ABLATION OF CONCEPTUS *PTGS2* PROVIDES A NEW UNDERSTANDING OF EARLY PREGNANCY EVENTS IN THE PIG

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

CAROLINE ANNE PFEIFFER

Dr. Rodney Geisert, Thesis Supervisor

JULY 2019

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

ABLATION OF CONCEPTUS *PTGS2* PROVIDES A NEW UNDERSTANDING OF EARLY PREGNANCY EVENTS IN THE PIG

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

Dr. Rodney Geisert – Thesis Advisor

Dr. Randall Prather

Dr. Thomas Spencer

Dr. Heide Schatten

ACKNOWLEDGMENTS

As a senior in high school applying to college, the University of Missouri was not even on my radar. However, as I reflect on the past 6 years here as both an undergraduate and graduate student, I realize how truly lucky I am to have been a student here at Mizzou. The students and professors that I have had the pleasure of knowing during my time here have changed my life in the best way possible. I would specifically like to thank all of the students, faculty, and staff in the Department of Animal Sciences for shaping me into the person, student, and scientist that I am today. I especially want to thank my thesis advisor, Dr. Rodney Geisert, for having full confidence in me while at the same time challenging me as a scientist. His guidance and wisdom have helped me become a more confident person, not only in the lab, but outside as well. I am still working on not saying ‘thank you’ as much, though. It is well understood that PI’s take on risks when hiring graduate students, and I am forever thankful that he took on this risk. After I had taken his undergraduate course, I looked up to Dr. Geisert as a role model and my favorite professor. Four years later, I am so lucky to continue to not only look up to Dr. Geisert as a role model and my favorite professor, but my friend and the best academic dad. I am forever grateful for the time I have spent with him.

I would also like to thank Dr. Don Spiers for helping me throughout the entire graduate school application process. I am very grateful for Dr. Prather for taking me on as an undergraduate researcher. The year I spent in his lab provided me with invaluable experiences and friendships as well. I look forward

continuing to learn and grow under such an accomplished, brilliant, and kind mentor. I would also like to thank Dr. Spencer for providing not only a space to conduct my research, but for challenging and pushing me to become the best scientist I can be.

I owe much of my laboratory understanding and confidence to Dr. Bethany Redel and Ashley Meyer. Thank you for being the best lab mentors that I could have asked for. Their leadership, patience, and kindness allowed me to have the best experiences as a researcher. I would not be the scientist I am today without either of their guidance, and I cherish the time that I was able to spend with you both. To all of the people that helped make my project possible: Lee Spate, Josh Benne, Raissa Cecil, Paula Chen, Dr. Murphy, Melissa Samuel, Sabrina Hammond, Elizabeth Blackstock, Jessica Foster, and Dr. Christine Spinka, your time and effort is so greatly appreciated. I would like to thank my fellow graduate students and friends, Eleanore O'Neil, Paula Chen, Lauren Ciernia, Jessica Foster, and Fayth Kumro for always having an open ear or a shoulder to cry on. There truly is no bond like the one between stressed graduate students.

Finally, I would like to thank my friends and family. I would not be where I am today without the love and support from my mom and dad. I am forever grateful for everything they have sacrificed to make me into the woman I am today. I would like to thank my sisters, Anna and Kathryn, for being my oldest and truest friends. To my friends, family, and boyfriend, Michael: thank you for being on the other end of the phone and always cheering me on. I am so blessed to be surrounded by so many incredible people; I owe it all to you.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

4-OHA: 4-Hydroxyandrostenedione

AI: Artificial Insemination

AKR1B1: Aldo-Keto Reductase Family 1 Member B

BSA: Bovine Serum Albumin

CASP1: Caspase 1

CL: Corpus Luteum

COX-1: Cyclooxygenase 1

COX-2: Cyclooxygenase 2

Cre/lox: Cyclization Recombination/loxP

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats

crRNA: CRISPR RNA

CYP19A1: Aromatase

DMEM: Dulbecco's Modified Eagle Medium

E_2: Estrogen

ER: Estrogen Receptor

ECG: Equine Chorionic Gonadotropin

FBS: Fetal Bovine Serum

FSH: Follicle Stimulating Hormone

GE: Glandular Epithelium

GnRH: Gonadotropin Releasing Hormone

gRNA: Guide RNA

hCG: Human Chorionic Gonadotropin

HDR: Homologous Directed Repair

ICM: Inner Cell Mass

IFND: Interferon-δ

IFNG: Interferon-γ

IFNT: Interferon-τ

IL-1: Interleukin 1

IL1β: Interleukin 1β

IL1β2: Interleukin 1β2

IL1RAP: IL1 Receptor Accessory Protein

IL1RI: Interleukin 1 Receptor Type I

IVF: *In Vitro* Fertilization

LAS X: Leica Application Suite X

LE: Luminal Epithelium

LH: Luteinizing Hormone

LIF: Leukemia Inhibiting Factor

mRNA: Messenger Ribonucleic Acid

NFKB: Nuclear Factor Kappa-B

NHEJ: Non-Homologous End Joining

P_4: Progesterone

PAF: Paraformaldehyde

PAM: Protospacer Adjacent Motif

PBS: Phosphate Buffered Solution

PG: Prostaglandin

PGE_1: Prostaglandin E_1

PGE_2: Prostaglandin E_2

PGE_3: Prostaglandin E_3

$PGF_{1\alpha}$: Prostaglandin $F_{1\alpha}$

$PGF_{2\alpha}$: Prostaglandin $F_{2\alpha}$

$PGF_{3\alpha}$: Prostaglandin $F_{3\alpha}$

PGFM: 13,14-dihydro-15-keto-$PGF_{2\alpha}$ Metabolite

PGFS: Prostaglandin F Synthase

PGG_2: Prostaglandin G_2

PGH_2: Prostaglandin H_2

PLA_2: Phospholipase A_2

PPAR: Proliferator-Activated Receptors

PTGES: Prostaglandin E Synthase

PTGES2: Prostaglandin E Synthase 2

PTGES3: Prostaglandin E Synthase 3

PTGS1: Prostaglandin Synthase 1

PTGS2: Prostaglandin Synthase 2

PVA: Polyvinyl Alcohol

SAL1: Salivary Lipocalin

SCNT: Somatic Cell Nuclear Transfer

SDS: Sodium Dodecyl Sulfate

SLCO2A1: Solute Carrier Organic Anion Transporter Family Member 2A1

SLCO5A1: Solute Carrier Organic Anion Transporter Family Member 5A1

TALEN: Transcription Activator Like Effector Nucleases

TBST: Tris Buffered Solution & Tween-20

TE: Trophectoderm

TL-HEPES: Tyrode Lactate HEPES

TNF: Tumor Necrosis Factor

tracrRNA: Trans-Activating RNA

ULF: Uterine Luminal Flush

UV: Ultraviolet

VEGF: Vascular Endothelial Growth Factor

ZFN: Zinc Finger Nuclease

ABSTRACT

ABLATION OF CONCEPTUS PTGS2 PROVIDES A NEW UNDERSTANDING OF EARLY PREGNANCY EVENTS IN THE PIG

Caroline Pfeiffer

Rodney Geisert, Thesis Advisor

Pig conceptuses produce and secrete estrogens (E_2), interleukin 1 beta 2 (IL1B2), and prostaglandins (PGs) during the period of rapid trophoblast elongation and establishment of pregnancy. Previous studies established that IL1B2 is essential for rapid conceptus elongation (Whyte et al., *Proc Natl Acad Sci USA* 2018), whereas E_2 is not essential for conceptus elongation or early maintenance of the corpora lutea (CL) (Meyer et al., 2019). Prostaglandin synthase 2 (*PTGS2*) expression increases during early pig conceptus development along with production of PGs. Blastocysts express *PTGS2* but not prostaglandin synthase 1 (*PTGS1*) during early development. To evaluate the role of conceptus PTGS2-derived PGs in early pregnancy, CRISPR/Cas9 gene editing was used to create a deletion in exon 1 of the *PTGS2* gene. Both $PTGS2^{+/+}$ and $PTGS2^{-/-}$ embryos were generated using edited fibroblast cells and somatic cell nuclear transfer. Culture media from $PTGS2^{+/+}$ and $PTGS2^{-/-}$ blastocysts was collected at day 7. Total PG was substantially lower ($P < 0.0001$) in the culture media of $PTGS2^{-/-}$ compared to $PTGS2^{+/+}$ blastocysts. PTGS2 was detectable by immunolocalization in $PTGS2^{+/+}$ but absent in $PTGS2^{-/-}$ blastocysts. Next, $PTGS2^{+/+}$ and $PTGS2^{-/-}$ blastocysts were transferred into the uterus of

surrogate gilts, and the reproductive tract was collected on either day 14 or 17 of pregnancy. Elongating and filamentous type conceptuses were recovered from the uterine lumen by flushing. The conceptuses were cultured for 3 hours to determine overall PG production. Total PG was lower ($P < 0.0001$) in media of *PTGS2*$^{-/-}$ conceptuses compared to *PTGS2*$^{+/+}$ conceptuses. However, total content of prostaglandin E_2 (PGE_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in the flushings of the uterine lumen containing either *PTGS2*$^{-/-}$ or *PTGS2*$^{+/+}$ conceptuses were not different (P = 0.99 and P = 0.15, respectively). Next, *PTGS2*$^{-/-}$ blastocysts were transferred into surrogate gilts to assess pregnancy establishment beyond 17 days. Of note, pregnancy was maintained beyond day 30 in gilts gestating *PTGS2*$^{-/-}$ embryos. Thus, conceptus-derived PGs do not have a biological role in early pregnancy in terms of conceptus elongation and pregnancy establishment.

CHAPTER ONE

1. INTRODUCTION

According to recent studies, the growing global population is driving an increase in meat production in which pork specifically is becoming higher in demand (Godfray et al., 2018). A 2018 USDA global report indicated that pork continues to lead the world in consumption rates compared to cattle and poultry. As of October of 2018, pig meat consumption in the world totaled 112,433 thousand metric tons, compared to 60,724 and 93,787 thousand metric tons for cattle and poultry, respectively (FAS/USDA, 2018). In addition to production for consumption, pigs are increasingly being utilized as biomedical models for both human and animal health research. Regarding human health research, pigs can provide an appropriate animal model as the pig is anatomically, physiologically, and metabolically similar to humans (Swindle et al., 1988). Having such an impact in both the agricultural and biomedical fields, fully understanding the biological and molecular processes that occur during early embryonic development and pregnancy in the pig is important for increasing piglet survivability to term. Pigs are reproductively highly efficient, ovulating on average 21 ova every 21 days (Da Silva et al., 2017) and producing litters that average from 10-12 piglets (USDA, 2018). Although large litter sizes are achievable, large litters tend to have an increase in smaller and less viable piglets at term. In addition, pregnancy rate and litter size is reduced significantly when cloning and *in vitro* maturation for gene editing are used to develop biological medical models and creation of disease resistant pigs.

As a litter bearing species, pigs have greater (~45%) embryonic mortality. It is understood that the majority of embryonic loss in the pig occurs during the peri-attachment period from days 10-30 of pregnancy (Kridli et al., 2016). Although there is a multiplicity of causes for early embryonic loss, failure of rapid conceptus elongation, stimulation of maternal recognition of pregnancy to maintain the lifespan of the corpora lutea (CL), and proper cell to cell communication between the fetal and maternal interfaces can be detrimental to the conceptus development and survival.

Because the pig has a true epitheliochorial placentation and does not undergo invasive implantation, the conceptus relies on rapid elongation of the trophoblast to obtain enough surface area ensuring adequate amounts of nutrient and blood transfer from the maternal endometrium (Spencer et al., 2016). The elongation of the trophectoderm (TE) is coincidental with the production of conceptus derived factors that are essential for maintaining the CL and therefore the production of progesterone (P_4), the hormone of pregnancy. Unlike other livestock species (cattle and sheep), rapid elongation is not achieved by mitotic cell division, but through trophoblast cellular remodeling. Perry (1981) indicated that conceptus elongation was analogous to elongating a ball of clay by rolling the clay between your hands. The rapid elongation of the conceptuses allows establishment of adequate surface area along the multiple complex folds of the maternal endometrium. The primary, secondary and tertiary folding in the endometrial surface of the pig allows greater surface area so multiple fetuses can obtain the necessary amount of blood flow and nutrients to support embryonic

development (Stroband and Van der Lende, 1990). Once the elongated conceptus is in close apposition to the maternal epithelium, microvilli from both the maternal and the fetal epithelium will intertwine. Therefore, the diffuse swine placenta does not undergo any invasion, but rather has a loose adhesion to the maternal endometrium (Geisert and Schmitt, 2002). Due to the lack of invasion, the developing conceptus is dependent on uterine gland histotrophic secretions, close communication between the maternal and fetal epithelium, and establishment of blood and nutrient flow from the diffuse placenta.

The window for maternal recognition of pregnancy in the pig occurs coincidentally with conceptus apposition and attachment. The conceptus must produce a signal to prevent luteolysis of the CL stimulated by endometrial release of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), which typically occurs on day 15 of the estrous cycle (Bazer et al, 1984). Since pigs have both local and systemic routes for luteolysis (Anderson et al., 1966), conceptuses need to cover a significant length of the uterine horns to be able to prevent $PGF_{2\alpha}$ from regressing the CL. For the past forty years, biphasic peaks of estrogen (E_2) produced by the conceptuses on days 11-12 and 15-30 have been proposed to redirect $PGF_{2\alpha}$ from the utero-ovarian artery into the uterine lumen, essentially acting as the maternal recognition signal (Bazer and Thatcher, 1977, Geisert et al., 1990). This endocrine/exocrine theory of redirecting $PGF_{2\alpha}$ away from the uterine vasculature and into the uterine lumen is dependent upon having at least two conceptuses in each uterine horn. In order to adequately protect the CL from luteolysis, it was

believed that two conceptuses were required in each uterine horn to cover enough surface area and produce enough E_2 to prevent systemic $PGF_{2\alpha}$ from reaching the ovary and regressing the CL (Dhindsa and Dziuk, 1968).

Recently a study indicated that E_2 is not necessarily essential for the early establishment of pregnancy in the pig (Meyer et al., 2019). Along with E_2, interleukin 1 beta 2 (IL1B2), prostaglandins (PGs), interferon-γ (IFNG), and interferon-δ (IFND) are all factors secreted by the conceptus to support conceptus elongation, maternal recognition, immune regulation, and embryonic and placental development. Although many of these conceptus factors have been well known, the essential role of each has been difficult to establish. Therefore, the following review will examine the conceptus factors released during early conceptus development, the molecular processes involved with early pregnancy, and utilization of gene editing to ultimately determine the role of conceptus factors in the successful establishment of pregnancy in the pig.

CHAPTER TWO

2. LITERATURE REVIEW

2.1 The Pig Estrous Cycle

The onset of sexual maturation (puberty) in the gilt occurs at approximately 6 to 8 months of age. Attempts to hormonally induce puberty before 100 days of age in pigs is difficult, as ovarian follicles have not fully developed at this time in prepubertal gilts (Dziuk, 1991). Studies have determined that the ovaries of prepubertal gilts become sensitive to gonadotropins, such as equine chorionic gonadotropin (eCG) between 4 to 6 months of age (Christenson et al., 1985). Treatment of 5 month old prepubertal gilts with eCG induces follicle development, estrus expression, ovulation, fertilization, and early conceptus development. However, few of the pregnancies are maintained beyond 24 days of gestation (Dziuk and Gehlbach, 1966). Thus, although estrus and ovulation can be induced earlier in prepubertal gilts, CL are not maintained beyond Day 15 and the pregnancy is lost. The 21 day estrous cycle in the pig consists of two phases: the luteal phase in which the ovaries contain CL from days 3 to 16 of the estrous cycle, and the follicular phase where developing new Graafian follicles begin to emerge between the CL following luteolysis on Day 15 for the induction of estrus and ovulation between days 17 to 2 of the estrous cycle (First expression of estrus = Day 0).

The estrous cycle can be divided into four different stages: proestrus, estrus, metestrus, and diestrus. Proestrus begins directly following uterine

release of multiple, frequent pulses of $PGF_{2\alpha}$ on day 15 and the subsequent regression of the CL decreasing the concentration of P_4 in the systemic blood. Proestrus is distinguished by rapidly growing ovarian Graafian follicles and increasing blood concentrations of E_2, stimulated by follicle stimulating hormone (FSH) until day 20. Estrus is characterized by the production of E_2 from the large, dominant Graafian follicles. The increased blood concentration of E_2 and decreased concentration of P_4 allows a positive feedback of E_2 on the hypothalamus, increasing pulsatile release of gonadotropin releasing hormone (GnRH) which stimulates a surge release of FSH and luteinizing hormone (LH) from the anterior pituitary. Increased concentrations of E_2 lead to stimulating the expression of behavioral estrus (standing heat) in the pig, around days 18 to 21 of the estrous cycle. First expression of standing heat is denoted as day 0 of the estrous cycle with the surge release of LH inducing ovulation 36 to 48 hours after the initiation of standing heat. As mentioned previously, commercial breeds of pigs will typically ovulate 21 to 30 oocytes during estrus (Da Silva et al., 2017). The LH surge causes an immediate decline in follicle E_2 production following ovulation as the granulosa and thecal cells in the ovulated follicles luteinize and increase P_4 synthesis. Metestrus is considered the period of transition from a corpus hemorrhagicum after ovulation to increased P_4 production by the functioning CL which occurs from Days 3 to 5 of estrous cycle. Diestrus, which immediately follows metestrus, is the stage in which increased P_4 plasma concentrations have a negative feedback on the hypothalamus and anterior pituitary lowering GnRH pulses allowing only secretion of basal amounts of the

gonadotropic hormones needed to support CL function. The suppression of E_2, FSH, and LH allows P_4 from the CL to be the dominant steroid hormone that regulates uterine function and the pig estrous cycle for about 11 days. Plasma progesterone reaches peak concentrations around day 12 to 14 of the estrous cycle. If females are not bred or no conceptuses are present, pulsatile release of uterine $PGF_{2\alpha}$ into the utero-ovarian vasculature is responsible for stimulating regression of the CL and the decrease of plasma progesterone concentrations after day 15 of the estrous cycle (Spencer and Bazer, 2004). After luteolysis, a new wave of ovarian follicles will resume and behavioral estrus will be expressed between 3 and 5 days following CL regression (Moeljono et al., 1976).

2.2 Early Pregnancy in the Pig

Early embryonic development in the pig relies on a receptive uterine environment for placental attachment and adequate production of endometrial secretory factors to support development throughout pregnancy. After fertilization and completion of Meiosis II in the ampullary-isthmic junction, the zygote quickly undergoes numerous mitotic cleavage cell divisions. The first cleavage division produces a 2-cell stage zygote, consisting of 2 blastomeres which undergoes further cleavage to a 4-cell zygote within 24 hours. While the first two mitotic divisions occur rather rapidly, within a day of fertilization, the cell division rate decreases and the blastomeres do not replicate for another 24-26 hours. Alteration in steroid hormonal secretion (shift from E_2 to P_4), relaxes the isthmus to allow the muscular contractions to propel the 8 to 16 cell embryos into the tip of the uterine horn by day 5 of pregnancy. Timing of embryo entry into the uterine

horns is hormonally regulated to allow the uterus time to become receptive to embryos following estrus. Administration of progesterone stimulates earlier passage through the oviduct in the pig (Day and Polge, 1968) while administration of exogenous estradiol causes the embryos to become restricted (tube locked) within the oviduct (Dziuk, 1985). Thus, progesterone from the developing CL regulates movement of fertilized and unfertilized eggs into the tip of the uterine horns.

Following egg transport into the uterus, cellular division continues to occur where upon reaching the 32 cell stage forming a morula (inside and outside cells), the outer cells undergo compaction through formation of apical tight junctions between one another. The process of compaction in the pig involves rearrangement and an increase in the amount of actin filaments around the areas of cell contact, suggesting that actin may play a role in distinguishing the different cell lineages (Albertini et al., 1987). Cell polarity and inside/outside theories state that any non-polar and centrally located cells within the embryo will differentiate into the inner cell mass (ICM) and the outer-most, polar cells transform into trophoblastic cells (Tarkowski and Wroblewska, 1967; Johnson and McConnell, 2004). The apical tight junctions form an outer seal between the trophoblastic cells allowing fluid to follow Na^+ movement through the action of Na^+/K^+ ATPase pumps present in the cell basal border into the center of the morula, creating a blastocoel cavity (Senger, 2003). The resulting zona pellucida enclosed blastocyst consists of trophoblast, inner cell mass, and a blastocoel cavity. As the blastocyst develops *in vivo*, endometrial and trophoblast enzymes weaken the

zona and the growth and expansion of the blastocyst increase pressure on the zona pellucida until the zona tears allowing hatching. Following hatching, the blastocyst is directly exposed to the surrounding endometrial luminal epithelium (LE) extracellular matrix and secretions on day 8 of pregnancy. Blastocyst hatching allows for expansion of the trophoblast cells, development of the epiblast, and proliferation of the extraembryonic membranes. The uterus undergoes rapid growth, doubling in length from days 2 to 6 of pregnancy to accommodate for embryonic development and placental expansion, (Perry and Rowlands, 1962).

Differentiation of the embryonic germ layers (ectoderm, mesoderm, and endoderm) within the ICM occurs shortly after hatching from the zona pellucida. The ectoderm in the ICM will give rise to the epidermis and the nervous system of the developing embryo. The mesoderm will develop the cardiovascular system, bones, muscles, and sex organs, while the endoderm will give rise to the primitive gut which leads to formation of the respiratory tract, lungs, stomach, liver, pancreas, and intestines (Patten et al., 1948). Because the pig has a diffuse, epitheliochorial type of placentation, the multiple conceptuses must migrate to not only establish their placental uterine surface area for development but covering a large area of the uterus is essential for preventing luteolysis. Conceptus migration, which is assisted by myometrial contractions, from the tip of the uterine horns is initiated shortly after blastocyst hatching from the zona pellucida (Day 8) and continues until trophoblast elongation occurs in the pig (Dziuk et al., 1964). By day 9 of pregnancy, some conceptuses will have

migrated and crossed over into the uterine horn opposite from the oviduct of origin (Dhindsa et al., 1967).

The process of uterine migration in the pig is mostly random, with most conceptuses remaining in the horn of origin. The conceptus may stimulate uterine migration through secretion of several factors. Pope et al. (1982) demonstrated that polysiloxane beads saturated with estradiol were more effective at migrating through the uterine horn than beads without estradiol. It is theorized that embryos producing greater amounts of estradiol may be more efficient at migrating through and between the uterine horns than embryos producing lower amounts of estradiol. By day 12 of pregnancy, migration will have ceased, and the embryos are adequately spaced out throughout the uterus before conceptus elongation occurs (Dziuk et al., 1964). In species such as the horse, blastocyst derived prostaglandins are essential for migration from the oviduct and into and throughout the uterine horns via peristaltic myometrial contractions. Without prostaglandin E_2 (PGE_2), the blastocyst would not be released from the oviduct nor would it be capable of migrating throughout the uterine horns to prevent luteolysis (Weber et al., 1991a; Weber et al., 1991b; Weber et al., 1995; Stout and Allen, 2001). Migration of the embryos becomes extremely important in early pregnancy establishment of the pig as the majority of the uterus needs to be occupied to prevent luteolysis beyond day 12. Even if one-eighth of the uterus is unoccupied, only 50-60% of pregnancies continue (Dhindsa and Dziuk, 1968). Further, intrauterine infusion of PGE_2 in the horse resulted in increased uterine tone and contractility while infusion of $PGF_{2\alpha}$ did not

cause the same stimulation for migration. This suggests that PGE_2 may play an essential role in embryonic migration and pregnancy establishment in the mare (Gastal et al., 1998a; Gastal et al., 1998b).

However, the role of conceptus prostaglandin production in uterine migration is unknown in the pig. It was determined that beads saturated with E_2 were capable of migrating farther and more efficiently than beads saturated with cholesterol (Pope et al., 1982). The same study investigated the result of injecting either a histamine inhibitor, cromolyn sodium, or both the inhibitor and histamine into the uterine tissue of day 6 pregnant gilts. Injection of cromolyn sodium resulted in restricted migration from the tip of the uterine horn, while uteri injected with both cromolyn sodium and histamine exhibited normal uterine migration. This indicated that both histamine and estradiol play important roles in embryonic migration in the pig (Pope et al., 1982). Administration of exogenous histamine on rat uteri resulted in increased production of endometrial PGE_2, possibly suggesting that histamine could stimulate a prostaglandin production response in the pig to assist with early embryonic migration (Viggiano et al., 1988).

2.3 Conceptus Elongation

While the early developmental stages for formation of the morula and blastocyst are mostly conserved across species, early conceptus development and rapid elongation between days 10 to 12 of pregnancy is somewhat a unique phenomenon in the pig. The peri-implantation pig conceptus, as first proposed by Heuser and Streeter (1929), undergoes 4 different critical morphological

changes: spherical, ovoid, tubular, and filamentous (Anderson, 1978). After hatching from the zona pellucida on day 8 of gestation, the conceptuses grow (cell division) to reach a spherical diameter of approximately 5 to 8 mm by days 9 to 10 of pregnancy (Heuser and Streeter, 1929). On day 10, conceptuses rely on stimulating peristaltic and anti-peristaltic uterine contractions to migrate within the lumen to distribute and equidistantly space themselves throughout the uterus (Pope et al., 1982). As migration occurs (Day 11 to 12), the elongation process begins when the spherical 9-10 mm conceptus transforms to an ovoid morphology. Although a greatly reduced, cellular proliferation continues as the ovoid conceptus develops into a tubular conceptus. Transformation from a tubular to a filamentous conceptus does not involve cellular proliferation but occurs through rapid cellular migration and remodeling on days 11 to 12 of pregnancy (Geisert et al., 1982a). At this point of pregnancy in the pig, the conceptus elongation terminates migration, establishing a portion of maternal luminal epithelium that the developing embryo and placenta will become closely apposed.

The transformation to a filamentous conceptus is an extremely rapid process with the conceptus reaching a long, thin, string-like (>100 mm) morphology in less than an hour (Geisert et al., 1982a; Geisert et al., 2015). Conceptus elongation and expansion throughout the uterus also occurs in ruminant species, however ruminant conceptuses do not elongate as quickly as the pig. It has been shown that trophoblastic elongation in ruminants does not involve rapid morphological changes, but rather an increase in cellular

hyperplasia (Wang et al., 2009). In contrast, elongation of the pig conceptus results from cellular remodeling rather than cellular proliferation (Perry, 1981) as cellular hyperplasia is greatly decreased during transformation from tubular to filamentous conceptuses (Geisert et al., 1982a). As previously stated, Perry (1981) compared rapid remodeling of the conceptus to rolling a ball of clay between your hands where the amount of clay does not change, but the shape of the ball becomes extended. Studies examining conceptus elongation indicated that the TE cells undergo a cellular change in shape. The TE of spherical and ovoid conceptus consisted of cuboidal shaped cells, the tubular conceptus of polygonal, and the filamentous conceptus of elongated cells (Mattson et al., 1990).

In addition to morphological changes to the trophectoderm, microvilli presence on apical cell-surface also change. The TE surfaces of spherical, ovoid, and tubular conceptuses have numerous microvilli present but were greatly reduced on filamentous conceptuses (Mattson et al., 1990). During this time, an accumulation of both endodermal and trophectoderm cells extending from the ICM will create the elongation zone of the trophectoderm (Geisert et al., 1982a). The change in TE cellular junctions and microfilaments allow the cellular shift and movement as the underlying endodermal cells migrate along the TE basement membrane to apply the force to elongate the trophoblast layer much like the clay model that Perry (1981) first proposed. Following conceptus elongation, the conceptus becomes closely apposed to the luminal epithelium and initiates

placental development. The decrease in TE surface microvilli allows for the conceptus to come in close contact with the maternal endometrium and acquire adequate amounts of uterine surface area for development (Bazer et al., 2009). Due to the pig's diffuse type placentation, obtaining enough uterine surface area is essential to the survival, growth, and development of the embryo throughout pregnancy. During the pre-implantation period in the pig, the developing embryo relies on the TE to obtain nutrients from the endometrium (Anderson, 1978). The fully developed placenta in the pig is quite long (~ 1 meter) while the length of the uterus it occupies is much shorter due to the massive amounts of endometrial folding allowing the placenta to acquire large amounts of surface area (Perry and Rowland, 1962).

2.4 Placentation

While cattle, sheep, horses, and pigs all have epitheliochorial type of placentation (Leiser, 1975; Guillomot et al., 1981; Hunter, 1980; Steven and Morriss, 1975), the attachment and implantation phases differ across species. King et al. (1982) described the differences in attachment, implantation and placentation among domestic species. Ruminants undergo a limited invasive process of implantation as binucleate cells formed from the trophectoderm fuse with the maternal luminal epithelium, thus creating multinucleated plaques in cattle or a syncytium in sheep (Bazer et al., 2009). Attachment, rather than invasive implantation, occurs in sows and mares. As mentioned above, commencement of conceptus elongation in the pig ceases migration through the uterine horns.

By day 13 of pregnancy in the pig, the elongated filamentous conceptuses have prominent embryonic disks (Anderson, 1978) and shortly after, there is growth of the trophectoderm, that is attached closely to the maternal epithelium (King et al., 1982). Following gastrulation, mesoderm expanding from the caudal end of the embryonic disc will proliferate and migrate between the trophectoderm and underlying endoderm, establishing the somatic mesoderm and initiating development of the chorion. At this time, formation of extra-embryonic membranes occurs with the primitive gut becoming surrounded by splanchnic mesoderm to form a functional yolk sac (Vallet, 2009). As mentioned previously, the early blastocyst is differentiated into two major cell lineages: the ICM and the TE. The inner cell mass will differentiate into either the epiblast, resulting in the embryo proper or the hypoblast which forms the primitive gut resulting in the yolk sac. The hypoblast, made entirely of endodermal cells, lines the base of the embryonic disc, establishing the primitive yolk sac (Amoroso, 1952). Again, the mesoderm derived from the embryonic disk will diverge and expand alongside the hypoblast, establishing the splanchnic mesoderm and vascularizing the developing yolk sac. The yolk sac will then expand, lining the inner surface of the chorion and functions as a temporary storage depot for nutritional molecules, obtained by the conceptus through histotrophic and hemotrophic transfer. Development of the amnionic membrane also occurs at this time. The expanding mesodermal ‘sac’ will extend upwards around the embryo, creating amniotic “folds.” The embryo will sink within the cavity, allowing the amniotic folds to extend completely around and above the embryo. The folds will eventually meet

above the embryo and fuse together, sealing the amnion. Expansion of these extra-embryonic membranes will allow apposition to the luminal epithelium. Apposition as defined by Enders and Schlafke (1969) is the stage of development in which the trophectoderm of the developing conceptus becomes fixed in close proximity to the luminal epithelium. Immobilization of the conceptus is due to the development of endometrial epithelial protrusions that become enclosed by chorionic caps (Dantzer, 1985). Close apposition and loose attachment of the conceptus trophectoderm to the luminal epithelium begins around day 14 of pregnancy in the pig. Similar to the morphological changes in the trophectoderm cells mentioned above, the luminal epithelial cells will also undergo changes in shape during the apposition and attachment period to provide a receptive uterine surface for the developing conceptus. During the conceptus elongation period in the pig, the luminal epithelium tends to be columnar in shape with nuclei located centrally within the LE cell. However, during the attachment period (days 14-16), the columnar cells increase in height and the nuclei will relocate closer to the apex of the cell (King et al., 1982). Firm adhesion of the chorion to the maternal endometrium involves the interdigitation of microvilli between the chorion and LE and begins on day 15 of gestation.

Following the initiation of the adhesion process, the yolk sac begins to regress and will be replaced by expansion of the allantois from the hindgut. Until this point in time, the developing conceptuses have been completely reliant on uptake of histotrophic secretions for nutrients, but by days 15-16 of pregnancy, transition to both histotrophic and hemotrophic nutrient transport (Geisert, 2015).

