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MECHANISMS REGULATING BLOOD VESSEL GROWTH IN THE EARLY BOVINE PLACENTA

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ABSTRACT

The U.S. dairy industry is among the highest milk-producing industries in the world. Improved fertility and healthy pregnancies are needed to keep up with this demand. A key aspect of pregnancy includes implantation and placentation, which involves attachment of the fetal membrane to the maternal endometrium and extensive angiogenesis. However, the process of bovine placental angiogenesis is poorly understood. Hypoxia-inducible factor-1 (HIF1a) induces blood vessel growth to ensure an adequate blood supply to hypoxic tissues via angiogenesis. We hypothesize that the presence of a conceptus will increase endometrial HIF1a expression, thus leading to increased placental angiogenesis. We assessed this hypothesis by inseminating dairy heifers and collecting uterine tissue samples on days 17 and 20 post-insemination. These samples were used to determine HIF1a protein localization within the tissue via immunofluorescence analysis and mRNA quantification of HIF1a, vascular endothelial growth factor A (VEGFA), and angiopoietin-like 4 (ANGPT4) via RT-qPCR analysis. Results were compared to uterine samples of estrus-synchronized dairy heifers collected on day 17 post-estrus. Immunofluorescence analysis showed stronger labeling of HIF1a in various stroma and intracellular spaces central to glands and blood vessels and little to no labeling in glands or luminal epithelium. RT-qPCR analysis found a low detectable amount of HIF1a mRNA in samples across the three groups. However, more abundant mRNA expression was detected for VEGFA and ANGPT4. Furthermore, VEGFA mRNA expression remained relatively consistent across all groups, while ANGPT4 mRNA expression was lower in the pregnant groups when compared to the cyclic group. Further work is needed to determine the effects of early pregnancy on HIF1a in the uterine endometrium.

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Chapter 1

Literature Review

Impact of the Dairy Industry

The United States dairy industry ranks as the second largest milk producer in the world (USDA-ERS, 2017). The U.S. dairy industry supports 3.2 million jobs, $49 billion in direct wages, and has a $794 billion annual economic impact in the U.S. (IDFA-DD, 2023). In the last two years alone, it has added 60,000 jobs and $41 billion to the U.S. economy (IDFA-PR, 2023). Increased productivity and efficiency are a significant reason behind this tremendous growth in the dairy industry. The annual milk production per cow increased by a rate of 1.53% from 2000-2020. In those two decades, farms increased their yearly milk production from 18,197 pounds (about 8,254 kilograms) to 23,777 pounds (about 10,785 kilograms) of milk produced per cow (Njuki, 2022). In 2023, the U.S. averaged 9.395 million dairy cattle and 24,113 pounds of milk per cow, producing 2.27 billion pounds of milk (USDA-DD, 2024). These remarkable achievements are a testament to the technological, industrial, and genetic improvements over the last century and our understanding of bovine physiology and fertility.

Fertility in the Dairy Industry

With the increase in milk productivity, there has been a subsequent decrease in fertility in the dairy cattle population. In 1951, the first-service conception rate in dairy cattle was approximately 65%, dropping to 40% in 1996 (Butler, 1998). In 2010, the median conception

rate of dairy cattle was 26-40% and slightly increased to 32-45% by 2022 (Hanks & Kossaibati, 2023). These conception rate percentages have climbed in the last decade thanks to improvements in genetics, management, and nutrition, but the percentages remain lower than in 1951. In 2006, the average value of a new pregnancy was around $278 (De Vries, 2006), translating roughly to $433 in 2024. With the dairy cow population at 9.395 million dairy cattle in the U.S. and a conception rate of 32-45%, the U.S. dairy industry loses between $2.2 billion and $2.7 billion annually. In addition, we are facing rising issues of climate change and increasing human population, so the dairy industry must be able to adapt and respond to those economic demands. Answers to increasing conception rates can be found in further understanding and researching the physiology of bovine fertility to improve the efficiency and profitability of the industry.

