ANTAGONISTIC ROLES OF MIR-199A-3P/MIR-214 AND THE MIR-200 FAMILY IN THE REGULATION OF UTERINE CONTRACTILITY DURING PREGNANCY AND LABOR

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DEDICATION

I would like to thank my Lord and Savior, Jesus Christ, by whom all things are possible. I thank my husband for being the man God designed him to be for our family. I thank my parents and my family members, who continue to love and support me. I thank Dr. Carole Mendelson for believing in me and inspiring me to be a great scientist and a great wife and mother. I also thank the UMBC Meyerhoff Program and the UTSW Medical Scientist Training Program, whose support has helped me to endure and inspired me to hold fast to my dreams.

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Progesterone (P_4) and estradiol-17β (E_2) play critical and opposing roles in regulating myometrial quiescence and contractility during pregnancy and labor (Kamel et al., 2010). While these contrasting hormonal effects are likely mediated via differential regulation of inflammatory and contractile genes, the underlying mechanisms remain incompletely understood.

Recently, we discovered that miR-200 family members, miR-200b and miR-429, and their target, transcription factor ZEB1, serve as P_4/progesterone receptor (PR)-mediated regulators of uterine quiescence during pregnancy (Renthal et al., 2010). In the present study, we identified a novel role for another miR-200 family member, miR-200a, to enhance local metabolism of P_4 in myometrium and, thus, decrease PR function during the progression towards labor (Williams et. al., 2012a). This occurs via miR-200a repression of signal transducer and activator of transcription (STAT)5b, a transcriptional repressor of the P_4-metabolizing enzyme 20α-hydroxysteroid dehydrogenase (20α-HSD). We observed that miR-200a expression increased and STAT5b expression coordinately decreased in myometrium of mice as they progressed to labor and in laboring myometrium from pregnant women. These changes were associated with a dramatic increase in expression and activity of 20α-HSD in laboring myometrium from mouse and human. In a progesterone-withdrawal mouse model of preterm labor, preterm labor was associated with increased miR-200a, decreased STAT5b and enhanced 20α-HSD expression.

In other studies, we also found that levels of the clustered miRNAs, miR-199a-3p and miR-214, were significantly decreased in laboring myometrium of pregnant mice and humans and in a inflammatory mouse

model of preterm labor, while the miR-199a-3p/miR-214 target, cyclooxygenase-2 (COX-2), a critical enzyme in synthesis of pro-inflammatory prostaglandins, was coordinately increased (Williams et al., 2012b). The physiological relevance of the labor-associated increase in miR-199a-3p/214 expression was highlighted by the finding that overexpression of miR-199a-3p and miR-214 in cultured human myometrial cells inhibited COX-2 protein and blocked TNF-α-induced myometrial cell contractility. Notably, estrogen and P_4 treatment of ovariectomized mice have opposing effects on uterine miR-199a-3p/214 expression that were mediated by ZEB1. Whereas, P_4 stimulated ZEB1 and upregulated miR-199a/214 expression in mouse and human myometrium (Renthal et al., 2010), estrogen had an opposing inhibitory effect. Notably, ZEB1/2 inhibit miR-200 family expression. Together, our findings point to the key pivotal roles of myometrial ZEB1 and its miRNA targets as a hormonally-controlled regulators of inflammatory and contractile gene expression in the pregnant uterus during term and preterm labor.

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1. Renthal NE, Chen CC, **Williams KC**, Gerard RD, Prange-Kiel J, Mendelson CR. miR-200 family and targets, ZEB1 and ZEB2, modulate uterine quiescence and contractility during pregnancy and labor. Proc Natl Acad Sci U S A. 2010 Nov 30;107(48):20828-33.
2. **Williams KC**, Renthal NE, Condon J, Gerard RD, Mendelson CR. miR-200a Serves a Key Role in the Decline of Progesterone Receptor Function Leading to Term and Preterm Labor. Proc Natl Acad Sci U S A. 2012 April.
3. **Williams KC**, Renthal NE, Gerard RD, Mendelson CR. The MicroRNA (miR)-199a/214 Cluster Mediates Opposing Effects of Progesterone and Estrogen on Uterine Contractility during Pregnancy and Labor. Manuscript in Review.

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1. **Williams KC**, Renthal NE, Mendelson CR.. *Decreased Expression of the miR-199a/214 Cluster Mediates Enhanced Cyclooxygenase-2 Expression and Contractility in Mouse Myometrium during Term and Preterm Labor*. Texas Forum for Reproductive Sciences 2011.
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Downregulation of STAT5 and Induction of 20alpha-Hydroxysteroid Dehydrogenase. Endocrine Reviews. 2011 Jun; 32(3): 154. (*co-first authors)

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Abbreviations	Definitions
20α-HSD	20alpha-hydroxysteriod dehydrogenase
20α-OHP	20alpha-dihydroprogesterone
β-Gal	Beta-galactosidase
BRG1	Brahma-related gene 1
CAP	Contraction-associated gene
ChIP	Chromatin immunoprecipitation
CMV	Cytomegalovirus
COS7	African Green Monkey SV40-transfected kidney fibroblast cell line
COX-2	Cyclooxygenase-2
CtBP	C-terminal binding protein
CX43	Connexin-43
DAPI	4,6-diamino-2-phenylindole
DGCR8	DiGeorge syndrome complex
Dnm3os	Dynamin 3, opposite strand
dpc	Days post coitum
DSP	Desmoplakin
E_2	Estrogen
EMT	Epithelial-mesenchymal transition
ER	Estrogen Receptor
FBS	Fetal Bovine Serum
HDAC	Histone deacetylase
HSD17β8	17-beta-hydroxysteroid dehydrogenase type 8
hTERT-HM	Human telomerase reverse transcriptase-Immortalized Myometrial Cells

IHC	Immunohistochemistry
IL-1β	Interleukin-1 beta
IκBα	Inhibitor of kappa B alpha
IKKβ	IkappaB Kinase beta
ISH	In situ hybridization
LPS	Lipopolysaccharide
MiRNA/miR	microRNA
mRNA	messenger RNA
NF-κB	Nuclear factor kappa B
NO	Nitric Oxide
Nur77	Nerve Growth factor IB
OXTR	Oxytocin receptor
P_4	Progesterone
PAF	Platelet activating factor
PGE_1	Prostaglandin-E_1
PGE_2	Prostaglandin-E_2
$PGF_2\alpha$	Prostaglandin-F_2alpha
PPARγ	Peroxisome proliferator-activated receptor gamma
PR	Progesterone receptor
Pri-miRNA	Primary miRNA
Pre-miRNA	Precursor miRNA
PTGFRN	Prostaglandin F_2 receptor negative regulator
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
RISC	RNA-induced silencing complex
siRNA	Small interfering RNA
STAT5b	Signal transducer and activator of transcription 5b

T47D	Human ductal breast epithelial tumor cell line
TNF-α	Tumor Necrosis Factor alpha
UTR	Untranslated Region
WT	Wild type
ZEB1	Zinc finger E-box-binding homeobox 1
ZEB2	Zinc finger E-box-binding homeobox 2

CHAPTER ONE
Introduction

The Dilemma of Preterm Birth

Preterm labor, defined as labor prior to 37 weeks of gestation, is a multifactorial phenomenon that is associated with increased infant mortality, an elevated risk of long-term developmental disabilities, respiratory distress syndrome, and chronic lung disease (Osterman et al., 2008). In fact, the rate of premature birth in the United States has steadily increased over the past two decades and has recently plateaued at ~12% of all births, or more than half a million preterm babies born per year (Allen et al., 2011). While late preterm birth, defined as delivery between 34-36 weeks gestation, accounts for majority of this rate increase, babies born during this period are still at an increased risk of several cognitive, motor, sensory, and behavioral deficits as well as poor growth (Klebanoff et al., 2011). In addition, preterm birth in the United States was estimated to cost our society at least $26.2 billion in 2005 and this estimate does not include the entire cost of care and management for these children (Klebanoff et al., 2011).

