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The Effect of Maternal Immune Activation on Placental and Fetal Development in Rats

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Abstract

Development of the placenta and embryo is a highly orchestrated process that is vulnerable to changes in the *in utero* environment. Maternal immune activation (MIA) during pregnancy is a major risk factor for many obstetric complications and predisposes offspring to growth restriction as well as cognitive and neurobehavioral deficits; however, the mechanisms underlying these risks remain unknown. In this thesis, I aimed to determine the effect of MIA on development of the placenta and fetal brain. By administering the viral mimetic polyinosinic:polycytidylic acid to pregnant rats as a model of MIA, I demonstrated that a maternal antiviral response is associated with impaired placental and fetal growth and altered fetal brain structure. Specifically, I showed that intraperitoneal injection of polyinosinic:polycytidylic acid into pregnant rats results in MIA, decreased placental and fetal size, and reduced expression of several paternally-expressed imprinted genes. Moreover, I determined that type I interferons impair differentiation of rat trophoblast stem cells. Secondly, by analyzing the cerebral cortex of midgestation rat fetuses one week following maternal injection of polyinosinic:polycytidylic acid or saline, I found that fetuses exposed to MIA had increased neural precursor cell self-renewal. This finding was recapitulated using neurospheres generated from these fetal brains and was associated with altered expression of Notch signaling components. Lastly, I found that uterine natural killer cells, the most prevalent immune cells in rat decidua in early pregnancy, produce the multifunctional protein osteopontin, which may contribute to decidual and placental development. Collectively, this work provides insight into the role of MIA and decidual immune cells in modulating development of the placenta and fetal brain.

Keywords

Maternal immune activation, antiviral response, PolyI:C, placenta, neurodevelopment, neural precursor cells, uterine natural killer cells, trophoblast, osteopontin

Summary for Lay Audience

The placenta is a necessity of human reproduction. Connecting a mother to her developing baby, the placenta sustains pregnancy by ensuring nutrients and oxygen reach the baby and by acting as a protective barrier. When the placenta does not develop properly, dangerous pregnancy complications can occur which put both mother and baby at risk of developing short and long-term illness or death. Factors that cause high risk of placental maldevelopment include infection and inflammation, and I believe that the mother's immune cells (which fight infections) play a role in the development of these complications during pregnancy. Using rats, I found that inflammation during early pregnancy altered the way the placenta forms and reduced the size of pups. Moreover, I found that inflammation during rat pregnancy changed the pups' brain structure, which could help explain why individuals exposed to infection while they are in the womb have an increased chance of problems with brain function. Additionally, I found that a protein called osteopontin is produced by immune cells close to the placenta and may have an important role in how the placenta forms. Collectively, these findings can help us understand how infections and inflammation lead to poor placental development and smaller babies.

Co-Authorship Statement

Chapter 2 is adapted from Baines, K. J et al (2020). Antiviral inflammation during early pregnancy reduces placental and fetal growth trajectories. The Journal of Immunology, 204(3), 694-706. Text and figures were reproduced for this thesis with permission from the American Association of Immunologists. KJ Baines designed all experiments with the assistance of Stephen J Renaud. All data was generated by KJ Baines in the laboratory of Stephen J Renaud and James C Lacefield. All figures were prepared by KJ Baines. Stephen J. Renaud contributed to the design and concept of the study and secured ethical approval for animal work. In addition, he provided critical feedback on the manuscript at all levels of completion.

Chapter 3 is adapted from Baines, K. J et al (2020). Maternal immune activation alters fetal brain development and enhances proliferation of neural precursor cells in rats. Frontiers in Immunology, 11, 1145. Text and figures were reproduced for this thesis with permission from Frontiers. KJ Baines and Dendra M Hillier designed the experiments with the assistance of SJ Renaud and KJ Baines and DM Hillier collected all data. Figures 1, 2 and 7 were designed by KJ Baines. Figures 3-6 were done in collaboration between KJ Baines and DM Hillier. SJ Renaud secured ethical approval for animal work. In addition, he provided critical feedback on the manuscript at all levels of completion.

Chapter 4 is entitled Gene Expression Profiling of Decidua from Interleukin-15 Deficient Rats Reveals Osteopontin as a Natural Killer Cell-Specific Marker. KJ Baines and Michelle S Klausner designed all experiments with the assistance of SJ Renaud. KJ Baines and MS Klausner collected and analyzed all data. Figures were designed by KJ Baines. SJ Renaud secured ethical approval for animal work. In addition, he provided critical feedback on the manuscript at all levels of completion.

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List of Abbreviations

ASD	autism-spectrum disorder
β3T	βIII-Tubulin
CM	cortical mantle
Ct	cycle threshold
drRNA	double stranded RNA
EdU	5-ethynyl-2′-deoxyuridine
EDV	end diastolic velocity
FGR	fetal growth restriction
GD	gestational day
IFN	interferon
IL	interleukin
IRF	IFN regulatory factor
MERS	Middle East respiratory syndrome
MIA	maternal immune activation
NICD	notch intracellular domain
NPC	neural precursor cell
NSC	neural stem cell
OPN	osteopontin
PA	placenta accreta

PAMP	pathogen-associate molecular patterns
PolyI:C	polyinosinic:polycytidylic acid
PSV	peak systolic velocity
qRT-PCR	quantitative RT-PCR
RI	resistance index
RPKM	reads per kilobase of transcript per million mapped reads
SD	sprague-dawley
SDH	sprague-dawley Holtzman
TLR	toll-like receptor
TS	trophoblast stem
uNK	uterine natural killer
VTI	velocity time integral
WT	wildtype
VZ	ventricular zone

Chapter 1

1 Introduction

1.1 The Placenta

The placenta is an intricate organ that connects a mother to the embryo during pregnancy playing a critical role in maintaining and protecting the developing baby. Functionally, the placenta ensures the transfer of nutrients and oxygen between maternal and fetal circulations, the removal of wastes from fetal circulation, as well as the synthesis and secretion of growth factors, hormones, and cytokines. In addition, it also acts as both a physical and endocrine barrier between maternal and fetal circulations, providing protection against pathogens, maternal hormones, and the maternal immune system. Impairments in placental development can result in fetal growth restriction (FGR) and other severe obstetric complications for both mother and baby (Brosens et al. 2011). While negative outcomes on fetal development are often observed during late gestation, the underlying causes of placental maldevelopment are thought to be due to events during early pregnancy.

Placentation is a complex process that begins prior to implantation of the blastocyst; early placental development is carried out primarily by trophoblast cells which originate from the trophectoderm (the outer layer) of the blastocyst. The trophectoderm can differentiate into several specialized trophoblast lineages which are involved in invasion, remodelling, endocrine functions, and immunity (Napso et al. 2018). Various animal models have been used to characterize and model placentation; in particular, the use of mice and rats has improved our understanding of early placentation since they exhibit a similar type of placentation as humans, they are multiparous with short gestations, and they are readily manipulated in laboratory settings.

1.1.1 Human Placental Development

In humans, the placenta is anatomically organized into specialized tree-like structures known as chorionic villi. Chorionic villi are comprised of a mesenchymal core containing fetal blood vessels, macrophages, and fibroblasts, and are surrounded by a layer of villous cytotrophoblast cells and an outer layer of fused trophoblast called syncytiotrophoblast (Figure 1-1). Within the mesenchymal core, fetal blood vessels develop and connect to fetal circulation through the umbilical vasculature, a process that does not complete until approximately three weeks into gestation (Knöfler et al. 2019). Chorionic villi can be classified into floating chorionic or anchoring villi, where floating villi emanate from anchoring villi and are small tree-like structures bathed in maternal blood – facilitating the majority of nutrient and gas exchange to take place. At the tips of anchoring villi, a stratified layer of extravillous cytotrophoblasts forms, which differentiate and invade into the maternal decidua and inner myometrium. Invading extravillous cytotrophoblasts play an important role in the remodelling of maternal spiral arteries, which is described below (Meekins et al. 1994). This process is essential for attachment of the placenta into the uterus as well as ensuring that an unimpeded supply of maternal blood reaches the placenta.

As pregnancy progresses, maternal spiral arteries need to be remodeled into large diameter, low resistance conduits which allows the space between floating villi (intervillous space) to fill with maternal blood (Brosens et al. 1967). This process is largely accomplished by extravillous trophoblast cells, which proliferate up into the distal columns of the anchoring villi and develop invasive properties (Weiss et al. 2016). As extravillous trophoblast cells become more invasive, they invade deep into the decidua basalis and through the uterine stroma or through the lumen of the maternal spiral arteries up into the inner third of the myometrium. During this early development of the placenta, extravillous trophoblasts plug the spiral arteries to allow blood plasma to seep into the intervillous space without red blood cells entering (Jauniaux et al. 2000). This plugging process creates a hypoxic environment which is essential to the regulation of trophoblast proliferation and invasion, and also protection of the placenta and fetus during critical phases of development (James et al. 2018). Trophoblast plugs disappear towards the end of the first trimester to allow for the increased volume of blood, which is required to create an environment capable of sustaining a growing fetus. As the structure of villi

develop and progress, unimpeded blood flow to the placenta is established around ten to twelve weeks of pregnancy (Burton et al. 2002). Poor placental development – a product of impaired trophoblast invasion and deficient spiral artery remodelling – results in the poor exchange of nutrients, oxygen, and hormones between maternal and fetal circulations and has been implicated in several obstetric complications including preeclampsia, FGR, placenta accreta, and stillbirth (Barrientos et al. 2017; Cotechini et al. 2014; Lyall et al. 2013; Kumer et al. 2021).

1.1.2 Preeclampsia, Fetal Growth Restriction, and Placenta Accreta

Numerous obstetric diseases have been linked to placental maldevelopment, including preeclampsia, FGR, and PA. In healthy pregnancies the maternal spiral arteries are remodeled into low-resistance high-flow conduits, however in pregnancies with impaired spiral artery remodelling there is reduced transfer of oxygen and nutrients to the fetus, resulting in placental insufficiency. Placental insufficiency can result from a number of factors including genetics, maternal undernutrition, and maternal hypertension, however over 60% of cases of placental insufficiency are idiopathic (Ghidini 1996).

Despite the advancements of modern medicine in the management of pregnancy, preeclampsia affected approximately 5% of pregnancies in Canada in 2012 (Auger et al. 2016). Women diagnosed with preeclampsia have higher mortality rates and are at an increased risk of developing cardiac, respiratory, renal, and hepatic failure (Abalos et al. 2014; Nerenberg et al. 2014). Additionally, offspring from pregnancies complicated by preeclampsia are at a disproportionate risk for further complications including a requirement for emergency caesarean section, pre-term birth, FGR, admission to intensive care, and long-term chronic morbidities (Abalos et al. 2014; Nerenberg et al. 2014; Bakker et al. 2011; Lisonkova et al. 2014). Preeclampsia is diagnosed as the sudden onset of new hypertension, in addition to at least one of: proteinuria, endothelial dysfunction, swelling, or headaches after the 20th week of pregnancy in mothers (Roberts et al. 1989; Williams et al. 2010). In many cases, delivery of the placenta is the only cure for preeclampsia and mothers remain at risk for long-term complications including diabetes, cardiovascular disease, and kidney disease (Williams et al. 2010). Placentas

from preeclamptic pregnancies have shown disruptions in the syncytial architecture as well as decreased size (Sankar et al. 2012; Chen et al. 2004; Chen et al. 2006). Mouse models of preeclampsia further show decreased vascular remodelling and increased vascular resistance indicating possible underlying mechanisms for the pathogenesis of preeclampsia (Dokras et al. 2006). Furthermore, in a subset of preeclampsia cases, the fetus stops growing in response to a lack of blood flow and results in growth deficiencies in the offspring.

Growth of the fetus is dependent on genetic and environmental factors and the ability of the placenta to transfer nutrients and oxygen between maternal and fetal circulations (Gardosi et al. 1992). Growth restriction is the failure of a fetus to achieve its pre-determined growth potential due to environmental or genetic factors, which manifests as suboptimal growth, and can develop with or without the presence of maternal preeclampsia (Figueras and Gratacos 2017; Mitani et al. 2009; Khalil et al. 2019). Several factors have been linked to the development of FGR including aneuploidies, maternal use of nicotine, infections, metabolic disorders, and placental maldevelopment or insufficiencies (Burton and Jauniaux 2018; Sharma, Shastri, et al. 2016; McCowan et al. 2009; Odibo et al. 2006; Shah and Knowledge Synthesis Group on Determinants of LBW/PT births 2010). In adulthood, offspring born with low birthweights face an increased risk for developing cardiovascular diseases, hypertension, diabetes, and metabolic and renal diseases (Murphy et al. 2006).

FGR is often defined as being born below the 10th percentile in weight for that gestational age, but this is not predictive of perinatal mortality as this definition encompasses many constitutionally small fetuses (considered small for gestational age). Current definitions of early onset FGR is defined as reduced growth before 32 weeks of gestation, with an estimated fetal weight (EFW) or fetal abdominal circumference (FAC) below the 3rd percentile with absent end diastolic flow, or EFW and FAC below the 10th percentile and a Doppler pulsatility index above the 95th percentile (Gordijn et al. 2016). Similarly, the definition of late gestation FGR includes a diagnosis of FAC or EFW below the 3rd percentile after 32 weeks of gestation or FAC and EFW below the 10th percentile with an impaired umbilical doppler flow or cerebro-placental ratio below the

5th percentile (Gordijn et al. 2016). Early onset FGR is considered to be the more severe phenotype and is associated with impairments in placental perfusion and subsequent fetal hypoxia, and offspring face an increased risk of preterm birth, morbidity, and mortality (Weiler et al. 2011; Lackman et al. 2001). FGR pregnancies with fetal weights below the 3rd percentile are at an increased risk of fetal mortality that correlates to the severity of the growth restriction observed at birth (McIntire et al. 1999). Doppler ultrasounds used throughout pregnancy can give a predictive value for poor outcomes per growth percentile, and those with diminished fetal arterial and venous doppler blood flow can be delivered early to prevent *in utero* fetal demise; however, premature birth introduces risks of perinatal morbidity and mortality (Zeitlin et al. 2000; Larkin et al. 2015).

Growth restriction is defined as an impairment in growth of the body, and therefore estimates of fetal growth throughout pregnancy can determine the possibility of organ sparing (asymmetrical FGR) or not (symmetrical FGR) (Briana and Malamitsi-Puchner 2018). Asymmetrical FGR accounts for the majority of FGR cases (~70%) and is typically linked to insults during mid or late gestation resulting in severe decreases in body size (Sharma, Shastri, et al. 2016; Sharma, Farahbakhsh, et al. 2016). The asymmetry results from a "brain-sparing effect," where blood is redirected towards the brain to preserve its functions while development of the other organs is compromised (Wu et al. 2006). Symmetrical FGR is associated with genetic abnormalities or placental insults during early pregnancy, accounting for the other 30% of FGR cases and is characterized by a proportional decrease in organ size (Sharma, Farahbakhsh, et al. 2016; Sharma, Shastri, et al. 2016).

Placenta accreta applies to conditions in pregnancy when the placenta attached abnormally into the maternal myometrium. The abnormal attachment of the placenta can be further broken down into three grades based on the degree of invasion, which include accreta (attachment to the myometrium), increta (invasion of trophoblast into myometrium), and percreta (invasion of trophoblasts through the myometrium) (Miller et al. 1997). In mice, hyper-invasive trophoblasts can be observed in a surgical model of PA (Burke et al. 2020). The excessive invasion of trophoblast past the decidua basalis and into the myometrium puts mothers at risk of severe bleeding following detachment of the

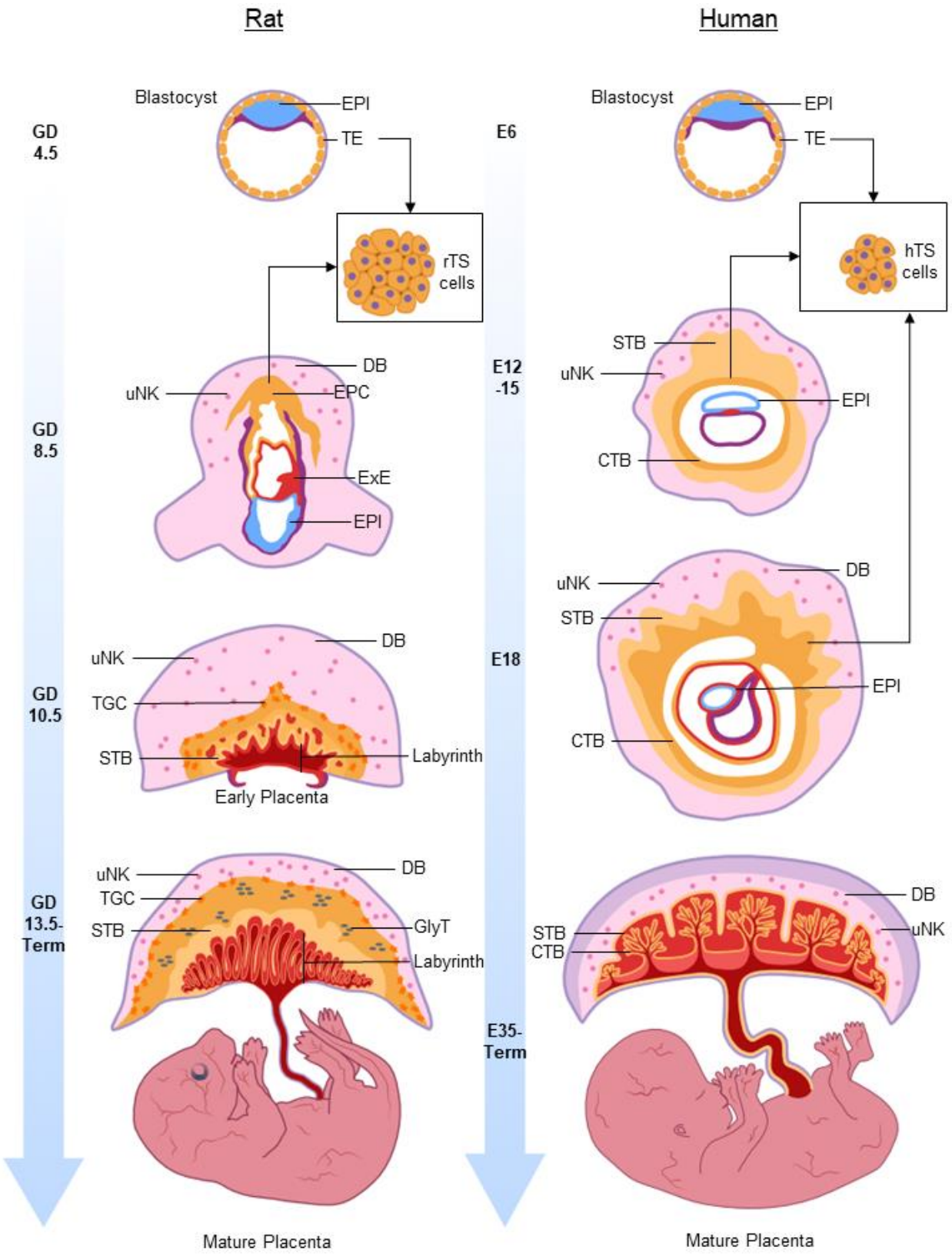
Rat
Human
Blastocyst
EPI
TE
GD 4.5
E6
rTS cells
hTS cells
DB
uNK
EPC
ExE
EPI
GD 8.5
E12 -15
STB
CTB
TGC
Labyrinth
Early Placenta
GD 10.5
E18
GlyT
GD 13.5- Term
E35- Term
Mature Placenta

Figure 1-1: Placental development in the rat and human.

Following fertilization, implantation of the blastocyst occurs by gestational day (GD) 4.5 in the rat and embryonic day (E) 6 in humans. The blastocyst gives rise to the trophectoderm (TE) and inner cell mass which will differentiate into the epiblast (EPI). By GD 8.5, the trophectoderm differentiates and gives rise to the ectoplacental cone (EPC) in rat placentation. In humans, the TE will develop into the syncytiotrophoblast (STB) and underlying proliferative cytotrophoblasts (CTB) around E 12. As the placenta develops a population of rat trophoblast stem (rTS) cells can be isolated from the TE and EPC, whereas human trophoblast stem (hTS) cells are isolated from the TE or CTB layer. From early in gestation uterine natural killer cells (uNK) can be found within the decidua basalis (DB) in both rat and human placentation. Around gestational day 10.5 in rats, the structure of the labyrinth begins to form, housing the syncytiotrophoblast (STB), and facilitating nutrient and gas exchange between maternal and fetal circulations. Trophoblast giant cells (TGC) line the interface between the (DB) and developing placenta. The structure of the definitive placenta is completed by GD 10 in rats, and E 35 in humans however it continues to grow throughout gestation until term.

placenta, which can lead to hemorrhage, multisystem organ failure, and death (Bailit et al. 2015; Warshak et al. 2010). Variations in the number of immune cells in placentas from PA pregnancies compared to healthy controls have been reported, in addition to an increase in the presence of inflammation, warranting further investigation (Ernst et al. 2017; Schwede et al. 2014; Hecht et al. 2020).

1.1.3 Rodent Placental Development

A variety of animal models have been studied to improve our understanding of the processes of implantation, placentation, and early pregnancy. Humans, primates, and rodents all feature hemochorial placentation which is characterized by the direct contact of maternal blood with trophoblast cells. Over the past decades, mice have been the preferred model for placental research due to their ease of use in transgenic approaches and have therefore greatly expanded our knowledge of genes essential for placental development, however more recently genetic editing tools have become available for rat work as well. Featuring spiral artery remodelling, humans, mice, and in particular rats, exhibit trophoblast invasion into the uterine lining (Soares et al. 2012). In addition, the recent development of rat TS cell models has enabled *in vitro* experimentation to complement *in vivo* models, furthering their use for experimental models (Asanoma et al. 2011). Despite their similarities, there are some differences in placental structure between humans and rats (Soncin et al. 2018). Even with these differences, their short gestations, large litter sizes, and deep trophoblast invasion make rats an excellent model for the study of placental development.

Following implantation in the rodent, the trophectoderm gives rise to the extra-embryonic ectoderm and the ectoplacental cone (Figure 1-1). The trophectoderm and subsequently the extraembryonic ectoderm and ectoplacental cone (EPC) differentiates into rat TS (rTS) cells, a self-renewing population of placental precursor cells that can be cultured *in vitro* (Asanoma et al. 2011). Further differentiation of the EPC forms the unique barrier that forms between the maternal decidua and the trophoblast layer in rodent placentation, known as the junctional zone. Positioned between the decidua and the underlying labyrinth layer, it primarily arises from the ectoplacental cone and together with the

labyrinth zone forms the fetal component of the mature rodent placenta. The junctional zone is comprised of three subtypes of trophoblast: spongiotrophoblast cells, glycogen cells, and trophoblast giant cells (TGC). Spongiotrophoblast cells comprise the bulk of the junctional zone and can be found between the giant cells and the labyrinth zone. While spongiotrophoblast may have an important endocrinological role and promote maternal physiological adaptations to pregnancy, their function is not entirely understood (Salbaum et al. 2011). The second specialized trophoblast subtype found within the junctional zone are termed glycogen trophoblast cells and they accumulate and metabolize large stores of glycogen, as well as invade into the decidua throughout gestation (Bouillot et al. 2006; Coan et al. 2006). The EPC also differentiates into TGC – cells known for their continuous endoreduplication (polyploidy) combined with the lack of cell division causing their large size (Simmons and Cross 2005). During early development, TGCs line the outside of the developing conceptus and are in direct contact with the maternal decidua. Along with spongiotrophoblast cells and glycogen trophoblast cells, TGCs provide the major endocrine functions of the placenta.

The site of gas and nutrient exchange in rodent placentas is functionally equivalent to human chorionic villi and termed the labyrinth zone due to its maze-like network of blood vessels (Baines and Renaud 2017). The labyrinth zone in rodents consists of three layers of trophoblast cells (two layers of syncytiotrophoblast and an outer mononucleated trophoblast layer termed sinusoidal trophoblast giant cells) (Hu and Cross 2010; Baines and Renaud 2017). The proximity of fetal trophoblast cells with maternal blood in the labyrinth layer of rodents ensures efficient nutrient and gas exchange between mother and developing fetus.

1.1.4 Uterine NK cells

The maternal portion of the maternal-placental interface in both rats and humans is comprised of the decidua basalis. There are variety of cell types within the decidua basalis including decidual stromal cells (from differentiation of endometrial stromal cells), blood vessels, glands, and a variety of immune cells. Immune cells comprise 30-40% of all decidual cells, which include uterine natural killer (uNK) cells, macrophages, dendritic cells, and T cells, each with unique functions and cytokine productions that

contribute to the maintenance of a successful pregnancy (Faas and de Vos 2017; Lash et al. 2010). Within the uterus the leukocyte population is primarily comprised of macrophages (20-25%) and uNK cells (70%) (Ander et al. 2019). In blood, peripheral NK cells are short-lived cells of the innate immune system which have the ability to eliminate cells through cytotoxic actions (Cerwenka and Lanier 2001). Healthy host cells are immune from the cytotoxic actions of NK cells by the interaction of their major histocompatibility complex (MHC) class I molecules with the inhibitory receptors on NK cells (Schmidt et al. 2004). While NK cells are found throughout the body, a unique subset is found within the mucosal tissues of the uterus, known as uterine NK (uNK) cells (King et al. 1996; Koopman et al. 2003; Whitelaw and Croy 1996). These uNK cells are unique from those found in blood, displaying a distinct $CD56^{bright}CD16^{dim}$ phenotype, and acting less cytotoxic than their peripheral NK counterparts (Cerdeira et al. 2013; Yang et al. 2019; Koopman et al. 2003).

While high in number during early pregnancy, uNK cells decline in number around mid-gestation (Jabrane-Ferrat 2019). Close in proximity to the uterine spiral arteries and invasive trophoblast cells, uNK cells are embedded in the decidua basalis and are thought to provide support for the process of trophoblast invasion and spiral artery remodelling (Smith et al. 2009). Co-culture experiments with uterine spiral artery segments implicate uNK cells in the restructuring of spiral arteries through loss of integrity of the tunica media (Robson et al. 2012), however the exact mechanism through which this process occurs remains unknown. Recent research has highlighted the wide range of factors that uNK cells can secrete – including the growth promoting factors: pleiotrophin, osteoglycin, and osteopontin, which each contribute to healthy fetal development (Fu et al. 2017). Changes in the number or activity of uNK cells has been associated with several pregnancy complications including FGR, preeclampsia, and spontaneous recurrent abortion (Wallace et al. 2013; Wallace et al. 2014), further implicating them as regulators of placental development.

1.1.4.1 uNK cells in Mouse

In mouse pregnancy, two distinct populations of uNK cells have been identified, which are most commonly defined by their reactivity to *Dolichos biflorous* agglutinin (DBA) (Paffaro et al. 2003). DBA+ uNK cells express high transcript levels of angiogenic factors, whereas DBA- uNK cells express a higher transcript level of interferon-gamma (IFN-γ) (Chen et al. 2012; A. A. Ashkar et al. 2000; Paffaro et al. 2003). Throughout murine pregnancy, mature uNK cells remain poorly cytotoxic despite containing pore-forming proteins such as perforin and granzymes (Parr et al. 1990). Murine uNK cells express lectin-like Ly49 receptors, which recognize MHC class I as well as killer-cell lectin like receptor G1 (Madeja et al. 2011; Kieckbusch et al. 2014). These two subsets have differing roles throughout murine pregnancy, with uNK IFN-γ playing a key role in the remodelling of maternal spiral arteries (Monk et al. 2005; Greenwood et al. 2000; Ashkar and Croy 1999)

Strong evidence that uNK cells contribute to spiral artery remodelling has come from studies using NK cell-deficient pregnant mice (Koopman et al. 2003; Kennedy et al. 2000; Mazurier et al. 1999; Kim et al. 2000; Gascoyne et al. 2009). Specifically, mouse models genetically lacking uNK cells consistently exhibit narrow spiral artery lumens (Ashkar and Croy 2001; Guimond et al. 1997; Hofmann et al. 2014). Impairments in spiral artery remodelling has also been associated with decreased blood flow and compromised fetal growth in mouse models genetically devoid of NK cells and those with reduced NK cell numbers or function (Barber and Pollard 2003; Ashkar et al. 2003; Kieckbusch et al. 2014). Together, these studies implicate the role of uNK cells in the initial stages of spiral artery remodelling and their importance in creating unimpeded blood flow to the developing mouse placenta.

1.1.4.2 uNK cells in Rat

Mice feature shallow trophoblast invasion with limited trophoblast-mediated spiral artery remodelling, whereas rats (like humans) have deep trophoblast invasion and extensive trophoblast-mediated spiral artery remodelling (Caluwaerts et al. 2005; Ain et al. 2003). The use of rats therefore provides an advantage to study this aspect of placental development. Like in mice, uNK cells comprise the majority of leukocytes within the decidua in rats, where they increase in number in the mesometrial compartment until

approximately midgestation (Acar et al. 2011; Gaynor and Colucci 2017). uNK cells accumulate around the spiral arteries prior to the onset of trophoblast invasion, and then subsequently decrease in number towards the end of pregnancy (Kusakabe et al. 1999). In both human and rat placentation, uNK cells initiate angiogenesis and a 'first wave' of spiral artery remodelling, since there is evidence of early spiral artery dilation without the presence of invasive trophoblast cells (Chakraborty et al. 2011; Smith et al. 2009). Depletion of NK cells in rats results in a transient delay in early aspects of spiral artery remodelling, and ultimately a compensatory over-invasive trophoblast phenotype (Chakraborty et al. 2011). Recent work has shown that a genetic knockout of interleukin-15 (IL-15) in rats, a cytokine required for NK cell survival, results in drastic alterations in placental structure. This includes an expanded junctional zone and early-onset and exacerbated trophoblast invasion and spiral artery remodelling (Renaud et al. 2017). Despite the underlying importance of uNK cells in spiral artery remodelling, studies in both rats and mice indicate that Il-15 deficiencies do not impair implantation, pregnancy success, or fetal growth (Renaud et al. 2017; Bany et al. 2012).

1.1.4.3 Interleukin-15

Interleukin-15 (IL-15) is a pleiotropic cytokine that can stimulate the growth of mature T, B, and NK cells, as well as act as a chemoattractant for T lymphocytes and promote the activation of NK cells (Armitage et al. 1995; Bamford et al. 1994; Carson et al. 1994). Therefore, the development of NK cells is reliant on the expression of IL-15. IL-15 is produced in bone marrow stromal cells (Ashkar et al. 2003; Grabstein et al. 1994), thymic epithelium (Leclercq et al. 1996), and fetal intestinal epithelium (Murray et al. 1998). *Il15* mRNA has been found in a wide range of tissues including placenta, skeletal muscle, kidney, lung, heart, and macrophages (Grabstein et al. 1994). The IL-15 receptor (IL-15R) is a heterotrimer composed of a unique α subunit that mediates specific and high affinity binding to IL-15, as well as a β and common γ subunit that transduce intracellular signals and are shared with several other interleukins such as IL-2 and IL-4 (Giri et al. 1995). Using adoptive transfer techniques, peripheral NK cells have been isolated from wild-type mice and transferred into IL-15-/- mice, however these cells did

not survive more than a few days in their new hosts, confirming the importance of IL-15 for NK cell survival (Ranson et al. 2003).

NK cells are dependent on IL-15 for their development and survival (Fehniger and Caligiuri 2001). In the uterus, endometrial IL-15 transcript levels are increased in the secretory phase of the menstrual cycle compared with the proliferative phase, reaching peak expression levels in the mid-secretory phase (Kitaya et al. 2000; Okada et al. 2000). This corresponds with an increased number of CD16- $CD56^{bright}$ NK cells in the endometrium during the secretory phase and early stages of pregnancy as well as in the early pregnant decidua (King et al. 1991). Trophoblasts inhibit IL-15 mediated uNK cell survival, proliferation, and cytotoxicity in *in vitro* studies using trophoblast conditioned media, by negatively regulating IL-15R signaling in uNK cells (Park et al. 2017). Inhibition of IL-15 gene expression results in a complete NK cell deficiency in animal models, including peripheral NK cells, tissue resident NK cells, and uNK cells, however IL-15 is not required for pregnancy success (Barber and Pollard 2003).

It has been suggested that uNK cells play a key role in regulating placental development through interactions with trophoblast cells and their purported involvement in spiral artery remodelling. In IL-15 deficient mice, spiral arteries have thicker walls and narrower lumens compared to wildtype animals (Renaud et al. 2017; Barber and Pollard 2003). Mice lacking IL-15 have no uNK cells in implantation sites in addition to a hypocellular decidua basalis, an absent metrial gland, and failed spiral artery remodelling (Ashkar et al. 2003; Mori et al. 2016). Despite poor placentation in mice devoid of uNK cells, there appears to be no impairments in pregnancy progression and no long term effects on offspring (Greenwood et al. 2000). Rats possessing a mutation in the gene encoding IL-15 have no detectable uNK cells and exhibit robust expansion of the junctional zone and precocious trophoblast invasion into spiral arteries. Interestingly, there is no observable change in fetal growth or litter size in these rats, suggesting that the placental adaptations that take place are sufficient to compensate for the lack of uNK cells (Renaud et al. 2017). These placental adaptations observed in uNK cell deficient rats may result from compensatory leukocytes or changes in the expression of various growth factors, cytokines or extracellular matrix proteins secreted by uNK cells.

1.1.5 Osteopontin

Osteopontin (OPN) is an extracellular matrix protein that is expressed in many tissues including teeth, brain, kidney, bone, muscle, chorionic villi, and decidua (Mazzali et al. 2002; Chen et al. 2014). In addition to structural functions, OPN can act as a cytokine and chemoattractant for macrophages and T-cells during inflammatory responses (S. Ashkar et al. 2000; Mazzali et al. 2002). OPN can also mediate many cellular functions including adhesion, migration, and cell survival (Wang and Denhardt 2008). OPN is a negatively charged protein encoded by a single gene on chromosome 4 with a promoter region that responds to several transcription factors (Fisher et al. 2001). While OPN is comprised of 314 amino acids, there are several cleaved products and splice variants. It is commonly expressed as a 33 kDa protein, but after post-translational modifications it can increase to 44 kDa (Sodek et al. 2000). The various post-translational modifications and cell sources of OPN provide a means for its numerous functions (Kazanecki, Uzwiak, et al. 2007; Kazanecki, Kowalski, et al. 2007). For example, tumor-derived OPN can inhibit the function of macrophages, while host-derived OPN acts as a chemoattractant (Crawford et al. 1998). OPN can interact with a variety of cell surface receptors, most notably integrins (αvβ1, αvβ3, αvβ5, αvβ6, and α5β1) (Barry et al. 2000; Yokosaki et al. 2005). Additionally, OPN can also be detected intracellularly on the inner surface of plasma membranes (iOPN), where it plays a role in motility, cell fusion, cell survival, and migration of fibroblasts, macrophages, and metastatic cells (Suzuki et al. 2002; Zohar et al. 2000). IFN-α expression may also be mediated by iOPN in dendritic cells, as observed in OPN deficient mice with impaired NK cell responses to tumors and decreased responses following herpes simplex virus 1 infection (Shinohara et al. 2006). Together, these studies implicate a role for OPN in mediating immune function.

OPN is produced by several immune cells including NK cells, though as an extracellular matrix protein it's primary roles are in tissue growth and regeneration through it's interactions with various cell surface receptors, growth factors, and cytokines (Fu et al. 2017). Increased levels of OPN have been implicated in pathological conditions such as cancer and inflammatory diseases and result in increased cellular apoptosis (Lund et al. 2009; Icer and Gezmen-Karadag 2018). However, contradicting reports note that OPN

can also act as a survival factor by inhibiting apoptosis as observed in stressed endothelial cells or in tumor development (Hsieh et al. 2006; Khan et al. 2002). Signalling through αvβ3 integrin allows OPN to modulate NFκB signaling, promoting endothelial cell survival in stress conditions, perhaps accounting for this variability (Figure 1-2) (Scatena et al. 1998; Rice et al. 2006). Interacting with MyD88 and TLR9, iOPN can activate interferon regulatory factor 7 and induce the production of IFN-α (Shinohara et al. 2006). Increased gene expression of OPN was observed in mouse intestines and epithelial cells infected with Listeria monocytogenes and rotavirus, however OPN-deficient mice exhibited prolonged symptoms of infection, leaving the role of OPN in immunological processes unclear (Rollo et al. 2005; S. Ashkar et al. 2000).

In pregnancy, OPN plays a role in several processes including: implantation (Nomura et al. 1988), acting as a component of histiotroph, decidualization, and immune regulation (Burton et al. 2002; Johnson et al. 2003). OPN has been found throughout the pregnant uterus in animal models, including in the glandular epithelium, metrial gland, and trophoblast cells (Johnson et al. 2003). Detectable throughout gestation at the transcript level in the mouse decidua (GD7.5-18.5), it is hypothesized that OPN is essential for proper placental progression (Nomura et al. 1988). In extravillous trophoblast cell lines, OPN is constitutively expressed, and inhibition of OPN hinders their proliferative and invasive capacity (Wu et al. 2015). Furthermore, inhibition of OPN compromises angiogenesis and decidualization, and decreased levels of OPN are found in cases of implantation failure (Wang et al. 2018). In pregnancy complications like preeclampsia, OPN plays a role in aberrant trophoblast invasion leading to pathogenic placental development (Gabinskaya et al. 1998). With possible implications in immunological functions and it’s presence in the uterus, OPN has been localized within uNK cells and found to be absent in mice lacking NK cells (Herington and Bany 2007).

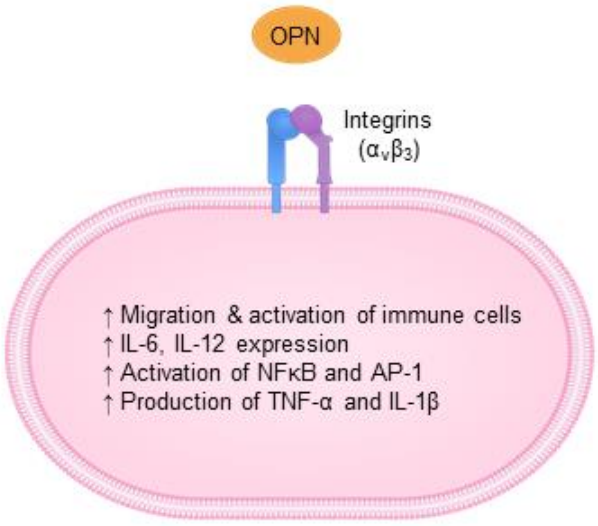

Figure 1-2: Schematic of osteopontin and receptor.

OPN is secreted by activated immune cells and has been shown to play a role in regulating early immune responses and inflammation. Upon binding to integrin αvβ3, OPN mediates the recruitment and activation of leukocytes at the sites of inflammation by inducing a pro-inflammatory response. OPN, osteopontin.

1.2 Inflammation and infections in pregnancy

Healthy pregnancies require a delicate immune balance to ensure a functional immune system capable of fighting pathogens while minimizing damage to the host and protecting the developing offspring. The unique cellular milieu within the pregnant decidua consists of a variety of immune cells, decidual stromal cells, glands, blood and lymph vessels, and trophoblast cells. These various cells must all interact with each other to ensure successful placental and fetal development. Mild inflammation is essential for the progression of a healthy pregnancy, including during implantation and parturition, however this process is tightly regulated by immunomodulatory mechanisms. Common to pregnancy complications such as preeclampsia, FGR, and preterm birth is an aberrant maternal immune response and impaired placental development (Kaufmann et al. 2003; Redman 1991). Increased maternal serum levels of the pro-inflammatory cytokines IL-6, IL-8, IL-12, and IFN-γ have been associated with recurrent pregnancy loss (Comba et al. 2015), however the relationship between inflammation and pregnancy loss remains elusive. Conditions associated with chronic low-level inflammation (e.g. diabetes and hypertension) have also been linked to increased hypoxia/ischemia, oxidative stress, and inflammation within the placenta as well as trophoblast cell death (Maxwell et al. 2015). While FGR has been associated with placental dysfunction, studies have found high levels of pro-inflammatory cytokines in maternal serum from women with FGR, and their offspring had a higher risk of brain morbidity (Gazzolo et al. 2006).

1.2.1 Viral Infections and Pregnancy

The effects of maternal viral infection during pregnancy can range from no impact on the fetus to pregnancy loss or congenital infection. Epidemiological data implicates pregnant women as a high risk for serious illness and mortality from viral infections, including influenza, Ebola, Lassa fever, and most recently, COVID-19 (Gervasi et al. 2012; Price et al. 1988; Moltner et al. 2021; Jamieson et al. 2014; Kwon et al. 2014). Including the well-known teratogenic TORCH infections (toxoplasmosis, others, rubella, cytomegalovirus, and herpes simplex virus), there remains no true standard of care for maternal or prenatal

management of infections, leaving pregnant women vulnerable to a wide range of symptoms and illnesses. For several decades researchers have been linking viral infections with pregnancy complications including preterm labor, preeclampsia and FGR, however the mechanisms of how infections can affect the progression of a healthy pregnancy remain poorly understood (Aldo et al. 2010; Gervasi et al. 2012; Arechavaleta-Velasco et al. 2008; Koga, Cardenas, et al. 2009).

