DEFECTS IN PERIIMPLANTATION SIGNALING CONTRIBUTE TO ADVERSE PREGNANCY OUTCOMES IN THE BPH/5 MOUSE MODEL OF PREECLAMPSIA

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DEFECTS IN PERIIMPLANTATION SIGNALING CONTRIBUTE TO ADVERSE PREGNANCY OUTCOMES IN THE BPH/5 MOUSE MODEL OF PREECLAMPSIA

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Preeclampsia (PE) is a devastating disorder of pregnancy that affects 10% of pregnancies worldwide. While it is a leading cause of perinatal morbidity and mortality, the cause of PE is unknown. To date the only definitive treatment is delivery of the placenta and the baby, which is often preterm and/or growth restricted. The origins of PE are generally regarded to be early in pregnancy at the time of placenta formation as placental developmental abnormalities often characterize PE pregnancies.

In these thesis studies, early pregnancy events required for proper placentation, implantation and decidualization, were investigated in the BPH/5 spontaneous mouse model of PE. We discovered profound developmental defects in implantation, decidualization, and placentation in BPH/5 mice. Along with evidence of decidual hypoxia, these defects were associated with a distinct molecular signature of aberrant embryo-uterine interactions. We identified significant dysregulation in the periimplantation period, particularly in the overexpression of cyclooxygenase 2 (Cox2) in the maternal uterine environment. Using pharmacological intervention at early pregnancy with a selective Cox2 inhibitor, we confirmed that defects in Cox2 signaling during the periimplantation period have “ripple effects” impacting downstream adverse pregnancy outcomes at mid and late gestation in BPH/5 mice, including fetal growth restriction and demise.

Finally, investigations in the periimplantation period lead us to discover deficiencies in immunoregulatory pathways crucial for placental development at the maternal-fetal interface in BPH/5 mice. We showed dramatic loss of decidual Natural Killer (dNK) cells and this was associated with overexpression of IL-15 in BPH/5 implantation sites. Furthermore, we linked

Cox2 inhibition with a reduction in IL-15. Altogether, this points towards inflammation in the maternal uterine environment during early pregnancy having a central role in the poor fetoplacental and pregnancy outcomes associated with this model and highlights Cox2 inhibitors as potential therapeutics to prevent fetal morbidity/mortality associated with PE. Importantly, our data supports the “ripple effect” hypothesis that defects in early pregnancy events are the source of downstream adverse pregnancy outcomes using the BPH/5 mouse model of PE.

BIOGRAPHICAL SKETCH

Jenny Liford Sones was born and raised in Baton Rouge, Louisiana in 1982. She is a proud alumnus of Louisiana State University receiving her Bachelor of Science and Doctor of Veterinary Medicine degrees there in 2004 and 2008, respectively. While in vet school she met her husband, Ryan Sones. She began her research career as a Howard Hughes undergraduate scholar while studying biology at Louisiana State University. Once in veterinary school she developed her love of the placenta and was awarded funding as a Merck Merial veterinary scholar to investigate placental inflammation in an equine model of ascending placentitis. This led to a Havemeyer fellowship with Dr. Douglas F. Antczak at Cornell University's Baker Institute in 2006. There she refined her appreciation for placental research and focused on investigating the molecular mechanisms that govern implantation in the horse. After graduating from veterinary school, she spent a year as an associate veterinarian in private practice before returning to academia as a post-DVM NIH research fellow in equine reproductive immunology at Cornell University again with Dr. Antczak. After embarking on a PhD in 2010 in the Department of Biomedical Sciences at Cornell University, she was introduced to the mouse placenta by Dr. Mark Roberson and gained a new appreciation for translational science. Jenny had the distinct privilege of joining Dr. Robin Davisson's laboratory in 2011 to complete her thesis research exploring placental defects in a mouse model of preeclampsia. During Jenny's PhD training she also gave birth to 2 beautiful children.

This dissertation is dedicated to my children, Sage and Hunter. We took this journey together and I couldn't have done it without your love, patience and inspiration.

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Finally, I must thank my husband, Ryan Sones. He has been my rock for the past 10 years, seeing me through vet school and now this PhD. His love, support, patience, and strength of character never cease to amaze me. Thank you for all that you do.

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LIST OF ABBREVIATIONS

BP	Blood pressure
Bmp2	Bone morphogenic protein 2
Cox2	Cyclooxygenase 2
dNK	decidual Natural Killer
E_2	Estrogen
FGR	Fetal Growth Restriction
GCM1	Glial Cells Missing homolog 1
HIF	Hypoxia Inducible Factor
Hoxa10	Homeobox a 10
IL	Interleukin
LIF	Leukemia Inhibitory Factor
MMP	Matrix metalloproteinase
P4	Progesterone
PG	Prostaglandin
SGA	Small-for-gestational age
Syn	Syncytin
VEGF	Vascular Endothelial Growth Factor

CHAPTER ONE:

INTRODUCTION

1.1 Preeclampsia

Preeclampsia (PE) is a devastating disorder of pregnancy that affects up to 8% of pregnant women in the United States[1]. The diagnosis of PE is made by the presentation of hypertension, ≥140mmHg systolic blood pressure (BP) or ≥90mmHG diastolic BP, and the presence of proteinuria, ≥300mg in 24 hours or 2+ on urine dipstick, after 20 weeks of gestation[2, 3]. These signs can occur suddenly and without warning. PE that presents before 34 weeks of gestation is considered early-onset and carries a greater risk for perinatal morbidity/mortality than late-onset PE that occurs at or after 34 weeks of gestation[2, 3]. Depending on the severity, PE can be accompanied by the presence of the HELLP (Hemolysis, Elevated Liver enzymes, Low Platelets) syndrome, indicative of multi-organ dysfunction[4]. At this time there is no cure for PE and the only definitive treatment is delivery of the baby and placenta. If allowed to progress to eclampsia (PE with neurologic involvement), death is inevitable. PE also carries the risk of significant fetal and neonatal morbidity/mortality in addition to long term health risks for mother and child[4]. Despite significant research efforts to accurately predict, diagnose, and treat PE, a cure eludes us. Elucidating the pathophysiological mechanisms that cause PE will aid in our ability to accurately prevent, mange, and treat PE in order to avoid maternal and fetal losses.

1.1.1 Epidemiology of PE

While PE characterizes approximately 300,000 pregnancies per year in the United States[1], it affects up to 10% of pregnancies worldwide[3]. Shockingly, PE is also responsible for 42% of maternal deaths worldwide killing an estimated 76,000 women every year[5, 6]. There appears to be a disparity in the incidence of maternal death associated with PE in developing countries with 99% occurring in low- to middle-income nations[1]. Although genetic susceptibility cannot be ruled out, this discrepancy may simply be due to differences in perinatal care available in those countries. Even though perinatal care in developed countries is constantly improving,

the incidence of PE is rising. From 1987 to 2004, the prevalence of PE diagnosis from admission to labor increased by 25% according to a United States National Hospital Discharge Survey[2].

In an effort to positively predict which women may develop PE during pregnancy, a number of conditions have been labeled as risk factors. Pre-existing maternal conditions include advanced age, African-American race, higher body mass index (BMI), pre-gestational diabetes, chronic hypertension, renal disease, autoantibody disease (ie antiphospholipid antibody syndrome and systemic lupus erythematosus), family or personal history of PE, and lack of smoking[2]. Pregnancy-specific factors include nulliparity, partner related factors (ie new paternity, limited sperm exposure, barrier contraception), multifetal gestation, and hydatidiform mole[2]. When considering all of these pre-existing conditions and risk factors, the increasing prevalence of PE could be attributed to the number of shifting trends in the United States and other countries. Women are looking to become mothers later in life and this often necessitates fertility treatments that result in multifetal gestations. Also, with the increased incidence of obesity worldwide, we see more women of childbearing age that have higher BMIs, diabetes, and hypertension as well as subfertility[2]. However, the fact that an estimated two-thirds of all PE cases occur in first pregnancies that develop beyond the first trimester is staggering[7]. This data strongly points toward defects in maternal adaptions to pregnancy that may be associated with first exposure of paternal antigens. The most surprising risk factor is the lack of smoking. This has been attributed to its effect on angiogenic factors[8], however, smoking before or during pregnancy would never be advised.

PE is widely regarded as having a familial disposition[9]. For example, the frequency of PE in daughters is reported to be 2-5 times greater than in daughters-in law[10]. Studies to identify a simple acting dominant gene have been unrewarding due in part to the heterogeneity of disease presentation with PE having early or late onset and symptoms ranging from mild to moderate to severe[3]. Thus PE is considered a complex trait with inputs from environmental factors as well as fetal (paternal) genes[9, 10]. Genome-wide searches are underway and again variations are seen

among and between populations[10]. Determining the genetic mechanisms of PE have been complicated and frustrating.

Importantly, there is evidence that PE predisposes women to further health risks later in life. In a Scandinavian population of healthy nulliparious women with severe PE that necessitated preterm delivery, the risk of developing cardiovascular disease increased eight-fold[11]. A 24- to 36-year follow-up of maternal health status in PE affected women in Jerusalem reported a two-fold increase in mortality rate from cardiovascular related causes[2, 4]. Hypertension, dyslipidemia, insulin resistance, endothelial dysfunction, and vascular impairment have all been diagnosed in women with a history of PE as early as months, and even years, after pregnancy[12]. It is unclear whether PE is the primary insult in these otherwise healthy women or if PE exacerbated an underlying pathology. Regardless of the trigger for subsequent cardiovascular disease, these women have to be monitored closely for the rest of their lives.

1.1.2 Consequences of PE on offspring

While PE presents with the signs of maternal hypertension and proteinuria, there are significant consequences on the offspring from PE pregnancies including perinatal, neonatal and infant morbidity/mortality[13]. The only definitive means to halt the progression of PE is delivery of the placenta and baby, which is often preterm. In fact PE is responsible for 15-20% of preterm births per year[2]. Prematurity often leads to neonatal or infant morbidity and mortality as the baby is not yet developmentally competent to live outside the uterus. One of the most serious outcomes of preterm birth is respiratory complications[13]. PE is also one of the leading causes of fetal growth restriction (FGR) with approximately 12-25% of growth restricted and small-for-gestational age (SGA) babies attributed to PE[2]. Fetal growth is a useful marker for *in utero* fetal well-being and when intrauterine FGR is present the risk of perinatal mortality rises[13]. Birth weight that is below the tenth percentile, as is the case with SGA babies, has been linked to increased neonatal and infant mortality[13]. Sadly, PE is also a risk factor for fetal demise and stillbirth at an estimated rate of 21 per 1000[13]. In cases of PE where the offspring are born and

survive infancy, children still have an increased risk of stroke, coronary heart disease, and metabolic syndrome in adult life[14]. One phenotypic modification that has been associated with FGR is reduced nephron number, which has been linked to development of hypertension later in life[15].

Thus, PE itself represents a significant risk factor for the development of cardiovascular disease in mothers affected as well as the babies born to them. There is a strong genetic component as females born to PE affected mothers have a greater chance of developing PE during their own pregnancies[13]. The epidemic-like nature of PE and its associated diseases warrants intense research efforts into the pathophysiology of this disorder and how it exerts its long-term effects.

1.1.3 Historical perspectives on PE & eclampsia

Interestingly, the maternal signs of PE/eclampsia have been observed and described for centuries. In ancient times, Hippocrates recorded the concept of eclampsia as "headache accompanied by heaviness and convulsions during pregnancy"[13]. Unfortunately any advances in understanding PE/eclampsia were halted due to the rise of Christianity and opposition to scientific and medical discovery during the Middle Ages[16]. Therefore PE/eclampsia, was not classified as a disorder of pregnancy until the Renaissance period[13]. During this time anatomists and artists accurately described and depicted the female reproductive tract. It is Fallopius that is credited with naming the ovaries, uterine tubes (Fallopian tubes) and the placenta[16]. In the 17th century, men such as Francois Mauriceau entered the field of obstetrics and established it as a specialty[16]. It is Francois Mauriceau that is credited with first systemically describing eclampsia and that primigravidas are at a greater risk for convulsions than multigravidas. By the end of the Renaissance Period, convulsions of pregnancy were labelled more uniquely different than epilepsy from other causes (ie the head, stomach and chilled extremities) and the word "eclampsia" first appeared in Varandaeus' treatise on gynecology[16]. Nineteenth century physicians such as Demanet, John Lever, Robert Johns, Vaquez and Nobecourt, made

observations of the premonitory symptoms of the eclamptic convulsions: edema, proteinuria, headaches, and hypertension, respectively[16]. Finally the concept of PE was widely recognized. The "preeclamptic" state began appearing in textbooks in 1903[16]. Over the last 50 years, PE has been placed in several different categories of pregnancy disorders. First, it was considered a toxemia of pregnancy and was often referred to as "pregnancy toxemia"[16]. After that it was categorized as a hypertensive disorder of pregnancy and its placement in this category was later refined as pregnancy-induced hypertension and specific criteria was defined to distinguish mild-moderate from severe PE[16]. Although there are some discrepancies in the field regarding the criteria, any evidence of hypertension during pregnancy is treated as emergent. In this regard, there is a need for uniformity of diagnostic criteria and early biomarkers of PE are essential to manage these women with the goal of halting the maternal hypertension as well as preventing the need for preterm delivery.

1.2 PE, a disease of theories

Given the longevity of PE in the medical literature and the mortality associated with it, PE has received significant attention as understanding its pathogenesis is crucial. The signs of PE resolve after delivery and thus pathologies with the placenta may hold the key to unraveling this complex syndrome (Figure 1.1). Placenta from cases of severe PE where the baby was delivered preterm are small, whereas placental weights from late gestation PE are variable[18]. However, certain placental microscopic pathologies are hallmarks of PE[18]. Several theories exist to describe the possible link between placental disease and development of the maternal syndrome.

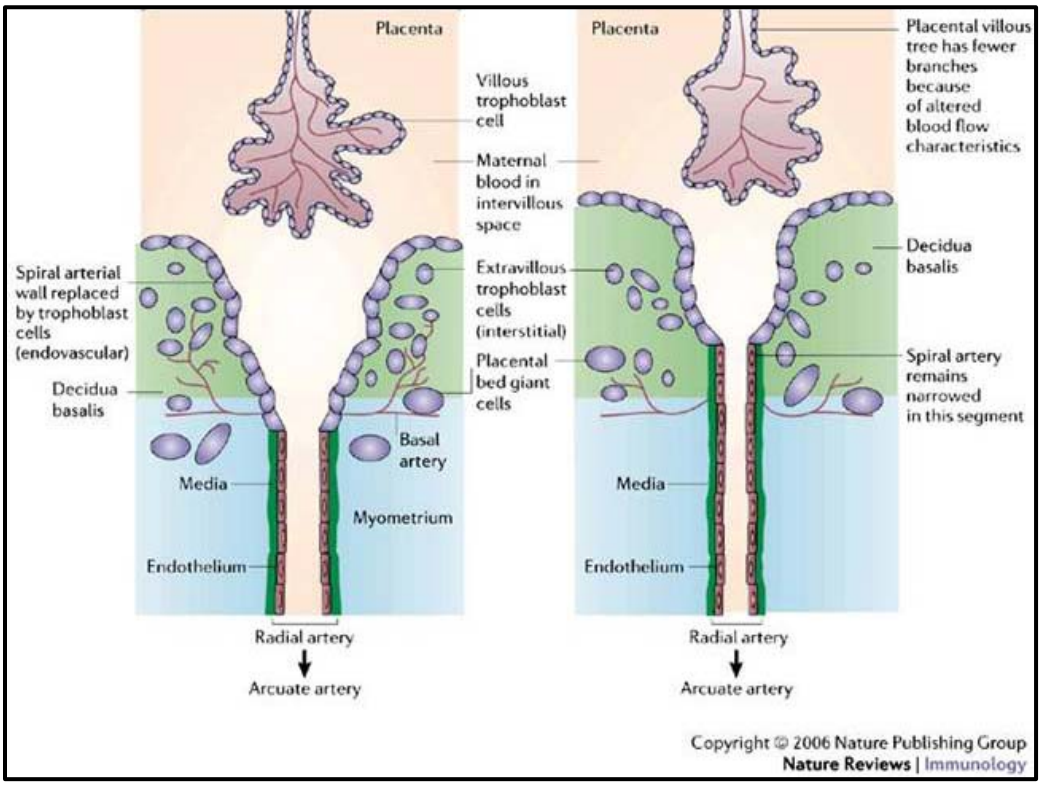


Figure 1.1 Schematic depicting the blood supply to the human placenta (chorionic villus). The left panel depicts adequate blood flow to the placenta with dilated decidual vessels supplying the branched villi. The right panel depicts uteroplacental blood flow during PE and FGR pregnancies where trophoblast invasion has not properly remodeled the decidual vessels and blood flow is diminished resulting in underdeveloped villi and poor fetal growth[17].

1.2.1 A two-stage disease model

A two-stage disease model has been proposed to describe the pathogenesis of PE[18]. The first stage involves abnormal placentation characterized by poor trophoblast invasion, incomplete vascular remodeling, and placental hypoxia. The second stage manifests as the maternal syndrome of hypertension and proteinuria with systemic endothelial dysfunction. The transition between the two stages is thought to be due to the release of factors from the abnormally developed placenta into the maternal circulation[19]. Recent evidence suggests an imbalance of pro- and anti-angiogenic factors in the pathophysiology of PE[20]; however, the cause and onset of these mechanisms are unknown.

1.2.2 Angiogenic factors in PE

In early pregnancy, trophoblast cells plug arterioles to protect the conceptus from excessively high oxygen levels and thus placenta formation begins in a hypoxic state[21]. The redox sensitive transcription factor, hypoxia inducible factor (HIF) 1α, plays a significant role in this process[22]. Once the intra-arterial plugs are removed, oxygenated blood perfuses the newly formed placenta. Prolonged hypoxia can have damaging effects, including induction of apoptosis, oxidative stress, and expression of anti-angiogenic factors and this is hypothesized to occur in PE[23]. In fact, placental explants subjected to hypoxic conditions secrete the anti-angiogenic factor, soluble fms-like tyrosine kinase 1 (sFlt)[22, 24, 25]. Some PE patients show increased levels of circulating sFlt early in pregnancy and this is thought to induce a state of angiogenic imbalance by binding to the pro-angiogenic factors, vascular endothelial growth factor (VEGF) and placental growth factor (PGF)[26]. Increased sFlt is not observed in all PE patients; therefore studies are ongoing to determine the origins of increased anti-angiogenic factors in PE and the apparent lack of pro-angiogenic factors. Inadequate uterine angiogenesis/vascularity at the time of implantation has been suggested[27].

1.2.3 Prostaglandins and PE

The precise signaling of implantation molecules before, during, and after the window of implantation is critical in establishing vascularization at the maternal-fetal interface during pregnancy[27]. Increased uterine vascular permeability and angiogenesis at the site of blastocyst attachment are the two hallmarks of implantation[28]. It is believed that estrogen (E_2) promotes uterine vascular permeability, while progesterone (P4) stimulates angiogenesis[28]. However, the *in vivo* roles of E_2 and P4 during implantation are not fully understood, but it is widely accepted that cyclooxygenase 2 (COX2)-derived prostanoids are important in uterine angiogenesis [28]; [29]. Prostacyclin (PGI_2) has vasodilator effects and is thought to have counter regulatory effects on thromboxane (TX) A_2, a vasoconstrictor, in order to maintain physiologic balance. It has been proposed that a PGI_2 deficiency or PGI_2/TX imbalance during pregnancy may be associated with PE[30]. However, the etiology of prostanoids and their effects during PE pregnancies is unclear.

Non-specific COX inhibition with low dose aspirin has been used to prevent PE for many years[30]. Early initiation of treatment with aspirin between 12-14 weeks of gestation has been shown to significantly decrease PE in high-risk women as compared with patients not receiving treatment and in women receiving aspirin later at 18 weeks of gestation. Recently, a consensus has been published in support of low-dose aspirin given prophylactically to high-risk women early in pregnancy to reduce the risk of PE and also FGR[31].

1.3 The placenta

As a disorder of pregnancy, the origins of PE are firmly rooted in the gestating baby. Because signs only resolve upon delivery of the fetus and the placenta, it is widely accepted that the developing fetoplacental unit plays a causal role in the pathogenesis of PE[9]. The importance of the placenta is further confirmed by the observation of PE-like signs during molar pregnancies where trophoblast cells are present, but not fetal tissue[32]. Placental studies in humans are limited to tissues collected by elective termination in the first trimester and those collected at delivery,

which is often late in gestation. Therefore, it is challenging to asses early placental defects that may lead to the development of the PE syndrome in women.

1.3.1 Human placental development

The placenta is an organ of pregnancy that is essential for the maintenance of gestation and fetal survival. It is responsible for maternal-fetal exchanges of nutrients and waste as well as maternal tolerance of the semi-allogeneic fetus that is composed of maternal and paternal antigens[17]. Defects in placental development can lead to adverse pregnancy outcomes that impact fetal and/or maternal health and survival.

Human placental development begins with implantation of the embryo into the uterus, which occurs approximately 7-10 days after the luteinizing hormone (LH) peak. By day 21 after ovulation (5 weeks gestation), the definitive structural and functional unit of the placenta, the chorionic villus, is apparent[21]. After blastocyst attachment trophoblast cells undergo rapid proliferation and differentiation into villous and extravillous subsets. The villous cytotrophoblasts will undergo further differentiation into multinucleated syncytiotrophoblast cells that are primarily involved in maternal-fetal exchanges, but also have specific endocrine functions[21]. The extravillous cytotrophoblasts are invasive and have two routes whereby they invade maternal tissues. First, interstitial invasion of the uterine stroma involves migration of the trophoblastic cells towards the decidual vessels and myometrium[21]. Next, endovascular invasion occurs with infiltration of spiral arterial lumens and walls by extravillous trophoblast cells that replace smooth muscle and endothelial cells[21]. This process results in the transformation of high-resistance, low-capacitance vessels into dilated conduits for establishing uteroplacental perfusion and bathing the chorionic villi in maternal blood. Intra-arterial plugs composed of trophoblast cells prevent this blood flow until the 12th week of gestation to protect the conceptus from excessively high oxygen levels during this critical phase of gestation[21]. By the end of the first trimester, the fetoplacental unit is formed with a functioning circulatory system.

1.3.2 Comparative placentation

All eutherian mammals form placenta(e) during pregnancy to nourish and support their growing offspring. Placental mammals are a diverse group that includes nearly 4000 species (ucmp.berkeley.edu). Classification of different placental types between species is often made on the macroscopic shape and the intimacy of maternal-fetal contact. The placenta of humans, primates, rodents (rat, mouse, rabbit, guinea pig, and beaver), and bats have a discoid shape (placentation.ucsd.edu). Carnivores (dogs, cats, seals, and bears) and elephants have a zonary placenta. While ruminants have a cotyledonary, and horses and pigs have a diffusely shaped placenta. Further distinction in the types of placentation between species is based on the number of tissue layers separating maternal and fetal blood. Epitheliochorial placentation occurs when trophoblast cells are opposed to uterine epithelium, endotheliochorial places trophoblast cells adjacent to the maternal endothelium, and hemochorial has trophoblast cells in direct contact with maternal blood[33]. Horses, swine, and ruminants have an epitheliochorial placenta and thus have the most separation between maternal and fetal blood. Dogs and cats have an endotheliochorial placenta and humans, primates, and rodents share a hemochorial type of placentation. By sharing the same shape and structure of placentation as humans, primates and rodents stand out as candidates for animal models of pregnancy research where the placenta is of interest. Non-human primates (Old World monkeys) have similar trophoblast invasion in to the spiral arteries, while rodents, especially mice, have slightly less aggressive remodeling of maternal vessels[34]. However, primate research carries significant ethical concerns as well as logistical limitations with the expense of maintaining primate colonies. Of the rodents, the guinea pig has been proposed as an excellent candidate for studying PE and FGR[34]. They have similar depth of trophoblast invasion into the spiral arteries as in people and they have been reported to develop pregnancy toxemia[34]. As they are larger in size than mice, there is a significant cost associated with investigating their gestations. Thus, the mouse with its similarities to human placentation and cost effectiveness as a laboratory animal, has many advantages as a model of pregnancy-related disorders.

1.3.3 Mouse placental development

Similar to humans, the development of the placenta in mice begins with implantation on embryonic day (e) 3.5. After the trophectoderm layer of the blastocyst comes in contact with the uterus, mural trophectoderm begins to differentiate into the invasive trophoblast giant cells (TGCs) that are analogous to the extravillous trophoblast cells in humans[35]. Polar trophectoderm gives rise to the extraembryonic ectoderm and the ectoplacental cone. The extraembryonic ectoderm further differentiates into the chorion layer before developing into the labyrinth region of the placenta. Spongiotrophoblast cells of the ectoplacental cone provide structural support to the labyrinth and there they provide the boundary between the labyrinth and the TGC layer, ie the junctional zone[35]. The human counter part of the mouse spongiotrophoblast cells are the column cytotrophoblast cells (Figure 1.2). Another subset of mouse trophoblastic cells, the glycogen trophoblast cells, later differentiates from the junctional zone to invade the uterine stroma. The allantois, extraembryonic mesoderm derived from the epiblast, joins the chorion at approximately e8.5 during chorioallantoic attachment[35]. Defects in chorioallantoic attachment are among the most common causes of midgestation embryonic lethality[35]. At approximately e9.0, primary villi form from the chorionic surface by branching morphogenesis to expand the labyrinth and fill the villous folds with blood vessels. Fusion of chorionic trophoblast cells of the labyrinth gives rise to the multinucleated syncytiotrophoblast cells, Type I and II, which line the fetal endothelium. Expansion of the labyrinth is ongoing during the second half of gestation until birth (e19.5)[35]. Defective patterning or branching of the labyrinth with inadequate vascularization leads to impaired placental perfusion and hypoxia. This is thought to be central in the pathogenesis of PE as placental biopsies from PE patients after delivery reveals evidence of hypoperfusion[18].

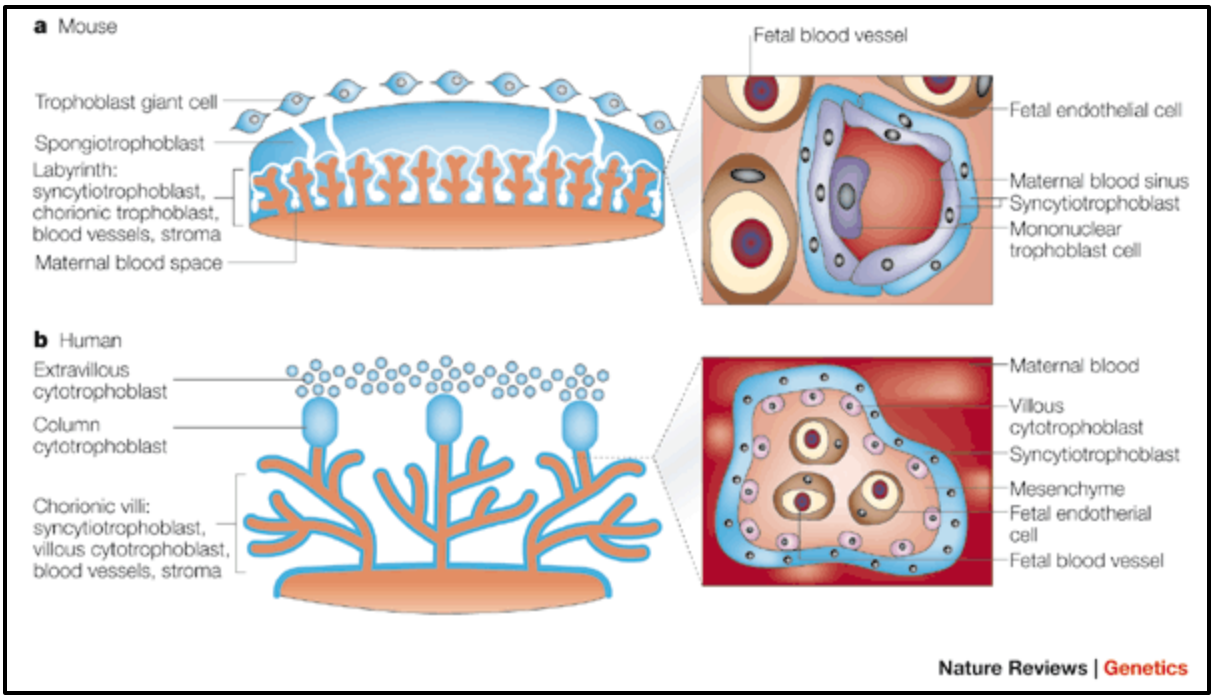


Figure 1.2 Schematic depicting the similarities in structure between the mouse and human placenta. (a) The trophoblast subtypes of the mouse placenta (trophoblast giant cells, spongiotrophoblast cells, and the trophoblast cells of the labyrinth region) are detailed on the left panel and the inset on the right details the two syncytiotrophoblast cell layers that separate maternal and fetal blood. **(b)** The trophoblast subtypes of the human placenta (extravillous cytotrophoblasts cells, column cytotrophoblasts cells, and the trophoblast cells of the chorionic villi) are detailed on the left panel and the inset on the right details the single syncytiotrophoblast cell layer that separates maternal and fetal blood[36].

1.4 Pre-placental events: implantation, decidualization, and the maternal-fetal interface

Although PE presents as maternal hypertension and proteinuria, it is a placental disease[18]. Implantation is a critical time point in pregnancy prior to placental development and marks the first encounter between the embryo and uterus[37]. The uterus must undergo decidualization for subsequent placenta formation. Proper embryo-uterine crosstalk during implantation and decidualization are critical for setting up the maternal-fetal interface during pregnancy[37-39].

1.4.1 The window of implantation

In rodents and humans, implantation is the first coordinated encounter between mother and baby[37]. Implantation success relies on the synchronization of uterine receptivity and embryonic development[37-39]. The uterine environment for blastocyst implantation is classified into 3 phases: pre-receptive, receptive and non-receptive[40]. Uterine receptivity is primarily directed by the ovarian steroid hormones E_2 and progesterone (P4)[41]. Under the influence of rising P4 and a very brief E_2 surge, implantation is initiated at e3.5 in mice. This time of uterine receptivity is short and thus has been termed the window of implantation. By the morning of e5.5, the window of implantation is closed and blastocysts must have attached and invaded the uterus in order to survive. Upon implantation, beginning on the morning of e4.5, uterine stromal cells surrounding the attached blastocyst proliferate extensively and differentiate into decidual cells through a process called decidualization[37-39]. Decidualization is a unique and transient process in the pregnant uterus that directs placental formation and appropriately regulates trophoblast invasion, modulates local vascular and immune responses, and confers resistance to environmental and oxidative stresses[37,42,43]. This transitory process involves angiogenic remodeling of the uterine environment which is preceded by increased uterine vascular permeability[37]. Critical to these periimplantation processes are vasoactive agents, such as leukotrienes, histamines, and prostaglandins (PGs), along with other signaling molecules, including cytokines, growth factors, transcription factors, and morphogens that act as autocrine, paracrine, and juxtacrine factors[38, 39].

1.4.2 The "ripple effect" hypothesis

The key events of implantation encompassing uterine receptivity, blastocyst attachment, and decidualization require precise cross-talk between the mother and embryo (Figure 1.3). It is hypothesized that dysregulation at any point in these events can terminate pregnancy or create adverse "ripple effects" throughout gestation. Failure of implantation and decidualization has been linked to infertility[39]. Defects in the timing of implantation or of decidualization may lead to abnormal placentation and retarded fetal development[39, 44-46]. Importantly, these pathologies characterize the PE phenotype. Placental sampling at 10-12 weeks of gestation revealed profound decidual gene dysregulation in women that went on to develop PE[47]. Furthermore, significant pregnancy loss has been shown to occur when implantation falls just 24 hours outside the optimal window in people[46]. It is hypothesized that defects in the periimplantation period play a key role in the pathogenesis of poor fetoplacental development and the maternal syndrome in PE[39]; however this has been difficult to address directly in people.

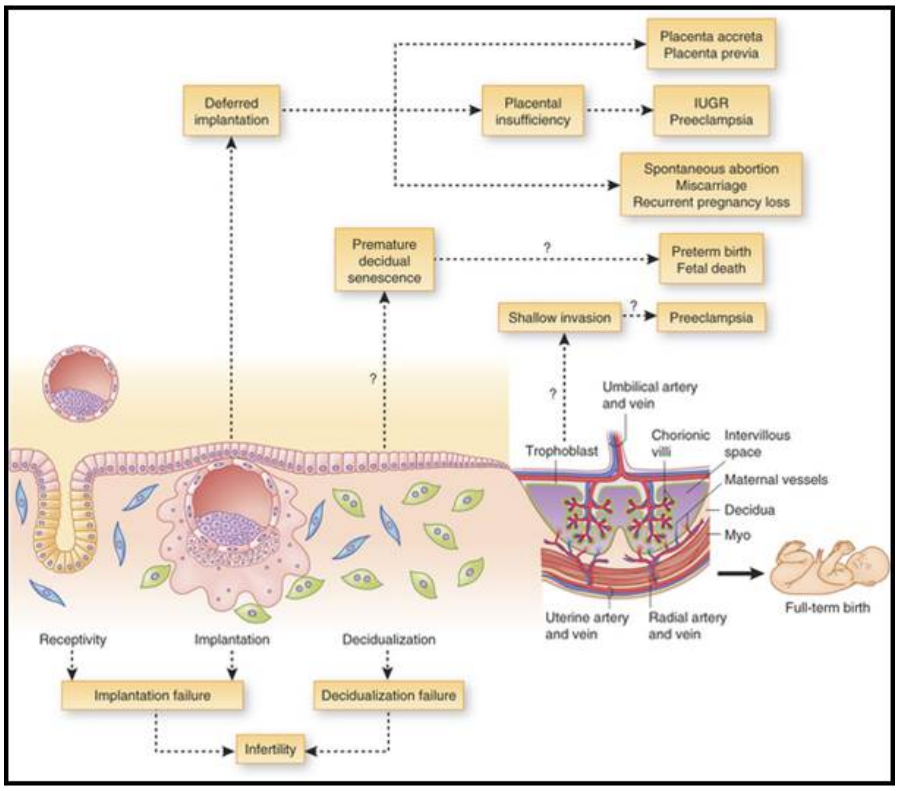


Figure 1.3 Schematic illustrating embryo-uterine interactions and downstream pregnancy outcomes. Coordinated embryonic development with uterine receptivity is required for implantation and decidualization. Aberrations in these processes are believed to impact pregnancy success and contribute to the above pregnancy pathologies, including placental insufficiency, PE and fetal loss[39].

