Conceptus Interferon Gamma is Essential for Pregnancy Maintenance in the Pig

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A Thesis presented to

the Faculty of the Graduate School

at the University of Missouri – Columbia

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

By

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MAY 2021

The undersigned, appointed by the Dean of the Graduate School, have examined the thesis entitled

CONCEPTUS INTERFERON GAMMA IS ESSENTIAL FOR PREGNANCY MAINTENANCE IN THE PIG

Presented by Destiny N. Johns
A candidate for the degree MASTER OF SCIENCE
We hereby certify that in our opinion it is worthy of acceptance

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ACKNOWLEDGEMENTS

In sitting down to write this segment of my thesis, I am overcome with gratitude for so many people who have made this journey possible. First and foremost, I want to thank my thesis advisor, Dr. Rod Geisert, for the constant support, ability to provide perspective, and unwavering faith in me even when I doubted myself. I will never forget the life lessons and countless laughs you shared with me during my time in this program and 'thank you' only begins to express a fraction of my appreciation for everything you've done for me. You have been the best academic dad any trainee could ask for.

To the students and faculty at the University of Missouri – Columbia, thank you for the greatest memories and lifelong friendships. Specifically, thank you to my committee: Dr. Randy Prather, Dr. Tom Spencer, and Dr. Heide Schatten, for their guidance and for challenging me as a trainee. Additionally, thank you Dr. Spencer for allowing me to use your lab and thank you to everyone who has worked in there during my time here and helped me troubleshoot protocols or simply filled in long incubation periods with good conversation. Also, a big thank you to Dr. Prather and all of his staff for generating my embryos and for taking care of the numerous gilts we utilized for this project. Thank you to each of my professors who fostered my love and enthusiasm for reproductive biology while teaching their courses. You all made this journey into more than just a degree checklist, but an entire experience I'll never forget or take for granted.

I would also like to thank Ashley Meyer and Caroline Pfeiffer for being such an immense help throughout my entire program from teaching me basic lab

techniques, to assisting me in pig collections and always answering my endless questions. Additionally, I wouldn't have been able to complete my research without the help and support of Shelbi Perry, Dr. Carol Gomez, Paula Chen, Katy Stoecklein, Lee Spate, Raissa Cecil, Carson Andersen, Jessica Foster, Eleanore O'Neil, Dr. Harriet Fitzgerald, and Melissa Samuel; thank you all so much for every contribution you made to this research project.

Finally, I want to thank my family. From my earliest memories, my parents instilled in me a keen desire to pursue every curiosity and understand how the systems in our would interact. Thank you, Mom and Dad, for never allowing me to settle. Without your unconditional love and support, I would not have developed the confidence and determination to follow every last one of my dreams, no matter how ambitious they seemed. To my three older brothers, I wouldn't be the strong and tenacious young woman I am were it not for each of you. Thank you for never allowing me to forget who I am and what I'm capable of. To Logan, thank you for always going above and beyond. No amount of distance or time has hindered your ability to love me, challenge me, and support me in all things. I am beyond blessed to have had this opportunity and to share my experience with all of you. Thank you all, from the bottom of my heart, for everything you've done.

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LIST OF ABBREVIATIONS

cDNA: Complementary DNA

CD3+: Lymphocyte

CIITA: Class II Major Histocompatibility Complex

CL: Corpus Luteum

COC: Cumulus Oocyte Complex

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats

CT: Cycle Threshold

CXCL: Cysteine-X-cysteine Chemokine Ligand

CYP19A1: Aromatase

DEG: Differentially Expressed Gene

ELISA: Enzyme-Linked Immunosorbent Assay

EN: Endoderm

E_2: Estrogen

ERK: Extracellular Signal-Regulated Kinase

ESR1: Estrogen Receptor-1

FASLG: Fas Ligand

FC: Fold Change

FSH: Follicle Stimulating Hormone

GAS: IFNG Activated Site

GE: Glandular Epithelium

GnRH: Gonadotropin Releasing Hormone

gRNA: Guide RNA

HE: Hematoxylin and Eosin

HIER: Heat Induced Epitope Retrieval

ICM: Inner Cell Mass

IDO1: Indoleamine 2,3-Dioxygenase 1

IFN: Interferon

IFNA: Interferon Alpha

IFNAR1: Interferon Alpha Receptor 1

IFNAR2: Interferon Alpha Receptor 2

IFNB: Interferon Beta

IFND: Interferon Delta

IFNE: Interferon Epsilon

IFNG: Interferon Gamma

IFNGR1: Interferon Gamma Receptor 1

IFNGR2: Interferon Gamma Receptor 2

IFNK: Interferon Kappa

IFNT: Interferon Tau

IFNW: Interferon Omega

IL1: Interleukin-1

IL18: Interleukin-18

IL1RT1: Interleukin 1 Receptor Type 1

IL1RT2: Interleukin 1 Receptor Type 2

IL1B: Interleukin 1 Beta

IL1B2: Interleukin 1 Beta 2

IRF: Interferon Regulatory Factor

IRF1: Interferon Regulatory Factor 1

IRF2: Interferon Regulatory Factor 2

IRF9: Interferon Regulatory Factor 9

ISG: Interferon Stimulated Gene

ISGF3: Interferon Stimulated Gene Factor 3

ISRE: Interferon-Stimulated Response Element

IVF: In Vitro Fertilization

JAK: Janus Activated Kinases

JAK1: Janus Activated Kinase 1

JAK2: Janus Activated Kinase 2

LE: Luminal Epithelium

LH: Luteinizing Hormone

MAPK: Mitogen-Activated Protein Kinase

MC: Mast Cells

MCP-1/CCL2: Monocyte Chemoattractant Protein-1

MHC: Major Histocompatibility Complex

mRNA: Messenger RNA

MRP: Maternal Recognition of Pregnancy

NFKB: Nuclear Factor Kappa B

NK: Natural Killer

OAS: (2',5') Oligoadenylate Synthetase

OXTR: Oxytocin Receptor

PBS: Phosphate Buffered Saline

PCNA: Proliferating Cell Nuclear Antigen

PCR: Polymerase Chain Reaction

PI3K: Phosphoinositol 3-Kinase

PKC: Protein Kinase C

PLA2: Phospholipase A2

P_4: Progesterone

PG: Prostaglandin

PGE: Prostaglandin E

PGE_2: Prostaglandin E2

$PGF_{2\alpha}$: Prostaglandin F2 Alpha

PTGS1: Prostaglandin Synthase 1

PTGS2: Prostaglandin Synthase 2

roIFNT: Recombinant Interferon Tau

RT-PCR: Reverse Transcribe-Polymerase Chain Reaction

SAL1: Salivary Lipocalin

SCNT: Somatic Cell Nuclear Transfer

SEM: Scanning Electron Microscopy

STAT: Signal Transducer and Activator of Transcription

STAT1: Signal Transducer and Activator of Transcription 1

STAT2: Signal Transducer and Activator of Transcription 2

TE: Trophectoderm

TNFSF10: Tumor Necrosis Factor Super Family 10

TYK2: Tyrosine Kinase 2

ULF: Uterine Lumen Flush

uMC: Uterine Mast Cells

uNK: Uterine Natural Killer

VMDL: MU CVM Veterinary Medical Diagnostic Laboratory

WT: Wild-Type

YWHAG: 14-3-3 Protein Gamma

ABSTRACT

CONCEPTUS INTERFERON GAMMA IS ESSENTIAL FOR PREGNANCY MAINTENANCE IN THE PIG

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Establishment and maintenance of pregnancy in the pig is a complex process that relies on adequate communication between the conceptus and maternal uterine endometrium. During the peri-implantation period, the conceptuses produce and secrete estrogens, interleukin 1 beta 2, prostaglandins and other biological factors into the uterine lumen that change the uterine epithelium to become receptive to the attaching conceptuses as well as promote proper conceptus development. Following elongation, beginning on day 12 of pregnancy, the conceptus is known to secrete two different types of interferons. The pig conceptus secretes both type I (interferon delta, *IFND*) and type II (interferon gamma, *IFNG*) interferons during this time. CRISPR/Cas9 gene editing and somatic cell nuclear transfer (SCNT) technologies were used to create an *IFNG* loss-of-function study in pigs. Blastocyst stage embryos that were $IFNG^{+/+}$ or $IFNG^{-/-}$ were transferred into recipient gilts and their reproductive tracts were collected on days 15 and 17 of pregnancy. Elongated conceptuses were flushed from recipient gilts on day 15 $IFNG^{+/+}$ (4/4) and $IFNG^{-/-}$ (4/4) recipient gilts. On day 17 of pregnancy, all $IFNG^{+/+}$ recipient gilts (4/4) contained elongated viable conceptuses; however, conceptuses were only recovered from 2 of 8 $IFNG^{-/-}$ embryo recipient gilts. In all $IFNG^{-/-}$ pregnancies, the conceptuses

were thin and fragmented compared to *IFNG*$^{+/+}$ conceptuses. Additionally, the reproductive tracts that received *IFNG*$^{-/-}$ conceptuses which were not pregnant on day 17 appeared hyperemic, inflamed and edematous. IFNG was localized to the trophectoderm of *IFNG*$^{+/+}$ conceptuses on both day 15 and 17 of pregnancy. However, IFNG expression was not detected in *IFNG*$^{-/-}$ conceptuses on either day 15 or day 17 of pregnancy. Conceptus *IFNG* mRNA expression was significantly affected by genotype (P=0.0006) and day (P<0.0001). Total IFNG was significantly lower (P=0.0018) in ULF of *IFNG*$^{-/-}$ embryo recipient gilts compared to *IFNG*$^{+/+}$ embryo recipient gilts. *IFNG*$^{+/+}$ conceptuses induced endometrial folding and recruited large numbers of immune cells to the endometrial stroma beneath the site of conceptus attachment compared to less endometrial folding and presence of immune cells in *IFNG*$^{-/-}$ pregnancies on day 15. An additional group of recipient gilts received either *IFNG*$^{+/+}$ embryos (n=6) or both *IFNG*$^{-/-}$ and *IFNG*$^{+/+}$ embryos (n=5) to determine if the presence of IFNG producing conceptuses could rescue the *IFNG*$^{-/-}$ embryos beyond day 17 of pregnancy. On day 30 of pregnancy, 3/6 of *IFNG*$^{+/+}$ embryo recipient gilts contained 3-4 viable embryos, however, only 1 of 5 *IFNG*$^{-/-}$ and *IFNG*$^{+/+}$ co-transferred recipient gilts maintained pregnancy to day 30. Genotyping indicated that all five (1 healthy, 4 degenerating) embryos were *IFNG*$^{+/+}$. These results indicate conceptus IFNG production is not essential for early conceptus development, rapid elongation or establishment of pregnancy. However, conceptus IFNG production does appear to be necessary for survival during the period of placental attachment beyond day 15.

CHAPTER ONE

1. INTRODUCTION

The United States Department of Agriculture inventory of all hogs and pigs was 79.1 million head as of September 1st, 2020 with 6.33 million head used as breeding inventory (https://usda.library.cornell.edu). The trend line for both market pigs and breeding stock has steadily increased over recent years to meet the demand for pork consumption as the US and world population grows. In addition to being important for agriculture, pigs are increasingly being used as biomedical models in human and animal research. Pigs are able to serve as an animal model for human research due to their similar anatomy, physiology, and metabolism to human biology (Swindle et al. 1988). To meet the increasing demand for pigs in both the agricultural and biomedical fields, it is important to minimize fetal loss. Approximately 20-45% of fetal loss occurs during embryonic development and attachment between days 10-30 of gestation (Kridli et al. 2016). It is during this window of early pregnancy when the developing conceptuses must synthesize and secrete several biological factors into the uterine lumen to prime the maternal system for attachment and stimulate maternal recognition of pregnancy.

During the peri-implantation period (days 10-12 of pregnancy), pig conceptuses begin a rapid elongation event in which the developing conceptus goes from spherical in morphology, to ovoid, then filamentous in a matter of a few hours. This is achieved primarily due to cellular remodeling and migration as opposed to cell proliferation. This morphological change is unique to the pig

compared to other mammals that undergo conceptus elongation over several days (Geisert et al. 1982b). Elongation in the pig likely occurs so rapidly due to being a litter bearing species where the size and length of the uterine horns limits uterine space for placental development. Rapid elongation allows each conceptus to attach and establish its territory within the uterus as it competes for uterine surface area to develop a true epitheliochorial placenta at a later stage.

The pig placenta is classified as diffuse forming an epitheliochorial type of placentation with the maternal endometrium. Close apposition and loose attachment of the placenta begins around day 14 of pregnancy in the pig and by day 15 firm attachment takes hold due to interdigitating microvilli between the trophoblast and luminal epithelium (LE) (Senger 2003). The pig placenta is divided into areolae and interareolae areas. Areolae begin forming over the opening of uterine glands around day 15 of pregnancy and take up nutrient dense histotroph secretion or “uterine milk” (Vallet et al. 2009). The interareolae areas of the placenta consist of a maternal LE layer tightly adhered to the fetal trophoblast, which begins to produce primary (villi) folds beginning on day 30-35 of pregnancy. During this period of conceptus elongation and attachment to the uterine surface, the conceptus must relay the maternal recognition of pregnancy (MRP) signal to the maternal system to extend the life of the corpora lutea (CL) and establish pregnancy.

Maternal recognition of pregnancy is defined as the process by which a chemical signal from the developing conceptus results in the establishment of pregnancy by protecting the CL from luteolysis and therefore maintain the

continued production of progesterone (P_4) by the CL beyond the length of a normal estrous cycle (Perry et al. 1976). The pig has both a local and systemic vascular route for transporting prostaglandin F2α ($PGF_{2\alpha}$), the luteolytic hormone, to the ovaries. During the estrous cycle, endometrial release of $PGF_{2\alpha}$ into the uterine vein leaving the uterine horn can diffuse into the ovarian artery via countercurrent exchange and act locally on the ipsilateral ovary as well as through the systemic vascular system to regress the contralateral CL after day 15 (Dhindsa & Dziuk 1968). Estradiol (E_2) has long been proposed as the signal for MRP when conceptuses synthesize and release E_2 during their rapid elongation event, shifting the release of $PGF_{2\alpha}$ away from the uterine vasculature and instead sequestering it into the uterine lumen (Bazer & Thatcher 1977). Once $PGF_{2\alpha}$ enters the uterine lumen, it can be metabolized to its inactive 13,14-dihydro-15-keto prostaglandin $F_{2\alpha}$ metabolite thereby preventing luteolysis. This endocrine/exocrine theory for MRP in the pig requires a minimum of two conceptuses in each horn for enough factor to be produced to shift $PGF_{2\alpha}$ away from the uterine vasculature and into the uterine lumen.

A recent study using CRISPR/Cas9 gene editing to inhibit aromatase gene expression in the conceptus indicated that E2 production is not essential for conceptus elongation or early CL maintenance (Meyer et al. 2019). Therefore, the maternal recognition of pregnancy signal in the pig must involve other conceptus-derived factors. Other known conceptus factors secreted during this time include interleukin 1 beta 2 (IL1B2), prostaglandins (PG), type I interferon delta (IFND) and type II interferon gamma (IFNG) in addition to many others.

These factors are believed to initiate rapid conceptus elongation, signal MRP, provide immune regulation, and support embryonic and placental development. The following review will provide further insight into the roles of these conceptus-derived factors during early development, maternal recognition of pregnancy, immune regulation, and the use of genomic editing to create specific research models.

CHAPTER TWO

2. LITERATURE REVIEW

2.1 The Pig Estrous Cycle

Sexual maturity of the gilt (puberty) occurs between 150 to 220 days of age depending on several factors which include body weight, body condition score and timing of boar exposure (Soede et al. 2011). The sexually mature gilt expresses reoccurring estrous cycles that are between 18 to 24 days (21 days on average) in length. The ability to synchronize the onset of estrus in pigs is possible with effective management and the use of several pharmaceuticals. However, not all synchronization techniques work the same for pre-pubertal, sexually mature, or recently weaned females. One synchronization technique is to provide mature boar (>12 months of age) exposure to prepubertal gilts (between 160 and 180 days of age), mature gilts, and lactating sows immediately after weaning. Boar exposure works to induce estrus for the prepubertal gilts (40% of estrus periods occur within 10-14 days of initial contact) and weaned sows, but it does not have an effect on mature gilts. Additionally, boar exposure paired with P.G. 600® (a combination of equine chorionic gonadotropin and human chorionic gonadotropin) leads to an even higher percentage of prepubertal gilts showing estrus (80% 5-10 days after P.G. 600® injection). Another synchronization technique in pigs is adding Matrix® (orally active synthetic progesterone-like compound) to the feed of mature sows and gilts for 16-18 days to induce estrus (4-9 days after removal on average). Matrix® does

not work in pre-pubertal gilts or recently weaned sows, however, 80-90% of mature gilts exhibit estrus 4-9 days after end of feeding (Flowers 2001).

The estrous cycle of the pig consists of two phases: The follicular phase, which occurs between cycle days 18-3; and the luteal phase, which occurs between cycle days 4-17. During the Follicular phase, pre-antral follicles mature to form large estrogen (E_2) active graafian follicles which leads to ovulation of a cohort of graafian follicles and the formation of corpora lutea (CL). During the Luteal phase, the ovaries now containing fully formed CL produce progesterone (P_4) and follicular development of large graafian follicles is suppressed (Soede *et al.* 2011).

The estrous cycle can be further divided into four specific stages- proestrus, estrus, metestrus and diestrus. Proestrus and estrus make up the follicular phase while metestrus and diestrus make up the luteal phase. During a 21-day estrous cycle, proestrus occurs between days 18-21 and is distinguished by rapidly growing ovarian graafian follicles which begin to increase blood plasma concentrations of E_2 in response to follicle stimulating hormone (FSH) and small pulses of luteinizing hormone (LH) from the anterior pituitary. Estrus occurs between days 0-3 of the estrous cycle which is distinguished by a period of sexual receptivity where the female displays behavioral estrus (standing heat) in response to peak secretion concentrations of E_2 and the LH surge. In addition to this visual behavioral change, vulvar changes such as increased redness, swelling and mucus production are also observed (Soede *et al.* 2011). Peak

concentrations of E_2 paired with low plasma P_4 levels allows for a positive feedback loop of E_2 on the hypothalamus to stimulate the release of multiple pulses of gonadotropin releasing hormone (GnRH) that act on the anterior pituitary to stimulate a surge release of LH. The surge (sustained release of which builds on itself) of LH stimulates pathways in the mature graafian follicles to breakdown the follicle wall and rupture, releasing the oocyte (ovulation) approximately 24-36 hours after the onset of estrus (Perry & Rowlands 1962). Following ovulation, estrus is terminated and metestrus occurs next between days 3-4 of the estrous cycle. At this time concentrations of E_2 and LH have all declined and the female is no longer exhibiting standing heat. The ruptured follicle's thecal and granulosa cells within the wall of the follicle form a corpus hemorrhagicum leading to luteinization and begins synthesizing and secreting P_4. The formation of fully functional CL leads into the longest stage of the estrous cycle, diestrus, which is considered to be days 5-18 of the estrous cycle. Plasma P_4 concentrations continue to increase until reaching a peak around days 12-14 of the estrous cycle. The sustained high concentrations of P_4 stimulate a negative feedback loop on the hypothalamus to decrease the GnRH pulses acting on the anterior pituitary and lowering FSH and LH to the basal levels necessary for CL maintenance. If females are bred, conceptuses must provide the MRP signal during this stage. During the estrous cycle, luteolytic pulses of prostaglandin F2 alpha ($PGF_{2\alpha}$) are released from the uterine endometrium after day 15 which induce CL regression and decrease the concentration of P_4. Following luteolysis, proestrus is reinitiated, prompting a new wave of ovarian

follicles to mature and ovulate to restart another 21-day estrous cycle (Hines 2018).

2.2 Early Pregnancy in the Pig

The pig is described as a polytocous species that ovulates anywhere from 15 to 30 oocytes on average in a single cycle. (Soede *et al.* 2011). The oocytes are surrounded by sticky cumulus cells to form a large cumulus-oocyte complex (COC) which is caught by the cilia of the fimbria cells within the infundibulum. Fertilization occurs at the ampulla-isthmic junction and within the first 24-36 hours following fertilization, the zygote undergoes its first and second cleavage cell divisions. The 4-8 cell embryos are able to rapidly travel down the isthmus toward the uterus when E_2 concentrations decrease and production of P_4 increases from the developing CL. The drop in E_2 and increase in P_4 relaxes the isthmus muscularis and stimulates muscle contractions toward the uterotubal junction and facilitate passage of the embryo into the tips of the uterine horns. If exogenous E_2 is administered before embryo transport in the oviduct, the embryos become restricted (tube locked) in the isthmus and do not pass through to the uterus (Dziuk 1985).

By day 4 post-fertilization, the uterine environment is receptive to the developing embryos which enter the uterus and remain near the tip of the uterine horn until day 6. The embryos continue to divide whereupon reaching the 32-cell stage, they transform into a morula stage embryo which contains inside and outside cells. The cells undergo compaction to form tight junctions between each other. According to cell polarity and inside-outside theories, any nonpolar and

centrally located cells will become the inner cell mass (ICM) while the outside, polar cells will become the trophoblast (Yamanaka et al. 2006). The tight junctions form a seal between the trophoblastic cells that allows fluid to follow Na^+ through the Na^+/K^+ ATPase pumps into the center of the morula to form a blastocoel cavity (Senger 2003). The zona pellucida still surrounds the blastocyst during this time until day 6-7 when endometrial and trophoblastic enzymes weaken the zona paired with the pressure from growth and expansion of the blastocoel allows it to hatch from the zona pellucida. The hatched blastocysts then begin to migrate away from the tip of the horn and toward the body of the uterus. By day 9, some blastocysts have migrated into the horn opposite of their origin while other blastocysts may only migrate within their horn of origin (Dziuk 1985). All blastocysts will continue to migrate until day 12. Uterine migration is necessary in the pig to allow for adequate placental-uterine surface area for nutrient exchange as well as establishing pregnancy. The majority of the uterus must be occupied by a minimum of four conceptuses (two in each horn) in order for pregnancy to maintain beyond day 12 (Polge et al. 1966). The speed of migration may be affected by secretions from the embryos acting on the uterus (Dziuk 1985). One study demonstrated that polysiloxane beads saturated with estradiol migrated more efficiently through the uterine horns than the same beads containing cholesterol suggesting that conceptus-derived estrogens aid in spacing and migration (Pope et al. 1982).

