High glucose alters fetal rat islet transcriptome and induces progeny islet dysfunction

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Abstract

Offspring of diabetic mothers are susceptible to developing type 2 diabetes due to pancreatic islet dysfunction. However, the initiating molecular pathways leading to offspring pancreatic islet dysfunction are unknown. We hypothesized that maternal hyperglycemia alters offspring pancreatic islet transcriptome and negatively impacts offspring islet function. We employed an infusion model capable of inducing localized hyperglycemia in fetal rats residing in the left uterine horn, thus avoiding other factors involved in programming offspring pancreatic islet health. While maintaining euglycemia in maternal dams and right uterine horn control fetuses, hyperglycemic fetuses in the left uterine horn had higher serum insulin and pancreatic beta cell area. Upon completing infusion from GD20 to 22, RNA sequencing was performed on GD22 islets to identify the hyperglycemia-induced altered gene expression. Ingenuity pathway analysis of the altered transcriptome found that diabetes mellitus and inflammation/cell death pathways were enriched. Interestingly, the downregulated genes modulate more diverse biological processes, which includes responses to stimuli and developmental processes. Next, we performed ex and in vivo studies to evaluate islet cell viability and insulin secretory function in weanling and adult offspring. Pancreatic islets of weanlings exposed to late gestation hyperglycemia had decreased cell viability in basal state and glucose-induced insulin secretion. Lastly, adult offspring exposed to in utero hyperglycemia also exhibited glucose intolerance and insulin secretory dysfunction. Together, our results demonstrate that late gestational hyperglycemia alters the fetal pancreatic islet transcriptome and increases offspring susceptibility to developing pancreatic islet dysfunction.

Introduction

Diabetes complicates 5.6–11.7% of all pregnancies (Hunt & Schuller 2007, DeSisto et al. 2014), with affected mothers and offspring vulnerable to adverse metabolic outcomes (Ratner et al. 2008, Fraser & Lawlor 2014, Tam et al. 2017, Das Gupta et al. 2018). Offspring of diabetic mothers suffer a four- to eight-fold increased risk of developing type 2 diabetes (Clausen et al. 2008) due to obesity (Raghavan et al. 2017), insulin resistance (Sauder et al. 2017) and pancreatic islet dysfunction (Gautier et al. 2001, Tam et al. 2017). In addition to increased adiposity,
recent human studies have shown that by the age of 7 years, children born to diabetic mothers had impaired glucose tolerance and decreased beta cell compensation (Tam et al. 2017). Animal offspring exposed to a diabetic milieu in utero also exhibited pancreatic islet dysfunction (Cerf et al. 2006, Han et al. 2007, Blondeau et al. 2011, Zambrano et al. 2016). While both human and animal studies confirmed that the altered in utero environment during diabetic pregnancy permanently reprograms the metabolic health of offspring, the underlying mechanisms modulating offspring pancreatic islet function remain poorly understood.

During maternal diabetes (type 1, type 2 and gestational diabetes), maternal hyperglycemia occurs secondary to inadequate insulin secretion and/or underlying insulin resistance. Commonly used animal models simulating diabetic pregnancy, such as chemically induced maternal diabetes (insulin deficiency model) (Han et al. 2007, Blondeau et al. 2011) or maternal high-fat diet model (maternal insulin resistance/obesity) (Cerf et al. 2006, 2009, Zambrano et al. 2016), expose developing fetuses to a multitude of maternal biochemical changes far beyond hyperglycemia during critical development periods (Xiang et al. 2007, Wang et al. 2010). Maternal hyperglycemia has been implicated as the primary contributing factor (Clausen et al. 2008, Tam et al. 2017, Martin & Sacks 2018) and has been shown to induce pancreatic islet dysfunction early during fetal life (Frost et al. 2012, Green et al. 2012). However, the exact means by which maternal hyperglycemia impacts offspring metabolic health is unknown due to (1) the absence of a rodent model capable of exposing the developing fetus to an exclusively excessive glucose supply and (2) a limited understanding in early transcriptome changes induced by maternal hyperglycemia.

Both rodents and humans undergo continuous pancreatic beta cell mass expansion and functional maturation postnatally until young adulthood (Bonner-Weir et al. 2016). This process of postnatal pancreatic beta cell mass expansion and functional maturation is tightly regulated by transcription factors (e.g. MAFB, UCN3) (Arttner et al. 2007, van der Meulen & Huising 2014), miRNA (Jacovetti et al. 2015, 2017) and growth factors (insulin, INGAP) (Barbosa et al. 2006). Changes in any of these factors could affect the biological and mechanistic pathways involved with diabetes-induced pancreatic islet dysfunction in offspring.