1.4.3 Immunoregulatory events at the maternal-fetal interface

Coincident with decidualization in humans and mice is the appearance of decidual Natural Killer (dNK) cells, the predominant immune cell at the maternal-fetal interface[48-50]. Mature dNK cells maintain decidual integrity and produce factors that directly modify decidual vessels[50]. Upon stimulation by IL-15, dNK cells begin secreting key angiogenic factors, including VEGF, PGF, and interferon-γ (IFN-γ)[51]. IFN-γ provides the signals that transiently change spiral arteries from constricted vessels to dilated vein-like structures necessary for adequate placental perfusion[49]. Another important function of dNK cells in pregnancy is their ability to recruit trophoblast cells as has been demonstrated in culture[52]. However, others have shown that acute dNK cell depletion in rats leads to more robust endovascular trophoblast invasion which they linked to poor decidual vessel remodeling earlier in pregnancy and increased hypoxia at the maternal-fetal interface that subsequently drove HIF1α mediated activation of the invasive trophoblast[53]. There is conflicting evidence regarding the significance of dNK cells in pregnancy outcomes. Some reports state that dNK cells are increased in PE, albeit with an altered phenotype[54], while others show dNK cells are decreased in preeclamptic placental samples[55]. Interestingly, others propose that dNK cell function, rather than number, may be altered in PE[56]. A very recent paper described carbon monoxide administration early in pregnancy enhanced proliferation of dNK cells and ameliorated the maternal syndrome in heme oxygenase deficient mice that exhibit a PE-like phenotype along with FGR and poor spiral artery remodeling[57]. This is very provocative as the *lack of smoking* has been documented as a risk factor for developing PE[2]. The role of dNK cells in PE is an area of great interest.

1.5 Rodent models to investigate PE

The initiation of fetoplacental pathologies has been extraordinarily difficult to study in humans due to logistical and ethical challenges associated with examining the first trimester. Therefore, animal models of PE are critically important for longitudinal investigation of early events that are essential for healthy pregnancy outcomes. Despite some differences in pregnancy

physiology, rodents have been widely used for studying human pregnancy disorders, such as PE. Some rodent models of PE involve surgical or pharmacological induction of the maternal syndrome at mid to late gestation[26, 58], thus making it impossible to study key early pregnancy events that drive placenta formation. Recently, several transgenic mouse strains have been developed that phenocopy the maternal syndrome along with other key features of PE[59, 60]. These mice allow the opportunity to investigate early pregnancy events in the context of PE, however due to the heterogeneity of the disease presentation it is unlikely that a single gene is responsible for its development.

1.5.1 The rat model of placental ischemia, RUPP (reduced uterine perfusion pressure)

Remodeling of spiral arteries during early pregnancy is essential to deliver adequate blood flow to the developing fetoplacental unit. In the case of PE, evidence points towards incomplete remodeling of spiral arteries by invasive trophoblast cells and thus inadequate uteroplacental blood flow to the placenta and fetus[18]. To mimic reduced uteroplacental perfusion *in vivo*, Crews and colleagues developed the RUPP rat model of PE[61]. At e14.5, silver clips are placed around the aorta proximal to the iliac bifurcation and also around the right and left uterine arcade to prevent compensatory blood flow to the placenta. Blood flow to the gravid uterus is reduced by 40%[62]. This surgical intervention results in late gestational hypertension and proteinuria as well as decreased litter sizes and evidence for FGR. In addition to these maternal and fetal consequences of PE, the RUPP rat model also recapitulates the angiogenic imbalance seen in women with PE, including increased anti-angiogenic factors sFlt1 and soluble endoglin (sENG) as well as decreased VEGF and PGF[63, 64]. Importantly though, these rodents do not have abnormal placentation[62]. Therefore the RUPP rat model provides a useful tool for studying placental ischemia and late gestational outcomes, including potential therapeutic approaches for managing the maternal syndrome, but not the origins of placental defects.

1.5.2 The chronic arginine vasopressin infusion model

The arginine vasopressin (AVP) pathway has recently come forward as a candidate for investigating PE. AVP-dependent hypertension is characterized by low circulating renin-angiotensin system activity, which is also seen is preeclamptic women relative to non-preeclamptic pregnant women[1]. Chronic infusion with AVP during pregnancy (e0.5 until e16.5) was sufficient to induce the PE-phenotype in normal healthy C57 female mice. Systolic BP and urinary protein excretion were increased at e15.0/16.0 and e17.0, respectively. This was associated with robust renal glomerular endotheliosis, a pathognomonic finding in PE[1]. There was also evidence for FGR in AVP-infused pregnancies. Fetal masses measured at late-gestation (e18.5) were significantly smaller after chronic AVP infusion[1]. This model supports the novel concept that central mediators of hypertension are activated in early pregnancy. Chronic AVP infusion during pregnancy warrants further exploration to determine if inhibition of AVP secretion or activation can prevent and/or treat PE.

1.5.3 STOX1 overexpressing mice

While animal models that mimic the maternal syndrome provide a tool for investigating late gestation pathologies and end-stage treatment strategies, they provide little insight into the origins of PE. Transgenic mouse models that phenocopy the maternal syndrome are emerging; one such is the STOX1-overexpressing mice[59]. *STOX1* has been found to be overexpressed in first trimester placenta from pregnancies that went on to develop PE[47]. The human *STOX1* cDNA was used to generate transgenic mice and *hSTOX1* was preferentially overexpressed in the placenta[59]. Wildtype (WT) female mice crossed with transgenic males were used to restrict transgene expression to the fetoplacental unit. A steady rise in systolic BP was observed in STOX1-overexpressing pregnancies beginning at the onset of pregnancy (e0.5), peaking at delivery and rapidly returning to baseline within days after parturition. Pregnant females also exhibit increases in urinary albumin/creatinine ratios at mid and late gestation. In addition to displaying the cardinal features of the PE maternal syndrome, WT female mice carrying fetuses

expressing the transgene had elevations in circulating levels of sFlt1 and sEng and significant renal pathologies at e16.5 compared to WT x WT females. As is often seen in human cases of PE, these transgenic mice appeared to suffer from fetal demise, as evident by smaller litter sizes, and structural abnormalities of the placentae. Interestingly, chronic aspirin therapy ameliorated the maternal syndrome in WT female mice carrying fetuses expressing the transgene[59]. Because this model does not require intervention to provoke the maternal syndrome, it provides a tool to assess treatment strategies beginning early in pregnancy. However, PE is a multifactorial disease that is unlikely to be monogenic and this must be considered when making any conclusions from these results.

1.5.4 MMP9-null mice

Animal models that mimic the maternal PE syndrome without intervention during pregnancy are critical to understanding the early origins of this disorder. Single gene mutations can be engineered in mice that then develop distinct pregnancy-associated pathologies, such as the matrix metalloproteinase 9 (MMP9)-null mouse that has been shown to phenocopy features of PE and IUGR[60]. MMP9-null mice were generated by a functional knockout of the active and zinc-binding domain of MMP9[65]. Homozygous matings between MMP9-null mice resulted in pregnancies with resorptions and poorly developed fetoplacental units at mid gestation (e10.5), which was attributed to impaired differentiation of trophoblast cells[60]. MMP9 production by trophoblast giant cells is needed for proper trophoblast invasion into the decidua; therefore it was an important finding that MMP-null implantation sites had inadequate remodeling of the decidua and morphologically abnormal maternal-embryonic connections at e7.5[60]. These seminal results are important in shifting the focus of PE research on the origins of this disease process rather than the late-gestational development of the maternal syndrome, which is believed to occur long after the initiating pathologies. However, MMP9-null mice exhibit increased systolic BP compared to heterozygotes at pre, early, mid and late gestation without a significant rise from pre-pregnancy to any stage of pregnancy[60]. Furthermore, they have pre-existing renal pathologies

including the pathognomic-PE sign of glomerular endotheliosis[60]. These two signs may complicate studies where interventions are made early in pregnancy prior to the onset of the maternal syndrome. Interestingly, increased maternal blood pressure early in pregnancy has been linked to lower birth-weight and SGA babies in women with PE[66], so this model may be promising for investigating not just PE but also FGR.

1.6 The BPH/5 mouse model of PE

Our laboratory discovered the first strain of mice to spontaneously recapitulate the maternal syndrome of PE, BPH/5[67]. These mice were one of several mouse strains developed at the University of Kansas by the geneticist Dr. Gunther Schlager[68]. Hypotensive and hypertensive mice were generated by inbreeding eight different strains of normotensive mice and selecting for low and high blood pressure over 23 generations. This resulted in two new and distinct genetically inbred strains of mice, BPL (Blood Pressure Low)/1 and BPH (Blood Pressure High)/2. Several sublines of BPH/2 were further developed with varying intermediate blood pressures. BPH/5 mice exhibited a mildly elevated blood pressure profile and because this is a known risk factor in the development of PE in women, Davisson et al. hypothesized that BPH/5 mice would develop the maternal syndrome of PE during gestation.

1.6.1 Discovery of a novel spontaneous mouse model of PE

BPH/5 mice are pre-hypertensive, without signs of renal disease, prior to pregnancy[67]. Beginning at e14.0, pregnant BPH/5 mice display a significant rise in mean arterial pressure that returns to baseline after delivery of the pups and placentae. These mice also exhibit late-gestational proteinuria with renal histopathological findings consistent with glomerulosclerosis[67]. Similar to PE in people that experience perinatal morbidity/mortality, BPH/5 mice also have small litter sizes due to *in utero* fetal demise at mid gestation. Pups that are born live are low-birth weight compared to C57 control mice, indicative of FGR[67]. Another feature of the maternal PE syndrome is widespread endothelial dysfunction[69]. Importantly, endothelium-intact

mesenteric arteries taken from BPH/5 pregnant females at e19.5 showed diminished relaxation in response to acetylcholine, suggestive of systemic endothelial dysfunction[67]. Thus the BPH/5 mouse emerged as a novel spontaneous genetic model of PE recapitulating the key pathophysiological findings in women with PE and their offspring.

1.6.2 Fetoplacental abnormalities in the BPH/5 mouse model

It is widely accepted that the placenta plays a causal role in the pathogenesis of PE[9]. In addition to the cardinal features of PE, BPH/5 mice also develop placental pathologies that precede the maternal syndrome[70]. Between e9.5 and e14.5, BPH/5 pregnant mice have a 40-50% reduction in placental mass as compared to C57 placenta[70]. Also, fetal weight is significantly decreased in BPH/5 pregnant females at e12.5, e14.5, and e18.5 versus C57[70]. Therefore, the lower-birth weight pups first documented by Davisson et al. are a result of *in utero* FGR. Histological analyses of the BPH/5 placentae at e10.5 revealed inadequately remodeled decidual vessels with thickened walls that retained actin-positive cells and thus narrowed lumens compared to C57 decidual vessels[70]. Placentae at e12.5 show decreased depth of trophoblast invasion into the maternal decidua and a decrease in the fractional area occupied by the junctional zone[70]. Both these findings are indicative of impaired endovascular invasion and interstitial invasion, respectively. These two pathologies of the BPH/5 placenta would suggest reduced placental perfusion and indeed this was reflected using Doppler ultrasound[70]. Uterine arteries from BPH/5 mothers at e16.5 had elevated pulsatility index which indicates placental vascular insufficiency and impairment of the maternal-fetal circulation as compared to C57[70]. Furthermore, the labyrinth region in BPH/5 placentae at e12.5 had attenuated and irregular branching morphogenesis and reduced expansion[70]. Therefore, defects in both the fetal and maternal compartments characterize the BPH/5 placenta and this occurs in pregnancy before the onset of the maternal syndrome.

1.6.3 BPH/5 mice have oxidative stress & an angiogenic imbalance during pregnancy

BPH/5 mice demonstrate the cardinal signs of the maternal PE syndrome[67] as well as placental abnormalities identified in women with PE[67, 70]. Having characterized this PE-like phenotype, studies were undertaken to demonstrate a causal role for pathways implicated in the pathogenesis of PE. First, oxidative stress was investigated during BPH/5 gestation. BPH/5 mid gestation placentae have increased levels of superoxide and reduced levels and activity of the antioxidant enzyme, superoxide dismutase (SOD)[71]. Placental oxidative stress was attenuated by chronic Tempol (SOD mimetic) treatment and this was associated with a decrease in BPH/5 resorptions, an increase in BPH/5 litter size and normalization of BPH/5 placental and fetal weights[71]. Furthermore, Tempol treatment ameliorated the maternal syndrome of late-gestational hypertension and proteinuria[71]. These findings provide evidence for a causal role of oxidative stress in the pathogenesis of the maternal PE syndrome as well poor pregnancy outcomes, such as fetal demise and poor fetoplacental development.

Next, angiogenic factors were considered as a potential mechanism behind the maternal syndrome of PE and poor fetoplacental outcomes in this model. BPH/5 females were found to have decreased circulating and placental levels of VEGF protein at early and mid gestation[72]. Another pro-angiogenic factor was measured, PGF, and found to be decreased in the circulation and in placental mRNA abundance at early gestation[72]. However, this was not associated with an increase in the anti-angiogenic factor, sFlt, but rather a decrease in plasma and placental mRNA[72]. Delivery of an adenovirus encoding $VEGF_{121}$ (Ad-VEGF) early in gestation at e7.5 blunted the maternal syndrome in BPH/5 females while also decreasing mid gestation resorptions and improving litter size at term versus C57[72]. Interestingly, serum collected at e12.5 from BPH/5 Ad-VEGF treated mothers showed a marked improvement in angiogenic potential as measured by the endothelial tube formation assay[72]. These data highlight the importance of angiogenic factors in the BPH/5 mouse model and supports the hypothesis that VEGF plays a key role in the maternal PE syndrome as well as fetal outcomes. Moreover, delivery of Ad-

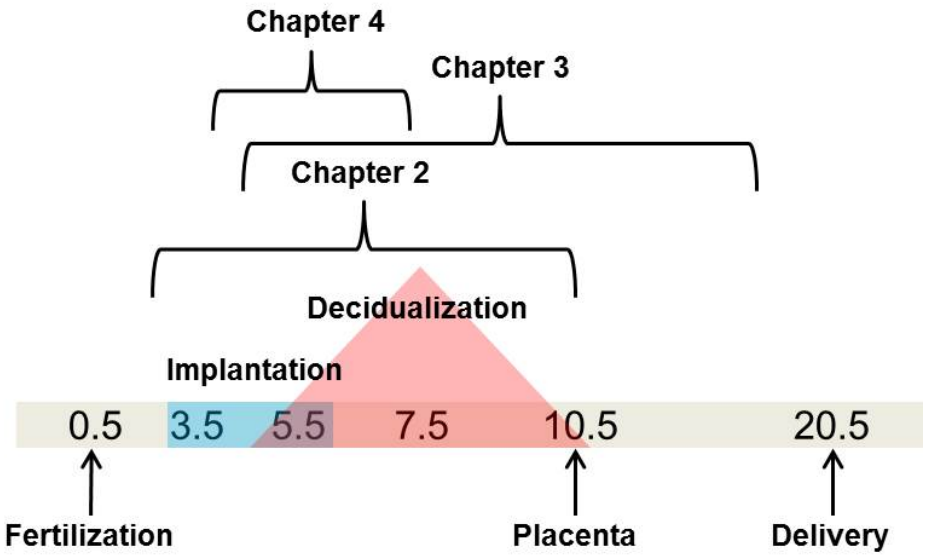
VEGF early in pregnancy provides strong rationale for investigating early pregnancy events and their relationship to downstream pregnancy outcomes in BPH/5.

The spontaneous BPH/5 mouse model provides us with the opportunity to interrogate early pregnancy events prior to the onset of the maternal syndrome. Thus we are able to assess any stage of pregnancy and probe for dysregulated events which may have "ripple effects" that promote poor pregnancy outcomes in this model.

1.7 Statement of the problem

PE is a widely recognized multifactorial disorder of pregnancy. While the diagnosis of PE is made by the clinical presentation of the maternal syndrome, the origins of PE begin much earlier in pregnancy. Even though this devastating disorder of pregnancy has been described for thousands of years, the mechanisms involved in PE still baffle clinical and basic scientists alike. Placenta formation occurs in the first trimester of pregnancy and thus this very critical time point in gestation may hold the key to the dysregulated events that lead to PE. The use of animal models affords researchers the opportunity to investigate key early pregnancy events that may contribute to the development of PE.

In these studies, early pregnancy events, such as implantation and decidualization, were investigated in the BPH/5 spontaneous mouse model of PE. Using a molecular approach, we identified dysregulation in key mediators of implantation and decidualization and functionally linked anomalies in Cox2 signaling to downstream fetoplacental defects in this model. We further addressed the maternal-fetal interface during this very early time point in pregnancy and uncovered defects in the immunoregulatory events that are required for adequate placental formation. Taken together our data supports the "ripple effect" hypothesis that defects in early pregnancy events are the source of downstream poor pregnancy outcomes, including PE.



1.8 Approach

First, we tested the hypothesis that BPH/5 mice have abnormalities in implantation and decidualization that precede fetoplacental development defects in this model. To test the hypothesis that the origins of adverse pregnancy outcomes in BPH/5 begin during the periimplantation period, we utilized molecular methods to assay key molecules at specific gestational time points that mediate implantation, decidualization, and placentation. By using this approach, we revealed significant dysregulation of the periimplantation period, particularly in the expression of *Ptgs2*/Cox2 in the maternal uterine environment. Using pharmacological intervention at early pregnancy, we confirmed that defects in Cox2 signaling during the periimplantation period have "ripple effects" impacting downstream pregnancy outcomes at mid and late gestation in BPH/5 mice. Finally, investigations in early pregnancy lead us to discover deficiencies in dNK cell populations at the maternal-fetal interface and this was associated with overexpression of IL-15. Altogether, this points towards inflammation in the maternal uterine environment during early pregnancy having a central role in the poor fetoplacental and pregnancy outcomes associated with this model.

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CHAPTER TWO:

THE PERIIMPLANTATION PERIOD IN BPH/5 MICE IS CHARACTERIZED BY PROFOUND DEFECTS IN IMPLANTATION, DECIDUALIZATION AND FETOPLACENTAL DEVELOPMENT

SUMMARY

Preeclampsia (PE) is a pregnancy-specific disorder that manifests as late gestational hypertension and proteinuria in the mother, however it is widely accepted that abnormal placentation in early gestation is responsible for the cascade of events leading to the presentation of the maternal syndrome. Defective implantation is thought to cause adverse “ripple effects” throughout pregnancy, leading to abnormal placentation, retarded fetal development, and poor pregnancy outcomes, such as PE. The precise implantation defect(s) initiating adverse pregnancy outcomes in the context of PE remains elusive. The BPH/5 mouse model, which spontaneously develops cardinal features of PE, was utilized to investigate the periimplantation period and fetoplacental development prior to the onset of the maternal PE syndrome.

The periimplantation period in BPH/5 mice is characterized by deferred timing of implantation along with abnormal embryo clustering along the uterine horns, which was associated with delayed and defective decidualization. BPH/5 implantation sites show evidence of hypoxia and dysregulated angiogenesis at the time of peak decidualization. By mid gestation BPH/5 pregnant females exhibit poor fetal health status. Using ultrasonography, fetuses can be categorized into three increasingly severe phenotypic classes (healthy, compromised, and resorbed) that are associated with a distinct molecular signature of ineffective syncytiotrophoblast differentiation and inadequate placental development. Taken together the BPH/5 mouse has significant periimplantation defects early in pregnancy, most notably in decidualization, and exhibits signs of poor fetoplacental development at mid gestation. These data suggest that the origins of abnormal placentation, prior to the maternal PE syndrome, begin in the periimplantation period in the BPH/5 mouse model of PE.

INTRODUCTION

Pregnancy success depends on the coordination of many developmental processes. Precise embryo-uterine crosstalk is required for post-fertilization events including implantation, decidualization, and placentation. Defects in any of these early pregnancy events are thought to have downstream effects on placental development and pregnancy outcomes, including fetal growth restriction (FGR) and preeclampsia (PE)[1]. Therefore, investigating these early developmental processes in the context of BPH/5 pregnancies is valuable in elucidating the early origins of PE in women.

Implantation, the initial event of placentation, is dependent on two things: 1) an activated blastocyst and 2) a receptive uterus. Blastocyst activation depends on precise cleavage of the fertilized egg and differentiation of embryonic cells to the blastocyst stage[2]. The uterine environment for blastocyst implantation can be classified into 3 phases: pre-receptive, receptive, and non-receptive[3]. Uterine receptivity is dependent on ovarian production of steroid hormones estrogen (E_2) and progesterone (P4)[4]. Pre-ovulatory E_2 stimulates proliferation and differentiation of luminal and glandular epithelium, while P4 initiates stromal cell proliferation[5]. Additionally, an E_2 surge just prior to implantation is the stimulus for blastocyst attachment. Optimal levels of ovarian hormones are necessary to adequately regulate the molecular mechanisms that govern uterine receptivity and thus, implantation[3]. In mice this begins with the uterine cytokine, Leukemia Inhibitory Factor (LIF), which is E_2 responsive[6]. *Lif* displays biphasic uterine expression to first confer receptivity at embryonic day (e) 3.5 and next at e4.5 to mediate implantation in the uterine glandular epithelium and stroma surrounding the blastocyst[7, 8]. It is believed that LIF is important in human implantation as increased uterine levels have been reported during implantation in women[9].

This precise pre-implantation hormonal milieu is required for uterine receptivity for the activated blastocyst as well as the attachment reaction on the evening e3.5 in mice, after which uterine stromal cells surrounding the implanting blastocyst proliferate extensively and

differentiate into decidual cells[5]. The decidual cell reaction, or decidualization, confers a unique phenotype on the pregnant uterus that allows for placenta formation, including the ability to regulate trophoblast invasion, modulate local vascular and immune responses, and resist environmental and oxidative stress[10]. The two hallmarks of successful implantation and decidualization are increased uterine vascular permeability and angiogenesis[11]. These processes are profoundly influenced by a number of cytokines, vasoactive agents, and growth factors, including vascular endothelial growth factor (VEGF)[2]. The key events of implantation, uterine receptivity, blastocyst attachment, and decidualization each require precise cross-talk between the mother and embryo to ensure proper fetoplacental development and a normal pregnancy.

Normal development of the placenta requires trophoblast cells, specialized cells of embryonic origin, to proliferate and differentiate to sub-serve unique molecular and functional roles[12]. Similar to humans, a subset of mouse trophoblast cells will terminally differentiate into multinucleate syncytiotrophoblast cells that populate the labyrinth, a region of the placenta responsible for maternal-fetal exchanges[13]. Expansion and patterning of the labyrinth is crucial for placental perfusion of nutrients and oxygen[14]. Transcription factors have been shown to play a key role in determination of trophoblast cell differentiation and organization[15]. Glial cells missing homologue 1 (GCM1), a placental transcription factor, has been reported as the crucial regulator of syncytiotrophoblast cell differentiation, with *Gcm1* null mutants experiencing embryonic lethality at approximately e10.0 due to blockage of syncytiotrophoblast formation and absence of a labyrinth layer[12]. Syncytins, fusion proteins expressed by syncytiotrophoblast cells, are involved in trophoblast cell fusion to form the syncytiotrophoblast cell layer and exchange surface[16]. GCM1 regulates syncytin gene expression in human and mouse placenta[17, 18] and both GCM1 and syncytin expression has been shown to be decreased in placenta from PE pregnancies[19, 20].

Dysregulation at any point in the events encompassing implantation and decidualization can create adverse “ripple effects” throughout pregnancy, leading to abnormal placental development, retarded fetal development, and maternal/fetal loss[1, 21-23]. Since these poor

outcomes are all associated with PE, we hypothesized that aberrations in periimplantation events and poor fetoplacental outcomes characterize BPH/5 mice. Indeed, we found that abnormal implantation and decidualization events precede placenta formation in these mice. The periimplantation period (e3.5-5.5) in BPH/5 mice is characterized by deferred timing of implantation along with abnormal embryo clustering along the uterine horns, which was associated with delayed and defective decidualization. Postimplantation, at peak decidualization (e7.5), BPH/5 implantation sites show evidence of hypoxia and dysregulated angiogenesis. Furthermore, BPH/5 pregnant females at mid gestation (e10.5) exhibit poor fetal health status at the ultrasonographic level which is associated with a distinct molecular signature of inadequate placental development that has been associated with the development of PE-like symptoms in women.

METHODS

Animals

Adult C57BL/6 (C57) and BPH/5 mice (8-12 weeks of age) obtained from in-house colonies were used for all experiments. Mice were housed in a temperature controlled room with a 12:12-hour light: dark cycle. Timed mating experiments were performed by singly housing strain-matched male and female mice overnight. Day of vaginal plug detection was denoted as e0.5. All animal procedures were approved by the Institutional Animal Care and Use Committee of Cornell University and were in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Pontamine blue perfusion studies

To visualize and collect implantation sites at precise time points, pregnant females at e3.5pm (20 00h), e4.5am (8 00h), e4.5pm (20 00h), and e5.5am (8 00h) were given a single injection of 100 µL of 1% pontamine blue (BDH, Franklin Lakes, NJ) dissolved in saline, administered via tail vein, and after 5 minutes mice were euthanized. Implantation sites were then collected and stored for histologic and molecular analyses. The incidence of embryo clustering was recorded and defined as an implantation site that is located less than the length of one implantation site (~1mm) away from an adjacent implantation site (Ashley Woods; PhD thesis).

Hormone Assays

Blood was collected every 12 hours beginning in the evening of e1.5 through e4.5 via cardiac puncture, allowed to clot at room temperature for 90 minutes, and centrifuged at 3500 RPM for 20 minutes. Serum was frozen at -80°C until assayed. A commercially available estradiol ELISA was performed according to manufacturer's instructions (Calbiotech, Spring Valley, CA). The sensitivity of this assay is 3pg/mL.

In situ hybridization with radioactive isotopes

In situ hybridization (ISH) was performed as previously described[24]. Implantation sites were collected and snap frozen. Tissue sections (12 μm) were mounted on poly-L-lysine coated slides, fixed in 4% PFA solution in PBS at 4°C, and acetylated. After pre-hybridization, sections were hybridized at 45°C for 4 hours in 50% formamide buffer containing ^{35}S-labeled sense or anti-sense cRNA probes. After hybridization, RNase A-resistant hybrids were detected by autoradiography using Kodak NTB-2 liquid emulsion. Parallel sections were hybridized with sense probes and served as negative controls.

Alkaline phosphatase activity assay

To assess decidualization of uterine tissues, alkaline phosphatase (ALP) activity was performed as previously described[25] in BPH/5 and C57 implantation sites at day e4.5am, e4.5pm, and e5.5am. Briefly, uteri with implantation sites were fixed in 4% paraformaldehyde (PFA) overnight at 4°C, followed by sucrose gradients in PBS (15 and 30%), then frozen in embedding medium O.C.T. (Optimal Cutting Temperature) and sectioned at 16 μm onto poly-L-lysine coated slides. Slides were postfixed in 0.2% glutaraldehyde, washed in phosphate-buffered saline (PBS), and incubated with a 100 mM Tris buffer (pH 9.5) containing chromogenic substrates for ALP (168.5 μL of 100mg/mL nitro blue tetrazolium salt in dimethylformamide and 175 μL of 50 mg/mL 5-bromo-4-chloro-3-indoyl phosphate/toluidinium salt (NBT/BCIP) in dimethylformamide added to 50 mL of Tris buffer; Roche, Penzburg, Germany). The development of a purple color is indicative of ALP activity. Liver sections incubated with and without substrate served as positive and negative controls, respectively. Quantification of ALP activity, with purple staining corresponding to the secondary decidual zone and lack of purple staining adjacent to the embryo corresponding to the primary decidual zone, was performed using the average of 3 adjacent sections from ≥3 implantation sites per time point. All measurements were done using Image J software (NIH).

Ultrasound

Timed mating experiments were performed as described above with strain matched C57 control and BPH/5 mice. Preliminary studies showed that e10.5 was the earliest time point in which the greatest variation in embryonic health could be observed prior to fetal death and resorption (Ashley Woods; PhD thesis). Pregnant C57 and BPH/5 mice at e10.5 were anaesthetized with 2% isofluorane. Maternal heart rate (HR), respiratory rate, and core temperature were monitored while fetoplacental units were assessed using a Vevo-770 high-frequency ultrasound with a 40MHz frequency scanhead (Visualsonics, Toronto, ON), according to published methods[26]. Fetal heart rates were measured using pulse-wave Doppler (expressed as fetal/maternal HR), and crown-rump measurements were taken when the orientation permitted the maximum distance between head and tail[27]. Fetuses were placed in one of 3 categories: Healthy (normal HR and development); Compromised (slow or irregular HR, reduced size); Resorbed (no fetal heart, often no fetus). Immediately after ultrasonographic categorization, the female was euthanized and individual fetoplacental units (fetus, placental tissue, and surrounding uterine wall) were excised, snap frozen in liquid nitrogen and stored at -80 °C for future molecular analyses.

Quantitative real-time PCR analysis of gene expression

Primers were designed using Primer Design software provided by Integrative DNA Technologies (www.idtdna.com; Coralville, IA). Total RNA was extracted using TriZol (Life Technologies, Grand Island, NY) as previously described[28]. Reverse transcription was performed using Quanta kits (Gaithersburg, MD) per the manufacturer's instructions, and quantitative real-time PCR (qRT-PCR) was performed with 25ng cDNA in triplicate using SybrGreen reagents (Quanta) on an ABI 7500 Fast System (Applied Biosystems, Life Technologies, Grand Island, NY). Data was expressed as 2^-ΔΔCt relative to housekeeper gene expression (*18S rRNA*). Primer sequences are listed in Table 2.1.

In situ hybridization with non-radioactive labels

The *Gcm1, SynA,* and *SynB* riboprobes for *in situ* hybridization (ISH) were a gift from Dr. James C. Cross (University of Calgary, Canada). C57 and classed BPH/5 fetoplacental units, categorized via ultrasound, were isolated at e10.5 and snap frozen in freezing media (Super Friendly Freeze IT; Life Technologies, Carlsbad, CA). Frozen fetoplacental units were cryosectioned at 12 μm and mounted onto Superfrost®Plus slides (Life Technologies). ISH experiments were performed simultaneously with C57 and all classed BPH/5 fetoplacental unit sections for each probe. At least 3 sections per fetoplacental unit were tested across at least 3 different pregnancies per strain. Briefly, sections were fixed in 4% paraformaldehyde for 10 minutes, acetylated for 10 minutes, pre-incubated in hybridization buffer for 2 hours, and then incubated in hybridization buffer containing 0.8 μg/mL digoxigenin-labeled riboprobes at 65°C for 14-16 hours. The sections were sequentially washed 2 times in 5x SSC for 20 minutes at 65°C, 3 times in 0.2x SSC for 20 minutes at 65°C, and 3x in TBS buffer for 5 minutes each at room temperature. After sections were blocked in blocking buffer for 2 hours at room temperature, they were incubated overnight with the alkaline-phosphatase conjugated sheep anti-digoxigenin antibody (Roche; 1:1000 dilution) in blocking buffer. The riboprobes labeled with digoxigenin were detected with NBT/BCIP (Roche). Once the appropriate level of signal intensity was seen in sections with antisense probe, slides were rinsed with TBS, counterstained with nuclear fast red and mounted under a cover glass with Permount. Slides were scanned using ScanScope Digital Scanner (Aperio, Vista, CA) to capture the images.

Western blot

C57 and BPH/5 implantations sites at e7.5 were isolated and processed for $VEGF_{164}$ protein analyses. C57 and classed BPH/5 fetoplacental units were isolated at e10.5 and the placenta was dissected free from the surrounding tissues for Gcm1 protein analyses. Both samples were processed as follows; tissues were homogenized in RIPA buffer containing protease inhibitors (Roche). The tissue lysate was quantified with Nanodrop ND-1000 spectrophotometer (Life

Technologies) using BCA protein assay kit (Life Technologies). Fifty micrograms of protein were separated by a 10% gradient SDS-polyacrylamide gel and transferred to nitrocellulose membrane (Life Technologies). The membrane was blocked in 5% skim milk in PBS for 1 hour at room temperature and subsequently probed with primary antibody (1:500 goat anti-$VEGF_{164,}$ Sigma Aldrich; 1:1000 rabbit anti-mGCM1, Millipore, Darmstadt, Germany) overnight at 4 °C. After three washes with PBS-T, the membrane was incubated with secondary antibody (1:5000 donkey anti-goat horse radish peroxidase (HRP)-conjugated, Santa Cruz Biotechnologies, Santa Cruz, CA; 1:5000 goat-anti-rabbit HRP-conjugated; Life Technologies). Blots were probed with mouse anti-beta actin primary antibody (1:5000, Sigma Aldrich, St. Louis, MO) and goat-anti-mouse HRP-conjugated secondary antibody (1:5000, Santa Cruz Biotechnologies). Signals were detected by chemiluminescence with Pierce ECL Western Blotting Substrate (Life Technologies). Band intensities were quantified and normalized to beta actin with ImageJ.

