Original Research

Increased alpha and beta cell mass during mouse pregnancy is not dependent on transdifferentiation

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Impact statement

Gestational diabetes mellitus (GDM) is an increasingly prevalent form of diabetes, resulting from suboptimal endocrine adaptations in pregnancy. Unfortunately, GDM results in both short- and long-term health repercussions for mother and child. The purpose of this study was to investigate α-cell plasticity and α- to β-cell trans-differentiation in order to provide insights into potential impairments of adaptations in endocrine pancreas in GDM. To the best of our knowledge, we are the first to implicate impairments in adaptations of pancreatic α-cells in pregnancy to glucose intolerance in a rodent model of GDM. Although we concluded no contribution of α- to β-cell transdifferentiation in pregnancy, we identified cells in a transitional stage of this process in pregnancy. This study provides insights into mechanisms of suboptimal endocrine pancreas adaptations in GDM. Our findings highlight strategies that could prove useful in evaluating pharmacological interventions aimed at safely decreasing hyperglycemia during GDM.

Abstract

Maternal pancreatic beta-cell mass (BCM) increases during pregnancy to compensate for relative insulin resistance. If BCM expansion is suboptimal, gestational diabetes mellitus can develop. Alpha-cell mass (ACM) also changes during pregnancy, but there is a lack of information about α-cell plasticity in pregnancy and whether α- to β-cell transdifferentiation can occur. To investigate this, we used a mouse model of gestational glucose intolerance induced by feeding low-protein (LP) diet from conception until weaning and compared pregnant female offspring to control diet-fed animals. Control and LP pancreata were collected for immunohistochemical analysis and serum glucagon levels were measured. In order to lineage trace α- to β-cell conversion, we utilized transgenic mice expressing yellow fluorescent protein behind the proglucagon gene promoter (Gcg-Cre/YFP) and collected pancreata for histology at various gestational timepoints. Alpha-cell proliferation increased significantly at gestational day (GD) 9.5 in control pregnancies resulting in an increased ACM at GD18.5, and this was significantly reduced in LP animals. Despite these changes, serum glucagon was higher in LP mice at GD18.5. Pregnant Gcg-Cre/ YFP mice showed no increase in the abundance of insulin$^+$YFP$^+$glucagon$^-$ cells (phenotypic β-cells). A second population of insulin$^+$YFP$^+$glucagon$^+$ cells was identified which also did not alter during pregnancy. However, there was an altered anatomical distribution within islets with fewer insulin$^+$YFP$^+$glucagon$^-$ cells but more insulin$^+$YFP$^+$glucagon$^+$ cells being present in the islet mantle at GD18.5. These findings demonstrate that dynamic changes in ACM occur during normal pregnancy and were altered in glucose-intolerant pregnancies.

Keywords: Pancreas, alpha-cell, beta-cell, pregnancy, mouse, gestational diabetes

Introduction

Pregnancy is a physiological state characterized by relative maternal insulin resistance.[1] This has been linked to the presence of placentally-derived hormones and cytokines in the maternal circulation in the second half of pregnancy.[2] In preparation for the increased demand for insulin, adaptive changes occur in the endocrine pancreas in order to maintain euglycemia whilst also supplying the growing fetus with an adequate nutrient supply. A reversible expansion of beta-cell mass (BCM) has been documented in both rodents and humans and is maximal at late gestation (around gestational day [GD] 18.5 in rodents).[3–8] In situations where BCM expansion is suboptimal, gestational diabetes mellitus (GDM) can develop. This has been demonstrated in both clinical studies[9] and animal models of GDM[3,8,10–13] implicating β-cell failure as a major driver to metabolic pathogenesis. GDM is described as diabetes that first appears during pregnancy, which regresses postpartum in most cases. Nonetheless, GDM is associated with adverse short- and long-term fetal and maternal health outcomes[14–18] necessitating the development of effective methods of intervention. Current treatments for GDM, such as

lifestyle behavioral change or administration of insulin or metformin, aim to decrease hyperglycemia but do not treat the underlying causes including a suboptimal BCM. Thus, a better understanding of mechanisms of BCM expansion in pregnancy is needed in order to effectively target potential therapeutic interventions.

The adaptive mechanisms of BCM expansion during rodent pregnancy have been shown to involve a re-entry of normally quiescent pre-existing β-cells into cell replication, mediated in part through prolactin receptor signaling in response to lactogenic hormones,[12,19,20] in addition to increased β-cell hypertrophy.[10] These processes are maximal around midgestation in rodents to prepare the pancreas for enhanced glucose-stimulated insulin release in late pregnancy.[7,8] Additional mechanisms of BCM expansion are likely to include the expansion and subsequent differentiation of a multipotent β-cell progenitor pool expressing some insulin but low levels of glucose-transporter 2 (Ins$^+$Glut2^{LO}).[7] Ins$^+$Glut2^{LO} cells are able to differentiate into mature β-cells under metabolic stress.[21,22] Pregnant mice were shown to have a higher proportion of proliferating Ins$^+$Glut2^{LO} cells at GD9.5, preceding maximal β-cell proliferation at GD12.5. This was concurrent with increased *Pdx1* mRNA expression, marking endocrine progenitor and mature β-cells, implicating this progenitor pool to BCM expansion during pregnancy. The contribution of non-β-cell progenitors to gestational BCM expansion has also been proposed and could contribute up to 25% of new β-cells in pregnancy.[23,24] An increase in the number of islets during rodent pregnancy has also been documented, providing further support for a contribution of islet neogenesis.[7,8,25] Furthermore, there were fewer small-sized islets throughout pregnancy in glucose-intolerant pregnant mice, implicating a potential critical role for a deficiency of β-cell neogenesis to the development of glucose intolerance.[8] Although evidence exists to support expansion of BCM in pregnant humans, due to the scarcity of human samples, the mechanisms involved in BCM expansion remain unclear and controversial.[6,26] As such, the scarcity of pregnant human pancreas samples implicates the reliance on animal models of diabetes in pregnancy.

Although there is evidence that α-cells contribute to hyperglycemia in patients with type 2 diabetes mellitus (T2DM) *via* hyperglucagonemia,[27-31] the dynamics of pancreatic α-cells in pregnancy have only recently been explored.[8,32] The changes in α-cell abundance during the endocrine adaptation to pregnancy were described with an expansion of α-cell mass (ACM) at GD18.5 in mice[8,32] which was impaired in glucose-intolerant pregnancies.[8] One source of new β-cells during pregnancy could derive from a molecular re-programming of glucagon-producing α-cells as part of dynamic changes in the α-cell population. Previously it was shown that α-cells can replenish β-cells following extreme β-cell loss or during β-cell stress by α- to β-cell transdifferentiation.[33,34] Quesada-Candela *et al.*[32] suggested that a negligible amount of α- to β-cell transdifferentiation was occurring at GD18.5 in normal pregnancy compared to non-pregnant mice; however, genetic lineage tracing of α-cells was not performed to confirm this, and earlier timepoints in pregnancy were not examined. As this study was performed in normal pregnancy, there remains a lack of information about α-cell plasticity in the development of GDM. In this study, we aimed to address these knowledge gaps by (1) documenting changes in the balance of α- and β-cells in control compared to glucose-intolerant mouse pregnancy and (2) elucidating any temporal changes in α- to β-cell transdifferentiation in normal mouse pregnancy using genetic lineage tracing. We hypothesized that one of the putative mechanisms related to the adaptational endocrine increase in β-cells in pregnancy could be the transdifferentiation of α-cells into β-cells and that any disbalance in this process will predispose to GDM.