The increase in inflammation seen with infections can lead to both systemic symptoms for mothers and fetal complications like preterm birth and miscarriage (Pazos et al. 2012; Fell et al. 2017). Infections like CMV, Zika virus, and Dengue have been associated with placental abnormalities including villi enlargement, intravillous hemosiderin deposition, and intervillositis (Ribeiro et al. 2017; Krauer et al. 2017; Martinez 2016; Villamil-Gómez et al. 2016). Respiratory infections like Middle East respiratory syndrome (MERS), while understudied, show records of stillbirths at 5 months of gestation (Alserehi et al. 2016). Additionally, congenital infections acquired during development may lead to disabilities later in life, like those seen due to congenital TORCH infections. The relatively recent outbreaks of Zika virus resulted in ZIKV syndrome, with offspring exhibiting microcephaly and lesions within the brain comparable to those seen in congenital CMV infections (de Vries 2019).

More recently, SARS-CoV-2 (severe acute respiratory syndrome coronavirus-2; COVID-19), a virus of the coronaviridae family, emerged in December 2019 and continues to grow as one of the most significant public health threats of this century. Swiftly spreading around the world, COVID-19 has caused over 250 million infections and over 5 million deaths as of November 2021. Presenting from asymptomatic cases to symptoms of fever, cough, pneumonia, acute respiratory distress syndrome, multisystem organ failure, and a cytokine storm, the case fatality rate of COVID-19 is approximately 2% (Wu and McGoogan 2020; Huang et al. 2020; Chen et al. 2020; Ye et al. 2020). Although the majority of pregnant women with COVID-19 have mild symptoms, women testing positive for COVID-19 during their pregnancy are at an increased risk of being admitted to the intensive care unit or requiring invasive ventilation, as well as delivering prematurely or maternal death (Allotey et al. 2020; Elsaddig and Khalil 2021;

Andrikopoulou et al. 2020; Wu and McGoogan 2020; Breslin et al. 2020). Vertical transmission of SARS-CoV-2 is uncommon, although there is some evidence that placental and fetal infection can occur (Kirtsman et al. 2020; Patanè et al. 2020; Dong et al. 2020; Mattar et al. 2020). The possibility of the intense cytokine storm produced by COVID-19 that could possibly pass through the placental barrier and the risk of severe symptoms in pregnant women may have future implications in fetal development that should continue to be monitored.

The maternal-fetal interface acts as an effective barrier against foreign pathogens while permitting oxygen, hormones, and nutrients to pass to the developing baby (Brett et al. 2014; Wong and Cox 2017). Viral infections can disturb the delicate immune balance that exists at the maternal-fetal interface, and aberrations in the immune cell population and function in the uterus may lead to fetal damage even without direct contact with the virus (Koga, Aldo, et al. 2009). Trophoblast cells (syncytiotrophoblast, cytotrophoblasts, and extravillous trophoblasts) express various pattern recognition receptors which can recognize a wide range of molecules common to many pathogens and are important for immune surveillance (Abrahams and Mor 2005; Abrahams 2008). These pattern recognition receptors stimulate distinct downstream pathways and, in the case of viral infections, can mediate a rapid antiviral response (Alexopoulou et al. 2001), and produce both type I and type III interferons (Bayer et al. 2016). An antiviral immune response has the ability to interrupt fetal and placental growth (Yockey and Iwasaki 2018) and has been associated with pregnancy complications like preeclampsia and FGR (Mor et al. 2011).

Viral infections and/or inflammation may affect the ability of trophoblasts to enter into and invade the uterine tissue and spiral arteries, thereby leading to placental dysfunction and pregnancy complications (Gierman et al. 2021; Chatterjee et al. 2012). Viruses require a living host where they recruit host machinery to replicate and create an infectious cycle of generating new viral particles. Viruses can enter cells through fusion with the plasma membrane or through cellular internalization, whereby viruses are encapsulated by endosomes and further release into the cytoplasm (Yamauchi and Greber 2016; Schneider-Schaulies 2000).

Double-hit rodent models of murine herpes virus (HPV) and lipopolysaccharide (LPS), detected no (HPV) infection in the placenta but observed robust increases in maternal levels of proinflammatory cytokines that correlated with an increased risk of preterm labor (Cardenas et al. 2011). The authors hypothesized that these results may stem from the maternal pro-inflammatory response to infection rather than from the response to the infectious agent itself (Cardenas et al. 2011). As shown in animal models, maternal immune activation (MIA) during pregnancy can lead to alterations in offspring behavior (Bakos et al. 2004), brain development (Patterson 2002; Wischhof et al. 2015; Urakubo et al. 2001), and metabolic health (Niklasson et al. 2006; Yu et al. 2017). Maternal fever alone has been linked to increased risk of neural disorders in offspring (Zerbo et al. 2013). By using single or double stranded-RNA, a potent type I interferon response can be generated in human first trimester trophoblast cell lines through TLR3 and TLR8, both of which are detectable in cells at the maternal fetal interface (Potter et al. 2015; Aldo et al. 2010). The cytokine storm associated with infections and proinflammatory events in pregnancy can result in increased maternal IL-2 and IL-17 levels which have been implicated in the pathogenesis of preeclampsia and miscarriage (Romero-Adrián et al. 2002; Wilson et al. 2003; Wu et al. 2014). Although TNF-α and its receptor are detectable throughout reproductive tissues, increased TNF-α levels can be used to diagnose possible health outcome depending upon gestational age, and can lead to tissue necrosis as well as hypoxia within the placenta (Azizieh and Raghupathy 2015; Carpentier et al. 2011).

1.2.2 Toll-Like Receptors in Pregnancy

Infections are commonly recognized through pathogen-associated molecular patterns (PAMPs), notably the TLRs (Kawai and Akira 2009). Ten of these receptors have been identified and described in humans, each recognizing distinct patterns present in bacteria, viruses, and other pathogens (Janeway and Medzhitov 2002). For example, TLR4 binds to LPS found on the outer membrane of invading gram-negative bacteria, while TLR2 responds to lipoteichoic acid within the cell wall of gram-positive bacteria. Within the endosome of infected cells, TLR3 is activated by double-stranded RNA (dsRNA), a common product of many viral replication pathways that does not exist in

uninfected mammalian cells (Muzio et al. 2000; Karikó et al. 2004; Cavassani et al. 2008). Once activated, TLRs employ various intracellular signaling cascades to initiate the production of pro-inflammatory cytokines or type I interferons (Akira and Takeda 2004). While commonly found on innate immune cells, mRNA expression of all 10 TLRs have been found within first trimester trophoblasts, and mRNA expression of TLRs 1-9 have been found within uterine epithelial cells (Tangerås et al. 2014; Schaefer et al. 2004). Several reports note that TLR3 had the highest expression across various gestational time points in the decidua and placenta (Pudney et al. 2016; Zarember and Godowski 2002; Gierman et al. 2021), which may indicate the ability of the placenta to recognize viral pathogens and initiate an immune response.

TLR3 is specifically activated by viral dsRNA and is expressed by a variety of cells, notably epithelial cells and innate immune cells like NK cells, dendritic cells, and macrophages (Muzio et al. 2000). TLR3 contains an extracellular domain and an intracellular domain, forming a horseshoe-like structure. Upon activation by dsRNA, TLR3 homodimerizes and initiates a signal transduction cascade by recruiting the adaptor protein TRIF at the intracellular domain (Gosu et al. 2019). Activation of TLR3 leads to the downstream activation of the NF-κB pathway, activator protein 1 (AP-1), or interferon regulatory factor (IRF3) pathways resulting in the production of pro-inflammatory cytokines and an antiviral response (Figure 1-3) (Yamamoto et al. 2003; Alexopoulou et al. 2001; Schmidt et al. 2004). TLR3 has the ability to activate NK cells with or without stimulation of a MHC molecule on an antigen presenting cell (Schmidt et al. 2004). In pregnant women, TLR3 is present on human endometrial epithelial cells, uterine primary epithelial cells, and trophoblast cells (Jorgenson et al. 2005; Schaefer et al. 2005; Abrahams et al. 2005). Studies looking at the differential expression of TLRs in the placenta throughout pregnancy highlighted that TLR3 has the highest expression compared to other TLRs in both first and second trimesters (Pudney et al. 2016). Increased TLR3 levels have been described in placentas from preeclampsia complicated pregnancies compared to healthy controls and in mouse models of preeclampsia (Chatterjee et al. 2012; Gierman et al. 2021; Pineda et al. 2011). The high expression of TLR3 in healthy placentas and increased expression in preeclampsia complicated pregnancies suggests that TLR3 mediates low-grade inflammation during normal

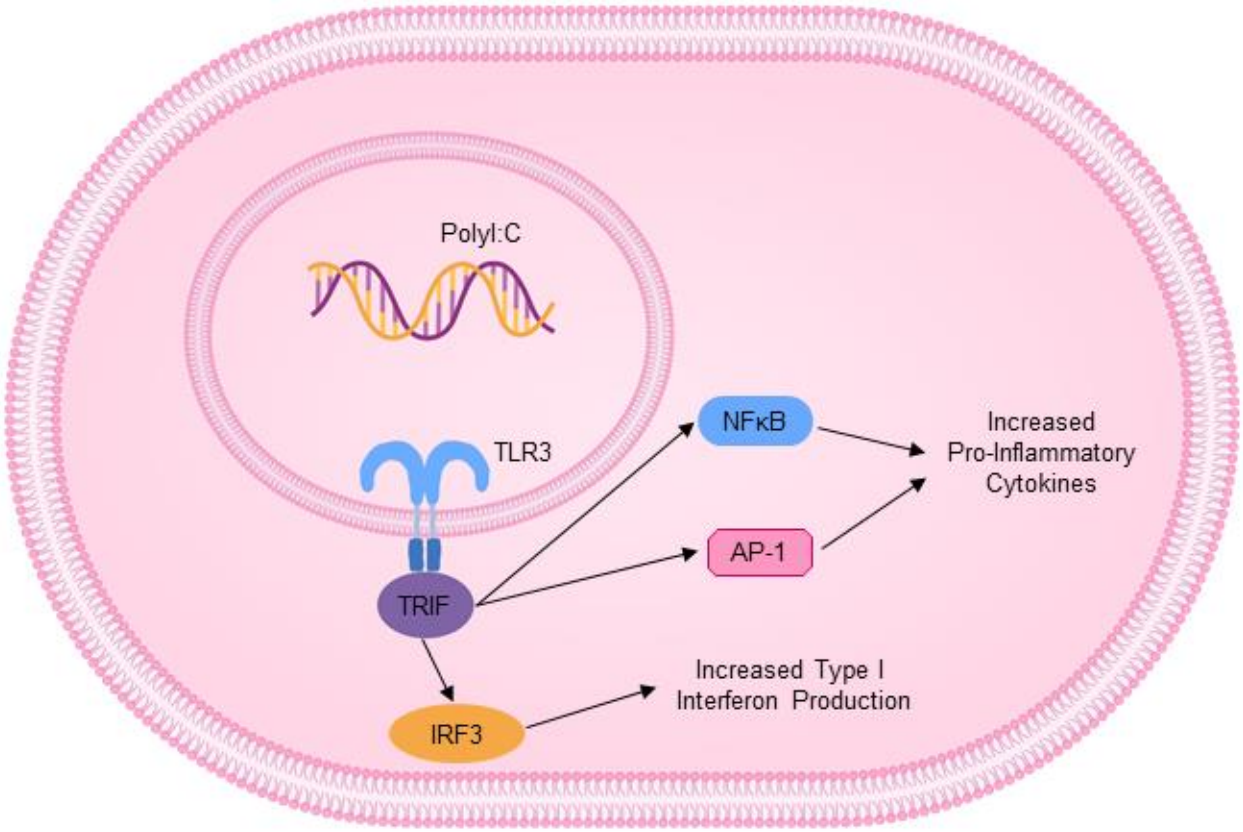

Figure 1-3: Activation of TLR by PolyI:C.

Upon dsRNA binding to TLR3 within the endosome of cells, TLR3 recruits the adaptor protein TRIF, which further activates NFκB, AP-1, to generate a proinflammatory response. Additionally, TRIF can activate IRF3 to stimulate the production of type I interferons. PolyI:C, polyinosinic:polycytidylic acid; TLR3, toll-like receptor 3; TRIF, TIR-domain-containing adapter-inducing interferon-β; IRF3, interferon regulatory factor 3; NFκB, nuclear factor kappa B; AP-1, activator protein 1.

pregnancy and may play a role in the increased inflammation and placental dysfunction seen in pregnancy complications such as preeclampsia.

In many animal models, inflammation is sufficient to cause symptoms similar to those seen in common obstetric complications (Scharfe-Nugent et al. 2012; Gomez-Lopez et al. 2016). Polyinosinic:polycytidylic acid (PolyI:C) is a known TLR3 agonist (Zhang et al. 2007) and a synthetic dsRNA molecule (Field et al. 1967). In addition to TLR3, PolyI:C can also activate retinoic acid-inducible gene 1 (RIG-1) and melanoma differentiation-associated protein 5 (MDA5), to initiate antiviral responses and the production of interferons (Kato et al. 2006). PolyI:C is often used to activate immune functions of cells both *in vivo* and *in vitro* (Sivori et al. 2004). The pro-inflammatory effects of PolyI:C are acute, with cytokine responses peaking rapidly and declining within 48 hours of exposure, depending on the dosage and route of administration (Fortier et al. 2007). Cytokines observed in this antiviral response include IL-6, IL-1β, TNF-α, as well as various type I interferons (Gitlin et al. 2006; Duluc et al. 2009; Fortier et al. 2007; Smith et al. 2007; Murray et al. 2015). Studies using human trophoblast cells *in vitro* have shown that exposure to PolyI:C results in an increase in secretion of chemokines, specifically chemokine (c-c motif) ligand 5 (CCL5) (Abrahams et al. 2005). In mice, injection of PolyI:C at GD 16.5 results in an increase in the production of pro-inflammatory chemokines and cytokines (such as CCL2 and IL-6) and causes preterm birth within 24 hours of injection (Koga, Cardenas, et al. 2009). Lower doses of PolyI:C did not appear to affect maternal wellbeing at all, however at a dose high enough to cause 100% preterm delivery and a significant increase in the number of deceased pups, and dams exhibited piloerection, tachypnea, and decreased movement (Koga, Cardenas, et al. 2009). Zhang et al. (2007) determined that when pregnant mice were injected with PolyI:C at GD 6.5, not only was there an increase in the secretion of TNF-α but there was also increased TLR3 expression, vessel to lumen ratios of spiral arteries, and fetal loss. In a study conducted by Tinsley et al. (2009), rats injected with PolyI:C at gestational day 10 experienced preeclampsia like symptoms with high systolic blood pressures and protein in the urine. Together, these results indicate PolyI:C elicits a potent immune response that leads to pregnancy complications in animal models.

1.3 Maternal Inflammation and Fetal Brain Development

Suboptimal *in utero* environments, including those affected by maternal inflammation, infection, hypoxia, diet, and age may have detrimental consequences on the development of the fetal brain as well as long-lasting effects on functioning of the adult brain (Knuesel et al. 2014). Mounting evidence has linked maternal infections during pregnancy to increased risk of psychiatric disorders with developmental origins, such as autism spectrum disorder (ASD) and schizophrenia (Brown et al. 2004; Han et al. 2021; Goines et al. 2011). Acquired infections or activation of the maternal immune system during early pregnancy may interfere with fundamental neurodevelopmental events leading to psychiatric illness in adulthood. Due to the complicated nature of studying prenatal neurodevelopment, preclinical animal models can provide mechanistic links to understanding possible *in utero* origins of disease. Using rodent models, research has shown that maternal inflammation during pregnancy can impede development of the hippocampus and skew stress responses, learning, and memory, key impairments seen in psychiatric illnesses (Ratnayake et al. 2012; Lins et al. 2019; Rasmussen et al. 2019). There is little known, however, about how activation of the maternal immune system can result in alteration in early brain development, resulting in neural dysfunction and psychiatric illnesses in adults.

1.3.1 Early Neural Development in Rats

Development of the brain is a highly orchestrated process and variations in the maternal environment due to factors such as inflammation can lead to long term consequences on neurodevelopmental outcomes of the offspring. The cerebral cortex is primarily involved in higher order processes like attention, awareness, memory, and behavior (Shipp 2007). Development of the fetal cortex occurs in an inside out fashion, initiating from a pool of neural precursor cells (NPCs) that resides in the ventricular zone (Van Essen et al. 2018). As these cells divide from NPCs to glial cells and definitive neurons, they migrate outwards to the cortical plate where they accumulate to form the cerebral cortex (Noctor et al. 2001). Many factors play a role in the successful progression of neurogenesis, including environmental, molecular, and genetic factors. Insults that disrupt neurogenesis can cause deficiencies in cortical development and lead to changes in brain volume.

Neurogenesis is responsible for the development of all definitive neurons in the brain. The timing of neurogenesis, however, varies quite widely between species. Rodents begin embryonic cortical neurogenesis around gestational day 12, peaking at gestational day 15.5, with expansive proliferation diminishing by gestational day 18.5 (Rice and Barone 2000). In humans, pre-natal cortical neurogenesis begins around gestational week 5, and ends around gestational week 15 (Rice and Barone 2000); however, in both species neurogenesis continues throughout adult life as NPC continually differentiate into functional neurons. The differentiation of neurons in the developing brain requires a large number of signaling pathways initiated at the correct time and intensity to ensure functional plasticity of neurons and limit the risk of neurodevelopmental disorders.

1.3.2 Notch Pathway in Neurogenesis

The Notch signaling pathway is an evolutionarily conserved signaling system found in most animals. There are four Notch receptors (NOTCH1, NOTCH2, NOTCH3, NOTCH4) and six ligands (DLL1, DLL2, DLL3, DLL4, JAG1, JAG2) (Fiúza and Arias 2007; Bray 2006). Important for cell-cell communication, the Notch signaling pathway offers a wide variety of cellular functions. Notch pathways play a critical role in developmental processes, controlling cell fate decisions, cell death, and maintaining cells in the stem state (Fiúza and Arias 2007; Ohishi et al. 2000). Activation of NOTCH by one of its ligands results in the cleavage of its intracellular domain (NICD), where it translocates to the nucleus to repress or activate transcription (Figure 1-4) (Lathia et al. 2008). The main target genes of NOTCH include two families of transcription factors, *Hes* and *Hey*, which have been implicated in neurodevelopment (Jalali et al. 2011; Sakamoto et al. 2003; Kandasamy et al. 2010). NOTCH proteins are highly prevalent in the telencephalon during early embryogenesis but can also be found in post-mitotic neurons in the adult CNS (Yoon et al. 2004). High levels of NOTCH1 and DLL1 have been localized to the neural tube of developing mouse fetuses (Dang et al. 2006; Gaiano et al. 2000), while human embryos show high expression of NOTCH1 and minimal NOTCH3 staining in the forebrain and spinal cord (Kostyszyn et al. 2004).

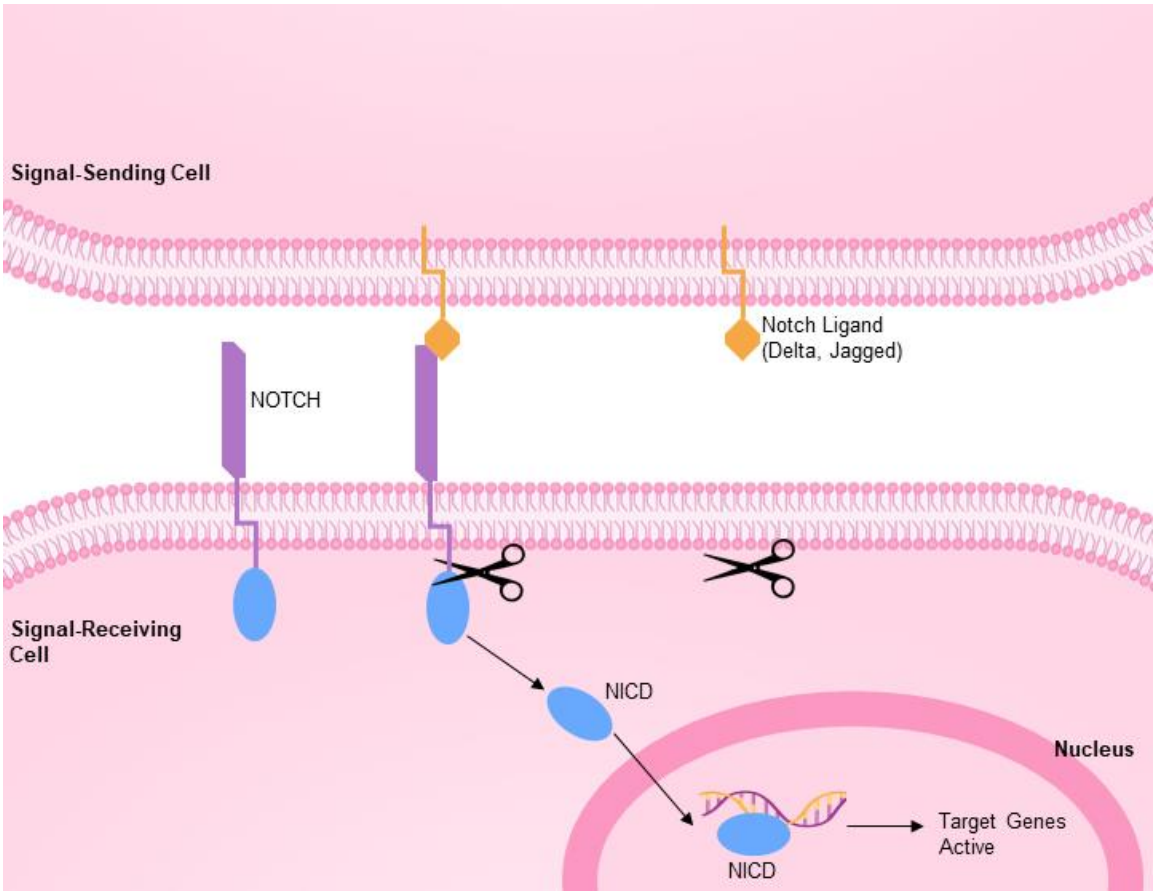

Figure 1-4: Schematic of NOTCH activation.

Binding of NOTCH ligands (Delta, Jagged) on one cell to the NOTCH receptor on another cell results in the cleavage of the Notch intracellular domain (NICD). NICD enters the nucleus and interacts with DNA to result in the activation of various target genes.

In neurodevelopment, NOTCH signaling has been implicated in maintenance of neural precursor cell populations within the ventricular zone, as well as controlling the balance between gliogenesis and neurogenesis. In mice, expression of all four NOTCH proteins can be found throughout various stages of CNS development, but their expression is predominantly found in undifferentiated cells (Lindsell et al. 1996; de la Pompa et al. 1997). Knockout of NOTCH1 leads to embryonic lethality at GD 9.5 and 11.5 in mice, with null embryos exhibiting exacerbated neuronal differentiation (Swiatek et al. 1994; de la Pompa et al. 1997). Loss of function models of NOTCH components can cause depletion of the neural precursor cell pools while simultaneously increasing neuronal differentiation (Bolós et al. 2007). Additionally, *in vitro* models demonstrate that expression of NOTCH1 can block neurogenesis but not gliogenesis (Nye et al. 1994), further indicating the importance of balanced NOTCH expression in neurogenesis.

1.3.3 Infections, Inflammation, and Neurodevelopment

There is mounting evidence that inflammation plays a role in central nervous system injuries in both the fetal and adult brain. During fetal life and infancy, the brain is vulnerable to insults, and several neurodevelopmental disorders have been linked to MIA including ASD, schizophrenia, epilepsy, and depression (Knuesel et al. 2014). Neurodevelopmental disorders include a wide range of conditions that emerge in early childhood and adolescence that are primarily associated with abnormal brain function. These disorders often result in persistent impairments in cognitive, social, academic, and occupational functioning, and can include ASD, schizophrenia, intellectual disabilities, attention-deficit/hyperactivity disorder, conduct disorders, cerebral palsy, as well as vision and hearing impairments. While there is considerable overlap in phenotypes between various disorders, there is considerable sexual dimorphism that is skewed towards males for neurodevelopmental disorders like ASD, while depression is more common in women (Motlagh et al. 2010; Weissman et al. 1996; Kessler et al. 1994).

Exposure to maternal infections during *in utero* development is associated with offspring exhibiting cognitive impairments later in life (Brown et al. 2001; Selten et al. 2010; Chess 1971; Lubinsky 1979; O'Callaghan et al. 1991; Sham et al. 1992). This hypothesis

dates back to the 1918 Spanish influenza pandemic where researchers documented evidence of the development of psychiatric disorders in offspring from mothers with influenza exposure during their pregnancy (Menninger 1926; Yudofsky 2009). Similar effects were observed following an outbreak of Rubella in the USA in 1964, where there was a 19% increase in cases of schizophrenia and a 12% increase in cases of ASD (Estes and McAllister 2016; Li et al. 2009). Additionally, maternal exposure to parasites (i.e., toxoplasma gondii) during early gestation leads neurological impairments in offspring like microencephaly, blindness, and developmental delays (Gleason and Back 2005; McAuley 2014). Administration of viruses like influenza to pregnant mice during early gestation results in an increase in the volume of the adult offspring brain, however exposure during late gestation results in decreased brain volumes (Fatemi et al. 2002; Shi et al. 2003). While there are varying reports of viruses causing brain overgrowth and undergrowth, it is important to note that route of administration, dose and duration, as well gestational timing may be responsible for these differing results (Meyer et al. 2006; Meyer et al. 2008).

Activation of the maternal immune system by infections, stress, smoking, obesity, autoimmunity, or inflammation during early pregnancy can affect both innate and adaptive immune systems. Inflammatory mediators, like cytokines and chemokines can influence the function, development, and maintenance of neurons within the brain (Zalcman et al. 1994; Ling et al. 1998; Dunn 2006). Cytokines play an essential role in the development of the central nervous system, acting as signaling molecules while maintaining their immunomodulatory roles, resulting in the developing CNS remaining susceptible to perturbations in cytokine levels (Ling et al. 1998; Dunn 2006). Animal models in which MIA is induced, such as injection of PolyI:C or LPS into pregnant rodents, has allowed for a deeper understanding of the impact of the maternal immune response on fetal brain development. Early studies of MIA and its effects on fetal neurodevelopment in rodents linked increased rates of behavioral abnormalities (similar to those seen in human ASD and schizophrenia cases) in offspring to mothers with prenatal influenza infections (Fatemi et al. 2002; Fatemi et al. 1999). Several animal studies utilize the effects of MIA to induce a wide range of brain and behavioral dysfunctions, including symptoms observed in ASD and schizophrenia (Meyer et al.

2007; Traynor et al. 2004). Similarity like exposing animals to true pathogens, the use of MIA methods allows for the potent activation of the maternal immune system, including proinflammatory cytokines. Both methods result in impairments in social behaviors and sensorimotor gating as well as increases in anxiety-like behaviors which are correlated to the similar phenotypes of altered neuronal migration, synaptic function and structure observed in cases of ASD and schizophrenia (Choi et al. 2016; Wu et al. 2017).

Studies using animal models have further shown that the maternal immune response is primarily responsible for the behavioral changes observed in offspring, rather than the pathogen itself (Meyer et al. 2005; Shi et al. 2003; Zuckerman et al. 2003). Additionally, animal models have indicated that variations in timing, intensity, and type of immune activation can result in a broad spectrum of behavioral phenotypes in offspring. It has been well documented that the effects of MIA on neurodevelopment can vary between strains of animal, the dosage given, and administration route of the MIA-inducing agent (Arsenault et al. 2014; Schwartzer et al. 2013).

Studies using PolyI:C as a model of MIA in mouse and rat models have consistently reported that offspring present with anxiety like behaviors coupled with disrupted growth in offspring, as well changes in the cerebral vasculature and decreases in brain volumes (Piontkewitz et al. 2011). As neural precursor cells differentiate and divide from the ventricular zone, they migrate outwards towards the cortical plate, a process that has been shown to be hindered by administration of PolyI:C and influenza (Fatemi et al. 1999; Meyer et al. 2006). Additionally, elevations in pro-inflammatory cytokines like IL-6 can drive neural precursor cells from neurogenic pathways to the gliogenic state (Nakanishi et al. 2007). While migration of cortical interneurons is delayed following PolyI:C administration in mice, inhibition of IL-6 does not rescue this effect (Gumusoglu et al. 2017). MIA from polyI:C has also demonstrated increased cell death in hippocampal and cortical neurons in neonatal pups (Zuckerman et al. 2003). Several studies have also shown the MIA at mid-gestation can lead to decreased synaptic transmission, impaired cortical connectivity, and an increase in the activity and number of microglia in postnatal rats (Coiro et al. 2015; Dean et al. 2011; Girard et al. 2010; Juckel et al. 2011). Microglia are the resident macrophages found throughout the brain not only serving immune

defense roles but also participating in normal neurodevelopmental functions. Changes in microglial activity and number following MIA may lead to decreased NPC cell numbers in the fetal brain and result in pathological behaviors later in life (Cunningham et al. 2013; Paolicelli and Gross 2011). It is possible, therefore, that changes in fetal brain morphology may result from the effects of MIA directly or through impairments in neurodevelopmental processes.

ASD is a complex neurodevelopmental disorder that is primarily characterized by impairments in social behaviors and the presence of repetitive behaviors (American Psychiatric Association 2013). According to the Canadian medical association journal approximately 1-2% of the population is on the autistic spectrum, and 1 in 66 children will be diagnosed with ASD (Anagnostou et al. 2014; Anon n.d.). Genetic disorders account for approximately 10% of ASD cases, while the other 90% have an unknown etiology (Muhle et al. 2004). Researchers noticed neurological abnormalities similar to those in ASD in MIA models, highlighting a possible link between maternal inflammation and early exposure to proinflammatory cytokines (Croonenberghs et al. 2002; Fatemi et al. 2008; Smith et al. 2007).

The association between maternal PolyI:C exposure and behavioral deficits in offspring are similar to the behavioral phenotypes observed in offspring exposed to viruses (i.e., influenza) during pregnancy (Reisinger et al. 2015). Pups exposed to PolyI:C *in utero* exhibit increased anxiety-like behaviors and decreased post-natal weights and decreased motor function (Arsenault et al. 2014). Administration of PolyI:C to pregnant mice results in transcriptional changes in the fetal brain and perturbations in key neurological developmental processes which can contribute to inflammation-associated neurodevelopmental disorders (Kalish et al. 2021). Many MIA studies have focused on male offspring in part due to the male predominance of human ASD (Coiro and Pollak 2019; Klein and Corwin 2002; Brown and Meyer 2018), but it is important to consider both sexes as a biological variable in MIA-associated responses.

It is of interest that many neurodevelopmental disorders affect a larger proportion of males. In autism diagnoses the incidence ratio of male-to-females is 4:1, schizophrenia

was 2:3, and the incidence of Parkinson's disease is a 2:1 ratio (Baird et al. 2000; Scott et al. 2002; Kirkbride et al. 2006; Van Den Eeden et al. 2003). It is believed that the regulation of cerebellar development varies between biological sexes, with various genes and proteins being expressed at different levels between sexes; however, the mechanisms in which environmental and genetic factors can affect CNS development remain poorly understood (Suárez et al. 1992; Ramírez and Jiménez 2002). Therefore, recognition of sex-related differences in development and function of the CNS is required to further understand the possible molecular mechanisms leading to a disproportionate incidence rate of neurodevelopmental disorder diagnoses in males.

1.4 Rationale & Study Aims

There is a strong association between infections during pregnancy and the development of placenta-associated disorders and obstetric complications like preeclampsia, FGR, preterm labor, and spontaneous abortion (Brown et al. 2000; Pereira et al. 2014; Conde-Agudelo et al. 2008). There are pathogen-specific immune responses responsible for impairments in both placenta and fetal development, however, epidemiological data implicates the maternal immune response as an increased risk factor for developing obstetric complications and impairments in fetal neurodevelopment (Scharfe-Nugent et al. 2012; Gomez-Lopez et al. 2016; Buka et al. 2008; Buka et al. 2001). The similar effects on fetal and placental development between infections and pathogen-free pro-inflammatory immune responses indicate a common underlying mechanism where the maternal immune response is the likely cause; however, the relationship between impaired fetal and placental development due to MIA is not yet known.

Thus, the overall hypothesis of my thesis is that MIA during pregnancy will disrupt normal placental and fetal development.

The specific objectives of this project were:

1) To determine the effects of a systemic maternal pro-inflammatory response on development of the placenta, and if this affects growth of the fetus.

2) To investigate the effects of early-gestation maternal immune activation on neurodevelopment of the offspring.

3) To compare gene expression profiles of *IL15Δ/Δ* and WT rat deciduas, in order to uncover novel regulatory factors produced by uNK cells.

Together, my work will further our understanding of the role of the maternal immune system in healthy and pro-inflammatory conditions on the progression of pregnancy at both the placental and fetal level.

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Chapter 2

2 Antiviral Inflammation during Early Pregnancy Reduces Placental and Fetal Growth Trajectories

Many viruses are detrimental to pregnancy and negatively affect fetal growth and development. What is not well understood is how virus-induced inflammation impacts fetal–placental growth and developmental trajectories, particularly when inflammation occurs in early pregnancy during nascent placental and embryo development. To address this issue, we simulated a systemic virus exposure in early pregnant rats (gestational day 8.5) by administering the viral dsRNA mimic polyinosinic:polycytidylic acid (PolyI:C). Maternal exposure to PolyI:C induced a potent antiviral response and hypoxia in the early pregnant uterus, containing the primordial placenta and embryo. Maternal PolyI:C exposure was associated with decreased expression of the maternally imprinted genes *Mest, Sfrp2,* and *Dlk1,* which encode proteins critical for placental growth. Exposure of pregnant dams to PolyI:C during early pregnancy reduced fetal growth trajectories throughout gestation, concomitant with smaller placentas, and altered placental structure at midgestation. No detectable changes in placental hemodynamics were observed, as determined by ultrasound biomicroscopy. An antiviral response was not evident in rat trophoblast stem (TS) cells following exposure to PolyI:C, or to certain PolyI:C-induced cytokines including IL-6. However, TS cells expressed high levels of type I IFNR subunits (*Ifnar1* and *Ifnar2*) and responded to IFN-α by increasing expression of IFN-stimulated genes and decreasing expression of genes associated with the TS stem state, including *Mest*. IFN-α also impaired the differentiation capacity of TS cells. These results suggest that an antiviral inflammatory response in the conceptus during early pregnancy impacts TS cell developmental potential and causes latent placental development and reduced fetal growth.

2.1 Introduction

Successful pregnancy requires a delicate balance between proinflammatory and anti-inflammatory pathways. Pathological inflammation disrupts this balance and is an underlying feature of a spectrum of prevalent pregnancy complications (Romero et al.

2007). In humans, it is difficult to discern whether inflammation is a cause or consequence of a compromised pregnancy, but circumstantial and epidemiological evidence implicate inflammation as a component of disease pathogenesis (Cotechini and Graham 2015; Mor et al. 2011). A direct link between inflammation and various pregnancy pathological conditions has been demonstrated in animal models in which dams are exposed to microbial products called pathogen-associated molecular patterns, resulting in changes in labor onset and alterations in fetal survival, growth, and developmental trajectories (Renaud et al. 2011; Chatterjee et al. 2012; Cotechini et al. 2014; Bakos et al. 2004). However, the underlying mechanisms through which inflammation compromises fetal growth and development are still unknown.

Fetal growth restriction (FGR; also known as intrauterine growth restriction) is a serious pregnancy complication associated with failure of a fetus to grow to its full potential. Although an accepted definition and consensus diagnosis of FGR are lacking, it is generally defined as fetal weight below the 10th percentile for that population and gestational age while considering sex, race, and genetic growth potential (Anon 2019). Identifying cases of morbid FGR versus healthy fetuses that are constitutionally small remains a significant problem. In general, FGR is one of the most important indicators of pregnancy outcome including prematurity, stillbirth, intrapartum fetal distress, hypoglycemia, and meconium aspiration pneumonia (Brodsky and Christou 2004), as well as long-term growth impairments and permanent pulmonary, metabolic, endocrine, and neurobehavioral sequelae (Sharma et al. 2016). FGR may be caused by multiple factors, including chromosomal aberrations, vascular abnormalities, and infections and in many cases is associated with inflammation as an underlying feature (Raghupathy et al. 2012). In 40–50% of cases, the cause of FGR is idiopathic and attributed to maldevelopment or dysfunction of the placenta.

The placenta is the extraembryonic organ that ensures an adequate nutrient and gas supply for the fetus and as such is a critical regulator of fetal growth and development. The efficiency of transferring substrates needed for fetal growth, including oxygen, amino acids, glucose, and fatty acids, to the fetal circulation is dependent on placental size, morphology, and blood flow (Sibley et al. 2010). Formation of the placenta begins

shortly after blastocyst implantation and is contingent on proper proliferation and differentiation of trophoblast stem (TS) cells. TS cells are multipotent progenitor cells that specialize into heterogeneous trophoblast subtypes that form the bulk of the placenta and perform various functions required for fetal growth and pregnancy success. In humans, TS cells differentiate into extravillous trophoblasts, which direct maternal resources to the conceptus, and villous trophoblasts, which specialize in nutrient transfer between maternal and fetal blood. A similar dichotomous organization of the placenta occurs in rodents, in which junctional zone and labyrinth zone trophoblasts perform analogous functions to extravillous and villous trophoblasts, respectively (Baines and Renaud 2017). TS cell development and differentiation are adaptable and respond to changes in the surrounding environment, including cellular stress and changes in oxygen and nutrient delivery, providing the placenta a degree of plasticity during its early development to facilitate embryo survival during extrinsic challenges (Soares et al. 2018). Trophoblasts cultured from rodent blastocysts and ectoplacental cones alter their survival and differentiation potential in response to IFNs (Ain et al. 2003; Athanassakis et al. 2000; Hoshida et al. 2007; Buchrieser et al. 2019), indicating that inflammation may be an additional extrinsic challenge that can impact placental morphology and fetal growth.

To determine the effect of systemic inflammation on placental development and fetal growth, we injected rats with the viral mimic polyinosinic:polycytidylic acid (PolyI:C) during early pregnancy. PolyI:C is a synthetic analogue of dsRNA: a molecular pattern produced by many viruses, either as the genetic material for some RNA viruses or as an intermediate for viral RNA synthesis. PolyI:C is a ligand for the pattern recognition receptors TLR3, retinoid acid inducible gene-1 (RIG1), and melanoma differentiation-associated gene 5, which enables mammalian cells to detect the presence of viruses and initiate cellular antiviral responses, such as production of IFNs (Palchetti et al. 2015). Given the purported immune tolerance mechanisms evident within the conceptus during early pregnancy, we were uncertain whether an antiviral response would be evident at this site. We found that PolyI:C exposure during early pregnancy induced a robust antiviral response and hypoxia within the conceptus, close to the location of placental development. The antiviral response was associated with reduced placental and fetal size

during middle and late pregnancy. Furthermore, we provide evidence that rat TS cells are responsive to IFN-α, and IFN-α exposure decreased expression of stem-related genes in these cells and reduced their differentiation capacity. Our results suggest that an IFN-mediated intrauterine antiviral response during early pregnancy causes transient placental dormancy by inhibiting TS cell developmental potential, resulting in smaller-than-normal placentas and fetuses.

2.2 Materials and Methods

2.2.1 Animals

Female (6–8 wk) and male (8–10 wk) Sprague Dawley rats were obtained from Charles River Laboratories and maintained in a 12-h light/12-h dark cycle with food and water available ad libitum. Females were cycled by daily inspection of cells within a vaginal lavage and mated when in proestrus with a fertile male. Gestational day (GD) 0.5 was defined as the day immediately following mating if spermatozoa were detected within the vaginal lavage. All protocols involving the use of rats were approved by the University of Western Ontario Animal Care and Use Committee.

2.2.2 Experimental protocol and tissue collection

In the first set of experiments, pregnant rats received PolyI:C (1, 5, or 10 mg/kg; Sigma-Aldrich) or sterile saline i.p. on GD8.5. Dams were sacrificed on GD13.5 to assess for fetal viability. Based on these results, 10 mg/kg PolyI:C was used in all subsequent experiments because there was no difference in litter size or number of resorptions with any of the doses tested, and it is a dose that has been previously used in pregnant dams to elicit longterm metabolic and neurodevelopmental problems in offspring (20–24). For experiments in which fetal and placental growth were analyzed, dams were sacrificed on GD8.5 (6 h after maternal saline or PolyI:C injection), GD13.5 (5 d after maternal saline or PolyI:C injection), or GD18.5 (10 d after maternal saline or PolyI:C injection) using mild carbon dioxide inhalation until respiratory failure, followed by cardiac puncture or decapitation. For tissues collected on GD8.5, whole conceptuses (containing decidua, placenta, and embryo) were isolated and either snap-frozen in liquid nitrogen or fixed in 10% neutral buffered formalin. For analysis of placental and fetal weights on GD13.5 or

GD18.5, uterine horns were dissected to isolate whole conceptuses, preserved in 10% neutral buffered formalin for 24 h, and then dissected to isolate and independently weigh the fetus and placenta. Preservation was used to enhance the structural integrity of fetal tissue, particularly for tissues isolated on GD13.5, and ensure that residual fetal membranes were completely removed. Fetal weights were consistent between fresh and fixed specimens. Fetal crown-rump length was also measured using a digital caliper. For cryosections, whole conceptuses were placed in dry ice–cooled heptane and stored at −80˚C until sectioned. For paraffin-embedded sections, whole conceptuses were fixed in 10% neutral buffered formalin for at least 24 h, then transferred to 70% ethanol and stored at 4˚C prior to sectioning.

