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# Placental Insufficiency Decreases Pancreatic Vascularity and Disrupts Hepatocyte Growth Factor Signaling in the Pancreatic Islet Endothelial Cell in Fetal Sheep

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**Hepatocyte growth factor (HGF) and vascular endothelial growth factor A (VEGFA) are paracrine hormones that mediate communication between pancreatic islet endothelial cells (ECs) and  $\beta$ -cells. Our objective was to determine the impact of intrauterine growth restriction (IUGR) on pancreatic vascularity and paracrine signaling between the EC and  $\beta$ -cell. Vessel density was less in IUGR pancreata than in controls. HGF concentrations were also lower in islet EC-conditioned media (ECCM) from IUGR, and islets incubated with control islet ECCM responded by increasing insulin content, which was absent with IUGR ECCM. The effect of ECCM on islet insulin content was blocked with an inhibitory anti-HGF antibody. The HGF receptor was not different between control and IUGR islets, but VEGFA was lower and the high-affinity VEGF receptor was higher in IUGR islets and ECs, respectively. These findings show that paracrine actions from ECs increase islet insulin content, and in IUGR ECs, secretion of HGF was diminished. Given the potential feed-forward regulation of  $\beta$ -cell VEGFA and islet EC HGF, these two growth factors are highly integrated in normal pancreatic islet development, and this regulation is decreased in IUGR fetuses, resulting in lower pancreatic islet insulin concentrations and insulin secretion.**

Pancreatic islet signaling between the endothelial cell (EC) and  $\beta$ -cell is critical for normal islet development and

function (1). However, how this signaling is disrupted in intrauterine growth restricted (IUGR) fetuses is understudied. IUGR fetuses develop  $\beta$ -cell defects in response to restricted supplies of nutrient substrates and oxygen. In severe cases, usually due to chronic placental insufficiency, IUGR fetuses have lower plasma glucose and insulin concentrations, and compared with appropriately growing fetuses, they also have decreased glucose-stimulated insulin secretion in late gestation (2). Consistent with this decreased glucose-stimulated insulin secretion, severely growth restricted fetuses have lower pancreatic islet vascularity and a smaller  $\beta$ -cell population, both of which are more severe than their decrement in body or pancreas weight (3). The mechanisms responsible for reduced  $\beta$ -cell number and function are incompletely understood but might explain the increased risk that IUGR offspring have for developing type 2 diabetes as adults (4).

Pancreatic and islet ECs provide signals responsible for the normal formation, maturation, and function of the pancreatic  $\beta$ -cells (5–11). Hepatocyte growth factor (HGF) secreted by the EC stimulates adult  $\beta$ -cell function and production of insulin in vitro (12,13). In transgenic mice with HGF overexpression, pancreatic islets have increased insulin secretion and increased  $\beta$ -cell mass and are resistant to experimental induction of diabetes (6,14,15). Furthermore, when the HGF receptor cMET is inactivated in

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$\beta$ -cells, mice develop glucose intolerance and diabetes due to decreased insulin production and secretion (16,17). In addition to HGF, ECs produce laminins and other components of the extracellular basement membrane that are recognized by integrin receptors to augment  $\beta$ -cell function (8,18). Nitric oxide (NO) is produced by endothelial NO synthase (eNOS) and may increase insulin secretion; autocrine actions of NO also have been described for  $\beta$ -cells (19).

An important relationship exists between  $\beta$ -cells and ECs to increase pancreatic islet vascularity. Insulin, for example, stimulates EC growth (20) along with other factors that establish and maintain normal islet vascularity, such as vascular endothelial growth factor A (VEGFA) (21). VEGFA is a potent angiogenic factor secreted by  $\beta$ -cells (22), and decreased pancreatic islet vascularity has been observed in mice with pharmacologic inhibition of VEGFA or genetic inactivation of VEGFA in pancreatic progenitor cells or  $\beta$ -cells (5,11,23,24). A majority of these mice develop glucose intolerance and diabetes as adults. Of note, an inducible system to inhibit VEGFA expression in adult  $\beta$ -cells has shown a very mild insulin secretory defect and no reduction in  $\beta$ -cell insulin production despite a significant reduction in islet vascularity (24). This observation demonstrates that decreased islet vascularity is not always coupled to impaired insulin secretion. Furthermore, inhibition of EC function and the reduced cross talk with  $\beta$ -cells that might occur in the prenatal and perinatal periods are potentially more deleterious to long-term  $\beta$ -cell function (24).

A majority of the research that demonstrates EC and  $\beta$ -cell cross talk is based on genetically manipulated mice or pharmacological inhibition. The relevance of these interactions to pathophysiological conditions, especially in the fetal period, is understudied. Decreased islet vascularity has been shown in two rodent models of IUGR (25,26). These models were characterized by lower  $\beta$ -cell mass, insulin secretion defects, and development of glucose intolerance and diabetes in adults (27–30). Additionally, both models have decreased perinatal islet VEGFA, indicating decreased islet  $\beta$ -cell-to-EC signaling (although HGF and other EC-to- $\beta$ -cell signals were not measured) (25,26). In this study, we tested the hypothesis that chronic and severe placental insufficiency decreases fetal pancreatic vascularity and inhibits islet HGF and VEGFA pathways. Experiments were performed in a sheep model of severe IUGR due to chronic placental insufficiency in which the late-gestation fetuses (0.7–0.9 gestation) have lower glucose and insulin concentrations, lower  $\beta$ -cell mass due to slower rates of  $\beta$ -cell mitosis, and reduced islet insulin secretion due to lower islet insulin content (31–34). Pancreatic and pancreatic islet vascularity were determined at both 0.7 and 0.9 gestation; isolated fetal sheep pancreatic islets and ECs were evaluated at 0.9 gestation.

## RESEARCH DESIGN AND METHODS

Studies were conducted in pregnant Columbia-Rambouillet ewes carrying a singleton fetus in compliance with the

Institutional Animal Care and Use Committees of the University of Colorado Denver and University of Arizona. These laboratories are accredited by the American Association for Accreditation of Laboratory Animal Care. Animals used for fetal pancreatic histology were housed at the University of Arizona, and those used for isolated islet experiments at the University of Colorado. We have not observed location as a confounding factor for this model (unpublished data).