Role of Early Placental Growth in Pregnancy

At the establishment of pregnancy, the bovine conceptus must signal the corpus luteum to maintain its progesterone production and ensure a successful pregnancy (Hughes et al., 2019). The conceptus elongates from a tubular to filamentous conceptus around days 12-14 after ovulation (Degrelle et al., 2005). The filamentous conceptus releases hormones, including interferon tau (IFNT), that act upon the uterine epithelium (Spencer & Bazer, 2004). IFNT is an anti-luteolytic signal found in ruminants that serves as a maternal recognition signal and prevents the pulsatile release of prostaglandin (PGF2 alpha) from the uterine endometrium that would otherwise trigger luteolysis of the corpus luteum (Bazer & Thatcher, 2017).

Implantation then begins around day 16-18 after ovulation, and placentation occurs around day 22 (Pedersen et al., 2017). Placentation occurs between the fetal membrane, known as the chorioallantois, and the maternal uterine tissue, known as the endometrium, at the caruncular areas of the endometrium. This fusion stimulates the growth of new blood vessels (angiogenesis), allowing for the exchange of nutrients and waste products from the conceptus and the mother. While there is no accurate data on the number of cows that lose pregnancies due to placentation and placental issues, we do know that any failure of implantation and placentation can result in pregnancy loss. Further research into angiogenesis during this stage of development can help to increase both conception rates and help to identify ways to improve fetal and maternal health.

Blood Vessel Growth Regulators

A connection was found between the signaling pathways for IFNT and HIF1alpha in the ovine uterus (Song et al., 2008). Hypoxia-inducible factor (HIF) protein is a transcription factor that regulates genes that ensure the survival of cells, tissues, organs, and organisms under hypoxia (Ke & Costa, 2006). HIF is a heterodimeric protein composed of three alpha and beta subunits. One subunit, HIF1alpha (HIF1a), was discovered to be upregulated under hypoxic conditions and trigger many physiologic responses such as erythropoiesis, glycolysis, and angiogenesis to create ways in which oxygen can be delivered to hypoxic cells or tissues (Semenza, 1998). Under normal oxygenated conditions, HIF1a is bound by the von Hippel-Lindau (VHL) protein that recruits a ubiquitin ligase that directs HIF1a for proteasomal degradation (Kaelin Jr. & Ratcliffe, 2008), keeping the concentration of HIF1a low. In hypoxic

conditions, however, ubiquitination does not occur, allowing HIF1a to accumulate in the cell, translocate to the nucleus, and bind with HIF1beta (HIF1b). Together, this complex activates hundreds of genes that regulate cell proliferation, metastasis, glycolysis, pH regulation, and, most notably, angiogenesis (Lee et al., 2021). Mouse embryos without HIF1a were found to have multiple cardiovascular defects that resulted in death (Iyer et al., 1998).

One notable gene upregulated by the HIF1 complex is vascular endothelial growth factor A (VEGFA), a major inducer of angiogenesis. HIF1a and VEGF were present in mice in the stroma after embryo implantation (Daikoku et al., 2003). HIF1a also upregulates the angiopoietin-like 4 (ANGPT4) gene, which plays a significant role in endothelial cell proliferation, survival, and angiogenesis (Hu et al., 2016; Zhou et al., 2023).

Placentation requires extensive angiogenesis in the maternal uterus for pregnancy. However, the process of bovine placental vasculature development is poorly described. A better understanding of angiogenesis should contribute to improved fertility and production efficiency in the dairy industry.

This study determined the localization and expression of HIF1a, VEGFA, and ANGPT4 during early bovine pregnancy to better understand placental and vascular growth. We hypothesize that the conceptus and its fetal membranes increase HIF1a expression in the endometrium, increasing blood vessel growth factors and leading to new blood vessel growth.