The aim of this study was to identify early transcriptome changes induced by maternal hyperglycemia on pancreatic islets of offspring, uncovering a primary mechanism of offspring pancreatic islet programming. We hypothesize that maternal hyperglycemia alters the offspring pancreatic islet transcriptome, consequently conferring increased offspring susceptibility to developing pancreatic islet dysfunction. To create the fetal hyperglycemic environment, we employed a model capable of inducing localized fetal hyperglycemia in rats (Yao et al. 2010, Gordon et al. 2015). While maintaining maternal euglycemia, this model targets glucose delivery to fetuses residing in the left uterine horn, allowing the use of fetuses in the right uterine horn as genetically similar controls as they remain normoglycemic (Yao et al. 2010, Gordon et al. 2015). Using an RNA sequencing approach, we identified early transcriptome alterations induced by late gestation hyperglycemia in fetal islets. Subsequently, we selected regenerating islet-derived protein 3-gamma (Reg3g) for validation due to its highest fold change and reported protective role as compensatory factor during islet stress (Marselli et al. 2010, Xia et al. 2016). Based on the biological processes enriched and functions of differentially expressed genes, we performed additional ex and in vivo studies evaluating weaning and offspring pancreatic islet cell viability and insulin secretory function. Together, our results showed that offspring exposed to late gestational hyperglycemia acutely developed pancreatic islet morphological changes with altered islet transcriptomes that have critical functions on pancreatic islet health, and subsequently developed persistent pancreatic islet dysfunction as early as weaning.

Methods

Animals

All procedures conformed to the regulations of the Animal Welfare Act and the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee. Rodents were housed in a temperature controlled, 12-h light–dark cycled animal care facility with free access to water and regular chow.

Localized fetomaternal hyperglycemia

On gestation day (GD) 20, a vascular catheter draining into the left uterine artery was placed in timed pregnant CD Sprague Dawley rats (Charles River, Wilmington, MA, USA) to infuse glucose directly into the left uterine artery (Yao et al. 2010, Gordon et al. 2015). Maternal tail vein blood...
glucose levels were measured prior to anesthesia \((n=13\text{ GD20 dams})\). Anesthesia was induced using isoflurane inhalation with oxygen. A 3 Fr Polyurethane Catheter (Norfolk Access, IL, USA) was inserted and secured 1.75 cm retrograde into the femoral artery, thus placing the tip of the catheter several millimeters proximal to uterine artery divergence from the common iliac artery. The left inferior peritoneal space was explored and superfine microclips (GEM 1521, Synovis Micro Companies Alliance Inc, AL, USA) were placed on the superior gluteal and hypogastric trunk arteries. The catheter was tunneled subcutaneously to exit at the mid-scaphular space and connected to a single channel infusion swivel (Instech, PA, USA), allowing rats to move freely. Following this procedure, glucose (D20W) was infused at 4 mg/min (20μL/min) until GD22 (term). All pregnant dams received the same postoperative analgesia. Topical bupivacaine was applied immediately after wound closure and subcutaneous buprenorphine SR (0.5 mg/kg) was given once preoperatively with Meloxicam (3 mg/kg) once daily until delivery. After measuring maternal tail vein glucose level \((n=13\text{ GD22 dams})\), GD 22 pregnant dams were anesthetized for laparotomy. Left and right uterine vein blood was collected prior to fetal extraction for glucose measurement using an Alphatrac Glucometer (Zoetis, NJ, USA) \((n=15\text{ GD22 dams})\). After delivery, pups exposed to hyperglycemic infusion (HG) and respective right uterine horn controls (Con) were either euthanized for sample collection and blood glucose measurement \((n=22\text{–26 fetus; 12–14 neonates per group})\) or resuscitated and cross-fostered to healthy dams who delivered a day apart. Negative control experiment was performed using the same surgical approach but dams were infused with normal saline \((n=3\text{ GD20 pregnant dams})\).

### Islet isolation

Fetal pups were euthanized immediately after delivery and the abdominal surface was sterilized with 70% ethanol. Laparotomy was performed and the fetal pancreas was separated from surrounding tissue starting from the spleen. One pancreas from each gender was pooled and cut into pieces smaller than 3 mm. Collagenase ( Worthington, 1 mL of 2 mg/mL concentration per pancreas) was added and the tissue/collagenase mixture was incubated at 37°C for 10–12 min with intermittent manual shaking. Subsequently, a 10 mL syringe attached to 20 G needle was used for aspiration–ejection to homogenize the tissue lysates. Next, HBSS/BSA was added to deactivate collagenase. The lysate was centrifuged, supernatant was removed and the digested pellet was resuspended with RPMI 1640 media. Lastly, islets were handpicked and cultured in RPMI 1640 media with 5.5 mM glucose. For weanlings, islets were isolated per standard ductal inflation technique \((Stull et al. 2012)\).