Materials and methods

Animals and sample collection

All animal procedures were approved by the Animal Care Committee of Western University in accordance with the guidelines of the Canadian Council for Animal Care. Mice were housed in a temperature-controlled room with 12-h light:dark cycle at Lawson Health Research Institute, London, ON, Canada. Water and food were given *ad libitum*.

Aim 1. Adult (six-week-old) C57BL/6 male and female (F0) mice were obtained from Charles River Laboratories (Wilmington, MA, USA). Mice showing gestational glucose intolerance at GD18.5 were generated using a previously described protocol involving a dietary insult during early life.[8] Briefly, F0 females underwent estrous cycling and were time-mated with males. Dams were randomly assigned to either a control (C, 20% protein, Bio-Serv, Frenchtown, NJ, USA) or a low-protein (LP, 8%) diet similar to that described by Snoeck *et al.*[35] The two diets were iso-calorific, the deficiency in calories in the LP diet being compensated by additional carbohydrate.[8] F0 dams were fed either the LP or C diet throughout gestation and lactation, and female offspring (F1) were weaned onto C diet. At maturity (postnatal day [PND] 42), female offspring (F1) of LP and C diet-fed mothers were randomly allocated into two study groups: pregnant or non-pregnant. All pregnant-grouped females were time-mated (GD9.5, 12.5, 18.5) with C diet-fed males. These timepoints were chosen based on previous studies where significant changes have been observed in the endocrine pancreas during mouse pregnancy, including proliferation of Ins$^+$Glut2^{LO} β-cell progenitors which is maximal at GD9.5[7] and β-cell proliferation which is maximal at GD12.5.[7,8] GD18.5 was chosen as this is the timepoint where BCM is maximal in a control pregnancy[7,8] but has been shown to be reduced in GDM pregnancy resulting in glucose intolerance.[8] Females (F1) were euthanized by CO_2 asphyxia for comparison to non-pregnant age-matched F1 females. The pancreas was removed at each assigned day of gestation ($n = 4$–6 C and LP animals for each timepoint during gestation and for the non-pregnant groups), fixed in 4% paraformaldehyde for histology and embedded in optical cutting temperature compound.

Aim 2. Glucagon-Cre[iCre] mice (stock #030663, Jackson Laboratories, Bar Harbor, Maine, USA) that express Cre in 93–95% of α-cells were crossed with a Rosa26-eYFP reporter mouse strain (stock #006148, Jackson Laboratories) to produce double transgenic Glucagon-Cre/Rosa26-eYFP (Gcg-Cre/YFP) mice. At maturity, double transgenic female offspring were randomly separated into two study groups: pregnant and non-pregnant. Pregnant-grouped females underwent estrous cycling in order to produce timed pregnancies.[36] Individual double transgenic female and wild-type C57BL/6 male mice (Charles River Laboratories, Wilmington, MA, USA) were housed together the morning of pro-estrous for mating and were separated the following morning. Females in the non-pregnant group were age-matched to animals in the pregnant group (GD9.5, 12.5, and 18.5). Animals were euthanized by CO_2 asphyxia, and the pancreas was removed at each assigned day of gestation ($n = 4$ animals for each timepoint during gestation and $n = 8$ animals for the non-pregnant group) and prepared for histology as described above.

Immunofluorescence staining

Fixed pancreas tissue was prepared and sectioned as previously described.[37] At least two 7-µm-thick cryosections (replicates) were cut from each pancreas for immunohistochemical analysis. The interval between each section was >100 µm, representing at least two longitudinal slices through the pancreas. Sections included both the head and tail of the pancreas. For aim 1, immunofluorescence immunohistochemistry was performed to localize insulin, glucagon, and Ki-67 as described previously.[7,8] Antibodies against insulin (1:2000, antimouse, Sigma-Aldrich, St. Louis, MO, USA) and glucagon (1:200, antirabbit, Santa Cruz Biotechnology, Dallas, TX, USA) were applied to tissues and incubated overnight at 4°C. To investigate α-cell proliferation, antibodies against glucagon (1:200, antirabbit, Santa Cruz Biotechnology) and Ki-67 (1:50, antimouse, Biosciences, Mississauga, ON, Canada) were applied to tissues and incubated overnight at 4°C. The following day, secondary antibodies (1:500 Thermo Fisher Scientific Waltham, MA, USA) were applied against the primary antibodies using 555 and 488 fluorophores, respectively, along with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (1:500, Thermo Fisher Scientific) to counterstain nuclei. ACM data were retrieved from our previous publication[8] and calculated by multiplying the fractional α-cell area (sum of all glucagon-expressing areas divided by the whole pancreas surface area) by pancreas weight. For aim 2, fluorescent immunohistochemistry was performed to localize insulin (phenotypic β-cells), glucagon (phenotypic α-cells), and YFP (α-cell origin) for cell counting analysis. Background Sniper (Biocare Medical, Concord, CA, USA) was applied to each tissue section for 8 min to reduce non-specific background binding. Subsequently, antibodies against insulin (1:50, anti-guinea pig, Abcam, Cambridge, UK), glucagon (1:2000, antimouse, Sigma-Aldrich), and YFP (1:1000, antirabbit, Abcam) were applied to tissue sections and incubated overnight at 4°C. The following day,

secondary antibodies (1:400 Thermo Fisher Scientific) were applied against the primary antibodies using 555, 647, 488 fluorophores, respectively, along with DAPI (1:500, Thermo Fisher Scientific) to counterstain nuclei.

Cell counting analysis

Tissue sections were visualized by a blinded technician at 20× using a Nikon Eclipse TS2R inverted microscope (Nikon, Minato, Tokyo, Japan) with the program NIS elements (Nikon, Minato, Tokyo, Japan), and images were captured and analyzed using cell counter on ImageJ software. Every insulin-, glucagon-, and YFP-expressing cell was imaged for each section and for each animal. In this study, an "islet" was considered to contain >5 β-cells and an extra-islet endocrine "cluster" as containing 1–5 β-cells.[37] For aim 1, manual cell counting analysis determined the percentage of Insulin+Glucagon+ (insulin and glucagon double-positive) cells as a marker for α- to β-cell transitional cells.[32,38–40] Alpha-cell proliferation was determined by manually counting glucagon and Ki-67 double-positive cells. For aim 2, manual cell counting analysis determined the percentage of Insulin+YFP+Glucagon+ cells as a marker for a possible intermediate, transitional cell type between an α-cell and a β-cell (Figure 1). The percent Insulin+YFP+Glucagon− cells was also determined to identify phenotypic β-cells arising from an α-cell origin. These cells were further localized as either being in the islet core or mantle, following comparable criteria to a study where the mantle was considered as a region ~20µm deep following the external perimeter of islets.[41] Co-localized cells that were part of the outermost layer of Insulin+ cells within each islet were classified as being part of the islet's mantle. Any co-localized cells that were closer to the middle of the islet, and therefore surrounded by this outer layer of Insulin+ cells, were classified as being part of the islet core. While islets are large enough to be able to break down into either core or mantle components, clusters are not. As each cluster of cells is only made up of 1–5 Insulin+ cells, no definitive outer layer of cells exists within this structure. Therefore, the division of co-localized cells into core and mantle layers was only feasible in "islets," which are each composed of 6 or more Insulin+ cells. The core and mantle analysis was completed for both Insulin+YFP+Glucagon+ cells and Insulin+YFP+Glucagon− cells. For the core and mantle calculations, the total number of Insulin+YFP+Glucagon− or Insulin+YFP+Glucagon+ cells that fell within either the core or the mantle were divided by the total number of Insulin+YFP+Glucagon+ or Insulin+YFP+Glucagon− cells counted for the tissue section.