2.2.3 RT-PCR

RNA was extracted from tissue by homogenizing in TRIzol (Thermo Fisher Scientific). The aqueous phase was then diluted with 70% ethanol, placed on RNeasy columns (Qiagen), treated with DNase I, and purified. RNA was extracted from cells by lysing in TRIzol and proceeding as directed by the manufacturer. cDNA was generated from purified RNA (50 ng/µl) using High-Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific), diluted 1:10, and used for conventional RT-PCR or quantitative RT-PCR (qRT-PCR). Conventional RT-PCR was conducted using primers described in Table I, and DreamTaq DNA Polymerase (Thermo Fisher Scientific) to amplify cDNA. Cycling conditions involved an initial holding step (95˚C for 3 min), followed by 34 cycles of PCR (95˚C for 30 s, 55˚C for 30 s, and 72˚C for 1 min), and a final elongation phase at 72˚C for 5 min. PCR products were resolved on 2% agarose gels and imaged using a ChemiDoc imaging system (Bio-Rad Laboratories). For qRT-PCR, cDNA was mixed with SensiFAST SYBR green PCR Master Mix (FroggaBio) and primers described in Table 2-1. A CFX Connect Real-Time PCR system (Bio-Rad Laboratories) was used for amplification and fluorescence detection. Cycling conditions were as follows: an initial holding step (95˚C for 3 min), followed by 40 cycles of two-step PCR (95˚C for 10 s,

Table 2-1: List of Primers used for RT-PCR.

Gene	Accession Number	Forward Sequence	Reverse Sequence
Actb	NM_031144.3	5’-agccatgtacgtagccatcc-3’	5’-ctctcagctgtggtggtgaa-3’
Cdx2	NM_023963.1	5’-gagcacggacactgtgagaa-3’	5’-agaagccccaggaatcactt-3’
Cxcl10	NM_139089.1	5’-tgtccgcatgttgagatcat-3’	5’-gggtaaagggaggtggagag-3’
Dio3	NM_017210.4	5’-gcctctacgtcatccagagc-3’	5’-gcccaccaattcagtcactt-3’
Dlk1	NM_053744.1	5’-gagctggcggtcaatatcat-3’	5’-gcatagagggagctgtgagg-3’
Ddx58	NM_001106645.1	5’-tcctcccacctgtttgagac-3’	5’-acaagccatgttaggggatg-3’
Eef2	NM_017245.2	5’-cgcttctatgccttcggtag-3’	5’-gtagtgatggtgccggtctt-3’
Gapdh	NM_017008.4	5’-agacagccgcatcttcttgt-3’	5’-cttgccgtgggtagagtcat-3’
Gcm1	NM_017186.2	5’-cctcaatgattgcctgtcct-3’	5’-tccaggtttgtgcttcatca-3’
Id2	NM_013060.3	5’-tgaaagccttcagtccggtg-3’	5’-gagcttggagtagcagtcgt-3’
Ido1	NM_023973.1	5’-cgtggatccagacacctttt-3’	5’-ggctggaggcatgtactctc-3’
Ifna	NM_001014786.1	5’-ggtggtggtgagctactggt-3’	5’-ttgagccttctggatctgct-3’
Ifnar1	NM_001105893.1	5’-attacgtgttctccccacca-3’	5’-cgattctcctctccagcaac-3’
Ifnar2	XM_006248107.3	5’-tggcccacaaatgttcttct-3’	5’-gtgcaccctaatgctgtcct-3’
Igf2	NM_001190162.1	5’-cggacgacttccccagatac-3’	5’-ctgaacgcttcgagctcttt-3’
Il1b	NM_031512.2	5’-aggcttccttgtgcaagtgt-3’	5’-tgagtgacactgccttcctg-3’
Il6	NM_012589.2	5’-gccagagtcattcagagcaa-3’	5’-cattggaagttggggtagga-3’
Il6r	NM_017020.3	5’-agggtgtctgcttcctgcta-3’	5’-ttgtgaaaaggcaagctcct-3’
Irf7	NM_001033691.1	5’-cctctgctttctggtgatgc-3’	5’-gcgctcagtcatcagaactg-3’
Isg15	NM_001106700.1	5’-gccatgacctggaacctaaa-3’	5’-ctgcctgataagggcaacac-3’
Isg20	NM_001008510.1	5’-tctgaaaggcaagctggtg-3’	5’-tggacgtgtcgtagatggtg-3’
Magel2	XM_001054803.6	5’-tccagcctgcctctactgat-3’	5’-gacttggaagcctctgcatc-3’
Mest	NM_001009617.1	5’-ccatcgtcctctccttctcc-3’	5’-tcccgtcattgttgcgaatc-3’
Mx1	NM_001271058.1	5’-cctgaggtaaggctgtggaa-3’	5’-aatacggccccacaaaacac-3’
Plagl1	NM_012760.3	5’-ggtgctgccttttcagagtc-3’	5’-ccacactcagtcttggagca-3’
Peg3	NM_001304816.1	5’-cagaatgaactggcagacga-3’	5’-ggtgtaggagggcccatatt-3’
Peg10	XM_008762737.1	5’-gttgaccgtgtccgtgtatg-3’	5’-gacgtctgatcttgcgtttg-3’
Pgf	NM_053595.2	5’-agacgacaaaggcagaaagg-3’	5’-tctcctctgagtggctggtt-3’
Prl3b1	NM_012535.3	5’-acctggagctgttggaagtg-3’	5’-gacagggatggctactcagc-3’
Rn18s	NM_046237.1	5’-gcaattattccccatgaacg-3’	5’-ggcctcactaaaccatccaa-3’
Rsad2	NM_138881.1	5’-gggatgctagtgcctactgc-3’	5’-ctgagtctccttgggctcac-3’
Sfrp2	NM_001276712.1	5’-tgtccgatagggacctgaag-3’	5’-cgagaagccactccactagg-3’
Tlr3	NM_198791.1	5’-ggtggcccttaaaagtgtgg-3’	5’-ggtttgcgtgtttccagagt-3’
Vegfa	NM_001110333.2	5’-caatgatgaagccctggagt-3’	5’-tttcttgcgctttcgttttt-3’
Ywhaz	NM_013011.3	5’-ttgagcagaagacggaaggt-3’	5’-cctcagccaagtagcggtag-3’

60˚C for 45 s), then a dissociation step (65˚C for 5 s and a sequential increase to 95˚C). Relative mRNA expression was calculated using the comparative cycle threshold (Ct) (ΔΔCt) method. The geometric mean of Ct values obtained from amplification of five constitutively expressed genes (*Rn18s*, *Ywhaz*, *Eef2*, *Gapdh*, and *Actb*) was used as reference RNA. Ct values from each of these genes were stable among the conditions tested.

2.2.4 Ultrasound

Ultrasound imaging and Doppler waveform recordings were used to compare blood flow patterns in the maternal uterine artery and fetal umbilical vessels. Dams were anesthetized with inhaled isoflurane (4% induction, 1-3% maintenance). Body temperature was maintained at 36-38˚C with a heating pad. Heart rate and respiratory physiology were monitored throughout the imaging procedure. Hair was removed from the abdomen by shaving, followed by application of a depilatory cream, after which a coupling gel (37˚C) was applied. Rats were imaged transcutaneously using a preclinical ultrasound imaging system (model Vevo 2100; FujiFilm VisualSonics) coupled with MS-250 (20 MHz, uterine artery) and MS-400 (30 MHz, umbilical artery) MicroScan transducers. The transducer beam angle was set to <45˚ for all measurements. Doppler waveforms were obtained from the uterine artery near the utero-cervical junction and from the umbilical artery spanning the fetal–placental junction. Peak systolic velocity (PSV) and end diastolic velocity (EDV) were manually measured from three sequential cardiac cycles, and data were averaged. The resistance index (RI) (RI = [PSV − EDV]/PSV) was then calculated to quantify the resistance of arterial blood velocity waveforms. Uterine artery measurements were recorded from nine dams per group; umbilical artery measurements were conducted in at least two conceptuses from each of these dams. The duration of anesthesia was limited to no more than 45 min. During this time, maternal and fetal circulatory parameters were not noticeably affected.

2.2.5 Western blotting

Protein expression was evaluated by Western blotting. Total protein in tissues was isolated by homogenizing snap-frozen GD 8.5 conceptuses in radioimmunoprecipitation

assay lysis buffer (50 mM Tris, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, pH 7.2) supplemented with protease inhibitor mixture (Sigma-Aldrich) and then sonicated (Sonic Dismembrator Model 100; Thermo Fisher Scientific). A modified bicinchoninic acid assay (Bio-Rad Laboratories) was used to measure total protein concentrations. Approximately 25 μg of tissue lysates were mixed with 4× loading buffer (final volume: 62.5 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 0.025% bromophenol blue, 50 mM DTT), boiled for 5 min, and subjected to SDS-PAGE. Proteins were then transferred to polyvinylidene difluoride (PVDF) membranes, blocked with 3% BSA in TBS containing 0.1% Tween-20, and probed with primary Abs specific for STAT1 (catalog no. 14994, 1:1000; Cell Signaling Technology), VIPERIN (catalog no. MABF106, 1:1000; Sigma-Aldrich), and IFN regulatory factor (IRF) 1 (catalog no. 8478, 1:1000; Cell Signaling Technology). Expression of GAPDH (catalog no. 5174, 1:1000; Cell Signaling Technology) was used as a loading control. Following incubation with species-appropriate, infrared-conjugated secondary Abs (Cell Signaling Technology), protein signals were detected using a LI-COR Odyssey imaging system (LI-COR Biotechnology).

2.2.6 Immunohistochemistry

Frozen conceptuses were embedded in Optimal Cutting Temperature compound and cryosectioned at 10-mm thickness. Sections were fixed in 10% neutral buffered formalin, permeabilized using 0.3% Triton-X and 1% BSA in PBS, and blocked in 10% normal goat serum (Thermo Fisher Scientific). Sections were immersed in primary Abs specific for cytokeratin (catalog no. 628602, 1:400; BioLegend) and vimentin (catalog no. MA5-11883, 1:50; Invitrogen) overnight at 4˚C. After washing, sections were immersed in species-appropriate biotin-conjugated secondary IgG Abs followed by Vectastain ABC Elite reagent (Vector Laboratories), and color was developed using 3-amino-9-ethylcarbazole (AEC) red (Thermo Fisher Scientific). Sections were counterstained with Gill No. 1 hematoxylin (Sigma-Aldrich) and mounted using Fluoromount-G mounting medium (SouthernBiotech). Images were acquired using a Nikon ECLIPSE Ni Series microscope equipped with a Ds-Qi2 camera. Placental sections that were immunostained using vimentin (which is detectable in labyrinth zone and uterine tissue but not in

junctional zone and is therefore useful for demarcating different placental zones) were subjected to morphometric analysis using ImageJ software (25). The mean thickness of the conceptus, placenta (junctional zone and labyrinth zone), and each zone was calculated following three independent measurements within the middle of the placenta. The mean area of each placenta and zone was calculated following three independent measurements of the perimeter. All measurements were conducted by two researchers who were blinded to the experimental treatments, and results between observers were averaged.

To detect localization of perforin-containing cells on GD8.5, whole conceptuses were collected, fixed in 10% neutral buffered formalin, embedded with paraffin, and sectioned at 5-mm thickness. Sections were dewaxed in Histo-clear and rehydrated using increasing dilutions of ethanol. Formaldehyde crosslinks were broken by immersing sections in a retrieval solution (Reveal Decloaker; BioCare Medical) heated to 95˚C for 20 min. Sections were then permeabilized using 0.3% Triton-X and 1% BSA in PBS and blocked in 10% normal goat serum. Samples were immersed in primary Ab specific for rat perforin (catalog TP251, 1:400; Torrey Pines Biolabs) overnight at 4˚C, followed by Alexa 555–conjugated anti-rabbit secondary Ab for 1 h at room temperature. Sections were then immersed in an Ab specific for cytokeratin (BioLegend) for 1 h, then exposed to Alexa 488–conjugated anti-mouse secondary Abs, and mounted using Fluoromount-G. Images were acquired using a Nikon ECLIPSE Ni Series microscope equipped with a Ds-Qi2 camera. Sections were subjected to localization analysis using ImageJ software (25). The distance between the bulk of perforin-positive cells and the tip of the ectoplacental cone was measured in arbitrary units (pixels) and calculated relative to the length of the ectoplacental cone.

2.2.7 Detection of hypoxia

To assess hypoxia in uterine and placental tissue after maternal PolyI:C exposure, immunohistochemical staining for pimonidazole was performed. Pimonidazole covalently binds to thiol groups in proteins of cells exposed to hypoxia (PO_2 < 10 mm Hg). Pimonidazole adducts can then be detected immunohistochemically to denote tissue areas exposed to hypoxic conditions. Rats were i.p. injected with pimonidazole

hydrochloride (hypoxyprobe-1, 60 mg/kg; Natural Pharmacia) 2.5 h before sacrifice. After euthanasia, whole conceptuses were collected, fixed in 10% neutral buffered formalin, and embedded with paraffin. Paraffin-embedded tissues were then sectioned at 5-μm thickness. Sections were dewaxed in Histo-clear and rehydrated using increasing dilutions of ethanol. Formaldehyde crosslinks were broken by immersing sections in Reveal Decloaker, as described above. Endogenous peroxidases were then inhibited using 3% hydrogen peroxide in PBS, and tissues were blocked in 10% normal goat serum. Sections were incubated in hypoxyprobe-1 mouse mAb (clone 4.3.11.3, 1:50; Natural Pharmacia), followed by biotin-conjugated anti-mouse secondary IgG Ab. Following wash, samples were then immersed in Vectastain (Vector Laboratories) for 30 min at room temperature, and color was developed using AEC red (Thermo Fisher Scientific). Samples were counterstained with Gill No. 1 hematoxylin (Sigma-Aldrich), mounted, and imaged as above.

2.2.8 Rat TS cell cultures

Blastocyst-derived rat TS cells (Asanoma et al. 2011) were used to evaluate the effects of PolyI:C, or selected PolyI:C-induced cytokines, on trophoblast cell behavior. Rat TS cells were generously provided by Michael Soares (University of Kansas Medical Center, Kansas City, KS). Cells were cultured in RPMI 1640 media (Life Technologies) supplemented with 20% (v/v) FBS (Life Technologies), 100 μM 2-ME (Sigma-Aldrich), 1 mM sodium pyruvate (Sigma-Aldrich), 50 μM penicillin, 50 U/ml streptomycin, fibroblast growth factor 4 (25 ng/ml; R&D Systems), and heparin (1 μg/ml; Sigma-Aldrich). A total of 70% of the media was preconditioned by mitomycin C–treated mouse embryonic fibroblasts prior to being added to rat TS cells. PolyI:C (50 mg/ml), recombinant rat IFN-α1 (100 U/ml or 1000 U/ml; R&D Systems), recombinant rat IL-6 (IL-6, 20 ng/ml; PeproTech), or recombinant rat IFN-γ (100 U/ml; PeproTech) was added to TS cells for 24 h. Transfection of PolyI:C (50 mg/ml) into rat TS cells was conducted using lipofectamine 2000 (Thermo Fisher Scientific). To determine the effect of IFN-α on trophoblast differentiation, rat TS cells were cultured for 3 d as above but without mouse embryonic fibroblast-conditioned media, fibroblast growth factor 4, activin A, or heparin

and in the presence or absence of recombinant rat IFN-α1 (1000 U/ml). Media were replaced daily.

2.2.9 Immunocytochemistry and EdU incorporation

Cells were fixed in 4% paraformaldehyde, permeabilized using 0.3% Triton-X, and blocked using 10% normal goat serum (Life Technologies). Cells were then immersed in anti-phospho-histone H3 Ab (1:1000, catalog no. 3377P; Cell Signaling Technology), followed by goat anti-rabbit Alexa Fluor 488–conjugated secondary Ab. Nuclei were then counterstained using DAPI (Thermo Fisher Scientific). For EdU, rat TS cells were treated with 10 μM EdU (ClickiT EdU Proliferation Kit; Thermo Fisher Scientific) for 6 h, followed by fixation as above. Detection of EdU was performed as per the manufacturer's instructions, and nuclei were detected by counterstaining with Hoechst. Cells were imaged with a Zeiss Axio fluorescence microscope. The total number of cells, and the number positive for phospho-histone H3 or EdU, was then counted in three random nonoverlapping fields of view per well. The percentage of phospho-histone H3 and EdU-positive cells was then calculated by dividing the total number of positive cells by the total number of cells, then multiplying by 100.

2.2.10 Statistical analysis

Values are expressed as mean ± SEM. Unless indicated otherwise, statistical significance was analyzed by unpaired *t* test when comparing two groups and one-way ANOVA with Tukey multiple comparisons test when comparing three or more groups. All statistical tests were two sided, and differences were considered significant when $p < 0.05$. All animal experiments were conducted with a minimum of three dams. The specific number of dams and conceptuses analyzed is indicated in the figure legends.

2.3 Results

2.3.1 Maternal PolyI:C exposure triggers a potent antiviral response within the conceptus during early pregnancy

PolyI:C is an analogue of dsRNA, which is a molecular pattern associated with viral infection and a well-known inducer of antiviral immune responses. The pregnant uterus

and fetal–placental unit are considered immune-privileged sites, so our first goal was to determine whether systemic PolyI:C administration to pregnant dams elicited an antiviral response within the conceptus (referring to decidualized uterine tissue along with primordial placenta and embryo). Compared with pregnant rats administered saline, expression of genes encoding various antiviral factors was increased within the conceptus 6 h after injecting pregnant rats with 10 mg/kg PolyI:C, including IFN-α (*Ifna*, 3.3-fold increase), *Il6* (11-fold increase), *Il1a* (17-fold increase), *Il1b* (6-fold increase), c-x-c motif ligand (Cxcl) 10 (*Cxcl10*; 323-fold increase), IRF 7 (*Irf7*, 22-fold increase), IDO (*Ido1*, 221-fold increase), radical SAM domain-containing 2 (*Rsad2*; 166-fold increase), and *Tlr3* (5-fold increase, Figure 2-1A, all $p < 0.05$, primer sequences provided in Table I). Myxovirus resistance 1 (*Mx1*), which is an IFN-inducible gene encoding a dynamin-like GTPase that confers an antiviral state against a wide range of RNA viruses (including influenza A) and some DNA viruses, was also highly upregulated in the conceptus following PolyI:C exposure (386-fold increase, Figure 2-1A, $p < 0.05$). A similar robust antiviral response was detected when transcript levels were evaluated within isolated decidua following maternal PolyI:C exposure.

Next, we determined whether PolyI:C altered expression of select proteins associated with antiviral immune responses. PolyI:C exposure to pregnant dams caused a rapid increase in RSAD2 (also known as VIPERIN, which is a multifunctional protein that inhibits viral processes) in the conceptus 2, 6, and 12 h after injection and increased IRF1 expression 2 and 6 h after injection. Both VIPERIN and IRF1 are directly and rapidly activated by both PolyI:C and IFNs and contribute to the cellular antiviral state (Stirnweiss et al. 2010). Injection of PolyI:C also increased expression of STAT1 in the conceptus 12 and 24 h after maternal exposure (Figure 2-1B). The relatively longer time required for increased STAT1 expression in comparison with VIPERIN and IRF1 likely relates to the time required for protein stabilization and accumulation following activation (Wenta et al. 2008). Collectively, these data indicate that maternal exposure to PolyI:C during early pregnancy provokes a robust antiviral response near the site of placental and embryonic development.

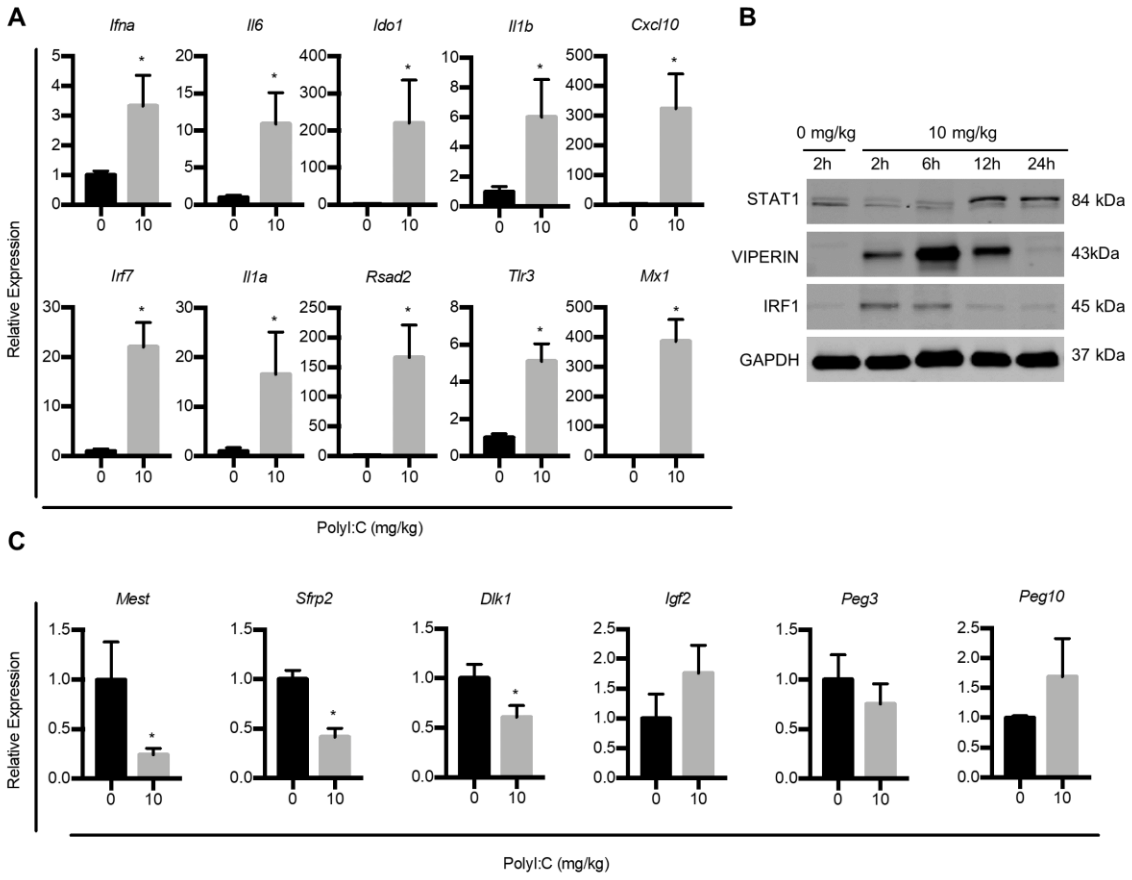

Figure 2-1: Exposure of pregnant rats to PolyI:C elicits a robust antiviral response in the conceptus.

RNA was isolated from the conceptus (containing decidua, primordial placenta, and embryo) 6 h following maternal injection of 10 mg/kg PolyI:C or saline (0 mg/kg PolyI:C) on GD8.5. (A) Expression of genes associated with antiviral responses after saline or PolyI:C exposure. (B) Protein levels of STAT1, VIPERIN, IRF1, and GAPDH are shown 2, 6, 12, and 24 h following maternal PolyI:C exposure (10 mg/kg) or 2 h following maternal saline exposure (0 mg/kg). (C) Expression of maternally imprinted genes associated with fetal and placental growth after saline or PolyI:C exposure. Results represent means ± SEM. Data significantly different ($p < 0.05$) from controls are indicated by an asterisk (*) (n = 9 from three dams per group).

We next assessed expression of nine maternally imprinted genes associated with embryonic and placental growth and development (Piedrahita 2011). In pregnant rats exposed to PolyI:C, we observed decreased expression of mesoderm-specific transcript (*Mest*, 4-fold decrease), a gene highly associated with placental growth, as well as secreted frizzled-related protein 2 (*Sfrp2*; 2.4-fold decrease) and d-like noncanonical notch ligand 1 (*Dlk1*, 1.7-fold decrease, Figure 2-1C, all $p < 0.05$). Expression of *Dio3* appeared to be decreased, although it did not reach statistical significance ($p = 0.10$, not shown). No change in expression of MAGE family member L2 (*Magel2*; not shown), paternally expressed gene 3 (*Peg3*), paternally expressed gene 10 (*Peg10*), or insulin-like growth factor 2 (*Igf2*) was detected in the conceptus after maternal exposure to PolyI:C (Figure 2-1C). Transcript levels of another maternally imprinted gene, PLAG1-like zinc finger 1 (*Plagl1*), did not meet the threshold detection levels (Ct > 30 cycles).

2.3.2 Maternal PolyI:C exposure is associated with acute hypoxia within the conceptus during early pregnancy

To assess whether maternal administration of PolyI:C increased tissue hypoxia within the conceptus, immunohistochemical detection of pimonidazole was used. Sections of the conceptus collected from saline-injected dams showed moderate levels of pimonidazole immunoreactivity, indicative of hypoxic regions proximate to the primordial placenta (Figure 2-2). In comparison, following maternal PolyI:C administration, pimonidazole immunoreactivity was noticeably more intense, suggesting that PolyI:C administration altered tissue oxygenation (Figure 2-2). Likewise, maternal administration of PolyI:C was also associated with a 1.7-fold increase in hypoxia-inducible factor 1 a (*Hif1a*) transcript levels, and a 2-fold increase in transcript levels of the hypoxia-inducible gene *Vegfa* within conceptuses (Figure 2-2C, $p < 0.05$). The pregnant uterus contains an abundant population of perforin containing uterine NK cells. PolyI:C alters the activation of uterine NK cells (Thaxton et al. 2013; Hsiao and Patterson 2011), so we sought to examine whether maternal PolyI:C exposure during early pregnancy alters localization of these cells. In dams injected with saline, perforin-containing cells were abundant within the decidua, but few cells were detectable close to the site of placental development. Following injection of PolyI:C, there appeared to be an accumulation of perforin-positive

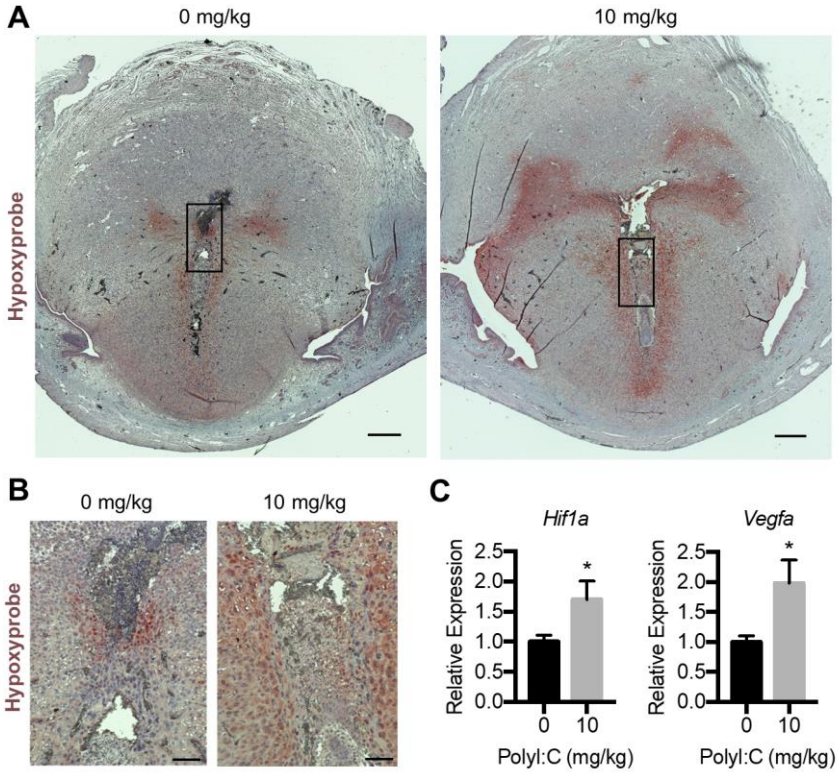

Figure 2-2: Detection of hypoxia in the conceptus following maternal exposure to PolyI:C.

(A) Hypoxia was detected in sections of the conceptus collected 6 h following maternal PolyI:C (10 mg/kg) or saline (0 mg/kg) exposure using hypoxyprobe. The black boxes surround the ectoplacental cone (part of the primordial placenta), and a higher magnification image is shown in (B). Scale bar in (A), 1000 μm. Scale bar in (B), 100 μm. (C) Transcript levels of *Hif1a* and *Vegfa* 6 h following saline or PolyI:C exposure. Results represent means ± SEM. Data significantly different ($p < 0.05$) from controls are indicated by an asterisk (*) (n = 9 from three dams per group).

cells adjacent to the nascent placenta in some conceptuses. However, the effect was variable, and we were unable to detect a statistically significant difference in the distance separating perforin-positive cells from the nascent placenta. Collectively, these results indicate that maternal PolyI:C exposure during early pregnancy alters the decidual microenvironment during development of the nascent placenta and embryo.

2.3.3 Maternal PolyI:C exposure during early pregnancy is associated with reduced fetal weight at mid and late gestation

After determining that maternal PolyI:C administration during early pregnancy triggered robust antiviral response and tissue hypoxia within the conceptus, our next goal was to examine fetal growth and survival 5 and 10 d after maternal exposure to PolyI:C. On GD13.5, average fetal weight was decreased by 10% in dams exposed to PolyI:C in early pregnancy compared with those exposed to saline (Figure 2-3A, $p < 0.05$). Similarly, fetal crown-rump length was reduced by 9% following maternal PolyI:C exposure (Figure 2-3B, $p < 0.05$). Because fetal growth is often referenced to percentiles for a given gestational age and babies smaller than the 10th centile are typically considered small for gestational age, we characterized the distribution of control GD13.5 fetuses using the saline-treated group to generate percentiles. Fetuses falling below the 10th percentile (0.068 g weight; 8.17 mm crown-rump length) were considered FGR. Strikingly, maternal PolyI:C exposure during early pregnancy caused 73% of fetuses to fall below the FGR threshold in weight and 52% to fall below the FGR threshold in crown-rump length (Figure 2-3A, 3B, $p < 0.05$). On GD18.5, fetal body weight and crown-rump length remained smaller in dams exposed to PolyI:C, although these effects were more modest in comparison with that which was observed on GD13.5 (decreased by 9 and 2% compared with saline-treated dams, respectively, Figure 2-3C, 2-3D, $p < 0.05$), suggesting that these fetuses had some degree of catch-up growth. Using the same percentile strategy to characterize distribution of control GD18.5 fetuses, we observed a greater percentage of fetuses with weights and crown-rump lengths below the 10th centile when dams were exposed to PolyI:C 10 d earlier (43% in weight, 16% in crown-rump length, Figure 2-3C, 2-3D, $p < 0.05$). In line with the

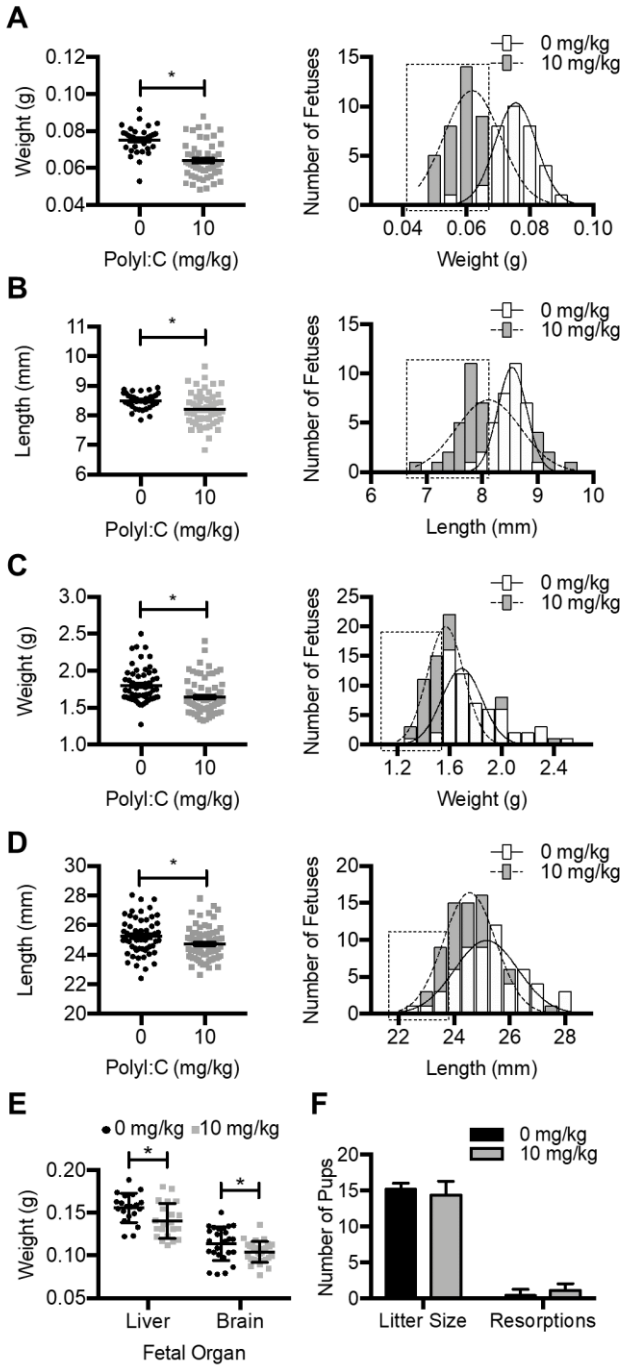

Figure 2-3: Maternal PolyI:C treatment early in pregnancy is associated with decreased fetal size at mid and late pregnancy.

Following maternal administration of saline (0 mg/kg) or PolyI:C (10 mg/kg) on GD8.5, litters were collected on GD13.5 (A, B, and F) or GD18.5 (C–E). Fetal weight (A and C) and crown-rump length (B and D) were analyzed. (E) Fetal brain and liver weights were

measured. (F) Litter size and number of resorptions are presented. Results represent means ± SEM. Data significantly different from controls ($p < 0.05$) are indicated by an asterisk (*) (n = 35 saline and n = 53 PolyI:C from at least six dams on GD13.5; n = 58 saline and n = 81 PolyI:C from at least six dams on GD 18.5)

smaller overall fetal size, maternal PolyI:C exposure was associated with smaller fetal liver weights (9% decrease, $p < 0.05$) and fetal brain weights (10% decrease, $p < 0.05$) compared with saline-injected dams (Figure 2-3E). Surprisingly, we did not observe any significant difference in litter size, as well as number of viable or resorbed fetuses, indicating that PolyI:C did not affect fetal survival (Figure 2-3F). Thus, maternal exposure to PolyI:C during early gestation is associated with delayed or impaired fetal growth at mid and late gestation without affecting viability.

2.3.4 Maternal PolyI:C exposure results in smaller placentas

Proper development of the placenta is fundamental for supporting fetal growth throughout pregnancy. Because maternal PolyI:C exposure induced a potent antiviral response within the conceptus and was associated with reduced fetal growth, we next evaluated the effect of PolyI:C on placental growth and development. As illustrated schematically in Figure 2-4A, the rodent placenta is composed of three distinct zones. The labyrinth zone is situated closest to the chorionic plate (and, hence, the fetus) and is specialized to promote nutrient and gas transfer between maternal and fetal blood. The middle layer is the junctional zone, which forms the interface between maternal and fetal tissue. The maternal portion of the placenta includes the decidua and mesometrial triangle, which contain a variety of cell types (e.g., stromal cells, blood vessels, leukocytes) that support placentation and pregnancy outcome. We did not observe a noticeable difference in the composition of the maternal portion of the placenta 5 d following exposure to PolyI:C. Specifically, there was no evident change in the area or thickness of the decidua or mesometrial triangle, number or localization of NK cells, blood vessels, or the depth of trophoblast invasion between saline- or PolyI:C-exposed dams on GD13.5. However, in dams exposed to PolyI:C on GD8.5, average placental weight, area, and thickness were reduced on GD13.5 (15, 10, and 8% decreased compared with saline-treated dams, respectively; Figure 2-4, $p < 0.05$ for all), concomitant with reduced area and thickness of the junctional zone (12 and 17%, respectively, Figure 2-4, $p < 0.05$) and labyrinth zone (14 and 6%, Figure 2-4, $p < 0.05$). Interestingly, the effects of maternal PolyI:C exposure on placental structure were

transient, as there were no differences in placental weights, zonal areas, or thicknesses on GD18.5. Collectively,

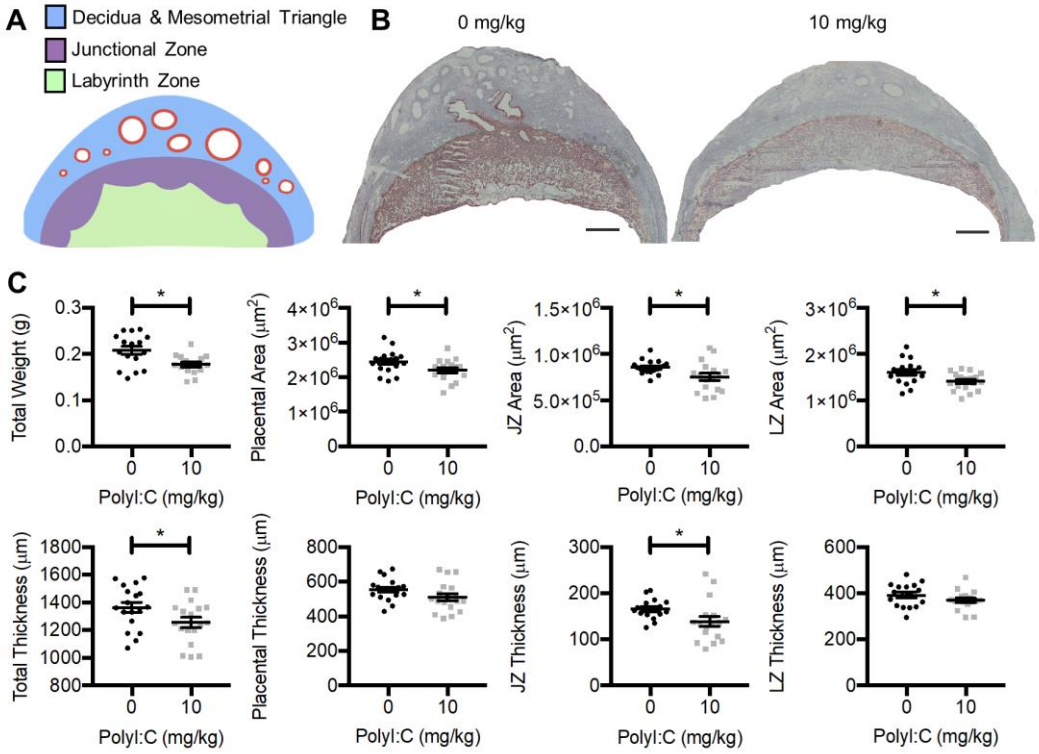

Figure 2-4: Changes in placental morphology and size following maternal PolyI:C exposure.

Representative images of placentas collected on GD13.5 5 d after dams were injected with saline (0 mg/kg) or PolyI:C (10 mg/kg). Scale bar, 1000 μm. (C) Placentas were collected on GD13.5 and weighed, and sections were immunostained with vimentin to analyze area and thickness of the placenta, labyrinth zone (LZ), and junctional zone (JZ). Results represent means ± SEM. Data significantly different from controls ($n \geq 18$ placentas from six dams, $p < 0.05$) are indicated by an asterisk (*).

these results suggest that PolyI:C exposure during early pregnancy is associated with a transient impairment or delay in development of the fetal components of the placenta.

To assess whether placental hemodynamics were altered on GD13.5 following maternal PolyI:C exposure, ultrasound biomicroscopy was performed to measure blood flow in uterine and umbilical vasculature. There were no statistically significant differences in blood flow through the uterine artery 5 d after maternal exposure to PolyI:C, as determined by average PSV, EDV, RI, and velocity time integral (VTI) (Figure 2-5A). In the umbilical artery, there was no difference detected when measuring EDV, RI, heart rate, and VTI. We did note a modest 23% increase in PSV 5 d after maternal exposure to PolyI:C (Figure 2-5B, $p < 0.05$), although in the absence of a statistical difference in other umbilical vessel parameters, we are unable to conclude whether this difference has important implications for placental function and fetal growth (Kessous et al. 2014). Collectively, these results demonstrate efficient hemodynamics of the placenta following early gestational exposure to PolyI:C despite their smaller size.

