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A role for placental kisspeptin in β -cell adaptation to pregnancy

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Abstract

During pregnancy the maternal pancreatic islets of Langerhans undergo adaptive changes to compensate for gestational insulin resistance. Kisspeptin has been shown to stimulate insulin release, through its receptor GPR54. The placenta releases high levels of kisspeptin into the maternal circulation suggesting a role in modulating the islet adaptation to pregnancy. In the present study we show that pharmacological blockade of endogenous kisspeptin in pregnant mice results in impaired glucose homeostasis. This glucose intolerance is due to a reduced insulin response to glucose as opposed to any effect on insulin sensitivity. A β -cell-specific GPR54 knockdown mouse line was found to exhibit glucose intolerance during pregnancy, with no phenotype observed outside of pregnancy. Furthermore, in pregnant women circulating kisspeptin levels significantly correlate with insulin responses to oral glucose challenge and are significantly lower in women with gestational diabetes (GDM) compared to those without GDM. Thus, kisspeptin represents a placental signal that plays a physiological role in the islet adaptation to pregnancy, maintaining maternal glucose homeostasis by acting through the β -cell GPR54 receptor. Our data suggest reduced placental kisspeptin production, with consequent impaired kisspeptin-dependent β -cell compensation, may be a factor in the development of GDM in humans.

Summary

Placental kisspeptin plays an important role in both mice and humans in the successful islet adaptation to pregnancy necessary for preventing gestational diabetes.

Keywords: Kisspeptin, Pregnancy, Glucose Homeostasis, Insulin, β -cell, Islets, Gestational Diabetes

Introduction

Kisspeptins are a family of peptides, encoded by the *KISS1* gene which are the endogenous ligands for the Gq-protein coupled receptor GPR54, also commonly known as KISS1R. Kisspeptin is well known for its permissive role acting locally in the hypothalamus to regulate puberty and reproductive function (1, 2) but its role in other physiological systems is much less understood. GPR54 is highly expressed in several peripheral tissues (3), including insulin-secreting pancreatic β -cells (4), consistent with a role for kisspeptin in regulating glucose homeostasis (4). However, there are discrepancies in the literature about the effects of exogenous kisspeptin on β -cell function. We have previously reported that exogenous kisspeptin enhances glucose-induced insulin secretion from rodent, porcine and human islets in vitro (5, 6), and in rats and mice in vivo (7), consistent with other reports of stimulatory effects in vitro (8), in vivo in a non-human primates (9) and in humans (10). However, others report predominantly inhibitory effects in vitro (11, 12). Similarly, studies investigating the physiological role(s) of endogenous kisspeptin in glucose homeostasis are seemingly contradictory. Thus, it has been suggested that low levels of hepatic kisspeptin are released to impair β -cell function and play a role in the development of type 2 diabetes (13), whilst GPR54 null mice become glucose intolerant, consistent with stimulatory effects of kisspeptin on β -cell function (14, 15). Aspects of the metabolic phenotype may also be a sex-specific, as female GPR54 null mice develop increased adiposity and impaired glucose tolerance that is not observed in males (16).

Circulating kisspeptin levels are extremely low under most physiological circumstances. The exception is in pregnancy when kisspeptin is released from the placenta into the maternal circulation such that circulating levels increase several thousand-fold in humans (17). The circulating kisspeptin during pregnancy in animal models is unclear due to the lack of reliable assays, however kisspeptin mRNA is expressed in rodent placenta and increases through pregnancy (18-20). Thus, one potential physiological role for β -cell GPR54 is in regulating β -cell adaptive responses to pregnancy. Pregnancy presents the maternal metabolism with the problem of providing for the energy requirements of the growing fetus while maintaining fuel homeostasis in the mother. In both rodents and humans there are reversible adaptive changes including a progressive increase in maternal insulin resistance which, under normal circumstances, is countered by increased maternal insulin secretion to maintain normoglycaemia. The increased insulin secretory capacity is provided through both functional and structural changes to the islets (21). Failure of these compensatory mechanisms may result in the development of glucose intolerance in pregnancy, known as gestational diabetes mellitus (GDM) in humans (22). At present our understanding of the signals regulating β -cell adaptations to pregnancy is incomplete. Previous studies have demonstrated a role for prolactin and placental lactogens (23, 24), but lactogenic hormones alone are unlikely to account for all of the β -cell adaptive responses. The timing of their increased secretion does not mirror that of the adaptive

responses and there is interest in identifying other contributing signals. Placental production of kisspeptin, and the consequent increased level of kisspeptin in the maternal circulation, closely reflect the time course of the increased insulin secretory capacity of maternal β -cells (25, 26).

We here demonstrate that placental kisspeptin acts as an important signal to amplify insulin secretory responses as needed to compensate for increased insulin resistance during pregnancy. We show that, in experimental animals, pharmacological blockade or genetic ablation of β -cell GPR54 during pregnancy results in a failure of normal homeostatic mechanisms and the consequent development of maternal glucose intolerance. Furthermore, we demonstrate that low circulating levels of kisspeptin during human pregnancy are associated with GDM. Therefore, placental kisspeptin acting via β -cell GPR54 is essential for normal glucose homeostasis during pregnancy.

Results

Effects of pharmacological activation or blockade of GPR54 on glucose homeostasis

Chronic activation of GPR54 improved glucose tolerance and insulin secretion in mice, consistent with a role for circulating kisspeptin in glucose homeostasis. Thus, kisspeptin administration to non-pregnant mice for 8 days resulted in a significant improvement in glucose tolerance as assessed by an intraperitoneal glucose tolerance test with significant reductions in blood glucose at 15-30 minutes after administration (Fig. 1A). However, this was not reflected in the overall area under curve (AUC) data (Fig. 1B) since blood glucose levels were similar in both treatment groups from 60-120 minutes. The difference in glucose profile was associated with significantly elevated plasma insulin levels in kisspeptin-treated mice 30 minutes after glucose administration, whilst basal insulin levels were not altered (Fig. 1C). There were no significant differences in plasma glucose between treatment groups in an insulin tolerance test (Fig. 1D, E). Thus, circulating kisspeptin enhances β -cell secretory responses to glucose challenge.

Pharmacological blockade of GPR54 caused impaired insulin secretion and impaired glucose tolerance in pregnant mice, as shown in Figure 2. Pregnancy in ICR mice is associated with impaired glucose tolerance and increased insulin resistance at both days 10-12 and days 16-18 of pregnancy when compared to non-pregnant controls (Supplementary Fig. S1A-C), and this was associated with enhanced glucose-induced insulin secretion, as assessed by significantly higher plasma insulin levels in the pregnant mice 30mins after a glucose load (Fig. 2B). Chronic administration of the GPR54 antagonist kisspeptin-234 to pregnant mice further impaired glucose tolerance by day 18 of pregnancy (Fig. 2A-B), which was associated with significant reductions in glucose-stimulated insulin secretion (Fig. 2C) but without any effect on basal plasma insulin levels (Fig. 2C). Blocking endogenous kisspeptin by antagonist administration had no detectable effects on the pregnancy-dependent increases in insulin resistance observed at either mid (day 12) or late (day 18) pregnancy (Fig. 2D-E). Thus, blocking activation of GPR54 by endogenous kisspeptin during pregnancy induces glucose intolerance by reducing glucose-induced insulin secretion rather than through effects on insulin target tissues.