Conventionally, preterm birth can be categorized by its clinical presentation: spontaneous or indicated. In the former, the presenting event either involves contractions, cervical softening and thinning, or

spontaneous membrane rupture (Bastek et al., 2011). On the other hand, ‘indicated preterm birth’ is initiated due to pregnancy complications, and labor is induced therapeutically or the baby is delivered by cesarean delivery. While there are several contributing factors to the progression of labor such as chorio-decidual membrane and cervical changes, historically, the focus of most tocolytic agents have been on mitigating uterine contractions. Specifically, myometrial contractility is a common target for tocolytic agents such as β-adrenergic receptor agonists, nitric oxide (NO) donors, magnesium sulfate and calcium channel blockers (Wisanskoonwong et al., 2011). Unfortunately, the safety and efficacy of these current therapies are inadequate. This is because the molecular events involved in myometrial contractility during term and preterm labor remain incompletely defined (Mendelson et al., 2009). Thus, further research on the multiple mechanisms that underlie both term and preterm labor is required to aid in the development of improved therapeutic strategies.

Hormonal and inflammatory Pathways in the Initiation of Parturition

The molecular mechanisms that maintain quiescence of the myometrium throughout most of pregnancy and mediate its conversion into a synchronously contractile unit culminating in parturition remain incompletely understood. Research in the field has shown that pregnancy and labor are multifactoral processes that involve various complex pathways (Kamel et al., 2009). In particular, studies reveal an intricate interaction that occurs between endocrine and immunological pathways in process of term and preterm labor (Vrachnis et al., 2012). It is clear that progesterone acting through progesterone receptor is essential for maintaining myometrial quiescence, while an enhanced inflammatory response caused by signals from mother and fetus promotes the progression to labor (Mendelson et al., 2009).

Over the past few decades it has been firmly established that throughout most of pregnancy, uterine quiescence is sustained by increased circulating progesterone (P_4) and progesterone receptor (PR) activity, while labor is initiated/facilitated by a concerted series of molecular events that impair PR function. In humans, the high circulating P_4 levels are maintained by the corpus luteum of the ovary during the early stages of pregnancy and then by the placenta in the second and third trimester of pregnancy. The existence and timing of this luteo-placenta shift is different

among the various mammalian species (Csapo et al., 1969). For instance, in the mouse the corpus luteum remains the major source of circulating P_4 throughout pregnancy, but during the transition to labor, there is a steep drop in serum P_4 levels. This finding in rodents that circulating maternal P_4 levels decline precipitously near term (Virgo et al., 1974) has led to the concept that labor is associated with P_4 withdrawal. On the other hand, in humans and guinea pigs, circulating P_4 levels remain elevated throughout pregnancy and into labor, as do myometrial levels of PR (Challis et al., 2000) (Smith et al., 2009). Nonetheless, treatment with PR antagonists, mifepristone (RU486) or onapristone can cause increased cervical ripening and spontaneous labor or enhanced sensitivity to labor induction by oxytocin or prostaglandins (Frydman et al., 1992) (Dudley et al., 1996).

Furthermore, the importance of elevated serum P_4 levels during pregnancy is also highlighted by the fact that removal of the corpus luteum during early pregnancy results in low circulating P_4 levels and, subsequently, early abortion in both mice and humans (Csapo et al 1973)(Abel et al., 1986). It should also be noted that, even in mice, in which maternal P_4 levels significantly decrease at term, the serum P_4 levels still remain well above the K_d for binding to PR. These collective findings have led to the concept that parturition in all species is initiated by a concerted series of biochemical mechanisms that antagonize the ability

of the P_4/PR to regulate target genes in the uterus and cervix that maintain myometrial quiescence. The decline in PR function near term has been associated with a decrease in PR coactivators (Condon et al., 2003), increased expression of inhibitory PR isoforms (Condon et al., 2005)(Mesiano et al., 2002), enhanced local metabolism of P_4 in the cervix (Andersson et al., 2008) and uterus (Condon et al., 2004)(Williams et al., 2012), and antagonistic interaction of PR with the inflammatory transcription factor, NF-κB (Kalkhoven et al., 1996) (Hardy et al., 2006), which is activated in the myometrium near term (Kalhoven et al., 1995; Hardy et al., 2006). Indeed, P_4/PR mediates uterine quiescence, in part, by directly interacting with NF-κB to suppress NF-κB activation of contraction-associated genes such as cyclooxygenase-2 (COX-2) and prostaglandin F_2α receptor (Olson, 2003) and indirectly by inducing the expression of the NF-κB inhibitor, IκBα (Hardy et al., 2006).

Interestingly, an increase in circulating estradiol-17β levels (Wu et al., 1995)(Tibbetts et al.,1999)(Challis et al., 1971)(Buster et al., 1979) and increased estrogen receptor (ER)α activity (Mesiano et al., 2002) have also been shown to be involved in the endocrine cascade leading to parturition. Furthermore, activation of ER is reported to facilitate labor by directly promoting the transcription of the contraction-associated genes, such as oxytocin receptor and cyclooxygenase 2 (COX-2) (Murata et al.,

2003)(Mesiano et al., 2002). Notably, estrogens have been found to cause an inflammatory response in the uterus to further promote the progression to labor. For example, estrogens have been shown to enhance the formation of gap junctions and expression of oxytocin receptor, myosin light chain kinases (MLCK) and calmodulin and to increase prostaglandin $F_{2\alpha}$ activity (Lockwood et al., 1999) in myometrium. Additionally, estrogens induce the influx of macrophages and neutrophils into the uterus; an action that is antagonized by P_4 acting via the progesterone receptor (Tibbetts et al., 1999). When compared to women who were not in active labor at term, patients with premature uterine contractions who delivered preterm had elevated serum and amniotic fluid estrogen levels (Mazor et al., 1996). Moreover, a premature increase in the synthesis of placental estrogen in rhesus monkeys resulted in preterm labor (Mecenas et al., 1996).

