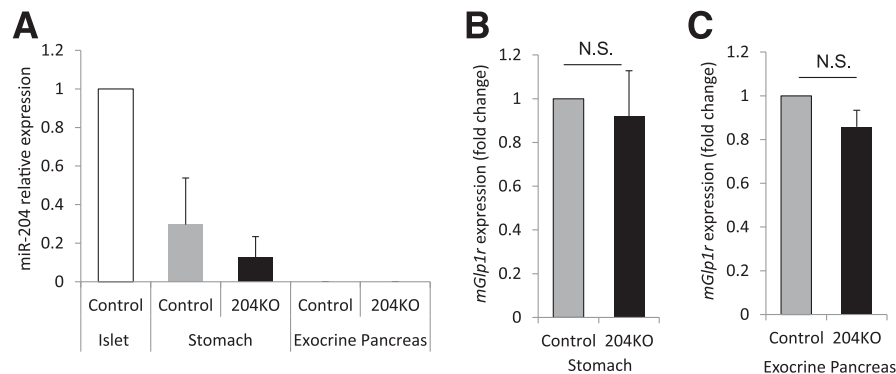


Figure 5—miR-204 expression is low or absent in stomach or exocrine pancreas, and GLP1R expression remains unaltered in response to miR-204 deletion in these tissues. **A**: Low or absent expression of miR-204 in stomach and exocrine pancreas of control and 204KO mice as compared with miR-204 expression in control islets. GLP1R expression in stomach (**B**) and exocrine pancreas (**C**) of 204KO mice and control mice. All data are shown as the mean \pm SEM of at least three mice.