Effects of β -cell GPR54 deletion on in vitro islet function

To assess whether the metabolic effects of pharmacological blockade of GPR54 were mediated primarily through β -cells we generated a β -cell specific GPR54 knockout mouse model (β -cell GPR54^{-/-}). Successful β -cell specific knockdown of GPR54 was confirmed and the model validated (Supplementary Fig. S2-3). Prior to in vivo experiments islets from β -cell GPR54^{-/-} mice were isolated for in vitro characterisation.

For studies in β -cell GPR54^{-/-} mice three different control groups were potentially necessary: Cre +ve mice not administered tamoxifen (Cre⁺/TMX⁻), Cre -ve mice administered tamoxifen (Cre⁻/TMX⁺) and Cre -ve mice not administered tamoxifen (Cre⁻/TMX⁻). There was no difference in insulin content between β -cell GPR54^{-/-} mice and any of these control groups (Cre⁺/TMX⁻; Cre⁻/TMX⁺; Cre⁻/TMX⁻; Fig. 3A). For subsequent in vitro characterisation the Cre⁺/TMX⁻ group was used, as the presence of the Cre gene was more likely to represent a confounding factor in terms of islet function. In static incubation experiments there was no significant difference between β -cell GPR54^{-/-} and Cre⁺/TMX⁻ islets in glucose stimulated insulin secretion across a physiological range of glucose concentrations from 5-11mM glucose (Fig. 3B). Similarly, in perfusion experiments there was no difference in basal insulin secretion at 2mM glucose nor in the first or second phase insulin response to a maximally-stimulating glucose concentration (20mM; Fig. 3C-D). In Cre⁺/TMX⁻ islets the addition of 1 μ M kisspeptin-10 also resulted in a significant potentiation of glucose stimulated insulin secretion, that was sustained for the duration of kisspeptin exposure. In contrast, β -cell GPR54^{-/-} islets showed an initial stimulatory insulin response to kisspeptin but this effect was brief and kisspeptin was unable to maintain a prolonged potentiating effect (Fig. 3C-D).

Effects of deletion of β -cell GPR54 on glucose homeostasis in vivo

Having confirmed normal glucose responsiveness, but impaired kisspeptin response, in β -cell GPR54^{-/-} islets the mice were used to assess whole-body glucose homeostasis. GPR54 ablation in β -cells had no significant effects in non-pregnant mice, as shown in Figure 4 (A-C). Thus, virgin female β -cell GPR54^{-/-} mice showed no significant differences in glucose tolerance (Fig. 4A), glucose-induced insulin secretion (Fig. 4B), or insulin resistance (Fig. 4C) when compared to any of the three control groups (Cre⁺/TMX⁻; Cre⁻/TMX⁺; Cre⁻/TMX⁻). In addition, we did not detect any significant differences in glucose tolerance, circulating insulin or insulin sensitivity (Fig. 4A-C) between any of the control groups, suggesting that neither the presence of the Cre transgene nor the use of tamoxifen as an activator had any significant effect on glucose homeostasis in this experimental model.

However, the β -cell GPR54^{-/-} mouse model confirmed the importance of kisspeptin signalling in β -cell adaptive responses to pregnancy, as shown in Figure 4 (D-H). Thus, by day 16 of pregnancy β -cell GPR54^{-/-} mice had significantly impaired glucose tolerance when compared to controls (Fig. 4D, E), and this was associated with reduced insulin secretory responses 30min after a glucose challenge (Fig. 4F), although basal plasma insulin levels were unaffected by the β -cell GPR54 deletion (Fig. 4F). In accordance with the studies using the kisspeptin antagonist in pregnant mice (Fig. 2D-E), pregnant β -cell GPR54^{-/-} mice showed no significant differences in insulin resistance (Fig. 4G-H) or in body weight when compared to controls (Supplementary Fig. S4A). Thus, deletion of β -cell GPR54 induces glucose intolerance in pregnancy by

reducing glucose-induced insulin secretion, consistent with placental kisspeptin acting to compensate for pregnancy-induced insulin resistance by enhancing β -cell function.

Effects of deletion of β -cell GPR54 on pregnant β -cell mass

Pregnant mice were administered BrdU from day 10-18 of pregnancy to label proliferating cells prior to pancreas samples being collected at day 18. The percentage of BrdU positive β -cells was significantly reduced in β -cell GPR54^{-/-} mice compared to Cre⁺/TMX⁻ controls, demonstrating an attenuation of the rapid β -cell proliferation normally observed during late pregnancy (Fig. 5C). Despite the change in β -cell proliferation there was no significant difference in islet cross-sectional area between day 18 pregnant β -cell GPR54^{-/-} mice and Cre⁺/TMX⁻ controls (Fig. 5D).

Rodent pregnancy is also associated with β -cell hypertrophy, so the mean individual β -cell area was calculated as total β -cell area divided by number of β -cell nuclei. This may slightly overestimate individual β -cell area because not all β -cell nuclei may be visualised in a given section, but it is still a useful measure for comparing changes in individual β -cell area between treatment groups. No significant difference was observed in the mean area of individual β -cells suggesting that deletion of β -cell GPR54 has no effect on pregnancy-induced hypertrophy (Fig. 5E).

Circulating kisspeptin and glucose tolerance in pregnant women

Our studies in a cohort of pregnant women further support a role for circulating placental kisspeptin in β -cell adaptive responses to pregnancy, as shown in Figure 6. Under basal (fasting) conditions there was a significant, albeit weak, positive correlation between kisspeptin and β -cell secretory function, as assessed by HOMA2-% β (Fig. 6G), although no significant correlation with fasting insulin (Fig. 6B). There was a highly significant medium-strength positive correlation between kisspeptin and oral glucose-stimulated insulin levels, as assessed by insulin AUC (Fig. 6F). There was a highly significant medium-strength positive correlation between kisspeptin and serum insulin at 60 min after oral glucose load (Fig. 6D), with a trend towards significance at 10 min (Fig. 6C) and no significant correlation at 120 min (Fig. 6E). There was a slight negative correlation between kisspeptin and fasting plasma glucose ($r^2=-0.064$, $p=0.016$) with no significant correlations at 10 min, 60 min, or 120 min (supplementary Fig. S5), suggesting the positive correlations between kisspeptin and insulin are not secondary to higher circulating glucose levels. There were no significant correlations between kisspeptin and HOMA2-IR (Fig. 6H) or the Matsuda index (Fig. 6I), suggesting the positive correlations between kisspeptin and insulin are not secondary to altered insulin resistance.