The vascularized allantoic sac is comprised of both mesoderm and endoderm expands throughout the elongated chorion, enlarging to replace the yolk sac as it regresses. The allantois expands and fills the chorion, until the fluid of allantois presses the chorioallantoic membrane against the uterine wall. Since the uterine horns can hold multiple fetuses, there is direct end to end chorionic contact with the adjoining fetuses within the horn. However, conceptuses do not overlap during elongation. Since there is contact among the fetal chorions, the allantois may not be able to expand into the entire chorionic cavity. The part of the chorion that is not in contact with the allantois will not be vascularized and will become necrotic, known as the 'necrotic tip' (Vallet, 2009). The remainder of the vascularized chorion will establish contact with the maternal vascular beds, beginning hemotrophic exchange of nutrients and blood flow. On the surface of the chorion, placental areolae develop over the mouths of uterine glands to absorb histotrophic secretions (Bielanska-Osuchowska, 1979). By day 18 of pregnancy in the pig, the yolk sac is completely regressed and the chorioallantoic surface microvilli will be attached and intertwined with the microvilli on the maternal epithelium (Dantzer, 1985).

2.5 Expression and Secretion of IL1B2 During Elongation

An important aspect of the maternal recognition in pigs is the successful elongation of the conceptuses by day 12 to 13 of pregnancy. During elongation, the pig conceptus will increase expression of many genes that may have roles in elongation, establishment of pregnancy, attachment, and embryonic development. Interleukin 1 beta (IL1B) has been proposed to play a role in the

pro-inflammatory processes of implantation across many species including humans, primates, mice, and cattle (De los Santos et al., 1996; Strakova et al., 2005; Bourdiec et al., 2014; Correia-Alvarez et al., 2015). Tou et al. (1996) first discovered *IL1B* as a result of the differential expression patterns in elongating pig conceptuses and this data was further supported by Ross et al. (2003). Interestingly, gene expression patterns revealed that *IL1B* was highest on days 11-12 of pregnancy, coincident with the rapid elongation of the conceptus trophectoderm, but decreased more than 2000-fold immediately after elongation (Ross et al., 2003). These results indicated that IL1B could be responsible for elongation, maternal recognition, or early maintenance of pregnancy in the pig.

In addition to examining differential gene expression, IL1B production was measured in the uterine luminal flush (ULF). Similarly, the production levels were greatest on days 11 to 12 and then decreased significantly by day 18 of pregnancy (Ross et al., 2003). While rapid elongation of the trophectoderm is unique to pigs, IL1B has been reported to play an important role in early embryonic development in other species as well. In humans, it is proposed that a polymorphism of *IL1B* can lead to recurrent pregnancy loss (Wang et al., 2002), while in mice, the absence of IL1B did not have any effect on reproduction (Zheng et al., 1995; Kuida et al., 1995; Li et al., 1995). Further studies trying to understand the role that IL1B plays in early pregnancy in the pig led to the discovery of a novel conceptus-specific *IL1B* isoform that is unique to the pig, *IL1B2* (Mathew et al., 2015) which was first suggested from an earlier study by Ross et al. (2003). *IL1B2* is 85% identical at the protein level to *IL1B1* and

caspase 1 (CASP1) is needed to cleave both pro-IL1B1 and pro-IL1B2 to become biologically active (Arend et al., 2008; Katebi et al., 2010). Expectedly, CASP1 content in the ULF is increased on day 13 of pregnancy in the pig. IL1B can stimulate phospholipase A_2 (PLA_2), which is responsible for increasing cell fluidity and liberating arachidonic acid from the cell membrane phospholipid bilayer (Kol et al., 2002).

PLA_2 plays an important role in the prostaglandin synthesis pathway, as prostaglandin synthase 1 (PTGS1) and prostaglandin synthase 2 (PTGS2) are the rate limiting enzymes that convert arachidonic acid into prostaglandin H_2 (PGH_2), which allows synthesis of many downstream prostaglandins. PLA_2's role in stimulating cell fluidity is consistent with the morphologic changes in the trophectoderm during the period of conceptus elongation in the pig. Gene editing studies were performed using the clustered regularly interspaced palindromic repeats (CRISPR)/Cas9 system to determine the role that IL1B2 may have specifically on rapid conceptus elongation and on establishment of pregnancy. Whyte et al. (2018) discovered that ablation of *IL1B2* was detrimental to conceptus elongation. *IL1B2*-null embryos were in fact capable of developing to the blastocyst and spherical stages of development but failed to elongate or survive *in vivo* (Whyte et al., 2018). Therefore, IL1B2 has been determined to be one of the essential factors for the successful rapid elongation of the conceptus during pregnancy in the pig.

2.5.1 Endometrial Stimulation by IL1B2

Although the role of IL1B2 is known to be essential for pig conceptus elongation, conceptus IL1B2 secretion is also important for regulating endometrial gene expression. As part of the (interleukin 1) IL-1 cytokine family, IL1B2 is a key component in priming the maternal endometrium to be receptive to the developing conceptus and to provide the proper inflammatory response that will initiate endometrial secretions important for maternal recognition of pregnancy, early embryonic development, and establishment of pregnancy in the pig. IL1B2 binds to the IL1B receptor, the interleukin 1 receptor type I (IL1RI) on the luminal epithelial surface and will initiate the stimulation of a cascade of inflammatory and immune response pathways (Dinarello, 2009).

Some of the genes of interest that IL1B2 stimulates are salivary lipocalin (SAL1), nuclear factor kappa-B (NFKB) and PTGS2. SAL1 is a member of the lipocalin family, which is responsible for transporting many hydrophobic molecules (Flower, 1996). Endometrial *SAL1* expression is higher on day 12 of pregnancy compared to day 12 of the estrous cycle in the pig (Ka et al., 2009). Localization of *SAL1* messenger RNA (mRNA) expression was only faintly detected in the glandular epithelium (GE) on days 12 and 15 of the estrous cycle but expression was significantly increased on day 12 of pregnancy (Seo et al., 2011). IL1B2 is proposed to induce *SAL1* expression in the maternal endometrium, as IL1B2 expression is spaciotemporally associated with increased expression of *SAL1* (Seo et al., 2011). It has also been proposed that SAL1 plays a role in prostaglandin transport, further assisting with the endometrial

attachment during early pregnancy in the pig (Seo et al., 2008).

Nuclear factor kappa-B is another critical pro-inflammatory pathway that is stimulated by IL1B2 (Hayden and Ghosh, 2012; Mathew et al., 2015). While NFKB is most notably known and understood as a mediator for immune responses, it also acts as an influential transcription regulator in cell survival, differentiation, and proliferation (Hayden and Ghosh, 2012). NFKB is localized in cell cytoplasm, which upon activation, is relocated into the nucleus of cells where it regulates gene expression by binding to kappa-B sites within the gene promoter (Hayden and Ghosh, 2012).

Following pig conceptus ILB2 production and elongation, NFKB is located in nucleus of LE adjacent to the attaching pig conceptuses (Ross et al., 2003; Mathew et al., 2011). Conceptus ILB2 production increases endometrial expression of IL1R1 and IL1 receptor accessory protein (IL1RAP). Activation of NFKB has been reported to stimulate an increase in expression of *PTGS2* (Ali and Mann, 2004). Endometrial expression of *PTGS2* is increased in the pig when stimulated by mature IL1B2 (Mathew et al., 2015). *PTGS2* expression increases on day 12 of both the estrous cycle and day 12 of pregnancy in the pig, indicating that endometrial PTGS2 is not pregnancy specific (Ross et al., 2010), as PTGS1 and PTGS2 can both contribute to the production of $PGF_{2\alpha}$ for luteolysis (Ashworth et al., 2006).

2.6 Maternal Recognition of Pregnancy

It was at one time believed that the conceptuses secreted a certain substance that would be diffused into the uterine milk and transported across the

lumen to alert the endometrial tissue (Perry et al., 1973). Roger Short (1969) first coined the term “maternal recognition signal” in reference to a chemical signal produced by the developing conceptus in communication with the maternal endometrium to establish and maintain pregnancy. In order for the embryo to be protected from luteolysis, the conceptus must first produce a chemical signal, a hormonal substance in many species, to extend the lifespan of the corpus luteum beyond the length of a normal estrous cycle (Perry et al., 1976). To maintain CL function, the semi-allograft conceptus must protect itself from the maternal immune system and synthesize additional factors to regulate changes in endometrial lymphocyte production and activity throughout pregnancy (Geisert et al., 2017).

As previously described, the luteolysin in pigs and other species is $PGF_{2\alpha}$ synthesized and release by the endometrium (Moeljono et al., 1976). Between days 14 and 17 of the pig estrous cycle, luminal and glandular epithelium begin secreting pulses of $PGF_{2\alpha}$ into the uterine vasculature, inducing the regression of the CL after day 15 (Moeljono et al., 1977). The pig has both a systemic and local vascular route for inducing luteolysis, meaning that uterine $PGF_{2\alpha}$ has two possible vascular pathways to reach the ovaries to regress the CL present on the ovaries (Anderson et al., 1966). Luteolysis occurs when the endometrial derived $PGF_{2\alpha}$ is released into the uterine venous drainage on Day 15 (Bazer, 1989) where it is routed into the general circulation towards the lungs. Endometrial $PGF_{2\alpha}$ can also transferred (diffused) directly across from the utero-ovarian vein to the ovarian artery to stimulate luteolysis in the ovary ipsilateral to the uterine

horn (local) (Dhindsa and Dziuk, 1968). Ruminants have a local route for $PGF_{2\alpha}$ transfer to the ipsilateral ovary but do not have a systemic route as the lungs metabolize 90% of the $PGF_{2\alpha}$ into 13,14-dihydro-15-keto-$PGF_{2\alpha}$ metabolite (PGFM) which inactivates the luteolytic activity before the heart pumps the arterial blood back towards the ovaries. Unlike ruminants, porcine lungs do not efficiently metabolize $PGF_{2\alpha}$. Therefore, any $PGF_{2\alpha}$ that has been released into the uterine vasculature will not be totally metabolized in one passage through lungs allowing a systemic pathway for $PGF_{2\alpha}$ to stimulate CL regression in both ovaries (Del Campo and Ginther, 1973; Davis et al., 1980; Ginther, 1981). Thus, pig conceptuses need to prevent release of $PGF_{2\alpha}$ from both uterine horns to maintain CL function for pregnancy.

Dhindsa and Dziuk (1968) discovered that flushing conceptuses from the gravid uterine horns before day 11 failed to extend CL function beyond the length of a normal estrous cycle (21 days). However, when conceptuses were flushed out of the uterus after day 12, the CL were maintained for 25 to 28 days. These data indicate that the maternal recognition signal in pigs is conceptus derived and occurs after day 12 of pregnancy. In an attempt to determine the conceptus factor(s) involved with rescuing the CL from luteolysis, Ball and Day (1982) examined the effects of placing gelatin implants containing embryonic extracts into individual CL's. Corpora lutea with implants containing both embryonic extracts and $PGF_{2\alpha}$ were still viable, weighed more, and produced more P_4 than other CLs that did not have implants containing embryonic extracts. These data supported the theory that factors derived from the developing conceptuses were

in fact, capable of preventing luteolytic action of $PGF_{2\alpha}$ and extending the lifespan of the CL.

Early research indicated that administering diethylstilbestrol, a synthetic and non-steroidal estrogen, on day 11 of the estrous cycle extend CL survival to day 25 (Kidder, 1955). Kidder's experiment was the first to suggest that estrogen may play a role in the maternal recognition signal in pigs. Gardner et al. (1963), reported that administration of estradiol to cycling gilts on day 11 of the estrous cycle was capable of extending the CL to day 25 confirming Kidder's original 1955 study. Studies then began looking into conceptus derived estrogen and the role that it may play in the maternal recognition signal in pigs. Perry et al. (1973) established that blastocysts through elongated conceptuses were in fact capable of synthesizing steroid hormones such as estrogen and progesterone. It was first believed that conceptuses secreted a single peak of estrogen around days 11 to 12 of pregnancy, coincident with the rapid elongation of the conceptus trophectoderm. However, it was later found that administration of estradiol from day 11 to 15 of the estrous cycle maintained luteal function for 60 days or longer (Frank et al., 1977) indicating that a more prolonged increase in E_2 was necessary to obtain long-term CL maintenance. Zavy et al. (1980) and Geisert et al. (1982b) established that pig conceptuses synthesize and secrete estrogens in biphasic peaks: one coincidental with the rapid conceptus elongation period (days 11-12) and again during the attachment period (days 15-18). Further studies revealed that induction of pseudopregnancy in cyclic gilts was only maintained for 25 days with administration of estradiol at either day 11 or days 14

to 18 (Geisert et al., 1987). However, when estradiol was administered on days 11 and 14 to 18 of the estrous cycle, pseudopregnancy was extended beyond 60 days indicating the critical importance of the conceptus estrogen biphasic peaks together.

To determine how conceptus estrogen functions as the maternal recognition signal, Frank et al. (1977) demonstrated that $PGF_{2\alpha}$ concentration in the utero-ovarian vasculature was greatly decreased in cyclic gilts treated with estradiol valerate compared to controls. Coincident with the decrease in $PGF_{2\alpha}$ detected in the uterine vasculature, there is an increase of $PGF_{2\alpha}$ accumulation in the uterine lumen of estradiol valerate gilts on days 15 to 18. Thus, unlike other species, the amount of $PGF_{2\alpha}$ endometrial synthesis was not decreased but sequestered in the uterine lumen away from the utero-ovarian vasculature (Bazer et al., 1982). Bazer and Thatcher, (1977) proposed that estrogen redirected $PGF_{2\alpha}$ from endocrine secretion (into uterine venous drainage) to an exocrine direction (into the uterine lumen). These data led to the 'endocrine/exocrine theory' for maternal recognition of pregnancy in the pig. While this theory has been widely accepted as the maternal recognition signal in the pig, alternate hypotheses for conceptus and uterine factors contributing to luteal maintenance and establishment of pregnancy in the pig have been published. Prostaglandin E_2 is proposed to assist in extending the lifespan of the CL by acting in a more direct, luteoprotective manner. Cyclic ewes that received infusions of both $PGF_{2\alpha}$ and PGE_2 into the ovarian artery did not exhibit CL regression, indicating that PGE_2 has the ability to successfully antagonize the luteolytic actions of $PGF_{2\alpha}$

(Henderson et al., 1977). A similar study looking at the direct effect of PGE_2 on preventing luteolysis of the pig CL was conducted by Ford and Christenson (1991). Silastic beads containing either (i) estradiol-17β, (ii) PGE_2, (iii) $PGF_{2\alpha}$, (iv) estradiol-17β and $PGF_{2\alpha}$, or (v) PGE_2 and $PGF_{2\alpha}$ were inserted into individual CLs of cyclic gilts. Expectedly, the beads containing only $PGF_{2\alpha}$ were successful in regressing the CL by day 19. Individual CLs containing beads comprised of $PGF_{2\alpha}$ and estradiol-17β were not significantly different from control silastic beads and exhibited luteolysis. However, individual CLs implanted with beads containing PGE_2 and $PGF_{2\alpha}$ prevented luteolysis, were heavier in weight, and produced more P_4 on day 19 (Ford and Christenson, 1991). These data suggest that PGE_2 plays an important luteoprotective role, directly preventing the actions of $PGF_{2\alpha}$ at the CL, whereas estradiol does not act directly at the CL. This led to further investigations into reconsidering the maternal recognition signal in the pig and fully understanding the possible mechanism(s) that may be involved. The following sections will briefly cover estrogens and prostaglandins and their role in early embryonic development, establishment of pregnancy, and maintenance of the corpus luteum in the pig.

2.7 Estrogen

As previously discussed, studies investigating the pattern of conceptus estrogen synthesis revealed that conceptus estradiol-17β is produced in biphasic peaks on days 11 to 12 and 14 to 18 of pregnancy (Zavy et al., 1980; Geisert et al., 1982b). It should be noted that endometrium is sensitive to the timing of estrogen stimulation as pregnant gilts treated with estradiol on days 9 or 10 of

pregnancy, 48 hours before normal conceptus estrogen synthesis, undergo fragmentation and early embryonic loss by day 18 of pregnancy (Morgan et al., 1987). Thus, early estrogen exposure does not induce early conceptus elongation but advances uterine environment which makes it unsuitable for conceptus attachment and survival (Geisert et al., 2004). Efforts to determine the role the biphasic peaks of estrogen have on early embryonic development and maintenance of pregnancy in the pig first involved attempts to use inhibitors to block both maternal and conceptus estrogen production (O'Neill et al., 1991). Previous studies utilizing estrogen inhibitors in other species determined that keoxifene prevented estrogen production in both rats (Black et al., 1983) and pig ovarian granulosa cells *in vitro* (Veldhuis et al., 1986). Clomiphene administered to mice, rats, and rabbits proved to be effective in preventing development of the decidua (Clark and Markaverich, 1981). Studies performed in the rat have also demonstrated that both 4-hydroxyandrostenedione (4-OHA) and LY56110 significantly hindered estrogen production and 4-OHA prevented implantation (Brodie et al., 1977; Hirsch et al., 1987). Niemann and Elsaesser (1986) reported that the anti-estrogen, Nafoxidene, prevented development of pig morulas to the blastocyst stage *in vitro*. However, administration of estrogen receptor (ER) antagonists (keoxifene and clomiphene) or estrogen inhibitors (LY56110 and 4-OHA) to pregnant gilts beginning on day 10 of pregnancy did not prevent conceptus elongation, or early embryonic development in the pig (O'Neill et al., 1991). There was an increase in calcium in uterine luminal contents of the antagonist treated gilts. The increase in uterine luminal calcium content is

stimulated by either the presence of estrogens produced by conceptuses during pregnancy or after exogenous estrogen administration to cyclic gilts (Geisert et al., 1982a; Geisert et al., 1982b; Geisert et al., 1987; O'Neill et al., 1991). These results indicate that the antagonists and inhibitors used may not have been sufficient at completely blocking the action of *in vivo* conceptus derived estrogens on the maternal endometrium (O'Neill et al., 1991). Inhibitor studies in the pig have proven to be difficult due to inaccuracy of systemic inhibitor and antagonist concentrations as well as the inability to infuse anything directly into the uterus without creating an endometrial inflammatory reaction. The development of recent genetic engineering technologies allows for a more accurate and precise evaluation of the role that specific embryonic derived factors may play in early embryonic development and maintenance of pregnancy in the pig.

A recent gene editing study utilizing the CRISPR/Cas9 system has questioned that conceptus estrogen production acts as the sole maternal recognition signal in the pig. Meyer et al. (2019) inhibited conceptus estrogen synthesis by inactivating the conceptus aromatase (*CYP19A1*) gene. The study created a biallelic *CYP19A1* edit in a pig fetal fibroblast cell line which was utilized for somatic cell nuclear transfer (SCNT). *CYP19A1*$^{-/-}$ cloned embryos which developed to the blastocyst stage *in vitro* were transferred into recipient gilts. *CYP19A1*$^{-/-}$ conceptuses elongated on day 14 of pregnancy and surprisingly the CL were maintained beyond day 15 despite the complete lack of conceptus estrogen production. However, *CYP19A1*$^{-/-}$ conceptus pregnancies did not survive beyond 30 days. Although CL could be maintained beyond day 30

through exogenous estrogen treatment, recipient gilts still lost *CYP19A1*$^{-/-}$ embryos. The failure of pregnancy maintenance beyond day 30 indicated that the ablation of the aromatase gene was possibly embryonic lethal, despite the extended lifespan of the CL. To determine if estrogen producing conceptuses (*CYP19A1*$^{+/+}$) could rescue the pregnancy, *CYP19A1*$^{-/-}$ embryos were co-transferred into recipient gilts with *CYP19A1*$^{+/+}$ *in vitro* fertilized (IVF) cultured embryos. Once transferred with embryos capable of synthesizing and producing estrogen, pregnancies were maintained, and viable fetuses were retrieved by day 35 of pregnancy (Meyer et al., 2019). The concentration of estrogen in the allantoic fluid was greatly reduced in the *CYP19A1*$^{-/-}$ placentas compared to *CYP19A1*$^{+/+}$ litter mates (Meyer, Pfeiffer, Geisert unpublished data) indicating that local estrogen placental production by *CYP19A1*$^{+/+}$ litter mates provided adequate support for *CYP19A1*$^{-/-}$ embryos *in utero*.

This data indicates that while estrogen may not be required for early embryonic development and continuation of corpora luteal function, it is essential for maintaining pregnancy beyond day 30. It is possible that high concentrations of estrogen at the placental-endometrial interface are needed to regulate the maternal immune system. The ability of *CYP19A1*$^{-/-}$ conceptuses to maintain pregnancy throughout the maternal recognition period in the pig strongly suggests additional conceptus derived factors can act to prevent luteolysis during early developments. Therefore, the role of conceptus derived prostaglandins, specifically PGE_2, on early embryonic development, establishment of pregnancy, and maintenance of the CL needs to be investigated.

2.8 Prostaglandins

Although there is considerable literature on the role of $PGF_{2\alpha}$ in stimulating luteolysis, it is not the only prostaglandin that plays an important role(s) in reproductive biology. PGE_2 has been shown to play a role with embryonic migration, protection from luteolysis directly at the CL, and early embryonic development across many species. In order to fully appreciate the many roles that prostaglandins play in reproduction and early maintenance of pregnancy in the pig, one must understand the history and chemical synthesis of these eicosanoids.

2.8.1 History of Prostaglandins

In the early 1900's, studies discovered that canine prostate extractions induced a rise in blood pressure in dogs when they were infused intravenously (Japelli and Scafa, 1906). Similarly, rabbits treated with prostate derived secretions from dogs and bulls also experienced an initial increase in blood pressure (Thaon, 1907). In some cases, rabbits injected with hedgehog and human prostate extracts resulted in the death due to blood clotting (Camus and Gley, 1896; Gotzl, 1910). Later studies demonstrated that fresh human semen was capable of inducing uterine contractions. Kurzrok and Lieb (1930) focused on sterility patterns of women and men by evaluating uterine tissue and seminal samples. This investigation demonstrated that uterine tissue sections reacted differently to various individual's seminal fluid while a seminal fluid sample could have different effects on uterine tissue collect from different females. This led the

researchers to believe that the uterus can either be relaxed or contractile in its response to the presence of semen. These early studies established that seminal fluid contains a component that acts as a contractile stimulant on the uterus. Further studies revealed that the effects of the seminal fluid on uterine contractibility was similar to treatment with acetylcholine (Cockrill et al., 1935). Goldblatt and von Euler (1935) also independently described a pharmacologically active substance in the extracts of human prostates, but the substance had an anti-hypertensive effect in rabbits, cats, and dogs. Von Euler noticed similarities between this prostate derived “P-Substance” to another hypotensive substance synthesized in monkey vesicular glands, vesiglandin (von Euler, 1935). However, this hypotensive substance was chemically different from other depressants such as choline, acetylcholine, and histamine. These results led to the belief that “P-Substance” was produced solely by the prostate gland and was thus named “prostaglandin.” Consistent with the previous research, it was understood that the human prostate contained biologically active components that could either have stimulatory or depressive effects on rabbit blood pressure. The stimulant was proven to be adrenaline, as adrenaline was known to be abundantly present in accessory sex glands. Inactivation of prostate derived adrenaline prevented the increase in blood pressure observed in the rabbit. Interestingly, once the adrenaline was removed, rabbit blood pressure decreased while intestinal contractions increased (von Euler, 1934). Human seminal fluid induced uterine smooth muscle contractions in rabbit similar to the effect prostaglandin had on the intestinal smooth muscle (von Euler, 1936).

Shortly after the discovery of the active compound capable of decreasing blood pressure and inducing smooth muscle contractions, Bergström and associates (1947) extracted thousands of sheep prostate glands. Sheep prostate tissue was homogenized, and secretions extracted for analysis of substance activity. Using chromatography and mass spectrometry, the researchers were able to isolate two different crystalline prostaglandin structures, prostaglandin E_1 (PGE_1) and prostaglandin $F_{1\alpha}$ ($PGF_{1\alpha}$), from the sheep prostate extracts (Bergström and Sjövall, 1957). It was determined that prostaglandins are derived from the polyunsaturated fatty acid, prostanoic acid, and all share a similar 20 carbon skeleton structure with a 5 membered ring. Shortly after the established structure of PGE_1 and $PGF_{1\alpha}$, the structures of PGE_2, prostaglandin E_3 (PGE_3), $PGF_{2\alpha}$, and prostaglandin $F_{3\alpha}$ ($PGF_{3\alpha}$) were elucidated (Bergström, 1965). Other prostaglandins were proposed to be derived from downstream oxidative cyclization reactions of PGE and PGF. Further investigations into the prostaglandins led researchers to discover that contrary to von Euler's initial belief, prostaglandins are widely distributed and produced at basal levels by most tissues in the body and not just the prostate. Isolation of individual prostaglandins led researchers to examine the role that each specific prostaglandin had on the body. PGE_1 was determined to act as a vasodilator and stimulator of smooth muscle contraction while prostaglandins belonging to the PGF family exhibit vasoconstrictive activity (Bergström et al., 1959; DuCharme et al., 1968). Clinical studies examining the effect of exogenous PGE_1 derived from sheep vesicular glands indicated that PGE_1 increased heart rate and stimulated tightening of the

chest in men. However, as previously proposed, blood pressure decreased (Bergström et al., 1959). When pure prostaglandin compounds became available, results demonstrated that PGE_1 and PGE_2 were efficient vasodilators and induced tachycardia. However, studies with $PGF_{2\alpha}$ demonstrated varying effects depending upon the species utilized. $PGF_{2\alpha}$ caused vasodilatory effects in cats and rabbits while it was vasoconstrictive in rats and dogs (DuCharme et al., 1968).

During the late 1960's, researchers began investigating the role that PGEs and PGFs have in reproduction and parturition. Studies confirmed that both PGEs and PGFs were capable of stimulating uterine contractions as earlier research had indicated (Eglinton et al., 1963). Because a strong correlation was established for increasing $PGF_{2\alpha}$ and intensity of the uterine contractions (Karim and Devlin, 1967), $PGF_{2\alpha}$ was utilized to determine the efficiency for inducing early labor in women. Research demonstrated that both PGE_2 and $PGF_{2\alpha}$ could successfully induce early labor and preterm delivery (Karim et al., 1969). Eventually, understanding the effect of prostaglandins on early to mid-gestation was pursued. $PGF_{2\alpha}$ had been demonstrated to terminate pregnancy in monkeys and rodents (Kirton et al.,1970a; Kirton et al., 1970b; Gutknecht et al., 1969). Administration of either $PGF_{1\alpha}$ or $PGF_{2\alpha}$ to women during mid-gestation resulted in termination of pregnancy when women were treated early in the pregnancy with an optimum dosage required for therapeutic abortion. However, most of the women experienced intense nausea, vomiting, and diarrhea as side effects of intense smooth muscle contractions (Karim and Filshie,1970). These researchers

determined that at term pregnancies required significantly less $PGF_{2\alpha}$ to induce early labor, whereas early to mid-gestational pregnancies required ten-fold the amount of $PGF_{2\alpha}$ to stimulate uterine contractions which resulted in severe side effects. During this same time period, other researchers were interested in the role that prostaglandins play during the estrous cycle in animals. It was soon determined that $PGF_{2\alpha}$ is the luteolysin in rats, humans, sheep, cattle, and pigs (Pharriss and Wyngarden, 1969; Wiqvist and Bygdeman, 1970; McCracken et al., 1972; Lauderdale, 1972; Liehr et al., 1972; Rowson et al., 1972; Moeljono et al., 1976). Research leading to the understanding of the role that prostaglandins play in luteolysis and the estrous cycle contributed to the development and use of $PGF_{2\alpha}$ for estrous synchronization and timed breeding in many species. However, much still remains to be known about the role that prostaglandins have in the establishment and maintenance of pregnancy.

2.8.2 Conceptus Prostaglandins

Synthesis of prostaglandins by developing blastocysts has been reported across a number of mammalian species including rabbits, mice, pigs, cows, and sheep (Dickmann and Spilman, 1975; Dey et al, 1980; Pakrasi and Dey, 1982; Harper et al., 1983; Watson and Patek, 1979; Davis et al., 1983; Shemesh et al., 1979; Lewis et al., 1982; Marcus, 1981; Hyland et al., 1982; Lacroix and Kann, 1982). Day 16 pig conceptuses synthesize significant amounts of PGF which was even greater than the prostaglandin synthesis by the maternal endometrium (Watson and Patek, 1979; Lewis and Waterman, 1985). The content of prostaglandin in early pig blastocysts and conceptuses indicates that there is an

increase in prostaglandin synthesis at the time of blastocyst formation with a significant increase around days 12 to 14 of pregnancy (Davis et al., 1983). The increase in conceptus prostaglandin synthesis is coincident with the time of maternal recognition of pregnancy in the pig when conceptuses elongate and increase production of E_2 and IL1B2 (Geisert et al., 1982a; Ross et al., 2003; Mathew et al., 2015). Data indicate that pig conceptuses produce about twice as much PGE_2 than $PGF_{2\alpha}$ during this period of increased prostaglandin synthesis (Davis et al., 1983). The ratio of PGE/PGF is about 2:1 which is similar to the content of PGE/PGF present in the uterine luminal contents during the maternal recognition period in the pig (Geisert et al., 1982b). These data suggest that PGE_2 may play an important luteoprotective role during this critical period of embryonic survival in the pig. In order to comprehend the role of conceptus prostaglandins in the pig, an understanding of prostaglandin synthesis is important.

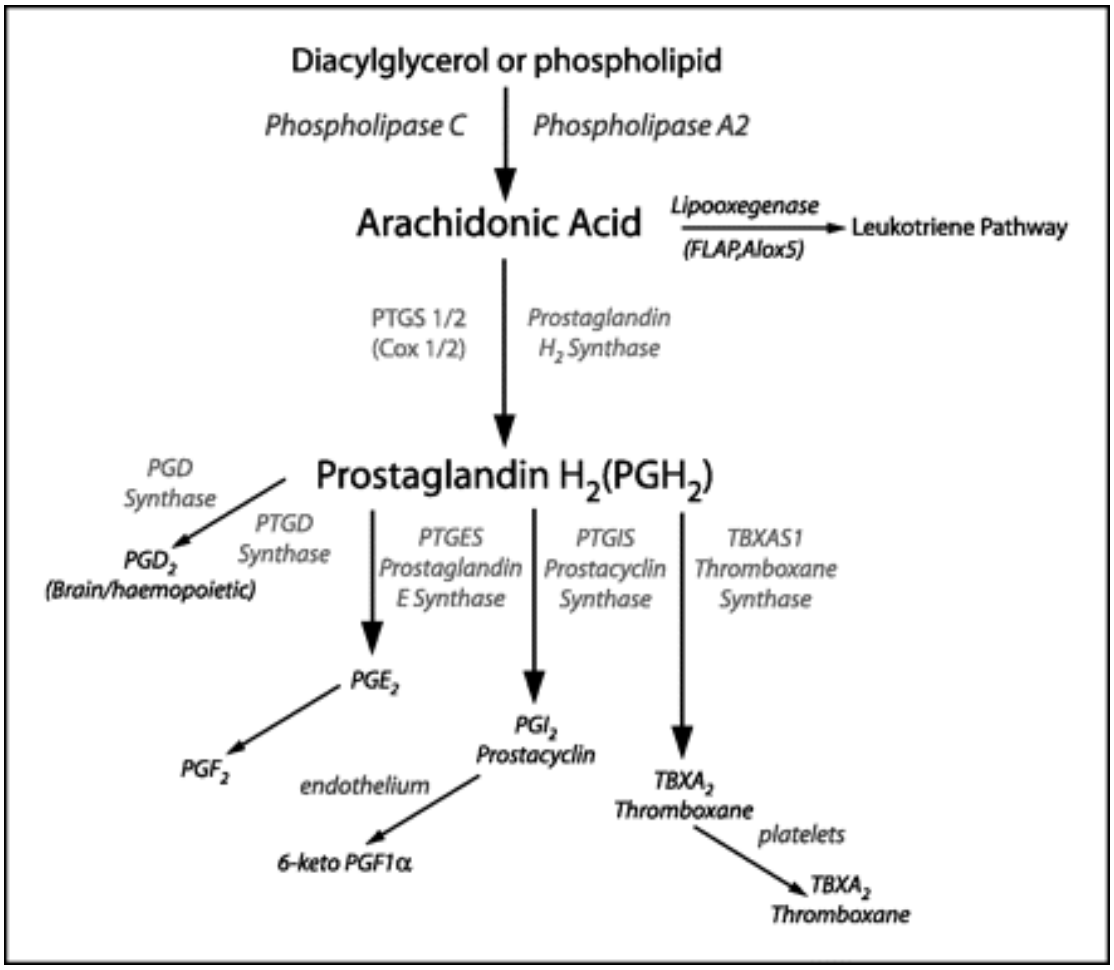

Figure 2.1: Diagram of the Prostaglandin Pathway. (Abraham et al., 2009). All prostaglandins are derived from arachidonic acid. The action of prostaglandin synthase 2/Cyclooxygenase 2 (PTGS2/COX2) converts arachidonic acid into PGH_2 and consequently into further downstream prostaglandins.