Chapter 2

Materials and Methods

Animals

All procedures involving animals were reviewed and approved by the Penn State Institutional Animal Care and Use Committee and complied with all regulations regarding the care and use of agricultural animals in research (Ag Guide; protocol #PRAMS 201044524). Holstein dairy heifers between 12-14 months of age were estrus synchronized using prostaglandin F2α and gonadotropin-releasing hormone (GnRH). Estrus activity was monitored. On the day of estrus (Day 0), the heifers were either inseminated or not inseminated to serve as pregnant (N=12) and non-pregnant (cyclic; N=6) controls, respectively. To monitor progesterone, a blood sample was taken via the tail vein the day before and on the day of sample collection. On day 17 of the cycle (D17C; N=6) or pregnancy (D17P; N=6), animals in each subgroup were moved into a procedure room with a chute system for tissue collection. On day 20 of pregnancy (D20P), a different subset of inseminated animals (N=6) was moved into a procedure room with a chute system for tissue collection.

Tissue Collection

On the day of tissue collection, a tube of blood was collected for plasma progesterone analysis and placed on ice. The animals were given epidural anesthesia using lidocaine (5-8 mL of 2% lidocaine hydrochloride), and the animals' perineal area was cleaned before the insertion of a cervical dilator. A sterile infusion tube was placed through the cervix, and the uterus was

flushed with 60 mL filtered sterile phosphate-buffered saline (PBS; pH 7.7) and collected in conical tubes for PAG assay or conceptus recovery. The uterine flushes were transported to the laboratory on ice and stored in a -20°C freezer. Four biopsies of uterine and endometrium tissue were then taken transcervically using sterilized custom biopsy forceps. Two biopsies per animal were placed in sterile cryotubes and snap-frozen with liquid nitrogen for RNA extraction. The other two biopsies per animal were incorporated into separate molds with an optimal cutting temperature (OCT) compound and frozen in isopentane cooled over liquid nitrogen for immunofluorescence analysis. All biopsies were stored in liquid nitrogen for transport to the laboratory and placed in a -80°C freezer. After tissue collection concluded, the animals were injected with prostaglandin F2α to induce estrus and minimize any possible intrauterine infections. Animals were monitored for abnormalities for three days and treated accordingly by a veterinarian if needed. Only one heifer received veterinary care for abnormal vaginal discharge, and 64% (N=18) of heifers conceived another pregnancy within two services following the collection.

PAG ELISA

Aliquots of the uterine flushes were shipped on dry ice to Biotracking LLC Inc. (Moscow, Idaho), and the concentration of PSPB (pregnancy-specific protein B; proteins of the pregnancy-associated glycoprotein family) was measured using a commercially available ELISA assay. If a high concentration of PAG was detected in the uterine flush, the corresponding bred animal was confirmed pregnant at the time of sample collection (N=12).

Immunofluorescence Analysis

Tissues frozen in OCT from D17C, D17P, and D20P heifers were sectioned at 5 µm thickness, thaw-mounted on positively charged slides, and stored at -80°C. For immunofluorescence labeling, sections were thawed at room temperature (RT) for 10 minutes and fixed with cold 100% ethanol for 10 minutes. Sections were washed twice in fresh PBS for 5 minutes and incubated with PBS containing 0.1% Triton X-100 and 20% goat serum (PBS-T/GS) for 30 minutes at RT for blocking and permeabilization. Slides were moved to a wet, dark chamber. They were incubated with a primary antibody solution containing either HIF1a (MA1-516, Invitrogen) and PBS-T/GS in a 1:200 dilution at 4°C for a minimum of 3 hours or maximum overnight. Sections were washed three times in fresh PBS for 5 minutes on a shaker platform and incubated with 0.01 µg/µL of a secondary antibody in PBS-T/GS for 1 hour at RT. Sections treated with HIF1a were incubated with Goat anti-mouse IgG Alexa Flour 488 (A-11001, Invitrogen) as the secondary antibody. After incubation, slides were washed three times in PBS on a shaker platform and protected from light. Cell nuclei were labeled using 4',6-diamidino-2-phenylindole (DAPI, P36931) and sealed with gold prolong anti-fade (Invitrogen) and a coverslip and stored at 4°C for 24 hours in a dark chamber. Isotype (MCA928, Bio-Rad) and 'no primary antibody' controls were treated similarly and used to establish background labeling.