In this cohort, 28.6% of women had GDM. There were no significant differences in age, ethnicity, multiple pregnancy, gestation, number of previous pregnancies, BMI, or blood pressure between women with and without GDM (supplementary table). However, women with GDM had significantly lower plasma kisspeptin levels than women without GDM (Fig. 6A).

The reported correlations are for all pregnant women, both with and without GDM. Correlation coefficients were also calculated for women with and without GDM separately and r^2 values for the independent groups were similar to those for the combined data. The correlation observed between kisspeptin and HOMA2- β was no longer significant when comparing women with GDM alone, however this is most likely due to the smaller n-number in the GDM group and the initial weak correlation. All other correlations that were significant in the combined data were also significant for women with and without GDM independently.

In summary, in pregnant women higher plasma kisspeptin levels are associated with enhanced insulin secretion, particularly after oral glucose load, and lower levels of plasma kisspeptin are associated with diagnosis of GDM.

Discussion

Pregnancy represents a unique physiological state in which the islet β -cells are exposed to very high levels of circulating kisspeptin for a prolonged period, and we have here demonstrated that placentally-derived kisspeptin is involved in β -cell adaptations to pregnancy. Chronic elevations in circulating kisspeptin in virgin mice improved glucose tolerance as a consequence of enhanced glucose-induced insulin secretion, consistent with previous *in vitro* studies of a direct stimulatory effect of β -cell GPR54 activation on insulin secretion (7). The significant effects of exogenous kisspeptin on glucose tolerance in non-pregnant mice were only apparent between 15-30 minutes post-glucose administration, most likely because the normal control of blood glucose leaves little room for improvement, but the chronic kisspeptin treatment had marked effects to enhance glucose-induced insulin secretion *in vivo*. During murine pregnancy, pharmacological blockade of GPR54 resulted in impaired glucose tolerance as a consequence of reduced glucose-induced insulin secretion. In normal mouse pregnancy, glucose tolerance is progressively impaired from mid-pregnancy (day 10-12) to late-pregnancy (day 16-18), associated with increased peripheral insulin resistance. Thus, pharmacological blockade of GPR54 induced a mild impairment of glucose tolerance at mid-pregnancy, which progressed to a more pronounced phenotype by late pregnancy suggesting a progressive role for endogenous kisspeptin in mediating β -cell adaptations to pregnancy-induced insulin resistance. This is consistent with the development of the mouse placenta which only becomes fully functional around days 7-9 (27), suggesting that levels of circulating kisspeptin are unlikely to be elevated before this stage, with a subsequent progressive increase in circulating kisspeptin for the remainder of the pregnancy, as has been reported during human pregnancy (17). The precise changes in circulating levels of kisspeptin in rodent pregnancy have not been clearly established because of continuing difficulties in developing accurate and specific rodent assays. However, rodent placenta expresses increasing levels of kisspeptin mRNA through pregnancy (18-20), consistent with observations in humans, suggesting placental release of kisspeptin into the maternal circulation.

The pharmacological data were supported by our observations using a β -cell specific GPR54 knockout mouse model. Hypothalamic GPR54 plays a critical role in maintaining reproductive function so the MIP-CreERT line was selected to avoid the off-target hypothalamic Cre expression that is a characteristic of the PDX1-Cre and RIP-Cre lines (28, 29). Whilst β -cell specific GPR54 knockout mice have been previously reported (13), they have not previously been studied during pregnancy. Validation of the β -cell GPR54^{-/-} mice demonstrated a tissue specific 70% reduction in islet GPR54 mRNA expression, with no detectable leakage in the hypothalamus (see Supplementary Figure S3). This reduction in GPR54mRNA expression is consistent with other inducible knockout models, where 100% gene knockout is not typically observed. Also, there is some controversy regarding whether GPR54 is also expressed in islet α -cells with some

previous studies suggesting it is expressed (4) and others finding it absent (13). If GPR54 is present in islet endocrine cells other than β -cells then this would not be deleted in the β -cell GPR54^{-/-} mice, resulting in an underestimation of GPR54 knockdown based on whole islet expression. In vitro studies in isolated islets confirmed that GPR54 knockdown had no effect on insulin content or on glucose-induced insulin secretion. Kisspeptin treatment of β -cell GPR54^{-/-} islets did still induce a brief transient potentiation of insulin secretion, which may be consistent with the presence of residual GPR54 expression in the islets. However, kisspeptin did not maintain elevated insulin release in β -cell GPR54^{-/-} islets suggesting that the effects are significantly attenuated in β -cell GPR54^{-/-} islets.

Based on the GPR54 blockade studies, β -cell GPR54^{-/-} mice were assessed during late pregnancy (day 16-18) to maximise any metabolic phenotype. As expected, pregnant β -cell GPR54^{-/-} mice had significantly impaired glucose tolerance during late pregnancy as a consequence of reduced glucose-induced insulin secretion, in accordance with the pharmacological studies and confirming a β -cell specific action of placental kisspeptin to regulate glucose tolerance. However, no difference in placental weight, pup weight or pup length were observed between β -cell GPR54^{-/-} mice and controls (Supplementary Figure S4B-D). The absence of macrosomia suggests that despite the significant glucose intolerance loss of β -cell GPR54 signalling is not sufficient to induce GDM.

These studies used several different controls to avoid any potentially confounding factors associated with the inducible transgene. Thus, the MIP-CreERT mouse has been reported to exhibit inappropriate human growth hormone (hGH) expression in β -cells leading to increased islet serotonin levels (30-32), an effect which has so far only been demonstrated in males, and which is relatively small compared to the normal increases in islet serotonin seen during pregnancy (3, 33). Nonetheless, we used Cre +ve control mice to demonstrate that the transgene itself had no effect on glucose tolerance in pregnant or non-pregnant female mice. Tamoxifen is a selective estrogen receptor modulator which can disrupt female reproductive cycles (34) and affect glucose tolerance through effects on lipid metabolism (30, 34, 35). To minimise these confounding effects mice were left for 6 weeks following tamoxifen administration by which time animals had regained fertility and no tamoxifen-dependent effects on glucose tolerance were detectable.

In addition to enhanced secretory responsiveness to glucose β -cells also adapt to pregnancy by increasing their functional β -cell mass, through a combination of an increased rate of proliferation and β -cell hypertrophy in rodents. This β -cell expansion primarily occurs after placentation in rodents over a relatively short and well-defined time span and is reversible post-partum, consistent with placental signals playing a major role in informing the β -cells of the gestational stage via circulating mediators. It is well established that the lactogenic hormones, prolactin and placental lactogen, play a key role in metabolic adaptations

during pregnancy, but lactogenic hormones are unlikely to account for all of the β -cell adaptive responses and other factors have been implicated in the successful islet adaptation to pregnancy (36). Our observations of reduced β -cell proliferation in pregnant β -cell GPR54^{-/-} mice suggest that placental kisspeptin does play a physiological role in regulating β -cell mass to compensate for the pregnancy-induced insulin resistance. However, the reduced proliferative rate in pregnant β -cell GPR54^{-/-} islets is not associated with a corresponding reduction in β -cell hypertrophy and overall is not sufficient to translate into a significant overall reduction in β -cell mass. It might be expected that the reduced rate of proliferation would result in a corresponding reduction in mean islet area. Whilst there is a trend towards reduced islet area in the pregnant β -cell GPR54^{-/-} mice it is likely that the relatively small change in proliferation rate combined with the natural variability in islet area means that any effect on islet area is not sufficient to achieve significance. It is worth noting that, though significant, the effects of β -cell GPR54 knockdown on proliferation are not as dramatic as the effect observed in response to β -cell specific knockdown of the prolactin receptor (37). Thus, whilst placental kisspeptin may play a contributory role in the upregulation of β -cell proliferation during pregnancy it does not appear to be a major driver of this response to the extent of the lactogenic hormones.

