Effects of genetics and *in utero* diet on murine pancreatic development

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Abstract

Intrauterine (IU) malnutrition could alter pancreatic development. In this study, we describe the effects of high-fat diet (HFD) during pregnancy on fetal growth and pancreatic morphology in an 'at risk' animal model of metabolic disease, the glucose transporter 4 (GLUT4) heterozygous mouse (G4+/−). WT female mice mated with G4+/− males were fed HFD or control diet (CD) for 2 weeks before mating and throughout pregnancy. At embryonic day 18.5, fetuses were killed and pancreata isolated for analysis of morphology and expression of genes involved in insulin (INS) cell development, proliferation, apoptosis, glucose transport and function. Compared with WT CD, WT HFD fetal pancreata had a 2.4-fold increase in the number of glucagon (GLU) cells ($P=0.023$). HFD also increased GLU cell size by 18% in WT pancreata compared with WT CD. Compared with WT CD, G4+/− CD had an increased number of INS cells and decreased INS and GLU cell size. Compared with G4+/− CD, G4+/− HFD fetuses had increased pancreatic gene expression of *Igf2*, a mitogen and inhibitor of apoptosis. The expression of genes involved in proliferation, apoptosis, glucose transport, and INS secretion was not altered in WT HFD compared with G4+/− HFD pancreata. In contrast to WT HFD pancreata, HFD exposure did not alter pancreatic islet morphology in fetuses with GLUT4 haploinsufficiency; this may be mediated in part by increased *Igf2* expression. Thus, interactions between IU diet and fetal genetics may play a critical role in the developmental origins of health and disease.

Key Words
- high-fat diet
- pancreas
- fetus
- programing
- glucagon cells

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Introduction

Metabolic syndrome (MetS) is a cluster of risk factors including obesity, dyslipidemia, insulin (INS) resistance, and hypertension. MetS increases the risk for type 2 diabetes mellitus (T2D), which is characterized by peripheral INS resistance and INS cell dysfunction (Grundy *et al*. 2004).

In Western Societies, fat and carbohydrate-dense foods have become increasingly abundant and easily accessible (Cordain *et al*. 2005), contributing to the increased prevalence of MetS in adults in the USA (Mozumdar & Liguori 2011). Increased globalization, associated with a nutritional transition toward Western diets, is thought to be a contributing factor to the increasing prevalence of obesity and T2D globally (Popkin 2006). In addition to poor diet and genetics, evidence indicates that an altered intrauterine (IU) environment

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plays a key role in the development of MetS (Vuguin *et al.* 2013), and that interactions between the IU environment and lifestyle can increase risk of MetS and T2D in people who are genetically susceptible (Hu 2011).

Poor nutrition during pregnancy affects fetal growth and development, particularly that of the endocrine pancreas (Snoeck *et al.* 1990, Vuguin *et al.* 2013). Specifically, alterations in the IU environment caused by a low-calorie, low-protein or a high-fat diet (HFD) affect the function of the endocrine pancreas by altering islet size, islet vascularization, number of INS cells, INS content, function, and parasympathetic innervation (Dahri *et al.* 1991, Garofano *et al.* 1997, Cerf *et al.* 2005, Ford *et al.* 2009, Ng *et al.* 2010, Rodriguez-Trejo *et al.* 2012, Vogt *et al.* 2014). In addition, it has been suggested that HFD during fetal development induces glucagon (GLU) cell hypertrophy and hyperplasia, resulting in an increase in GLU cell number and volume in the neonatal offspring (Cerf *et al.* 2005).

These findings could be partially explained by altered expression of growth factors, such as insulin-like growth factors (IGFs) and regulatory proteins involved in endocrine cell differentiation, such as the transcription factor pancreatic and duodenal homeobox 1 (*PDX1*; Park *et al.* 2008, Chen *et al.* 2012). Specifically, HFD exposure during development reduced *Pdx1* immunoreactivity in a rodent model, indicating that *Pdx1* is susceptible to IU environment (Cerf *et al.* 2009).

The IGF system, an important metabolic and mitogenic factor, is the major regulator of fetal growth and development. *Igf2* mRNA is highly expressed in islet cells and some ductal epithelial cells in late fetal life (Hill *et al.* 1999) and co-localizes with INS cells and GLU cells in human fetal pancreas (Portela-Gomes & Hoog 2000). *Igf2* is mitogenic for INS cells (Hill *et al.* 1999, Calderari *et al.* 2007) and inhibits apoptosis of INS cells (Hill *et al.* 1999, Raile *et al.* 2003, Cornu *et al.* 2009). Fetal expression of *Igf2* has been shown to be increased by a HFD during gestation (Zhang *et al.* 2009), indicating that changes in *Igf2* expression, in response to the altered IU environment, may play a role in the programming of the endocrine pancreas.