2.3 Conceptus Elongation

During the peri-implantation period (days 10-12), porcine conceptuses undergo dramatic morphological changes. An event first elucidated by Heuser and Streeter (1929), the spherical conceptus grows in diameter from 1-3 mm to 9-10 mm via cell proliferation turning ovoid in shape. Once the ovoid conceptus becomes tubular in morphology (12-30 mm), it rapidly elongates to a filamentous form at a rate of more than 40 mm/hour reaching over 100 mm in length in approximately one hour (Anderson 1978; Geisert et al. 1982a; Geisert *et al.* 1982b; Geisert et al. 2015). It was suggested by Perry et al. (1981) that in order for pig conceptuses to achieve this unique, rapid elongation, the conceptuses must undergo rapid cellular remodeling and cellular migration. Results from Geisert et al. (1982b) provided evidence for this claim. In the study, they utilized scanning electron microscopy (SEM) to look at elongating blastocysts and discovered that cellular remodeling was occurring during the transition from spherical to filamentous blastocysts. They further examined Feulgen-stained conceptuses which revealed changes in cellular organization during elongation. Cell migration results in the formation of an elongation zone where extra-embryonic endoderm (En) cells and trophectoderm (Tr) cells migrate towards the ends of the blastocyst further reducing the diameter and increasing the conceptus length. The same group also demonstrated that the mitotic index of blastocysts at the onset of elongation (10-20 mm tubular blastocysts) was reduced by nearly 40%. This process for elongation allows each conceptus to attach and establish its territory within the uterus as it becomes very competitive,

and then begin to develop a true epitheliochorial placenta at a later stage. Additionally, SEM showed that during the transition from spherical to tubular and filamentous conceptuses, the trophectodermal cells flatten and the number of microvilli on the cell surface increases. In conclusion, pig conceptus elongation appears to primarily be a result of both surface and ultrastructural changes to the trophectoderm and endoderm cells and not solely cellular hyperplasia (Anderson 1978; Perry 1981; Geisert *et al.* 1982b; Geisert *et al.* 2015). After day 12, migration halts and the elongated conceptuses begin to adhere to the uterine luminal epithelium (LE). The uterus also lengthens throughout the first 18 days of pregnancy to provide adequate space for the conceptuses and this growth occurs most rapidly between days 2-6 when a 50% increase in horn length is observed (Perry & Rowlands 1962).

2.3.1 IL1B2 Role in Elongation

Transcriptome analysis identified interleukin 1 beta (IL1B) to be one of the most abundantly expressed genes during the peri-implantation period (Days 10-12) of pregnancy when conceptuses elongate from spherical to filamentous morphology (Tuo et al. 1996; Ross et al. 2003; Ross et al. 2009). IL1B is a pro-inflammatory cytokine which has been proposed to play a role in the inflammatory response associated with a variety of cellular activities such as cell proliferation and apoptosis. Additionally, IL1B has the ability to increase the expression of cell adhesion molecules (Dinarello 2009). *IL1B* gene expression precipitously increases on day 12 of pregnancy during the period when pig conceptuses initiate their unique, rapid elongation and declines by more than

2,000-fold immediately following elongation (Ross *et al.* 2003). Absence of the IL1B protein in the uterine lumen during the estrous cycle compared to high levels of protein detected during conceptus elongation suggests that IL1B in the lumen of pregnant pigs originates from the elongating conceptuses (Ross *et al.* 2003). Peri-implantation expression of IL1B has been reported to play an important role in early pregnancy of other species as well. There is an increase in IL1B expression prior to blastocyst implantation in mice (Takacs & Kauma 1996), and IL1B is also suggested as the initiator of crosstalk at the maternal-fetal interface during pregnancy in humans (Lindhard et al. 2002).

The pig conceptus is unique in that it expresses an IL1B isoform which has not been detected in other mammals (Mathew et al. 2015). Interleukin 1 beta 2 (IL1B2) is 85% identical to IL1B at the protein level and has been suggested as the factor responsible to initiate cell signaling pathways for rapid conceptus remodeling during elongation in the pig. IL1B induces the expression of phospholipase A2 (PLA2) (Kol et al. 2002), which is proposed to regulate the release of arachidonic acid from the phospholipid bilayer, allowing membrane fluidity necessary for rapid remodeling of the trophectoderm during elongation (Ross *et al.* 2003). In a study done by Whyte et al. (2018), CRISPR/Cas9 gene editing technology was used to successfully create a loss of function deletion in the *IL1B2* gene and demonstrated that conceptus elongation in the pig does not occur in the absence of *IL1B2* conceptus expression. The IL1B2 null conceptuses were able to develop to the spherical blastocyst stage but failed to elongate and developed an abnormal morphology. In the same study, they

observed that conceptus production of IL1B2 regulates conceptus E_2 synthesis, which has a potential role in regulating the proinflammatory response of the endometrium during conceptus elongation and placental attachment to the uterine luminal surface (Whyte *et al.* 2018).

In addition to being essential for proper conceptus elongation in the pig, expression of IL1B is also proposed to play a role in implantation and establishment of pregnancy in cattle, mice, women, and non-human primates (De los Santos et al. 1996; Strakova et al. 2005; Bourdiec et al. 2014; Correia-Alvarez et al. 2015). As a member of the interleukin 1 (IL1) cytokine family, IL1B2 serves an important role in preparing the uterine endometrium to become receptive to the attaching conceptus by activating expression of a variety of genes. The IL1 system consists of converting enzymes, two receptors: IL1RT1 (functional) and IL1RT2 (pseudo receptor), receptor accessory proteins, and isoforms of receptor agonists (Mantovani et al. 1998; Ross *et al.* 2003).

As a cytokine, IL1B stimulates several proinflammatory and immune response pathways (Dinarello 2009). During elongation, IL1B2 induces an increase of salivary lipocalin *SAL1* expression in the glandular epithelium as well as SAL1 protein secreted from the uterine glands (Seo et al. 2010). SAL1 is a transporter of hydrophobic molecules and could potentially play a role in binding to lipids and prostaglandins during placental attachment and establishment of pregnancy (Seo et al. 2008). SAL1 can be found in the uterine lumen during day 12 of the estrous cycle but the concentrations are more abundant in the uterine lumen on day 12 of pregnancy coinciding with the time of peak IL1B2 production

by the conceptus (Ka et al. 2009). A critical pro-inflammatory pathway that is stimulated by IL1B2 is nuclear factor kappa B (NFKB) (Mathew *et al.* 2015). Endometrial tissue treated with IL1B is shown to activate the NFKB pathway in epithelial cells localized to the uterine LE directly in contact with the conceptus (Mathew *et al.* 2015). NFKB, however, is tightly regulated as it is known to stimulate transcription factors for cell survival, proliferation, cell adhesion, and inflammation (Hayden & Ghosh 2012). Type I interferons (IFN) have also been reported to activate the NFKB signaling pathway which then regulates the cellular response to interferons (Pfeffer 2011). Alongside the secretion of IL1B2 from the conceptus is a mirrored secretion of conceptus E_2 which is believed to be a possible mediator of the NFKB pathway therefore regulating the proinflammatory response (Geisert *et al.* 2015). Additionally, NFKB stimulates an increase in the expression of prostaglandin synthase 2 (PTGS2) (Ali & Mann 2004).

In addition, by the third week of pregnancy, the fetal-maternal interface is greatly increased in area by formation of primary and secondary folds (Perry 1981). Due to the diffuse nature of true-epitheliochorial placentas, it is essential for each conceptus to acquire a large enough uterine surface area for proper growth, development and survival of the embryo throughout pregnancy. The roles of E_2, PG, and IFN in early pregnancy of the pig will be reviewed in more depth later.

2.4 Placentation

Vertebrate species can be separated into three reproductive categories: viviparous, oviparous and ovoviviparous. Viviparous animals (pigs, humans, etc.)

give birth to live young while oviparous animals (birds, turtles, etc.) lay eggs and ovoviviparous animals (salamanders, rays, etc.) produce living young from eggs that hatched within the body. During pregnancy, viviparous vertebrates develop a complex system of membranes around the fetus for long term gas and nutrient exchange during gestation called the placenta. Mossman (1937) defines placenta as “an apposition or fusion of the fetal membranes to the uterine mucosa for physiological change”. There are a number of different types of placentas which can be categorized together based on similar characteristics. The pig placenta is classified as diffuse forming an epitheliochorial type of placentation with the maternal endometrium. Although placentation is noninvasive in the pig, conceptuses are actually very invasive. Pig conceptuses that are transferred outside of their *in vivo* environment have proven to be very proteolytic and highly invasive, however, *in utero* there are a number of endometrial protease inhibitors which limit pig conceptuses to their superficial epitheliochorial, diffuse type of placentation (Samuel & Perry 1972; Geisert et al. 2014). The placental membranes begin to form following rapid elongation and after embryo migration has ceased. The extraembryonic placental membranes that form following attachment to the endometrium consist of the yolk sac, chorion, allantois and amnion. The elongated conceptus consists of an embryonic disc (containing endoderm, mesoderm and ectoderm cells) and the trophectoderm. The endoderm from the embryonic disk begins to grow downward against the basal surface of the trophoblast, forming a cavity. This cavity is called the yolk sac and it is the first functional placental membrane to develop (Senger 2003). The yolk

sac serves to provide nutrients to the developing embryo while the rest of the placenta continues to form. The mesoderm begins to grow around the yolk sac as it sinks down and pushes up the mesoderm to form amniotic folds. The mesoderm and the trophectoderm will begin to combine and form the chorion while the amniotic folds continue to grow outward to form and seal the amniotic sac (Senger 2003). The amniotic sac fills with a viscous yellowish-transparent fluid suspending and protecting the embryo (Bertassoli et al. 2015). Then, the allantois begins to form the primitive gut within the embryo replacing the yolk sac which rapidly decreases in size between days 14-20 of pregnancy (Patten & Carlson 1974; Vallet *et al.* 2009). The allantois continues to expand outward toward the chorion and where they meet, they fuse together to form the chorioallantois. The chorioallantois fills with a watery, amber-transparent fluid with peak accumulation occurring around day 30 of pregnancy, a decline in fluid until day 45, and another peak of fluid volume at day 60 then recedes to a minimal volume for the remainder of gestation (Vallet *et al.* 2009; Bertassoli *et al.* 2015). The chorioallantois becomes vascularized and functions for hematotrophic nutrient exchange after day 16 of pregnancy in addition to histotrophic nutrient transport (Geisert *et al.* 2015). The portion of the chorion that the allantois expansion does not reach will not vascularize and is termed the “necrotic tips” (Friess et al. 1980).

Close apposition and loose attachment of the placenta begins around day 14 of pregnancy in the pig and by day 15 firm attachment takes hold due to interdigitating microvilli between the trophoblast and LE (Senger 2003). The pig

placenta is divided into areolae and interareolae areas. Areolae begin forming at the opening of uterine glands around day 15 of pregnancy and take up nutrient dense histotroph secretion or “uterine milk” (Vallet *et al.* 2009). The interareolae areas of the placenta consist of a maternal LE layer tightly adhered to the fetal trophoblast, which begins to produce primary (villi) folds beginning day 30-35 of pregnancy. The folds continue to grow and become more elaborate as pregnancy advances. At mid gestation the primary folds are relatively close, then, by late gestation the fetal placental stroma has grown in between the primary folds and secondary (microvilli) folds begin to develop in this space therefore increasing the interactive surface area of the placenta (Vallet et al. 2014). This close apposition of the fetal trophoblast and maternal epithelium is crucial for proper growth and survival of the pig conceptus due to the nature of their true epitheliochorial, diffuse type placenta. In addition to proper formation of the placenta, the developing conceptus must provide a signal to the maternal system to prolong the life of the CL and establish pregnancy.

2.5 Maternal Recognition of Pregnancy

Maternal recognition of pregnancy is defined as the process by which a chemical signal from the developing conceptus results in the establishment of pregnancy by protecting the CL from luteolysis and therefore the continued production of P_4 by the CL beyond the length of a normal estrous cycle (Perry *et al.* 1976). As previously discussed, the luteolytic hormone in most mammalian species, including pigs, is $PGF_{2\alpha}$ which is secreted in pulses by the luminal and glandular epithelium of the uterus and carried by the uterine vasculature to the

ovaries to regress the CL after day 15 of the estrous cycle (Moeljono et al. 1976; Moeljono MP 1977). The pig has both a local and systemic vascular route for transporting $PGF_{2\alpha}$ to the ovaries. During the estrous cycle, endometrial release of $PGF_{2\alpha}$ into the uterine vein leaving the uterine horn can diffuse into the ovarian artery via countercurrent exchange and act locally on the ipsilateral ovary to regress the CL (Dhindsa & Dziuk 1968). Systemically, the portion of $PGF_{2\alpha}$ secreted into the uterine vein that does not diffuse and act locally will pass through the lungs where only 40% of the hormone will be metabolized to 13,14-dihydro-15-keto prostaglandin $F_{2\alpha}$ in the pig leaving enough active hormone to continue travelling through the blood to act on the contralateral ovary. The metabolism in the pig is low compared to cattle where 90% of $PGF_{2\alpha}$ is metabolized by the lungs to 13,14-dihydro-15-keto prostaglandin $F_{2\alpha}$ therefore inactivating most luteolytic activity in a single pass and consequently preventing luteolysis occurring from a systemic route (Del Campo & Ginther 1973; Ginther 1981).

In the pregnant pig, the endometrium continues to produce and secrete similar concentrations of $PGF_{2\alpha}$ during the estrous cycle, however, it is sequestered away from the uterine vasculature toward the uterine lumen (Bazer et al. 1984). The endocrine/exocrine hypothesis has previously been proposed by Bazer et al. (1982) as the mechanism for maternal recognition of pregnancy in the pig. The hypothesis suggests that the life of the CL are prolonged when the endometrial secretion of $PGF_{2\alpha}$ changes from an endocrine fashion (into the uterine vasculature) during the estrous cycle to an exocrine fashion (sequestered

into the uterine lumen) of a pregnant pig at day 15, the time of maternal recognition of pregnancy (Bazer 1982). By moving the $PGF_{2\alpha}$ into the lumen, it can be metabolized by the endometrium and conceptuses therefore preventing luteolysis and allowing the CL to continue producing P_4 (Spencer & Bazer 2002). In order for $PGF_{2\alpha}$ to be sequestered and pregnancy to be established, a minimum of two elongated conceptuses must be present in each horn after day 12 of pregnancy (Dhindsa & Dziuk 1968).

2.5.1 Estrogen Role in Maternal Recognition of Pregnancy

Estrogen synthesis and secretion by the developing conceptuses during rapid elongation and early attachment coincides with the timing of $PGF_{2\alpha}$ being sequestered into the uterine lumen and away from the uterine vasculature (Bazer & Thatcher 1977). Earlier research using a nonsteroidal, synthetic estrogen (diethylstilbestrol) documented that when administered on day 11 of the estrous cycle, CL life was extended to day 25 (Kidder et al. 1955) which was later confirmed by Gardner et al. (1963) when they administered estradiol on day 11 of the estrous cycle and also reported CL life to be extended to day 25. This prompted other researchers to begin looking at estrogen as a possible signal for maternal recognition of pregnancy in the pig. A study completed by Perry et al. (1973) established that blastocysts are capable of producing steroid hormones such as E_2 and P_4. Pig conceptuses were also found to synthesize and secrete estrogen in two biphasic peaks: the first occurring during the period of rapid elongation (days 11-12) and the second during the attachment period (days 15-18) (Zavy et al. 1980; Geisert et al. 1982c). This was evaluated even further by

Geisert et al. (1987) when they administered E_2 on either day 11 or days 14-16 of the estrous cycle and found that pseudopregnancy was maintained to day 25; however, when E_2 was administered on both day 11 and days 14-16 of the estrous cycle, pseudopregnancy was extended beyond day 60. This study demonstrated the importance of the conceptus derived biphasic peaks of E_2 on days 11 and 14-16. However, E_2 can have a negative effect if pregnant pigs are treated with E_2 on days 9 and 10 of pregnancy, 48 hours earlier than normal conceptus synthesis of E_2. Premature exposure of pregnant gilts to E_2 does not affect conceptus elongation on day 12, but the developing conceptuses undergo fragmentation and early embryonic loss by day 18 of pregnancy (Morgan et al. 1987). To directly address the role of conceptus estrogen production in the establishment of pregnancy, Meyer et al. (2019) used CRISPR/Cas9 gene editing technology to inhibit conceptus estrogen synthesis by creating a loss of function biallelic edit in the aromatase (*CYP19A1*) gene of porcine fetal fibroblast cells and then through somatic cell nuclear transfer (SCNT) created viable, cloned embryos. These *CYP19A1*$^{-/-}$ embryos developed to the blastocyst stage *in vitro* and then were surgically transferred in recipient gilts where they continued to develop *in vivo* until they were collected on day 14. *CYP19A1*$^{-/-}$ conceptuses elongated on day 14 and CL were still present after day 15 despite the lack of conceptus estrogen production. However, *CYP19A1*$^{-/-}$ conceptuses did not survive beyond 30 days. Although treatment of *CYP19A1*$^{-/-}$ pregnancies with exogenous estradiol could rescue the CL beyond 30 days of gestation, the *CYP19A1*$^{-/-}$ embryos were still lost at day 30. To determine if the *CYP19A1*$^{-/-}$ edit

was embryonic lethal, they co-transferred *CYP19A1*$^{+/+}$ in vitro fertilized (IVF) embryos with *CYP19A1*$^{-/-}$ cloned embryos. Pregnancy was maintained and viable *CYP19A1*$^{-/-}$ embryos were collected with *CYP19A1*$^{+/+}$ embryos capable of synthesizing and secreting conceptus estrogen during early pregnancy (Meyer *et al.* 2019). This study indicated that conceptus derived estrogen may not be essential for early conceptus development and establishing pregnancy, but is essential for pregnancy maintenance beyond day 30. While E_2 cannot be ruled out entirely as a signal for maternal recognition of pregnancy, it is likely not the only signal. Further studies are needed to gain more understanding of this system in pigs.

2.5.2 Prostaglandin Role in Maternal Recognition of Pregnancy

An alternate theory for maternal recognition of pregnancy in the pig suggests that endometrial and/or conceptus-derived prostaglandin E (PGE) may assist in establishing pregnancy by acting as a luteoprotective agent. Constant intrauterine infusion of prostaglandin E2 (PGE_2) from days 7-23 of the estrous cycle were proven to extend the life of the CL (Akinlosotu et al. 1986). Another study looking at the effect of PGE_2 preventing luteolysis in the CL of the pig conducted by Ford and Christenson (1991) used silastic beads containing either (i) estradiol-17β, (ii) PGE_2, (iii) $PGF_{2\alpha}$, (iv) estradiol-17β + $PGF_{2\alpha}$, (v) PGE_2 + $PGF_{2\alpha}$ or control silastic beads. These beads were inserted accordingly into individual CL of cyclic gilts and the beads containing $PGF_{2\alpha}$ alone stimulated regression of the CL by day 19. The CL with the beads containing estradiol-17β + $PGF_{2\alpha}$, were not significantly different from the control silastic beads and also

exhibited luteolysis. However, CL containing silastic beads with PGE_2 + $PGF_{2\alpha}$ successfully prevented luteolysis, were heavier in weight and produced more P_4 on day 19 compared to controls (Ford & Christenson 1991). Production of prostaglandins increases the ratio of $PGE_2/PGF_{2\alpha}$ found in the uterine vasculature and lumen during the period of maternal recognition of pregnancy suggesting that the increase of PGE_2 occurs to help protect the CL from $PGF_{2\alpha}$ and by extension, luteolysis (Waclawik et al. 2009). A study completed by Pfeiffer et al. (2019) used CRISPR/Cas9 gene editing technology to inhibit conceptus prostaglandin synthase 2 (PTGS2) synthesis by creating a loss of function biallelic edit in the *PTGS2* gene in porcine fetal-derived fibroblast cells and then through SCNT created viable, cloned embryos. These *PTGS2*$^{-/-}$ embryos developed to the blastocyst stage *in vitro* and then were surgically transferred into recipient gilts where they continued to develop *in vivo* until they were collected. Pig conceptuses also express *PTGS1*, however, expression is not detected until loose trophoblast attachment on day 13 of pregnancy (Waclawik & Ziecik 2007). Despite the decreased PG synthesis, *PTGS2*$^{-/-}$ conceptuses were able to elongate, establish pregnancy, and maintain pregnancy to day 35 of gestation. Although *PTGS2*$^{-/-}$ PG production was significantly decreased on day 14 and 17 of pregnancy, uterine lumen flush (ULF) content of PG was not affected (Pfeiffer et al. 2019). ULF content of PG was expected to be similar between control and edited conceptus pregnancies as the maternal endometrium is the major contributor of PG into the uterine lumen. Additionally, conceptus estrogen stimulates endometrial prostaglandin production (PGE) and therefore is

able to act as MRP in the absence of conceptus PG. While the *PTGS2*$^{-/-}$ model was able to establish and maintain pregnancy to day 35, the question remains as to whether PGE can serve as the signal for maternal recognition of pregnancy or possibly another signal altogether.

2.6 Conceptus Synthesis and Secretion of Interferons

While interferon research began over sixty years ago, it wasn't until the late 1980's that interferons were implicated in the establishment and maintenance of pregnancy. Since then, there has been considerable literature published on the role of conceptus-derived interferons in sheep, cattle, and the pig. The early pig conceptuses produce large quantities of interferons but their role in early pregnancy has not yet been clearly elucidated. In order to fully appreciate the significance of interferons and their importance in early pregnancy of the pig, one must understand interferon's history, pathways and biological roles as we understand it at this time.

2.6.1 A History of Interferons

Interferons were first discovered by a British bacteriologist Alick Isaacs and Swiss microbiologist Jean Lindenmann in 1957 (Isaacs et al. 1957). Isaacs and Lindenmann found that incubation of the heat-inactivated virus within the chick chorio-allantoic membrane stimulated release of a new factor that was coined "interferon" for its ability to induce interference in the multiplication of new active virus particles. The interference phenomena was first described in plants, followed by bacteria, then animals and humans (Henle 1950) before interferons were first identified in 1957. In 1965, Wheelock described a functionally related

protein produced by mitogen activated human T-lymphocytes that is now called interferon gamma (IFNG), the only type II interferon. Following their discovery, interferons were hypothesized to be a very useful antiviral drug. However, attempts to purify and molecularly define IFN proteins were unsuccessful for nearly 20 years causing many scientists to be skeptical of not only their use but their existence altogether. Nevertheless, two different groups of researchers were able to successfully clone interferon complementary DNA (cDNA) and identify interferon genes which propelled interferon research back into popularity (Taniguchi et al. 1979; Nagata et al. 1980).

Initially, interferons were classified by the secreting cell type but are now classified into type I and type II according to their structure, sequence homology and receptor specificity. There are numerous type I interferons, many of which are also composed of multiple subtypes, which all bind to the same heterodimeric receptor. As summarized in a review by Pestka and colleagues in 2004, type I interferons consist of IFN-alpha (IFNA), IFN-beta (IFNB), IFN-epsilon (IFNE), IFN-kappa (IFNK), IFN-omega (IFNW), IFN-tau (IFNT), and IFN-delta (IFND). Type I interferons were recognized early to have many pleiotropic activities in addition to their ability to inhibit virus replication including the enhancement of the lytic action of cytotoxic T cells for tumor target cells and enhancement of major histocompatibility complex (MHC) antigen expression (Lindahl et al. 1972; Lindahl et al. 1976). Interferon gamma (IFNG), which has its own unique amino acid sequence, is the only type II interferon and binds to its own specific receptor. Interferon gamma, like type I interferons, functions primarily as an

immunomodulatory cytokine but was also shown to have pleiotropic activities. It took several years to prove interferons had activities beyond that of a selective antiviral protein and ultimately that breakthrough didn't come until their receptors had been defined and their signal transduction pathways were elucidated. The two interferon receptors and their downstream pathways will be reviewed more in depth in the following sections.