Our mouse data suggests that kisspeptin signalling via GPR54 facilitates metabolic control by enhancing the insulin secretory responses of the β -cells and to some extent by increasing the number of β -cells. Less information is available about the regulation of the β -cell adaptation to human pregnancy, particularly with regard to β -cell mass. Post-mortem studies indicate that β -cell mass certainly increases in human pregnancy, though the underlying mechanisms are unclear and may differ from rodent pregnancy (38). Regardless, the core characteristics involved in the β -cell adaptation in rodents appear to be conserved to some extent in human pregnancy.

There is great interest in determining whether signals identified in rodent pregnancy are also involved in regulating human β -cells. Our previous studies suggested that the effects of kisspeptin in pregnancy may translate to humans given that kisspeptin is able to potentiate glucose stimulated insulin secretion from human islets in vitro (6). Our studies in pregnant women undergoing OGTTs as part of their routine clinical care suggest that placental kisspeptin may have a similar function in humans to that revealed in our mouse models. In accordance with our animal data, glucose-induced insulin secretion was positively correlated with plasma kisspeptin. This is consistent with placentally-derived kisspeptin exerting an “incretin-like” (39) action in pregnancy to facilitate greater β -cell insulin secretion in response to glucose ingestion, to compensate for pregnancy-associated insulin resistance. We also observed a weak positive correlation between kisspeptin and basal β -cell secretory function as assessed by HOMA2-% β in pregnant women, though there was no significant correlation between kisspeptin and fasting insulin levels in either pregnant

women or in animal studies. In addition, we demonstrated that women with GDM had significantly lower plasma kisspeptin levels than women without GDM, which supports one previous report of lower mean kisspeptin in GDM (40). Low placental production of kisspeptin has been suggested as a risk factor for pre-eclampsia and early pregnancy bleeding (41, 42) and it is of interest that GDM is a risk factor for pre-eclampsia (43). These data are constrained by the obvious ethical limits of intervening during human pregnancy, and there should be some caution when drawing conclusions from correlative results. However, it is noteworthy that all of the significant correlations observed in these pregnant women are between kisspeptin levels and markers of β -cell function (e.g. robust insulin response to glucose, HOMA2-% β), with no correlations observed between kisspeptin and other markers (e.g. HOMA2-IR). Considered with our animal data, this suggests reduced placental kisspeptin production, with consequent impaired kisspeptin-dependent β -cell compensation, may be a factor in the development of GDM in humans.

It is less clear what, if any, role kisspeptin plays in metabolic control outside of pregnancy since we detected no phenotype in non-pregnant β -cell GPR54^{-/-} mice. Previous studies have reported both potential beneficial and inhibitory effects of kisspeptin on glucose tolerance in non-pregnant, usually male, mice (13, 14, 44), but these effects may not be β -cell specific, and are most often observed in metabolically stressed animals. Furthermore a recent human study reports that elevated plasma kisspeptin correlates with impaired glucose tolerance in both male and female non-pregnant, non-diabetic individuals (45), though it is well established that plasma kisspeptin levels are several-thousand fold lower outside of pregnancy (17). Whilst we cannot rule out that kisspeptin may play a role in regulating β -cell function outside of pregnancy, the impaired glucose tolerance seen in these β -cell GPR54^{-/-} mice appears to be a pregnancy-specific phenomenon. It has been suggested that the different length kisspeptin peptides may have differing effects, and it is worth noting that the placenta releases all forms of kisspeptin into the circulation. There is precedent for the differing forms of kisspeptin having specific effects as kisspeptin-10, but not longer forms of kisspeptin, plays a role in trophoblast invasion (46). Several studies have put forward the hypothesis that higher concentrations of kisspeptin may potentiate glucose-induced insulin release whilst lower concentrations may be inhibitory (47). As such our findings in pregnant women and the β -cell GPR54^{-/-} mouse model do not necessarily contradict previous studies demonstrating inhibitory effects of kisspeptin on insulin secretion, but support the possibility that kisspeptin may have different effects under different conditions though the precise mechanisms involved remain to be established.

Together, our data are consistent with placentally-derived kisspeptin in the maternal circulation exerting an effect on maternal β -cells during pregnancy to facilitate enhanced glucose-induced insulin secretion and increase β -cell mass to compensate for maternal insulin resistance, thus avoiding overt hyperglycaemia. The expression of GPR54 by β -cells allows them to detect elevations in kisspeptin during pregnancy,

informing them about the state of pregnancy, and enabling them to respond to the peculiar metabolic demands of pregnancy. We have recently reported that mouse placenta expresses ~80 different ligands for β -cell GPCRs (48) suggesting that kisspeptin may be one of many novel placental signals regulating β -cell function during pregnancy.

In conclusion, we have demonstrated an important role for placental kisspeptin in amplifying glucose-induced insulin secretion across species, consistent with a physiological role for kisspeptin in the β -cell adaptation to pregnancy. Our data in pregnant women, considered with our animal data, suggests reduced placental kisspeptin production, with consequent impaired kisspeptin-dependent β -cell compensation, may be a factor in the development of GDM in humans. Kisspeptin may offer a promising biomarker for developing GDM, or a tractable target for therapeutic intervention, or both.

Methods

Animals

Female ICR mice (Envigo, Bicester, UK) at 8 weeks of age were used for in vivo pharmacological studies. The β -cell GPR54^{-/-} mouse model was generated on a C57Bl/6 background using Cre-Lox recombination. GPR54-LoxP mice (49) (obtained from Prof. Tena-Sempere, University of Cordoba, Spain) were cross-bred with MIP-Cre/ERT mice ((50), B6.Cg-Tg(Ins1-cre/ERT)1Lphi/J, Jackson Labs). Tamoxifen was administered to induce GPR54 knockdown in the relevant mice at 8 weeks of age through daily intraperitoneal (i.p.) injection (100 μ l of 20mg/ml tamoxifen in peanut oil, Sigma-Aldrich) for 4 consecutive days. Mice were then left for 6 weeks to allow tamoxifen washout. Further details on the validation of the β -cell GPR54^{-/-} mice are available within supplementary data.

All animals were housed under controlled conditions (14:10h light/dark; lights on at 07:00h; temperature at 22 \pm 2°C) and provided with food and water ad libitum.