To develop T2D, pancreatic dysfunction has to be accompanied by a state of peripheral INS resistance. Peripheral INS resistance can be defined as a reduction in the ability of target tissues such as skeletal muscle, white adipose tissue, and heart to respond to INS. One response of INS resistance is a reduction in INS-stimulated glucose uptake, mediated via the glucose transporter 4 (GLUT4; Zierath *et al.* 1996, Bryant *et al.* 2002). In animal models, GLUT4 haploinsufficiency (G4+/−) results in peripheral INS resistance and T2D (Charron & Kahn 1990, Rossetti *et al.* 1997, Stenbit *et al.* 1997, Li *et al.* 2000). In addition, as G4+/− mice age, they develop islet cell hyperplasia due to an increase in INS cell number (Brissova *et al.* 2005).

Studies have demonstrated that GLUT4 mRNA and GLUT4 protein are expressed in the GLU cells and INS cells of mouse, rat, and human endocrine pancreas (Kobayashi *et al.* 2004, Bahr *et al.* 2012). GLUT4 expression in pancreatic endocrine cells seems to be regulated by glucose and INS (Bahr *et al.* 2012). Specifically, in GLU cells, high glucose levels decrease and high INS levels increase GLUT4 expression. In contrast, the opposite occurs in INS cells. In addition, pancreatic GLUT4 expression is elevated in T2D patients and decreased in animal models of T1D, indicating that alterations in GLUT4 expression in the endocrine pancreas may play a role in the regulation of pancreatic cell function during disease states (Bahr *et al.* 2012).

While effects of maternal nutrition on INS cell development and function have been extensively studied, very few studies have examined the effects of HFD during gestation on GLU cell development and function (Cerf *et al.* 2005). We have previously demonstrated that exposure to a HFD *in utero* and during lactation lead to the development of MetS in G4+/− and WT offspring (Hartil *et al.* 2009, Vuguin *et al.* 2013). Although both animal models developed features of metabolic disease, genotype-dependent differences were observed. Specifically, WT fetuses exposed to a HFD *in utero* have significantly higher glucose levels compared with similarly exposed G4+/− fetuses. We propose the hypothesis that GLUT4 haploinsufficiency protects the pancreatic endocrine cell function from the effects of HFD, thus protecting the G4+/− fetus from becoming hyperglycemic.

The animal model used in this study provides a unique opportunity to study the influence of genotype and diet on fetal pancreatic islet development (Vuguin *et al.* 2013). The results of this study indicate that the interaction between a HFD and a genotype during gestation 'programs' the fetus for increased susceptibility to T2D in part by altering pancreatic islet cell composition leading to an impaired ability to optimize glucose homeostasis.

Materials and methods

The animal protocols were approved by the Institute for Animal Care and Use Committee at the Albert Einstein College of Medicine. As previously described (Hartil *et al.*

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2009), age and body weight (BW)-matched female WT mice (CD1 background) were maintained on control PicoLab Mouse Diet #5058 (CD: 9% fat as soybean oil and animal fat, 20% protein, 53% carbohydrate, 3.59 kcal/g) or switched to high-fat Bio-Serv Product #F3282 (HFD: 35.5% fat as lard, 20% protein, 36.3% carbohydrate, 5.29 kcal/g) 2 weeks before mating and throughout pregnancy (IU). The females were bred with non-littermate G4+/− males. Pregnancy was determined by the detection of a copulatory plug and defined as embryonic day (e) 0.5. Pregnant mice were killed on e18.5.

Following dissection from the uterine horn a total of 196 fetuses were killed by immediate decapitation. Fifty-six fetuses were exposed to CD and 140 were exposed to HFD during gestation. This cohort was produced from a total of four WT females exposed to CD and 11 WT females exposed to HFD. The number of fetuses per litter and fetal weights were recorded while the animals were killed. The numbers of abnormal and dead pups were also noted. Genotyping and sex determination of fetuses were carried out as described previously (Hartil *et al*. 2009, Vuguin *et al*. 2013).

Tissue processing for immunostaining

These techniques have been previously described (Vuguin *et al*. 2006, Kedees *et al*. 2007). The fetuses were placed in ice-cold PBS and decapitated. The dorsal pancreas was removed by dissection and fixed overnight by immersion in a fixative solution of 4% paraformadehyde in 0.1 M phosphate buffer. The fixed tissues were infiltrated with 30% sucrose and mounted in an embedding matrix (Lipshaw Co., Pittsburgh, PA, USA). The cryosections (10–20 micron) were mounted onto the glass slides coated with a solution of 1% gelatin containing 0.05% chromium potassium sulfate.