During the first decade of IFN research, the general hypothesis for their antiviral activity was thought to be a result of the interferon protein entering the cell and possibly undergoing some metabolic changes to inhibit the virus replication itself. Indirect evidence for cell surface binding of the IFN molecule was first reported in the late 1960's (Friedman 1967). Binding studies with radiolabeled interferon proteins were completed several years later that indicated there are specific, high-affinity cell surface receptors for interferons and that different subspecies of type I interferons share the same receptor whereas type II interferon gamma binds to a second and distinctly different receptor (Branca & Baglioni 1981; Aguet et al. 1982). Further research supported the finding that all type I interferons bind to the same dimeric receptor, although type I interferon subspecies can elicit different biological responses (van Boxel-Dezaire et al. 2006). Two type I interferons, IFND and IFNT, are not predominantly regulated by responding to viral infections but rather are expressed constitutively by the developing conceptus of pigs and ruminants respectively.

Early research indicated that IFN were believed to be a highly selective antiviral agent devoid of any actions in uninfected cells and assumed to be

completely nontoxic to the rest of the body. This promoted the belief that IFN's could serve as therapeutic agents for the treatment of viral infections and cancers in the 1970's. By the mid 1970's, a study showed that injection of large doses of type I mouse interferon to newborn mice caused growth retardation, and eventually, death due to steatosis and necrosis of the liver. In addition, mice given sublethal doses developed glomerulonephritis later in life (Gresser et al. 1975). Endogenous interferon produced by the body during the course of an infection was also found to be harmful as demonstrated when studying lymphocytic choriomeningitis virus infection in newborn mice (Rivière et al. 1977). Most of the infected pups died within 2-3 weeks and those that survived developed glomerulonephritis later in life. However, infected pups treated with a neutralizing antibody to type I mouse IFN both survived and had a lower incidence of glomerulonephritis later in life. Gradually, more data became available further suggesting that interferons produced to prevent virus replication can also potentially cause morbidity. Early clinical studies administering natural or recombinant IFNA or IFNB to patients produced fever, fatigue, anemia and other "flu-like" symptoms indicating that the symptoms seen in many common acute virus infections are due in part to endogenous interferon production (Vilcek 1984). Additionally, interferon activity was found in the sera of patients suffering from various autoimmune diseases suggesting a pathogenetic effect rather than a protective one (Hooks 1979).

As interferon research continued, a new family of transcription factors termed interferon regulatory factors (IRF) were discovered as a mechanism for

interferon regulation by binding to the upstream regulatory region of the interferon gene and mediating virus-induced transcription (Miyamoto et al. 1988). Two members of this family, IRF1 and IRF2, were found to be important in a variety of innate and adaptive immune responses including T helper 1 responses and natural killer (NK) cell differentiation (Taniguchi et al. 2001). Additionally, IRF3 and IRF7 were found to play vital roles in type I interferon gene activation (Juang 1998; Sato et al. 1998). Another investigation demonstrated that the binding of interferons to their specific receptors initiated the synthesis of several unique polypeptides and their corresponding mRNA (Larner et al. 1984). Today, hundreds of these IFN stimulated genes (ISG) have been identified, the products of which exert numerous antiviral functions, many of which still haven't been fully described. Collectively, ISG can target and inhibit nearly any stage of the virus life cycle while some even go so far as to further induce IFN or ISG (Schoggins & Rice 2011). Additionally, as reviewed by Lee & Ashkar in 2018, type I IFN in the innate immune response are secreted in response to a viral infection and through autocrine and paracrine signaling place the surrounding cells into an antiviral state. The continued production of type I IFNB also increases the production of monocyte chemoattractant protein-1 (MCP-1/CCL2) between days 1 and 2 post infection which results in an inflammatory monocyte recruitment and has been implicated in NK cell recruitment. The recruited inflammatory monocytes result in the release of IL18 which stimulates NK cells to produce IFNG around 48 hours post infection (Lee & Ashkar 2018). However, much still remains to be known about conceptus IFN, IRF, ISG and their downstream applications.

2.6.2 Conceptus Interferons in Ruminant Species

It wasn't until nearly 25 years after the discovery of interferons that conceptus interferons were first reported to be secreted during early pregnancy. Unlike leucocytic interferons, conceptus interferons are not produced in response to pathogens or antigens, but rather, their induction is developmentally programmed. In the beginning, interferon tau (IFNT) was given a number of names including trophoblastin, protein-X and ovine trophoblast protein-1 (Martal et al. 1979; Godkin et al. 1982; Godkin et al. 1984). Martal and colleagues (1979) first reported IFNT in the ewe by injecting homogenates from trophoblasts between days 14-16 of pregnancy into the uterus of cyclic ewes which extended the life of the CL for more than one month. However, homogenates from embryos and their membranes between days 21-23 of pregnancy did not extend the life of the CL suggesting that INFT synthesis only occurs for a short period of time. Additionally, surgical removal of embryos at 21-23 days also resulted in luteal maintenance for more than one month in 50% of the females. In females that maintained their CL, the CL weight was 50% smaller than females containing conceptuses suggesting the presence of other luteotrophic factors being secreted during pregnancy (Martal *et al.* 1979). In another early study of IFNT, sheep conceptuses (day 13-21) were cultured *in vitro* with radiolabelled amino acids to determine and track specific protein synthesis and release. Analysis of polypeptides present in the medium were carried out by two-dimensional PAGE and fluorography of the dried gels which revealed to investigators that IFNT had a relatively small molecular weight (estimated to be approximately 17 kDa)

(Godkin *et al.* 1982). The same group successfully purified IFNT by culturing day 14-16 conceptuses *in vitro* for 24 hours. Immunocytochemistry determined that IFNT was localized to trophectoderm cells of the elongated conceptus as well as the luminal and upper glandular epithelium of the maternal endometrium (Godkin *et al.* 1984). IFNT is believed to have arisen from a duplication event of the IFNW gene approximately 36 million years ago. The IFNT genes have continued to duplicate since that time and acquired the ability to be transcribed uniquely in the trophectoderm (Roberts et al. 2003). It was the conclusion of each of these studies that IFNT function is not immune related, but rather it serves as the signal for maternal recognition of pregnancy in sheep.

Unlike the rapid conceptus elongation phenomenon seen in pigs, sheep conceptus elongation occurs over several days (11-16) and it involves the proliferation of mononuclear trophectoderm cells of the conceptus which synthesize and secrete IFNT during the peri-implantation period (Roberts et al. 1999). Increasing levels of IFNT coincide with increasing proliferation of mononuclear trophectoderm cells; as the cell number multiplies so do the levels of IFNT. IFNT acts in a paracrine manner on the uterine endometrium to inhibit the development of the luteolytic mechanism. IFNT does this in sheep by inhibiting transcription of the estrogen receptor alpha (ESR1) gene directly and the oxytocin receptor (OXTR) gene indirectly in the LE and GE (Spencer & Bazer 1996). In that study, they analyzed endometrial total RNA to indicate the amount of endometrial ESR1 and OXTR mRNA in pregnant ewes and found that it was approximately two-fold lower ($P<0.05$) than levels found in cyclic ewes on day 15.

They also treated non-pregnant ewes with intrauterine injections of recombinant ovine IFNT (roIFNT) on days 11 – 14 and found that their endometrial ESR1 and OXTR mRNA levels on day 15 were also two-fold lower ($P<0.05$) than the cyclic control ewes. The authors indicated that the anti-luteolytic mechanism of IFNT in sheep is to suppress transcription of the ESR1 gene which prevents estrogen associated increases of the OXTR in the endometrial epithelium. This sequence of events initiated by sheep conceptus IFNT inhibits production of luteolytic pulses of $PGF_{2\alpha}$, thereby continuing the lifespan of the CL to continue producing P_4 to maintain pregnancy (Spencer & Bazer 1996).

Studies in other ruminant species indicated that IFNT serves as the MRP signal not only in sheep, but in cattle and goats as well. In cattle, elongation occurs between days 14-19 via proliferation of the mononuclear trophectoderm cells which synthesize and secrete IFNT, similarly to sheep (Thatcher et al. 1989). However, in contrast to sheep, IFNT in cattle inhibits transcription of the *OXTR* without causing any change in transcription of *ESR1* gene in the uterine endometrium (Mann et al. 1999; Robinson et al. 1999). Therefore, the mechanism by which IFNT acts to inhibit the OXTR development remains to be determined. Even less is understood about IFNT function in goats. Goat IFNT was detected in trophoblast cells as early as day 14 of pregnancy but no later than day 17 confirming that goat conceptuses secrete IFNT during the period of MRP. However, this rapid decrease suggest that other factors are likely needed to be present by day 18 to take over its role in maintenance of luteal function (Guillomot et al. 1998).

2.6.3 Conceptus Interferons in the Pig

In the pig, substantial antiviral activity has been detected during the peri-implantation period. Further analysis revealed pig conceptus trophectoderm cells, the external monolayer of the trophoblast, synthesize and secrete type I IFND and type II IFNG beginning on day 12 of pregnancy (La Bonnardiere et al. 1991). The predominant interferon responsible for the antiviral activity detected in early pregnancy of the pig is type II IFNG which is secreted in large amounts upwards of 250 µg per uterine horn (La Bonnardière 1993). The possible roles of conceptus-derived interferons in the pig are currently only speculative. Pig conceptus interferons are not believed to have an antiluteolytic effect but rather paracrine effects which are suggested by the localization of their receptors on the maternal endometrium to regulate several ISG that aid in the complex communication between the maternal and fetal system (Johnson et al. 2009). In one study, pig conceptus secretory proteins obtained from medium by culturing day 15 conceptuses for 30 hours were pooled and concentrated for intrauterine infusion and the results showed that these proteins had no antiluteolytic effect but they do, however, stimulate the secretion of PGE2 by uterine cells which is thought to enhance the structural integrity of the CL (Harney & Bazer 1989). A study by D'Andrea et al. (1994) observed the local effects of conceptus IFN on endometrial cells and on the trophoblast by measuring antiviral activity and the induction of (2',5') oligoadenylate synthetase (OAS) activity. Their results showed no (2',5') OAS activity on the trophoblast, even after IFN treatment, however, endometrial epithelial and stromal cells in primary cultures were sensitive to IFN

activity. Therefore, they reported that there is no believed autocrine activity of conceptus IFN in the pig trophoblast as it did not appear to possess any receptors for either IFN during the time of their expression (D'Andrea et al. 1994). However, the same group detected *IFNGR1* mRNA by RT-PCR not only in uterine epithelial and stromal cells but also in embryonic tissues from as early as day 10 *in vitro*. They indicated expression of *IFNGR1* in the trophoblast was developmentally regulated; although expression was weak on days 12 and 15 of gestation, it reached a level similar to that found on some IFNG sensitive cells on day 16 (D'Andréa & La Bonnardière 1998b). This suggests a possible delayed autocrine effect on the trophoblast by conceptus IFNG in addition to its paracrine effects on the endometrial epithelial and stromal cells.

IFNG has been hypothesized to play a role in conceptus attachment by assisting other factors in their roles such as integrins and heparin sulphate proteoglycans (Farrar & Schreiber 1993). It has been suggested that either IFNG or IFND, or both, contribute to a remodeling of the endometrial epithelium, affecting its polarity and receptivity to the trophoblast, thus favoring trophoblast attachment (D'Andrea *et al.* 1994). It has been reported that IFN increase expression of many endometrial genes, including class I and II major histocompatibility complexes, STAT1, and IRF1 which are all involved in immune regulation (Joyce et al. 2007a; Joyce et al. 2007b; Joyce et al. 2008). Additionally, it has been reported that conceptus IFN are responsible for induction of endometrial FASLG, TNFSF10, and FAS expression on day 15 of pregnancy in the pig (Yoo et al. 2020b). Furthermore, the same study showed

that IFNG increases the expression of cysteine-X-cysteine chemokine ligands (CXCL) 9, 10, 11, and 12 which in turn, increase migration of T and NK cells, suggesting that IFN may play a role in recruitment of immune cells into the endometrium during the implantation period in pigs (Yoo *et al.* 2020b). While very little is known about conceptus interferons during early pregnancy in the pig, understanding their pathways could lead to more insight.

2.6.4 Type I and Type II IFN Receptors

Type I IFND and type II IFNG bind to two different cell surface receptors, both of which belong to the class II cytokine receptor subfamily. Both the type I IFN receptor and type II IFN receptor have transmembrane structures which are composed of two distinct subunits. IFND binds to the heterodimeric receptor composed of IFNAR1 and IFNAR2 while IFNG binds to IFNGR1 and IFNGR2 to transmit their signals to the cell. The initial step in both type I IFN and type II IFN mediated signaling is the activation of the receptor-associated Janus activated kinases (JAK), which occurs in response to a ligand-dependent dimerization of the receptor subunits, followed by autophosphorylation and activation of the associated JAK (Figure 2.1) (Darnell et al. 1994; Ihle 1995). The JAK-STAT signaling cascade, first discovered in the 1990's (Silvennoinen et al. 1993) is the most extensively studied IFN-dependent pathway. Classical JAK-STAT signaling involves rapid nuclear translocation and initiation of gene transcription by STATs that have been activated at the plasma membrane in response to JAK-mediated phosphorylation. However, as research continued, it became very obvious that the JAK-STAT pathway alone was not responsible for all the biological activities

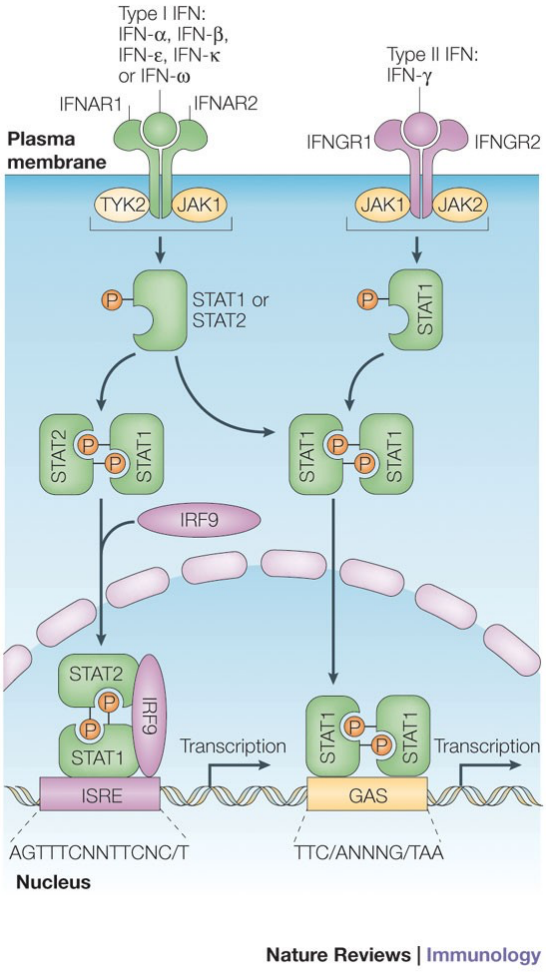

Figure 2.1: Diagram of Type I IFN Receptor and Type II IFN Receptor

(Platanias 2005). Activation of JAKs associated with the type I IFN receptor results in tyrosine phosphorylation of STAT2 and STAT1; this leads to the formation of STAT1-STAT2-IRF9 complexes which can translocate to the nucleus and bind ISRE in DNA to initiate transcription. Type II IFNG binding to its receptor can induce the formation of STAT1-STAT1 homodimers that translocate to the nucleus and bind GAS (IFNG activated site) elements that are present in the promoter of certain ISG, thereby initiating the transcription of these genes. Type I IFN binding to their receptors can also induce STAT1 homodimers which can bind to GAS elements and initiate ISG transcription as well.

of IFN. In addition to activation of classical JAK-STAT signaling pathways, activation of receptor-associated JAK regulated, either directly or indirectly, a number of other downstream signaling cascades. As mentioned previously, this signaling diversity is consistent with the pleiotropic biological effects IFN have on target cells and tissues early researchers documented. The type I and type II IFN receptors will be discussed separately in the paragraphs below.

As previously stated, all type I IFN bind to the two receptor subunits at the same sites and form very similar structural ternary complexes (Piehler et al. 2012). Following ligand-induced dimerization of IFNAR1 and IFNAR2, the JAK family TYK2 and JAK1 which are associated with the membrane-proximal part of the cytoplasmic domain of IFNAR1 and IFNAR2, respectively, are activated by reciprocal transphosphorylation (Cohen et al. 1995). Then, they phosphorylate several tyrosine residues in the intracellular domains of IFNAR1 and IFNAR2, which serve as docking sites for effector proteins of the STAT family. Upon phosphorylation, STAT1 and STAT2 form homo- and heterodimers which subsequently translocate to the nucleus to activate gene transcription (Piehler *et al.* 2012). One prominent type I IFN signaling pathway involves the STAT1-STAT2 heterodimer, which alongside IRF9 forms the transcription factor ISGF3 that binds interferon-stimulated regulatory elements (ISRE) within the promotor region of ISG. These ISG encode for a multitude of proteins that are responsible for antiviral, antiproliferative, and immunoregulatory cellular responses. In addition to the classical JAK-STAT pathway, other signaling factors regulate IFN activity. Some of these include protein kinase C (PKC) and the multifunctional

adapter protein CrkL, members of the p38 mitogen-activated protein kinase (MAPK) pathway, the phosphoinositol 3-kinase (PI3K) signaling pathway, and the extracellular signal-regulated kinase (ERK) MAPK pathway as reviewed by de Weerd and Nguyen (2012). IRF1 is induced by type I and type II IFN and activates ISG by binding ISRE of the ISG promoters whereas IRF2 acts as a transcriptional repressor of ISG due to a repression domain in its C-Terminus (Taniguchi *et al.* 2001). A study on pig endometrial explant tissues revealed that the abundance of IFNAR1 and IFNAR2 mRNAs was increased by IL1B and the abundance of IFNAR2 mRNAs was increased by estradiol treatment on day 12 of the estrous cycle in endometrial explant tissues (Jang et al. 2017b). These data suggest that E_2 and IL1B prepare the endometrium by inducing receptors for IFND and possibly IFNG for the developing conceptus which subsequently begin secreting IFND and IFNG following E_2 and IL1B production (Yoo et al. 2019).

As mentioned previously, IFNG is the only type II IFN, and it binds to its two receptor subunits IFNGR1 and IFNGR2. Following ligand binding, dimerization of IFNGR1 and IFNGR2 occurs and activates JAK1 and JAK2, respectively, causing subsequent activation of STAT1. Activated STAT1 forms a homodimer which enters the nucleus and activates transcription of many IFNG-regulated genes containing the IFNG-activation site (GAS) element in their promotor regions (Figure 2.1) (Bach et al. 1997; Schroder et al. 2004). IRF1, a transcription factor produced by STAT1, activates many other genes alone or together with STAT1 by binding to the ISRE of those genes (Decker et al. 1997; Murphy et al. 2009; Isom et al. 2013). As previously discussed, in pigs the

endometrial epithelial and stromal cells both possess both type I and type II IFN receptors whereas the trophoblast only indicated IFNGR1 *in vitro* strongly suggesting that IFND and IFNG secreted by the peri-implantation conceptus does have paracrine effects but it is still unclear whether or not conceptus IFN exhibit autocrine effects (D'Andréa & La Bonnardière 1998b). The expression of IFN signaling molecules STAT1, STAT2, IRF1, AND IRF2 has been reported in the endometrium in pigs (Joyce *et al.* 2007a; Joyce *et al.* 2007b). Additionally, stromal cell expression of STAT1 is induced by conceptus-secretory proteins which suggests the possibility that conceptus IFND or IFNG or both affects stromal expression of STAT1. In a study done by Yoo et al. (2019) researchers analyzed the expression and regulation of IFNGR1 and IFNGR2 in the endometrium during the estrous cycle and pregnancy in pigs. Their results revealed that levels of IFNGR1 and IFNGR2 mRNA changed in the endometrium with the highest levels during mid-pregnancy for IFNGR1 and on day 12 of pregnancy for IFNGR2 which corresponds to the time when the conceptus initiates attachment to the endometrium. The expression of IFNGR1 and IFNGR2 mRNAs was also detected in conceptuses during early pregnancy and chorioallantoic tissues during mid to late pregnancy. They also showed that IFNGR1 and IFNGR2 mRNAs were localized to endometrial epithelial and stromal cells as well as the chorionic membrane during pregnancy. Yoo and others (2019) also performed endometrial explant culture studies and found that estrogen increased levels of IFNGR2, but not IFNGR1 mRNAs while IL1B did not affect levels of either IFNGR1 or IFNGR2 mRNAs. Moreover, IFNG increased

levels of IRF1, IRF2, STAT1, and STAT2 mRNAs in the endometrial explants (Yoo *et al.* 2019). More research is needed to better understand the expression pattern and localization of IFNGR1 and IFNGR2 during pregnancy and their regulatory mechanism in the endometrium and potentially trophoblast as well.

2.7 Utilizing CRISPR/Cas9 Genomic Editing Technology to Generate a Pig Conceptus IFNG Null Research Model

The majority of embryonic loss in the pig occurs during early pregnancy, typically between days 10 to 30 of gestation (Pope 1994). Several major developmental events occur during this time, such as rapid conceptus elongation, maternal recognition and establishment of pregnancy, conceptus apposition and attachment to the uterine luminal epithelium and placentation. While previous research has studied the effects of various conceptus secretory factors during the peri-implantation period, the role of interferons in the pig still remains to be elucidated. Previous studies of conceptus IFNG in the pig were able to identify and roughly quantify the amount of this secretory protein present in the uterine horn during pregnancy and estrous. However, in more recent years there has been increased interest and availability of genomic editing technologies which can be used in advanced investigations of specific genes and the effects they have on biological systems. The present research will utilize the CRISPR/Cas9 system to target and edit pig conceptus IFNG to generate a loss-of-function deletion to determine the role of conceptus-derived IFNG during early embryonic development and establishment of pregnancy in the pig.

CHAPTER THREE

Conceptus Interferon Gamma is Essential for Pregnancy Maintenance in the Pig

3.1 Introduction

The establishment and maintenance of pregnancy in viviparous mammals involves an intricate crosstalk at the maternal-fetal interface throughout pregnancy (Bazer et al. 2010; Bazer & Johnson 2014; Roberts et al. 2016; Nair et al. 2017). During the establishment of pregnancy, the rapidly developing conceptus must extend the function of the corpus luteum (CL), attach to the endometrial luminal surface epithelium (LE), stimulate the release of endometrial secretions for continued development, initiate formation of its extramural placental membranes and prevent maternal immune-mediated rejection. Initial extended attachment of the conceptus to the uterine surface may have evolved from regulating the induction of inflammatory responses derived from therian mammals (Griffith et al. 2017). The semiallogeneic conceptus (later fetus and placenta) must regulate or suppress conceptus-induced proinflammatory stimulation of the uterus during conceptus attachment while modulating the maternal immune system to inhibit classical pathways involved with tissue rejection (Horton et al. 2002; Mor et al. 2011). Maternal and conceptus secretion of steroids (estrogens, progestogens and corticosteroids), cytokines and chemokines immunomodulate the cellular interactions at the maternal-placental

interface during conceptus attachment (Nair *et al.* 2017; Złotkowska & Andronowska 2019). Peri-implantation porcine conceptuses produce estrogens (E_2), an interleukin (IL1B2), type 1 and type 2 interferons (IFND, IFNG) and prostaglandins (PG) (Bazer & Johnson 2014; Geisert *et al.* 2014; Geisert *et al.* 2015), which function to regulate endometrial responses to establish pregnancy in the pig.