In vivo studies

For pregnant mice, females were housed with a male and checked daily for the presence of a vaginal plug. The day a vaginal plug was observed was designated day 0 of pregnancy. Age matched female mice were used for studies in non-pregnant animals.

For pharmacological studies, animals were implanted with a subcutaneous osmotic minipump (Alzet, Cupertino, Ca, USA) under isofluorane anaesthesia. Minipumps contained either 1mM of kisspeptin-10 (Alta Biosciences, Birmingham, UK) or 2mM of the GPR54 antagonist kisspeptin-234 (51) (Sigma-Aldrich, Poole, UK). In pregnant animals minipumps were implanted at either day 2 or day 8 of pregnancy. Osmotic minipumps released either 0.25 nmol/hr kisspeptin-10 or 0.4 nmol/hr kisspeptin-234 at an infusion rate of 0.4 nmol/hr.

For glucose tolerance tests mice were fasted for 6 hours and administered with glucose (2g/kg) via intraperitoneal injection. Blood samples (~1 μ l) were taken by tail prick for the measurement of blood glucose levels. Larger blood samples were taken from the tail vein for the measurement of plasma insulin levels. Plasma insulin levels were subsequently measured using a mouse ultrasensitive insulin ELISA kit (Merckodia, Sweden).

For insulin tolerance tests mice were also fasted for 6 hours and were administered with insulin (0.75iU/kg, Sigma) via intraperitoneal injection. Small blood samples were taken by tail prick for the measurement of blood glucose levels.

In β -cell GPR54^{-/-} mice, from day 10 of pregnancy onwards BrdU (1mg/ml) was administered in the drinking water to label proliferating cells. Following the insulin tolerance test animals were culled and the pancreas collected for histology.

β -cell GPR54^{-/-} in vitro studies

Islets of Langerhans were isolated from female β -cell GPR54^{-/-} and control mice by collagenase digestion of the exocrine pancreas. Islets were incubated at 37°C in RPMI (supplemented with 10% fetal calf serum, 2mM glutamine and 100unit/ml penicillin/0.1mg/ml streptomycin) for at least 24 hrs before use.

Insulin secretion from mouse islets was assessed using either a temperature-controlled perfusion apparatus or in static incubations of islets. For perfusion experiments groups of 40 islets were perfused with a bicarbonate-buffered physiological salt solution (0.5ml/min, 37°C,) containing 2mM glucose, 2mM CaCl₂ and 0.5mg/ml BSA and supplemented with treatments of interest. Perifusate was collected at two-minute intervals for insulin content. For static incubations islets were pre-incubated in 2mM glucose RPMI. Groups of 10 size-matched islets were incubated at 37°C for 1 hr in physiological salt solution as described above, supplemented with agents of interest. After 1 hr samples were taken and assayed for insulin content. For islet insulin content, groups of 10 islets were transferred into acid-ethanol and sonicated. Insulin levels were determined using an in-house radioimmunoassay.

β -cell mass analysis

Pancreas samples were fixed in 4% paraformaldehyde (Sigma-Aldrich) and embedded in paraffin wax. Sections (5 μ m) were rehydrated followed by antigen retrieval with 2N hydrochloric acid for 20 min at 37°C and subsequent 0.05% trypsin solution (Sigma-Aldrich) for 15 min at 37°C. Sections were incubated in blocking buffer (0.25% BSA, 0.25% Triton X100 in PBS) before incubation with both a mouse monoclonal anti-BrdU antibody (1:100, Sigma-Aldrich, B8434) and guinea-pig polyclonal anti-insulin antibody (1:200, Dako, A0564) for 2 h at 37°C.

Alexa-488 conjugated donkey anti-mouse (1:100, Jackson Immuno Research, USA) and Alexa-594 conjugated donkey anti-guinea-pig (1:100, Jackson Immuno Research) were used to visualise staining. A Nikon eclipse TE2000-U microscope was used for acquiring images at x200 magnification using a Nikon DS-Qi1MC camera.

Sections were analysed from across the pancreas for each animal and all islets on each section were analysed. ImageJ image analysis software was used to count the number of BrdU positive β -cells, total number of β -cells and cross-sectional area for each islet.

Plasma kisspeptin and glucose tolerance in pregnant women

Pregnant women between 26-34 weeks gestation referred for an oral glucose tolerance test (OGTT) at King's College Hospital as part of routine care were invited to participate. Women were referred for an OGTT if they had GDM in a previous pregnancy, BMI $\geq 40\text{kg/m}^2$ or random plasma glucose $\geq 6.7\text{mmol/l}$. Exclusion criteria were being unable to give informed consent and known major medical problems. One hundred women were recruited and data are presented for 91 women for whom blood samples were obtained. Further participant details are available within supplementary data.

Participants underwent a standard 2hr 75g OGTT with additional blood sampling. After overnight fasting ($>9\text{hrs}$), an intravenous cannula was inserted in an arm vein for blood sampling, the participant drank 75g glucose in 300ml and rested for 2 hours. Venous blood samples were taken prior to glucose consumption for the measurement of plasma kisspeptin, plasma glucose and serum insulin. Further blood samples were taken at 10min, 60min and 120min for the measurement of plasma glucose and serum insulin. For the purposes of this research GDM was diagnosed according to the International Association of Diabetes and Pregnancy Study Groups (IADPSG) criteria (52): one or more of fasting venous plasma glucose $\geq 5.1\text{mmol/l}$, 60 min $\geq 10.0\text{mmol/l}$, 120 min $\geq 8.5\text{mmol/l}$. Weight, height and blood pressure were measured.

Preparation and assay of human samples

For plasma kisspeptin, 5ml venous blood samples were collected into tubes containing ethylene-diamine-tetra-acetic acid (EDTA, BD Vacutainer Blood Collection Tubes) (53). Samples were centrifuged at 855g for 10min at 4°C. Plasma was collected and stored at -80°C until assay. Plasma kisspeptin was measured using a commercially available ELISA kit (Phoenix Pharmaceuticals Inc., Karlsruhe, Germany). Peptide extraction was performed on all kisspeptin samples based on the manufacturer's protocol (Phoenix Pharmaceuticals Inc., Karlsruhe, Germany) and absorbances measured using a Chameleon plate reader.

For serum insulin, 5ml venous blood samples were collected into SST tubes (BD Vacutainer Blood Collection Tubes), allowed to stand for a minimum of 20 min at room temperature before centrifugation at 3000 rpm for 10 min at 4°C. Serum was collected and stored at -80°C until assay. Serum insulin levels were measured using a commercially available ELISA kit (Mercodia, Uppsala, Sweden). The ELISA was performed according to the manufacturer's instructions and absorbances measured using a Chameleon plate reader.

For plasma glucose measurement, 2ml venous blood samples were collected into tubes containing Fluoride EDTA (BD Vacutainer Blood Collection Tubes). Samples were centrifuged at 3000 rpm for 10 min at 4°C. Plasma glucose was measured immediately using a YSI 2300 Stat Analyser (YSI Life Sciences).