Immunoflourescence

As previously described (Vuguin *et al*. 2006, Kedees *et al*. 2007), the sections were incubated sequentially in empirically derived optimal dilutions of control serum or primary antibody overnight at 4 °C and with a 1:200 dilution of the secondary antibody (in 0.01 M PBS). After completion of the staining procedure, the sections were covered with two to three drops of Vectashield solution (Vector Labs, Inc., Burlingame, CA, USA; Vuguin *et al*. 2006).

Source of antibodies

Guinea pig anti-bovine INS antibodies were purchased from Linco Research, Inc. (Eureka, MO, USA). Rabbit anti-human GLU antibodies were purchased from Calbiochem, Inc. (San Diego, CA, USA). Anti-rabbit GLUT2 sera was purchased from Chemicon, Inc. (Temecula, CA, USA). Rabbit antiserum to *Pdx1* was a generous gift from C.V.E. Wright (Vanderbilt University, Nashville, TN, USA). Antibodies were used at the following dilutions: anti-INS, 1:400; anti-GLU, 1:4000; anti-Pdx1, 1:5000; and anti-GLUT2, 1:1000. Secondary antibodies: Alexafluor 488 anti-mouse, anti-rat, and anti-rabbit IgG; Alexafluor 594 anti-guinea pig, anti-rabbit, and anti-mouse IgG were purchased from Molecular Probes, Inc. (Eugene, OR, USA).

Confocal microscopy

Confocal images were obtained using a Radiance 2000 confocal microscope (Bio-Rad, Inc.) attached to a Zeiss Axioskop microscope (Carl Zeiss, Inc, Thornwood, NY, USA). Images of 540×540 pixels were obtained and processed using Adobe Photoshop 6.0 (Adobe Systems; Vuguin *et al*. 2006).

Morphometric analysis

The number of islets, size of individual endocrine cells, and cell number ratio for cells expressing INS and GLU were calculated as previously described (Vuguin *et al*. 2006, Kedees *et al*. 2007). Consecutive 10–20 μm sections were obtained from each pancreas (n=4–8/fetal genotype per diet from at least four litters per diet). Between 12 and 15 sections at 200–300 mm apart/pancreas per genotype per diet were examined. Cell counting and area measurements were made using the National Institute of Health Image J Software (http://rsb.info.nih.gov/ij/).

Definitions of parameters measured

Total endocrine area ($\mu m^2/mm^2$) Total endocrine area was defined as the sum of INS- and GLU-expressing areas normalized to the total pancreatic area.

INS- and GLU-expressing areas (μm^2) INS- and GLU-expressing areas were calculated as the product of the cell number and the cell size for either INS- or GLU-expressing cells.

Total pancreatic area (mm^2) Total pancreatic area was determined by manually defining the perimeter of the exocrine and endocrine pancreatic tissues in each section examined.

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Islet number (no. of islets/mm^2) Islet number was defined as aggregates of at least five INS- or GLU-expressing cells (Garofano *et al.* 2000). The islet numbers were normalized to total pancreatic area.

INS and GLU cell numbers (no. of cells/mm^2) The relative number of INS and GLU cells per islet was determined by the point sampling method and normalized to total pancreatic area in each section as previously described (Garofano *et al.* 2000). At least 5000 points were scored in 30 islets/genotype per diet.

INS:GLU cell ratio INS:GLU cell ratio was determined by dividing the total number of INS-expressing cells by the total number of GLU-expressing cells.

INS and GLU cell size (μm^2) The area of individual INS- or GLU-expressing cells was determined by manually defining the perimeter of the cells immunostained by either INS or GLU. More than 50 cells/pancreas per genotype per diet from multiple litters were measured and averaged.

RNA isolation and quantitative real-time PCR analysis

Total RNA was prepared from fetal pancreata, liver (GLUT4 expression negative control), gastrocnemius, and adipose tissue (GLUT4 expression positive controls) ($n=6$–10 diet/genotype, $n=4$–8 litters) as previously described (Hartil *et al.* 2009, Vuguin *et al.* 2013). The RNA was checked for DNA contamination, using PCR with control primers as described previously (Ouhilal *et al.* 2012).

Quantitative real-time PCR was the method of choice to determine the expression of genes of interest (Ouhilal *et al.* 2012). Four commonly used housekeeping genes, ubiquitin, β-actin, hypoxanthine guanine ribotransferase, and cyclophilin B were used for normalization, as described previously (Ouhilal *et al.* 2012). For quantitative analysis, all samples were normalized to the genes described above using the ΔΔCT value method. Each sample was measured in triplicate to assess technical variability (Ouhilal *et al.* 2012).