Images were viewed and captured using an Olympus BX51 microscope fitted with a DP71 camera and microscope filters for green (U-MNB2), red (U-N41004), and blue (U-MNU2) wavelengths. Two sections were analyzed per animal (N=6) for a qualitative comparison across the D17C, D17P, and D20P groups. Images of luminal endometrium, shallow and deep stroma, shallow and deep glands, and blood vessels were taken. Image exposure time and gain settings

were held consistent based on the negative controls (isotype and 2°Ab only). DAPI and HIF1a images were merged to create a singular image.

RT-qPCR

RNA from the endometrial biopsies and some additional endometrium collected in 2014 from D17C, D17P, and D20P heifers were extracted, purified, and quantitively analyzed using procedures described by Kamat et al. (2016). Twelve samples were analyzed per group (D17C, D17P, D20P). Primers were validated (see Table 1 in Appendix A) and used in reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis.

Standard curves showed between 90% and 100% efficiency, and any samples with cycle thresholds below the detection limit of the standard curve were removed from the data analysis. The geometric mean of threshold cycles (C_T) from the reference genes ribosomal protein L19 (RPL19) and Beta-actin (BACT) were calculated for each sample.

Statistical Analysis

Data were analyzed with the $2^{-\Delta\Delta C_T}$ (fold change) in a one-way ANOVA using D17C as the control group (Kamat et al., 2016). C_T of the reference gene was calculated using the geomean of C_T mean RPL19 and C_T mean Beta-actin. The average ΔC_T of the D17C group was used as a calibrator group to calculate $\Delta\Delta C_T$. $2^{-\Delta\Delta C_T}$ was calculated, and the values were graphed using GraphPad 9.3 (GraphPad Software Inc.) to represent fold change across each group (see Figure 2 and Figure 3).

Chapter 3

Results

Immunofluorescence Analysis

Images for D17C, D17P, and D20P were semi-qualitatively compared to the negative control images, as shown in Figure 1. Stronger labeling of HIF1a was seen in some areas of the stroma (ST; Fig. 1 B2), especially around areas containing blood vessels (BV; Fig. 1 C2) and some glands (GL; Fig. 1 B3). Faint labeling was observed within the vasculature (BV; Fig. 1 D1). Minor to no staining of HIF1a was found in the intraglandular space (IGS; Fig. 1 B4) or the luminal epithelium (LE; Fig. 1 D1). These observations were generally consistent across all three groups, with the pregnant groups having slightly more surface area labeling than the cyclic group.

RT-qPCR Analysis

Quantitative analysis of RT-qPCR data was performed. The expression of HIF1a was under the limit of the qPCR assay. Valid data for VEGFA and ANGPT4 were obtained in all three groups (D17C, D17P, and D20P). VEGFA mRNA was consistent in expression across all three groups, but ANGPT4 mRNA expression was lower in the endometrium of pregnant heifers than the cyclic heifers. Based on the one-way ANOVA, neither VEGFA nor ANGPT4 were different between pregnancy statuses ($P > 0.05$). F and R square values were calculated for VEGFA and ANGPT4 ($F=1.368$, $R^2=0.1203$; $F=0.8672$, $R^2=0.0674$).