In light of the anti-inflammatory activity of P_4/PR throughout most of pregnancy and the pro-inflammatory actions of estrogen/ERα near term, it is not surprising that inflammation and/or infection have also been shown to play an essential role in the initiation of term and preterm labor. Notably, with the onset of labor there is a significant increase in leukocyte migration to and invasion of the myometrium, cervix and fetal membranes (Osman et al., 2003)(Thomson et al., 1999)(Yuanet al., 2009). This

contributes to the surge in cytokine expression in the myometrium and cervix during labor (Osman et al., 2003). Importantly, the inflammatory transcription factor, NF-κB, which is activated by this increase in pro-inflammatory factors, directly interacts with the progesterone receptor to inhibit its function. This decline in PR function contributes to a further increase in NF-κB activation, resulting in an exacerbation of the inflammatory response and the expression of pro-inflammatory factors in the myometrium (Allport et al., 2001). These pro-inflammatory cytokines stimulate uterine contractility and enhance tissue remodeling and leukocyte migration to the myometrium to further promote the parturition process. Specifically, interleukin-1β (IL-1β) is a pro-inflammatory cytokine that enhances myometrial contractility by inducing the influx of calcium into myometrial smooth muscle cells (Tribe et al., 2003).

IL-1β acts together with another pro-inflammatory cytokine, tumor necrosis factor α (TNF)-α, to induce the expression of an important contraction-associated protein (CAP) gene, cyclooxygenase-2 (COX-2) (Huang et al., 2000). COX-2 is an inducible gene that is expressed at undetectable to low levels under normal conditions, but is highly upregulated in inflammation and by the pro-inflammatory hormone estrogen (Engstrøm et al., 2001). COX-2 catalyzes the production of prostaglandins, which are bioactive lipids that play a crucial physiological

role in the inflammatory process of labor by acting as potent uterine contractility agents (Gibb et al., 1998). Prostaglandins are synthesized from arachidonic acid via the eicosanoid pathway. Interestingly, TNF-α and IL-1β both stimulate the release of arachidonic acid as well, thereby, emphasizing the causal link between inflammation and labor (Liu et al., 1998). Reports have also shown that uterine prostaglandin concentrations increase before and during labor and contribute to myometrial contractility (Romero et al., 1996)(Allport el al., 2001). The importance of COX-2 and prostaglandins in labor is highlighted by the fact that COX-2 inhibitors have been used to mitigate uterine contractions during pre-term labor (Borna et al., 2007), whereas, vaginal administration of prostaglandins PGE_2 and $PGF_{2}\alpha$, is used to induce labor at term in women (Mozurkewich et al., 2011).

Thus, the process of parturition involves a complex interplay between hormonal and inflammatory factors that influence one another and their respective effects on the uterus. Important events, such as progesterone withdrawal, estrogen receptor activation and enhanced inflammatory response, activate the contractile nature of the myometrium and enhance the myometrium's responsiveness to key contractile factors such as prostaglandins and oxytocin, thereby encouraging the progression of labor and expulsion of the fetus.

MicroRNAs in Female Reproduction

Female reproduction involves many complex processes. The mechanisms behind these processes are not completely understood. Recent research investigating the roles of miRNAs in the physiology of female reproduction identified novel roles for these post-transcriptional regulators of gene function (Carletti et al., 2009). MicroRNAs are 22-nucleotide molecules that serve particularly important roles in female reproductive physiology and have been identified as promising potential drug targets for a variety of pathological conditions (Creighton et al., 2010)(Montenegro et al., 2007)(Fabbri et al., 2011).

MicroRNAs are encoded within the genome either in clusters or individually (Lee et al., 2001). Those miRNAs that are found in clusters are often regulated by the same promoter (Lagos-Quintana et al., 2001)(Lim et al., 2003). The beginning of miRNA biogenesis involves transcription of primary miRNA (pri-miRNA). The pri-miRNA then undergoes processing by a nuclear core microprocessor complex that includes the RNase III enzyme, Drosha, and the DiGeorge syndrome critical region gene 8 protein (DGCR8) in order to generate the precursor miRNA (pre-miRNA). The pre-miRNA is then exported to the cytoplasm by Exportin-5 to be processed into single-stranded mature miRNAs by the type III ribonuclease, Dicer. During cytoplasmic processing by Dicer, a double-

stranded RNA molecule with a 3’ overhang of 1-4 nucleotides is generated. Typically, only one strand is incorporated into the RISC complex, while the other strand is either degraded or expressed at low levels (Hutvagner et al, 2002). In the case where both strands of the pre-miRNA are equivalently processed to mature miRNAs and incorporated into a RISC complex, the miRs are termed miR-XXX-3p and miR-XXX-5p (Schwarz et al., 2003). The mature miRNA is then loaded onto the RNA-induced silencing complex (RISC) complex in order to inhibit expression of specific mRNA targets. MicroRNAs repress gene expression by using their 6-8 nucleotide seed sequence to bind to the complementary sequence in the 3’untranslated region (UTR) of their specific mRNA targets, resulting either in degradation of the mRNA or its translational repression. The seed sequence of the miRNA is essential for target binding and is often well conserved across various species.

Findings from numerous studies indicate that alterations in the expression pattern of miRNAs often correlate with essential physiologic processes within the female reproductive organs. For example, in a study investigating miRNA changes during the receptive phase of implantation in mice, 32 miRNAs were found to be upregulated (Hu et al., 2008). Notably, two miRNAs, miR-101a and miR-199a-3p were observed to target COX-2, which contributes to the inflammatory processes that adjust the

immunological receptivity of the uterus prior to implantation. The relevance of miRNAs in female reproduction is highlighted by the finding that the reproductive tissues express key proteins in the miRNA biogenesis pathway and that loss of these proteins tend to adversely affect the development of the female reproductive organs. For instance, Müllerian duct mesenchyme-specific *Dicer* conditional null female mice are infertile due to a reduction in oviduct and uterine horn size, as well as a defect in embryo transit (Gonzalez et al., 2009). These findings highlight the importance of miRNAs in postnatal development of the female reproductive tract and in fertility. Furthermore, since the Müllerian duct mesenchyme is believed to be the progenitor of the uterine myometrium, this study also emphasizes the significance of miRNA expression in myometrium for a successful pregnancy. Interestingly, loss of Argonaute2, the catalytic component of the RNA-induced silencing complex (RISC) that promotes miRNA-mediated miRNA silencing, results in a similar phenotype to the Dicer mutant; however, embryonic lethality occurs soon after implantation (Kaneda et al., 2009).

Interestingly, several components of the miRNA processing pathway have been shown to be modulated by steroids in the mouse uterine tissues. It has been reported that both estrogen and progesterone increase Exportin-5 expression, but only progesterone increased Dicer

expression in the mouse uterus (Nothnick et al., 2010). In uterine tissues from mice lacking ERα there is an upregulation in small subsets of miRNAs (Macias et al., 2009). The decreased production of certain mature miRNAs was attributed to the inhibitory indirect association of activated ERα with Drosha. On the other hand, uterine expression of miR-125a, miR-195, miR-143, miR-145, miR-16, miR-190, miR- 181b and miR-204 was found to be significantly reduced following estradiol treatment of ovariectomized mice (Nothnick et al., 2010). Taken together, these studies suggest that estrogen negatively regulates the production of certain miRNAs in the uterus of mice. Collectively, hormonal modulation of miRNA expression at various stages of miRNA processing allows for additional levels of regulation that impact uterine gene expression and female reproductive physiology.