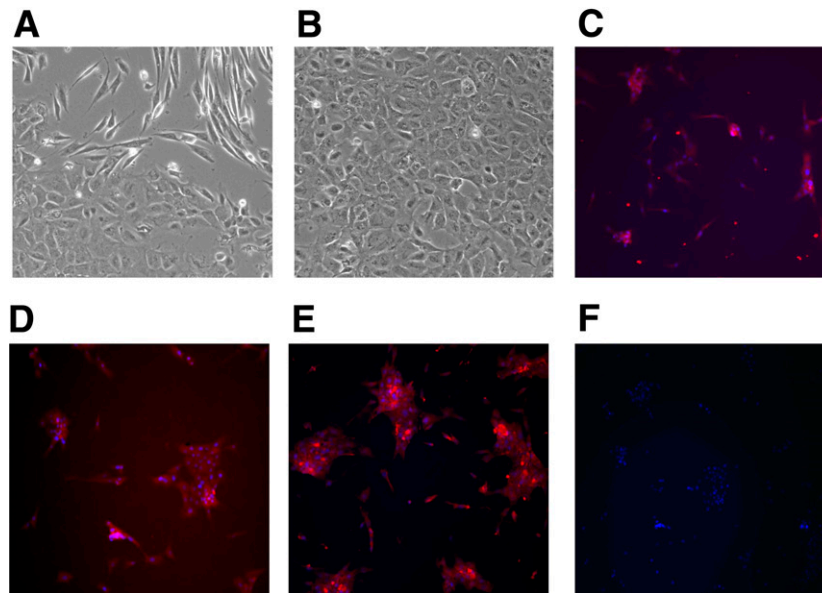
### IUGR Model and Organ Isolation

Placental insufficiency was induced by exposing pregnant ewes to elevated ambient temperatures for  $66.9 \pm 2.8$  days ( $40^\circ\text{C}$  for 12 h,  $35^\circ\text{C}$  for 12 h) beginning on  $38.7 \pm 0.3$  days gestation age (dGA) as previously described (32–34). Control ewes were gestational age matched, pair fed to treated ewe average intake, and maintained at ambient temperatures. At necropsy, the pancreas was weighed and the splenic portion fixed overnight in 4% paraformaldehyde (weight for volume [w/v]) and frozen in optimal cutting temperature freeze media as described previously (31–33,35).

Pancreatic islets were isolated with collagenase digestion at  $133.9 \pm 0.8$  dGA for control and  $133.1 \pm 0.6$  dGA for IUGR fetuses (33,36). In 10 control and 4 IUGR fetuses, islets were isolated and immediately frozen for protein analysis. In three control and four IUGR fetuses, islet ECs were isolated. Briefly, islets were dispersed on a 10-cm collagen-coated (rat tail type 1; BD Biosciences, Bedford, MA) plate with DMEM with 10% FBS for 48–72 h. Polymorphic cell populations originated from the pancreatic islets (Fig. 1A). Groups of cells with a cobblestone appearance, characteristic of ECs, were preserved and subsequently grown in Endothelial Cell Growth Medium MV2 (PromoCell, Heidelberg, Germany). Manual removal of non-phenotypic ECs was repeated every 1–3 days until the enriched EC population was  $\sim 75\%$  confluent (2–2.5 weeks) (Fig. 1B). Purity of the ECs ( $>95\%$ ) was confirmed after removal of cells from the primary dish with trypsin and dispersion on glass slides followed by immunostaining for EC antigens (vascular endothelial growth factor receptor 2 [VEGFR2], vascular endothelial cadherin, and von Willebrand factor) (Fig. 1C–E). Anti-desmin antibody immunoreactivity was unable to detect smooth muscle cells in cultures (Fig. 1F) (20). Islet EC-conditioned media (ECCM) comprised 10 mL of RPMI medium with 1% FBS (volume for volume [v/v]) and 1.1 mmol/L glucose (normal fetal sheep plasma glucose concentration) for 24 h. Media were collected and cleared by centrifugation, and the supernatants stored at  $-20^\circ\text{C}$ . Islet ECs were washed with PBS, collected in lysis buffer and stored at  $-80^\circ\text{C}$  (20).

### Fetal Pancreatic Histology

Fetal pancreatic islet vascularity was measured by morphometric analysis following previously described immunostaining procedures (31,37,38). Pancreatic vascularity was demarcated with fluorescein isothiocyanate (FITC)-conjugated *Griffonia simplicifolia* agglutinin (GS-1)



**Figure 1**—Pancreatic islet EC isolation. Following incubation for 48–72 h, polymorphic cell types can be identified migrating away from isolated pancreatic islets (A). Repetitive scraping of noncobblestone-appearing cells results in a >95% pure population of cells (B) that stain positive for EC markers after removal of cells from the primary dish (B) with trypsin and dispersion on glass slides followed by immunostaining for EC antigens (C: red, VEGFR2; D: red, vascular endothelial cadherin; E: red, von Willebrand factor; C–E: blue, DAPI). Cells did not stain for the smooth muscle antigen desmin (F: red, desmin; blue, DAPI).

(15  $\mu\text{g}/\text{mL}$ ) (Vector Laboratories, Burlingame, CA), and mature endocrine cells were identified with the following primary antibodies: guinea pig anti-porcine insulin (1:500) (Dako, Carpinteria, CA), mouse anti-porcine glucagon (1:250) (Sigma-Aldrich, St. Louis, MO), rabbit anti-human somatostatin (1:500) (Dako), and rabbit anti-human pancreatic polypeptide (1:500) (Dako). Immunocomplexes were detected with the following secondary antiserum: anti-guinea pig IgG conjugated to 7-amino-4-methylcoumarin-3-acetic acid (blue), anti-rabbit IgG conjugated to Texas Red, and anti-mouse IgG conjugated to Texas Red (all 1:500) (Jackson ImmunoResearch Inc.).

Fluorescent images were visualized on a Leica DM5500 microscope equipped with a 10 $\times$  objective lens and a SPOT Pursuit Camera. Morphometric analysis was performed with Image-Pro 6.3 software (Media Cybernetics, Silver Spring, MD) by an individual blinded to treatment groups. Pancreatic vessel density was determined by dividing the GS-1<sup>+</sup> area by the total pancreatic area in a field of view. Pancreatic islet vessel density was determined by dividing the GS-1<sup>+</sup> area within the islet by the area of that islet (37). Islets were defined as endocrine cell clusters with a minimum of two endocrine cell types and an area of at least 500  $\mu\text{m}^2$  (37). For pancreatic vessel density, 10–25 fields of view were evaluated in each of two sections. For pancreatic islet vessel density, 35  $\pm$  2 islets were evaluated for each animal.

#### Pancreatic Islet Incubation

Pancreatic islets were isolated from four late-gestation control fetal sheep for incubation with islet ECCM

(control and IUGR). Conditioned media from control or IUGR islet ECs were combined in equal volumes to provide one control test medium and one IUGR test medium for overnight incubations of the freshly isolated islets. Freshly isolated islets also were incubated in nonconditioned medium, which was generated the same way with the exception of an overnight incubation on a plate without cells. HGF was measured in each animal's islet ECs and ECCM.