Statistical analysis

Data are expressed as the mean±S.E.M. Statistical analyses were performed using JMP IN 5.1 Software (SAS Institute, Cary, NC, USA). ANOVA was used to test the difference between the means of two (*t*-test) or more groups. Significance was defined as $P<0.05$.

Results

Our previous studies revealed that maternal exposure to HFD decreased fetal BW at e18.5 and increased blood glucose levels without altering plasma INS levels (Hartil *et al.* 2009, Vuguin *et al.* 2013). Consistent with those results HFD IU decreased fetal growth by 10% independent of fetal genotype (Table 1). To determine whether HFD exposure affected pancreatic morphology or islet cell composition, we examined e18.5 WT and G4+/− pancreata. No significant differences were seen between the groups when analyzed based on fetal sex with regards to pancreatic morphology or gene expression.

Pancreatic morphology

Total endocrine area (the sum of INS- and GLU-expressing areas normalized to the total pancreatic area) was increased almost twofold in WT HFD fetal pancreata (Table 1) compared with those of WT CD ($P=0.0015$).

Table 1 Metabolic parameters and pancreatic morphology seen in WT and G4+/− fetuses exposed to either CD or HFD during development. Data is expressed as mean±S.E.M., $n=5$–19/genotype

	WT fetus		G4+/− fetus	
	CD	HFD	CD	HFD
Body weight (g)	1.32±0.019*	1.18±0.015†	1.34±0.017*	1.17±0.019†
Total endocrine area (μm^2/mm^2)	0.0104±0.001*	0.0204±0.009†	0.0124±0.001*	0.0106±0.003*
Islet number (no. of islets/mm^2)	6.2±0.4*	6.2±0.6*	5.8±0.7*	7.5±0.9*
GLU cell number (no. of cells/mm^2)	38±7*	91±20†	64±13*,†	54±17*,†
INS cell number (no. of cells/mm^2)	88±10*	110±20*,†	153±15†	123±37*,†
INS:GLU cell number	2.6±0.3*	1.3±0.2†	2.5±0.2*	2.3±0.2*
INS cell size (μm^2)	79±7*	91±11*	58±1†	65±4*,†
GLU cell size (μm^2)	81±1*	96±7†	61±2‡	72±6*,†,‡

Values that do not share the same symbol are significantly different from each other, $P<0.05$.

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In contrast, there was no significant difference in total endocrine area in G4+/− HFD compared with G4+/− CD fetal pancreata. Despite a twofold increase in total endocrine area in WT HFD fetal pancreata, HFD did not increase the number of islets (defined as aggregates of at least five INS- or GLU-expressing cells) in either genotype (Table 1). Islets, independent of the genotype and diet, comprised a mantle of GLU cells surrounded by a core of INS cells (Fig. 1A, B, C and D).

To determine whether the increase in total endocrine area in WT HFD pancreata was associated with an increase in the relative abundance or size of endocrine cells within the islets, the number and area of GLU- and INS-immunoreactive cells were quantified.

Morphometric analysis revealed that the mean number of GLU cells per pancreata was 2.4-fold higher in WT HFD than that in WT CD ($P=0.023$; Fig. 1A and B). In contrast, no change was observed in the mean number of GLU cells per pancreata between G4+/− CD and G4+/− HFD (Fig. 1C and D). Similarly, no change in the relative number of INS cells was observed when each genotype was compared with the same genotype on the alternative diet. However, the mean number of INS cells per pancreata was 1.7-fold higher in G4+/− CD than that in WT CD ($P=0.007$; Table 1 and Fig. 1C).

G4+/− HFD and G4+/− CD fetal pancreata had a significantly higher INS:GLU cell ratio when compared with WT HFD; however, this was not significantly different