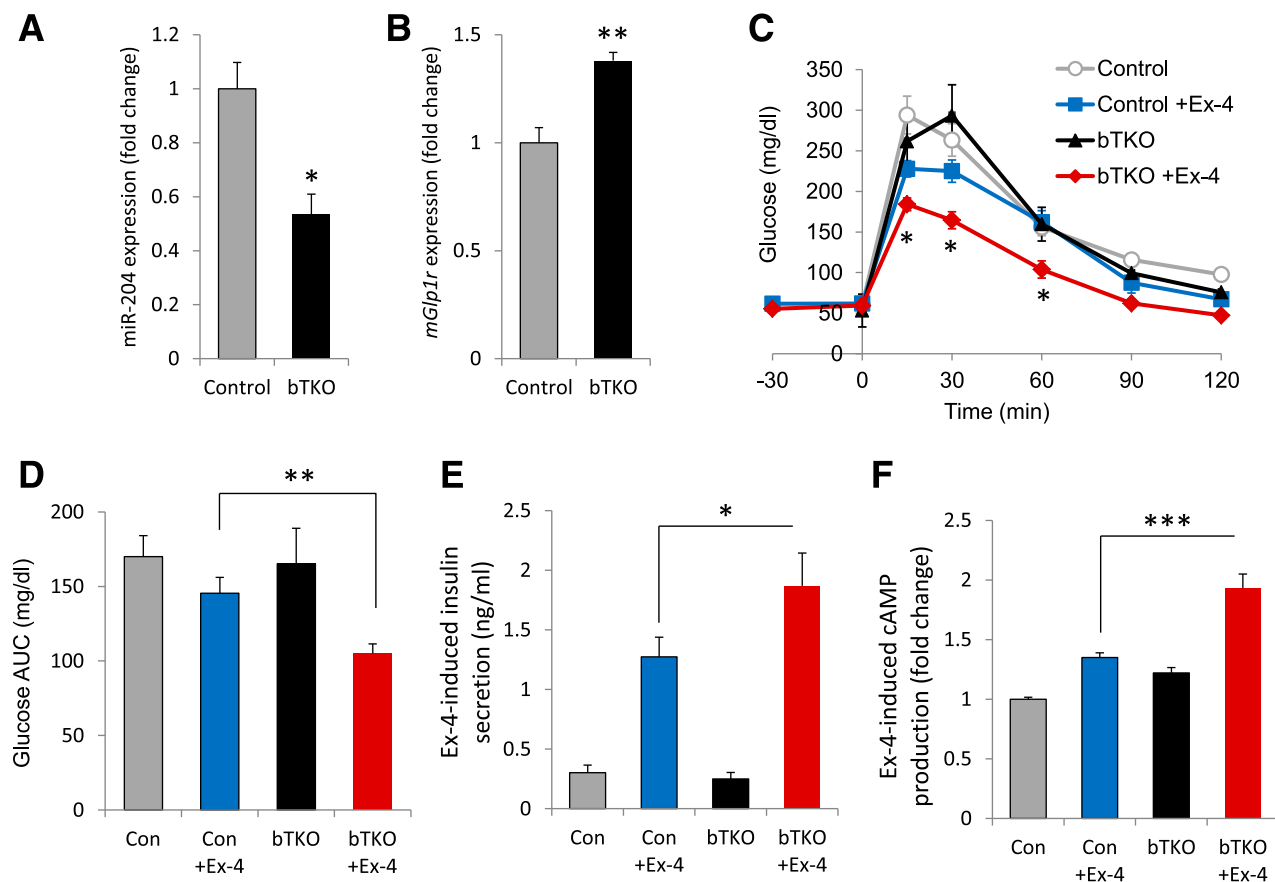


Figure 6— β -Cell-specific TXNIP knockout mice have lower miR-204 and higher GLP1R expression and exhibit enhanced insulin secretion and glucose control in response to Ex-4. miR-204 (A) and GLP1R (B) expression was measured in islets of bTKO mice and control mice using qRT-PCR. C: Glucose tolerance tests were performed in age- and sex-matched bTKO or control mice by glucose administration (1.5 g/kg i.p. at 0 min) and with or without Ex-4 (10 nmol/kg i.p. at -30 min); blood glucose was measured at the designated time points; glucose AUC (D) and Ex-4-induced changes in serum insulin (E). F: Intracellular cAMP production in response to Ex-4 was assessed in isolated islets of bTKO or control mice; $n = 3$ –9 mice per group. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

incretin-mediated insulin secretion, is in line with this notion, as are our previous observations of miR-204 targeting the major insulin transcription factor MafA and downregulating insulin production (4). It is also interesting to note that by doing so, miR-204 can inhibit both insulin synthesis and GLP1R-mediated insulin secretion. Together with our recent finding that miR-204 also regulates β -cell ER stress (7), these results indeed suggest that this miRNA may act as a linchpin in the control of β -cell function and stress response. Moreover, deletion of miR-204 also had protective effects in the context of diabetes and, like deletion of TXNIP, resulted in improved glucose control and increased insulin levels, suggesting that at least some of the previously described beneficial effects of TXNIP inhibition (23) might be mediated by miR-204 downregulation. Furthermore, the fact that the protective effects of miR-204 deletion were dependent on intact GLP1R signaling and blunted by a GLP1R antagonist underlines the important role that GLP1R regulation plays in mediating the miR-204 effects.

We have previously shown that TXNIP is upregulated by glucose and diabetes and induces β -cell apoptosis (21–23,

37,39) and have established TXNIP inhibition as a novel approach to protect against β -cell death, promote β -cell mass, and prevent diabetes (21–23,37), and this was validated by us and others in different models (43–45). Recently, we further found that TXNIP regulates β -cell miRNA expression and in particular induces expression of miR-204 and thereby inhibits insulin transcription (4). Our current results now indicate for the first time that by controlling GLP1R, TXNIP can also regulate GLP1R agonist-mediated insulin secretion, revealing a novel link between TXNIP and incretin action.