Islets were isolated from six additional control fetuses and incubated in pulmonary artery ECCM. Previously collected large-vessel ECs were used (20). The conditioned medium was prepared from large-vessel ECs at passages 4–6 as described previously, with the modifications that cells were grown on a 25-cm plate and that 20 mL of medium was conditioned. Experiments were performed with this test medium or nonconditioned medium. Additions to conditioned and nonconditioned media included an inhibitory anti-HGF IgG antibody 10  $\mu\text{g}/\text{mL}$  (R&D Systems, Minneapolis, MN), normal IgG 10  $\mu\text{g}/\text{mL}$  (R&D Systems), and HGF 100 ng/mL (ProSpec, East Brunswick, NJ), respectively. In addition, aliquots of isolated islets were incubated in wells in which ECs were allowed to form a monolayer that was removed using PBS to leave components of the extracellular matrix (ECM). We also evaluated fetal sheep skin fibroblasts that were isolated, propagated, and maintained as previously described for the conditioned medium and ECM as a control to compare EC experiments (39).

For all islet incubation studies, entire batches of isolated islets were washed in Krebs-Ringer bicarbonate

**Table 1—Animal characteristics**

	0.7 Gestation			0.9 Gestation		
	Control (n = 6)	IUGR (n = 5)	P value	Control (n = 16)	IUGR (n = 15)	P value
Gestational age (days)	104.3 ± 1.6	102.4 ± 0.7		133.8 ± 0.5	133.3 ± 0.4	
Male sex (%)	67	100	0.15	56	71	0.39
Fetal weight (g)	1,233 ± 132	887 ± 118	0.09	3,578 ± 123	1,570 ± 118	<0.01
Pancreas weight (g)	1.32 ± 0.14	1.11 ± 0.15		3.58 ± 0.3	2.07 ± 0.52	<0.05
Placenta weight (g)	442 ± 42	219 ± 39	<0.01	386 ± 26	177 ± 22	<0.01

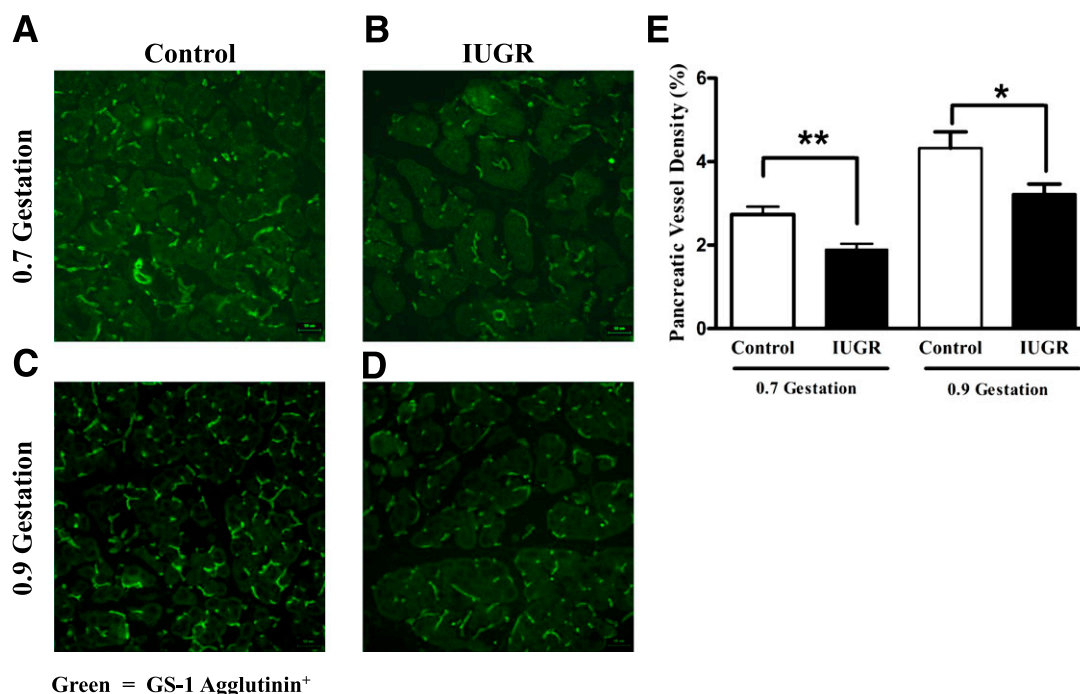
Data are mean ± SE unless otherwise indicated. Sex was not recorded for one 0.9 gestation fetus in both the control and the IUGR groups.

buffer with 0.5% (w/v) BSA and divided into equal volumes for incubation in the test medium. Islets were then incubated in test conditions overnight and collected by centrifugation for 5 min at 4°C and 5,000g. Supernatant was removed, and total islet insulin content determined as previously described (33). Results were normalized to islet insulin content that was determined for the batch of islets incubated in the nonconditioned medium. Late-gestation animals were used for all islet isolations to obtain adequate numbers of isolated islets.

#### Western Blotting

Islets were lysed by subjecting them to one freeze-thaw cycle followed by the addition of lysis buffer with 1% (v/v) Triton X and sonication (20). Islet proteins (15 µg), islet

EC proteins (15 µg), and islet ECCM (20 µL) were separated by gel electrophoresis, and Western blotting was performed as previously described (20). The following primary antibodies were diluted in Tris-buffered saline with 0.1% Tween 20 (v/v) with 5% BSA (w/v) except as noted: goat anti-HGF (0.2 µg/mL diluted in 2% ECL Advance) (R&D Systems), rabbit anti-eNOS (0.2 µg/mL) (Cell Signaling, Danvers, MA), rabbit anti-phosphorylated ser1177 eNOS (0.2 µg/mL) (Cell Signaling), rabbit anti-collagen IV (1 µg/mL) (Abcam, Cambridge, MA), rabbit anti-laminin α1 (1 µg/mL) (Santa Cruz, Dallas, TX), rabbit anti-laminin α4 (0.1 µg/mL) (Sigma-Aldrich), mouse anti-laminin α5 (0.5 µg/mL) (Abcam), rabbit anti-laminin β1 (1:1,000) (Abcam), rabbit anti-insulin receptor β-subunit (0.4 µg/mL



**Figure 2—Pancreatic vessel density in IUGR fetuses.** Pancreatic vessel density was measured by FITC-conjugated GS-1 staining and was lower in IUGR fetuses (B, D) than in controls (A, C) at both 0.7 (A, B) and 0.9 (C, D) gestation. E: Data are mean ± SE for 0.7 gestation control (n = 6) and IUGR (n = 5) fetuses and for 0.9 gestation control (n = 5) and IUGR (n = 5) fetuses. \*P < 0.05, \*\*P < 0.01 for comparisons between IUGR and controls of similar gestational ages.