During early development, porcine conceptuses expand throughout the uterus by transforming from 9-10 mm ovoid shaped blastocysts between Days 10 and 12 and then rapidly transitioning to tubular and filamentous forms by elongating to >100 mm in length prior to initiating attachment of trophectoderm (TE) to the uterine LE (Geisert *et al.* 1982b). Rapid trophoblastic elongation involves the presumptive placental membranes (trophectoderm and extra-embryonic endoderm) that drive cellular changes required to form a filamentous conceptus (Geisert *et al.* 1982b). Elongation of the conceptus is initiated through conceptus expression of *IL1B2* (Mathew *et al.* 2015). Selective knockout of *IL1B2* gene expression indicated that IL1B2 is essential for the rapid transformation of elongating pig conceptuses (Whyte *et al.* 2018). During elongation, conceptuses produce E_2 and PG on Days 11 and 12 function as conceptus signals for pregnancy recognition (Geisert *et al.* 2015). However, while disruption of expression of either porcine conceptus CYP19A1 (aromatase) or prostaglandin-endoperoxide synthase 2 (PTGS2) inhibited conceptus E_2 and PG synthesis, the early conceptuses established pregnancy and the CL were maintained beyond day 20 of gestation (Meyer *et al.* 2019; Pfeiffer *et al.* 2019). These results

suggest that either conceptus E_2 or PG (PGE) are capable of supporting early pregnancy in the absence of the other or it may be possible that other conceptus factors that contribute to early survival. Although loss of CYP19A1 expression did not affect placental development, all gilts aborted soon after day 25 of pregnancy (Meyer *et al.* 2019).

Although many studies have investigated the role and function of conceptus estrogens and PG in the establishment and maintenance of pregnancy, the role of conceptus IFN in modulating the endometrium following the incremental attachment of the trophectoderm to the uterine LE (Burghardt et al. 1997) has only recently been more intensely investigated. Interferons consist of three distinct families (type I, II and III) which stimulate cellular function through different membrane receptors (Cochet et al. 2009; Casazza et al. 2020). Although there are multiple forms of type I IFN, including ruminant conceptus IFNT and porcine IFND, all type I IFN signal through the same receptor. The pig genome contains 10 to 11 IFND genes (Cochet *et al.* 2009). In contrast to type I and III IFN, IFNG is the only type II IFN. IFNG signals through the tetrameric receptor composed of IFNGR1 and IFNGR2 that activates kinases to phosphorylate STAT1 which dimerizes to activate promoters containing Gamma-activated sites (GAS) to transcribe IFNG-stimulated genes (Casazza *et al.* 2020). Although all IFN have antiviral activity to inhibit viral infection, IFNG has proinflammatory and immunomodulatory roles in the activation of innate and adaptive immune responses and inhibition of cell proliferation and apoptosis (Boehm et al. 1997). In addition, IFNG increases endometrial angiogenesis and

vascular remodeling during implantation in mice and humans through effects of IFNG from the developing trophoblast and migrating leukocytes (T cells) into the site of implantation (Ashkar & Croy 2001; Murphy *et al.* 2009; Kim et al. 2012; McLendon et al. 2020; Yoo *et al.* 2020b).

Pig conceptus trophectoderm synthesizes and secretes both type I, IFND, and type II, IFNG, during the peri-implantation period (day 12 to 20) of pregnancy (La Bonnardiere *et al.* 1991; Cencic & La Bonnardiere 2002). The conceptus trophectoderm and endometrial LE express both the type I interferon receptor 1 (IFNAR1) and type II interferon gamma receptor (IFNGR1) (Niu et al. 1995; D'Andréa & La Bonnardière 1998a; Lefèvre et al. 1998). Endometrial IFNAR1 and IFNAR2 expression increases on day 12 of pregnancy which is during the period of rapid conceptus elongation (Jang et al. 2017a). Although pig conceptuses secrete IFND and IFNG, IFNG is the major IFN expressed by pig conceptuses during the peri-implantation period. IFNG alters endometrial LE tight junctions and stimulates stromal cell expression of MHC class II molecules at the points of conceptus attachment to uterine LE suggesting a role for IFNG in local regulation of maternal immune activity during early pregnancy (Cencic & La Bonnardiere 2002; Joyce *et al.* 2008; Kim *et al.* 2012; Han et al. 2017; Yoo et al. 2020a). Transcriptomic analysis of day 12 endometrial explants cultured with IFNG revealed that IFNG-stimulated expression of many IFNG-regulated genes are involved with activation of the immune system, chemokine signaling and antigen processing and presentation (Yoo *et al.* 2020a). During early pregnancy, trophoblast and endometrial cells express chemokines CCL2, CCL5, CCL11,

CXCL9, CXCL10 and CXCL12 that may play roles in trophoblast attachment to uterine LE, increasing endometrial vascularity and recruiting immune cells (Złotkowska & Andronowska 2019; McLendon *et al.* 2020). Expression of endometrial chemokines may initiate the localized increase in the accumulation of leukocytes beneath the interface of the attachment site of trophoblast and endometrial LE (Ashkar & Croy 2001; Murphy *et al.* 2009; Kim *et al.* 2012; McLendon *et al.* 2020; Yoo *et al.* 2020a). McLendon et al. (2020) demonstrated that IFNG increased the number of CD3+ T cells within the endometrium at sites of implantation.

Previous research established the role of IFNG on regulation of endometrial transcription and regulation of pregnancy in the pig. However, early conceptus development and attachment involve a network of conceptus factors (E_2, IL1B2, PG and IFND), all of which interact at the placental interface to establish pregnancy. The ability to ablate IFNG synthesis by the pig conceptus via targeting the IFNG expression would provide a more direct and tissue-specific method to analyze the biological roles and functions of conceptus IFNG in the establishment and maintenance of pregnancy. Therefore, the objective of the present study was to specifically determine the role of conceptus IFNG expression in early conceptus development and establishment of pregnancy in the pig. CRISPR/Cas9 gene editing was used to directly target conceptus *IFNG* expression to generate *IFNG*$^{-/-}$ embryos to evaluate the effects on conceptus attachment, immunomodulation of immune cells in the maternal endometrium, and survival of conceptuses in the pig.

3.2 Materials & Methods

All procedures in this study were approved by the University of Missouri – Columbia Institutional Animal Care and Use Committee.

Animals

Recipient gilts utilized for embryo transfer were large white by Landrace crossbred gilts of similar age (8-10 months) and weight (100-130 kg). Gilts were observed for estrous behavior twice daily with the onset of estrus designated Day 0 of the estrous cycle.

CRISPR/Cas9 Design and Transfection of Porcine Fetal-Derived Fibroblast Cells

Two pairs of guides RNAs (gRNA) were designed to specifically target the start codon within exon 1 of the *Sus scrofa IFNG* gene by using the Broad Institute design tool (http://www.broadinstitute.org/rnai/public/analysis-tools/sgrna-design). The pig *IFNG* gene is located on chromosome 5 and contains four exons. To minimize off targeting events, each gRNA was tested by using NCBI Nucleotide Blast Tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Annealed gRNA oligonucleotides were cloned into the pX330 vector (Addgene) that contains expression cassettes for human codon-optimized *Cas9* and the chimeric gRNA. The ability of the gRNA pairs to cleave was tested *in vitro*. The two pairs of guides were transfected together to optimize cutting efficiency. To determine optimal concentrations of gRNAs, porcine fetal-derived fibroblasts were

transfected with the gRNA pairs in various amounts (4 µg, 7.5 µg, or 15 µg total). Following transfection, fibroblast cells were cultured for 2-3 days, lysed, and DNA used for PCR amplification by using primers specifically designed to amplify the location of gRNA design. The PCR amplicon was evaluated on a 2% ethidium bromide agarose gel for multiple bands and sequenced to determine which guides were cutting the most efficiently. The optimal guide pair (647 and 3527) was determined the most efficient and optimal at a concentration of 15 µg (7.5 µg per gRNA).

Clonal Expansion and Colony Screening

Transfected fibroblast cells were plated using a dilution curve to promote colony growth from a single cell. Ten days following the transfection, individual colonies were collected, lysed, and submitted to PCR amplification to determine editing. The PCR product was run on a 2% ethidium bromide agarose gel and bands were screened for monoallelic and biallelic edits. The PCR amplicon from individual cell colonies with biallelic edits were purified using the PureLink PCR Purification Kit (ThermoFisher Scientific, K310001) and submitted to the University of Missouri DNA Core Facility for Sanger Sequencing to confirm biallelic edits. Out of 1,754 cell colonies screened, 12 cell colonies were identified by PCR (Figure 3.1) and Sanger Sequencing to have a biallelic deletion in exon 1 of *IFNG* containing the start codon. Of those 12 biallelically edited colonies, one cell line designated ‘DNJ5G’ proliferated better than the other edited colonies and was selected for cloning. The PCR amplicon from this cell line was cloned into a

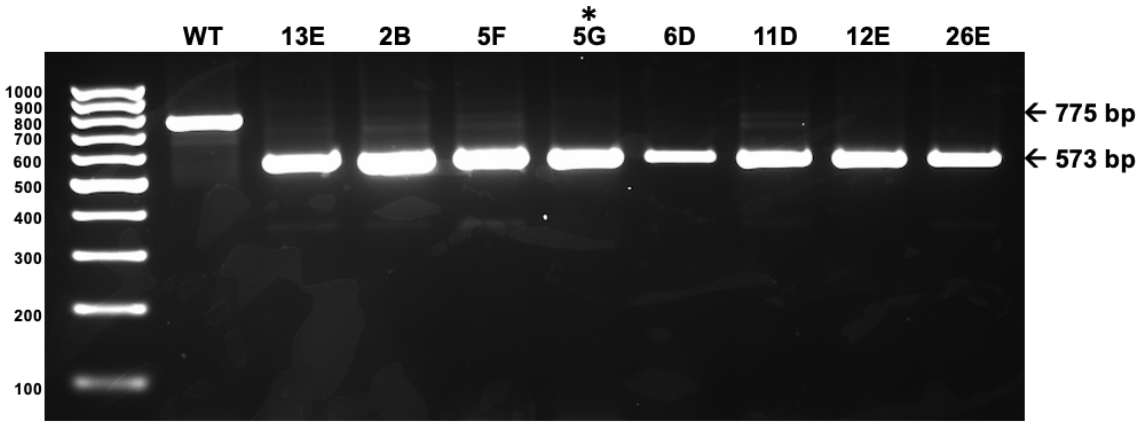

Figure 3.1: Colony screening for biallelic modifications. Individual cell colonies were picked, lysed, and used for PCR amplification. The resulting PCR was run on a 2% agarose gel with ethidium bromide and imaged. Cell colonies identified with a modification were cleaned and submitted to sequencing (MU DNA Core, University of Missouri-Columbia). Each band represents an individual cell colony or control wild-type (WT) *IFNG*$^{+/+}$ cells. The image demonstrates the identification of the biallelic edited cell line 5G (202 bp deletion), designated DNJ5G, used for somatic cell nuclear transfer.

PCRTM4-TOPO® TA vector (ThermoFisher Scientific, 450071) which was used to identify the location of the modification on each allele. Both alleles in this cell line had the same modification, a 202 base pair deletion including the start codon (Figure 3.2).

Somatic Cell Nuclear Transfer (SCNT)

Fibroblast cell colonies *IFNG*$^{+/+}$ (unedited control), or *IFNG*$^{-/-}$ (biallelically edited) were grown to ~80% confluency and used as donor cells for SCNT as previously described (Whitworth et al. 2014) with modifications to the maturation system (Yuan et al. 2017). To produce embryos *in vitro*, ovaries from pre-pubertal gilts were obtained from a local slaughterhouse (Smithfield Food, Milan, MO). Aspirated oocytes were matured *in vitro*, and the cumulus cells were removed with 0.1% hyaluronidase and gentle vortexing. Due to the COVID-19 pandemic, the source of the ovaries (Smithfield Food) was disrupted, and oocytes were purchased from Desoto Biosciences (Seymore, TN) to generate cloned embryos. The polar body and adjacent cytoplasm, containing the metaphase II plate, were removed from the oocyte. A donor fibroblast cell (*IFNG*$^{+/+}$ or *IFNG*$^{-/-}$) was inserted into the perivitelline space (Lai & Prather 2003) and electrically fused with two DC pulses at 1.2kV/cm for 30 µsec using a BTX Electro Cell Manipulator (Harvard Apparatus). After fusion, the cloned zygotes were chemically activated (Macháty et al. 1997) and cultured for 14-16 h (Spate et al. 2015).

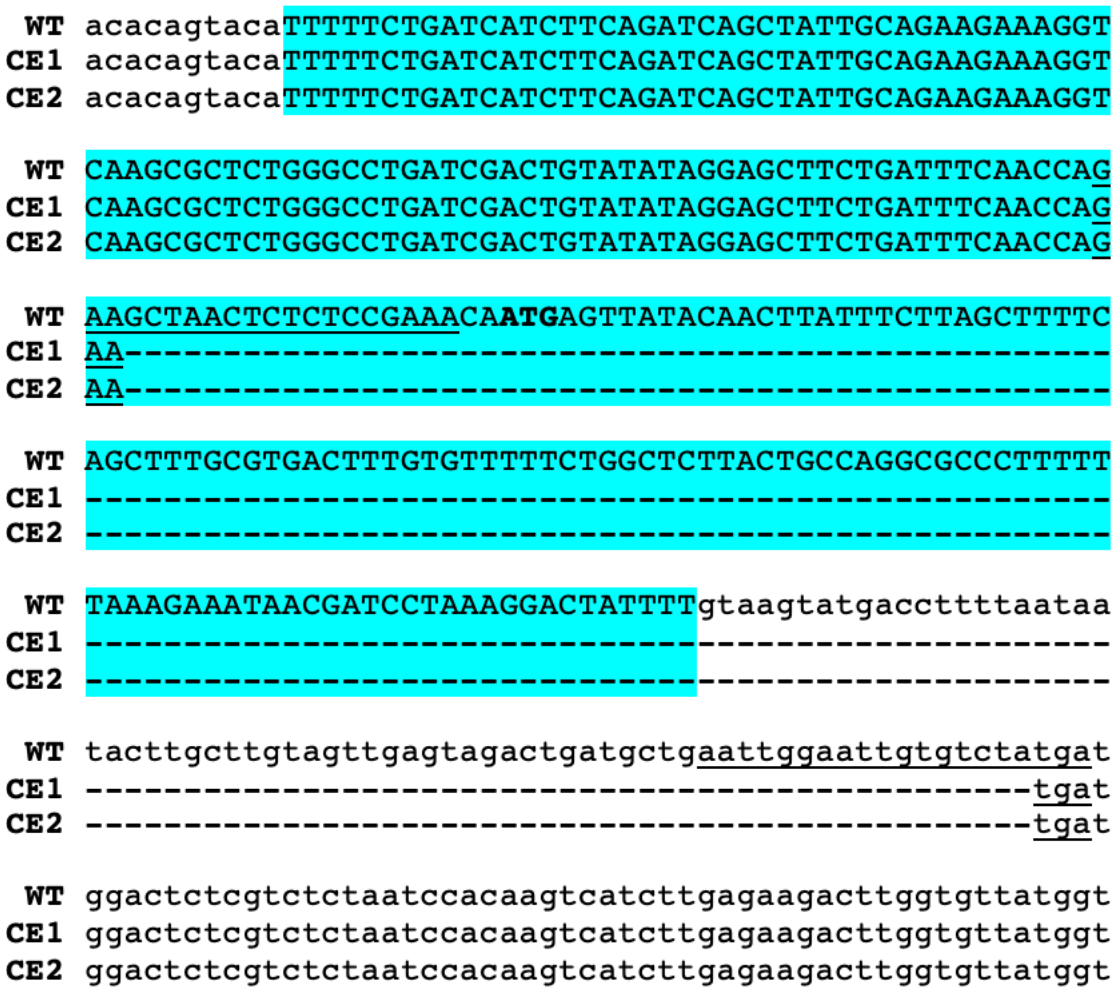

Figure 3.2: Biallelic modification on exon 1 of the porcine *IFNG* gene.

The sequence shows that Exon 1 (uppercase, highlighted in blue) of the *IFNG*$^{-/-}$ cell line has a biallelic 202 base pair deletion (CE1 and CE2) including the start codon (**ATG**) compared to the *IFNG*$^{+/+}$ sequence (WT). Guide RNAs are underlined. Introns are displayed in lowercase.

Embryo Transfer

Recipient gilts were monitored for signs of estrus and used for embryo transfer. Day 6 morula to blastocyst stage embryos (n=30-50) were surgically transferred into the oviduct of the recipient gilts near the ampullary–isthmic junction on day 3, 4, 5, or 6 after first standing estrus as previously described (Lee et al. 2013).

Embryo Collection

Recipient gilts were euthanized via jugular injection of Euthasol (Virbac AH, Inc; Fort Worth, TX) on either day 15 (n=4 *IFNG*$^{+/+}$, n=4 *IFNG*$^{-/-}$) or day 17 (n=4 *IFNG*$^{+/+}$, n=8 *IFNG*$^{-/-}$) of gestation. The uteri and ovaries were excised from the abdomen, placed on ice and transported to the laboratory for processing. The reproductive tract was rinsed and trimmed free of the mesometrium. Day 15 and 17 conceptuses were recovered from the uterus by flushing each uterine horn twice with 30 mLs of Phosphate Buffered Saline (PBS). Conceptuses retrieved from the uterine lumen flushing (ULF) were examined and the morphology and viability assessed. Prior to flushing, parts of each uterine horn near the uterine body were clamped, cut transversely, and the section fixed in 4% paraformaldehyde (PFA) for 24 h. These tissue sections were then stored in 70% ethanol until used for immunohistochemistry. Conceptuses and endometrial tissue samples (~ 5 g) collected from five random sections along the mesometrial side of each uterine horn were placed into individual 1.5 mL Eppendorf tubes, flash frozen in liquid nitrogen and stored at -80°C until used for RNA, DNA, and protein extraction. ULF was centrifuged at 4,000 x g at 4°C for

10 min to separate fluid and cellular debris. The ULF was flash frozen and stored at -80°C until used for analyses of total PG, IL18, estradiol-17β (E_2), and IFNG. An additional group of recipient gilts received either *IFNG*$^{+/+}$ embryos (n=6) or both *IFNG*$^{-/-}$ and *IFNG*$^{+/+}$ embryos (n=5). These gilts were allowed to continue pregnancy to day 30 before the uteri were excised from the abdomen, placed on ice, and transported to the lab for immediate processing. Co-transfer of *IFNG*$^{-/-}$ and *IFNG*$^{+/+}$ was used to determine if the presence of IFNG producing WT conceptuses could rescue the *IFNG*$^{-/-}$ conceptuses beyond day 17 of pregnancy. The broad ligament was removed from the uterus and the uterine horns were then opened along the antimesometrial border to dissect out each individual placenta and fetus. Fetal, placental, and endometrial tissues were collected, flash frozen, and stored at -80°C until RNA/DNA/protein was extracted.

Total RNA, Genomic DNA, and Protein Isolation

Endometrial total RNA was obtained by using TRIzol-RNA lysis reagent (ThermoFisher Scientific, 15596018). Direct-zol RNA Miniprep Plus kits (Zymo Research, R2070) were used for purification of RNA from the endometrium. Conceptus RNA, DNA, and protein was extracted simultaneously by using the AllPrep DNA/RNA/Protein Mini Kit (Qiagen, 80004). Extracted RNA was treated with RNase Free DNase Set (Qiagen, 79256) to eliminate genomic DNA contamination. Quantity and quality of RNA and DNA in the samples were established by using a NanoDrop ND-1000 spectrophotometer and visualization of conceptus DNA bands on a 2% ethidium bromide agarose gel and RNA with a

1% ethidium bromide agarose and a 1% bleach integrity gel as previously described (Pfeiffer *et al.* 2019).

Reverse Transcriptase and RT-PCR Gene Expression Analysis

Total conceptus and endometrial RNA was reverse transcribed in a 20 µL reaction mix using iScript RT Supermix (BioRad, 1708841). Amplification of cDNA was performed in an Eppendorf Mastercycler Pro and incubated at 25°C for 5 min, reverse transcribed at 42°C for 30 min, and finally heat-inactivated at 85°C for 5 min. Negative controls for each sample were prepared without the iScript RT Supermix to test for DNA contamination. Stocks of cDNA were stored at -20°C until used for RT-PCR. First strand cDNA for RT-PCR was synthesized from 2.5 µg of total RNA and RT-PCR was performed and quantified by using the CFX384 Real-Time System (Bio-Rad). Conceptus gene expression was measured by using primers (Table 3.1) specific for *IFNG* (Joyce *et al.* 2007a), and *IFND* (Joyce *et al.* 2007a), *PTGS2* (Blitek et al. 2006), and *CYP19A1* (Ebeling et al. 2011). Similarly, endometrial gene expression was measured by using primers specific for *IFNG* (Joyce *et al.* 2007a), *IFND* (Joyce *et al.* 2007a), *TNFSF10* (Yoo *et al.* 2020b), *IDO1* (Yoo *et al.* 2019), and *CIITA* (Yoo *et al.* 2019) (Table 3.1). RT-PCR analysis determined that porcine *YWHAG* gene expression was not different ($P > 0.05$) among the conceptus and endometrial total RNA samples and was therefore used as the housekeeping gene. Relative expression of sequence-specific products was quantified by using the $^{-2\Delta}$CT method. The ΔCT was

Table 3.1 Endometrial and Conceptus RT-PCR Primers

Gene	RefSeq gene	Primers 5' → 3'	Source
PTGS2	**AY028583.1**	ATGATCTACCCGCCTCACAC	(Blitek et al., 2006)
	Provisional	AAAAGCAGCTCTGGGTCAAA	
CYP19A1	**NM_214429.1**	GCTAATTGCAGCACCAGACA	(Ebeling et al., 2011)
	Provisional	TGTTGGTTCCCTTTTTCACC	
IFNG	**NM_213948.1**	CCATTCAAAGGAGCATGGAT	(Joyce et al., 2007)
	Provisional	TTCAGTTTCCCAGAGCTACCA	
IFND	**NM_001002832.1**	ATGGATTGTCCCCATGTAGG	(Joyce et al., 2007)
	Provisional	CTGAGCTACCAGGGTTACCG	
TNFSF10	**NM_001024696.1**	TGATTTTGAGAACCTATGAG	(Yoo et al., 2020)
	Provisional	GATGTAGTAAAACCCTGTTTGATGG	
CIITA	**NM_001315695.1**	GCCAAGTCCATGAAGGATGT	(Yoo et al., 2020)
	Provisional	GATGCTGCAGGGAAGAAAAG	
IDO1	**NM_001246240.1**	TCAATGTTCTTCGCATATAC	(Yoo et al., 2020)
	Provisional	CTGGTGGATATAGGTTCTC	
YWHAG	**XM_005661962.3**	TCCATCACTGAGGAAAACTGCTAA	(Whitworth et al., 2015)
	Model	TTTTTCCAACTCCGTGTTTCTCTA	

determined as the difference between the cycle threshold (CT) for the gene of interest and geometric mean of the CT for *YWHAG*. Fold change was calculated compared to day 17 *IFNG*$^{-/-}$ ΔCT average.

Measurement of Total Uterine IFNG, IL18, Estradiol-17β, and PG in Uterine Luminal Flushings (ULF)

A pig IFNG ELISA Kit (Sigma Aldrich, RAB0226-1KT), PG ELISA kit (Cayman Chemical Company, 514012), and IL18 ELISA kit (Sigma-Aldrich, RAB0871-1KT) were used according to the manufacturer's directions to determine concentrations and total amounts (pg) of IFNG, total PG, and IL18, respectively, in ULF. Concentrations of estradiol-17β in ULF were quantified in a single RIA as described previously (Kirby et al. 1997; Pfeiffer *et al.* 2019).