Statistical Analysis

All data are expressed as mean ± SEM using Student's unpaired *t* test or one-way ANOVA followed by Newman-Keuls test for significance. A value of $p < 0.05$ was considered statistically significant.

Table 2.1 Primer sequences used for quantitative Real Time PCR (qRT-PCR) analyses

Gene name	Forward and reverse sequences (5’ to 3’)
Lif	TCAGCGACAAAGTTACTCCACCGT
	AAGTGATGACAAAGCCCAACAGGC
Hif1α	CCTTAACCTGTCTGCCACTTT
	TGCTGCAATAATGTTCCAATTCC
Gcm1	TGACACCTGGACAAGAAGCTGACA
	CAAGAGCAGCTGAAGGGCTTGTTT
SynA	GCGCCTCCAGAATTTCCAACCATT
	TGTCACGCTGGTGATCTGAAGGAA
SynB	TGCCCAACAGACATCAAACACTGA
	ACTGACATGGTAACAGGGTGGGTT
18S	GTAACCCGTTGAACCCCATT
	CCATCCAATCGGTAGTAGCG3

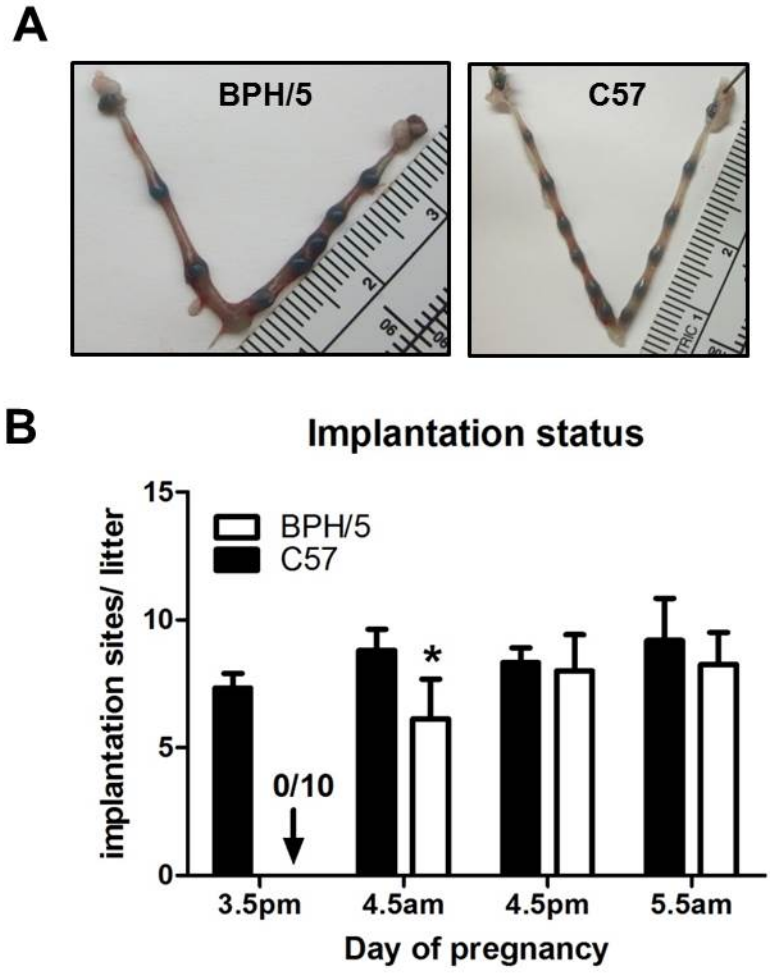
RESULTS

BPH/5 females show deferred timing of implantation.

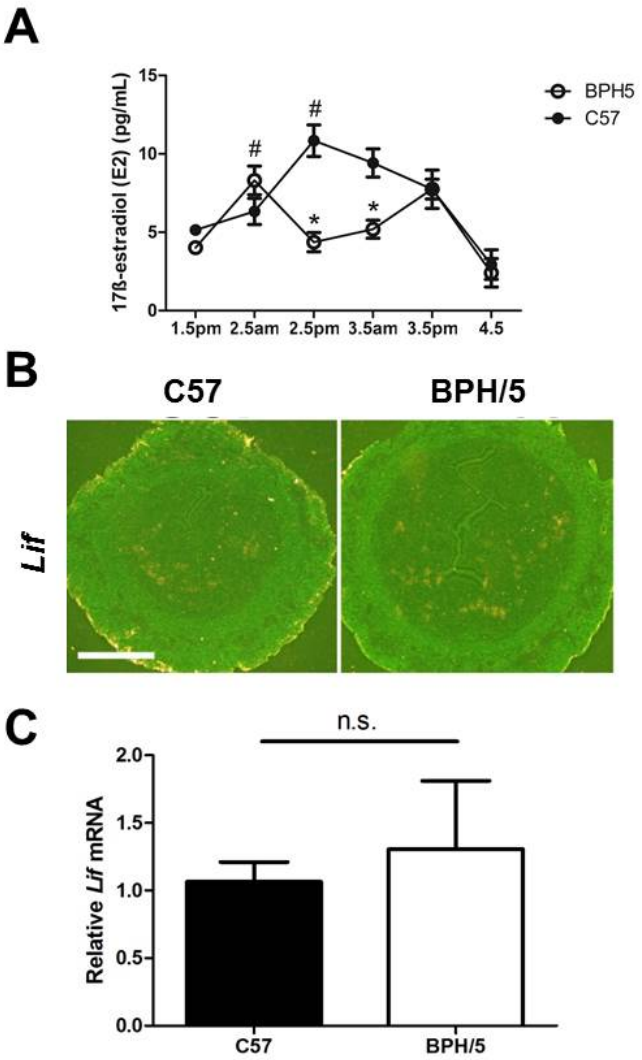
It is hypothesized that the development of PE begins with abnormal placentation as a result of defective implantation[29]. Since we have shown that BPH/5 mice have PE-like phenotypes[30, 31], we investigated implantation events in these mice. One of the earliest signs of implantation is increased endometrial vascular permeability at the site of blastocyst attachment, which can be identified by injecting a macromolecular blue dye[32]. Using this method, implantation sites were visualized in pregnant C57 and BPH/5 females during the periimplantation period, e3.5pm-e5.5am (Figure 2.1A). BPH/5 females exhibited deferral of implantation timing as determined by the complete absence of blue bands (n=10 BPH/5 females) at e3.5pm, when implantation is first evident in C57 females (Figure 2.1B). Twelve hours later at e4.5am, faint blue bands were observed in BPH/5 uteri; however, there was a significant reduction in the number of implantation sites in BPH/5 females (Figure 2.1B). BPH/5 and C57 females had comparable numbers of implantation sites by e5.5am (Figure 2.1B). However, at every time-point examined, BPH/5 implantation sites showed abnormal embryo clustering along the uterine horn.

Uterine receptivity is adequate in BPH/5 females prior to implantation

Successful implantation requires the interaction of an implantation-competent blastocyst and a receptive uterus[1, 33, 34]. It has previously been shown that BPH/5 embryos are developmentally delayed at the time of implantation (Ashley Woods; PhD thesis), however uterine receptivity in BPH/5 females was unknown. Uterine receptivity is dependent on ovarian hormones, estrogen (E_2) and progesterone (P_4) [4]. Therefore, serum levels of estradiol-17β and P_4 were measured before, during, and after implantation. Between e1.5 and e4.5 of pregnancy, BPH/5 females showed lower serum estradiol-17β levels prior to the anticipated time of receptivity, while C57 females show higher serum estradiol-17β levels at e2.5pm (Figure 2.2A).



<u>Figure 2.1:</u> Implantation timing is deferred in BPH/5 mice. (A) Representative images of pontamine blue-stained implantation sites within BPH/5 (left) and C57 (right) uteri at e5.5. **(B)** Time-course of implantation sites counted per litter in pregnant BPH/5 and C57 females during the periimplantation period (n=7-10 females). *$p<0.05$ vs time matched C57.

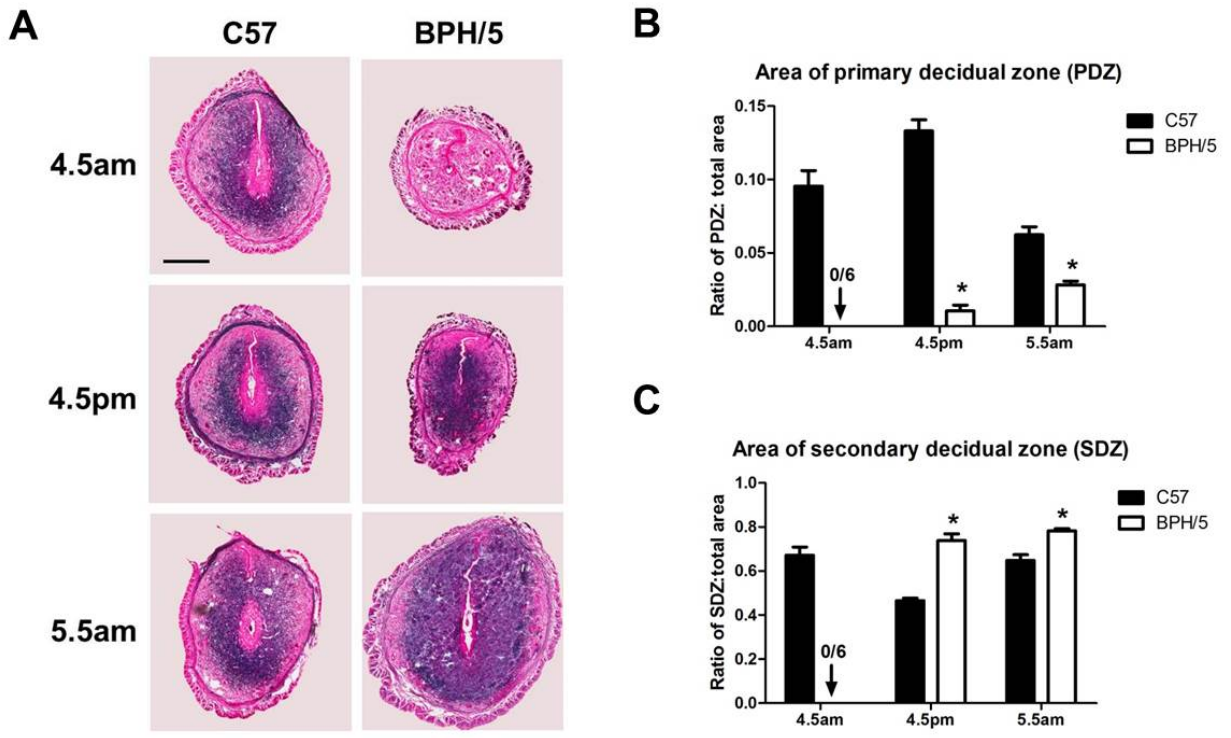


<u>Figure 2.2:</u> Circulating 17β estradiol levels are altered, but receptivity is adequate in BPH/5 uteri at e3.5am. (A) Serum 17β estradiol levels before and during the periimplantation period in pregnant C57 and BPH/5 (n=3-6 females). *p<0.05 vs time matched C57 and #p<0.05 vs strain matched baseline values. **(B)** Representative ISH and **(C)** qRT-PCR measurements of the uterine receptivity marker, *Lif*, in e3.5am uteri (n=4-8). n.s.= non-significant. Scale bar= 500µm.

During this time, serum P4 levels were comparable between BPH/5 and C57 females with a significant increase in P4 levels at e2.5pm in BPH/5 (15.805±0.763 vs C57: 11.371±0.773, n=7, $p<0.05$; Ashley Woods PhD thesis). Because of disparate serum estradiol-17β levels between C57 and BPH/5 females, receptivity-specific gene expression was examined in C57 and BPH/5 gravid uteri prior to the time of implantation. The expression profile of *Lif*, an E_2-sensitive marker of uterine receptivity[35, 36], was assessed by ISH and qRT-PCR in e3.5am uteri. Surprisingly, *Lif* showed similar expression patterns in BPH/5 and C57 uteri as determined by ISH (Figure 2.2B) and this was mirrored in qRT-PCR analyses (Figure 2.2C). Therefore, despite the aberrant estradiol-17β levels in BPH/5 females, the expression of *Lif* was unaltered. These findings suggest that while the preimplantation uterine environment of BPH/5 females is receptive to implantation, embryo-uterine crosstalk at the time of implantation may be dysregulated.

BPH/5 exhibit delayed and defective decidualization events

The observation of deferred implantation timing led us to further characterize the implantation phenotype in BPH/5 mice. Successful embryo-uterine crosstalk during pregnancy is required for decidualization, as the attachment reaction serves as the stimulus[33]. Thus, we next assessed the activity of alkaline phosphatase (ALP), the classical decidualization marker[25], in BPH/5 and C57 implantation sites during the periimplantation period. BPH/5 implantation sites show delayed decidualization with no ALP activity at e4.5am, followed by increased ALP activity from e4.5pm through e5.5am (Figure 2.3A). In addition to the temporal differences in ALP activity within BPH/5 implantation sites, a spatial difference in the decidual zones was also observed. The primary decidual zone (PDZ) is avascular and ALP-negative, and is thought to protect the developing embryo from oxidative stresses in the surrounding uterus[33].



<u>Figure 2.3:</u> Decidualization is delayed and defective in BPH/5 mice during the periimplantation period. (A) Representative alkaline phosphatase (ALP)-stained implantation sites harvested from C57 and BPH/5 females during the periimplantation period (e4.5am-e5.5am). Scale bar= 500µm. **(B)** Area of the primary decidual zone (PDZ; pink/ALP-negative) surrounding the embryo was measured using ImageJ and area was expressed as a ratio of total implantation site area. (n=3-6 implantation sites/group) *p<0.05 vs time matched C57. **(C)** Area of the secondary decidual zone (SDZ; purple/ALP-positive) was measured using ImageJ and area was expressed as a ratio of total implantation site area. (n=3-6 implantation sites/group) *p<0.05 vs time matched C57.

The ALP-positive secondary decidual zone (SDZ) is the site of uterine stromal cell differentiation and transformation into decidual cells, which have a unique morphological and biochemical profile that allows for uterine angiogenesis and vascular development of the placental bed[33]. Beginning with the onset of decidualization in BPH/5 implantation sites at e4.5pm and also at e5.5am, the PDZ area is smaller than in C57 time-matched implantation sites (Figure 2.3B). Additionally, the SDZ area is significantly greater in BPH/5 implantation sites at e4.5pm and e5.5am (Figure 2.3C). To determine whether these observations stemmed from the inability of BPH/5 uteri to undergo decidual cell transformation, experimentally-induced decidualization experiments were performed in pseudopregnant females. This procedure produced comparable deciduoma formation between BPH/5 and C57 (data not shown).

BPH/5 females show evidence for increased hypoxia at the peak of decidualization

After implantation is complete at e5.5, extensive angiogenesis during decidualization, which peaks at e7.5, is required to ensure adequate blood flow to the developing placenta by e10.5 (Figure 2.4A). We hypothesized that angiogenesis would be dysregulated during decidualization as delayed and defective decidualization events predominate in BPH/5 females. In mice, this phenomenon is influenced by P4-induced expression of hypoxia inducible factor (HIF) and its subsequent binding to the hypoxia response element in the *Vegf* promoter[37]. It has been shown that *Hif1α* and *Hif2α* are upregulated during the postimplantation period in association with *Vegf* as decidual angiogenesis increases in mice[37]. However, hypoxia is the most potent inducer of HIF1α expression. We examined *Hif1α* mRNA expression by qRT-PCR and observed significant upregulation in BPH/5 implantation sites at e7.5 as compared to C57 implantation sites at this time point (Figure 2.4B). The functional consequence of this gene expression profile was supported by Western blot analyses showing increased expression of $VEGF_{164}$ protein in e7.5 BPH/5 implantation sites compared to C57 (Figure 2.4C & D).

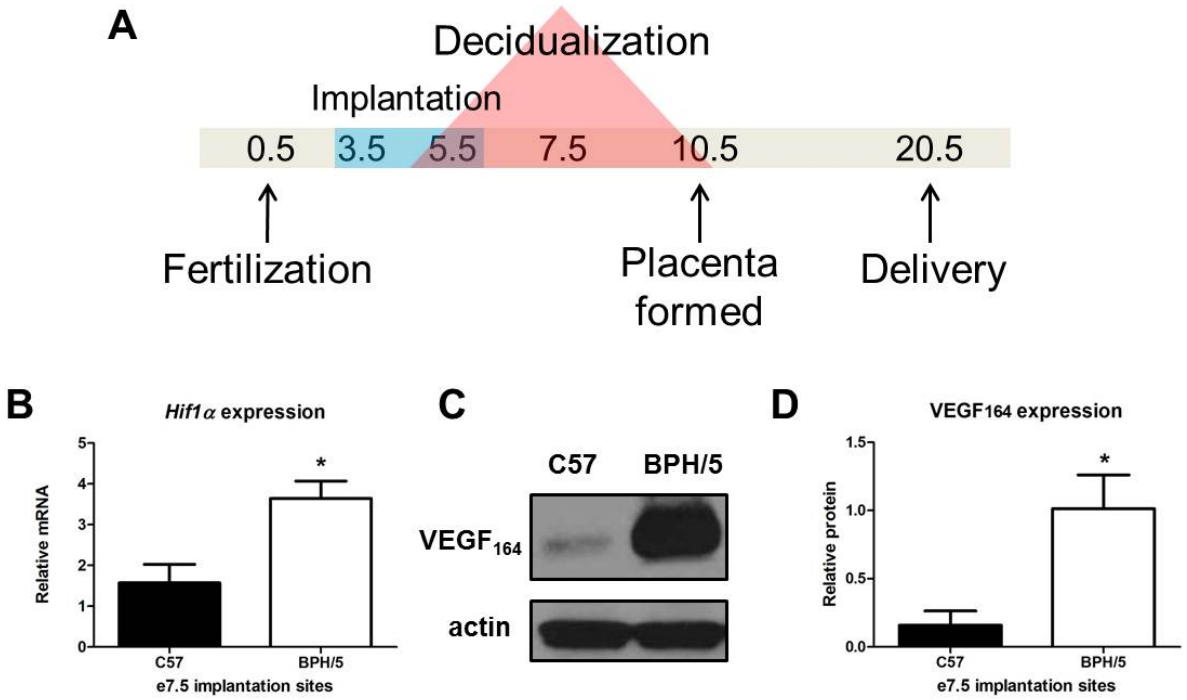


Figure 2.4: Postimplantation decidualization events precede placenta formation and are marked by hypoxia in BPH/5 female mice at e7.5. (A) Schematic representing the timeline of crucial pregnancy events (fertilization, implantation, decidualization, placental formation, and delivery) during mouse gestation. **(B)** Quantification of *Hif1α* mRNA by qRT-PCR in e7.5 implantation sites from C57 and BPH/5 females (n=7/group) *p<0.05 vs C57. **(C)** Representative Western blot gel and **(D)** quantitative summary of $VEGF_{164}$ protein expressed relative to actin in e7.5 implantation sites from C57 and BPH/5 females (n=7/group) *p<0.05 vs C57.

BPH/5 females exhibit poor fetoplacental health and development at mid gestation

BPH/5 fetoplacental development was next considered as its outcome relies heavily on implantation and decidualization events. Our lab has previously described smaller litter sizes in BPH/5 females beginning at mid gestation[30]. Using ultra high frequency ultrasound during pregnancy, fetal resorptions were first identified at e10.5 in BPH/5 females (Ashley Woods; PhD thesis). Incidentally at this time point, the greatest variation in fetal health was also observed, including the ratio of fetal to maternal heart rate (HR ratio) and crown-rump length (CRL). Based on these criteria, we categorized BPH/5 fetuses into three categories: healthy, compromised, and resorbed (Table 2.2). The HR ratio in BPH/5 healthy fetuses was found to be similar to C57 fetuses, whereas the BPH/5 compromised fetuses had a significantly lower HR ratio. The CRL in all live BPH/5 fetuses was significantly lower than C57. We hypothesized that coincident with these three phenotypic classes of fetal health is a unique molecular signature of variation in placental development. The expression patterns of trophoblastic markers that play a role in placental development have recently been implicated in the pathogenesis of PE; notably, glial cells missing homologue 1 (GCM1) as a regulator of syncytiotrophoblast differentiation[38]. GCM1, a placental specific transcription factor, is essential for proper placental development in human and mouse pregnancy[15, 39]. It is also known to regulate expression of Syncytin B (*SynB*) to ensure proper differentiation and formation of the syncytiotrophoblast (SynT)-II layer of the labyrinth for adequate maternal-fetal exchanges[40]. Syncytin A (*SynA*) serves as a marker for the SynT-I layer and, although it is not regulated by *Gcm1*, its development is interdependent on SynT-II and *SynB*[40]. Therefore, *Gcm1*, *SynA*, and *SynB* expression was investigated in classed BPH/5 fetoplacental units at e10.5. ISH was used to visualize *Gcm1* expression patterns within whole fetoplacental units at e10.5.

Table 2.2 Ultrasound classification of C57 and BPH/5 fetuses at e10.5

	Fetal : maternal HR	CRL
C57	0.125±0.011	4.124±0.076
BPH/5 healthy	0.133±0.001	3.571±0.118*
BPH/5 compromised	0.101±0.020†	2.385±0.125*
BPH/5 resorbed	N/A	N/A

n=40-138; ; HR=heart rate, CRL= crown rump length
*p<0.05 vs C57; †p<0.05 vs C57 and BPH/5 healthy
N/A= not applicable (no viable fetus present)

Compromised and resorbed BPH/5 fetoplacental units showed very little to no signal of the *Gcm1* riboprobe within the labyrinth region of the placenta (Figure 2.5A). Using qRT-PCR, *Gcm1* mRNA was found to be significantly decreased in whole BPH/5 compromised and resorbed fetoplacental units as compared to C57 fetoplacental units at e10.5 (Figure 2.5B). Western analyses were then performed on dissected classed fetoplacental units to assess GCM1 protein expression only in the placenta at e10.5. A defined placenta was not identifiable within the resorbed BPH/5 fetoplacental units and therefore GCM1 protein could not be accurately assessed in those tissues. GCM1 protein was significantly reduced in both BPH/5 healthy and compromised placentae as compared to C57 placentae (Figure 2.5C&D). Furthermore, *SynA* mRNA expression was also significantly decreased in compromised and resorbed BPH/5 labyrinth as compared to C57 at e10.5 by ISH (Figure 2.6A) and in whole fetoplacental units by qRT-PCR (Figure 2.6B). Similarly, *SynB* mRNA was also significantly decreased in BPH/5 compromised and resorbed labyrinth as compared to C57 at e10.5 by ISH (Figure 2.7A) and in whole fetoplacental units by qRT-PCR (Figure 2.7B). These results demonstrate that *Gcm1* mRNA as well as protein along with *SynA* and *SynB* mRNA are downregulated in BPH/5 fetoplacental units at e10.5 and their expression patterns within the placenta were associated with degree of phenotypic severity of the fetal health. This indicates defects in placental development of the labyrinth region, and more specifically, ineffective syncytiotrophoblast differentiation may be a factor that impacts fetoplacental health in this mouse model of PE.

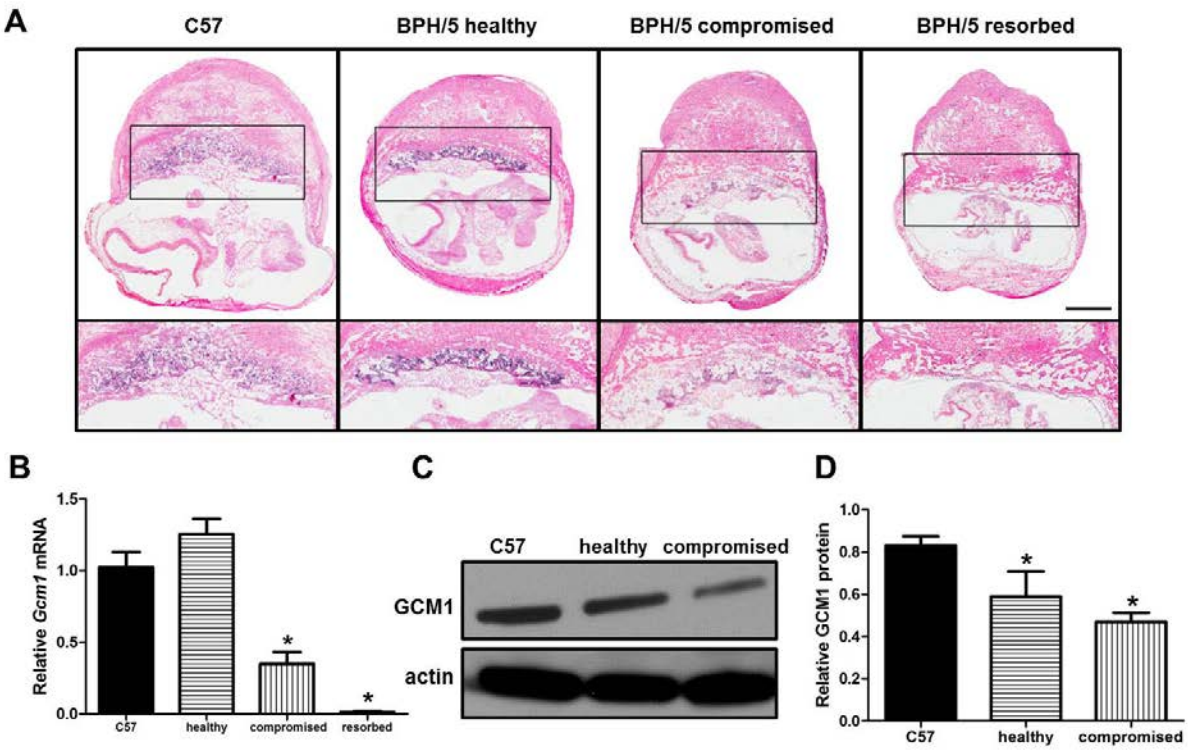


Figure 2.5: Molecular analyses of placental *Gcm1* expression reveals downregulation associated with severity of fetal health status at e10.5 in BPH/5 female mice. (A) Representative ISH images of *Gcm1* expression within whole fetoplacental units from C57 and BPH/5 females after ultrasonographic categorization. Scale bar= 1000μm. **(B)** Quantification of *Gcm1* mRNA by qRT-PCR in e10.5 whole fetoplacental units from C57 and BPH/5 females after ultrasonographic categorization (n=3-6/group) *p<0.05 vs C57 and BPH/5 healthy. **(C)** Representative Western blot gel and **(D)** quantitative summary of GCM1 protein in e10.5 placenta from C57 and BPH/5 healthy and compromised fetoplacental units (n=4-6/group) *p<0.05 vs C57.

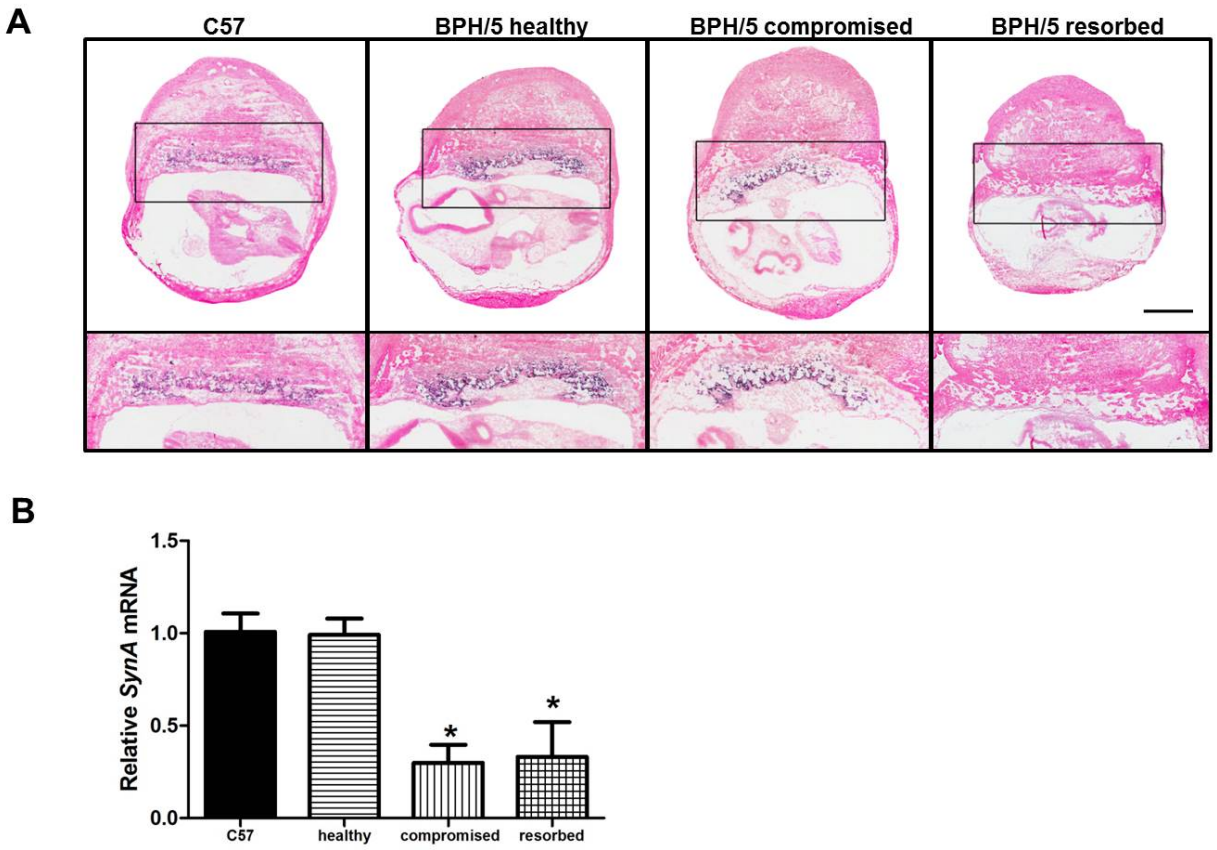
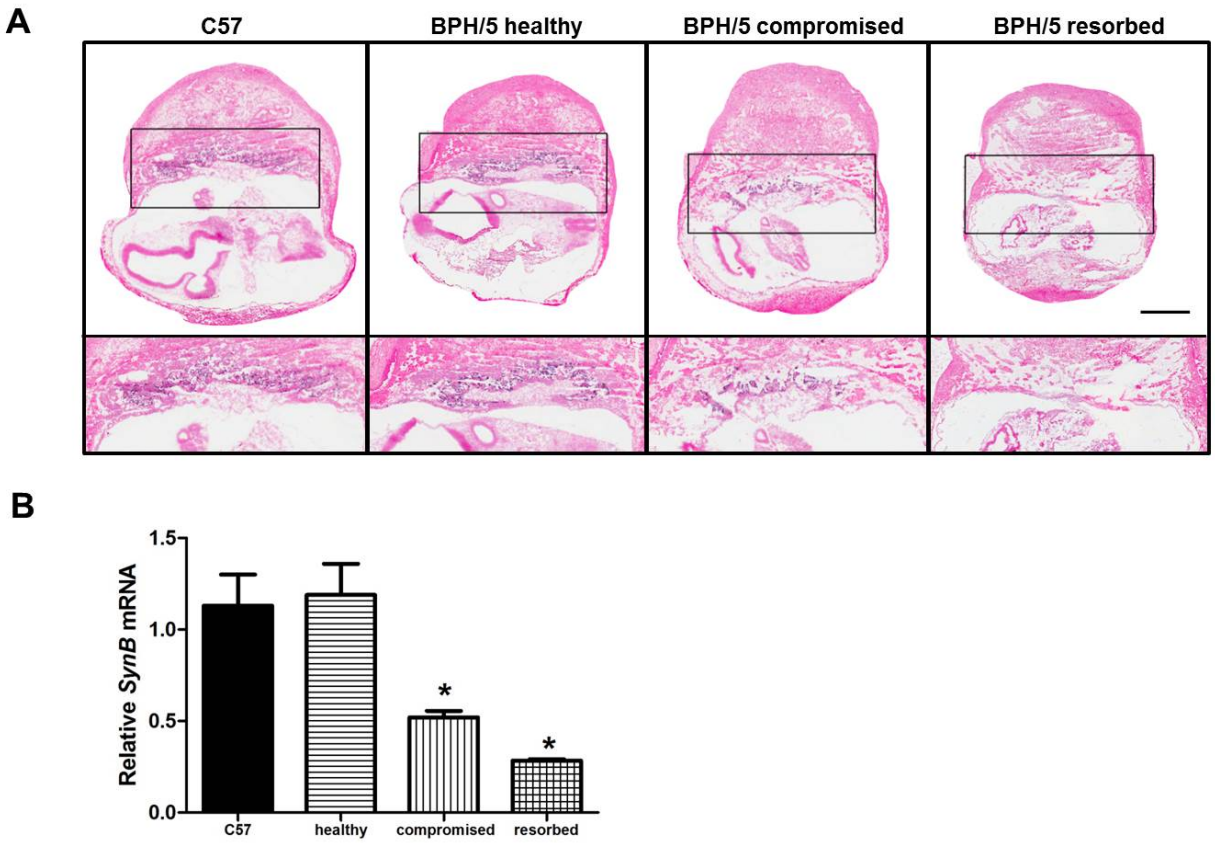


Figure 2.6: Molecular analyses of *SynA* expression reveals downregulation associated with severity of fetal health status at e10.5 in BPH/5 female mice. (A) Representative ISH images of *SynA* expression within whole fetoplacental units from C57 and BPH/5 females after ultrasonographic categorization. Scale bar= 1000µm. **(B)** Quantification of *SynA* mRNA by qRT-PCR in e10.5 whole fetoplacental units from C57 and BPH/5 females after ultrasonographic categorization (n=3-6/group) *p<0.05 vs C57 and BPH/5 healthy.