Serum enzyme-linked immunosorbent assay

For aim 1, maternal (F1) postprandial blood samples were collected *via* cardiac puncture after euthanasia following an intraperitoneal glucose tolerance test (IPGTT) in order to quantify serum insulin and glucagon using an Ultra-Sensitive Mouse Insulin Enzyme-Linked Immunosorbent Assay (ELISA) kit (Crystal Chem, Downers Grove, IL, USA) or Mouse Glucagon ELISA kit (Crystal Chem),
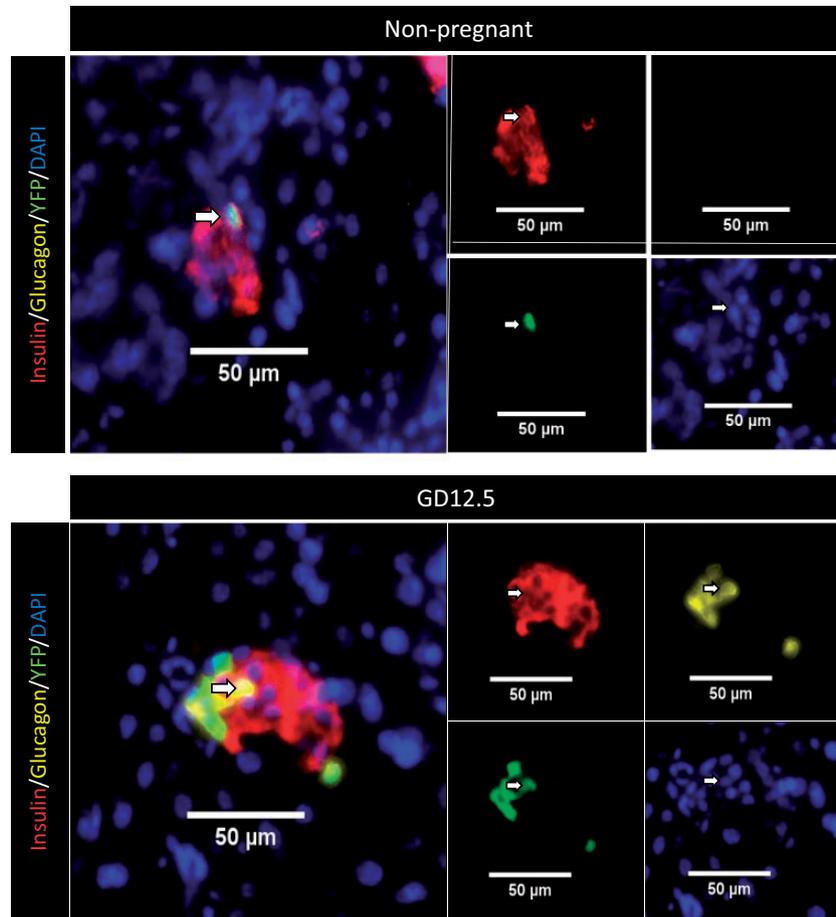
Figure 1. Representative images demonstrating staining for insulin (red), glucagon (yellow), YFP (green), and nuclei (DAPI, blue) in pancreatic sections from GlucagonCre-YFP transgenic female mice. The arrow in the non-pregnant islet represents a β-cell arising from an α-cell that no longer expresses glucagon (Insulin$^+$YFP$^+$Glucagon$^-$). The arrow in the GD12.5 islet represents an Insulin$^+$YFP$^+$Glucagon$^+$ cell. (A color version of this figure is available in the online journal.) YFP: yellow fluorescent protein; DAPI: 4′,6-diamidino-2-phenylindole dihydrochloride; GD: gestational day.

respectively. The insulin assay has a sensitivity of 0.05 ng/ mL using a 5 µL sample with precision coefficient of variation (CV) $\leq 10.0\%$. The glucagon assay has a sensitivity of 1.1 pg/mL using a 10 µL sample with precision CV $\leq 10\%$. Samples were run in duplicate. Data were collected using a BioRad iMark plate reader and analyzed using Microplate Manager Software. Data throughout pregnancy were compared to non-pregnant animals, as a previous study[32] found that mice showed hypoglucagonemia as they entered pregnancy. Therefore, we compared the data as a percent change relative to non-pregnant animals, to determine how the animals adapt pancreatic α-cells in response to pregnancy.

Statistical analysis

The sample size of four to six animals per variable in either the LP or C groups was calculated based on achieving a statistically significant difference with an expected standard deviation around mean values for BCM and glucose tolerance of 15% or less based on our previous studies.[7] Data are presented as mean $\pm$ standard error of the mean (SEM), with statistics analyzed using GraphPad Prism software (Version 5.0). An unpaired two-tailed Student's *t* test, one-way analysis of variance (ANOVA), or two-way

ANOVA were applied according to the set of groups that were compared. A Tukey's post hoc test or a Bonferroni post hoc test was performed after one-way ANOVA or two-way ANOVA analysis, respectively. Non-parametric tests were performed when data did not meet the assumption of normality. Significant outliers were determined using Grubbs' test for each parameter. Each animal presented as a single unit of analysis (*n*). Statistical significance was determined as $P < 0.05$.