2.8.3 Prostaglandin Synthesis

Prostaglandins are synthesized through the eicosanoid pathway (Figure 2.1). As previously described, prostaglandin biosynthesis is achieved from oxidative cyclization of the 20 carbon skeleton with a 5-membered ring polyunsaturated fatty acid. Arachidonic acid is esterified to the phospholipids present within the cell membrane. The phospholipase A_2 is responsible for liberating the arachidonic acid from the fatty acid structures (Flower and Blackwell, 1976). Once freed from the phospholipid bilayer, arachidonic acid is released into the cytoplasm of the cell where prostaglandin synthase enzymes (PTGS1 and PTGS2) act as the rate limiting step to begin the process of converting arachidonic acid into further downstream prostaglandins (Dubois et al., 1998). Prostaglandin synthase 1 and 2 act enzymatically to catalyze the conversion of arachidonic acid into prostaglandin G_2 (PGG_2) and PGH_2. PGH_2 is subsequently converted further into a variety of prostaglandins through the oxidative cyclization reaction. These prostaglandins will bind and react with one of two receptor types: G protein-coupled receptors or nuclear peroxisome proliferator-activated receptors (PPAR). Prostaglandins typically belong to the cell surface G protein-coupled receptor family but can act as ligands for the PPARs (Kliewer et al., 1992; Kliewer et al., 1994; Forman et al., 1995; Forman et al., 1997). While PTGS1 and PTGS2 are both biologically similar when active, the two enzymes are expressed in response to different stimuli. PTGS1 is the conserved form of the prostaglandin synthase meaning that it is expressed in most body tissues at all times. However, PTGS2 is the inducible form, increasing

in expression in response to inflammation, cell growth, immune stimuli, or tumorigenesis (Smith and Dewitt, 1996; Dubois et al., 1996; Oshima et al., 1996).

2.8.4 Prostaglandin Synthase 2

Similar to the purification and isolation of prostaglandins, PTGS1 was purified from sheep and cow seminal vesicles (Hemler and Lands, 1976; Miyamoto et al., 1976). However, it first was believed that PTGS1 was the only prostaglandin synthase until cell division studies discovered PTGS2 in 1991 (Xie et al., 1991; Kujubu et al., 1991). According to recent pig genome annotation (ENSSSCG00000015579), the pig genome contains one *PTGS2* gene that spans approximately 10 kb of chromosome 9 (127,848,603-127,859,287 forward strand) containing 10 exons and can express 2 different transcripts through alternative splicing. PTGS2 increases prostaglandin production in response to inflammation, and therefore was intensely targeted during the pursuit of establishing an effective anti-inflammatory drug (Copeland et al., 1994; Kurumbail et al., 1996).

Prostaglandins are very abundant in the endometrium during important reproductive events such as implantation and decidualization in rodents and humans (Chakraborty et al., 1996). In mice, *PTGS2* expression is localized to the luminal epithelium and stromal cells in close apposition to the attaching blastocyst. Expression of *PTGS2* is then relocated to the mesometrial side of the uterus where placentation and decidualization occurs during implantation in the mouse (Lim and Dey, 1997). Because the pig has diffuse epitheliochorial placentation, endometrial expression *PTGS2* in the pig plays no role in

decidualization. However, the luminal and glandular epithelium highly express *PTGS2* throughout pregnancy in the pig. *PTGS2* expression in the upper glandular epithelium and luminal epithelium increases significantly after day 10 of pregnancy in the pig, coincident with conceptus elongation and the period of maternal recognition (Dubois et al., 1993; Ashworth et al., 2006; Blitek et al., 2006). Similarly, conceptus *PTGS2* expression increases significantly during the period of rapid conceptus elongation on day 12 of pregnancy (Wilson et al., 2002). While the transcriptional activity of conceptus *PTGS2* is understood in the pig, the role that PTGS2 induced prostaglandins play remain largely unknown.

2.8.5 Inflammatory Response of Prostaglandins

During the period of elongation and attachment, developing pig conceptuses secrete prostaglandins and other factors to assist with regulating the uterine inflammatory induced by the attaching conceptuses (Modrić et al., 2000; Ross et al., 2003; Joyce et al., 2007; Waclawik and Ziecik, 2007). IL1B2 produced during the period of rapid elongation in the pig stimulates PLA_2 and PTGS2 expression. The increase of IL1B2, PLA_2 and PGs during the rapid conceptus elongation is thought to increase cell fluidity which allows cellular remodeling. During the window of uterine receptivity, the endometrium is responsive to conceptus derived factors which can stimulate an inflammatory response. The endometrium increases production of Leukemia Inhibiting Factor (LIF) and Tumor Necrosis Factor (TNF) between days 10 to 15 to allow conceptus adhesion and attachment to the uterine LE in the pig (Modrić et al., 2000; Blitek et al., 2010; Yu et al., 1998). TNF has also been proposed to induce

PTGS2 and PGE_2 production from LE during elongation in the pig (Waclawik et al., 2010). As mentioned previously, prostaglandins can induce vascular changes among different species. Because the pig does not have an invasive form of placentation, the maintenance and success of pregnancy rely on increasing maternal blood flow and endometrial vascularity to transfer nutrients from mother to fetuses. Expression of the vascular endothelial growth factor (*VEGF*) is significantly increased in the stromal cells around day 12 of pregnancy in the pig (Kaczmarek et al., 2008). It is believed that PGE_2 stimulates VEGF production and angiogenesis from days 15-18 of pregnancy which is the critical period of apposition, attachment, and adhesion of the conceptus to the maternal endometrium (Kaczmarek et al., 2008).

2.9 Prostaglandin and PTGS2 Inhibition

Prostaglandin synthase inhibitor studies have been utilized to determine the biological and physiological role that prostaglandins may have on early pregnancy. Once it was understood that blastocysts were capable of synthesizing prostaglandins, researchers strived to determine what role prostaglandins had on early embryonic development. Anti-inflammatory drugs and an acetylenic analog were utilized *in vitro* to examine the role of prostaglandins on blastocyst development and hatching in the mouse. Addition of indomethacin, meclofenamic acid, phenidone, and 7-oxa-13-prostynoic acid to culture media containing mouse blastocysts, inhibited blastocyst prostaglandin production *in vitro* and blocked hatching from the zona pellucida (Biggers et al., 1978). Results indicated that prostaglandins play a role in mouse blastocyst

expulsion from the zona pellucida. However, studies in rat and hamster treated *in vivo* with indomethacin (Kennedy, 1977; Evans and Kennedy, 1978) on day 4 or 5 of pregnancy did not exhibit any loss of pregnancy. Although embryonic survival was the same, pregnant rats administered indomethacin contained implantation sites with less mass and had a longer duration of gestation. These results indicate that inhibition of uterine and blastocyst prostaglandin synthesis causes delayed implantation in rodents (Kennedy, 1977; Evans and Kennedy, 1978). Administration of indomethacin also inhibits decidualization in rats (Sananes et al., 1976; Tobert, 1976).

Inhibition of prostaglandin synthesis during pregnancy has also been reported in large domestic farm species. Administration of the prostaglandin inhibitor, meloxicam, to ewes from days 8 to 14 of pregnancy resulted in the failure of conceptuses to elongate properly (Dorniak et al., 2011). However, unlike the sheep conceptus, although treatment of pregnant gilts with either indomethacin and flunixin meglumine block uterine and conceptus prostaglandin synthesis, pig conceptuses were capable of undergoing elongation (Geisert et al., 1986). Further investigation of the effect of prostaglandin inhibitors in the establishment of pregnancy in the pig indicated a majority of pregnant pigs treated with indomethacin from days 10 to 25 of gestation failed to maintain CL function and pregnancy beyond day 26 of gestation (Kraeling et al., 1985). This study established that overall prostaglandin production by the developing conceptus and maternal endometrium are important for maintenance of pregnancy. While these prostaglandin synthase inhibitor studies prevent

prostaglandin production from both the mother and the developing conceptuses, they do not address the specific role that conceptus prostaglandin synthesis plays in early establishment of pregnancy in the pig.

New technologies have allowed scientists to more directly understand the roles of biologically active substances at specific tissues which cannot be resolved using inhibitors. For example, recent studies in mice using cyclization recombination/loxP (cre/lox) technology to generate a fetal cyclooxygenase 1 ($COX\text{-}1^{-/-}$)/$COX\text{-}2^{-/-}$ double null mutation discovered that embryos incapable of synthesizing prostaglandins develop to the time of parturition but fail to survive shortly after birth (Reese et al., 2000). Similar studies revealed that dams without PTGS1 resulted in smaller placental and fetal development, while removal of the PTGS2 gene essentially reared dams infertile preventing ovulation, fertilization, implantation, and decidualization (Langenbach et al., 1995; Dinchuk et al., 1995; Morham et al., 1995; Lim et al., 1997). The cre/lox system works efficiently and accurately in mice, but due to the longer generational time periods in larger animals, CRISPR/Cas9 gene editing technology could be more efficient at determining the role PTGS2 has on early pregnancy in livestock species. Similar research utilizing the CRISPR/Cas9 system was conducted by O'Neill et al. (2017) to establish the biological role of PTGS2 in early pregnancy of sheep. Surprisingly, ablating conceptus *PTGS2* resulted in normal elongation and maintenance of the CL to day 14 of pregnancy in the ewe. These results are different from the inhibitor study of Dorniak et al. (2011) which indicated a possible role of prostaglandins in elongation in the ewe. However, inhibitors are

capable of blocking maternal and conceptus prostaglandins but do not analyze the effect conceptus prostaglandin plays in early pregnancy. A similar strategy was conducted to determine the role that PTGS2 derived prostaglandins play in early embryonic development and pregnancy establishment in the pig.

2.10 CRISPR/Cas9 Genomic Editing Technology

The discovery of the double helix has led to further research endeavors to understand more about the genome and ways to manipulate the genetic makeup of many different organisms (Doudna and Charpentier, 2014). Once more was learned about the genetic structure, increased interest in genetic engineering resulted in many different gene targeting technologies such as oligonucleotides (Khorana,1968), transgenes (Chang and Cohen, 1974), zinc finger nucleases (ZFNs) (Kim et al., 1996), and transcription activator like effector nucleases (TALENs) (Li et al., 2011) that were utilized to generate desired genetic edits (Wells and Prather, 2017). One of the newer, more readily accessible, and efficient gene editing technologies, CRISPR/Cas9, has advanced investigations into the role of specific genes in many biological systems. Jennifer Doudna and Emmanuelle Charpentier are credited with the technological application of DNA repair found in prokaryotic species (Doudna and Charpentier, 2014). The genomes of these bacteria and yeast cells contain clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) proteins to help protect and conserve the genome from foreign nucleic acids (Jinek et al., 2012). This CRISPR defense system which Doudna and Charpentier observed consisted of 2 parts: Cas9 protein and guide RNA (gRNA).

The Cas9 protein is responsible for creating a double stranded cut in the genome. The gRNA complex consists of two types of RNA: CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA), both which are synthesized naturally by the cell. The crRNA will act as a guide and bring the endonuclease enzyme, Cas9, to the targeted foreign bacterial DNA. The tracrRNA is responsible for then holding the crRNA in place within the Cas9 protein. Together, gRNA (crRNA/tracrRNA hybrid) and the Cas9 protein will locate, cut, and remove the foreign DNA.

This process was then translated into a gene editing technology that can target any region of the genome and with the Cas9 protein, create a double stranded break within the genomic helix. Scientists are now able to design crRNA specific to any gene of interest. Once the target gene is located and broken, the genome undergoes one of two repairs: non-homologous end joining (NHEJ) and homologous directed repair (HDR) (Doudna and Charpentier, 2014). The CRISPR/Cas9 system allows for not only knocking out specific genes, but inserting, swapping out, or repairing certain regions of the genome. When donor genes are used with the CRISPR/Cas9 system, the genome undergoes HDR by gene replacement. However, when no donor genes are applied, the genome undergoes gene editing by NHEJ to result in either a deletion or an insertion (Doudna and Charpentier, 2014). Since the development of CRISPR/Cas9, the genetic engineering technique has been increasingly utilized and has led to new optimization applications. Software and online databases have made the CRISPR/Cas9 gRNA design procedure significantly easier, requiring only that the

gRNA sequence is followed by a protospacer adjacent motif (PAM), 5'-NGG-3' (Sorek et al., 2013). This readily available gene editing technology allows scientists to efficiently edit the genome of many mammalian species. In order to accurately determine the role that conceptus derived PTGS2 has on early embryonic development and pregnancy establishment and maintenance in the pig, the CRISPR/Cas9 system was utilized.

2.11 Utilizing CRISPRs for the Generation of Pig Conceptus PTGS2 Null Research Model

The majority of embryonic mortality occurs during between days 10 to 30 of gestation in the pig (Pope, 1994). This period of embryonic development is also synonymous with the uterine migration and spacing of embryos, rapid conceptus elongation, maternal recognition of pregnancy, conceptus apposition and attachment to the endometrial luminal epithelium, and placentation. While the effects of many conceptus derived factors on early pregnancy events in the pig are understood, much is still unknown about the maternal recognition signal (aromatase deficient conceptuses maintain CL function) and causes for embryonic loss during this time. Previous studies have examined the effect of prostaglandins on conceptus elongation, maternal recognition of pregnancy, attachment, and placentation. However, many of these previous studies utilized inhibitors or antagonists that have been successful in preventing both maternal and conceptus prostaglandin production as previously described. Recent gene editing studies allow for specific targeting of genes to analyze function. The present research will utilize the CRISPR/Cas9 system to target and edit the

porcine conceptus *PTGS2* and conduct a loss-of-function study to determine the role of conceptus PTGS2 derived prostaglandin production during early embryonic development and establishment of pregnancy in the pig.

CHAPTER THREE

ABLATION OF CONCEPTUS *PTGS2* PROVIDES A NEW UNDERSTANDING OF EARLY PREGNANCY EVENTS IN THE PIG

3.1 Introduction

Maintenance of pregnancy in mammals relies on creating favorable maternal conditions for conceptus development and attachment to the uterine surface epithelium. Secretions from the maternal endometrium, which are influenced by the production of multiple factors from the developing conceptus, provide the proper milieu and environment for conceptus and fetal development to term (Geisert et al. 2017). During early development, the conceptus must regulate endometrial synthesis and/or movement of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) to prevent luteolysis to maintain progesterone (P_4) secretion during pregnancy. In 1969, Roger Short coined the term 'maternal recognition of pregnancy' and inspired the pursuit to determine the conceptus derived biochemical signal(s) resulting in corpus luteum (CL) maintenance beyond the length of the estrous cycle across species.

The signal, pathway, and timing for maternal recognition of pregnancy varies across mammalian species. In cattle and sheep, interferon-τ (IFNT), a type I interferon produced by conceptuses during trophoblast expansion, prevents the pulsatile release of $PGF_{2\alpha}$ from the endometrium which occurs to stimulate CL regression during the estrous cycle (Bazer et al., 1994). In primate species, human chorionic gonadotropin (hCG) produced by the

syncytiotrophoblast acts directly on the CL to prevent luteolysis from ovarian derived $PGF_{2\alpha}$ (Ross, 1978). Although endometrial $PGF_{2\alpha}$ release is attenuated during the period of maternal recognition in the mare, the conceptus signal remains unclear (Klein, 2016). Spatiotemporal synthesis and release of estrogen (E_2) by the rapidly expanding conceptuses has long been considered the maternal recognition signal in the pig (Bazer and Thatcher, 1977).

Pig conceptuses undergo rapid trophoblast elongation concurrent with the time of maternal recognition of pregnancy (Perry and Rowlands, 1962; Anderson, 1978; Geisert et al., 1982a,b). Since the pig has true epitheliochorial placentation, the developing fetus relies on nutrients and blood flow from the mother to be diffused across the attached placenta. Whyte et al. (2018) demonstrated that rapid conceptus elongation (1 to 2h) involves conceptus expression and synthesis of interleukin 1 beta 2 (IL1B2). Expansion of multiple conceptuses throughout the long uterine horns of the pig is essential to preventing luteolysis during the establishment of pregnancy.

Studies indicated that conceptus E_2 stimulated the redirection of $PGF_{2\alpha}$ away from the uterine vasculature and into the uterine lumen to prevent luteolysis (Bazer, 1977). The ‘endocrine/exocrine theory’ has been the accepted model as the maternal recognition signal in the pig (Bazer et al., 1982). However, recent CRISPR/Cas9 editing of conceptus aromatase gene expression indicated that conceptus E_2 production is not essential for conceptus elongation or early CL maintenance (Meyer et al., 2019). Therefore, the maternal recognition signal in the pig involves alternate conceptus secretions beyond just estrogens. In addition

to E_2, prostaglandins (PGs) and interferons produced by the conceptuses during the period of elongation and placentation may stimulate pathways to maintain CL function during early pregnancy (Geisert et al. 2017).

Inhibition of prostaglandin synthesis causes conceptus loss during early pregnancy in the pig (Kraeling et al. 1985) and ewe (Dorniak et al., 2011), implying that conceptus and endometrial prostaglandins play a pivotal role in early embryonic development and CL maintenance. Prostaglandins have been proposed to play a role in the maternal recognition signal in the pig. Uterine luminal concentrations of $PGF_{2\alpha}$ increase during the period of trophoblastic elongation and attachment to the uterine luminal surface (Zavy et al., 1980, Geisert et al. 1982b). The luminal content of PGE_2 in the uterus also increases more than double the amount of $PGF_{2\alpha}$ (Davis and Blair, 1993; Christenson et al., 1994). The higher ratio of $PGE_2/PGF_{2\alpha}$ has been proposed to play a luteoprotective role in preventing CL regression in the pig. Ford and Christenson (1991) indicated that individually injected CL with PGE_2 and $PGF_{2\alpha}$ were larger and produced more progesterone than CL treated with E_2 and $PGF_{2\alpha}$ suggesting that PGE_2 may play a direct role in protecting the CL.

Conceptus prostaglandin synthesis is dependent on the expression of prostaglandin synthase (*PTGS*) 1 and 2. PTGS1 is well conserved in many tissues across species, whereas PTGS2 is induced by inflammatory and immune responses (Xie et al., 1992; Herschman, 1996; Smith et al., 1996). While conceptus *PTGS1* expression is low to undetectable until the time of placental attachment (Day 14-18) in the pig (Waclawik and Ziecik, 2007), conceptus

PTGS2 is highly expressed concurrently with the period of trophoblastic elongation on Day 12 (Wilson et al., 2002).

Although the studies utilizing PTGS1 and 2 inhibitors in the gilt (Kraeling et al. 1985) and ewe (Dorniak et al., 2011) demonstrated a negative effect on early embryonic development and CL maintenance, the inhibitors have been shown to interfere with both endometrial and conceptus prostaglandin synthesis. Therefore, the objective of the present study is to determine the role of conceptus PTGS2 expression on early conceptus development and establishment of pregnancy in the pig. CRISPR/Cas9 gene editing is utilized to directly target conceptus *PTGS2* expression to specifically engineer *PTGS2*$^{-/-}$ embryos to evaluate the effects on embryonic development and luteal function in the pig.

3.2 Materials and Methods

All procedures used 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 crossbred gilts of similar age (8-10 months) and weight (100-130 kg). Gilts were observed for estrus behavior twice daily with the onset of estrus designated Day 0 of the estrous cycle.

CRISPR/Cas9 Design and Transfection of Porcine Fetal Fibroblast Cells. The sus scrofa *PTGS2* gene spans 10kb of chromosome 9 (127,848,603-

127,859,287 forward strand) and contains 10 exons. Broad Institute design tool (https://broadinstitute.org/gpp/public/analysis-tools/sgrna-design) was used to design two pairs of guide RNAs (gRNAs) that specifically targeted exon 1 of the porcine *PTGS2* gene. To minimize off targeting events, each gRNA was tested using NCBI's Nucleotide BLAST Tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Annealed gRNA oligonucleotides were inserted into a pX330 plasmid containing the Cas9 protein (Addgene) which was cloned into amphicillin resistant E.coli. Cutting efficiency of the two selected gRNAs was tested through transfections of porcine fetal fibroblast cells at different concentrations (2, 4, or 8 µg). Following transfection, fetal fibroblast cells were cultured for 2-3 days, lysed, and DNA used for PCR amplification using primers specifically designed to amplify the location of gRNA design. PCR amplicon was visualized on a 2% ethidium bromide agarose gel to determine which guides had the greatest cutting efficiency. The optimal guide pair was confirmed and used at a concentration of 4 µg (2 µg per gRNA).

Clonal Expansion and Colony Screening. Fetal fibroblast cells transfected with the selected optimal pair of gRNAs were plated using a dilution curve to promote colony growth from a single cell. One week after transfection, individual colonies were collected, lysed, submitted to PCR amplification and the PCR product run on a 2% ethidium bromide agarose gel to screen for monoallelic or biallelic edits. The PCR amplicon of individual cell colonies with biallelic edits were purified using the PureLink PCR Purification Kit (ThermoFisher Scientific, K310001) and

submitted to the University of Missouri Life Sciences DNA Core for Sanger Sequencing. Out of 264 screened cell colonies, one biallelically edited cell line designated 'CAP81' was identified by PCR (Fig. 3.1) and confirmed with Sanger Sequencing to have a 128 base pair deletion on one allele and a 131 base pair on the second allele of *PTGS2* exon 1 (Fig. 3.2). The PCR amplicon from the 'CAP81' cell line was cloned into a pCR™4-TOPO® TA vector (ThermoFisher Scientific, 450071) which was sequenced to identify variations in the modification on each allele.

Somatic Cell Nuclear Transfer (SCNT). Fetal fibroblast cell lines, *PTGS2*$^{+/+}$ (unedited) and *PTGS2*$^{-/-}$ (biallelically edited), were grown to 80% confluency and used as donor cells for SCNT as previously described (Whitworth et al., 2014). 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. The polar body and adjacent cytoplasm, containing the metaphase II plate, were removed from the oocyte. A donor fibroblast cell (*PTGS2*$^{+/+}$ or *PTGS2*$^{-/-}$) was inserted into the perivitelline space (Lai and Prather, 2003) and electrically fused with two DC pulses at 1.2 kV/cm for 30 µsec using a BTX Electro Cell Manipulator (Harvard Apparatus). After fusion, the cloned zygotes were chemically activated (Machaty, Wang, Day, and Prather, 1997) and cultured for 14-16 h (Spate et al., 2015). Culture media (500 µL/well) was collected to determine prostaglandin synthesis following development to

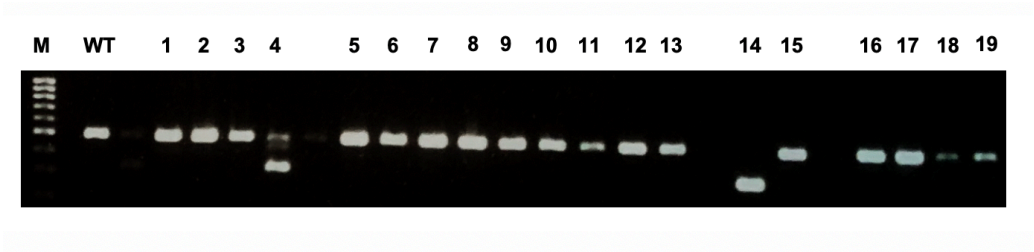

Figure 3.1: Colony Screening for Biallelic Modifications. Individual cell colonies were picked, lysed, and used for PCR amplification. The resulting PCR was run on a 2% agarose gel with ethidium bromide and imaged. Cell colonies identified with a modification were cleaned and submitted to sequencing (MU DNA Core, University of Missouri-Columbia). Each band represents an individual cell colony (1-19) or control wild-type *PTGS2*$^{+/+}$ cells (WT). The image demonstrates the identification of the biallelic edited cell line #14, CAP81, used for somatic cell nuclear transfer.

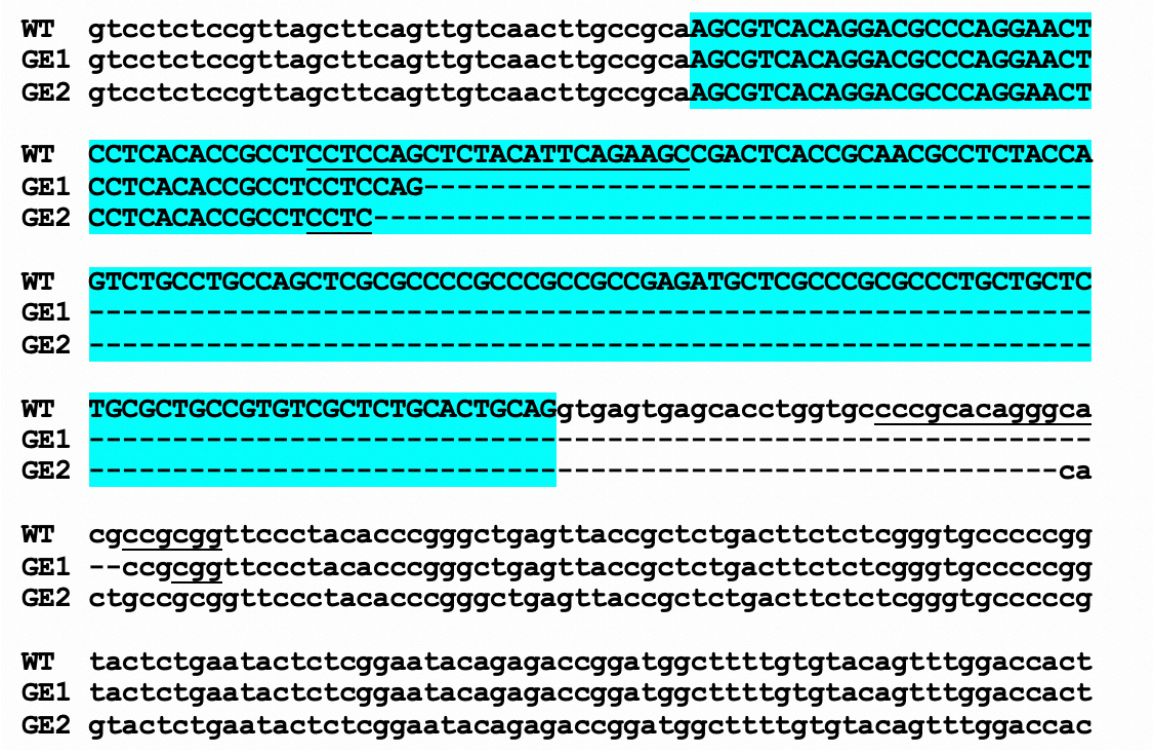

Figure 3.2: Biallelic Modification on Exon 1 of the Porcine *PTGS2* gene. Sequence shows that Exon 1 (highlighted in blue) of the *PTGS2*$^{-/-}$ cell line has a 128 base pair deletion in one allele (GE1) and a 131 base pair deletion in the second allele (GE2) compared to the *PTGS2*$^{+/+}$ WT sequence (WT). The locations of the gRNAs are underlined. Introns are displayed in lowercase.

blastocyst stage on day 5 to 6 (60 blastocysts/well; *PTGS2*$^{+/+}$ n=5 and *PTGS2*$^{-/-}$ n=5). A subset of Day 6 *PTGS2*$^{+/+}$and *PTGS2*$^{-/-}$ blastocysts were used for whole-mount immunohistochemistry. Blastocysts were fixed in 4% paraformaldehyde (PAF) for 20 min at room temperature and stored in Polyvinyl Alcohol (PVA) treated Tyrode Lactate-HEPES (TL-HEPES) for PTGS2 immunohistochemistry.

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 recipient gilt oviduct near the ampullary-isthmic junction on day 3, 4, or 5 after initiation of 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 14 (n=4 *PTGS2*$^{+/+}$, n=4 *PTGS2*$^{-/-}$) or day 17 (n=3 *PTGS2*$^{+/+}$, n=4 *PTGS2*$^{-/-}$). The uteri and ovaries were excised from the abdomen, placed on ice and brought to the laboratory for processing. The reproductive tract was rinsed and trimmed free of the mesometrium. Day 14 and 17 conceptuses were recovered from the uterus by flushing each uterine horn 2X with 30 mLs of Phosphate Buffered Saline (PBS). Conceptuses retrieved from the uterine lumen flushing (ULF) were examined and the morphology and viability assessed. A subset of day 14 and 17 filamentous conceptus tissue (~ 60 mg wet weight) were immediately placed into 2 mLs of Dulbecco's Modified Eagle Medium (DMEM; ThermoFisher Scientific, 11995-

065) culture media supplemented with fetal bovine serum (FBS) and penicillin streptomycin (Pen Strep; ThermoFisher Scientific, 15140-122) following flushing from the uterine horn. Conceptuses placed in the culture media were placed in a 5% O_2, 5% CO_2 incubator at 37°C. Media (200 µL) was collected and snap frozen at 3 and 12 h of culture for analysis of conceptus prostaglandin production. 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 utilized 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 PG, IL1B, and estradiol-17β (E_2) analysis.

Uteri from a group of artificially inseminated (AI) gilts (n=3) and recipient gilts containing *PTGS2*$^{-/-}$ conceptuses (n=3) were collected on day 35 of gestation. The pregnant uteri were excised immediately following euthanasia, placed on ice, and taken into the lab for immediate processing. 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 extraction. Allantoic fluid from each placenta was collected, flash frozen, and stored at -80°C until use for analysis of E_2. Uterine sections containing attached placental tissue were removed and fixed

in 4% PAF for 24 h. Tissue sections were then stored in 70% ethanol until utilized for immunohistochemistry.

Measurement of Blastocyst and Conceptus Prostaglandin Production. A commercial prostaglandin screening ELISA kit (Cayman Chemical Company, 514012) was utilized to measure prostaglandin production in both blastocyst and conceptus (day 14 and 17) culture media (3 h post collection). All samples were analyzed in duplicate using an EL808 Ultra Microplate Reader (Bio-Tek).

Total RNA, Genomic DNA, and Protein Isolation. Endometrial total RNA was obtained using TRIzol-RNA lysis reagent (ThermoFisher Scientific, 15596018). Direct-zol RNA Miniprep Plus kits (Zymo Research, R2070) were used for RNA purification of the endometrial homogenate. Conceptus RNA, DNA, and protein was extracted simultaneously 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 with 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 1% bleach integrity gel.

Reverse Transcription 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 1000 ng of total RNA and RT-PCR was performed and quantified using the CFX384 Real-Time System (Bio-Rad). Conceptus gene expression was measured using primers (Table 3.1) specific for *PTGS2* (Blitek et al., 2006), *PTGS1* (Ashworth et al., 2006), prostaglandin E synthase (*PTGES;* Choi et al., 2019), prostaglandin E synthase 2 (*PTGES2;* Choi et al., 2019), prostaglandin E synthase 3 (*PTGES3;* Choi et al., 2019), prostaglandin F synthase (*PGFS;* Waclawik et al., 2006), *CYP19A1* (Ebeling et al., 2011), *IL1B2* (Whyte et al., 2018), *IFNG* (Joyce et al., 2009), and *IFND* (Joyce et al., 2009). Similarly, endometrial gene expression was measured using primers specific for aldo-keto reductase family 1 member b (*AKR1B1*; Choi et al., 2019), *PTGES* (Choi et al., 2019), *PTGES2* (Choi et al., 2019), *PTGES3* (Choi et al., 2019), *PTGS1* (Ashworth et al., 2006), *PTGS2* (Blitek et al., 2006), solute carrier organic anion transporter family member 2A1 (*SLCO2A1*; Choi et al., 2019), and solute carrier organic anion transporter family member 5A1 (*SLCO5A1*; Choi et al., 2019) (Table 3.1). RT-PCR analysis determined that porcine *YWHAG* gene expression was not statistically different ($P > 0.05$) among the conceptus and endometrial

Table 3.1. Endometrial and Conceptus RT-PCR Primers.