<u>Figure 2.7:</u> Molecular analyses of *SynB* expression reveals downregulation associated with severity of fetal health status at e10.5 in BPH/5 female mice. (A) Representative ISH images of *SynB* expression within whole fetoplacental units from C57 and BPH/5 females after ultrasonographic categorization. Scale bar= 1000µm. **(B)** Quantification of *SynB* mRNA by qRT-PCR in e10.5 whole fetoplacental units from C57 and BPH/5 females after ultrasonographic categorization (n=3-6/group) *$p<0.05$ vs C57 and BPH/5 healthy.

DISCUSSION

Preeclampsia (PE), a pregnancy associated disorder that affects 8-10% of pregnant women worldwide, is characterized by hypertension and proteinuria in late gestation[41]. PE is a leading cause of maternal and fetal morbidity/mortality and the only known treatment is delivery of the baby and placenta[42]. Although the etiology of PE remains unknown, research on abnormal placentation, including implantation and decidualization prior to placenta formation, has provided insight into the pathophysiologic mechanisms of the disorder[1]. However, early placental development in the context of PE is difficult to investigate in women. The BPH/5 mouse model that spontaneously develops the cardinal signs of PE along with fetoplacental abnormalities also seen in women with PE is a useful tool to examine critical early pregnancy events. Here we provide evidence for periimplantation defects in BPH/5 mice including deferment of implantation timing, delayed and defective decidualization along with evidence of increased hypoxia in implantation sites postimplantation, followed by poor placental development at mid gestation.

In previous preliminary studies, it was observed that BPH/5 mice abnormally space their embryos within the uterine horns at the time of implantation (Ashley Woods; PhD thesis). Embryo crowding in mice is a contributing factor to improper placentation[8, 23, 43]. As poor placental development is a hallmark of PE pregnancies, further work characterizing the periimplantation period in BPH/5 mice was performed. First, it was observed that BPH/5 mice have deferred timing of implantation, approximately 12 hours behind C57 mice, with implantation only being apparent at e4.5am. Additionally, fewer implantation sites are evident at e4.5am and implantation site numbers are only comparable to C57 after another 12 hours at e4.5pm. This important finding of implantation deferment in BPH/5 mice is remarkable considering in humans significant pregnancy loss has been shown to occur when implantation occurs just 24 hours outside the optimal window[21]. By measuring urinary estrogen and progesterone metabolites in healthy women attempting to conceive, ovulation days were determined and this was followed by detection of human chorionic gonadotropin in the urine,

indicative of implantation. It was determined the conceptus implants 6 to 12 days after ovulation and the risk of early pregnancy loss increased with later implantation (8 days post ovulation and beyond)[21]. This work highlighted the importance of implantation timing and pregnancy success.

We went on to further characterize the periimplantation period in BPH/5 mice by next assessing uterine receptivity to the implanting embryo. Previously, it was determined that embryonic development was retarded in BPH/5 mice at e3.5am (Ashley Woods; PhD thesis), but receptivity had not been determined at this time. It has long been known that steroid hormones govern uterine receptivity[4], therefore, maternal serum levels of estradiol-17β and progesterone (P4) were measured before, during, and after the time of receptivity. Unexpectedly, BPH/5 demonstrated their estrogen (E_2) surge by peak serum estradiol-17β prematurely at e2.5am, which was 12 hours ahead of C57 females. More importantly, BPH/5 females showed lower serum estradiol-17β levels compared to C57 females between e2.5pm and e3.5am, prior to the anticipated time of implantation (e3.5pm). C57 females show peak serum estradiol-17β levels at e2.5pm, indicative of their E_2 surge. Taken together, this would suggest deficient uterine receptivity in BPH/5 females. Optimal levels of ovarian hormones are critical in regulating the molecular mechanisms that govern uterine receptivity and thus, implantation in mice[3]. To test the hypothesis that altered serum estradiol-17β profiles in BPH/5 females would interfere with uterine receptivity, we measured the expression of *Lif* in BPH/5 and C57 uteri at e3.5am. The uterine cytokine *Lif* was chosen as it is E_2 responsive and absolutely required for conferring uterine receptivity and implantation in mice[44]. LIF is also believed to be important in human implantation as increased uterine levels have been reported during implantation in women[9]. However, we found comparable amounts of *Lif* mRNA in the uteri of BPH/5 and C57 at e3.5am by ISH and qRT-PCR. This was surprising, but not unexpected as we do indeed see implantation in BPH/5 mice, albeit later than in C57 females. *Lif*-null mice display complete implantation failure[45]. Because of the biphasic nature of *Lif* expression in mice at the time of uterine receptivity and implantation, analysis of *Lif* at e4.5 is also warranted. It is interesting that LIF

can replace the requirement for nidatory E_2[44] and this suggests that its expression in the uterus is more important than the E_2 surge in conferring uterine receptivity.

Upon implantation, uterine stromal cells undergo decidualization in response to the embryo[1, 33, 34]. To further characterize this implantation phenotype in BPH/5 mice, we next examined decidualization events. Using the alkaline phosphatase (ALP) activity assay, we observed dramatic differences in this process between BPH/5 and C57 mice. At the beginning of decidualization (e4.5), uterine stromal cells surrounding the blastocyst begin to proliferate to form the primary decidual zone (PDZ)[46]. The secondary decidual zone (SDZ) is formed by proliferation of the stromal cells surrounding the PDZ and stain ALP-positive[46]. While C57 showed robust ALP activity at e4.5am, this response was completely absent in BPH/5 implantation sites. This was not unexpected as BPH/5 implantation sites at this time stained weakly with pontamine blue, indicative that signaling events involved in increasing uterine vascular permeability were inadequate. Together this suggests that the signaling molecules needed for triggering decidualization were also insufficient. While decidualization requires appropriate ovarian hormone priming of the uterus, it also requires coordinated embryo-uterine crosstalk during attachment. We observed comparable deciduoma formation in BPH/5 and C57 pseudopregnant females. This suggests that the decidualization delay in BPH/5 females may be a result of unsuccessful embryo-uterine crosstalk rather than inadequate ovarian hormone signaling.

Subsequent robust overcompensation of ALP activity was observed at e4.5pm and e5.5am in BPH/5 implantation sites compared to C57. Differentiated decidual cells demonstrate ALP activity; however, over-exuberant ALP activity has not yet been reported in mouse implantation sites. The functional consequence of this finding is unknown, but suggests that decidualization events are no longer regulated in BPH/5 females. Thorough analyses of the signaling molecules involved in mediating decidualization are needed to fully understand these aberrations in decidual cell differentiation in BPH/5 mice.

Following implantation in mice, uterine angiogenesis during decidualization is primarily regulated by vascular endothelial growth factor (*Vegf*) signaling[37]. A number of *Vegf* isoforms are expressed in the mouse uterus, but the *Vegf* isoform 164 has been shown to be most abundant during implantation and decidualization[47]. The predominance of P4 during the postimplantation period of decidualization has been shown to induce *Hif1α* as demonstrated by localization in the uterus of ovariectomized mice after P4 injection[37]. The same expression patterns could not be observed in intact or ovariectomized progesterone receptor (PR)-/- mice treated with E_2 or P4. In addition to P4, hypoxia is also a potent inducer of HIF expression which can bind to the hypoxia response element in the *Vegf* promoter. BPH/5 implantation sites show increased *Hif1α* expression at the peak of decidualization, e7.5, as compared to C57 implantation sites. It is unclear if this increase is due to P4 or hypoxia, but increased $VEGF_{164}$ protein in BPH/5 implantation sites would suggest that increased hypoxia is present as this is one mechanism by which VEGF is associated with ischemic retinal neovascularization in the mouse[48] as well as uterine angiogenesis during decidualization in the mouse[37]. Upregulation of *Hif1α* and $VEGF_{164}$ protein in BPH/5 implantation sites predates the previously described mid gestation placental increases in reactive oxygen species in BPH/5[49]. Because it is known that placental hypoxia is a hallmark of PE, we next investigated fetal health status and the associated placental development at the very earliest stages in fetoplacental development.

Our lab has previously reported smaller litter sizes in BPH/5 mice due to fetal demise at mid gestation[30]. This was attributed to resorptions that were first evident by e12.5[30]. However, using ultrahigh frequency ultrasound, longitudinal examination of BPH/5 pregnancies revealed 3 phenotypic variations in fetal health status as early as e10.5 (Ashley Woods; PhD thesis). These findings were confirmed herein. Healthy BPH/5 fetuses could be found at e10.5 that had similar fetal HRs to C57 fetuses at this time point, while compromised BPH/5 fetuses had significantly slower fetal HRs and importantly, both groups of BPH/5 fetuses had shorter CRLs as compared to C57. The resorbed BPH/5 fetuses were classified as such by the lack of a visible fetus within a gestational sac and/or lack of a fetal heartbeat. These fetal pathologies mirror those seen in PE

pregnancies that exhibit fetal growth restriction (FGR) with or without fetal demise[50, 51]. This time point in mouse pregnancy is crucial to fetal health as extraembryonic tissues fuse during chorioallantoic attachment to form the placenta proper. It is defects in this process that account for the majority of mid gestation embryonic lethality[14]. Primary villi form by branching morphogenesis to expand the labyrinth and fill the villous folds with blood vessels to facilitate maternal-fetal exchanges[52]. In human and mouse, the *Gcm1* homolog has been demonstrated to regulate placental labyrinth formation[53] and trophoblast differentiation[15]. We therefore examined *Gcm1* expression in BPH/5 healthy, compromised, and resorbed fetoplacental units at e10.5.

Gcm1 and its target gene, *SynB*, and the closely related, *SynA* showed significant downregulation in compromised and resorbed BPH/5 fetoplacental units at e10.5 by qRT-PCR and this was confirmed to be due to decreased expression of these genes within the labyrinth region of the placenta. Western blot analysis of protein expression in BPH/5 and C57 placenta showed GCM1 was significantly decreased in BPH/5 healthy and compromised fetoplacental units. It is curious that BPH/5 healthy fetoplacental units showed similar mRNA expression of *Gcm1* to C57 fetoplacental units, but significantly decreased expression of GCM1 protein. Our lab has been investigating placental microRNAs in BPH/5 mice and has data supporting the upregulation of the microRNAs that specifically target *Gcm1* mRNA (Sones & Zhou, unpublished data). Therefore, posttranslational modifications may be at work to decrease GCM1 in all BPH/5 placentae. It would make sense that all BPH/5 placentae have decreased GCM1 as all BPH/5 fetuses demonstrate smaller CRLS at e10.5, indicative of *in uteri* FGR, and defects in the labyrinth region would be a contributing factor to this phenomenon.

GCM1 expression is decreased in placenta of human PE patients[20]. Moreover, studies have shown that GCM1 degradation is a result of hypoxia *in vitro* using human trophoblast cell lines[54]. Furthermore, reduced placental expression of syncytins was reported to correlate with severity of PE in women[19]. *SynA* deficient placentae have altered architecture of the syncytiotrophoblast-containing labyrinth and reduction of fetal blood vessel spaces[55]. *SynB* knockout mice exhibit impaired formation of the SynT-II layer and late-onset FGR[16]. The

Adamson laboratory has recently shown that wildtype female mice carrying *Gcm1* hypomorph embryos demonstrate increased late gestational mean arterial pressure along with placental pathologies indicative of dysregulated trophoblast differentiation and abnormal placental vascularity[38]. These findings support a strong role for GCM1 and placental defects in the pathogenesis of PE.

These results presented here establish that BPH/5 mice have profound periimplantation defects, including embryo clustering along with deferred implantation but without alterations in expression of the uterine receptivity-specific gene, *Lif*. Furthermore, BPH/5 mice have delayed and defective decidualization marked by overexpression of *Hif1α* and its downstream product, $VEGF_{164}$. BPH/5 mice also have significant abnormalities in fetoplacental development after implantation at mid gestation. *Gcm1* mRNA as well as protein is dysregulated in BPH/5 fetoplacental units at e10.5 and its downregulation is associated with degree of phenotypic severity of fetal health status. Syncytin genes, *SynA* and *SynB*, are also decreased, suggesting that disrupted syncytiotrophoblast differentiation in the labyrinth may be a factor that impacts fetal health and placental development in this mouse model of PE.

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CHAPTER THREE:

DYSREGULATED COX2 SIGNALING DURING THE PERIIMPLANTATION PERIOD HAS MARKED "RIPPLE EFFECTS" ON ADVERSE PREGNANCY OUTCOMES IN BPH/5 MICE

SUMMARY

Although preeclampsia (PE) is diagnosed during the second half of gestation with the onset of maternal disease (hypertension and proteinuria), this disorder of pregnancy also has significant consequences on fetal outcomes. PE pregnancies are often characterized by fetal demise, fetal growth restriction (FGR), and frequently results in the delivery of a preterm baby. Pregnancy success relies on coordinated embryo-uterine interactions, and defects in these events are thought to have adverse "ripple effects" that can lead to poor outcomes, such as PE and FGR. The BPH/5 mouse model spontaneously develops the maternal PE syndrome along with abnormal placentation and poor fetal outcomes. We used BPH/5 mice to investigate the molecular mediators of implantation and decidualization, and test if aberrations in their signaling had "ripple effects" throughout pregnancy. Abnormalities during the periimplantation period in this model, including deferred implantation and defective decidualization (see Chapter 2), are associated with an aberrant signature of uterine signaling molecules, *Lif*, *Hoxa10*, *Bmp2*, and *Ptgs2* (the gene encoding Cox2). In particular, overexpression of *Ptgs2* and Cox2, a pivotal molecule during the periimplantation period, was observed in BPH/5 implantation sites. Administration of a selective Cox2 inhibitor, celecoxib, early in pregnancy normalized Cox2 expression as well as downstream $VEGF_{164}$ and PGE_2 levels in BPH/5 implantation sites. Celecoxib administration in early pregnancy also improved mid gestation fetoplacental health and ameliorated late gestation FGR in BPH/5 mice. These results implicate Cox2 as a causal factor in the poor fetal status in BPH/5 mice and provide evidence that the fetal morbidity/mortality associated with PE originate in the periimplantation period.

INTRODUCTION

Early pregnancy events, including implantation and decidualization, depend on tightly regulated signaling pathways to ensure pregnancy success. Aberrations in the expression of implantation and decidualization molecular mediators can have "ripple effects" that may lead to poor pregnancy outcomes, including abnormal placentation, fetal loss or growth restriction, and even infertility[1]. The role of implantation and decidualization molecules in the pathogenesis of preeclampsia (PE) and PE-associated outcomes, such as fetal growth restriction (FGR) is undefined. Identifying molecular dysregulations during the periimplantation period in BPH/5 mice would help elucidate the mechanisms by which early pregnancy defects contribute to downstream poor fetal outcomes in PE pregnancies.

In rodents and humans, implantation is the first coordinated encounter between mother and baby[1,2]. Decidualization, or the differentiation of uterine stromal cells into epithelial-like decidual tissue, is required in both species to complete implantation. Unlike humans that decidualize every month during the luteal phase of the menstrual cycle, mice require the physical interaction of the blastocyst with the uterus to undergo decidualization during pregnancy[2]. It first begins during the periimplantation period after the attachment reaction at embryonic day (e) 4.5 in mice[1]. Decidual cells first aggregate around the implanting embryo and then dominate the mesometrial pole of the uterus, the entry point of the uterine vasculature[4, 5]. This transitory process is characterized by significant vascular remodeling of the uterus between e5.5 and e7.5 to ensure proper placental blood flow[1]. Therefore, decidualization is absolutely necessary for pregnancy success.

Critical to implantation and decidualization are vasoactive agents, such as leukotrienes, histamines, and prostaglandins (PGs), along with other signaling molecules, including cytokines, growth factors, transcription factors, and morphogens that act as autocrine, paracrine, and juxtacrine factors[3]. In particular, cyclooxygenase 2 (Cox2)-derived PGs are crucial for implantation, decidualization, placental formation and angiogenesis early in pregnancy[1, 4, 5]. The pathways involved in these processes are initiated by ovarian steroid hormone signaling to

estrogen (E_2) and progesterone (P4)-responsive genes, Leukemia inhibitory factor (*Lif*) and Homeobox A10 (*Hoxa10*) respectively[4]. *Lif*-null mice experience implantation failure, which is believed to be partially due to dysregulation of downstream implantation signaling molecules, including prostaglandin synthase 2 (*Ptgs2*; the gene encoding Cox2)[6]. *Hoxa10*-/- female mice are infertile primarily due to reduced stromal cell proliferation and consequent failure to undergo decidualization despite blastocyst attachment[1]. Bone morphogenic protein 2 (*Bmp2*) is an important intermediary as it is downstream of *Lif* and *Hoxa10*, and directly regulates *Ptgs2*[7]. Cox2 is thought to be a final common factor upon which the upstream implantation and decidualization molecules including *Lif*, *Hoxa10* and *Bmp2* converge [4, 7].

Expression of *Ptgs2* is first detected in the uterine luminal epithelium and stroma around the blastocyst beginning at e4.5 with the attachment reaction[8]. Its expression increases with decidualization, peaking at e7.5 in the uterine stroma and becoming nearly undetectable by the time the placenta is formed. *Ptgs2*-/- mice are subfertile with defects in ovulation and fertilization[9]. Furthermore, *Ptgs2*-/- mice have compromised decidualization and implantation, indicating that Cox2-derived PGs are essential for these processes[9].

The predominant PG in the mouse implantation site is prostacyclin (PGI_2), and due to its potent vasoactive properties, is thought to be associated with the increased vascular permeability localized in the uterus around the blastocyst at the time of implantation and decidualization[5]. Importantly, its expression is found to be in the same stromal compartment as *Ptgs2*[5]. Moreover, *Ptgs2*-/- mice show marked improvement in decidualization and implantation when given carbaprostacyclin (cPGI), a stable analog of PGI_2[5]. PGE_2 is the second most abundant PG in the mouse implantation site during implantation and decidualization and it is a potent vasoconstrictor in the placental vasculature[10]. The other PGs, thromboxane (TX) B_2 and $PGF_{2\alpha}$, are present in lower levels[5]. PGI_2 has vasodilator effects and is thought to have counter regulatory effects on TXA_2, a vasoconstrictor, in order to maintain physiologic balance. Some have proposed that a PGI_2 deficiency or PGI_2/TX imbalance during pregnancy may be associated with PE[10]. Defective implantation and decidualization in *Ptgs2*-/- mice is thought to be due to dysregulated vascular

events and defective vascular endothelial growth factor (VEGF) signaling[11]. Importantly, women with PE often display angiogenic imbalances early in pregnancy and inadequate uterine angiogenesis/vascularity at the time of implantation has been proposed as a mechanism[12]. Therefore, it is of great importance to investigate the period of decidualization and the Cox2 signaling pathway in the context of PE.

Our laboratory showed that the BPH/5 strain of mice spontaneously develops a PE-like syndrome during pregnancy, including late-gestational hypertension and proteinuria, which resolve upon delivery[13]. BPH/5 pregnancies are further characterized by abnormal placentation and fetal loss, with approximately one third of all implantation sites resorbed by mid to late gestation[13, 14]. This phenomenon results in smaller live litter sizes and lower birth-weights in those neonatal pups that do survive[13, 14]. BPH/5 mice also demonstrate placental oxidative stress and angiogenic imbalances[15, 16]. At early gestational time points (e9.5-e12.5), approximately the time of early fetal demise, BPH/5 mice have decreased circulating and placental VEGF protein[16]. This suggests that the initial angiogenic insult in BPH/5 mice is likely to occur early in pregnancy.

Periimplantation events, including implantation and decidualization, precede placenta formation and dysregulation at any point during these events may create adverse "ripple effects" throughout pregnancy. Because these initiating pathologies occur during a woman's first trimester, it is extraordinarily difficult to study due to logistical and ethical concerns. Therefore, the BPH/5 model is critically important for longitudinal investigation of these early pregnancy events. Because BPH/5 mice have defects in implantation and decidualization as well as abnormal placentation, we tested the hypothesis that BPH/5 implantation sites would show a unique molecular signature that could be linked to the retarded fetoplacental development and poor pregnancy outcomes in this model. Here we show that abnormal periimplantation processes accompanied by dysregulated expression of critical implantation-specific genes were evident in BPH/5 pregnancies. Furthermore, early correction of Cox2 levels with the selective inhibitor celecoxib improved fetal morbidity and mortality in this model.

METHODS

Mice

Virgin BPH/5 and control C57Bl/6 (C57) mice (8-12 weeks old) were obtained from in-house colonies. Strain-matched matings were performed with BPH/5 and C57 to induce pregnancy. The day of vaginal plug detection is defined as e0.5. Celecoxib (Sigma Aldrich, St. Louis, MO) was administered to pregnant mice at e6.5 by oral gavage at a dose of 10 mg/kg body weight (dissolved in 100uL sesame oil), an anti-inflammatory dosage tested safe during early rodent pregnancy[17]. For analysis of pregnancy outcomes, C57 and BPH/5 pregnant females treated with vehicle (sesame oil) or celecoxib at e6.5 were euthanized at e7.5, e10.5, e12.5, or e18.5. Uterine horns were exposed and litter size was counted. Placentae and fetuses were dissected free from the gestational sacs and weights were recorded. Resorptions were visualized by ultrasound and/or lack of a fetus within a gestational sac. Care of the mice met or exceeded the standards set forth by the National Institutes of Health Guide for the Care and Use of Laboratory Animals, USDA regulations, and the AVMA Panel on Euthanasia.

Pontamine blue perfusion studies

To collect implantation sites at precise time points, pregnant females at e4.5am (8 00h) and e5.5am (8 00h) were given a single injection of 100 uL of 1% pontamine blue (BDH; Radnor, PA) dissolved in saline, administered via tail vein, and after 5 minutes mice were euthanized. Implantation sites were then collected and stored for histologic and molecular analyses.

Ultrasound assessment of fetoplacental health

Pregnant C57 and BPH/5 mice from intra-strain matings were anesthetized using 2% isofluorane. A Vevo 770 ultra-high frequency ultrasound (Visualsonics, Toronto, ON) was used to examine fetal health at e10.5. Preliminary studies showed that e10.5 was the earliest time point in which the greatest variation in embryonic health could be observed prior to fetal death and resorption (Ashley Woods; PhD thesis). A ventral incision was made to exteriorize the uterus and examine

individual implantation sites using a 40MHz probe according to published methods[18]. Body temperature of the pregnant females was maintained and monitored throughout the recording procedure. Fetal heart rates (HR) were measured and reported as a ratio of fetal heart rate to maternal heart rate (HR ratio) to control for minor variations in anesthesia. Fetal crown rump lengths (CRL) were recorded when orientation permitted[19]. BPH/5 fetuses that had similar HR ratios as C57 were classified as healthy, while BPH/5 fetuses that had significantly lower HR ratios and reduced CRLs compared to C57 were considered compromised. Resorptions were classified by the lack of a fetus within a gestational sac and/or a fetal heart.

Quantitative real-time PCR

Primers were designed using Primer Design software provided by Integrative DNA Technologies (www.idtdna.com, Coralville, IA). Total RNA was extracted using TriZol (Life Technologies, Grand Island, NY) as previously described[20]. Reverse transcription was performed using Quanta kits (Gaithersburg, MD) per the manufacturer's instructions, and quantitative real-time PCR (qRT-PCR) was performed with 25ng cDNA in triplicate using SybrGreen reagents (Quanta) on an ABI 7500 Fast System (Applied Biosystems, Life Technologies, Grand Island, NY). Data was expressed as 2^-dCt relative to housekeeper gene expression (*18S rRNA*). Primer sequences can be found in Table 3.1.

***In situ* hybridization with radioactive isotopes**

In situ hybridization (ISH) was performed as previously described[9]. Implantation sites were collected and snap frozen. Tissue sections (12 μm) were mounted on poly-L-lysine coated slides, fixed in 4% PFA solution in phosphate-buffered saline (PBS) at 4°C and acetylated. After pre-hybridization, sections were hybridized at 45°C for 4 hours in 50% formamide buffer containing ^{35}S-labeled sense or anti-sense cRNA probes. After hybridization, RNase A-resistant hybrids were detected by autoradiography using Kodak NTB-2 liquid emulsion. Parallel sections were hybridized with sense probes and served as negative controls.

Western Blotting

Whole implantation sites were visualized and collected at e7.5 from untreated as well as vehicle and celecoxib treated intra-strain timed matings. Tissues were homogenized in 10mM sodium phosphate buffer containing protease inhibitors (Roche). Protein concentrations were determined by bicinchoninic acid (BCA) reagents followed by Nanodrop ND-1000 spectrophotometer readings (Life Technologies). Equal amounts of protein (50ug) were separated on 10% SDS-PAGE gel and transferred to nitrocellulose membrane. Membranes were blocked in 5% non-fat milk in PBST. Probing with primary antibody was performed for Cox2 protein levels (1:500 rabbit anti-Cox2 Cayman Chemicals, Ann Arbor, MI) and $VEGF_{164}$ protein levels (1:500 goat anti-$VEGF_{164,}$ Sigma Aldrich), followed by incubation with a secondary antibody (1:5000 goat anti-rabbit and donkey anti-goat IgG peroxidase respectively, Santa Cruz Biotechnologies, Santa Cruz, CA). Blots were also probed with a monoclonal anti-actin antibody (1:5000, Sigma Aldrich) and goat-anti-mouse HRP-conjugated secondary antibody (1:5000, Santa Cruz Biotechnologies). Signals were detected by chemiluminescence with Pierce ECL Western Blotting Substrate (Life Technologies). Densitometric analysis was performed using ImageJ software (NIH) and band intensities were normalized to actin.

Measurement of Prostaglandins

The amount of prostaglandin E_2 (PGE_2) and prostacyclin (PGI_2; stable metabolite 6-keto Prostaglandin $F_{1\alpha}$) in each sample was measured by ELISA (Cayman Chemicals) as previously described[21]. Individual implantation site tissues were weighed and homogenized in buffer (0.1M PBS, pH 7.4 containing 1mN EDTA and 10μM indomethacin). Ethanol was then added to the sample, vortexed, and protein precipitation was removed by centrifugation at 3000g for 10 minutes. The supernatant was removed and evaporated by vacuum centrifugation. Samples were reconstituted with 1M acetate buffer to pH 4.0 and passed through a solid phase extraction cartridge (Cayman Chemicals) for purification. Samples were evaporated under a gentle stream of nitrogen and reconstituted with 500 μL enzyme immunoassay (EIA) buffer. The amount of

PGE_2 or 6-keto Prostaglandin F_1 was measured by the ELISA kit as per manufacturer's instructions. The intra- and inter-assay coefficients of variation were <10%. Using monoclonal antibodies the detection limit was 15pg/mL.

Measurement of Total Urinary Protein

Urine protein levels were measured in voided urine collected at the time of euthanasia from pregnant C57 and BPH/5 mice at e18.5. Twenty microliters were pipetted onto protein indicator of a Multistix 10SG dipstick (Siemens, Berlin, Germany) and colorimetric readings were recorded after 60 seconds. Semiquantitative amounts of protein in urine were assigned with a '+' symbol, with +=30 mg/dL, 2+=100 mg/dL, 3+= 300 mg/dL, and 4+= ≥2000 mg/dL, based on color change. Trace and negative amounts of protein were also determined based on the lack of color change. Readings are sensitive to 15-30 mg/dL albumin.

Statistical Analyses

All data are expressed as mean ± SEM. Multiple comparisons were made using a one or two-way ANOVA and where appropriate, followed by Newman-Keuls test for significance. A student's *t* test was used for all others. Statistical significance was defined as $p<0.05$.

Table 3.1: Primer sequences used for quantitative Real Time PCR (qRT-PCR) analyses

Gene name	Forward and reverse sequences (5' to 3')
Lif	TCAGCGACAAAGTTACTCCACCGT
	AAGTGATGACAAAGCCCAACAGGC
Bmp2	TGTGGGCCCTCATAAAGAAGCAGA
	AGCAAGCTGACAGGTCAGAGAACA
Hoxa10	TTAGCTAAAGGGCTTGACCTGGCT
	AGAGAGGTTTCCTTCTCTTGCCCA
Ptgs2	ACTGGGCCATGGAGTGGACTTAAA
	AACTGCAGGTTCTCAGGGATGTGA
Ptgs1	CTTTGCACAACACTTCACCCACCA
	TTGAAGAGCCGCAGGTGATACTGT
18S	GTAACCCGTTGAACCCCATT
	CCATCCAATCGGTAGTAGCG3

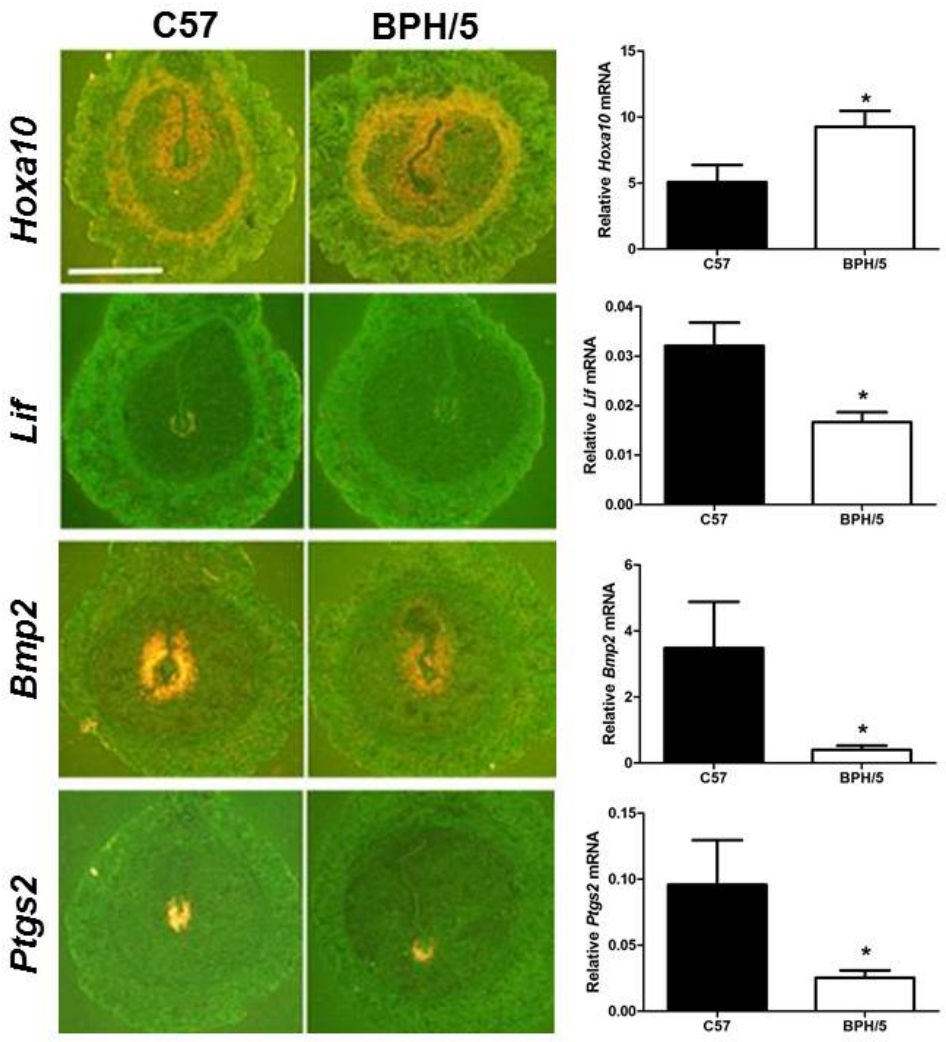
RESULTS

BPH/5 mice show aberrant gene expression in the periimplantation uterus

To better understand dysregulated implantation and decidualization events in BPH/5 females, we assessed the status of Homeobox a 10 (*Hoxa10*), Leukemia inhibitory factor (*Lif*), Bone morphogenic protein 2 (*Bmp2*), and Prostaglandin synthase 2 (*Ptgs2*) expression, key signaling molecules in the gravid uterus during the periimplantation period[1, 4]. Using qRT-PCR of whole implantation sites and ISH of implantation site sections adjacent to the embryo, we could measure gene expression and also localize that expression within the implantation site. Our results show that e4.5am implantation sites in BPH/5 females have increased levels of *Hoxa10*, whereas the expression of *Lif*, *Ptgs2*, and *Bmp2* was significantly lower compared to C57 controls (Figure 3.1). *Lif* has biphasic expression during early pregnancy: first on the morning of e3.5 (receptive phase) and again at the time of attachment[8]. We have shown earlier by ISH that *Lif* expression in the BPH/5 uterus at e3.5 was comparable to that of C57 females, suggesting that uterine receptivity in BPH/5 females with respect to *Lif* expression is adequate (Figure 2.1B & C). However, we show here that *Lif* expression at e4.5am was lower in BPH/5 implantation sites. The observation of downregulated *Ptgs2* and *Bmp2* in BPH/5 implantation sites at e4.5am was not unexpected in light of the faint staining of pontamine blue and reduced decidualization seen at this time point, corroborating the lack of ALP activity in BPH/5 e4.5am implantation sites (Chapter 2). Interestingly, we found that *Bmp2* and *Hoxa10* expression were comparable in BPH/5 and C57 implantation sites by e5.5am, but *Ptgs2* was now significantly upregulated (Figure 3.2).