While the majority of research concerning miRNAs in reproductive biology has focused on their roles in reproductive tract development, endometrial biology and implantation, and early pregnancy, we have obtained evidence to support essential roles for members of the miR-200 family of miRNAs in the uterine myometrium during pregnancy and labor (Renthal et al., 2010). In our studies, we have focused on the roles of miRNAs in the regulation of contraction-associated genes by progesterone and estrogen.

The microRNA-200 Family

The miR-200 family is composed of two clusters of miRNAs (Figure 1-1.1). The first cluster contains miR-200b, miR-200a, and miR-429 and is located in chromosome 1 of *homo sapiens* and in chromosome 4 of *mus musculus*. The second cluster contains miR-200c and miR-141 and is located in chromosome 12 of homo sapiens and in chromosome 6 of mus musculus (Mongroo et al., 2010). There is nearly complete homology between the miR-200 family members of mouse and human suggesting that these miRNAs are highly conserved and potentially target a similar set of mRNA targets. Analysis of the individual miR-200 family members revealed that these miRNAs share almost identical seed sequences, with only a one-nucleotide difference between the seed sequences of miR 200a and miR-141 *vs.* those of miR-200b, miR-200c and miR-429 (Fig. 1-1.1).

Previously, we found a significant induction in the expression of two members of the miR-200 family, miR-200b and miR-429, at term in myometrium of laboring mice and humans, as well as a coordinate downregulation of two miR-200 family targets, zinc finger E-box binding homeobox proteins, ZEB1 and ZEB2 (Renthal et al., 2010). Interestingly, there are eight miR-200 binding sites in the 3'UTR of ZEB1 and nine miR-200 binding sites in the 3'UTR of ZEB2 (Renthal et al., 2010). These allow

for reciprocal repression between ZEB1/2 and the miR-200 family in various tissues (Brabletz et al., 2010).

Furthermore, we noted this reciprocal change in expression in miR-200 cluster and ZEB1/2 at 17.5 dpc, at which time miR-200 family members increase and ZEB1/2 decline significantly. This represents a critical time in the gestation of the mouse that corresponds to the beginning of the decline in P_4/PR function (Virgo et al., 1974). Additionally, Shynlova et al. report that at the corresponding time within the pregnant rat, the myometrium undergoes a "synthetic to contractile switch," during which structural and organizational changes occur within the myometrium in preparation for labor (Shynlova et al., 2009). These changes include an increase in basement membrane attachment and reorganization and a decrease in myometrial myocyte proliferation. In studies from our lab we have observed that prior to 17.5 dpc, when P_4 levels and PR function is high, ZEB1 levels are relatively high. Our data suggest that the high levels of ZEB1 during pregnancy inhibit miR-200 expression, which further increases ZEB1 and induces ZEB2 expression (Renthal et al. 2010). Collectively, the high levels of ZEB1 and ZEB2 repress miR-200 family expression in the myometrium, thereby, reducing the inhibitory effects of miR-200 family on ZEB1 and ZEB2 and helping to maintain the high levels of ZEB proteins. Interestingly, we found that enhanced expression of

ZEB1 and ZEB2 inhibited expression of the contraction-associated genes, oxytocin receptor (OXTR) and connexin-43 (CX43) in myometrial cells, and blocked oxytocin-induced myometrial contractility in a collagen gel contraction assay (Renthal et al., 2010). Thus, the high levels of ZEB1 and ZEB2 expression during pregnancy inhibit the expression of OXTR and CX43.

Once pregnant mice pass the critical 17.5 dpc time point after which there is a decline in P_4/PR function, there is a decrease in myometrial ZEB1 and ZEB2 levels by two mechanisms. First, since PR directly interacts with the *ZEB1* promoter to induce ZEB1 expression (Renthal et al., 2010), the decline in PR function results in a decrease in transcriptional activation of the *ZEB1* gene. Second, as a consequence of the decline in PR function and the subsequent decline in ZEB1 levels, this releases ZEB1 repression of the miR-200 family. The increased levels of miR-200 family expression further repress the expression of ZEB1 and also repress ZEB2 expression in the myometrium (Figure 1-1.2). This is supported by our findings that miR-200 overexpression inhibits ZEB1 and ZEB2 expression in cultured myometrial cells (Renthal et al., 2010). Furthermore, we observed a decline in ZEB1 and ZEB2 when PR function was blocked in the RU486 mouse model of preterm labor. This is likely due to the fact that RU486 blocked PR induction of ZEB1, which

subsequently allows for the induction of miR-200 family, which results in the decline in ZEB2 expression. Together, the decline in ZEB1 and ZEB2 expression near term and during labor released inhibition of OXTR and CX43, resulting in increased myometrial contractility. Increase myometrial contractility is promoted by two different pathways: in the first, oxytocin induces smooth muscle contraction by increasing intracellular calcium levels (Carsten et al., 1977), and in the other pathway, CX43 operates in gap junctions to allow the myometrium to function as a coordinated contractile unit (Döring et al., 2006).

As previously mentioned, miR-200b and miR-429 are expressed within the same primary transcript with another miR-200 family member, miR-200a. In the present study, we show that, as we observed for miR-200b and miR-429, miR-200a is highly induced in the myometrium of pregnant mice and humans during late gestation and labor. Moreover, miR-200a not only inhibits the expression of ZEB1, but also targets expression of signal transducer and activator of transcription (STAT)5b, a transcription factor known to inhibit expression of *20α-hydroxysteroid dehydrogenase* (*20α-HSD)*, a major P_4 metabolizing enzyme in the uterus (Figure 1-1.3).

The microRNA-199a Cluster

The miR-199a cluster is a conserved cluster of miRNAs comprised of miR-199a-5p, miR-199a-3p and miR-214. The miR-199a cluster is synthesized from the miR-199a-2 primary transcript that is encoded within the 7.9-kb anti-sense transcript of *dynamin 3* opposite strand (*Dnm3os*), which is highly expressed in uterus during pregnancy (Loebel et al., 2005). In support of this, we previously found high levels of all three miRNAs in the miR-199a cluster within pregnant myometrium in a miRNA microarray (Renthal et al., 2010). The miR-199a cluster is located in chromosome 1 of mus musculus and chromosome 1 in homo sapiens (Figure 1-1.4). The seed sequences for miR-199a-5p, miR-199a-3p and miR-214 are highly conserved from mouse to human. Whereas, seed sequences of miR-199a-3p and miR-214 are highly similar, the seed sequence of miR-199a-5p differs significantly from the other two miRNAs in the cluster, suggesting that miR-199a-5p targets a different set of messenger RNAs.

Interestingly, miR-199a-3p and 214 are known and predicted, respectively, to target the mRNA encoding COX-2 (Chakrabarty et al., 2007) (Figure 1-1.5), the key regulatory enzyme in the synthesis of prostaglandins, which are potent stimulators of uterine contractility (O'Brien et al., 1995) (Terzidou et al., 2007). Although, miR-199a-5p is not

predicted to target COX-2, it has been shown to target one of the catalytic subunits of the IkappaB kinase (IKK) complex, IkappaB kinase beta (IKKβ), thereby, suppressing nuclear factor-kappa B (NF-κB) pathway activation. Furthermore, studies revealed that miR-199a-5p inhibition of the NF-κB pathway resulted in a decrease in pro-inflammatory and invasion mediators (Lee et al., 2012).