Consistent with this notion, the effects of TXNIP or miR-204 deletion on glucose tolerance were primarily observed in response to Ex-4 and therefore seem to be dependent on the upregulation of GLP1R. In fact, the results of regular glucose tolerance tests in the absence of Ex-4 were not significantly altered in our bTKO or 204KO mice showing no significant improvement in blood glucose, glucose AUC, insulin secretion, or cAMP production as compared with control mice. Nevertheless, we cannot exclude the possibility that additional targets of miR-204, such as MafA (4) or PERK (7), might contribute to the overall improvement in β -cell function in

response to miR-204 deletion. In future studies, it therefore will be interesting to further explore the effects this miRNA may have on the dynamics of intracellular cAMP production, as it has previously been linked to granular priming and exocytosis (46). Conversely, it is also conceivable that the higher expression level of GLP1R in the bTKO mice as compared with controls may facilitate endogenous GLP-1 function and thereby may have contributed to the improved glucose homeostasis observed in these TXNIP-deficient mice (23).

Also, we have previously shown that GLP1R agonist treatment results in decreased islet TXNIP expression (47). Now our data indicate that TXNIP inhibits GLP1R expression, suggesting that by doing so, it may diminish the effects of GLP-1, which theoretically would promote TXNIP expression. Interestingly, we have recently described a TXNIP-positive feed-forward loop that would be consistent with such an idea (48).

Type 2 diabetes is a growing public health problem, and inadequate β -cell function and insulin secretion are key factors in its pathogenesis (49). Many new diabetes drugs have therefore been developed over the last several decades that attempt to address these issues. However, many have significant side effects, and aside from metformin, incretin mimetics are the only class of antidiabetes drugs that is unlikely to cause hypoglycemia and that is not associated with any weight gain. GLP-1 analogs and DPP-4 inhibitors have therefore become widely used in patients with type 2 diabetes (9,10,12,25). Obviously, a prerequisite for their function is an adequate expression level of GLP1R, and this is also true for some newly developed synthetic small molecule stimulators of GLP-1 release (50). However, GLP1R expression has been shown to be downregulated in response to hyperglycemia (14,15), and less GLP1R staining was observed in islets of patients with type 2 diabetes as compared with control subjects without diabetes (13). Given this challenge, it is not surprising that mechanisms that might enhance GLP1R activity and lead to targets that could help overcome this hurdle have received significant attention (51,52).

In the meantime though, incretin mimetic drugs are just used at high enough doses to elicit the desired effects on β -cell insulin secretion and glucose control. Although incretin mimetics have many advantages, they are also associated with dose-dependent adverse effects, most commonly nausea and delayed gastric emptying (30,31), as well as some rare and still controversial side effects, including the elevated risk for pancreatitis and pancreatic cancer, even though the latter seems now to have been effectively rebutted (32–34). These side effects are not necessarily surprising given the fact that GLP1R is expressed not only in pancreatic β -cells but also in the exocrine pancreas, stomach, and brain (28,29). Therefore, being able to selectively promote β -cell GLP1R activity would be highly beneficial in terms of avoiding some of these adverse effects.

Intriguingly, like GLP1R, miR-204 is also highly expressed in pancreatic β -cells (2,4,6); however, we found very little

miR-204 expression in other GLP1R-expressing tissues, such as stomach or exocrine pancreas, and consistent with that, deletion of miR-204 did not affect GLP1R expression in these tissues, even though it effectively increased β -cell GLP1R expression. This raises the attractive possibility that by inhibiting miR-204, for example with an RNA therapeutic, one could confer β -cell-specific induction of GLP1R expression and, when combined with an incretin mimetic, safely enhance the effectiveness of this diabetes treatment. Moreover, this novel concept of inhibiting an miRNA in a nontargeted manner, but taking advantage of its restricted tissue distribution, and thereby selectively upregulating its target genes in that tissue may have far reaching implications for miRNA biology and tissue-specific gene targeting in general.

Conclusion

Thus, the results of the present studies establish GLP1R as a novel target of miR-204, uncover a previously unappreciated link between TXNIP and incretin action, and reveal potential novel strategies to optimize incretin mimetic diabetes drug therapy.

Funding. This work was supported by grants to A.S. from the National Institute of Diabetes and Digestive and Kidney Diseases (R01DK078752 and UC4DK104204) and JDRF (3-SRA-2014-302-M-R). Services provided by the UAB TGEMs facility were supported by NIH awards P30CA13148, P30AR048311, P30DK074038, P30DK05336, and P60DK079626.