2.3.5 IFN-a promotes an antiviral response in rat TS cells and disrupts stem cell traits

PolyI:C is capable of directly inducing expression of genes associated with cellular antiviral responses. PolyI:C also robustly stimulates production of cytokines, such as type I IFNs and IL-6, which have pleiotropic effects on various cell types and mediate antiviral inflammation. Therefore, our next goal was to determine whether PolyI:C, type I IFNs, or IL-6 induce an antiviral response in TS cells. Treatment of rat TS cells with PolyI:C or IL-6 did not alter expression of genes associated with antiviral responses, including *Rsad2*, *Cxcl10*, *Irf7*, *Isg15*, and *Isg20*. Because some cell types only respond to PolyI:C if it is present within the cytosol (Palchetti et al. 2015), we transfected PolyI:C into rat TS cells but still did not observe any change in expression of these genes (data not shown). Likewise, exposure of rat TS cells to 100 U/ml IFN-γ (a type II IFN that is constitutively expressed in the conceptus during early pregnancy, owing to the prevalence of uterine NK cells) did not affect expression of any of these genes in TS cells.

Conversely, exposure of rat TS cells to 100 U/ml IFN-α stimulated expression of *Cxcl10* (2.6-fold), *Rsad2* (7.1-fold), *Irf7* (11.6-fold), *Isg15* (24-fold), and *Isg20* (2-fold, Figure 2-6A, all $p < 0.05$). In line with these results, robust expression of type I IFNR subunits *Ifnar1* and *Ifnar2* was detected in rat TS cells, whereas expression of IL-6R and the PolyI:C receptors *Tlr3* and *Ddx58* (which encodes RIG1) were low or undetectable (Figure 2-6B). To further evaluate the responsiveness of TS cells to IFN-α, cells were exposed to low-dose (100 U/ml) or high-dose (1000 U/ml) IFN-α. IFN-α stimulated expression of *Cxcl10*, *Rsad2*, *Irf7*, and *Isg20* in a dose-dependent manner, with 1000 U/ml IFN-α increasing expression of these genes by 7.34-fold, 39-fold, 38-fold, and 2.97-fold, respectively. Both doses of IFN-α stimulated *Isg15* expression ~30-fold (Figure 2-6C, $p < 0.05$).

After deducing that IFN-α stimulates an antiviral response in TS cells, our next goal was to determine whether IFN-α disrupts TS cell behavior. First, we evaluated proliferation in TS cells and found that there was no statistically significant change in proliferation as determined by percentage of cells that incorporated EdU (Figure 2-7A, 2-7B) or that were positive for phospho-histone H3 (data not shown). We next evaluated expression of genes associated with maintaining the TS cell stem state and found that 1000 U/ml IFN-α decreased transcript levels of *Cdx2* (22%) and *Mest* (14%, Figure 2-7C, $p < 0.05$). There was no statistically significant change in the expression of *Id2*. When TS cells were cultured in differentiation conditions, expression of the labyrinth-associated gene glial cells missing-1 (*Gcm1*) was increased by 5.3-fold, and the trophoblast giant cell–associated gene prolactin family 3, subfamily b, member 1 (*Prl3b1*; also called placental lactogen 2) was increased by 2.4-fold (Figure 2-7D, both $p < 0.05$). However, in the presence of IFN-α, expression of Gcm1 was reduced by 78%, and Prl3b1 levels were reduced by 63% compared with cells not exposed to IFN-α (Figure 2-7D, both $p < 0.05$). Thus, IFN-α disrupts expression of a subset of genes required to maintain TS cells in a stem state and inhibits their capacity to undergo differentiation into specialized trophoblast lineages.

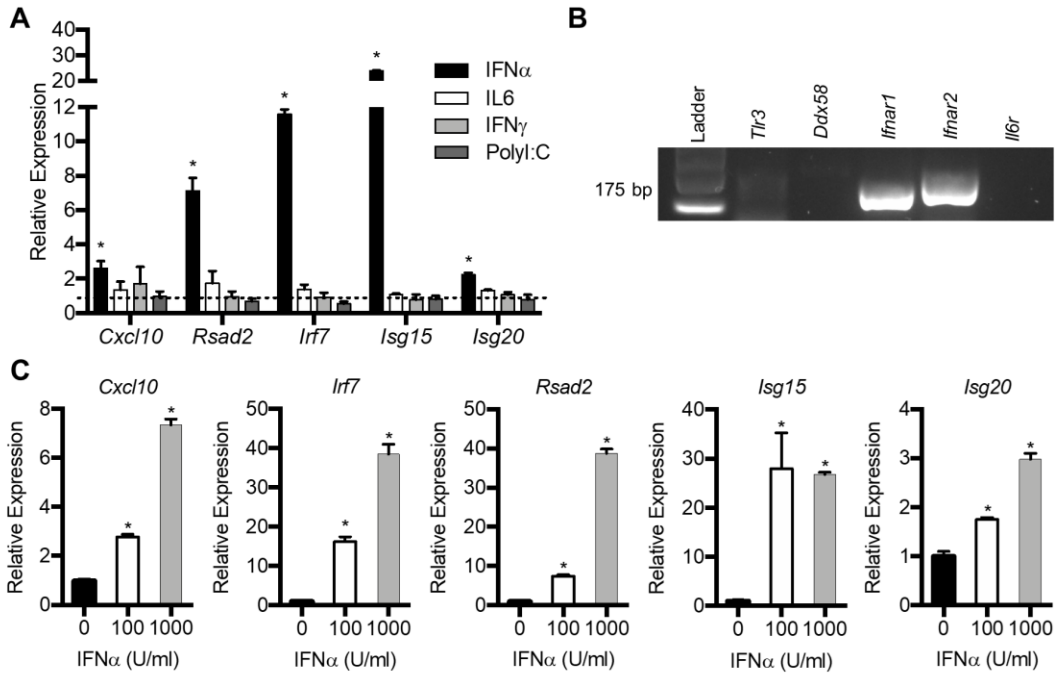

Figure 2-5: IFN-α promotes an antiviral response in rat TS cells.

(A) Rat TS cells were exposed to 100 U/ml IFN-α, 20 ng/ml IL-6, 100 U/ml IFN-γ, and 50 mg/ml PolyI:C for 24 h. The expression of five representative antiviral genes (*Cxcl10*, *Rsad2*, *Irf7*, *Isg15*, and *Isg20*) was analyzed using qRT-PCR. Expression levels were calculated relative to five transcripts (*Rn18S*, *Ywhaz*, *Eef2*, *Gapdh*, *Actb*). The horizontal dotted line represents control rat TS cells that were not exposed to cytokines. (B) Expression of receptors that interact with PolyI:C (*Tlr3*, *Ddx58*), type I IFNs (*Ifnar1*, *Ifnar2*), and IL-6 (*Il6r*) were examined in rat TS cells by RT-PCR. (C) Rat TS cells were exposed to low-dose (100 U/ml) and high-dose (1000 U/ml) IFN-α, and expression of antiviral genes was assessed by qRT-PCR. Results represent means ± SEM. Data significantly different from controls (n = 3, $p < 0.05$) are indicated by an asterisk (*).

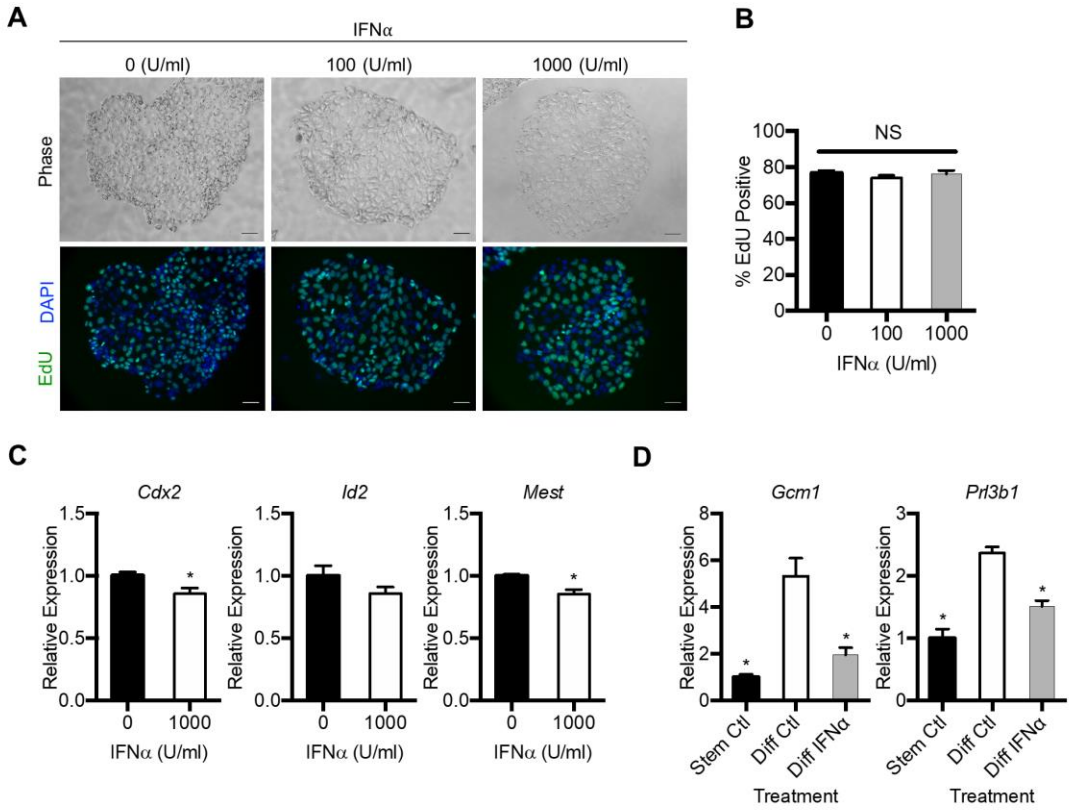

Figure 2-6: IFN-α inhibits expression of stem-related genes in rat TS cells.

(A) Representative images of rat TS cells exposed to 1000 U/ml IFN-α. Proliferation of rat TS cells was evaluated by EdU incorporation. Scale bar, 50 μm. (B) Percentage of cells that incorporated EdU. (C) Expression of transcripts associated with TS cell stem state after rat TS cells were exposed to IFN-α for 24 h. (D) Expression of *Gcm1* and *Prl3b1* in rat TS cells following culture in stem conditions or in differentiation (Diff) conditions in the presence or absence of IFN-α. Data significantly different from controls [n = 3 for (A)–(C), n = 4 for (D); $p < 0.05$] are indicated by an asterisk (*).

2.4 Discussion

In this study, we show that maternal exposure to a viral stimulus during formation of the nascent placenta is associated with a robust antiviral response and hypoxia at the site of placental development, concomitant with reduced placental and fetal growth trajectories. We further found that TS cells express type I IFNR and respond to IFN-α by increasing IFN regulatory gene expression and decreasing expression of genes associated with the TS cell stem state. TS cells also exhibit impaired differentiation potential in the presence of IFN-α. Together, these results suggest that antiviral inflammation early in pregnancy impairs proper TS cell function and causes latent placental development and fetal growth.

Viral infections during pregnancy can result in a variety of perinatal outcomes. In some cases, a viral infection may have no apparent effect on the health of a fetus or newborn, whereas in other cases, spontaneous abortion, FGR, or severe congenital anomalies may result. The different outcomes likely relate to the type of virus, the time during pregnancy when the infection is active, and whether the pathogen is transmitted to the fetus (such as with toxoplasmosis, other, rubella, CMV, and HSV infections, which are major causes of congenital anomalies) (Silasi et al. 2015). Aside from toxoplasmosis, other, rubella, CMV, and HSV infections, the effect of viral inflammation on fetal and placental growth and development is poorly understood. Consequently, there is currently no standard of care for managing viral infections during pregnancy, despite the increased susceptibility of pregnant women to severe illness and mortality from viral infections (Kourtis et al. 2014). In this study, we sought to investigate the effect of viral inflammation on pregnancy outcome using the synthetic dsRNA molecule, PolyI:C. Because PolyI:C is a TLR3 ligand and robust stimulator of antiviral inflammation (Palchetti et al. 2015), the use of PolyI:C provides a means to elucidate the contribution of antiviral inflammation to pregnancy outcome in the absence of a pathogen. Previous studies have reported adverse pregnancy outcomes after administering PolyI:C to pregnant rodents, including embryonic lethality (Buchrieser et al. 2019; Thaxton et al. 2013; Lin et al. 2006; Yockey et al. 2018), preeclampsia-like symptoms (Chatterjee et al. 2012), preterm birth (Ilievski et al. 2007), and neurodevelopmental impairments in offspring (Reisinger et al. 2015). The novelty of our study is that we administered PolyI:C during early pregnancy prior to

formation of the definitive placenta and then evaluated acute inflammation in the conceptus as well as placental and fetal growth throughout pregnancy. Because administration of PolyI:C to mice early in pregnancy results in embryonic lethality (Buchrieser et al. 2019; Thaxton et al. 2013; Lin et al. 2006; Yockey et al. 2018), it was surprising that, in our hands, fetal viability was unaffected when rats were exposed to a similar dose per weight of PolyI:C, despite a robust antiviral response and hypoxia at the placentation site. Species differences in PolyI:C-TLR3 signaling responses have been reported (Lundberg et al. 2007), so these results may reveal differing susceptibility or sensitivity to PolyI:C between mice and rats. The use of rats may thus provide an advantage to study the effects of prenatal exposure to viral inflammation early in pregnancy on long-term placental function, fetal development, and offspring health without confounding embryonic demise. Our results that inflammation rather than viral burden affects early placental development and pregnancy outcome are consistent with other studies. For instance, exposure of pregnant immunocompetent mice to Zika virus early in pregnancy, prior to formation of the definitive placenta, causes placentation defects and adverse pregnancy outcomes despite rare detection of virus in the fetus or placenta. Interestingly, these adverse effects were limited to infection during early pregnancy and were less robust when the virus was administered at midpregnancy, when the placenta is functional (Szaba et al. 2018). Thus, antiviral inflammation during early pregnancy may be a strong independent contributor to deficient placentation and adverse pregnancy outcomes regardless of pathogenic burden.

Proper fetal growth is an essential determinant of prenatal and perinatal health. Babies born growth restricted have a decreased chance of surviving their first year and have a higher risk of cardiovascular, metabolic, and neurodevelopmental sequelae in later life (Sharma et al. 2016). FGR can be classified as symmetric (whole body) or asymmetric (head sparing). Asymmetric FGR is often due to extrinsic events (e.g., placental insufficiency, uterine anomalies) occurring late in pregnancy that cause more severe growth impairments in abdominal organs than the head. Symmetric FGR, in contrast, is usually attributed to an early embryonic event that causes all growth parameters to be below normal for gestational age. We observed that fetal weight was symmetrically decreased in later pregnancy when dams were exposed to PolyI:C during early gestation.

We found no evidence of aberrant placental hemodynamics based on Doppler ultrasound recordings; thus, PolyI:C-induced FGR is unlikely to be attributed to alterations in placental blood flow dynamics. Therefore, viral inflammation reduced placental and fetal growth but did not impair placental hemodynamics, which are phenotypes consistent with symmetric FGR (Fleiss et al. 2019).

Viruses can be a major detriment to placental development. Viral infections have been associated with increased trophoblast apoptosis, decreased trophoblast invasion, production of proinflammatory responses, disruption of placental endocrine function, and increased risk of obstetric complications (Griffiths and Baboonian 1984; Liu et al. 2015; Chou et al. 2006; Arechavaleta-Velasco et al. 2006; Miner et al. 2016). Our findings using PolyI:C suggest that antiviral inflammation is sufficient to impair placentation and is consistent with other studies. For example, administration of PolyI:C to pregnant mice elicits maternal decidual leukocyte activation, decidual necroptosis, and production of TNF-α and IL-6 that induces STAT3 activation and endocrine disruption in placental cells (Thaxton et al. 2013; Hsiao and Patterson 2011; Yu et al. 2017). Prenatal PolyI:C exposure early in pregnancy also stimulates expression of IFN-induced transmembrane proteins, which interferes with trophoblast syncytialization and labyrinth zone formation, leading to embryo demise. Interestingly, mice lacking these transmembrane proteins are protected from PolyI:C-induced placental maldevelopment and embryonic loss, implicating placental maldevelopment as a critical determinant causing adverse pregnancy outcomes during viral inflammation (Buchrieser et al. 2019). In rats, PolyI:C administration at midgestation is associated with altered expression of amino acid and drug transporters in the placenta, resulting in a corresponding change in amino acid concentrations in the fetus (McColl and Piquette-Miller 2019; Petrovic and Piquette-Miller 2010). Term human placental explants also exhibit altered expression and function of multidrug resistance transporters following challenge with PolyI:C (Lye et al. 2015). In our study, we noted decreased expression of several maternally imprinted genes associated with placental and fetal growth after maternal PolyI:C exposure, including *Sfrp2*, *Dlk1*, and *Mest*. *Sfrp2* encodes a cysteine-rich protein that acts as a regulator of placental development through its modulation of the Wnt signaling pathway (Guilleret et al. 2009). *Dlk1* encodes a cell-surface transmembrane protein highly expressed in

placental villi and exhibits reduced expression in FGR (Wylie et al. 2000). *Mest* is expressed mainly by the placenta. Mice lacking *Mest* exhibit FGR because of a severe placental growth defect (Lefebvre et al. 1998), and decreased expression of *Mest* is evident in human placentas during FGR (McMinn et al. 2006). Our results are consistent with studies in which pregnant mice were challenged with a bacterial infection (*Campylobacter rectus*) early in pregnancy, resulting in FGR associated with repression of key developmental genes, including several imprinted genes (Bobetsis et al. 2010; Bobetsis et al. 2007). Thus, it is possible that the latent fetal and placental growth observed after PolyI:C exposure is at least partially due to altered expression of key placental growth-promoting genes like *Mest*.

Recognition of dsRNA by pattern recognition receptors triggers an antiviral response that features synthesis of type I (α, β) IFNs. Type I IFNs are a family of 13 cytokines that bind to a single heterodimeric IFN-αR composed of two subunits, IFNAR1 and IFNAR2. Binding of type I IFNs to IFNAR initiates downstream signaling cascades that regulate activation and repression of hundreds of genes (Schreiber and Piehler 2015). The products of these genes promote a cellular antiviral state designed to limit replication and spread of viruses and other pathogens and can also alter cell proliferation, health, and viability in a cell-type and environment-specific context. The importance of type I IFNs for mediating systemic responses to viruses (and PolyI:C) in pregnant rodents is elegantly demonstrated in *Ifnar*$^{-/-}$ mice (Yockey et al. 2018; Racicot et al. 2017; Cappelletti et al. 2017). Type I IFNs also alter stem cell traits in pluripotent and hematopoietic stem cells (Pietras et al. 2014; Smith et al. 2018; Eggenberger et al. 2019), so we postulated that type I IFNs impact TS cell behavior. We found that rat TS cells expressed high levels of *Ifnar1* and *Ifnar2* and robustly responded to IFN-α by increasing expression of various IFN-stimulated genes. IFN-α also decreased expression of genes associated with the TS cell stem state and placental growth, including *Mest*, and impaired their differentiation potential, as shown by reduced expression of *Gcm1* and *Prl3b1*. Thus, similar to reports with pluripotent and hematopoietic stem cells, type I IFN signaling may interfere with signal transduction pathways that maintain TS cell stem traits and temporarily disrupt normal TS cell function in the nascent placenta. Conversely, rat TS cells expressed low levels of *Tlr3*, *Ddx58*, and *Il6r*, and no detectable changes in IFN-stimulated genes were

observed after rat TS cells were exposed to PolyI:C, IL-6, or to another IFN constitutively expressed at the placentation site, IFN-γ. These results are interesting because human and murine trophoblasts respond directly to PolyI:C, IL-6, and IFN-γ (Abrahams et al. 2006; Jones et al. 2009; Hu et al. 2008). Multiple cell types at the maternal-placental interface are capable of recognizing pathogen-associated molecular patterns, such as PolyI:C, and promote an antiviral state in part by producing type I IFNs. Our data suggest that TS cells are unable to recognize PolyI:C and some other inflammatory cytokines but possess the capacity to respond to type I IFNs. The narrow scope of TS cell responsiveness to viral patterns and cytokines other than type I IFNs may be a protective mechanism to avoid unwanted disruption of normal cellular functioning or viability while still contributing to an aggregate antiviral state. As they differentiate into specialized trophoblast lineages, trophoblasts may gain the capacity to recognize and respond to a broader range of immune mediators to limit pathogen replication and vertical transmission. Nonetheless, production of IFN-α in response to a viral infection early in pregnancy may be a determinant of acute changes in TS cell function, which could have long-lasting impacts on placental and fetal growth.

This study has several limitations. First, although humans and rats exhibit hemochorial placentation featuring regions with analogous structure and function (Soares et al. 2012), there are key differences in early placental development between these species. Therefore, we cannot conclude whether viral inflammation early in pregnancy affects human placental development in the same way as we observed for rat placentation. Of note, women diagnosed with primary CMV infection between 11 and 15 weeks of pregnancy exhibit progressively increased placental thickness, as demonstrated through ultrasound examination (La Torre et al. 2006), rather than decreased placental size, as would be suggested by our study. Potential explanations for this discrepancy are that CMV infection was detectable only after the placenta was formed and functional. In our study, the viral stimulus was provided during early placental development prior to chorioallantoic fusion. Thus, the timing of viral stimulus may be an important consideration. Moreover, the chronic CMV infection and relatively long duration of pregnancy in humans may have facilitated either a progressive edematous and

inflammatory response or a placental compensatory response, resulting in a thicker placenta that would not be apparent in our postmortem analyses in rats.

A second limitation is that we cannot ascertain that altered TS cell function in response to type I IFNs is directly responsible for the reduction in placental and fetal size after maternal PolyI:C. In future studies, these experiments could be performed using a line of rats in which the *Ifnar* gene is compromised by targeted genomic editing (Qaisar et al. 2017). Third, because PolyI:C was administered to dams systemically, we cannot conclude whether the concentration of dsRNA at the maternal-placental interface in our model represents the amount present within a typical virus infection. We attempted to evaluate the presence of dsRNA at the maternal-placental interface by immunofluorescence using a mAb (J2), but we were unable to detect robust staining. However, the profile of antiviral factors induced at the maternal-placental interface that was observed in our study is consistent with previous results that have used live viruses, such as Zika and influenza A (Bayer et al. 2016; Steuerman et al. 2018; Sheridan et al. 2017), suggesting that the dose of PolyI:C used in our study elicits antiviral inflammation at the maternal-placental interface that is comparable to inflammation present during a virus infection. Exposure of pregnant rodents to PolyI:C is frequently used as a model of brain maldevelopment and cognitive deficiencies in offspring (Reisinger et al. 2015), which is consistent with the known association between viral infections during pregnancy and increased risk of neurobehavioral impairments in humans (Kratimenos and Penn 2019). FGR is also an established risk factor for neurodevelopmental and behavioral problems in childhood (Murray et al. 2015). Thus, our findings provide a new dimension to the link between viral infections and FGR and raise the question of whether altered placental and fetal growth trajectories are causally linked to long-term neurobehavioral consequences. Our results may also help to stratify those pregnancies at risk for poor fetal growth following exposure to a viral infection by evaluating expression of genes associated with placental growth (e.g., *Mest*) so that targeted management strategies can be implemented to alleviate long-term health sequelae.

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Chapter 3

3 Maternal Immune Activation Alters Fetal Brain Development and Enhances Proliferation of Neural Precursor Cells in Rats

Maternal immune activation (MIA) caused by exposure to pathogens or inflammation during critical periods of neurodevelopment is a major risk factor for behavioral deficits and psychiatric illness in offspring. A spectrum of behavioral abnormalities can be recapitulated in rodents by inducing MIA using the viral mimetic, PolyI:C. Many studies have focused on long-term changes in brain structure and behavioral outcomes in offspring following maternal PolyI:C exposure, but acute changes in prenatal development are not well-characterized. Using RNA-Sequencing, we profiled acute transcriptomic changes in rat conceptuses (decidua along with nascent embryo and placenta) after maternal PolyI:C exposure during early gestation, which enabled us to capture gene expression changes provoked by MIA inclusive to the embryonic milieu. We identified a robust increase in expression of genes related to antiviral inflammation following maternal PolyI:C exposure, and a corresponding decrease in transcripts associated with nervous system development. At mid-gestation, regions of the developing cortex were thicker in fetuses prenatally challenged with PolyI:C, with females

displaying a thicker ventricular zone and males a thicker cortical mantle. Along these lines, neural precursor cells (NPCs) isolated from fetal brains prenatally challenged with PolyI:C exhibited a higher rate of self-renewal. Expression of Notch1 and the Notch ligand, delta-like ligand 1, which are both highly implicated in maintenance of NPCs and nervous system development, was increased following PolyI:C exposure. These results suggest that MIA elicits rapid gene expression changes within the conceptus, including repression of neurodevelopmental pathways, resulting in profound alterations in fetal brain development.

3.1 Introduction

Development of the brain is an intricately orchestrated process that commences early in gestation and is particularly vulnerable to changes in the prenatal environment. There is now substantial evidence that maternal infection during windows of susceptibility can affect critical aspects of fetal brain development, leading to a wide range of neuronal dysfunctions and behavioral outcomes in offspring (Gumusoglu and Stevens 2019). For example, epidemiological evidence implicates maternal infection as a major risk factor for neurodevelopmental disorders in offspring, including schizophrenia (Brown et al. 2009), autism-spectrum disorder (ASD) (Parker-Athill and Tan 2010), and epilepsy (Sun et al. 2008), as well as neurodegenerative disorders including Alzheimer's disease and Parkinson's disease (Carvey et al. 2003; Hoeijmakers et al. 2016). Altered brain development is recapitulated in offspring born to pregnant mice and rats exposed to different types of infections, resulting in a spectrum of behavioral abnormalities apparent in adolescent and adult offspring (Boksa 2010). Despite considerable progress identifying behavioral phenotypes in juvenile and adult offspring exposed prenatally to infection, the impact of infection on neuronal development and brain structure, particularly during fetal life, is not well understood.

Maternal immune activation (MIA) in the absence of a pathogen is sufficient to elicit brain maldevelopment and behavioral abnormalities in offspring, indicating that the maternal response to infection, rather than the infection itself, is a likely culprit. One of the most recognized and established models of neurodevelopmental deficiencies resulting from acute exposure to MIA involves prenatal exposure to polyinosinic:polycytidylic

acid (PolyI:C) (Meyer and Feldon 2012). PolyI:C is a synthetic analog of double-stranded RNA, which resembles the molecular pattern of certain viruses. PolyI:C induces the generation of a cellular antiviral response, and stimulates the production of inflammatory cytokines including type I interferons, interleukin (IL)-6, and IL-1β, all of which may participate in MIA-induced neurodevelopmental impairment (Field et al. 2010; Smith et al. 2007). PolyI:C therefore effectively mimics the acute cellular response to viral infections and is advantageous as a model because the response is transient, and the stimulus intensity and timing can be precisely controlled to evaluate the impact of MIA during particular phases of brain development. Administration of PolyI:C to pregnant mice or rats during early-to-mid gestation or late gestation has revealed differing levels of sensitivity to fetal cytokines, corresponding to unique behavioral phenotypes later in life (Meyer et al. 2008). These altered behaviors include deficits in social interaction, attention, memory, and sensorimotor function that have construct and face validity towards human neuropsychiatric disorders such as schizophrenia and ASD (Careaga et al. 2017), suggesting that MIA may interfere with structural and developmental programming during prenatal brain development leading to behavioral changes in postnatal life.

The cerebral cortex integrates sensory and motor information and is responsible for shaping behavior, perception, higher-order thought, and reasoning in mammals. It develops from a single layer of neuroepithelium that lines the ventricles. As early as embryonic day 9 (GD 9) in rats, neural stem cells (NSCs) within the neuroepithelium undergo symmetric divisions to provide the early NSC pool that is crucial for determining cell number and adult brain size (Goldman-Rakic 1995). By GD 11, NSCs start dividing asymmetrically to form neural precursor cells (NPCs), which then produce neurons that migrate away from the ventricular zone to form the preplate, and subsequent volley of neurons form the remainder of cortical layers. Recent evidence indicates that cortical neurogenesis is orchestrated by highly complex signaling mechanisms involving precise, temporally-regulated expression of transcription factors as well as the action of signaling molecules belonging to the Notch and Wnt pathways (Martynoga et al. 2012). Disruptions in maintenance of the NSC pool and cortical neurogenesis impact neuronal number and brain size, and also result in malformations in layering (heterotopia) and

neuronal connections. It is also important to note that in rats, cortical angiogenesis begins at GD 10 and the blood-brain barrier is functional by GD 16. Consequently, early developmental periods prior to neuronal migration on GD 11 is a potentially vulnerable period for MIA-mediated abnormalities (Rice and Barone 2000).

In this study, we injected pregnant rats with saline or PolyI:C at GD 8.5 and generated a transcriptional profile on whole conceptus tissue that revealed both the response of the nascent uterine environment to MIA and changes in gene expression critical for neurodevelopmental processes. We then evaluated cortical structure on GD 15.5 and isolated neurospheres to assess the self-renewal capacity of NSCs and NPCs. We discovered a robust antiviral inflammatory response following PolyI:C administration that correlated with impaired progression of neurodevelopmental events. Surprisingly, we also identified a "rebound effect" at mid-to-late gestation, in which cortical thickness was increased suggesting an expanded NSC pool that correlated to increased NSC/NPC proliferation capacity and altered expression of receptors and ligands regulating Notch signaling – a pathway crucial for NSC proliferation and nervous system development.

3.2 Materials and Methods

3.2.1 Animals

Female (6-8 weeks old) and male Sprague Dawley rats were obtained from Charles River Laboratories and maintained in a 12:12 hour light-dark cycle (lights on at 7:00 am) at constant temperature/humidity and food and water available *ad libitum*. Females were cycled by daily inspection of cells within the vaginal lavage and mated when in proestrus with a fertile male. Gestational day (GD) 0.5 was defined as the day following mating if spermatozoa were detected within the vaginal lavage. All experiments were in compliance with guidelines outlined by the Canadian Council of Animal Care, using protocols approved by the University of Western Ontario Animal Care Committee.

3.2.2 Injection protocol and tissue collection

Pregnant females were injected intraperitoneally on GD 8.5 with sterile saline (0.9% NaCl) or an equal volume of saline containing 10 mg/kg PolyI:C sodium salt (Sigma-

Aldrich). The 10 mg/kg dose of PolyI:C is based on our previous study (Baines et al. 2020), and other reports in which this dose of PolyI:C elicited an inflammatory response and altered behavioral phenotypes in offspring (Bloise et al. 2017; Forrest et al. 2012). For experiments evaluating the acute impact of PolyI:C on gene expression in the conceptus (encompassing maternal decidua as well as nascent embryo and placenta), dams were euthanized 6 h following saline or PolyI:C injection (early gestation). All other tissue collections were performed on GD 15.5 (mid-to-late gestation; total pregnancy duration in rats is ~22 days). Euthanasia was conducted using mild carbon dioxide asphyxiation, followed by thoracotomy. For samples collected on GD 8.5, whole conceptuses were isolated and snap-frozen in liquid nitrogen. For tissues collected on GD 15.5, embryos were collected, and whole brains were removed and fixed in 4% paraformaldehyde for immunohistochemical analysis, or cortical tissue was minced and processed for NPC and neurosphere culture.

3.2.3 RNA Sequencing

RNA was extracted from tissue by homogenizing in RiboZol (Amresco). The aqueous phase was diluted with 70% ethanol and placed on RNeasy columns (Qiagen), treated with DNase I, and purified. RNA integrity was assessed using an Agilent 2100 bioanalyzer (Agilent Technologies), and indexed libraries were generated following rRNA reduction. Libraries were then sequenced with NextSeq High Output 75 cycle sequencing kit (Illumina) at the London Regional Genomics Centre. Reads from *.fastq files were aligned to the rat reference genome (Rnor_6.0.91) using CLCBio Genomics Workbench (Qiagen version 10.1.2), and transcript abundance was expressed as reads per kilobase of transcript per million mapped reads (RPKM). Analysis was restricted to transcripts in which average RPKM was >0.1 among either saline or PolyI:C-exposed conceptuses. Statistical significance was calculated by empirical analysis of digital gene expression, followed by Bonferroni's correction. Data are available on the Gene Expression Omnibus (GSE145167). Gene ontology pathway analysis was completed using DAVID Functional Annotation Bioinformatics (Huang et al. 2009).

3.2.4 Quantitative RT-PCR

RNA was extracted from cells and tissues by lysing in RiboZol (Amresco) as directed by the manufacturer. Complementary DNA was made from purified RNA using High Capacity complementary DNA Reverse Transcription kit (ThermoFisher Scientific), diluted 1:10, and used for quantitative RT-PCR. Complementary DNA was mixed with SensiFAST SYBR green PCR Master Mix (FroggaBio) and primers (Table 1). Amplification and fluorescence detection were conducted using a CFX Connect Real-Time PCR system (Bio-Rad Laboratories). Cycling conditions included an initial holding step (95℃ for 3 min), followed by 40 cycles of two-step PCR (95℃ for 10 s, 60℃ for 45 s), and a dissociation step (65℃ for 5 s, and a sequential increase to 95℃). The comparative cycle threshold (ΔΔCt) method was used to calculate relative mRNA

Table 3-1: List of Primers used for RT-PCR.

Gene	*Accession Number*	*Forward Sequence*	*Reverse Sequence*
Actb	NM_031144.3	5'-AGCCATGTACGTAGCCATCC-3'	5'-CTCTCAGCTGTGGTGGTGAA-3'
Ccl5	NM_031116.3	5'-CCTTGCAGTCGTCTTTGTCA-3'	5'-GAGTAGGGGGTTGCTCAGTG-3'
Cxcl10	NM_139089.1	5'- TGTCCGCATGTTGAGATCAT-3'	5'-GGGTAAAGGGAGGTGGAGAG-3'
Dll1	NM_032063.2	5'-GCACGAGAAAACCAGAAAGC-3'	5'-TCTTCAAAGACCCAGGGATG-3'
Dll3	XM_006228611.3	5'- GACTCACAGCGCTTCCTTCT-3'	5'- TCTTCGGGATGATTCCAGTC-3'
Dll4	NM_001107760.1	5'- ACCTTTGGCAATGTCTCCAC-3'	5'- TTGGATGATGATTTGGCTGA-3'
Doc2b	NM_031142.1	5'-GAGCCAGCAAGGCAAATAAG-3'	5'-GTGTGGTTGGGTTTCAGCTT-3'
Eef2	NM_017245.2	5'-CGCTTCTATGCCTTCGGTAG-3'	5'-GTAGTGATGGTGCCGGTCTT-3'
Gapdh	NM_017008.4	5'- AGACAGCCGCATCTTCTTGT-3'	5'-CTTGCCGTGGGTAGAGTCAT-3'
Il1b	NM_031512.2	5'-AGGCTTCCTTGTGCAAGTGT-3'	5'-TGAGTGACACTGCCTTCCTG-3'
Jag1	NM_019147.1	5'- ATCGCATCGTACTGCCTTTC-3'	5'- GGCAATCCCTGTGTTCTGTT-3'
Jag2	NM_001375303.1	5'- TGCACTTTAACCGTGACCAA-3'	5'- AAAGACACAGCCACCTCCAC-3'
Notch1	NM_001105721.1	5'-GTTTGTGCAAGGATGGTGTG-3'	5'-CCTTGAGGCATAAGCAGAGG-3'
Notch2	NM_024358.2	5'- TCAATCGCTTCCAGTGTCTG-3'	5'- TGCAGATGCAGGTGTAGGAG-3'
Notch3	NM_020087.2	5'- CCCACAAGCCCTGTAGTCAT-3'	5'- TCACATTGATGACCCTGGAA-3'
Notch4	NM_001002827.1	5'- CACCTGCCTGAGGCTATCTC-3'	5'- GAGCTCTTCCAGAGGGCTTT-3'
Rn18s	NM_046237.1	5'-GCAATTATTCCCCATGAACG-3'	5'-GGCCTCACTAAACCATCCAA-3'
Rnf112	NM_138613.1	5'-CGCCAAGAAGGAGTTTGAAG-3'	5'-CAGAAGCGCATTGTGTAGGA-3'

Rsad2	NM_138881.1	5’-GGGATGCTAGTGCCTACTGC-3’	5’-CTGAGTCTCCTTGGGCTCAC-3’
Sema5c	NM_017308.1	5’-ACTGTCTTTCCCCCTGTCCT-3’	5’-GTGGGGGCTCCTACACAGTA-3’
Ywhaz	NM_013011.3	5’-TTGAGCAGAAGACGGAAGGT-3’	5’-CCTCAGCCAAGTAGCGGTAG-3’

expression, using the geometric mean of Ct values obtained from amplification of five genes (*Rn18s*, *Ywhaz*, *Eef2*, *Gapdh*, and *Actb*) as reference RNA.

3.2.5 Isolation of NPCs and neurosphere culture

Single-cell suspensions of cortical cells can be isolated from the embryonic telencephalon and propagated as spherical NPC-containing masses called neurospheres (Campos 2004). NPC culture was based on the method described by Ghosh et al., with modifications (Ghosh and Greenberg 1995). Embryos were collected on GD 15.5, and DNA isolated from tails using reagents from REDExtract-N-Amp DNA isolation kit (Sigma-Aldrich) to evaluate sex of embryos, based on presence or absence of the *Sry* gene. Primers used for *Sry* genotyping were 5'-TGGGAATGTATGCTGGCATA-3' and 5'-CCTCTCATGCCCAGAGTGAC-3'. Using a dissecting microscope, skin, skull, and meninges were removed to access the cortex, then cortical tissue was removed and placed in a dish containing fresh primary culture media (NeuroCult Proliferation Kit, Stem Cell Technologies). Cells were gently dissociated, plated at a density of 120,000 cells/ml, and incubated at 37ºC and 5% CO2 initially for 4 days. Under these conditions, cells efficiently proliferated to generate neurospheres. To determine their proliferation capacity, after three days in culture, neurospheres were treated with 10 mM 5-ethynyl-2'-deoxyuridine (EdU) for 24 h, followed by immunohistochemistry as described below.

After four days, primary cortical NPCs were isolated from neurospheres. In brief, neurospheres were mechanically dissociated, centrifuged, and resuspended in either proliferation or differentiation medium. Cell viability was determined through trypan blue exclusion, and 25,000 viable cells/ml were distributed into 24-well plates containing sterile coverslips precoated with poly-D lysine (10 μg/ml) and laminin (10 μg/ml; both from Sigma-Aldrich). For experiments in which male and female embryos were individually analyzed, cells from each embryo were cultured in their own well until genotyping was performed, then male or female cells were combined into a single well. For all other experiments, cells from 3-5 embryos were combined. Cells plated for proliferation experiments received NeuroCult proliferation medium, and cells plated for differentiation experiments were cultured in NeuroCult differentiation medium (both

from Stem Cell Technologies). Cells were cultured for 3 days prior to treatment with 10 mM EdU for 24 h, followed by immunofluorescence as described below.

3.2.6 Immunohistochemistry

Whole fetal brains were collected on GD 15.5 and placed in 4% paraformaldehyde overnight. Brains were transferred to 30% sucrose for at least 24 h, and then embedded in O.C.T. compound and stored at –80°C until sectioning. Coronal cryosections were obtained at 12 μm thickness. For neurosphere cryosectioning, media were carefully removed from neurospheres after allowing them to settle to the bottom of a conical centrifuge tube, and then 4% paraformaldehyde was added to the tube. Neurospheres were transferred to 30% sucrose for at least 24 h, embedded in O.C.T. compound, and cryosectioned at 10 μm thickness.

Sections of fetal brain and neurospheres were post-fixed for 10 min in 4% paraformaldehyde, permeabilized in PBS containing 1% bovine serum albumin and 0.3% Triton-X and blocked using 10% normal goat serum to reduce non-specific antibody binding. Sections were immersed in primary antibodies as described below. The following day, species-appropriate, fluorescent-conjugated secondary antibodies were applied for 1 h, followed by Hoechst nuclear stain (ThermoFisher Scientific). For detection of EdU, sections of EdU-treated neurospheres were probed with an Alexa488-conjugated EdU monoclonal antibody (ClickIt EdU proliferation kit, ThermoFisher Scientific), followed by Hoechst nuclear stain. Sections were then mounted using Fluoromount G (SouthernBiotech), and fluorescence detected using a Nikon ECLIPSE Ni series microscope equipped with a Ds-Qi2 camera. Confocal microscopy was performed using a Zeiss LSM800 series confocal laser scanning microscope.

3.2.7 Immunofluorescence

Cell culture media were removed, and cells fixed in 4% paraformaldehyde. Detection of EdU-positive cells was performed using an Alexa488-conjugated EdU monoclonal antibody. Nuclei were subsequently counterstained using Hoechst nuclear stain. Coverslips were then mounted, and fluorescence detected as described above. The percentage of EdU-positive cells was calculated by dividing the number of EdU-positive

cells by the total number of cells (as determined by detection of nuclei by Hoechst staining), multiplied by 100.

For detection of various proteins, antibodies targeting Sox2 (1:100, 48-1400, ThermoFisher Scientific), βIII-Tubulin (β3T; 1:1000, 60052, Stem Cell Technologies), Pax6 (1:250, 42-6600, ThermoFisher Scientific), NeuN (1:200, MAB377, EMD Millipore), Phospho-Histone H3 (1:800, 3377, Cell Signaling Technology), GFAP (1:200, 12389, Cell Signaling Technology), and Notch1 (1:200, 4380, Cell Signaling Technology) were diluted in PBS and applied overnight at 4ºC. The following day, species-appropriate, fluorescent-conjugated secondary antibodies (AlexaFluor anti-mouse 488 and anti-rabbit 555, ThermoFisher Scientific) were applied for 1 h, followed by Hoechst nuclear stain (ThermoFisher Scientific). Coverslips were then mounted, and fluorescence detected as described above.