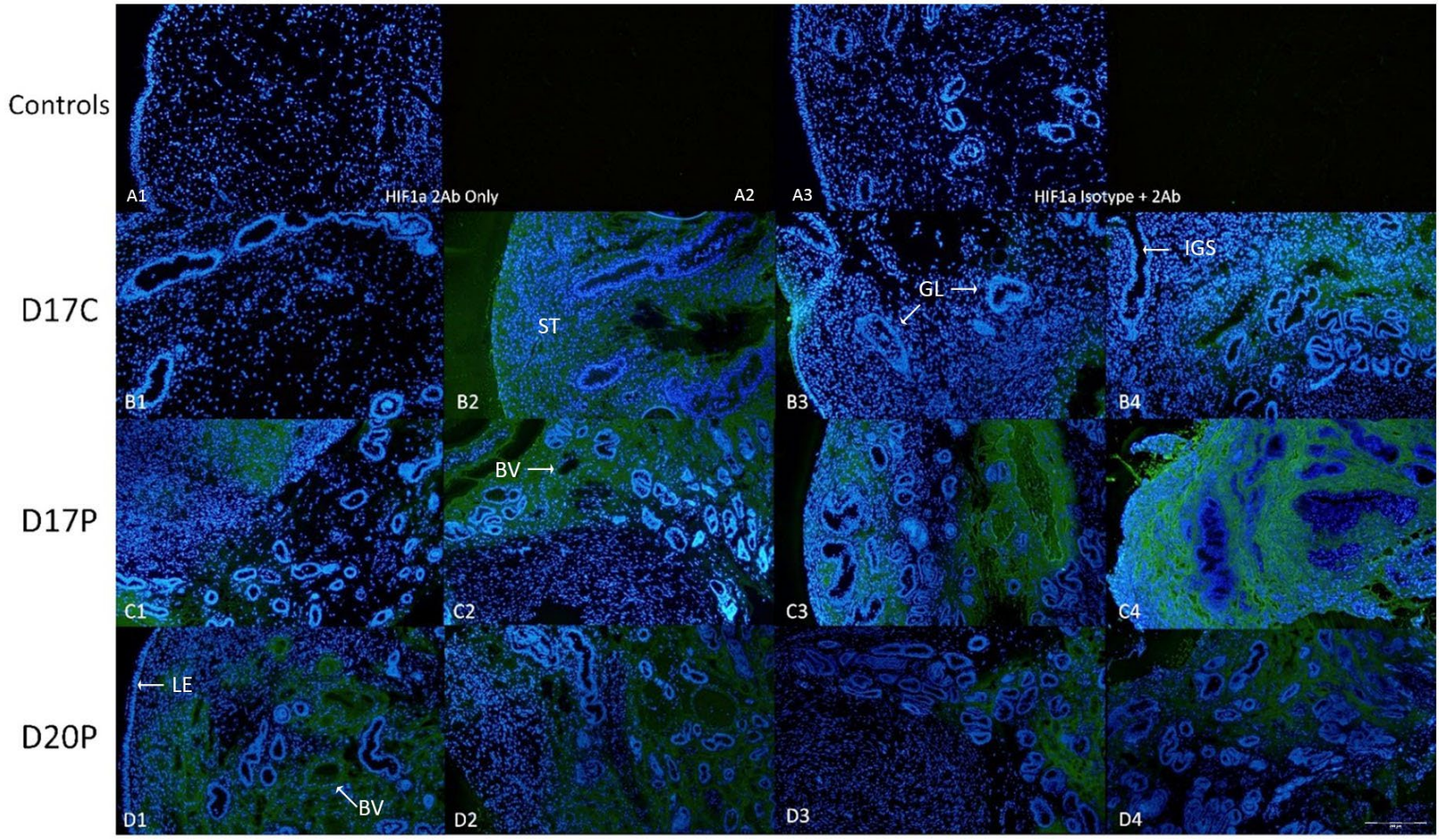

Figure 1: HIF1a immunofluorescence images of bovine endometrium for D17C, D17P, and D20P. (A1) DAPI and (A2) fluorescence signal from negative control HIF1a isotype. (A3) DAPI and (A4) fluorescence signal from negative control HIF1a isotype and secondary antibody (2° Ab). HIF1a labeling in biopsies collected from 4 different animals on (B1-B4) day 17 of the estrous cycle [D17C], (C1-C4) day 17 of pregnancy [D17P], and (D1-D4) day 20 of pregnancy [D20P]. Stronger labeling of HIF1a was seen in some areas of the stroma (ST; B2), especially around areas containing blood vessels (BV; C2) and some glands (GL; B3). Faint labeling can be observed within the vasculature (BV; D1). Minor to no staining of HIF1a was found in the intraglandular space (IGS; B4) or the luminal epithelium (LE; D1). All images with original magnification of 100x. Scale: 200 µm.