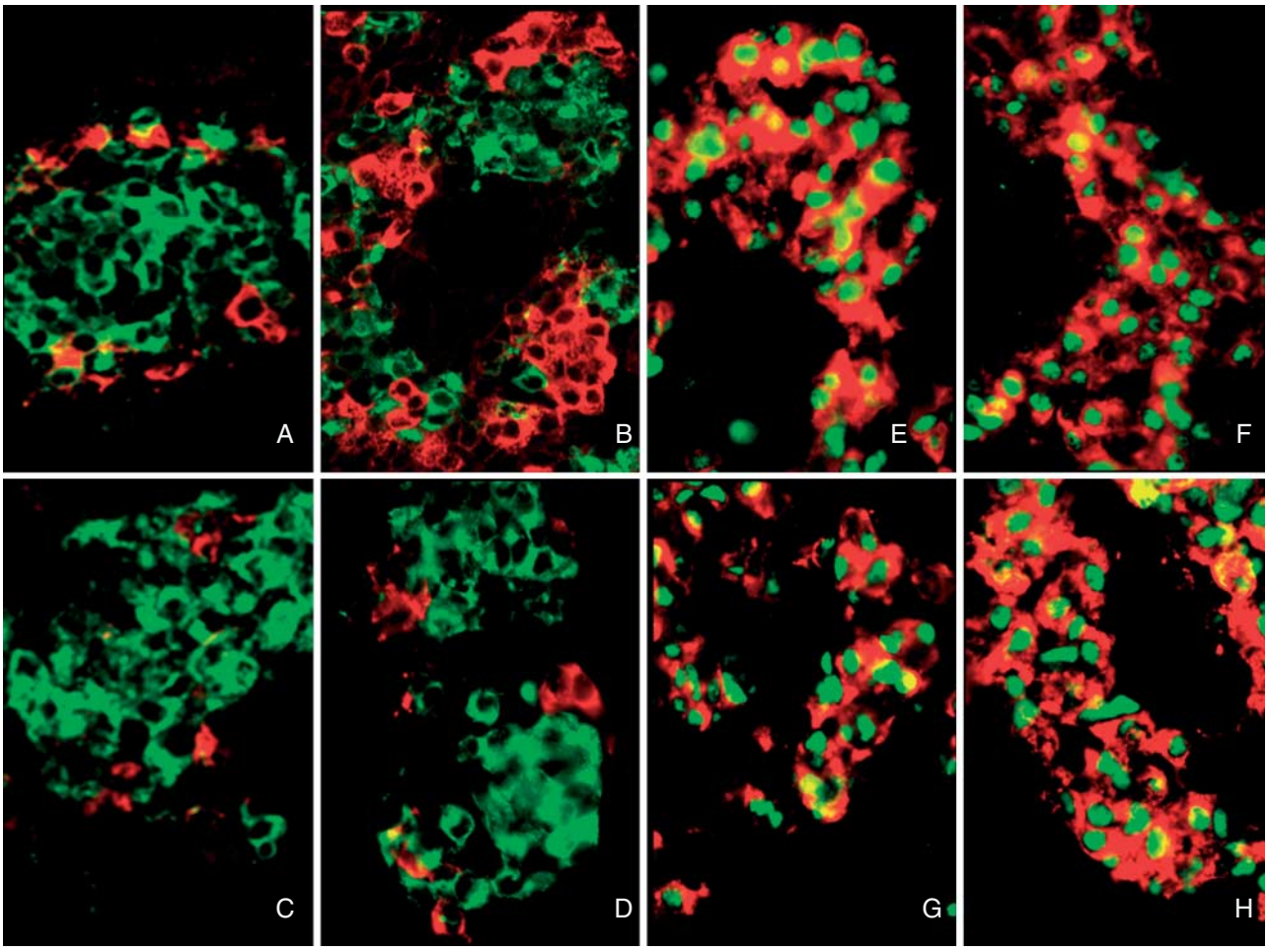

Figure 1
Photomicrographs of confocal images of representative islets from embryos at e18.5 immunostained for insulin (green; A, B, C and D) and glucagon (red; A, B, C and D), or insulin (red; E, F, G and H) and *Pdx1* (green; E, F, G and H) cells in WT C (A and E), WT HFD (B and F), G4+/− CD (C and G), and G4+/− HFD fetusus (D and H). Pancreata from WT HFD (B) fetuses exhibit an increased number of GLU cells (red) as compared with WT CD (A), G4+/− CD (C), and G4+/− HFD (D) fetuses. HFD did not alter the expression or the localization of *Pdx1* (green) in WT (E and F) or G4+/− (G and H) fetal pancreata compared with those of CD pancreata. Scale bar=40 μm.

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when compared with WT CD fetal pancreata (Table 1 and Fig. 1A, B, C and D).

Exposure to HFD did not affect INS cell size in WT or G4+/− fetal pancreas when compared with the fetuses exposed to CD. However, G4+/− CD pancreata had smaller INS cells compared with WT independent of the IU diet ($P=0.03$ vs WT HFD and $P<0.002$ vs WT CD) but were not significantly different when compared with G4+/− HFD (Table 1).

Exposure to HFD increased GLU cell size by 18% in both WT and G4+/− fetal pancreata, although this only reached statistical significance in WT HFD vs WT CD ($P=0.03$; Table 1). Interestingly, similar to reduced INS cell size, G4+/− CD had smaller GLU cells compared with WT independent of diet ($P=0.0033$ vs WT HFD and $P<0.0001$ vs WT CD; Table 1).

GLUT2 and *Pdx1* localization

To determine whether IU HFD regulates factors involved in the regulation of INS cell development, differentiation, and function, we determined *Pdx1* and GLUT2 localization in the islets of fetuses exposed to both diets. IU HFD did not alter the localization of *Pdx1* in WT or G4+/− fetal pancreata compared with a CD (Fig. 1 E, F, G and H). Similarly, HFD did not alter the GLUT2 localization in WT or G4+/− fetuses (data not shown).