3.2.8 Western blotting

Protein expression was evaluated by western blotting. Total protein was isolated by immersing cells in radioimmunoprecipitation assay lysis buffer (50 mM Tris, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, pH 7.2) supplemented with protease inhibitor cocktail (Sigma-Aldrich). A modified bicinchoninic acid assay (Bio-Rad Laboratories) was used to measure total protein concentrations. Approximately 10 µg of cell lysates were mixed with 4× sample loading buffer (final concentration: 62.5 mM Tris pH 6.8, 2% SDS, 10% glycerol, 0.025% bromophenol blue, 50 mM dithiothreitol), boiled for 10 min, and subjected to SDS-polyacrylamide gel electrophoresis. Proteins were then transferred to polyvinylidene difluoride membranes, blocked with 5% bovine serum albumin in TBS containing 0.1% Tween-20, and probed with primary antibodies specific for phospho-histone H3 (1:1000, 3377, Cell Signaling Technology), Notch1 (1:1000, 4380, Cell Signaling Technology), and β-actin (1:500, sc-47778, Santa Cruz Biotechnology). Following incubation with species-appropriate, infrared-conjugated secondary antibodies (Cell Signaling Technology), protein signals were detected using a LI-COR Odyssey imaging system (LI-COR Biosciences).

3.2.9 Statistical analysis

Statistical comparisons between two means were conducted using unpaired, two-tailed Student's t-tests, whereas statistical comparisons of three or more means were conducted using analysis of variance, followed by Tukey's *post-hoc* test. Sex-dependent effects were compared using two-way analysis of variance followed by Sidak's multiple comparison. Assessment of changes in transcript abundance was conducted using nine conceptuses collected from three dams per group. Data were independently validated with an additional nine conceptuses from three dams per group. Fetal cortical thickness was analyzed by obtaining images of the cortex from three distinct regions (anterior, middle, posterior) separated from each other by approximately 100 μm, using at least ten male and ten female brains collected from a total of three different litters per group. Total cortical thickness, as well as thickness of each layer (Sox2+, β3T+), was measured in three independent areas of each image using ImageJ (Schneider et al. 2012). Data are presented as individual length measurements in μm ± SEM. For comparison of proliferation capacity between neurospheres isolated from saline and PolyI:C-treated dams, at least 1655 cells per dam prepared from four different litters per treatment were used for analyses. Data are presented as percent EdU-positive cells per neurosphere ± SEM. For comparing proliferation capacity in NPCs following disaggregation, five fields of view were randomly imaged across two technical replicates, and cell numbers were determined using an in-house automated cell-counting program (MATLAB) to obtain total cell counts (based on the number of Hoechst-positive nuclei) and EdU-positive cells. At least 300 cells per treatment obtained from 3 dams per group were counted. The percent EdU-positive cells in each image was then obtained. All differences were considered statistically significant when $p < 0.05$. Graphing and statistical analyses were performed using GraphPad Prism 7.0.

3.3 Results

3.3.1 Maternal PolyI:C exposure during early pregnancy induces an antiviral response in the conceptus and alters expression of genes associated with nervous system development

PolyI:C is a synthetic analog of dsRNA and has been used to stimulate cellular antiviral responses in lieu of an active pathogen. In rodents (Smith et al. 2007) and primates (Rose et al. 2017), exposure of dams to PolyI:C during early or mid-pregnancy elicits long-term cognitive and behavioral deficits in offspring, suggesting that PolyI:C, or the antiviral response incited by PolyI:C, interferes with normal brain developmental processes. Transcriptome profiles have been generated using neonatal or postnatal brain tissue, days or weeks following prenatal exposure to PolyI:C (Richetto et al. 2017; Weber-Stadlbauer et al. 2017). However, acute gene expression changes in the conceptus (encompassing decidua, embryo, and extraembryonic tissues) triggered by PolyI:C have not been extensively characterized. Therefore, our first goal was to use RNASeq to profile global gene expression changes in the conceptus following acute PolyI:C treatment. Overall, from a total of 15,135 transcripts examined, exposure of dams to PolyI:C induced significant changes in 1,032 transcripts in the conceptus (≥2-fold increased or decreased compared to dams injected with saline, FDR $p < 0.01$). Of these, 771 genes were upregulated, and 261 genes were downregulated (Figure 3-1). The 20 transcripts exhibiting the highest increase and decrease following maternal PolyI:C exposure are listed in Table 2 and Table 3, respectively. Among upregulated transcripts, maternal PolyI:C exposure induced expression of interferon-stimulated genes (e.g. *Isg15*, 101.5-fold; *Ifit3*, 140.5-fold; *Mx1*, 196.8-fold; *Mx2*, 108.2-fold), anti-viral molecules (e.g., Rsad2, 139.9-fold; *Stat1*, 9.5-fold; *Stat2*, 15.1-fold), and inflammatory chemokines (e.g. *Cxcl10*, 196.3-fold; *Cxcl11*, 255.7-fold; *Ccl2*, 65.9-fold; Figure 3-1C, all FDR $p < 0.01$). Interestingly, a considerable number of transcripts that were decreased after PolyI:C exposure are associated with neurulation and neural development (e.g. *Ranbp1*, 13.4-fold; *Sema6c*, 7.8-fold; *Pnmal2*, 12.1-fold; *Nxph1*, 4.5-fold; Figure 3-1C, all FDR $p < 0.01$). To validate results obtained from RNASeq on a separate cohort of samples, quantitative RT-PCR was conducted on conceptuses exposed to saline or PolyI:C. Expression of *Cxcl10*,

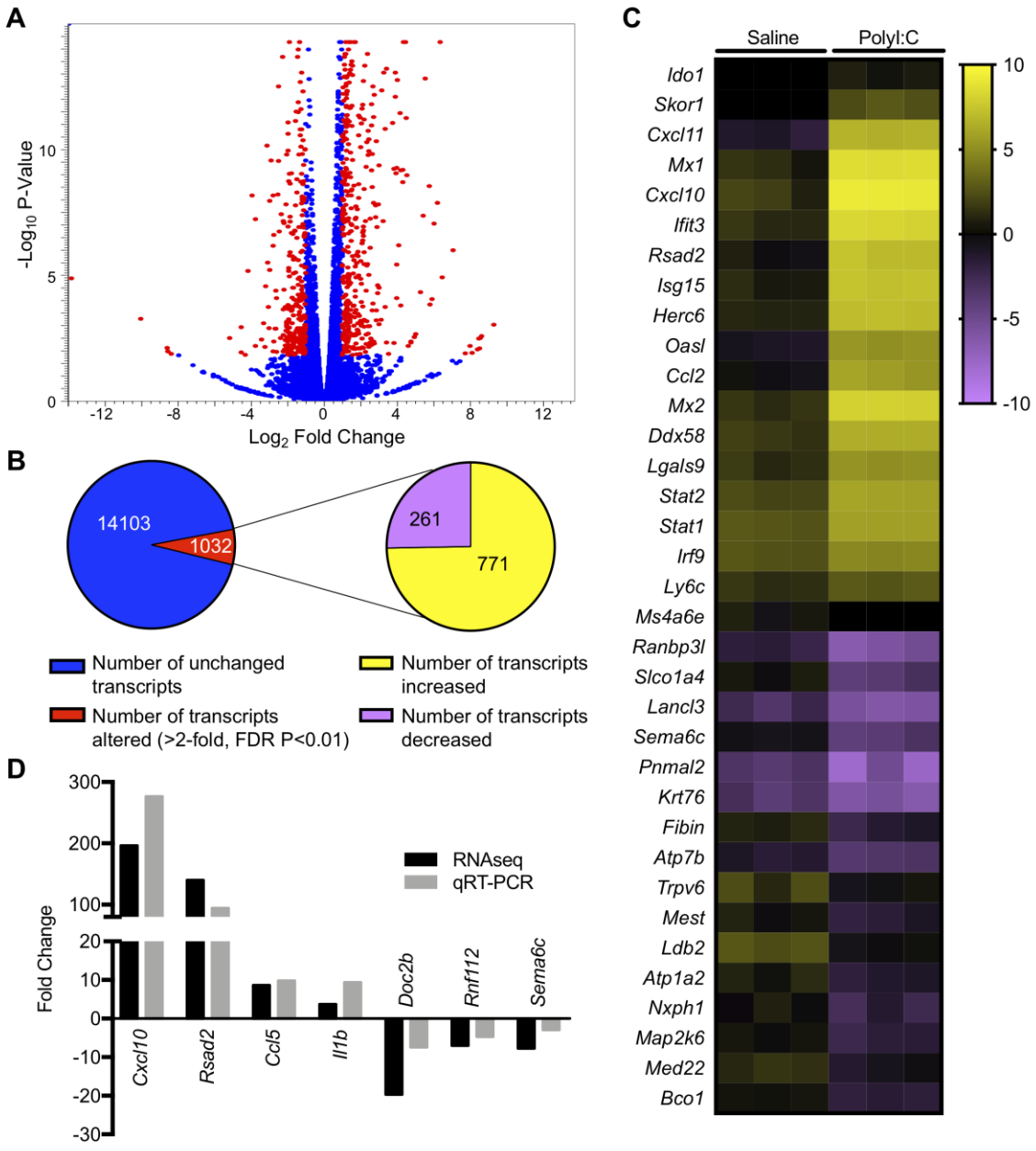

Figure 3-1: Gene expression changes in the conceptus following maternal exposure to PolyI:C.

(A) Volcano plot depicting number of unique transcripts expressed in the conceptus following PolyI:C exposure relative to conceptuses exposed to saline. The x-axis represents magnitude of fold-change (log_2), and the y-axis shows P-value (log_{10}). Transcripts altered two-fold or more (false discovery rate $p < 0.01$) following PolyI:C are

shown in red. (B) Pie chart showing number of transcripts upregulated and downregulated in conceptuses exposed to PolyI:C compared to those exposed to saline. (C) Heat map showing RPKM values (log_2) of select transcripts in conceptuses following maternal exposure to saline or PolyI:C. (D) Quantitative RT-PCR validation of select transcripts in relation to values obtained using RNA-Seq. Data are normalized to values obtained from saline-exposed conceptuses. All data are based on lysates from three conceptuses pooled from each of three dams (nine conceptuses total) per treatment.

Rsad2, *Ccl5*, and *Il1b* was increased in conceptuses following maternal PolyI:C exposure, and expression of *Doc2b*, *Rnf112*, and *Sema6c* was decreased, with fold changes consistent with results obtained using RNASeq (Figure 3-1D).

Next, we performed gene ontology analysis to identify pathways that were significantly altered in the conceptus following maternal PolyI:C exposure. Not surprisingly, pathways that were upregulated after PolyI:C exposure included those associated with antiviral pathways and inflammation. Examples of upregulated pathways include: negative regulation of viral replication pathway (p = 8.2E-16, 17 genes), innate immune response (p = 4.5E-19, 47 genes), and inflammatory response (p = 1.8E-24, 57 genes, Figure 3-2A). Intriguingly, pathways associated with decreased gene expression were related to nervous system function and development, including central nervous system development (p = 3.3E-02, 5 genes); axon guidance (p = 7.6E-03, 7 genes); and neuronal action potential (p = 7.7E-04, 5 genes, Figure 3-2B). Thus, maternal PolyI:C exposure during early pregnancy induces robust transcriptome changes in the conceptus, including increased expression of genes associated with antiviral and inflammatory pathways and reduced expression of genes associated with neurological development.

3.3.2 Maternal PolyI:C exposure during early pregnancy alters cortical architecture in the fetus

Since offspring born to dams exposed to PolyI:C during early pregnancy are predisposed to neurobehavioral impairments, and we found decreased expression of various genes associated with central nervous system development following maternal PolyI:C exposure, we sought to elucidate whether cortical architecture was altered in developing fetal brains. Cortical neurogenesis begins around GD 11 in rats, peaks at E15, and slows by E17 (Workman et al. 2013). Consequently, we measured cortical thickness, including thickness of the cortical mantle (CM; delineated using the neuronal marker β3T) and ventricular zone (VZ; identified using Sox2, which is highly expressed in NPCs, Figure 3A,B) in coronal sections of fetal brains on GD 15.5, one week following maternal saline

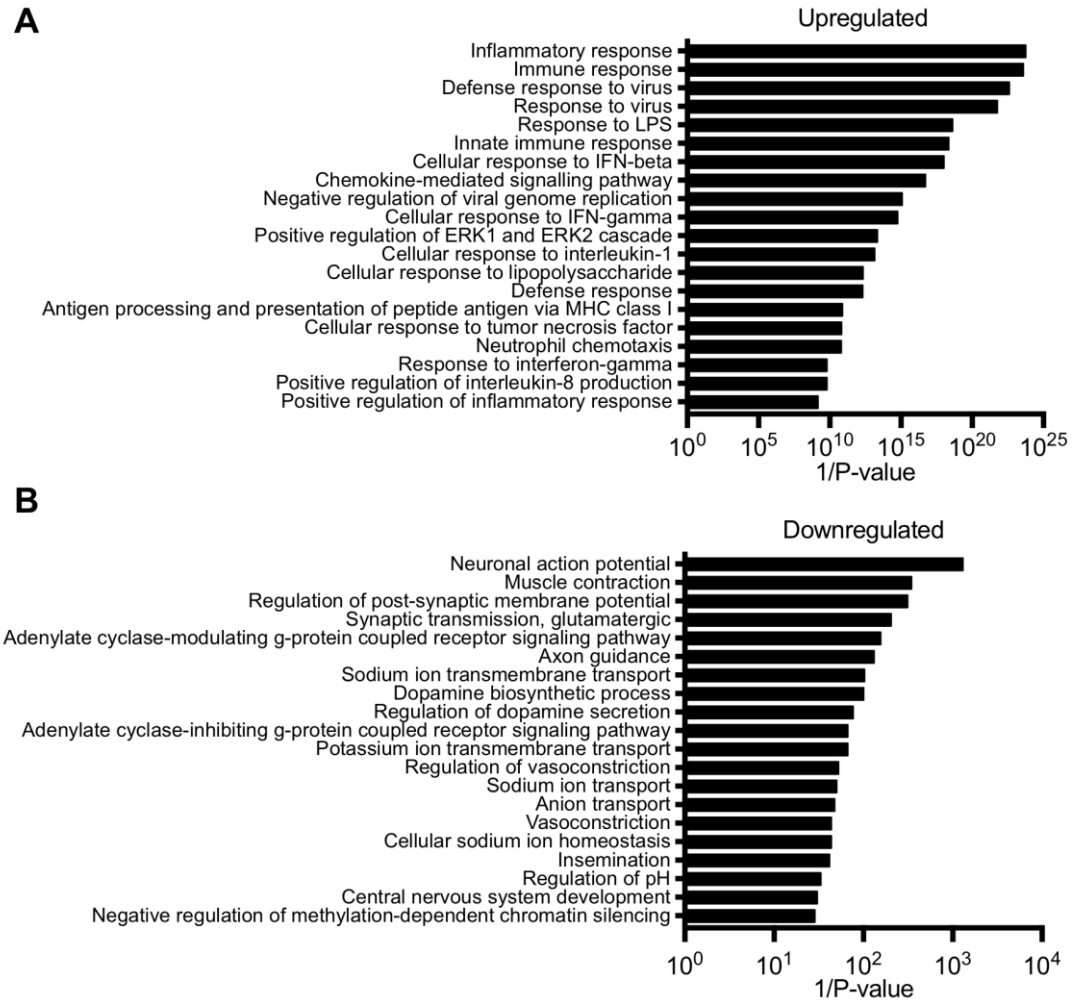

Figure 3-2: Pathway analysis of unique gene signatures upregulated and downregulated in the conceptus following maternal exposure to PolyI:C.

(A) Top 20 gene pathways upregulated in the conceptus following administration of PolyI:C to pregnant dams. (B) Top 20 gene pathways downregulated in the conceptus following administration of PolyI:C to pregnant dams. Pathway analysis was conducted by inputting genes changed more than two-fold compared to conceptuses collected following maternal exposure to saline, and with a false discovery rate < 0.01.

Table 3-2: List of top 20 increased genes following maternal PolyI:C exposure.

Gene	Fold	Saline RPKM	PolyI:C RPKM
Ido1	636.88	0	0.68
Skor1	398.92	0	0.18
Defb52	378.92	0	1.30
Tnf	364.57	0	0.29
RGD1305184	280.10	0.18	44.85
Cxcl11	255.69	0.41	91.90
Iqcf3	255.49	0	0.26
Gbp3	212.40	0.02	4.65
Mx1	196.83	2.06	358.59
Cxcl10	196.34	3.12	541.00
Gbp1	194.82	0.22	38.16
Ubp	38.16	0.86	142.26
Ifit3	140.50	2.35	291.69
Rsad2	139.88	1.13	137.84
Olr1735	134.79	0	0.68
Mx2	108.19	2.58	246.85
Isg15	101.53	1.71	154.19
Usp18	97.96	0.64	55.25
Cxcl9	96.43	2.84	243.99
Olr1734	89.75	0	0.42

Table 3-3: List of top 20 decreased genes following maternal PolyI:C exposure.

Gene	Fold Change	Saline RPKM	PolyI:C RPKM
Ms4a6e	-1046.99	1.28	0
LOC103690002	-15.47	2.84	0.16
Slco1a4	-13.43	1.30	0.09
Ddo	-13.43	0.12	0.01
RGD1561034	-9.23	0.87	0.01
Grcc10	-8.79	4.54	0.47
Clec4a	-8.52	0.43	0.04
Lancl3	-7.83	0.68	0.08
Sema6c	-7.76	0.16	0.02
Btnl9	-7.45	0.72	0.08
Myom2	-6.93	0.21	0.03
Muc16	-6.88	0.16	0.02
Abcg4	-6.77	0.61	0.08
Gria2	-6.71	0.63	0.08
Shc3	-6.25	0.32	0.05
Kcng1	-6.16	0.13	0.02
Scn7a	-5.81	0.39	0.06
Ldb2	-5.72	5.73	0.89
Etv1	-5.67	0.29	0.05
Rbp7	-5.57	2.37	0.38

or PolyI:C treatment. Male fetuses prenatally challenged with PolyI:C showed an 8.3% increase in total cortical thickness on GD 15.5 compared to saline-exposed male fetuses (Figure 3-3C, $p < 0.05$). The increased cortical thickness in male fetuses was primarily attributed to a thicker CM (20% increase in PolyI:C-exposed fetuses compared to saline, Figure 3-3C, $p < 0.05$). The VZ also appeared to be increased in PolyI:C-exposed male fetuses, but it was not statistically significant. Interestingly, thickness of the VZ in female fetuses prenatally challenged with PolyI:C was increased by 10% (Figure 3-3C, $p < 0.05$), whereas there was no change in thickness of the CM. Total cortical thickness appeared to be thicker in females exposed to PolyI:C, but did not reach statistical significance ($p = 0.08$). Our results indicate that maternal PolyI:C exposure during early pregnancy caused sex-specific alterations in cortical architecture at mid-to-late gestation, and in general was associated with increased thickness of the developing cerebral cortex.

3.3.3 Increased proliferation of fetal NPCs following maternal exposure to PolyI:C

Our results indicate that maternal PolyI:C treatment at early gestation results in altered thickness of cortical layers in fetuses. This might be mediated through abnormal functioning of NSCs/NPCs. Thus, for our next series of experiments, pregnant dams were administered saline or PolyI:C on GD 8.5, and NPCs were isolated from fetal cortices on GD 15.5 (Figure 3-4A). Cells isolated from fetal cortices expressed Pax6, Nestin, and Sox2, and displayed morphology consistent with NPCs, including formation into neurospheres within 4 days, at which point they were either fixed and cryosectioned, or disaggregated and plated into monolayers. Interestingly, neurospheres isolated from fetuses exposed prenatally to PolyI:C showed rapid growth and formed into larger colonies, suggesting that they may have a higher proliferative index compared to neurospheres isolated from fetuses collected from saline-treated dams (Figure 3-4B). To examine the proliferative capacity of NPCs, we measured the percentage of cells within neurospheres that incorporated EdU and found that neurospheres prepared from fetal cortical tissue collected from PolyI:C-treated dams exhibited a 9.3% increase in EdU incorporation compared to neurospheres prepared from saline-exposed fetuses (Figure 3-4C, $p < 0.05$). Furthermore, a greater percentage of neurospheres from fetuses collected

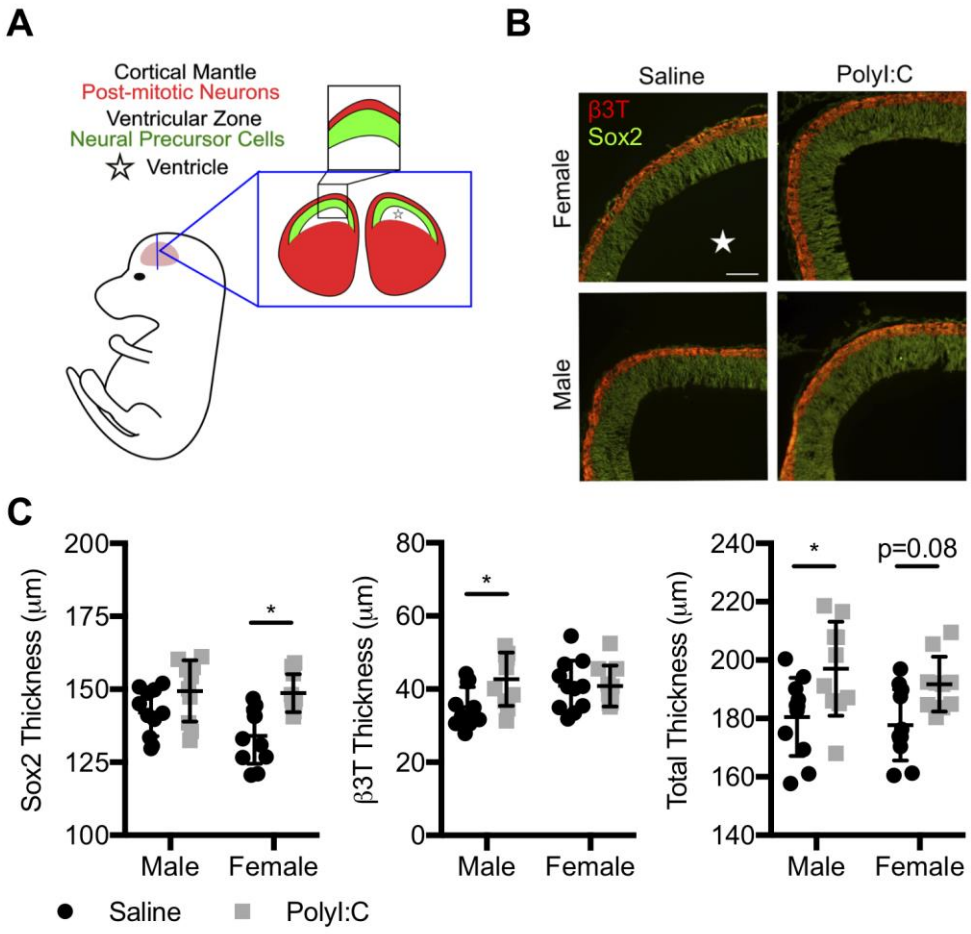

Figure 3-3: Prenatal exposure to PolyI:C prompts changes in cortical architecture.

Dams were injected with either saline or PolyI:C on GD 8.5, and whole fetal brains were collected one week later. (A) Schematic depiction of the layers of the rat fetal cortex at GD 15.5. (B) Coronal sections of the cortex at GD 15.5 were stained for β3T (red) and Sox2 (green) to delineate the CM and VZ, respectively. (C) Measurements showing total cortical thickness and thickness of the CM and VZ in male and female fetuses. Graphs represent means ± SEM. A star (☆) denotes the ventricle. Statistical analyses were performed using two-way analysis of variance and Sidak's multiple comparison. Data are represented as mean ± SEM and asterisks denote statistical significance (*; $p < 0.05$; N ≥ 10 fetuses per sex from at least three dams per group). Scale bar = 100 μm.

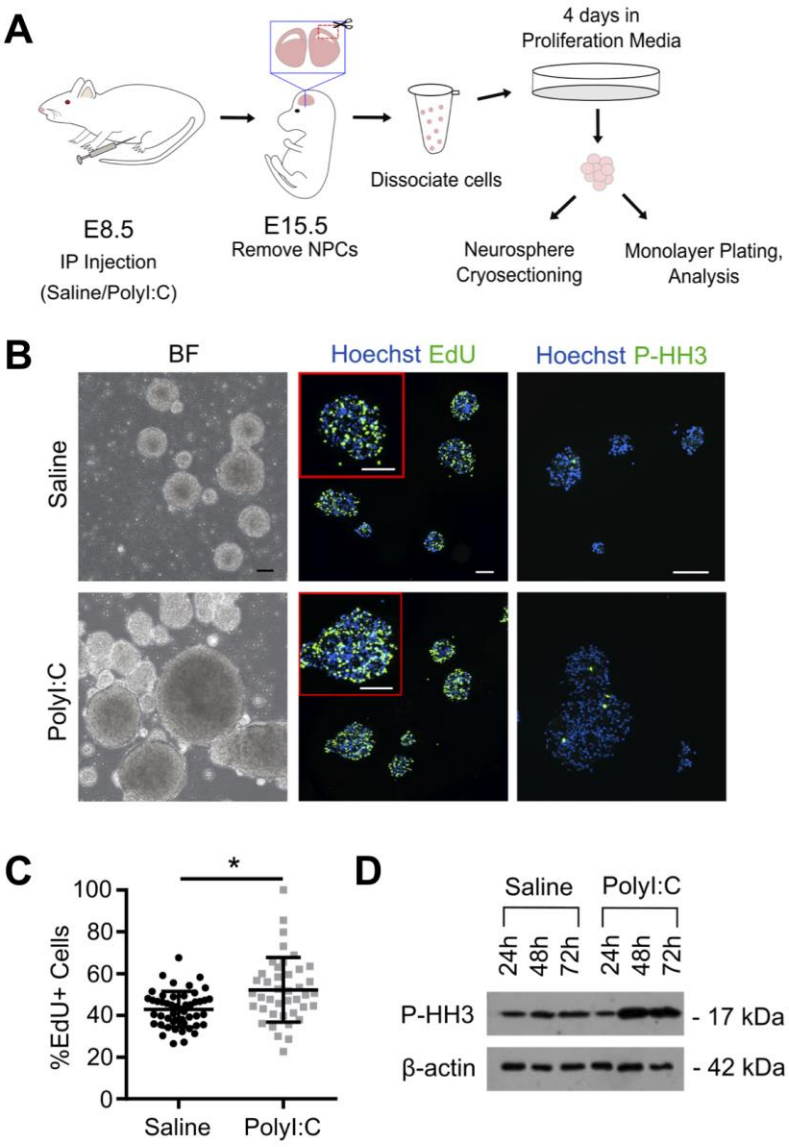

Figure 3-4: Maternal exposure to PolyI:C increases proliferation potential of neurospheres prepared from fetal cortices.

Pregnant rats were administered saline or PolyI:C on GD 8.5, and NPCs were isolated from GD 15.5 fetal cortices for neurosphere culture. (A) Schematic depiction of experimental protocol. (B) The two left panels show neurospheres produced from cortical NPCs imaged using brightfield. The middle two panels show EdU incorporation in cryosectioned neurospheres. The right two panels show immunofluorescence for

phospho-histone H3 (P-HH3). In the middle and right panels, Hoechst is used to counterstain nuclei. Scale bar = 100 μm. (C) Percent EdU positive cells in each neurosphere were quantified from each treatment group. (D) Western blot analysis of P-HH3 expression in neurospheres following 24, 48, and 72 h culture. β-actin was used as a loading control. Statistical analyses were performed using Student's *t*-test. Data are represented as mean ± SEM. Data significantly different ($p < 0.05$) from controls are indicated by an asterisk (*; 5-6 fetuses from each of 3 dams per treatment were used for neurosphere generation).

from PolyI:C-treated pregnancies had increased staining for phospho-histone H3 (P-HH3), a marker of mitotically active cells, as shown using both immunofluorescence (Figure 3-4B) and western blotting (Figure 3-4D).

When neurospheres are disaggregated and cells plated as monolayers, NPCs can be maintained as a proliferative population expressing Nestin, Pax6, and Sox2, or induced to differentiate into NeuN+ neurons and GFAP+ glia (Figure 3-5A). Thus, to determine the proliferative capacity of NPCs using monolayer cultures, neurospheres prepared from individual fetuses were disaggregated, sex determined through *Sry* genotyping, and male or female NPCs combined and re-plated as monolayers. In NPCs prepared from GD 15.5 fetal brains one-week following maternal PolyI:C exposure, approximately 15% more NPCs incorporated EdU regardless of whether they were derived from a male or female fetus compared to NPCs from saline-exposed fetuses (Figure 3-5B and C, $p < 0.05$), which is consistent with the increased proliferation of NPCs in neurospheres and thicker cortical layers in fetal brains prenatally challenged with PolyI:C (Figures 3-3 and 3-4). The increased EdU incorporation was also evident if cells were cultured in conditions that favor differentiation (Figure 3-5D, $p < 0.05$). Additionally, we used NeuN to identify cells that underwent differentiation into neurons. In NPCs prepared from fetal cortices one week following maternal PolyI:C exposure, there was a 26.3% ($p = 0.0003$) and 20.2% ($p = 0.0212$) increase in NeuN-labelled cells in male and female NPCs, respectively (Figure 3-5E). Although both male and female NPCs prepared from fetuses exposed to PolyI:C have a higher differentiation potential compared to fetuses exposed to saline, the effect was more robust in male fetuses. Collectively, these results show that exposing pregnant rats to MIA during early pregnancy disrupts the normal proliferation potential and behavior of fetal NPCs.

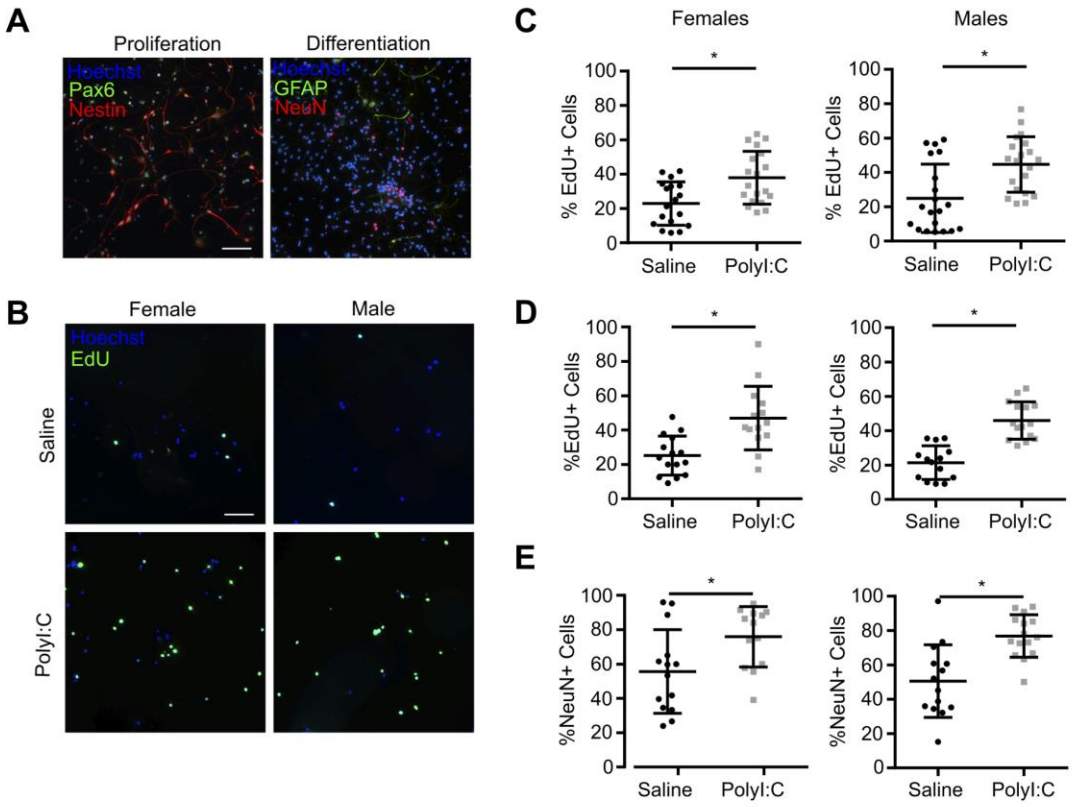

Figure 3-5: NPCs isolated from fetal cortices challenged prenatally with PolyI:C exhibit increased proliferation potential.

Pregnant rats were administered saline or PolyI:C on GD 8.5, and NPCs were isolated from GD 15.5 cortices for neurosphere culture. Neurospheres from male or female embryos were pooled, and then mechanically dissociated to form monolayers. Monolayers were subsequently cultured in media to promote stem or differentiated states. (A) Representative images of NPC monolayers cultured in proliferation conditions stained for Pax6 (green) and Nestin (red) or cultured in differentiation conditions stained for GFAP (green) and NeuN (red). Hoechst (blue) was used to counterstain nuclei. (B) Representative images of EdU incorporation in NPCs prepared in proliferation conditions from male and female cortices challenged prenatally with saline or PolyI:C one week prior. (C) Percentage of male and female NPCs that incorporated EdU during culture in proliferation conditions. (D) Percentage of male and female NPCs maintained in differentiation media that incorporated EdU. (E) Percentage of male and female NPCs

maintained in differentiation media immunoreactive for NeuN indicating their differentiation capacity. Statistical analyses were performed using Student's *t*-test. Data are represented as mean ± SEM. Data significantly different ($p < 0.05$) from controls are indicated by an asterisk (*; 2-3 fetuses per sex collected from at least 3 dams). Scale bar = 50 μm.

3.3.4 Altered expression of components of the Notch signaling pathway in conceptuses following maternal exposure to PolyI:C.

The Notch signaling pathway is intricately involved in shaping the cell number and function of the vertebrate nervous system and is well known to facilitate binary cell fate choices, including the regulation of NPC maintenance and differentiation (reviewed in (Pierfelice et al. 2011)). In mammals, Notch signaling is controlled by cell-cell interactions, with Notch receptors (Notch1-4) on one cell activated by ligands (Delta-like (Dll)-1, Dll-3, Dll-4, Jagged (Jag)-1, and Jag-2) expressed on adjacent cells. Ligand-receptor interactions facilitate cleavage of the Notch intracellular domain, which translocates to the nucleus to activate target genes influencing cell fate in the nervous system. Since there are no intermediates between activation of Notch receptors in the cell membrane and their target site in the nucleus, Notch signaling is principally regulated by the expression of receptors, ligands and enzymatic activity at the cell membrane. In our RNA-Seq analysis, we noted increased expression of *Notch1* (2.2-fold, FDR $p < 0.01$) and *Dll1* (4.72-fold, FDR $p < 0.01$) in conceptuses following MIA, which prompted us to evaluate expression of all receptors and ligands that participate in Notch signaling. Using quantitative RT-PCR, we confirmed that following prenatal challenge with PolyI:C, conceptuses exhibited increased expression of *Notch1* and *Dll1* compared to conceptuses exposed to saline, with fold-changes comparable to results obtained using RNA-Seq (Figure 3-6A, both $p < 0.05$). We also found reduced expression of *Notch3* and *Jag2* (51% decreased and 41% decreased, respectively, $p < 0.05$). Expression of Notch3 and Jag2 appeared to be similarly reduced in our RNA-Seq analysis, but the *p*-values did not reach our stringent cut-off (FDR *p*-value = 0.10 and 0.06, respectively). There was no change in *Notch2*, *Dll4*, or *Jag1* expression between saline or PolyI:C-exposed conceptuses, whereas *Notch4* and *Dll3* levels were below the threshold for detection (Cq>30 in both groups).

Notch1 is the prototypical Notch receptor implicated in maintaining NPC function. Since we found increased *Notch1* expression in conceptuses following MIA, we sought to determine whether sustained *Notch1* expression is evident in the developing fetal cortex,

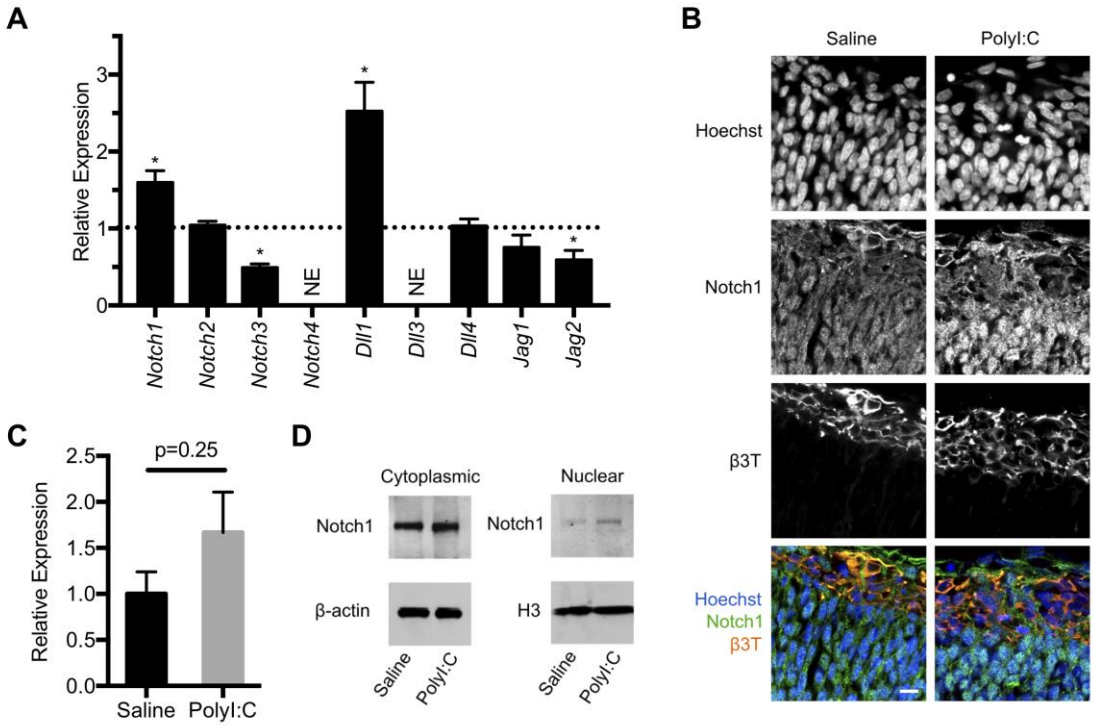

Figure 3-6: Maternal exposure to PolyI:C alters expression of Notch signaling components in the conceptus.

(A) Transcript levels of Notch receptors (*Notch1-4*) and ligands (*Dll1*, *Dll3*, *Dll4*, *Jag1*, *Jag2*) in conceptuses 6 h following maternal exposure to PolyI:C. Transcript levels were normalized to levels in conceptuses from saline-exposed dams. NE = Not expressed (below threshold of detection). (B) Immunofluorescence showing Notch1 expression in GD 15.5 fetal cortices one-week following maternal exposure to saline or PolyI:C. β3T was used to delineate the CM. Hoechst was used to stain nuclei. Note that Notch1 is expressed throughout the cortices, but qualitatively increased expression and nuclear accumulation of Notch1 is evident following prenatal challenge with PolyI:C. Scale bar = 10 μm. (C) Transcript levels of Notch1 expression in neurospheres collected from cortices of GD 15.5 fetuses 6 h following maternal exposure to PolyI:C or saline. (D) Cytoplasmic and nuclear levels of Notch1 in NPCs collected from fetal cortices one week after maternal exposure to saline or PolyI:C. β-actin was used as a loading control for cytoplasmic lysates; histone H3 was used as a loading control for nuclear lysates.

Statistical analyses were performed using Student's t-test. Data are represented as mean ± SEM. Data significantly different ($p < 0.05$) from controls are indicated by an asterisk (*; 3 fetuses collected from each of 3 dams per treatment).

one-week following maternal exposure to saline or PolyI:C. We found that NOTCH1 was expressed in cells throughout the cortex, with more intense immunoreactivity in nuclei located within the VZ in fetuses prenatally challenged with PolyI:C (Figure 3-6B). To determine whether increased *Notch1* expression was evident in NPCs following MIA, we isolated NPCs from fetal cortices following maternal exposure to saline or PolyI:C one week earlier, and generated neurospheres. Although there was no change in *Notch1* mRNA expression in neurospheres collected from fetal brains one-week following maternal exposure to PolyI:C, Notch1 protein was increased, particularly in nuclear lysates (Figure 3-6C and D). Collectively, our results indicate that maternal exposure to PolyI:C results in abnormalities in Notch signaling in the conceptus and fetal brain (schematically depicted in Figure 3-7).