Immunohistochemistry

Immunohistochemistry (IHC) was performed on fixed uterine tissue sections embedded in paraffin wax and sectioned by the MU CVM Veterinary Medical Diagnostic Laboratory (VMDL). The paraffinized tissue samples were trimmed and loaded into cassettes then processed overnight in the Sakura Tissue-Tek VIP 6 AI vacuum infiltration processor. After the tissues were processed, they were embedded in paraffin wax by using the Sakura Tissue-Tek Embedding center. Sections from the paraffin blocks were cut between 4-6 microns using Leica RM2255 Microtomes. Sections were mounted on charged glass slides and heated to melt the wax before being processed for IHC, Hematoxylin and Eosin

(H&E) staining, or toluidine blue (T-blue) staining. For H&E staining, slides were deparaffinized in xylene, rehydrated in a graded alcohol series, stained with Hematoxylin and Eosin, and then processed backwards through a graded alcohol series before being covered with a glass slip with a synthetic mounting medium (in accordance with VMDL protocols). For T-blue staining, the slides were deparaffinized in xylene and rinsed in distilled water, then stained with 0.1% Toluidine Blue solution for 10 min, rinsed in distilled water rinse and dehydrated in 95% ethanol, 100% ethanol, and xylene to clear the tissue before it was covered with a glass slip (in accordance with VMDL protocols).

Slides for CD3 IHC staining were heated in a Fisher Oven for at least 13 min at 80ºC until the paraffin was melted. Slides were processed through a Leica AutoStainer XL for deparaffinization in xylene and rehydration through an alcohol gradient. The slides were then placed in a Coplin jar with Diva Decloaker solution pretreatment (diluted 1:10; BioCare Medical, Concord, CA) for heat-induced epitope retrieval (HIER). The Coplin jars of slides were placed in the Decloaking chamber (BioCare Medical) for HIER. Following HIER, slides were loaded on a Biocare intelliPATH FLX Automated IHC Slide Stainer and blocked with Background Sniper Block (BioCareMedical, Concord, CA) for 10 min, washed and incubated with rabbit anti-CD3 antibody (1:150 dilution; BioCare Medical, Concord, CA Cat# A0452 DAKO) for 45 min. Slides were then washed and incubated with Rabbit EnVision+ system-HRP (DAKO, Carpinteria, CA) for 30 min, washed, and incubated with Chromogen-Romulin Red AEC Buffer (BioCare Medical, Concord, CA) for 10 min. Sections were counterstained with IP FLX

Hematoxylin (IPCS5006 diluted 1:10) for 5 min and covered with a glass slip with Synthetic mounting medium before imaging (in accordance with VMDL protocols).

For dual immunofluorescent staining, the slides were baked at 60°C for 10 min, deparaffinized in xylene substitute, and rehydrated in a graded alcohol series. The sections then underwent antigen retrieval for immunolocalization by incubating sections for 30 min in boiling Reveal Decloaker 10x (pH 6.0; RV1000M, Biocare Medical). After cooling to room temperature (RT), sections were rinsed once in Milli Q H_2O for 10 min, once in PBS-tween (PBST, 0.1%) for 20 min, and twice in PBS (pH 7.5) for 10 min each time all at RT on a rotor. Sections were then outlined with a wax pen and blocked with 10% normal goat serum at RT for 30 min and then incubated overnight at 4°C with rabbit anti-IFNG polyclonal antibody (1:200 dilution; US Biological, Salem, MA; USA, Cat# I7662-16N8) and mouse anti-PCNA polyclonal antibody (1:200 dilution; Abcam, Cambridge, MA; ab29). Sections were washed twice with PBS and incubated for 1 h at RT with secondary antibodies Alexa Fluor Plus 488-conjugated secondary antibody (1:400 dilution; ThermoFisher Scientific, A-32731) and Alexa Fluor Plus 555-conjugated secondary antibody (1:400 dilution; ThermoFisher Scientific, A-32727) in the dark. Sections were then incubated with Hoechst Fluorescent Stain 33342 (ThermoFisher Scientific, H3570) diluted in Milli Q H_2O (1:2000) for 5 min at RT on the rotor and then mounted for imaging. Images for fluorescence were captured with a Leica DM5500 B upright microscope and Leica DFC450 C camera by using LAS X.

Statistical Analysis

Statistical analyses were performed by using SAS software, Version 9.4 (SAS Institute Inc., Cary, NC, USA). ULF data were analyzed using two-way analysis of variance, while gene expression data were analyzed using two-way analysis of variance for gilt level data and a repeated measures two-way analysis of variance for conceptus level data. Square root and natural logarithm transformations of the response variables were considered, when appropriate, to better satisfy the assumptions of the analysis of variance procedure.

3.3 RESULTS

In Vitro Blastocyst Development from IFNG$^{-/-}$ and IFNG$^{+/+}$ Fibroblasts Following Somatic Cell Nuclear Transfer

The *IFNG$^{-/-}$* fibroblast cell line established was utilized for SCNT to generate *IFNG$^{-/-}$* morulae or blastocyst stage embryos. Porcine fetal fibroblast cells transfected without the gRNA/Cas9 complex were used as donor cells for SCNT to establish *IFNG$^{+/+}$* control embryos. Embryos were genotyped and verified to contain the expected *IFNG$^{-/-}$* gene biallelic edit (Figure 3.3).

Uterine Flushing of Recipient Gilts Containing Either IFNG$^{+/+}$ or IFNG$^{-/-}$ Conceptuses on Days 15 and 17 of Pregnancy

The uterine horns of recipient gilts were flushed on days 15 and 17 of pregnancy to collect conceptuses and uterine luminal contents. Elongated conceptuses were flushed from all day 15 *IFNG$^{+/+}$* (4/4) and *IFNG$^{-/-}$* (4/4) recipient gilts

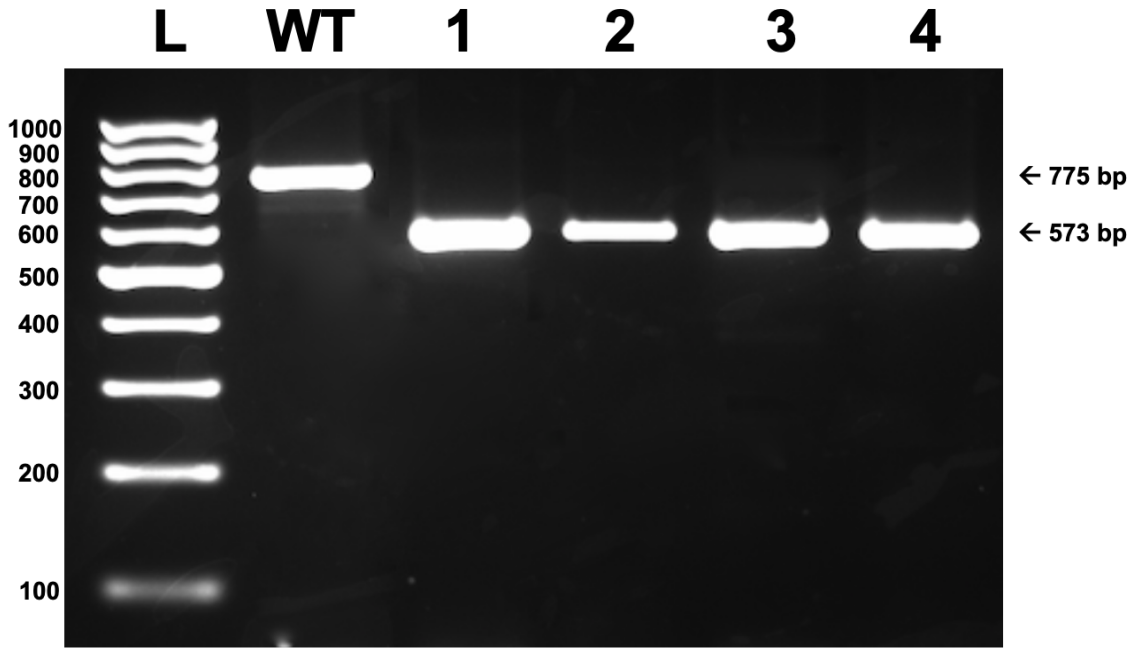

Figure 3.3: Genotyping of *IFNG*$^{-/-}$ blastocysts. Blastocysts derived from *IFNG*$^{-/-}$ fetal fibroblast cells via SCNT (1-4) were collected from *in vitro* culture on day 7 and genotyped by PCR to confirm 202 bp deletion. Ladder (L), *IFNG*$^{+/+}$ wildtype (WT) blastocyst.

(Figure 3.4 A, B). Although all *IFNG*$^{-/-}$ embryo recipient gilts were pregnant, the trophoblast of elongated conceptuses appeared to be less developed compared to conceptuses flushed from gilts who received *IFNG*$^{+/+}$ embryos. On day 17 of pregnancy, all control gilts (4/4) who received *IFNG*$^{+/+}$ embryos contained elongated viable conceptuses; however, conceptuses were only recovered from 2 of 8 gilts who received *IFNG*$^{-/-}$ embryos (Figure 3.4 C, D). In the two gilts who received *IFNG*$^{-/-}$ embryos that contained conceptus material on day 17, the trophoblast of the conceptuses was thin and fragmented compared to *IFNG*$^{+/+}$ conceptuses (Figure 3.4 B). Endometrium of the gilts who received *IFNG*$^{-/-}$ embryos on day 15 (Figure 3.5 B, C) was very hyperemic compared to gilts who received *IFNG*$^{+/+}$ embryos (Figure 3.5 A). Uterine horns of day 17 gilts who received *IFNG*$^{-/-}$ embryos that were pregnant (Figure 3.5 D, E) and did not contain conceptuses (Figure 3.5 F, G) were hyperemic, inflamed and edematous. The CL on ovaries of 5 nonpregnant gilts who received *IFNG*$^{-/-}$ embryos were undergoing luteolysis which normally occurs shortly after day 15 in cyclic gilts (Figure 3.6). Ovaries of the one remaining nonpregnant gilt who received *IFNG*$^{-/-}$ embryos contained functional CL and the lumen of uterine horns were closed as would occur in the presence of conceptus estrogen secretion when flushing the uterine horns of pregnant or pseudopregnant females. This was consistent with the concept that conceptuses had provided the maternal recognition signal on day 15 to extend CL function but conceptuses failed to survive to day 17. Endometrial samples collected from day 17 gilts who received *IFNG*$^{-/-}$ embryos

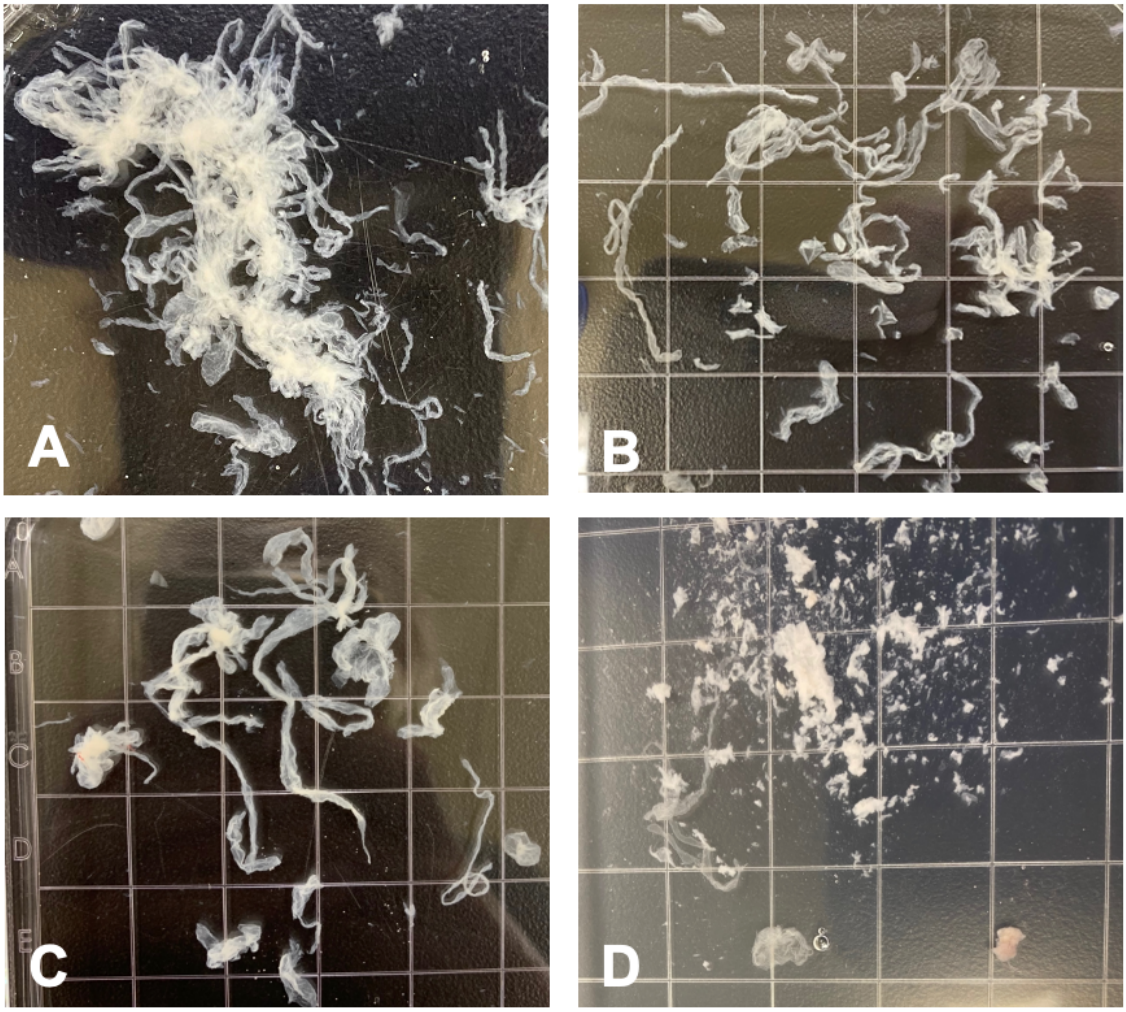

Figure 3.4: Conceptus morphology. Conceptuses flushed from the uterine horn of recipient gilts containing either *IFNG*$^{+/+}$ or *IFNG*$^{-/-}$ conceptuses on day 15 and 17 of pregnancy. **A)** Day 15 *IFNG*$^{+/+}$ elongated conceptuses. **B)** Day 15 *IFNG*$^{-/-}$ elongated conceptuses. **C)** Day 17 *IFNG*$^{+/+}$ elongated conceptuses. **D)** Day 17 *IFNG*$^{-/-}$ fragmented conceptuses.

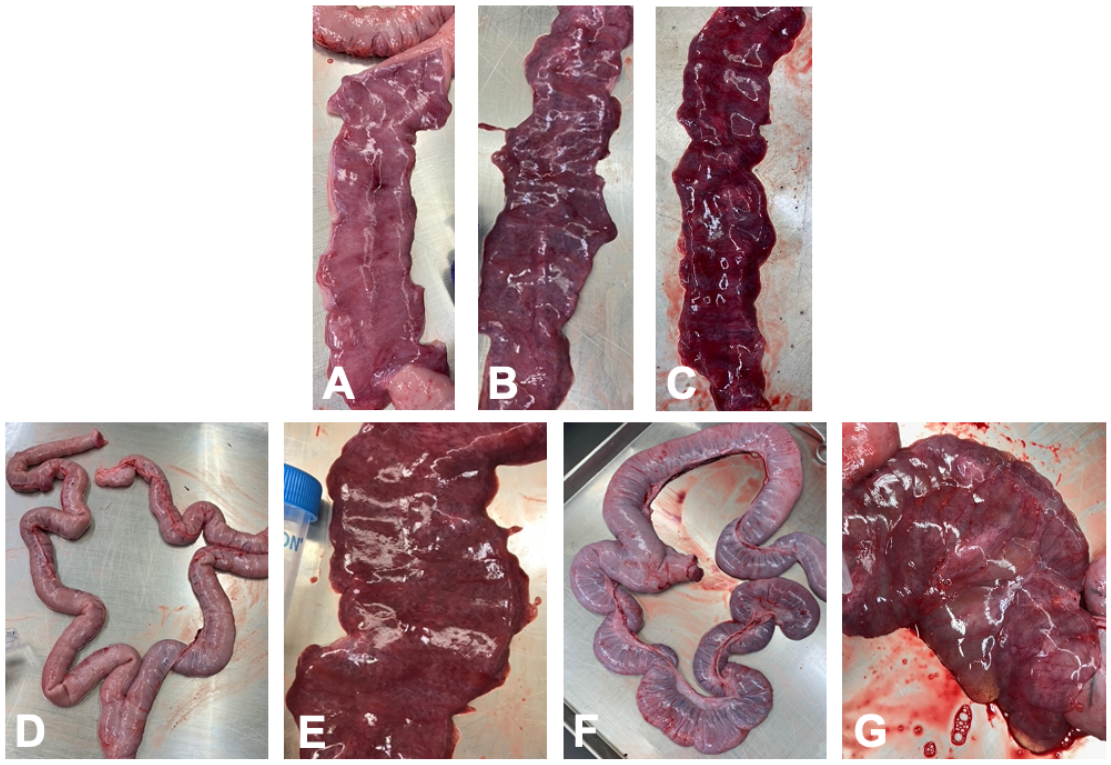

Figure 3.5: Uterine morphology. Reproductive tracts of *IFNG*$^{-/-}$ recipient gilts were hyperemic, inflamed and edematous compared to reproductive tracts of gilts who received *IFNG*$^{+/+}$ embryos. **A)** Endometrium of gilts who received *IFNG*$^{+/+}$ embryos appeared healthy on day 15 and 17. **B,C)** Pregnant reproductive tracts of gilts who received *IFNG*$^{-/-}$ embryos were inflamed and hyperemic on day 15. **D,E)** Endometrium of pregnant gilts who received *IFNG*$^{-/-}$ embryos appeared hyperemic on day 17. **F,G)** Non-pregnant reproductive tracts of gilts who received *IFNG*$^{-/-}$ embryos were highly inflamed, hyperemic, and edematous on day 17.

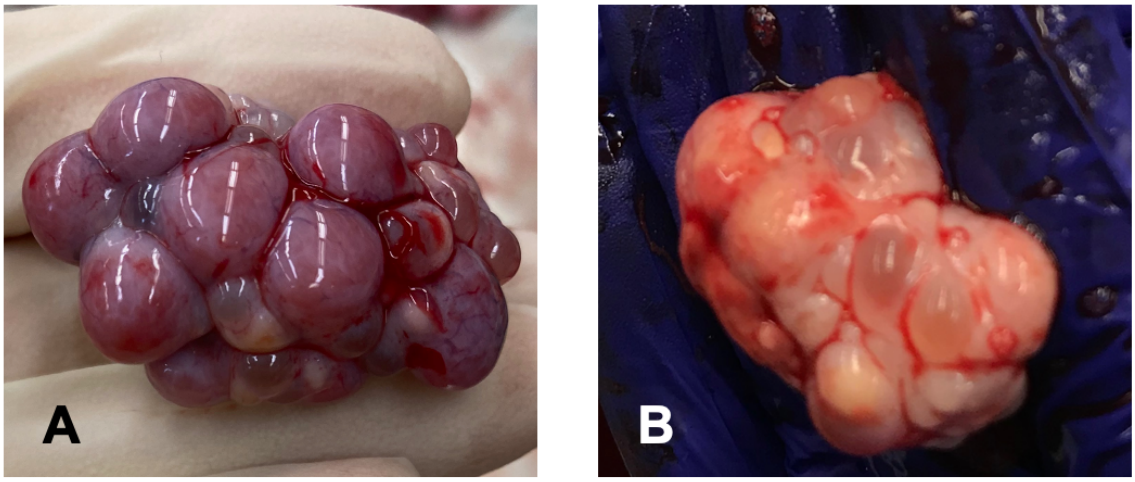

Figure 3.6: Corpora lutea of gilts who received *IFNG*$^{-/-}$ embryos. **A)** Ovaries of gilts who received *IFNG*$^{-/-}$ embryos contained healthy and viable corpora lutea on day 15 of pregnancy. **B)** Luteolysis had begun in ovaries of gilts who received *IFNG*$^{-/-}$ embryos on day 17 of pregnancy resulting in pale, corpora albicantia.

which did not contain conceptuses (n=6) were excluded from the analysis of gene expression data.

Conceptus IFNG Gene and Protein Expression

Expression of *IFNG* mRNA expression was affected by genotype of conceptus (P=0.0006) and day (P<0.0001). Conceptus *IFNG* mRNA expression was ~18-fold greater for day 15 $IFNG^{+/+}$ conceptuses and ~8-fold greater for day 17 $IFNG^{+/+}$ conceptuses compared to day 17 $IFNG^{-/-}$ conceptuses (Figure 3.7). Localization of IFNG expression in day 15 and day 17 $IFNG^{-/-}$ *and* $IFNG^{+/+}$ conceptuses and surrounding endometrial tissue was performed by using immunofluorescence (Figure 3.8). Expression of IFNG was localized to the trophectoderm of $IFNG^{+/+}$ conceptuses on both day 15 and 17 of pregnancy. However, IFNG expression was not detected in $IFNG^{-/-}$ conceptuses on either day 15 (Figure 3.9) or day 17 (Figure 3.10) of pregnancy. Expression of PCNA (proliferating cell nuclear antigen) was abundant in the trophectoderm and endoderm of the $IFNG^{+/+}$ conceptuses, as well as within the endometrial stroma near the site of conceptus attachment to uterine LE for gilts who received $IFNG^{+/+}$ embryos on day 15 and day 17 of pregnancy. However, endometrial expression of PCNA in the stroma was less on day 17. Expression of PCNA was significantly reduced in $IFNG^{-/-}$ conceptuses indicating that cells were not proliferating as rapidly and/or conceptus development was failing on days 15 and 17. The PCNA positive cells in the endometrial stroma were likely immune cells (round) and they were present in reduced numbers of endometria of gilts who received $IFNG^{-/-}$

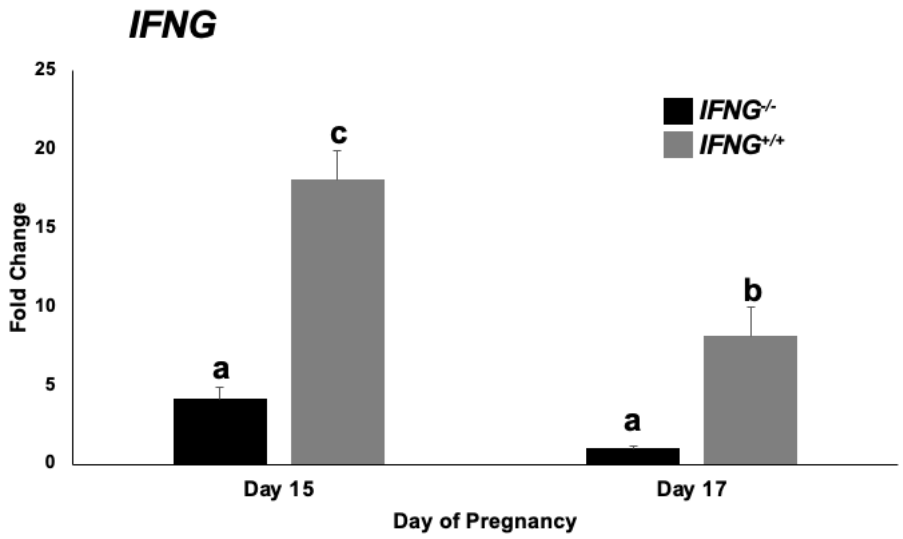

Figure 3.7: Fold change in expression of *IFNG* mRNA in conceptuses. *IFNG*$^{-/-}$ (black bar) and *IFNG*$^{+/+}$ (grey bar) fold change in gene expression is relative to the average ΔCT of day 17 *IFNG*$^{-/-}$. Fold change of *IFNG* (day P<0.0001), (genotype P=0.0006). *IFNG* gene expression data were log transformed to better satisfy the assumptions of the analysis of variance procedure. Data are presented as means with standard errors.