HOMA2-%B and HOMA2-IR were calculated using the HOMA2 calculator (version 2.2.3, specific insulin) downloaded from www.dtu.ox.ac.uk/homacalculator/ on 4 August 2018 (54). Matsuda index was calculated using the online calculator available at www.mmatsuda.diabetes-smc.jp/MIndexsi.html on 4th August 2018 using time 0, 60 and 120 min data (55).

Statistical analysis

All data are expressed as mean and standard error of the mean (SEM). Each in vitro and in vivo study was repeated at least twice with consistent results. For comparison between two groups un-paired two-tailed Student's *t*-test was used. For comparisons between 3 or more groups a one-way analysis of variance (ANOVA) was used followed by Tukey's post-hoc test. For analysis of individual IPGTT and IPITT data points two-way repeated measures ANOVA was used, followed by Tukey where appropriate. For analysis of correlations the Pearson product-moment correlation coefficient was used. Differences with $p < 0.05$ were considered significant.

Study approval

All animal procedures were approved by the local King's College London Animal Welfare and Ethical Review Board and were undertaken in accordance with United Kingdom Home Office Regulations.

The research in humans was conducted in accordance with the Declaration of Helsinki (2013) and was approved by the London-Westminster Research Ethics Committee (13/LO/0539). Written informed consent was obtained from all participants.

Author Contributions

JB was involved in designing the research studies, conducting animal experiments and writing the manuscript. TH conducted the majority of the β -cell GPR54^{-/-} experiments, assisted with assaying human samples and analysed data. KH was involved in designing and conducting the translational human studies and with writing the manuscript. LS and SS assisted with β -cell GPR54^{-/-} experiments. SA assisted with designing and conducting the translational human studies. PJ was involved in designing the study and writing the manuscript.

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Declaration of Interests

The authors declare that no conflict of interest exists.

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Figure Legends

Figure 1: In vivo effects of chronic kisspeptin administration on glucose homeostasis in non-pregnant mice. (A) In non-pregnant female ICR mice, chronic subcutaneous administration of kisspeptin resulted in improved glucose tolerance after intraperitoneal (i.p.) glucose administration (2g/kg) when compared with untreated controls (two-way RM ANOVA, 15min $p < 0.001$). (B) There was no significant difference in overall 120min area under curve (AUC), but kisspeptin treatment did result in a significant reduction in glucose AUC over 0-60min (students t-test, $p = 0.0321$). (C) Whilst chronic kisspeptin treatment had no significant effect on fasting plasma insulin levels, kisspeptin treated animals had significantly increased insulin release in response to i.p. glucose administration (2g/kg) after 30min when compared to controls (one-way ANOVA, $p = 0.033$). (D) Kisspeptin had no effect on the plasma glucose response to i.p. insulin administration (0.75IU/kg) when compared with controls, through both comparison of individual time points and (E) glucose AUC. Mean \pm SEM, $n = 11-12$, * $P < 0.05$ vs control.

Figure 2: Effects of kisspeptin-234 on glucose homeostasis during pregnancy in mice. (A) At day 10 of pregnancy chronic kisspeptin-234 (KP-234) administration had no significant effect on glucose tolerance, however at day 16 of pregnancy mice treated with KP-234 had significantly impaired glucose tolerance following intraperitoneal (i.p.) glucose administration (2g/kg) when compared with untreated pregnant controls, as determined by both comparison of individual time points (two-way RM ANOVA, 60min $p = 0.022$, 90min $p = 0.046$) and (B) glucose area under curve (AUC) over the course of the test (one-way ANOVA, $p = 0.031$). (C) Blocking endogenous kisspeptin signalling with KP-234 did not have any effect on fasting plasma insulin levels at either day 10 or day 16 of pregnancy. At day 16 chronic KP-234 treatment resulted in significantly reduced insulin release in response to i.p. glucose administration (2g/kg) after 30min when compared with pregnant controls (one-way ANOVA, $p = 0.009$). A similar trend was observed at day 10, however this was not significant. (D) KP-234 had no effect on the plasma glucose response to i.p. insulin administration (0.75IU/kg) when compared with untreated controls at either day 12 or day 18 or pregnancy, through both comparison of individual time points and (E) glucose AUC. Mean \pm SEM, panels A-E: $n = 6$, * $P < 0.05$ vs control.

Figure 3: Effects of β -cell GPR54 deletion on islet function in vitro. (A) There were no significant differences in islet insulin content between β -cell GPR54^{-/-} mice and any control groups (Cre+/TMX-, Cre-/TMX+, Cre-/TMX-). Mean \pm SEM, $n = 9$. (B) In static incubation experiments there was no significant difference in the insulin secretory response to physiological glucose concentrations between β -cell GPR54^{-/-} and Cre+/TMX- islets. (C) In perfusion experiments there was no significant difference in basal insulin secretion at 2mM glucose or first or second phase insulin secretion in response to 20mM glucose in β -cell GPR54^{-/-} compared to Cre+/TMX- islets. Exposure of Cre+/TMX- islets to kisspeptin (1 μ M, 30-50min) resulted in a sustained enhancement of second phase insulin secretion for the duration of kisspeptin administration. However, in β -cell GPR54^{-/-} islets this response was transient and not maintained beyond 10 minutes as determined by comparison of individual time points and (D) area under curve over 10 minute phases of the perfusion (students t-test, KP – prolonged response $p = 0.007$). Mean \pm SEM, panel A: $n = 8$, panel B: $n = 7-8$, panels C-D: $n = 4$, data representative of experiments conducted 2-3 times, * $P < 0.05$.

Figure 4: Glucose tolerance in female β -cell GPR54^{-/-} mice. (A) There was no significant difference in glucose tolerance between non-pregnant adult female β -cell GPR54^{-/-} mice and any of the female control groups (Cre+/TMX-, Cre-/TMX+, Cre-/TMX-). Similarly, the β -cell GPR54^{-/-} mice did not have (B)

significantly altered plasma insulin levels, either fasted or post-glucose, or (C) any change in insulin resistance when compared to any control group. (D) At day 16 of pregnancy β -cell GPR54^{-/-} mice had significantly impaired glucose tolerance at 15 and 30 minutes post-glucose administration (two-way RM ANOVA, 15min $p=0.028$, 30min $p=0.014$) and (E) glucose area under curve (AUC) over the course of the test when compared to all control groups (one-way ANOVA, 0-120min AUC $p=0.02$, 0-60min AUC $p<0.001$). (F) Pregnant β -cell GPR54^{-/-} mice did not have significantly altered basal fasting plasma insulin levels at day 16 of pregnancy, however GPR54 knockdown did result in significantly reduced insulin release in response to i.p. glucose administration (2g/kg) after 30min when compared with all controls (one-way ANOVA, $p=0.045$). (G) Pregnant β -cell GPR54^{-/-} mice did not have significantly different insulin sensitivity either at any individual time point or in (H) glucose AUC. Mean \pm SEM, panels A-C: $n=7-9$, panels D-H: $n=7-12$, * $P<0.05$.