Members of the miR-199a cluster also have been linked to several disorders of the female reproductive system, such as endometriosis and ovarian cancer (Dai et al., 2011)(Chen et al., 2008). Interestingly, Twist1, a known positive regulator of the miR-199a cluster, and ZEB1, a known regulator of miR-200 family, exert a number of similar functions. Both bind to E-boxes in their target gene promoters (Postigo et al., 1997; Lee et al., 2009) and cause tumor progression by potently inhibiting E-cadherin expression, thus promoting epithelial to mesenchymal transition (EMT) (Peinado et al., 2007; Yang et al., 2008). In the present study, we discovered that miR-199a-3p and miR-214 expression declined in pregnant myometrium toward term in a pattern highly coordinately with the decreased expression of ZEB1. Furthermore, we found that ZEB1 mediates the opposing actions of progesterone and estrogen in the regulation of miR-199a/214 cluster and miR-200 family and of CAP gene expression during pregnancy and labor.

The Hormonally-Regulated Transcription Factors, ZEB1 and STAT5b

Zinc finger E-Box-binding homeobox 1 (ZEB1) is an important transcription factor in development and disease (Hurt et al., 2008). ZEB1 is encoded by the *ZFHX1a* gene, and is composed of two clusters of C_2H_2-type zinc fingers that bind to paired CAGGTA/G E-box-like promoter elements. In between these two zinc-finger domains, there is a POU-like homeodomain that is believed to play a role in protein-protein interactions (Smith et al., 2003). Notably, ZEB1 can function as a transcriptional repressor or activator depending upon a number of poorly understood conditions, such as expression levels, conformation, gene target and the presence of certain cofactors (Fontemaggi et al., 2001)(Ikeda et al.,1995). Co-repressors such as CtBPs, HDACs, and BRG1, allow ZEB1 to act as a repressive transcription factor, whereas, the co-activators p300 and pCAF interact with ZEB1 to activate gene transcription (Vandewalle et al., 2009). ZEB1 is highly expressed in the uterus, with expression levels second only to the another smooth muscle organ, the bladder (Hurt et al., 2008). In *ZFHX1a*$^{+/-}$ mice expressing a LacZ reporter inserted into the *ZFHX1a* first exon, it was observed that ZEB1 promoter activity was selectively expressed in myometrium of virgin mice and was upregulated in myometrium and stroma during pregnancy (Spoelstra et al., 2006).

As previously mentioned, ZEB1 and ZEB2 exist in a double-negative feedback loop with miR-200 family (Bracken et al., 2008). This reciprocal relationship is critical for regulation of miR-200 and ZEB levels within tissues and plays a key role in the cellular plasticity that occurs during epithelial-to-mesenchymal transition (EMT), a critical feature of normal development and cancer. Considering the complex cellular changes that occur in the uterus during pregnancy and into labor, it was of great interest to find that ZEB1 and ZEB2 levels, which are elevated during pregnancy, decrease significantly during the transition to labor (Rental et al., 2010). Findings from that study revealed that the decline in PR function leading to term and preterm labor contributed to the marked decline in ZEB1 expression in the myometrium. This caused an upregulation of miR-200 family expression, which resulted in repression of ZEB2. Conversely, P_4 treatment of ovariectomized mice upregulated uterine ZEB1 expression by the direct action of P_4/PR at the *ZFHX1a* promoter (Renthal et al., 2010). The induction of ZEB1 caused repression of miR-200 family expression, resulting in de-repression and upregulation of ZEB2. Progesterone also was observed to induce ZEB1 expression in uterine cancers and breast carcinoma cell lines (Spoelstra et al., 2006)(Richer et al., 2002)(Renthal et al., 2010). On the other hand, the effect of estrogen on ZEB1 expression is controversial. In studies of

ovariectomized mice, ZEB1 was reported to be induced by estrogen treatment (Spoelstra et al., 2006). On the other hand, ZEB1 expression in LbetaT2 gonadotrope cells was found to be suppressed by estrogen (Kowase et al., 2007).

Interestingly, one of the miR-200 family members, miR-200a, is predicted to target the mRNA encoding another progesterone-regulated transcription factor, signal transducer and activator of transcription 5b (STAT5b) (Figure 1-1.4). STAT5b is a member of the STAT transcription factor family. Cytokine signaling activates these latent cytoplasmic transcription factors by tyrosine phosphorylation, which leads to the formation STAT heterodimers or homodimers that translocate to the nucleus to transcriptionally regulate the expression of specific target genes (Carvajal et al 2005). STAT dimers use the palindromic core motif, $TTCN_{2-4}GAA$, to recognize their gene targets. Similar to ZEB1, STAT5b mRNA and protein expression is induced by P_4 in the mammary gland and breast cancer cells (Philip et al., 1996) (Carvajal et al., 2005). STAT5b is known to regulate a number of biological processes, such as tumor growth and progesterone metabolism. Indeed, STAT5b is known to inhibit gene expression of 20α-hydroxysteroid dehydrogenase (20α-HSD), a P_4-metabolizing enzyme within the uterus and ovaries (Piekorz et al., 2005).

20α-HSD is a member of the AKR superfamily of NAD(P)(H)-dependent oxidoreductases, comprising >100 proteins, which catalyze the reduction of a wide variety of substrates, including steroid hormones, prostaglandins, carbohydrates and xenobiotics (Penning et al., 2009)(http://www.med.upenn.edu/akr/index.html). 20α-HSD belongs to the steroid metabolizing HSD subgroup of the AKR1 family. In the human, there are four clustered HSD genes on chromosome 10, corresponding to 20α-HSD (AKR1C1), 3α-HSD, type III (AKR1C2), 17β-HSD, type V (AKR1C3) and 3α-HSD type I (AKR1C4). In the mouse, there are four distinct HSD genes that correspond to their human gene counterparts in the order listed above (AKR1C18, AKR1C6, AKR1C12 and AKR1C13), as well as 4 novel HSDs, all clustered on chromosome 13 (Vergnes et al., 2003). The AKR family members in human and mouse that metabolize P_4 with the highest activity are AKR1C1 and AKR1C18, respectively (Velica et al., 2009). The physiological relevance of AKR181C is highlighted in studies involving gene targeted mice deficient in 20α-HSD, STAT5b, or both. Mice that lack 20α-HSD manifest a significant delay in labor (Piekorz et al., 2005). By contrast, *STAT5b* knockout mice, which manifest increased levels of 20α-HSD expression and decreased levels of circulating P_4, abort during midgestation. Notably, when *STAT5b* null mice were crossed with *20α-HSD* null mice, midgestation abortions caused by

STAT5b deficiency were partially rescued (Pierkorz et al., 2005), suggesting that the inhibitory effects of STAT5B on 20α-HSD expression during most of pregnancy is crucial for pregnancy maintenance.