BPH/5 mice show aberrant *Ptgs2* and Cox2 expression in the post-implantation decidua

The molecular periimplantation defects observed in BPH/5 females ultimately converge on persistently dysregulated *Ptgs2* expression. Using the same approach, qRT-PCR of whole implantation sites and ISH of implantation site sections adjacent to the embryo, we examined gene expression of *Hoxa10*, *Bmp2*, and *Ptgs2* in e7.5am implantation sites from BPH/5 and C57



<u>Figure 3.1:</u> BPH/5 uteri exhibit altered expression of molecules critical to implantation at e4.5am of pregnancy. Representative ISH (left panels) and quantification by qRT-PCR (right panels) of key transcripts (*Hoxa10, Lif, Bmp2, and Ptgs2*) in implantation sites at e4.5am (n=3-9) *p<0.05. Scale bar= 500 µm.

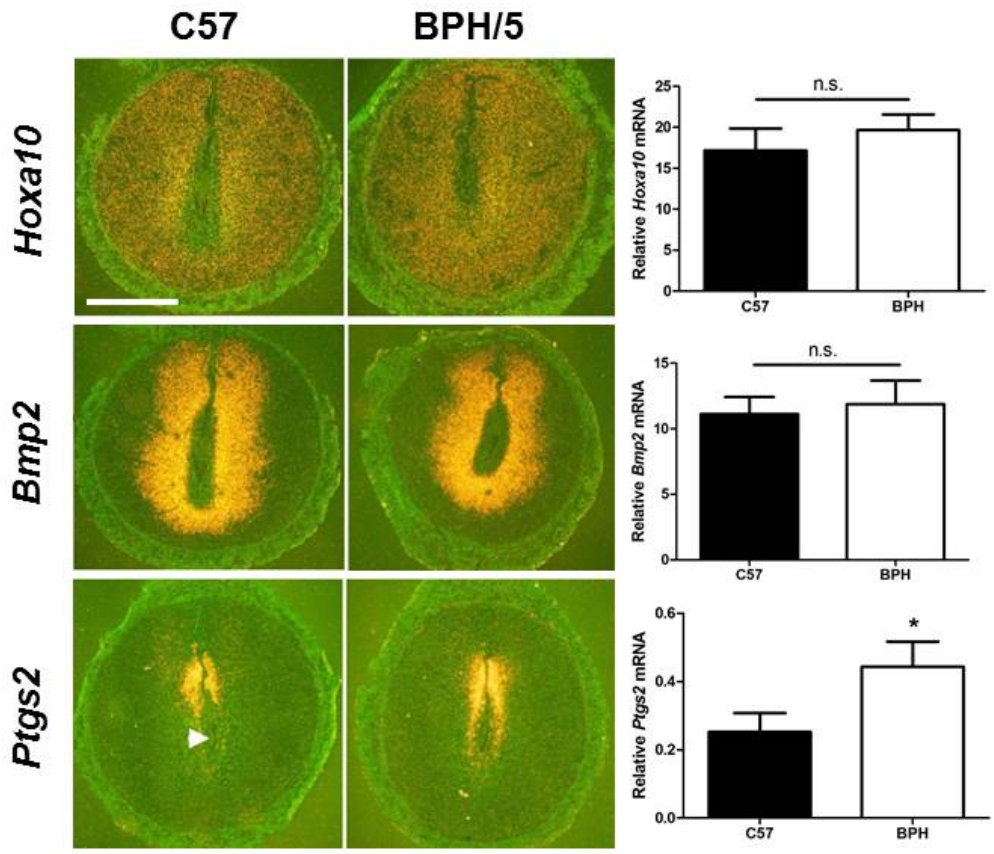


Figure 3.2: ***Ptgs2*** **is overexpressed in BPH/5 uteri at e5.5am of pregnancy.** Representative ISH (left panels) and quantification by qRT-PCR (right panels) of *Hoxa10, Bmp2, and Ptgs2* in implantation sites at e4.5am (n=3-9) *p<0.05. Scale bar= 500 µm. White arrow indicates the embryo.

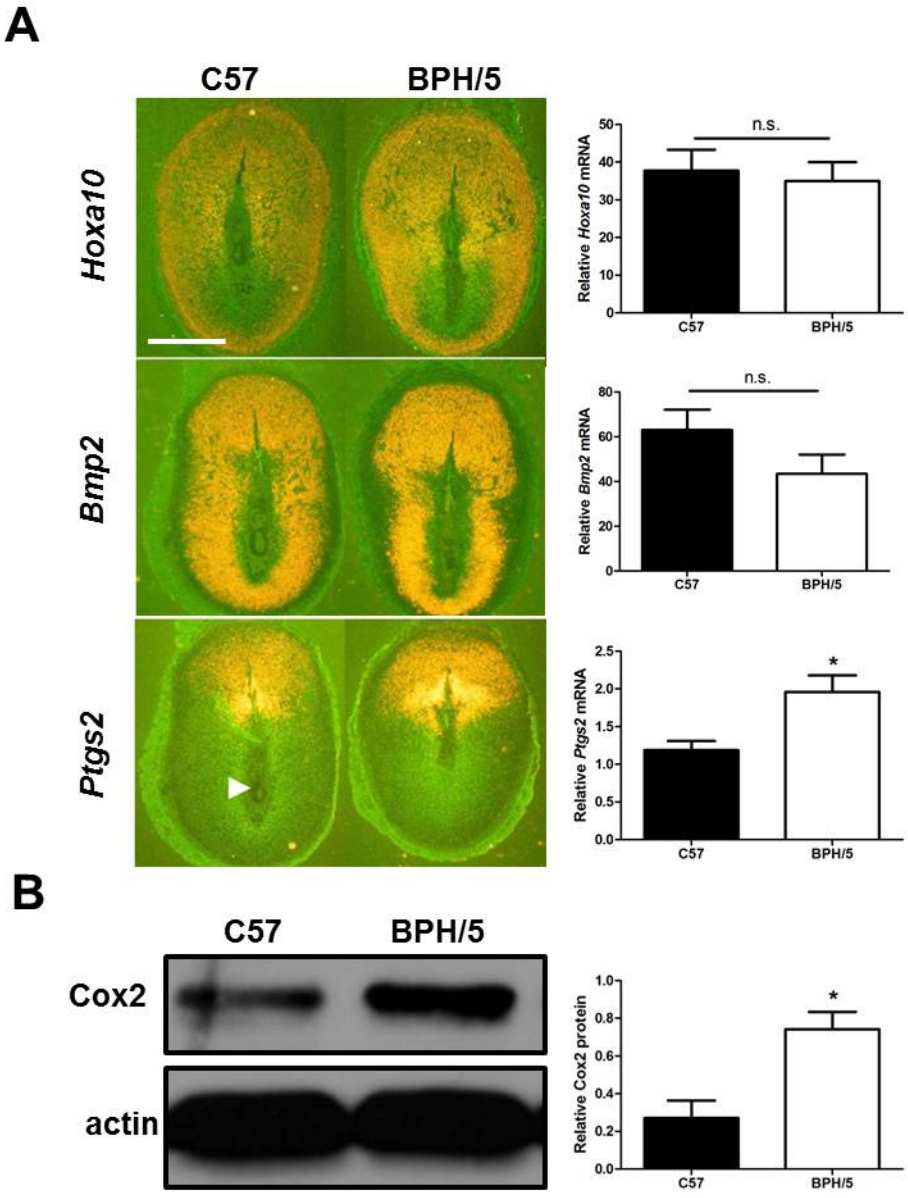
control females. Again we found that *Bmp2* and *Hoxa10* expression were still comparable between BPH/5 and C57 implantation sites by e7.5am, but *Ptgs2* had persistent upregulation in BPH/5 implantation sites concentrated at the mesometrial pole of the decidua (Figure 3.3A). This finding was supported by increased expression of Cox2 protein within whole implantation sites from BPH/5 females at e7.5 compared to C57 (Figure 3.3B).

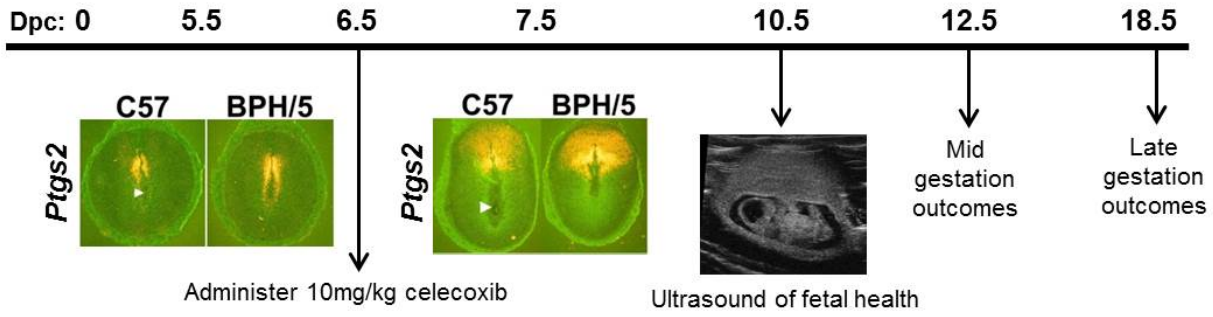
Selective inhibition of Cox2 has efficacious effects on BPH/5 implantation sites

Since Cox2-derived PGs are crucial for decidualization, placentation and placental angiogenesis[1, 4, 9, 22] and we observe defects in all these processes during pregnancy in BPH/5 mice, we next tested the effect of a selective Cox2 inhibitor (celecoxib) on fetoplacental development in BPH/5 females. Celecoxib is classified as a non-steroidal anti-inflammatory drug (NSAID) that selectively inhibits Cox2 and not Cox1[17]. It is of note that prostaglandin synthase 1 (*Ptgs1*; the gene encoding Cox1) mRNA expression was similar between BPH/5 and C57 whole implantation sites at e7.5 (data not shown), the time at which we see maximal *Ptgs2* expression in BPH/5 implantation sites. Studies were conducted using a dose of celecoxib found to be compatible with implantation and decidualization in female rats[17]. Anti-implantation effects and decreased decidualization of uterine horns was seen at 80 times the dose we administered here[17]. The timing of celecoxib administration on e6.5 was selected so as to not interfere with implantation and early decidualization, since Cox2 is essential for these processes, but rather to target misregulated Cox2 during peak decidualization[17] (Figure 3.4).

First, we confirmed that our dose of celecoxib did not negatively impact implantation in C57 or BPH/5 females (Figure 3.5A). Cox2 protein levels were normalized in BPH/5 e7.5 implantation sites just 24 hours after celecoxib administration (Figure 3.5B & C). We also tested the downstream effects of Cox2 inhibition by measuring $VEGF_{164}$ protein, PGE_2 and 6-keto $PGF_{1\alpha}$ levels in BPH/5 and C57 implantation sites at e7.5 after celecoxib or vehicle administration. Celecoxib normalized $VEGF_{164}$ protein levels in BPH/5 implantation sites after celecoxib administration (Figure 3.5D &E). We also found a reduction in PGE_2 levels in BPH/5

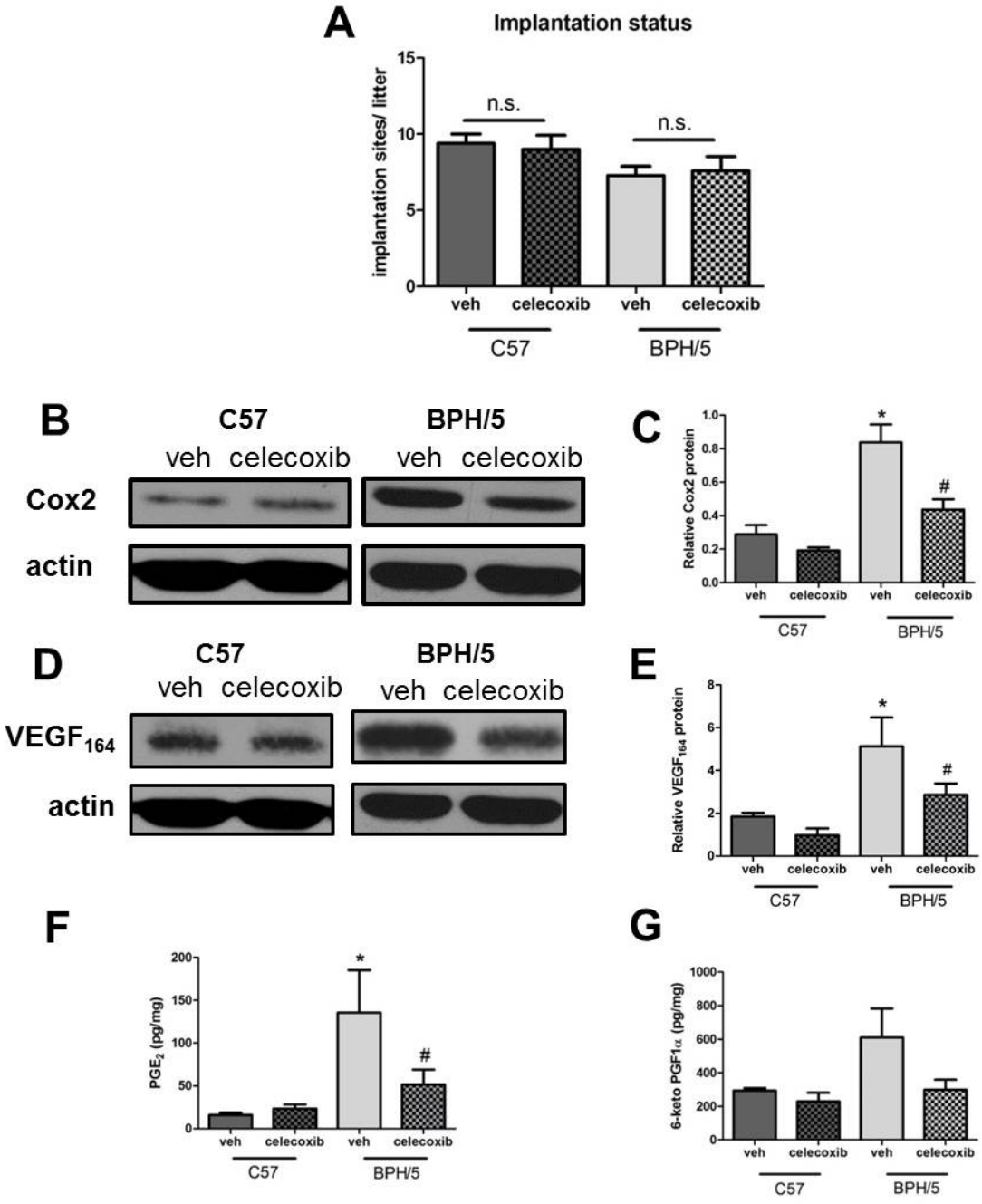
Figure 3.3: ***Ptgs2*** **and Cox2 is persistently upregulated in postimplantation BPH/5 implantation sites at e7.5am of pregnancy. (A)** Representative ISH (left panels) and quantification by qRT-PCR (right panels) of *Hoxa10, Bmp2, and Ptgs2* in implantation sites at e7.5am (n=3-9). **(B)** Representative Western blot (left panel) and quantitative summary (right panel) of Cox2 protein expression in implantation sites at e7.5. Data are expressed as Cox2 protein relative to actin (n=4),*$p<0.05$. Scale bar= 500 μm.


A
C57
BPH/5
Hoxa10
Bmp2
Ptgs2
n.s.
Relative Hoxa10 mRNA
Relative Bmp2 mRNA
Relative Ptgs2 mRNA
*
B
Cox2
actin
Relative Cox2 protein



<u>Figure 3.4:</u> Schematic showing experimental strategy used to selectively inhibit Cox2. Celecoxib (10 mg/kg body weight) was administered by oral gavage to pregnant BPH/5 and C57 females on e6.5 after implantation but before peak decidualization (e7.5). Separate cohorts were analyzed at e10.5 via ultrasound and mid gestation (e12.5) and late gestation (e18.5) litter sizes were counted along with fetal and placental weight measurements. Dpc= days post-coitus.

Figure 3.5: Celecoxib administration targets postimplantation Cox2 and $VEGF_{164}$ expression, and PGE_2 synthesis in e7.5 BPH/5 implantation sites. (A) Number of implantation sites counted per litter at e7.5 in C57 and BPH/5 females after vehicle (veh) and celecoxib administration (n=4-7 litters). **(B)** Representative western blot gels of Cox2 and actin protein in C57 e7.5 implantation sites (left panels) and in BPH/5 e7.5 implantation sites (right panels) after veh and celecoxib administration. **(C)** Quantitation of Cox2 protein relative to actin in C57 and BPH/5 e7.5 implantation sites after veh and celecoxib administration. (n=3 implantation sites per veh group and n=4 implantation sites per celecoxib group). **(D)** Representative western blot gels of $VEGF_{164}$ and actin protein in C57 e7.5 implantation sites (left panels) and in BPH/5 e7.5 implantation sites (right panels) after veh and celecoxib administration. **(E)** Quantitation of $VEGF_{164}$ protein relative to actin in C57 and BPH/5 e7.5 implantation sites after veh and celecoxib administration. (n=3 implantation sites per veh group and n=4 implantation sites per celecoxib group). **(F)** Measurement of endogenous prostaglandin E_2 (PGE_2) in C57 and BPH/5 e7.5 implantation sites after veh and celecoxib administration (n=4 implantation sites per veh group and n=5 implantations sites per celecoxib group). **(G)** Measurement of endogenous prostacyclin (PGI_2) via the stable metabolite 6-keto Prostaglandin $F_{1\alpha}$ (6-keto $PGF_{1\alpha}$) in C57 and BPH/5 e7.5 implantation sites after veh and celecoxib administration (n=4 implantation sites per veh group and n=5 implantations sites per celecoxib group). *$p<0.05$ vs C57 veh and # $p<0.05$ vs BPH/5 veh.


A
Implantation status
implantation sites/ litter
15
10
5
0
n.s.
n.s.
veh
celecoxib
veh
celecoxib
C57
BPH/5
B
C57
BPH/5
veh celecoxib
veh celecoxib
Cox2
actin
C
Relative Cox2 protein
1.0
0.8
0.6
0.4
0.2
0.0
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#
veh
celecoxib
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C57
BPH/5
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BPH/5
veh celecoxib
veh celecoxib
VEGF164
actin
E
Relative VEGF164 protein
8
6
4
2
0
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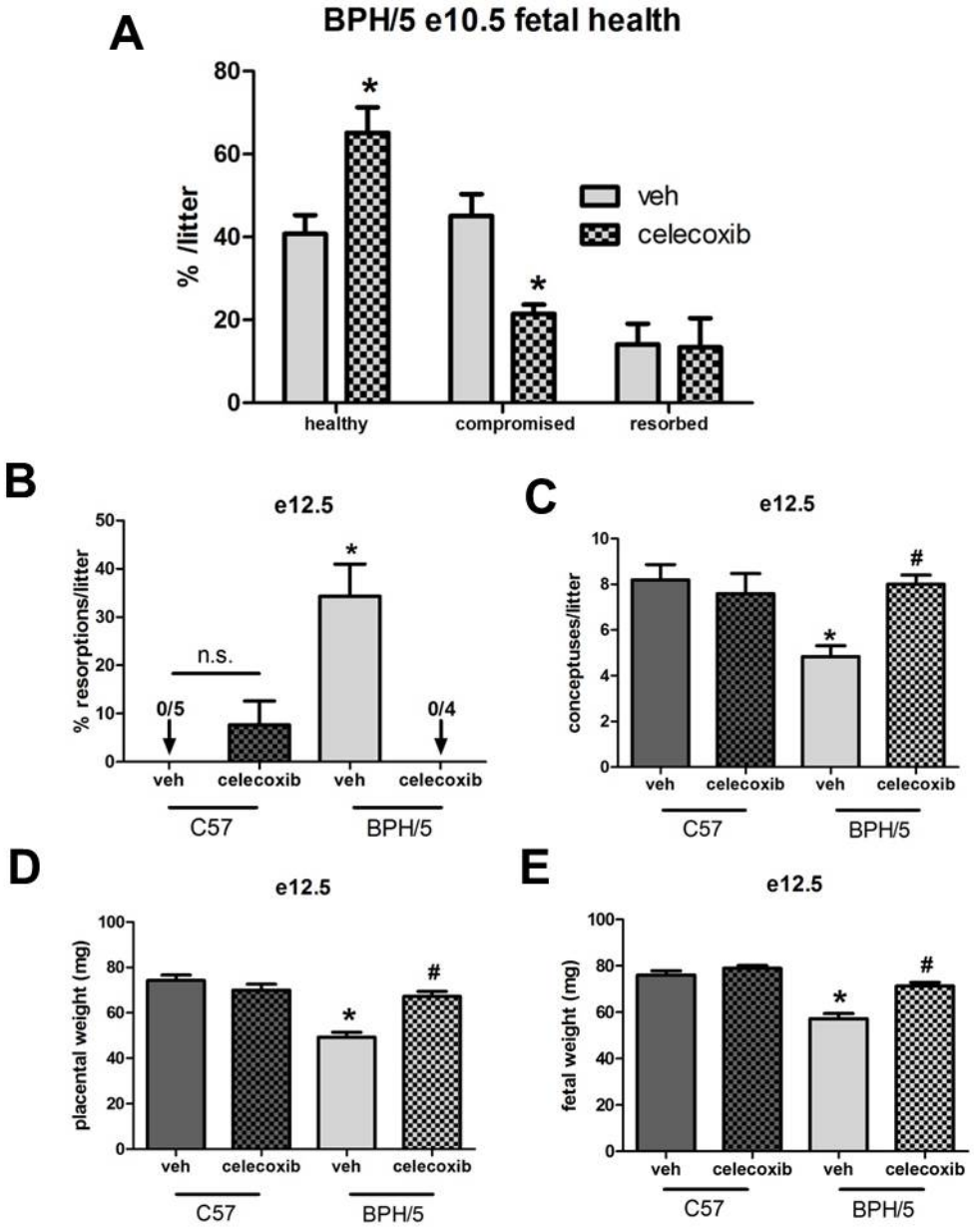
veh
celecoxib
veh
celecoxib
C57
BPH/5
F
PGE2 (pg/mg)
200
150
100
50
0
*
#
veh
celecoxib
veh
celecoxib
C57
BPH/5
G
6-keto PGF1α (pg/mg)
1000
800
600
400
200
0
veh
celecoxib
veh
celecoxib
C57
BPH/5

implantation sites from celecoxib-treated versus vehicle-treated mothers (Figure 3.5F) with a trend for reducing 6-keto $PGF_{1\alpha}$ levels (Figure 3.5G). These results indicated that celecoxib had an efficacious effect at e7.5 and that Cox2 signaling was being altered in the BPH/5 implantation site by our treatment strategy.

Cox2-specific inhibition early in BPH/5 implantation sites improves downstream fetoplacental outcomes at mid and late gestation

Our lab has previously described smaller litter sizes in BPH/5 females beginning at midgestation[13] and using ultrasound during pregnancy we can observe fetal resorptions as early as e10.5 in BPH/5 females. Incidentally at this time point, the greatest variation in fetal health can also be observed, including the ratio of fetal to maternal heart rate (HR ratio) and crown-rump length (CRL). Based on these criteria, we categorized BPH/5 fetuses into three categories: healthy, compromised, and resorbed. To test the hypothesis that Cox2 inhibition would improve pregnancy outcomes in BPH/5 mice, we administered celecoxib to pregnant BPH/5 and C57 mice at e6.5 and assessed mid and late gestation fetoplacental development. First, we observed an improvement in overall pregnancy health in BPH/5 females with an increased percentage of healthy fetuses and a reduction in the percentage of compromised fetuses within BPH/5 litters at e10.5 after celecoxib administration (Figure 3.6A). In these mice the percentage of resorbed fetoplacental units per litter was unchanged from vehicle-treated BPH/5 (Figure 3.6A), however we observed a significant reduction in the percentage of resorptions in celecoxib-treated BPH/5 litters at e12.5 (Figure 3.6B) and this translated to an improvement in litter size (Figure 3.6C). Furthermore, celecoxib normalized placental and fetal weight in BPH/5 females at e12.5 (Figure 3.6D & E). This improvement in fetal health and development at mid gestation was also observed at late gestation (e18.5) by a significant (~5%) increase in pup weight in BPH/5 celecoxib-treated mothers compared to vehicle-treated (Figure 3.7A & B). This increase in pup weight was associated with an increase in placental weight, but not with an improvement in placental efficiency or the fetal: placental weight ratio (Figure 3.7C). Provocatively, these late

<u>Figure 3.6:</u> Selective inhibition of Cox2 early in pregnancy improves pregnancy outcomes in BPH/5 females at mid gestation. (A) Ultrasonography was used to identify 3 distinct phenotypic classes of fetal health in BPH/5 females at e10.5: healthy, compromised, and resorbed. The incidence of each fetal health class was recorded in BPH/5 pregnancies at e10.5 and recorded as a percentage per litter after vehicle (veh) and celecoxib administration (n=4 litters/group). **(B)** Resorptions were observed at e12.5 in BPH/5 and C57 females after vehicle (veh) and celecoxib administration and recorded as %resorptions per litter (n=4-6 litters per group). **(C)** Litter size was counted in BPH/5 and C57 at e12.5 after veh (n=6 BPH/5 mothers, n=5 C57 mothers) or celecoxib (n=4 BPH/5 mothers, n=5 C57 mothers) administration. **(D)** Placental weight and **(E)** fetal weight was measured in BPH/5 and C57 pregnancies at e12.5 after vehicle (n=29 BPH/5, n=40 C57) or celecoxib (n=32 BPH/5, n=36 C57) administration. *$p<0.05$ vs C57 veh, # $p<0.05$ vs BPH/5 veh. n.s.= non-significant.


A
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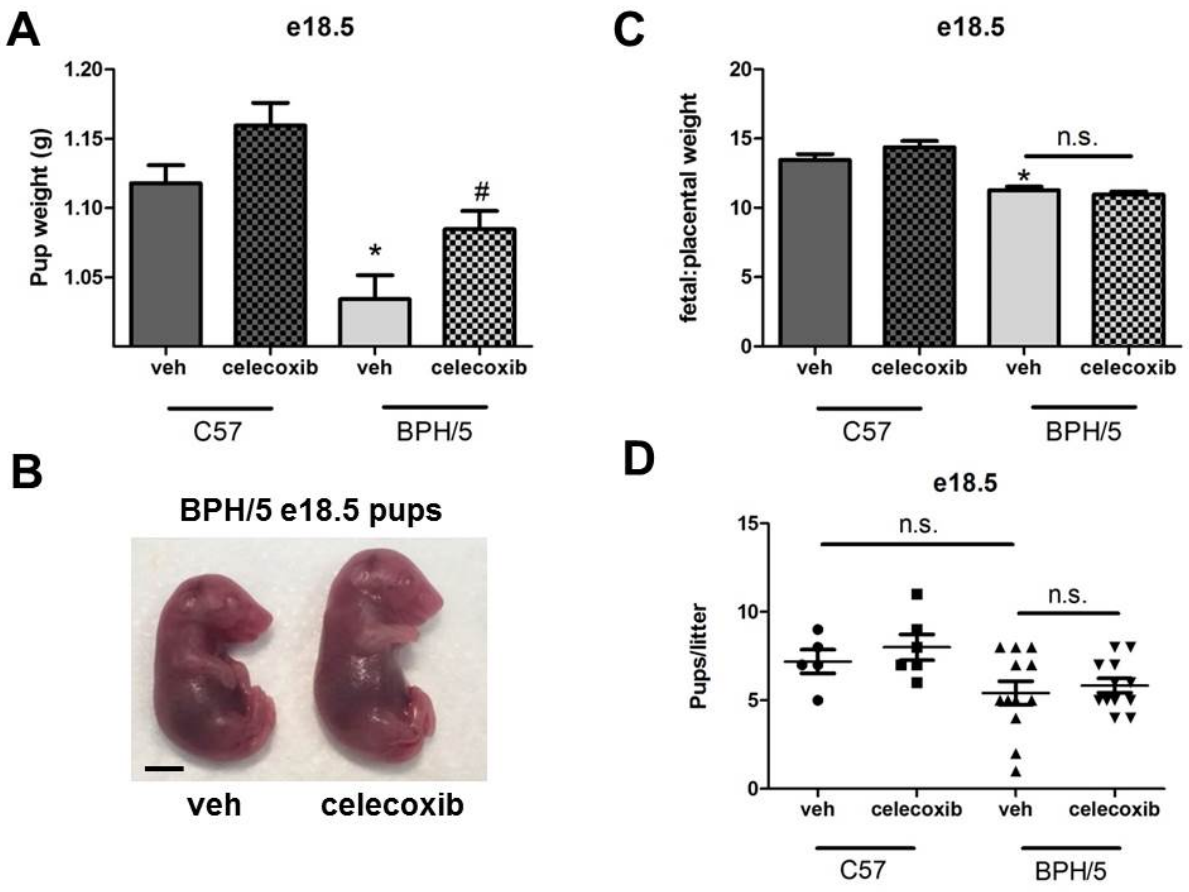

<u>Figure 3.7:</u> Late-gestational fetal growth is improved in BPH/5 mice with selective inhibition of Cox2 early in pregnancy. (A) Pup weight was measured in BPH/5 and C57 at e18.5 after vehicle (n=49 BPH/5, n=36 C57) or celecoxib administration (n=51 BPH/5, n=40 C57). **(B)** Representative image of BPH/5 pup size at e18.5 after veh (left) and celecoxib (right) administration. Scale bar= 4mm. **(C)** Placental efficiency was calculated as a ratio of fetal to placental weight in BPH/5 and C57 at e18.5 after vehicle (n=49 BPH/5, n=36 C57) or celecoxib administration (n=51 BPH/5, n=40 C57). **(D)** Litter size was counted in BPH/5 and C57 at e18.5 after veh (n=12 BPH/5 mothers, n=5 C57 mothers) or celecoxib (n=12 BPH/5 mothers, n=6 C57 mothers) administration. *$p<0.05$ vs C57 veh, # $p<0.05$ vs BPH/5 veh. n.s.= non-significant.

gestational findings in celecoxib-treated BPH/5 mothers was not associated with a significant increase in litter size at this time point (Figure 3.7D). However, late gestational urinary protein levels in celecoxib-treated BPH/5 mothers, as measured by dipstick, appear to be similar to C57 mothers (vehicle and celecoxib-treated) and show only trace to 1+ levels (Table 3.2). These results provide evidence that Cox2 plays a key role in fetoplacental development in a mouse model of PE and that celecoxib improves pregnancy outcomes in BPH/5 females, most notably in ameliorating FGR in BPH/5 offspring.

Table 3.2: Urine dipstick measurements of urinary protein from e18.5 C57 and BPH/5 vehicle (veh) and celecoxib treated female mice.

C57 veh	1+	n=3
C57 celecoxib	trace-1+	n=3
BPH/5 veh	1-3+	n=4
BPH/5 celecoxib	trace-1+	n=4

DISCUSSION

This study provides evidence that molecular dysregulation during early pregnancy events, such as implantation, decidualization and placentation, underlie the deleterious pregnancy outcomes in the BPH/5 mouse model of spontaneous preeclampsia (PE). PE is hypothesized to begin early in pregnancy with abnormal placentation and this is thought to play a causal role in abnormal fetal development and the maternal syndrome[1, 23, 24]. In fact, recent reports link increased maternal blood pressure early in pregnancy to lower birth-weight and small-for-gestational age babies in women with PE[25]. Thus the origins of poor fetal outcomes in PE likely begin early in pregnancy.

Although abnormal implantation and placentation have been linked to the pathogenesis of PE, the exact mechanisms are still unknown[23, 24, 26]. Early pregnancy events associated with PE have been difficult to study in humans. A recent microarray study analyzed gene expression in chorionic villus samples taken from women in their first trimester that went on to have healthy pregnancies or develop PE[27]. Several genes involved in decidual regulation were found to be dysregulated. However, gene expression validation by qRT-PCR was largely unsuccessful. Therefore, animal models that spontaneously mimic the PE syndrome seen in women allow for longitudinal studies that are vital to elucidating the origins of this disease of pregnancy prior to presentation of the maternal syndrome. In this study, we investigated molecular dysregulations during the periimplantation period in BPH/5 mice and present evidence to support our hypothesis that aberrations in early pregnancy events influence fetoplacental outcomes associated with the PE syndrome. Furthermore, our data implicate misexpressed uterine Cox2 as a causal factor in the poor fetal health status in BPH/5 mice.

BPH/5 females, which experience fetal loss during pregnancy, demonstrate deferred timing of implantation along with abnormal spacing of implantation sites (embryo clustering) and defective decidualization in comparison to C57 females (see Chapter 2). Wilcox et al. also observed increased incidence of early pregnancy loss in humans when implantation occurred outside the window of receptivity[28]; however, the association of deferred implantation with

adverse pregnancy outcomes such as PE and fetal growth restriction (FGR) has not been defined in women. Molecular analyses during the periimplantation period in BPH/5 mice revealed defects in the expression of critical mediators of implantation and decidualization in implantation sites compared to C57.

First, e4.5am implantation sites from BPH/5 mothers showed increased expression of *Hoxa10*, a progesterone (P4)-sensitive transcription factor[4]. Since BPH/5 females have a brief, but significant rise in circulating P4 before implantation (Ashley Woods; PhD thesis), it was not unexpected to see an early increase in *Hoxa10* mRNA expression in the uterus. However, we saw a significant reduction in *Lif*, *Bmp2*, and *Ptgs2* expression in BPH/5 implantation sites at this time compared to C57. Because *Lif* induction in the uterus during implantation requires estrogen (E_2)[8, 29] and BPH/5 mice have reduced circulating levels of estradiol-17β prior to implantation (Figure 3.1A), the reduced expression of *Lif* in BPH/5 implantation sites may possibly contribute to the deviation in appropriate implantation timing. Similar to BPH/5 mice, implantation sites of *Lif*$^{-/-}$ females exhibit dysregulation of downstream signaling molecules that are essential for implantation[8]. Appropriate timing of uterine *Lif* expression could stand to improve the aberrant molecular profile seen in BPH/5 implantation sites. It would be interesting to see if correcting the temporal expression of *Lif* in the BPH/5 uterus would also improve other distal pregnancy outcomes. Decidualization in the context of ALP expression was absent in BPH/5 implantation sites at this time despite increased expression of *Hoxa10.* One possibility is that overexpression of *Hoxa10* was not enough to provoke decidualization in BPH/5 females at e4.5am, because *Bmp2* and *Ptgs2* are also critical for decidualization in mice[1, 7, 9]. *Bmp2* is downstream of *Lif* and knockout mice that have *Bmp2* deleted specifically in progesterone receptor (PR)-expressing cells show profound decidualization defects[7]. Thus *Bmp2* may be more important in mediating decidualization than *Hoxa10*.