Duality of Interest. No potential conflicts of interest relevant to this article were reported.

Author Contributions. S.J. performed and analyzed most of the experiments. J.C. generated the mouse lines, performed the islet isolations, and helped with the mouse studies. G.X. was responsible for the initial findings, performed the cloning and insulin secretion studies, and helped with the data analysis. T.B.G. and L.A.T. helped with the genotyping and maintenance of the mouse lines. A.S. conceived the project, supervised the work, and wrote the manuscript. A.S. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

References

1. Fernandez-Valverde SL, Taft RJ, Mattick JS. MicroRNAs in β -cell biology, insulin resistance, diabetes and its complications. *Diabetes* 2011;60:1825–1831
2. Filios SR, Shalev A. β -Cell microRNAs: small but powerful. *Diabetes* 2015;64:3631–3644
3. Guay C, Roggli E, Nesca V, Jacovetti C, Regazzi R. Diabetes mellitus, a microRNA-related disease? *Transl Res* 2011;157:253–264
4. Xu G, Chen J, Jing G, Shalev A. Thioredoxin-interacting protein regulates insulin transcription through microRNA-204. *Nat Med* 2013;19:1141–1146
5. van de Bunt M, Gaulton KJ, Parts L, et al. The miRNA profile of human pancreatic islets and beta-cells and relationship to type 2 diabetes pathogenesis. *PLoS One* 2013;8:e55272
6. Klein D, Misawa R, Bravo-Egana V, et al. MicroRNA expression in alpha and beta cells of human pancreatic islets. *PLoS One* 2013;8:e55064
7. Xu G, Chen J, Jing G, Grayson TB, Shalev A. miR-204 targets PERK and regulates UPR signaling and β -cell apoptosis. *Mol Endocrinol* 2016;30:917–924
8. Landgraf P, Rusu M, Sheridan R, et al. A mammalian microRNA expression atlas based on small RNA library sequencing. *Cell* 2007;129:1401–1414
9. Lamont BJ, Li Y, Kwan E, Brown TJ, Gaisano H, Drucker DJ. Pancreatic GLP-1 receptor activation is sufficient for incretin control of glucose metabolism in mice. *J Clin Invest* 2012;122:388–402