In the present study, we investigated the role of ZEB1-regulated miR-200 family and the miR-199a/214 cluster in the regulation of the 20α-HSD transcriptional inhibitor, STAT5b, and the contraction associated gene, COX-2. Our investigations indicate that myometrial ZEB1, which is upregulated by P_4/PR throughout most of pregnancy and declines with the decrease in P_4/PR function and the increase in E_2/ERα activity during the progression to labor (Mesiano et al., 2002), serves a key pivotal role in the regulation of myometrial quiescence and contractility. Our collective findings indicate that P_4 upregulation of ZEB1 maintains myometrial quiescence by suppressing the miR-200 family and the expression of CAP genes, such as the OXTR and CX43 (Rental et al., 2010), on the one hand, and by enhancing miR-199a-3p/214 expression and causing suppression of COX-2, on the other. Furthermore, suppression of the miR-200 family by ZEB1 decreases miR-200a levels, thereby, allowing for increased expression of STAT5b, which inhibits 20α-HSD expression to maintain increased local levels of P_4 within the myometrium. Near term, enhanced ERα activity and the increased inflammatory response in myometrium promote a decline in PR function causing decreased

expression of ZEB1. This results in de-repression of CAP genes and the miR-200 family with an associated increase in 20α-HSD expression, resulting in enhanced local P_4 catabolism within the myometrium. The decline in ZEB1 levels also results in a downregulation of *miR-199a/214* expression with an associated increase in COX-2, causing further upregulation of the inflammatory response and amplification of the contractile state of the uterus for progression into active labor. These findings not only highlight unique roles for miR-200a, miR-199a-3p and miR-214 in the regulation of uterine P_4 metabolism and uterine contractility during pregnancy and labor, but also implicate ZEB1 as a central regulator of miRNAs in this novel pathway of P_4-regulated uterine contractility.

CHAPTER TWO – Section 1

Identifying a Novel Role for the MiR-200 Family in the Decline in PR Function Leading to Labor

Introduction and Experimental Design

In order to identify miRNAs involved in the transition of the myometrium from a quiescent state to a contractile unit, we previously conducted a miRNA microarray analysis of quiescent pregnant mouse myometrium at 15.5 dpc *vs.* contractile myometrium just before labor at 18.5 dpc (Renthal et al., 2010). In these studies we observed that the miR-200 family was significantly upregulated towards term, but only the most significantly increased miR-200 family members in the array, miR-200b and miR-429, were validated using qRT-PCR and investigated in subsequent studies (Renthal et al., 2010). Interestingly, the third most significantly increased miR-200 family member in the miRNA microarray, miR-200a, is encoded within the same 7.5-kb polycistronic primary miRNA (pri-miR) transcript as miR-200b and miR-429. This transcript was shown to be repressed by ZEB1 (Bracken et al., 2008), suggesting their coordinate regulation (Figure 2-1.1).

In the present study, we turned our attention to miR-200a because it was predicted by TargetScan analysis (http://www.targetscan.org/) to

target the transcription factor, signal transducer and activator of transcription (STAT)5b, which serves as a P_4-responsive transcriptional repressor of *20α-HSD* in reproductive tissues (Richer et al., 1998)(Pierkoz et al., 2005). Stat5b-deficiency in mice resulted in pregnancy loss during mid-gestation. This was correlated with increased expression of ovarian 20α-HSD and decreased circulating P_4 (Pierkorz et al., 2005). In the present study, we demonstrated for the first time that STAT5b is a bonafide target of miR-200a and observed that upregulation of miR-200a within the myometrium of laboring mice and humans was associated with decreased STAT5b expression and increased expression and activity of 20α-HSD (Williams et al. 2012a). In an effort to further investigate the role of the miR-200a during the transition to labor, we used quantitative reverse transcriptase PCR (qRT-PCR) to assess miR-200a expression within pregnant mouse myometrium collected from late gestation to active labor (which includes gestation days 15.5, 16.5, 17.5, 18.5 and 19.0).

Results: Regulation of the miR-200a in Late Gestation and Active Labor

The results of the qRT-PCR analysis revealed that miR-200a expression significantly increased between 15.5 dpc and labor (Figure 2-1.1A) in a pattern similar to that of miR-200b and miR-429 and in a manner reciprocal to the expression of ZEB1 (Renthal et al., 2010). Notably, these three miR-200 family members are clustered together on mouse chromosome 4 and on human chromosome 1. Additionally, the 3'UTR of ZEB1 contains binding sites for miR-200b/200a/429, which allow these miRNAs to collectively contribute to inhibition of ZEB1 expression in various tissues (Renthal et al., 2010). In previous studies, we observed that adenoviral overexpression of miR-200b/200a/429 in cultured human myometrial cells (hTERT-HM) suppressed ZEB1 expression (Renthal et al., 2010) and in recent studies we found that overexpression of miR-200a alone was sufficient to repress ZEB1 expression in cultured human myometrial cells (Williams et al., 2012a). This suggests that the reciprocal expression of miR-200a and ZEB1 in the myometrium during the transition to labor is due to their existence in a double negative feedback loop (Williams et al., 2012a).

Discussion

Our finding of the upregulation of miR-200a expression towards term agrees with our previous findings of miR-200b and miR-429 expression within the pregnant myometrium (Renthal et al., 2010). This also supports the hypothesis that these miRNAs are similarly regulated in the myometrium during pregnancy and labor. Additionally, since miR-200b, miR-429 and miR-200a share similar seed sequences and have a similar expression pattern, it's likely that these miRNAs target a similar set of messenger RNA targets that function to regulate changes in the pregnant myometrium during the transition to labor.

During gestation in the mouse, serum P_4 levels remain high throughout most of pregnancy to maintain the uterus in a quiescent state. After 17.5 dpc, circulating P_4 levels begin to decline and the uterus switches from a quiescent state to a contractile state (Virgo et al., 1974). Interestingly, a significant increase in the expression of miR-200a, miR-200b and miR-429 was first observed at this time point, suggesting that changes in P_4 may influence the expression of these miRNAs in the uterus.

Having established the temporal expression pattern of miR-200a in the myometrium during late gestation, we next sought to identify specific

mRNA targets of miR-200a and examine how they might influence the contractile state of the uterus during the progression towards labor.

Materials and Methods

Mouse Myometrial Preparations. All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center. Eight-week-old timed-pregnant ICR/CD1 female mice were purchased from Harlan Laboratories. Timed-pregnancies were achieved by housing female mice with male mice overnight, beginning at 1800 hours. Mice found to have vaginal plugs at 0600 hours were considered to be 0.5 dpc. Uterine tissues were isolated from pregnant mice at 15.5, 16.5, 17.5, and 18.5 dpc and on delivery of the first pup (labor). The uterus was cleared of all embryonic material and maternal decidua and was further enriched for myometrium by sterile scraping and blotting with a paper towel. The remaining myometrial tissue was washed in 1× PBS and flash-frozen for subsequent protein and mRNA analysis.

Reverse Transcriptase-Quantitative PCR (qRT-PCR) Analysis of miR-200a. cDNA was reverse-transcribed from total RNA using specific miRNA primers from the TaqMan miRNA assay and reverse transcription kit (Applied Biosystems). PCR products were amplified from cDNA samples using the TaqMan miRNA assay. PCR for each sample was performed in triplicate using a miRNA-specific TaqMan probe and TaqMan Universal

PCR Master Mix (Applied Biosystems) on an ABI Prism 7700 Detection System (Applied Biosystems). miRNA expression was normalized to U6 snRNA using the ΔΔCt method.