diluted in 5% BSA + 1% nonfat dried milk) (Santa Cruz), rabbit anti-VEGFR2 (0.2  $\mu\text{g}/\text{mL}$  diluted in 2% ECL Advance) (Cell Signaling), rabbit anti-VEGFA (1  $\mu\text{g}/\text{mL}$  diluted in 2% ECL Advance) (Santa Cruz), rabbit anti-cMET (1:1,000 diluted in 2% ECL Advance) (Millipore, Billerica, MA), rabbit anti-integrin  $\alpha 4$  (0.2  $\mu\text{g}/\text{mL}$ ) (Cell Signaling), rabbit anti-integrin  $\alpha 5$  (0.2  $\mu\text{g}/\text{mL}$ ) (Cell Signaling), rabbit anti-integrin  $\alpha 6$  (1:1,000) (Abcam), rabbit anti-integrin  $\beta 1$  (0.2  $\mu\text{g}/\text{mL}$ ) (Cell Signaling), mouse anti-actin (1:10,000) (MP Biomedicals, Solon, OH), and rabbit anti-proliferating cell nuclear antigen (PCNA) (1  $\mu\text{g}/\text{mL}$ ) (Santa Cruz). Before incubation, membranes were blocked in Tris-buffered saline with 0.1% Tween 20 (v/v) with 5% nonfat dried milk (w/v) with the exception of membranes probed for HGF, VEGFR2, VEGFA, and cMET, which were blocked in 2% ECL Advance. Results are expressed as the ratio of the densitometry for the protein of interest and actin, except for HGF measured in conditioned media.

#### Statistical Analysis

Statistical analysis was performed using SAS version 9.1 or GraphPad Prism software. Data are presented as mean  $\pm$  SE. Comparisons between groups of two were made using Student *t* test or the Mann-Whitney test for nonparametric data. For islet incubation experiments, repeated-measures

ANOVA was used to account for the measurements made in islets derived from the same animal, and individual means were compared using Fisher least significant difference method. The proportion of male fetuses in each group was compared using the two-sample test of equality of proportions.  $P \leq 0.05$  was considered significant.

## RESULTS

### Fetal Measurements and Organ Weights in IUGR and Control Fetuses

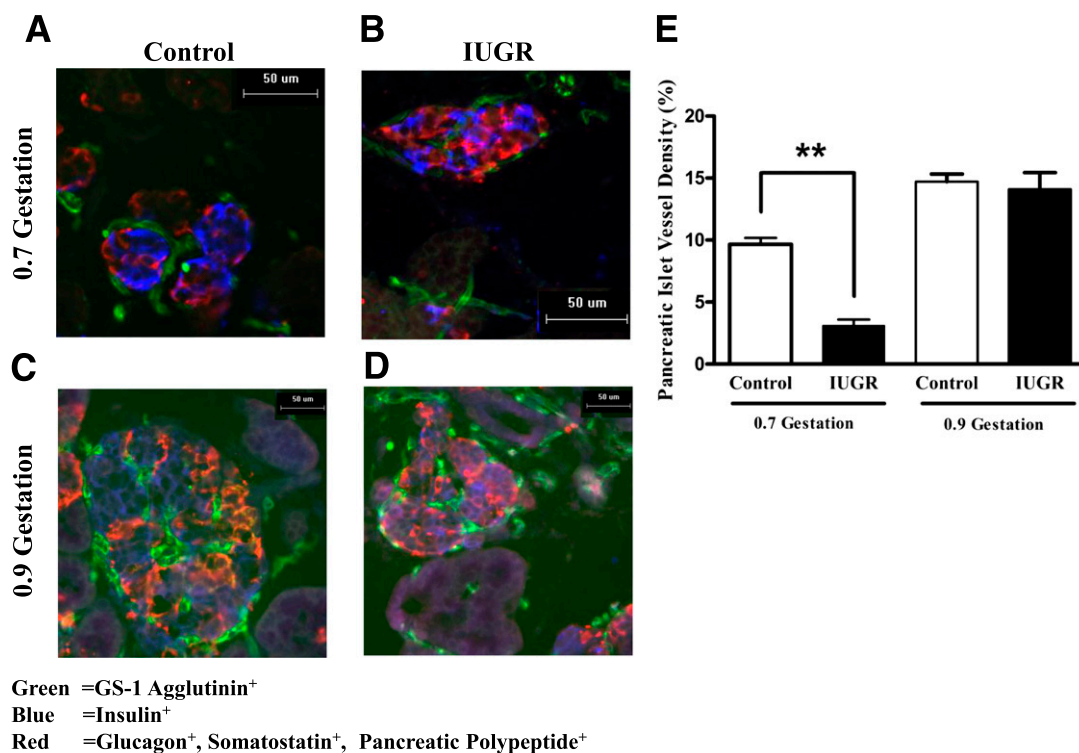
Fetal ages, sex, and weights are presented in Table 1.

### Pancreatic and Pancreatic Islet Vessel Density in IUGR Fetuses

In IUGR fetuses at both 0.7 and 0.9 gestation, pancreatic vessel density was 30% and 25% lower, respectively, than in control fetuses ( $P < 0.05$ ) (Fig. 2). Pancreatic islet vessel density was 68% lower at 0.7 gestation in IUGR fetuses ( $P < 0.01$ ) but was not different from control fetuses at 0.9 gestation (Fig. 3).

### ECCM

Islet-derived ECCM pooled from four IUGR or three control fetuses were evaluated in islet cultures from four other control fetuses and presented relative to nonconditioned media incubations. Following overnight incubation in control islet-derived ECCM, islet insulin content was



**Figure 3**—Pancreatic islet vessel density in IUGR fetuses. Pancreatic islet vessel density was measured by immuno- and FITC-conjugated GS-1 staining and was lower in IUGR fetuses (B, D) than in controls (A, C) at 0.7 (A, B) gestation but not at 0.9 (C, D) gestation. Costaining also was performed for insulin as well as glucagon, somatostatin, and pancreatic polypeptide. E: Data are mean  $\pm$  SE for 0.7 gestation control ( $n = 6$ ) and IUGR ( $n = 5$ ) fetuses and for 0.9 gestation control ( $n = 5$ ) and IUGR ( $n = 5$ ) fetuses.  $**P < 0.01$  for comparisons between IUGR and controls of similar gestational ages.

increased  $\sim 1.8$ -fold relative to nonconditioned media and greater than IUGR islet-derived ECCM ( $P < 0.01$ ) (Fig. 4A).