Gene	RefSeq gene	Primers 5' → 3'	Source
PTGS2	**AY028583.1**	ATGATCTACCCGCCTCACAC	(Blitek et al.,
	Provisional	AAAAGCAGCTCTGGGTCAAA	2006)
PTGS1	**AF207823**	CAACACTTCACCCACCAGTTCTTC	(Ashworth et
	Model	TCCATAAATGTGGCCGAGGTCTAC	al., 2006)
PTGES	**NM_001038631.1**	AGAGCATGAAAACCATCACTCC	(Choi et al.,
	Provisional	CTCAAGGACATTCTGTCAGGTTC	2019)
PTGES2	**XM_001927936.5**	ATACCAGTACAAGACGTGTCCATTC	(Choi et al.,
	Model	ATGATGACAGAGGAGTCATTCAGTT	2019)
PTGES3	**XM_001929413.6**	CAAATGATTCCAAGCATAAAAGAAC	(Choi et al.,
	Model	GGTAAATCTACATCCTCATCACCAC	2019)
PGFS	**AY863054.1**	GGACTTGGCACTCTCGTCTC	(Waclawik et
	Validated	GAACAGCTCCTCCCTCTTCA	al., 2006)
CYP19A1	**NM_214429.1**	GCTAATTGCAGCACCAGACA	(Ebeling et al.,
	Provisional	TGTTGGTTCCCTTTTTCACC	2011)
IL1B2	**NM_001302388.1**	GCCAATGGTTTTCTCTGTGATGCC	(Whyte et al.,
	Validated	CTCATGCAGAACACCACTTCTCTC	2018)
IFNG	**NM_213948.1**	CCATTCAAAGGAGCATGGAT	(Joyce et al.,
	Provisional	TTCAGTTTCCCAGAGCTACCA	2009)
IFND	**NM_001002832.1**	ATGGATTGTCCCCATGTAGG	(Joyce et al.,
	Provisional	CTGAGCTACCAGGGTTACCG	2009)
AKR1B1	**NM_001001539**	ATGTGTACCAGAACGAGAACGAG	(Choi et al.,
	Provisional	GGATAAGGTAGAGGTCCAGGTAGTC	2019)
SLCO2A1	**NM_001123195**	TGCTGCAGATCTTTGTGGAC	(Choi et al.,
	Provisional	GGCAATGACAGAGGAGAAGG	2019)
SLCO5A1	**XM_005663031.3**	CTGTTCATAGTGACCTTCATCACAG	(Choi et al.,
	Model	TAAAAATAAAGCCAACGAATTTGAG	2019)
YWHAG	**XM_005661962.3**	TCCATCACTGAGGAAAACTGCTAA	(Whitworth et
	Model	TTTTTCCAACTCCGTGTTTCTCTA	al., 2015)

total RNA samples and was therefore used as the housekeeping gene. Relative expression of sequence-specific products was quantified by the $^{-2\Delta}$CT method. The Δ CT was 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 14 *PTGS2*$^{+/+}$ Δ CT average.

Western Blot Analysis. Conceptus protein concentrations were determined using the Qubit 4 Fluorometer (Invitrogen). Equal amounts of protein (20 µg) were loaded and separated on a 4-20% Mini Protean TGX Precast Protein Gel (Bio-Rad, 4561096). Separated proteins were transferred onto a PVDF membrane (0.2 µm pore size) using the iBlot Transfer Stack, PVDF (ThermoFisher Scientific, IB401001) and an iBlot Dry Blotting System (Invitrogen). Non-specific binding sites were blocked by incubating the membrane with 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) and 3 mg/mL bovine serum albumin (BSA) for 4 h at room temperature (rt). Membranes were then rinsed and incubated at 4°C overnight with polyclonal PTGS2 (dilution 1:500; Cayman Chemicals, 160107). After washing (4X) in TBST buffer for 10 min, membranes were incubated at rt for 1.5 h in Goat anti-Rabbit IgG (H+L) secondary antibody, HRP (dilution 1:5000; Invitrogen, 31466) or Rabbit anti-Mouse IgG (H+L) secondary antibody, HRP (dilution 1:5000; Invitrogen, 31450), respectively. Membranes were washed (4X) in TBST buffer for 10 min, placed onto a Fotodyne Imaging System (Fisher Scientific, F71720DLN) platform and reacted with Pierce ECL 2 Western Blotting Substrate (ThermoFisher, 80196) for 3 min. The membrane was then exposed to

ultraviolet (UV) light for 10 min and images of the membrane were captured and stored within the FOTO/Analyst PC Image Acquisition Software. Once images were taken, the membrane was stripped with a mild stripping buffer (15 g glycine, 1 g sodium dodecyl sulfate (SDS), 10 mL Tween20, and 1 L ultrapure H_2O, pH 2.2) and reprobed with mouse THE™ alpha Tubulin Antibody (dilution 1:5000; GenScript, A01410). Densitometry of the protein bands was analyzed with Fiji Is Just ImageJ (FIJI).

Measurement of IL1B and E_2. A pig IL1B ELISA kit (Abcam, 100754) was utilized to measure the ULF content of IL1B. ULF and allantoic concentrations of E_2 were quantified on individual RIAs as described previously (Kirby et al., 1997). The sensitivity of the E_2 assay was 0.5 pg/mL. Standards and pooled aliquots of ULF were linear and parallel over a mass of 0.25 to 20 pg. All samples were measured in duplicate with an intraassay coefficient of variation of 3.84%. Standards and pooled aliquots of allantoic fluid were linear and parallel over a mass of 0.25 to 20 pg. Due to the high concentrations of E_2, allantoic fluid was diluted 1:100 with 100 µL of ultrapure H_2O supplemented with 1 g BSA, 0.12 g PO_4, 0.88 g NaCl, and 0.01 g Sodium Azide in 100 mL double distilled H_2O. The allantoic fluid E_2 intraassay coefficient of variation was 4.08%.

Prostaglandins Assay. Conceptus culture media (12 h post collection) and ULF were analyzed with a Parent Prostaglandin assay by the Eicosanoid Core Laboratory at Vanderbilt University Medical Center in Nashville, TN. To quantify

eicosanoids in conceptus culture media and ULF samples, 100 µL of fluid was placed in a microcentrifuge tube containing 25% methanol in water (500 µL) and internal standard (d_4-PGE_2 and d_4-LTB_4, 1 ng each). The sample was vortexed and then extracted on an Oasis MAX uElution plate (Waters Corp., Milford, MA) as follows. Sample wells were first washed with methanol (200 µL) followed by 25% methanol in water (200 µL). The sample was then loaded into the well and washed with 600 µL 25% methanol. Eicosanoids were eluted from the plate with 30 µL 2-propanol/acetonitrile (50/50, v/v) containing 5% formic acid into a 96-well elution plate containing 30 µL water in each well. Samples were analyzed on a Waters Xevo TQ-S micro triple quadrupole mass spectrometer connected to a Waters Acquity I-Class UPLC (Waters Corp., Milford, MA USA). Separation of analytes was obtained by using an Acquity PFP column (2.1 x 100 mm) with mobile phase A being 0.01% formic acid in water and mobile phase B acetonitrile. Eicosanoids were separated by using a gradient elution beginning with 30% B going to 95% B over 8 minutes at a flow rate of 0.250 mL/min.

Immunohistochemistry. Immunohistochemistry was performed on SCNT blastocysts and day 35 placental-uterine sections to visualize PTGS2 cellular localization. The fixed *PTGS2*$^{+/+}$and *PTGS2*$^{-/-}$ blastocysts were permeabilized with 0.1% Triton X-100 in PVA TL-HEPES for 30 min at rt and then washed in PVA treated TL-HEPES. Blastocysts were then placed at rt in a blocking solution with 2% goat serum and BSA (3mg/ml) for 4 h. *PTGS2*$^{+/+}$ and *PTGS2*$^{-/-}$ blastocysts were incubated with COX-2 (mouse) primary polyclonal antibody

(1:50 dilution; 160106, Cayman Chemical) overnight at 4°C. Negative controls were not incubated with primary antibody. Blastocysts were washed (3X) in PVA treated TL-HEPES and incubated 1 h with the Alexa Fluor Plus 488-conjugated secondary antibody (1:200 dilution; ThermoFisher Scientific, A-32731). Blastocysts were washed again with PVA treated TL-HEPES and stained with 0.5μL/mL Hoescht Fluorescent Stain 33342 (ThermoFisher Scientific, H3570) for 5 min. The blastocysts were mounted onto glass slides with VectaShield Antifade Mounting Medium (Vector Laboratories, H-1000) and imaged for fluorescence using a Leica DM5500 B upright microscope and Leica DFC450 C camera using Leica Application Suite X (LAS X).

Fixed placental/uterine tissue sections were embedded in paraffin wax and sectioned (6 μm). Sections were mounted on slides, baked at 37°C for 10 minutes, placed under UV light for 15 minutes to quench tissue autofluorescence, deparaffinized in xylene substitute, and rehydrated in a graded alcohol series. The sections then underwent antigen retrieval for immunolocalization by incubating sections for 20 mins in boiling Reveal Decloaker 10x (pH 6.0; RV1000M, Biocare Medical). After cooling to room temperature, sections were rinsed twice in PBS (pH 7.5) and blocked with 10% normal goat serum in PBS at rt for 30 min and then incubated overnight at 4°C with COX-2 (mouse) polyclonal antibody (1:250 dilution; 160106, Cayman Chemical). Sections were washed with PBS and incubated for 1 h with Alexa Fluor Plus 488-conjugated secondary antibody (1:400 dilution; ThermoFisher Scientific, A-32731). Uterine sections were counterstained with VectaShield Antifade Mounting Medium (Vector

Laboratories, H-1000) containing 0.5µL/mL Hoescht Fluorescent Stain 33342 (ThermoFisher Scientific, H3570). Images for fluorescence were captured with a Leica DM5500 B upright microscope and Leica DFC450 C camera using LAS X.

Statistical Analysis. Statistical analysis of quantitative data was analyzed using the PROC GLM and PROC GLIMMIX model in the Statistical Analysis Software 9.4 program (SAS Institute Inc., Cary, NC). The statistical model used to evaluate ULF content, conceptus culture media, and conceptus and endometrial gene expression included the conceptus genotype (*PTGS2*$^{+/+}$ or *PTGS2*$^{-/-}$), day of pregnancy (day 14 or 17), and the genotype x day interaction. Blastocyst culture media was statistically evaluated for genotype differences with a T-Test. Skewness, kurtosis, and Shapiro-Wilk tests were analyzed for all data. Log and square root transformations were performed to test for homogeneity and correct for normality.

3.3 Results

Experiment 1:

In Vitro Blastocyst Development from *PTGS2*$^{-/-}$ and *PTGS2*$^{+/+}$ Fibroblasts Following Somatic Cell Nuclear Transfer. The *PTGS2*$^{-/-}$ fibroblast cell line established was utilized for SCNT to generate *PTGS2*$^{-/-}$ blastocysts. Porcine fetal fibroblast cells transfected without gRNA/Cas9 complex were used as the *PTGS2*$^{+/+}$ control blastocysts. The resulting embryos derived from the *PTGS2*$^{-/-}$ fibroblast cell line had a blastocyst development rate of 48% which is similar to

the development rate of 35% for *PTGS2*$^{+/+}$ embryos. Blastocysts were genotyped and verified to have the *PTGS2* gene biallelic edit (Figure 3.3).

Immunofluorescence and Prostaglandin Production Analysis of *PTGS2*$^{-/-}$ and *PTGS2*$^{+/+}$ Blastocysts. Localization of PTGS2 expression in day 7 *PTGS2*$^{-/-}$ and *PTGS2*$^{+/+}$ blastocysts was performed using immunofluorescence. Expression of PTGS2 was localized in the trophectoderm cytoplasm of *PTGS2*$^{+/+}$ blastocysts while staining was absent in *PTGS2*$^{-/-}$ blastocysts similar to deletion of primary antibody following immunostaining with *PTGS2*$^{+/+}$ blastocysts (Figure 3.4). Total prostaglandin content was significantly ($P < 0.0001$) decreased in the culture media (50-60 blastocysts/well) containing *PTGS2*$^{-/-}$ (n=4 wells) compared to *PTGS2*$^{+/+}$ (n=4 wells) blastocysts (Figure 3.5). The concentration of PG's in culture media of *PTGS2*$^{-/-}$ blastocysts was below the sensitivity of the assay. However, *PTGS2*$^{-/-}$ embryos exhibited normal development to the blastocyst stage and hatching from the zona pellucida.

Uterine Flushing of Recipient Gilts Containing Either *PTGS2*$^{+/+}$ or *PTGS2*$^{-/-}$ Conceptuses on Days 14 and 17 of Pregnancy. The uterine horns of recipient gilts were flushed on days 14 and 17 of pregnancy to obtain *PTGS2*$^{+/+}$ (n=4, day 14; n=3, day 17) or *PTGS2*$^{-/-}$ (n=4, day 14; n=4, day 17) conceptuses and uterine luminal contents. Pregnant tracts with either *PTGS2*$^{+/+}$ or *PTGS2*$^{-/-}$ embryos contained normally elongated, filamentous conceptuses on both days 14 and 17. By day 17 of pregnancy, both *PTGS2*$^{+/+}$ and *PTGS2*$^{-/-}$ conceptuses had

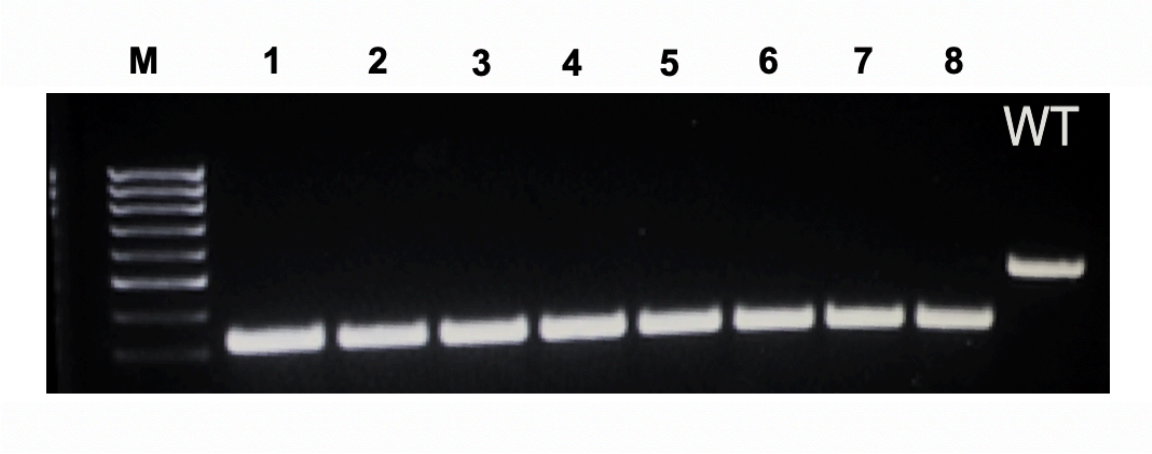

Figure 3.3: Genotyping of *PTGS2*$^{-/-}$ blastocysts. Blastocysts derived from *PTGS2*$^{-/-}$ fetal fibroblast cells via SCNT (1-8) were collected from *in vitro* culture on day 7 and genotyped by PCR. Wildtype (WT) unedited *PTGS2*$^{+/+}$ blastocyst.

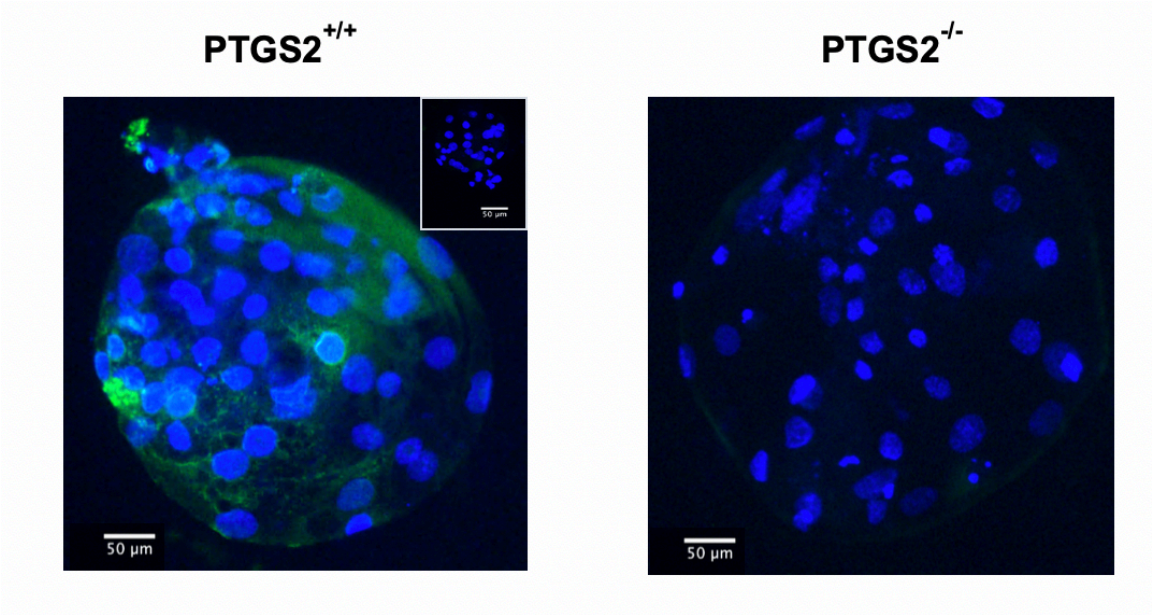

Figure 3.4: Immunolocalization of PTGS2 in day 7 *PTGS2*$^{+/+}$ and *PTGS2*$^{-/-}$ blastocysts. Immunofluorescence was performed on SCNT blastocysts to localize PTGS2 with a polyclonal PTGS2 primary antibody. Inset image is *PTGS2*$^{+/+}$ blastocyst incubated without the primary antibody.

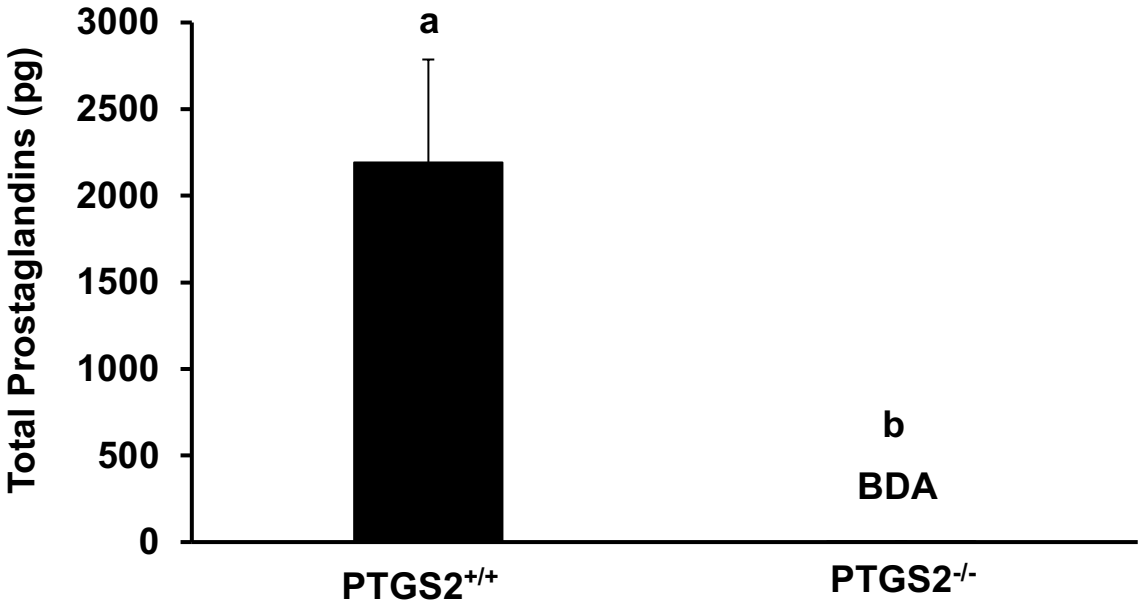

Figure 3.5: Total blastocyst prostaglandin production (pg/500µL) collected from blastocyst culture media containing either *PTGS2*$^{+/+}$ (Black bar) or *PTGS2*$^{-/-}$ (Grey bar) blastocysts on day 7 of culture. A genotype effect ($P < 0.0001$) was detected for total prostaglandins. Bars without a common superscript represent a statistical difference. (BDA = below detection of assay)

elongated completely, began development of the extraembryonic membranes, and ovaries maintained corpora lutea function.

PTGS2 gene and protein expression. Gene editing of pig conceptus *PTGS2* significantly ($P > 0.0001$) decreased *PTGS2* mRNA expression in *PTGS2*$^{-/-}$ conceptuses compared to *PTGS2*$^{+/+}$ conceptuses. Expression of *PTGS2* was ~7-fold lower between conceptus genotypes on day 14 and ~59-fold decrease between genotypes on day 17 (Figure 3.6 A). The PTGS2 primary antibody utilized recognized the mature 75 kD form of PTGS2 but also lower 45 and 32 kD bands that represent possible immature, non-glycosylated forms of PTGS2 or PTGS2 splice variants. Day 14 (Figure 3.6B) and 17 (Figure 3.6C) conceptus protein western blot analysis revealed expression of PTGS2 in *PTGS2*$^{+/+}$ conceptus tissue but detected only faint to no PTGS2 expression in *PTGS2*$^{-/-}$ conceptuses. The intensity of the 75 kD band was decreased in day 17 *PTGS2*$^{+/+}$ conceptus tissue compare to day 14. The lighter intensity of a 75 kD band detected in several *PTGS2*$^{-/-}$ conceptuses was caused by endometrial epithelial contamination following flushing the conceptus which was confirmed by detection of uteroferrin (endometrial specific gene) mRNA expression in the conceptus samples (Figure 3.7).

Analysis of Day 14 and 17 Conceptus Prostaglandin Production. Conceptus tissue was immediately cultured for 3 h to measure prostaglandin synthesis

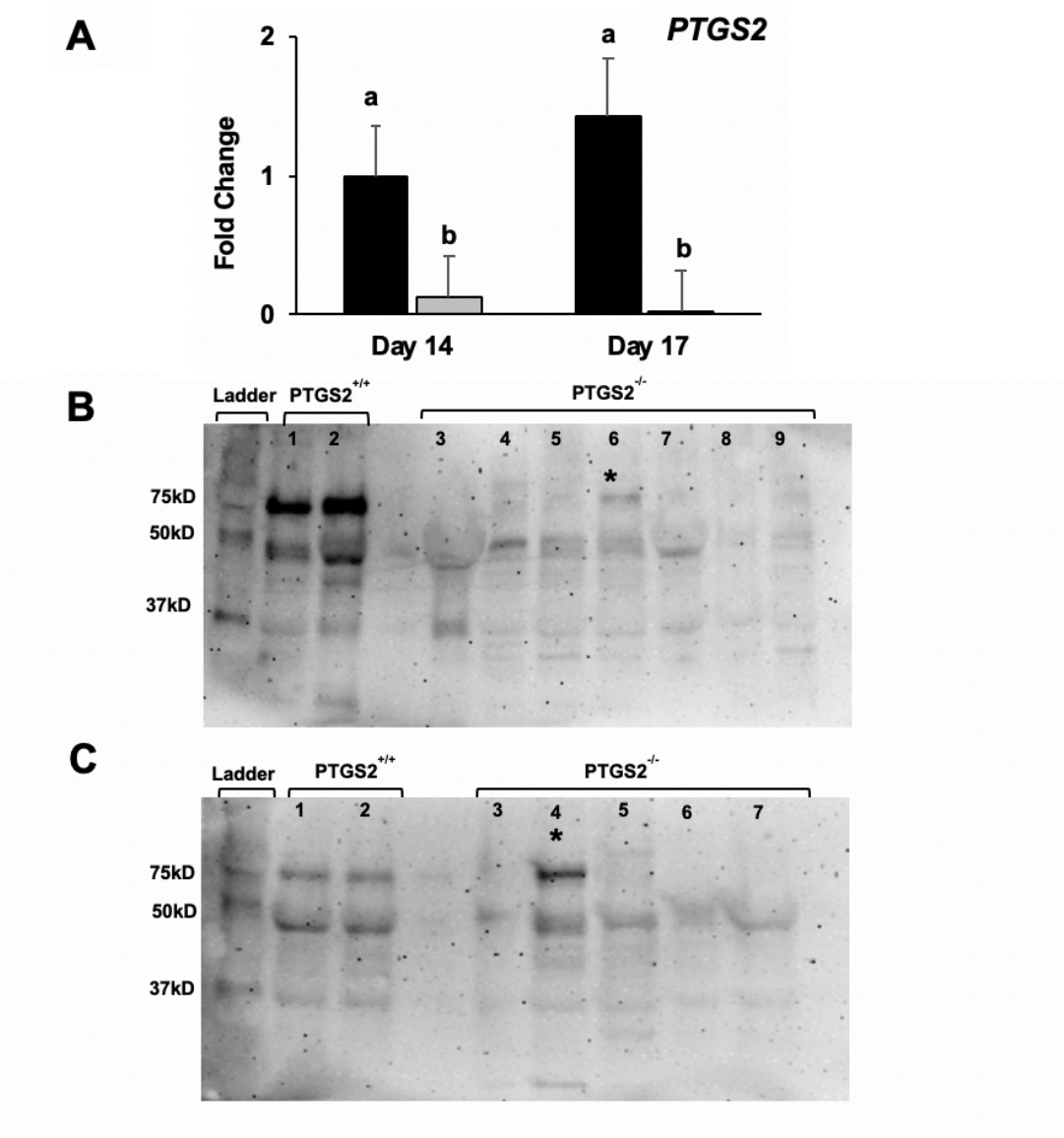

Figure 3.6: Gene and protein analysis of *PTGS2* in *PTGS2*$^{+/+}$ and *PTGS2*$^{-/-}$ conceptuses on day 14 and 17 of pregnancy. A) *PTGS2*$^{+/+}$ (black bar) and *PTGS2*$^{-/-}$ (grey bar) conceptus tissue *PTGS2* expression was lower in *PTGS2*$^{-/-}$ conceptuses on day 14 and 17*(genotype P < 0.0001)*. Western blots of day 14 (B) and 17 (C) conceptus PTGS2 protein expression detected bands of 75, 42 and 35 kD. The mature form of PTGS2 (75 kD) is present in *PTGS2*$^{+/+}$ conceptus tissues, but absent in *PTGS2*$^{-/-}$ conceptus tissue. Asterisk (Lanes 6 and 4, respectively) denotes presence of 75 kD band in conceptus sample confirmed to be contaminated with endometrial epithelial cells.

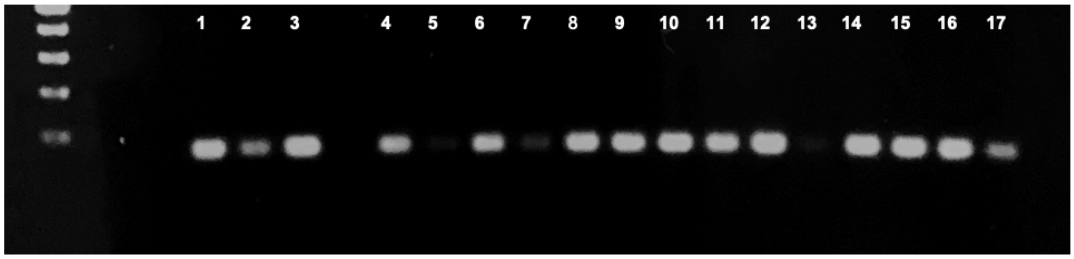

Figure 3.7: PCR analysis of uteroferrin expression in conceptus tissue samples. Uteroferrin gene expression (endometrial specific gene) by samples of conceptus tissue indicates the presence of maternal endometrial epithelial cells contained within the entangled elongated conceptuses following flushing from the uterine horns.

following flushing the conceptuses from the uterine horns. Total prostaglandins released into the culture media was significantly ($P < 0.0001$) decreased with *PTGS2*$^{-/-}$ compared to *PTGS2*$^{+/+}$ conceptus tissue on both day 14 and 17 (Figure 3.8). There was a conceptus genotype by day interaction ($P = 0.003$) for the total content of PGE_2 in culture media. The content of PGE_2 in media containing day 14 *PTGS2*$^{-/-}$ conceptuses was decreased compared to *PTGS2*$^{+/+}$ conceptuses but similar between conceptus genotypes on day 17 (Figure 3.9). A conceptus genotype effect ($P = 0.004$) was detected for the content of $PGF_{2\alpha}$ in culture media (Figure 3.10).

Uterine Luminal Flushing Analysis. Total PGE_2 content in the recipient gilt ULF increased ($P = 0.002$) from day 14 to 17 of pregnancy (Figure 3.11). Similarly, there was a day effect ($P = 0.002$) detected for total $PGF_{2\alpha}$ in the uterine luminal flush (Figure 3.12). Content of ULF 15-keto-PGE_2 increased from day 14 of pregnancy to day 17 of pregnancy ($P = 0.02$), while there was no significant difference ($P = 0.86$) in conceptus genotype (Figure 3.13). Total uterine content of estradiol-17β in the ULF exhibited a conceptus genotype by day effect ($P = 0.002$) as estradiol-17β in the ULF from recipient gilts was similar between conceptus genotypes on day 14 but was lower in the ULF of recipients containing day 17 *PTGS2*$^{-/-}$ conceptuses compared to recipient gilts containing *PTGS2*$^{+/+}$ conceptuses (Figure 3.14). There was a day effect ($P = 0.003$) for IL1B content in ULF but conceptus genotype was not significantly different ($P = 0.86$) (Figure 3.15).

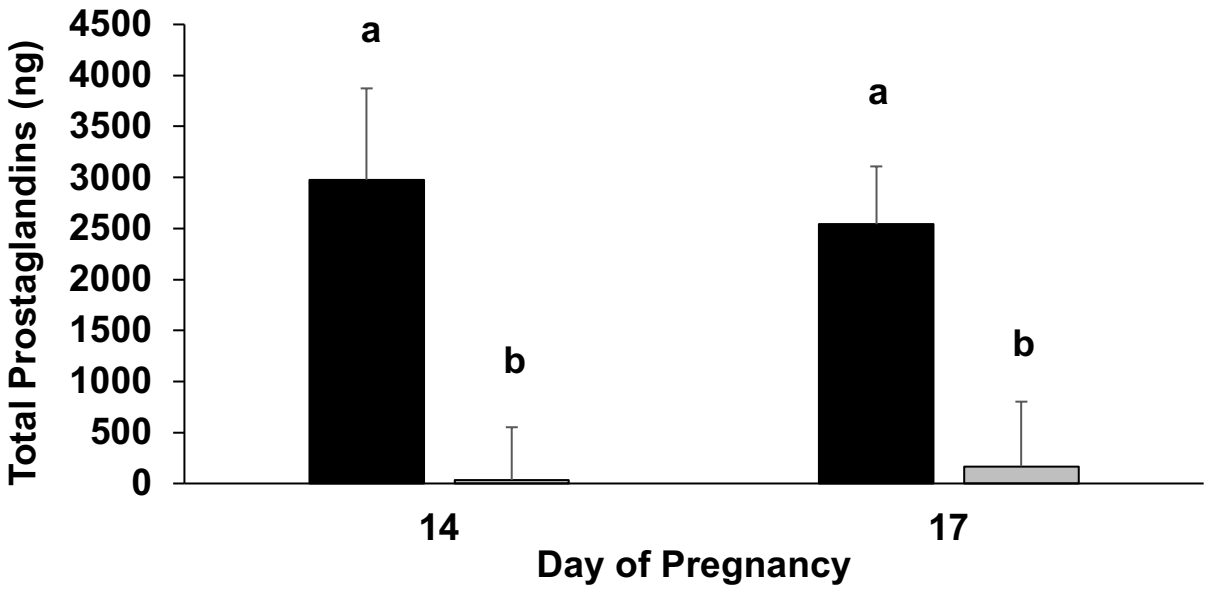

Figure 3.8: Total conceptus prostaglandin production (ng/2mL) in conceptus culture media containing either *PTGS2*$^{+/+}$ (Black bar) or *PTGS2*$^{-/-}$ (Grey bar) conceptuses on either days 14 or 17 of pregnancy. A genotype effect ($P < 0.0001$) was detected for total prostaglandin. Bars without a common superscript represent a statistical difference.