Figure 5: Effects of deletion of β -cell GPR54 on pregnant β -cell mass. Representative illustrative images of immunostaining for the measurement of β -cell proliferation in pregnant (A) β -cell GPR54^{-/-} and (B) Cre⁺/TMX⁻ islets showing merged bromodeoxyuridine (BrdU) staining (green) and insulin staining (red). Scale bars = 100 μ m. (C) β -cell GPR54^{-/-} mice administered with BrdU from day 10-18 of pregnancy had significantly reduced levels of BrdU labeling compared to pregnant Cre⁺/TMX⁻ mice (students t-test, $p=0.041$). At day 18 of pregnancy there was no significant difference in either (D) mean overall islet area or (E) size of individual β -cells between β -cell GPR54^{-/-} and Cre⁺/TMX⁻ mice. Mean \pm SEM, panels C-E: $n=6$, * $p<0.05$.

Figure 6: Relationships between kisspeptin and insulin response to oral glucose and presence of GDM in pregnant women. In pregnant women undergoing a routine 75g oral glucose tolerance test (OGTT), women with gestational diabetes mellitus (GDM, IADPSG 2010 criteria, $n=26$) had significantly lower kisspeptin than women without GDM ($n=62$, * $p=0.0022$, students t-test) (A). Analyzing all pregnant women ($n=91$) there was no significant correlation between kisspeptin levels and fasting serum insulin (B), but there was a significant positive correlation between kisspeptin and serum insulin at 60 min (D, $r^2=0.1757$, $p<0.0001$) and between kisspeptin and area under curve serum insulin over the OGTT (F, $r^2=0.1279$, $p=0.0013$). There was no significant correlation between kisspeptin and serum insulin at 10min (C) or 120min (E). There was a significant positive correlation between kisspeptin and HOMA2-%B (G, $r^2=0.0656$, $p=0.0411$), but no significant correlations between kisspeptin and HOMA2-IR (H) or the Matsuda index (I). Women diagnosed with GDM are represented by white markers, women without GDM are represented by black markers. Pearson product-moment correlation coefficient was used for analysing correlation data and presented correlation data is based on all women.