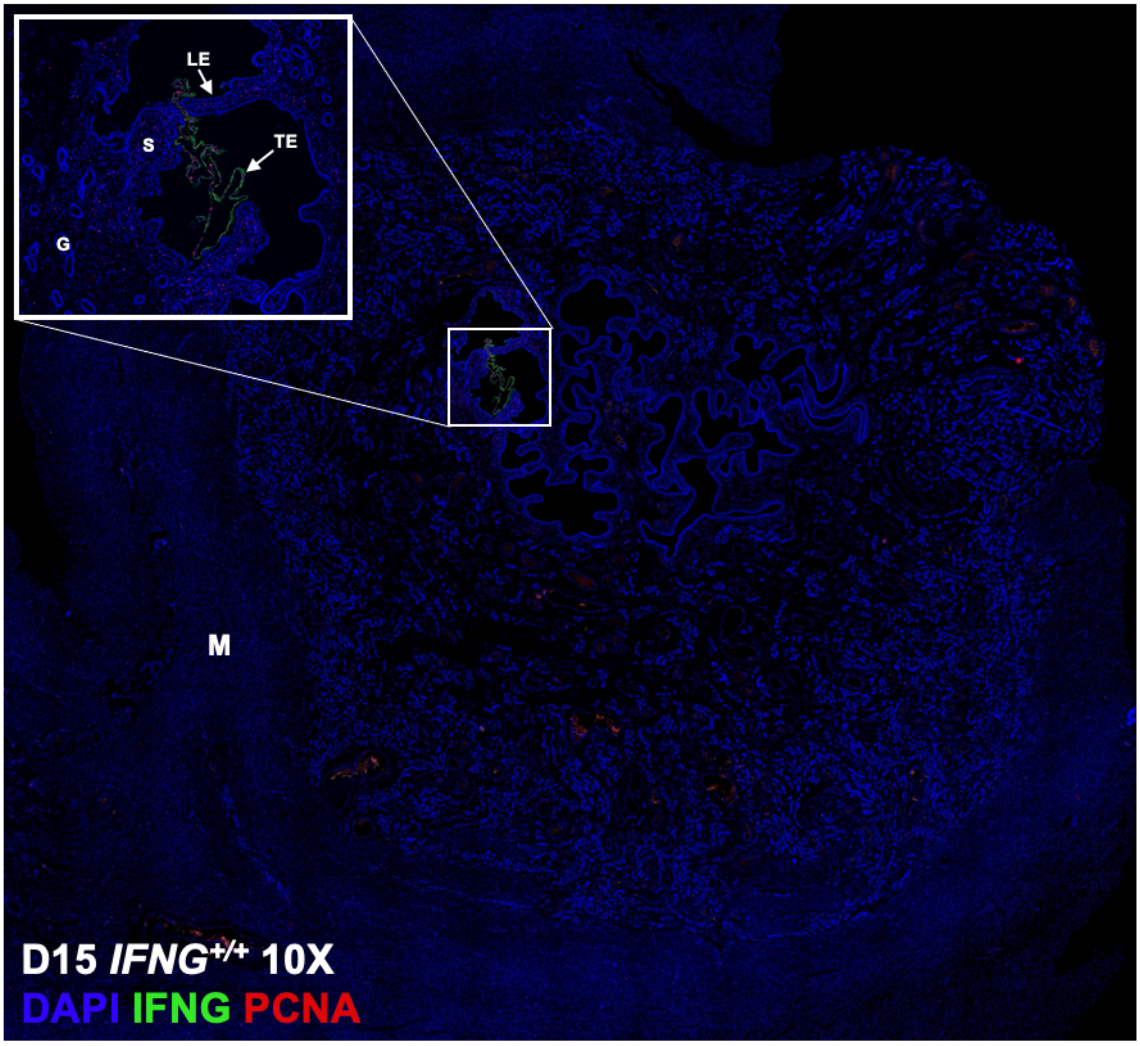

Figure 3.8: Immunofluorescence localization of *IFNG*$^{+/+}$ in conceptus and uterus on day 15 of pregnancy. Localization of IFNG and PCNA was limited to the conceptus and endometrium at the site of attachment of trophectoderm to uterine LE. Note the small area that the developing conceptus occupies on the mesometrial side of the endometrial folds in the uterine horn following rapid elongation. LE, endometrial luminal epithelium; TE, trophectoderm; S, endometrial stroma; G, uterine gland; M, myometrium.

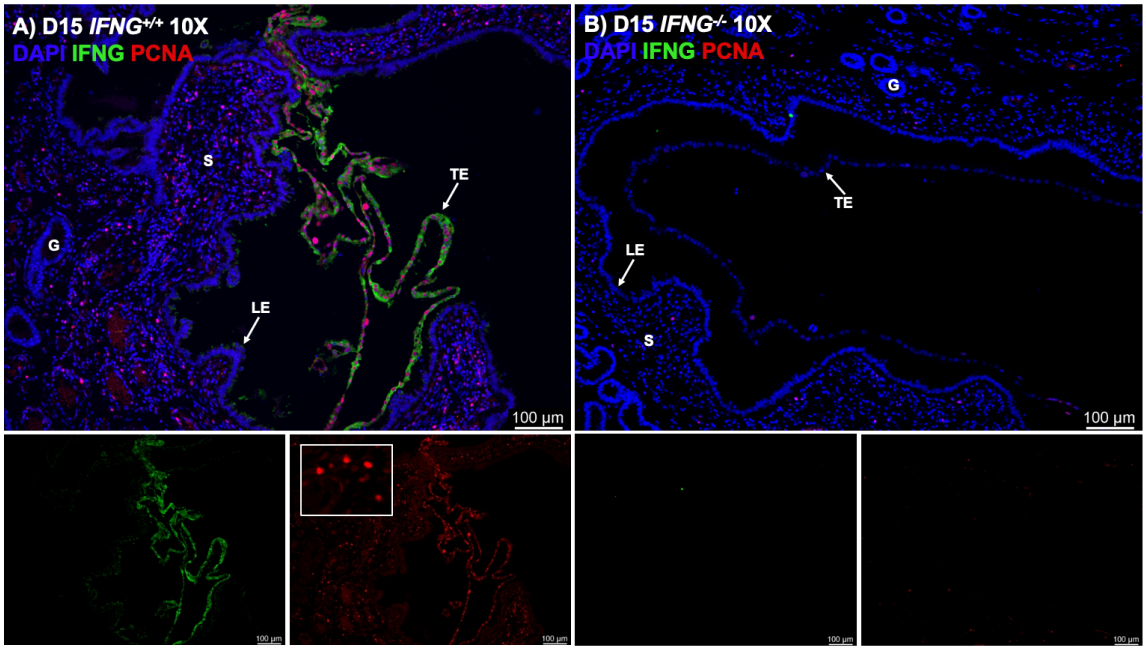

Figure 3.9: Immunolocalization of IFNG and PCNA in day 15 (**A**) *IFNG*$^{+/+}$ and (**B**) *IFNG*$^{-/-}$ conceptuses and surrounding uterine tissues. Immunofluorescence analyses were performed to localize IFNG (green) and PCNA (red) by using polyclonal primary antibodies. IFNG expression was localized to the TE of the *IFNG*$^{+/+}$ conceptuses (**A**) but was not detected in the *IFNG*$^{-/-}$ conceptuses (**B**) on day 15 of pregnancy. Similarly, PCNA is abundant in the conceptus TE and endoderm, as well as the endometrial stroma of the gilts who received *IFNG*$^{+/+}$ embryos (**A**). Several PCNA positive cells in the endometria of gilts who received *IFNG*$^{-/-}$ embryos were large and round (white box) indicating they are likely immune cells. There was relatively low PCNA detected in the conceptuses and endometria of the gilts who received *IFNG*$^{-/-}$ embryos (**B**). LE, endometrial luminal epithelium; TE, trophectoderm; S, endometrial stroma; G, uterine gland.

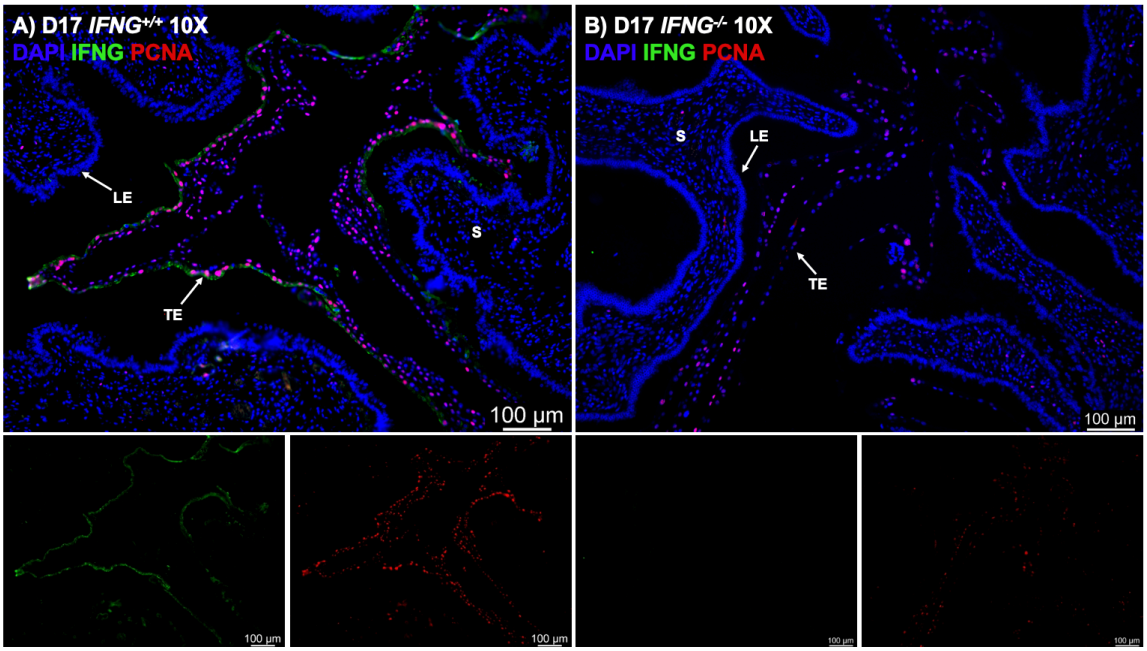

Figure 3.10: Immunolocalization of IFNG and PCNA in day 17 (**A**) *IFNG*$^{+/+}$ and (**B**) *IFNG*$^{-/-}$ conceptuses and surrounding uterine tissues. IFNG was expressed in the *IFNG*$^{+/+}$ conceptuses (**A**) but was not detected in the *IFNG*$^{-/-}$ conceptuses (**B**) on day 17 of pregnancy. Similarly, PCNA was expressed in the TE of the *IFNG*$^{+/+}$ conceptuses and endometria of the recipient gilts (**A**) whereas PCNA is almost undetectable in the *IFNG*$^{-/-}$ conceptuses and endometria of the recipient gilts (**B**). LE, endometrial luminal epithelium; TE, trophectoderm; S, endometrial stroma.

embryos on days 15 (Figure 3.9) and day 17 (Figure 3.10) of pregnancy. Total IFNG content in ULF of recipient gilts was less (P<0.002) when they had *IFNG*$^{-/-}$ conceptuses on days 15 and 17 of pregnancy (Figure 3.11). However, there was no effect of day (P=0.1104) on amounts of IFNG in the ULF in gilts who received *IFNG*$^{+/+}$ embryos between days 15 and 17 of pregnancy. There was a trend for a decrease in IFNG in ULF between day 15 and day 17 of pregnancy that was consistent with results of immunofluorescent localization of IFNG (Figure 3.10, Figure 3.11).

Expression of IFND, CYP19A1, and PTGS2 mRNA in Conceptuses

Expression of *PTGS2* mRNA was not affected statistically by genotype of conceptus; however, there was an effect of day (P < 0.0041) as expression increased ~2.5-fold from day 15 to day 17 for *IFNG*$^{-/-}$ conceptuses and ~5-fold for *IFNG*$^{+/+}$ conceptuses (Figure 3.12 A). There were no differences (P>0.05) in expression of mRNA for *IFND* and *CYP19A1* on days 15 and 17 of pregnancy (Figure 3.12 B, C).

Total Content of Estradiol-17β, IL18, and PG in Uterine Lumen Flush

The ULF collected on day 17 from recipient gilts who received *IFNG*$^{-/-}$ embryos that were not pregnant (n=6) were excluded from analysis of data. Total content of estradiol-17β in ULF of gilts who received *IFNG*$^{-/-}$ embryos was not affected by genotype (P>0.05) but increased (P=0.008) from day 15 to day 17 of pregnancy

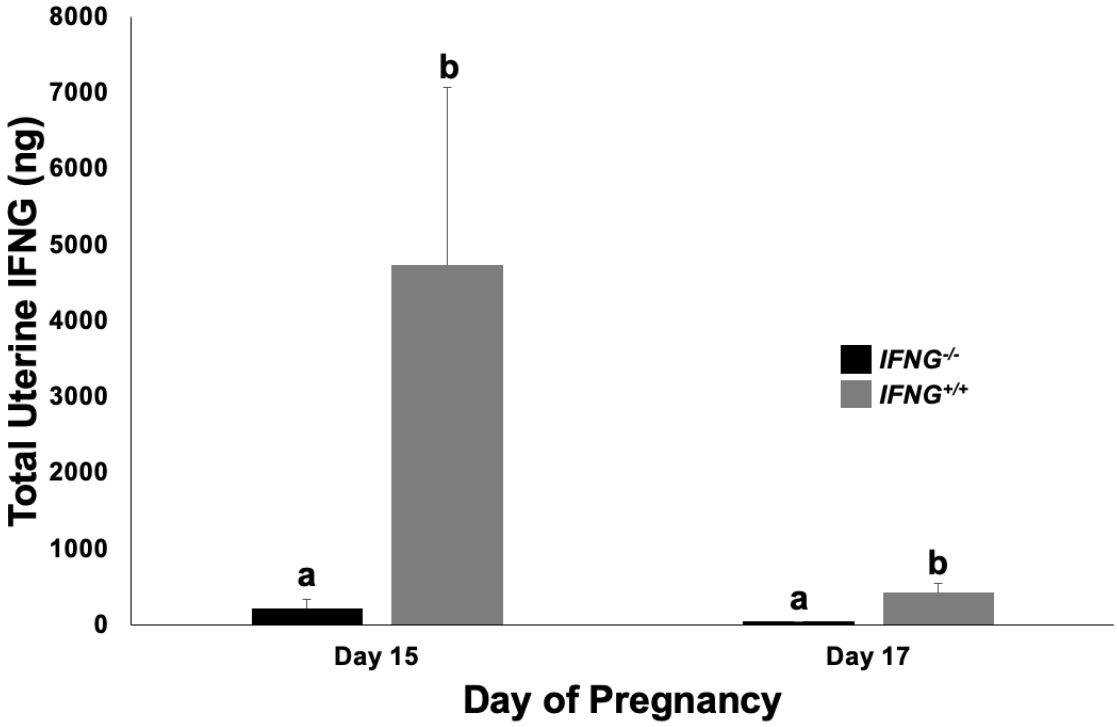

Figure 3.11: Total amounts of IFNG (ng) in uterine flushings collected from recipient gilts with either *IFNG*$^{-/-}$ (black bar) or *IFNG*$^{+/+}$ (grey bar) conceptuses on days 15 and 17 of pregnancy. An effect of genotype (P = 0.0018) was detected for total IFNG. Bars without a common superscript represent a statistical difference. Data on IFNG in ULF were log transformed to better satisfy the assumptions of the analysis of variance procedure. Data are presented as the means with standard errors.

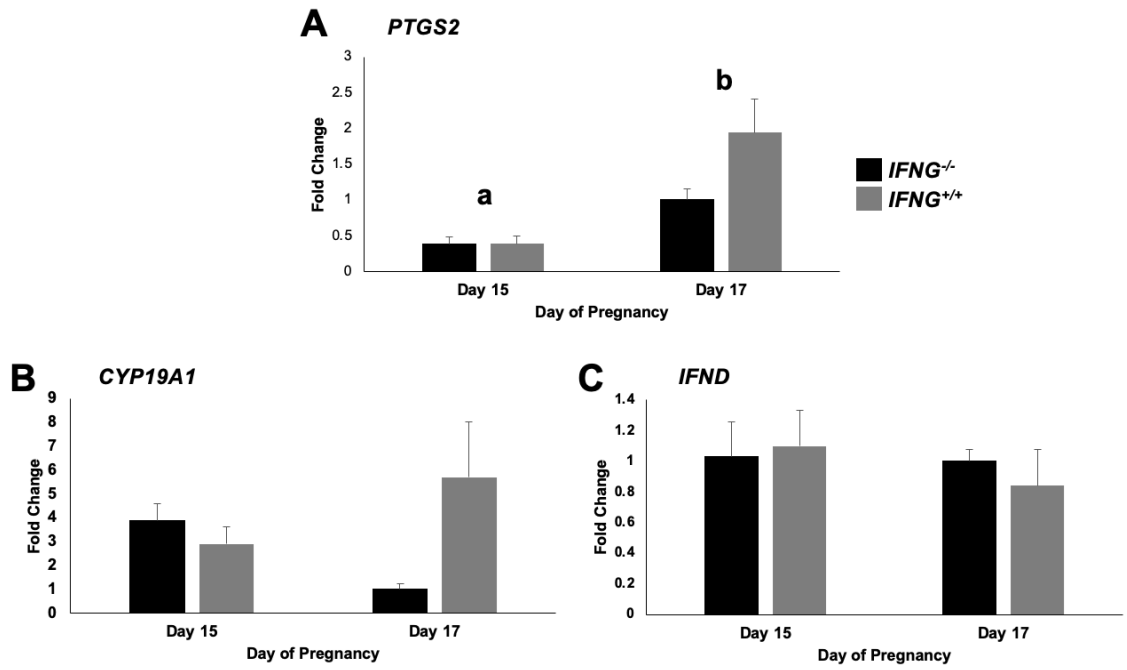

Figure 3.12: Fold changes in expression of *PTGS2*, *CYP19A1*, and *IFND* mRNA by conceptuses on days 15 and 17 of pregnancy. *IFNG*$^{-/-}$ (black bar) and *IFNG*$^{+/+}$ (grey bar) fold change in gene expression is relative to the average ΔCT of day 17 *IFNG*$^{-/-}$. **A)** Expression of *PTGS2* was affected by day ($P<0.0041$). **B, C)** Fold-changes in expression of *CYP19A1* and *IFND* mRNA were not different ($P>0.05$). Bars without a common superscript represent a statistical difference. The data for *CYP19A1* and *IFND* were square root transformed while the PTGS2 data was log transformed to better satisfy the assumptions of the analysis of variance procedure. Data are presented as the means with standard errors.

(Figure 3.13 A). There were no differences (P>0.05) in ULF of IL18 and total PG on days 15 and 17 of pregnancy (Figure 3.13 B, C).

Endometrial TNFSF10, IDO1, CIITA mRNA Expression

Yoo et al. (2020) indicated that porcine endometrial explants treated in culture with IFNG had increased expression of *TNFSF10*, *IDO1*, and *CIITA*. However, in the present study, expression of *TNFSF10*, *IDO1* and *CIITA* mRNA was not affected by day of pregnancy or genotype of conceptus (Figure 3.14).

Conceptus Development and Immunolocalization of CD3+ and Mast Cells in the Uterus

Evaluation of H&E sections of the uteri containing a conceptus indicated that the morphology of cells of the conceptuses and the presence of immune cells in the underlying endometrium were different when *IFNG*$^{-/-}$ conceptuses were present. Elongated, filamentous *IFNG*$^{+/+}$ conceptuses induced endometrial folding on day 15 of pregnancy. The endometrial stroma beneath the site of conceptus attachment contained a large number of immune cells. The endometrium directly opposite to the site of conceptus attachment appeared to recruit fewer immune cells (Figure 3.15). On day 17, the elongating conceptuses continued to attach to the uterine LE which formed pinopodes, but the numbers of immune cells were reduced (Figure 3.16). In contrast, although filamentous *IFNG*$^{-/-}$ conceptuses were present, cellular morphology suggested that these conceptuses were deteriorating, there was less endometrial folding and fewer immune cells at

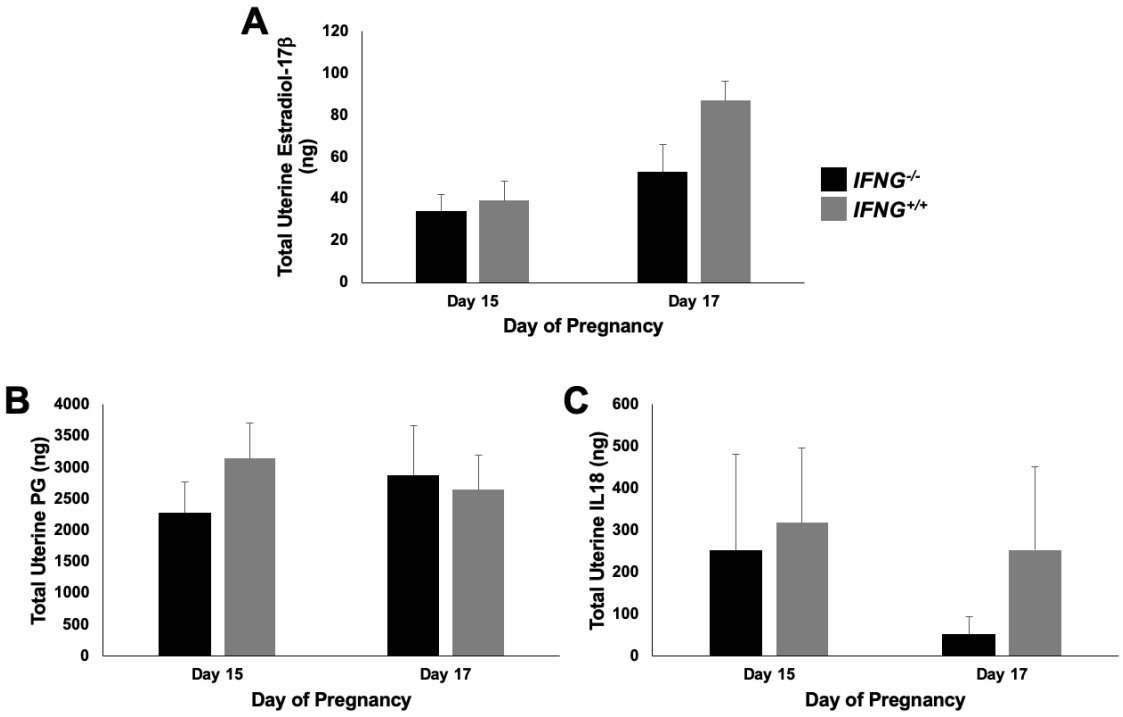

Figure 3.13: Total content (ng) of estradiol-17β, prostaglandins (PG), and interleukin 18 (IL18) in uterine flushings collected from recipient gilts containing either *IFNG*$^{-/-}$ (black bar) or *IFNG*$^{+/+}$ (grey bar) conceptuses on days 15 and 17 of pregnancy. **A)** The abundance of *estradiol-17β* was affected by day *(P = 0.008).* **B, C)** The amounts of PG and IL18 were not different due to day or genotype of conceptus (P>0.05). Bars without a common superscript represent a statistical difference. The IL18 data were log transformed to better satisfy the assumptions of the analysis of variance procedure. Data for PG and estradiol-17β did not require transformation. Data are presented as the means with standard errors.