Gene expression

To determine whether IU HFD regulates factors involved in INS cell differentiation, proliferation, survival, apoptosis, and function, we determined mRNA expression of *Pdx1*, sex determining region Y-box 9 (*Sox9*), baculoviral IAP repeat containing 5 (*Birc5–survivin*), B-cell CLL/lymphoma 2 (*Bcl2*), and apoptosis-related cysteine peptidase (*Casp3*) (Table 2). HFD IU did not alter mRNA expression of *Pdx1*, *Sox9*, *Birc5*, *Bcl2*, and *CasP3* in WT or G4+/− fetal pancreata compared with a CD.

To determine whether IU HFD regulates the expression of genes involved in INS secretion, pancreatic growth, and development we determined mRNA expression of the potassium inwardly rectifying channel (*Kir6.1*), sulfonylurea receptor (*Sur1*), glucocorticoid receptor (*Nr3c1*), and the IGFs, *Igf1* and *Igf2*. HFD IU did not alter mRNA expression of *Kir6.1*, *Sur1*, *Nr3c1*, or *Igf1* in the pancreas of WT and G4+/− fetuses.

HFD significantly increased the expression of *Igf2* mRNA, a potent INS cell growth and anti-apoptotic factor, in G4+/− HFD pancreas compared with G4+/− CD. In contrast, no significant difference in *Igf2* expression was observed in WT HFD fetal pancreata compared with WT CD. To determine whether IU HFD regulates the expression of genes involved in facilitated glucose transport in pancreatic islets, mRNA expression of the main facilitated GLUT expressed in pancreatic islets, GLUT2, was measured. In addition, GLUT4 mRNA expression was determined. HFD did not alter mRNA expression of GLUT2 in WT or G4+/− fetuses. GLUT4 mRNA expression was not detected in fetal pancreata at e18.5.

Discussion

Diabetes mellitus is a complex disease resulting from the interplay between genetics and the environment. It results from dysfunctional pancreatic INS cells that cannot compensate for the metabolic demands imposed by peripheral tissues such as skeletal muscle and adipose tissue. Owing to the role of both genetics and the IU environment, and the importance of the pancreas in the development of diabetes, we sought to determine the effect of a maternal HFD on pancreatic development in a mouse model genetically predisposed to develop the features of diabetes, the G4+/− mouse (Charron & Kahn 1990, Rossetti *et al.* 1997, Stenbit *et al.* 1997, Li *et al.* 2000).

Consistent with results from our previous studies, HFD exposure decreased fetal BW (Vuguin *et al.* 2013). Similar to a rat model of IU HFD that resulted in poor fetal growth, exposure to HFD during development was accompanied by an increase in GLU cell volume and number in WT, but not G4+/−, pancreata (Cerf *et al.* 2009).

HFD did not alter the islet morphology in G4+/− fetuses despite an increase in RNA expression of the mitogenic growth factor *Igf2* when compared with CD. These data indicate that increased *Igf2* could be a compensatory response to maintain normal islet morphology.

There were no discernible structural differences in the islets of fetuses exposed to HFD. INS cells were present at the core and GLU cells were present at the mantle, indicating normal regulation of islet formation. In contrast to other models of IU programing, IU HFD did not alter gene expression and or localization of the transcription factor, *Pdx1*, thought to be relevant for INS cell adaptation to an altered IU environment (Gesina *et al.* 2006, Chen *et al.* 2012, Rodriguez-Trejo *et al.* 2012). In addition, the expression of genes implicated in pancreatic growth (*Igf1*) or INS secretory capacity (*Kir6.1* and *Sur1*) was not altered by maternal HFD.

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Table 2 Effect of IU HFD exposure on fetal pancreatic gene expression. Gene expression was determined for genes involved in cell differentiation, cell survival and apoptosis, glucose transport, cortisol receptor and cell function, and growth factors. The increase in mRNA (fold change) measured by quantitative real-time PCR for IU HFD compared with IU CD in WT and G4+/− fetal pancreata (n=6–10/genotype diet at e18.5) is given

