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

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Abstract

Hepatocyte growth factor (HGF) and vascular endothelial growth factor A (VEGFA) are paracrine hormones that mediate communication between pancreatic islet endothelial cells (EC) and β-cells. Our objective was to determine the impact of intrauterine growth restriction (IUGR) on pancreatic vascularity and paracrine signaling between the EC and β-cell. Vessel density was less in IUGR pancreata than 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 higher in IUGR islets and ECs, respectively. These findings show that paracrine actions from EC increase islet insulin content, and in IUGR EC secretion of HGF was diminished. Given the potential feed forward regulation of β-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.
Introduction

Pancreatic islet signaling between the endothelial cell and β-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 β-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 to appropriately growing fetuses, they also have decreased glucose stimulated insulin secretion (GSIS) in late gestation.(2) Consistent with this decreased GSIS, severely growth restricted fetuses have lower pancreatic islet vascularity and a smaller β-cell population, both of which are more severe than their decrement in body or pancreas weight.(3) The mechanisms responsible for reduced β-cell number and function are incompletely understood, but might explain the increased risk that IUGR offspring have for developing type 2 diabetes mellitus as adults.(4)

Pancreatic and islet endothelial cells provide signals that are responsible for the normal formation, maturation, and function of the pancreatic β-cell.(5-11) Hepatocyte growth factor (HGF) secreted by the endothelial cell stimulates adult β-cell function and production of insulin in vitro.(12;13) In transgenic mice with HGF over-expression, pancreatic islets have increased insulin secretion, increased β-cell mass, and are resistant to experimental-induction of diabetes.(6;14;15) Furthermore, when the HGF receptor cMET is inactivated in β-cells, mice develop glucose intolerance and diabetes due to decreased insulin production and secretion.(16;17) In addition to HGF, endothelial cells also produce laminins and other components of the extracellular basement membrane that are recognized by integrin receptors to
augment β-cell function. Nitric oxide (NO) is produced by endothelial cell nitric oxide synthase (eNOS) and may increase insulin secretion; autocrine actions of NO also have been described for β-cells.

There is an important relationship between β-cells and endothelial cells to increase pancreatic islet vascularity. Insulin, for example, stimulates endothelial cell growth along with other factors that act to establish and maintain normal islet vascularity such as vascular endothelial growth factor A (VEGFA). VEGFA is a potent angiogenic factor secreted by β-cells, 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 β-cells. A majority of these mice develop glucose intolerance and diabetes as adults. Interestingly, an inducible system to inhibit VEGFA expression in adult β-cells has shown a very mild insulin secretory defect and no reduction in β-cell insulin production despite a significant reduction in islet vascularity. This observation demonstrates that decreased islet vascularity is not always coupled to impaired insulin secretion. Furthermore, inhibition of endothelial cell function and reduced cross talk with β-cells that might occur in the prenatal and perinatal periods are potentially more deleterious to long term β-cell function.

A majority of the research that demonstrates endothelial cell and β-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. These models were characterized by lower β-cell mass, insulin secretion defects, and development of glucose
intolerance and diabetes in adults. Additionally, both models have decreased perinatal islet VEGFA, indicating decreased islet β-cell to endothelial cell signaling (though HGF and other endothelial cell to β-cell signals were not measured). In this study we test 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 of gestation) have lower glucose and insulin concentrations, lower β-cell mass due to slower rates of β-cell mitosis, and reduced islet insulin secretion due to lower islet insulin content. Pancreatic and pancreatic islet vascularity were determined at both 0.7 and 0.9 gestation; isolated fetal sheep pancreatic islets and endothelial cells 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 Committee, 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±2.8 days (40°C for 12h; 35°C for 12h) beginning on 38.7±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 was fixed overnight in 4% paraformaldehyde (w/v) and frozen in optimal cutting temperature (OCT) freeze media, as described previously. (31-33;35)