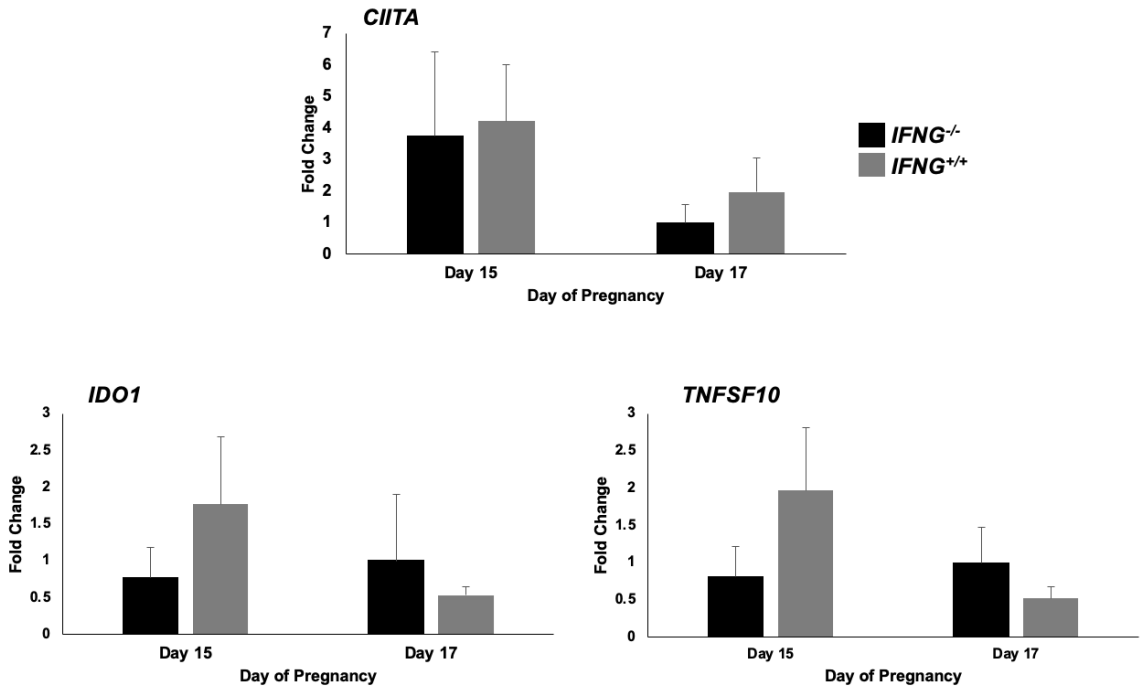

Figure 3.14: Fold changes in endometrial expression of *CIITA*, *IDO1*, and *TNFSF10* mRNA. *IFNG*$^{-/-}$ (black bar) and *IFNG*$^{+/+}$ (grey bar) fold change in gene expression is relative to the average ΔCT of day 17 recipient gilts with *IFNG*$^{-/-}$ conceptuses. Effects of day and genotype of conceptus were not different (P>0.05) for any of these mRNA. The *CIITA* data were log transformed while data on *IDO1* were square root transformed to better satisfy the assumptions of the analysis of variance procedure. The *TNFSF10* data did not require transformation. Data are presented as the means with standard errors.

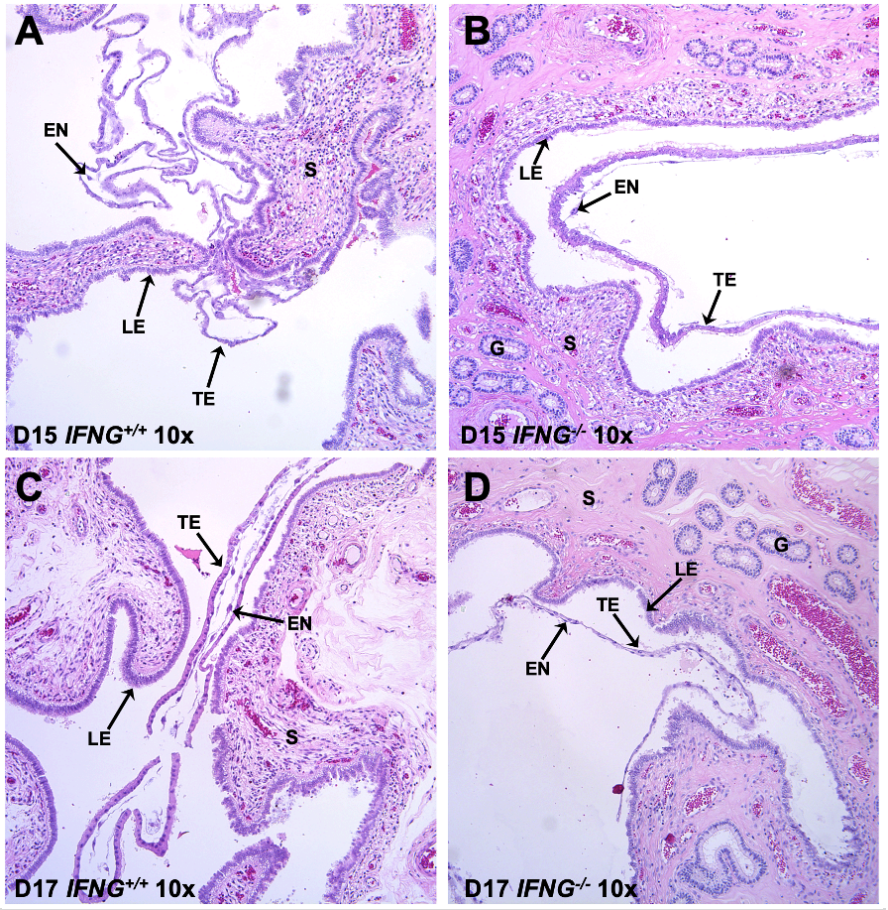

Figure 3.15: Pregnant uteri were examined following H&E staining to assess effects of genotype of conceptus and day of pregnancy for gilts with *IFNG*$^{+/+}$ and *IFNG*$^{-/-}$ conceptuses. There appeared to be more immune cells (round, dark purple) present in the endometrial stroma at areas of conceptus attachment in *IFNG*$^{+/+}$ conceptus pregnancies (**A, C**) compared to *IFNG*$^{-/-}$ conceptus pregnancies (**B, D**). There appeared to be increased endometrial folding in association with attachment of *IFNG*$^{+/+}$ conceptuses (**A, C**) compared to *IFNG*$^{-/-}$ conceptuses (**B, D**). Uteri of *IFNG*$^{-/-}$ embryo recipient gilts (**B, D)** appeared very hyperemic compared to *IFNG*$^{+/+}$ recipient gilts (**A, C**). LE, endometrial luminal epithelium; TE, trophectoderm; EN, endoderm; G, uterine gland; S, endometrial stroma.

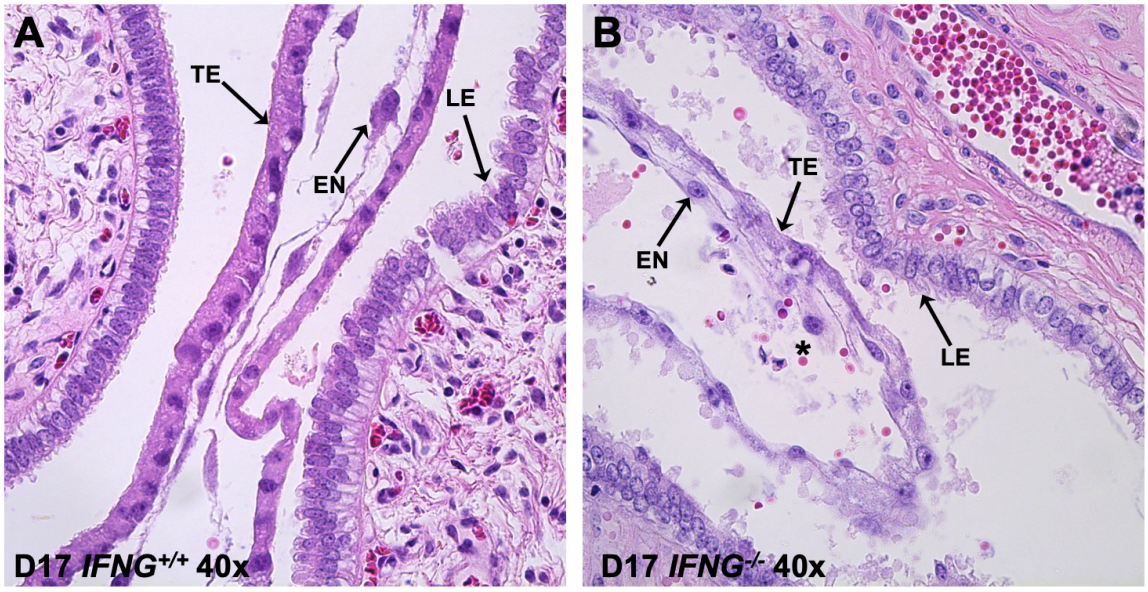

Figure 3.16: Conceptuses were subjected to H&E staining to assess differences in morphology between *IFNG*$^{+/+}$ and *IFNG*$^{-/-}$ conceptuses on day 17 of pregnancy. The *IFNG*$^{+/+}$ conceptuses were well developed with normal appearing cells, intact, and viable (**A**) compared to *IFNG*$^{-/-}$ conceptuses with degenerating cells, cellular debris, and appearing to be unviable (**B**). Erythrocytes (*****) were present in the uterine lumen surrounding the *IFNG*$^{-/-}$ conceptuses which is degenerating along with alternations in the morphology of the uterine LE (**B**) compared to normal attachment of *IFNG*$^{+/+}$ conceptuses (**A**). Additionally, there appeared to be fewer immune cells (round, dark purple) being recruited to the site of implantation of *IFNG*$^{-/-}$ conceptuses (**B**) compared to *IFNG*$^{+/+}$ conceptuses (**A**). There was also an endometrial hyperemic response in gilts with *IFNG*$^{-/-}$ conceptuses compared gilts with *IFNG*$^{+/+}$ conceptuses. LE, endometrial luminal epithelium; TE, trophectoderm; EN, endoderm.

attachment sites of conceptuses on day 15 compared to *IFNG*$^{+/+}$ conceptus pregnancies. By day 17, the TE of *IFNG*$^{-/-}$ conceptuses was degenerating and undergoing fragmentation, while the endometrium was very hyperemic. There were a large number of erythrocytes present in the uterine lumen in contact with the conceptus (Figure 3.16). Additionally, there were fewer conceptuses present in the uteri of gilts that received *IFNG*$^{-/-}$ embryos as compared to that for gilts that received *IFNG*$^{+/+}$ embryos.

The numbers of CD3+ cells and mast cells visually appeared greater in the endometrial stroma beneath the conceptus attachment sites for gilts who received *IFNG*$^{+/+}$ embryos on days 15 (Figure 3.17) and 17 (Figure 3.18) compared to gilts who received *IFNG*$^{-/-}$ embryos. Although the numbers of CD3+ cells and mast cells appeared to decline between day 15 and day 17 (Figure 3.18), they remained less abundant in the stroma of *IFNG*$^{-/-}$ embryo recipient gilts.

Co-Embryo Transfer of IFNG$^{-/-}$ and IFNG$^{+/+}$ Blastocysts

In an attempt to rescue the development of *IFNG*$^{-/-}$ embryos, gilts received either *IFNG*$^{+/+}$ embryos (n=6) or both *IFNG*$^{-/-}$ and *IFNG*$^{+/+}$ embryos (n=5). Recipient gilts were euthanized on day 30 of pregnancy and their reproductive tracts removed for collection and genotyping of embryos. On day 30 of pregnancy, 50% of *IFNG*$^{+/+}$ embryo recipient gilts (3/6) contained 3-4 healthy, viable embryos, however, only 1 of 5 *IFNG*$^{-/-}$ and *IFNG*$^{+/+}$ co-transferred recipient gilts maintained pregnancy to day 30. Of the four failed *IFNG*$^{-/-}$ and *IFNG*$^{+/+}$ co-transferred

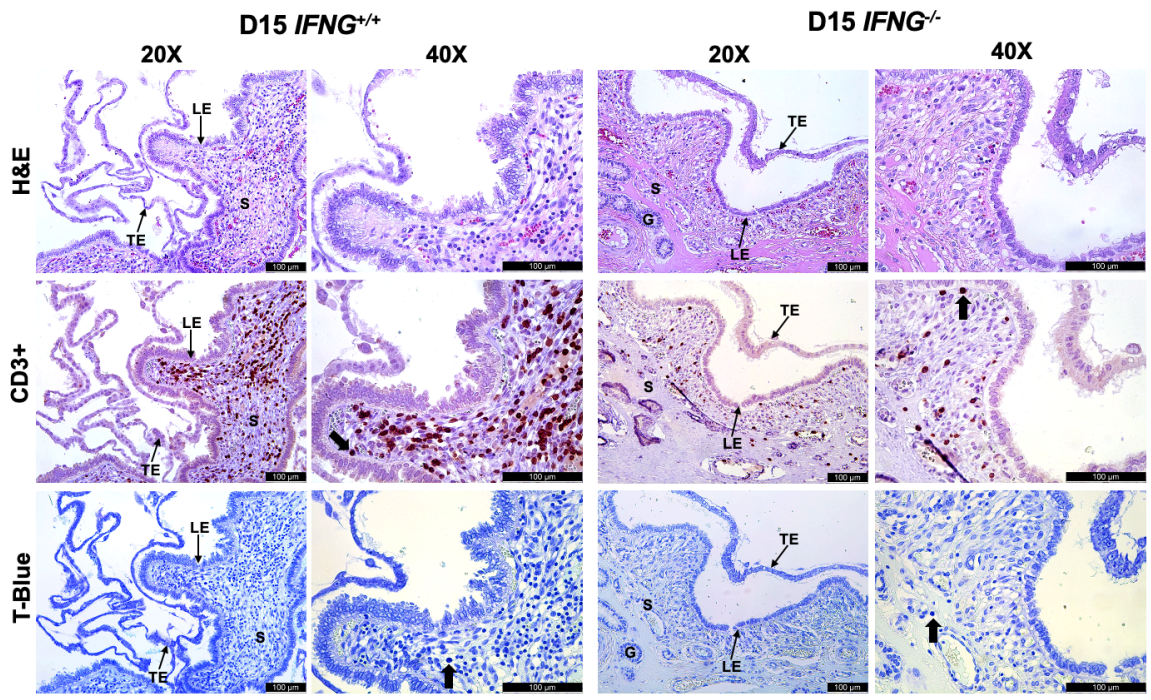

Figure 3.17: Localization of immune cells in the endometria of gilts who received *IFNG*$^{+/+}$ and *IFNG*$^{-/-}$ embryos on day 15 of pregnancy. Immune cells were localized to the endometrial stroma at areas of conceptus attachment in both *IFNG*$^{+/+}$ and *IFNG*$^{-/-}$ pregnancies. There appeared to be increased numbers of CD3+ cells (black arrow) and mast cells (black arrow) localized to the endometrial stroma beneath the uterine LE of gilts who received *IFNG*$^{+/+}$ embryos, but not gilts who received *IFNG*$^{-/-}$ embryos. LE, endometrial luminal epithelium; TE, trophectoderm; EN, endoderm; G, uterine gland; S, endometrial stroma.

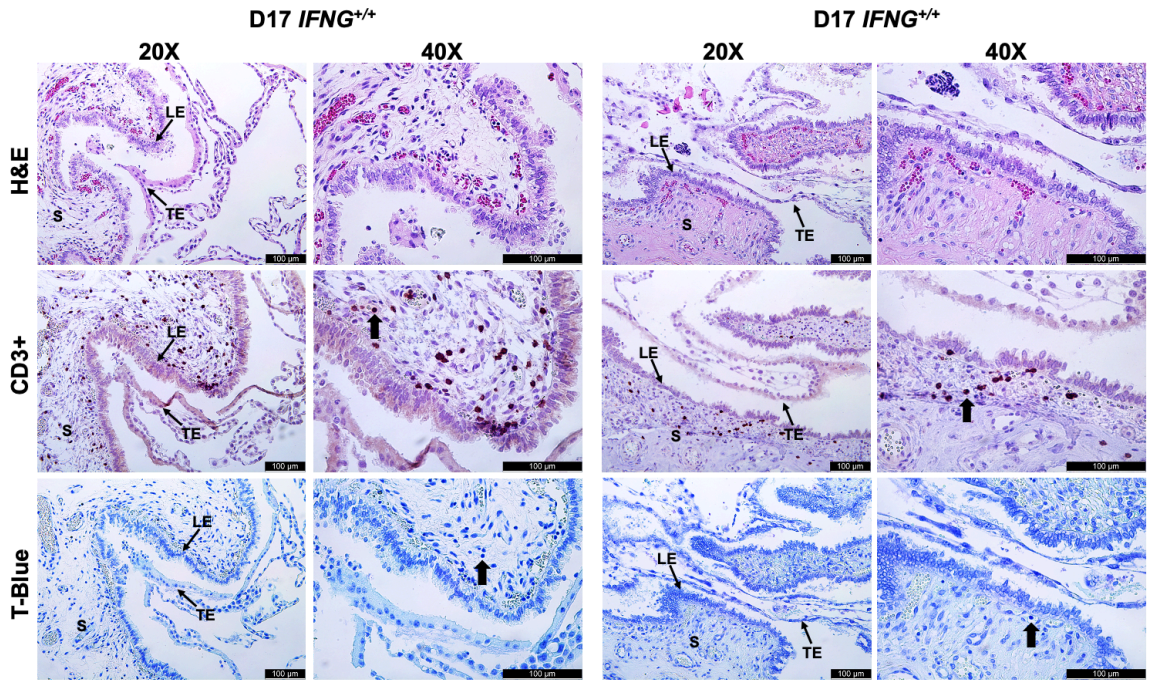

Figure 3.18: Localization of immune cells in the endometria of gilts who received *IFNG*$^{+/+}$ and *IFNG*$^{-/-}$ embryos on day 17 of pregnancy. Immune cells were localized to areas of conceptus attachment in both *IFNG*$^{+/+}$ and *IFNG*$^{-/-}$ pregnancies. The numbers of CD3+ cells (black arrow) and mast cells (black arrow) appeared to be less on day 17 than day 15 but remained more abundant in the endometria of gilts who received *IFNG*$^{+/+}$ embryo than gilts who received *IFNG*$^{-/-}$ embryos. LE, endometrial luminal epithelium; TE, trophectoderm; EN, endoderm; S, endometrial stroma.

pregnancies, two recipient gilts returned to estrus by day 20. The uteri of the other two gilts contained fluid, but there was no evidence of embryos when ultrasound was performed on day 28. The uteri of these two gilts euthanized on day 30 did not contain embryos and the uterus was highly edematous, hyperemic and inflamed. The one pregnant *IFNG*$^{-/-}$ and *IFNG*$^{+/+}$ co-transferred recipient gilt contained 5 embryos, 4 of which were degenerating (Figure 3.19). Genotyping indicated that all five remaining embryos were *IFNG*$^{+/+}$ (Figure 3.20).

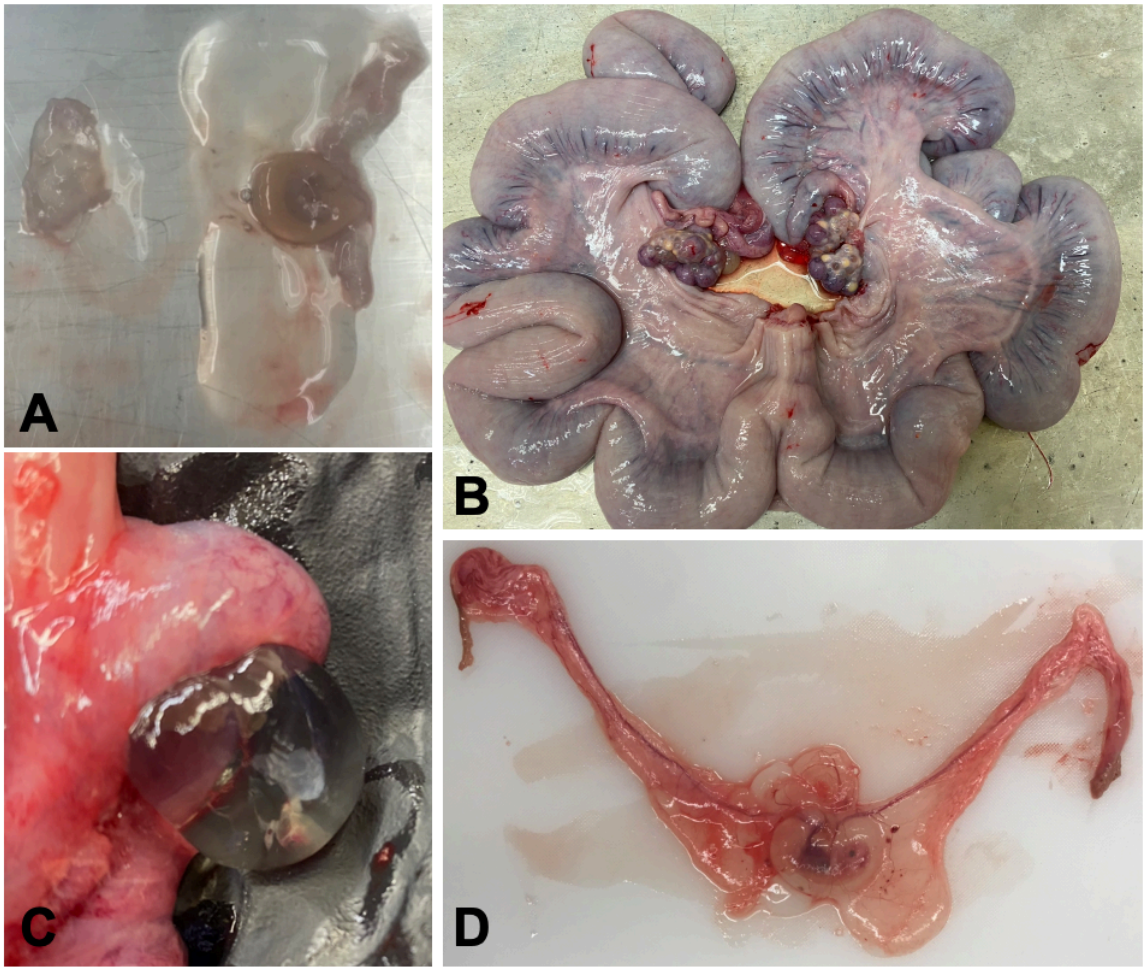

Figure 3.19: Embryos collected on day 30 of pregnancy from recipient gilts following *IFNG*$^{-/-}$ and *IFNG*$^{+/+}$ co-embryo transfer. Five embryos were collected, one embryo appeared normal (**D**) and four embryos were degenerating (**A, C**). The reproductive tract of the recipient gilt was hyperemic and edematous (**B**).