Next, we found *Bmp2* and *Hoxa10* expression were comparable in BPH/5 and C57 implantation sites at e5.5am, but *Ptgs2* alone was significantly upregulated in e5.5am BPH/5 implantation sites compared to C57. *Ptgs2* expression mirrored the robust decidual response seen

in BPH/5 implantation sites by ALP activity at e5.5am. Indeed, *Ptgs2* and Cox2 represent a final common factor upon which the upstream implantation and decidualization molecules converge[4, 7]. Lastly, *Ptgs2* expression was persistently upregulated in BPH/5 implantation sites at e7.5 as well as the protein it encodes, Cox2. Persistent upregulation of Cox2 in BPH/5 implantation sites at e7.5, the time of peak decidualization in mice, strongly suggests that the molecular phenotype of BPH/5 decidualization is aberrant and that normalization of Cox2 signaling might improve pregnancy outcomes in BPH/5 females.

The use of transgenic mice has greatly improved our molecular understanding of periimplantation events[1, 22]. Several studies have shown that dysregulation of PG pathways results in embryo clustering and deferred implantation. Mice deficient in cytosolic phospholipase A2α (cPLA2α), which releases arachidonic acid from membrane phospholipids to serve as a substrate for Cox2 to generate PGs, show deferred implantation and defective embryo spacing[30]. Consistent with these results, mice deficient in lysophosphatidic acid (LPA), which is generated by PLA2α, also showed a similar phenotype in embryo spacing and implantation defects working via altered Cox2 expression[31]. Since *Ptgs2* and Cox2 are highly dysregulated in BPH/5 implantation sites, this underscores the significance of exploring further the role of PG signaling in BPH/5 females during pregnancy. To causally link our findings that periimplantation defects in Cox2 signaling play a key role in initiating downstream fetoplacental defects in a mouse model of spontaneous PE, we devised a strategy to specifically target Cox2 within the implantation site by administering a selective Cox2 inhibitor (celecoxib) to BPH/5 and C57 females at e6.5 in pregnancy.

The two hallmarks of successful implantation and decidualization are increased uterine vascular permeability and angiogensis[32]. Cox2-derived PGs markedly influence these processes by directing vascular endothealial growth factor (VEGF) signaling in conjunction with the angiopoietin system[33]. This is corroborated by the fact that *Ptgs2*$^{-/-}$ mice show compromised uterine angiogenesis during implantation and decidualization partially due to defective *Vegf* signaling[5, 9, 11]. A number of *Vegf* isoforms are expressed in the mouse uterus during implantation

and decidualization, but the *Vegf* isoform 164 has been shown to be the most abundant[34]. Importantly, the human isoform 165b has been shown to increase vascular permeability in PE women[35]. The selective Cox2 inhibitor celecoxib normalized $VEGF_{164}$ protein expression in the BPH/5 implantation site at e7.5. *Vegf* expression in the mouse uterus is influenced by hypoxia inducible factor (HIF) as well as Cox2 during implantation and decidualization[4]. By inhibiting Cox2 we are not directly targeting excessive hypoxia, which we provide evidence of in BPH/5 implantation sites at e7.5 (Figure 2.4B), but rather impeding Cox2-mediated overexpression of $VEGF_{164}$. Hypoxia can induce Cox2 expression in other cell types besides the uterus[36]. Additional work is needed to elucidate the mechanism by which excessive hypoxia in the decidua may alter uterine Cox2 and VEGF signaling in BPH/5 and its contribution to downstream fetoplacental outcomes. It would be intuitive to hypothesize that all these factors impact the decidual vasculature and contribute to inadequate spiral artery remodeling. However, studies in $Rag2^{-/-}Il2rg^{-/-}$ mice that do not adequately remodel their spiral arteries during pregnancy showed that oxygen delivery to the fetus was not impaired[37]. If this is in fact true, it would represent a paradigm shift in the hypothesized mechanism of placental defects in PE-mediated poor fetal outcomes, such as FGR. This is an important concept and further investigation is warranted to understand uteroplacental perfusion in the context of PE and FGR.

In addition to having an effect on $Vegf_{164}$ protein, celecoxib also had an effect on PG levels in the BPH/5 implantation site. The two principle PGs involved in mouse decidualization at this time in pregnancy are Cox2-derived PGE_2 and prostacyclin (PGI_2)[21]. Levels of PGE_2, a potent vasoconstrictor in the placental vasculature, were shown to be increased in BPH/5 implantation sites at e7.5 in vehicle-treated mothers and this was normalized after celecoxib administration. Measurements of the vasodilatory PGI_2 metabolite (6-keto PGF1α) revealed an insignificant reduction in synthesis after celecoxib. Our findings that celecoxib administration at e6.5 did not impair implantation but rather had a significant reduction in Cox2 and PGE_2 levels in e7.5 BPH/5 implantation sites indicated not only the appropriate dose and timing of celecoxib administration to see a physiologic outcome, but also that decreased local PGE_2 may provide a

mechanism by which BPH/5 pregnancy outcomes were improved. These results are intriguing, since altered levels of Cox2-derived PGs are implicated in the pathogenesis of PE, and abnormal umbilical blood flow in women with pregnancy-induced hypertension and PE[10, 38]. Therefore further investigating PG signaling in the decidua just prior to placenta formation may provide novel insights to the origins of abnormal placentation in PE.

Improved pregnancy outcomes at mid gestation were seen in BPH/5 mice after celecoxib with respect to an increase in the percentage of healthy fetuses and a decrease in the percentage of compromised fetuses per litter at e10.5 that lead to fewer resorptions and larger litter sizes at e12.5 and this was associated with an improvement in fetal and placental weights. We know that the compromised fetuses have decreased expression of *Gcm1*, *SynA*, and *SynB* in the labyrinth indicative of poor placental development (Chapter 2). It will be interesting to explore if celecoxib has an effect on decidual expression of genes involved in trophoblast invasion, such as matrix metalloproteinases (MMP) and if treatment early in pregnancy allows for improved labyrinth formation, suggesting enhanced maternal-fetal exchange. This would be of particular interest given that MMP9 deficient mice phenocopy key features of PE as well as display implantation defects[39]. Although there is no doubt of redundancy in the pathways modified by celecoxib, this could be one way in which fetal health is improved in BPH/5 mice beginning at mid gestation, and continuing throughout gestation as we also see an increase in e18.5 pup weight in BPH/5 celecoxib-treated mothers. It is very exciting that normalization of Cox2 and PGE_2 with celecoxib is associated with improved BPH/5 fetal health and placental development here as we know Cox2-derived PGs are directly responsible for regulating fetal and placental vascularity[5, 40]. This data supports a causative role for abnormal uterine Cox2 signaling during the periimplantation period in mediating poor fetoplacental health and growth restriction in this mouse model of PE.

It is confusing that neither placental efficiency nor litter size was changed at e18.5 after celecoxib treatment. Although it does make sense that the ratio of fetal to placental weight would not be changed between BPH/5 treatment groups due to both of those specimens increasing size

proportionally. However, the BPH/5 e18.5 vehicle litter sizes ranged from one pup to eight pups per litter in this study. This underscores the heterogeneity of disease presentation in BPH/5 mice, which is also observed in women with PE[41]. Some PE pregnancies present as early onset, while others are late onset. There is also a gradient of severity of disease presentation from mild to moderate to severe. It is important to note that celecoxib did not have an adverse effect on late gestation litter size as data indicates celecoxib given to pregnant ewes in late gestation can have adverse fetal outcomes, such as premature closure of the ductus arteriosus[42]. Despite this caveat in timing of administration during pregnancy, there is evidence that celecoxib has a role in combinatorial therapy to prevent preterm labor[43, 44]. Our strategy specifically avoids celecoxib administration in the second half of pregnancy and studies of our pups given celecoxib during early pregnancy have not yielded any signs of systemic pathology (data not shown). The most profound pregnancy outcome seen in BPH/5 females after celecoxib treatment was the consistent improvement in fetal health and FGR. Preliminary data showing a reduction in proteinuria at late gestation in BPH/5 mothers after celecoxib treatment holds exciting promise for an improvement in the maternal syndrome of PE. These data provide a link between the fetoplacental defects observed prior to the onset of the maternal syndrome in BPH/5 mice and the dysregulated molecular implantation and decidualization events that characterize this model.

Periimplantation anomalies have adverse “ripple effects” on fetoplacental development and pregnancy outcomes. While many of these fetal pathologies often characterize PE and other diseases of pregnancy, it has been difficult to study the impact of these early events. Here we present strong evidence that dysregulation of molecular signaling during implantation and decidualization results in the undesirable fetal and pregnancy outcomes in a mouse model of PE. To our knowledge, this is the first report of Cox2 being a central player in normal fetal development and growth in pregnancies affected by PE, making it a potential candidate for therapeutic targeting to prevent fetal morbidity/mortality associated with PE and perhaps other disorders of pregnancy. Furthermore, our findings with celecoxib point toward insights into

recent reports that low-dose aspirin is associated with reduced risk of PE and FGR when given to high-risk women early in pregnancy[45].

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CHAPTER FOUR:

EVIDENCE FOR PERTURBED IMMUNOREGULATORY SIGNALING AT THE BPH/5 MATERNAL-FETAL INTERFACE DURING EARLY PREGNANCY

SUMMARY

Preeclampsia (PE) is a hypertensive proteinuric disorder of pregnancy and a leading cause of maternal and fetal morbidity/mortality. The only definitive treatment is delivery of the fetus and placenta, therefore the fetoplacental unit is implicated as the causative agent. Despite this, the underlying mechanism(s) of this devastating condition remains elusive. To determine the origins of PE, investigations of early maternal-fetal interactions involved in placentation are crucial. The BPH/5 mouse spontaneously develops the maternal signs of PE (hypertension and proteinuria) and abnormalities in placentation that are also seen in women with PE, including inadequate remodeling of spiral arteries. Immune cells at the maternal-fetal interface, particularly decidual Natural Killer (dNK), are thought to be highly involved in the transformation of spiral arteries for sufficient uteroplacental perfusion. We investigated dNK cells and associated cytokines in BPH/5 and C57 implantation sites to test the hypothesis that dysregulation of key immunoregulatory pathways at the maternal-fetal interface pre-dates the placental pathologies seen in BPH/5 mice. In Chapter 3, we demonstrated evidence of increased inflammation in BPH/5 implantation sites at e7.5 with increased Cox2 expression. However, this time point in BPH/5 pregnancy was associated with a decrease in interferon-γ (*Ifn-γ*) mRNA expression. Since dNK cells are a source of IFN-γ, this provided evidence of dNK cell dysregulation. We found BPH/5 implantation sites had significantly fewer activated dNK cells and this was preceded by increased IL-15, a uterine cytokine that is thought to activate dNK cells. We functionally linked elevated IL-15 to aberrant dNK cell loss in C57 implantation sites and provide evidence for anti-inflammatory therapy mediating decreased IL-15 in BPH/5 implantation sites. In summary, the BPH/5 maternal-fetal interface is characterized by dysregulated dNK cell activation and function prior to placenta formation and IL-15 overexpression plays a key role in this process.

INTRODUCTION

One of the most remarkable features of pregnancy is maternal tolerance of the semi-allogeneic fetus[1, 2]. However, there is not only tolerance but acceptance of foreign fetoplacental antigens. The maternal uterine environment undergoes significant physiologic adaptations to ensure survival of the developing conceptus. Embryo-uterine interactions, beginning with implantation, trigger the recruitment and activation of key immune cells at the maternal-fetal interface[2]. The decidua represents the initial site of maternal-fetal interactions and decidual vessels (spiral arteries) must be transformed during pregnancy to facilitate placental blood flow to the growing baby. Decidual immune cell constituents play an important role in this process[2]. Pregnancies characterized by preeclampsia (PE) have inadequate remodeling of spiral arteries within the maternal vasculature and poor placental perfusion[3]. This is thought to create placental hypoxia and trigger the maternal syndrome[3]. Because the placenta is formed in the first trimester, the placental pathologies seen in PE are thought to originate early in pregnancy. However, perturbations in the immunoregulatory pathways involved are not easily studied at these gestational time points. Therefore, investigating key immune cells at the maternal-fetal interface in the spontaneous BPH/5 mouse model of PE is advantageous in identifying dysregulated pathways prior to placenta formation and the development of the maternal syndrome.

The decidua is formed in women and female rodents in order to accept and protect the implanting embryo[1,2]. The process of uterine decidualization involves the differentiation of stromal cells into epithelial-like decidual tissue that has a distinct morphological and biochemical phenotype[4]. Cycling women decidualize every month during the luteal phase of the menstrual cycle in preparation of fertilization. If implantation does not occur, these decidual cells are shed at the time of menstruation[5]. Interestingly, mice require the physical interaction of the blastocyst to undergo decidualization during pregnancy[6]. Beginning at embryonic day (e) 4.5, after the attachment reaction, decidual cells first appear in the mouse uterus[1]. They are present around the implanting embryo and then populate the mesometrial pole of the uterus, which is the entry point of the uterine vasculature. The process of decidualization is marked by the recruitment of

immune cells to the decidua that aid in uterine vascular remodeling to facilitate adequate uteroplacental perfusion by the time the functioning placenta is formed at e9.5-10.5[4].

Coincident with decidualization in humans and mice is the appearance of decidual Natural Killer (dNK) cells, the predominant immune cell at the maternal-fetal interface[2, 7, 8]. In women, dNK cells appear in the endometrium prior to implantation[2]. While in mice common lymphoid progenitor cells in the bone marrow become pre-dNK cells in secondary lymphoid tissues and migrate to the uterus where they proliferate and differentiate further into mature dNK cells[8]. Proliferation requires progesterone (P4)[8], while differentiation of mature dNK cells has been linked to uterine interleukin (IL)-15[7]. In humans, dNK cells are identified by the lack of CD16 and positive expression of $CD56^{bright}$ in first trimester decidua[9]. In mice, mature dNK cells are recognized histologically by their lymphoid shape and cytoplasmic granules that react with *Dolichos biflorus* agglutinin (DBA)[10]. DBA^{+} cells have been identified as early as e5.5 in the decidua. They continue to proliferate and accumulate at the base of the placenta, obtaining peak numbers at mid gestation (~e12.5) and declining thereafter[11]. In women the appearance of activated dNK cells is also restricted to the first half to two-thirds of pregnancy[11].

Mature dNK cells maintain decidual integrity and produce factors that directly modify decidual vessels[8]. Upon stimulation by IL-15, dNK cells begin secreting angiogenic factors, including vascular endothelial growth factor (VEGF), placental growth factor (PGF), and interferon-γ (IFN-γ)[12]. VEGF and PGF promote angiogenesis in the implantation site during early pregnancy, while dNK cell-derived IFN-γ is believed to directly modify the uterine vasculature[12]. It has been shown in mice that IFN-γ provides the signals that transiently change spiral arteries from constricted vessels to dilated vein-like structures, which is necessary for adequate placental perfusion[7]. IFN-γ is also thought to work in an autocrine manner on dNK cells to promote their physiologic senescent decline after mid gestation[11]. The production of IFN-γ by dNK cells is critical for normal gestational changes in the mouse decidua and uterine vasculature. However, the precise role of IFN-γ in human pregnancy success is still under investigation.

PE is often described as a two-stage disease model with the first stage involving abnormal placentation characterized by poor trophoblast invasion, incomplete vascular remodeling, and placental hypoxia[3]. The second stage then presents as the maternal syndrome of hypertension and proteinuria presumably due to the release of factors, from an abnormally developed placenta, into the maternal systemic circulation[13]. The cause and onset of these mechanisms are unclear. One possibility is inadequate uterine angiogenesis and placental vasculogenesis at the time of implantation[14]. Because dNK cells are critical for uterine angiogenesis and spiral artery remodeling, gestational processes that are often defective in PE pregnancies, it is of the utmost importance to investigate this immunoregulatory pathway in the context of PE.

Over a decade ago, Davisson et al. first discovered that the BPH/5 strain of mice spontaneously developed a PE-like syndrome during pregnancy characterized by late-gestational hypertension and proteinuria that resolved upon delivery[15]. Importantly, BPH/5 mice demonstrate abnormal placentation with placental pathologies similar to those seen in PE, including shallow trophoblast invasion and inadequate remodeling of spiral arteries[15, 16]. BPH/5 mice also demonstrate increased vascular resistance in uterine arteries, an indicator of placental vascular insufficiency and impairment of uteroplacental circulation[16]. BPH/5 pregnancies are marked by poor fetal outcomes, including fetal demise and *in utero* fetal growth restriction (FGR), which translates to smaller live litter sizes and lower birth-weight pups compared to control mice[15, 16]. Importantly, BPH/5 mice also demonstrate deficiencies in circulating and placental angiogenic factors (VEGF and PGF) at early gestational time points[17]. We hypothesized that dysregulated dNK cells and associated immunoregulatory molecules would precede defects in placental vascular remodeling and angiogenesis seen in BPH/5 mice.

Decidualization and the recruitment of dNK cells at the maternal-fetal interface are required for the remodeling of decidual vessels and establishing placental blood flow. These placental defects are hallmarks of PE and thought to be linked to the development of the maternal syndrome. We investigated dNK cells and associated cytokines during the

periimplantation period in BPH/5 mice. Indeed we found that BPH/5 implantation sites have fewer dNK cells in the decidua and that this was associated with decreased *Ifn-γ* mRNA. These observations were preceded by overexpression of IL-15 in BPH/5 implantation sites, which we functionally linked to dNK cell loss in C57 pregnant mice. Furthermore, we provide evidence here that anti-inflammatory therapy reduces IL-15 expression in the BPH/5 implantation site. Our mechanistic data with BPH/5 mice provides novel insights in the physiologic adaptations of the maternal-fetal interface in early pregnancy and how they may be dysregulated in the context of PE.

METHODS

Mice

Virgin BPH/5 and control C57Bl/6 (C57) mice (8-12 weeks old) were obtained from in-house colonies. Strain-matched matings were performed with BPH/5 and C57 to induce pregnancy. The day of vaginal plug detection is defined as e0.5. To collect implantation sites before they could be identified grossly (earlier than e6.5), pontamine perfusion was performed. Pregnant females at e4.5 and e5.5 were given a single injection of 100 uL of 1% pontamine blue (BDH; Radnor, PA) dissolved in saline, administered via tail vein, and after 5 minutes mice were euthanized. Implantation sites were then collected and stored for histologic and molecular analyses. Celecoxib (Sigma Aldrich, St. Louis, MO) was administered to a cohort of pregnant mice at e6.5 by oral gavage at a dose of 10 mg/kg body weight (dissolved in 100uL sesame oil), an anti-inflammatory dosage tested safe during early rodent pregnancy[18]. Care of the mice met or exceeded the standards set forth by the National Institutes of Health Guide for the Care and Use of Laboratory Animals, USDA regulations, and the AVMA Panel on Euthanasia.

Intrauterine delivery of recombinant IL-15

A recombinant IL-15-antibody complex (IL-15R/IL-15Ra-Fc; R&D Systems, Minneapolis, MN) was administered to C57 female mice at e2.5 by intrauterine injection. Two days after positive plug detection, C57 females were anaesthetized with an intraperitoneal injection of ketamine (100mg/kg) and xylazine (10mg/kg). Two dorsal incisions were made over the ovarian fat pads. The uterine horns were identified and elevated by manipulating the ovarian fat pad. 15ug of IL-15R/IL-15Ra-Fc was dissolved in 20uL of saline, divided into two aliquots and injected into the distal tip of each uterine horn. Controls were given similar intrauterine injections of saline. For analysis of outcomes, C57 females were allowed to recover, and then sacrificed three days later at e5.5 to collect implantation sites.

Quantitative real-time PCR

Primers were designed using Primer Design software provided by Integrative DNA Technologies (www.idtdna.com). Total RNA was extracted using TriZol (Life Technologies, Grand Island, NY) as previously described[19]. Reverse transcription was performed using Quanta kits (Gaithersburg, MD) per the manufacturer's instructions, and quantitative real-time PCR (qRT-PCR) was performed with 25ng cDNA in triplicate using SybrGreen reagents (Quanta) on an ABI 7500 Fast System (Applied Biosystems, Life Technologies, Grand Island, NY). Data was expressed as 2^-dCt relative to housekeeper gene expression (*18S rRNA*). Primer sequences can be found in Table 4.1.

Flow cytometry

Animals were euthanized and cleared of blood by perfusion with 10% heparin phosphate-buffered saline (PBS). Whole implantation sites were collected from untreated pregnant C57 and BPH/5 female mice at e5.5 as well as at e5.5 from pregnant C57 females administered vehicle and IL-15R/IL-15Ra-Fc at e2.5. Tissue was digested using collagenase type IX (125 U/ml; Sigma Aldrich), collagenase type IV (450 U/ml; Sigma Aldrich) dissolved in 20 mmol/L 4-(2-hydroxyethyl)-1- piperazineethanesulfonic acid-PBS buffer containing calcium for 15 minutes. The digested tissue was then passed through a 70 mm sterile cell strainer (Becton Dickinson, Franklin Lakes, NJ), yielding single cell suspensions. This was followed by a red blood cell lysis (5 minutes, room temperature) and wash (400*g*, 10 minutes). The white blood cell containing pellet was resuspended in fixable Near-IR dead cell staining solution (Invitrogen, Grand Island, NY). After 15 min on ice 1% bovine serum albumin/PBS buffer (FACS buffer) was added to the cells. Following another wash, cells were stained for 25 minutes at 4°C with the following antibodies: PE rat anti-mouse CD122 (clone: TM-β1, BD Bioscience), FITC anti-Dolichos biflorus agglutinin Lectin (EY Laboratories, San Mateo, CA); PerCP anti-CD45 (Clone: 30-F11, BD Biosciences); APC anti-CD3 (Clone: 145-2C11, BD Biosciences, Franklin Lakes, NJ). After immunostaining, cells were resuspended in FACS buffer containing fluorescent counting beads

(CountBright™, Invitrogen) and analyzed on a LSR-II flow cytometer with DIVA software (Becton Dickinson). Only live cells positive for CD45 staining were used to identify decidual Natural Killer cells (CD3-/CD122+/DBA+). All data were analyzed as total cell number.

Immunofluorescence

Implantation sites were collected from pregnant C57 and BPH/5 at e5.5, rinsed in PBS, and fixed in 4% paraformaldehyde (PFA) overnight at 4°C followed by sucrose gradients in PBS (15 and 30%). Afterwards the samples were embedded in Optimal Cutting Temperature and sectioned at 12 μm onto poly-L-lysine coated slides. Prior to staining, tissue sections were fixed with ice-cold acetone for 10-20 minutes and rinsed in 10mM PBS before blocking in a humidity chamber at room temperature for 1 hour. After blocking, slides were incubated for 1 hour with 1:1000 primary antibody (FITC anti-Dolichos biflorus agglutinin Lectin; EY Laboratories) at room temperature**,** rinsed in PBS for 5 minutes. Post incubation, slides were rinsed in PBS for 5 minutes, cleared by administering xylene and mounted using VectaShield (Burlingame, CA).

Western Blotting

Whole implantation sites were collected from untreated pregnant C57 and BPH/5 female mice at e4.5, e5.5, and e7.5 as well as at e7.5 from pregnant BPH/5 females administered vehicle and celecoxib at e6.5. Individual implantation sites were homogenized in 10mM sodium phosphate buffer containing protease inhibitors (Roche, Penzburg, Germany). Protein concentrations were determined by bicinchoninic acid (BCA) reagents followed by Nanodrop ND-1000 spectrophotometer readings (Life Technologies). Equal amounts of protein (50ug) were separated on 10% SDS-PAGE gel and transferred to nitrocellulose membrane. Membranes were blocked in 5% non-fat milk in PBST, then probed with 1:500 goat polyclonal anti-IL-15 (Santa Cruz Biotechnologies, Santa Cruz, CA) overnight at 4C followed by incubation with (1:2500 donkey anti-goat IgG-horseradish peroxidase (HRP) (Santa Cruz Biotechnologies). An IL-15 blocking peptide (Santa Cruz Biotechnologies) was used to confirm specificity of IL-15 antibody

binding. Blots were also probed with a monoclonal anti-actin antibody (1:5000, Sigma Aldrich, St. Louis, MO) and goat-anti-mouse HRP-conjugated secondary antibody (1:5000, Santa Cruz Biotechnologies). Signals were detected by chemiluminescence with Pierce ECL Western Blotting Substrate (Life Technologies). Densitometric analysis was performed using ImageJ software (NIH) and band intensities were normalized to actin.

Statistical Analyses

All data are expressed as mean ± SEM. Multiple comparisons were made using a one or two-way ANOVA, where appropriate, followed by Newman-Keuls test for significance. A student's *t* test was used for all others. Statistical significance was defined as $p \leq 0.05$.

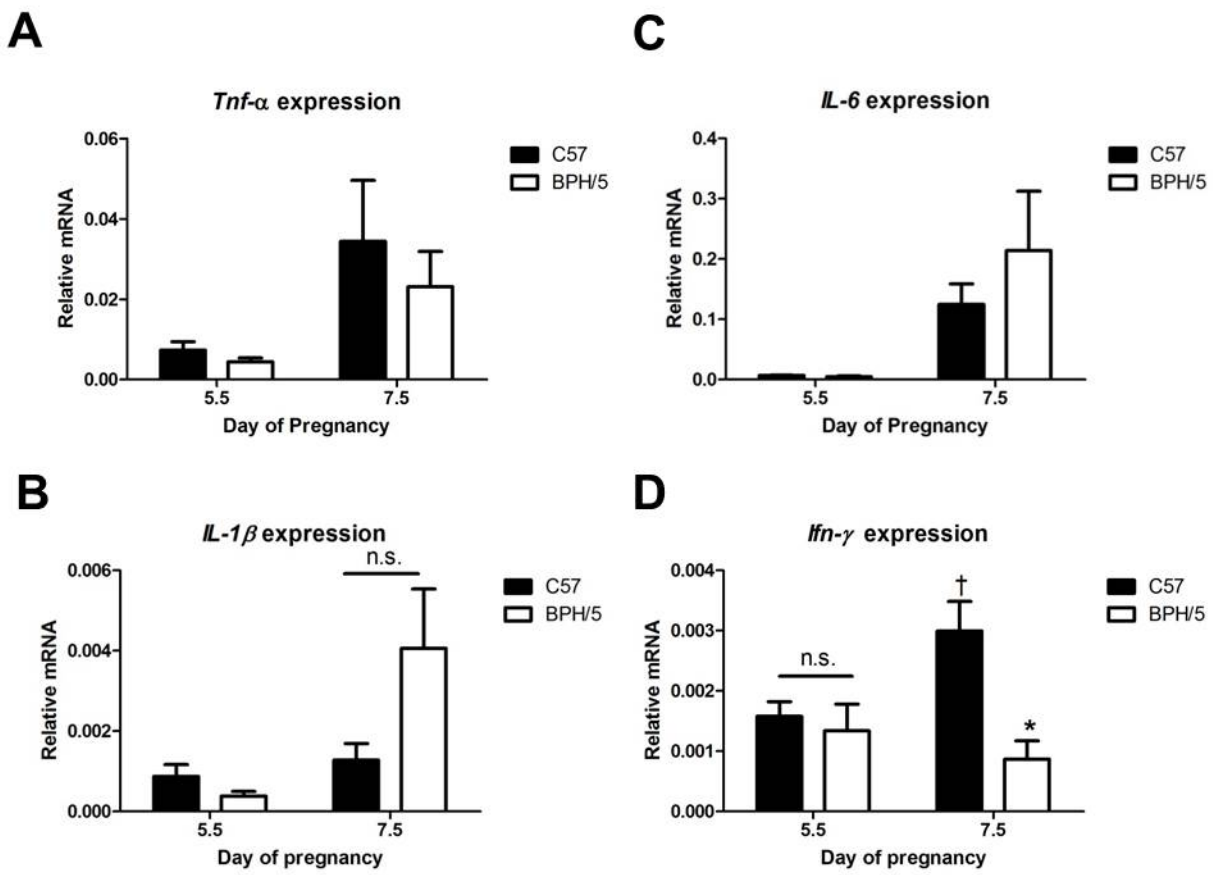
Table 4.1: Primer sequences used for quantitative Real Time PCR (qRT-PCR) analyses

Gene name	Forward and reverse sequences (5' to 3')
IL-1β	ACGGACCCCAAAAGATGAAG
	TTCTCCACAGCCACAATGAG
IL-6	TGCCTAAGGACCAAGACCATCCAA
	AACGCACTAGGTTTGCCGAGTAGA
Tnf-α	TCTCATGCACCACCATCAAGGACT
	ACCACTCTCCCTTTGCAGAACTCA
Ifn-γ	CACACTGCATCTTGGCTTTG
	GCTGTTGCTGAAGAAAGTA
IL-15	CTGCAAGTCTCTCCCAATTCTC
	CCTCCTGTAGGCTGGTTATCT
NKp46	CTACCGACCCTACTTCTTCTGT
	TTCTCCATCTTCTCCTGCATTC
18S	GTAACCCGTTGAACCCCATT
	CCATCCAATCGGTAGTAGCG

RESULTS

BPH/5 implantation sites have aberrant gene expression of IFN-γ, but not other pro-inflammatory cytokines early in pregnancy

The periimplantation period in BPH/5 mice is characterized by increased *Ptgs2*/Cox2 expression at the maternal-fetal interface (Chapter 3). Suppressing local inflammation during the periimplantation period is essential for embryo survival and a successful pregnancy[20]. To further explore the local inflammatory profile in BPH/5, we measured gene expression of pro-inflammatory cytokines, tumor necrosis factor-α (*Tnf-α*), interleukin (IL)-6 (*IL-6*), *IL-1β*, and interferon-γ (*Ifn-γ*) in BPH/5 and C57 implantation sites. Using qRT-PCR of whole implantation sites, we performed high through-put screening of pro-inflammatory genes at the time of increased *Ptgs2* expression (e5.5 and e7.5). Our results revealed similar expression of *Tnf-α*, *IL-6*, and *IL-1β* in BPH/5 implantation sites compared to time-matched C57 implantation sites (Figure 4.1A-C) However, expression of *Ifn-γ* was found to be dysregulated in BPH/5. While *Ifn-γ* expression was comparable between BPH/5 and C57 at e5.5, it was significantly lower in e7.5 BPH/5 implantation sites compared to C57, which showed maximal *Ifn-γ* expression at this time point (Figure 4.1D). This is a critical time point in mouse gestation that represents the time of peak decidualization at the mesometrial pole[6]. It has been reported that decidual Natural Killer (dNK) cells provide approximately 90% of the IFN-γ at the mesometrial pole of the mouse implantation site[11]. Our data showing decreased *Ifn-γ* at the time of peak decidualization suggested dysregulation in dNK cells in BPH/5 implantation sites. Thus we sought to examine the signaling pathway involved in dNK cell activation, including IL-15 expression, during the periimplantation period (Figure 4.2).



<u>Figure 4.1:</u> BPH/5 implantation sites have aberrant *Ifn-γ* expression at e7.5. (A) Quantification by qRT-PCR of pro-inflammatory genes in C57 and BPH/5 implantation sites at e5.5 and e7.5, including *Tnf-α*, **(B)** *IL-1β*, **(C)** *IL-6* and **(D)** *Ifn-γ*. (n=3-4) *p<0.05 vs time-matched C57, † p<0.05 vs e5.5 C57. n.s.= non-significant.

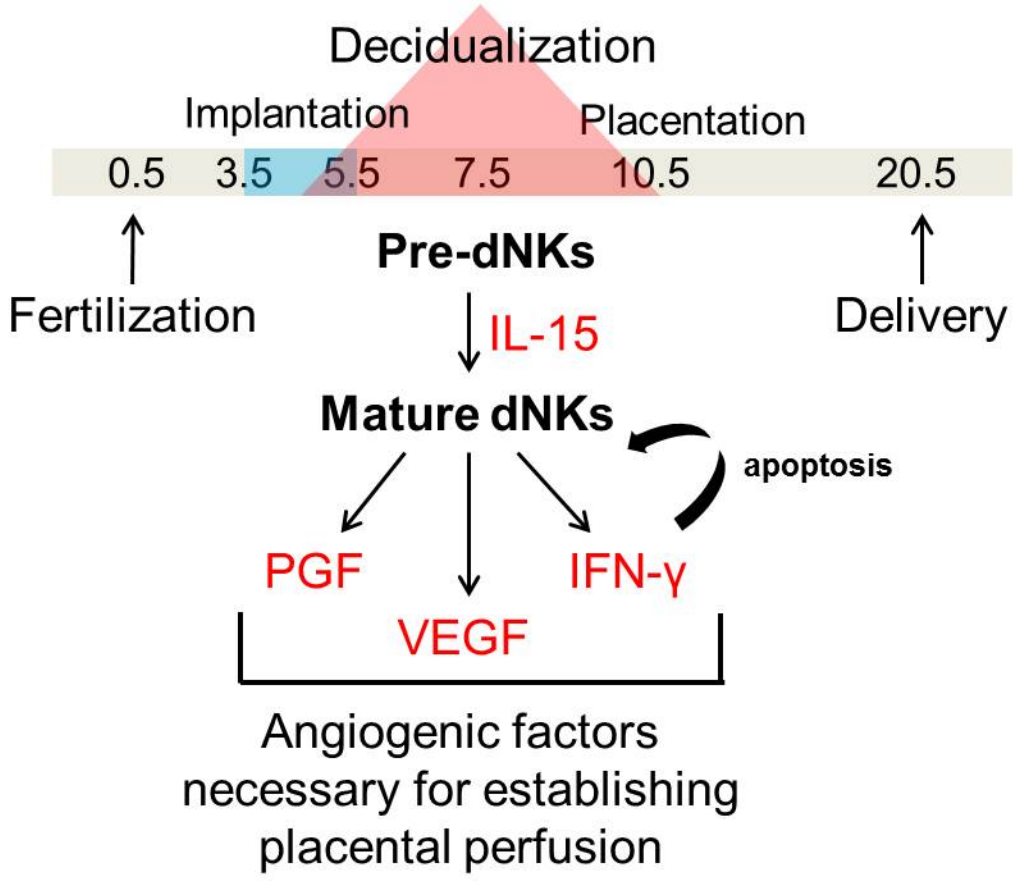


Figure 4.2: Schematic depicting the decidual Natural Killer (dNK cell) signaling pathway. During decidualization (e5.5-e7.5) pre-dNK cells are recruited to the maternal-fetal interface where they are activated by IL-15 to become mature dNK cells that secrete cytokines and angigenic factors, such as placental growth factor (PGF), vascular endothelial growth factor (VEGF), and IFN-γ. It is IFN-γ that induces apoptosis of dNK cells at mid gestation.