### RNA sequencing

The mRNA sequencing was performed by the Center for Medical Genomics at the Indiana University School of Medicine. Fetal islets were isolated as described earlier. Three set of paired fetal islet samples collected from fetuses from three independent infusions were used for RNA sequencing. Total islet RNA was extracted using an RNeasy micro kit (Qiagen) following the manufacturer’s instructions. Purified total RNA was first evaluated for its quantity and quality using the Agilent Bioanalyzer 2100. A RIN (RNA Integrity Number) of five or higher was required to pass the quality control. 65–150 ng of total RNA per sample were used for library preparation. cDNA library was generated and indexed individually. The cDNA library preparation included mRNA purification/enrichment, RNA fragmentation, cDNA synthesis, ligation of index adaptors and amplification following the TruSeq Stranded mRNA Sample Preparation Guide (RS-122-9004DOC, Part# 15031047 Rev. E; Illumina, Inc.). Each resulting indexed library was quantified and its quality assessed by Qubit and Agilent Bioanalyzer, and then pooled in equal molarity according to the guide. Average size of library insert was about 150 b. Five microliters of 2 nM pooled libraries per lane were then denatured, neutralized and applied to the cBot for flow cell deposition and cluster amplification, before loading on to HiSeq 4000 for 75 b paired-end sequencing (Illumina, Inc.). A Phred quality score (Q score) was used to measure the quality of sequencing. More than 90% of the sequencing reads reached Q30 (99.9% base call accuracy). Median raw reads were 41 million per sample. The sequencing data were mapped to the rat genome (UCSC mm6) using a STAR RNA-seq aligner \((Dobin et al. 2013)\) and read counts were summarized using featureCounts (subread) \((Dobin et al. 2013, Liao et al. 2014)\) to get gene expression data. Seventy three percent of the reads were mapped to the gene area. The genes with no/low expression were removed and the expression data were normalized using the trimmed mean of \(M\) values (TMM) method. Differential expression analysis was performed using edgeR \((Robinson et al. 2010, McCarthy et al. 2012)\), and the false discovery rate (FDR) was computed from \(P\) values using the Benjamini–Hochberg procedure \((n=3\text{ paired replicates per group})\).
from three separate infusions). Sequencing data can be found at GEO (GSE118323).

**Ingenuity pathway analysis (IPA) package and GO biological process enrichment**

Differentially expressed genes (FDR <0.05 and FC >1.5) were analyzed using two pathway analyses. IPA package was used to identify enriched pathways and disease processes (adjusted P value <0.05 after Bonferroni correction). Next, the GO biological processes enriched by upregulated and downregulated genes were identified separately using the PANTHER overrepresentation test (database version: GO Ontology database Released 2018-05-21, Reference List: Rattus Norvegicus, Annotation Data Set: GO Biological Process complete, Test Type: Fisher’s Exact With FDR multiple test correction, FDR <0.05 as significant) (Mi et al. 2017) and visualized using REVIGO (Supek et al. 2011).

**Quantitative RT-PCR (RT-qPCR)**

Additional sets of paired fetal islet samples collected as described above were used to validating RNA sequencing findings (n=5 paired replicates from five mothers, of those one was technical replicate from RNA-seq). For RT-qPCR, total RNA was purified and reverse transcribed at 37°C with 15μg of random hexamers, 0.5 mM dNTPs, 5x first strand buffer, 0.01 mM dithiothreitol and 200U of M-MLV reverse transcriptase (Invitrogen) in a final reaction volume of 20μL. RT-qPCR was performed using a SYBR Green-based methodology and primers that were synthesized commercially (Table 1). Briefly, 0.4μL of forward primer (5μM), 0.4μL of reverse primer (5μM), 5μL of 2x SYBR Green PCR Master mix (Applied Biosystems, NBY), and 4.2μL of cDNA were mixed. Next, the reactions were amplified for 40 cycles using Applied Biosystems QuantStudio 3 Real-Time PCR system (Applied Biosystems).

**Immunohistochemistry/immunofluorescence**

Pancreas was removed from animals after euthanasia and fixed rapidly, embedded in paraffin and sectioned into 5μm thick slices, with one section of each group on the same slide to avoid slide-to-slide variation. Two to three sections per animal were analyzed for all immunohistochemistry/immunofluorescence studies. The pancreatic sections were deparaffinized and rehydrated through a series of graded ethanol solutions. Endogenous peroxidase activity blockade was performed and antigens were retrieved by microwaving slides with unmasking solution (Vector Laboratories). To identify pancreatic endocrine cell areas (fetus: 5–6 pups/group from five mothers; adult offspring: 4 males/group from four mothers), sections were incubated with anti-insulin (Santa Cruz sc-9168, 1:500) or anti-glucagon antibodies (Santa Cruz sc-13091, 1:500) overnight. Digital images depicting whole pancreatic tissue sections were obtained using an Axios-Scan Z1 inverted microscope (Zeiss). The area of insulin- or glucagon-positive cells (calculated using Zen Pro) was divided by the total area of whole pancreatic sections to obtain the beta- or alpha-cell cross-sectional area as a percentage of total pancreatic area. For adult offspring, beta cell mass is calculated by multiplying percent insulin-positive area with total pancreatic mass. To assess relative REG3G distribution in beta-cells (GD22 fetus: 5 pups/group from five mothers), we quantified the ratio of Reg3g-stained volume to that of insulin. Pancreatic sections were incubated with anti-insulin antibody (Santa Cruz sc-9168, 1:500) and anti-REG3G antibody (Antibodies Online, ABIN3023039, 1:200) overnight. Pancreatic histological samples were scanned bidirectionally with a Leica TCS SP8 laser-scanning confocal microscope system equipped with a 405 nm diode laser and 488 nm and 552 nm semiconductor lasers and an HC PL APO CS2 40×/1.30 oil objective lens through a 68.06μm pinhole (1.0 Airy unit). Emission bandwidths were set to 415–480 nm for blue emission, 495–545 nm for green emission and 560–700 nm for red emission. Twelve-bit 1024×1024 voxel images were collected at a voxel volume of 0.212μm×0.212μm×0.502μm with a line average setting of two using LAS X v3.1.5.16308 software. These settings were applied to all acquired images. All image processing was performed with Fiji, version 1.51 (Schindelin et al. 2012). First, Reg3g and insulin images were thresholded with the Li algorithm in Fiji and a region of interest (ROI) was drawn around insulin-stained cells. All signal outside the ROI was removed and the insulin signal volume (μm³) contained within the ROI was quantified with the 3D object counting function in Fiji. This ROI outline was transferred to the corresponding REG3G image and all outside signal was removed. Subsequently, the signal volume (μm³) within the insulin-defined ROI that was stained positively for REG3G was measured. Finally, the relative expression of Reg3g in beta-cells was determined by calculating the ratio of Reg3g signal volume to insulin volume.
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Ex vivo islet glucose-stimulated insulin secretion (GSIS)

