

Insulin-like growth factor and fibroblast growth factor expression profiles in growth-restricted fetal sheep pancreas

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Abstract

Placental insufficiency results in intrauterine growth restriction (IUGR), impaired fetal insulin secretion and less fetal pancreatic β -cell mass, partly due to lower β -cell proliferation rates. Insulin-like growth factors (IGFs) and fibroblast growth factors (FGFs) regulate fetal β -cell proliferation and pancreas development, along with transcription factors, such as pancreatic and duodenal homeobox 1 (PDX-1). We determined expression levels for these growth factors, their receptors and IGF binding proteins in ovine fetal pancreas and isolated islets. In the IUGR pancreas, relative mRNA expression levels of IGF-I, PDX-1, FGF7 and FGFR2IIIb were 64% ($P < 0.01$), 76% ($P < 0.05$), 76% ($P < 0.05$) and 52% ($P < 0.01$) lower, respectively, compared with control fetuses. Conversely, insulin-like growth factor binding protein 2 (IGFBP-2) mRNA and protein concentrations were 2.25- and 1.2-fold greater ($P < 0.05$) in the IUGR pancreas compared with controls. In isolated islets from IUGR fetuses, IGF-II and IGFBP-2 mRNA concentrations were 1.5- and 3.7-fold greater ($P < 0.05$), and insulin mRNA was 56% less ($P < 0.05$) than control islets. The growth factor expression profiles for IGF and FGF signaling pathways indicate that declines in β -cell mass are due to decreased growth factor signals for both pancreatic progenitor epithelial cell and mature β -cell replication.

Keywords: placental insufficiency, intrauterine growth restriction, islets of Langerhans, β -cell, insulin, fetal programming

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Introduction

Several epidemiological and experimental studies have demonstrated a relationship between intrauterine growth restriction (IUGR) and impaired glucose homeostasis in later life, indicating that β -cell dysfunction was established *in utero*.^{1–4} IUGR fetuses have lower plasma insulin concentrations and glucose-stimulated insulin secretion,⁵ which are partially explained by a diminished number of pancreatic β -cells in severe cases.⁶ Pancreas development in fetal sheep parallels human fetal pancreas development,^{7,8} and complications of IUGR in human fetuses are similar to those in a comparative sheep model, with placental insufficiency produced by maternal exposure to a high-temperature environment during the bulk of gestation.^{9–11} Two reasons for decreased β -cell mass in near-term placental insufficiency-induced IUGR (PI-IUGR) sheep fetuses are slower rates of β -cell replication⁹ and less pancreatic progenitor epithelium at the beginning of the third trimester (SW Limesand *et al.*, unpublished). In normal pancreas development, these processes are regulated by insulin-like growth factors (IGFs) and fibroblast growth factors (FGFs), respectively.^{12,13} However, the impact of placental insufficiency on pancreatic IGFs and FGFs is not defined.

IGFs regulate cell proliferation, survival and metabolism, while their binding proteins (IGFBPs) can inhibit or potentiate IGF activity, depending on subtypes and target cells.^{14–17} Furthermore, pancreatic progenitor epithelial cell proliferation is increased by fibroblast growth factor receptor (FGFR) 2IIIB ligands, FGF7 and 10.^{13,18–20} Expansion of these pancreatic and duodenal homeobox 1 (PDX-1)-positive progenitor cells will also influence the absolute number of β -cells formed. PDX-1 expression is high in β -cells and regulates insulin and glucose transporter 2 (Glut2), which is required for proper β -cell function.^{21,22} Therefore, we postulated that reduced expression of IGFs and FGFs or greater expression of IGFBPs in the IUGR fetal pancreas will explain the decline in β -cell mass and insulin secretion in the PI-IUGR sheep fetus. Furthermore, the expression profile for IGFs may occur in fetal islets of Langerhans and reduce other factors required for β -cell function.