Results

Glucagon presence and α-cell proliferation in control versus glucose-intolerant pregnancies

We examined the changes in α-cell presence and function during pregnancy, and particularly the cells co-staining for insulin and glucagon, comparing normal pregnancies and those previously shown by us to have impaired gestational glucose tolerance with a decreased BCM.[8] First, we measured postprandial insulin and glucagon samples from serum collected after an IPGTT throughout pregnancy (GD9.5, 12.5, and 18.5) and compared these values to non-pregnant animals. Raw values, not expressed relative to non-pregnant animals, are shown in Supplemental Figure
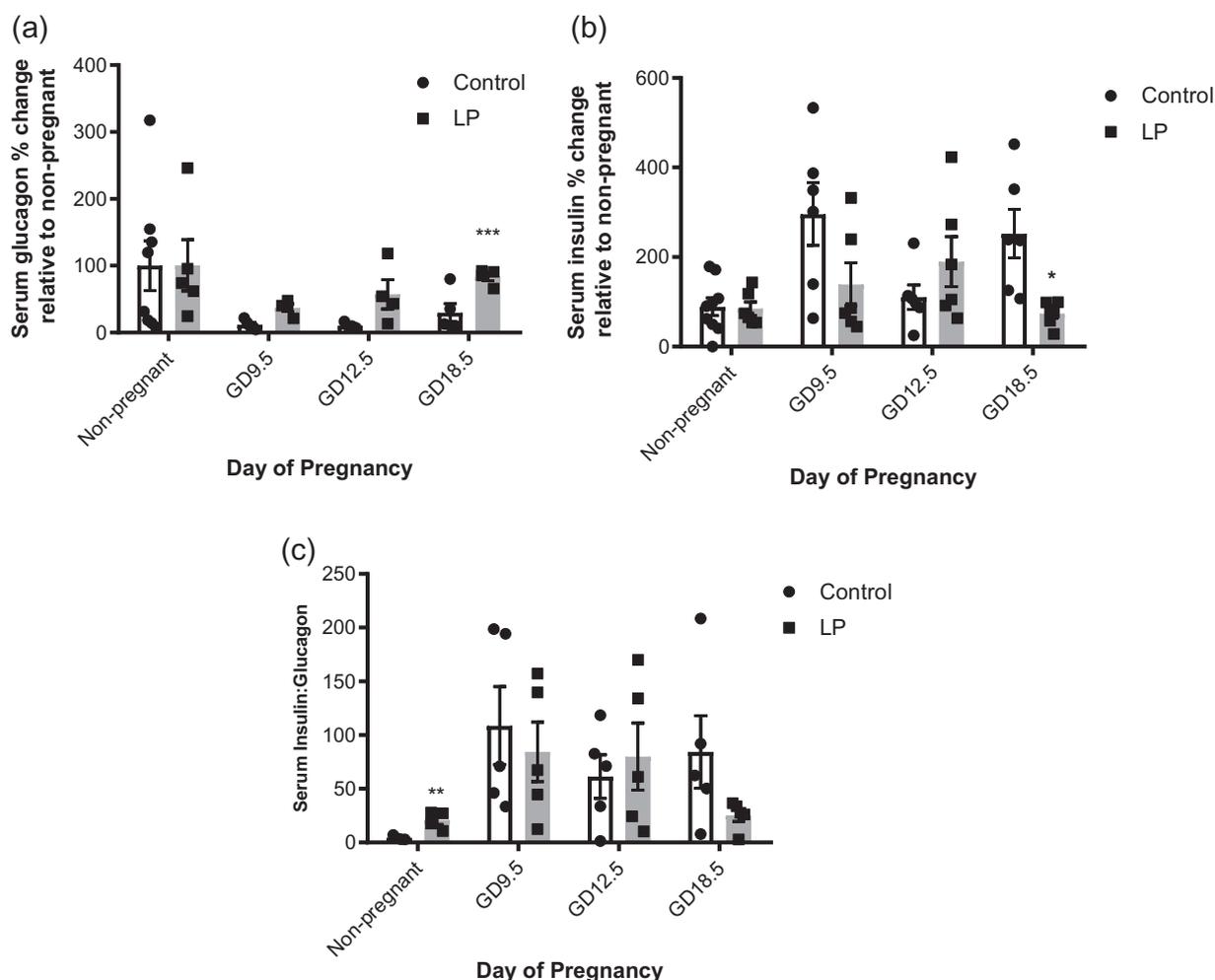
Figure 2. Circulating levels of glucagon and insulin for various timepoints throughout pregnancy in control and LP animals, and changes in ratio of insulin to glucagon. (a) Serum glucagon and (b) insulin levels are shown as a percentage change relative to non-pregnant animals for the gestational days indicated. (c) Serum insulin to glucagon ratio throughout pregnancy. Samples were collected after euthanasia *via* cardiac puncture following an intraperitoneal glucose tolerance test. Mean $\pm$ SEM values are shown together with individual data points, $n = 4$–6 C and LP animals, ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$. GD: gestational day; LP: low protein.

1. During pregnancy, both control and LP mice exhibited hypoglucagonemia relative to non-pregnant animals (Figure 2(a)). However, the LP diet group showed a significantly greater serum glucagon presence (Figure 2(a)) and lower serum insulin (Figure 2(b)) in late gestation compared to control-diet animals when expressed relative to the values in treatment-matched non-pregnant animals. Nonetheless, the overall serum insulin/glucagon ratio did not change during pregnancy between treatment groups, although values were higher throughout pregnancy compared to non-pregnant animals (Figure 2(c)). This indicates that an increase in both circulating insulin and glucagon occurs during pregnancy but with relatively more insulin.

When the ontogeny of α-cell proliferation was examined during pregnancy, a significant increase was seen at GD9.5 across the whole pancreas compared to prepregnancy in control animals, although this subsequently declined (Figure 3(a)). However, proliferating α-cells were significantly reduced in extra-islet clusters at GD9.5 in the LP diet group relative to controls (Figure 3(b)). ACM changed across gestation in both control and LP groups ($P = 0.01$,

Figure 3(c)). However, ACM was significantly reduced in the LP group compared to control animals at GD18.5 (Figure 3(c)).

The balance of pancreatic α- and β-cells in control versus glucose-intolerant pregnancies