10. Mondal P, Song WJ, Li Y, Yang KS, Hussain MA. Increasing β -cell mass requires additional stimulation for adaptation to secretory demand. *Mol Endocrinol* 2015;29:108–120
11. Brubaker PL, Drucker DJ. Minireview: glucagon-like peptides regulate cell proliferation and apoptosis in the pancreas, gut, and central nervous system. *Endocrinology* 2004;145:2653–2659
12. Ahrén B. Islet G protein-coupled receptors as potential targets for treatment of type 2 diabetes. *Nat Rev Drug Discov* 2009;8:369–385
13. Shu L, Matveyenko AV, Kerr-Conte J, Cho JH, McIntosh CH, Maedler K. Decreased TCF7L2 protein levels in type 2 diabetes mellitus correlate with down-regulation of GIP- and GLP-1 receptors and impaired beta-cell function. *Hum Mol Genet* 2009;18:2388–2399
14. Xu G, Kaneto H, Laybutt DR, et al. Downregulation of GLP-1 and GIP receptor expression by hyperglycemia: possible contribution to impaired incretin effects in diabetes. *Diabetes* 2007;56:1551–1558
15. Rajan S, Dickson LM, Mathew E, et al. Chronic hyperglycemia downregulates GLP-1 receptor signaling in pancreatic β -cells via protein kinase A. *Mol Metab* 2015; 4:265–276
16. Fehmann HC, Jiang J, Pitt D, Schweinfurth J, Göke B. Ligand-induced regulation of glucagon-like peptide-1 receptor function and expression in insulin-secreting beta cells. *Pancreas* 1996;13:273–282
17. Maida A, Lamont BJ, Cao X, Drucker DJ. Metformin regulates the incretin receptor axis via a pathway dependent on peroxisome proliferator-activated receptor- α in mice. *Diabetologia* 2011;54:339–349
18. Pan QR, Li WH, Wang H, et al. Glucose, metformin, and AICAR regulate the expression of G protein-coupled receptor members in INS-1 beta cell. *Horm Metab Res* 2009;41:799–804
19. Whitaker GM, Lynn FC, McIntosh CH, Accili EA. Regulation of GIP and GLP1 receptor cell surface expression by N-glycosylation and receptor heteromerization. *PLoS One* 2012;7:e32675
20. Minn AH, Couto FM, Shalev A. Metabolism-independent sugar effects on gene transcription: the role of 3-O-methylglucose. *Biochemistry* 2006;45:11047–11051
21. Minn AH, Hafele C, Shalev A. Thioredoxin-interacting protein is stimulated by glucose through a carbohydrate response element and induces beta-cell apoptosis. *Endocrinology* 2005;146:2397–2405
22. Chen J, Saxena G, Mungrue IN, Lusis AJ, Shalev A. Thioredoxin-interacting protein: a critical link between glucose toxicity and beta-cell apoptosis. *Diabetes* 2008;57:938–944
23. Chen J, Hui ST, Couto FM, et al. Thioredoxin-interacting protein deficiency induces Akt/Bcl-xL signaling and pancreatic beta-cell mass and protects against diabetes. *FASEB J* 2008;22:3581–3594
24. Chen D, Liao J, Li N, et al. A nonpeptidic agonist of glucagon-like peptide 1 receptors with efficacy in diabetic db/db mice. *Proc Natl Acad Sci U S A* 2007;104:943–948
25. Habener JF. The incretin notion and its relevance to diabetes. *Endocrinol Metab Clin North Am* 1993;22:775–794
26. De Marinis YZ, Salehi A, Ward CE, et al. GLP-1 inhibits and adrenaline stimulates glucagon release by differential modulation of N- and L-type Ca²⁺ channel-dependent exocytosis. *Cell Metab* 2010;11:543–553
27. Li Y, Hansotia T, Yusta B, Ris F, Halban PA, Drucker DJ. Glucagon-like peptide-1 receptor signaling modulates beta cell apoptosis. *J Biol Chem* 2003;278:471–478
28. Pyke C, Heller RS, Kirk RK, et al. GLP-1 receptor localization in monkey and human tissue: novel distribution revealed with extensively validated monoclonal antibody. *Endocrinology* 2014;155:1280–1290
29. Williams DL. Minireview: finding the sweet spot: peripheral versus central glucagon-like peptide 1 action in feeding and glucose homeostasis. *Endocrinology* 2009;150:2997–3001
30. Thazhath SS, Marathe CS, Wu T, et al. The glucagon-like peptide 1 receptor agonist exenatide inhibits small intestinal motility, flow, transit, and absorption of glucose in healthy subjects and patients with type 2 diabetes: a randomized controlled trial. *Diabetes* 2016;65:269–275
31. Kanoski SE, Rupprecht LE, Fortin SM, De Jonghe BC, Hayes MR. The role of nausea in food intake and body weight suppression by peripheral GLP-1 receptor agonists, exendin-4 and liraglutide. *Neuropharmacology* 2012;62:1916–1927
