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NEUTROPHIL PRODUCTS CONTROL THE EXPRESSION OF PROGESTERONE RECEPTORS AND MATRIX METALLOPROTEINASE-1 IN THE DECIDUAL AND MYOMETRIUM AND ARE POSSIBLE REGULATORS OF PREMATURE LABOR

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

by

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List of Abbreviations

17P	17 alpha-hydroxyprogesterone caproate
2-ME	2-Mercaptoethanol
5-AZA	5-aza-2'-deoxycytidine
cAMP	Cyclic Adenosine Monophosphate
Ct	Cycle Threshold
Cx43	Connexin43
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic Acid
ER	Endoplasmic Reticulum
FBS	fetal bovine serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	granulocyte-macrophage colony-stimulating factor
HOX	Homeobox
hTERT	Human Telomerase Reverse Transcriptase
HX	Hypoxanthine
IL	Interleukin
IP3	Inositol 1,4,5-trisphosphate
MLCK	Myosin Light Chain Kinase
MMP	Matrix Metalloproteinase

NFκB	Nuclear Factor κ B
NSAID	Non-steroidal anti-inflammatory drug
OT	Oxytocin
PAR	Protease Activated Receptor
PBS	Phosphate Buffered Saline
PG	Prostaglandin
PKA	Protein Kinase A
PLC	Phospholipase C
PPROM	Preterm Premature Rupture of Membranes
PR	Progesterone Receptor
PR-A/B	Progesterone Receptor Ratio A/B
PR-A+B	Progesterone Receptor Primer A+B
PTL	Preterm Labor
qRT-PCR	Quantitative Reverse Transcriptase – Polymerase Chain Reaction
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
TL	Term Labor
TNF	Tumor Necrosis Factor
TNL	Term Not in Labor
XO	Xanthine Oxidase

Abstract

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Neutrophils infiltrate myometrium and decidual tissue prior to parturition. Activated neutrophils release reactive oxygen species (ROS) and tumor necrosis factor α (TNFα), which might increase expression of pro-labor genes such as matrix metalloproteinase-1 (MMP-1), progesterone receptor (PR) A/B ratio, and cause demethylation of DNA. These changes might cause labor.

Decidual tissue was obtained from consented, healthy women at term (37+ weeks of gestation) not in labor (no contractions, without cervical effacement), term labor and preterm labor (under 37 weeks of pregnancy). Decidual and myometrial cells in culture

were treated with (1) ROS, (2) TNFα, or (3) 5-aza-2'-deoxycytidine. Total RNA was extracted, converted to cDNA and evaluated by qRT-PCR for MMP-1, PR-A+B and PR-B.

TNFα increased MMP-1 by 17 fold in decidual cells and more than 12 fold in myometrial cells. PR-A/B was increased by 5.6 fold in decidua. ROS up-regulated MMP-1 by 6 fold and elevated the PR-A/B ratio by 4.5 fold in decidual tissue. DNA demethylation increased MMP-1 by about 4 and 11 fold in decidual and myometrium, respectively. The PR-A/B ratio was increased by 4 fold in decidua and the PR-B was decreased by 40% in the myometrium due to DNA demethylation. Decidual tissue in preterm labor showed a 7-fold increase in MMP-1 over term laboring and over a 15-fold increase over term not in labor tissue.

In conclusion, MMP-1 expression and PR-A/B ratio was increased by neutrophil products possibly through a mechanism of DNA methylation in decidua and myometrium. Preterm decidua showed a dramatic increase in MMP-1 over normal labor tissue. TNFα and ROS increased expression of MMP-1 to possibly initiate parturition. These data might help explain mechanisms responsible for preterm labor unrelated to infection or premature rupture of membranes.

CHAPTER 1: Introduction

A. General Background

Normal birth takes place after the 37th week of the baby's growth. Preterm birth is defined as the delivery of a baby before 37 weeks of gestation. According to the latest statistics compiled in 2006, 12.8% of all pregnancies within the United States end up with the baby being born premature [1]. In other words, out of a total of 4,265,555 births, 1 in 8, or almost 550,000 babies are born preterm [1]. Minority women are at an increased risk for preterm labor (PTL), with a rate of 18.5% for African Americans and 12.2% for Hispanics compared to 11.7% for Caucasian [2, 3]. Not only is preterm birth readily prevalent within the population, the percentage of babies delivered before 37 weeks has risen over 36% since the early 1980s and efforts to curb the rate of prematurity have been unsuccessful [1]. Children who are born premature are also in need of extensive post natal care and may suffer from a number of problems such as cognitive and behavioral abnormalities, cerebral palsy, problems with vision and hearing and emotional disturbances, among other things [4]. Additionally, over 75% of post natal deaths are the result of preterm delivery [4]. The toll of premature birth is both emotionally and financially expensive, resulting in loss of economic productivity for mothers due to bed rest and hospitalization, the expense in diagnosing and treating mothers and the long term costs associated with health care, child care and education for low birth weight children.

The financial costs range into billions of dollars [5]. Premature delivery is the result of changes in the myometrium and cervix, which allow for the expulsion of the fetus. It is associated with a host of known risk factors such as tobacco and drug use, low socioeconomic class, inadequate access to prenatal care and poor nutrition [6]. A number of pathways responsible for early parturition are well studied and substantiated by clinical and experimental research. These include infection and/or inflammation of the genital tract, urinary tract or fetal membranes, myometrial tissue and fetal membrane overstretching, placental abruption [7] and maternal and fetal stress [8]. Activation of such mechanisms may occur over months, weeks or days either individually, as with women carrying multiple babies, or in concert with one another, such as with unexplained bleeding accompanied by inflammation [7]. Predictors of preterm birth remain sparse. These include measurements of fibronectin levels in vaginal fluid and cervical ultrasound to establish cervical dilation, shortening and ripening which are sometimes useful in predicting who is at acute risk for PTL [7]. Women who have had prior PTL are at greatest risk for future preterm delivery, yet this is only a small subset of pregnancies which will end delivery prior to 37 weeks of gestation; a full 45% of all PTL is spontaneous and of an unknown etiology [9]. No tests or procedures exist for evaluating women who might be at risk for PTL in the future.

As seen with the consistent rise in PTL in the United States, treatment options for women at risk and in the midst of early labor are limited. Studies on the efficacy of tocolytic agents have shown that despite prolonging labor, neonatal outcomes have not improved [10]. Current tocolytic agents include prostaglandin (PG) synthetase inhibitors, betamimetics and calcium channel blockers. Because a large portion of PTL is associated

with infection and inflammation, PG synthetase inhibitors (NSAIDs) are widely used. NSAIDs such as indomethacin have a low incidence of maternal side-effects but are associated with serious fetal and neonatal complications such as oligohydramnios and constriction of the ductus arteriosus which may result in hypertrophy of fetal pulmonary vasculature [10]. Betamimetics function by increasing myometrial concentrations of cAMP which in turn lowers calcium levels within the cell and also diminish actin-myosin sensitivity to calcium [10]. These β-adrenergic receptor agonists are associated with several severe maternal side effects such as pulmonary edema, arrhythmia and even death [10, 11]. Calcium channel blockers are also increasingly used to prolong labor. These drugs work by decreasing calcium influx into myometrial cells and thereby reducing smooth muscle tone of the tissue [12]. In most cases, all of these drugs do not prolong labor for more than 48 hours and their extended use is associated with a much higher incidence of side effects, both for the mother and baby [10]. Additionally, tocolytics are counterindicated for many frequent PTL causes such as placental abruption, chorionamnionitis and fetal distress [11]. The utility of these medications lies in providing a window of opportunity to administer corticosteroids, to stimulate fetal lung development and to transfer the mother and baby to a location with a neonatal intensive care unit.

Recently, 17 alpha-hydroxyprogesterone caproate (17P) treatment, a naturally occurring metabolite of the hormone progesterone has been proposed as a prophylactic medication in certain women who already experienced prior preterm birth [13]. Progesterone maintains uterine quiescence by serving as a potent anti-inflammatory agent and inhibitor of oxytocin (OT) action by mediating both nuclear and non-genomic pathways [14]. 17P is applied as a cream directly onto the cervix, being a highly lipid

soluble molecule it can therefore effect the most critical tissues involved in pregnancy, mainly limiting cervical effacement and myometrial contractility [14]. Additionally, a recent review of six studies on progesterone use to prevent PTL by Rode and colleagues concluded that for women with singleton pregnancy and prior preterm delivery, progesterone treatment lowered preterm delivery before 32 weeks, reduced postnatal death and diminished certain complications in infants [4]. However, further studies are needed to support wide spread recommendation of this therapy to more women.

Early labor can be the result of an aberrant mechanism of parturition or activation of an abnormal pathway and characterized by an anomaly of uterine quiescence and/or the stimulation of contractile processes. In humans, parturition can be initiated through functional progesterone withdraw [15], activation of inflammatory processes [16] and via thrombin activation of protease activated receptor – 1 (PAR1) in the myometrium [17].

B. Functional Progesterone Withdraw

During pregnancy, progesterone is a key element maintaining the uterus in a relaxed state until term [18]. It suppresses uterine contractility by decreasing the expression of the oxytocin and prostaglandin receptors [19]. Another key role of progesterone is to reduce the translocation and assembly of Connexin43 (Cx43). Cx43 is a gap junction myometrial protein necessary to synchronize uterine contraction within the myometrium [19]. Progesterone also acts to supplement the effectiveness of protein kinase A (PKA) to inhibit phospholipase C (PLC), and therefore limit the levels of Ca^{2+} available for contraction within the smooth muscle of the uterus [19]. Progesterone inhibits matrix

metalloproteinase (MMP) 1 and 3 expression in decidual cells, which are collagenases responsible for weakening the cervix and causing rupture of the fetal membranes. In this way progesterone also protects against preterm birth [16]. Progesterone is essential for maintaining uterine quiescence because the drug RU486 (Mifepristone), a progesterone antagonist, can trigger parturition at any point during pregnancy [20].

In humans, the placenta serves as the largest generator of progesterone while for other animals the corpus luteum of the ovary is the principal source of the hormone. Many animals experience labor with a preceeding drop in circulating progesterone levels. For example, some species undergo a decrease in placental progesterone secretions while other species rely on the regression of the corpus luteum to decrease overall progesterone available in the circulation and consequently the myometrial tissue [18]. In higher level primates and in humans, levels of progesterone remain high until the placenta is expelled. Therefore, labor is not initiated by a drop in circulating progesterone [18].

One of the best-studied mechanisms to explain functional progesterone withdrawal in humans has been the altered expression of inhibitory receptor types, especially as parturition is approached and labor begins. The progesterone receptor exists in two major subtypes: the inhibitory form (PR-A) and the activator of most progesterone response genes (PR-B). Both receptors bind progesterone with equal affinity and produce two distinct mRNA transcripts [21]. PR-B is longer by 164 amino acids at its amino terminus and has its own, distinct promoter region and activation factors [22]. Although PR-A is known to influence the transcription of some genes, it also functions to sequester available progesterone away from PR-B and even act as a trans-dominant inhibitor [23].

During normal pregnancy, the expression of PR-A is barely detectable before labor [24]. In order to have a significant reduction in progesterone response, the levels of PR-A must be much higher than that of PR-B [21]. With labor, Pieber et. al. showed that PR-A is found in human myometrium cells only after the onset of labor [24]. A recent review by Messiano and colleagues demonstrated that non-laboring samples of myometrial tissue showed almost a 1:1 ratio of PR-A/B [22]. The following review also established that during labor, this ratio can increase over 2:1 [22]. In a separate study, Messiano et. al. was able to confirm the increase in the ratio of PR-A/B associated with labor demonstrated an analogous increase in expression of estrogen receptor-α and the Homeobox gene HOXA10, both of with are normally down-regulated by progesterone in the myometrium[22, 25]. Furthermore, equivalent changes have been observed in fetal membranes and decidual tissue with significant increases in PR-A over PR-B for women in labor compared to those not in labor [26].

C. Inflammatory Processes and Labor

Inflammatory processes are some of the first hallmarks of labor and may possibly instigate other more profound changes associated with parturition [20]. Thompson et. al. used immunohistochemistry to illustrate that neutrophils and macrophages are the predominant cells infiltrating the myometrium at term [27]. Leukocytes are also found in great density within decidual and fetal membrane tissue [28]. Analysis of samples from term in labor women has shown the presence of neutrophil specific recruitment and activation factors [29]. Not only are neutrophils found in great numbers in myometrial,

decidual and cervical tissue, but these cells are the primary source of tumor necrosis factor α (TNFα) and other inflammatory cytokines, such as interleukin (IL) -1β, IL-6 and IL-8 during spontaneous labor compared to non-laboring tissue samples [30]. Immune cells of women in labor also produce other inflammatory factors such as reactive oxygen species (ROS) which can oxidize lipids, alter protein function and change DNA by modifying bases, shifting the deoxyribose backbone, and cross-linking to other molecules and in general modify cell function [31]. As inflammation drives the production of PGs and their analogues, progesterone withdraw can be initiated through a mechanism of nuclear factor (NF) κ B regulation to increase the inhibitory form of the PR [32, 33]. Inflammatory cytokines have been known to induce the expression of MMP genes and proteins, serum PG concentrations and decrease collagen synthesis [34]. The increase of inflammatory cytokines with infiltrating neutrophils and in decidual tissue result in an enhanced inflammatory response by recruiting and activating more neutrophils, softening and dilation of the cervix and weakening the fetal membranes [16, 35]. Studies in women experiencing PTL show increased sensitivity to inflammatory signals and up-regulation of many pro-labor genes such as MMPs, PGs and a number of ILs [16].