3.4 Discussion

Ample epidemiological and experimental evidence supports the notion that MIA during early pregnancy may interfere with the development of the brain leading to potential long-term neuropsychiatric and behavioral abnormalities. While many studies have focused on long-term changes on brain development and/or behavioral outcomes following MIA, molecular and structural changes in prenatal brain development are not clear, and such changes may be central for postnatal consequences. In this study, we identified significant changes to the transcriptome in conceptuses immediately following MIA in rats, including increased expression of genes involved in inflammatory and antiviral responses and suppression of genes involved in brain development. Increased thickness of the VZ was evident in female fetuses one-week following MIA; total cortical thickness and thickness of the CM were increased in male fetuses. Furthermore, NPCs prepared from fetal cortices one-week following MIA exhibited enhanced proliferation *in vitro*, which may contribute to increased thickness of the cerebral cortex and is likely mediated by altered Notch signaling. Together, these results suggest that MIA during early pregnancy causes rapid alterations in signaling pathways required for normal neurogenesis, leading to profound and persistent changes in cortical development.

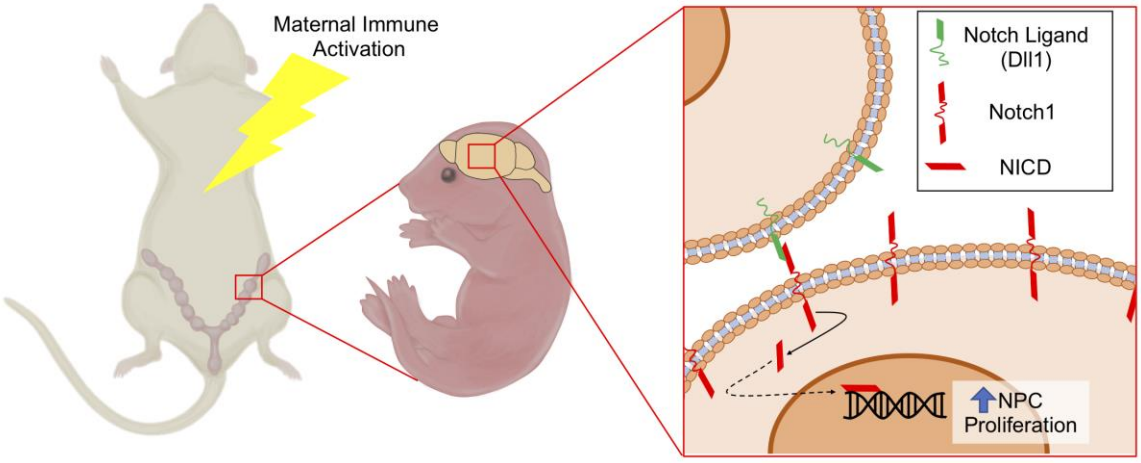

Figure 3-7: Schematic depicting increased NPC proliferation mediated by Notch1 signaling following MIA.

MIA elicits increased expression of Notch1 and its ligand Dll1 in NPCs, which may contribute to altered NPC proliferation and differentiation dynamics leading to changes in cortical structure. NICD = Notch Intracellular Domain.

Exposure of pregnant rodents or primates to the synthetic dsRNA mimetic PolyI:C is a well-established tool for modelling MIA (Meyer 2014). Many viruses produce dsRNA as part of their replication cycle. Eukaryotic cells have evolved the capacity to recognize dsRNA through at least three receptors: toll-like receptor 3 (TLR3), retinoic acid-inducible gene 1 (RIG1), and melanoma differentiation-associated protein-5 (MDA5), which instigate an antiviral response to combat viral infection and propagation (Mian et al. 2013). Since PolyI:C is sufficient to activate these receptors and trigger cellular antiviral responses, the use of PolyI:C provides a means to investigate the impact of MIA without confounding variability in the severity, spread, and duration of viral infections. Molecular and cellular analyses conducted on brains of adult offspring challenged prenatally with PolyI:C show profound and reproducible impairments that align with behavioral phenotypes of clear relevance to neuropsychiatric disorders including ASD, schizophrenia, and depression (Careaga et al. 2017; Malkova et al. 2012). Moreover, behavioral abnormalities in offspring can be recapitulated by direct maternal treatment with PolyI:C-induced cytokines (e.g. IL-6), whereas blocking key pathways prevents neural and behavioral deficiencies following MIA (Smith et al. 2007; Murray et al. 2015; Choi et al. 2016). In our study, we exposed dams to PolyI:C during early gestation (GD 8.5), which is a sensitive timepoint for neurogenesis in rats, and can also indirectly impact brain development by disrupting formation and function of supporting organs like the placenta (Baines et al. 2020; Hsiao and Patterson 2011). Although previous studies have analyzed the transcriptome of the fetal brain following maternal PolyI:C exposure (Garbett et al. 2012), our study is the first to characterize global transcriptome changes in the whole conceptus (including the primordial nervous system and placenta as well as maternal decidua, which is a likely source of cytokines deleterious for brain development). A limitation of this approach was the inability to ascertain changes happening specifically in the nascent central nervous system following MIA, however, it did provide us the opportunity to identify molecules activated within the conceptus and correlate these changes to pathways integral for brain development. In future studies, the use of single cell transcriptomics may facilitate identification of cell type-specific responses to MIA.

Our study revealed broad antiviral and inflammatory responses triggered within the conceptus rapidly following PolyI:C exposure. Among genes robustly upregulated following maternal PolyI:C exposure, those conventionally associated with MIA-associated neurological impairments (e.g., *Il6*, *Il1b*, and interferon-stimulated genes) were evident. A variety of other highly upregulated genes were identified in our analysis that may have important relevance for nervous system development and embryogenesis. For example, indoleamine 2,3-dioxygenase (*Ido1*) encodes a potent immunomodulatory protein that catabolizes tryptophan – a critical amino acid for synthesis of neuromodulators such as kynurenine and serotonin. High levels of IDO1 are associated with depressive behavior in rats as well as pain and depression in humans (Kim et al. 2012). Increased expression of the genes encoding C-X-C motif chemokine (CXCL)-10 and CXCL11 occurs during central nervous system inflammation (McColl et al. 2004). We also identified increased expression of a family of genes encoding interferon-induced transmembrane proteins, which restrict cellular viral entry and spread, but may exacerbate MIA and contribute to PolyI:C-induced embryonic lethality in mice (Buchrieser et al. 2019). Thus, the conceptus competently produces a powerful immune response that may be required to combat viral infections, but this response may have consequences on processes essential for embryogenesis.

Previous studies have described an inverse relationship between inflammation and expression of genes associated with brain development and function. For example, a single intracerebroventricular injection of lipopolysaccharide into adult mice decreases expression of genes associated with memory and learning (e.g., *Egr1* and *Arc*) in the cortex (Bonow et al. 2009). Systemic inflammation also reduces gene expression of cholinergic components within various brain structures (Silverman et al. 2015), homeostatic genes in microglia (Sousa et al. 2018), and pathways canonically associated with nervous system development and function, including genes penetrant to ASD (Oskvig et al. 2012; Lombardo et al. 2018). Consistent with these studies, our transcriptomics analysis identified decreased expression of numerous genes including *Doc2b*, *Sema6c*, *Gria1*, *P2rx1*, *Scn10a*, and *Rnf112*, that are associated with neural induction, neurogenesis, and central nervous system development following maternal PolyI:C exposure. *Doc2b* encodes a protein that contributes to release of

neurotransmitters and synaptic transmission (Pang et al. 2011). *Sema6c* encodes the transmembrane protein Semaphorin-6c, which plays an integral role in central nervous system connectivity and formation of the peripheral nervous system (Masuda and Taniguchi 2016). Altered levels of glutamate receptor 1, encoded by *Gria1*, is a risk factor for schizophrenia (Parekh et al. 2018). P2X purinoceptor 1, encoded by *P2rx1*, potentiates neurite outgrowth, while mutations in sodium voltage-gated channel alpha subunit 10, encoded by *Scn10a*, has been implicated in neurological disorders like multiple sclerosis and Pitt-Hopkins (Rannals et al. 2016). RING finger protein 112, encoded by *Rnf112*, regulates neuronal differentiation during embryo development and maintains neural function in adults. Mice lacking Rnf112 exhibit severe growth retardation and impaired neurocognitive development characterized by deficiencies in motor balance, learning, and memory (Tsou et al. 2017). Collectively, the rapid repression of multiple genes critical for neurogenesis following MIA may interfere with the normal progression of nervous system development and predispose to long-term deficiencies in cognition and behavior. However, questions remain about specific neurogenic pathways in the primordial nervous system that are sensitive to MIA, and their relevance to long-term pathology in humans with neurobehavioral or cognitive impairments.

Since we found that MIA represses expression of many genes associated with nervous system development, we predicted that structural changes would be evident in the cerebral cortex – the area of the brain responsible for higher order thought, perception, and reason. Proper cortical development and expansion are dependent on coordinated regulation of NPC proliferation and cell fate specification; accumulating evidence indicates that these processes are disrupted following MIA. For example, MIA enhances expression of cell cycle-related genes and alters NPC proliferation patterns, resulting in increased cortical thickness and neuron density, brain overgrowth, regions of cortical dysplasia, and layering defects (Choi et al. 2016; Ben-Reuven and Reiner 2021; Smith et al. 2012; Le Belle et al. 2014). In this context, our findings of increased cortical thickness and enhanced NPC proliferation in fetuses one-week following MIA support previous findings and suggest endogenous dysregulation of NPC self-renewal and differentiation. Although the thickness of the cerebral cortex in both male and female brains was

increased by prenatal exposure to PolyI:C, structural changes exhibited a degree of sexual dimorphism, with males displaying a thicker CM and females a thicker VZ. In line with the increased CM thickness in male fetuses prenatally challenged with PolyI:C, NPCs isolated from male fetuses also had a more pronounced tendency to differentiate *in vitro*. The structural discrepancies between male and female brains following prenatal challenge with PolyI:C may reflect distinct responses to inflammation, including a heightened and prolonged surge of inflammatory cytokines such as IL-6 and IL-1β in male brains, and increased susceptibility to apoptosis in female brains, which could differentially predispose male and female offspring to distinct neuropsychiatric phenotypes (Chavez-Valdez et al. 2019; Loram et al. 2012). Additionally, the duration of time in which NPCs exhibit dysregulated proliferation following maternal inflammation is not known. Whereas our study focused on changes occurring within one week of PolyI:C exposure, a transient surge of maternal IL-6 given on E13.5 in mice is sufficient to cause an expanded NPC population in two-month-old offspring, indicating that dysregulated NPC behavior may endure long after *in utero* exposure to inflammation (Gallagher et al. 2013). The ramifications of dysregulated NPC behavior and altered corticogenesis are not fully established, but one possible repercussion is exhaustion of NPC pools in older adult offspring (Storer et al. 2018). Another possible consequence is that cortical structural defects resulting from aberrant NPC behavior may be an etiological factor linking MIA with poor neurobehavioral outcomes in offspring. In support of this possibility, increased brain volume, head circumference, and number of cortical neurons are found in a subset of patients diagnosed with ASD (Courchesne et al. 2011; Pramparo et al. 2015).

The evolutionarily conserved Notch signaling pathway is a major contributor to cell fate determination and patterning in the developing nervous system, including neurogenesis in embryonic brains, maintenance of NPC populations, and specification of glia (Pierfelice et al. 2011). Mice lacking *Notch1*, *Dll1,* or *Rbpj* (which encodes a key nuclear component of the canonical Notch pathway) exhibit early embryonic lethality with compromised nervous system development, including rapid depletion of NPCs and precocious neuronal differentiation (Imayoshi et al. 2010; Hitoshi et al. 2002), suggesting that signaling through Notch1 and Dll1 is critical for proliferation and maintenance of NPCs. The role

of Notch3 in NPC biology is less clear. Notch3 is expressed in NPCs, but mice lacking Notch3 are viable, and their brain development appears normal (Krebs et al. 2003). In some studies, Notch3 is found to promote NPC proliferation similar to Notch1; other studies have reported Notch3 antagonizes Notch1, thereby inhibiting NPC proliferation and stimulating differentiation of NPCs (Rusanescu and Mao 2014). Intriguingly, we observed increased expression of *Notch1* and *Dll1*, and decreased expression of *Notch3* in conceptuses following MIA. Consistent with our findings, elevated expression of several Notch signaling components (e.g. *Notch1*, *Dll1*) is evident in inflammatory conditions such as rheumatoid arthritis and systemic lupus erythematosus, and de novo production of Notch ligands and receptors is increased following activation of TLR pathways (Shang et al. 2016). Moreover, direct infection of human NPCs with Zika virus is sufficient to induce Notch signaling; proper differentiation and improved viability of Zika-infected NPCs is restored using a Notch pathway inhibitor, DAPT (Ferraris et al. 2019). We therefore propose that dysregulated expression of Notch signaling components following MIA may contribute, at least in part, to the sustained increase in NPC proliferation and increased thickness of cortical layers during fetal development.

In conclusion, we have uncovered a comprehensive profile of gene expression changes in the rat conceptus following an early gestational exposure to PolyI:C and correlated these changes to sustained dysregulation of cortical structure and NPC function. PolyI:C and other immunogens (e.g., lipopolysaccharide) elicit strong, short-lived innate immune responses, and have an advantage of controlling the onset and severity of MIA within defined periods of embryogenesis. A limitation of these models is that they may not fully recapitulate the broad and potentially long-lasting immune response characteristic of live pathogens. Despite this limitation, many distinct types of bacterial and viral infections predispose to neuropsychiatric deficiencies in humans at various stages in pregnancy, and behavioral phenotypes are comparable in rodents challenged prenatally with PolyI:C, lipopolysaccharide, and active pathogens (e.g., influenza A) (Arsenault et al. 2014). This suggests that maternal immune responses provoked by distinct pathogens and immunogens may converge to impact development of the primordial nervous system, with implications for long-term cognitive function and behavioral phenotype. In future studies, it will be enticing to dissect the impact that MIA exerts on pathways vital for

nascent nervous system development, such as dysregulated expression of Notch signaling components, which may underlie the pathogenesis of neurodevelopmental disorders caused by distinct stimuli. For example, in another rodent model that elicits behaviors reminiscent of ASD, inhibition of excessive Notch signaling alleviates ASD-like behaviors in offspring (Zhang et al. 2019). Therefore, this pathway-specific approach may yield new therapeutic strategies that selectively target the molecular underpinnings of abnormalities in cortical development and offspring behavior following prenatal exposure to immunogens.

3.5 References

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Chapter 4

4 Gene Expression Profiling of Decidua from Interleukin-15 Deficient Rats Reveals Osteopontin as a Natural Killer Cell-Specific Marker

Uterine Natural Killer (uNK) cells are the most prevalent immune cells within the uterus during early pregnancy. These cells are thought to contribute to the regulation of decidualization and placentation, but their functions remain poorly characterized. Previously, we use targeted genomic to edit the *Interleukin-15* (*Il15*) locus in rats, resulting in a frameshift mutation in *Il15*. Since IL15 is required for NK cell survival, these *Il15*-deficient (*IL15Δ/Δ*) rats exhibit a severe deficiency of NK cells including uNK cells, which caused robust changes in placental morphology including precocious trophoblast invasion. Therefore, we hypothesize that uNK cells produce factors during early pregnancy that control the depth of placentation. This study aimed to profile gene expression differences in the conceptus between wild-type (WT) and *IL15Δ/Δ* rats during early pregnancy to identify regulatory factors produced by uNK cells involved in controlling placental invasion. Global transcript changes in the conceptus were assessed between pregnant *IL15Δ/Δ* and WT Sprague-Dawley Holtzman rats at gestational day (GD) 9.5 using Clariom S gene expression profiling. There were 257 genes differentially expressed between WT and *IL15Δ/Δ* rats. Notably, we detected a significant decrease in *Prf*, which encodes the uNK cell marker perforin, and *Spp1*, which encodes osteopontin (OPN) in *IL15Δ/Δ* rats. Using immunohistochemistry, OPN was localized to the uterine glands, decidua, and primitive placenta at GD 9.5 in WT rats. Conversely, *IL15Δ/Δ* rats had strong OPN expression in the uterine glands, but no detectable OPN in the decidua. Isolated uNK cells from GD 9.5 decidua expressed *Spp1* mRNA; OPN and perforin staining were co-localized *in vitro* and *in vivo*, strongly indicating that OPN is produced by uNK cells. This study provides the first comprehensive characterization of rat uNK cells and identifies OPN as a uNK cell marker in rats. These findings will ultimately provide a deeper understanding of uNK cell biology and how uNK cells contribute to placental development.

4.1 Introduction

Decidualization involves morphological and functional changes to the uterine endometrium in preparation for an implanting embryo. Decidualization facilitates implantation of a blastocyst and is critical for proper regulation of placentation, which in humans requires deep invasion of trophoblasts into the maternal endometrium and inner myometrium. The decidua basalis contains a variety of specialized cell-types that have a vital role in regulating the extent to which trophoblasts can invade towards the maternal myometrium (Godbole et al. 2011; Zhu et al. 2009; Lash et al. 2006; Smith et al. 2009). In particular, during early pregnancy there is a marked infiltration and proliferation of immune cells at the decidual-placental interface (Jabrane-Ferrat 2019). Uterine natural killer (uNK) cells are the predominant immune cell population of the decidua in both humans and rodents, comprising over 70% of uterine leukocytes during early pregnancy (Jabrane-Ferrat 2019). These cells are thought to be important for placental and decidual development (Cerdeira et al. 2013). For example, uNK cells are found in close proximity to the uterine spiral arteries (the feeder vessels of the placenta) prior to trophoblast cell invasion, thereby contributing to the regulation of trophoblast invasion (Robson et al. 2012; Chakraborty et al. 2011). Studies primarily using transgenic mice devoid of NK cells indicate a contribution of uNK cells to the widening of the spiral arteries during pregnancy to accommodate the increased blood flow to the placenta required for rapid fetal growth in late gestation (Moffett and Colucci 2014). The role of uNK cells at the maternal-placental interface in other species with hemochorial placentation, such as rats and humans, is controversial since invading trophoblast cells emanating from the placenta are primarily responsible for spiral artery remodelling in these species (Gaynor and Colucci 2017). It is possible that uNK cells have conserved functions in initiating spiral artery remodelling, and that invasive trophoblasts are required for complete arterial transformation in these species.

Interleukin-15 (IL-15) is a cytokine required for NK cell development and survival and is produced by several tissues including: the heart, appendix, testes, skeletal muscle, lungs, placenta, and peripheral blood mononuclear cells (Huntington et al. 2007; Patidar et al. 2016; Grabstein et al. 1994). IL-15 also plays a role in regulating the activation of innate

immune cells in response to certain stimuli such as viral infections (Carson et al. 1995; Ma et al. 2000). To examine the role of uNK cells as regulators of decidualization and placentation, several mouse studies have exploited deleted the gene encoding IL-15 or its receptor to generate NK cell-deficient animals. Consistently, IL-15-deficient mouse models feature uterine abnormalities that result from an absence of uNK cells, including a hypocellular decidua and failed spiral artery remodelling, and have been instrumental in furthering our understanding regarding uNK cell function during pregnancy (Ashkar et al. 2003; Barber and Pollard 2003; Mori et al. 2016). However, since a deeply invasive trophoblast is not a feature of mouse placentation, these models have been less informative about the functional role of uNK cells in the regulation of trophoblast invasion during placentation.

To gain insight into the role of uNK cells in the regulation of trophoblast invasion and placentation, we previously generated an IL-15 mutant (*IL15Δ/Δ*) rat model (Soares et al. 2018; Renaud et al. 2017). Rats, like humans, have a deeply invasive trophoblast, so the generation of *IL15Δ/Δ* rats provided for us an opportunity to study the regulatory role of uNK cells in trophoblast invasion and placentation. *IL15Δ/Δ* rats have a severe deficiency of uNK cells, and exhibit placental morphological changes including an expanded junctional zone (the portion of the rodent placenta from which invasive trophoblasts arise) at midgestation. Additionally, these rats present with widened spiral arteries with increased internal luminal diameter compared to wild-type (WT) rats due to precocious trophoblast invasion (Renaud et al. 2017). These findings are consistent with other studies using asialo GM1 antibody-mediated NK cell depletion in rats (Chakraborty 2011). Therefore, we hypothesize that uNK cells produce regulatory factors that promote decidualization and negatively regulate the depth of trophoblast invasion. In this study, we performed gene expression profiling of rat WT and *IL15Δ/Δ* uteri on GD 9.5, a time period when uNK cells are prevalent in the decidua and the placenta is in a nascent state, to better characterize transcriptional signatures of rat uNK cells and identify gene expression differences that may account for the robust changes in placental development. We found a variety of differentially expressed transcripts in uteri between WT and *IL15Δ/Δ* dams, notably *Spp1*, which encodes the multifunctional phosphoprotein osteopontin (OPN). Additionally, we identified uterine glands and uNK cells as the

primary sources of OPN in the GD 9.5 rat conceptus. Further, we were able to successfully isolate and culture rat uNK cells which expressed significant levels of OPN. Collectively, our findings highlight robust gene expression differences between WT and IL15Δ/Δ deciduas in rats including many factors expressed by uNK cells and suggest that OPN may be a critical uNK cell-derived factor capable of dampening trophoblast invasion.

4.2 Materials and Methods

4.2.1 Animals

WT male (8-10 weeks old) Holtzman Sprague-Dawley rats were obtained from Envigo and bred with in-house generated WT or *IL15Δ/Δ* Holtzman Sprague-Dawley females (6-8 weeks old). NK cell isolation experiments were conducted with WT Sprague-Dawley animals (Charles River). All animals were maintained in a 12 h light: 12 h dark cycle with water and food available ad libitum. Females were cycled by daily inspection of cells within the vaginal lavage and mated with a fertile male when in pro-estrus. Cells were identified under a microscope as outlined by Goldman et al. (Goldman et al. 2007). Gestational day (GD) 0.5 was defined as the day immediately following mating if spermatozoa were detected within the vaginal lavage. All protocols involving the use of rats were approved by the University of Western Ontario Animal Care and Use Committee.

4.2.2 Tissue Collection

To obtain tissue for immunohistochemical or molecular analysis, pregnant dams were sacrificed on GD 9.5 using mild carbon dioxide inhalation until respiratory failure, followed by thoracotomy. Whole GD 9.5 conceptuses (containing decidua, placenta, and embryo) were isolated and placed in sterile saline for uNK cell isolations. For immunohistochemistry, whole conceptuses were fixed in 10% neutral buffered formalin for paraffin-embedded sections, or tissues were snap-frozen in liquid nitrogen and stored at −80°C for RNA and protein extraction.

4.2.3 Clariom S Gene Expression Array

RNA was isolated from snap-frozen conceptuses (encompassing maternal decidua as well as nascent embryo and placenta), of WT and *IL15Δ/Δ* rats at GD 9.5. RNA was collected by homogenizing whole conceptuses in RiboZol (VWR; 3 dams of each WT and mutant, 3 conceptuses from each dam), followed by collection of the aqueous phase which was applied to RNeasy columns (Qiagen) with DNase I (Qiagen). Purified RNA (30 µg per sample) was submitted to Hamilton Health Sciences Centre (Hamilton, ON, Canada) for transcriptome analysis on a GeneChip Scanner 3000 using the Clariom S rat array (Thermo Scientific). Samples were normalized using the Signal Space Transformation-Robust Multiarray Analysis data normalization algorithm to reduce background and data files were generated and processed for analysis using Transcriptome Analysis Console Software 4.0 (ThermoFisher Scientific) to analyze global expression patterns of genes. Gene ontology pathway analysis was completed using DAVID Functional Annotation Bioinformatics (Huang et al. 2009).

4.2.4 Quantitative RT-PCR

To confirm the validity of the array results at the transcript level and in a larger number of conceptuses, quantitative RT-PCR (qRT-PCR) was performed. RNA was extracted from cells and tissues by lysing in RiboZol (Amresco) as directed by the manufacturer. Purified RNA was used to make complementary DNA using High-Capacity complementary DNA Reverse Transcription kit (ThermoFisher Scientific), diluted 1:10, and used for qRT-PCR. Complementary DNA was mixed with SensiFAST SYBR green PCR Master Mix (FroggaBio) and primers detailed in Table I. To amplify DNA and detect fluorescence, a CFX Connect Real-Time PCR system (Bio-Rad Laboratories) was used. Cycling conditions involved an original holding step (95℃ for 3 minutes), followed by 40 cycles of two-step PCR (95℃ for 10 s, 60℃ for 45 s), and a dissociation step (65℃ for 5 s, and a sequential increase to 95℃). The comparative cycle threshold (ΔΔCt) method was used to calculate relative mRNA expression, using the geometric mean of Ct values obtained from amplification of four genes (*Rn18s*, *Ywhaz*, *Actb*, and *Gapdh*) as reference RNA.

4.2.5 Protein Extraction and Western Immunoblot

Total tissue protein was isolated by homogenizing snap frozen GD 9.5 conceptuses in radioimmunoprecipitation assay lysis buffer (50 nM Tris, 150 nM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, pH 7.2) supplemented with protease inhibitor cocktail (Sigma-Aldrich), then sonicated (Sonic Dismembrator Model 100, Thermo Fisher Scientific). To measure total protein concentrations, a modified bicinchoninic acid assay (Bio-Rad Laboratories) was used according to the manufacturer's instructions. Approximately 25 µg of tissue lysates were mixed with 4× reducing loading buffer (62.5 nM Tris, pH 6.8, 10% glycerol, 2% SDS, 50 nM DTT, and 0.025% bromophenol blue). Samples were boiled for 5 minutes and then subjected to SDS-polyacrylamide gel electrophoresis. Proteins were transferred to a nitrocellulose membrane, blocked with 3% BSA in TBS that contained 0.1% Tween-20, and then probed with primary antibodies specific for OPN (AKm2A1, 1:500, Santa Cruz Biotechnology), Perforin (PRF; TP251, 1:1000, Torrey Pines Biolabs), and GAPDH (5174, 1:1000, Cell Signaling Technology). Following incubation with species-appropriate, infrared-conjugated secondary antibodies (Cell Signaling Technology), protein signals were detected using a LI-COR Odyssey imaging system (Li-COR Biotechnology). Resulting bands were analyzed using ImageJ software, where band intensities of target proteins were normalized to corresponding GAPDH signals.

Table 4-1: Forward and reverse primers used for quantitative RT-PCR amplification.

Gene	Accession Number	Forward Sequence	Reverse Sequence
Rn18s	NM_046237.1	5'-GCAATTATTCCCCATGAACG-3'	5'-GGCCTCACTAAACCATCCAA-3'
Actb	NM_031144.3	5'-GCCATGTACGTAGCCATCC-3'	5'-CTCTCAGCTGTGGTGGTGAA-3'
Ccl5	NM_031116.3	5'-CCTTGCAGTCGTCTTTGTCA-3'	5'- GAGTAGGGGGTTGCTCAGTG-3'
Crym	NM_053955.2	5'-GCTGTTGGAGCCAGTAGACC-3'	5'-TCAGCCCCTGACAACAGAAC-3'
Eomes	XM_039082697.1	5'-TTCACCCAGAATCTCCCAAC-3'	5'-TGGAAGGCTCATTCAAGTCC-3'
Gapdh	NM_017008.4	5'-AGACAGCCGCATCTTCTTGT-3'	5'-CTTGCCGTGGGTAGAGTCAT-3'
Prf1	NM_017330.2	5'-GGCACTCAAGGAACCTTCC-3'	5'-CTCAAGCAGTCTCCTACC-3'
Spp1	NM_012881.2	5'-AGACTGGCAGTGGTTTGCTT-3'	5'-TGTAATGCGCCTTCTCCTCT-3'
Vim	NM_031140.1	5'-ATGCTTCTCTGGCACGTCTT-3'	5'-TGGCAGCCACACTTTCATAC-3'
Ywhaz	NM_013011.3	5'-TTGAGCAGAAGACGGAAGGT-3'	5'-CCTCAGCCAAGTAGCGGTAG-3'

4.2.6 Immunohistochemistry

GD 9.5 whole conceptuses were collected, fixed in 10% neutral buffered formalin, embedded with paraffin, and sectioned at 5 µm thickness. Serial sections were deparaffinized in Histoclear (National Diagnostics) and rehydrated using increasing dilutions of ethanol washes. Formaldehyde crosslinks were fragmented by placing slides in Reveal Decloaker (Biocare Medical) at 95°C for 20 minutes. Following rehydration, endogenous peroxidases were blocked by treating tissues with 0.3% hydrogen peroxide in methanol. Sections were then permeabilized using 0.3% Triton-X and 1% BSA in PBS and blocked with 10% normal goat serum (Life Technologies). Sections were immersed in primary antibodies specific for OPN (0.5 µg/ml, Santa Cruz Biotechnology) or PRF (2.5 µg/ml, Torrey Pines Biolabs), overnight at 4°C. Subsequently for chromogenic staining, sections were incubated with species-appropriate biotinylated secondary antibodies, followed by Vectastain (MJS Biolynx). Colour was developed using 3-amino-9-ethylcarbazole (AEC) red (Vector Laboratories), counterstained with hematoxylin (Sigma Aldrich) and mounted with Fluoromount-G mounting medium (Southern Biotech). For fluorescent staining, sections were incubated with species-specific Alexa 488– or Alexa 555–conjugated secondary antibodies followed by DAPI nuclear stain (ThermoFisher Scientific. Sections were imaged using a Nikon ECLIPSE Ni series microscope equipped with a Ds-Qi2 camera.

4.2.7 uNK Cell Isolation

Deciduas were removed from GD 9.5 implantation sites under a dissection microscope and extraembryonic ectoderm and ectoplacental cones were removed. Isolated decidual tissue was minced and then placed in gentle collagenase/hyaluronidase in DMEM (StemCell Technologies) for 2 hours at 37°C on a gentle shaker as per the manufacturer's instructions. Digested tissue was passed through a 100 µm cell strainer and the resulting single cell suspension was layered onto Lymphoprep™ (StemCell Technologies) and subjected to density-gradient centrifugation as per manufacturer's instructions. Isolated mononuclear cell layer was washed, and cells were counted for flow cytometry or plated

for cell culture experiments. For cell culture, isolated mononuclear cells were resuspended in RPMI supplemented with 1% FBS and 10 ng/ml rat recombinant IL-15 (PeproTech) and plated. For coculture experiments cells were placed directly into incubators for 24 h. For separation conditions, resuspended cells were incubated for 2 hours at 37°C. After 2 hours, cells in suspension were removed from those adhered and placed in separate wells or analyzed by flow cytometry. For all conditions, after 24 hours (plus 6 h and 12 h for separation experiments) RNA was isolated from both adhered and suspended cells for expression of NK cell markers.

4.2.8 Immunofluorescence

After resuspension, a small number of isolated mononuclear cells were isolated and fixed in 4% paraformaldehyde. Following fixation, cells were smeared on gelatin-coated microscope slides. Cell smears were permeabilized using 0.3% Triton-X and 1% BSA in PBS and blocked with 10% normal goat serum (Life Technologies). Slides were then immersed in primary antibodies specific for OPN (0.5 µg/ml, Santa Cruz Biotechnology) and PRF (2.5 µg/ml, Torrey Pines Biolabs) overnight. Slides were then incubated with Alexa 555–conjugated anti-rabbit secondary (Invitrogen), Alexa 488–conjugated anti-mouse secondary (Invitrogen) antibodies, and DAPI (ThermoFisher Scientific) prior to mounting with Fluoromount-G. Images were acquired using a Nikon ECLIPSE Ni Series microscope equipped with a Ds-Qi2 camera.

4.2.9 Flow Cytometry

Following isolation, freshly resuspended cells or suspended cells plated for two hours were subsequently diluted to 1 x 10^6 cells per ml and incubated for 30 minutes with PE-conjugated mouse anti-rat anti-CD161 (BD Biosciences) or PE-conjugated mouse IgG1 (κ isotype control; BD Biosciences), and then fixed and permeabilized using BD Cytofix/Cytoperm Fixation/Permeablization Kit (BD Biosciences) as per manufacturer's instructions. Fixed cells were then analyzed using a Cytomics FC500 flow cytometer with CXP software (Beckman Coulter).

4.2.10 Statistical Analysis

Unless indicated otherwise, statistical significance was analyzed by Student's *t*-test when comparing two groups, and Analysis of Variance followed by a Tukey's post-hoc test when comparing three or more groups. Differences were considered statistically significant when $p \leq 0.05$. GraphPad Prism 8.0 was used for all graphing and statistical analysis. All animal experiments were conducted with a minimum of three dams. The specific number of dams and conceptuses analyzed is indicated in the figure legends, with "N" representing the number of dams and "n" representing the number of conceptuses used in each cohort of samples.

4.3 Results

4.3.1 Altered Gene Expression in Decidua of *IL15Δ/Δ* Rats

Our previous study utilizing *IL15Δ/Δ* rats found an expanded junctional zone, extensive trophoblast invasion into spiral arteries and enhanced dilation of spiral arteries associated with a lack of uNK cells compared to WT rats (Renaud et al. 2017); however, the mechanisms underlying these changes have yet to be elucidated. We set out to examine global gene expression changes between WT and *IL15Δ/Δ* rats, which would allow us to determine candidate genes that may be driving changes in decidualization and placentation in response to the absence of IL-15. Moreover, since *IL15Δ/Δ* rats do not possess uNK cells, this approach may uncover genes expressed specifically by rat uNK cells. Thus, we used a microarray analysis to profile global gene expression changes on GD 9.5 between WT and *IL15Δ/Δ* conceptuses. Overall, from a total of 23,418 transcripts examined, there were significant ($\geq$ 2-fold increased or decreased compared to WT, $p \leq 0.05$) differences in the expression of 257 genes between *IL15Δ/Δ* and WT conceptuses (Figure 4-1A, B). Of these, 91 transcripts were upregulated more than 2-fold in *IL15Δ/Δ* rats, and 156 transcripts were downregulated more than 2-fold (Figure 4-1B). Transcripts upregulated in *IL15Δ/Δ* deciduas include those associated with neurotransmission and central nervous system development (e.g., *Gria4*, 3.92-fold; *Crym*, 3.61-fold) and various glycoproteins involved in implantation or inflammatory responses (e.g., *Zp2*, 31.18-fold; *Orm1*, 24.17-fold; *Prom1*, 5.26-fold). Unsurprisingly, many of the downregulated genes

Table 4-2: List of top 20 increased genes in *IL15Δ/Δ* animals.

Gene	Fold Change	WT Reads (log_2)	*IL15Δ/Δ* Reads (log_2)
Zp2	31.18	3.27	8.23
Hp	6.84	8.42	11.19
Gria4	3.92	5.23	7.2
LOC102553649	3.61	7.87	9.72
Crym	3.61	5.13	6.98
Olr1220	3.36	4.34	6.09
Olr1468	3.29	2.92	4.63
Chpt1	3.17	12.26	13.93
Olr1081	3.09	3.15	4.78
Olr84	2.98	2.32	3.9
LOC685828	2.93	6.98	8.53
LOC100911939	2.93	3.33	4.88
Olr1237	2.93	3.33	4.88
Olr1237	2.93	3.33	4.88
Fis1	2.91	8.62	10.16
Samt4	2.87	2.84	4.36
Hormad1	2.82	4.25	5.75
Qpct	2.72	7.83	9.27
Vom2r10	2.72	3.36	4.81
Olr1063	2.67	4.55	5.97

Table 4-3: List of top 20 decreased genes in *IL15Δ/Δ* animals.

Gene	Fold Change	WT Reads (log_2)	*IL15Δ/Δ* Reads (log_2)
Gzmc	-1798.9	15.71	4.9
Prf1	-318	13.13	4.82
LOC100911163	-140.18	11.64	4.51
Gzmb	-80.41	11.12	4.79
Gzmbl2	-75.92	11.32	5.08
Nkg7	-49.08	8.74	3.13
Il2rb	-42.81	9.34	3.92
Spp1	-23.94	12.21	7.63
Fcrl6	-18.35	8.44	4.24
Cd96	-13.34	7.42	3.68
Ccl5	-7.97	6.93	3.94
Eomes	-7.86	8.27	5.29
Laptm5	-5.57	9.17	6.69
Mcpt10	-5.39	8.07	5.64
LOC100910060	-5.36	5.54	3.12
Plek	-5.19	5.42	3.04
Lcp1	-5.17	10.04	7.67
Clnk	-5.16	6.0	3.63
Mcpt8	-4.86	8.35	6.06
Sh2d1b	-4.49	5.56	3.4

encoded proteins associated with inflammatory processes and immune cell (namely NK cell) development (e.g., *Gzmc*, 1798.9-fold; *Prf1*, 318-fold; *Gzmb*, 80.41-fold).

Gene ontology analysis identified downregulated pathways in *IL15Δ/Δ* conceptuses including immune response (p = 4.2E-6, 12 genes), immune system process (p = 3.3E-4, 10 genes), and leukocyte cell-cell adhesion (p = 3.0E-5, 5 genes, Figure 4-1C). Interestingly, pathways associated with upregulated genes were related to neurological processes and sensory perceptions including chemical stimulus (p = 1.2E-3, 13 genes) and olfaction (p = 2.5E-3, 12 genes, Figure 4-1D). To further confirm microarray results, qRT-PCR was conducted to detect a subset of these transcripts using conceptuses collected from a separate cohort of WT or *IL15Δ/Δ* dams. Compared to WT rats, the relative expression of *Crym* was significantly ($p \leq 0.05$) increased in *IL15Δ/Δ* rats. The relative expression of the genes encoding the NK cell associated proteins eomesodermin (*Eomes*) and chemokine ligand 5 (*Ccl5*) were significantly decreased ($p \leq 0.05$, $N \geq 5$, $n \geq 13$) (Figure 4-1E). Thus, conceptuses from *IL15Δ/Δ* rats exhibit robust gene expression Changes, including reduced expression of many genes associated with NK cell development and function.

4.3.2 *IL15*Δ/Δ Rats Do Not Possess Uterine Natural Killer Cells

Since IL-15 is required for NK cell development, our next goal was to confirm the absence of uNK cells in conceptuses collected from *IL15Δ/Δ* rats. First, we conducted qRT-PCR on GD 9.5 conceptuses collected from WT and *IL15Δ/Δ* dams ($N \geq 5$, $n \geq 13$ for each group) to detect *Prf*, the gene that encodes the pore-forming cytolytic protein PRF, which is highly expressed in NK cells and cytotoxic T lymphocytes. Since cytotoxic T lymphocytes are typically rare in the decidua (Erlebacher 2013; Yang et al. 2019), we concluded that *Prf* expression can be used as a proxy for NK cells. Not surprisingly, *Prf* transcript expression was significantly downregulated (35-fold, $p \leq 0.05$) in conceptuses from *IL15Δ/Δ* rats (Figure 4-2A), which is consistent with results from our microarray analysis. To confirm these changes at the protein level, we performed western blotting, and observed an 86% decrease in PRF protein levels in *IL15Δ/Δ* conceptuses compared with WT conceptuses (Figure 4-2B). To confirm the

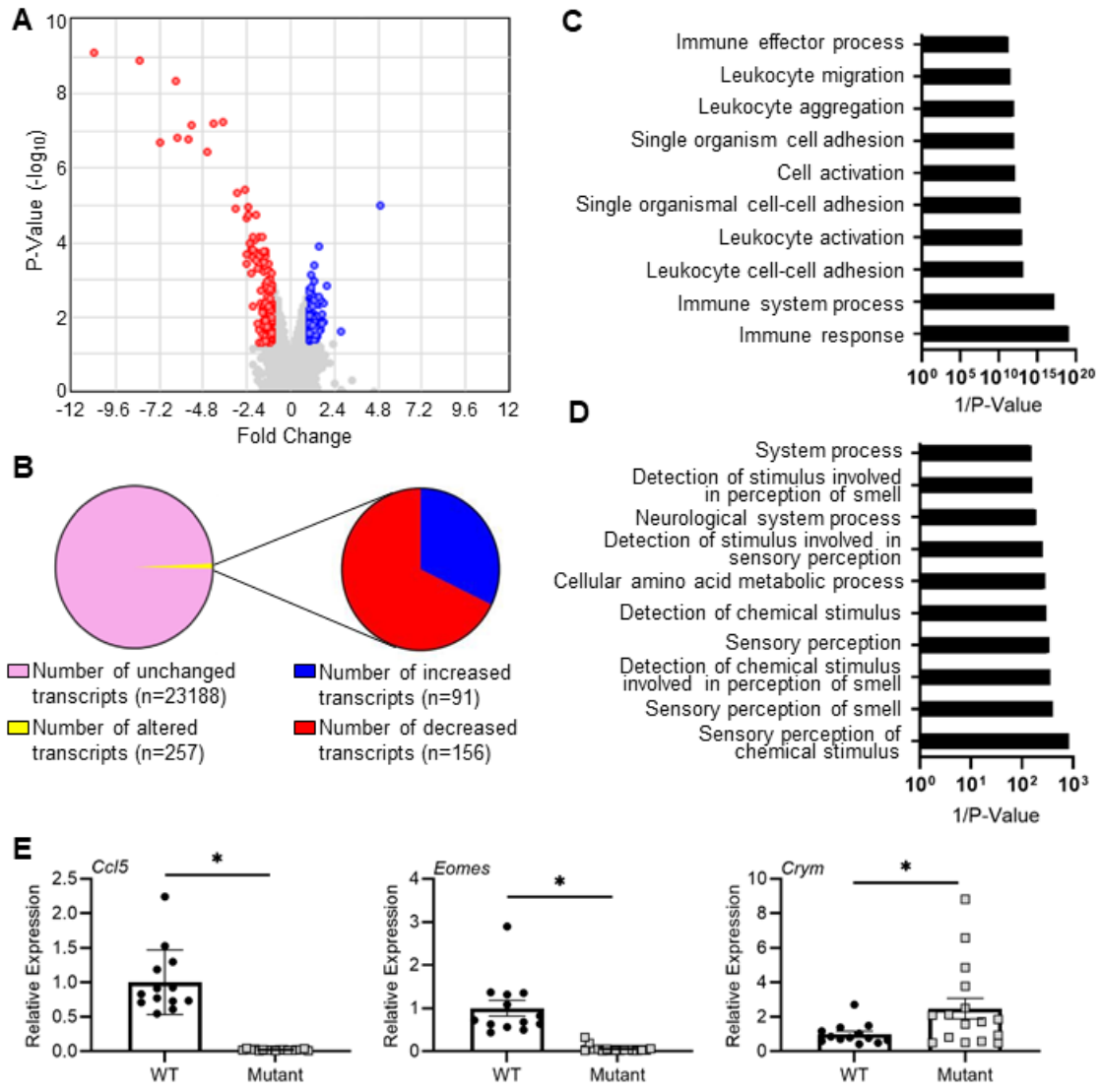

Figure 4-1: Gene expression changes in conceptuses isolated from WT and *IL15Δ/Δ* rats.