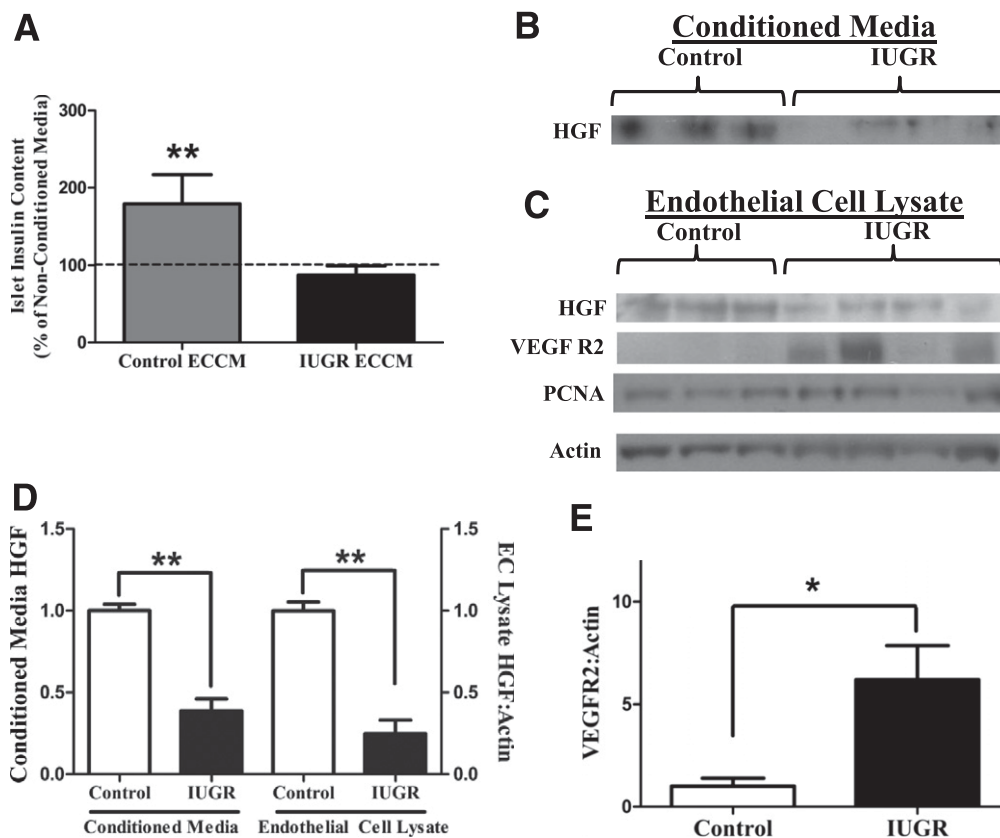
In the IUGR islet-derived ECCM, HGF concentrations were lower than in control islet-derived ECCM ( $P < 0.01$ ) (Fig. 4B and D). ECs also had reduced HGF protein expression in IUGR islet-derived EC lysates compared with control lysates ( $P < 0.01$ ) (Fig. 4C and D). Insulin receptor, eNOS, collagen type IV, and laminin  $\alpha 1$ ,  $\alpha 4$ ,  $\alpha 5$ , and  $\beta 1$  protein expression were not different between IUGR and control EC lysates (data not shown). VEGFR2 was increased in IUGR islet-derived EC lysates ( $P < 0.05$ ) (Fig. 4C and E). There was no difference in PCNA expression between the control and the IUGR islet-derived EC lysates (Fig. 4C).

To further explore the role of HGF in EC-to- $\beta$ -cell cross talk, isolated large-vessel-derived ECCM and fibroblast-conditioned media were used in overnight incubations with a separate set of isolated fetal pancreatic islets ( $n = 6$ ). Similar to control islet-derived ECCM following overnight incubation in conditioned media from large-vessel-derived

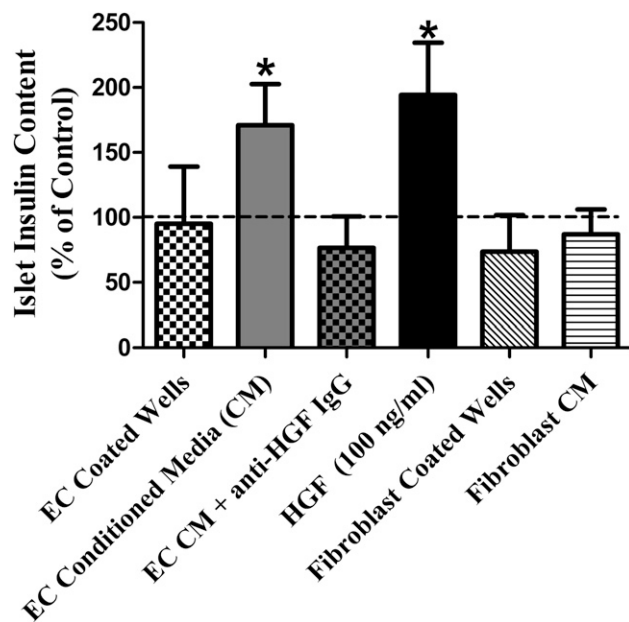
ECs, isolated islet insulin content was 1.7-fold relative to nonconditioned media ( $P < 0.05$ ). This effect was associated with increased HGF in the ECCM and blocked by the addition of an inhibitory anti-HGF antibody ( $P < 0.05$ ). Furthermore, following overnight incubation with HGF (100 ng/mL) in nonconditioned media, islet insulin content was 1.9-fold relative to nonconditioned media ( $P < 0.05$ ). Islet insulin content was not different following overnight incubations in wells coated with ECM from ECs or in fibroblast-conditioned media and ECM (Fig. 5).

#### Pancreatic Islet Cell Protein Expression

VEGFA and integrin  $\beta 1$  protein expression were lower ( $P < 0.05$ ) in pancreatic islets isolated from IUGR fetuses ( $n = 4$ ) compared with controls ( $n = 10$ ), but there was no change in the HGF receptor cMET or integrin  $\alpha 5$  (Fig. 6). Although integrins  $\alpha 4$  and  $\alpha 6$  could be identified in whole pancreatic lysates, we could not identify them in lysates from either control or IUGR fetal islets (data not shown).



**Figure 4**—Islet ECCM increases pancreatic islet insulin content. **A**: Freshly isolated fetal sheep islets were divided equally into different incubation conditions (*x*-axis) and were collected the following day for pancreatic islet insulin content measurement by ELISA. Results are normalized to the nonconditioned media incubation for each batch of islets (100% on the *y*-axis). Pancreatic islet insulin content ( $n = 4$ ) increased after overnight incubation in media conditioned by isolated control pancreatic islet ECs (control ECCM), but no difference for islet insulin content was found with media conditioned by isolated IUGR pancreatic islet ECs (IUGR ECCM, combined conditioned media from four fetuses). \*\* $P < 0.01$ . **B**: HGF could be detected by Western blot in control ECCM ( $n = 3$ ), but the expression was less or absent in IUGR ECCM ( $n = 4$ ). **C**: Western blotting of EC lysates revealed less expression for HGF, more expression for VEGFR2, and no difference in the expression of PCNA in the IUGR ECs ( $n = 4$ ) compared with control ECs ( $n = 3$ ). **D**: Densitometry quantification of HGF in conditioned media and EC lysates. Data are mean  $\pm$  SE. \*\* $P < 0.01$ . **E**: Densitometry quantification of VEGFR2 in EC lysates. Data are mean  $\pm$  SE. \* $P < 0.05$ .