D. Thrombin and Preterm Labor

It has long been known that placental abruption, a premature separation of the placenta before delivery, is associated with spontaneous labor. Fareed et. al. first identified thrombin as the principal initiator of uterine contractility [17]. Thrombin is best known as a coagulation protein present in the blood and a key enzyme involved in

haemostasis [36]. It is a serine protease which is also capable of catalyzing many other reactions via its interaction with a set of proteinase-activated receptors (PAR) [37]. PARs belong to a family of membrane G-protein coupled receptors with 7 transmembrane domains [37]. Thrombin is capable of cleaving a specific site on the extracellular N-terminus of the PAR, allowing the new amino terminus to act as a transactivator of the receptor in order to initiate numerous intracellular signaling events [38].

In the case of smooth muscle myometrial cells, Elovitz et. al. has shown that thrombin is capable of eliciting phasic uterine contractions via thrombin activation of the phosphatidylinositol signaling pathway [36]. Once the receptor is activated, the coupled G-protein complex stimulates phosphoinositide-specific phospholipase C (PLC) which hydrolyzes phosphatidylinositol 4,5-bisphosphate releasing inositol 1,4,5-trisphosphate (IP3) which turns on IP3 mediated channels in the endoplasmic reticulum causing a release of sequestered calcium stores [36]. Additional calcium can enter the cytosol via ryanodine receptors on the ER and L-type calcium channels [36]. Cycles of emptying and refilling of ER calcium stores and periodic extracellular influx of Ca^{2+} produces episodic activation of calmodulin, myosin light chain kinase (MLCK) and actin and myosin creating phasic uterine contractions [36]. O'Sullivan et. al. has shown that thrombin uterotonic effects are mediated by specific activation of PAR1 in human tissue, confirming earlier studies done in the rat myometrium [39, 40]. Shintani et. al. has demonstrated that thrombin's enhanced effects on intracellular calcium influx in pregnant rats compared to non-pregnant animals is the result of PAR up-regulation in the myometrium [41]. Recent findings by O'Brien et. al. have confirmed the findings of Shintani and colleagues in human myometrial tissue samples which also demonstrated an

increase in PAR1 during pregnancy and even greater up-regulation of the receptor with labor [42].

E. MMP-1 and Pregnancy

A number of MMPs have been identified as instrumental in the progression of normal labor in order to rupture fetal membranes and ripen the cervix to allow for birth [43]. Inflammatory cytokines, such as ILs and TNFα as well as ROS, are capable of enhancing MMP expression [44, 45]. MMP-1 is critical in digesting type I and III collagens [46] and is released by many different cell types, including smooth muscle tissue [47], fetal membranes [44, 48], decidual tissue [35] and the placenta [49]. Over-expression of MMP-1 in amniotic fluid has been associated with both preterm premature rupture of membranes (PPROM) and PTL [43, 46]. In 2005 Boire et. al. first described that MMP-1 is capable of acting as a PAR1 agonist in transformed breast cancer cells [50]. This was further confirmed, in breast cancer cells by Yang et. al. [51], in platelets by Trivedi et. al. [52] and endothelial cells by Goerge and colleagues [53]. MMP-1 cleaves the N-terminus of the PAR1 receptor at a site two amino acids upstream of the thrombin cleavage site, producing a longer tether, yet one that is still able to generate a PAR1 agonist [52]. The resulting activated PAR1 can act through $G_{\alpha/\beta}$, and in turn, Rho kinase [54]. Finally, Agarwal and colleagues were able to show that PAR1 cleavage by MMP-1 activates the same downstream effects as thrombin in tumor cells [55]. Since myometrial cells express PAR1, it is possible that MMP-1 might initiate labor contractions through a previously

unknown and unrecognized mechanism and could potentially serve as a key factor responsible for PTL.

F. Summary

A number of key interactions known to cause cervical ripening, rupture of the fetal membranes and myometrial contractions are represented in Figure 1. Neutrophil products are known to affect MMP-1 through activation of the mitogen-activated protein kinases (MAPK/MEK) as well as extracellular signal-regulated kinases (ERK) [56, 57]. However these effects have not been shown in either myometrial cells or decidual cells. Additionally, even though PR-A/B ratio is known to increase other pro-labor genes, the mechanisms responsible for affecting PR expression are not known.

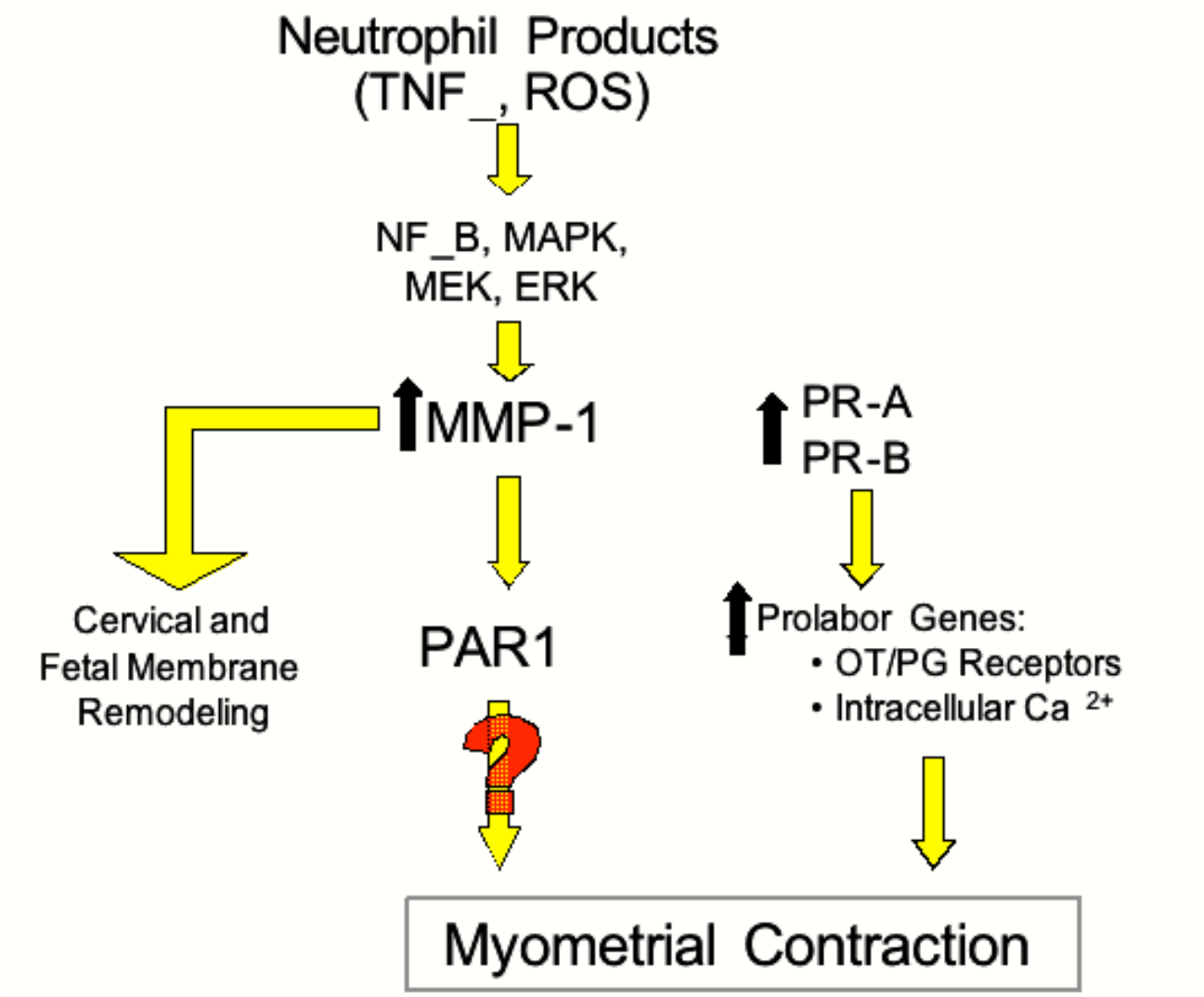

Figure 1: Neutrophil Products and PR Effects on Labor

G. Purpose of Study and Hypotheses

The goal of our study is to show that neutrophils and neutrophil products (ROS, TNFα) are capable of mimicking conditions present during preterm labor in decidua and myometirum. We hypothesize that: (1) MMP-1 will be up-regulated by neutrophils and neutrophil products in both myometrial and decidual cells; (2) The ratio of PR-A/PR-B will be increased by neutrophils, ROS and TNFα; (3) MMP-1 and PR-B will be epigenetically regulated; and (4) women in PTL will show an increase in MMP-1 expression over those in term labor (TL) and term not in labor (TNL).

CHAPTER 2: Materials and Methods

A. Chemicals

Phosphate buffered saline (PBS; Invitrogen, Grand Island, NY), fetal bovine serum (FBS; Invitrogen, Dulbecco's modified Eagle's medium: nutrient mixture F-12 (DMEM/F12; Invitrogen), antibiotic-antimycotic containing 10,000 units of penicillin, 10,000 μg of streptomycin, and 25 μg of amphotericin B/ml utilizing penicillin G (sodium salt), streptomycin sulfate, and amphotericin B as Fungizone Antimycotic in 0.85% saline (Invitrogen), Turbo DNAse (Ambion; Austin, TX), 2-mercaptoethanol (2-ME; Fisher Scientific, Fair Lawn, NJ), arachidonic acid (Cayman Chemical Co., Ann Arbor, MI), granulocyte-macrophage colony-stimulating factor (GM-CSF; R&D Systems, Minneapolis, MN), Histopaque 10771 and 11191 (Sigma-Aldrich, St. Louis, MO), cDNA synthesis kit (BioRad, Hercules, CA), Hypoxanthine (HX; Sigma-Aldrich), Xanthine Oxidase (XO; Calbiochem, La Jolla, CA), FujiFilm RNA cell and tissue extraction kit (Autogen, Holliston, MA), Trypan Blue 0.4% solution (Sigma-Aldrich), recombinant TNFα (R&D Systems), 5-aza-2'-deoxycytidine (5-AZA; Sigma-Aldrich), Potassium Chloride (KCl; Sigma-Aldrich), 100% ethanol (Pharmco-AAPER, Shelbyville, KY), blood collection tubes with 143 USP units of sodium heparin (BD Vacutainer, Franklin Lakes, NJ), Laboratory designed PCR Primers (Integrated DNA

Technologies, IDT, San Diego, CA), PAR1 Primer Set (SA Biosciences, Frederick, MD), qPCR SYBR Green Master Mix (SA Biosciences).

B. Equipment

Nano Drop 2000 (Thermo Scientific, Madison, WI), Bambino hybridization oven (Boekel Scientific, Feasterville, PA), QuickGene Mini-80 (AutoGen, Holliston, MA), Eppendorf Mastercycler ep Realplex real time PCR system (Westbury, NY), Agilen 2100 – Bioanalyzer (Santa Clara, CA), 96-well PCR plate (Applied Biosystems, Foster City, CA), PCR plate caps (Applied Biosystems).

C. Isolation of Decidual Tissue Samples

The placenta and accompanying fetal and maternal membranes were collected from PTL, TL and TNL pregnant women undergoing either vaginal or cesarean section deliveries at the Medical College of Virginia Hospital, Virginia Commonwealth University Health System. The PTL women included in the study were in active labor, based on the presence of uterine contractions and cervical effacement and gave birth between 24-37 weeks of pregnancy. Those in TL were experiencing active contractions with cervical effacement and were delivering over 37 weeks of gestation. Those at TNL were not in active labor, showing no cervical dilation and no contractions. Patients were excluded based on the following criteria: prior diagnosis of incompetent cervix, diabetes, preeclampsia, HIV or other active infection, carrying multiple fetuses, had fetuses with

congenital abnormalities or were under the age of 18. Consent was obtained prior to delivery and the study was approved by the Virginia Commonwealth University Office of Research Subjects Protection (VCU IRB #: HM 10906). Decidual tissue was collected by gentle scraping of the decidual cells from the chorion into a conical 50 ml tube filled with ice cold PBS. After collection, cells were centrifuged at 4° C and 300 x g for 10 minutes. PBS was aspirated and the tissue was washed with 25 ml ice cold PBS, briefly vortexed, and re-spun at 4° C and 300 x g for another 10 minutes. The remaining pellet contained both decidual tissue and residual erythrocytes. Contaminating red blood cells were lysed with 3 ml of ice cold ddH_2O followed by an addition of 1 ml of 0.6 M solution of KCl after 30 seconds to restore tonicity. The remaining re-suspension of tissue was centrifuged at 4° C and 300 x g for 4 minutes. The supernatant was removed and total RNA was extracted immediately.