The BPH/5 maternal-fetal interface shows reduced dNK cell activation and proliferation

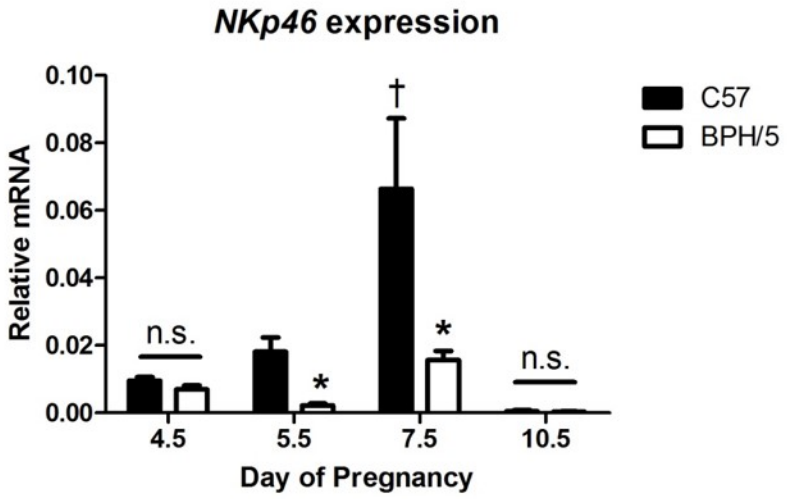
The defects observed in BPH/5 spiral artery remodeling at e10.5[16], and our data indicating reduced *Ifn-γ* prior to that at e7.5 in BPH/5 implantation sites (Figure 4.1D) led us to ask the question if dNK cells would also be dysregulated at the maternal-fetal interface in BPH/5 mice during the periimplantation period. We used a screening approach with qRT-PCR to first determine the expression pattern of *NKp46*, an activating receptor present in the gravid mouse uterus[21]. During critical early pregnancy time points in BPH/5 and C57 females, *NKp46* mRNA increases with gestation in C57 implantation sites with peak expression at e7.5 (Figure 4.3A). It is of note that e7.5 was also the time of maximal *Ifn-γ* expression in C57 implantation sites (Figure 4.1D). BPH/5 implantation sites did not show this rise in *NKp46,* but rather showed significantly lower expression at e5.5 and e7.5 versus C57 (Figure 4.3A). We next measured dNK cell numbers by flow cytometry to see if reduced *NKp46* corresponded to reduced staining of DBA^+ cells within BPH/5 implantation sites. Indeed, flow cytometry confirmed fewer $CD122^+/DBA^+$ cells in the BPH/5 implantation site at e5.5 compared to C57 (Figure 4.3B). Immunofluorescence localized DBA^+ cells to the decidua in C57 e5.5 implantation sites, while BPH/5 had few positive cells which were mostly outside of the decidua (Figure 4.3C).

BPH/5 implantation sites have profound upregulation of IL-15 prior to dNK cell loss

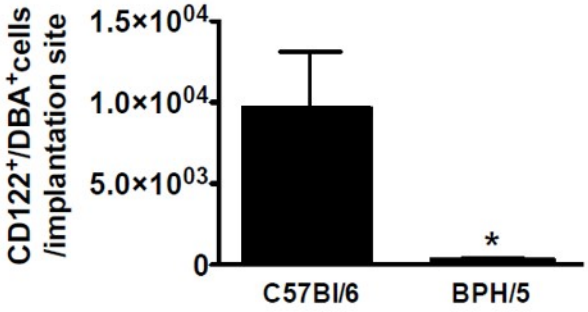
IL-15 is expressed by the pregnant uterus in mice and women[11]. It is also required for dNK cell activation[7]. Since there is a dramatic reduction in dNK cells at the maternal-fetal interface in BPH/5 mice (Figure 4.3), we next assessed the expression of IL-15 in BPH/5 and C57 implantation sites. Using qRT-PCR, we saw significant upregulation of *IL-15* mRNA in BPH/5 implantation sites compared to C57 beginning at e4.5, and this persisted at e5.5 and e7.5 (Figure 4.4A). Similarly, Western blot analyses showed IL-15 protein was also significantly upregulated in BPH/5 implantation sites versus C57 at e4.5, e5.5, and e7.5 (Figure 4.4B-E). This finding was unexpected as IL-15 signaling promotes proliferation and maturation of dNK cells[11].

<u>Figure 4.3:</u> The BPH/5 maternal-fetal interface shows profound dNK cell loss at e5.5 and e7.5. (A) Quantitation by qRT-PCR of *NKp46* mRNA in C57 and BPH/5 implantation sites during periimplantation and postimplantation time points. **(B)** Quantitation of $CD122^{+}/DBA^{+}$ cells per implantation site from C57 and BPH/5 at e5.5. **(C)** Immunofluorescent staining of DBA^{+} cells in e5.5 C57 and BPH/5 implantation sites at 40x magnification. (n=3-12) *$p<0.05$ vs time-matched C57, † $p<0.05$ vs e4.5 C57. n.s.= non-significant.

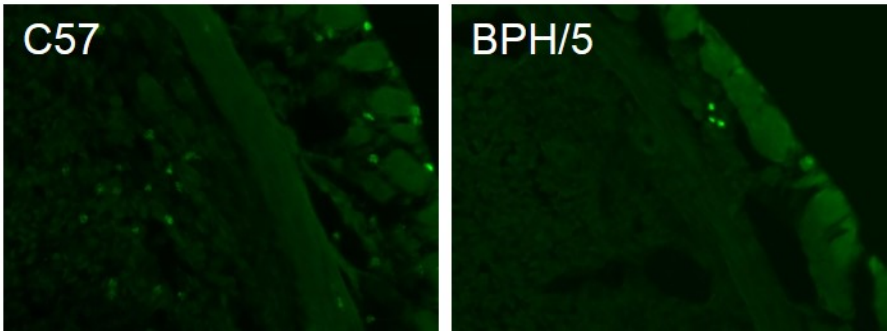
A


NKp46 expression
Relative mRNA
0.10
0.08
0.06
0.04
0.02
0.00
n.s.
*
†
*
n.s.
4.5
5.5
7.5
10.5
Day of Pregnancy
C57
BPH/5

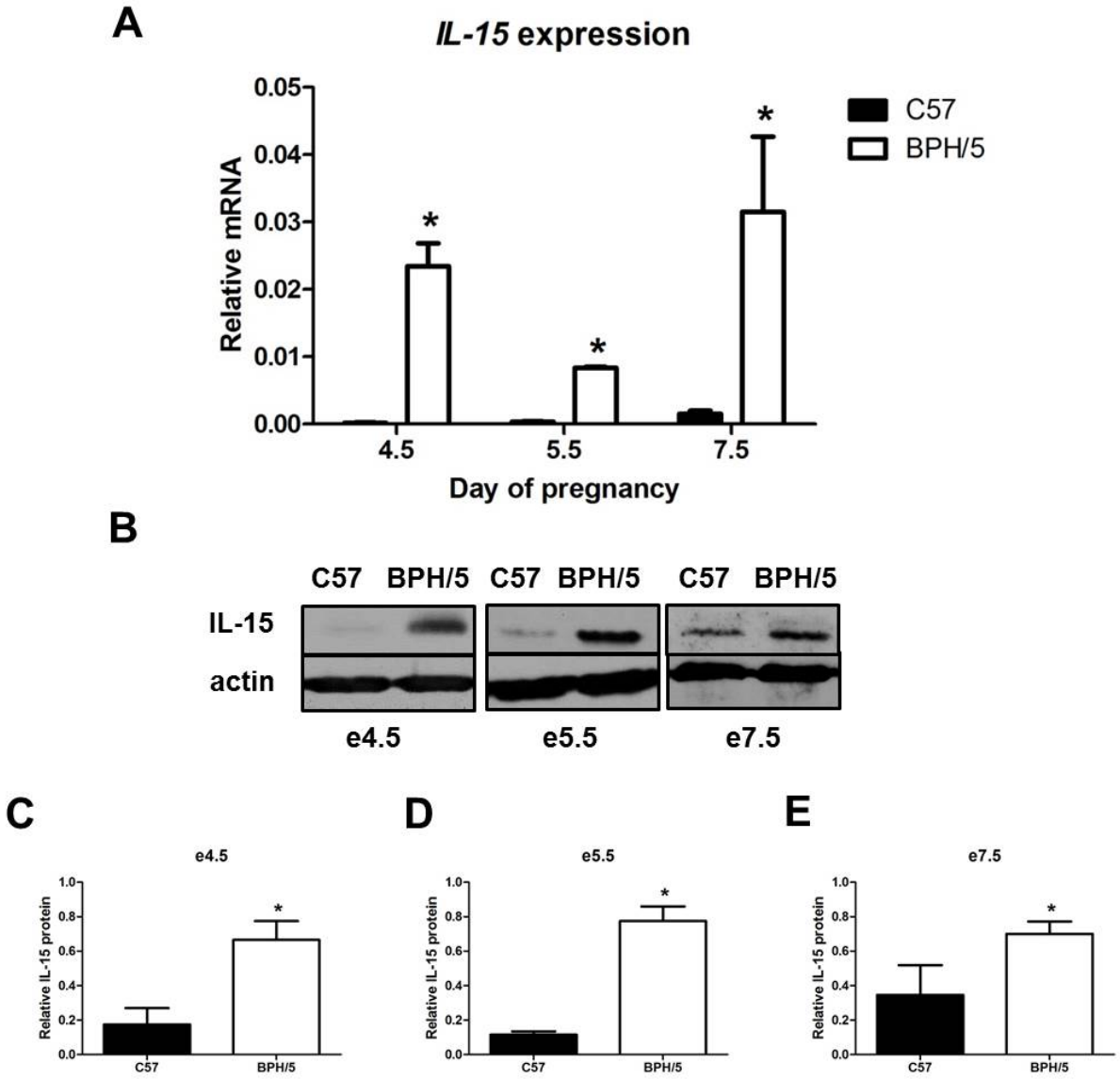
B


CD122+/DBA+cells /implantation site
1.5×10^04
1.0×10^04
5.0×10^03
0
*
C57Bl/6
BPH/5

C


C57
BPH/5

<u>Figure 4.4:</u> IL-15 is significantly increased in BPH/5 implantation sites throughout the periimplantation period. (A) Quantitation by qRT-PCR of *IL-15* mRNA in C57 and BPH/5 implantation sites at e4.5, e5.5, and e7.5. **(B)** Representative western blot gels of IL-15 protein expression in C57 and BPH/5 implantation sites at e4.5, e5.5, and e7.5. **(C)** Quantitative summary of IL-15 protein expression in implantation sites at e4.5, **(D)** e5.5, and **(E)** e7.5. Data are expressed as IL-15 protein relative to actin (n=4),*$p \leq 0.05$.


A
IL-15 expression
Relative mRNA
0.05
0.04
0.03
0.02
0.01
0.00
4.5
5.5
7.5
Day of pregnancy
C57
BPH/5
*
B
C57 BPH/5 C57 BPH/5 C57 BPH/5
IL-15
actin
e4.5
e5.5
e7.5
C
D
E
e4.5
e5.5
e7.5
Relative IL-15 protein
1.0
0.8
0.6
0.4
0.2
0.0
C57
BPH/5

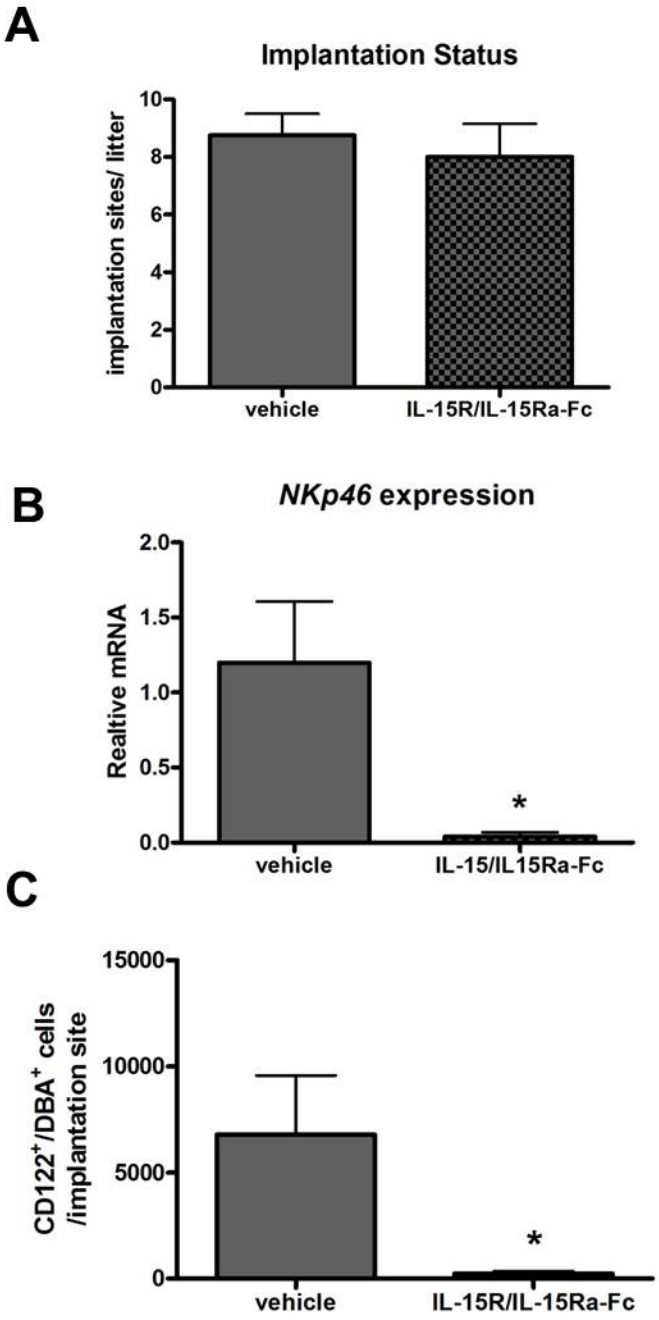
We next sought to functionally link overexpression of local IL-15 to dNK cell loss in C57 control mice. Pregnant C57 mice were anesthetized and given an intrauterine injection of a recombinant IL-15-antibody complex (IL-15R/IL-15Ra-Fc) or vehicle (saline) on e2.5. Implantation sites were then collected at e5.5 to assess dNK cell activation status and dNK cell numbers within implantation sites. First, we determined that our strategy was not interfering with implantation status as we were able to visualize similar numbers of implantation sites between C57 vehicle and IL-15R/IL-15Ra-Fc-treated females (Figure 4.5A). *NKp46* mRNA was measured in implantation sites collected from both groups and found to be significantly decreased in the IL-15R/IL-15Ra-Fc treated group (Figure 4.5B). Furthermore, fewer $CD122^{+}/DBA^{+}$ cells were observed by flow cytometry in implantation sites from the IL-15R/IL-15Ra-Fc treated group (Figure 4.5C).

Selective Cox2 inhibition early in pregnancy reduces IL-15 in BPH/5 implantation sites

We have provided evidence that the BPH/5 maternal-fetal interface is characterized by striking defects in the Cox2 signaling pathway (Chapter 3). Cox2 plays a key role in fetoplacental development as celecoxib (a selective Cox2 inhibitor) improved pregnancy outcomes in BPH/5 females, including amelioration of fetal growth restriction (FGR) in BPH/5 offspring. IL-15 has been shown to increase Cox2 expression in a prostaglandin (PG) E_2 dependent manner[22]. We have demonstrated that celecoxib administration at e6.5 reduces Cox2 protein and PGE_2 levels in BPH/5 implantation sites just 24 hours later at e7.5 (Figure 3.5C & F). We next hypothesized that celecoxib would also have an effect on IL-15 expression in BPH/5 implantation sites. First, *IL-15* mRNA was measured by qRT-PCR in BPH/5 and C57 vehicle and celecoxib-treated e7.5 implantation sites. Expression levels were undetectable (Ct values >35) after celecoxib in C57 implantation sites, whereas a trend was present for the reduction in *IL-15* in BPH/5 celecoxib-treated implantation sites (data not shown). Therefore, we measured IL-15 protein expression by Western blot in BPH/5 vehicle and celecoxib-treated e7.5 implantation sites only. There was a reduction ($p=0.07$) in IL-15 protein from BPH/5 celecoxib-

treated versus vehicle-treated implantation sites (Figure 4.6). This data suggests that downregulation of IL-15 may be an off-label effect of Cox2 inhibition with celecoxib in BPH/5 implantation sites and that celecoxib could be improving pregnancy outcomes in BPH/5 mice by also working through the IL-15 signaling pathway.

Figure 4.5: Uterine IL-15 is functionally linked to dNK cell loss during pregnancy in C57 mice. (A) Implantation status at e5.5 was measured in C57 female mice after intrauterine injection of a recombinant IL-15-antibody complex (IL-15R/IL-15Ra-Fc) or vehicle (saline) on e2.5. **(B)** Quantitation by qRT-PCR of *NKp46* mRNA in C57 e5.5 implantation sites after IL-15R/IL-15Ra-Fc and vehicle administration. **(C)** Quantitation of $CD122^{+}/DBA^{+}$ cells per e5.5 implantation site from C57 IL-15R/IL-15Ra-Fc and vehicle-treated mice. (n=3-4),*$p<0.05$.


A
Implantation Status
implantation sites/ litter
10
8
6
4
2
0
vehicle
IL-15R/IL-15Ra-Fc
B
NKp46 expression
Realtive mRNA
2.0
1.5
1.0
0.5
0.0
*
vehicle
IL-15/IL15Ra-Fc
C
CD122+/DBA+ cells /implantation site
15000
10000
5000
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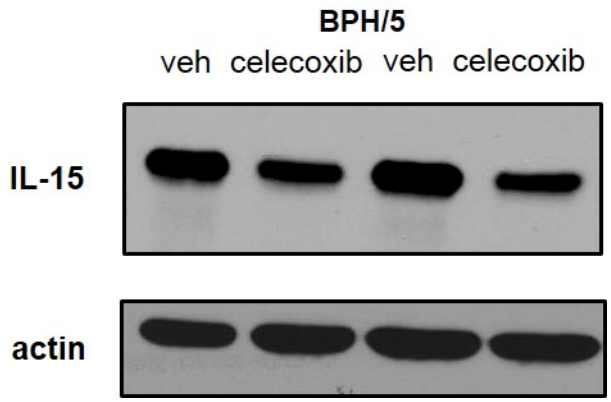
vehicle
IL-15R/IL-15Ra-Fc



<u>Figure 4.6:</u> A selective Cox2 inhibitor (celecoxib) reduces IL-15 protein in BPH/5 implantation sites at e7.5. Western blot analysis of IL-15 and actin protein in e7.5 BPH/5 implantation sites after celecoxib (10 mg/kg body weight) administration on e6.5.

DISCUSSION

The data presented here supports the hypothesis that the BPH/5 maternal-fetal interface has defects in key immunoregulatory pathways early in pregnancy that are crucial for proper placenta formation. Remodeling of spiral arteries is necessary for establishing uteroplacental perfusion and decidual Natural Killer (dNK) cells at the maternal-fetal interface aid in this process[2]. As defects in spiral artery remodeling are a hallmark of PE in women[3] and BPH/5 mice[16], we measured dNK cells and associated cytokines in BPH/5 implantation sites. We found fewer dNK cells in the BPH/5 decidua along with reduced activation status and function. These observations were preceded by overexpression of IL-15 in BPH/5 implantation sites, which we functionally linked to dNK cell loss in C57 pregnant mice. To gain further insight into the mechanism by which IL-15 promotes dNK cell loss, we determined celecoxib treatment reduced IL-15 in BPH/5 implantation sites. Our study provides novel mechanistic insight into the origins of these PE-associated placental defects in the BPH/5 mouse model.

Investigating the role of dNK cells in PE has gained importance in recent years. Not only are dNK cells the most abundant immune cell at the maternal-fetal interface, constituting approximately 70%, they also have important regulatory functions during pregnancy[2]. They are highly involved two developmental processes crucial for placentation, trophoblast invasion and vascular remodeling[9]. Chemokines, such as IL-8 and interferon-inducible protein-10, produced by dNK cells have been shown to regulate trophoblast invasion *in vitro* (human) and *in vivo* (mouse)[9]. Furthermore, their absence has been linked to robust endovascular invasion mediated by hypoxia at the maternal-fetal interface[23]. Therefore, a delicate balance in dNK cell abundance in the decidua is required for proper trophoblast invasion. Contrary to their name, dNK cells are poorly cytolytic, but rather are a source of cytokines, chemokines, and growth factors with potent angiogenic activity, such as vascular endothelial growth factor (VEGF) and placental growth factor (PGF)[11].

The literature presents confusing and contradictory data about the role of dNK cells in PE. One report describes increased numbers of $CD56^{+}$ dNK cells in the decidua of PE patients[24].

However, these samples were collected at the time of delivery from healthy controls and PE patients (26-42 weeks of gestation). We know that dNK cells reach maximal expression in the human placenta by the end of the 2nd trimester[11], thus it is hard to make conclusions from these results. Another source reports fewer $CD56^+$ dNK cells in the decidua from both PE pregnancies and pregnancies with FGR alone[25]. Again these placental biopsies were taken at the time of delivery and therefore it is difficult to interpret the significance of these findings. A possibly more relevant finding is an association of impaired chemoattraction of trophoblasts by dNK cells from pregnancies that had high uterine artery Doppler resistance index (RI), an indicator of impaired spiral artery remodeling[26]. These data hold promise as women with high uterine artery RI have increased risk of developing PE[26]. Interestingly, the BPH/5 mouse also demonstrates increased uterine artery RI during pregnancy further corroborating the importance of investigating dNK cells in BPH/5 mice.

The BPH/5 mouse develops the maternal PE syndrome of late-gestational hypertension and proteinuria, as well as abnormal placentation with shallow trophoblast invasion and inadequate remodeling of spiral arteries[15, 16]. Importantly, BPH/5 pregnant mice also demonstrate increased vascular resistance in uterine arteries, an indicator of placental vascular insufficiency and impairment of uteroplacental circulation[16]. Circulating and placental angiogenic factors (VEGF and PGF) are reduced in BPH/5 mice at early gestational time points and delivery of an adenovirus encoding $VEGF_{121}$ at e7.5 blunted the maternal syndrome[17]. Taken together, it is likely that there would be perturbations in dNK cell populations at the maternal-fetal interface in BPH/5 mice. Therefore, we hypothesized that key immunoregulatory pathways involving dNK cells and their associated cytokines would be dysregulated early in BPH/5 pregnancy.

Defective angiogenesis and vascularity during the periimplantation period has been proposed as a possible mechanism for abnormal placentation in PE[3, 14, 27, 28]. We have demonstrated significant periimplantation defects in the BPH/5 mouse; therefore we investigated the immunoregulatory cells and cytokines early in BPH/5 pregnancy. First, we examined the expression of pro-inflammatory genes (*IL-6*, *IL-1β*, *Tnf-α*, and *Ifn-γ*) in the periimplantation

period when we also see significant increases in *Ptgs2* and Cox2 (Chapter 3). While implantation and decidualization are considered inflammatory pregnancy events, local suppression of excessive inflammation is essential for embryo survival and pregnancy success[20]. Implantation sites from BPH/5 and C57 at e5.5 showed comparable expression of *IL-6, IL-1β, Tnf-α,* and *Ifn-γ* (Figure 4.1). However, at e7.5 we saw a significant reduction in *Ifn-γ* expression in BPH/5 implantation sites (Figure 4.1D). In mice, IFN-γ provides the signals that transiently vasodilate spiral arteries to increase blood flow for proper placental perfusion[7]. Endometrial IFN-γ expression mirrors dNK cell localization in the uterus[12]. This expression profile was not observed in a mouse strain (tgε26; $NK^-T^-B^+$) having much reduced levels (only 1%) of normal dNK cell numbers[29]. Implantation sites from *Ifng* null mice exhibit excessive numbers of incompletely differentiated dNK cells, widespread decidual necrosis, a lack of appropriate spiral artery modifications[12] and significant fetal loss[29]. Treatment of alymphoid mice with recombinant IFN-γ results in normal decidual and arterial morphology[12]. Therefore, the production of IFN-γ by dNK cells is critical for gestational changes in the decidua and uterine vasculature.

We next went on to explore the dNK cell profile in BPH/5 during the periimplantation period. Using NKp46 as a marker of activated dNK cells, we discovered that C57 show a progressive increase in implantation site *NKp46* expression that peaks at e7.5 (Figure 4.3A). BPH/5 mice do not show this rise in expression and have significantly lower *NKp46* expression compared to C57 at e5.5 and e7.5 (Figure 4.3A). These qRT-PCR findings of downregulated activated dNK cells were confirmed by flow cytometry and immunohistochemical analyses of the mouse dNK cell marker, *Dolichos biflorus* agglutinin (DBA) in BPH/5 e5.5 implantation sites (Figure 4.3B & C). Because dNK cells are thought to provide ~90% of the IFN-γ in the mouse implantation site[11], it makes sense that a reduction in dNK cells would precede lower *Ifn-γ* expression in the BPH/5 implantation site. IL-15 is required to activate dNK cells in the uterus of women and mice; therefore we next looked at IL-15 in the BPH/5 implantation site at these critical gestational time pints.

Similar to BPH/5 mice, *IL-15*$^{-/-}$ mice show inappropriate decidualization, lack mature dNK cells and, interestingly, produce low-birth-weight pups [30]. Exogenous IL-15 administration in these mice restores dNK cell populations, thus confirming its importance in dNK cell maturation. Therefore, we hypothesized that BPH/5 mice would show decreased expression of IL-15 in implantation sites during the periimplantation period. Very unexpectedly, BPH/5 implantation sites show robust expression of both mRNA and IL-15 protein at e4.5, e5.5, and e7.5 compared to C57 (Figure 4.4A-E). Uterine IL-15 is upregulated at the time of decidualization to activate dNK cells[11]. This robust overexpression may be a consequence of defective decidualization we see in BPH/5 mice (Chapters 2 & 3) or it could be a physiologic response of the uterus to produce more and more IL-15 as adequate numbers of pre-dNK cells are not being recruited to the BPH/5 decidua. Ovarian hormones are thought to play a prominent role in this recruitment process[11]. BPH/5 females have aberrations in ovarian hormone profiles during pregnancy (Chapter 2) as well as in the non-pregnant state (data not shown). Further investigation of dNK cell recruitment to the decidua and the endocrine profile in BPH/5 mice is thus warranted.

Increased decidual IL-15 expression has been linked to recurrent miscarriage in women, suggesting impaired implantation and vascularization of the placenta[31]. Other reports have demonstrated increased circulating IL-15 levels in the serum of preeclamptic mothers as compared to healthy controls, where IL-15 levels were proportional to severity of disease presentation[32]. These findings provide evidence that IL-15 maybe involved in poor pregnancy outcomes. Thus overexpression of IL-15 in BPH/5 implantation sites may be another feature of the decidualization defects we see in this model. We next sought to determine if increased IL-15 in BPH/5 implantation sites was having a direct effect on dNK cell activation and proliferation. To test this, C57 mice given an intrauterine injection of a recombinant IL-15- antibody complex (IL-15R/IL-15Ra-Fc) on e2.5 to increase local IL-15 at the time of implantation (when we first see increased IL-15 in BPH/5). Strikingly, *NKp46* mRNA was reduced and CD122^{+}/DBA^{+} cells were less abundant compared to C57 vehicle (Figure 4.5B & C). This is a novel finding and

needs further study. Some reports describe increased placental dNK cells at delivery in PE patients that also have increased serum levels of IL-15[24]. Furthermore, these dNK cells have an altered phenotype that may be responsible for dysregulated dNK signaling in these tissues. This would suggest that the dNK phenotype would be important to examine in BPH/5 and could provide even further insight into the altered immunoregulatory mechanisms during PE pregnancies. For instance, assessing the angiogenic potential of BPH/5 dNK cells and if they produce adequate VEGF and PlGF could provide further mechanistic insight into the early angiogenic imbalance seen in BPH/5 mice.

We have identified key molecular defects during the periimplantation period in BPH/5 mice, including dysregulated IL-15 and Cox2 signaling (Chapter 3 & 4). IL-15 is crucial for dNK cell-mediated adaptations in establishing placental blood flow[11]. However, we have functionally linked its overexpression to the downregulation of dNK cells in BPH/5 implantation sites prior to placenta formation. Conversely, *IL-15*-/- mice lack mature dNK cells[30], indicating that a sensitive equilibrium in its expression during pregnancy it critical for successful outcomes. Fetal and placental outcomes were improved in BPH/5 mice after inhibition of Cox2 with celecoxib in implantation sites early in pregnancy (Chapter 3). An interesting study using fibroblast-like synoviocytes taken from patients with rheumatoid arthritis showed that Cox2 is increased by IL-15 in a PGE_2 dependent manner[22]. Therefore we next tested the hypothesis that Cox2 inhibition with celecoxib would also have an effect on IL-15 expression as PGE_2 was reduced in BPH/5 implantation sites after celecoxib treatment. Indeed, there was a reduction in IL-15 protein in BPH/5 celecoxib-treated versus vehicle-treated implantation sites (Figure 4.6). This data would suggest that PGE_2 may have feedback on IL-15 to lower its expression and subsequent Cox2 expression after celecoxib administration. This downregulation of IL-15 may be contributing to the improvement in pregnancy outcomes seen in BPH/5 mice after celecoxib treatment. Much work is needed to elucidate the precise mechanisms, but a strong upstream candidate that regulates both IL-15 and Cox2 is nuclear factor Κ Β (NFKB)[22]. Furthermore,

inhibition of Cox2, prostaglandins and NFKB has been proposed as potential therapeutic approaches for preterm labor, a pregnancy disorder characterized by placental inflammation[33].

Recruitment and activation of dNK cells early in pregnancy during the periimplantation period is crucial for placental development and pregnancy outcomes. While PE-associated placental defects are known to originate early in pregnancy, it is difficult to accurately study these events in the context of PE. We present here novel mechanistic findings on the immunoregulatory dNK cells and cytokines that play essential roles in placental development and how aberrations in their signaling precede placental pathologies in this model of PE. Furthermore, we provide exciting new insights into pharmacological approaches for managing dysregulations at the maternal-fetal interface in the context of PE. We know that anti-inflammatory therapy early in pregnancy is associated with reduced risk of PE in high risk women[34], but a defined mechanism of action is still unknown.

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CHAPTER FIVE:

DISCUSSION

Summary of findings

Over the past decade the Davisson laboratory has made significant advances in characterizing the phenotype of the BPH/5 mouse model of preeclampsia (PE). Beginning with the maternal syndrome and working backwards towards early pregnancy events. BPH/5 females were first observed to develop late-gestational hypertension and proteinuria[1]. They also exhibited poor fetal outcomes, including fetal demise and *in utero* fetal growth restriction (FGR). Prior to the appearance of the maternal syndrome in BPH/5, abnormalities in placental development could be detected, including shallow trophoblast invasion and inadequate remodeling of spiral arteries[1, 2]. Importantly these placental pathologies are also seen in women with PE[3]. Therefore, the placenta is likely a central player in the adverse pregnancy outcomes seen in both women with PE and in our model. To confirm this, studies were undertaken to identify a causal role for the placenta in the development of the BPH/5 PE-like syndrome and associated adverse pregnancy outcomes. To date, placental oxidative stress and an angiogenic imbalance have been linked to the maternal syndrome in BPH/5 mice[4, 5]. These studies provided strong evidence that the dysregulated placenta is central to the pathogenesis of PE in this model. Therefore, examining placentation at the very earliest stages of development in BPH/5 is crucial to elucidate the origins of PE.

The studies presented in this thesis were designed to further define the causal role of the placenta in the pathogenesis of adverse pregnancy outcomes in BPH/5 mice and test 3 main hypotheses. 1) BPH/5 mice have periimplantation developmental defects before placenta formation. 2) Periimplantation defects have “ripple effects” that impact pregnancy outcomes in BPH/5 mice. 3) Dysregulated immunoregulatory mechanisms working at the level of the maternal-fetal interface are associated with periimplantation defects.