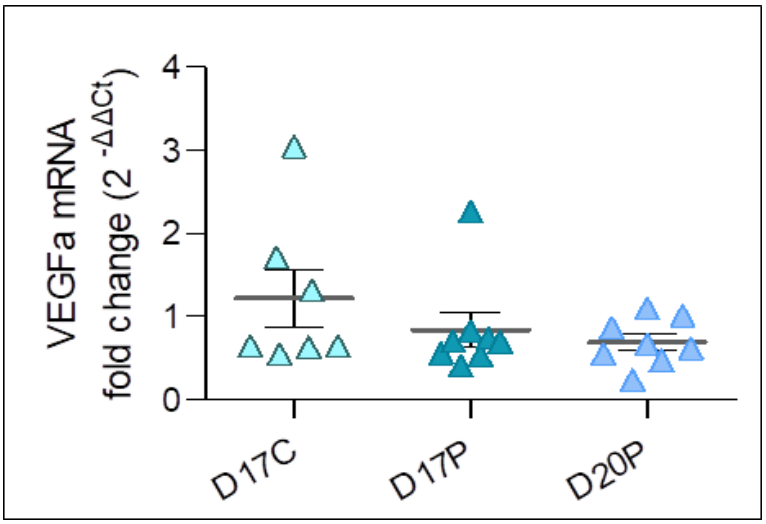

Figure 2: **Fold change in VEGFA mRNA abundance in endometrium from days 17 and 20 pregnant (D17P and D20P) compared to day 17 cyclic (D17C). The graph shows the Least Squares mean (horizontal line and SEM).**

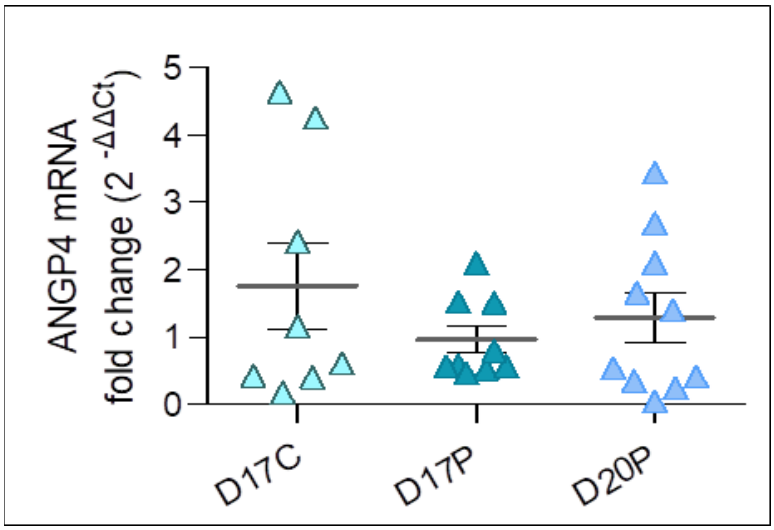

Figure 3: **Fold change in ANGPT4 mRNA abundance in endometrium from days 17 and 20 pregnant (D17P and D20P) compared to day 17 cyclic (D17C). The graph shows the Least Squares mean (horizontal line and SEM).**

Chapter 4

Discussion

While the topic has been researched in labs in species such as humans and mice, the process and regulation of angiogenesis in the bovine placenta is relatively unknown. This study aimed to determine the expression of HIF1a, VEGFA, and ANGPT4 in the bovine endometrium to further understand the process of blood vessel growth in the early bovine placenta. We discovered that while HIF1a protein was detected in the stroma and around glands and blood vessels by immunofluorescence analysis, HIF1a mRNA was below the detection threshold for the RT-qPCR. Blood vessel growth factors VEGFA and ANGPT4, on the other hand, were detectable by RT-qPCR in each of the three groups (D17C, D17P, D20P). While VEGFA mRNA stayed relatively consistent across each group, ANGPT4 mRNA was lower in both pregnant groups than in the cyclic group. This suggests low expression of HIF1a and some angiogenic factors during the early stage of placental growth between days 17-20 after conception.

It is possible that angiogenesis in the bovine placenta may not involve HIF1a protein, at least a high level, until after day 20 of pregnancy. A study on gene expression in cattle throughout gestation identified the greatest HIF1a expression at day 241 of gestation (Rotta et al., 2015). In addition, samples were taken only of the maternal endometrium, not the fetal membranes. The fetal membranes may be the source of HIF1a expression, which is not yet connected to the endometrium at the analyzed stages. A sample of the fetal membrane may need to be taken, perhaps also later in gestation.