D. Cell Culture

i. Myometrial Cell Line

A human telomerase reverse transcriptase (hTERT) immortalized human myometrial cell line was provided by R. Ann Word (Southwestern Medical Center at Dallas; Dallas, TX) (2002 – Condon Telomerase Paper). Cell culture was maintained in DMEM/F12 media containing 10% FBS, an antibiotic-atimicotic, and incubated at 37° C in 95% air and 5% CO_2. Media was replaced every 48 hours.

ii. Primary Decidual Cell Culture

Primary culture decidual cells were isolated with the method of Lockwood et. al. [58]. After decidual tissue was scraped from the maternal side of the fetal membranes, it was digested with a collagenase, treated with DNase three times and remaining cell clusters were dissociated by passing the mixture through a 25 gauge needle. The entire procedure was repeated twice more. The resulting cells were centrifuged in two different Percoll gradients and quantified. Cells were grown to confluence on culture dishes, harvested and evaluated for leukocyte contamination by flow cytometry with anti-CD45 and anti-CD14 antibodies. Cells were passaged three more times until leukocyte free (<1%). To make sure that cells were purely decidual and without trophoblast contamination, purified samples were evaluated by immunostaining for vimentin (positive) and cytokeratin (negative). All treatments were done on cells passaged a maximum of 6 times.

E. Cell Culture Treatment

i. ROS Treatment

Decidual cells were seeded at 250,000 per T-25 flask. Once cells reached 70% confluency a superoxide generating system of 0.05 mM HX and 0.003

units/ml XO in DMEM/F12 plus 10% FBS was added for 24 hours. After treatment, the media was aspirated and the cells were washed two times with 5 ml PBS, scraped and total RNA was extracted.

ii. TNFα treatment

Myometrial and decidual cells were seeded at 250,000 cells per T-25 flask with DMEM/F12 medium and 10% FBS. When cells reached about 70% confluency, a 1ng/ml concentration of TNFα was added with fresh media and 10% FBS. After 24 hours, media was removed and the cells were washed twice with 5 ml PBS, scraped and total RNA extracted.

iii. 5-AZA-treatment

Both primary culture decidual cells and hTERT myometrial cells were separately seeded into T-25 flasks with about 250,000 cells in each. Once the cells reached 30% confluency, the DMEM/F12 media with 10% FBS was refreshed with 5 μM 5-AZA and allowed to grow for two days. After 48 hours, the media was aspirated and the cells were washed two times with 5 ml PBS. Fresh media and 10% FBS was replenished and the cells were allowed to grow for another 24 hours before they were washed twice with 5 ml of PBS, scraped and collected for total RNA extraction.

iv. Neutrophil co-culture treatment

Twenty ml of blood was obtained from consented adults into two heparin treated tubes. Neutrophils were purified using the method first described by Boyum [59]. In brief, 10771 histopaque was layered on top of 11191 histopaque followed by a layer of 20 ml of whole blood in a conical tube. The tube was centrifuged at 700 x g at 25˚ C for 30 minutes. The upper layers of plasma, monocytes, lymphocytes and histopaque were aspirated and discarded. The granulocyte layer was transferred into a new tube, washed with PBS and centrifuged at 300 x g at 25˚ C for 10 minutes. The wash was then repeated. Remaining erythrocytes were lysed with ddH_2O and followed by re-suspension in 0.6 M KCl and the solution was centrifuged at 300 x g at 4˚ C for 4 minutes. The supernatant was discarded and the cell pellet resuspended in 1 ml DMEM/F12 media. Cells were counted using 50 μl of cell suspension in 350 μl of trypan blue on a hemocytometer. The remaining neutrophils were activated with 50 μM arachidonic acid and incubated for 30 minutes at 37˚ C in a bambino hybridization oven. Activated neutrophils were added to previously started cell culture of either decidual or myometrial cells which had been growing until 70% confluency in DMEM/F12 media with 10% FBS. Additionally, 5 ng/ml of GM-CSF was added to each flask to promote neutrophil adhesion and prevent apoptosis, as described by Stanford et. al. [60]. After 24 hours of incubation, the media was removed and cells were washed two times with PBS before total RNA was collected.

F. RNA extraction

Total RNA was purified using either the cell culture or tissue FujiFilm total RNA extraction kit and protocol on a QuickGene Mini-80 machine. For decidual tissue, 600 μl of lysis buffer containing a 1% solution of 2-ME was added to a 20 mg sample. The tissue was homogenized at the lowest speed for about 2-4 seconds with a rotor-stator homogenizer and then centrifuged at 16,000 x g for 3 minutes at room temperature. The supernatant (350 μl) was removed and used for total RNA extraction. Both myometrial and decidual cell culture samples were lysed in 350 μl lysis buffer and 1% 2-ME. A solubilization buffer included in the kit (50 μl) was added to each sample. The samples were vortexed for 15 seconds and briefly centrifuged. Pure ethanol was added (170 μl) to each sample and the samples vortexed for 1 minute briefly centrifuged. The solution was transferred to a sterile column and pressurized to allow the RNA to adhere to the filter. The samples were washed with the included wash solution (750 μl), and treated with Turbo DNAse for 5 minutes. The samples were then washed twice more with 750 μl of wash solution. An elution buffer included in the kit was added to the column (50 μl), left to incubate for 4 minutes at room temperature and eluted into sterile 1.5 ml collection tubes.

G. Quantitative Reverse Transcriptase – Polymerase Chain Reaction (qRT-PCR)

RNA integrity was determined via Agilent 2100 Bioanalyzer kit to verify the intactness of both the 18S and 28S rRNA bands. RNA was quantified using NanoDrop and converted into cDNA using a reverse transcriptase kit from BioRad. Primers of human PR-B and PR-A+B and the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from IDT, based on prior published sequences [25, 61]. PR-B primers were targeted for the unique 164 amino acid region present only in PR-B. PR A+B primers were targeted towards the common progesterone hormone binding site present in both PR-A and PR-B RNA.

MMP-1 primers were designed in our lab by Renato Capello, PhD. A commercially available primer for the PAR1 receptor was used. Plates were loaded with 10.5 μl of nuclease free water, 12.5 μl of master mix, 1 μl of diluted sample cDNA and 1 μl of equal parts forward and reverse primers in each well. Each sample was run in duplicate. The plate was centrifuged at 1500 x g at 4˚ C for 5 minutes to remove any remaining bubbles. qRT-PCR was performed by measuring SYBR green fluorescence. Cycling was monitored on an Eppendorf Mastercycler ep Realplex. Cycling conditions were 50˚ C for 2 min, 95˚ C for 10 min, and 40 cycles of 95˚ C for 15 sec, 60˚ C for 1 min. All primer sets were functional, producing single amplicons of the expected type, verified via a melting curve analysis of the PCR products run at the end of the cycles. Cycle threshold (Ct) values for each sample well were aligned so that the exponential increase in the abundance of the amplicon remained parallel for all samples. RNA abundance was expressed relative to the quantity of the constitutively expressed GAPDH transcripts and controls based on the ΔΔCt method. GAPDH Ct value replicates were averaged for each sample and subtracted from each control and experimental Ct value to

obtain a ΔCt. Then, each control ΔCt value was subtracted from the experimental ΔCt value to obtain a ΔΔCt. For calculating the expression of PR-A, the ΔCt values of PR-B were subtracted from the ΔCt values of PR-A+B for each control and experimental samples. The subsequent new ΔCt for PR-A was obtained. Subsequently the control ΔCt for PR-A was subtracted from the experimental treatment ΔCt value in order to obtain a new ΔΔCt value for PR-A. The fold changes in mRNA expression were compared to the control value and calculated by the following formula:

$$\text{FOLD CHANGE} = 2^{(-\Delta\Delta Ct)}$$

Table 1: qRT-PCR Primer sequences

Gene	Primer Sequence	Genbank Reference No.
PR A/B	F: 5'-AGCCCACAATACAGCTTCGAG-3'	NM000926
	R: 5'-TTTCGACCTCCAAGGACCAT-3'	
PR B	F: 5'-CCTGAAGTTTCGGCCATACCT-3'	NM000926
	R: 5'-AGCAGTCCGCTGTCCTTTTCT-3'	
MMP-1	F: 5'-GGGAGATCATCGGGACAACTC-3'	NM002421.2
	R: 5'-GGGCCTGGTTCAAAAGCAT-3'	
GAPDH	F: 5'-CAATGCCTCCTGCACCACCAA-3'	BC 020308
	R: 5'-GAGGCAGGGATGATGTTCTGGA-3'	

H. Statistical Analysis:

mRNA expression for MMP-1, PR-B and PR-A+B was evaluated for decidual tissue, primary culture decidual cells and hTERT transformed myometrial cells. The sample number for each treatment and cell type represents a separate T-25 flask. For cell culture analysis, statistical differences in mRNA expression were compared to an analogous control sample on the same PCR plate by a two tailed, unpaired t test. For

decidual tissue samples from TNL, TL and PTL women, statistical variation and significance was evaluated using a one way ANOVA. Statistical significance is reported as * $p < 0.05$.

CHAPTER 3: Results

A. Neutrophils, TNFα and ROS Effect on PR, MMP-1 and PAR1

TNFα is an inflammatory cytokine capable of activating mitogen-activated protein kinases and extracellular signal-regulated kinases [56, 57]. The ROS generating system used in our cell culture produces two superoxide molecules through the conversion of HX to xanthine and subsequently uric acid by XO. The superoxide anion is then catalyzed by extracellular superoxide dismutase (EC-SOD)[62] or spontaneously dismutates into hydrogen peroxide which can freely cross lipid membranes and activate varying signaling cascades through mitogen-activated protein kinases and NFκB [63, 64] as well as potentially cause changes in DNA methylation [65].

i. PR

A significant increase in PR-A was seen with TNFα treatment (Figure 3). This treatment showed an average increase of 2.6 ± 0.5 fold in 8 different flasks ($p<0.01$). The same treatment decreased the expression of PR-B by an average of 0.5 fold ± 0.06 in 10 different flasks ($p<0.0001$) (

Figure 4). The ratio increase in PR-A/B was 5.6-fold over normal controlled flasks.

ROS treatment in decidual cells produced an increase in the PR-A of an average 3.9 ± 1.3 fold in 6 flasks ($p < 0.05$) (

Figure 5). ROS did not significantly affect the expression of PR-B, as the decrease was an average of only 0.8 ± 0.1 in 8 samples and ($p<0.1$, Figure 6). In total, ROS increased the expression of the PR-A/B ratio by 4.5 fold over the control. The hTERT myometrial cell line produced similar results with TNFα treatment with an average decrease of expression for PR-B of 50% ± 0.05 in 5 samples, $p<0.001$ (Figure 7). Pursuasant to the literature, PR-A was not expressed in these cells[61]. Additionally, this particular cell line was extremely resistant to oxidative stress with concentrations as high as 1.6 mM HX and 0.01 units/ml XO not producing any induction in oxidative stress genes in the nuclear transcription factor κB (NFκB) family, such as NFκB1 or RELB. Concentrations of 1.9 mM HX and 0.01 units/ml XO produced cell death.

ii. MMP-1

A significant increase in MMP-1 was seen with treatment by TNFα and ROS in decidual cells. For TNFα 12 different flasks were treated and the mean fold increase was 17.0 ± 4.0, $p<0.001$ (Figure 8). ROS also produced significant increases in the expression of the MMP-1 gene in these cells with an average increase of 6 ± 1.3 fold in 10 samples, $p<0.01$ (Figure 9). In myometrial cells, similar increases with TNFα treatment were observed with an average fold increase of 12.4 ± 2.3, $p < 0.001$ (Figure 10).

iii. PAR1

hTERT myometrial cells were also screened for expression of PAR1 (Figure 11), although the expression of the thrombin receptor did not vary as a result of treatment, the gene was highly expressed with an average Ct value of 23.7.

B. Effect of DNA Demethylation on PR and MMP-1

5-AZA is a hypomethylating agent. The molecule itself is a modified cytosine which gets incorporated into cellular DNA upon cellular and consequently DNA replication [66]. The modified cytosine, prevents the action of a DNA-methyltransferase which inhibits methylation in previously hypermethylated regions of the DNA, making it easier for those DNA sequences to be transcribed [66].

i. PR

With 5-AZA treatment PR-A was increased in decidual cells by an average of 1.9 ± 0.5 for 6 samples ($p=0.2$) (Figure 12). PR-B was significantly decreased by an average of 0.5 ± 0.1, $p<0.01$ (Figure 13) in primary culture decidual cells. In total, with 5-AZA treatment, these changes reduced the PR-A/PR-B ratio by 4-fold compared to control. A similar reduction in the expression of PR-B was also observed in myometrial cells (

Figure 14) with a mean average decrease of 0.6 ± 0.2 fold in 6 treatment flasks (p=0.06).

ii. MMP-1

In primary culture decidual cells, 5-AZA treatment increased MMP-1 gene expression by an average of 3.8 ± 0.6 (p=0.0002) in 8 sample flasks (Figure 15). hTERT transformed myometrial cells also showed significant increase of MMP-1 expression with an average of 11.2 ± 3.3 in a sample of 9 (p=0.007) (Figure 16).