In Chapter 2, we examined key periimplantation events leading up to placental development during BPH/5 pregnancy. We observed a deferment in the timing of implantation along with significant decidualization defects. This was followed by evidence of hypoxia at the time of peak decidualization just before placenta formation. We identified variation in fetal

health status in BPH/5 mice at mid gestation that was associated with downregulated trophoblast differentiation markers within the placenta. Defective implantation is thought to cause adverse "ripple effects" throughout pregnancy, leading to abnormal placentation, retarded fetal development, and poor pregnancy outcomes, such as PE[6-8]. This data provided preliminary evidence that defects in periimplantation events may lead to adverse pregnancy outcomes in the BPH/5 model of PE.

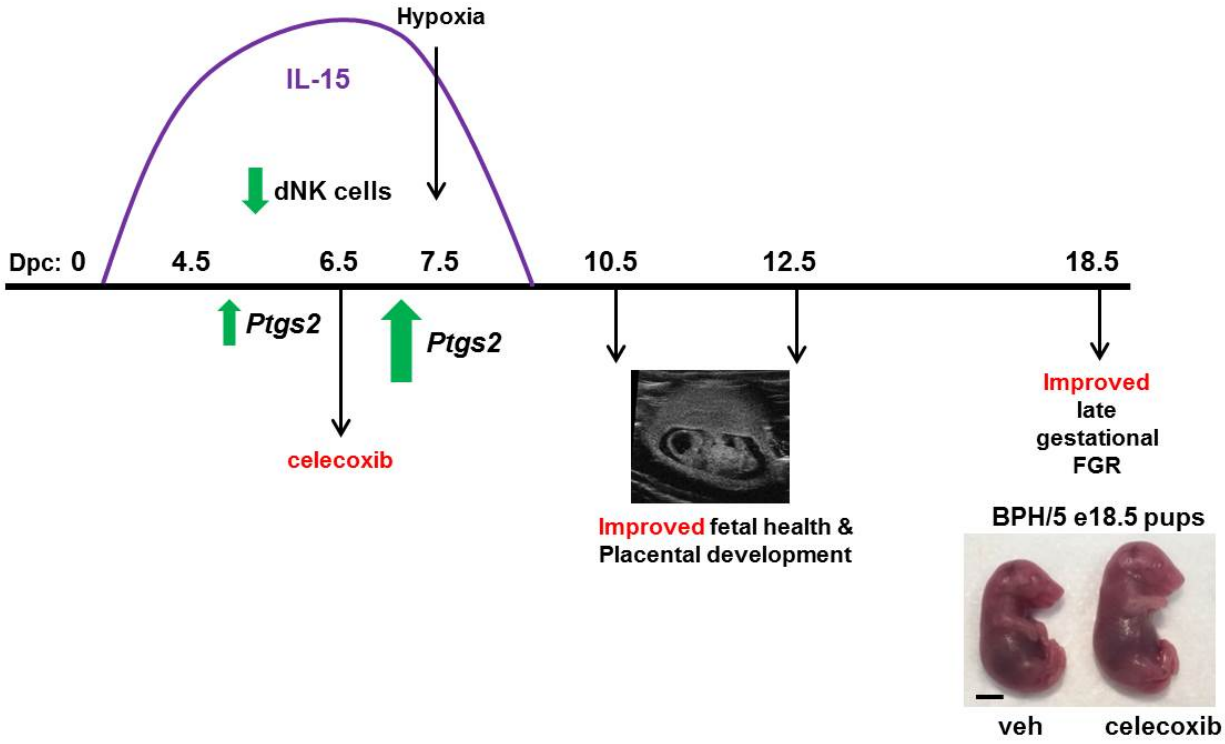
In Chapter 3, we explored the causal role of defective periimplantation signaling on adverse pregnancy outcomes in BPH/5 mice. Aberrations in the expression of implantation and decidualization molecular mediators can have "ripple effects" that may lead to poor pregnancy outcomes[8]. Their role in PE-associated adverse pregnancy outcomes is undefined. Beginning at the very onset of the periimplantation period, we observed dysregulation of key implantation and decidualization molecular mediators in BPH/5 implantation sites. However by the end of implantation, *Ptgs2*/Cox2 alone was dysregulated and showed robust overexpression in the decidua, the site of placenta formation. Because Cox2 is a pivotal molecule during the periimplantation period, we used pharmacologic targeting with a Cox2 inhibitor to selectively blunt its overexpression in the implantation site. This strategy corrected fetoplacental developmental abnormalities (Chapter 2) and FGR in this mouse model. Preliminary data show an improvement in late-gestational proteinuria in BPH/5 mice, which may indicate that correction of defective periimplantation signaling improves fetal health, placental development, and the maternal syndrome in this model.

In Chapter 4, we explored potential immunoregulatory mechanisms for BPH/5 placental pathologies in the periimplantation period. Implantation and decidualization trigger the recruitment and activation of key immune cells, decidual Natural Killer (dNK) cells, at the maternal-fetal interface that aid in the transformation of decidual spiral arteries[9]. We present here novel mechanistic findings on dNK cells loss at the maternal-interface being mediated by uterine IL-15 expression. Furthermore, administration of a selective Cox2 inhibitor early in BPH/5 pregnancy reduced IL-15 expression in the implantation site. This data provides evidence for

new pharmacological approaches for managing dysregulations at the maternal-fetal interface in the context of PE.

Proposed model

The findings presented in this thesis provide a greater depth of understanding into the origins of adverse pregnancy outcomes in a mouse model of PE. We provide strong evidence to support the hypothesis that defects in the periimplantation period have “ripple effects” on poor fetoplacental development in BPH/5 mice and that dysregulated maternal-fetal interactions play a causative role. The salient findings of this thesis are presented diagrammatically (Figure 5.1).



<u>Figure 5.1:</u> Periimplantation signaling involving inflammation and hypoxia have adverse "ripple effects" on pregnancy outcomes in the BPH/5 mouse model of PE. Key dysregulations early in BPH/5 pregnancy involve delayed implantation and decidualization coincident with overexpression of interleukin (IL)-15, prostaglandin synthase 2 (*Ptgs2*), and hypoxia inducible factor 1α (*Hif1α*) at the maternal-fetal interface with few decidual Natural Killer (dNK) cells. This thesis discusses a role for anti-inflammatory therapy early in pregnancy with celecoxib and its potential for improving adverse pregnancy outcomes seen in this model of PE. Dpc= Days post coitus.

Periimplantation developmental and molecular defects

The periimplantation period involves coordinated embryo-uterine interactions in order to establish and maintain a healthy pregnancy[6-8]. These studies presented in this thesis provide evidence that dysregulation of early pregnancy events, such as implantation and decidualization, underlie adverse pregnancy outcomes in the BPH/5 mouse model of spontaneous PE.

It was first observed by Dr. Ashley Woods, that BPH/5 embryos space abnormally within the uterus and appear clustered during the periimplantation period (PhD thesis). This was the first indicator that implantation was aberrant in BPH/5 mice and may be linked to placental defects, as embryo crowding has been associated with abnormal placentation[10, 11, 12]. Mice deficient in cytosolic phospholipase A2α (cPLA2α) not only have defects in embryo spacing, but also in placental development and fetal growth[10]. Interestingly, *cPLA2α-/-* mice have deferred timing of implantation. By performing pontamine perfusion studies in a time course during the periimplantation period, we identified a deferment in implantation timing in BPH/5 mice by 12 hours. In the evening of embryonic day (e) 3.5, we observe pontamine blue stained implantation sites within the C57 uterus. Pontamine blue staining is not apparent in BPH/5 until e4.5am, at which time implantation sites are weakly stained and fewer in number than C57. This was quite an extraordinary initial finding as significant pregnancy loss in women has been shown to occur when implantation occurs just 24 hours outside the optimal window of receptivity[13]. Perhaps if BPH/5 mice had even longer deferment in implantation they would show signs of early pregnancy loss. Both our data and findings in the human literature suggest that timing is everything.

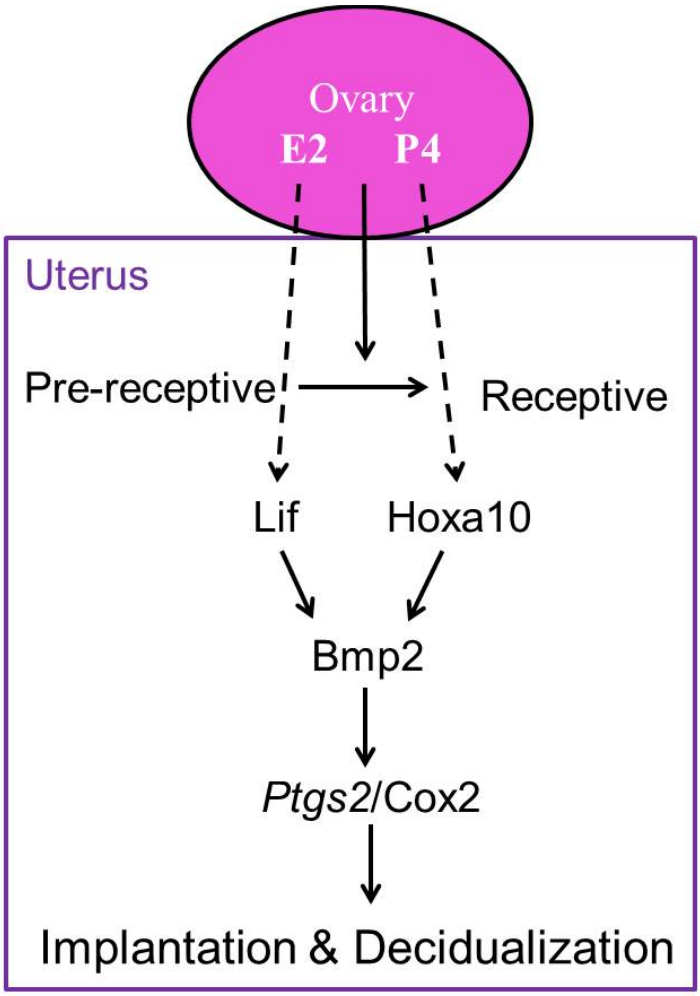


Figure 5.2: Schematic of implantation and decidualization signaling pathway. Ovarian hormones initiate the transformation of the pre-receptive uterus to the receptive state while signaling to estrogen (E_2) and progesterone (P4)-responsive genes, Leukemia inhibitory factor (*Lif*) and Homeobox A10 (*Hoxa10*), respectively. Both these factors input on Bone morphogenic protein 2 (Bmp2), which is upstream of prostaglandin synthase 2 (*Ptgs2*) and the protein it encodes, cyclooxygenase 2 (Cox2). Cox2-derived prostaglandins (PGs) are essential in mediating vascular changes involved in implantation and decidualization. Figure adapted from Lee et al. 2007 Mol Cell Bio.

An important cytokine involved in the timing of implantation is Leukemia inhibitory factor (LIF). LIF is also believed to be important in people as increased uterine levels have been reported during implantation in women[14]. *Lif* has biphasic expression in the mouse uterus to confer uterine receptivity first and then facilitate the attachment reaction by signaling to downstream implantation and decidualization signaling molecules[12]. Although BPH/5 mice have lower circulating estradiol-17β during the time of anticipated receptivity, uterine expression of the estrogen (E_2) - sensitive cytokine, *Lif*, was unchanged. However, 24 hours later at the time of implantation in BPH/5 mice, *Lif* was significantly decreased in BPH/5 implantation sites. This was very curious as we know *Lif* deficient mice have complete implantation failure with the embryo remaining in a dormant-like state[12]. Because we do see implantation in BPH/5 mice, albeit 12 hours delayed, the defects in periimplantation signaling stemming from reduced *Lif* expression would likely be more discreet and seen as a "ripple effect".

Thorough analyses of the signaling molecules involved in mediating subsequent decidualization were performed to link these aberrations in periimplantation events with a molecular signature. The pathways involved in decidualization rely on ovarian steroid hormone signaling via E_2 and progesterone (P4)-responsive genes, *Lif* and Homeobox A10 (*Hoxa10*) respectively[6]. While *Lif*-null mice experience implantation failure, *Hoxa10*-/- female mice are infertile due to defective decidualization[8]. We saw overexpression of *Hoxa10* in BPH/5 implantation sites at e4.5, but at the time when we see a lack of decidualization. This would suggest that *Hoxa10* is not the central mediator of decidualization and other downstream factors may be more important. Bone morphogenic protein 2 (*Bmp2*) is an important downstream factor of both *Lif* and *Hoxa10*, and upstream of prostaglandin synthase 2 (*Ptgs2*)[15]. *Bmp2* and *Ptgs2* expression was downregulated in BPH/5 implantation sites initially at e4.5am. This finding pointed towards a very central role for *Bmp2* and *Ptgs2* in the decidualization delay seen in BPH/5 mice. Cox2 is thought to be a final common factor upon which the upstream implantation and decidualization molecules including *Lif*, *Hoxa10* and *Bmp2* converge [6, 15]. Indeed by the end of implantation at e5.5am, *Ptgs2* alone is dysregulated in BPH/5 implantation sites and showed

increasing expression around the embryo moving towards the mesometrial pole of the decidua. Postimplantation at the time of peak decidualization (e7.5) we observed persistent *Ptgs2* expression strongly within the decidua at the mesometrial pole. Additionally, Cox2 protein showed corresponding increased expression in whole BPH/5 implantation sites. Cox2-derived prostaglandins (PGs) are crucial for implantation, decidualization, placental formation and angiogenesis early in pregnancy[6, 8, 16]. Thus Cox2 would seem to be the master regulator for periimplantation defects seen in BPH/5 mice.

Periimplantation defects and Cox2 signaling

An overwhelming amount of evidence in this thesis points towards the role of Cox2 and Cox2-derived PGs in the periimplantation defects seen in BPH/5 mice. *Ptgs2*-/- mice have compromised implantation and decidualization[17]. Mice that have reduced levels of PGs (*cPLA2α*-/-) have periimplantation and fetoplacental developmental defects similar to BPH/5 mice. Furthermore, mice deficient in lysophosphatidic acid (LPA), which is generated by PLA2α, also show similar adverse pregnancy outcomes via altered Cox2 signaling[11]. Since *Ptgs2* and Cox2 are highly dysregulated in BPH/5 implantation sites, we strongly believe this pathway is a key player in adverse pregnancy outcomes seen in BPH/5 mice.

Cox2-derived prostacyclin (PGI_2) has been shown to be necessary for implantation[16]. *Ptgs2*-/- mice show marked improvement in periimplantation processes when given carbaprostacyclin (cPGI), a stable analog of PGI_2[16]. The scenario in BPH/5 mice however is more complex. We first see decreased expression of *Ptgs2* in the implantation site at e4.5am and then increased expression at e5.5am and e7.5am. Over exuberant *Ptgs2* expression in the periimplantation period has not been characterized in the context of PE. Furthermore, most of the literature focuses on targeting Cox2 during the periimplantation period as a potential contraceptive[18]. Defective implantation and decidualization in *Ptgs2*-/- mice is partially due to dysregulated vascular events and defective vascular endothelial growth factor (*Vegf*) signaling[19].

$VEGF_{164}$ is downstream of Cox2 in the periimplantation period and its robust expression in BPH/5 implantation sites further confirm dysregulated Cox2 signaling in BPH/5 decidua.

To functionally link periimplantation signaling defects with adverse pregnancy outcomes in BPH/5 mice, studies were performed using a non-steroidal anti-inflammatory drug (NSAID) that selectively inhibits Cox2 (celecoxib)[20]. Because Cox2 is a contraception target and inhibition can have anti-implantation effects, a dose of celecoxib was used that had been tested safe during early pregnancy[20]. Likewise, this recommended dose had also been reported as an adjunct therapy in an experimental mouse model to prevent preterm labor[21]. The timing of celecoxib administration on e6.5 was selected so as to not interfere with implantation and early decidualization, since Cox2 is essential for these processes, but rather to target misregulated Cox2 during peak decidualization. This strategy normalized Cox2, $VEGF_{164}$ protein and reduced PGE_2 levels in BPH/5 implantation sites just 24 hours later at e7.5, that very critical time in mouse gestation before placenta formation. PGE_2 is the second most abundant PG in the mouse implantation site during implantation and decidualization and it is a potent vasoconstrictor in the placental vasculature[22]. Therefore, reducing local levels of it could improve decidual blood flow as the fetoplacental unit is being established. Advances in high frequency ultrasonography allows for quantitative real time assessment of placental perfusion[23]. Longitudinal studies looking at placental perfusion after celecoxib therapy has the potential to be informative in this regard. Women with PE display angiogenic imbalances early in pregnancy and inadequate uterine angiogenesis/vascularity at the time of implantation has been proposed as a mechanism[24]. This may be due to Cox2 signaling given its profound impacts on angiogenesis and the uterine vasculature.

The significance of hypoxia in the periimplantation period

A potential upstream mechanism for increased Cox2 expression is hypoxia. While a definitive study hasn't been performed in the mouse periimplantation uterus, hypoxia can induce Cox2 expression in other cell types[25]. The *Vegf* isoform 164 is downstream of not only Cox2 but

also hypoxia inducible factor (HIF) during implantation and decidualization in mice[6]. HIF can bind to the hypoxia response element in the *Vegf* promoter to initiate gene expression. We saw significant $VEGF_{164}$ protein expression in BPH/5 e7.5 implantation sites, coincident with maximal *Ptgs2*/Cox2 expression. While Cox2 signaling alone could provoke this expression pattern, the fact that *Hif1α* was also found to be increased at this critical time point in pregnancy was an interesting finding. Hypoxia to an extent is physiologic during early pregnancy[26], but the over expression of *Hif1α* in BPH/5 implantation sites may have a more prominent role in adverse pregnancy outcomes seen in this model than is addressed here in this thesis. Prolonged hypoxia can have profound damaging effects, including induction of oxidative stress and this is hypothesized to occur in PE[27]. BPH/5 mice have increased amounts of reactive oxygen species (ROS) in the placenta and this has been causally linked to fetoplacental defects and the maternal syndrome[4]. Upregulation of *Hif1α* and $VEGF_{164}$ protein in BPH/5 implantation sites shown here predates the timing of placental oxidative stress in this model (mid gestation) and thus the hypoxia that drives the generation of placental ROS may have origins in the periimplantation period. This avenue of research warrants further investigation and has the potential to greatly enhance our understanding of early placental hypoxia and placenta defects in the context of PE.

Linking decidualization defects with poor placental development

Dysregulation at any point in the events encompassing implantation and decidualization can create adverse "ripple effects" throughout pregnancy, leading to abnormal placental development, retarded fetal development, and adverse pregnancy outcomes[8, 10, 11, 13]. Because these pregnancy pathologies are also seen in PE, it is hypothesized that periimplantation defects are involved in the pathogenesis of this devastating pregnancy disorder. Defective patterning of the labyrinth, the maternal-fetal exchange region of the placenta, can lead to impaired placental perfusion and hypoxia[28]. This is thought to be central in the pathogenesis of PE as placental biopsies from PE patients after delivery reveals evidence of hypoperfusion[3]. In the human and mouse placenta, Glial cells missing homolog 1 (GCM1) has been demonstrated to regulate

labyrinth formation[29] and trophoblast differentiation[30]. Importantly, GCM1 expression is decreased in the placenta of human PE patients[31]. Moreover, *in vitro* studies have shown that GCM1 degradation occurs in a human trophoblast cell line after exposure to hypoxia[32]. Perhaps hypoxia during decidualization, as seen in BPH/5 mice, plays a key role in poor placental development by working directly on placental GCM1, leading to poor syncytiotrophoblast differentiation and defective patterning of the labyrinth. Hypoxia could induce expression of apoptotic genes or anti-angiogenic factors, as it's known to induce sFLT1[33], and make for a hostile environment for the implantation embryo. It would be interesting to expose C57 trophoblast cells to hypoxic conditions and examine not only their invasion potential *in vitro*, but also what factors might be induced in the decidua *in vivo*. A certain amount of decidual-trophoblast crosstalk occurs during early pregnancy at the maternal-fetal interface[9, 34]. Defective trophoblast invasion and differentiation in PE may be a consequence of exposure to an excessively hypoxic decidua. Autophagy, a physiologic response to hypoxia, occurs during placental development and its impairment has been linked to PE[35]. We are currently exploring this process in BPH/5 pregnancy and investigating if this may be another pathway that can be targeted to improve adverse pregnancy outcomes in BPH/5 mice.

Perturbations in the maternal-fetal interface

Essential to placentation in women and mice are decidual Natural Killer (dNK) cells[9, 36, 37]. In both species they are transiently present in the decidua and number decline by mid gestation[38]. These cells which are present in high numbers around maternal spiral arteries produce chemokines and cytokines that recruit trophoblast cells and modify decidual vasculature[34]. An imbalance in circulating angiogenic factors exists early in BPH/5 pregnancy (e9.5-e12.5)[5]. The source of this imbalance is presumably the placenta; however the cell type is not precisely defined. As both trophoblast cells and dNK cells produce VEGF and placental growth factor (PGF), either or both may be dysregulated and thus involved in this angiogenic imbalance. The fact that BPH/5 mice have inadequate remodeling of spiral arteries is clearly

defined at e12.5 as well as increased vascular resistance in uterine arteries at e16.5[2]. Both of these features would suggest placental insufficiency in BPH/5 mice due to poorly remodeled spiral arteries. Because dNK cells are critical for uterine angiogenesis and spiral artery remodeling, it is not surprising that we see fewer dNK cells at the maternal-fetal interface at e5.5 and e7.5. What is exciting is that overexpression of IL-15 in the BPH/5 implantation site may be mediating this dNK cell loss. We demonstrated this functionally in C57 control mice given intrauterine recombinant IL-15 just prior to implantation. However, there is debate that uterine stromal cells or macrophages or both produce IL-15 to activate dNK cells. Macrophage deficient *Csf1*$^{op/op}$ mice still show dNK cell activation[9]. Thus even though macrophages are the second most abundant immune cell at the maternal-fetal interface, dNK cells being the most abundant, their importance in implantation is undefined. Whatever the source of IL-15 in the implantation site, we were able to blunt its expression in BPH/5 implantation sites after Cox2 inhibition. This was a surprising finding and opened the door for further studies in the pleiotropic effects of anti-inflammatory therapy with celecoxib early in pregnancy.

Celecoxib improving adverse pregnancy outcomes

Cox2 inhibition with celecoxib improved pregnancy outcomes at mid and late gestation. This was determined by increasing the percentage of healthy BPH/5 fetuses and reducing the percentage of compromised BPH/5 fetuses (fetal bradycardia and *in utero* FGR) per litter at e10.5. Compromised fetuses have placental developmental anomalies, including decreased expression of *Gcm1*, *SynA*, and *SynB* in the labyrinth indicating poor syncytiotrophoblast differentiation and formation of the labyrinth. These compromised fetuses may be programmed to die and thus why we see a high percentage of resorption (40%) in BPH/5 litters at e12.5. Periimplantation Cox2 is ostensibly playing a role in these adverse outcomes as celecoxib improved these defects and was associated with larger litter sizes at e12.5. We also saw an improvement in fetal and placental weights at e12.5 which too may be a result of fewer compromised fetuses at e10.5. A direct connection between Cox2 and Gcm1 as not been

reported, but evidence provided here would suggest hypoxia is a strong candidate. A recent study was published to address the claim that smoking during pregnancy reduces the risk of PE[39]. They showed *in vivo* and *in vitro* that nicotine exposure inhibited trophoblast invasion, increased placental hypoxia, decreased GCM1 and reduced placental vascularization while decreasing MMP9 activity[40]. These findings highlight the intersection of hypoxia and placenta formation.

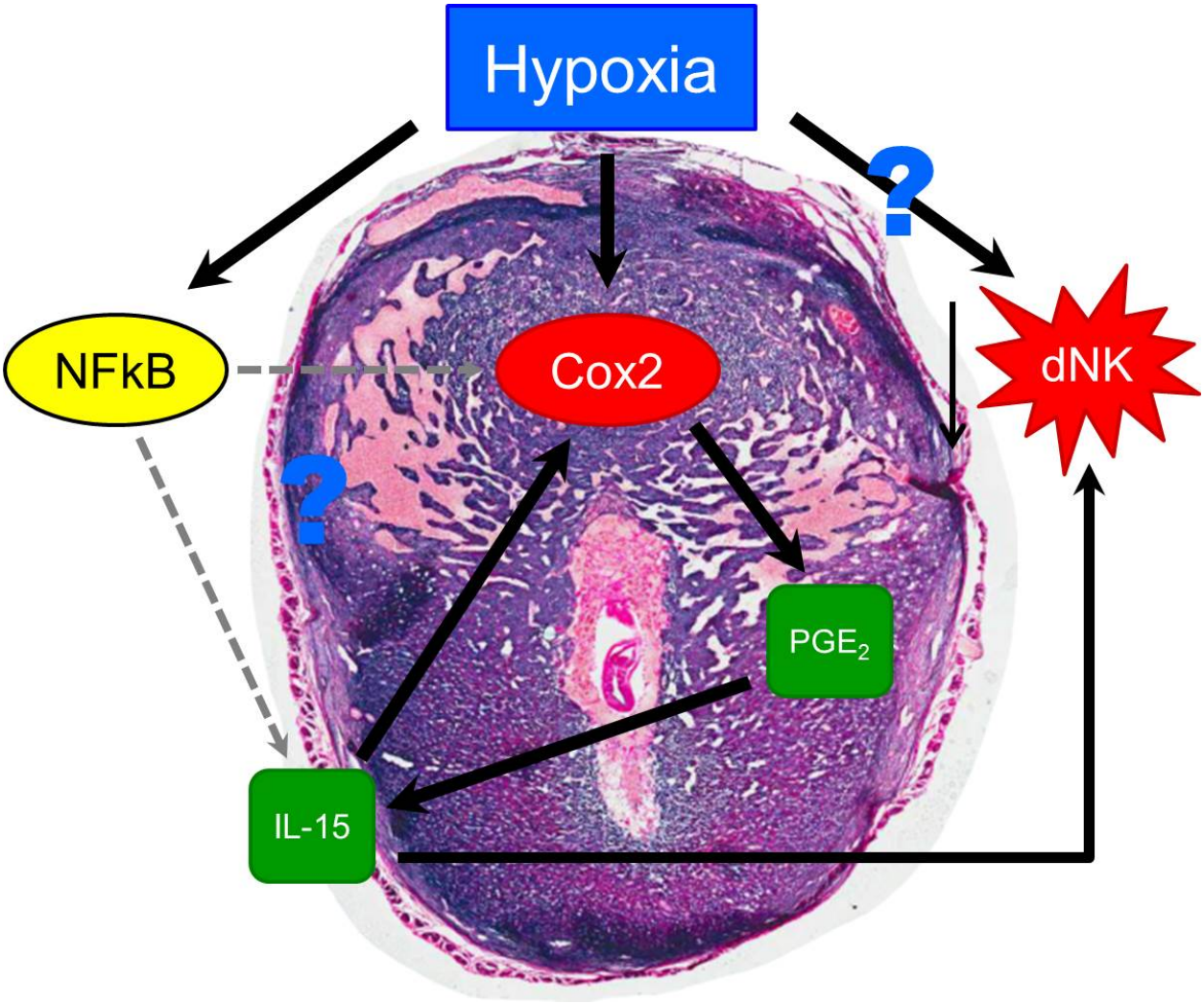
The "ripple effects" of early Cox2 inhibition extended throughout gestation as we also saw an increase in e18.5 pup weight in BPH/5 celecoxib-treated mothers. It is very exciting that normalization of Cox2 and PGE_2 with celecoxib is associated with improved BPH/5 fetal health and placental development here as we know Cox2-derived PGs are directly responsible for regulating fetal and placental vascularity[16, 41]. However, we also provide evidence for redundancy in the pathways modified by celecoxib. We showed off-label effects such as reducing IL-15 protein in BPH/5 implantation sites. Unfortunately this was not associated with an increase in *NKp46*, indicating normalization of dNK cell activation status. Though the precise mechanism is unknown, this data supports a causative role for abnormal uterine Cox2 signaling during the periimplantation period in mediating poor fetoplacental health and FGR in the BPH/5 mouse model of PE.

T regulatory cells, a candidate for dysregulated immunoregulatory mechanisms

The consequence of this potentially hypoxic periimplantation condition in BPH/5 mice is ultimately the generation of a highly proinflammatory decidual environment. Immune cells at the maternal-fetal interface are involved in local suppression of inflammation during pregnancy to ensure embryo survival[42], including dNK cells. One proposed mechanism by which dNK cells mediate this is the antagonism of T helper 17 (Th17) cells and the expansion of T regulatory cells[42]. We provide evidence here for decreased dNK cells at the maternal-fetal interface as well as lower production of *Ifn-γ*. It is the dNK cell derived *Ifn-γ* that is thought to dampen inflammatory Th17 cells at the maternal-fetal interface[42]. Preeclamptic women have been shown to have a decrease in circulatory T regulatory cells and an increase in Th17 cells[43]. Furthermore,

studies using the reduced uterine perfusion pressure (RUPP) rat indicate that increased circulating CD4+ cells stimulate increased pro-inflammatory cytokine production of IL-17 in a rodent model of PE. Moreover, human T lymphocytes can be stimulated to produce Cox2 and other inflammatory cytokines[44]. We already know CD4+ T cells are increased during BPH/5 pregnancies (unpublished data), therefore investigating T regulatory cells as an essential anti-inflammatory component in BPH/5 pregnancy would be very promising.

<u>Figure 5.3:</u> Potential mechanistic model for dysregulated pathways seen early in BPH/5 pregnancy. Hypoxia is reported to upregulate nuclear factor K B (NFKB) and Cox2 among other pro-inflammatory molecules. Hypoxia is actually thought to stimulate dNK cell recruitment to the maternal-fetal interface, but a direct relationship between hypoxia and dNK cell absence has not been established. NFKB can induce IL-15 expression as well as Cox2. We have demonstrated that excessive IL-15 mediated dNK cell loss in the decidua and that Cox2 inhibition with celecoxib reduces IL-15. We present here a mechanism for feedback from PGE_2 derived Cox2 on IL-15 along with NFKB. It is our hypothesis that this loop is activated in the decidua prior to placenta formation to mediate adverse pregnancy outcomes in BPH/5 mice.


Hypoxia
?
NFkB
Cox2
dNK
?
PGE2
IL-15

A potential upstream master regulator of BPH/5 adverse pregnancy outcomes

Previous work performed in the lab confirmed a causal role for oxidative stress in the pathogenesis of the BPH/5 PE-like syndrome[4]. At mid gestation, BPH/5 placenta have increased levels of superoxide and reduced levels and activity of the antioxidant enzyme, superoxide dismutase (SOD)[4]. Importantly, placental oxidative stress was attenuated by chronic Tempol (SOD mimetic) treatment beginning before and continuing throughout pregnancy. Furthermore, this was associated with a decrease in BPH/5 resorptions, an increase in BPH/5 litter size and normalization of BPH/5 placental and fetal weights[4]. We saw improvement in these same pregnancy outcomes after celecoxib treatment in BPH/5 mice early in pregnancy. Placental hypoxia is a key feature of PE pregnancies in women[3]. Further investigation is warranted, but a strong upstream candidate that is involved in hypoxia and regulates both IL-15 and Cox2 is nuclear factor K B (NFKB)[45]. Strikingly, inhibition of Cox2, PGs and NFKB has been proposed as potential therapeutic approaches for preterm labor, a pregnancy disorder characterized by placental inflammation[46].

Hypoxia is reported to upregulate nuclear factor K B (NFKB) and Cox2 among other pro-inflammatory molecules[47]. Interestingly, NFKB can mediate IL-15 signaling in the microvasculature of the brain[48]. Cox2 is increased by IL-15 in a PGE_2 dependent manner in arthritic synoviocytes[45]. We have demonstrated that excessive IL-15 mediated dNK cell loss in the decidua and that Cox2 inhibition with celecoxib reduces IL-15. We have not established a direct relationship between hypoxia and dNK cell absence, but NFKB- induced IL-15 overexpression could be a potential mechanism. We present here a mechanism for feedback from PGE_2 derived Cox2 on IL-15 along with NFKB. It is our hypothesis that this loop is activated in the decidua prior to placenta formation to mediate adverse pregnancy outcomes in BPH/5 mice.

Celecoxib treatment earlier in BPH/5 pregnancy, at e4.5 when we first see rises in IL-15, may increase the benefits seen with anti-inflammatory therapy in BPH/5 pregnancy. One can speculate that IL-15 downregulation could be crucial to improving placental defects not improved at late gestation by the single dose of celecoxib by working on dNK cells. Taken

together, data published from the Davisson laboratory using BPH/5 mice along with this thesis data and information combed from the literature all suggest a complex interplay between the dysregulated signaling molecules seen in BPH/5 during early pregnancy.

New working model for the pathogenesis of adverse pregnancy outcomes in BPH/5

During the preparation of this thesis, it became overwhelmingly apparent that a common denominator may exist to coordinate the dysregulation beginning in implantation and rippling throughout pregnancy in BPH/5 mice. Placental hypoxia is a hallmark of PE pregnancies in women[3] and we also show strong evidence of it in BPH/5 pregnancies playing a causal role in the maternal syndrome[4]. Defects in periimplantation signaling in BPH/5 mice eventually converge on what is an inflamed and hypoxic decidua. Trophoblast cells are then forced to invade and differentiate within this hostile maternal environment; thus leading to placental defects in trophoblastic patterning of the labyrinth and remodeling of spiral arteries. Poor uteroplacental perfusion and fetoplacental circulation then deprive the fetus of blood, oxygen, and nutrients and promotes *in utero* FGR. Factors from the abnormally developed placenta leak into circulation to stimulate systemic disease in the mother and the clinical presentation of the maternal syndrome is made. Using the BPH/5 mouse we can test our hypotheses regarding periimplantation dysregulation and hopefully move the field forward in our knowledge of the decidual origins of PE.

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