The frequency of insulin-staining cells that also contained glucagon was approximately 15% in non-pregnant control-diet mice (Figure 4(a)), but the abundance of such bihormonal cells in whole pancreas, islets, or extra-islet clusters did not change in control animals during pregnancy and also did not differ in the LP diet group (Figure 4(a) to (c)). However, the LP mice did enter pregnancy with a pre-existing reduction in the number of such cells compared to controls. Furthermore, there was a trend towards fewer dual-stained cells at GD12.5 in LP versus control animals, suggesting that this potential lack of plasticity remained throughout pregnancy (Figure 4(a), $P = 0.087$). Notably, there was a transient decrease of ~50% of such cells in control pregnancies at GD9.5 (non-pregnant 16 ± 3% to GD9.5 7 ± 1%) prior to replenishment of these cells by GD12.5/
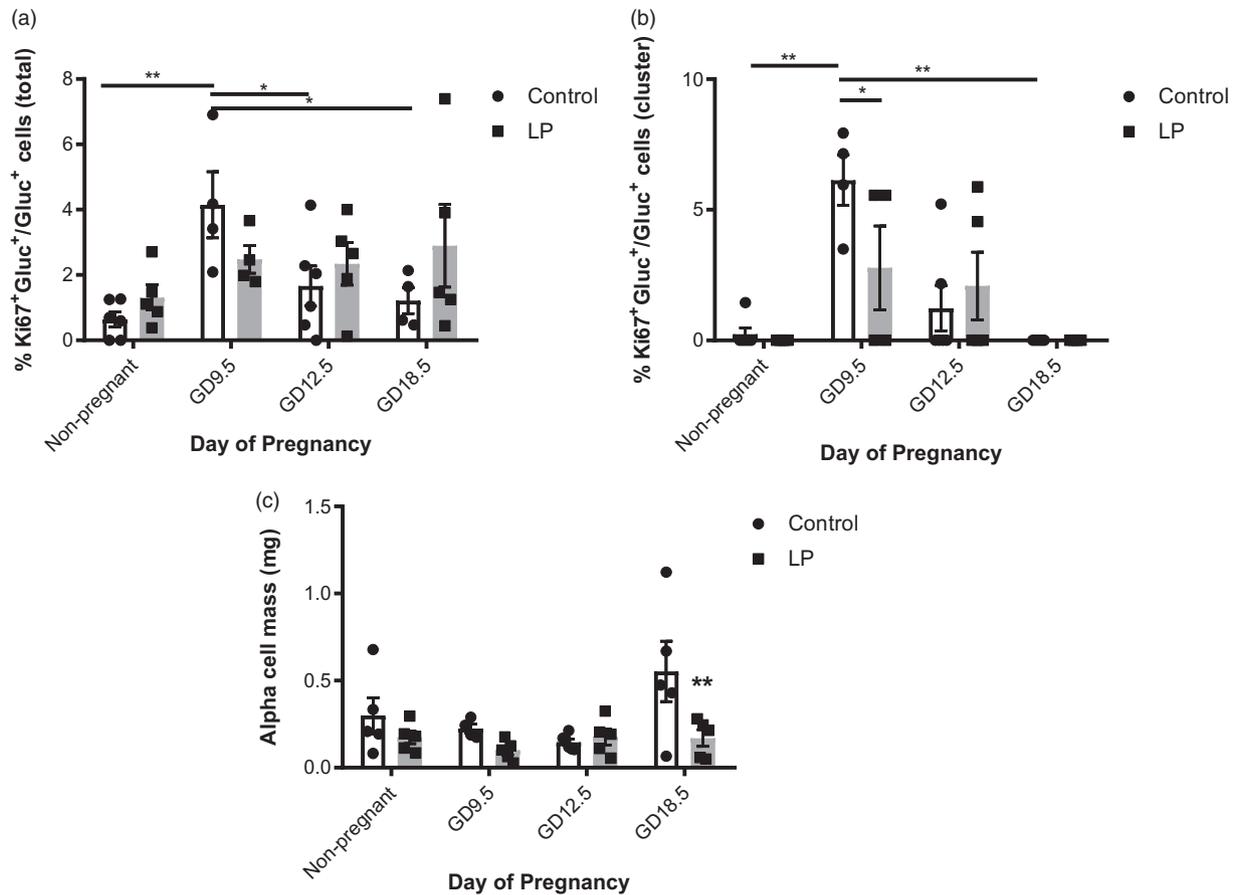
Figure 3. Alpha-cell proliferation measured by the nuclear presence of Ki67 in control and glucose-intolerant (LP-treated) mouse pregnancy at the gestational ages indicated. The percentage of proliferating α-cells in (a) whole pancreas and (b) extra-islet clusters. (c) α-cell mass in control and LP pregnancy is shown relative to all glucagon immunopositive cells. Mean $\pm$ SEM values are shown together with individual data points, $n = 4$–6 C and 4–5 LP animals, **$P < 0.01$, *$P < 0.05$. GD: gestational day; LP: low protein.

18.5, potentially implicating a burst of α- to β-cell transdifferentiation at GD9.5. This relative decrease in dual-stained cells was absent in LP dams at GD9.5 and could implicate α- to β-cell transdifferentiation as a mechanism to increased BCM expansion during gestation in control animals that was impaired in LP females.

The contribution of α- to β-cell transdifferentiation to new β-cells in control pregnancy

To address this question, we investigated the contribution of α- to β-cell transdifferentiation to BCM expansion in the pancreas during pregnancy by immunostaining histological sections of Gcg-Cre/YFP mouse pancreata for YFP, glucagon, and insulin. By using Gcg-Cre/YFP transgenic mice, we were able to accurately lineage trace changes in the fate of glucagon-expressing pancreatic α-cells during the course of pregnancy to determine if some cells transdifferentiate to express insulin but not glucagon. First, co-localization of YFP with insulin in cells that did not contain glucagon (Insulin$^+$YFP$^+$Glucagon$^-$) was examined within the pregnant mouse pancreas at various timepoints throughout pregnancy. Co-localization was seen in a minority of cells in both islets and small extra-islet endocrine clusters. In non-pregnant mice, approximately 8% of insulin-staining cells also expressed YFP, and this did not

alter significantly throughout pregnancy when examined for the whole pancreas (Figure 5(a)) or considering islets alone (Figure 5(b)). Furthermore, the pattern for fold change relative to non-pregnant animals was also negligible in whole pancreas (Supplemental Figure 2). In extra-islet clusters, the frequency of insulin-staining cells co-expressing YFP was significantly reduced during pregnancy compared with non-pregnant animals (Figure 5(c)). Whilst the relative number of insulin-YFP dual-stained cells in islets did not change during pregnancy, the distribution did alter, with a relative reduction being seen in late gestation in the outer mantle of the islets relative to the islet core (Figure 5(d)).

Cells in a transitional stage of α- to β-cell transdifferentiation increase in the islet mantle at GD18.5