C. Decidual Tissue MMP-1 Expression

Decidual tissue from TL (n=12) women compared to TNL (n=13) patients showed over an 8.4 ± 2.6 fold increase in MMP-1 (Figure 17). Tissue from TL vs. PTL (n=10) women showed a 7 ± 3.4 fold increase in MMP-1 (Figure 17). Finally, when PTL was compared to TNL samples, women who were in PTL showed a 15.4 ± 2.5 fold increase (Figure 17)

CHAPTER 4: Discussion

A. Effects on PR-A and PR-B

The decidua and myometrium are invaded by neutrophils during labor. These white blood cells secrete a number of cytokines and signaling molecules, which could trigger labor. Increasing the PR-A/B ratio is a primary way to suppress uterine quiescence during pregnancy [15]. There are two ways to increase this ratio, either amplify the expression of the inhibitory isoform of the receptor (PR-A) and/or decrease the expression of the functional form, PR-B. Our experiments showed that in decidual cells neutrophil products (TNFα and ROS) were able to significantly magnify the ratio of PR-A/B. TNFα seems to work through the mechanism of both decreasing the functional PR-B, and increasing PR-A while leaving the total amount of PR-A+B the same as control (figure not shown). On the other hand, ROS significantly increased PR-A expression while leaving PR-B untouched similar to control. Therefore, in both conditions the overall ratio of PR-A/B increased 5.6-fold for TNFα and 4.5-fold for ROS. Similar data were seen for TNFα treatments in myometrial cells, with a significant down-regulation of the active form of the PR. Neutrophil products could be one of the primary mechanisms to alter this ratio, possibly through a mechanism of DNA de-methylation [65, 67]. Our experiments were able to show a down-regulation of PR-B in both the myometrium and decidua, as well as an upregulation of PR-A in the decidual. Decidual cells had a total increase of the PR-A/B ratio of about 4-fold over the control, far above

what is seen with previously published in vivo studies showing increases of about 2-fold within tissue collected at labor [22].

The alterations in the PR ratios can increase the expression of many pro-labor genes and likely affect a wide range of tissue types, not just decidua and myometrium. In the myometrial muscle, increases in the proliferation of the oxytocin receptors, greater translocation and assembly of the Cx43 gap junction protein and the enhanced function of PLC, allowing more Ca^{2+} to enter cells, all serve to begin and maintain contractions of the uterine smooth muscle tissue. Additionally, the decreased availability of the functional PR allows for an indirect increased expression of MMPs causing the cervix to weaken and soften, ultimately allowing the passage of the baby through the birth canal [16]. A drop in available progesterone in the decidua has also been shown to increase the production of MMPs [68]. The secreted MMPs will breakdown the maternal-fetal membranes and cause their rupture. Within both the decidua and myometrium, a lack of functional PR will cause an increased production of PGs [69]. PGs, in concert with other inflammatory cytokines produced within the invaded tissue by leukocytes, will promote inflammation and further recruitment of neutrophils, which will in turn release even more ROS and TNFα to accelerate and enhance the changes triggered by these cytokines.

Because even normal pregnancy ultimately follows inflammatory pathways, follow up experiments could focus on the potential of other inflammatory cytokines to initiate positive alterations of the PR-A/B ratio in both the decidua and myometrium. Finding out a more successful way to co-culture neutrophils along with both decidual and myometrial cells would help confirm the results observed with pure TNFα and ROS treatments. These results would also be more robust if these experiments were repeated

with decidual and myometrial cells from other TNL women. Further studies will also continue to obtain samples from TNL, TL, and PTL women to screen these samples for both PR-B and PR-A expression.

B. Alterations in MMP-1 Production

MMP-1 has a number of known regulatory elements within the promoter region, many of which can be induced with ROS and TNFα treatments [56]. One of the mechanisms with which this could occur is through regulating DNA methylation. As these experiments showed, MMP-1 is upregulated as a result of DNA de-methylation in both cell types studied. The decidua is situated between the myometrial smooth muscle tissue on one side and the chorion on the other side. Since MMP-1 is an extracellular collagenase and is a secreted molecule, by virtue of the anatomical position of the decidual tissue, MMP-1 is perfectly positioned to facilitate both the rupture of fetal membranes and the softening of the cervix, particularly because the myometrium itself is capable of increasing MMP-1 production. In vivo results confirmed the fact that MMP-1 plays a major role in PTL as we saw a tremendous up-regulation with preterm labor (15.4 fold) and term labor (8.4 fold) compared to term not in labor tissue. It is likely that neutrophil products can alter the normal expression of MMP-1 in both the decidua and myometrium, through known mechanisms as well as epigenetic modulation. Also, we predict that in the uterus, MMP-1 has the potential to affect myometrial contractility through its actions on the PAR1 receptor. Additionally, because the myometrium expresses PAR1 receptors, it is possible that in the upper uterine segments, those most

responsible for phasic uterine contractions, MMP-1 could be activating contractile mechanisms via PAR1 receptors, utilizing methods similar to those of thrombin. MMP-1 activated PAR1 receptors could potentially activate PLC to increase Ca^{2+} within the cell.

This area of research is new and could prove to be the most exciting. First, it would be valuable to link MMP-1 gene expression results with protein expression. Future work could explore whether MMP-1 can cause myometrial contraction. MMP-1 activation of Ca^{2+} enhancing mechanisms within the myometrium could also be observed by measuring Ca^{2+} influx and whether or not these results are mediated through PLC activation and ultimately PAR1 activation. Further linking PTL with the state of MMP-1 gene methylation, compared to TL and TNL, would also be an interesting avenue of exploration. The confirmation of these studies in human myometrium strips and tissue would be crucial.

C. Epigenetic Modification of MMP-1 and PR

One of the primary predictors of preterm birth is a prior history of PTL, either a mother's previous preterm delivery or within the family. Our results showed that MMP-1 and PR are likely modified through DNA methylation in both the myometrium and the decidua. These findings could have significant implications for women living in poor environmental conditions because DNA methylation can be modified by exposure to toxic chemicals, pollution, poor nutrition, drugs, tobacco and alcohol [70, 71]. An already existent condition of de-methylation could cause an increase in the PR-A/B ratio. This would leave the woman vulnerable to further alterations within this receptor and help

explain some of the causes of PTL. Additionally, the situation is similar to MMP-1, with an already increased expression of the gene due to DNA de-methylation could leave the woman vulnerable to early membrane rupture, cervical dilation and possibly increased preterm contractions of the uterus. Because methylation states of the genes are inherited from the parents, even if a mother shows no prior risk factors for PTL, she could still be at potential risk for preterm parturition. Women exposed to such conditions could be putting their future fertility at risk and passing on their modified genes to their offspring because epigenetic modifications can be inherited.

In the future, tissue samples from women who had experienced preterm premature rupture of membranes could be screened for the state of DNA methylation within the MMP-1 and PR genes. It is also possible that assessing the state of DNA methylation of the parents could one day serve as a screen for risk of PTL in pregnant mothers.

D. Conclusions

The contributions of our study are summarized in Figure 2. We were able to show that MMP-1 and PR-A/B were up-regulated with two key neutrophil products (TNFα and ROS) in the decidua and myometrium through a possible mechanism of DNA demethylation. PTL tissue showed an increase in MMP-1 and could possibly cause myometrial contraction through MMP-1 action on the PAR1 receptor in uterine smooth muscle tissue.

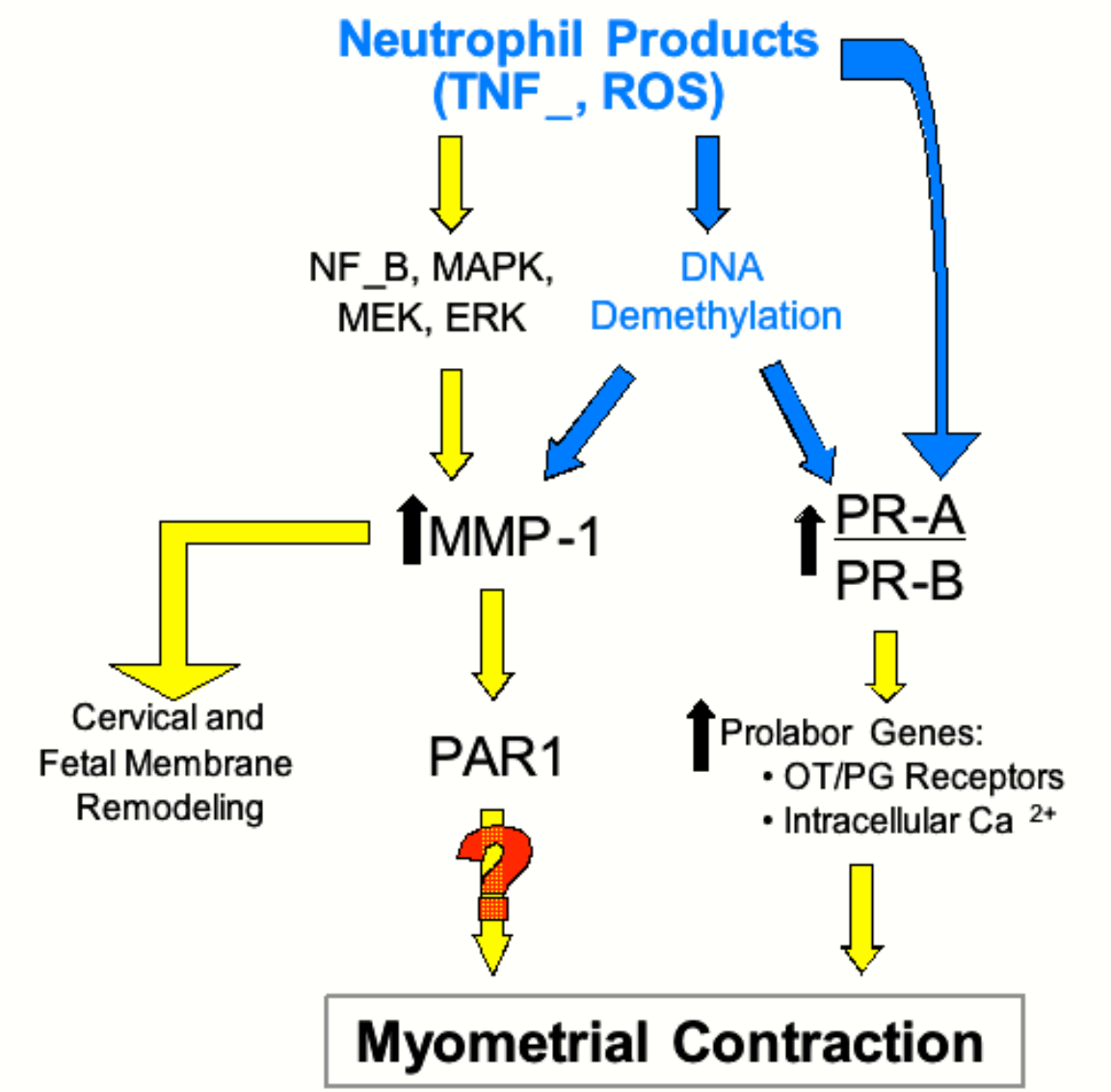

Figure 2: Neutrophil Products and DNA Methylation Effect on PR and MMP-1

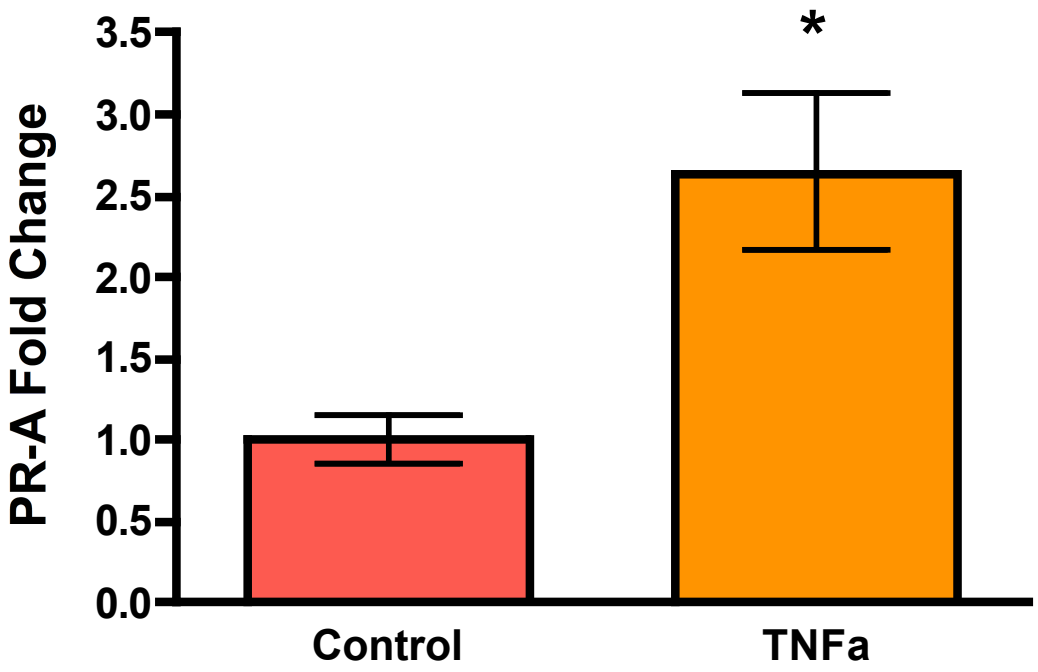

Figure 3: Fold Changes in PR-A Due to TNFα in Primary Culture Decidual Cells
Gene expression of PR-A increased significantly with TNFα treatment. ($p<0.01$, $n=8$, mean = 2.6 ± .5)