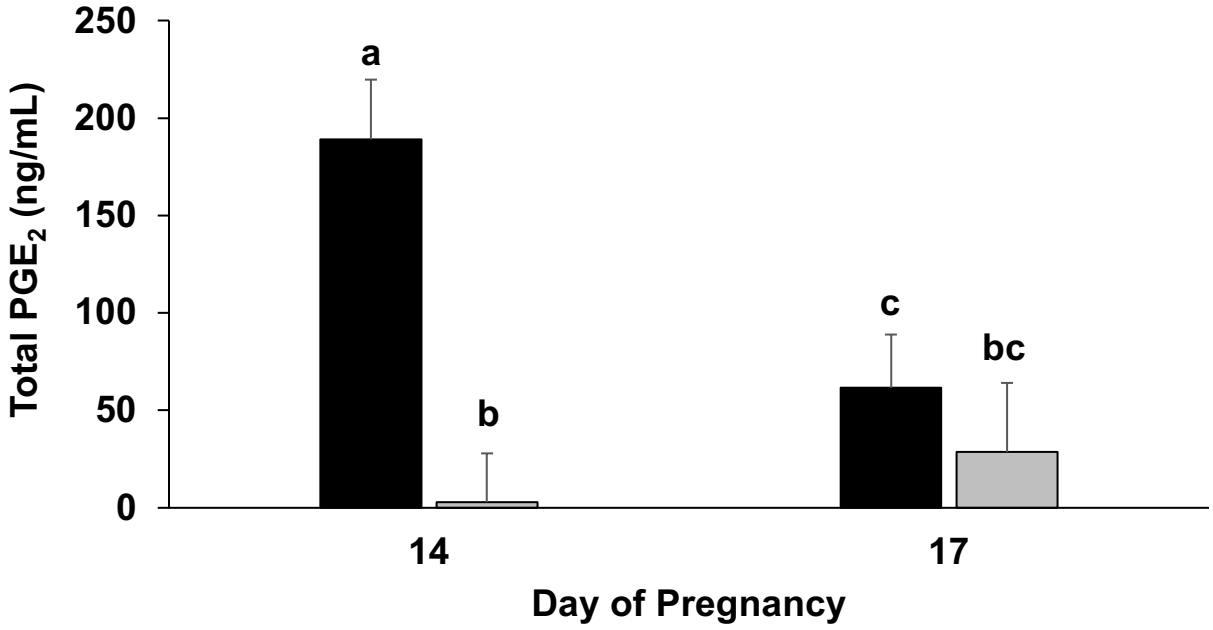

Figure 3.9: Total conceptus PGE_2 production (ng/2mL) in conceptus culture media containing either *PTGS2*$^{+/+}$ (Black bar) or *PTGS2*$^{-/-}$ (Grey bar) conceptuses on either days 14 or 17 of pregnancy. A genotype*day effect (P = 0.003) was detected for total PGE_2. Bars without a common superscript represent a statistical difference.

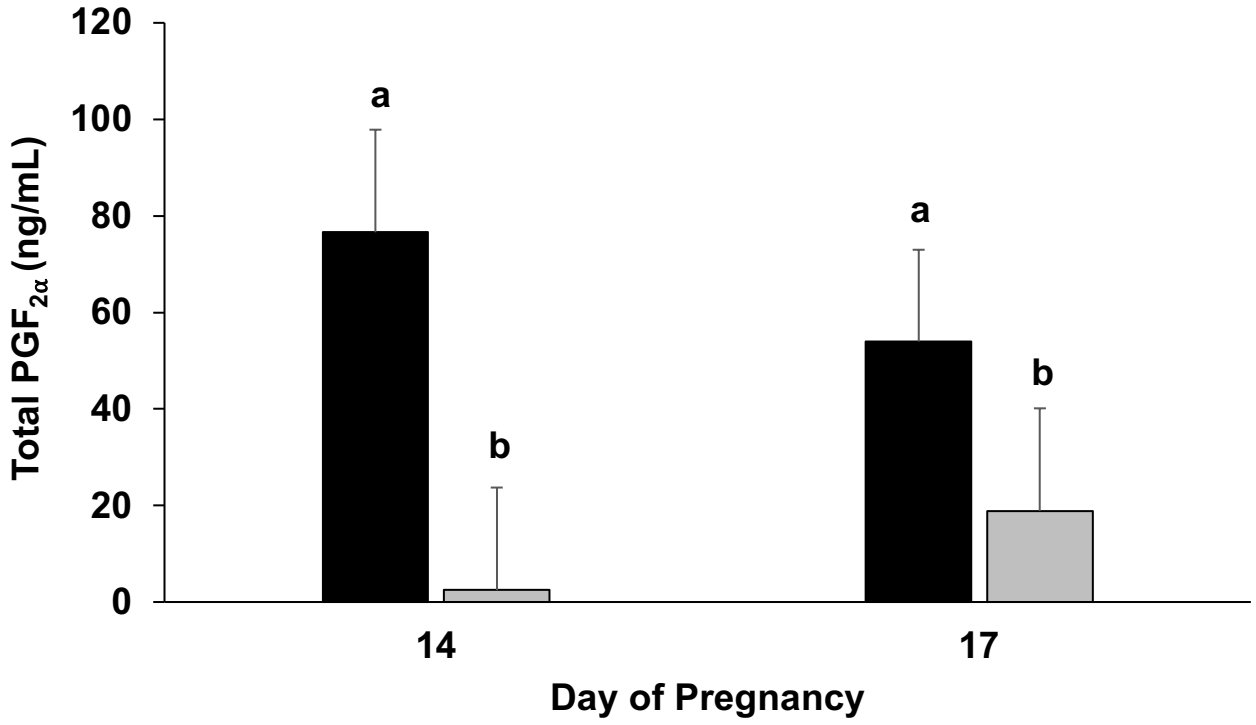

Figure 3.10: Total conceptus $PGF_{2\alpha}$ production (ng/2mL) in conceptus culture media containing either *PTGS2*$^{+/+}$ (Black bar) or *PTGS2*$^{-/-}$ (Grey bar) conceptuses on either days 14 or 17 of pregnancy. A genotype effect (P = 0.004) was detected for total $PGF_{2\alpha}$. Bars without a common superscript represent a statistical difference.

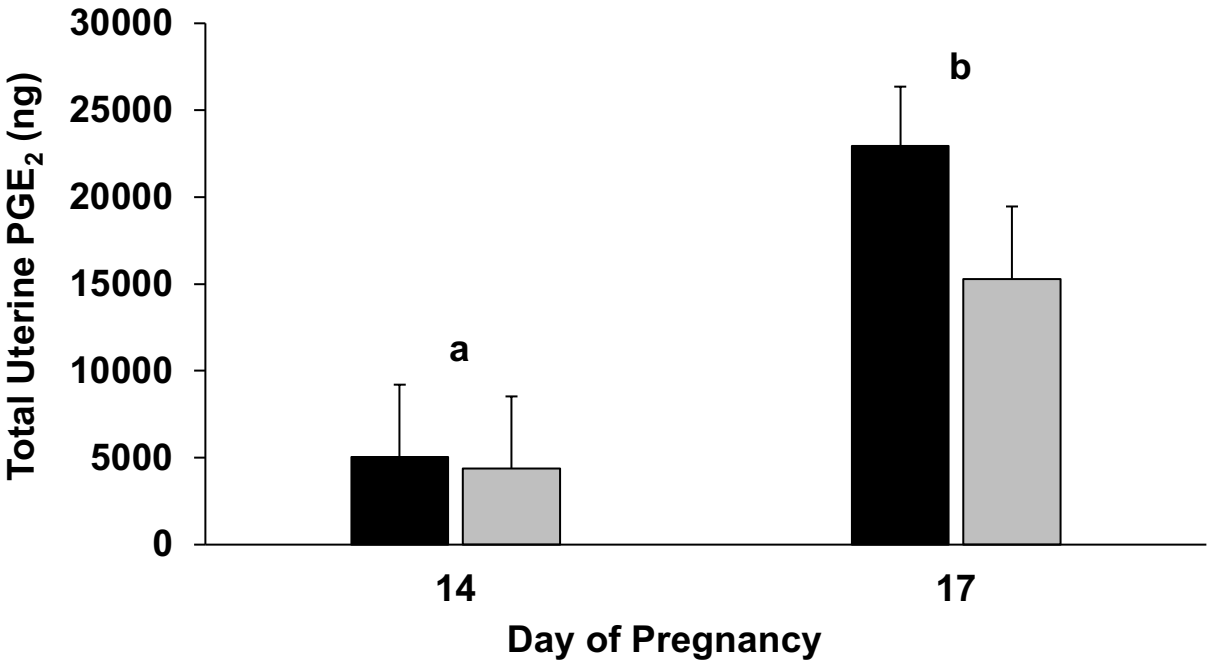

Figure 3.11: Total content PGE_2 (ng) in uterine flushings collected from recipient gilts containing either *PTGS2*$^{+/+}$ (Black bar) or *PTGS2*$^{-/-}$ (Grey bar) conceptuses on either days 14 or 17 of pregnancy. A day effect (P = 0.002) was detected for total PGE_2. Bars without a common superscript represent a statistical difference.

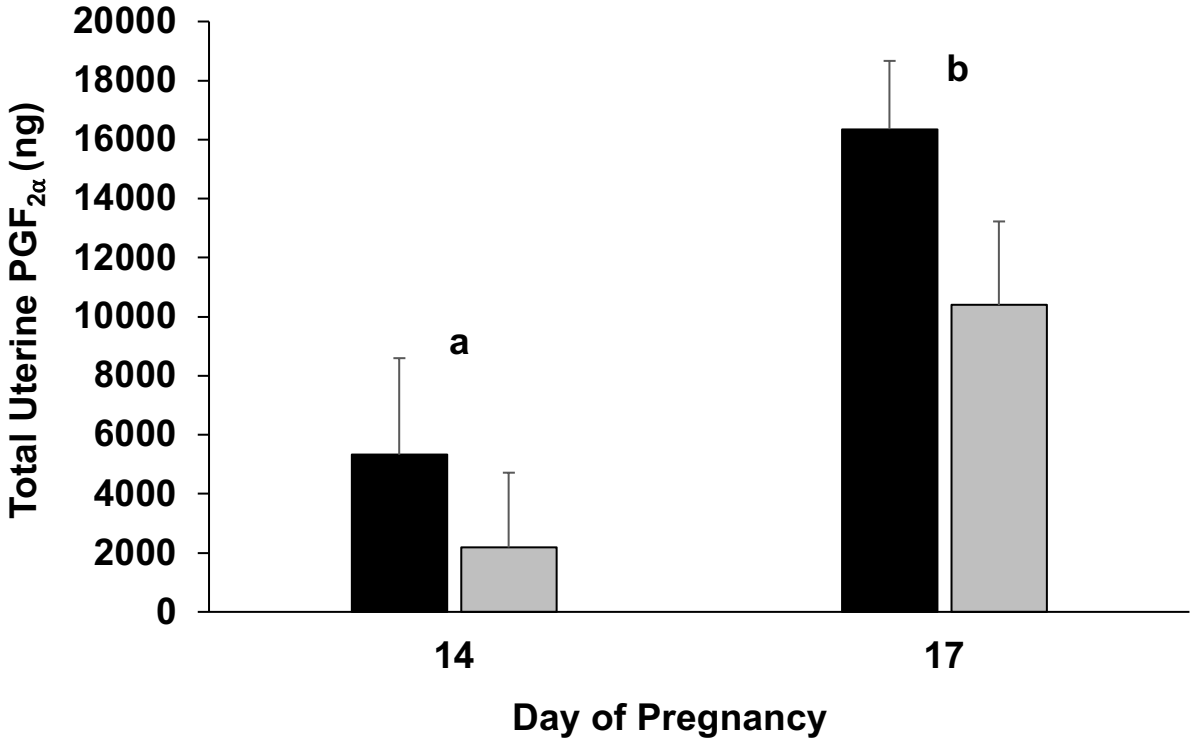

Figure 3.12: Total content $PGF_{2\alpha}$ (ng) in uterine flushings collected from recipient gilts containing either *PTGS2*$^{+/+}$ (Black bar) or *PTGS2*$^{-/-}$ (Grey bar) conceptuses on either days 14 or 17 of pregnancy. A day effect (P = 0.002) was detected for total $PGF_{2\alpha}$. Bars without a common superscript represent a statistical difference.

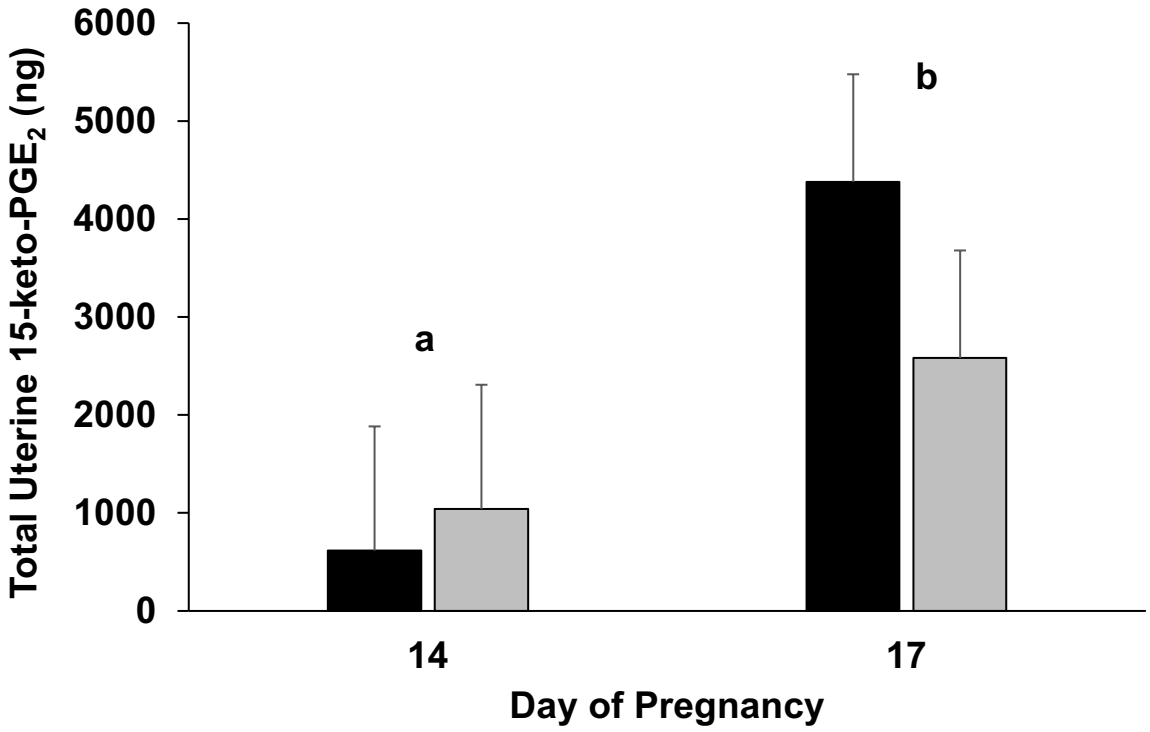

Figure 3.13: Total content 15-keto-PGE_2 (ng) in uterine flushings collected from recipient gilts containing either *PTGS2*$^{+/+}$ (Black bar) or *PTGS2*$^{-/-}$ (Grey bar) conceptuses on either days 14 or 17 of pregnancy. A day effect (P = 0.02) was detected for total 15-keto-PGE_2. Bars without a common superscript represent a statistical difference.

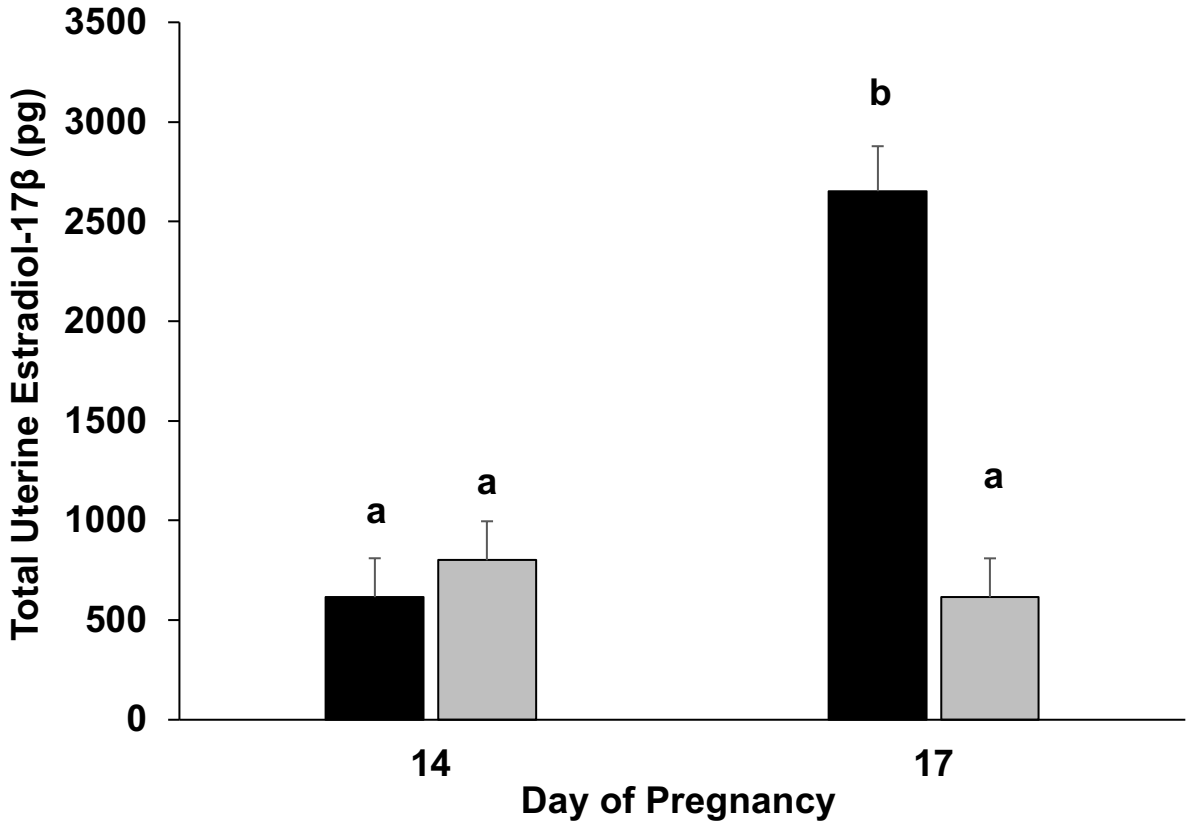

Figure 3.14: Total content estradiol-17β (pg) in uterine flushings collected from recipient gilts containing either *PTGS2*$^{+/+}$ (Black bar) or *PTGS2*$^{-/-}$ (Grey bar) conceptuses on either days 14 or 17 of pregnancy. A conceptus genotype*day effect (P = 0.002) was detected for total estradiol-17β. Bars without a common superscript represent a statistical difference.

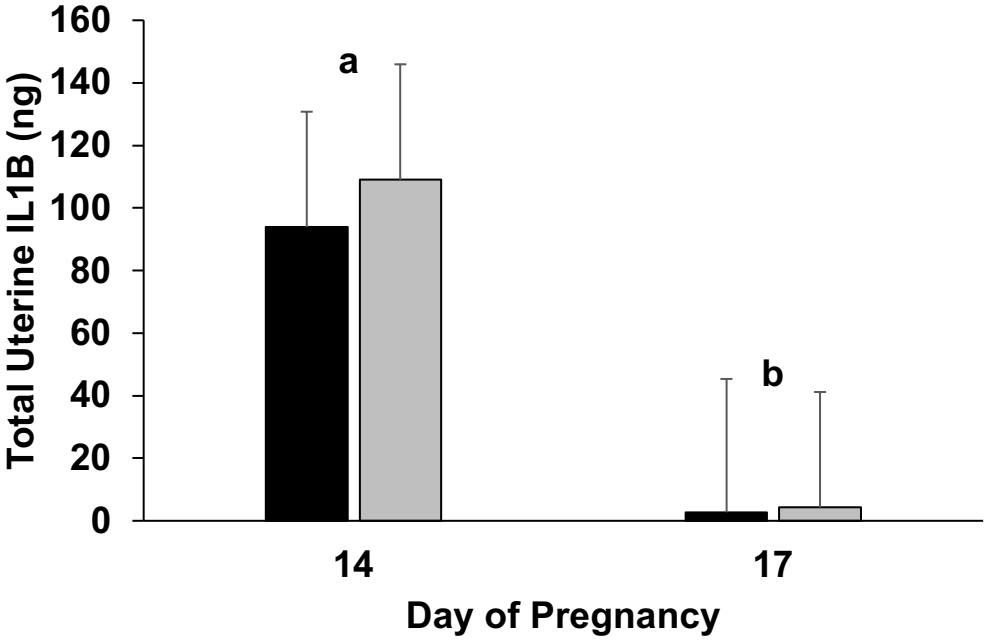

Figure 3.15: Total content IL1B (ng) in uterine flushings collected from recipient gilts containing either *PTGS2*$^{+/+}$ (Black bar) or *PTGS2*$^{-/-}$ (Grey bar) conceptuses on either days 14 or 17 of pregnancy. A day effect (P = 0.003) was detected for total IL1B. Bars without a common superscript represent a statistical difference.

Conceptus Gene Expression: Conceptus *PTGS1* and *PGFS* mRNA expression was not different between conceptus genotypes but there was a significant day effect ($P < 0.0001$) as expression increased ~ 50 fold from day 14 to day 17 (Figure 3.16 A,B). A significant conceptus genotype by day interaction ($P = 0.0002$) was detected for *PTGES2* expression as conceptus mRNA synthesis was ~2.5-fold greater in day 14 *PTGS2*$^{-/-}$ conceptuses compared to *PTGS2*$^{+/+}$ conceptuses but the difference in expression reversed as *PTGES2* expression was greater in *PTGS2*$^{+/+}$ conceptuses on day 17 (Figure 3.16 C). *PTGES3* expression decreased ($P = 0.002$) from day 14 to 17 of pregnancy (Figure 3.16 D). *CYP19A1* and *IL1B2* mRNA synthesis exhibited day effects ($P = 0.0008$ and $P = 0.003$, respectively) as expression decreased from day 14 to 17 of pregnancy (Figure 3.17 A,B). Both *IFND* and *IFNG* increased significantly ($P = 0.0001$ and $P < 0.0001$, respectively) in *PTGS2*$^{-/-}$ conceptuses compared to *PTGS2*$^{+/+}$ conceptuses. *IFND* was ~6-fold greater in day 14 *PTGS2*$^{-/-}$ conceptuses and ~2.5-fold in day 17 *PTGS2*$^{-/-}$ conceptuses. *IFNG* increased ~3-fold in day 14 *PTGS2*$^{-/-}$ conceptuses and ~4-fold in day 17 *PTGS2*$^{-/-}$ conceptuses (Figure 3.17 C&D). There were no differences ($P > 0.05$) in conceptus gene expression for *PTGES* on days 14 and 17 of pregnancy.

Endometrial Gene Expression Analysis. Endometrial *AKR1B1*, *PTGES*, *PTGES2*, *PTGS1*, *PTGS2*, *SLCO2A1*, and *SLCO5A1* expression on days 14 and 17 were not significantly affected by conceptus genotype or day. However, there was a conceptus genotype effect ($P = 0.053$) for *PTGES3*. *PTGES3* expression

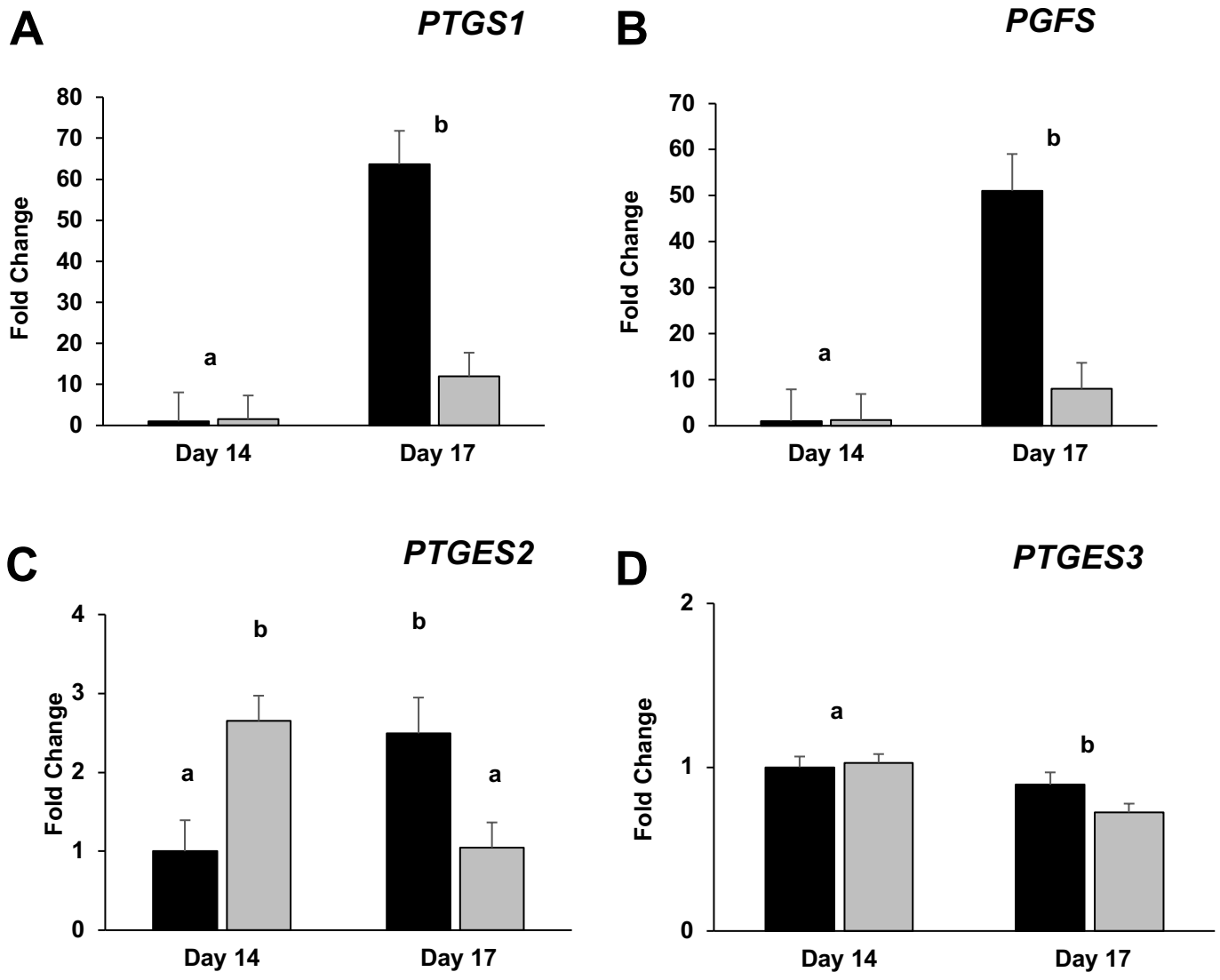

Figure 3.16: Fold change of conceptus gene expression of *PTGS1*, *PGFS*, *PTGES2, and PTGES3*. *PTGS2*$^{+/+}$ (black bar) and *PTGS2*$^{-/-}$ (grey bar) fold change in gene expression is relative to the average ΔCT of day 14 *PTGS2*$^{+/+}$. Fold change of **A)** *PTGS1 (day P < 0.0001)*, **B)** *PGFS (day P < 0.0001)*, **C)** *PTGES2 (genotype*day P < 0.0001)*, **D)** PTGES3 *(day P = 0.002)*.

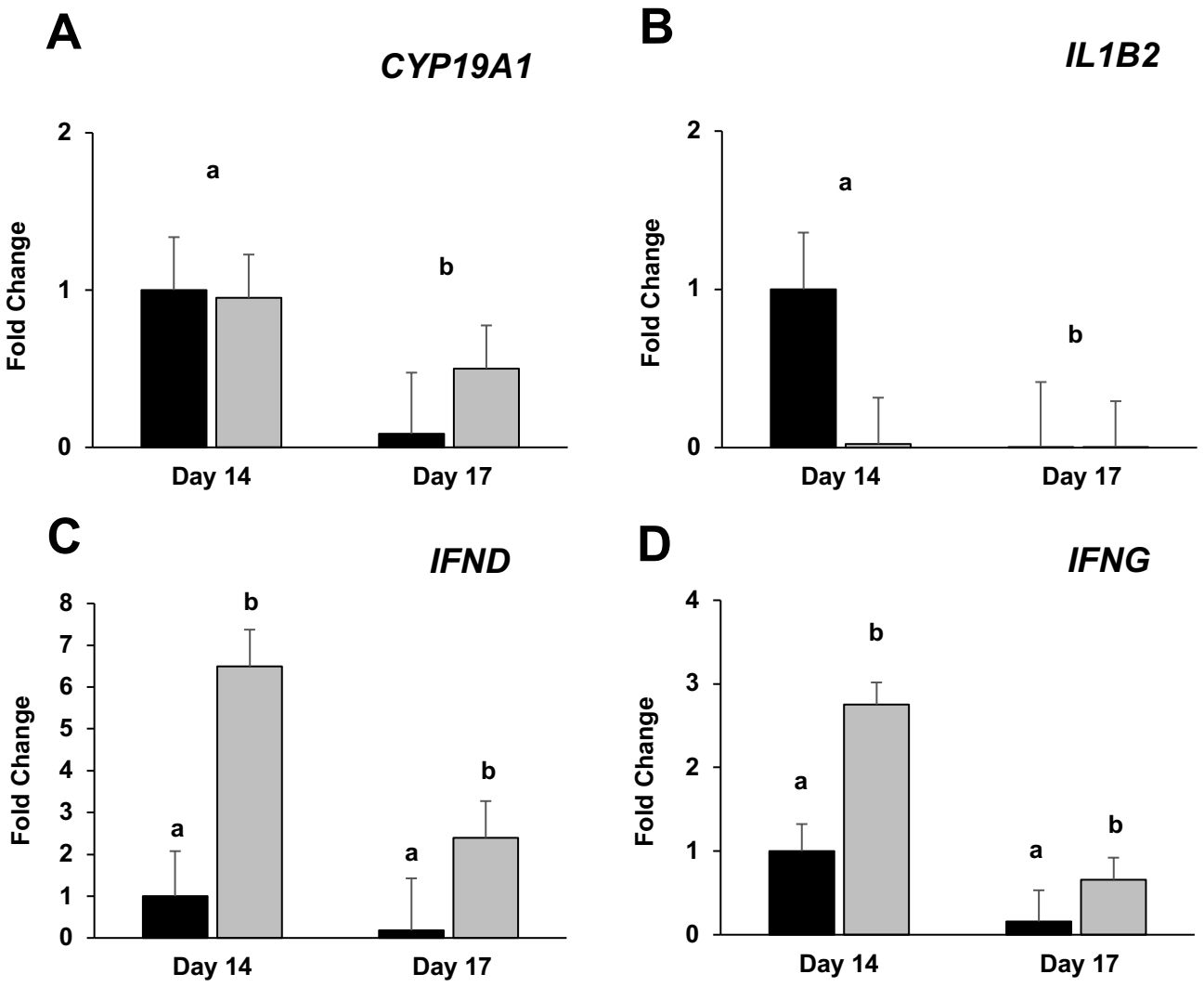

Figure 3.17: Fold change of conceptus gene expression of *CYP19A1, IL1B2, IFND*, and *IFNG*. *PTGS2*$^{+/+}$ (black bar) and *PTGS2*$^{-/-}$ (grey bar) fold change in gene expression is relative to the average ΔCT of day 14 *PTGS2*$^{+/+}$. Fold change of **A)** *CYP19A1 (day P = 0.0008)*, **B)** *IL1B2 (day P = 0.003)*, **C)** *IFND (genotype P = 0.0001)*, **D)** *IFNG (genotype P < 0.0001).*

in endometrium from recipients containing *PTGS2*$^{-/-}$ conceptuses was ~4-fold greater than endometrium from recipients with *PTGS2*$^{+/+}$ conceptuses on day 14 and 17 (Figure 3.18).

Experiment 2:

Day 35 Pregnancy. Since *PTGS2*$^{-/-}$ conceptuses survived and pregnancies were successfully maintained until day 17, recipient gilts gestating *PTGS2*$^{-/-}$ conceptuses were allowed to continue pregnancy. All *PTGS2*$^{-/-}$ recipient gilts (n=3) maintained pregnancy beyond 30 days, were euthanized on day 35 of pregnancy, and collectively resulted in the development of 13 (3,5,4) viable fetuses (Figure 3.19). The maintenance of pregnancy, embryonic development, and *PTGS2* derived production of prostaglandins were analyzed in *PTGS2*$^{-/-}$ conceptuses compared to the collective 42 (15,9,18) normal, wild-type bred *PTGS2*$^{+/+}$ fetuses obtained.