Isolated islets from weanlings (n = 5 male weanlings/group from five mothers) were recovered in RPMI overnight prior to measuring insulin secretion under static glucose incubation (Komatsu et al. 1995, Mehta et al. 2016). 20–25 size-matched weanling islets were incubated in Krebs–Ringer bicarbonate buffer (129 mM NaCl, 5 mM NaHCO₃, 4.8 mM KCl, 1.2 mM KH₂PO₄, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 0.1% BSA, 10 mM Hepes, pH 7.4) containing 2.8 mM glucose for 1 h at 37°C (pre-incubation). Next, the incubation medium was removed by aspiration and 1 mL of fresh KRB buffer containing different concentrations of glucose was added (5.5 mM and 16.7 mM) to determine islet insulin secretory function at different glucose levels. At the end of incubations, the medium was aspirated and stored at −80°C until measurement. At the end of the static culture, total insulin of pancreatic islets was extracted using acid-ethanol extraction (1.5% HCl, 75% EtOH, 0.1% Triton). Weanling ex vivo islet GSIS media insulin concentration was measured using Stellux Chemi Rodent Insulin ELISA (ALPCO, NH, USA). Results were analyzed and presented as a percentage of total insulin.

Alamar blue cell viability assay

Isolated islets from weanlings (n = 4 pups/group from four mothers) were recovered in RPMI 1640 media containing 11 mM glucose for 24 h prior to evaluation. Seven to eight size-matched islets were handpicked into 96 wells with 100 μL of fresh RPMI media. Alamar blue was subsequently added in 1:10 dilution and then read hourly with a FlexStation 3 Multi-Mode microplate reader (Molecular Devices, San Jose, CA, USA) at an excitation wavelength of 535 nm and emission wavelength of 585 nm. Between reads, islets were incubated in a humidified, warm tissue culture chamber. The fluorescence value produced by Con/HG wells was obtained by subtracting the relative fluorescence unit (RFU) of the negative control well (media and Alamar blue, no islets) from the measured RFU in each well at different timepoints.

In vivo metabolic evaluations of offspring

Metabolic phenotypes of weanlings and adult offspring were evaluated using intraperitoneal glucose tolerance testing (GTT) (weanling: total 14–19 males/group from nine mothers; 2-month-old adult: 6–7 males/group from five mothers) and intraperitoneal insulin tolerance testing (ITT) (n = 4–6 male weanlings/group from three mothers). Animals were fasted for 6 h prior to both tests. For GTT, 1 or 2 g/kg of glucose was injected intraperitoneally and blood was collected from animals via tail vein at 0, 10, 20, 30, 60, 90 and 120-min time points. Additional blood was collected at 10 and 30 min from adult offspring for serum insulin measurement. For ITT, 0.75 U/kg of Humulin R (Eli Lilly) was administered and blood glucose was measured at 0, 15, 30, 45 and 60-min time points. Animal blood glucose levels were measured with an Alphatrak Glucometer (Zoetis, NJ, USA). Fetal serum insulin concentrations were measured with an Alphatrak Glucometer (Zoetis, NJ, USA). Adult serum insulin levels were measured using Stellux Chemi Rodent Insulin ELISA (ALPCO).