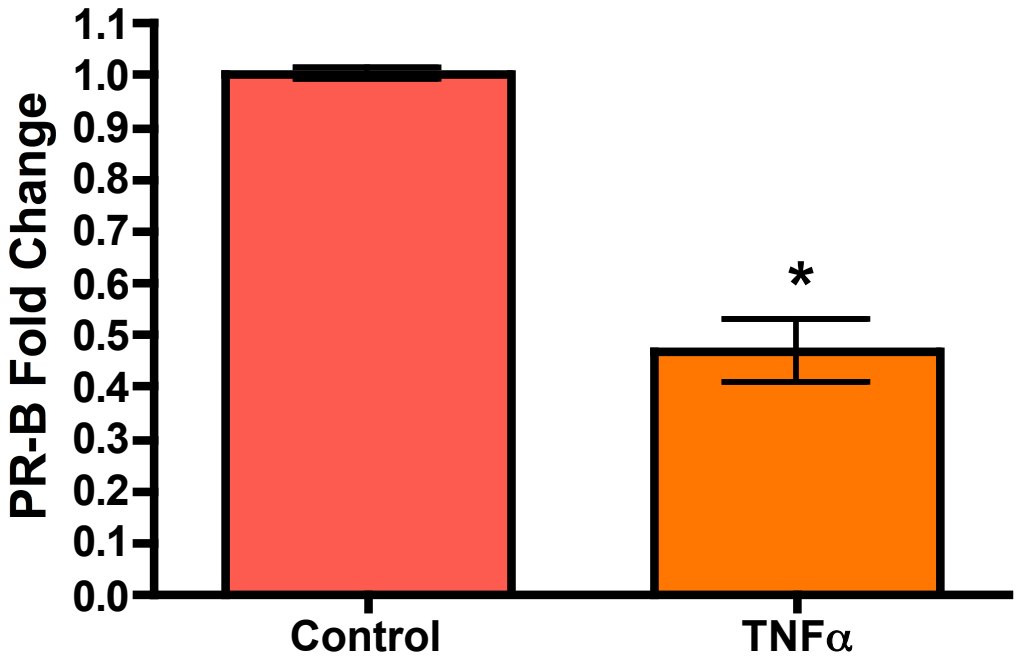

Figure 4: Fold Change in PR-B Due to TNFα in Primary Culture Decidual Cells
Gene expression of PR-B decreased significantly with TNFα treatment. ($p<0.0001$, $n=10$, mean 0.5 ± 0.06). As a result of TNFα treatment, the ratio of PR-A/B increased 5.6-fold.

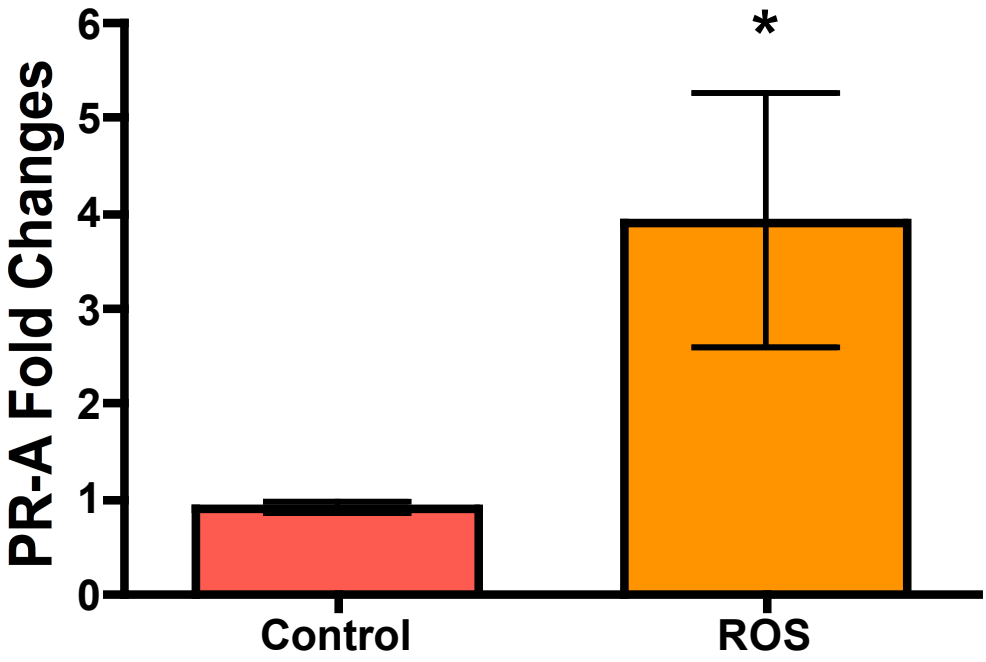

Figure 5: Fold Changes in PR-A Due to ROS in Primary Culture Decidual Cells
Gene expression of PR-A increased significantly with ROS treatment. ($p<0.05$, $n=6$, mean 3.9 ± 1.3).

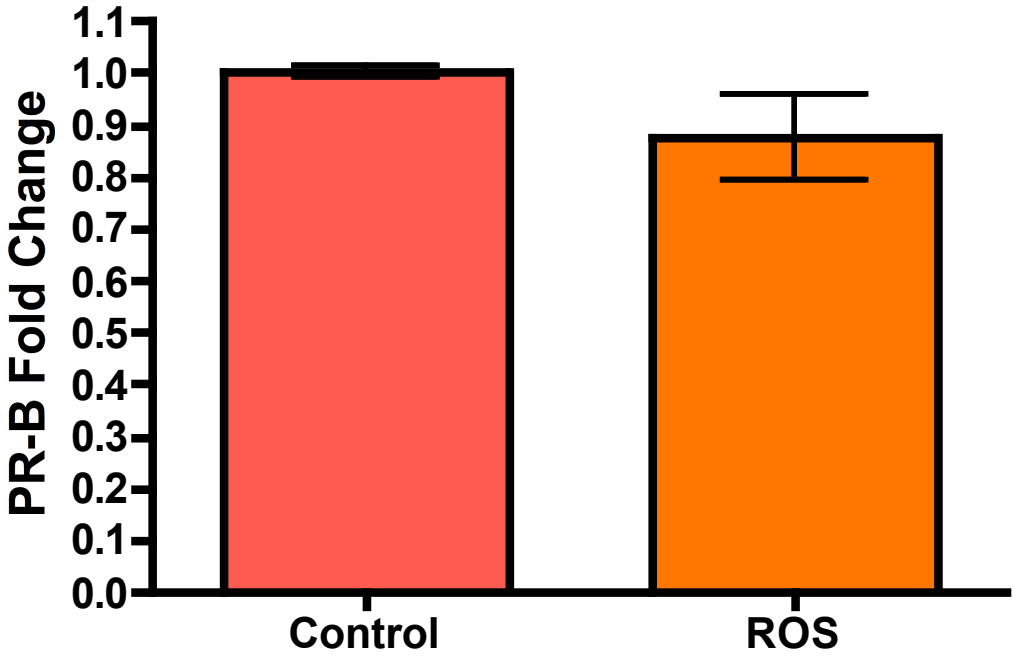

Figure 6: Fold Changes in PR-B Due to ROS in Primary Culture Decidual Cells
Gene expression of PR-B decreased with ROS treatment. ($p<0.1$, $n=8$, mean 0.8 ± 0.1). As a result of ROS treatment, the ratio of PR-A/B increased 4.5-fold.

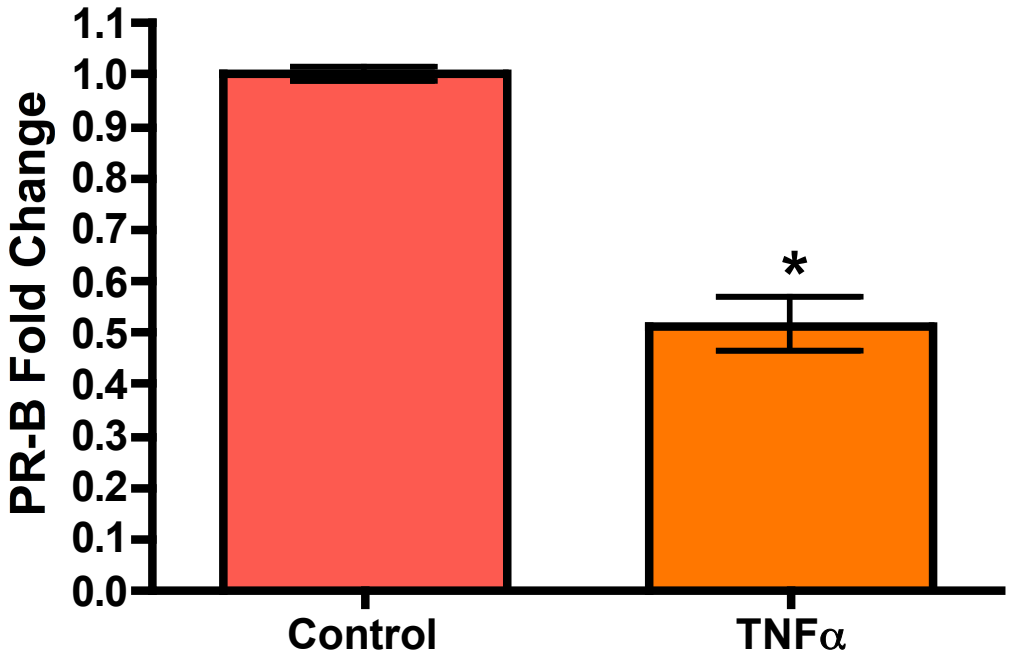

Figure 7: Fold Changes in PR-B Due to TNFα in hTERT Myometrial Cells
PR-B gene expression was significantly decreased by almost 50% with TNFα treatment in an hTERT transformed myometrial cell line. ($p < 0.001$, n=5, mean=0.5 ± 0.05)

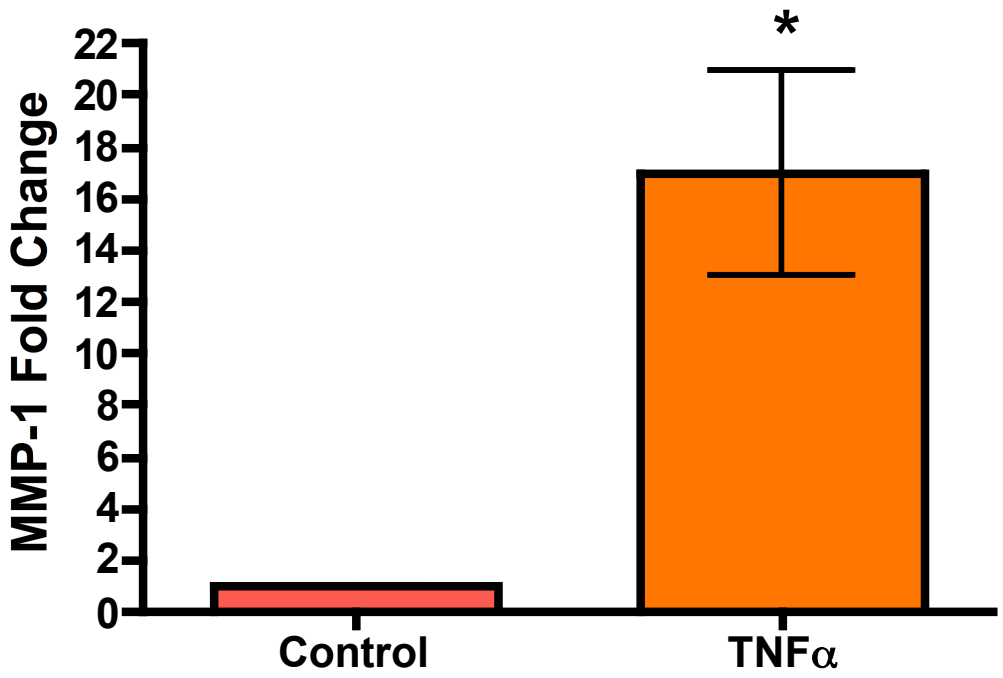

Figure 8: Fold Changes in MMP-1 Due to TNFα in Primary Culture Decidual Cells
TNFα treatment significantly increased gene expression of MMP-1 by over 16 fold in primary culture of decidual cells. ($p < 0.001$, n=12, mean=17.0 ± 4.0)