32. Azoulay L, Filion KB, Platt RW, et al.; Canadian Network for Observational Drug Effect Studies Investigators. Incretin based drugs and the risk of pancreatic cancer: international multicentre cohort study. *BMJ* 2016;352:i581
33. Butler PC, Elashoff M, Elashoff R, Gale EA. A critical analysis of the clinical use of incretin-based therapies: are the GLP-1 therapies safe? *Diabetes Care* 2013;36: 2118–2125
34. Nauck MA. A critical analysis of the clinical use of incretin-based therapies: the benefits by far outweigh the potential risks. *Diabetes Care* 2013;36:2126–2132
35. Nishiyama A, Masutani H, Nakamura H, Nishinaka Y, Yodoi J. Redox regulation by thioredoxin and thioredoxin-binding proteins. *IUBMB Life* 2001;52:29–33
36. Patwari P, Higgins LJ, Chutkow WA, Yoshioka J, Lee RT. The interaction of thioredoxin with Txnip. Evidence for formation of a mixed disulfide by disulfide exchange. *J Biol Chem* 2006;281:21884–21891
37. Chen J, Fontes G, Saxena G, Poitout V, Shalev A. Lack of TXNIP protects against mitochondria-mediated apoptosis but not against fatty acid-induced ER stress-mediated beta-cell death. *Diabetes* 2010;59:440–447
38. Xu G, Chen J, Jing G, Shalev A. Preventing β -cell loss and diabetes with calcium channel blockers. *Diabetes* 2012;61:848–856
39. Shalev A, Pise-Masison CA, Radonovich M, et al. Oligonucleotide microarray analysis of intact human pancreatic islets: identification of glucose-responsive genes and a highly regulated TGF β signaling pathway. *Endocrinology* 2002;143:3695–3698
40. Sun Y, Koo S, White N, et al. Development of a micro-array to detect human and mouse microRNAs and characterization of expression in human organs. *Nucleic Acids Res* 2004;32:e188
41. Krol J, Busskamp V, Markiewicz I, et al. Characterizing light-regulated retinal microRNAs reveals rapid turnover as a common property of neuronal microRNAs. *Cell* 2010;141:618–631
42. Roldo C, Missiaglia E, Hagan JP, et al. MicroRNA expression abnormalities in pancreatic endocrine and acinar tumors are associated with distinctive pathologic features and clinical behavior. *J Clin Oncol* 2006;24:4677–4684
43. Lerner AG, Upton JP, Praveen PV, et al. IRE1 α induces thioredoxin-interacting protein to activate the NLRP3 inflammasome and promote programmed cell death under irremediable ER stress. *Cell Metab* 2012;16:250–264
44. Osowski CM, Hara T, O'Sullivan-Murphy B, et al. Thioredoxin-interacting protein mediates ER stress-induced β cell death through initiation of the inflammasome. *Cell Metab* 2012;16:265–273
45. Chutkow WA, Birkenfeld AL, Brown JD, et al. Deletion of the alpha-arrestin protein Txnip in mice promotes adiposity and adipogenesis while preserving insulin sensitivity. *Diabetes* 2010;59:1424–1434
46. Renström E, Eliasson L, Rorsman P. Protein kinase A-dependent and -independent stimulation of exocytosis by cAMP in mouse pancreatic B-cells. *J Physiol* 1997;502:105–118
47. Chen J, Couto FM, Minn AH, Shalev A. Exenatide inhibits beta-cell apoptosis by decreasing thioredoxin-interacting protein. *Biochem Biophys Res Commun* 2006; 346:1067–1074
48. Chen J, Jing G, Xu G, Shalev A. Thioredoxin-interacting protein stimulates its own expression via a positive feedback loop. *Mol Endocrinol* 2014;28:674–680
49. Poitout V, Robertson RP. Minireview: secondary beta-cell failure in type 2 diabetes—a convergence of glucotoxicity and lipotoxicity. *Endocrinology* 2002;143: 339–342
50. Chepurny OG, Leech CA, Tomanik M, et al. Synthetic small molecule GLP-1 secretagogues prepared by means of a three-component indole annulation strategy. *Sci Rep* 2016;6:28934
51. Kimple ME, Keller MP, Rabaglia MR, et al. Prostaglandin E2 receptor, EP3, is induced in diabetic islets and negatively regulates glucose- and hormone-stimulated insulin secretion. *Diabetes* 2013;62:1904–1912
52. Huang X, Dai FF, Gaisano G, et al. The identification of novel proteins that interact with the GLP-1 receptor and restrain its activity. *Mol Endocrinol* 2013;27: 1550–1563