Statistics

Each group of fetuses and male offspring originating from one biological mother was considered as n = 1. In instances where more than one pups from the same mother were analyzed, the average of the acquired data would then

### Table 1  Primer sequences of target genes.

<table>
<thead>
<tr>
<th>Gene (accession number)</th>
<th>Sequence (5’–3’)</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reg3g (NM_173097.1)</td>
<td>Fwd TGTGCCCACTTCACGTATCA</td>
<td>IDT</td>
</tr>
<tr>
<td></td>
<td>Rev GGATCATGGAGGCCAATCCA</td>
<td></td>
</tr>
<tr>
<td>Reg3b (NM_053289.1)</td>
<td>Fwd GGAACAGCTACCAATATACC</td>
<td>Sigma</td>
</tr>
<tr>
<td></td>
<td>Rev CTCCATCTTAAGAATCCAGAAG</td>
<td>Sigma</td>
</tr>
<tr>
<td>Gabrp (NM_031029.1)</td>
<td>Fwd AGATGCGAGTCAAGATAGG</td>
<td>Sigma</td>
</tr>
<tr>
<td></td>
<td>Rev GTTAAAGCCTGAGATGGAG</td>
<td></td>
</tr>
<tr>
<td>Mmp7 (NM_012864.2)</td>
<td>Fwd ACAGACTTGCCCTGGTTCTT</td>
<td>IDT</td>
</tr>
<tr>
<td></td>
<td>Rev GTTCCGATCCTCCCCTTG</td>
<td></td>
</tr>
<tr>
<td>Actb (NM_031144.3)</td>
<td>Fwd AGGTCATCTACTATGGGCAACGA</td>
<td>Eurofins</td>
</tr>
<tr>
<td></td>
<td>Rev CACCTCATGTGAGATGGATAGTT</td>
<td></td>
</tr>
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</table>
be used as a single data (Vieleisis & Oh 1983, Roest et al. 2004, Gordon et al. 2015). All results were represented as mean±s.e.m., while fold changes of RT-qPCR results were represented as log2FC in comparison to RNA-seq results. For single timepoint measurement, the difference between two groups was assessed using a paired two-tailed t-test. For repeated measures (glucose level during GTT, ITT, GSIS, Alamar blue cell viability assay), two-way ANOVA tests followed by Bonferroni multiple comparison tests correction were performed to assess the difference between two groups. Results were defined as statistically different when $P<0.05$.

**Results**

**Localized fetomaternal hyperglycemia induces fetal pancreatic islet perturbations**

The localized fetomaternal hyperglycemia model allows for dosage and temporal control over glucose delivery to left uterine horn fetuses (Fig. 1A). To determine the effects of late gestation hyperglycemia, glucose was infused from GD20 to GD22, with offspring evaluated at different timepoints (Fig. 1B). On GD22, maternal blood glucose concentrations were unchanged with ongoing 4mg/min glucose infusion (Fig. 1C). To validate the specific targeting of glucose delivery, we measured both the maternal uterine vein and fetal blood glucose level. Indeed, blood glucose concentrations from the maternal left uterine vein were higher than those from the right (Fig. 1C). Compared to internal control fetuses from the right uterine horn (Con), left uterine horn fetal rats (HG) also had higher blood glucose levels (Fig. 1D). As with previously published report (Gain et al. 1981), blood glucose levels of control pups were higher 30 min after birth (Fig. 1D). In contrast, this increase in glucose level was not apparent in HG pups (Fig. 1D). In fact, when compared to control, newborn pups that received glucose infusion had lower blood glucose levels (Fig. 1D). Not surprisingly, left uterine pups exposed to glucose infusion also had higher serum insulin levels (Fig. 1E) and beta cell areas (Fig. 1F). The higher number of cells within insulin-positive area in hyperglycemic pups (Supplementary Fig. 1, see section on supplementary data given at the end of this article) indicating that the increase in beta cell area likely resulted from cellular hyperplasia. Taken together, fetal pups exposed to transient (48h) hyperglycemia developed both hyperinsulinemia and pancreatic beta cell hyperplasia.

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**Figure 1**

(A) Schematic representing localized fetomaternal hyperglycemia model. (B) Experimental timeline. Fetal pups were exposed to hyperglycemia from GD20 to 22, delivered via Cesarean section and cross-fostered to healthy dams. The cross-fostered pups were evaluated at weaning and at adulthood. (C) Maternal blood glucose was unchanged before (GD20) and during infusion (GD22) (left panel, $n=15$ mothers). During infusion, the glucose level in blood returning from the left uterine vein was higher than that of the right uterine vein (right panel, $n=15$ mothers). (D) The glucose levels of fetuses residing in left uterine horn (HG) were higher than those of their respective controls (Con) during glucose infusion while the placenta was intact ($n=22–26$ fetus from 12 mothers), but lower 30 min after birth (right panel, 12–14 pups from seven mothers). (E) Insulin levels of HG pups were higher as well ($n=5$ pups/group from five mothers). (F) Pancreatic beta- and alpha-cell area in the HG fetal pups ($n=5–6$ pups/group from five mothers).
RNA-seq identified differentially expressed genes known to mediate inflammation and pancreatic islet function