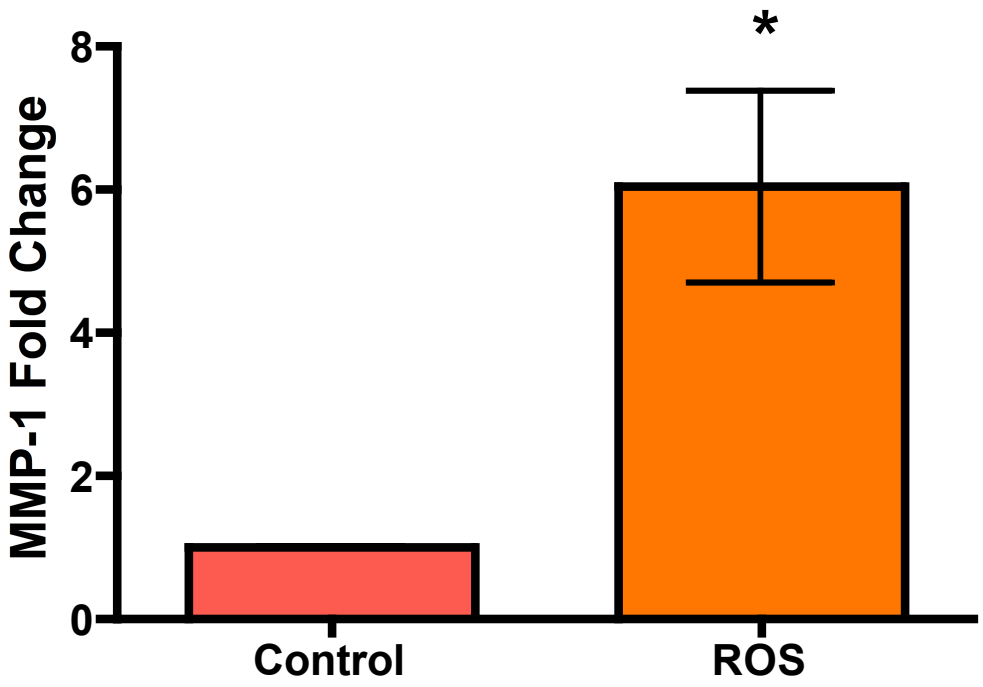

Figure 9: Fold Changes in MMP-1 Due to ROS in Primary Culture Decidual Cells
ROS treatment significantly increased the gene expression of MMP-1 by over 6 fold in primary culture decidual cells. ($p < 0.01$, n=10, mean=6.0 ± 1.3)

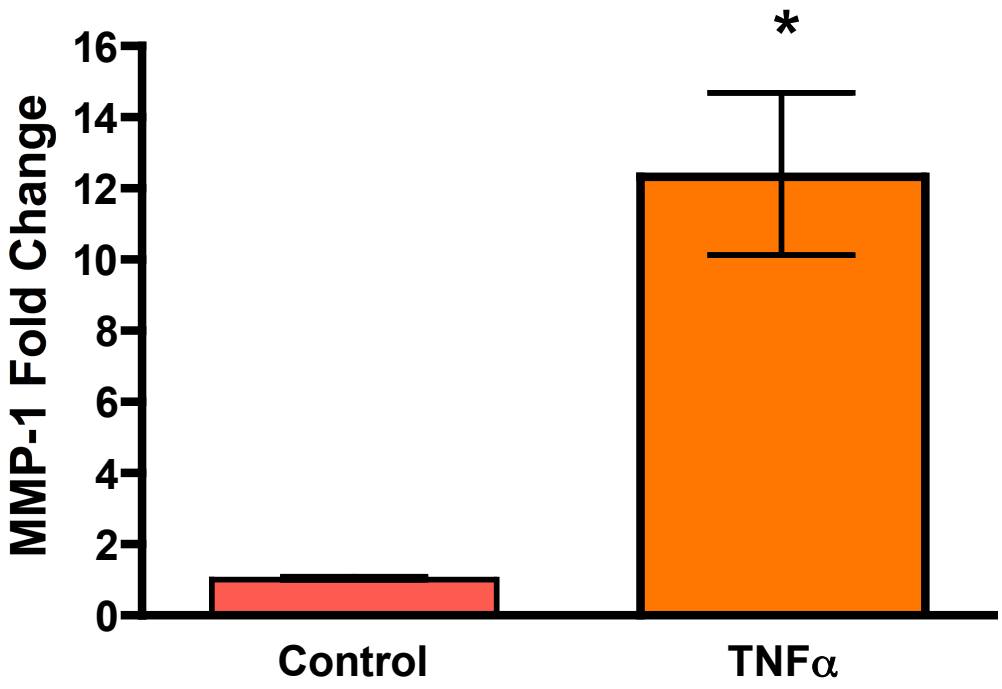

Figure 10: Fold Changes in MMP-1 Due to TNFα in hTERT Myometrial Cells
MMP-1 gene expression significantly increased by over 11 fold with 5-AZA treatment in an hTERT transformed myometrial cell line. ($p < 0.001$, n=7, mean=12.4 ± 2.3)

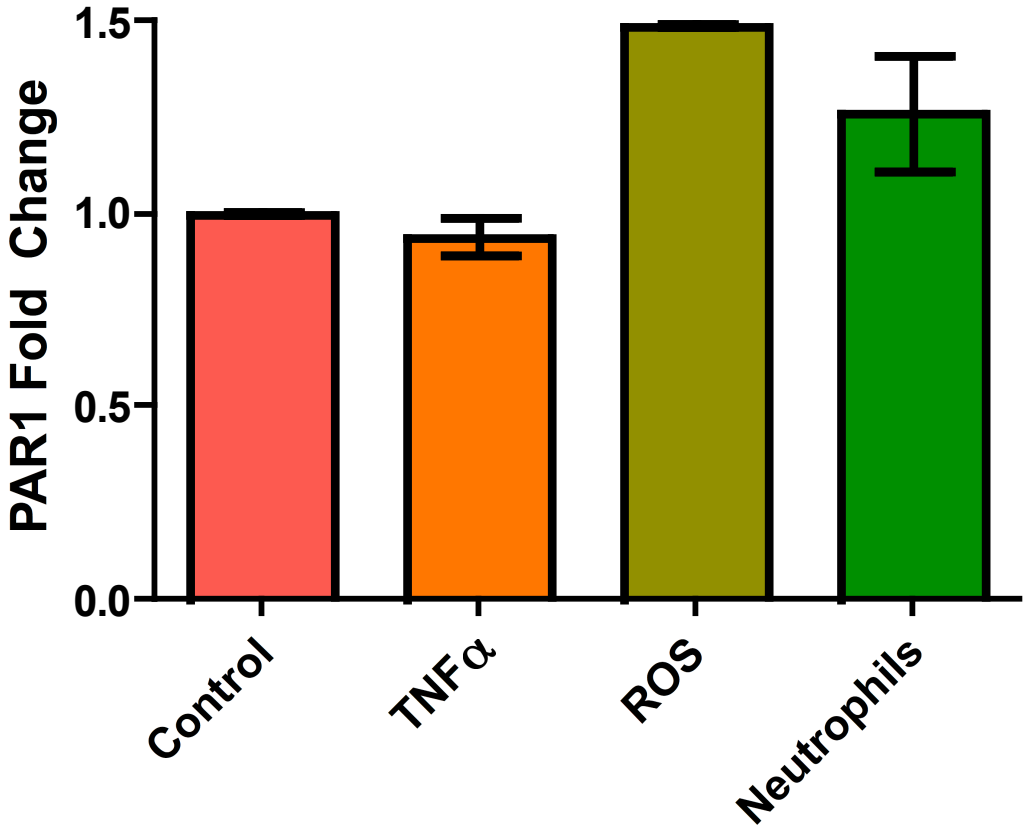

Figure 11: Fold Changes in PAR1 in hTERT Myometrial Cells
None of the treatments produced a significant change over the control conditions (n=2 for each treatment type).

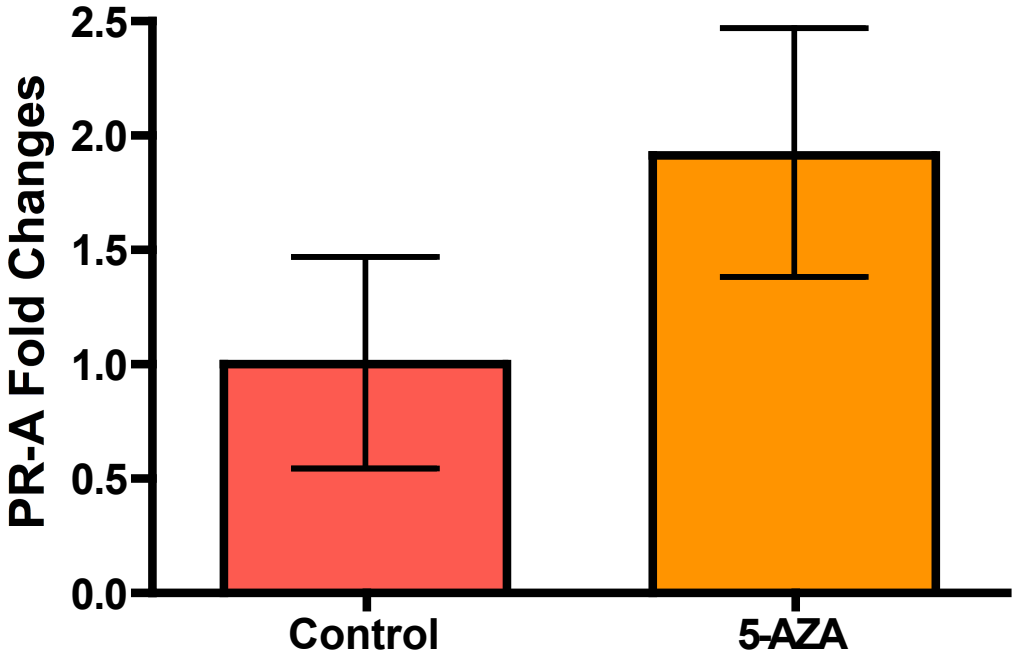

Figure 12: Fold Changes in PR-A Due to 5-AZA in Primary Culture Decidual Cells
PR-A gene expression was increased by almost 2 fold with 5-AZA treatment. (p=0.2, n=6, mean=1.9±.5)

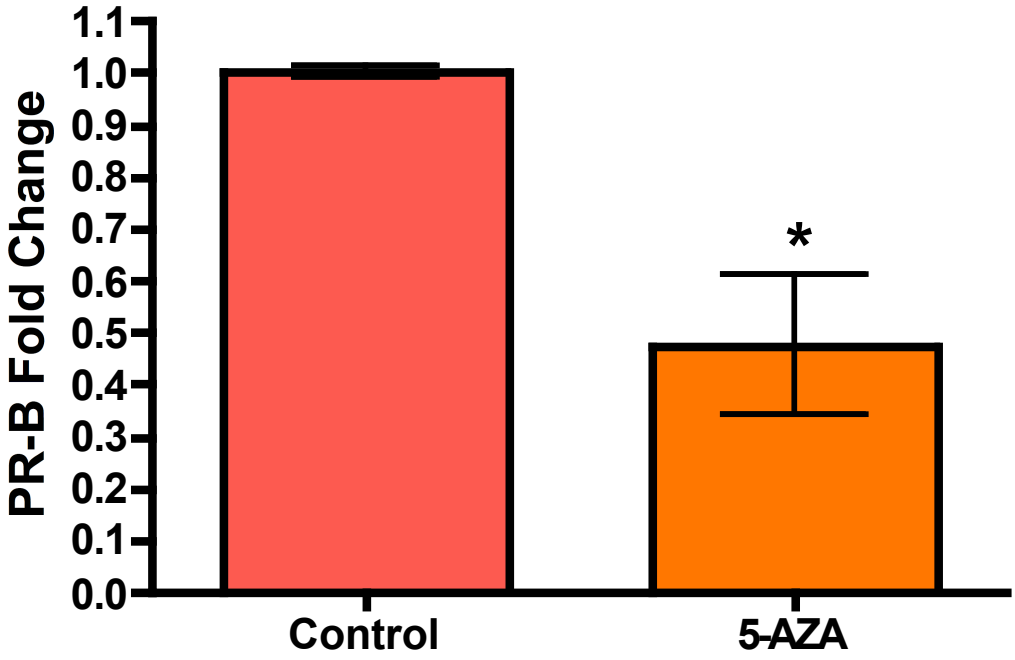

Figure 13: Fold Changes in PR-B Due to 5-AZA in Primary Culture Decidual Cells
Gene expression of PR-B decreased significantly with 5-AZA treatment. ($p < 0.01$, n=8, mean=0.5 ± 0.1)