Volcano plot of genes significantly ($p \leq 0.01$) upregulated or downregulated in conceptuses from *IL15Δ/Δ* rats compared to WT rats. (B) Pie chart showing number of transcripts upregulated and downregulated in conceptuses from *IL15Δ/Δ* rats compared to WT rats. (C) Top 10 gene pathways downregulated in conceptuses from *IL15Δ/Δ* rats. (D) Top 10 gene pathways upregulated in conceptuses from *IL15Δ/Δ* rats. Pathway analysis was conducted by inputting transcripts changed more than 2-fold with a $p \leq 0.01$ in *IL15Δ/Δ* rats compared to WT rats into DAVID Bioinformatics Resource. (N = 3, n = 9

for each group). (E) Quantitative RT-PCR was used to compare the relative expression of select transcripts in GD 9.5 conceptuses from *IL15Δ/Δ* rats compared to WT rats. N = 9, n ≥ 13 per group. Results represent means ± SEM. Data significantly different from controls ($p \leq 0.05$) are indicated by an asterisk (*).

localization of PRF, and thus NK cells, we conducted immunohistochemistry on WT and *IL15Δ/Δ* conceptuses, staining for PRF on GD 9.5. At GD 9.5, WT rats had increased PRF staining in the decidua basalis (DB), including moderate staining proximal to the ectoplacental cone (EPC, which contributes to formation of the placental junctional zone), whereas no staining was observed in the decidua of *IL15Δ/Δ* rats (Figure 4-2C). Quantifying the number of PRF positive cells highlighted a significant 98.5% decrease in PRF positive cells in the DB (Figure 4-2D) and 97.2% decrease in PRF positive cells in proximity to the EPC (Figure 4-2E) in *IL15Δ/Δ* rats compared to WT controls ($p \leq 0.05$, $N \geq 5$, $n = 7$ for each group). Collectively, these results demonstrate that deciduas from *IL15Δ/Δ* have greatly reduced numbers of uNK cells in the decidua and near the nascent placenta.

4.3.3 *IL15*Δ/Δ Rats Have Decreased Osteopontin Expression

OPN has previously been implicated in the homeostasis and function of NK cells (Leavenworth et al. 2015). Moreover, decreased levels of OPN have been reported in cases of placenta accreta, a condition characterized by deficient decidualization and over invasive placentation (Özer et al. 2018). As *Spp1*, the gene encoding OPN, was one of the most significantly downregulated genes in our gene expression analysis, we further evaluated expression and localization of *Spp1* in deciduas of WT and *IL15Δ/Δ* rats. Expression of *Spp1* was significantly decreased (2.45-fold) at the RNA level in GD 9.5 *IL15Δ/Δ* implantation sites compared to WT (Figure 4-3A, $p \leq 0.05$, $N \geq 5$, $n \geq 13$ for each group). Interestingly, four conceptuses from *IL15Δ/Δ* rats (out of a total of X) had comparable levels of *Spp1* as WT. These same four conceptuses had robustly downregulated PRF (Figure 3) indicating that these results were not due to an unexpected influx of NK cells, so we reasoned that the *Spp1* in these four conceptuses may be due to contamination of samples by uterine glands. Indeed, it has been reported that OPN is secreted by uterine glands in various species (Johnson et al. 2003; Chaen et al. 2012; Burton et al. 2002; Qi et al. 2014), and following immunohistochemistry for OPN, staining was readily apparent in both WT and *IL15Δ/Δ* uterine glands (Figure 4-3B,G).

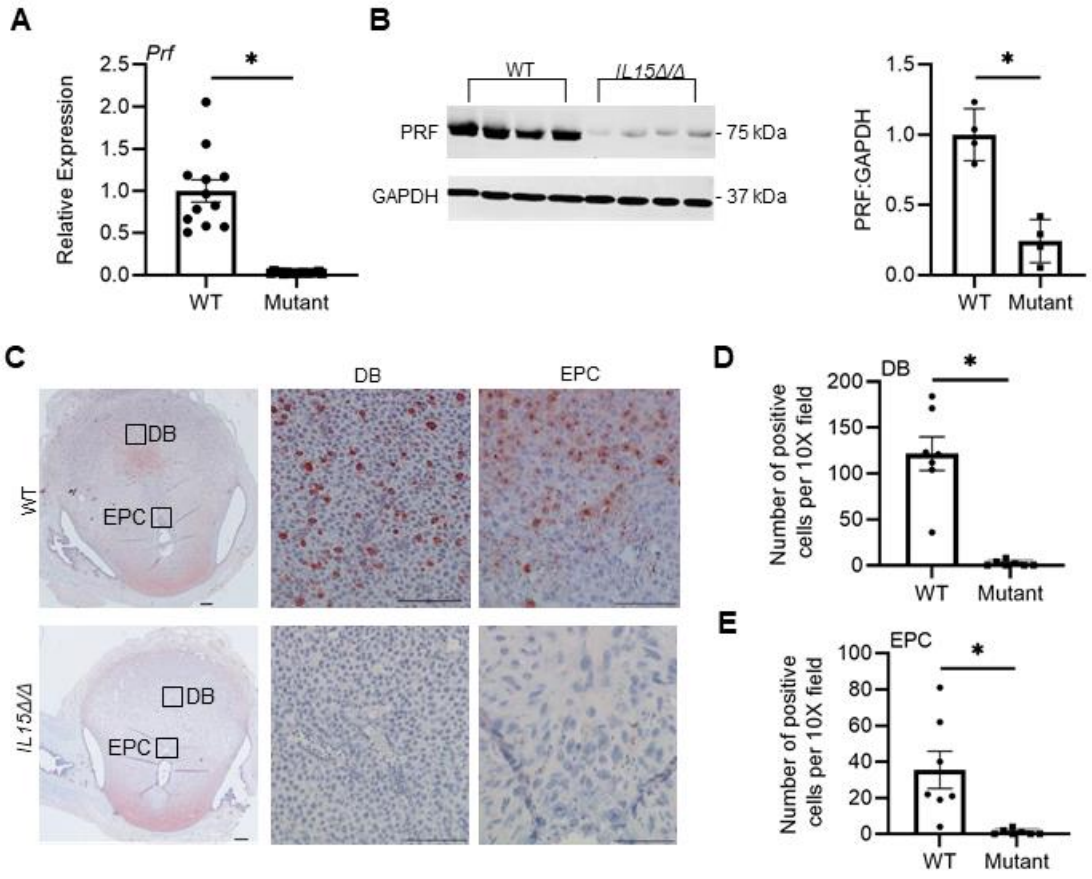

Figure 4-2: *IL15Δ/Δ* rats are devoid of PRF-containing uNK cells on GD 9.5.

(A) Quantitative RT-PCR comparing *Prf* levels between conceptuses from WT and *IL15Δ/Δ* rats ($N \geq 5$, $n \geq 13$). (B) Western blot showing decreased PRF levels in conceptuses from *IL15Δ/Δ* rats compared to conceptuses from WT rats. GAPDH was used as a loading control. The histogram on the right shows results from densitometric analysis comparing PRF levels relative to GAPDH ($n = 4$ per group) (C) Immunohistochemistry showing PRF localization in conceptuses on GD 9.5 ($N = 9$, $n = 12$ per group). Please note the lack of PRF detection in *IL15Δ/Δ* deciduas. Scale bar in whole conceptus, 250 µm. Scale bar in higher magnification images, 50 µm. (D, E) Quantification of PRF-positive cells in WT and *IL15Δ/Δ* conceptuses ($N \geq 5$, $n = 7$ per group) in the decidua (D) and near the EPC (E). Results represent means ± SEM. Data significantly different from controls ($p \leq 0.05$) are indicated by an asterisk (*).

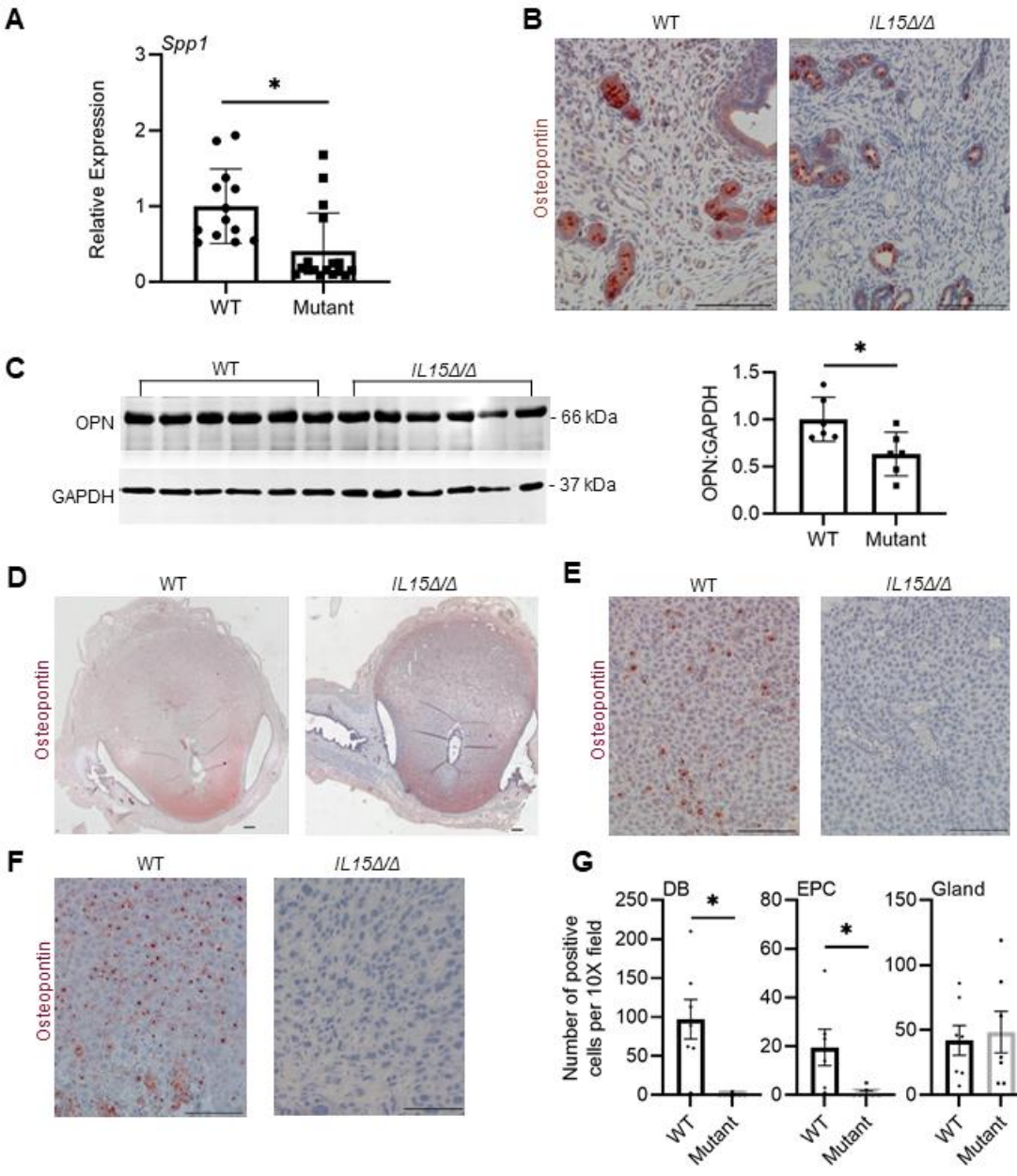

Figure 4-3: Decreased expression of decidual OPN in *IL15Δ/Δ* rats.

(A) Relative gene expression of *Spp1* in WT versus *IL15Δ/Δ* conceptuses on GD 9.5 ($N \geq 5$, $n \geq 13$). (B) Images showing OPN localization in uterine glands of WT and *IL15Δ/Δ* conceptuses on GD 9.5. (C) Western blot showing levels of OPN in lysates from GD 9.5 conceptuses of WT and *IL15Δ/Δ* rats. GAPDH was used as a loading control. The histogram on the right shows results from densitometric analysis comparing OPN levels

relative to GAPDH (n = 6 per group). Uncropped images of western blots are provided in Figure S2. (D) Immunohistochemical images of OPN staining in WT versus *IL15Δ/Δ* conceptuses. (E) Higher magnification images of OPN staining in the decidua (DB) of WT versus *IL15Δ/Δ* conceptuses. (F) Higher magnification image of OPN staining in the ectoplacental cone (EPC) of WT versus *IL15Δ/Δ* conceptuses. Please note that OPN staining is observed in sections of WT decidua, but not in *IL15*Δ/Δ decidua (N = 9, n = 12). Scale bar in D, 250 μm. Scale bar in E and F, 50 μm. (G) Quantification of OPN^+ cells in WT and *IL15Δ/Δ* conceptuses ($N \geq 5$, n = 7 per group). Results represent means ± SEM. Data significantly different from controls ($p \leq 0.05$) are indicated by an asterisk (*).

We next sought to determine whether changes in OPN expression were detectable at the protein level. Western blotting was performed using tissue lysates of whole GD 9.5 conceptuses collected from WT and *IL15Δ/Δ* rats. Compared to WT conceptuses, a 37% decrease in OPN was observed in *IL15Δ/Δ* conceptuses (Figure 4-3C, N = 3, n = 6 for each group, $p \leq 0.05$). To determine localization of OPN expression on GD 9.5, immunohistochemical detection of OPN was used. Sections of conceptuses collected from *IL15Δ/Δ* rats showed no OPN staining in or around the developing placenta (Figure 3D-F). In comparison, WT conceptuses had pronounced OPN staining both in the decidua basalis (DB) and near the ectoplacental cone (EPC). After counting the number of OPN-positive cells present in the decidua and glands of conceptuses from WT and *IL15Δ/Δ* rats ($N \geq 5$, n = 7 for each group), a significant decrease was seen in the levels of OPN staining in the DB (99.5% decrease) including near the EPC (95% decrease), while levels remained similar between the two groups in uterine glands (Figure 4-3G), indicating that any changes in OPN expression observed at the RNA or protein levels are likely due to changes in the decidua and not the uterine glands. To determine if OPN co-localizes with PRF and is therefore likely produced by uNK cells, we co-stained for PRF and OPN in GD 9.5 conceptuses and observed positive staining for both in WT conceptuses (Figure 4-4). Thus, OPN is expressed by uNK cells and it may play a role in modulating uNK cell functions.

4.3.4 Isolated uNK cells express OPN

While uNK cells have previously been isolated from both humans and mice, isolation of uNK cells in rats has yet to be reported (Paffaro et al. 2003; Timonen and Saksela 1980). Here, we demonstrate a protocol to isolate and culture NK cells from GD9.5 deciduas of pregnant rats. Isolated mononuclear cells were plated for 2 hours to allow the heterogenous population of cells time to separate by adherence. After 2 hours, cells were separated into suspended versus adherent populations. Adherent cells consistently exhibited reduced expression of genes encoding the NK cell markers perforin (*Prf1*), killer cell lectin-like receptor subfamily b 1F (*Klrb1f*), and *Ccl5* over 24 hours (Figure 5a). Compared to adherent cells, suspended cells expressed significantly ($p \leq 0.05$) higher levels of *Ccl5, Klrb1f, Prf1* and *Spp1,* which continuously increased over time in culture.

(Figure 4-5A). Culturing suspended cells with adherent cells for 24 h yielded significantly higher expression levels of NK cells markers *Ccl5* (31.52-fold), *Prf1* (5.83-fold), and *Klrb1f* (18.77-fold) as well as *Spp1* (11.17-fold) in suspended cells compared to adherent (Figure 4-5B). Interestingly, the transcript expression of *Vim*, a gene encoding vimentin, a protein found within decidual stromal cells (Glasser and Julian 1986), was significantly decreased (2.9-fold) in suspended cells compared to adherent controls (Figure 4-5B). Collectively, these results indicate enrichment of NK cells within the population of suspended cells isolated from GD 9.5 decidua, as compared to those adhering to tissue culture plastic.

Further confirmation of the presence of NK cells can be accomplished by analyzing the expression of CD161 (Arase et al. 1997). When evaluating surface expression of CD161 in decidual mononuclear cells, approximately 7.24% were positive for CD161 (Figure 4-6A). After allowing adherent cells to attach for 2 h and collecting the suspended cells, we successfully enriched the population of $CD161^+$ cells to approximately 25% (Figure 4-6B). Comparatively, allowing isolated decidual mononuclear cells to attach for 2 h allowed us to significantly enrich the population of $CD161^+$ cells by 18.34% (Figure 4-6C). Immunofluorescence conducted on suspended cells for the NK cell secreted protein PRF allowed us to visualize that a large number of these cells were likely uNK cells. PRF positive cells also co-stained for the expression of OPN indicating a colocalization of OPN expression and PRF in isolated uNK cells. Collectively, results from these experiments indicate that a population of uNK cells can be successfully isolated from rat deciduas and maintained in culture for at least 24 h. Furthermore, these results indicate that isolated rat uNK cells from GD 9.5 deciduas express OPN.

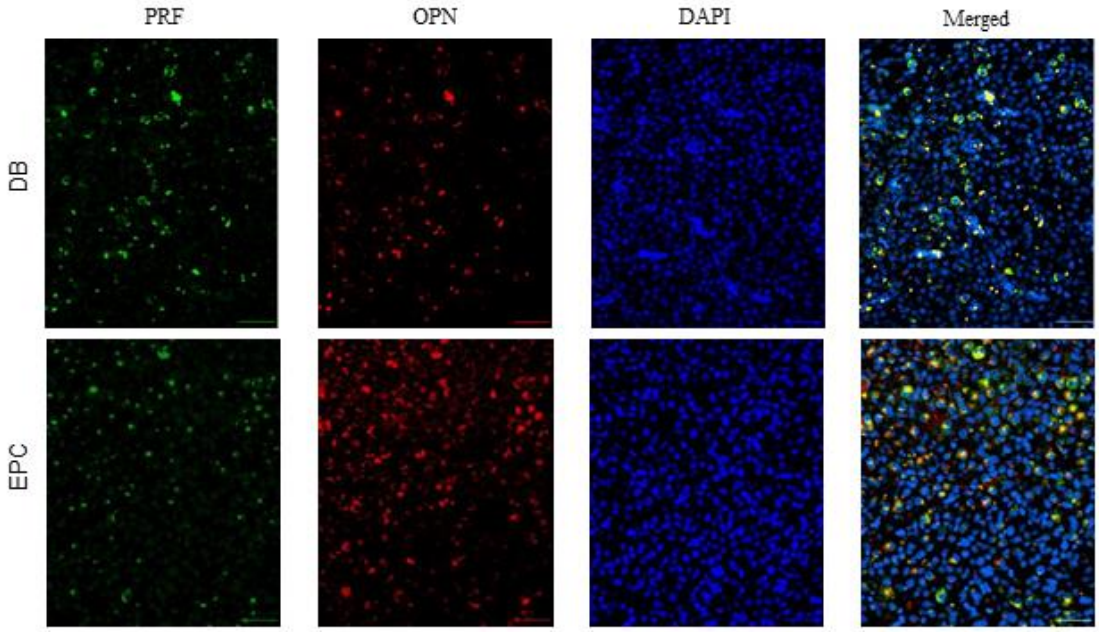

Figure 4-4: Co-localization of OPN in WT GD 9.5 conceptuses.

Co-localized staining for PRF (green) and OPN (red) in a representative GD 9.5 conceptus. PRF staining was used to detect uNK cells in the decidua basalis (DB) and ectoplacental cone (EPC). Images taken at 40x magnification and scale bars represent 50 μm.

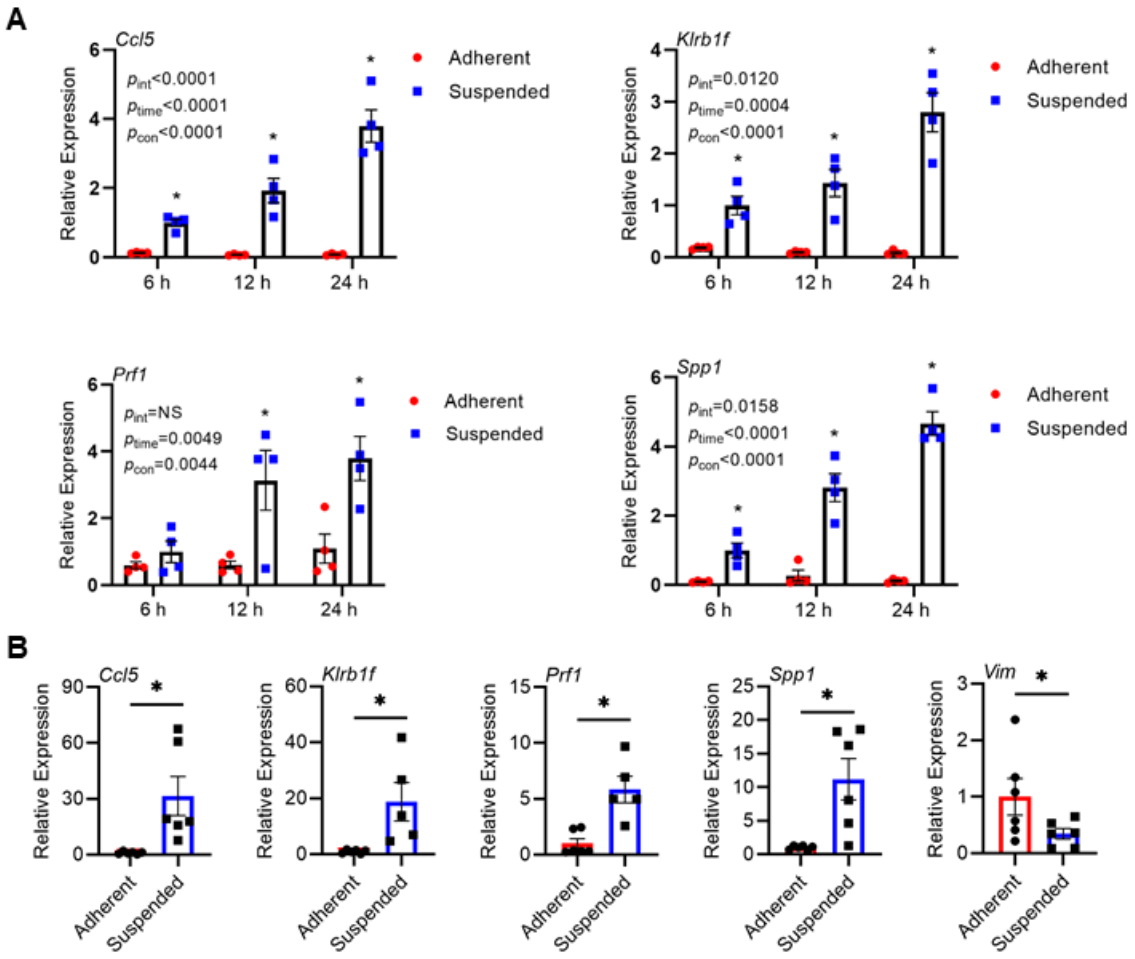

Figure 4-5: Isolated suspended cells express NK cell markers and *Spp1*.

(A) 24-hour time course of relative gene expression for *Ccl5*, *Klrb1f*, *Prf1*, and *Spp1* in suspended and adherent cells isolated from GD 9.5 deciduas (N=4). (B) Relative gene expression of *Ccl5*, *Klrb1f*, *Prf1*, *Spp1*, and *Vim* in adherent and suspended cells cultured together for 24 hours (N=5). Results represent means ± SEM. Data significantly different from controls ($p \leq 0.05$) are indicated by an asterisk (*).

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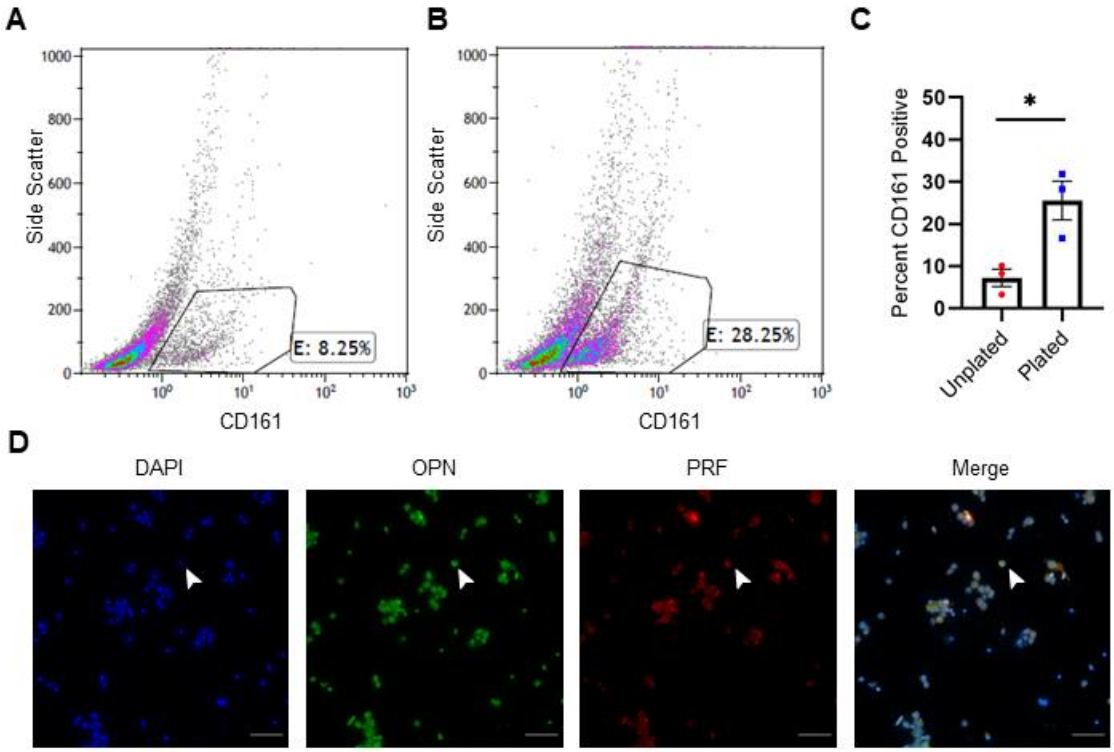

Figure 4-6: Isolated suspended cells express the NK cell-surface marker CD161.

(A, B) Flow cytometry for isolated uNK cells confirms the presence of the NK cell marker CD161 on 8.25% of cells **(A)**. **(B)** After plating for 2 h cells in suspension were stained for CD161 and the population of CD161 positive cells was enriched to 28.5%. **(C)** Percent values for CD161 positive cells with and without plating adherent cells for enrichment of suspended cell population. **(D)** uNK cells isolated from GD9.5 deciduas express both PRF and OPN. Arrows indicate cell positive for PRF and OPN staining. Images taken at 40x magnification and scale bars represent 50 μm.

suspended cells for the NK cell secreted protein PRF allowed us to visualize that a large number of these cells were likely uNK cells. PRF positive cells also co-stained for the expression of OPN indicating a colocalization of OPN expression and PRF in isolated uNK cells. Collectively, the results from these experiments indicate that a population of uNK cells can be successfully isolated from rat deciduas and maintained in culture for 24 hours. Furthermore, these results indicate that isolated rat uNK cells from GD 9.5 deciduas express OPN.

4.4 Discussion

Proper placental and decidual development is critical for healthy pregnancy outcomes; failure of these processes results in adverse pregnancy outcomes such as preeclampsia and PA (Sahu et al. 2019; Garrido-Gomez et al. 2017; Goh and Zalud 2016). While various cell types play a critical role in placentation, uNK cells are thought to play a key role in the regulation of placental development. Although several studies have examined the role of uNK cells on various aspects of pregnancy, most studies have been conducted using mouse models (Barber and Pollard 2003; Fu et al. 2017; Bany et al. 2012). The use of mice has provided invaluable insight into the potential functions of uNK cells; however, unlike placental development in humans and rats, placentation in mice is inherently shallow (Ain et al. 2003). Thus, the importance of uNK cells in species that exhibit deep placentation and extensive trophoblast invasion, such as in humans, is not well understood. Since IL-15 is required for the maturation of NK cells and as one of the few labs possessing an IL-15 deficient rat model, this study complements findings from mouse studies and provides unprecedented insight into the potential contribution of IL-15 and uNK cells in controlling placental trophoblast invasion. This has important translational significance for pregnancy complications associated with dysregulated trophoblast invasion such as preeclampsia and PA.

In this study, we conducted the first comprehensive characterization of rat uNK cells, identifying significant transcriptomic changes in conceptuses from *IL15Δ/Δ* rats compared to WT rats on GD 9.5. There were 257 genes that had significantly (> 2-fold up/down regulated and $p \leq 0.05$) changed expression between WT and *IL15Δ/Δ*

conceptuses. Genes that were upregulated in *IL15Δ/Δ* rats compared to WT rats were typically associated with functional pathways such as neurological processes and sensory pathways. One possibility is that upregulated genes between *IL15Δ/Δ* and WT rats may be associated with neurological and/or sensory pathways but have other functions in the decidua or during pregnancy. One example of this is the gene *Crym*, which encodes a heat shock protein called crystallin. Although functions of crystallin have been studied mostly in the context of central nervous system and lens development (Slingsby and Wistow 2014), upregulated levels of phosphorylated crystallin have been found in rat and mouse deciduas, with the proposed role of protecting decidualization against stress conditions (Iwaki et al. 1990; Zuo et al. 2014). Moreover, knocking down crystallin increases stromal cell death and inhibits decidualization under stress conditions (Zuo et al. 2014). Taken together, the upregulation of crystallin in *IL15Δ/Δ* rat conceptuses may therefore indicate that a lack of uNK cells results in a form of decidual stress similar to the effects seen following hypoxia exposure (Zuo et al. 2014). Additionally upregulated was *Gria4*, a gene that encodes the glutamate receptor 4 protein (GRIA4). While GRIA4 has primarily been implicated in neurotransmission and variations in *Gria4* expression leads to cognitive impairments, GRIA4 has been observed within the trophoblast and has been implicated in the context of pathogenic pregnancies (Martin et al. 2017; Yeung et al. 2016; Sagata et al. 2010; Uhlén et al. 2015). Genes that were significantly downregulated in *IL15Δ/Δ* rat conceptuses compared to WT included *Prf1*, *Ccl5*, *Eomes*, *Gzmb* and *Il2rb*, which have all been implicated in NK cell function (Gordon et al. 2012; Kim et al. 2011; Maghazachi et al. 1996; Fernandez et al. 2019), supporting the notion that the absence of uNK cells is the key difference between WT and *IL15Δ/Δ* rat deciduas.

The gene *Prf1* encodes perforin (PRF), a pore-forming cytolytic protein found in both T cells and NK cells (Kägi et al. 1994). Since the decidua lacks a prominent T-cell population, we utilized PRF as a marker of uNK cells, which was consistently decreased at both the transcript and protein level in *IL15Δ/Δ* rats (Head et al. 1994; Parr et al. 1990). Specifically, the absence of PRF observed in the decidua confirmed that *IL15Δ/Δ* rats are indeed devoid of uNK cells, as previously reported (Renaud et al. 2017). Comparably, studies that have knocked out or mutated either IL-15 or the IL-15R in mice, confirm that IL-15 function is required to maintain uNK cell populations (Barber and Pollard 2003;

Kennedy et al. 2000) and PRF staining is also absent in these deciduas (Herington and Bany 2007). Akin to what was seen in our study, uNK cells in mice are mainly localized to the decidua (Croy et al. 2003). In humans however, uNK cells are thought to directly interact with trophoblast cells in the decidua, controlling the depth of extravillous trophoblast invasion into spiral arteries (Hanna et al. 2006; Soares et al. 2014), but whether this is the same case in rats remains unknown. In the present study. We demonstrated that PRF is present at the ectoplacental cone, the location of trophoblast cells and the nascent placenta, which may indicate that interactions between uNK cells and trophoblast cells are occurring. This is supported by previous studies demonstrating that on GD 8.5 in mice (which approximates to GD 9.5 in rats), trophoblast cells at the ectoplacental cone are surrounded by a large number of uNK cells, some of which infiltrate into the cone (Madeja et al. 2011). Future studies can aim to explore this relationship and determine if uNK cell deficiency alters trophoblast invasion or function throughout pregnancy.

One of the most robustly downregulated genes in decidua from *IL15Δ/Δ* rats was *Spp1*, the gene encoding the protein OPN. OPN is a prime candidate as a potential regulator of the degree of placental invasion, due in part to its known role as a regulator of cell invasion (Wu et al. 2015; Huang et al. 2017; Qi et al. 2014) and its altered expression patterns in PA (Özer et al. 2018; Long et al. 2019). A well-characterized role for OPN is as a matricellular protein that is both produced and secreted into the extracellular space (Viloria and Hill 2016) to modulate cell function through interacting with molecules such as structural matrix proteins and integrins (i.e., αvβ3) (Bornstein 2009; Lessey et al. 1994). In pregnancy, OPN is thought to bind to integrins to mediate events including implantation, early blastocyst attachment trophoblast invasion and proliferation in various species (Wu et al. 2015; von Wolff et al. 2004; Johnson et al. 2014; Qi et al. 2014; Zhou et al. 2016). In cases of PA in humans, elevated expression levels of the integrin αvβ3 are observed in complicated placentas compared to normal controls, particularly in extravillous trophoblast cells regions (Weitzner et al. 2021). Since OPN plays are role in regulating cell adhesion, reduced OPN levels may decrease the ability for trophoblasts to adhere to their appropriate location at the end of their migration (Zhou et al. 2016). Therefore, a lack of OPN could result in the ability for trophoblast cells to

migrate more freely, causing the excessive invasion observed at mid-gestation in *IL15Δ/Δ* rats.

Since most downregulated genes in *IL15Δ/Δ* conceptuses are associated with NK cell function, it is logical that uNK cells are the main cellular source of decidual OPN and the absence of uNK cells in *IL15Δ/Δ* rats is the reason for reduced *Spp1* expression. In our study, decreased *Spp1* mRNA levels were consistently detected in *IL15Δ/Δ* conceptuses, although in a few cases notably high *Spp1* mRNA expression was observed, which we attribute to possible contamination with uterine glands which showed equally high OPN staining in both WT and *IL15Δ/Δ* conceptuses. Together, these results indicate that uNK cells are the likely cellular source of decidual OPN, which may be important for controlling placentation, and reveal the potential use of OPN as a uNK cell-specific marker. Since OPN was highly expressed in uterine glands and its remained unchanged between WT and *IL15Δ/Δ* rats, our results indicate that there are at least two different sources of OPN production (glandular and uNK cell-derived) in mid-gestation rat uteri.

To determine if uNK cells are the source of decidual OPN, we isolated uNK cells from the deciduas of WT animals. Isolation and culture of uNK cells has been successfully completed in both human and mouse models and while in rats peripheral NK cells have been collected from placentas, the isolation of uNK cells has yet to be reported (Koopman et al. 2003; Hanna et al. 2006; Yadi et al. 2008; Paffaro et al. 2003; Shields et al. 2018). Previous isolation of human uNK cells note the expression of various NK cells markers including CD56, NKP46, and KIR2DL1, plus the expression of granzymes A and B (Vacca et al. 2006). Here, we report isolated rat uNK cells express transcripts for the NK cell markers *Ccl5*, *Klrb1f*, and *Prf1* as well as the marker for OPN (*Spp1*), as well as provide evidence of uNK cells as the main source of OPN in the GD 9.5 rat decidua. Further supporting this, OPN was localized specifically to areas populated by uNK cells, and staining of OPN co-localized with the uNK cell marker PRF. Furthermore, isolated rat uNK cells expressed CD161, PRF, and OPN, further implicating uNK cells as the primary cellular source of decidual OPN. In mice, intracellular OPN has been found in peripheral NK cells and implicated in IL-15 signaling, therefore it is logical that it is logical that conceptuses from *IL15Δ/Δ* rats lack both PRF and OPN in uNK cells

(Leavenworth et al. 2015). Therefore, it is likely that uNK cells are the cellular source of decidual OPN which regulates placentation and reveals the potential use of OPN as a uNK cell-specific marker.

OPN has been implicated in inflammatory environments, and while it exists as a component of the extracellular matrix, it also functions as a soluble cytokine (Lund et al. 2009). In humans, isolated decidual tissues indicate that there is a correlation to the number of uNK cells to the levels of secreted OPN, indicating a relationship between uNK and OPN and the possible role OPN plays in recruiting uNK cells to the pregnant uterus (Qu et al. 2008). Combined with our findings that PRF and OPN are co-localized, it is possible that the secreted and intracellular forms of OPN work together to respectively recruit and maintain uNK cells during pregnancy. Consistent with this possibility, OPN in mice promotes the expansion of common lymphoid progenitor cells, which give rise to NK cells (Kanayama et al. 2017). Determining the subcellular distribution of OPN in uNK cells to define the expression of secreted versus intracellular *Spp1* isoforms is needed to confirm the localization and type of OPN expression in these cells.

In the present study, we successfully isolated a population of uNK cells from pregnant rat deciduas. The co-expression of OPN and PRF as well as the expression of CD161 on the surface of these cells; however, the identity of these cells remains unknown. To enrich our population of uNK cells, isolated MNC were allowed to adhere for 2 hours to separate decidual stromal cells and macrophages (adherent) from uNK cells found in suspension, methods similar to those used in human uNK cell isolation (Koopman et al. 2003). Separating out suspended cells from those that had adhered, allowed the population of uNK cells to express more NK cells markers and OPN over time ($p_{time} \leq 0.05$ for *Ccl5*, *Klrb1f*, *Prf1*, and *Spp1*). Due to normalization of RNA values prior to transcript level analysis, it is not known if isolated uNK cells are proliferating over 24 hours in cell culture, however these findings warrant further investigation. Additionally, it was observed that levels of NK cell markers had a larger relative expression when cells were cultured directly with those that adhered in a co-culture system. Previous NK cell cultures indicate the increased expression of various cytokines and growth factors (i.e.,

Vegfc, *Cxcl10*, *Cxcl11*), when co-cultured with trophoblast cells or endothelial cells, warranting further investigation into the cytotoxicity of uNK cells and interactions between adherent and suspended cells in a co-culture method (Gong et al. 2014; Lash et al. 2011). While our adherent enrichment strategy increased the number of $CD161^+$ cells by ~18%, future experiments are needed to further enrich isolated uNK cell population and determine the identify of additional present cells. Upon further enrichment, future experiments on the interaction of uNK cells and trophoblast cells will allow for genetic manipulation techniques like shRNA and CRISPR to decrease the expression of OPN and determine its importance in uNK cell proliferation and survival as well as trophoblast invasion.

This study has several limitations. First, the population of uNK cells throughout pregnancy is dynamic, additional experiments over the course of rat pregnancy would clarify the expression of OPN over time and provide an indication of its role in placental and decidual development. For example, determining if levels of OPN peak at the same time or prior to the commencement of certain decidualization processes (i.e., spiral artery remodelling may indicate a potential involvement of OPN in guiding these changes. This is further warranted by previous studies demonstrating that human decidual samples collected from later weeks during first trimester (weeks 8-9) pregnancies had dramatically increased expression of OPN compared to samples collected from earlier in the first trimester (weeks 4-5) (Qu et al. 2008). Second, we cannot ascertain that altered OPN expression in *IL15Δ/Δ* rats is directly a result of a lack of uNK cells. While we were able to use *IL15Δ/Δ* rats to identify a significant lack of both OPN and uNK cells, the use of single cell transcriptomics may help facilitate the identification of cell-specific OPN production in the decidua.

In summary, we have conducted a comprehensive profile of gene expression changes in the rat conceptus between WT and *IL15Δ/Δ* rats. We further determined that OPN is likely expressed by uNK cells, and we speculate that it may play a role in controlling processes related to placentation and decidualization. Due to the known role of OPN in promoting cell adhesion, reduced OPN levels may decrease trophoblast adhesion resulting in their ability to migrate more freely, leading to the excessive invasion seen at

mid-gestation. The effect of hyper-invasive trophoblasts has long been studied in PA, with emerging studies linking decreased OPN in placentas to the diagnoses of PA (Illsley et al. 2020; Miller et al. 1997; Özer et al. 2018). Thus, our findings provide a new aspect to investigate the association between decreased OPN levels and PA, allowing future studies to utilize isolated uNK cells for functional assays. Collectively, our results provide insight into the roles of IL-15, and more specifically uNK cells in pregnancy, which could ultimately aid in understanding mechanisms underlying common pregnancy complications so that targeted therapies can be implemented to alleviate long-term complications for both mothers and their babies.