**Figure 5**—Addition of an inhibitory anti-HGF antibody blocks increased islet insulin content following incubation in ECCM. Freshly isolated fetal sheep islets were divided equally into different incubation conditions (*x*-axis) and were collected the following day for pancreatic islet insulin content measurement by ELISA. Results are normalized to the nonconditioned media incubation for each batch of islets (100% on the *y*-axis). Isolated islets ( $n = 6$ ) were incubated overnight in control media in normal wells, control media in wells following removal of a monolayer of large-vessel ECs or skin fibroblasts with PBS, control media conditioned by large-vessel ECs with and without the addition of an inhibitory anti-HGF antibody, control media with the addition of HGF, and control media conditioned by skin fibroblasts. \* $P < 0.05$ , islet insulin content vs. control media.

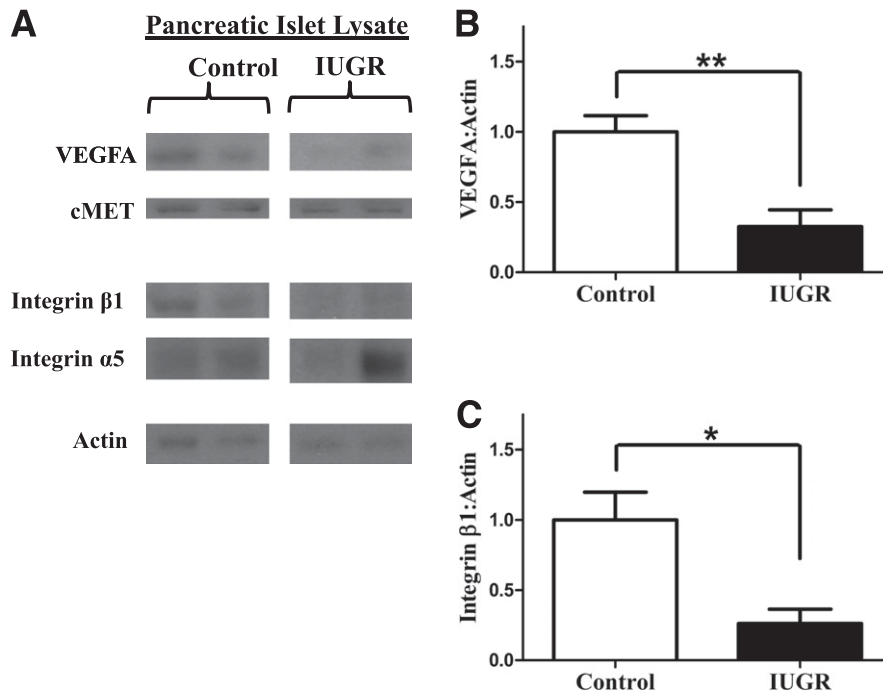
## DISCUSSION

In the current study, we show that placental insufficiency-induced IUGR fetuses have lower pancreatic vascularity and decreased islet paracrine hormone production. We also show that functional deficits in islet paracrine signaling may be just as important as overall islet vascularization in explaining how IUGR produces reductions in  $\beta$ -cell function and insulin content. An important finding in the current study is that conditioned media from control islet-derived ECs increased islet insulin content, but media conditioned by IUGR islet-derived ECs was ineffective, demonstrating a defect in the capacity of IUGR islet ECs to promote  $\beta$ -cell insulin production. A potential mechanism for this defect is the observation that IUGR islet-derived ECCM and IUGR islet-derived EC lysates had lower HGF than did controls. The importance of HGF for stimulating islet insulin content was confirmed in experiments with normal fetal sheep large-vessel-derived ECs and addition of HGF to nonconditioned media. Media conditioned by the large-vessel-derived ECs and nonconditioned media with supplemental HGF, like the normal islet ECs, both increased islet insulin content. This stimulatory action was completely blocked by the

addition of an inhibitory anti-HGF antibody. Previous studies using genetic manipulation to increase or decrease islet HGF signaling in mice have demonstrated its importance in normal  $\beta$ -cell development and function, but none of these studies investigated the role of this signaling in pathological conditions such as IUGR (6,7,15–17). Although HGF actions have been shown to increase maternal  $\beta$ -cell mass and function during pregnancy (9), the present findings are the first in our knowledge to demonstrate decreased pancreatic islet HGF in IUGR, a pathophysiological condition characterized by decreased fetal pancreatic  $\beta$ -cell mass and function (31–33).

Although these studies uniquely show that EC HGF plays a central role in promoting pancreatic  $\beta$ -cell function, islet ECs also stimulate  $\beta$ -cell function by non-HGF pathways. These include production of certain basement membrane components, laminins and collagen IV, which are ligands for  $\beta$ -cell receptors (8,18). The best characterized of these receptors are the integrins (8). The present results show no differences between control and IUGR islet EC laminins or collagen type IV, but integrin  $\beta 1$  was significantly decreased in islets isolated from IUGR fetuses compared with controls. The model of IUGR used in this study is characterized by decreased  $\beta$ -cell replication, insulin production, and secretion, and previous studies have demonstrated that integrin  $\beta 1$  stimulates these critical  $\beta$ -cell processes (8,31–33). It is, therefore, reasonable to hypothesize that decreased integrin  $\beta 1$  plays a significant functional role in the pathogenesis of fetal pancreatic  $\beta$ -cell dysfunction in IUGR caused by placental insufficiency. However, we were unable to show that basement membrane components produced by normal large-vessel ECs stimulate an increase in islet insulin content like ECCM do. Future studies will be required to more specifically test the role of this and other integrins in IUGR  $\beta$ -cell dysfunction. NO also has been reported to stimulate  $\beta$ -cell function in certain situations (19), but there were no differences between control and IUGR ECs for the EC isoform eNOS.

Islet VEGFA was lower in the present IUGR fetuses, implicating impaired  $\beta$ -cell-to-EC paracrine signaling in their islets. The predominant islet VEGF isoform VEGFA stimulates pancreatic and pancreatic islet vascularity as well as increases EC function (9–11,20). VEGFA also increases HGF expression and secretion in liver sinusoidal ECs, and in isolated islet ECs, both VEGFA and insulin stimulate HGF production (9,40). Although HGF can stimulate VEGFA in several cell types (41–43), direct confirmation of HGF-stimulated  $\beta$ -cell production of VEGFA is lacking. However, given the potential feed-forward regulation of  $\beta$ -cell VEGFA and islet EC HGF, it is likely that these two growth factors are highly integrated in normal pancreatic islet development and that complications resulting from placental insufficiency can have a major adverse impact on this integrated regulation. However, pancreatic islet cMET and islet EC VEGFR2 were normal and higher, respectively, in islets from IUGR fetuses, suggesting that



**Figure 6**—Pancreatic islet VEGFA and integrin  $\beta 1$  are decreased in IUGR fetuses. A: VEGFA, cMET, and integrin  $\beta 1$  were measured by Western blotting in pancreatic islets from control ( $n = 10$ ) and IUGR ( $n = 4$ ) fetal sheep. Representative Western blot is shown. There was significantly less VEGFA (B) (\*\* $P < 0.01$ ) and integrin  $\beta 1$  (C) (\* $P < 0.05$ ) in IUGR pancreatic islets compared with controls.

these pathways may remain responsive if provided appropriate paracrine signals. This possibility is currently being tested.