Materials and methods

Animals and experimental protocol

Protocols involving animals were approved by the Institutional Animal Care and Use Committee at the

University of Arizona and the University of Colorado Anschutz Medical Center. Both laboratories are accredited by the American Association for Accreditation of Laboratory Animal Care. Pregnant Columbia-Rambouillet crossbred ewes ($n = 32$) carrying singleton fetuses were purchased from Nebekar Ranch (Lancaster, CA, USA). PI-IUGR ewes ($n = 16$) were housed in environmentally controlled rooms with elevated ambient temperatures (40°C for 12 h and 35°C for 12 h; 30% relative humidity) from 37 ± 3 days of gestational age (dGA) to 120 ± 3 dGA ($0.24\text{--}0.82$ gestation). Pair-fed control ewes ($n = 16$) were maintained at thermoneutral conditions (25°C ; 25% relative humidity). At 133.4 ± 0.7 dGA, ewes were euthanized with an overdose of sodium pentobarbital (10 mmol/L, intravenously; Euthasol, Virbac Animal Health, Fort Worth, TX, USA). Fetal pancreases were removed and islets were isolated from a subset of these fetuses (PI-IUGR, $n = 7$; controls, $n = 6$) as previously described.^{10,23} The head of the pancreas was collected from the remaining PI-IUGR ($n = 9$) and control ($n = 10$) fetuses, snap-frozen in liquid nitrogen, and stored at -80°C for RNA and protein extraction.

Biochemical analysis

Fetuses were instrumented with arterial catheters as described previously.^{10,23} Fetal blood was collected in syringes lined with ethylenediaminetetraacetic acid or heparin. Plasma glucose concentrations were measured with a YSI model 2700 SELECT Biochemistry Analyzer (Yellow Springs Instruments, Yellow Springs, OH, USA). Blood oxygen content was measured with an ABL 520 (Radiometer, Copenhagen, Denmark).

Quantitative realtime polymerase chain reaction

RNA was isolated and reverse transcribed as described previously.^{23,24} Synthetic oligonucleotide primers for IGFs, FGFs, PDX-1, insulin and Glut2 (Table 1) were designed from conserved sequences. Polymerase chain reaction (PCR) products were cloned into pCR II (Invitrogen Life Technologies, Carlsbad, CA, USA) and verified by nucleotide sequencing. Quantitative PCR was performed using a master-mix of SYBR[®] Green JumpStart[™] Taq Ready Mix[™] (Sigma-Aldrich, St Louis, MO, USA), $0.3 \mu\text{mol/L}$ 5' and 3' primers, and $2 \mu\text{L}$ of cDNA in a DNA Engine Opticon II[™] System (MJ Research, Waltham, MA, USA). The standard thermocycler protocol began with an initial denaturation (96°C) for two minutes, plus 35 cycles of denaturation (96°C) for 45 s, annealing temperature (Table 1) for 1.5 min, extension (72°C) for 45 s and fluorescent detection (72°C). After DNA amplification, melting curve analysis ($72\text{--}99.9^{\circ}\text{C}$ with detection every 0.1°C/s) was used to verify the product purity. In addition, a Southern hybridization was performed on a sample of amplified DNA to confirm PCR products, as described previously.²⁵ Results were normalized to a reference gene (β -actin for the pancreas, S15 for the islet) using the comparative change of threshold cycles (ΔC_t) method (C_t gene of interest– C_t reference gene) and fold change was determined by the Pfaffl's and Livak's method.^{26,27} Values are presented as means $\pm$ SEM.