As would be expected in rodents, there were significantly more α-cells (70–80%) in the mantle compared to the core (Supplemental Figure 3). In addition to insulin-staining cells expressing YFP in the absence of glucagon (Insulin$^+$YFP$^+$Glucagon$^-$), an approximately equal number of cells co-stained for insulin, YFP, and glucagon in non-pregnant animals (Figure 6(a)). As observed above with the C and LP-diet animals, the relative abundance of
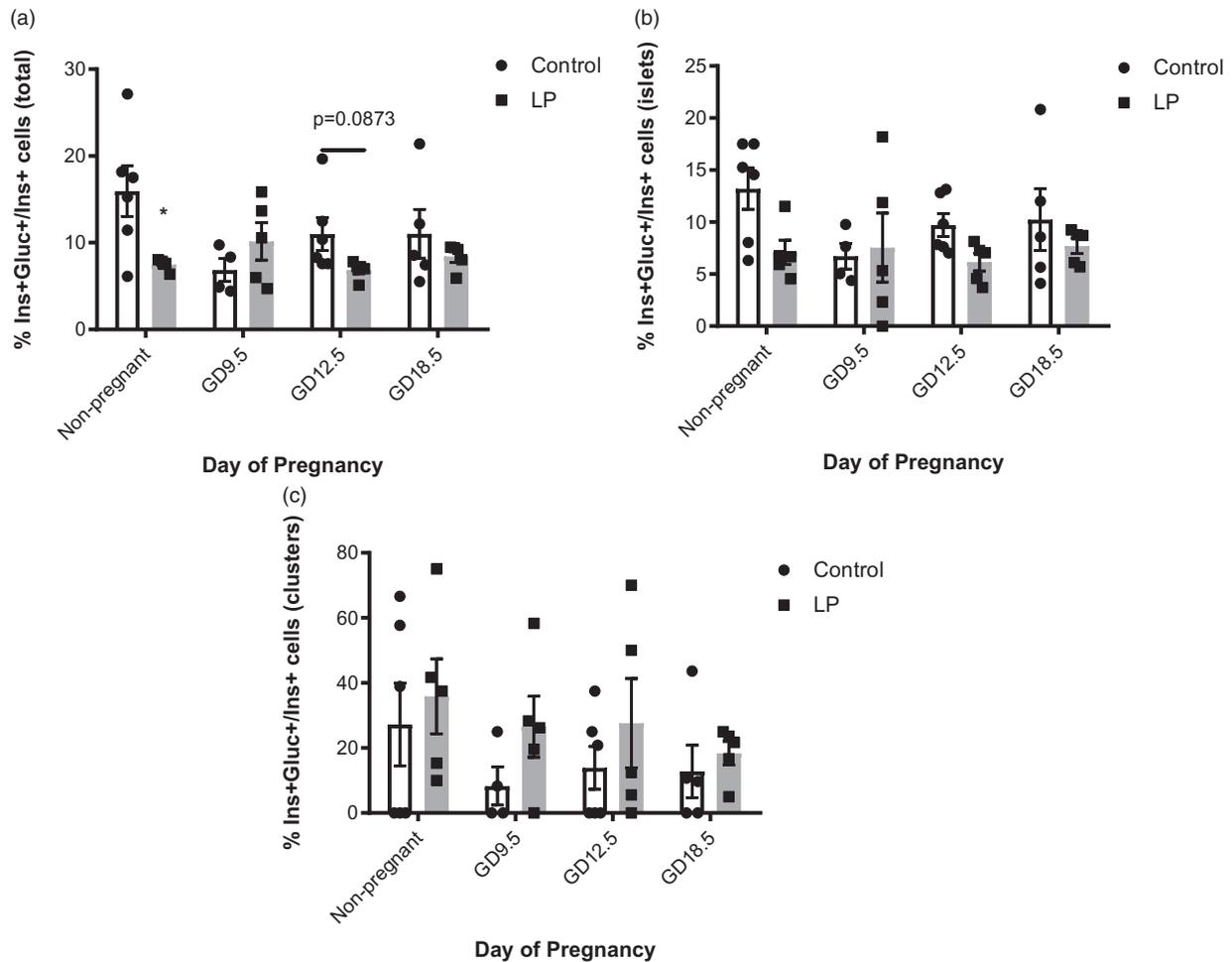
Figure 4. Ontogeny of bihormonal cells containing both glucagon and insulin in control and glucose-intolerant (LP-treated) pregnancies. The percentage of bihormonal cells is shown in (a) whole pancreas, (b) islets, and (c) extra-islet clusters relative to the total insulin immunopositive cells. Mean ± SEM values are shown together with individual data points, $n = 4–6$ C and 4–5 LP animals, *$P < 0.05$.
GD: gestational day; LP: low protein.

these cells did not change during pregnancy in either islets or extra-islet clusters (Figure 6(b) and (c)), but their relative anatomical distribution within islets did alter in late gestation with significantly more being observed in the islet mantle (Figure 6(d)).

Discussion

Pregnancy displays a remarkable reversible adaptation of BCM in order to maintain euglycemia; otherwise, pathologies such as GDM can arise. Although β-cells make up the majority of the islet, α-cells are the next most abundant cell type in the pancreas. It was originally believed that the development of mature α- and β-cells was static and encompassed a definitive lineage. However, studies have discovered that both α- and β-cells are able to interconvert between one another.[42] Remarkable pioneer experiments demonstrated that expression of β-cell specific transcription factors, such as *Pax4*, can cause conversion of α-cells into β-cells.[43] These two endocrine cells play a critical role in maintaining glucose homeostasis by functioning in an antagonistic manner, whereby the intra-islet hypothesis states that insulin inhibits glucagon secretion.[44] The

contribution of α-cells to hyperglycemia in patients with T2DM *via* hyperglucagonemia has been well documented.[27–31] However, much less is known regarding the plasticity of pancreatic α-cells in pregnancy, and this has yet to be investigated in glucose-intolerant pregnancy. In the present study, we demonstrated that pancreatic α-cells adapt to pregnancy in a healthy mouse pregnancy. However, dynamic changes in pancreatic α-cells were altered in glucose-intolerant mouse pregnancy. Since α-cells can act as a reservoir to increase β-cell regeneration *via* α- to β-cell transdifferentiation in non-pregnant animals,[42,45–47] it was also important to elucidate the role of this transdifferentiation in pregnancy. Although there were no temporal differences in α- to β-cell transdifferentiation during pregnancy, a second population of Insulin⁺YFP⁺Glucagon⁺ cells was identified and found to be enriched in the islet mantle at late gestation. This study provides mechanistic insights of suboptimal endocrine pancreas adaptations in GDM while also revealing an essential contribution of α-cells to these adaptations that was previously overlooked.

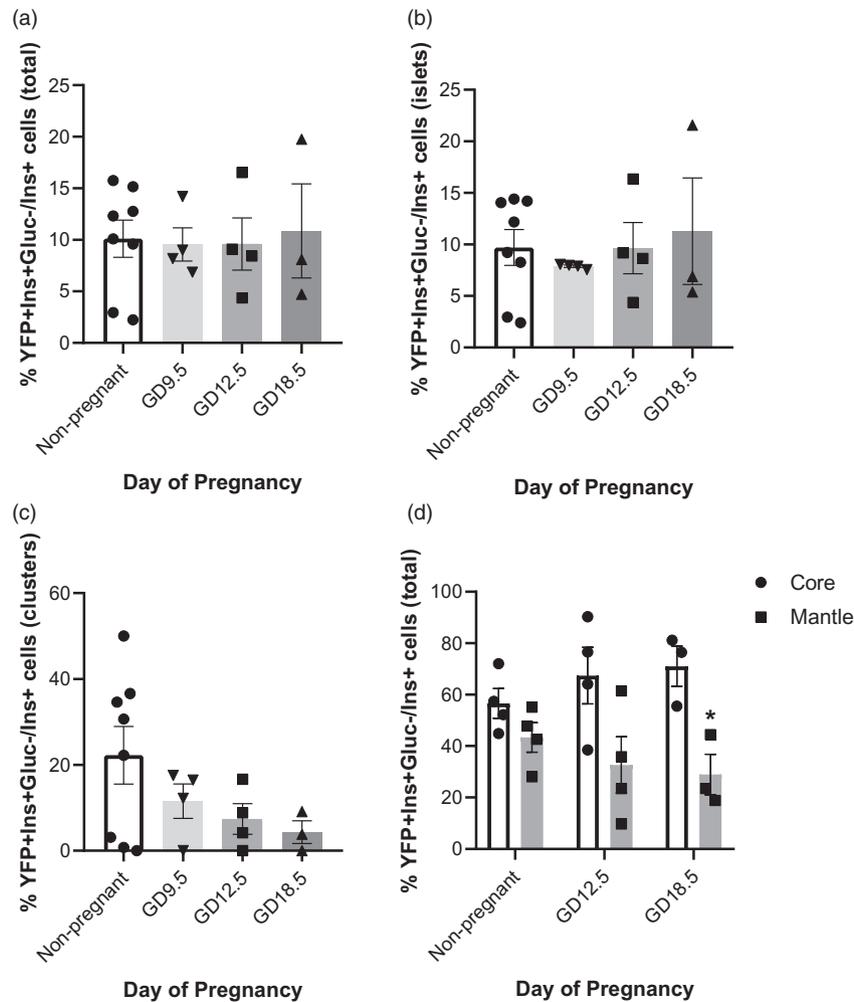We first investigated changes in pancreatic α-cells in glucose-intolerant pregnancy using a previously