Pancreatic islets were isolated with collagenase digestion at 133.9±0.8 dGA for controls and 133.1±0.6 dGA for IUGR fetuses. (33;36) In ten control and four IUGR fetuses islets were isolated and immediately frozen for protein analysis. In three control and four IUGR fetuses islet endothelial cells were isolated. Briefly, islets were dispersed on a collagen (Rat Tail Type 1, BD Biosciences, Bedford, MA) coated 10 cm plate with Dulbecco’s Modified Eagle’s Media (DMEM) with 10% Fetal Bovine Serum (FBS) for 48-72 hours. Polymorphic cell populations originated from the pancreatic islets (Figure 1a). Groups of cells with a cobblestone appearance, characteristic of endothelial cells (ECs), were preserved and subsequently grown in Endothelial Cell Growth Medium MV2 (PromoCell, Heidelberg, Germany). Manual removal of non-phenotypic EC cells was repeated every one to three days until the enriched EC population was approximately 75% confluent (2-2.5 weeks, Figure 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, Vascular Endothelial Cadherin, and von Willebrand Factor, Figure 1c-e). Anti-Desmin antibody immunoreactivity was unable to detect smooth muscle cells in cultures (Figure 1f). (20) Islet EC conditioned media was performed with 10 ml of RPMI with 1% Fetal Bovine Serum (FBS, v/v) and 1.1 mmole/L glucose (normal fetal sheep plasma glucose concentration) for 24 hours. Media was collected, cleared by centrifugation and the supernatant stored at -20°C. Islet ECs were
washed with PBS, collected in lysis buffer and stored at -80°C.(20)

_Fetal Pancreatic Histology_

Fetal pancreatic islet vascularity was measured by morphometric analysis after immunostaining procedures previously described.(31;37;38) Pancreatic vascularity was demarcated with FITC-conjugated Griffonia simplicifolia (GS-I) Agglutinin (15 µg/ml, Vector Labs, Burlingame, CA) and mature endocrine cells were identified with primary antibodies: guinea pig anti-porcine insulin (Dako, Carpinteria CA, 1:500), mouse anti-porcine glucagon (Sigma-Aldrich, 1:250), rabbit anti-human somatostatin (Dako, 1:500), and rabbit anti-human pancreatic polypeptide (Dako, 1:500). Immunocomplexes were detected with the following secondary antiserum: anti-guinea pig IgG conjugated to 7-amino-4-methylcoumarin-3-acetic acid (AMCA, blue), anti-rabbit IgG conjugated to Texas Red, and anti-mouse IgG conjugated to Texas Red (Jackson ImmunoResearch Laboratories, all 1:500).

Fluorescent images were visualized on a Leica DM5500 microscope equipped with a 10X objective lens and a Spot Pursuit Camera. Morphometric analysis was performed with ImagePro 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 Agglutinin+ area by the total pancreatic area in a field of view. Pancreatic islet vessel density was determined by dividing the GS-1 Agglutinin+ 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 µm².(37) For pancreatic vessel density 10-25 fields of view were evaluated in each of two sections. For pancreatic islet vessel density at 35±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 EC conditioned media (control and IUGR). Conditioned media from control or IUGR islet ECs were combined in equal volumes to provide one control test media and one IUGR test media for overnight incubations of the freshly isolated islets. Freshly isolated islets also were incubated in non-conditioned media 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 EC conditioned media.