Protein immunoblot analysis

Immunoblots were performed for IGF-I and IGFBP-2 in fetal pancreatic tissue (sc-9013 and sc-13096, respectively; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Protein was extracted, separated by polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane as previously described.²⁸ Non-specific binding was blocked in phosphate-buffered saline with 0.05% Tween-20 (PBST) with 5% non-fat dry milk for one hour, and then membranes were incubated with either IGF-I or IGFBP-2 antibody diluted (1:1000) in PBST with 5% non-fat dry milk at 4°C overnight. Immunocomplexes were detected with anti-goat immunoglobulin G horseradish peroxidase-conjugated secondary antibody (1:10,000; Bio-Rad Laboratories, Hercules, CA, USA) using enhanced chemiluminescence-Plus (Amersham Pharmacia Biotech, Arlington Heights, IL, USA) and exposed to X-ray film. IGF-I or IGFBP-2 immunocomplexes were removed with a 62.5 mmol/L Tris, pH 6.8, 2% sodium dodecyl sulfate and 100 mmol/L β -mercaptoethanol solution for 30 min at 50°C . Western blot analysis for β -actin (1:40,000; ICN Biomedicals, Burlingame, CA, USA) was used to normalize for loading differences. Densitometry value was measured with ImageJ software version 1.41 (National Institutes of Health, Bethesda, MD, USA) and the analysis was completed by comparing individual density value against mean value of control blots. Data are presented as means $\pm$ SEM.

Statistical analysis

One-way analysis of variance was performed using the general linear model procedure of SAS (SAS v. 9.1; SAS Institute Inc, Cary, NC, USA), and pair-wise comparisons were made using Student's *t*-test. Analysis of quantitative realtime PCR data was performed on ΔC_t values.

Results

Fetal parameters

Mean fetal body weight was lower ($1.7 \pm 0.2 \text{ kg}$ versus $3.4 \pm 0.1 \text{ kg}$, $P < 0.01$) in PI-IUGR fetuses ($n = 16$) compared with controls ($n = 16$). Gestation ages were similar (134 ± 1 days in PI-IUGRs versus 134 ± 1 days in controls). Mean plasma glucose concentrations were lower in IUGR fetuses compared with controls ($0.65 \pm 0.04 \text{ mmol/L}$ versus $1.11 \pm 0.04 \text{ mmol/L}$; $P < 0.01$). Mean blood oxygen contents were also lower in IUGR than control fetuses ($1.26 \pm 0.20 \text{ mmol/L}$ versus $3.00 \pm 0.13 \text{ mmol/L}$; $P < 0.01$). An equal number of fetal pancreases from each treatment group (control and PI-IUGR) were randomly collected for either pancreatic RNA and protein extraction or isolation of islets of Langerhans. Fetal characteristics (weight, glucose and oxygen) within treatments were not different between collection assignments.

mRNA expression in fetal pancreatic tissue

Relative IGF-I mRNA concentrations were 64% lower ($P < 0.01$) in PI-IUGR pancreases compared with control pancreases (Figure 1). IGFBP-2 mRNA was 2.25-fold higher ($P < 0.01$)