Gene name	Gene symbol	Gene sequence (forward and reverse)	WT IU HFD vs WT IU CD	G4+/− HFD vs G4+/− CD
Cell differentiation				
Pancreatic and duodenal homeobox 1	*Pdx1*	CGCGTCCAGCTCCCTTT CCTGCCCACTGGCCTTT	NS	NS
Sex determining region Y (SRY)-box 9	*Sox9*	GTACCCGCATCTGCACAAC CTCCTCCACGAAGGGTCTCT	NS	NS
Cell survival and apoptosis				
Baculoviral IAP repeat containing 5	*Birc5–survivin*	CCCGATGACAACCCGATA CATCTGCTTCTTGACAGTGAGG	NS	NS
B-cell CLL/lymphoma 2	*Bcl2*	AGTACCTGAACCGGCATCTG GGGGCCATATAGTTCCACAAA	NS	NS
Apoptosis-related cysteine peptidase	*Casp3*	GAGGCTGACTTCCTGTATGCTT AACCACGACCCGTCCTTT	NS	NS
Cortisol receptor and cell function				
Nuclear receptor subfamily 3, group C, member 1	*Nr3c1*	TGGAGCTACAGTCAAGGTTTCT GCTTGGAATCTGCCTGAGA	NS	NS
ATP-binding cassette, sub-family C, member 8–sulfonylurea receptor subunit	*Sur1*	GACGGCTGGGCAGATCTG GAGGTTTGGGCATAAGAAGAAAAA	NS	NS
Potassium inwardly rectifying channel, subfamily J, member 86.1	*KcnJ8*; *Kir6.1*	AGCCGCCATGCTGTGATT CCCACCCGGAACATGAAG	NS	NS
Glucose transport				
Solute carrier family 2 (facilitated glucose transporter), member 4	*Glut4*	CTGCAAAGCGTAGGTACCAA CCTCCCGCCCTTAGTTG	ND	ND
Solute carrier family 2 (facilitated glucose transporter), member 2	*Glut2*	TTGACTGGAGCCCTCTTGATG CACTTCGTCCAGCAATGATGA	NS	NS
Growth factors				
Insulin-like growth factor 1	*Igf1*	AACAAGCCCACAGGCTATGG AAGCAACACTCATCCACAATGC	NS	NS
Insulin-like growth factor 2	*Igf2*	CATCGTCCCCTGATCGTGTT CACTGATGGTTGCTGGACATCT	NS	5*

*P<0.001 for IU HFD versus IU CD. NS, non significant; ND, no detected.

In animal models, the most profound changes in islet cell morphology seen in response to IU HFD were the number and the volume of INS cells, which were significantly reduced in neonatal rats (Cerf *et al*. 2009). In contrast, no detectable change in INS cell number was observed. These findings may represent differences in the species or differences in the INS cell adaptation to a different metabolic environment.

INS cell mass adapts to changes in metabolic homeostasis. These adaptations can occur through increases or decreases in INS cell number, through changes in proliferation, neogenesis, apoptosis, or cell size. INS cell number was not significantly altered by diet, but was increased 1.7-fold in G4+/− CD compared with WT CD fetuses. Based on gene expression at e18.5 data, we did not find evidence of increased proliferation and/ or apoptosis. Future studies done at earlier developmental stages may be necessary to confirm these findings.

INS cell size was significantly reduced in G4+/− CD fetuses compared with WT CD. Adaptations in INS cell size have been observed during pregnancy (Dhawan *et al*. 2007) and in response to persistent hyperinsulinemia (Anlauf *et al*. 2005). Larger INS cells containing more protein per cell are known to be highly glucose-responsive (Martens *et al*. 2010). Our finding that G4+/− CD fetuses exhibit smaller but more INS cells indicates an adaptation to the metabolic environment associated with GLUT4 haploinsufficiency.

HFD decreased the INS:GLU cell ratio in WT HFD fetuses. Further analysis revealed a significant increase in GLU cell number and size in WT HFD pancreata. When the cell number was multiplied by the individual cell surface area, to approximate the endocrine cell mass, an increase in the total endocrine area was observed.

The mechanism that leads to GLU cell hyperplasia in WT HFD fetal pancreas is not clear. GLU cell hyperplasia has been seen in models with impaired GLU synthesis/

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signaling (Vuguin *et al.* 2006), GLU receptor expression (Chen *et al.* 2011), and GLU action (Chen *et al.* 2005); inactivation of *Pax4* (Sosa-Pineda *et al.* 1997); circulating low levels of INS (Rahier *et al.* 1983, Sosa-Pineda *et al.* 1997, Thyssen *et al.* 2006); mild hypoglycemia; and chronic HFD (Fiori *et al.* 2013). As circulating INS levels were normal and glucose levels were elevated in our HFD IU model, other factors must be responsible for the increase in GLU cell number (Jiang & Zhang 2003).