Islets were isolated from six additional control fetuses and incubated in pulmonary artery EC conditioned media. Previously collected large vessel ECs were utilized(20) Conditioned media was prepared from large vessel ECs at passages 4-6 as described above with the modifications that cells were grown on a 25 cm plate and 20 ml of media was conditioned. Experiments were performed with this test media or non-conditioned media. Additions to conditioned and non-conditioned media included an inhibitory anti-HGF IgG antibody 10 µg/ml (R&D Systems, Minneapolis, MN), normal IgG 10 µg/ml (R&D Systems), and HGF 100 ng/ml (ProSpec, East Brunswick, NJ), respectively. In addition, aliquots of isolated islets also were incubated in wells in which ECs were allowed to form a monolayer and then 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 conditioned media and ECM as a control to compare EC experiments.(39)
For all islet incubation studies, entire batches of isolated islets were washed in Kreb’s Ringer Bicarbonate Buffer (KRB) with 0.5% (w/v) bovine serum albumin (BSA) solution and divided into equal volumes for incubation in test media. Islets were then incubated in test conditions overnight and collected by centrifugation for 5 min at 4°C and 5000 X g. 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 that were incubated in the non-conditioned media. 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 addition of lysis buffer with 1% (v/v) Triton-X and sonication.(20) Islet proteins (15 ug), islet EC proteins (15 ug) and islet EC conditioned media (20ul) were separated by gel electrophoresis and western blotting performed as previously described.(20) The following primary antibodies were diluted in Tris buffered saline with 0.1% Tween 20 (vol/vol; TBST) with 5% BSA (wt/vol) 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, St. Louis, MO), mouse anti-laminin α5 (0.5 µg/ml; Abcam), rabbit anti laminin β1 (1:1000; Abcam), rabbit anti-Insulin Receptor β subunit (IR; 0.4 µg/ml 1:500 diluted in 5% BSA + 1% nonfat dried milk [NFDM]; Santa Cruz), rabbit anti-vascular endothelial growth factor receptor 2 (VEGFR2; 0.2 µg/ml diluted in 2% ECL Advance, Cell Signaling), rabbit anti-VEGFA (1 µg/ml diluted in 2%
ECL Advance; Santa Cruz), rabbit anti-cMET (1:1000 diluted in 2% ECL Advance; Millipore, Billerica, MA), rabbit anti-integrin α4 (0.2 µg/ml; Cell Signaling), rabbit anti-integrin α5 (0.2 µg/ml; Cell Signaling), rabbit anti-integrin α6 (1:1000; Abcam), rabbit anti-integrin β1 (0.2 µg/ml; Cell Signaling), mouse anti-actin (1:10,000; MP Biomedicals, Solon, OH), rabbit anti-proliferating cell nuclear antigen (PCNA; 1 µg/ml; Santa Cruz). Prior to incubation membranes were blocked in TBST with 5% NFDM (wt/vol) 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. Data is presented as mean ± SE. Comparisons between groups of two were made using student’s t test or the Mann-Whitney test for non-parametric 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 Fischer’s LSD method. The proportion of male fetuses in each group was compared with the two sample test of equality of proportions. P values ≤ 0.05 were considered significant.

Results

Fetal measurements and organ weights in IUGR and control fetuses

Fetal ages, sex, and weights are presented in the table.
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, Figure 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 (Figure 3).

Endothelial cell conditioned media

Islet derived EC conditioned media (ECCM) pooled from four IUGR or three control fetuses were evaluated in islet cultures from four other control fetuses and presented relative to non-conditioned media incubations. Following overnight incubation in control islet derived ECCM, islet insulin content was increased ~1.8 fold relative to non-conditioned media and greater than IUGR islet derived ECCM (P<0.01; Figure 4a).

In the IUGR islet derived ECCM, HGF concentrations were lower than in control islet derived ECCM (P<0.01; Figure 4b,d). Endothelial cells also had reduced HGF protein expression in IUGR islet derived EC lysates compared to control lysates (P<0.01; Figure 4c,d). Insulin receptor, eNOS, collagen type IV, and laminin α1, α4, α5, and β1 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; Figure 4c,e). There was no difference in PCNA expression between the control and IUGR islet derived EC lysates (Figure 4c).

To further explore the role of HGF in EC to β-cell crosstalk, 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 non-conditioned 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 (100ng/ml) in non-conditioned media, islet insulin content was 1.9 fold relative to non-conditioned 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 (Figure 5).

Pancreatic islet cell protein expression

VEGFA and integrin β1 protein expression were lower (P<0.05) in pancreatic islets isolated from IUGR fetuses (n=4) compared to controls (n=10), but there was no change in the HGF receptor, cMET, or integrin α5 (Figure 6). Although integrins α4 and α6 could be identified in whole pancreatic lysates, we could not identify them in lysates from either control or IUGR fetal islets (not shown).