4.5 References

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Chapter 5

5 Discussion

5.1 Discussion and Significance of Research

Maternal immune cells can be found in close proximity and interacting with placental cells throughout gestation and can secrete various cytokines and growth factors suggesting an important role in regulating placental development. The overall hypothesis of my thesis is that maternal immune activation during pregnancy will disrupt normal placental and fetal growth and development. With this work, I have advanced our knowledge of the detrimental impact that MIA can have on the development of the placenta and fetal brain. The main findings that support my hypothesis and further advance this knowledge are that:

1. Administration of MIA during early gestation results in aberrations in placental and fetal neurodevelopment.

2. OPN is produced by uNK cells, which as a multifunctional cytokine could modulate trophoblast invasion and placental development.

In this work, I have provided insight into the possible mechanisms by which MIA impairs placental development. This placental maldevelopment possibly occurs through the increased expression of type I interferons and their ability to impair differentiation of trophoblast stem cells. Additionally, MIA can lead to alterations in NPC cell proliferation and cortical thickness which may result aberrant activation of Notch signaling pathways. Finally, the presence of uNK cells at the placental-fetal interface results in the presence of decidual OPN, a secreted protein that may possibly regulate development of the placenta.

5.1.1 Administration of MIA during early gestation results in aberrations in placental and fetal neurodevelopment

In chapters 2 and 3, I administered PolyI:C during early gestation in rats as a model of MIA to analyze the downstream consequences on placental development and fetal

growth. MIA due to PolyI:C exposure altered placental and fetal growth trajectories (Chapter 2), and neurodevelopment in offspring (Chapter 3).

MIA during early gestation results in a rapid and extensive proinflammatory response in implantation sites (Chapter 2), which was further confirmed by transcriptome analysis 6 hours after administration of maternal PolyI:C exposure (Chapter 3). Whole viruses (like Zika and Coxsackie) pose additional obstacles due to the occurrence of symptoms of infection (i.e., fever and lethargy) compared to MIA models. Therefore, since PolyI:C is capable of eliciting MIA in the absence of a viral infection, my work suggests that changes in placental morphology could result from an antiviral response, rather than the pathogen itself. In animal models, sterile inflammation is sufficient to recreate many of the features found in various obstetric complications such as: embryonic loss, preterm birth, and preeclampsia (Scharfe-Nugent et al. 2012; Gomez-Lopez et al. 2016), further implicating MIA as a cause of pregnancy complications. Since PolyI:C is frequently used as a model of MIA-induced neurocognitive deficits, we wanted to explore the possibility that PolyI:C affected placental development, since the placenta provides oxygen and nutrients needed for healthy brain development. While I detected a robust immune response in the pregnant uterus near the developing placenta based on increased expression of several well-established antiviral genes, it was unclear whether the immune response at this site was propagated directly due to accumulation of PolyI:C or indirectly because of cytokines induced in other tissues. In an attempt to address this issue, I collected tissue at several time points (GD 8.5, 13.5, & 18.5) following maternal exposure to PolyI:C and performed immunohistochemistry on implantation sites and/or placentas using a J2 monoclonal antibody, which detects for dsRNA (Millipore Sigma, MABE1134). Unfortunately, the staining was inconclusive, so this remains an unanswered question. Despite the lack of positive staining of dsRNA, I determined that the expression of pro-inflammatory cytokines and chemokines within the uterus provides strong evidence of an anti-viral, type I interferon response near the developing placenta. Therefore, upon confirmation of a maternal antiviral response, I sought to determine if these effects impaired placental development. I found that MIA during early gestation in rats resulted in smaller placentas and fetuses. Additionally, I found that type I interferons

impaired rTS cell differentiation *in vitro*, which is consistent with other reports (Devendra et al. 2005; Longhi et al. 2009; Farina et al. 2010).

The finding that MIA during early gestation decreased placental size at midgestation are consistent with Zika and Coxsackie infections that result in damage to the placental barrier and growth retardation in offspring (Euscher et al. 2001; Miner et al. 2016). Furthermore, work with West Nile virus illustrates the vulnerability of trophoblast cells to infection by viruses after implantation and prior to formation of the definitive placenta, suggesting a window of susceptibility to viral insults (Julander et al. 2006). This work complements previous studies which indicate the placenta has a window of susceptibility to insults between GD 6.5 and GD 9.5 in rats, which resulted in aberrant placental morphology (Rosario et al. 2008). In a similar manner, our MIA model, which utilizes PolyI:C administration during early placental development (GD 8.5) occurs in the middle window of susceptibility to insults which creates a suboptimal *in utero* environment for the progression of a healthy pregnancy.

Using RNA sequencing (Chapter 3), I discerned that PolyI:C caused a substantial increase in expression of inflammatory genes at the implantation site. Additionally, many downregulated genes were involved in CNS development, but since whole tissue homogenates were used, the identity of the cells causing these changes changing genes remains unknown. To identify the effects of MIA on the developing fetal brain more clearly, single cell RNA-sequencing could be used in future experiments, in which single cells are isolated from the cerebral cortex at GD 15.5 following maternal exposure to PolyI:C or saline. This heterogenous population of cells could then be single-cell-barcoded and sequenced, allowing for clustering and analysis of expression of various genes at single cell resolution. Thus, NPCs within various stages of differentiation can be determined, and the identity of cells expressing potentially interesting genes such as *Notch1* can be identified.

Taken together, these results demonstrate the negative impact of MIA on placental development and fetal growth. Maternal PolyI:C injection results in a rapid and robust increase in inflammation near the site of the developing placenta during early gestation

that further results in placental impairments and reduced fetal growth. Additionally, decreased fetal brain weights were correlated with altered NPC proliferation in MIA animals compared to controls. Therefore, early gestation MIA results in long term consequences on placental morphology and fetal development that warrants further investigation to understand the link between aberrations in normal development of the placenta and neurological impairments in offspring (Figure 5-1).

5.1.2 OPN is produced by uNK cells, which as a multifunctional cytokine could modulate trophoblast invasion and placental development

Knockout of *Il15* in rats results in the complete loss of NK cells and specifically uNK cells as previously reported (Renaud et al. 2017). While *IL15* deficiency appears not to affect the number of T cells, B cells, or macrophages, it is possible that survival or presence of various subsets of these immune cells are altered, which warrants further investigation (Renaud et al. 2017). Previous studies utilizing NK cell deficient rat and mouse models consistently demonstrate impairments in uterine vascular development with spiral arteries featuring narrow lumens and thick vascular walls (Renaud et al. 2017; Mazurier et al. 1999; Wang et al. 1994; Kennedy et al. 2000). Although these findings in mice and rats indicate an important role for uNK cells initiating and contributing to pregnancy-dependent changes in the uterine environment, it is important to note that uNK cells are not required for pregnancy success or fetal development.

A common finding in both rat and mouse studies utilizing IL-15 deficient animals is the increased depth of trophoblast invasion into the maternal uterine tissue. These findings of hyper-invasive trophoblasts in animal models are consistent with the life-threatening obstetric complication, placenta accreta (PA). PA is a serious complication in human pregnancies in which invasive trophoblasts invade too deeply into the uterus due to a decidual deficiency and thereby increasing the risk of severe maternal hemorrhage during labour (Publications Committee, Society for Maternal-Fetal Medicine and Belfort 2010). Interestingly, recent studies have found decreased levels of uNK cells in PA suggesting that uNK cells play a role in preventing the deficient decidualization and deep placental invasion, characteristic of the disease (Colucci 2019; Laban et al. 2014).

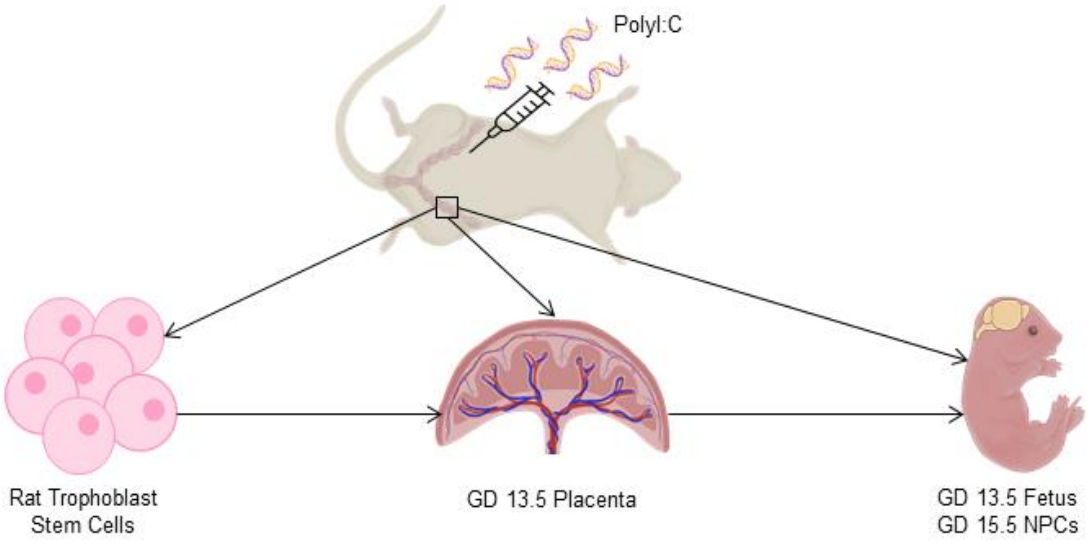

Figure 5-1: Proposed relationship between impairments in placental development with neurodevelopmental impairments observed in offspring.

Administration of PolyI:C to pregnant dams at GD 8.5 results in reduced placental development and fetal growth at GD 13.5. Inflammatory responses generated by PAMP (TLR3, RIG1, or MDA5) activation impaired the differentiation of rTS cells which form the definitive placenta. PolyI:C-exposed fetuses also had increased proliferative capacity of NPCs on GD15.5, although it is unclear whether these responses are due directly to MIA or indirectly due to impaired placental development.

This work implicates the production of OPN by uNK cells. Moreover, OPN is secreted from uterine glands as a component of histiotroph to supply early nutrition for the fetus and as such it has an essential role in implantation and reproduction (Nomura et al. 1988). Consistent with reports in mouse models, my work indicates the presence of OPN, an extracellular structural protein, in rat uNK cells (Chapter 4). While OPN was significantly downregulated at the mRNA level and undetectable via immunohistochemistry within the decidua in *IL15Δ/Δ* rats, immunblotting results indicate the presence of OPN at reasonably high levels in both WT and *IL15Δ/Δ* animals. Despite the knowledge that OPN is produced by uterine glands as histotrophic nutrition for the developing embryo, it is possible that the lack of uNK cell-produced OPN is being compensated for by the presence of other OPN producing cells like DSC or innate lymphoid cells that are not IL-15 dependent (von Wolff et al. 2004). Future studies can be done to determine the possible additional sources of OPN in the decidua, by isolating decidual tissue from surrounding tissues for protein analysis.

NK cells have long been hypothesized to play a role in trophoblast invasion and trophoblast-mediated spiral artery remodelling. While some reports indicate that chemokines and growth factors produced by uNK cells enhance trophoblast invasion (Hanna et al. 2006; Xiong et al. 2013; Lash et al. 2010), other reports indicate that factors produced by uNK cells inhibit trophoblast invasion (Hu et al. 2006; Lash, Otun, et al. 2006). Consistent with the latter reports, *IL15Δ/Δ* rats exhibit hyper-invasive trophoblasts, indicating a restrictive effect by uNK cells on trophoblast invasion and trophoblast-mediated spiral artery remodelling (Renaud et al 2017). Since my findings in Chapter 4 indicate that uNK cells produce OPN, it is possible that OPN produced and secreted by uNK may play a role in regulating invasion and migration of trophoblast cells. Over invasive trophoblasts pose both obstetric risks and serious long term health risks to a mother, like those observed in disorders such as placenta accreta (Tantbirojn et al. 2008). Compared to healthy term placental tissues, placentas from percreta cases exhibit significantly reduced OPN expression (Özer et al. 2018), and *in vitro* work implicates decreased OPN expression with impaired trophoblast growth and invasion (Zhou et al. 2016). Furthermore, in humans decreased uNK cell numbers has been

associated with the development of placenta accreta (Laban et al. 2014). Therefore, it is possible that uNK cell secreted OPN regulates trophoblast invasion, and the depth of placental attachment into the myometrium can be exacerbated in pregnancies lacking sufficient uNK cell numbers due to decreased OPN expression.

5.2 Limitations & Future Directions

Following the results observed in chapter 3, I sought to determine the contribution of uNK cells to the PolyI:C-induced antiviral response in the pregnant uterus. The initial strategy was to use an immunodepletion strategy using injection of anti-asialo-GM1 antibodies into pregnant rats (ThermoFisher). Use of asialo-GM1 antibodies was able to successfully deplete uNK cells as determined by qPCR and immunohistochemistry, which was associated with impaired immune response to PolyI:C and significant changes in immune responses between control animals and immunodepleted animals were not observed (Appendix A). Following up on these experiments, on GD 9.5 I exposed pregnant *IL15Δ/Δ* or WT dams to PolyI:C and another potent MIA stimulant, LPS, at doses previously shown to cause complete embryonic loss. In both *IL15Δ/Δ* or WT rats, neither PolyI:C nor LPS caused even partial embryonic loss, even when used at concentrations twice as high (Appendix B; Figure B-3). Since WT and *IL15Δ/Δ* rats are on a Sprague-Dawley Holtzman background, we attributed this lack of response to strain differences in the immune responses of these rats. It has previously been observed in mice (Sellers et al. 2012) and rats (Becker 2016) that there are varying immune responses between strains, however immune responses during pregnancy between Sprague-Dawley and Sprague-Dawley Holtzman rats has yet to be studied. It has also been noted that various rat strains have differing basal levels of immune cells within the blood, as well as varying levels of viral titers following adenovirus infection (Li et al. 2003). Together, our results along with previous work indicate possible strain differences in immune responses to pathogens or MIA models and these effects during pregnancy warrant further exploration. Unfortunately, because of this limitation we were unable to ascertain the contribution of NK cells in MIA-induced placental and brain maldevelopment.

While human and mouse uNK cells have previously been isolated and characterized (Lash et al. 2011; Koopman et al. 2003; Felker and Croy 2017), the isolation of uNK

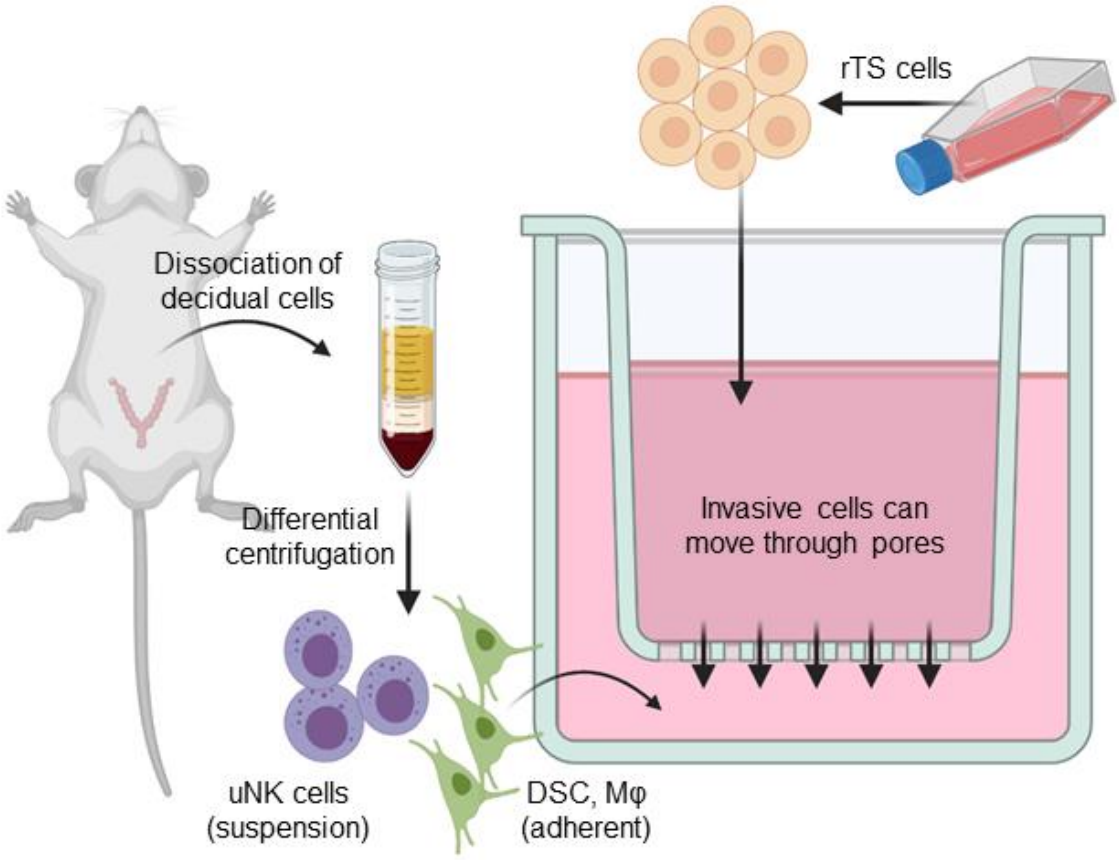

Figure 5-2: Proposed experimental methodology for co-culture of isolated decidual cells.

Gestational day 9.5 rats are sacrificed using mild-carbon dioxide inhalation and implantation sites are removed. Deciduas are isolated from the implantation sites and following removal of the primitive placenta and embryo, cells are dissociated and separated on a density gradient. Mononuclear cells are collected following differential centrifugation and cells are plated for 2 hours at 37°C to enrich the suspended population. Following plating, uNK cells can be separated as suspended cells in the cell culture medium, while DSC and macrophages remain adhered to the tissue culture plastic. Invasion assays will be conducted using rTS cells in the upper chamber and the bottom chamber can be filled with isolated cells prior to plating, following plating enrichment, or with conditioned media collected from separated cells.

cells from rat decidua provides an opportunity to further our understanding of mechanisms and roles of uNK cells in pregnancy. Therefore, because of the impact of uNK cells in regulating trophoblast invasion observed in *IL15Δ/Δ* rats (Renaud et al. 2017), as well as the decreased expression of OPN, the possibility of the relationship between these two features necessitates more investigation. To further assess the impact of uNK cell secreted OPN on placental development, it would be interesting to co-culture isolated rat uNK cells with rTS cells. My method of isolating uNK cells resulted in a higher number of $CD161^+$ cells following an adhering enrichment protocol, however further enrichment would allow for clearer results. Future studies could further enrich an isolated population of mononuclear cells with biotinylated CD161 beads (ThermoFisher) to allow for positive selection of $CD161^+$ uNK cells. Nevertheless, based on my experience, isolated uNK cells can be kept in culture for approximately 72 hours, although they appear to decline in number (as measured by the quantity of RNA and integrity of cells in culture; data not shown) after 24 hours. In transwell chambers, rTS cells can be plated above an extracellular matrix such as Matrigel (Corning) or Cultrex (R&D Systems) with enriched uNK cells or uNK cell conditioned media plated below the transwell (Figure 5-2). Based on the hyperinvasive trophoblast observed in *IL15Δ/Δ* rat placentation, I predict that rTS cell cultured in the presence of uNK cells or uNK cell conditioned media will have restricted trophoblast invasion compared to those cultured in control conditions. This model would allow us to determine how uNK cells modulate rTS cell differentiation and invasion. Additionally, we could quantify the amount of OPN secreted by isolated rat uNK cells throughout gestation using an enzyme-linked immunosorbent assay, which will help inform future experiments in which a specific dose of recombinant rat OPN can be applied to rTS cells to evaluate the resulting effects on differentiation and in invasion. Together, these experiments will further implicate uNK cells as a source of decidual OPN, as well as the role of uNK cell-derived products (including OPN) on the differentiation and invasive capacity of rTS cells.

To further evaluate the role of OPN in placental development, our lab is in the process of acquiring OPN deficient rats from the University of Glasgow. To discern the importance of decidual OPN, tissues will be isolated from pregnant *Spp1Δ/Δ* or WT rats throughout

gestation and stained for changes in the extent of trophoblast invasion and placental morphology. Furthermore, isolated uNK cells from *Spp1Δ/Δ* rats will be cultured for 24 hours, RNA and protein from isolated cells will be isolated and used for gene expression and an enzyme-linked immunosorbent assay (ELISA) of OPN, and uNK cells from OPN deficient rats will be cultured in invasion assay chambers to determine the role of uNK-derived OPN on rTS cell invasion. Together, this work will further evaluate both the role of OPN on placental development and trophoblast invasion as well as the relationship between uNK cells and decidual OPN.

While the use of PolyI:C as a model of MIA allows for an understanding of the basic mechanisms of viral infections, it has some limitations in its translatability to viral infections. While PolyI:C does cause similar immune responses in the pregnant mouse compared to influenza A infection, the responses in all cytokines are not comparable (Jacobsen et al. 2021). Specifically, the administration of PolyI:C results in a rapid and robust increase in proinflammatory cytokines (i.e., IL-6 & TNFα) in the maternal serum, however administration of influenza A takes longer to generate a similar immune response (Jacobsen et al. 2021). Therefore, it is important to take the activation time and degree of symptoms into consideration when utilizing PolyI:C. Furthermore, the use of PolyI:C to mimic the activation of TLR3 as seen during infection with dsRNA viruses does not factor in the additional immune cascades and side effects that live pathogens can initiate. For example, in addition to temperature changes, rats infected with influenza exhibit signs of respiratory distress, weight loss, and increased respiration (Ottolini et al. 2005), while the pathogen itself can activate TLR3, TLR7, TLR8, and RIG1 (Lund et al. 2004; Le Goffic et al. 2007; Le Goffic et al. 2006). In rats, the number of studying influenza A during pregnancy is limited, however increases in maternal cytokines like IFN-γ and IL-4 have been previously observed (Gu et al. 2011). These influenza-induced maternal cytokines in rats, paired with the impairments of placental development in mice exposed to influenza (Liong et al. 2020; Van Campen et al. 2020), warrant further investigation into the effects of whole pathogens like influenza, on the development of the placenta in rats. As influenza A is a respiratory virus, intranasal inoculation is most analogous to the primary route of infection in humans. Furthermore, work with intranasal influenza A infection in mice displays a prominent cytokine storm in the mother which

can reach the placenta (Liong et al. 2020; Van Campen et al. 2020). Therefore, to determine the effect of viral infection on development of the rat placenta, I propose infection with influenza A through intranasal inhalation to GD 9.5 pregnant rats. Following maternal early-gestation exposure to influenza A, placentas will be collected at mid and late-gestation for histological analysis to observe change in placental morphology. Additionally, doppler ultrasound microscopy will be performed on influenza A exposed dams compared to controls to determine if viral infection during early pregnancy will impair functionality of the definitive placenta. Furthermore, to explore the impact of viral stimuli on the developing cortex, additional experiments will be performed where NPCs will be isolated from fetuses from influenza A treated pregnancies compared to controls. NPCs will be cultured as previously described, and the effect of influenza A on the proliferative and self-renewal capacity of NPCs will be analyzed. The expression of NOTCH1 and its ligands will also be analyzed at the gene and protein level. I predict that influenza A infection during early pregnancy will result in impairments in placental development, however less robust effects on NPC differentiation and proliferation due to the longer incubation period of the virus and an increased window before a cytokine response is observed in the mother (Jacobsen et al. 2021). With the prevalence of SARS-CoV-2, additional future work assessing the impact of maternal COVID19 exposure would prove beneficial; however, these experiments are currently limited by the availability of feasible animal models (Reviewed by: Shou et al. 2021). Rat models have yet to be developed, which like mice have an incompatible ACE2 receptor to SARS-CoV-2 S protein and therefore would require the use of a rat-adapted SARS-CoV-2 strain or human-expressing ACE2 rats.

Since influenza has been observed to result in placental abnormalities in the placentas of infected dams (Van Campen et al. 2020; Littauer et al. 2017), it is possible that early gestation exposure can alter the growth trajectories due to MIA. To determine the effect of influenza A on the uterine immune environment I propose the use of intranasal inoculation of influenza A to pregnant WT SD dams during early gestation (approximately GD 7.5), I then plan to collect uNK cells from deciduas at GD9.5 as described above. Collecting uNK cells exposed *in utero* to influenza A may alter the expression of NK-cell like surface receptors. Reports of the ability of influenza to infect

NK cells themselves (Mao et al. 2009), as well as respond to infection through the NKp46 receptor in humans (Arnon et al. 2004) indicates a possibility of uNK cells to respond to influenza A infection near the developing placenta. Therefore, secreted anti-viral cytokines and granzymes from uNK cells can also be determined using ELISAs (IL-6, IFN-γ, Granzyme B) in response to influenza A infection compared to vehicle controls to further determine the role of uNK cells in the *in utero* immune response to viral infections. Isolated influenza A exposed uNK cells can additionally be cultured in invasion chambers with rTS cells as described above to understand the effect of viral infections on immune-mediated trophoblast invasion. With this work, I predict that influenza A infection will result in an increase in MIA at the maternal-fetal interface and an increase in the expression of uNK cell markers. Furthermore, I expect that uNK cells isolated from infection exposed dams will impair the ability of trophoblast invasion due to the increased secretion of pro-inflammatory cytokines. Together, these studies will further emphasize the detrimental effects of infection and maternal inflammation during early pregnancy on the development of the placenta and the fetal brain.

Single-cell work in the human decidua describes the presence of three major populations of uNK cells, each with distinct expression of immunomodulators and chemokines (Vento-Tormo et al. 2018). Previous work with supernatants from isolated human uNK cells, shows the increased proliferation of placental explants cultured in uNK conditioned media (Lash et al. 2010); however, the effects on early placental development remain unknown. Therefore, to further elucidate the effects of human uNK cells on placental development, I propose the isolation of human uNK cells as previously described (Lash, Schiessl, et al. 2006), to be cultured in transwell chambers with human TS (hTS) cells. Like rTS cells, hTS cells have the ability to differentiate into various trophoblast lineages, therefore assessment of the stem and differentiation capacity of hTS when cultured will indicate the effect of uNK secreted factors on early placental development. I predict that presence of uNK cells will result in the preferential differentiation of hTS cells into an invasive lineage.

5.3 Conclusions and Significance

Development of the placenta is a tightly regulated process in which aberrations in the maternal immune system can alter the intra-uterine environment and result in impairments in trophoblast differentiation. The overall conclusions of these studies are that *in utero* exposure to maternal inflammation during early gestation results in a significant decrease in the size of the junctional zone and decreased fetal growth. Additionally, reduced placental and fetal size also correlate to impairments in fetal neurodevelopment, with an increase in the proliferative capacity of NPCs from the cortex at mid-gestation. Furthermore, a lack of uNK cells during pregnancy is associated with decreased levels of OPN, which could contribute to the altered placental morphology in these rats. Our knowledge of the impact of MIA during pregnancy has been further advanced by this work. Collectively, this work expands on both the regulatory and protective roles of the maternal immune system during pregnancy and how aberrations in normal immune processes can lead to impairments in placental and fetal growth and development.

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Appendix A

Appendix A: Fetal weights and lengths following immunodepletion.

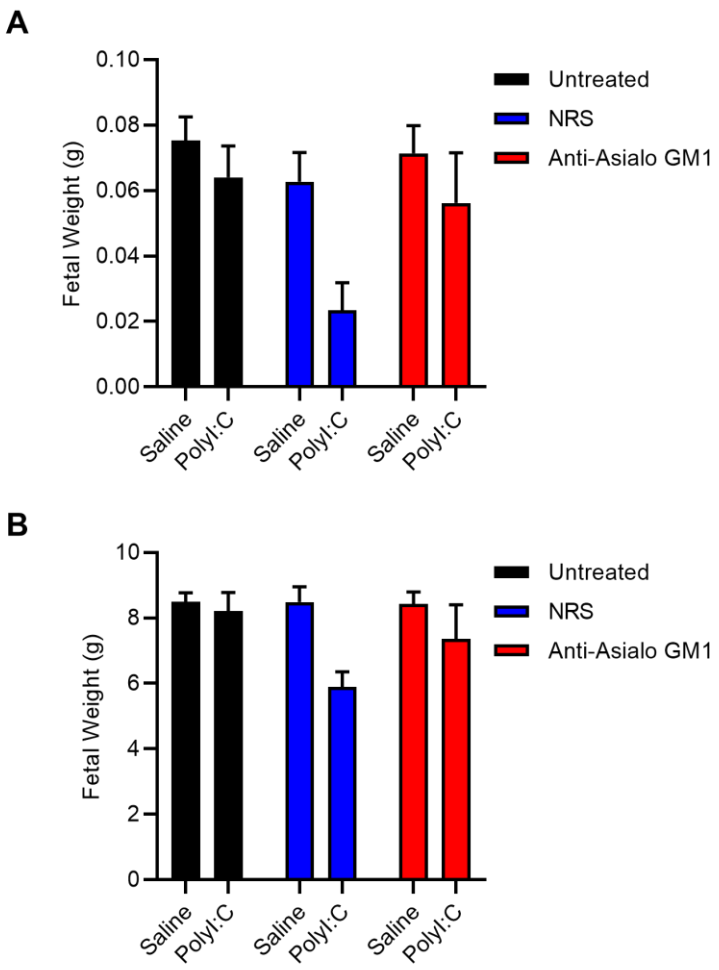

Figure A-1: Fetal weight and length following immunodepletion with anti-asialo GM1 and treatment with PolyI:C or saline.

When treated with PolyI:C, fetuses collected untreated animals show a significant decrease in fetal weight and length. Normal rabbit serum (NRS) exposure, a control for anti-asialo GM1 treatment resulted in a more severe growth restricted phenotype in pups compared to both untreated and anti-asialo GM1 litters. Results represented as means ± SEM (N = 3).

Appendix B

Appendix B: MIA in *IL15Δ/Δ* rats.

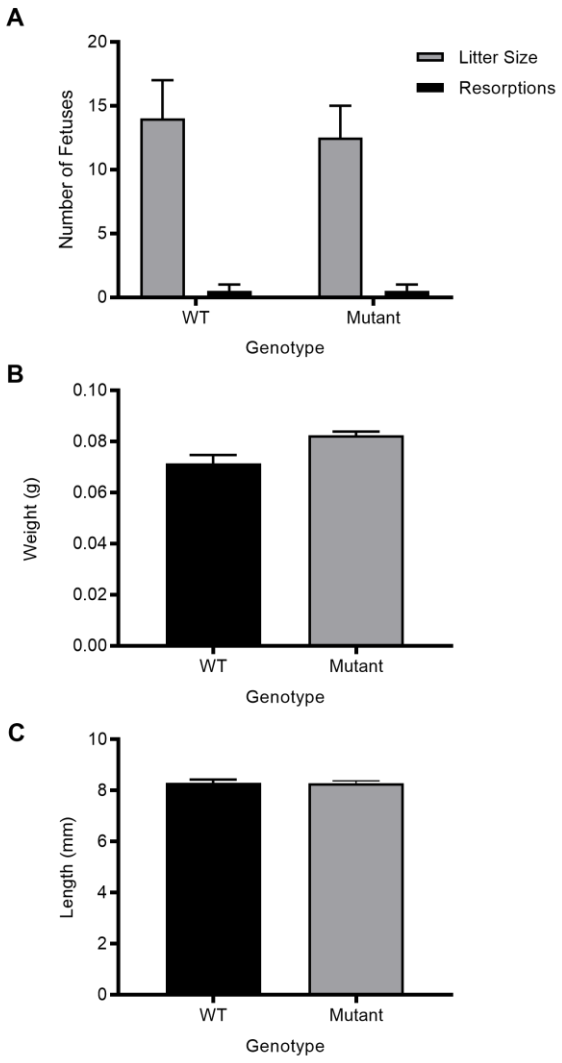

Figure B-1: Comparison of fetal development at mid-gestation in *IL15Δ/Δ* animals.

(A) Litter sizes and number of resorptions collected from GD 13.5 WT or *IL15Δ/Δ* dams had no significant differences. Both fetal weights (B) and fetal length (C) were not significantly different between genotypes. Results represented as means ± SEM (N = 3).

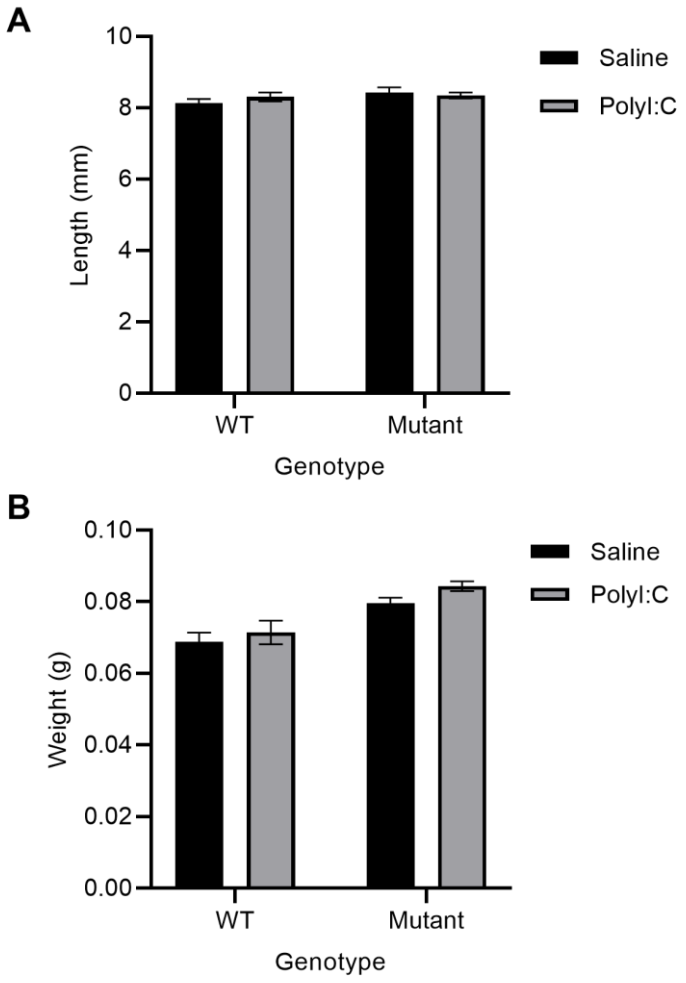

Figure B-2: Effect of early-gestation PolyI:C exposure on fetal growth in *1L15Δ/Δ* transgenic animals.

Fetal length (A) and fetal weight (B) at GD 13.5 in SDH pups showed no impairments following maternal PolyI:C exposure as compared to saline controls in either genotype. Results represented as means ± SEM (N = 3).

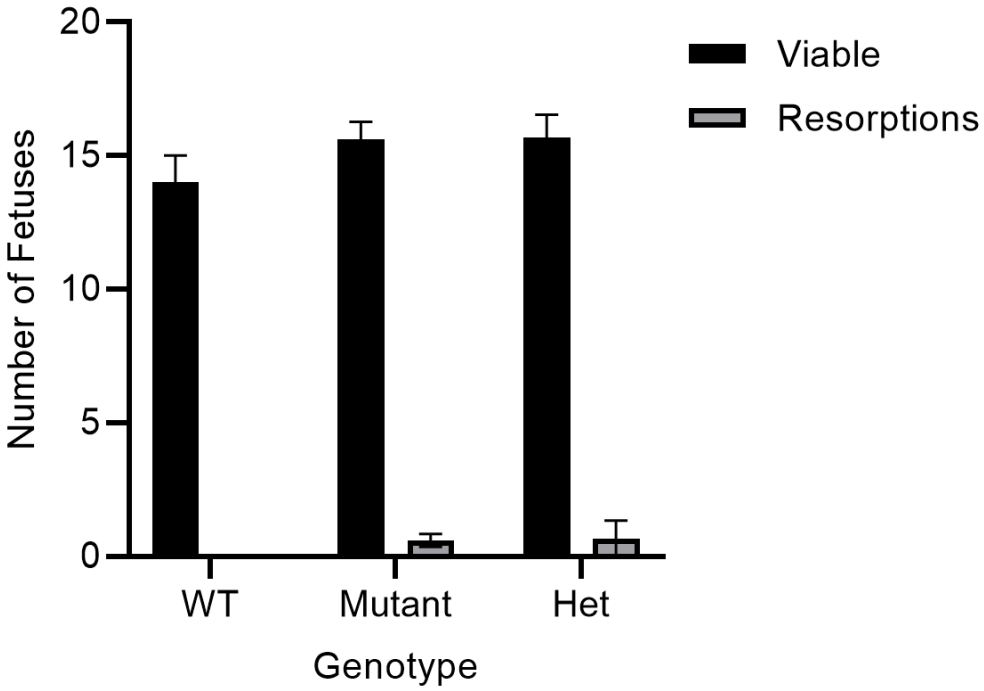

Figure B-3: LPS does not affect fetal viability between maternal *IL-15* genotypes.

Maternal treatment with 50ng/mL of LPS did not affect viability or the number of resorptions despite maternal genotype. Results represented as means ± SEM (N = 3).

Appendix C

Appendix C: Animal ethics approval

AUP Number: 2019-060
PI Name: Renaud, Stephen
AUP Title: Investigating the Role of Immune Cells in Rat Pregnancy
Approval Date: 09/01/2019

Official Notice of Animal Care Committee (ACC) Approval:
Your new Animal Use Protocol (AUP) 2019-060:1: entitled " Investigating the Role of Immune Cells in Rat Pregnancy"
has been APPROVED by the Animal Care Committee of the University Council on Animal Care. This approval, although valid for up to four years, is subject to annual Protocol Renewal.

Prior to commencing animal work, please review your AUP with your research team to ensure full understanding by everyone listed within this AUP.

As per your declaration within this approved AUP, you are obligated to ensure that:

1) Animals used in this research project will be cared for in alignment with:
   a) Western's Senate MAPPs 7.12, 7.10, and 7.15
   http://www.uwo.ca/univsec/policies_procedures/research.html

   b) University Council on Animal Care Policies and related Animal Care Committee procedures
   http://uwo.ca/research/services/animalethics/animal_care_and_use_policies.htm

2) As per UCAC's Animal Use Protocols Policy,
   a) this AUP accurately represents intended animal use;
   b) external approvals associated with this AUP, including permits and scientific/departmental peer approvals, are complete and accurate;
   c) any divergence from this AUP will not be undertaken until the related Protocol Modification is approved by the ACC; and
   d) AUP form submissions - Annual Protocol Renewals and Full AUP Renewals - will be submitted and attended to within timeframes outlined by the ACC.
   e) http://uwo.ca/research/services/animalethics/animal_use_protocols.html

3) As per MAPP 7.10 all individuals listed within this AUP as having any hands-on animal contact will
   a) be made familiar with and have direct access to this AUP;
   b) complete all required CCAC mandatory training (training@uwo.ca); and
   c) be overseen by me to ensure appropriate care and use of animals.

4) As per MAPP 7.15,

   a) Practice will align with approved AUP elements;
   b) Unrestricted access to all animal areas will be given to ACVS Veterinarians and ACC Leaders;
   c) UCAC policies and related ACC procedures will be followed, including but not limited to:
      i) Research Animal Procurement
      ii) Animal Care and Use Records
      iii) Sick Animal Response
      iv) Continuing Care Visits

5) As per institutional OH&S policies, all individuals listed within this AUP who will be using or potentially exposed to
hazardous materials will have completed in advance the appropriate institutional OH&S training, facility-level training, and reviewed related (M)SDS Sheets,
http://www.uwo.ca/hr/learning/required/index.html

Curriculum Vitae

Name: Kelly Baines

Post-secondary Education and Degrees:
Trent University
Peterborough, Ontario, Canada
2011-2015 B.Sc.

Honours and Awards:
Province of Ontario Graduate Scholarship
2017-2018

CHRI Quality of Life Fellowship
2016-2017, 2018-2019

Related Work Experience
Academic Assistant
Trent University
2015

Teaching Assistant
The University of Western Ontario
2015-2020

Publications:

5. Roberts H, Woodman AG, **Baines KJ**, Jeyarajah MJ, Bourque SL, Renaud SJ. (2021). Maternal iron deficiency alters trophoblast differentiation and placental development in rat pregnancy. Endocrinology.

4. Haddad FL, Lu L, **Baines KJ**, Schmid S. (2020). Sensory filtering disruption caused by poly I:C - timing of exposure and other experimental considerations. Brain, Behavior, & Immunity - Health.

3. **Baines KJ**, Hillier DM, Haddad FL, Rajakumar N, Schmid S, Renaud SJ. (2020). Maternal immune activation alters fetal brain development and enhances proliferation of neural precursor cells in rats. Front Immunol.

2. **Baines KJ**, Rampersaud AM, Hillier DM, Jeyarajah MJ, Grafham GK, Eastabrook G, Lacefield JC, Renaud SJ. (2020). Antiviral Inflammation during Early Pregnancy Reduces Placental and Fetal Growth Trajectories. J Immunol.

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