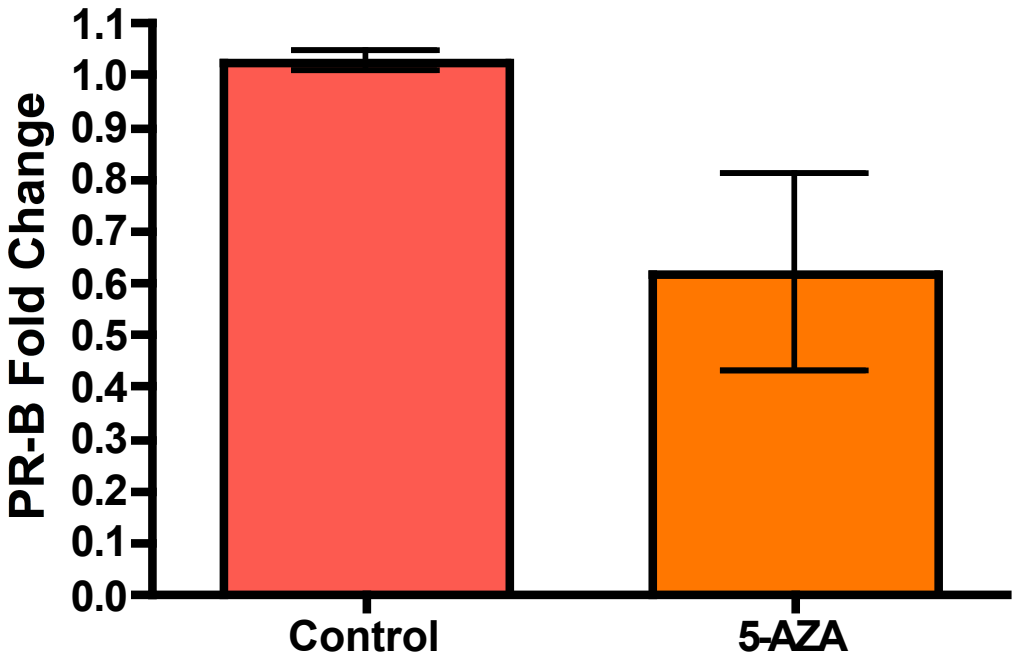

Figure 14: Fold Changes in PR-B Due to 5-AZA in hTERT Myometrial Cells
5-AZA did not significantly decrease PR-B gene expression. (p=0.06, n=6, mean=0.6 ± 0.2)

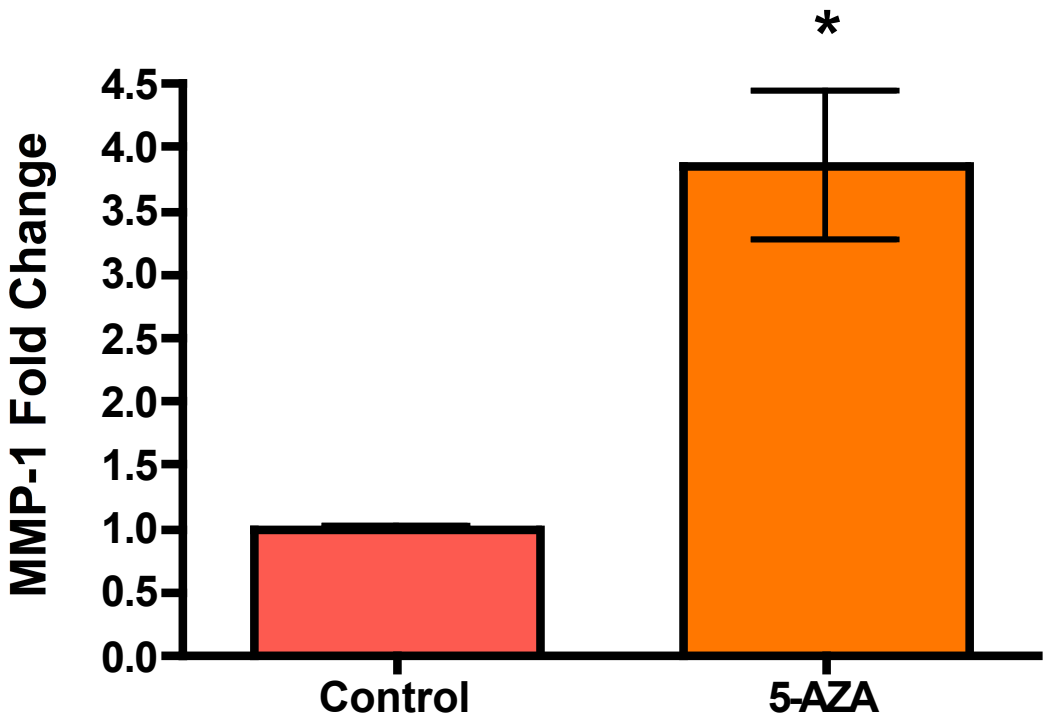

Figure 15: Fold Changes in MMP-1 Due to 5-AZA in Primary Culture Decidual Cells

5-AZA treatment significantly increased MMP-1 gene expression by almost 4 fold. ($p < 0.001$, n=8, mean=3.8 ± 0.6)

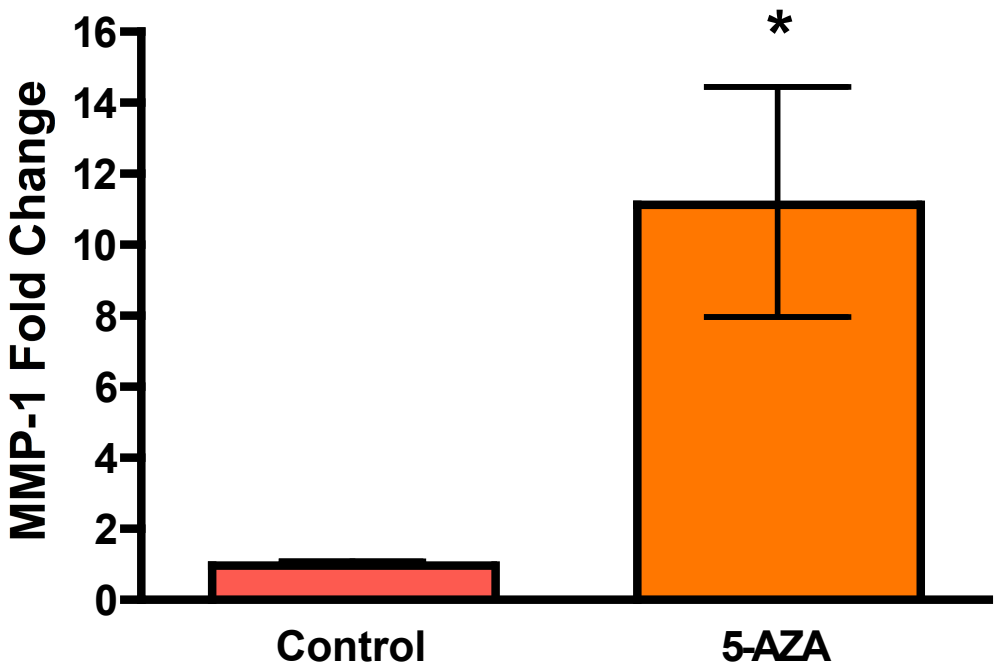

Figure 16: Fold Changes in MMP-1 Due to 5-AZA in hTERT Myometrial Cells
MMP-1 gene expression significantly increased by over 11 fold with 5-AZA treatment in an hTERT transformed myometrial cell line. ($p < 0.01$, $n=9$, mean$=11.2 \pm 3.3$)

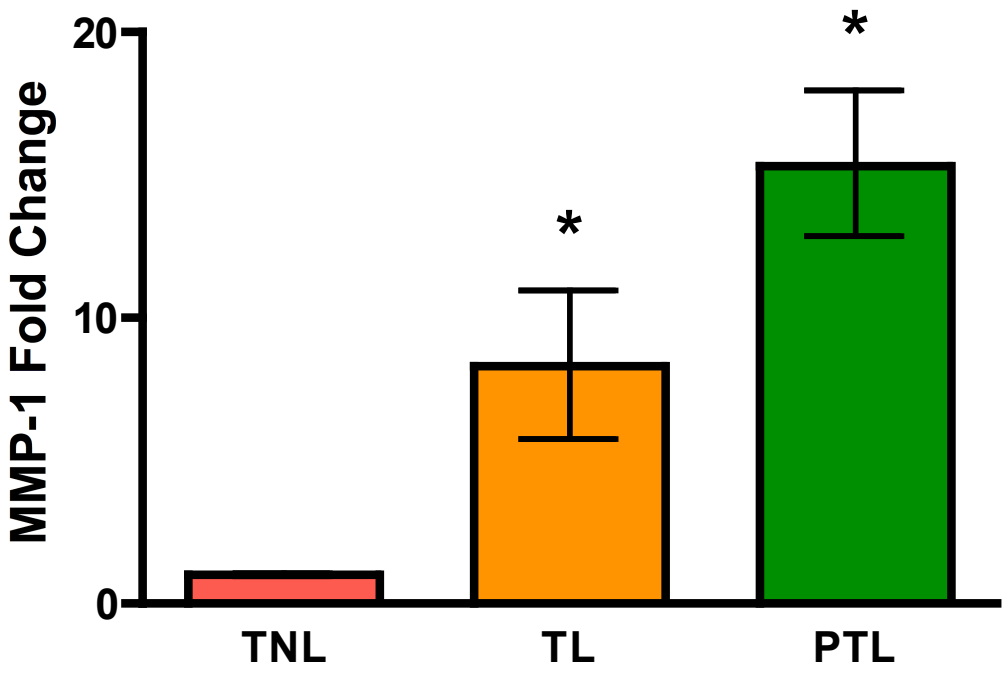

Figure 17: Fold Changes in MMP-1 in Decidual Tissue
Changes in MMP-1 compared to TNL vs. TL vs. PTL decidual tissue samples. TNL (mean=1 ± 0.05, n=13); TL (mean=8.4 ± 2.6, n=12) and PTL (mean=15.4 ± 2.5, n=10).

Literature Cited

1. Martin JA, Hamilton BE, Sutton PD, Ventura SJ, Menacker F, Kirmeyer S, et al. Births: Final Data for 2006. National Vital Statistics Reports 2009;57:102.

2. The Kaiser Family Foundation so. Data Source: Centers for Disease Control and Prevention. National Center for Health Statistics. VitalStats. http://www.cdc.gov/nchs/vitalstats.htm.

3. The Kaiser Family Foundation so. Data Source: The Center for Disease Control and Prevention, National Vital Statistics Reports. Births: Final Data for 2006, Vol. 57 No 7, 1/7/2009. http://www.cdc.gov.nchs/data/nvsr/nvsr57/nvsr57_07.pdf.

4. Rode L, Langhoff-Roos J, Andersson C, Dinesen J, Hammerum MS, Mohapeloa H, et al. Systematic review of progesterone for the prevention of preterm birth in singleton pregnancies. Acta Obstet Gynecol Scand 2009;88:1180-9.

5. Kornhauser M, Schneiderman R. How plans can improve outcomes and cut costs for preterm infant care. Manag Care 2010;19:28-30.

6. Voelker R. US preterm births: "D" is for dismal. Jama 2010;303:116-7.

7. Simhan HN, Caritis SN. Prevention of preterm delivery. N Engl J Med 2007;357:477-87.

8. Kramer MS, Lydon J, Seguin L, Goulet L, Kahn SR, McNamara H, et al. Stress pathways to spontaneous preterm birth: the role of stressors, psychological distress, and stress hormones. Am J Epidemiol 2009;169:1319-26.

9. Goldenberg RL, Culhane JF, Iams JD, Romero R. Epidemiology and causes of preterm birth. Lancet 2008;371:75-84.

10. Tan TC, Devendra K, Tan LK, Tan HK. Tocolytic treatment for the management of preterm labour: a systematic review. Singapore Med J 2006;47:361-6.

11. Meloni A, Melis M, Alba E, Deiana S, Atzei A, Paoletti AM, et al. Medical therapy in the management of preterm birth. J Matern Fetal Neonatal Med 2009;22 Suppl 3:72-6.

12. Sanborn BM. Relationship of ion channel activity to control of myometrial calcium. J Soc Gynecol Investig 2000;7:4-11.

13. Meis PJ, Klebanoff M, Thom E, Dombrowski MP, Sibai B, Moawad AH, et al. Prevention of recurrent preterm delivery by 17 alpha-hydroxyprogesterone caproate. N Engl J Med 2003;348:2379-85.

14. Sfakianaki AK, Norwitz ER. Mechanisms of progesterone action in inhibiting prematurity. J Matern Fetal Neonatal Med 2006;19:763-72.