Discussion

In the present 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 β-cell function and insulin content. An important finding in the current study was 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 β-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 compared to 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 non-conditioned media. Media conditioned by the large vessel derived ECs and non-conditioned 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 β-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 β-cell mass and function during pregnancy, (9) our findings are the first to demonstrate decreased pancreatic islet HGF in IUGR, a pathophysiological condition characterized by decreased fetal pancreatic β-cell mass and function. (31-33)

While our studies uniquely show that EC HGF plays a central role in promoting pancreatic β-cell function, islet ECs also stimulate β-cell function by non-HGF pathways as well. These include production of certain basement membrane components, laminins and collagen IV, which are ligands for β-cell receptors. (8;18) The best characterized of these receptors are the integrins. (8) Our results showed that there were no differences between control and IUGR islet EC laminins or collagen type IV, but integrin β1 was significantly decreased in islets isolated from IUGR fetuses compared to controls. The model of IUGR used in this study is characterized by
decreased β-cell replication, insulin production, and secretion; and previous studies have demonstrated that integrin β1 stimulates these critical β-cell processes.(8,31-33) It is, therefore, reasonable to hypothesize that decreased integrin β1 has a significant functional role in the pathogenesis of fetal pancreatic β-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 EC conditioned media does. Future studies will be required to more specifically test the role of this and other integrins in IUGR β-cell dysfunction. NO also has been reported to stimulate β-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 our IUGR fetuses, implicating impaired β-cell to EC paracrine signaling in their islets. The predominant islet VEGF isoform, VEGFA, stimulates pancreatic and pancreatic islet vascularity, as well as increases endothelial cell 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 β-cell production of VEGFA is lacking. However, given the potential feed forward regulation of β-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 these pathways may remain responsive if provided appropriate paracrine signals. This possibility is
currently being tested.

Our 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 also 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 to rodent models (e.g. the late gestation bilateral uterine artery ligation model and 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 β-cells in these situations can expand and develop a stable relationship with their reduced vascularity, though 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 β-cell function and mass compared to control fetuses as gestation progresses from 0.7 to 0.9.(31;32) It is important to consider that these IUGR sheep have fewer β-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 β-cell VEGFA production highlight the paradigm that factors other than simple islet vessel density, like paracrine hormones, are important for normal β-cell function.(24) Such
factors also can have significant effects on the cross talk between ECs and β-cells, which then can be disrupted by 0.9 gestation in IUGR sheep. 100% of the IUGR fetuses at 0.7 gestation were male, while only 67% of the controls were male at this age; removing the two females from the data set and comparing only males does not affect the findings.

Although the current study demonstrated functional defects in EC to β-cell HGF signaling in IUGR fetuses, the functional consequences of other changes, such as lower integrin β1 and VEGFA, have yet to be determined. Furthermore, in vivo studies in which these pathways are up or down regulated, both acutely and chronically, will provide further insight into their relevance for the pathophysiology of β-cell dysfunction in IUGR and their roles in coordinating normal β-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 different 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 β-cell to coordinate anabolic signals for growth with nutrient supply.

**Conclusions**

In conclusion, we have demonstrated that placental insufficiency that leads to IUGR produces significant defects in the HGF pathway mediating EC to β-cell signaling in the fetus. Additionally, placental insufficiency results in decreased fetal pancreatic islet VEGFA and integrin β1. These findings are associated with reductions in pancreatic islet and pancreatic islet vascularity. Although it is unknown if decreased pancreatic islet vascularity observed in the fetus persists in human adults who were IUGR, we speculate that defects in EC to β-cell cross talk
partly explain the increased risk of β-cell failure and type 2 diabetes that these individuals
develop later in life compared to individuals who were not born IUGR.

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P.J.R. is the guarantor of this work and, as such, had full access to all the data in the study and
takes responsibility for the integrity of the data and the accuracy of the data analysis.

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No conflicts of interest exist for this manuscript.

P.J.R conceived of the project, designed and performed experiments, interpreted data, wrote the
first draft of the manuscript. M.A., M.M., A.F., A.R.M., J.K., G.J.S. designed and performed
experiments and reviewed/edited the manuscript. S.A., W.W.H., and S.W.L. designed
experiments, interpreted data, contributed to the discussion, and reviewed/edited the manuscript.
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### Table. Animal Characteristics

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<td>Gestational Age (days)</td>
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<td></td>
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Values are mean ± standard error. Sex was not recorded for one 0.9 gestation fetus in both control and IUGR groups.
Figure 1. Pancreatic islet endothelial cell isolation. Following incubation for 48-72 hours, polymorphic cell types can be identified migrating away from isolated pancreatic islets (A). Repetitive scraping of non-cobblestone appearing cells results in >95% pure population of cells (B) that stain positive for endothelial cell 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).