In addition to the GLU cell hyperplasia, WT fetuses exposed to a HFD displayed an increased GLU cell size that could lead to increased GLU levels (Huang *et al.* 2013). Increased GLU levels could explain the increase in expression of genes involved in gluconeogenesis and the increased glucose levels in the livers of WT HFD fetuses, which we previously reported (Vuguin *et al.* 2013). Unfortunately, we were not able to determine serum GLU levels. Accurate determination of fetal GLU serum levels usually requires pooling serum from several fetuses to collect at least 50–100 μl of serum per sample (www.biotrend.com/download/gl-32k.pdf). Because of the technical difficulties associated with the adequate determination of serum GLU levels, alterations in pancreatic morphology were assessed instead. Thus, further studies are needed to confirm the role of GLU in the GLU cell phenotype observed in WT fetal pancreata exposed to HFD.

Increased GLU cell number and cell size could also occur in response to lower glucose levels during fetal development with an IU HFD. Decreased glucose levels would signal the CNS to increase GLU cell number and secretion leading to GLU cell hyperplasia that may result in hyperglycemia (Brunicardi *et al.* 1995, Furuta *et al.* 1997). Alternatively, high-glucose levels and GLU cell hyperplasia could be the result of cell transformation of GLU-expressing cells into INS cells (Thorel *et al.* 2010). GLU cells are the earliest identifiable cells of the pancreatic endocrine lineage, capable of becoming INS-expressing cells (Gromada *et al.* 2007). It is possible that the GLU cells in our model are incapable of being transformed into INS cells. This seems unlikely, because a decrease in INS cell number or size, as seen in other models of GLU cell hyperplasia, is not a feature found in this model (Cerf *et al.* 2009).

Increased INS:GLU endocrine cell ratio was seen only in WT HFD fetal pancreas while increased *Igf2* gene expression was seen in HFD G4+/− fetal pancreas which had normal islet architecture. It is possible that different signals regulate GLU cell number in islets of WT HFD and G4+/− mice.

GLUT4 has previously been found to be expressed at low levels in pancreatic GLU and INS cells of adult mouse, rat, and human endocrine pancreas (Kobayashi *et al.* 2004, Bahr *et al.* 2012) and its expression is regulated by glucose and INS levels (Bahr *et al.* 2012). Unfortunately, *Glut4* mRNA expression was not detected in fetal pancreata at e18.5. *Glut4* expression is turned on in brown adipose tissue, heart, and skeletal muscle toward the end of pregnancy (Santalucia *et al.* 1992). Its expression increases progressively after birth, and it has been found to be sensitive to alterations in maternal nutrient intake (Gardner *et al.* 2005, Thamotharan *et al.* 2005). Thus, the developmental timing of its expression in the pancreas could explain why we were not able to detect the presence of *Glut4* mRNA.

The exact role of *Igf2* expression in the fetal pancreas is unclear. In genetic models, decreased *Igf2* expression in fetal pancreata led to lower INS cell proliferation rates and increased INS cell apoptosis (Kulkarni 2005). Overexpression of *Igf2* in INS cells is associated with an increase in INS cell mass (Devedjian *et al.* 2000), while global overexpression of *Igf2* causes islet GLU cell hyperplasia with an abnormal INS:GLU ratio (Petrik *et al.* 1999).

In one model, maternal under-nutrition decreased pancreatic *Igf2* expression and was associated with increased INS cell mass (Martin *et al.* 2005). In contrast, another study demonstrated that maternal under-nutrition increased pancreatic *Igf2* expression with no alteration in INS cell mass (de Miguel-Santos *et al.* 2010).

In rats, a high-carbohydrate diet during the neonatal period decreased *Igf2* mRNA expression and GLU cell number (Petrik *et al.* 2001). Thus, the lack of consistency between studies makes it difficult to determine the precise role of *Igf2* in the pancreatic phenotype and INS:GLU cell ratio. Some differences may be related to compensatory increases in *Igf1* expression that could lead to increased INS cell mass (Martin *et al.* 2005). *Igf1* expression was not different in our study. In addition, gene expression was determined in whole fetal pancreatic tissue and it may be that expression levels are regulated differently in response to genotype and/or HFD IU in different (pancreatic) islet cell types.

In conclusion, nature (genotype) modifies the effects of nurture (HFD) on fetal pancreatic development. We propose that changes in endocrine cell ratio in islets induced by IU nutritional and genetic manipulation may affect islet cell responsiveness to physiological or pathological stimuli. Further investigation is needed to confirm whether the IU adaptations that occur in WT HFD islets are maladaptive in later life and whether developing in a

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IU HFD milieu affects the capacity of GLU cells to respond to INS resistance with increasing age and/or progressive metabolic deterioration. In addition, it is not known whether the lack of GLU cell hyperplasia in G4+/− pancreata in response to HFD might be detrimental to development during times when glucose delivery is interrupted, such as during time of delivery. Understanding the molecular basis of an altered INS:GLU-expressing cell ratio is an attractive objective, particularly in light of new treatments that may directly act on these pathogenic mechanisms of T2D.

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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