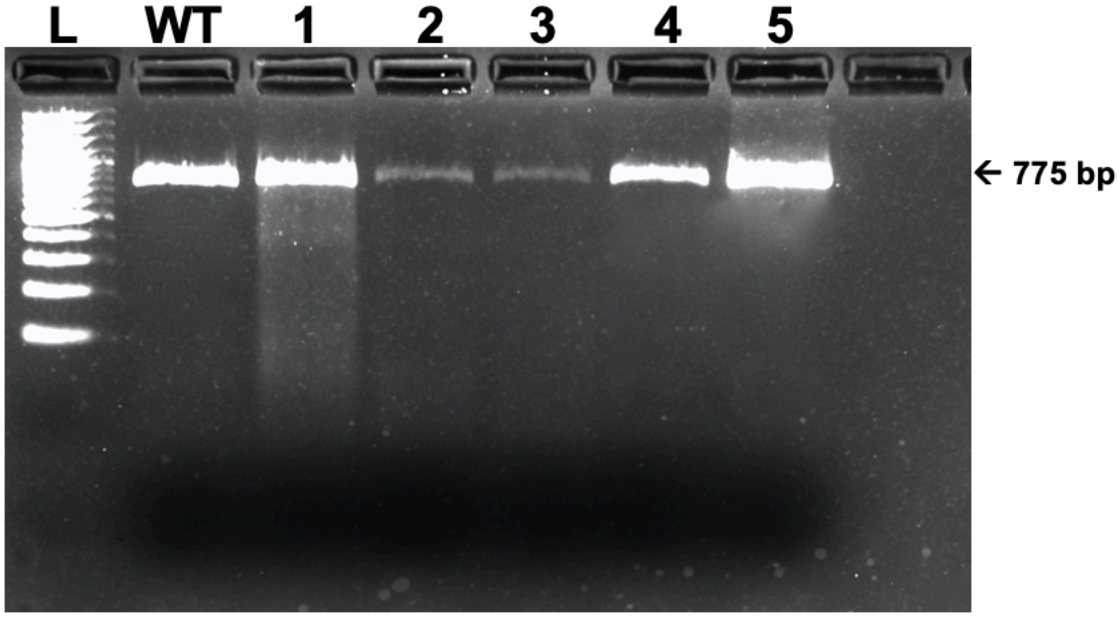

Figure 3.20: Genotyping of *IFNG*$^{-/-}$ and *IFNG*$^{+/+}$ embryos following co-embryo transfer. The four degenerating embryos were all *IFNG*$^{+/+}$ (wells 1-4), as was the one healthy embryo (well 5).

3.4 DISCUSSION

During early pregnancy, developing pig conceptuses synthesize and secrete biological factors into the uterine lumen to prime the maternal system for implantation of the conceptus and to signal maternal recognition of pregnancy. The early conceptus and later the placenta must also provide protection from maternal immune-mediated rejection of the developing semi-allogeneic conceptus. Peri-implantation pig conceptuses produce interferons, that are proposed to regulate a number of endometrial responses to establish pregnancy in the pig (Bazer & Johnson 2014; Geisert *et al.* 2014; Geisert *et al.* 2015).

Interferons were originally discovered for their ability to 'interfere' in the multiplication of new active viruses (Isaacs et al. 1952). Since then, there has been considerable literature published on the role of conceptus-derived interferons in sheep, cattle, and pigs. While the ruminant conceptus produced only type I IFNT (Martal *et al.* 1979; Roberts 1991), pig conceptuses produce both a type I (IFND) and type II (IFNG) interferon between days 12 to 20 of pregnancy (La Bonnardiere *et al.* 1991). Conceptus-derived IFNG accounts for the majority (~75%) of pig conceptus IFN antiviral activity which peaks on days 15-16 of pregnancy (Lefèvre et al. 1990).

While IFNG is presently the only type II IFN documented to be produced in mammals, the novel IFND first described in the pig may consist of 10 to 11 *IFND* genes or transcripts (Cochet *et al.* 2009). Conserved sequences among the multiple *IFND* genes makes it difficult to exactly determine the specific porcine IFND expressed during early conceptus development. Cochet et al. (2009)

suggested that the porcine *IFND-1* gene is most likely the only one expressed by the conceptus during early development. Grouping the different *IFND* genes together based on high sequence homology (Supplement Table 1) indicated that *IFND-1* and *IFND-2* (high sequence homology) were the only *IFND* genes expressed by pig conceptuses on days 14 and 17 of pregnancy (Supplement Figure 1) (Johns, Wells and Geisert unpublished data).

The roles of IFNG in regulating activation of innate and adaptive immune responses, inhibition of cell proliferation, apoptosis and increasing endometrial angiogenesis and vascular remodeling, suggests that conceptus IFNG expression plays a major role in the establishment and maintenance of pregnancy in the pig. The present study utilized CRISPR/Cas9 genome editing to successfully ablate expression of type II IFNG by pig conceptuses during the peri-implantation period. This *in vivo* model allowed determination of the importance of IFNG in regulating the maternal uterine endometrium to influence development of the conceptus during pregnancy.

IFNG$^{-/-}$ embryos developed to blastocysts, hatched and were capable of undergoing rapid trophoblast elongation. Normal embryonic and conceptus development would be expected as *IFNG* gene expression and protein synthesis are initiated immediately following the period of rapid elongation by pig conceptuses (Ross *et al.* 2003). Although gilts that recieved *IFNG*$^{+/+}$ and *IFNG*$^{-/-}$ embryos contained elongated conceptuses on day 15, the trophoblast of *IFNG*$^{-/-}$ conceptuses appeared to be less developed with much less mitotic activity and evidence of early stages of degeneration. The lack of normal TE development

and degeneration of the *IFNG*$^{-/-}$ conceptuses continued to day 17 of pregnancy when uteri of only 2 of 8 *IFNG*$^{-/-}$ embryo recipients contained conceptuses. In the two *IFNG*$^{-/-}$ recipient gilts containing conceptuses, the trophoblast of the conceptuses was thin and fragmented, and TE cells were not proliferating as rapidly and/or conceptus development was failing. Although IFNG expression was inhibited in *IFNG*$^{-/-}$ conceptuses, *CYP19A1* and *PTGS2* gene expression and uterine luminal content of E2 and PG were not different from that for *IFNG*$^{+/+}$ conceptuses. Interleukin 18, also known as IFNG inducing factor, is a member of the IL1 family of pro-inflammatory cytokines believed to play a significant role in implantation in pigs (Ashworth et al. 2010). Endometrial secretion of IL18 was not different between *IFNG*$^{+/+}$ and *IFNG*$^{-/-}$ embryo recipients. This is consistent with the ability of *IFNG*$^{-/-}$ conceptuses to secrete estrogen which enhances endometrial release of IL18 during early pregnancy (Ashworth *et al.* 2010). These results suggest that on days 15 and 17 of pregnancy, the lack of activation for the downstream pathways of IFNG that are responsible for modulating the endometrium during conceptus attachment results in endometrial inflammation and degeneration of the conceptuses. In addition, although E2 and PG in uterine flushings were similar on day 15, the CL were regressing in a number of the *IFNG*$^{-/-}$ embryo recipient gilts. The inflammation and hyperemic effects were even more evident on day 17 when a large number of erythrocytes were present in the uterine lumen and in contact with the conceptuses. Interestingly, transcriptomic analysis of conceptus mRNA revealed no difference in expression of type I IFND due to conceptus genotype indicating that there is no ‘rescue effect’ of a type I

IFN in the absence of type II IFNG. Since type I and type II IFN bind to different receptors and activate many separate cell-signaling pathways (Casazza *et al.* 2020), the loss of conceptuses and uterine inflammation during early pregnancy appears to be directly due to lack of expression of IFNG by pig conceptuses. The loss of *IFNG*$^{-/-}$ conceptuses is not only local, but there is an effect on the entire uterus as co-transfer with *IFNG*$^{+/+}$ embryos resulted in pregnancy failure and all embryos recovered on day 30 were *IFNG*$^{+/+}$.

Previous research investigated the possible role of IFNG in regulating maternal endometrial immunity during the implantation period in pigs by treating endometrial explants of cyclic animals with IFNG *in vitro* (Yoo *et al.* 2020a; Yoo *et al.* 2020b). Endometrial explants cultured with IFNG upregulated *CIITA*, *IDO1*, and *TNFSF10* mRNA among several other IFNG-regulated genes and MHC class II molecules. Expression of these genes is greatest on day 15 of pregnancy when peak *IFNG* expression by the pig conceptus occurs. IDO1 metabolizes the essential amino acid L-tryptophan into N-formylkynurenine (Shimizu et al. 1978) that inhibits T cell responses through the local depletion of tryptophan which is necessary for proliferation of T cells (Grohmann et al. 2003; Yoo *et al.* 2020a). Endometrial expression of IDO is believed to be involved in maternal immune tolerance in pigs because in human pregnancy, IDO is expressed in the placenta and contributes to fetal tolerance (Kudo et al. 2004). Pregnant mice treated with an IDO inhibitor experience activation of T cells at the maternal-fetal interface, and rejection of conceptuses (Munn et al. 1998). Additionally, *TNFSF10* is expressed in endometrial LE cells in response to IFNG on day 15 (Yoo *et al.*

2020a). *TNFSF10* mediates the apoptosis pathway by binding to its death-domain containing receptors (TNFSFR10A and TNFSFR10B) (Azahri & Kavurma 2013). Although there was a trend for greater expression of *CIITA*, *IDO1*, or *TNFSF10* there was no difference in endometrial mRNA expression between *IFNG*$^{-/-}$ and *IFNG*$^{+/+}$ embryo recipient gilts on either day 15 or day 17 of pregnancy. The failure to detect differences in endometrial expression of *CIITA*, *IDO1*, or *TNFSF10* may be related to directly stimulating the entire endometrial explant *in vitro* with IFNG compared to the very small localized endometrial response to conceptus IFNG during pregnancy (Joyce *et al.* 2007a). The expression of these IFNG stimulated genes could be diluted with the vast amount of endometrium that is not in contact with the conceptus *in vivo* (Figure 3.9). It is also possible that other conceptus-derived factors such as IFND are affecting endometrial gene activation in the absence of IFNG.

Both type I and type II IFN can induce STAT1 through the classical JAK-STAT signaling pathway wherein IFNG activation of cells via the IFNGR1:IFNGR2 receptor complex leads to activation GAS elements and induction of transcription (Decker *et al.* 1997). Joyce et al. (2007) determined that endometrial STAT1 is differentially regulated in a temporal and cell type-specific manner in response to conceptus estrogen and IFN in the pig. Specifically, estrogen treatment alone increases STAT1 in LE whereas infusion of conceptus secretory proteins containing conceptus IFN increased STAT1 in the stroma but not LE. It is possible that in the pig, the high levels of IFNG secretion act on the stromal cells and synergize with IFND to up-regulate STAT1 in close proximity to

the implanting pig conceptus (Joyce *et al.* 2007a). Interestingly, early administration of estrogen to pregnant gilts results in degeneration of conceptuses in pigs between day 14 and day 18 of pregnancy which is similar to that noted in the present study (Blair et al. 1991). Joyce et al. (2007) proposed that conceptus degeneration induced by inappropriate endometrial stimulation by exogenous estrogen compromised the paracrine release of IFNG, required for stromal expression of STAT1 leading to conceptus loss.

Previous studies have shown that IFNG alters tight junctions of uterine LE and stimulates expression of MHC class II by stromal cells at points of conceptus attachment to uterine LE suggesting a role for IFNG in local regulation of maternal immune cell activity during early pregnancy (Cencic & La Bonnardiere 2002; Joyce *et al.* 2008; Kim *et al.* 2012; Han *et al.* 2017; Yoo *et al.* 2020a). Natural killer (NK) cells, macrophages, dendritic cells, and mast cells (MC) are key players in the innate immune response at the fetal-maternal interface (Nair *et al.* 2017). In the mouse, uterine NK (uNK) cells are the predominant leukocyte (70%) present in the uterus during early pregnancy and are responsible for producing 90% of pregnancy-associated IFNG (Ashkar & Croy 2001). The same study found that implantation sites in uNK cell-deficient and IFNG-signal-disrupted mice display anomalies in the decidua and its spiral arteries. However, Ashkar and Croy (2001) reported that reconstitution of uNK cell-deficient females with bone marrow containing normal NK cell progenitors, established uNK cells and reversed the anomalies. Additionally, uterine mast cells (uMC) have a unique phenotype compared to peripheral mast cells as they increase and expand in the

uteri of women during pregnancy (Mori et al. 1997). Another study found that implantation and spiral artery remodeling are severely impaired in the absence of uNK cells in mice (Woidacki et al. 2013).

In contrast to the mouse, the pig conceptus is the major source of IFNG in the uterus, although there are numerous IFNG positive T cells present in the uterine stroma. During the period of implantation in pigs, there is a localized increase in the accumulation of leukocytes beneath the site of implantation (Ashkar & Croy 2001; Murphy *et al.* 2009; Kim *et al.* 2012; Yoo *et al.* 2019). Conceptus IFNG positively influences the expression of chemokines in endometrial cells (Złotkowska & Andronowska 2019). McLendon et al. (2020) indicated that IFNG upregulates expression of chemokines CXCL9, CXCL10, and CXCL11 in the endometrium. Conceptus INFG was associated with the accumulation of proliferating PCNA positive T cells and T cell co-signaling receptor. The major population of T cells were CD4+ with small numbers of CD8+, CD4+CD25+ and CD4+CD8+ cells (McLendon *et al.* 2020). These data suggest that conceptus secretion of IFNG promotes a highly modulated inflammatory response in the endometrium that recruits T cells to the sites of conceptus attachment.

Results from our *IFNG*$^{+/+}$ recipient gilts in the present study are consistent with those reported by McLendon et al. (2020). The number of CD3+ cells and mast cells were greatly increased in the endometrial stroma beneath the conceptus attachment sites of *IFNG*$^{+/+}$ recipient gilts on day 15 of pregnancy, but their numbers decreased by day 17. The number of stromal PCNA positive T

cells in the uterine stroma were also greater on day 15 than day 17 of pregnancy. The accumulation of CD3+ cells and mast cells were localized to the site of conceptus attachment as endometrium directly opposite the site of conceptus attachment contained far fewer immune cells as was noted for the endometrium not associated with implantation of the conceptus. Recruitment of immune cells to the endometrial stroma at the site of conceptus attachment was dependent upon conceptus IFNG secretion as the stroma beneath the attaching *IFNG*$^{-/-}$ conceptuses appeared to recruit fewer numbers of CD3+ cells and mast cells. In fact, the numbers of CD3+ cells and mast cells were similar to those in areas of the endometrium in which there was no conceptus. In addition to lack of recruitment of immune cells to the site of attachment, there was an absence of conceptus-induced endometrial folding and formation of LE pinopodes by uterine LE when *IFNG*$^{-/-}$ conceptuses were present. Thus, loss of conceptus expression IFNG results in an absence of immune cell recruitment to the implantation site, abnormal attachment to the uterine LE, and an inflammatory response that results in degeneration of the conceptuses.

It is possible that the lack of IFNG expression results in an imbalance with IFND and possible activation of IFNG-secreting T cells. Elevated type I IFN levels during pregnancy may be pathogenic as seen in diseases that increase the production of type I IFN leading to miscarriage (Crow & Manel 2015; Mor et al. 2017). IFNG assists in implantation, endometrial vascular remodeling, angiogenesis at sites of implantation, as well as maintenance of the decidual component of the placenta in murine pregnancies (Murphy *et al.* 2009), however,

excessive amounts of IFNG can be detrimental to embryonic survival in the mouse. Intriguingly, *IFNG*$^{-/-}$ mice survive to term (Ashkar & Croy 1999), whereas in the present study with pigs, *IFNG*$^{+/+}$ embryos were unable to rescue pregnancies when *IFNG*$^{-/-}$ embryos were co-transferred with *IFNG*$^{+/+}$ embryos.

The present study establishes that expression of IFNG by porcine conceptuses is not essential for their early development and rapid elongation, or establishment of pregnancy. However, conceptus IFNG is essential for immune cell recruitment to sites of implantation of the conceptus after day 15 of pregnancy. Thus, IFNG from pig conceptuses elicits a critical, localized immune response at the site of conceptus attachment that is essential for the establishment of successful pregnancies.

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Supplemental Data

MATERIALS & METHODS

Acquisition of Total Conceptus RNA

Conceptus RNA samples used to synthesize complementary DNA (cDNA) were obtained from day 14, 17, and 30 wild-type (WT) cloned conceptuses used in thesis research performed by (Pfeiffer *et al.* 2019). Conceptus RNA was isolated, quantified, and visualized for integrity as described previously (Pfeiffer *et al.* 2019).

Reverse Transcriptase and Primer Design for Type I Interferon Delta and Type II Interferon Gamma Gene Expression Analysis

Total conceptus RNA was reverse transcribed in a 20 µL reaction mix using iScript RT Supermix (BioRad, 1708841). Amplification of cDNA was performed in an Eppendorf Mastercycler Pro and incubated at 25°C for 5 min, reverse transcribed at 42°C for 30 min, and finally heat-inactivated at 85°C for 5 min. Negative controls for each sample were prepared without the iScript RT Supermix to test for DNA contamination. Stocks of cDNA were stored at -20°C until used for PCR amplification. Primers for porcine beta-actin were used as a housekeeping gene to confirm that all conceptus cDNA was viable. One unique primer pair was designed to target Exon 3 of *IFNG* which is synthesized from a single gene. Three unique primer pairs were designed to target three groupings of the 11 different IFND genes based on high similarity to each other (Table S.1).

Table S.1 RT-PCR Primers for IFND and IFNG

Gene	RefSeq Gene	Primers 5' → 3'	Source
BETA-ACTIN	**DQ178122.1**	TCTGGCACCACACCTTCT	(Erkins et al., 2006)
	Provisional	TGATCTGGGTCATCTTCTCAC	
IFNG	**NM_213948.1**	CCATTCAAAGGAGCATGGAT	(Joyce et al., 2007)
	Provisional	TTCAGTTTCCCAGAGCTACCA	
IFND-1,2	**NC_010443.5**	CTCGATCAACTACTCTCTAG	(Johns et al.,
	Provisional	CTACTCTGACAATCTCCC	unpublished data)
IFND-3, 4, 10	**NC_010443.5**	GATCAACTGCTCTCTAGG	(Johns et al.,
	Provisional	GCACAGAGGCTATATTCC	unpublished data)
IFND-5, 6, 7,	**NC_010443.5**	AGAACTGACTTCCAATTTC	(Johns et al.,
8, 9, 11	**Provisional**	TGTTGAAGAGGTTGATGAT	unpublished data)

The primer pair for Group 1 amplified sequence for *IFND-1* and *IFND-2*; the primer pair for Group 2 amplified sequence for *IFND-3, IFND-4*, and *IFND-10*; the primer pair for Group 3 amplified sequence for *IFND-5*, *IFND-6*, *IFND-7*, *IFND-8*, *IFND-9*, and *IFND-11*. The different IFND genes were grouped together based on high similarity to each other. These primers were used to amplify conceptus cDNA and visualized on a 2% ethidium bromide agarose gel to determine conceptus expression on day 14, 17, and 30 of pregnancy.

Results

Pig Conceptus Expression of Type I Interferon Delta and Type II Interferon Gamma During Early Pregnancy

Conceptus cDNA shows expression for *IFNG* on days 14, and 17 of pregnancy. Conceptus cDNA shows expression for primer Group 1 (*IFND-1*, and *IFND-2*) on days 14, 17, and 30 of pregnancy. Conceptus cDNA shows expression for primer Group 2 (*IFND-3*, *IFND-4*, and *IFND-10)* only on day 30 of pregnancy. Conceptus cDNA showed no expression for primer group 3 (*IFND-5*, *IFND-6*, *IFND-7*, IFND-8, *IFND-9*, and *IFND-11)* across day 14, 17 and 30 (Figure S.1).

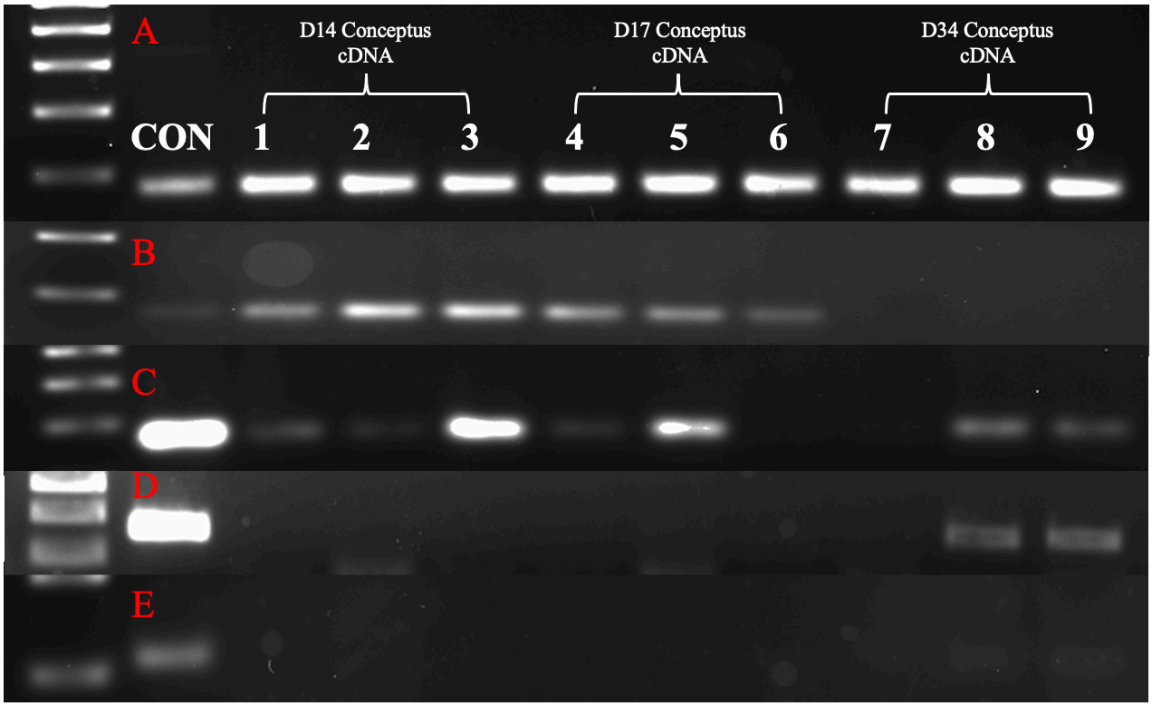

Figure S.1: PCR amplification of type I interferon deltas and type II interferon gamma cDNA. **A)** Porcine beta-actin confirmed all cDNA was viable **B)** *IFNG* is expressed on days 14 and 17 of pregnancy. **C)** *IFND-1* and *IFND-2* are expressed on days 14, 17, and 30 of pregnancy. **D)** *IFND-3*, *IFND-4*, and *IFND-10* are expressed only at day 30 of pregnancy. **E)** *IFND-5*, *IFND-6*, *IFND-7*, *IFND-8*, *IFND-9*, and *IFND-11* show no expression across days 14, 17 and 30.