The present data combined with previously published rodent and human data (3,25,26) also support the concept that reduced pancreatic islet vascularity is a common response to decreased fetal nutrient supply. In addition to reduced pancreatic islet vascularity, we observed decreased pancreatic vascularity, which was not reported with the rodent models of IUGR (25,26). It is possible that the sheep placental insufficiency model of IUGR, which represents a more-chronic condition of nutrient and oxygen restriction compared with rodent models (e.g., the late-gestation bilateral uterine artery ligation model, the low-maternal-protein diet model of IUGR), results in a more global reduction of pancreatic vascularity. Another interesting finding in the sheep placental insufficiency model of IUGR is that pancreatic islet vascularity is only lower at 0.7 gestation and not at 0.9 gestation. Studies in genetically manipulated mice with reduced vascularity due to inactivating mutations in VEGFA show that islets and  $\beta$ -cells in these situations can expand and develop a stable relationship with their reduced vascularity, although at the expense of normal insulin secretion (11,24). In other words, decreased islet vascularity persists in these genetically modified mice. IUGR sheep do not have a persistent decrease in islet vascularity at the very end of gestation despite a progressive decrement of  $\beta$ -cell function and mass compared with control fetuses as gestation progresses from 0.7 to 0.9 (31,32). It is important to consider

that these IUGR sheep have fewer  $\beta$ -cells and smaller islets (31,32). Furthermore, because islet vessel density is calculated relative to total islet area, there is an overall decrease in islet vessels in IUGR fetuses that is proportional to the decrease in islet area. Recent evidence from mice with genetic defects in pancreatic and  $\beta$ -cell VEGFA production highlight the paradigm that factors other than simple islet vessel density, like paracrine hormones, are important for normal  $\beta$ -cell function (24). Such factors also can have significant effects on the cross talk between ECs and  $\beta$ -cells, which then can be disrupted by 0.9 gestation in IUGR sheep. One hundred percent of the IUGR fetuses at 0.7 gestation were male, whereas only 67% of the controls were male at this age; removing the two females from the data set and comparing only males did not affect the findings.

Although the current study demonstrated functional defects in EC-to- $\beta$ -cell HGF signaling in IUGR fetuses, the functional consequences of other changes, such as lower integrin  $\beta 1$  and VEGFA, have yet to be determined. Furthermore, in vivo studies in which these pathways are up- or downregulated, both acutely and chronically, will provide more insight into their relevance for the pathophysiology of  $\beta$ -cell dysfunction in IUGR and their roles in coordinating normal  $\beta$ -cell function and development with normal variations in placental nutrient supply. Future studies in which specific components of the fetal nutrient supply (amino acids, glucose, and oxygen) are experimentally manipulated during various periods of gestation are required to determine the ability of the



pancreatic islet EC to act as a fetal nutrient sensor working with the pancreatic  $\beta$ -cell to coordinate anabolic signals for growth with nutrient supply.

In conclusion, we have demonstrated that placental insufficiency that leads to IUGR produces significant defects in the HGF pathway mediating EC-to- $\beta$ -cell signaling in the fetus. Additionally, placental insufficiency results in decreased fetal pancreatic islet VEGFA and integrin  $\beta$ 1. These findings are associated with reductions in pancreatic islet and pancreatic islet vascularity. Although it is unknown whether the decreased pancreatic islet vascularity observed in the fetus persists in human adults who were IUGR, we speculate that defects in EC-to- $\beta$ -cell cross talk partly explain the increased risk of  $\beta$ -cell failure and type 2 diabetes that these individuals develop later in life compared with those who were not born IUGR.

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## References

- Peiris H, Bonder CS, Coates PT, Keating DJ, Jessup CF. The  $\beta$ -cell/EC axis: how do islet cells talk to each other? *Diabetes* 2014;63:3–11
- Nicolini U, Hubinont C, Santolaya J, Fisk NM, Rodeck CH. Effects of fetal intravenous glucose challenge in normal and growth retarded fetuses. *Horm Metab Res* 1990;22:426–430
- Van Assche FA, De Prins F, Aerts L, Verjans M. The endocrine pancreas in small-for-dates infants. *Br J Obstet Gynaecol* 1977;84:751–753
- Hales CN, Barker DJ. The thrifty phenotype hypothesis. *Br Med Bull* 2001; 60:5–20
- Brissova M, Shostak A, Shiota M, et al. Pancreatic islet production of vascular endothelial growth factor—a is essential for islet vascularization, revascularization, and function. *Diabetes* 2006;55:2974–2985
- García-Ocaña A, Takane KK, Syed MA, Philbrick WM, Vasavada RC, Stewart AF. Hepatocyte growth factor overexpression in the islet of transgenic mice increases beta cell proliferation, enhances islet mass, and induces mild hypoglycemia. *J Biol Chem* 2000;275:1226–1232
- Mellado-Gil J, Rosa TC, Demirci C, et al. Disruption of hepatocyte growth factor/c-Met signaling enhances pancreatic beta-cell death and accelerates the onset of diabetes. *Diabetes* 2011;60:525–536
- Nikolova G, Jabs N, Konstantinova I, et al. The vascular basement membrane: a niche for insulin gene expression and Beta cell proliferation. *Dev Cell* 2006;10:397–405
- Johansson M, Mattsson G, Andersson A, Jansson L, Carlsson PO. Islet endothelial cells and pancreatic beta-cell proliferation: studies in vitro and during pregnancy in adult rats. *Endocrinology* 2006;147:2315–2324
- Konstantinova I, Lammert E. Microvascular development: learning from pancreatic islets. *BioEssays* 2004;26:1069–1075
- Lammert E, Gu G, McLaughlin M, et al. Role of VEGF-A in vascularization of pancreatic islets. *Curr Biol* 2003;13:1070–1074
- Johansson A, Lau J, Sandberg M, Borg LA, Magnusson PU, Carlsson PO. Endothelial cell signalling supports pancreatic beta cell function in the rat. *Diabetologia* 2009;52:2385–2394
- Johansson M, Andersson A, Carlsson PO, Jansson L. Perinatal development of the pancreatic islet microvasculature in rats. *J Anat* 2006;208:191–196
- Dai C, Li Y, Yang J, Liu Y. Hepatocyte growth factor preserves beta cell mass and mitigates hyperglycemia in streptozotocin-induced diabetic mice. *J Biol Chem* 2003;278:27080–27087
- García-Ocaña A, Vasavada RC, Cebrian A, et al. Transgenic overexpression of hepatocyte growth factor in the beta-cell markedly improves islet function and islet transplant outcomes in mice. *Diabetes* 2001;50:2752–2762
- Dai C, Huh CG, Thorgeirsson SS, Liu Y. Beta-cell-specific ablation of the hepatocyte growth factor receptor results in reduced islet size, impaired insulin secretion, and glucose intolerance. *Am J Pathol* 2005;167:429–436
- Roccisana J, Reddy V, Vasavada RC, Gonzalez-Pertusa JA, Magnuson MA, García-Ocaña A. Targeted inactivation of hepatocyte growth factor receptor c-met in beta-cells leads to defective insulin secretion and GLUT-2 downregulation without alteration of beta-cell mass. *Diabetes* 2005;54:2090–2102
- Weber LM, Hayda KN, Anseth KS. Cell-matrix interactions improve beta-cell survival and insulin secretion in three-dimensional culture. *Tissue Eng Part A* 2008;14:1959–1968
- Nakata M, Yada T. Endocrinology: nitric oxide-mediated insulin secretion in response to citrulline in islet beta-cells. *Pancreas* 2003;27:209–213
- Rozance PJ, Seedorf GJ, Brown A, et al. Intrauterine growth restriction decreases pulmonary alveolar and vessel growth and causes pulmonary artery endothelial cell dysfunction in vitro in fetal sheep. *Am J Physiol Lung Cell Mol Physiol* 2011;301:L860–L871
- Duvillié B, Currie C, Chrones T, et al. Increased islet cell proliferation, decreased apoptosis, and greater vascularization leading to beta-cell hyperplasia in mutant mice lacking insulin. *Endocrinology* 2002;143:1530–1537
- Vasir B, Aiello LP, Yoon KH, Quicquel RR, Bonner-Weir S, Weir GC. Hypoxia induces vascular endothelial growth factor gene and protein expression in cultured rat islet cells. *Diabetes* 1998;47:1894–1903
- Kamba T, Tam BY, Hashizume H, et al. VEGF-dependent plasticity of fenestrated capillaries in the normal adult microvasculature. *Am J Physiol Heart Circ Physiol* 2006;290:H560–H576
- Reinert RB, Brissova M, Shostak A, et al. Vascular endothelial growth factor-a and islet vascularization are necessary in developing, but not adult, pancreatic islets. *Diabetes* 2013;62:4154–4164
- Ham JN, Crutchlow MF, Desai BM, Simmons RA, Stoffers DA. Exendin-4 normalizes islet vascularity in intrauterine growth restricted rats: potential role of VEGF. *Pediatr Res* 2009;66:42–46
- Boujendar S, Arany E, Hill D, Remacle C, Reusens B. Taurine supplementation of a low protein diet fed to rat dams normalizes the vascularization of the fetal endocrine pancreas. *J Nutr* 2003;133:2820–2825
- Simmons RA, Templeton LJ, Gertz SJ. Intrauterine growth retardation leads to the development of type 2 diabetes in the rat. *Diabetes* 2001;50:2279–2286
- Dahri S, Snoeck A, Reusens-Billen B, Remacle C, Hoet JJ. Islet function in offspring of mothers on low-protein diet during gestation. *Diabetes* 1991; 40(Suppl. 2):115–120