Figure 5. Alpha- to β-cell transdifferentiation in non-pregnant mice and at various gestational days during normal pregnancy as expressed by the percentage of phenotypic β-cells (Insulin⁺YFP⁺Glucagon⁻) relative to the total insulin immunoreactive cells. The percentage of such cells in (a) the entire pancreas, (b) islets, and (c) clusters are shown. (d) Localization of Insulin⁺YFP⁺Glucagon⁻ cells to the islet mantle versus the core. Mean ± SEM values are shown together with individual data points, $n = 8$ non-pregnant and 4 pregnant animals, *$P < 0.05$.
YFP: yellow fluorescent protein; GD: gestational day.

established mouse model involving a dietary (LP diet) insult.[8] Substantial inter-animal variation in glucagon levels in blood samples of non-pregnant female mice and rats has been reported (~60-150pg/mL),[32,48] which is supported by the spread of data presented in the control animals in the present study. Interestingly, both dietary groups (LP and C) exhibited lower glucagon levels at the onset of pregnancy. This supports findings from a previous study that showed pregnant animals exhibited hypoglucagonemia and impaired glucagon secretion at GD18.5.[32] This likely occurs as a protective effect to prevent hyperglycemia in the presence of insulin resistance at late pregnancy. However, in our study, there was less suppression of serum glucagon in LP mice at GD18.5, contributing to glucose intolerance in these animals as has been shown to occur at late pregnancy in women with GDM.[49,50] Importantly, higher glucagon levels persisted after parturition in women with GDM, and it has been shown that this can contribute to dysglycemia and eventual development of T2DM. While treatment for GDM currently focuses on administering blood glucose lowering agents, such as

insulin, management of uncontrolled glucagon secretion in GDM could theoretically also serve as a mechanism to reverse blood glucose levels in hyperglycemic women, by means of suppressing these levels. In contrast, levels of insulin were lower in LP mice at GD18.5, further contributing to glucose intolerance in these animals, occurring due to reduced BCM and insulin secretion.[8] These findings demonstrate the sophisticated integrative islet communication between pancreatic α- and β-cells, functioning to balance levels of insulin and glucagon to accommodate metabolic homeostasis in pregnancy, which becomes dysregulated in GDM.

To further elucidate the role of pancreatic α-cells in control and glucose-intolerant (LP-diet mice) pregnancies, the ontogeny of α-cell proliferation throughout pregnancy was assessed. We found that α-cell proliferation was highest at GD9.5 in controls and subsequently declined. The decline in α-cell proliferation likely follows similar progesterone-mediated inhibition that has been shown to occur in β-cells at late pregnancy.[32,51] A previous study determined that α-cell proliferation is mediated by placental lactogens and
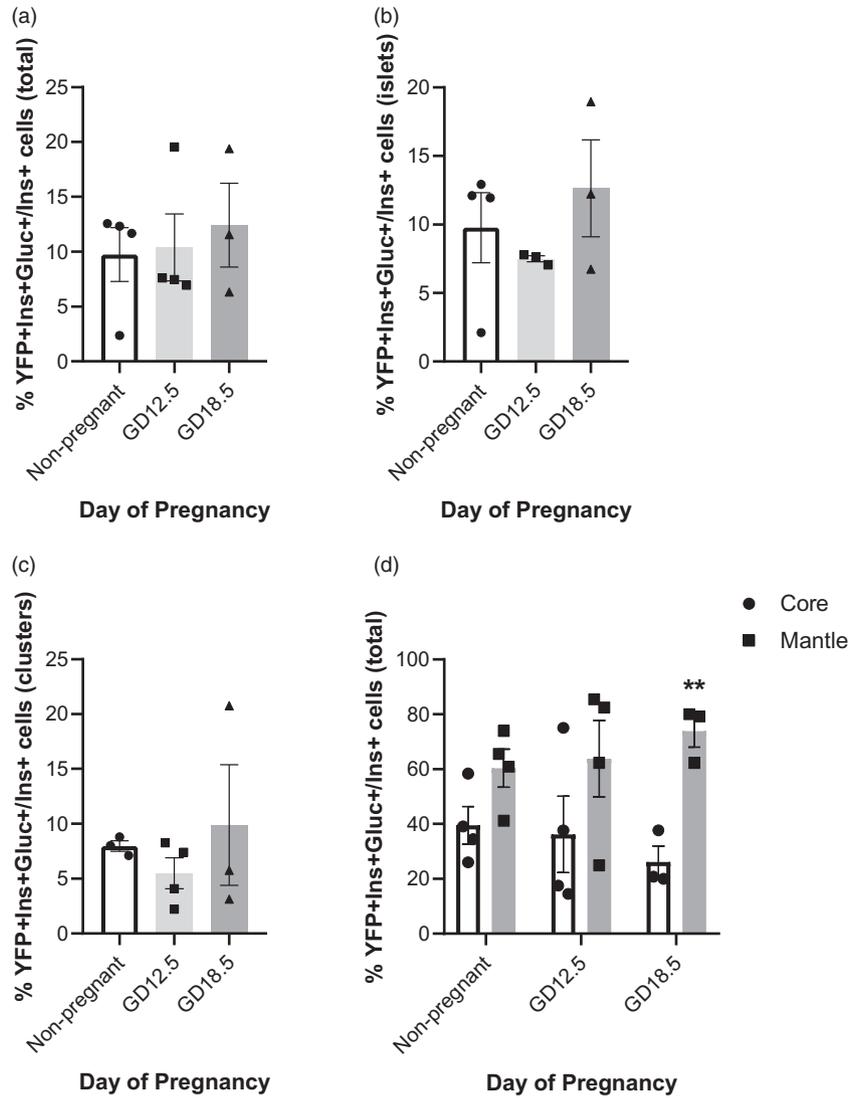
Figure 6. Presence of Insulin$^+$YFP$^+$Glucagon$^+$ cells in the pancreas of non-pregnant and pregnant mice at gestational days 12.5 and 18.5. The percentage of cells present in (a) whole pancreas, (b) islets, and (c) clusters is shown relative to all insulin immunoreactive cells. (d) Localization of Insulin$^+$YFP$^+$Glucagon$^+$ cells to the islet mantle versus the core. Mean ± SEM values are shown together with individual data points, $n = 4$ non-pregnant and 4 pregnant animals, **$P < 0.01$.
YFP: yellow fluorescent protein; GD: gestational day.

prolactin, similarly to what has been observed in β-cells.[32] However, earlier timepoints were not examined in this study[32] which could have provided crucial information as pregnancy hormones have been shown to mediate changes in pancreatic β-cells at GD9.5 to prepare the pancreas for adaptive BCM expansion at GD18.5. Thus, our results demonstrate an earlier onset of α-cell proliferation during gestation in control-diet animals at GD9.5, which is a significant temporal difference that could have important implications for therapeutics. This study provides histological evidence that α-cells follow similar temporal dynamics to β-cells in early pregnancy, which also reach maximal proliferation early in gestation.[7,8] Proliferating α-cells were subsequently localized to islets or clusters within the pancreas, as it has been shown that Ins$^+$Glut2^{LO} β-cell progenitors are enriched in clusters.[37] In contrast to control-diet animals, glucose-intolerant animals (LP) exhibited less α-cell proliferation in clusters at GD9.5. These data could implicate a contribution for α-cell neogenesis from small

endocrine clusters to the adaptive expansion of ACM at GD18.5, which has also been shown to be a mechanism of BCM expansion.[7,8,25] However, our data suggest that adaptive α-cell mechanisms were impaired in GDM.