Table 1 Quantitative realtime polymerase chain reaction parameters

Gene	Forward primer (5'–3')	Reverse primer (5'–3')	Product size (bp)	Annealing temperature (°C)	Accession #
IGF-I	TGCGATCTCTCTTCTATCTGGCCCTGT	GCAGTACATCTCCAGCCTCCTCAGA	240	62	M30653
IGF-II	GACCGCGGCTTCTACTTCAG	AAGAACTTGCCACGCGGGTAT	203	62	X55638
IGFBP-1	TGATGACCGAGTCCAGTGAG	GTCCAGCGAAGTCTCACA	248	62	X54979
IGFBP-2	CAATGGCGAGGAGCACTC	TGGGGATGTGTGTAGGGAAT	335	55	S44612
IGFBP-3	GGCATATTGAGCTCCACA	TCTCAGAGCACAGACACCCA	337	54	M76478
IGFBP-4	TGTGCGTGTGTGTTGATG	GAGGGAGCCAAGATGAGT	229	62	S77394
IGFBP-5	TCGTGCGGCGTCTACACTGAG	GAGTAGGTCTCTCCGCCATC	203	61	EU727460
IGFBP-6	AGGAGAGTAAGCCCAAGCAG	GTCACAATTGGCGAGGTAGAG	221	62	EU862545
IR	CAGCTGAGCTGAAGCCAAC	CCATTTCAGCAAGATCTTGTC	416	57	AY157728
IGF-1R	AACTGTCATCTCCAACCTC	CAAGCCTCCCACTATCAAC	490	53	X54980
IGF-2R	GAAGAGCAACGTGCACGA	TTGTCCAGGGAGATCAGCA	368	54	AF342811
FGF7	TCAGGACAGTGGCTGTTGGAA	CAACATTTCCTCCGCTGTG	195	65	EU626202
FGF10	CCCTCGATCAGGACATGGTG	AAGGCAAGGAGACGGACGAG	87	65	AF213396
FGFR2IIIB	CCGAGTGCCTTCAGAACCTTGA	AGAGGCGATGTGGAGTTTGTC	136	65	DQ908953
PDX-1	TTTCCCGTGGATGAAGCTAC	CGGTGCGTGTCCGCTTGTCT	101	60	JF728303
Glut2	CTTTGCAGTTGGTGAATGAT	GCTGATGAAGAGCACCGATAG	272	60	AJ318925
Insulin	TCAGCAACAGGTCTCTCGCAAG	GGGCCAGGTCTAGTTACAGTAG	352	60	U00659
β -Actin	GGGACCTGACCGACTACC	GACAGCGAGGCAGGATGG	502	62	U39357
S15	ATCATTCTGCCGAGATGGTG	TGCTTTACGGGCTTGATGGTG	134	60	AY949774

Glut2, glucose transporter 2; FGF, fibroblast growth factor; FGFR2IIIB, fibroblast growth factor receptor 2IIIB; IGF-I, insulin-like growth factor I; IGF-II, insulin-like growth factor II; IGFBP, insulin-like growth factor binding protein; IGF-1R, insulin-like growth factor type 1 receptor; IGF-2R, insulin-like growth factor type 2 receptor; IR, insulin receptor; PDX-1, pancreatic and duodenal homeobox 1

in PI-IUGR pancreases. IGFBP-1 mRNA transcripts were not detected in the pancreas by PCR-Southern blot hybridization procedures, but were detected in fetal liver (data not shown²⁹). No differences were observed in the expression of IGF-II, IGFBP-3, -4 and -5, insulin receptor (IR), IGF type 1 receptor (IGF-1R) or IGF type 2 receptor (IGF-2R) between PI-IUGR and control fetuses (data not shown). IGFBP-6 mRNA concentrations tended to be 46% lower ($P = 0.07$) in PI-IUGR pancreases than controls (data not shown). Expression levels of PDX-1 were 76% lower ($P < 0.05$) in PI-IUGR pancreases (Figure 1). FGF7 and FGFR2IIIB mRNA concentrations were 76% ($P < 0.05$) and

78% ($P < 0.01$) lower in PI-IUGR pancreases compared with controls, but FGF10 concentrations were not different (data not shown).

mRNA expression in isolated fetal pancreatic islets

Islets of Langerhans isolated from PI-IUGR fetuses had higher ($P < 0.05$) IGF-II (1.5-fold) and IGFBP-2 (3.7-fold) mRNA concentrations than controls (Figure 2). IGF-I and IGFBP-6 were not different between groups (data not shown). Insulin expression was 66% lower ($P < 0.05$) in PI-IUGR islets than control islets (Figure 2). However,