29. Petry CJ, Doring MW, Pawlak DB, Ozanne SE, Hales CN. Diabetes in old male offspring of rat dams fed a reduced protein diet. *Int J Exp Diabetes Res* 2001;2:139–143
30. Snoeck A, Remacle C, Reusens B, Hoet JJ. Effect of a low protein diet during pregnancy on the fetal rat endocrine pancreas. *Biol Neonate* 1990;57:107–118
31. Limesand SW, Jensen J, Hutton JC, Hay WW Jr. Diminished beta-cell replication contributes to reduced beta-cell mass in fetal sheep with intrauterine growth restriction. *Am J Physiol Regul Integr Comp Physiol* 2005;288:R1297–R1305
32. Limesand SW, Rozance PJ, Macko AR, Anderson MJ, Kelly AC, Hay WW Jr. Reductions in insulin concentrations and  $\beta$ -cell mass precede growth restriction in sheep fetuses with placental insufficiency. *Am J Physiol Endocrinol Metab* 2013;304:E516–E523
33. Limesand SW, Rozance PJ, Zerbe GO, Hutton JC, Hay WW Jr. Attenuated insulin release and storage in fetal sheep pancreatic islets with intrauterine growth restriction. *Endocrinology* 2006;147:1488–1497
34. Macko AR, Yates DT, Chen X, et al. Elevated plasma norepinephrine inhibits insulin secretion, but adrenergic blockade reveals enhanced  $\beta$ -cell responsiveness in an ovine model of placental insufficiency at 0.7 of gestation. *J Dev Orig Health Dis* 2013;4:402–410
35. Cole L, Anderson M, Antin PB, Limesand SW. One process for pancreatic beta-cell coalescence into islets involves an epithelial-mesenchymal transition. *J Endocrinol* 2009;203:19–31
36. Chen X, Green AS, Macko AR, Yates DT, Kelly AC, Limesand SW. Enhanced insulin secretion responsiveness and islet adrenergic desensitization after chronic norepinephrine suppression is discontinued in fetal sheep. *Am J Physiol Endocrinol Metab* 2014;306:E58–E64
37. Gadhia MM, Maliszewski AM, O'Meara MC, et al. Increased amino acid supply potentiates glucose-stimulated insulin secretion but does not increase  $\beta$ -cell mass in fetal sheep. *Am J Physiol Endocrinol Metab* 2013;304:E352–E362
38. Leos RA, Anderson MJ, Chen X, Pugmire J, Anderson KA, Limesand SW. Chronic exposure to elevated norepinephrine suppresses insulin secretion in fetal sheep with placental insufficiency and intrauterine growth restriction. *Am J Physiol Endocrinol Metab* 2010;298:E770–E778
39. Rozance PJ, Limesand SW, Zerbe GO, Hay WW Jr. Chronic fetal hypoglycemia inhibits the later steps of stimulus-secretion coupling in pancreatic beta-cells. *Am J Physiol Endocrinol Metab* 2007;292:E1256–E1264
40. LeCouter J, Moritz DR, Li B, et al. Angiogenesis-independent endothelial protection of liver: role of VEGFR-1. *Science* 2003;299:890–893
41. Moriyama T, Kataoka H, Hamasuna R, et al. Up-regulation of vascular endothelial growth factor induced by hepatocyte growth factor/scatter factor stimulation in human glioma cells. *Biochem Biophys Res Commun* 1998;249:73–77
42. Van Belle E, Witzenbichler B, Chen D, et al. Potentiated angiogenic effect of scatter factor/hepatocyte growth factor via induction of vascular endothelial growth factor: the case for paracrine amplification of angiogenesis. *Circulation* 1998;97:381–390
43. Wojta J, Kaun C, Breuss JM, et al. Hepatocyte growth factor increases expression of vascular endothelial growth factor and plasminogen activator inhibitor-1 in human keratinocytes and the vascular endothelial growth factor receptor flk-1 in human endothelial cells. *Lab Invest* 1999;79:427–438