To identify the earliest pathway and biological processes altered by hyperglycemia exposure, we performed RNA sequencing to examine whole islets transcriptome changes in GD22 fetal islets immediately after completing 48h of glucose infusion. Compared to controls, HG fetal islets had 87 differentially expressed genes (DEGs) (69 up- and 18 down-regulated) (Supplementary Table 1). The result of Ingenuity Pathway Analysis revealed 22 enriched pathways, the majority of which were related to inflammation (Supplementary Table 2). IPA also identified diabetes mellitus as a relevant disease process (24 DEGs) and also predicted the activation of cell death (41 DEGs) (Fig. 2A). Next, the biological processes enriched by up and downregulated genes were identified using the PANTHER classification system (Mi et al. 2017) and visualized using REVIGO (Supek et al. 2011). As shown in the semantic similarity-based scatterplots, the up- and down-regulated genes were involved in different biological processes (Fig. 2B). While the upregulated DEGs were heavily enriched in inflammatory and immune system-related biological processes, the downregulated genes were involved in more diverse biological processes (Fig. 2B). In addition to inflammation, the downregulated genes were also enriched in processes involving cellular responses to stimuli and developmental processes (table in Fig. 2B). To further understand the specific biological implications of these broad terms, we performed a literature review and identified a significant number of down-regulated genes (Ctgf, Clu, Cfr, Fgf3, Gabpr, Mmp7, Reg3b, Reg3g) that are involved in early pancreatic islet development (proliferation, new islet formation) (Crawford et al. 2009, Koivula et al. 2016), adult pancreatic islet function, neogenesis and anti-apoptotic effects during stress (Table 2). Further RT-qPCR validation confirmed both Reg3g and Reg3b, along with two additional downregulated genes (Gabpr and Mmp7), were consistently decreased in GD22 islets exposed to in utero hyperglycemia (Fig. 3A). Furthermore, HG pups revealed a decrease in the percentage of area positive for REG3G staining within insulin-positive cells (Fig. 3B), indicating that HG beta-cells had diminished REG3G protein expression. Collectively, RNA sequencing results revealed that fetal hyperglycemia induces the islet transcriptome associated with diabetes mellitus and activated islet inflammation/cell death pathways. Interestingly, the downregulated genes are involved in various biological processes that modulate pancreatic islet health.

Pancreatic islet dysfunction occurs in weanlings exposed to hyperglycemia in utero

Given that late gestational hyperglycemia rapidly alters fetal pancreatic islet phenotypes and transcriptome, it was thus of interest to determine whether offspring were vulnerable to developing pancreatic islet dysfunction. Particularly, we aimed to determine if late gestation hyperglycemia exposure altered offspring pancreatic islet viability and function as predicted by transcriptome analysis. At weaning (P21), HG pups developed impaired glucose tolerance as evidenced by higher glucose levels at 10min during IPGTT and higher incremental AUC (iAUC) (Fig. 4A, B, C and D). Using the same surgical approach, we performed a separate negative control experiment by infusing normal saline to left uterine horn fetal pups. We observed no difference in IPGTT of saline-infused offspring from the left uterine horn when compared to their internal controls from the right uterine horn (Supplementary Fig. 2). Next, we evaluated weanling pancreatic islet cell viability using the Alamar blue cell viability assay (Muthyala et al. 2017) and insulin secretory function via ex vivo static GSIS. Under basal conditions, pancreatic islets extracted from HG weanlings had lower cell viability suggesting an increased susceptibility to cell death (Fig. 4E). Additionally, HG weanling islets had decreased insulin secretion at both 5.6 mM and 16.7 mM stimulatory phases (Fig. 4F). In the absence of overt insulin resistance during insulin tolerance testing (Fig. 4I), these findings assert that offspring exposed to late gestation hyperglycemia developed glucose intolerance secondary to pancreatic islet dysfunction as observed by decreased cell viability and static glucose-stimulated insulin release.

Adult offspring exposed to HG had decreased beta cell mass and insulin secretory dysfunction without altered growth or increased inflammatory mediators

We sought to determine if pancreatic islet changes at weaning would impact adult offspring. As beta cell mass and function increase drastically after weaning (Bonner-Weir et al. 2016), we hypothesized that increased susceptibility to cell death and decreased glucose responsiveness in weanling islets would impact both pancreatic beta cell mass and insulin secretory function in HG adults. Indeed, HG adult offspring remained glucose intolerant with an increased glucose tolerance curve divergence (Fig. 5A and B).
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Figure 2

(A) Heatmap showing differentially expressed genes regulating diseases and biofunction predicted by IPA (n = 3 fetal islet samples/group from three mothers; each islet samples prepared from pool of one pancreas from each gender). Left panel: 24 genes that enriched diabetes mellitus disease process (adjusted P-value = 2.43 × 10^-6). Right panel: Cell death process was upregulated by 41 genes (adjusted P-value = 2.38 × 10^-4). (B) Upregulated and downregulated DEGs were analyzed separately using PANTHER and enriched GO BP was further summarized using REVIGO with the following parameters - database: whole UniProt; semantic similarity measure: Resnik; similarity allowed: Small (0.5). Note that upregulated genes enriched immune and inflammatory processes (red circles) and downregulated genes enriched different biological processes (blue circles). There were two commonly enriched GO BP (G4 – response to stimulus, purple circle in C1 – immune responses/humoral immune response). The table on right shows the summarized list of GO biological processes (BP) and number/percentage of genes annotated to the GO BP.
Despite a higher serum glucose level, HG adults had diminished in vivo insulin release 10 min after glucose injection (Fig. 5C). Additionally, HG adults also had lower pancreatic beta cell mass (Fig. 5D, E and F). To determine if altered growth or increased adiposity contributed to glucose intolerance, we measured offspring weight and adiposity at weaning and at 2 months. There were no differences between offspring who received in utero hyperglycemic infusion and their respective controls (Fig. 5G and H). Since the inflammatory pathways were overrepresented, we also measured five inflammasomes in fetal, neonatal and 2-month-old offspring, in which three were IPA predicted (IFNG, TNF-alpha, IL1B) and two were associated with the upregulated DEGs (CXCL10, IL-17). However, the levels of these inflammatory mediators in the serum both during early life and at 2 months were unchanged (Supplementary Fig. 3). These findings indicated that pancreatic islet dysfunction was not mediated by altered growth, increased adiposity or systemic inflammation.