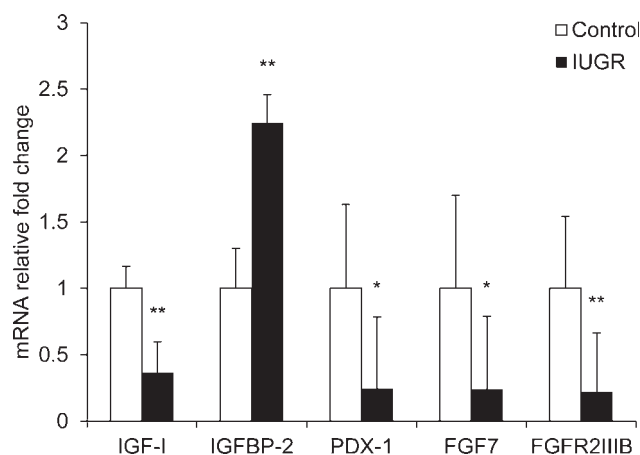


Figure 1 Pancreas expression profiles for IGF-I, IGFBP-2, PDX-1, FGF7 and FGFR2IIIB. Relative mRNA concentrations for the genes listed on the abscissa are normalized to a reference gene (β -actin) and expressed as the fold change from control calculated by the $2^{-\Delta\Delta C_t}$ in pancreatic tissue collected from control (□) and IUGR (■) fetuses. Mean $\pm$ SEM values for animals are presented. Significance is indicated by * $P < 0.05$ and ** $P < 0.01$. IGF-I, insulin-like growth factor I; IGFBP-2, insulin-like growth factor binding protein 2; PDX-1, pancreatic and duodenal homeobox 1; FGF7, fibroblast growth factor; FGFR2IIIB, fibroblast growth factor receptor 2IIIB; IUGR, intrauterine growth restriction

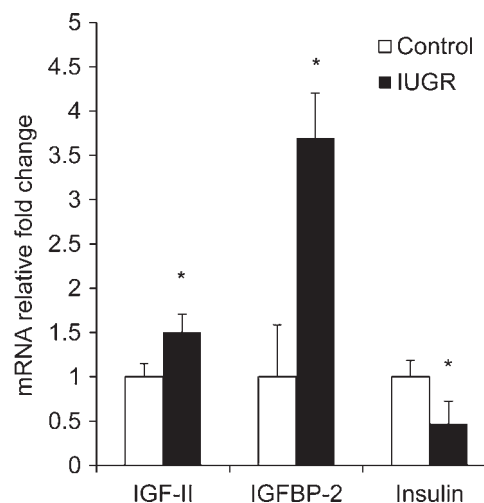


Figure 2 Pancreatic islet expression profiles for IGF-II, IGFBP-2 and insulin. Relative mRNA concentrations for the genes listed on the abscissa are normalized to a reference gene (S15) and expressed as the fold change from control calculated by the $2^{-\Delta\Delta C_t}$ in pancreatic tissue collected from control (□) and IUGR (■) fetuses. Mean $\pm$ SEM values for animals are presented. Significance is indicated by * $P < 0.05$ and ** $P < 0.01$. IUGR, intrauterine growth restriction. IGF-II, insulin-like growth factor II; IGFBP-2, insulin-like growth factor binding protein 2

PDX-1 and Glut2 islet expression levels were not different between groups (data not shown).

IGF-I and IGFBP-2 protein concentrations in pancreatic tissue

IGF-I protein content was not different between PI-IUGR and control pancreases. However, IGFBP-2 protein content was 1.2-fold greater ($P < 0.01$) in PI-IUGR pancreases compared with controls (Figure 3).

Discussion

Two scenarios could explain how the IGF and FGF signaling pathways act independently to reduce β -cell mass in IUGR fetuses following placental insufficiency. First, greater IGFBP-2 mRNA and protein concentrations can slow β -cell replication rates by antagonizing IGF action. Second, decreased FGF7 and FGFR2IIIb signaling can lower PDX-1 expression which, along with decreased IGF signaling, will contribute to reduced pancreatic progenitor epithelial cells and lower the proportion of cells available for differentiation into mature β -cells.^{13,30,31} Factors influencing β -cell mass are replication of mature β -cells, neoformation from pancreatic progenitor epithelium and apoptosis.^{32,33} Previously, we showed that mitotic rates, but not apoptotic rates, of β -cells are lower in IUGR sheep fetuses at 0.9 of gestation, which is consistent with IGFBP-2 inhibition of IGF-induced proliferation.⁹ We also showed that rates of β -cell neoformation are similar between IUGR and control