Next, we co-localized insulin and glucagon double-positive cells whose presence outside of pregnancy has been demonstrated previously.[32,38–40] A minority (~15%) of β-cells were bihormonal in non-pregnant, control-diet females suggesting that these cells are present in controls as a normal feature of pancreas morphology and could represent functionally immature cell types. However, there was a transient decrease of bihormonal cells in controls at GD9.5 that was absent in LP dams, potentially implicating a burst of α- to β-cell transdifferentiation as a mechanism to increase BCM expansion during gestation in C animals that was impaired in LP females. Transdifferentiation may be reduced in these mice due to fewer Insulin$^+$Glucagon$^+$ cells being present in the non-pregnant LP animal. Therefore, this dietary insult *in utero* may impair the plasticity of the

α- and β-cell endocrine lineages and reduce α- to β-cell transdifferentiation.[52] Nonetheless, there were also fewer Insulin+Glucagon+ cells at GD12.5 in LP animals versus controls, potentially implicating a deficit of α- to β-cell transdifferentiation in glucose-intolerant pregnancy specifically at this time.

Accordingly, to elucidate the role of α- to β-cell transdifferentiation to BCM expansion in pregnancy, we used transgenic mice to lineage track α-cells. Our data suggested that α- to β-cell transdifferentiation does not significantly contribute to BCM expansion in pregnancy. Interestingly, a minority (∼10%) of β-cells in non-pregnant females expressed an α-cell label (Insulin+YFP+) suggesting that these cells are present as a normal feature of pancreas morphology. These findings are in contrast to studies that investigated α- to β-cell transdifferentiation in unchallenged mice, where baseline values for transdifferentiation were only around 1%.[33,53,54] However, it is important to acknowledge that these studies used an inducible method for tagging α-cells that was initiated after pancreatic development, which would not take into account the significant pancreatic remodeling that occurs during postnatal development. Importantly, studies suggest that it is possible that some β-cells undergo a bihormonal, glucagon-expressing progenitor stage during embryonic/postnatal development. One study used Gcg-Cre/YFP mice and reported that 10% of β-cells expressed an α-cell label at PND 5, and 20% at PND 21, which is comparable to values reported in the present study. Likewise, an additional study found comparable values, where 5–10% of β-cells were tagged with an α-cell label at PND 1, and 12% at PND 7–14, in a similar model using Gcg-Cre/YFP mice where α-cells were also labeled during pancreas development.[55] Although direct lineage tracing of transdifferentiated β-cells from an α-cell lineage would not be feasible in human samples, clinical data also suggest that it is possible that some β-cells undergo a bihormonal, glucagon-expressing progenitor stage during embryonic/postnatal development as bihormonal cells were also found in the developing human pancreas.[56–58] Since our model is a conditional Cre that is present from conception, the higher baseline values in our study compared to what has been published in many α- to β-cell transdifferentiation studies could be explained by the different lineage tracing models used. One way to address this discrepancy to elucidate the effects of the pregnancy time window would be to use an inducible Gcg/CreER model.[59] Alternatively, using the model in the present study the fold change can be calculated and compared to baseline in the non-pregnant animals. As we found no temporal differences in the percentage of β-cells that underwent transdifferentiation, the pattern for fold change was unsurprisingly also negligible.

Although the relative number of Insulin+YFP+ dual-stained cells did not change during pregnancy, we found that there were fewer of these cells in the islet mantle compared to the core at GD18.5. Previous studies have suggested that the mantle of the islet of Langerhans (where α-cells predominantly reside in mouse) contains a neogenic niche of β-cell progenitors.[54,60] It is suggested that this group of cells is persistent throughout life and could represent a transitional cell type between an α-cell and a β-cell phenotype, perhaps within a process of α- to β-cell transdifferentiation. If so, then it does not appear that the metabolic stress of pregnancy enables a further differentiation of these cells to become unihormonal insulin-expressing. Using the lineage tracking molecule YFP, subpopulations of cells were also identified within this model that co-expressed both insulin and glucagon. While their relative abundance did not change during pregnancy, their anatomical distribution did. In contrast to phenotypic β-cells (Insulin+YFP+Glucagon−) that were predominantly located in the islet core at GD18.5, the Insulin+YFP+Glucagon+ cells were found predominantly in the mantle. This supports previous findings that lineage-flexible α-cells may be most abundant in the mantle of the islets of Langerhans[54] and that they are present during pancreatic remodeling and endocrine adaptation in pregnancy. It has been previously reported that β-cell maturation begins from the islet mantle and propagates to the islet core, being coordinated by islet vascularization.[61] Our data would support the notion that transitional endocrine cell types originate at the islet mantle and then likely propagate towards the centre of the islet once lineage committed. This process occurs as pregnancy progresses, in order to coordinate optimal islet function and facilitate cell-to-cell communication at GD18.5 when metabolic stress is highest.[62]

In summary, we present novel data showing that there is an early onset of α-cell proliferation during pregnancy in controls, contributing to ACM expansion. This was impaired in glucose-intolerant pregnancies (LP) resulting in reduced ACM expansion and possibly fewer α-cells for α- to β-cell transdifferentiation to occur. However, using lineage tracing, the process of transdifferentiation did not appear to dynamically alter during pregnancy. Nonetheless, both cell phenotypes examined (Insulin+YFP+Glucagon−, Insulin+YFP+Glucagon+) underwent anatomical changes in distribution within the islets in late gestation and in opposing directions. These data provide support for a potential transitional cell type in a pancreatic neogenic niche.

AUTHORS' CONTRIBUTIONS

SKS, BJS, and DJH designed the experiments. DJH provided resources, supervision, and acquired funding. SKS performed data curation and conducted the experiments in cooperation with JB and BJS. SKS, JB, and DJH analyzed the data. SKS and DJH interpreted the data. SKS wrote the original draft. All authors participated in the revision of the manuscript and approved the final version of the manuscript.

ACKNOWLEDGMENTS

DECLARATION OF CONFLICTING INTERESTS

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SUPPLEMENTAL MATERIAL

Supplemental material for this article is available online.

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