However, the question of the role that HIF1a plays in early placental growth and regulation still needs to be answered. HIF1a was observed near glandular and vascular structures in the stroma. Because glandular secretions are a primary source of nutrients for survival and

development of the conceptus at the time of attachment (Filant & Spencer, 2014), HIF1a could influence uterine secretions rather than angiogenesis during placental attachment. HIF1a protein was found to be involved in repair and in the secretory phase during the human menstrual cycle (Critchley et al., 2006), and another study confirmed the effect of decreased progesterone on the induction and secretion of angiogenic factors, including VEGF (Maybin et al., 2011).

IFNT levels during this time may also affect the HIF1a regulation. According to research in ovine uterine endometrium, the presence of conceptus IFNT does not cause an increase in HIF1a mRNA (Song, 2008), but further research will need to be performed to examine whether the amount of IFNT in the system is indicative of HIF1a expression in bovine uterine endometrium.

As stated previously, knowledge of bovine placental vasculature development is incomplete. HIF1a plays a role in angiogenesis and placental development in several species. Based on the results collected, HIF1a was present but not abundant during day 17 and day 20 of pregnancy, despite implantation and placentation initiating around that time. VEGFA and ANGPT4 were identified and quantified to better understand the effects of pregnancy status on their expression. More work is needed to determine how IFNT and progesterone affect HIF1a expression and its role in placental angiogenesis. Analyzing placental tissue under various levels of hypoxia in the uterus and fetal membranes may be an approach for determining when these angiogenic factors are present and facilitating angiogenesis. To fully understand the role that HIF1a plays in fertility and subsequent pregnancy, these research topics must be expanded upon.

Appendix A

Table 1: Primers used in RT-qPCR analysis

Gene	Primer Sequence (5' to 3')	Ta (°C)	Amplicon (bp)	Accession
ARNT	F: AGACAGCTTCCAACAGGTCG R: TTCGACAGTGAAGGTCGGTGG	60	204	NM_173993.1
HIF1A	F: CCTCTGATCTCACGAGGGGT R: TCGACGTTCAGAACTTATCTTTTTC	60	204	NM_174339.3
VEGFA	F: GACCCTGGTGGACATCTT R: CTCCTATGTGCTGGCTTT	60	188	NM_001316992.1
ANGPT4	F: AGATTGGGAAGGCAACGAGG R: CAAACCACCACCCTCCAGAC	60	215	NM_001076483.2
BACT	F: CTGGACTTCGAGCAGGAGAT R: GGATGTCGACGTCACACTTC	60	208	NM_173979.3
RPL19	F: ATCGATCGCCACATGTATCA R: GCGTGCTTCCTTGGTCTTAG	60	168	NM_001040516.2

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- Maple Ridge Club Pigs – Volunteer (Jan. 2021)
- Central PA Humane Society – Volunteer (June 2020)

Honors and Awards

- Dean's List (8 semesters)
- American Society of Animal Science Undergraduate Scholastic Achievement Award – 2023, 2024
- 2023-24: Anna K. Eaton Sch., Craola Sch. Award, Class of 1922 Sch., T. & S. Spring Sch., Keller Family Honors Sch.
- 2022-23: C. & M. Shallcross Sch., Anna K. Eaton Sch., J. & J. Darlington Sch., E.M. & M.M. Turner Sch.
- 2021-22: Anna K. Eaton Sch., Horace T. Woodward Sch., William H & M and Jean Coleman Sch.
- 2020-21: R.F. Russell Memorial Sch., Bayard and Ethel Kunkle Sch.

Activities

- Disciple Makers Christian Fellowship (DCF) – Leadership Team, PSU University Park, PA
- Center for Reproductive Biology and Health (CRBH) Research Team, PSU University Park, PA
- PSU Altoona for Animal Welfare (PAAWs) – Club President, PSU Altoona, PA
- Agriculture Club, PSU Altoona, PA