Placental Gene Expression Analysis. Chorioallantois *PTGS2* expression was decreased (P = 0.005) in the placenta's of *PTGS2*$^{-/-}$ compared to *PTGS2*$^{+/+}$ embryos on day 35 of pregnancy (Figure 3.20 A). *PTGS2*$^{-/-}$ placentas had ~15-fold greater *PGFS* expression (P = 0.05) on day 35 of pregnancy compared to *PTGS2*$^{+/+}$ conceptuses. (Figure 3.20 B). There was no significant (P = 0.2) difference in CYP19A1 expression among *PTGS2*$^{-/-}$ or *PTGS2*$^{+/+}$ placental tissue (Figure 3.20 C). *IFND* was ~4-fold greater (P = 0.005) in placental tissue of *PTGS2*$^{-/-}$ embryos than *PTGS2*$^{+/+}$ embryos (Figure 3.20 D). The chorioallantois gene expression was not significantly (P > 0.05) different for *IFNG*, *PTGES*, *PTGES2*, *PTGES3*, or *PTGS1*.

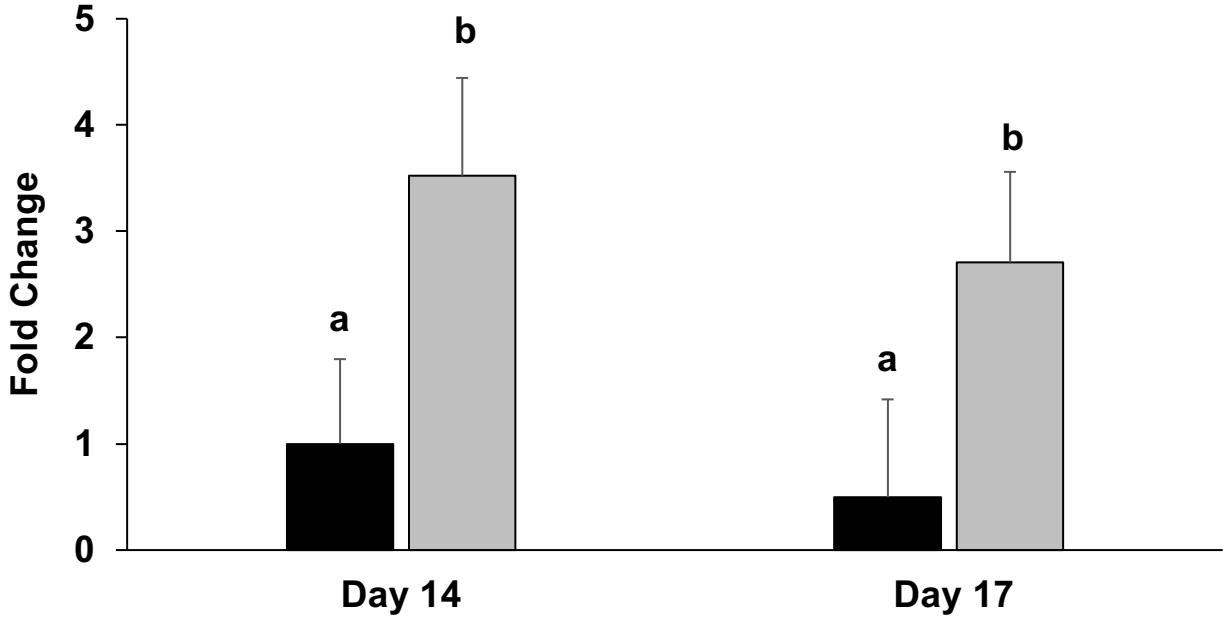

Figure 3.18: Fold change of endometrial gene expression of *PTGES3*. *PTGS2*$^{+/+}$ (black bar) and *PTGS2*$^{-/-}$ (grey bar) fold change in gene expression is relative to the average ΔCT of day 14 *PTGS2*$^{+/+}$. Fold change of *PTGES3 (genotype P = 0.053)*.

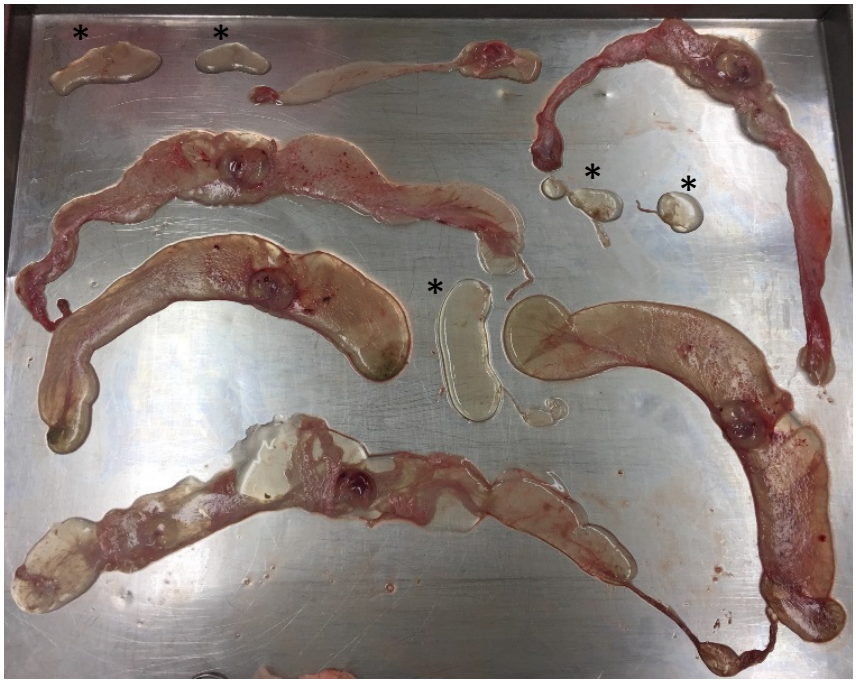

Figure 3.19: Day 35 placentas of *PTGS2*$^{-/-}$ clone embryos. Fully developed and vascularized placentas were seen in both genotypes. Undeveloped, unvascularized, degenerate conceptuses (denoted by asterisk) were present only in the *PTGS2*$^{-/-}$ pregnancies.

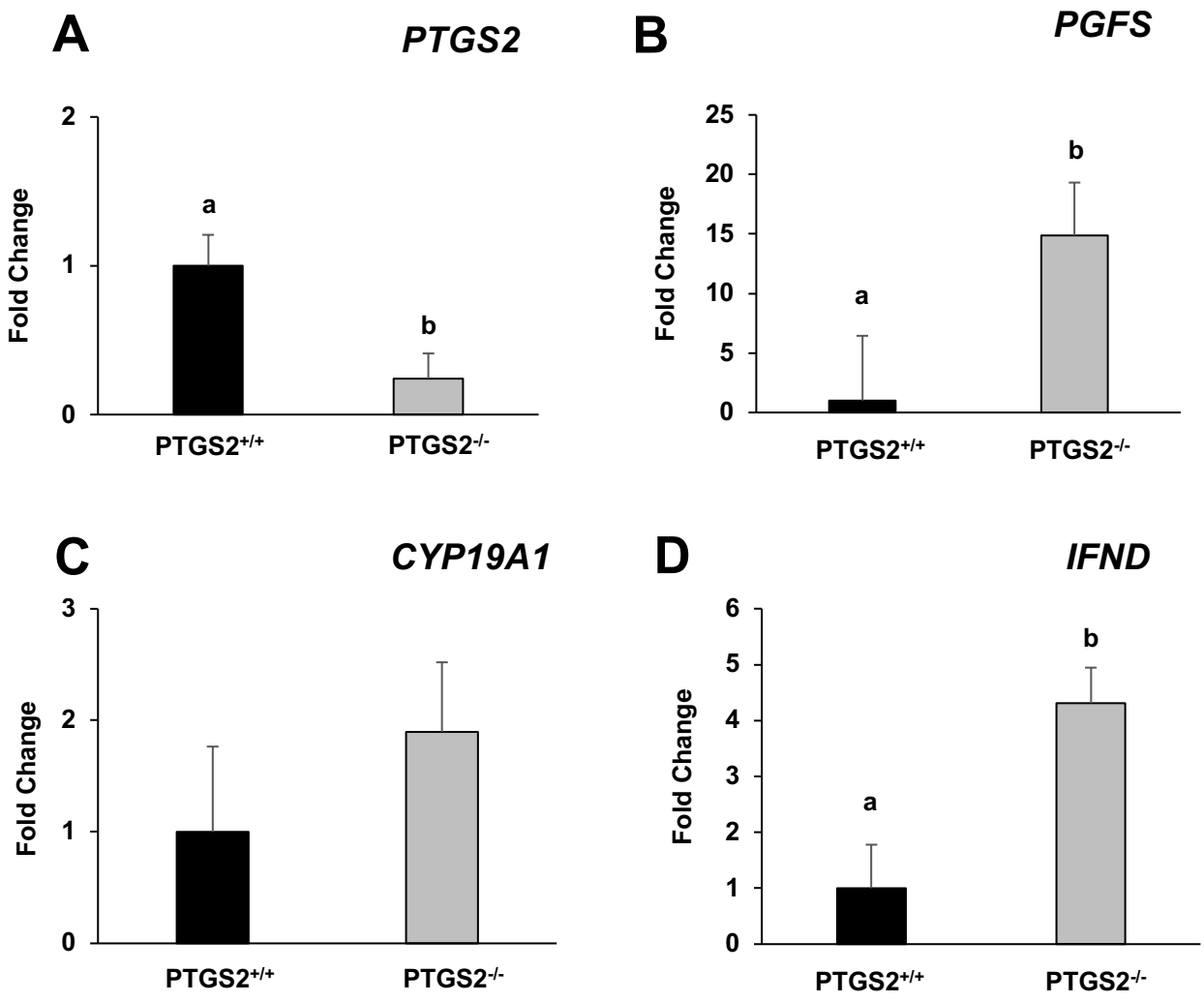

Figure 3.20: Fold change of placental gene expression of *PTGS2*, *IFND,* and *PGFS*. *PTGS2*$^{+/+}$ (black bar) and *PTGS2*$^{-/-}$ (grey bar) fold change in gene expression is relative to the average ΔCT of day 35 *PTGS2*$^{+/+}$. Fold change of **A)** *PTGS2 (genotype P = 0.005)*, **B)** *PGFS (genotype P = 0.05)*, and **C)** *CYP19A1 (genotype P = 0.2), **D** IFND (genotype P = 0.005)*.

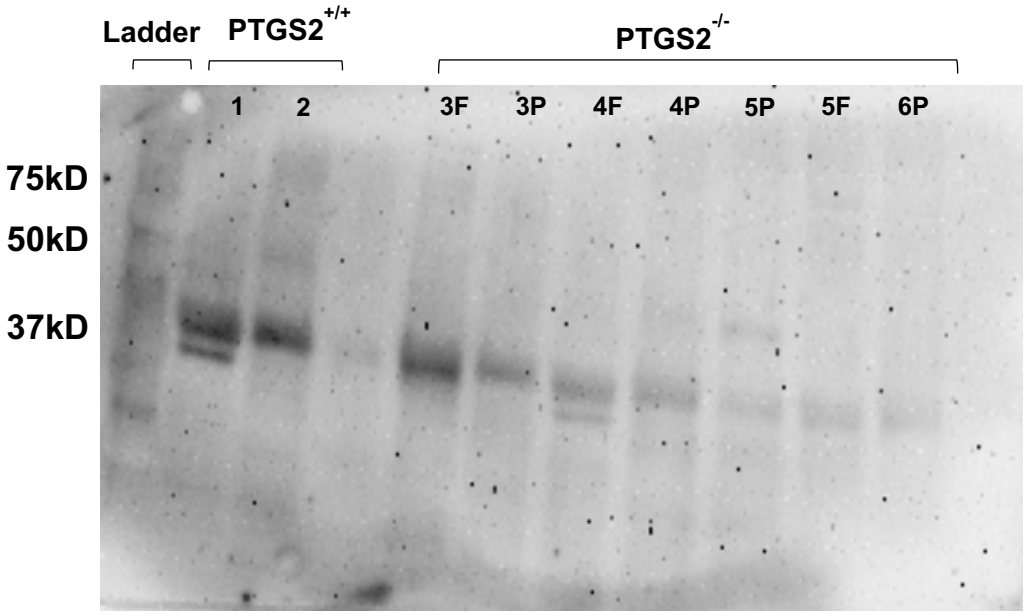

Figure 3.21: Western blot analysis of PTGS2 expression in *PTGS2*$^{+/+}$ and *PTGS2*$^{-/-}$ fetal or placental tissue on day 35 of pregnancy. Expression of the 75 KD band of PTGS2 is not detected in either *PTGS2*$^{+/+}$ (1 and 2) or *PTGS2*$^{-/-}$ (3-6) fetal and placental tissue. (F=fetal tissue and P=placental tissue)

PTGS2 Western Blot Analysis. Analysis of conceptus PTGS2 expression indicated that PTGS2 expression is barely detected by day 35 of pregnancy. *PTGS2*$^{+/+}$ and *PTGS2*$^{-/-}$ fetal and placental western blot protein analysis (Figure 3.21) revealed no detectable 75 kD bands present on day 35.

Analysis of Allantoic Fluid Prostaglandin and Estradiol-17B Content. The concentration of prostaglandins in the allantoic fluid was significantly (P = 0.007) less in placentas from *PTGS2*$^{-/-}$ fetuses compared to normal bred *PTGS2*$^{+/+}$ placentas (Figure 3.22). The concentration of estradiol-17β was significantly (P < 0.0001) greater in the allantoic fluid of *PTGS2*$^{-/-}$ fetuses compared to *PTGS2*$^{+/+}$ (Figure 3.23).

Endometrial Gene Expression Analysis. Gene editing of pig conceptus *PTGS2* did not alter endometrial *AKR1B1*, *PTGES*, *PTGES2*, *PTGS1*, *PTGS2*, *SLCO2A1*, or *SLCO5A1* expression among genotypes on day 35 of pregnancy. However, *PTGES3* mRNA exhibited a conceptus genotype effect (P =0.04) as endometrium containing *PTGS2*$^{-/-}$ conceptuses had ~2-fold less *PTGES3* mRNA expression than endometrium containing *PTGS2*$^{+/+}$ conceptuses (Figure 3.24).

Immunofluorescence of *PTGS2*$^{-/-}$ and *PTGS2*$^{+/+}$ Placental-Uterine Tissues. Localization of PTGS2 expression in day 35 *PTGS2*$^{-/-}$ and *PTGS2*$^{+/+}$ placental-uterine tissues was performed using immunofluorescence. Expression of PTGS2 was localized in the uterine GE and LE as well as the chorion of *PTGS2*$^{+/+}$ placenta. However, while PTGS2 was localized in the uterine GE and LE, PTGS2 expression was not localized in the chorion of *PTGS2*$^{-/-}$ conceptuses (Figure 3.25).

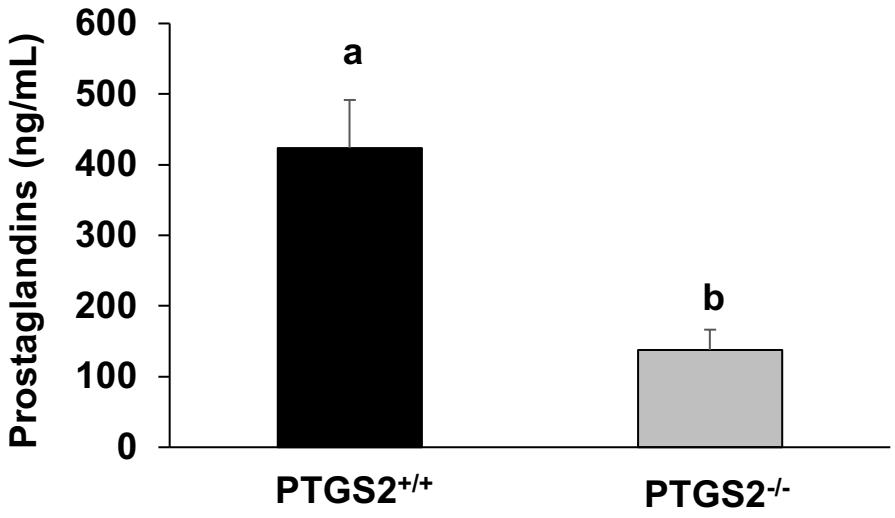

Figure 3.22: Prostaglandin concentrations (ng/mL) in allantoic fluid collected from *PTGS2*$^{+/+}$ (Black bar) or *PTGS2*$^{-/-}$ (Grey bar) placentas on day 35 of pregnancy. A genotype effect (P = 0.007) was detected for total prostaglandin. Bars without a common superscript represent a statistical difference.

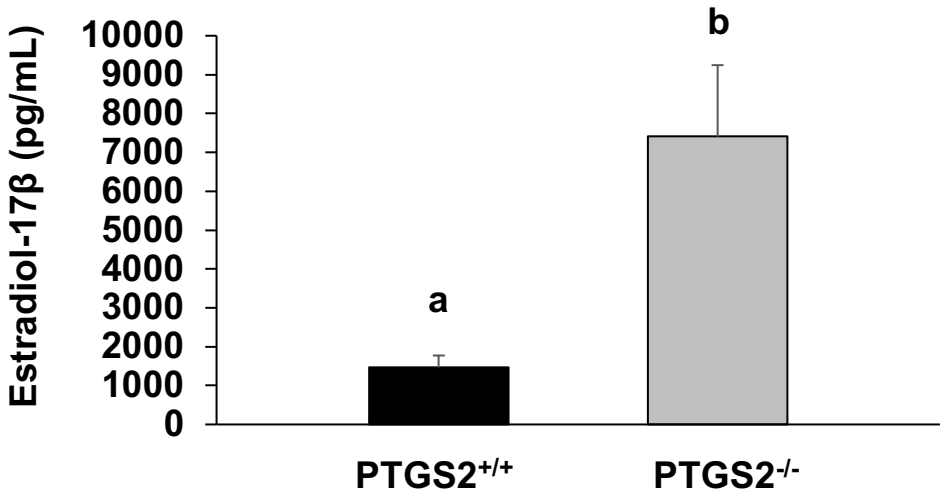

Figure 3.23: Estradiol-17β concentrations (pg/mL) in allantoic fluid collected from *PTGS2*$^{+/+}$ (Black bar) or *PTGS2*$^{-/-}$ (Grey bar) placentas on day 35 of pregnancy. A genotype effect ($P < 0.0001$) was detected for total estradiol-17β. Bars without a common superscript represent a statistical difference.

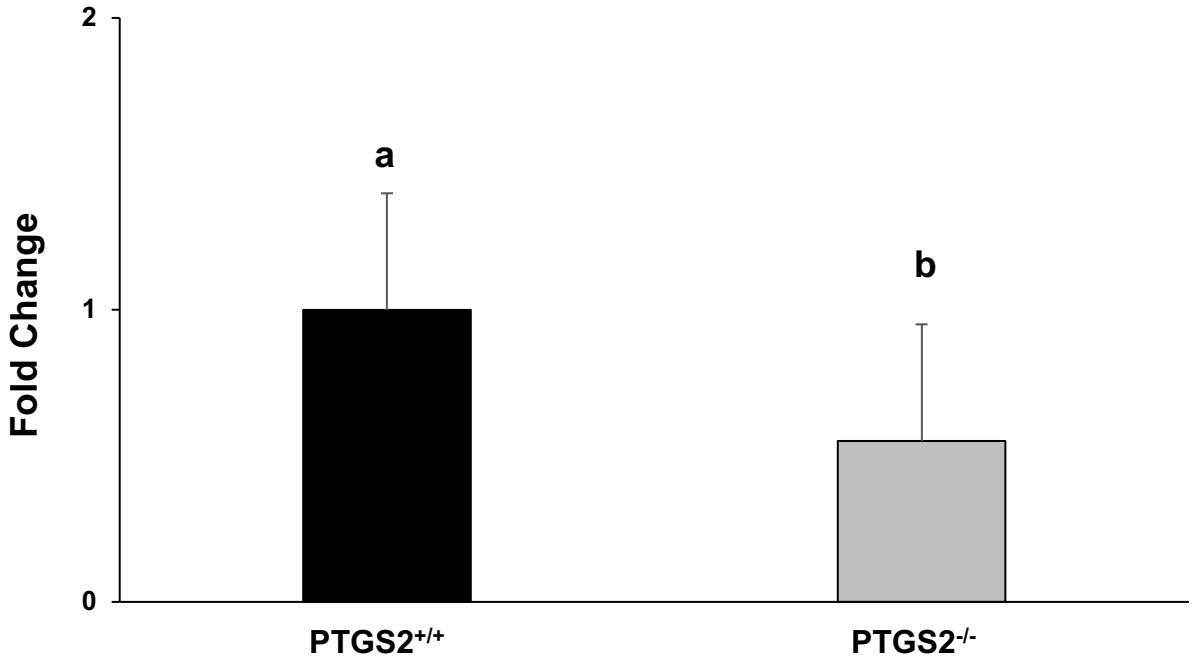

Figure 3.24: Fold change of endometrial gene expression of *PTGES3*. *PTGS2*$^{+/+}$ (black bar) and *PTGS2*$^{-/-}$ (grey bar) fold change in gene expression is relative to the average ΔCT of day 35 *PTGS2*$^{+/+}$. Fold change of *PTGES3 (genotype P = 0.04)*.

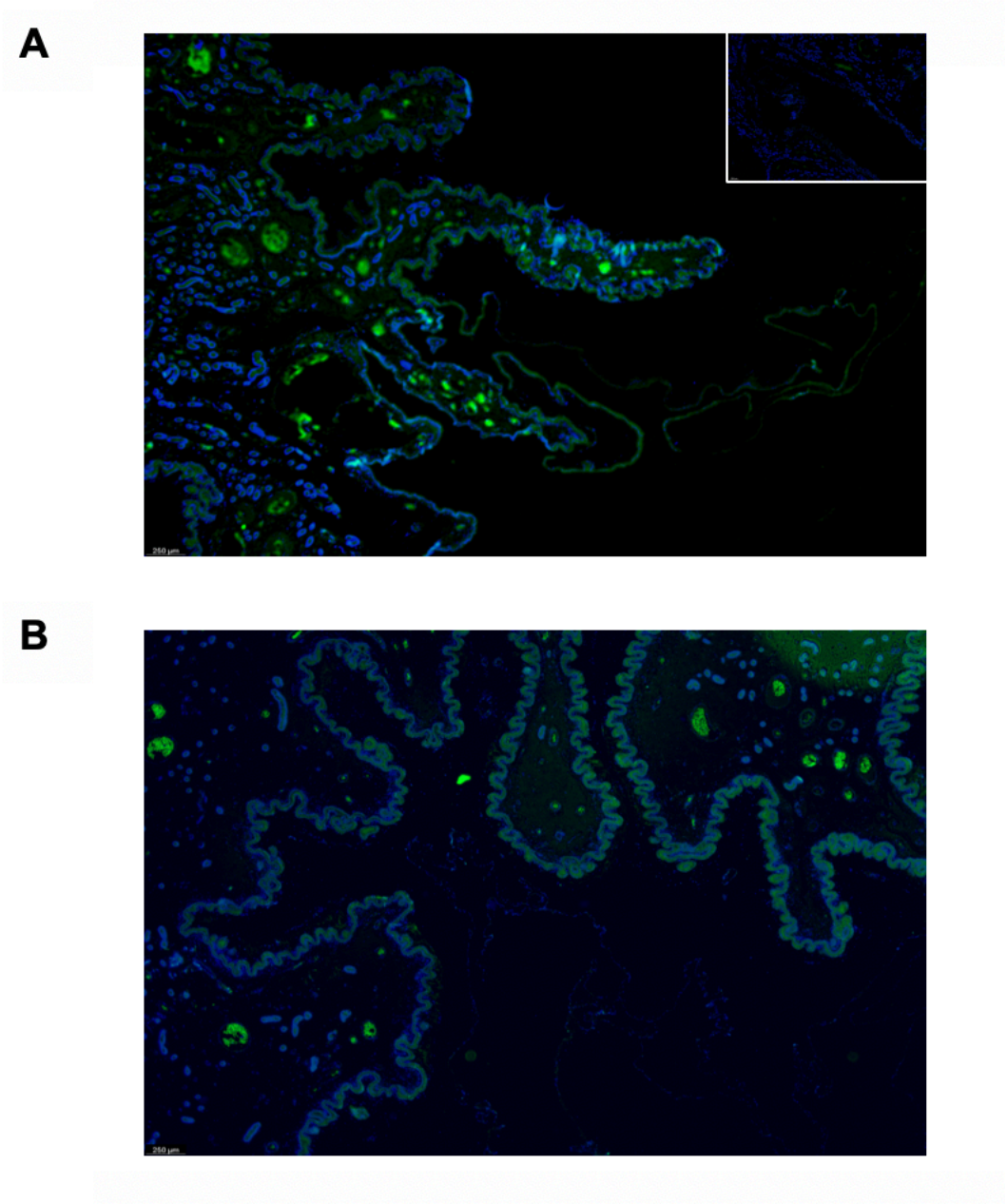

Figure 3.25: Immunolocalization of PTGS2 in day 35 (A) *PTGS2*$^{+/+}$ and (B) *PTGS2*$^{-/-}$ placental-uterine tissues. Immunofluorescence was performed on placental-uterine tissues to localize PTGS2 with a polyclonal PTGS2 primary antibody. Localization of PTGS2 in GE is denoted with arrow heads and localization of the chorion is denoted with arrows. Inset image is *PTGS2*$^{+/+}$ placental-uterine section incubated without the primary antibody.

3.4 Discussion

Events in early pig pregnancy require timely and close communication between the developing conceptus and the maternal uterine environment. In order for pregnancy to be successful in domestic species, the early conceptus must expand across the uterine surface, prevent $PGF_{2\alpha}$ from regressing the CL, attach to the luminal epithelium, and establish surface area and blood flow from maternal vasculature to the developing placenta. Previous research has established that pig conceptuses produce many factors such as IL1B2, estrogens, and PG's during the period of rapid elongation and maternal recognition in the pig. Conceptus synthesis of IL1B2 is essential for inducing rapid cellular remodeling for trophoblast expansion on day 12 of pregnancy (Whyte et al., 2018). Conceptus elongation across the uterine surface is important as the pig has a non-invasive placental development and therefore relies on close cell to cell contact between the trophectoderm and the luminal epithelium to establish a pathway for nutrient and blood flow to the developing fetus. IL1B, the proinflammatory cytokine belonging to the interleukin superfamily has been shown to upregulate expression of PLA_2, PTGS1, and PTGS2. Phospholipase A_2 is responsible for increasing cell fluidity to liberate arachidonic acid from the phospholipid bilayer. The increased free arachidonic acid is then converted by PTGS1 and PTGS2 into further downstream prostaglandins.

Coincident with the increased expression of *IL1B2*, conceptus E_2 production increases in biphasic peaks on days 11-12 and days 15-30 (Zavy et al., 1980; Geisert et al., 1982b). Studies proved that E_2 was capable of

sequestering PG from the uterine vasculature and into the uterine lumen to prevent regression of the CL (Bazer et al., 1977). The increased production of estradiol and the resulting 'exocrine/endocrine' action was proposed to be the maternal recognition signal in the pig. However, a recent gene editing study determined that conceptus aromatase (CYP19A1) and estrogen production is not essential for embryonic development, early maintenance of the CL, and establishment of the extraembryonic membranes (Meyer et al., 2019). Although the *CYP19A1*$^{-/-}$ conceptuses aborted by day 30 of pregnancy, these data indicate that estradiol is not the only conceptus derived factor involved in the extension of luteal function in the pig. Previous research has demonstrated that conceptus PGE_2 can maintain CL progesterone production, therefore suggesting that PGE_2 has a direct luteoprotective role (Henderson et al., 1977; Ford and Christenson, 1991). While utilization of prostaglandin inhibitors prevented conceptus elongation in the sheep (Dorniak et al., 2011) and maintenance of the CL during early pregnancy in the pig (Kraeling et al., 1985), the inhibitors cannot separate the maternal and conceptus effects.

The CRISPR/Cas9 gene editing system successfully ablated pig PTGS2 gene and protein expression preventing the synthesis of prostaglandins through this pathway. Pig conceptuses do produce PTGS1, but expression is not detected until after the initiation of trophoblast attachment on day 13 (Waclawik et al., 2007). Therefore, disrupting conceptus *PTGS2* expression allowed for a greater understanding for the function of the inducible form of prostaglandin synthase during the period of elongation and maternal recognition in the pig.

PTGS2 has been shown to be expressed in blastocysts across many species (Marshburn et al., 1990; Gurevich et al., 1994; Van-der-Weiden et al.,1996; Chakraborty et al., 1996; Charpigny et al., 1997) including pigs (Wilson et al., 2002). Prostaglandins have been shown to reactivate mouse blastocysts during delayed implantation (Paria et al., 1998) while hamsters require *PTGS2* for blastocyst hatching from the zona pellucida (Roy and Sheshagiri, 2013). Pig *PTGS2*$^{-/-}$ blastocysts were incapable of synthesizing prostaglandins but underwent normal development and hatching indicating that PTGS2 expression and prostaglandin production is not essential for early embryo development. Conceptus migration and spacing throughout the uterine horns is important in establishment of pregnancy in the pig (Dziuk et al., 1964). Establishment of early pregnancy in the mare also requires that the conceptus continually migrates between the uterine horns to block the release of $PGF_{2\alpha}$ (McDowell et al., 1985). The equine conceptus synthesizes PGE_2 to not only induce blastocyst movement from the oviduct into the tip of the uterine horn, but PGE_2 assists in stimulating myometrial contractions to move the conceptus around the uterus to maintain CL function (Weber et al., 1991a; Weber et al., 1991b; Weber et al., 1995; Stout and Allen, 2001). Pope et al. (1982) demonstrated that both estradiol and histamine assisted with efficient embryonic migration throughout the uterine horns in the pig. Interestingly, histamine has been shown to increase production of endometrial PGE_2 in the rat uterus (Viggiano et al., 1988).

As *PTGS2*$^{-/-}$ conceptuses were not only capable of developing to blastocysts and undergoing hatching, but successfully migrated and elongated

throughout both uterine horns. It is possible that endometrial PG's could stimulate conceptus migration and elongation. However, our data is consistent with indomethacin inhibition of total uterine and conceptus PG synthesis which did not affect conceptus migration or elongation on day 12 of pregnancy (Geisert et al., 1986). These results indicate that conceptus and endometrial prostaglandin production does not have an essential function at this early stage of pregnancy in the pig. Since conceptus elongation was not affected or stunted in *PTGS2*$^{-/-}$ conceptuses, it was expected that there would be no genotype difference for IL1B2 mRNA expression or production on day 12 of pregnancy. Neither IL1B2 mRNA nor protein expression was altered in *PTGS2*$^{-/-}$ conceptuses.

Despite the decrease in PG synthesis, *PTGS2*$^{-/-}$ conceptuses were not only capable of elongating and maintaining CL function but establishing maintaining pregnancy to day 35 of gestation. A recent study by Meyer et al. (2019) altered the theory that conceptus estrogen production was the major early maternal recognition signal in the pig. Although *CYP19A1*$^{-/-}$ conceptuses were successful in maintaining CL function beyond 24 days of pregnancy, *CYP19A1*$^{-/-}$ conceptus pregnancies were lost by day 35 of pregnancy. Several studies have proposed that PGE_2 plays an important role as a maternal recognition signal in the pig. Plasma concentration of PGE_2 in the utero-ovarian vein is significantly greater than cyclic gilts during the period of maternal recognition of pregnancy (Christenson et al., 1994). Individual CL implanted with PGE_2 but not E_2, are protected the effects of $PGF_{2\alpha}$ demonstrating a direct luteoprotective effect at the

CL (Ford and Christenson, 1991). PGE_2 can be directly at the CL due to the increase $PGE_2/PGF_{2\alpha}$ ratio which is produced by the conceptuses and endometrium. The conceptus produces about twice as much PGE_2 as $PGF_{2\alpha}$ to successfully protect luteal cells from regression (Gregoraszczuk and Michas, 1999). Thus, conceptus PGE synthesis could protect CL until day 24 in the absence of conceptus estrogen synthesis in the study of Meyer et al. (2019). Despite the inhibition or decrease of *PTGS2*$^{-/-}$ conceptus PGE synthesis, there were no effects on CL maintenance and establishment of pregnancy in the present study. Therefore, conceptus production of estradiol-17β could possibly compensate for the lack of PTGS2 derived prostaglandins as estrogens have been proven to increase endometrial *PTGS2* expression and production of PGE_2 (Waclawik et al., 2009b).