Discussion

Independent of genetic risk, offspring born from diabetic pregnancies experience a greater risk of insulin resistance, pancreatic islet dysfunction and type 2 diabetes (Ratner et al. 2008, Fraser & Lawlor 2014, Tam et al. 2017, Das Gupta et al. 2018). Such risk of transmission is thought to result from maternal hyperglycemia (Kubo et al. 2014, Tam et al. 2017, Kawasaki et al. 2018); however, no direct evidence exists elucidating the exact role of maternal hyperglycemia in programming offspring metabolic health. Our fetomaternal hyperglycemia model, capable of inducing localized maternal and fetal hyperglycemia, addresses this knowledge gap. Using this model, where late gestation fetal pups were exposed to mild-moderate hyperglycemia (<350 mg/dL) (Aerts & van Asche 1977, Blondeau et al. 2011, White et al. 2015), we first showed that hyperglycemic rodent offspring acutely developed a pancreatic islet phenotype similar to that of an infant of a diabetic mother (Helwig 1940, Cardell 1953) and identified DEGs that modulate pancreatic islet inflammation, cell viability and function. Along with transcriptome changes, metabolic testing during weaning showed that offspring exposed to hyperglycemia in utero developed glucose intolerance due to increased pancreatic islet susceptibility to cell death and decreased glucose-induced insulin secretion. Finally, consistent with the altered fetal islet transcriptome and findings in weanlings,
adult offspring exposed to late gestation hyperglycemia showed decreased beta cell mass and insulin secretory function.

There are a number of investigations that performed targeted molecular studies on offspring exposed to different diabetic pregnancy models. These studies identified that pancreatic islets collected from young offspring exposed to diabetic milieu in utero had altered IGF2/insulin receptor signaling (Ding et al. 2012, Bringhenti et al. 2016), altered glucose metabolism (Han et al. 2007, Cerf et al. 2009), and/or increased oxidative stress/inflammation (Wang et al. 2014, Yokomizo et al. 2014). While the hyperglycemic islet transcriptome predicted heightened inflammation, the DEGs and pathway analysis did not show changes in genes related to IGF2/insulin receptor signaling or enzymes regulating pancreatic islet glucose metabolism. This discrepancy could be due to multiple reasons, the first being the difference in our model and timepoint examination of offspring islets. Our model addressed the direct effects of hyperglycemia during late gestation, as opposed to other models that exposed the fetus to more complex metabolic perturbations throughout pregnancy and subsequently addressing the molecular pathways altered in offspring later in life. Additionally, all of the aforementioned studies, except for the study by Ding et al. (2012), examined the effects of maternal diabetes/overnutrition in young adult offspring.

Overall, the transcriptome analysis and literature review on individual DEGs predicted three major processes that regulate offspring pancreatic islet health: increased inflammation, susceptibility to cell death and decreased pancreatic islet insulin secretion. Particularly, the upregulated genes were heavily enriched in inflammatory pathways and an activated cell death process. Since there was absence of systemic inflammation, we reasoned that increased inflammation is not the primary mechanism inducing offspring pancreatic islet dysfunction. Rather, we hypothesized that the observed increased in inflammatory-related transcriptome in offspring exposed to late gestation hyperglycemia is stimulated by the increased in pancreatic islet susceptibility to cell death. The downregulated genes are closely related to pancreatic islet cells (Supplementary Table 3) and appear to have more diverse biological roles ranging from modulating pancreatic islet development, inflammatory response, anti-apoptotic effects and normal/compensatory beta cell insulin secretion (Table 2). The cystic fibrosis transmembrane conductance regulator (Cftr) has been increasingly recognized for its importance in cystic fibrosis-related diabetes (CFRD): the pathogenesis of which involves altered early life pancreatic islet morphogenesis (Rotti et al. 2018) and beta cell loss and intra-islet inflammation (Hart et al. 2018). Connective tissue growth factor (Ctgf) and fibroblast growth factor receptor 3 (Fgfr3) are another two downregulated genes that can affect early postnatal pancreatic islet development both morphologically and functionally. Both these genes are expressed only in late embryonic beta cell development and emerging islets (Arnaud-Dabernat et al. 2007, Crawford et al. 2009). Particularly, Ctgf inactivation during
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Embryogenesis caused decreased insulin-positive cells (Crawford et al. 2009), while Ctgf haploinsufficiency mice had decreased beta cell proliferation during pregnancy (Pasek et al. 2017).