Figure 1

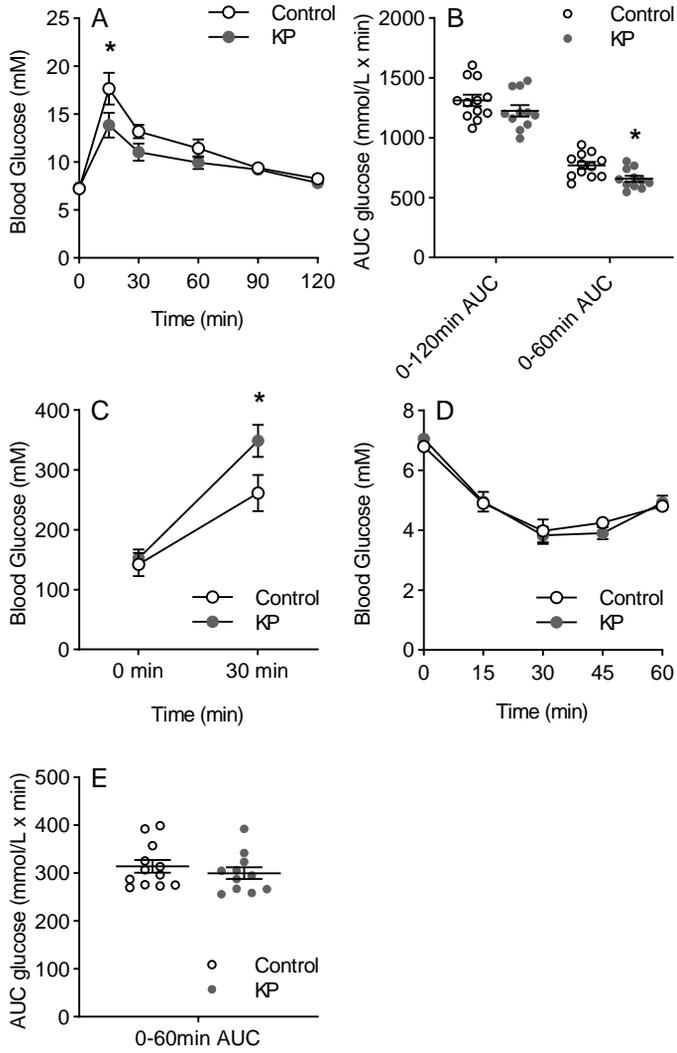


Figure 2

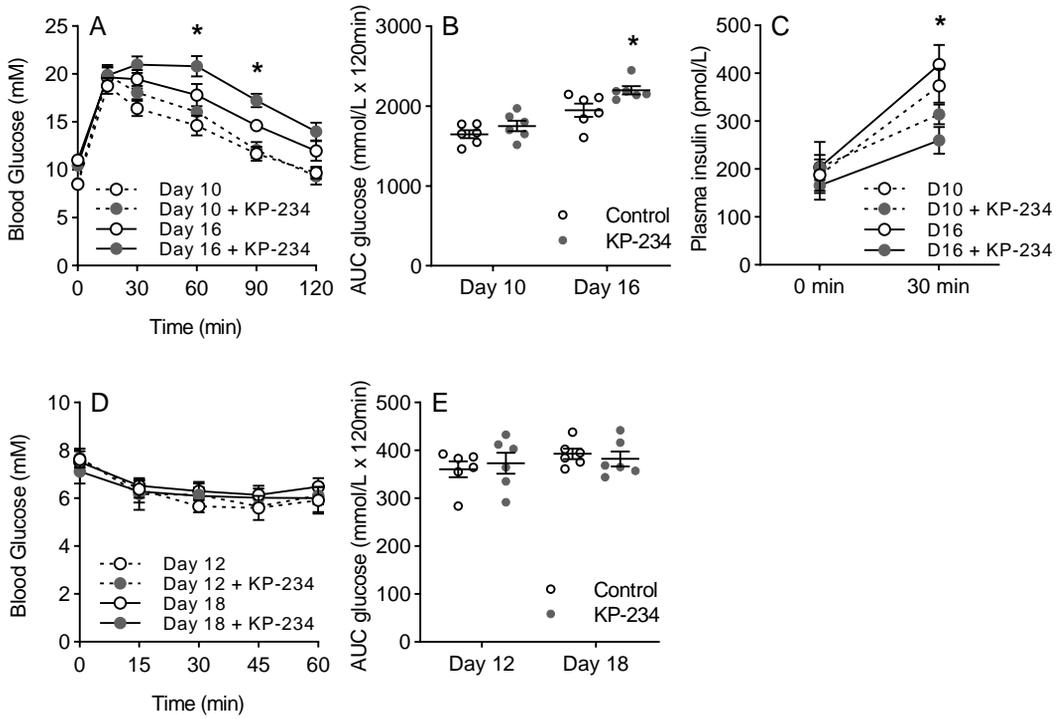


Figure 3

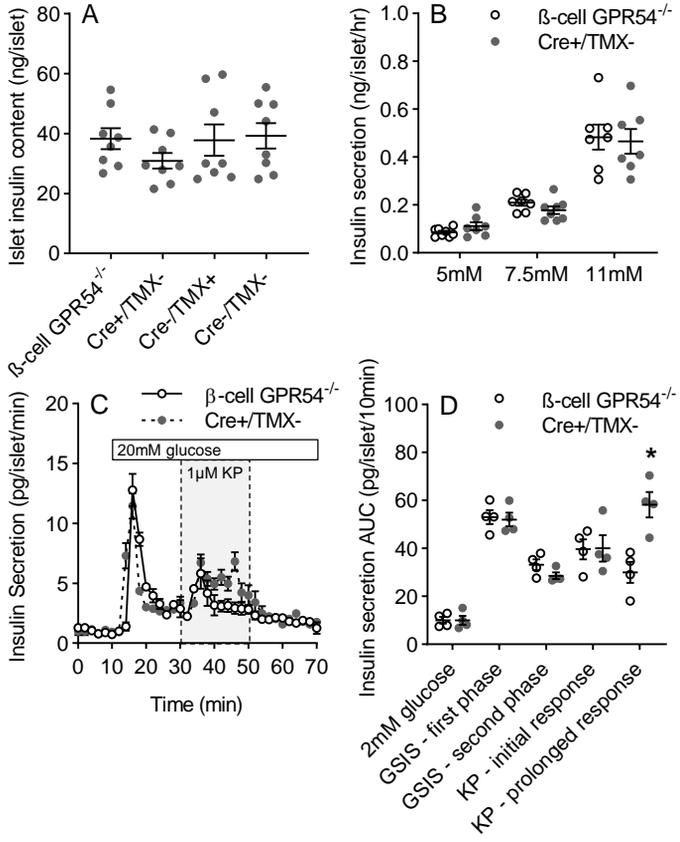


Figure 4

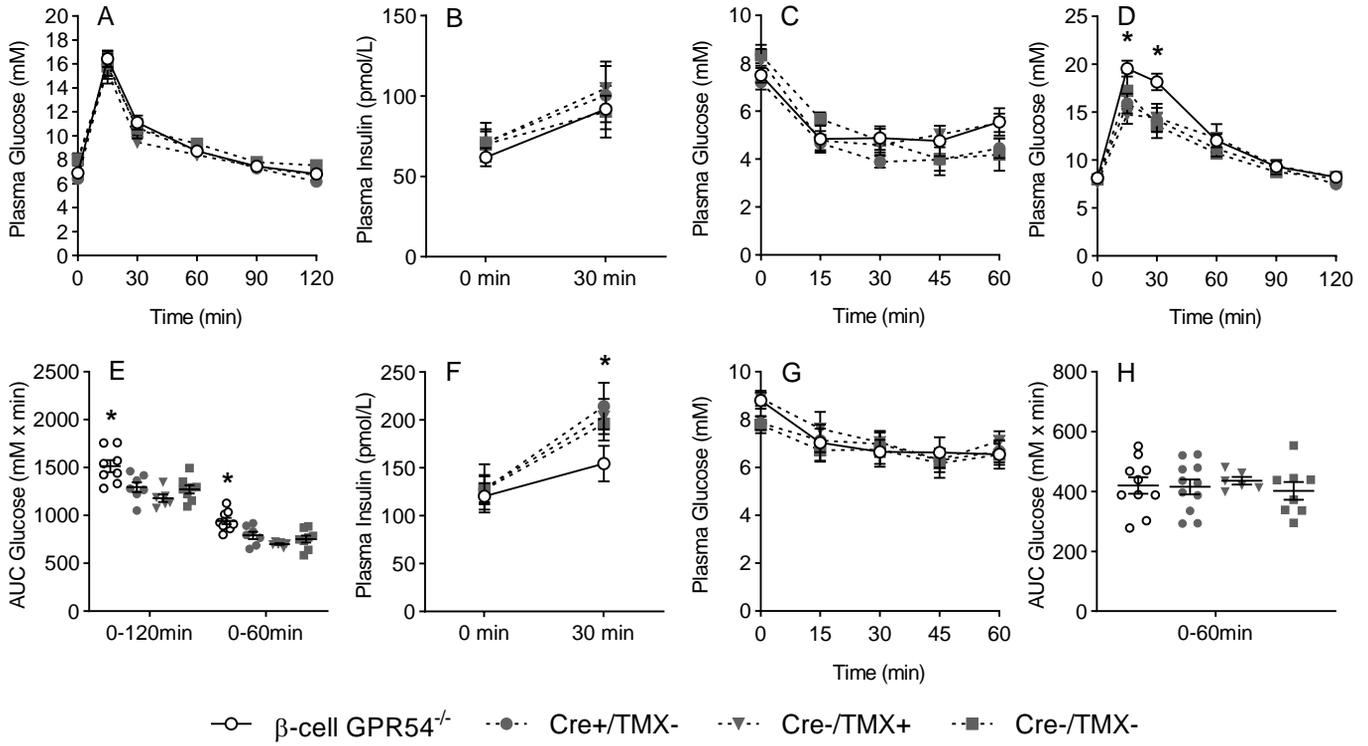


Figure 5

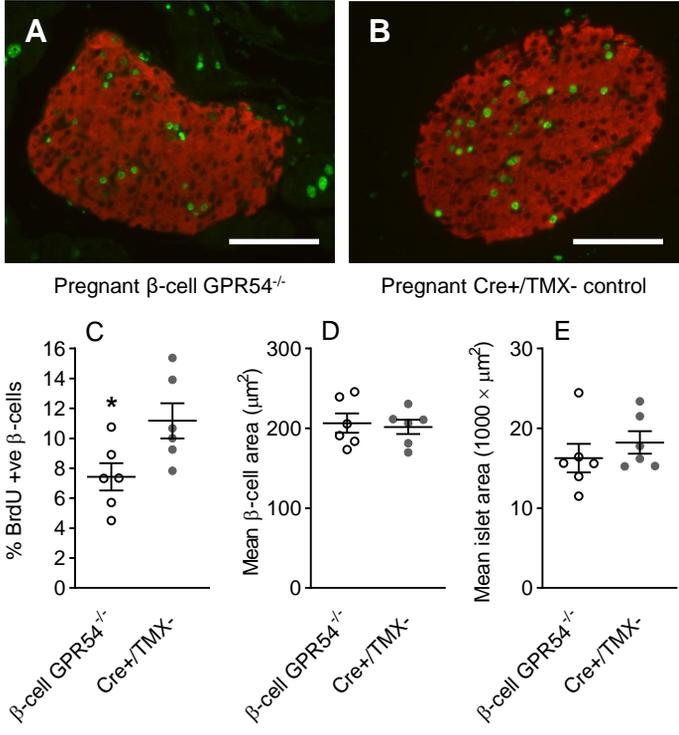


Figure 6