Although *PTGS2*$^{-/-}$ conceptus PG production was significantly decreased on day 14 and 17 or pregnancy, the content of PGs in ULF was not affected. The ULF content of PG was expectedly similar across genotypes as the maternal endometrium is contributing the major amount of prostaglandin released into the uterine lumen. There was also a day effect in prostaglandin production, as uterine luminal content of prostaglandins greatly increased from day 14 to 17, further indicating that prostaglandins are redirected into the uterine lumen to prevent luteolysis. Analysis of ULF from uteri containing *CYP19A1*$^{+/+}$ or *CYP19A1*$^{-/-}$ conceptuses revealed that the content of PGE_2 was different among the conceptus genotypes. ULF containing *CYP19A1*$^{+/+}$ conceptuses contained greater concentrations of PGE_2 than $PGF_{2\alpha}$, maintaining the 2:1 $PGE_2/PGF_{2\alpha}$

ratio which is consistent with previous research (Geisert et al., 1982b; Waclawik et al., 2009a).

To determine if *PTGS2*$^{-/-}$ conceptuses then had an effect on E_2 production, content of estradiol-17β was measured in the ULF of pigs gestating either *PTGS2*$^{-/-}$ or *PTGS2*$^{+/+}$ conceptuses. While there was no difference in conceptus *CYP19A1* expression, there was an unexpected decrease in estradiol-17β content in the ULF containing *PTGS2*$^{-/-}$ conceptuses on day 17. Previous literature regarding prostaglandin inhibition during early pregnancy indicated no difference in ULF estradiol content compared to vehicle treated controls on day 12 (Geisert et al., 1986). The cause of the decreased estradiol in the ULF but not *CYP19A1* expression of *PTGS2*$^{-/-}$ conceptuses is not known. Interestingly, the allantoic fluid concentrations of estradiol-17β on day 35 were significantly increased in the placentae of *PTGS2*$^{-/-}$ compared to *PTGS2*$^{+/+}$. While this is an interesting result, we cannot say that an increase in allantoic estradiol-17β content is the result of the ablation of the *PTGS2* expression or an effect that occurs in all cloned pig embryos since normal wild-type bred *PTGS2*$^{+/+}$ embryos were used as controls on day 35. Cloned embryos are typically developmentally delayed by about 2 days in comparison to normal wild-type bred embryos (Whyte et al., 2018). However, in future cloned *PTGS2*$^{+/+}$ fetuses need be evaluated to determine if this is the result of a clone effect or *PTGS2* gene edit.

In the present study not only were endometrial prostaglandins available to stimulate the conceptuses, but the *PTGS2*$^{-/-}$ conceptuses are capable of synthesizing prostaglandins after day 13 when *PTGS1* is expressed (Waklawik

and Ziecik, 2007). *In vitro* production of prostaglandins by day 14 *PTGS2*$^{-/-}$ conceptuses was significantly decreased compared to *PTGS2*$^{+/+}$ conceptus tissue, which confirms that not only had conceptus *PTGS2* activity been successfully inhibited but *PTGS1* expression was not initiated at this time. However, *in vitro* day 17 *PTGS2*$^{-/-}$ conceptus prostaglandin production was still lower compared to *PTGS2*$^{+/+}$ conceptuses when *PTGS1* expression increased ~10 to 60-fold from day 14 to 17. Despite the inhibition of PTGS2 derived prostaglandins, establishment of extra-embryonic membranes, growth of internal organs, and the initiation of placental development was not affected in day 17 *PTGS2*$^{-/-}$ conceptuses.

Endometrial gene expression was analyzed to determine if the developing *PTGS2*$^{-/-}$ conceptuses altered endometrial production of factors that may be assisting in the establishment and maintenance of pregnancy in the pig. Endometrial gene expression in pregnant gilts on days 14 and 17 showed no differential expression among genotypes and days in the genes evaluated. However, *PTGES3* expression was increased in endometrium containing *PTGS2*$^{-/-}$ conceptuses. This possibly indicates that endometrial PTGES3 is attempting to produce more PGE_2 in the absence of conceptus PGE_2, potentially being stimulated by conceptus E_2. As discussed earlier, PGE_2 has proven to play an important role in early pregnancy across many species as it can assist with early embryonic development and maintenance of the CL. Due to the diminished levels of PTGS2 derived PGE_2, it is possible that PGE_2 from the endometrium was stimulated by conceptus estrogen to increase *PTGES3* expression.

Previous studies have shown that inhibition of both embryonic *PTGS1* and *PTGS2* in mice did not affect conceptus development and demonstrated the successful fetal development to parturition. While conceptus prostaglandin production was prevented, prostaglandins from the maternal endometrium were proposed to play a role in the success of the maintenance of pregnancy in the mouse (Reese et al., 2000). Similar to inhibition of PTGS studies in domestic animals, inhibition of endometrial PTGS1 and PTGS2 derived prostaglandins in *PTGS1*$^{-/-}$ and *PTGS2*$^{-/-}$ mice result in implantation failure, smaller placental development or infertility (Langenbach et al., 1995; Dinchuk et al., 1995; Morham et al., 1995; Lim et al., 1997). These data indicate that endometrial prostaglandin production is essential for establishment of pregnancy and is capable of compensating for the absence of embryonic prostaglandins.

Earlier research has suggested that conceptus PGE_2 may have an effect on the immune system as a protective measure for the developing semi-allograft embryo (Kennedy et al., 1986; Parhar et al., 1989; Hamilton and Kennedy, 1994; Yang et al., 1997). Interestingly, *IFND* and *IFNG* expression were upregulated in day 14 and 17 *PTGS2*$^{-/-}$ conceptuses. It is possible that the inhibition of PTGS2 and lower production of PG's locally by the conceptus and chorioallantois on day 15 may induce an increased immune response which stimulates *IFND* and *IFNG* expression.

To determine if *PTGS2*$^{-/-}$ conceptuses could develop proper vascularization and placental formation, pregnancies were allowed to continue pregnancy to day 35. Although *PTGS2* expression is absent from the placenta of

PTGS2$^{-/-}$ embryos and there is a significant decrease in the concentration of total prostaglandins in the allantoic fluid, pregnancy was maintained and appear to have normal placental development.

This present study establishes that porcine conceptus PTGS2 derived prostaglandin production is not essential for early embryonic development, conceptus elongation, maternal recognition of pregnancy, attachment to the maternal epithelium, placentation, and maintenance of pregnancy to day 35. However, the interaction between PGE_2 and E_2 in both the *CYP19A1*$^{-/-}$ (Meyer et al. 2019) and our *PTGS2*$^{-/-}$ conceptus model indicate that both PGE_2 and E_2 can serve as maternal recognition signals, which in the absence of the other can establish pregnancy beyond 17 days. However, conceptus production of estrogen is essential for successful maintenance of pregnancy beyond day 30 as both conceptus and endometrial PGs are not successful in compensating for lost estrogen production at this time.

BIBLIOGRAPHY

Abraham, J. E., P. Harrington, K. E. Driver, J. Tyrer, D. F. Easton, A. M. Dunning, and P. D. Pharoah. 2009. Common polymorphisms in the prostaglandin pathway genes and their association with breast cancer susceptibility and survival. Clin Cancer Res 15(6):2181-2191. doi: 10.1158/1078-0432.CCR-08-0716

Albertini, D. F., E. W. Overstrom, and K. M. Ebert. 1987. Changes in the organization of the actin cytoskeleton during preimplantation development of the pig embryo. Biol Reprod 37(2):441-451. doi: 10.1095/biolreprod37.2.441

Ali, S., and D. A. Mann. 2004. Signal transduction via the NF-kappaB pathway: a targeted treatment modality for infection, inflammation and repair. Cell Biochem Funct 22(2):67-79. doi: 10.1002/cbf.1082

Amoroso, E. 1952. Placentation. In: A. Parkes, editor, Marshall's Physiology of Reproduction. Little Brown & Co., Boston. p. 127-311.

Anderson, L. L. 1978. Growth, protein content and distribution of early pig embryos. Anat Rec 190(1):143-153. doi: 10.1002/ar.1091900112

Anderson, L. L., R. P. Rathmacher, and R. M. Melampy. 1966. The uterus and unilateral regression of corpora lutea in the pig. Am J Physiol 210(3):611-614. doi: 10.1152/ajplegacy.1966.210.3.611

Arend, W. P., G. Palmer, and C. Gabay. 2008. IL-1, IL-18, and IL-33 families of cytokines. Immunol Rev 223:20-38. doi: 10.1111/j.1600-065X.2008.00624.x

Ashworth, M. D., J. W. Ross, J. Hu, F. J. White, D. R. Stein, U. Desilva, G. A. Johnson, T. E. Spencer, and R. D. Geisert. 2006. Expression of porcine endometrial prostaglandin synthase during the estrous cycle and early pregnancy, and following endocrine disruption of pregnancy. Biol Reprod 74(6):1007-1015. doi: 10.1095/biolreprod.105.046557

Ball, G. D., and B. N. Day. 1982. Local effects of PGF2 alpha and embryonic extracts on luteal function in swine. J Anim Sci 54(1):150-154. doi: 10.2527/jas1982.541150x

Bazer, F., S. R. Marengo, R. Geisert, and W. W. Thatcher. 1984. Exocrine versus endocrine secretion of prostaglandin F2α in the control of pregnancy in swine. Anim Reprod Sci 7(1-3):115-132. (Scientific)

Bazer, F. W. 1989. Establishment of pregnancy in sheep and pigs. Reprod Fertil Dev 1(3):237-242.

Bazer, F. W., R. D. Geisert, W. W. Thatcher, and R. M. Roberts. 1982. Establishment and Maintenance of Pregnancy. Proceedings - Easter School in Agricultural Science, University of Nottingham:227-252.

Bazer, F. W., T. L. Ott, and T. E. Spencer. 1994. Pregnancy Recognition in Ruminants, Pigs and Horses: Signals from the Trophoblast. Theriogenology 41(1):79-94.

Bazer, F. W., T. E. Spencer, G. A. Johnson, R. C. Burghardt, and G. Wu. 2009. Comparative aspects of implantation. Reproduction 138(2):195-209. doi: 10.1530/REP-09-0158

Bazer, F. W., and W. W. Thatcher. 1977. Theory of maternal recognition of pregnancy in swine based on estrogen controlled endocrine versus exocrine secretion of prostaglandin F2alpha by the uterine endometrium. Prostaglandins 14(2):397-400.

Bergstrom, S., L. A. Carlson, L. G. Ekelund, and L. Oro. 1965. Cardiovascular and metabolic response to infusions of prostaglandin E1 and to simultaneous infusions of noradrenaline and prostaglandin E1 in man. Prostaglandin and related factors 35. Acta Physiol Scand 64(4):332-339. doi: 10.1111/j.1748-1716.1965.tb04187.x

Bergstrom, S., R. Eliasson, E. U. von, and J. Sjovall. 1959. Some biological effects of two crystalline prostaglandin factors. Acta Physiol Scand 45:133-144. doi: 10.1111/j.1748-1716.1959.tb01685.x

Bergström, S., and J. Sjövall. 1957. The Isolation of Prostaglandin. Acta Chemica Scandinavica 11(6):1086.

Bergström, S., H. Theorell, and H. Davide. 1947. On a Metabolic Product of Ps. Pyocyanea, Pyolipic Acid, Active Against Nycobact. Tuberculosis. Arkiv for Kemi, Mineralogi och Geologi Journal 23(4-5):1-12.

Bielanska-Osuchowska, Z. 1979. Ultrastructure of the Trophoblast of the Pig Placenta. Acta Medica Polona 20(4):359-360.

Biggers, J. D., B. V. Leonov, J. F. Baskar, and J. Fried. 1978. Inhibition of hatching of mouse blastocysts in vitro by prostaglandin antagonists. Biol Reprod 19(3):519-533. doi: 10.1095/biolreprod19.3.519

Black, L. J., C. D. Jones, and J. F. Falcone. 1983. Antagonism of estrogen action with a new benzothiophene derived antiestrogen. Life Sci 32(9):1031-1036.

Blitek, A., M. M. Kaczmarek, J. Kiewisz, and A. J. Ziecik. 2010. Endometrial and conceptus expression of HoxA10, transforming growth factor beta1, leukemia inhibitory factor, and prostaglandin H synthase-2 in early pregnant pigs with gonadotropin-induced estrus. Domest Anim Endocrinol 38(4):222-234. doi: 10.1016/j.domaniend.2009.11.001

Blitek, A., A. Waclawik, M. M. Kaczmarek, T. Stadejek, Z. Pejsak, and A. J. Ziecik. 2006. Expression of cyclooxygenase-1 and -2 in the porcine endometrium during the oestrous cycle and early pregnancy. Reprod Domest Anim 41(3):251-257. doi: 10.1111/j.1439-0531.2006.00646.x

Bourdiec, A., V. Martel, and A. Akoum. 2014. Synchronous regulation of the determinants of endometrial receptivity to interleukin 1 at key stages of early embryo implantation in vivo. Fertil Steril 101(4):1183-1193. doi: 10.1016/j.fertnstert.2014.01.011

Brodie, A. M., W. C. Schwarzel, A. A. Shaikh, and H. J. Brodie. 1977. The effect of an aromatase inhibitor, 4-hydroxy-4-androstene-3,17-dione, on estrogen-dependent processes in reproduction and breast cancer. Endocrinology 100(6):1684-1695. doi: 10.1210/endo-100-6-1684

Camus, L., and E. Gley. 1896. Action Coagulante du Liquid Prostatique sur le Contenu des Vesicules Seminales. Mem. de la Soc. Biol. XLVIII:787-788.

Chakraborty, I., S. K. Das, J. Wang, and S. K. Dey. 1996. Developmental expression of the cyclo-oxygenase-1 and cyclo-oxygenase-2 genes in the peri-implantation mouse uterus and their differential regulation by the blastocyst and ovarian steroids. J Mol Endocrinol 16(2):107-122.

Chang, A. C., and S. N. Cohen. 1974. Genome construction between bacterial species in vitro: replication and expression of Staphylococcus plasmid genes in Escherichia coli. Proc Natl Acad Sci U S A 71(4):1030-1034. doi: 10.1073/pnas.71.4.1030

Charpigny, G., P. Reinaud, J. P. Tamby, C. Creminon, and M. Guillomot. 1997. Cyclooxygenase-2 unlike cyclooxygenase-1 is highly expressed in ovine

embryos during the implantation period. Biol Reprod 57(5):1032-1040. doi: 10.1095/biolreprod57.5.1032

Choi, Y., H. Jang, H. Seo, I. Yoo, J. Han, M. Kim, S. Lee, and H. Ka. 2019. Changes in calcium levels in the endometrium throughout pregnancy and the role of calcium on endometrial gene expression at the time of conceptus implantation in pigs. Mol Reprod Dev doi: 10.1002/mrd.23166

Christenson, L. K., D. B. Farley, L. H. Anderson, and S. P. Ford. 1994. Luteal maintenance during early pregnancy in the pig: role for prostaglandin E2. Prostaglandins 47(1):61-75.

Christenson, R. K., J. J. Ford, and D. A. Redmer. 1985. Maturation of ovarian follicles in the prepubertal gilt. J Reprod Fertil Suppl 33:21-36.

Clark, J. H., and B. M. Markaverich. 1981. The agonistic-antagonistic properties of clomiphene: a review. Pharmacol Ther 15(3):467-519.

Cockrill, J. R., E. G. Miller, and R. Kurzrok. 1935. The Substance in Human Seminal Fluid Affecting Uterine Muscle. Am J Physiol 122(5):77-80.

Copeland, R. A., J. M. Williams, J. Giannaras, S. Nurnberg, M. Covington, D. Pinto, S. Pick, and J. M. Trzaskos. 1994. Mechanism of selective inhibition of the inducible isoform of prostaglandin G/H synthase. Proc Natl Acad Sci U S A 91(23):11202-11206. doi: 10.1073/pnas.91.23.11202

Correia-Alvarez, E., E. Gomez, D. Martin, S. Carrocera, S. Perez, J. Otero, N. Peynot, C. Giraud-Delville, J. N. Caamano, O. Sandra, V. Duranthon, and M. Munoz. 2015. Expression and localization of interleukin 1 beta and interleukin 1 receptor (type I) in the bovine endometrium and embryo. J Reprod Immunol 110:1-13. doi: 10.1016/j.jri.2015.03.006

Da Silva, C. L. A., M. Broekhuijse, B. F. A. Laurenssen, H. A. Mulder, E. F. Knol, B. Kemp, and N. M. Soede. 2017. Relationship between ovulation rate and embryonic characteristics in gilts at 35 d of pregnancy. J Anim Sci 95(7):3160-3172. doi: 10.2527/jas.2017.1577

Dantzer, V. 1985. Electron microscopy of the initial stages of placentation in the pig. Anat Embryol (Berl) 172(3):281-293.

Davis, A. J., I. R. Fleet, F. A. Harrison, and F. M. M. Walker. 1980. Pulmonary Metabolism of Prostaglandin F2a in the Conscious Non-Pregnant Ewe and Sow J Physiol 301

Davis, D. L., and R. M. Blair. 1993. Studies of uterine secretions and products of primary cultures of endometrial cells in pigs. J Reprod Fertil Suppl 48:143-155.

Davis, D. L., P. L. Pakrasi, and S. K. Dey. 1983. Prostaglandins in swine blastocysts. Biol Reprod 28(5):1114-1118.

Day, B. N., and C. Polge. 1968. Effects of progesterone on fertilization and egg transport in the pig. J Reprod Fertil 17(1):227-230.

De los Santos, M. J., A. Mercader, A. Frances, E. Portoles, J. Remohi, A. Pellicer, and C. Simon. 1996. Role of endometrial factors in regulating secretion of components of the immunoreactive human embryonic interleukin-1 system during embryonic development. Biol Reprod 54(3):563-574. doi: 10.1095/biolreprod54.3.563

Del Campo, C. H., and O. J. Ginther. 1973. Vascular anatomy of the uterus and ovaries and the unilateral luteolytic effect of the uterus: angioarchitecture in sheep. Am J Vet Res 34(11):1377-1385.

Dey, S. K., S. M. Chien, C. L. Cox, and R. D. Crist. 1980. Prostaglandin synthesis in the rabbit blastocyst. Prostaglandins 19(3):449-453.

Dhindsa, D. S., and P. J. Dziuk. 1968. Influence of varying the proportion of uterus occupied by embryos on maintenance of pregnancy in the pig. J Anim Sci 27(3):668-672.

Dhindsa, D. S., P. J. Dziuk, and H. W. Norton. 1967. Time of transuterine migration and distribution of embryos in the pig. Anat Rec 159(3):325-330. doi: 10.1002/ar.1091590309

Dickmann, Z., and C. H. Spilman. 1975. Prostaglandins in rabbit blastocyst. Science 190(4218):997-998. doi: 10.1126/science.1188381

Dinarello, C. A. 2009. Immunological and inflammatory functions of the interleukin-1 family. Annu Rev Immunol 27:519-550. doi: 10.1146/annurev.immunol.021908.132612

Dinchuk, J. E., B. D. Car, R. J. Focht, J. J. Johnston, B. D. Jaffee, M. B. Covington, N. R. Contel, V. M. Eng, R. J. Collins, P. M. Czerniak, and et al. 1995. Renal abnormalities and an altered inflammatory response in mice lacking cyclooxygenase II. Nature 378(6555):406-409. doi: 10.1038/378406a0

Dorniak, P., F. W. Bazer, and T. E. Spencer. 2011. Prostaglandins regulate conceptus elongation and mediate effects of interferon tau on the ovine uterine endometrium. Biol Reprod 84(6):1119-1127. doi: 10.1095/biolreprod.110.089979

Doudna, J. A., and E. Charpentier. 2014. Genome editing. The new frontier of genome engineering with CRISPR-Cas9. Science 346(6213):1258096. doi: 10.1126/science.1258096

Dubois, D. H., L. C. Smith, and F. W. Bazer. 1993. Determination of porcine endometrial phospholipase A2 activity and detection of immunoreactive cyclooxygenase during the oestrous cycle and early pregnancy. Reprod Fertil Dev 5(5):531-543.

Dubois, R. N., S. B. Abramson, L. Crofford, R. A. Gupta, L. S. Simon, L. B. Van De Putte, and P. E. Lipsky. 1998. Cyclooxygenase in biology and disease. FASEB J 12(12):1063-1073.

DuBois, R. N., A. Radhika, B. S. Reddy, and A. J. Entingh. 1996. Increased cyclooxygenase-2 levels in carcinogen-induced rat colonic tumors. Gastroenterology 110(4):1259-1262.

Ducharme, D. W., J. R. Weeks, and R. G. Montgomery. 1968. Studies on the mechanism of the hypertensive effect of prostaglandin F2-alpha. J Pharmacol Exp Ther 160(1):1-10.

Dziuk, P. 1985. Effect of migration, distribution and spacing of pig embryos on pregnancy and fetal survival. J Reprod Fertil Suppl 33:57-63.

Dziuk, P. J. 1991. Reproduction in the Pig. In: P. T. Cupps, editor, Reproduction in Domestic Animals. Academic Press, University of California, Davis. p. 471-490.

Dziuk, P. J., and G. D. Gehlbach. 1966. Induction of Ovulation and Fertilization in the Immature Gilt. J Anim Sci 25(2):410-413.

Dziuk, P. J., C. Polge, and L. E. Rowson. 1964. Intra-Uterine Migration and Mixing of Embryos in Swine Following Egg Transfer. J Anim Sci 23(1):37-42.

Ebeling, S., D. Topfer, J. M. Weitzel, and B. Meinecke. 2011. Bone morphogenetic protein-6 (BMP-6): mRNA expression and effect on steroidogenesis during in vitro maturation of porcine cumulus oocyte complexes. Reprod Fertil Dev 23(8):1034-1042. doi: 10.1071/RD11027

Eglinton, G., R. A. Raphael, G. N. Smith, W. J. Hall, and V. R. Pickles. 1963. Isolation and Identification of Two Smooth Muscle Stimulants from Menstrual Fluid. Nature 200:960 PASSIM. doi: 10.1038/200960a0

Enders, A. C., and S. Schlafke. 1969. Cytological aspects of trophoblast-uterine interaction in early implantation. Am J Anat 125(1):1-29. doi: 10.1002/aja.1001250102

Evans, C. A., and T. G. Kennedy. 1978. The importance of prostaglandin synthesis for the initiation of blastocyst implantation in the hamster. J Reprod Fertil 54(2):255-261.

Flower, D. R. 1996. The lipocalin protein family: structure and function. Biochem J 318 (Pt 1):1-14. doi: 10.1042/bj3180001

Flower, R. J., and G. J. Blackwell. 1976. The importance of phospholipase-A2 in prostaglandin biosynthesis. Biochem Pharmacol 25(3):285-291. doi: 10.1016/0006-2952(76)90216-1

Ford, S. P., and L. K. Christenson. 1991. Direct effects of oestradiol-17 beta and prostaglandin E-2 in protecting pig corpora lutea from a luteolytic dose of prostaglandin F-2 alpha. J Reprod Fertil 93(1):203-209.

Forman, B. M., B. Ruan, J. Chen, G. J. Schroepfer, Jr., and R. M. Evans. 1997. The orphan nuclear receptor LXRalpha is positively and negatively regulated by distinct products of mevalonate metabolism. Proc Natl Acad Sci U S A 94(20):10588-10593. doi: 10.1073/pnas.94.20.10588

Forman, B. M., P. Tontonoz, J. Chen, R. P. Brun, B. M. Spiegelman, and R. M. Evans. 1995. 15-Deoxy-delta 12, 14-prostaglandin J2 is a ligand for the adipocyte determination factor PPAR gamma. Cell 83(5):803-812.

Frank, M., F. W. Bazer, W. W. Thatcher, and C. J. Wilcox. 1977. A study of prostaglandin F2alpha as the luteolysin in swine: III effects of estradiol valerate on prostaglandin F, progestins, estrone and estradiol concentrations in the utero-ovarian vein of nonpregnant gilts. Prostaglandins 14(6):1183-1196.

Gardner, M. L., N. L. First, and L. E. Casida. 1963. Effect of Exogenous Estrogens on Corpus Luteum Maintenance in Gilts. J Anim Sci 22(1):132-134.

Gastal, M. O., E. L. Gastal, C. A. Torres, and O. J. Ginther. 1998a. Effect of oxytocin, prostaglandin F2 alpha, and clenbuterol on uterine dynamics in mares. Theriogenology 50(4):521-534.

Gastal, M. O., E. L. Gastal, C. A. Torres, and O. J. Ginther. 1998b. Effect of PGE2 on uterine contractility and tone in mares. Theriogenology 50(7):989-999.

Geisert, R., and R. A. Schmitt. 2002. Early embryonic survival in the pig: Can it be improved? J Anim Sci 80(E-suppl_1):E54-E65. (Scientific) doi: 10.2527/animalsci2002.0021881200800ES10009x

Geisert, R. D., J. W. Brookbank, R. M. Roberts, and F. W. Bazer. 1982a. Establishment of pregnancy in the pig: II. Cellular remodeling of the porcine blastocyst during elongation on day 12 of pregnancy. Biol Reprod 27(4):941-955.

Geisert, R. D., G. A. Johnson, and R. C. Burghardt. 2015. Implantation and Establishment of Pregnancy in the Pig. Adv Anat Embryol Cell Biol 216:137-163. doi: 10.1007/978-3-319-15856-3_8

Geisert, R. D., R. J. Rasby, J. E. Minton, and R. P. Wetteman. 1986. Role of prostaglandins in development of porcine blastocysts. Prostaglandins 31(2):191-204.

Geisert, R. D., J. W. Ross, M. D. Ashworth, and J. R. Malayer. 2004. Estrogen Regulator and/or Endocrine Disruptor in Establishment of Pregnanct. . Current Topics in Steroid Research 4:69-84.

Geisert, R. D., W. W. Thatcher, R. M. Roberts, and F. W. Bazer. 1982b. Establishment of pregnancy in the pig: III. Endometrial secretory response to estradiol valerate administered on day 11 of the estrous cycle. Biol Reprod 27(4):957-965. doi: 10.1095/biolreprod27.4.957

Geisert, R. D., J. J. Whyte, A. E. Meyer, D. J. Mathew, M. R. Juarez, M. C. Lucy, R. S. Prather, and T. E. Spencer. 2017. Rapid conceptus elongation in the pig: An interleukin 1 beta 2 and estrogen-regulated phenomenon. Mol Reprod Dev 84(9):760-774. doi: 10.1002/mrd.22813

Geisert, R. D., M. T. Zavy, R. J. Moffatt, R. M. Blair, and T. Yellin. 1990. Embryonic steroids and the establishment of pregnancy in pigs. J Reprod Fertil Suppl 40:293-305.

Geisert, R. D., M. T. Zavy, R. P. Wettemann, and B. G. Biggers. 1987. Length of pseudopregnancy and pattern of uterine protein release as influenced by time and duration of oestrogen administration in the pig. J Reprod Fertil 79(1):163-172.

Ginther, O. J. 1981. Local versus systemic uteroovarian relationships in farm animals. Acta Vet Scand Suppl 77:103-115.

Godfray, H. C. J., P. Aveyard, T. Garnett, J. W. Hall, T. J. Key, J. Lorimer, R. T. Pierrehumbert, P. Scarborough, M. Springmann, and S. A. Jebb. 2018. Meat consumption, health, and the environment. Science 361(6399)doi: 10.1126/science.aam5324

Goldblatt, M. W. 1935. Properties of human seminal plasma. J Physiol 84(2):208-218. doi: 10.1113/jphysiol.1935.sp003269

Gotzl, A. 1910. Versuche über die Wirkung von Prostatapreßsäften. Zeitschr. f. Urol

Gregoraszczuk, E. L., and N. Michas. 1999. Progesterone and estradiol secretion by porcine luteal cells is influenced by individual and combined treatment with prostaglandins E2 and F2alpha throughout the estrus cycle. Prostaglandins Other Lipid Mediat 57(4):231-241.

Guillomot, M., J. E. Flechon, and S. Wintenberger-Torres. 1981. Conceptus attachment in the ewe: an ultrastructural study. Placenta 2(2):169-182.

Gurevich, M., and M. Shemesh. 1994. Induction of cyclooxygenase and prostaglandin E2 production by the bovine pre-embryo. Reprod Fertil Dev 6(6):687-691.

Gutknecht, G. D., J. C. Cornette, and B. B. Pharriss. 1969. Antifertility properties of prostaglandin F 2. Biol Reprod 1(4):367-371. doi: 10.1095/biolreprod1.4.367

Hamilton, G. S., and T. G. Kennedy. 1994. Uterine vascular changes after unilateral intrauterine infusion of indomethacin and prostaglandin E2 to rats sensitized for the decidual cell reaction. Biol Reprod 50(4):757-764. doi: 10.1095/biolreprod50.4.757

Harper, M. J., C. J. Norris, and K. Rajkumar. 1983. Prostaglandin release by zygotes and endometria of pregnant rabbits. Biol Reprod 28(2):350-362. doi: 10.1095/biolreprod28.2.350

Hayden, M. S., and S. Ghosh. 2012. NF-kappaB, the first quarter-century: remarkable progress and outstanding questions. Genes Dev 26(3):203-234. doi: 10.1101/gad.183434.111

Hemler, M., and W. E. Lands. 1976. Purification of the cyclooxygenase that forms prostaglandins. Demonstration of two forms of iron in the holoenzyme. J Biol Chem 251(18):5575-5579.

Henderson, K. M., R. J. Scaramuzzi, and D. T. Baird. 1977. Simultaneous infusion of prostaglandin E2 antagonizes the luteolytic action of prostaglandin F2alpha in vivo. J Endocrinol 72(3):379-383.

Herschman, H. R. 1996. Prostaglandin synthase 2. Biochim Biophys Acta 1299(1):125-140. doi: 10.1016/0005-2760(95)00194-8

Heuser, C. H. S., G.L. 1929. Early stages in the development of pig embryos, from the period of initial cleavage to the time of the appearance of limb-buds. Contrib Embryol (20):3-29. (Journal Article)

Hirsch, K. S., C. D. Jones, T. D. Lindstrom, N. B. Stamm, G. P. Sutton, H. M. Taylor, and D. E. Weaver. 1987. Discovery and development of a novel class of nonsteroidal aromatase inhibitors. Steroids 50(1-3):201-217.

Hunter, R. H. F. 1980. Physiology and Technology of Reproduction in Female Domestic Animals. Sch. Agric., Univ. Edinburgh, Edinburgh, UK.

Hyland, J. H., J. G. Manns, and W. D. Humphrey. 1982. Prostaglandin production by ovine embryos and endometrium in vitro. J Reprod Fertil 65(2):299-304.

Japelli, G., and G. M. Scafa. 1906. Sur les effets des injections intra-veineuses d'estrait prostatique de chien. Arch. Ital. Biol. 45:165.

Jinek, M., K. Chylinski, I. Fonfara, M. Hauer, J. A. Doudna, and E. Charpentier. 2012. A programmable dual-RNA-guided DNA endonuclease in adaptive

bacterial immunity. Science 337(6096):816-821. doi: 10.1126/science.1225829

Johnson, M. H., and J. M. McConnell. 2004. Lineage allocation and cell polarity during mouse embryogenesis. Semin Cell Dev Biol 15(5):583-597. doi: 10.1016/j.semcdb.2004.04.002

Joyce, M. M., R. C. Burghardt, R. D. Geisert, J. R. Burghardt, R. N. Hooper, J. W. Ross, M. D. Ashworth, and G. A. Johnson. 2007. Pig conceptuses secrete estrogen and interferons to differentially regulate uterine STAT1 in a temporal and cell type-specific manner. Endocrinology 148(9):4420-4431. doi: 10.1210/en.2007-0505

Ka, H., H. Seo, M. Kim, Y. Choi, and C. K. Lee. 2009. Identification of differentially expressed genes in the uterine endometrium on day 12 of the estrous cycle and pregnancy in pigs. Mol Reprod Dev 76(1):75-84. doi: 10.1002/mrd.20935

Kaczmarek, M. M., A. Blitek, K. Kaminska, G. Bodek, M. Zygmunt, D. Schams, and A. J. Ziecik. 2008. Assessment of VEGF-receptor system expression in the porcine endometrial stromal cells in response to insulin-like growth factor-I, relaxin, oxytocin and prostaglandin E2. Mol Cell Endocrinol 291(1-2):33-41. doi: 10.1016/j.mce.2008.04.020

Karim, S. M., and J. Devlin. 1967. Prostaglandin content of amniotic fluid during pregnancy and labour. J Obstet Gynaecol Br Commonw 74(2):230-234.