Figure 2. Pancreatic vessel density in IUGR fetuses. Pancreatic vessel density was measured by FITC-conjugated Griffonia simplicifolia-I Agglutinin staining (green) and was lower in IUGR fetuses (B,D) than controls (A,C) at both 0.7 (A,B) and 0.9 (C,D) gestation. E) Mean ± SE are shown 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. ** and * refer to P < 0.01 and 0.05, respectively for comparisons between IUGR and controls of similar gestational ages.

Figure 3. Pancreatic islet vessel density in IUGR fetuses. Pancreatic islet vessel density was measured by immuno and FITC-conjugated Griffonia simplicifolia-I Agglutinin staining (green) and was lower in IUGR fetuses (B,D) than controls (A,C) at 0.7 (A,B) gestation but not at 0.9 (C,D) gestation. Co-staining also was performed for insulin (blue), as well as glucagon, somatostatin, and pancreatic polypeptide (all red). E) Mean ± SE are shown 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. ** refers to P < 0.01 for comparisons between IUGR and controls of similar gestational
Figure 4. Islet endothelial cell conditioned media increases pancreatic islet insulin content.

A) Freshly isolated fetal sheep islets were divided equally into different incubation conditions (x-axis) and the following day were collected for pancreatic islet insulin content measurement by ELISA. Results are normalized to the non-conditioned media incubation for each batch of islets (100% on the y-axis). Pancreatic islet insulin content (n = 4) increased following an overnight incubation in media conditioned by isolated control pancreatic islet endothelial cells (Control ECCM), but no difference for islet insulin content was found with media conditioned by isolated IUGR pancreatic islet endothelial cells (IUGR ECCM, combined conditioned media from four fetuses). ** refers to 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, and more expression for VEGFR2, and no difference in the expression of PCNA in the IUGR ECs (n = 4) than in control ECs (n = 3). D) Densitometry quantification of HGF in conditioned media and EC lysates. Means with SE are shown, ** refers to P<0.01. E) Densitometry quantification of VEGFR2 in EC lysates. Means with SE are shown, * refers to P<0.05. (hepatocyte growth factor, HGF; VEGFR2, vascular endothelial growth factor receptor 2).

Figure 5. Addition of an inhibitory anti-HGF antibody blocks increased islet insulin content following incubation in endothelial cell conditioned media. Freshly isolated fetal sheep islets were divided equally into different incubation conditions (x-axis) and the following day were collected for pancreatic islet insulin content measurement by ELISA. Results are
normalized to the non-conditioned 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 endothelial cells or skin fibroblasts with PBS, control media conditioned by large vessel endothelial cells with and without the addition of an inhibitory anti-HGF antibody, in control media with the addition of HGF, and in control media conditioned by skin fibroblasts. * refers to differences (P<0.05) in islet insulin content versus control media.

**Figure 6. Pancreatic islet VEGFA and integrin β1 are decreased in IUGR fetuses.** A) VEGFA, cMET, and integrin β1 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 β1 (C; P<0.05) in IUGR pancreatic islets compared to controls.
Figure 1. Pancreatic islet endothelial cell isolation.
Figure 2. Pancreatic vessel density in IUGR fetuses.

Green = GS-1 Agglutinin$^+$
Figure 3. Pancreatic islet vessel density in IUGR fetuses.

Green = GS-1 Agglutinin$^+$
Blue = Insulin$^+$
Red = Glucagon$^+$, Somatostatin$^+$, Pancreatic Polypeptide$^+$
Figure 4 (A-C). Islet endothelial cell conditioned media increases pancreatic islet insulin content.
Figure 4 (D,E). Islet endothelial cell conditioned media increases pancreatic islet insulin content.
Figure 5. Addition of an inhibitory anti-HGF antibody blocks increased islet insulin content following incubation in endothelial cell conditioned media.
Figure 6. Pancreatic islet VEGFA and Integrin β1 is decreased in IUGR fetuses.