CHAPTER 2 – Section 2

Transcriptional Repressor STAT5b is Reciprocally Regulated with miR-200a in Late Gestation and Labor

Experimental Design

Using computational analysis (Target Scan; http://www.targetscan.org/), we found that miR-200a is predicted to target over 700 mRNAs. In order to refine the list of potential targets, we first excluded those targets that were not conserved in both mice and humans. Next, we only included those targets with at least two putative binding sites and a predictive context score (which predicts the likelihood of miRNAs to bind a target) less than -0.20 since these are more likely to be valid targets (Table 2-2.1). Then, we performed a literature review to search for those remaining putative targets that function in processes associated with the initiation of labor, such as inflammation or hormonal regulation.

To determine whether the expression of the possible miR-200a targets were inversely related to the expression of miR-200a, quantitative reverse transcriptase PCR (qRT-PCR) and immunoblotting analysis were employed using myometrial tissues from pregnant mice across late

gestation. Subsequently, we conducted miRNA adenoviral and miRNA mimic overexpression studies in cultured human myometrial cells (hTERT-HM) to confirm the miRNA/target relationship in the myometrium. Then, to assess whether miR-200a directly targets and represses the expression of our prospective target(s), we transfected COS7 cells with miR-200a mimics and a luciferase reporter plasmid comprised of a portion of the predicted target's 3'UTR that contains the putative miR-200a binding sites subcloned downstream of the *luciferase* gene (Figure 2-1.2E).

Results: STAT5b is Suppressed by miR-200a in Uterus

Using our exclusion criteria, only 5 of the 744 potential targets were considered for further study (Table 2-2.1). After evaluating the relevance of these putative targets to parturition, we selected signal transducer and activator of transcription (STAT)5b since it is known to transcriptionally repress expression of the gene encoding the P_4-metabolizing enzyme, *20α-hydroxysteroid dehydrogenase (20α-HSD)*, in reproductive tissues (Piekorz et al., 2005). Using TargetScan prediction software, we identified two putative binding sites for miR-200a in the 3'UTR of STAT5b and found that STAT5b has a context score of -0.20. Notably, Stat5b-deficiency in mice resulted in pregnancy loss during mid-gestation. This was correlated with increased expression of ovarian 20α-HSD and decreased circulating P_4 (Piekorz et al., 2005). Furthermore, the abortion rate in Stat5b-deficient mice was partially corrected by crossing them with mice deficient in 20α-HSD. 20α-HSD-deficient mice maintain high serum P_4 levels throughout pregnancy, which results in a delay of labor greater than 2 days (Ishida et al., 2007). This is quite significant considering that gestation in the mouse is only 19 days long. Collectively, these data highlight the importance of STAT5b in the timing of labor. While these published studies focused on STAT5b regulation of 20α-HSD in the corpus luteum and on the functional luteolysis that occurs near the end of pregnancy in mice, we postulated

that local metabolism of P_4 within the myometrium might be of even greater importance. This led us to investigate the role of miR-200a in STAT-5b/20α-HSD regulation within the myometrium of mice and humans during pregnancy and labor. As shown in Figure 2-1.1A, miR-200a is upregulated in the myometrium of pregnant mice beginning at 17.5 dpc. qRT-PCR and immunoblot analysis of STAT5b mRNA and protein expression reveal that during late gestation STAT5b expression remains relatively high until 17.5 dpc, at which time protein levels begin to decline with a significant decrease in STAT5b mRNA from 18.5 dpc into labor (Figure 2-1.1B-C).

As mentioned, the 3'UTR of STAT5b contains two putative binding sites for miR-200a; however, direct binding of miR-200a to the STAT5b 3'-UTR and miR-200 inhibition of STAT5b expression have not previously been tested. To determine whether miR-200a inhibits endogenous STAT5b expression in human hTERT immortalized myometrial cells, hTERT-HM (Condon et al., 2002) were infected with recombinant adenovirus expressing miR-200b/200a/429. Following transduction of miR-200 family members, STAT5b expression was found to be repressed at the mRNA (Figure 2-1.2A) and protein (Figure 2-1.2B) levels. Moreover, transfection of the hTERT-HM cells with miR-200a mimics also significantly decreased STAT5b mRNA expression (Figure 2-1.2D). In

subsequent studies we employed luciferase reporter assays to investigate whether STAT5b is a bonafide target of miR-200a. For these experiments, miR-1 mimic was cotransfected as a negative control, since miR-1 is not predicted to target STAT5b by TargetScan analysis (http://www.targetscan.org/). Enhanced expression of miR-200a significantly repressed luciferase reporter activity, while transfection of miR-1 had no effect (Figure 2-1.2F). In cells transfected with a luciferase reporter construct in which both miR-200a binding sites in the STAT5b 3'UTR were mutated, this repression was lost (Figure 2-1.2F).

Discussion

These findings establish a novel relationship between miR-200a and STAT5b in the murine myometrium during late gestation. Although most of the past research on STAT5b in reproductive organs has focused on the mammary gland and ovary, STAT5b expression has previously been reported in the uterus (Hewitt et al., 2010). Additionally, P_4 was shown to induce STAT5b expression and nuclear localization in breast cancer cells (Carvajal et al., 2005) and to inhibit 20α-HSD expression *in vitro* (Bao et al., 2007).

As previously stated, STAT5b is a transcription factor that homo- or heterodimerizes in the cytoplasm upon phosphorylation/activation and then translocates to the nucleus to regulate the expression of target genes by binding to a $TTCN_{2-4}GAA$ motif in the promoter region of the gene target. Due to its role as a transcriptional regulator and its suggested role in the regulation of 20α-HSD and in the timing of labor, we hypothesized that during pregnancy, inhibition of 20α-HSD by STAT5b within the myometrium helps to maintain high local P_4 levels and to maintain uterine quiescence.

Materials and Methods

Reverse Transcriptase-Quantitative PCR (qRT-PCR) Analysis. RNA was DNase-treated (Invitrogen), and 2 µg were reverse-transcribed using the SuperScript III-RT kit (Invitrogen). Gene expression analysis was conducted using SYBR Green (Applied Biosystems) on an ABI Prism 7700 Detection System (Applied Biosystems). Relative gene expression was calculated using the comparative cycle threshold (ΔΔCt) method. qPCR cycling conditions were 50°C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. The cycle threshold was set at a level at which the exponential increase in PCR amplification was approximately parallel between all samples. All primer sets produced amplicons of the expected size and sequence. The primer sequences for analysis were as follows: mouse GAPDH (Forward: 5' AGG TCG GTG TGA ACG GAT TTG '3; Reverse: 5' TGT AGA CCA TGT AGT TGA GGT CA '3), mouse STAT5b (Forward: 5' CAC CCG CAA TGA TTA CAG CG '3; Reverse: 5'CTC TTG ATT CGT TTC AGG GAC A '3), mouse Nur77 (Forward: 5' TTG AGT TCG GCA AGC CTA CC '3; Reverse 5' GTG TAC CCG TCC ATG AAG GTG '3). Gene expression was normalized to GAPDH using the comparative cycle threshold (ΔΔCt) method.