fetuses.⁹ Contrary to reduced pancreatic IGF-II, isolated PI-IUGR islets have greater IGF-II expression. This may represent a local compensatory mechanism to sustain a minimal β -cell mass by inhibiting apoptosis. Together, these findings begin to identify several mechanisms that define how reductions in selected growth factors in the PI-IUGR pancreas contribute to decreased β -cell mass in response to placental insufficiency.

Plasma IGF-I concentrations are lower in the IUGR fetus;³⁴ however, IGF regulation of somatic cell growth may not be dependent on circulating IGF-I concentrations but instead on local autocrine/paracrine action. This has been shown in mice with a liver-specific IGF-I mutation, where circulating IGF-I concentrations were reduced but growth and development were unaffected.^{35,36} Thus, IGF production within the pancreas most likely regulates pancreatic β -cell development, expansion and survival by local autocrine/paracrine actions.^{15,16,30,37} Increased expression of IGF-I coincides with increased rates of β -cell mitosis during regeneration,³⁸ whereas disruption of pancreatic IGF signaling decreases β -cell mass and pancreas development,³⁰ which might explain the reduction in fetal β -cell mass in the IUGR pancreas.

The greater expression of IGF-II in our IUGR fetal pancreatic islets contradicts reports in fetal rat models of IUGR, including the experimental model of IUGR produced by a maternal low protein diet and the genetic β cell-deficient Goto-Kakizaki rat model, which exhibit reduced pancreatic IGF-II mRNA, lower β -cell proliferation rates and greater apoptotic rates.^{39,40} However, elevated IGF-II expression supports the survival of β -cells, which is consistent with our observations of similar apoptotic rates between control and IUGR β -cells.^{9,15,41}

We identified pancreatic IGFBP-2-6 transcripts in the present study, but IGFBP-1 expression was not found, comparable to previous observations in the fetal rat pancreas.³⁵ The six high-affinity IGF binding proteins positively or negatively influence IGF actions, adding an additional level of regulation.^{14,42,43} Transgenic mice that globally over-express IGFBP-2 have lighter carcass weights, showing that IGFBP-2 negatively influences IGF growth actions.¹⁷ Therefore, as a negative regulator, greater IGFBP-2 levels in the pancreas and islets would act to reduce IGF signaling despite normal IGF-I and increased IGF-II concentrations. Additionally, maximal IGF stimulation of β -cell proliferation requires adequate glucose concentrations, which play an important and specific role in β -cell development by activating the endocrine progenitors.^{16,44} Therefore, due to placental insufficiency, fetal hypoglycemia may further inhibit IGF-potiated β -cell proliferation.⁹

In sheep and humans, β -cells differentiate from the pancreatic progenitor epithelium from at least 0.24 of gestation until term.⁷ During this period, pancreatic progenitor epithelial cells also proliferate, which is regulated by FGF signaling.^{19,20,45} Although FGF10 mRNA was not different, lower pancreatic FGF7 and FGFR2IIIb mRNA concentrations would act to reduce pancreatic progenitor epithelial cell expansion and subsequent β -cell neoformation in PI-IUGR fetuses.^{19,20,45} In genetically engineered mice, transient ablation of PDX-1-positive pancreatic progenitor cells