Karim, S. M., and G. M. Filshie. 1970. Therapeutic abortion using prostaglandin F2alpha. Lancet 1(7639):157-159. doi: 10.1016/s0140-6736(70)90402-2

Karim, S. M., R. R. Trussell, K. Hillier, and R. C. Patel. 1969. Induction of labour with prostaglandin F2a. J Obstet Gynaecol Br Commonw 76(9):769-782.

Katebi, A. R., A. Kloczkowski, and R. L. Jernigan. 2010. Structural interpretation of protein-protein interaction network. BMC Struct Biol 10 Suppl 1:S4. doi: 10.1186/1472-6807-10-S1-S4

Kennedy, T. G. 1977. Evidence for a role for prosaglandins in the initiation of blastocyst implantation in the rat. Biol Reprod 16(3):286-291. doi: 10.1095/biolreprod16.3.286

Kennedy, T. G., J. L. Keys, and G. J. King. 1986. Endometrial prostaglandin E2-binding sites in the pig: characterization and changes during the estrous cycle and early pregnancy. Biol Reprod 35(3):624-632. doi: 10.1095/biolreprod35.3.624

Khorana, H. G. 1968. Synthetic nucleic acids and the genetic code. JAMA 206(9):1978-1982.

Kidder, H. E., L. E. Casida, and R. H. Grummer. 1955. Some Effects of Estrogen Injections on the Estrual Cycle of Gilts. J Anim Sci 14(2):470-474.

Kim, Y. G., J. Cha, and S. Chandrasegaran. 1996. Hybrid restriction enzymes: zinc finger fusions to Fok I cleavage domain. Proc Natl Acad Sci U S A 93(3):1156-1160. doi: 10.1073/pnas.93.3.1156

King, G. J., B. A. Atkinson, and H. A. Robertson. 1982. Implantation and early placentation in domestic ungulates. J Reprod Fertil Suppl 31:17-30.

Kirby, C. J., M. F. Smith, D. H. Keisler, and M. C. Lucy. 1997. Follicular function in lactating dairy cows treated with sustained-release bovine somatotropin. J Dairy Sci 80(2):273-285. doi: 10.3168/jds.S0022-0302(97)75935-6

Kirton, K. T., B. B. Pharriss, and A. D. Forbes. 1970a. Luteolytic effects of prostaglandin F2a in primates. Proc Soc Exp Biol Med 133(1):314-316. doi: 10.3181/00379727-133-34464

Kirton, K. T., B. B. Pharriss, and A. D. Forbes. 1970b. Some effects of prostaglandins E 2 and F 2 on the pregnant rhesus monkey. Biol Reprod 3(2):163-168. doi: 10.1093/biolreprod/3.2.163

Klein, C. 2016. Early pregnancy in the mare: old concepts revisited. Domest Anim Endocrinol 56 Suppl:S212-217. doi: 10.1016/j.domaniend.2016.03.006

Kliewer, S. A., B. M. Forman, B. Blumberg, E. S. Ong, U. Borgmeyer, D. J. Mangelsdorf, K. Umesono, and R. M. Evans. 1994. Differential expression and activation of a family of murine peroxisome proliferator-activated

receptors. Proc Natl Acad Sci U S A 91(15):7355-7359. doi: 10.1073/pnas.91.15.7355

Kliewer, S. A., K. Umesono, D. J. Noonan, R. A. Heyman, and R. M. Evans. 1992. Convergence of 9-cis retinoic acid and peroxisome proliferator signalling pathways through heterodimer formation of their receptors. Nature 358(6389):771-774. doi: 10.1038/358771a0

Kol, S., I. Kehat, and E. Y. Adashi. 2002. Ovarian interleukin-1-induced gene expression: privileged genes threshold theory. Med Hypotheses 58(1):6-8. doi: 10.1054/mehy.2001.1389

Kraeling, R. R., G. B. Rampacek, and N. A. Fiorello. 1985. Inhibition of pregnancy with indomethacin in mature gilts and prepuberal gilts induced to ovulate. Biol Reprod 32(1):105-110.

Kridli, R. T., K. Khalaj, M. Bidarimath, and C. Tayade. 2016. Placentation, maternal-fetal interface, and conceptus loss in swine. Theriogenology 85(1):135-144. doi: 10.1016/j.theriogenology.2015.08.001

Kuida, K., J. A. Lippke, G. Ku, M. W. Harding, D. J. Livingston, M. S. Su, and R. A. Flavell. 1995. Altered cytokine export and apoptosis in mice deficient in interleukin-1 beta converting enzyme. Science 267(5206):2000-2003. doi: 10.1126/science.7535475

Kujubu, D. A., B. S. Fletcher, B. C. Varnum, R. W. Lim, and H. R. Herschman. 1991. TIS10, a phorbol ester tumor promoter-inducible mRNA from Swiss

3T3 cells, encodes a novel prostaglandin synthase/cyclooxygenase homologue. J Biol Chem 266(20):12866-12872.

Kurumbail, R. G., A. M. Stevens, J. K. Gierse, J. J. McDonald, R. A. Stegeman, J. Y. Pak, D. Gildehaus, J. M. Miyashiro, T. D. Penning, K. Seibert, P. C. Isakson, and W. C. Stallings. 1996. Structural basis for selective inhibition of cyclooxygenase-2 by anti-inflammatory agents. Nature 384(6610):644-648. doi: 10.1038/384644a0

Kurzrok, R., and C. C. Lieb. 1930. Biochemical Studies of Human Semen. II. The Action of Semen on the Human Uterus. Exp Biol Med 28(3)

Lacroix, M. C., and G. Kann. 1982. Comparative studies of prostaglandins F2 alpha and E2 in late cyclic and early pregnant sheep: in vitro synthesis by endometrium and conceptus effects of in vivo indomethacin treatment on establishment of pregnancy. Prostaglandins 23(4):507-526.

Lai, L., and R. S. Prather. 2003. Production of cloned pigs by using somatic cells as donors. Cloning Stem Cells 5(4):233-241. doi: 10.1089/153623003772032754

Langenbach, R., S. G. Morham, H. F. Tiano, C. D. Loftin, B. I. Ghanayem, P. C. Chulada, J. F. Mahler, C. A. Lee, E. H. Goulding, K. D. Kluckman, H. S. Kim, and O. Smithies. 1995. Prostaglandin synthase 1 gene disruption in mice reduces arachidonic acid-induced inflammation and indomethacin-induced gastric ulceration. Cell 83(3):483-492.

Lauderdale, J. W. 1972. Effects of PGF2α on Pregnancy and Estrous Cycle of Cattle. J Anim Sci 35:246.

Lee, S. H., H. S. Lee, C. K. Lim, Y. S. Park, K. M. Yang, and D. W. Park. 2013. Comparison of the clinical outcomes of day 4 and 5 embryo transfer cycles. Clin Exp Reprod Med 40(3):122-125. doi: 10.5653/cerm.2013.40.3.122

Leiser, R. 1975. [Development of contact between trophoblast and uterine epithelium during the early stages on implantation in the cow]. Zentralbl Veterinarmed C 4(1):63-86.

Lewis, G. S., W. W. Thatcher, F. W. Bazer, and J. S. Curl. 1982. Metabolism of arachidonic acid in vitro by bovine blastocysts and endometrium. Biol Reprod 27(2):431-439. doi: 10.1095/biolreprod27.2.431

Lewis, G. S., and R. A. Waterman. 1985. Metabolism of arachidonic acid in vitro by ovine conceptuses recovered during early pregnancy. Prostaglandins 30(2):263-283.

Li, J., M. A. Perrella, J. C. Tsai, S. F. Yet, C. M. Hsieh, M. Yoshizumi, C. Patterson, W. O. Endege, F. Zhou, and M. E. Lee. 1995. Induction of vascular endothelial growth factor gene expression by interleukin-1 beta in rat aortic smooth muscle cells. J Biol Chem 270(1):308-312. doi: 10.1074/jbc.270.1.308

Li, T., S. Huang, X. Zhao, D. A. Wright, S. Carpenter, M. H. Spalding, D. P. Weeks, and B. Yang. 2011. Modularly assembled designer TAL effector nucleases for targeted gene knockout and gene replacement in eukaryotes. Nucleic Acids Res 39(14):6315-6325. doi: 10.1093/nar/gkr188

Liehr, R. A., G. B. Marion, and H. H. Olson. 1972. Effects of Progstaglandin on Cattle Estrous Cycles J Anim Sci. p Abstract.

Lim, H., and S. K. Dey. 1997. Prostaglandin E2 receptor subtype EP2 gene expression in the mouse uterus coincides with differentiation of the luminal epithelium for implantation. Endocrinology 138(11):4599-4606. doi: 10.1210/endo.138.11.5528

Lim, H., B. C. Paria, S. K. Das, J. E. Dinchuk, R. Langenbach, J. M. Trzaskos, and S. K. Dey. 1997. Multiple female reproductive failures in cyclooxygenase 2-deficient mice. Cell 91(2):197-208.

Machaty, Z., W. H. Wang, B. N. Day, and R. S. Prather. 1997. Complete activation of porcine oocytes induced by the sulfhydryl reagent, thimerosal. Biol Reprod 57(5):1123-1127. doi: 10.1095/biolreprod57.5.1123

Marcus, G. J. 1981. Prostaglandin formation by the sheep embryo and endometrium as an indication of maternal recognition of pregnancy. Biol Reprod 25(1):56-64. doi: 10.1095/biolreprod25.1.56

Marshburn, P. B., R. B. Shabanowitz, and M. R. Clark. 1990. Immunohistochemical localization of prostaglandin H synthase in the embryo and uterus of the mouse from ovulation through implantation. Mol Reprod Dev 25(4):309-316. doi: 10.1002/mrd.1080250402

Mathew, D. J., E. M. Newsom, J. M. Guyton, C. K. Tuggle, R. D. Geisert, and M. C. Lucy. 2015. Activation of the transcription factor nuclear factor-kappa B in uterine luminal epithelial cells by interleukin 1 Beta 2: a novel interleukin 1 expressed by the elongating pig conceptus. Biol Reprod 92(4):107. doi: 10.1095/biolreprod.114.126128

Mathew, D. J., E. M. Sellner, J. C. Green, C. S. Okamura, L. L. Anderson, M. C. Lucy, and R. D. Geisert. 2011. Uterine progesterone receptor expression, conceptus development, and ovarian function in pigs treated with RU 486 during early pregnancy. Biol Reprod 84(1):130-139. doi: 10.1095/biolreprod.110.086843

Mattson, B. A., E. W. Overstrom, and D. F. Albertini. 1990. Transitions in trophectoderm cellular shape and cytoskeletal organization in the elongating pig blastocyst. Biol Reprod 42(1):195-205. doi: 10.1095/biolreprod42.1.195

McCracken, J. A., J. C. Carlson, M. E. Glew, J. R. Goding, D. T. Baird, K. Green, and B. Samuelsson. 1972. Prostaglandin F 2 identified as a luteolytic hormone in sheep. Nat New Biol 238(83):129-134.

McDowell, K. J., D. C. Sharp, L. S. Peck, and L. L. Cheves. 1985. Effect of Restricted Conceptus Mobility on Maternal Recognition of Pregnancy in Mares. Equine Vet J 3:23-24.

Meyer, A. E., C. A. Pfeiffer, K. E. Brooks, L. D. Spate, J. A. Benne, R. Cecil, M. S. Samuel, C. N. Murphy, S. Behura, M. K. McLean, L. A. Ciernia, M. F. Smith, K. M. Whitworth, K. D. Wells, T. E. Spencer, R. S. Prather, and R. D. Geisert. 2019. New perspective on conceptus estrogens in maternal recognition and pregnancy establishment in the pigdagger. Biol Reprod doi: 10.1093/biolre/ioz058

Miyamoto, T., N. Ogino, S. Yamamoto, and O. Hayaishi. 1976. Purification of prostaglandin endoperoxide synthetase from bovine vesicular gland microsomes. J Biol Chem 251(9):2629-2636.

Modric, T., A. A. Kowalski, M. L. Green, R. C. Simmen, and F. A. Simmen. 2000. Pregnancy-dependent expression of leukaemia inhibitory factor (LIF), LIF receptor-beta and interleukin-6 (IL-6) messenger ribonucleic acids in the porcine female reproductive tract. Placenta 21(4):345-353. doi: 10.1053/plac.1999.0493

Moeljono, M. P., F. W. Bazer, and W. W. Thatcher. 1976. A study of prostaglandin F2alpha as the luteolysin in swine: I. Effect of prostaglandin F2alpha in hysterectomized gilts. Prostaglandins 11(4):737-743.

Moeljono, M. P., W. W. Thatcher, F. W. Bazer, M. Frank, L. J. Owens, and C. J. Wilcox. 1977. A study of prostaglandin F2alpha as the luteolysin in swine: II Characterization and comparison of prostaglandin F, estrogens and progestin concentrations in utero-ovarian vein plasma of nonpregnant and pregnant gilts. Prostaglandins 14(3):543-555.

Morgan, G. L., R. D. Geisert, M. T. Zavy, and A. T. Fazleabas. 1987. Development and survival of pig blastocysts after oestrogen administration on day 9 or days 9 and 10 of pregnancy. J Reprod Fertil 80(1):133-141.

Morham, S. G., R. Langenbach, C. D. Loftin, H. F. Tiano, N. Vouloumanos, J. C. Jennette, J. F. Mahler, K. D. Kluckman, A. Ledford, C. A. Lee, and O. Smithies. 1995. Prostaglandin synthase 2 gene disruption causes severe renal pathology in the mouse. Cell 83(3):473-482.

Niemann, H., and F. Elsaesser. 1986. Evidence for estrogen-dependent blastocyst formation in the pig. Biol Reprod 35(1):10-16. doi: 10.1095/biolreprod35.1.10

O'Neill, L. A., R. D. Geisert, M. T. Zavy, G. L. Morgan, and R. P. Wettemann. 1991. Effect of estrogen inhibitors on conceptus estrogen synthesis and development in the gilt. Domest Anim Endocrinol 8(1):139-153.

O'Neil, E. V., J. A. Benne, K. E. Brooks, G. Burns, and T. E. Spencer. 2017. Conceptus Derived Prostaglandin Synthase 2 (PTGS2) Regulates Embryonic Development in Sheep.

Oshima, M., J. E. Dinchuk, S. L. Kargman, H. Oshima, B. Hancock, E. Kwong, J. M. Trzaskos, J. F. Evans, and M. M. Taketo. 1996. Suppression of intestinal polyposis in Apc delta716 knockout mice by inhibition of cyclooxygenase 2 (COX-2). Cell 87(5):803-809.

Pakrasi, P. L., and S. K. Dey. 1982. Blastocyst is the source of prostaglandins in the implantation site in the rabbit. Prostaglandins 24(1):73-77.

Parhar, R. S., S. Yagel, and P. K. Lala. 1989. PGE2-mediated immunosuppression by first trimester human decidual cells blocks activation of maternal leukocytes in the decidua with potential anti-trophoblast activity. Cell Immunol 120(1):61-74.

Paria, B. C., W. Ma, D. M. Andrenyak, P. C. Schmid, H. H. Schmid, D. E. Moody, H. Deng, A. Makriyannis, and S. K. Dey. 1998. Effects of cannabinoids on preimplantation mouse embryo development and implantation are mediated by brain-type cannabinoid receptors. Biol Reprod 58(6):1490-1495. doi: 10.1095/biolreprod58.6.1490

Patten, B. M. 1948. Embryology of the Pig. 3 ed. The Blakiston Company, Philadelphia and Toronto.

Perry, J. S. 1981. The mammalian fetal membranes. J Reprod Fertil 62(2):321-335.

Perry, J. S., R. B. Heap, and E. C. Amoroso. 1973. Steroid hormone production by pig blastocysts. Nature 245(5419):45-47. doi: 10.1038/245045a0

Perry, J. S., R. B. Heap, R. D. Burton, and J. E. Gadsby. 1976. Endocrinology of the blastocyst and its role in the establishment of pregnancy. J Reprod Fertil Suppl (25):85-104.

Perry, J. S., and I. W. Rowlands. 1962. Pathways to pregnancy and parturition. J Reprod Fertil 4:175-188.

Pharriss, B. B., and L. J. Wyngarden. 1969. The effect of prostaglandin F 2alpha on the progestogen content of ovaries from pseudopregnant rats. Proc Soc Exp Biol Med 130(1):92-94. doi: 10.3181/00379727-130-33495

Pope, W. F. 1994. Embryonic Mortality in Swine. In: R. D. Geisert and M. T. Zavy, editors, Embryonic Mortality in Domestic Species. CRC Press. p. 236.

Pope, W. F., R. R. Maurer, and F. Stormshak. 1982. Intrauterine migration of the porcine embryo: influence of estradiol-17 beta and histamine. Biol Reprod 27(3):575-579. doi: 10.1095/biolreprod27.3.575

Reese, J., B. C. Paria, N. Brown, X. Zhao, J. D. Morrow, and S. K. Dey. 2000. Coordinated regulation of fetal and maternal prostaglandins directs successful birth and postnatal adaptation in the mouse. Proc Natl Acad Sci U S A 97(17):9759-9764. doi: 10.1073/pnas.97.17.9759

Ross, G. T. 1978. Human chorionic gonadotropin and maternal recognition of pregnancy. Ciba Found Symp (64):191-208.

Ross, J. W., M. D. Ashworth, D. Mathew, P. Reagan, J. W. Ritchey, K. Hayashi, T. E. Spencer, M. Lucy, and R. D. Geisert. 2010. Activation of the transcription factor, nuclear factor kappa-B, during the estrous cycle and early pregnancy in the pig. Reprod Biol Endocrinol 8:39. doi: 10.1186/1477-7827-8-39

Ross, J. W., J. R. Malayer, J. W. Ritchey, and R. D. Geisert. 2003. Characterization of the interleukin-1beta system during porcine trophoblastic elongation and early placental attachment. Biol Reprod 69(4):1251-1259. doi: 10.1095/biolreprod.103.015842

Rowson, L. E., R. Tervit, and A. Brand. 1972. The use of prostaglandins for synchronization of oestrus in cattle. J Reprod Fertil 29(1):145.

Sananes, N., E. E. Baulieu, and C. Le Goascogne. 1976. Prostaglandin(s) as inductive factor of decidualization in the rat uterus. Mol Cell Endocrinol 6(2):153-158.

Sen Roy, S., and P. B. Seshagiri. 2013. Expression and function of cyclooxygenase-2 is necessary for hamster blastocyst hatching. Mol Hum Reprod 19(12):838-851. doi: 10.1093/molehr/gat063

Senger, P. 2003. Pathways to Pregnancy and Parturition. 2 ed. Current Conceptions, Pullman, WA.

Seo, H., M. Kim, Y. Choi, and H. Ka. 2011. Salivary lipocalin is uniquely expressed in the uterine endometrial glands at the time of conceptus

implantation and induced by interleukin 1beta in pigs. Biol Reprod 84(2):279-287. doi: 10.1095/biolreprod.110.086934

Seo, H., M. Kim, Y. Choi, C. K. Lee, and H. Ka. 2008. Analysis of lysophosphatidic acid (LPA) receptor and LPA-induced endometrial prostaglandin-endoperoxide synthase 2 expression in the porcine uterus. Endocrinology 149(12):6166-6175. doi: 10.1210/en.2008-0354

Shemesh, M., F. Milaguir, N. Ayalon, and W. Hansel. 1979. Steroidogenesis and prostaglandin synthesis by cultured bovine blastocysts. J Reprod Fertil 56(1):181-185.

Short, R. V. 1969. Implantation and the Maternal Recognition of Pregnancy. In: G. E. W. Wolstenholme and M. O'Connor, editors, Foetal Autonomy. J. & A. Churchill LTD., London. p. 2-25.

Smith, W. L., and D. L. Dewitt. 1996. Prostaglandin endoperoxide H synthases-1 and -2. Adv Immunol 62:167-215.

Sorek, R., C. M. Lawrence, and B. Wiedenheft. 2013. CRISPR-mediated adaptive immune systems in bacteria and archaea. Annu Rev Biochem 82:237-266. doi: 10.1146/annurev-biochem-072911-172315

Spate, L. D., A. Brown, B. K. Redel, K. M. Whitworth, and R. S. Prather. 2015. PS48 can replace bovine serum albumin in pig embryo culture medium, and improve in vitro embryo development by phosphorylating AKT. Mol Reprod Dev 82(4):315-320. doi: 10.1002/mrd.22474

Spencer, T. E., and F. W. Bazer. 2004. Conceptus signals for establishment and maintenance of pregnancy. Reprod Biol Endocrinol 2:49. doi: 10.1186/1477-7827-2-49

Spencer, T. E., N. Forde, and P. Lonergan. 2016. Insights into conceptus elongation and establishment of pregnancy in ruminants. Reprod Fertil Dev 29(1):84-100. doi: 10.1071/RD16359

Steven, D. H., and G. M. Morriss. 1975. Development of the Foetal Membranes In: D. H. Steven, editor, Compararive Placentation. Essays in Structure and Function. Academic Press. p. 328.

Stout, T. A., and W. R. Allen. 2001. Role of prostaglandins in intrauterine migration of the equine conceptus. Reproduction 121(5):771-775.

Strakova, Z., P. Mavrogianis, X. Meng, J. M. Hastings, K. S. Jackson, P. Cameo, A. Brudney, O. Knight, and A. T. Fazleabas. 2005. In vivo infusion of interleukin-1beta and chorionic gonadotropin induces endometrial changes that mimic early pregnancy events in the baboon. Endocrinology 146(9):4097-4104. doi: 10.1210/en.2005-0380

Stroband, H. W., and T. Van der Lende. 1990. Embryonic and uterine development during early pregnancy in pigs. J Reprod Fertil Suppl 40:261-277.

Swindle, M. M., A. C. Smith, and B. J. Hepburn. 1988. Swine as models in experimental surgery. J Invest Surg 1(1):65-79.

Tarkowski, A. K., and J. Wroblewska. 1967. Development of blastomeres of mouse eggs isolated at the 4- and 8-cell stage. J Embryol Exp Morphol 18(1):155-180.

Thaon, P. 1907. Note sur la sécrétion de l'hypophyse et ses vaisseaux évacuateurs. CR Soc. Biol. 63:111.

Tobert, J. A. 1976. A study of the possible role of prostaglandins in decidualization using a nonsurgical method for the instillation of fluids into the rat uterine lumen. J Reprod Fertil 47(2):391-393.

Tuo, W., D. Zhu, and F. W. Bazer. 1996. Transfer of heterologous immunoglobulin into the uterine lumen of pigs. J Reprod Immunol 32(2):145-155.

Vallet, J. L., J. R. Miles, and B. A. Freking. 2009. Development of the pig placenta. Soc Reprod Fertil Suppl 66:265-279.

van der Weiden, R. M., L. J. Wisse, F. M. Helmerhorst, M. J. Keirse, and R. E. Poelmann. 1996. Immunohistochemical and ultrastructural localization of prostaglandin H synthase in the preimplantation mouse embryo. J Reprod Fertil 107(2):161-166.

Veldhuis, J. D. 1986. Mechanisms subserving the bipotential actions of estrogen on ovarian cells: studies with a selective anti-estrogen, LY156758, and the sparingly metabolizable estrogen agonist, moxestrol. J Steroid Biochem 24(5):977-982.

Viggiano, M., A. M. Franchi, A. Faletti, M. A. Gimeno, and A. L. Gimeno. 1988. Histamine alters prostaglandin output from diestrous rat uteri. Involvement of H2-receptors and 9-keto-reductase. Prostaglandins 36(3):317-328.

Von Euler, U. S. 1934. Zur Kenntnis der pharmakologischen Wirkungen von Nativsekreten und Extrakten männlicher accessorischer Geschlechtsdrüsen. Naunyn Schmiedebergs Arch Pharmacol 175(1):78-84.

Von Euler, U. S. 1935. A Depressor Substance in the Vesicular Gland. J Physiol 84(2):1-2.

Von Euler, U. S. 1936. On the Specific Vaso-Dilating and Plain Muscle Stimulating Substances from Accessory Genital Glands in Man and Certain Animals (Prostaglandin and Vesiglandin). J Physiol 88(2):213-234.

Waclawik, A., A. Rivero-Muller, A. Blitek, M. M. Kaczmarek, L. J. Brokken, K. Watanabe, N. A. Rahman, and A. J. Ziecik. 2006. Molecular cloning and spatiotemporal expression of prostaglandin F synthase and microsomal prostaglandin E synthase-1 in porcine endometrium. Endocrinology 147(1):210-221. doi: 10.1210/en.2005-0880

Waclawik, A., A. Blitek, M. M. Kaczmarek, J. Kiewisz, and A. J. Ziecik. 2009a. Antiluteolytic mechanisms and the establishment of pregnancy in the pig. Soc Reprod Fertil Suppl 66:307-320.

Waclawik, A., A. Blitek, and A. J. Ziecik. 2010. Oxytocin and tumor necrosis factor alpha stimulate expression of prostaglandin E2 synthase and secretion of prostaglandin E2 by luminal epithelial cells of the porcine endometrium during early pregnancy. Reproduction 140(4):613-622. doi: 10.1530/REP-10-0092

Waclawik, A., H. N. Jabbour, A. Blitek, and A. J. Ziecik. 2009b. Estradiol-17beta, prostaglandin E2 (PGE2), and the PGE2 receptor are involved in PGE2 positive feedback loop in the porcine endometrium. Endocrinology 150(8):3823-3832. doi: 10.1210/en.2008-1499

Waclawik, A., and A. J. Ziecik. 2007. Differential expression of prostaglandin (PG) synthesis enzymes in conceptus during peri-implantation period and endometrial expression of carbonyl reductase/PG 9-ketoreductase in the pig. J Endocrinol 194(3):499-510. doi: 10.1677/JOE-07-0155

Wang, J., M. Guillomot, and I. Hue. 2009. Cellular organization of the trophoblastic epithelium in elongating conceptuses of ruminants. C R Biol 332(11):986-997. doi: 10.1016/j.crvi.2009.09.004

Wang, Z. C., E. J. Yunis, M. J. De los Santos, L. Xiao, D. J. Anderson, and J. A. Hill. 2002. T helper 1-type immunity to trophoblast antigens in women with a history of recurrent pregnancy loss is associated with polymorphism of the IL1B promoter region. Genes Immun 3(1):38-42. doi: 10.1038/sj.gene.6363812

Watson, J., and C. E. Patek. 1979. Steroid and prostaglandin secretion by the corpus luteum, endometrium and embryos of cyclic and pregnant pigs. J Endocrinol 82(3):425-428.

Weber, J. A., D. A. Freeman, D. K. Vanderwall, and G. L. Woods. 1991a. Prostaglandin E2 hastens oviductal transport of equine embryos. Biol Reprod 45(4):544-546. doi: 10.1095/biolreprod45.4.544

Weber, J. A., D. A. Freeman, D. K. Vanderwall, and G. L. Woods. 1991b. Prostaglandin E2 secretion by oviductal transport-stage equine embryos. Biol Reprod 45(4):540-543. doi: 10.1095/biolreprod45.4.540

Weber, J. A., G. L. Woods, and A. B. Lichtenwalner. 1995. Relaxatory Effect of Prostaglandin E2 on Circular Smooth Muscle Isolated from the Equine Oviductal Isthmus Biol Reprod 52(1):125-130.

Wells, K. D., and R. S. Prather. 2017. Genome-editing technologies to improve research, reproduction, and production in pigs. Mol Reprod Dev 84(9):1012-1017. doi: 10.1002/mrd.22812

Whitworth, K. M., K. Lee, J. A. Benne, B. P. Beaton, L. D. Spate, S. L. Murphy, M. S. Samuel, J. Mao, C. O'Gorman, E. M. Walters, C. N. Murphy, J. Driver, A. Mileham, D. McLaren, K. D. Wells, and R. S. Prather. 2014. Use of the CRISPR/Cas9 system to produce genetically engineered pigs from in vitro-derived oocytes and embryos. Biol Reprod 91(3):78. doi: 10.1095/biolreprod.114.121723

Whitworth, K. M., J. Mao, K. Lee, W. G. Spollen, M. S. Samuel, E. M. Walters, L. D. Spate, and R. S. Prather. 2015. Transcriptome Analysis of Pig In Vivo, In Vitro-Fertilized, and Nuclear Transfer Blastocyst-Stage Embryos Treated with Histone Deacetylase Inhibitors Postfusion and Activation Reveals Changes in the Lysosomal Pathway. Cell Reprogram 17(4):243-258. doi: 10.1089/cell.2015.0022

Whyte, J. J., A. E. Meyer, L. D. Spate, J. A. Benne, R. Cecil, M. S. Samuel, C. N. Murphy, R. S. Prather, and R. D. Geisert. 2018. Inactivation of porcine interleukin-1beta results in failure of rapid conceptus elongation. Proc Natl Acad Sci U S A 115(2):307-312. doi: 10.1073/pnas.1718004115

Wilson, M. E., S. C. Fahrenkrug, T. P. Smith, G. A. Rohrer, and S. P. Ford. 2002. Differential expression of cyclooxygenase-2 around the time of elongation in the pig conceptus. Anim Reprod Sci 71(3-4):229-237.

Wiqvist, N., and M. Bygdeman. 1970. Induction of therapeutic abortion with intravenous prostaglandin F. Lancet 1(7652):889. doi: 10.1016/s0140-6736(70)91710-1

Xie, W., D. Robertson, and D. Simmons. 1992. Mitogen-Inducible Prostaglandin G/H Synthase: A New Target for Nonsteroidal Antiinflammatory Drugs. Drug Dev Res 25(4):249-265.

Xie, W. L., J. G. Chipman, D. L. Robertson, R. L. Erikson, and D. L. Simmons. 1991. Expression of a mitogen-responsive gene encoding prostaglandin

synthase is regulated by mRNA splicing. Proc Natl Acad Sci U S A 88(7):2692-2696. doi: 10.1073/pnas.88.7.2692

Yang, Z. M., S. K. Das, J. Wang, Y. Sugimoto, A. Ichikawa, and S. K. Dey. 1997. Potential sites of prostaglandin actions in the periimplantation mouse uterus: differential expression and regulation of prostaglandin receptor genes. Biol Reprod 56(2):368-379. doi: 10.1095/biolreprod56.2.368

Yu, Z., J. R. Gordon, J. Kendall, and P. A. Thacker. 1998. Elevation in tumour necrosis factor-alpha (TNF-alpha) messenger RNA levels in the uterus of pregnant gilts after oestrogen treatment. Anim Reprod Sci 50(1-2):57-67.

Zavy, M. T., F. W. Bazer, W. W. Thatcher, and C. J. Wilcox. 1980. A study of prostaglandin F2 alpha as the luteolysin in swine: V. Comparison of prostaglandin F, progestins, estrone and estradiol in uterine flushings from pregnant and nonpregnant gilts. Prostaglandins 20(5):837-851.

Zheng, H., D. Fletcher, W. Kozak, M. Jiang, K. J. Hofmann, C. A. Conn, D. Soszynski, C. Grabiec, M. E. Trumbauer, A. Shaw, and et al. 1995. Resistance to fever induction and impaired acute-phase response in interleukin-1 beta-deficient mice. Immunity 3(1):9-19.

VITA

Caroline Anne Pfeiffer was born in Oak Lawn, Illinois to Dan and Laura Pfeiffer on August 30, 1995. She was raised in Lockport, Illinois and resided there with her parents and sisters, Anna and Kathryn, until attending university. From a young age, she was always interested in animal health, behavior, and anatomy. She would spend countless hours watching animal planet, attending routine visits to the veterinary clinic with her pets, and observing animals in nature. Caroline's curiosity and love for animals drove her to complete a Bachelor of Science degree in Animal Science at the University of Missouri. As an undergrad at University of Missouri, she worked as a researcher in Dr. Randall Prather's lab learning about porcine *in vitro* fertilization and embryo culture. Shortly after graduating in 2017, she started her work towards a Master of Science degree with Dr. Rodney Geisert in the Division of Animal Science of the College of Agriculture, Food, and Natural Resources at the University of Missouri. Her research was conducted at the Animal Science Research Center at Mizzou with the help of Drs. Prather and Spencer. This thesis is the result of that research program and was defended in July of 2019.