15. Zakar T, Hertelendy F. Progesterone withdrawal: key to parturition. Am J Obstet Gynecol 2007;196:289-96.

16. Challis JR, Lockwood CJ, Myatt L, Norman JE, Strauss JF, 3rd, Petraglia F. Inflammation and pregnancy. Reprod Sci 2009;16:206-15.

17. Fareed J, Kindel G, Kumar A. Modulation of smooth muscle responses by serine proteases and related enzymes. Semin Thromb Hemost 1986;12:265-76.

18. Brown AG, Leite RS, Strauss JF, 3rd. Mechanisms underlying "functional" progesterone withdrawal at parturition. Ann N Y Acad Sci 2004;1034:36-49.

19. Mesiano S, Welsh TN. Steroid hormone control of myometrial contractility and parturition. Semin Cell Dev Biol 2007;18:321-31.

20. Smith R. Parturition. N Engl J Med 2007;356:271-83.

21. Thijssen JH. Progesterone receptors in the human uterus and their possible role in parturition. J Steroid Biochem Mol Biol 2005;97:397-400.

22. Mesiano S. Myometrial progesterone responsiveness and the control of human parturition. J Soc Gynecol Investig 2004;11:193-202.

23. Graham JD, Clarke CL. Expression and transcriptional activity of progesterone receptor A and progesterone receptor B in mammalian cells. Breast Cancer Res 2002;4:187-90.

24. Pieber D, Allport VC, Hills F, Johnson M, Bennett PR. Interactions between progesterone receptor isoforms in myometrial cells in human labour. Mol Hum Reprod 2001;7:875-9.

25. Mesiano S, Chan EC, Fitter JT, Kwek K, Yeo G, Smith R. Progesterone withdrawal and estrogen activation in human parturition are coordinated by progesterone receptor A expression in the myometrium. J Clin Endocrinol Metab 2002;87:2924-30.

26. Oh SY, Kim CJ, Park I, Romero R, Sohn YK, Moon KC, et al. Progesterone receptor isoform (A/B) ratio of human fetal membranes increases during term parturition. Am J Obstet Gynecol 2005;193:1156-60.

27. Thomson AJ, Telfer JF, Young A, Campbell S, Stewart CJ, Cameron IT, et al. Leukocytes infiltrate the myometrium during human parturition: further evidence that labour is an inflammatory process. Hum Reprod 1999;14:229-36.

28. Osman I, Young A, Jordan F, Greer IA, Norman JE. Leukocyte density and proinflammatory mediator expression in regional human fetal membranes and decidua before and during labor at term. J Soc Gynecol Investig 2006;13:97-103.

29. Haddad R, Tromp G, Kuivaniemi H, Chaiworapongsa T, Kim YM, Mazor M, et al. Human spontaneous labor without histologic chorioamnionitis is characterized by an acute inflammation gene expression signature. Am J Obstet Gynecol 2006;195:394 e1-24.

30. Young A, Thomson AJ, Ledingham M, Jordan F, Greer IA, Norman JE. Immunolocalization of proinflammatory cytokines in myometrium, cervix, and fetal membranes during human parturition at term. Biol Reprod 2002;66:445-9.

31. Orrenius S, Gogvadze V, Zhivotovsky B. Mitochondrial oxidative stress: implications for cell death. Annu Rev Pharmacol Toxicol 2007;47:143-83.

32. Madsen G, Zakar T, Ku CY, Sanborn BM, Smith R, Mesiano S. Prostaglandins differentially modulate progesterone receptor-A and -B expression in human

myometrial cells: evidence for prostaglandin-induced functional progesterone withdrawal. J Clin Endocrinol Metab 2004;89:1010-3.

33. Bisits AM, Smith R, Mesiano S, Yeo G, Kwek K, MacIntyre D, et al. Inflammatory aetiology of human myometrial activation tested using directed graphs. PLoS Comput Biol 2005;1:132-6.

34. Farina L, Winkelman C. A review of the role of proinflammatory cytokines in labor and noninfectious preterm labor. Biol Res Nurs 2005;6:230-8.

35. Oner C, Schatz F, Kizilay G, Murk W, Buchwalder LF, Kayisli UA, et al. Progestin-inflammatory cytokine interactions affect matrix metalloproteinase-1 and -3 expression in term decidual cells: implications for treatment of chorioamnionitis-induced preterm delivery. J Clin Endocrinol Metab 2008;93:252-9.

36. Elovitz MA, Ascher-Landsberg J, Saunders T, Phillippe M. The mechanisms underlying the stimulatory effects of thrombin on myometrial smooth muscle. Am J Obstet Gynecol 2000;183:674-81.

37. Bohm SK, McConalogue K, Kong W, Bunnett NW. Proteinase-Activated Receptors: New Functions for Old Enzymes. News Physiol Sci 1998;13:231-240.

38. Grand RJ, Turnell AS, Grabham PW. Cellular consequences of thrombin-receptor activation. Biochem J 1996;313 (Pt 2):353-68.

39. Shintani Y, Hirano K, Nakayama T, Nishimura J, Nakano H, Kanaide H. Mechanism of trypsin-induced contraction in the rat myometrium: the possible involvement of a novel member of protease-activated receptor. Br J Pharmacol 2001;133:1276-85.

40. O'Sullivan CJ, Allen NM, O'Loughlin AJ, Friel AM, Morrison JJ. Thrombin and PAR1-activating peptide: effects on human uterine contractility in vitro. Am J Obstet Gynecol 2004;190:1098-105.

41. Shintani Y, Hirano K, Nishimura J, Nakano H, Kanaide H. Enhanced contractile response to thrombin in the pregnant rat myometrium. Br J Pharmacol 2000;131:1619-28.

42. O'Brien M, Morrison JJ, Smith TJ. Expression of prothrombin and protease activated receptors in human myometrium during pregnancy and labor. Biol Reprod 2008;78:20-6.

43. Cockle JV, Gopichandran N, Walker JJ, Levene MI, Orsi NM. Matrix metalloproteinases and their tissue inhibitors in preterm perinatal complications. Reprod Sci 2007;14:629-45.

44. So T, Ito A, Sato T, Mori Y, Hirakawa S. Tumor necrosis factor-alpha stimulates the biosynthesis of matrix metalloproteinases and plasminogen activator in cultured human chorionic cells. Biol Reprod 1992;46:772-8.

45. Rice GE. Cytokines and the initiation of parturition. Front Horm Res 2001;27:113-46.

46. Maymon E, Romero R, Pacora P, Gervasi MT, Bianco K, Ghezzi F, et al. Evidence for the participation of interstitial collagenase (matrix metalloproteinase 1) in preterm premature rupture of membranes. Am J Obstet Gynecol 2000;183:914-20.

47. Watari M, Watari H, DiSanto ME, Chacko S, Shi GP, Strauss JF, 3rd. Pro-inflammatory cytokines induce expression of matrix-metabolizing enzymes in human cervical smooth muscle cells. Am J Pathol 1999;154:1755-62.

48. Menon R, Fortunato SJ. The role of matrix degrading enzymes and apoptosis in rupture of membranes. J Soc Gynecol Investig 2004;11:427-37.

49. Vu TD, Yun F, Placido J, Reznik SE. Placental matrix metalloproteinase--1 expression is increased in labor. Reprod Sci 2008;15:420-4.

50. Boire A, Covic L, Agarwal A, Jacques S, Sherifi S, Kuliopulos A. PAR1 is a matrix metalloprotease-1 receptor that promotes invasion and tumorigenesis of breast cancer cells. Cell 2005;120:303-13.

51. Yang E, Boire A, Agarwal A, Nguyen N, O'Callaghan K, Tu P, et al. Blockade of PAR1 signaling with cell-penetrating pepducins inhibits Akt survival pathways in breast cancer cells and suppresses tumor survival and metastasis. Cancer Res 2009;69:6223-31.

52. Trivedi V, Boire A, Tchernychev B, Kaneider NC, Leger AJ, O'Callaghan K, et al. Platelet matrix metalloprotease-1 mediates thrombogenesis by activating PAR1 at a cryptic ligand site. Cell 2009;137:332-43.

53. Goerge T, Barg A, Schnaeker EM, Poppelmann B, Shpacovitch V, Rattenholl A, et al. Tumor-derived matrix metalloproteinase-1 targets endothelial proteinase-activated receptor 1 promoting endothelial cell activation. Cancer Res 2006;66:7766-74.

54. Pei D. Matrix metalloproteinases target protease-activated receptors on the tumor cell surface. Cancer Cell 2005;7:207-8.

55. Agarwal A, Covic L, Sevigny LM, Kaneider NC, Lazarides K, Azabdaftari G, et al. Targeting a metalloprotease-PAR1 signaling system with cell-penetrating pepducins inhibits angiogenesis, ascites, and progression of ovarian cancer. Mol Cancer Ther 2008;7:2746-57.

56. Overall CM, Lopez-Otin C. Strategies for MMP inhibition in cancer: innovations for the post-trial era. Nat Rev Cancer 2002;2:657-72.

57. Tasaki K, Shintani Y, Saotome T, Andoh A, Fujiyama Y, Hozawa S, et al. Pro-inflammatory cytokine-induced matrix metalloproteinase-1 (MMP-1) secretion in human pancreatic periacinar myofibroblasts. Pancreatology 2003;3:414-21.

58. Lockwood CJ, Arcuri F, Toti P, Felice CD, Krikun G, Guller S, et al. Tumor necrosis factor-alpha and interleukin-1beta regulate interleukin-8 expression in third trimester decidual cells: implications for the genesis of chorioamnionitis. Am J Pathol 2006;169:1294-302.

59. Boyum A. Isolation of mononuclear cells and granulocytes from human blood. Isolation of monuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g. Scand J Clin Lab Invest Suppl 1968;97:77-89.

60. Stanford SJ, Pepper JR, Burke-Gaffney A, Mitchell JA. Cytokine-activated human vascular smooth muscle delays apoptosis of neutrophils: relevance of interactions between cyclo-oxygenase-2 and colony-stimulating factors. Faseb J 2001;15:1813-5.

61. Condon JC, Hardy DB, Kovaric K, Mendelson CR. Up-regulation of the progesterone receptor (PR)-C isoform in laboring myometrium by activation of nuclear factor-kappaB may contribute to the onset of labor through inhibition of PR function. Mol Endocrinol 2006;20:764-75.

62. Skrzycki M, Czeczot H. [Extracellular superoxide dismutase (EC-SOD)--structure, properties and functions]. Postepy Hig Med Dosw (Online) 2004;58:301-11.

63. Kim KS, Oh da H, Choi HM, Bang JS, Ryu CJ, Kim JH, et al. Pyrrolidine dithiocarbamate, a NF-kappaB inhibitor, upregulates MMP-1 and MMP-13 in IL-1beta-stimulated rheumatoid arthritis fibroblast-like synoviocytes. Eur J Pharmacol 2009;613:167-75.

64. Nelson KK, Subbaram S, Connor KM, Dasgupta J, Ha XF, Meng TC, et al. Redox-dependent matrix metalloproteinase-1 expression is regulated by JNK through Ets and AP-1 promoter motifs. J Biol Chem 2006;281:14100-10.

65. Franco R, Schoneveld O, Georgakilas AG, Panayiotidis MI. Oxidative stress, DNA methylation and carcinogenesis. Cancer Lett 2008;266:6-11.

66. Patra SK, Bettuzzi S. Epigenetic DNA-(cytosine-5-carbon) modifications: 5-aza-2'-deoxycytidine and DNA-demethylation. Biochemistry (Mosc) 2009;74:613-9.

67. Weitzman SA, Turk PW, Milkowski DH, Kozlowski K. Free radical adducts induce alterations in DNA cytosine methylation. Proc Natl Acad Sci U S A 1994;91:1261-4.

68. Goldman S, Shalev E. Progesterone receptor isoforms profile, modulate matrix metalloproteinase 2 expression in the decidua. Am J Obstet Gynecol 2007;197:604 e1-8.

69. Facchinetti F, Vaccaro V. Pharmacological use of progesterone and 17-alpha-hydroxyprogesterone caproate in the prevention of preterm delivery. Minerva Ginecol 2009;61:401-9.

70. Chmurzynska A. Fetal programming: link between early nutrition, DNA methylation, and complex diseases. Nutr Rev 2010;68:87-98.

71. Volanis D, Kadiyska T, Galanis A, Delakas D, Logotheti S, Zoumpourlis V. Environmental factors and genetic susceptibility promote urinary bladder cancer. Toxicol Lett 2010;193:131-7.

Vita

Anna Viktorovna Solotskaya was born on June 10, 1984 in Kiev, Ukraine. She came to this country in 1994 and soon became a U.S. citizen. In May 2006, she received her Bachelor's degree in Psychology and a minor in Biology with *cum laude* honors from Boston University. She worked for two years before enrolling in the Physiology Certificate program at Virginia Commonwealth University in August 2008. The following year she entered the Physiology Master's Program. Anna was an active member of the Virginia Commonwealth University community working as a Graduate Residence Director for the office of Residential Life and Housing and serving as a teaching assistant for the undergraduate human physiology course as well as participating in the Women's Health Research Day symposium.