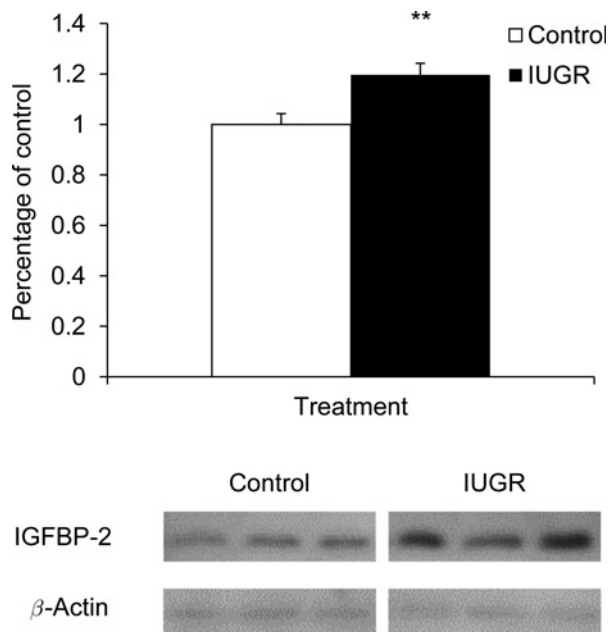


Figure 3 Immunoblot analysis for IGFBP-2 in pancreas tissue. IGFBP-2 protein concentrations were measured in control (□) and IUGR (■) pancreatic tissue. The graph summarizes band intensities from each treatment group. Significance from the Student's *t*-test analyses are depicted by ** $P < 0.01$. Representative immunoblots are shown for three control and three PI-IUGR pancreas samples from both IGFBP-2 and β -actin. IUGR, intrauterine growth restriction; IGFBP-2, insulin-like growth factor binding protein 2

was shown to be a critical and limiting determinant of pancreas size and β -cell area.⁴⁷ Decreased FGFR2IIIB signaling also lowers PDX-1 expression in pancreatic epithelial cells.^{13,46} Thus, while decreased pancreatic PDX-1 mRNA concentrations in PI-IUGR fetuses may be partially due to decreased numbers of β -cells,⁹ it may also be due to the lower expression of pancreatic FGF7 and FGFR2IIIB (Figure 1). Together, these conditions will reduce the pancreatic progenitor cell pool, and lower β -cell mass in PI-IUGR fetuses. In the β -cells, PDX-1 transactivates insulin and Glut2 genes.^{19,48,49} In isolated pancreatic islets in the present studies, PDX-1 and Glut2 mRNA concentrations were similar between IUGR and control fetuses (Figure 2). This normal expression of PDX-1 mRNA, however, was not associated with normal insulin mRNA (Figure 2), which was also reduced by 66% in the IUGR pancreas.⁹ These findings reflect differential regulation of insulin and Glut2 expression by factors other than PDX-1. For example, normal glucose concentrations are required for the normal regulation of insulin gene expression and so fetal hypoglycemia may be responsible for decreased islet insulin mRNA.⁵⁰

In summary, we showed reductions in IGF-I, FGF7, FGFR2IIIB and PDX-1 mRNA expression in PI-IUGR fetal pancreatic tissue, and increased IGFBP-2 mRNA and protein in both pancreatic tissue and isolated islets in PI-IUGR fetuses. Reductions in IGF and FGF signaling in the PI-IUGR pancreas act to decrease expansion of the pancreas progenitor epithelium, leading to fewer cells differentiating into mature β -cells.^{13,30,31} Furthermore, decreased IGF signaling will also reduce β -cell replication. Decreased differentiation of new β -cells and reduced replication of mature β -cells will result in less overall β -cell mass, consistent with our previous observations.⁹ These findings are the first to define how growth factor signaling pathways reduce β -cell mass in fetuses with placental insufficiency-induced IUGR and provide a mechanistic foundation for the development of type 2 diabetes. In addition, the new data point out that the response of the pancreas in the IUGR fetus is complex and potentially treatable.

Author contributions: All authors participated in the experimental design, interpretation of the findings and manuscript review. XC, PJR and SWL conducted the experiments and analyzed the data; XC wrote the manuscript; and PJR, WWH Jr and SWL contributed constructive suggestions to the writing of the manuscript.

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