Interestingly, the two most downregulated genes, Reg3g and Reg3b, were from the common regenerating islet-derived protein (REG protein) family. Based on DNA sequence and protein structure similarities, these two REG proteins are classified under type 3 REG (Abe et al. 2000), which is expressed in pancreatic tissue (Parikh et al. 2012) and suggested to pattern embryonic endocrine cells (Hamblet et al. 2008). REG gene expression levels correlate with insulin secretory function (Madrid et al. 2013) and treatment using INGAP, one of the subtypes of REG protein, enhances neonatal islet insulin secretion (Barbosa et al. 2006, Madrid et al. 2009). More importantly, REG protein expression is upregulated in diabetic human islets (Marselli et al. 2010, Planas et al. 2010), with animal models supporting their role as a compensatory factor during islet stress (Siddique & Awan 2016, Xia et al. 2016). In regard to the downstream signaling pathway, ex vivo (keratinocytes) (Barbosa et al. 2008, Lai et al. 2012, Wu et al. 2016) and in vivo (Xia et al. 2016) studies have indicated that REG3G/REG3A protein binds to EXTL3, which subsequently activates AKT and/or STAT3 downstream signaling. While we have not performed further protein evaluation in our model, IPA indicated the involvement of STAT3 pathway (−log P value = 3.28) with STAT3 predicted as an inhibited upstream regulator with the lowest Z score (Exp FC: −1.214, Z-score = -3.65, −log P value = 16.8, −log adjusted P value = 14.0). Considering these reported roles of REG protein, our findings indicate that decreased Reg3g and/or Reg3b in pups exposed to hyperglycemia in utero would negatively impact postnatal pancreatic islet formation and/or functional maturation leading to decreased offspring islet cell viability and function. Therefore, future studies are warranted to determine the implication of decreased Type 3 REG during early postnatal pancreatic islet development (Lai et al. 2012, Wu et al. 2016).

Both pancreatic beta cell mass and glucose responsiveness increase most significantly after weaning (Jacovetti et al. 2015, Bonner-Weir et al. 2016); changes in islet susceptibility to cell death and decrease in function could determine pancreatic islet mass and function in adulthood. Therefore, it is not surprising that the findings describing increased susceptibility to cell death and

Figure 4
H6 weanlings developed glucose intolerance and pancreatic islet insulin secretory defect. (A) 1 g/kg intraperitoneal glucose tolerance testing showing increased blood glucose level at ten timepoint and (B) higher incremental glucose area under the curve (IAUC) (*P < 0.05, n = 7–10 male weanlings from six mothers, internal pairs were connected with dashed lines). (C) 2 g/kg intraperitoneal glucose tolerance testing yielded the same result where HG pups continued to have a higher glucose level at 10 min and (D) a higher incremental glucose AUC (*P < 0.05, n = 7–9 male weanlings/group from four mothers, internal pairs were connected with dashed lines). (E) Alamar blue cell viability assay showing the cell viability of HG islets was decreased (*P < 0.05, 7–8 islets per replicate, n = 4 pups/group from four mothers). (F) Ex vivo static GSIS showing decreased HG islets insulin secretion at 5.6 mM glucose and 16.7 mM glucose phase (*P < 0.05, 20–25 islets/group collected from n = 5 weanlings/group from five mothers). (G) Insulin tolerance testing of weanling males (n = 4–6 HG male/group from three mothers).
the insulin secretory defect during weaning negatively impacted HG offspring pancreatic beta cell mass and insulin secretory defect. This finding is consistent with both human epidemiological data (Tam et al. 2017) and an animal model mimicking diabetic pregnancy with mild-to-moderate maternal hyperglycemia (<350 mg/dL) (Aerts & van Assche 1977, Blondeau et al. 2011, White et al. 2015). Most importantly, we showed that late gestation hyperglycemia, even for a short duration (<10% of pregnancy), exhibited long-lasting negative impacts on offspring pancreatic islet function. These findings stress not only the critical role of maternal hyperglycemia, but also the importance of examining the metabolic outcome of offspring early in life for both human and animal studies.

**Conclusion**

In conclusion, late gestation hyperglycemia perturbs fetal pancreatic islet morphology and diminishes insulin secretory function in young offspring. Transcriptome analysis indicated that GD22 islets exposed to in utero hyperglycemia displayed heightened inflammatory responses, increased susceptibility to cell death and decreased pancreatic islet insulin secretory function. This finding guided our study to identify pancreatic islet dysfunction in weanlings, which predisposed adult offspring to decreased beta cell mass and insulin secretion. Our transcriptome analysis provides a paradigm for elucidating the programming mechanism resulting from excessive glucose exposure. Future studies validating the targets in modulating postnatal pancreatic islet neogenesis and function are warranted.

**Supplementary data**
This is linked to the online version of the paper at https://doi.org/10.1530/JOE-18-0493.

**Declaration of interest**
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.
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