Immunoblot Analysis. Tissues were lysed using the NE-PER extraction reagent kit (catalog no. 78833; Pierce) according to the manufacturer's instructions. Protein concentration was determined by a Bradford assay (BCA Protein Assay Kit, catalog no. 23227; Pierce). Equivalent amounts of protein were added to 2× Laemmli buffer, and samples were heated to 95°C for 10 min, loaded on 10% (wt/vol) SDS-polyacrylamide gels, run at 70–110 V, and transferred to a PVDF membrane. Membranes were incubated in blocking buffer composed of Tris-buffered saline [0.15 M NaCl, 0.05 M Tris·HCl (pH 8.0), 0.05% (vol/vol) Tween 20 (TBST)] containing 3% (wt/vol) nonfat dry milk for 60 min at room temperature before addition of primary antibodies. The expression of proteins of interest was determined using primary polyclonal antibodies against STAT5b (Abcam) at 1:1000 dilutions, respectively. β-Actin (Abcam) was analyzed as a loading control. Antibodies were diluted in blocking buffer. Primary antibodies were incubated with membranes overnight at 4 °C with rocking. Membranes were washed four times with TBST for 5 min each time at room temperature. Horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (catalog no. 62-1820; Zymed Invitrogen) was diluted in blocking buffer (1:6,000) and incubated with membranes for 60 min. Membranes were washed four times in TBST for 5 min each and visualized using SuperSignal West Pico Chemiluminescent substrate

(Thermo Scientific) according to the manufacturer's protocol. Quantitative measurement of immunoblots was performed using Image J (National Institutes of Health).

Transfection and Transduction of Human Myometrial Cells. Immortalized human myometrial cells (hTERT-HM) (Condon et al., 2002) were cultured in DMEM-F12 medium (Gibco) with 10% FBS (vol/vol). In the miR-200 overexpression experiments, hTERT-HM cells were infected overnight with recombinant adenoviruses expressing miR-200b/200a/429 or β-galactosidase (control) at a multiplicity of infection of 500. The cells were harvested 72 h after infection. β-Gal staining (β-Gal Staining Kit, Invitrogen) was performed to assess transduction efficiency. Other dishes of cells were transfected with miR-200a mimics. Briefly, hTERT-HM cells were transfected with miR-200a or scramble mimics (20 nM, Qiagen) using HiPerFect Transfection Reagent (Qiagen). Cells were harvested after 24 h for mRNA and protein analysis. The hTERT-HM cells were at ~80% confluency at the time of these experiments.

Luciferase Reporter Assays. TargetScan software was used to identify putative miR-200a binding sites in STAT5b. STAT5b 3'UTR containing potential miR-200a binding sites was amplified from human genomic DNA

using the following primers: forward: 5'-GGGACTAGTCTTCAGCTTCTTCATCTTCACCAGAGGAAT-3'; reverse: 5'- GGGAAGCTTTTTAAAAAAGTCATCTTCCAATAAATAATT-3'. The 530 bp fragment was then cloned 3' of luciferase in pMIR-REPORT (Invitrogen); sequence of the recombinant plasmid was confirmed prior to transfection. For mutation analysis, QuikChange II Site-Directed Mutagenesis Kit (Stratagene) was used to mutate three nucleotides in both putative miR-200a binding sites (AGUGUU to AGUCCC). COS-7 cells were cotransfected with these reporter plasmids and 20 nM miR-200a or miR-1 (control) and then assayed for relative luciferase activity 48 h later. To normalize for transfection efficiency, cells were cotransfected with a β-galactosidase expression plasmid.

CHAPTER 2 – Section 3

STAT5b Transcriptionally Represses 20α-HSD in the Myometrium During Late Gestation and Labor

Experimental Design

While the detailed mechanisms that culminate in labor are incompletely defined, there is growing evidence to support the concept that parturition in all species is initiated by a concerted series of biochemical mechanisms that antagonize the ability of the P_4/PR to regulate target genes in the uterus and cervix that maintain myometrial quiescence. In particular, enhanced local metabolism of P_4 to inactive products has been implicated in the process of parturition. Increased P_4 metabolism within the cervix (Mahendroo et al., 1999) and pregnant uterus approaching term has been observed in a number of species (Mahendroo et al., 1999)(Puri et al., 1982)(Power et al., 1987)(Csapo et al., 1981)(Runnebaum et al., 1971). In myometrium of pregnant women at term, there is a dramatic increase in the ratio of the inactive P_4 metabolite, 20α-dihydroprogesterone (20α-OHP), to P_4 (Runnebaum et al., 1971). Since 20α-OHP is generated by 20α-HSD, this finding suggests there is an increase in 20α-HSD expression during the transition to labor. To investigate this further, we used qRT-PCR, immunoblotting and enzyme

activity assays to determine the changes in 20α-HSD expression and activity across late gestation.

While previous studies indicate that STAT5b negatively regulates 20α-HSD expression in the ovary (Piekorz et al., 2005), this relationship has not been established in the myometrium. To investigate whether STAT5b regulates 20α-HSD expression in the myometrium, we transfected the immortalized human myometrial cell line, hTERT-HM with a CMV-driven expression plasmid for STAT5b, and then assessed 20α-HSD mRNA expression using qRT-PCR. Effects of siRNA-mediated STAT5b knockdown in the hTERT-HM cells also were used to assess the role of endogenous STAT5b on 20α-HSD expression in myometrium.

It was previously suggested that STAT5b inhibits 20α-HSD expression by binding to a putative STAT5b response element(s) within the *20α-HSD* promoter (Bao et al., 2007). In those studies, cotransfection of a reporter construct containing -2,500 bp of the *20α-HSD* 5'-flanking region fused to luciferase together with an expression vector for constitutively active Stat5b caused substantial inhibition of *20α-HSD* promoter activity. Using computational analysis (MatInspector) and visual inspection, we found two putative STAT5b response elements within this genomic region at -155 and -547 bp. To assess gestational changes in

binding of endogenous STAT5b to the proximal region of the *20α-HSD* promoter that contains these putative STAT5b response elements, we performed chromatin immunoprecipitation (ChIP) assays comparing myometrial tissues from pregnant mice at 15.5 dpc to myometrium collected just prior to labor at 18.5 dpc.

Results: STAT5b Suppresses 20α-HSD Expression

In the present study, qRT-PCR and immunoblotting revealed a decline in STAT5b mRNA and protein expression in the mouse myometrium between 15.5 dpc and labor (Figure 2-1.1B-C). This gestational decline in STAT5b expression during late gestation was associated with a dramatic, but transient increase in 20α-HSD mRNA (Figure 2-3.1A) that resulted in a sustained increase in both 20α-HSD protein (Figure 2-3.1B) and enzymatic activity (Figure 2-3.1C).

In our investigation of STAT5b regulation of 20α-HSD expression in the myometrium, we found that transfection of hTERT-HM cells with a CMV-driven expression plasmid for STAT5b augmented STAT5b expression at the mRNA and protein levels and caused a decrease in 20α-HSD mRNA levels (Figure 2-3.2A-C). Conversely, 20α-HSD mRNA expression was increased as a consequence of siRNA-mediated knockdown of STAT5b mRNA and protein expression in cultured human myometrial cells (Figure 2-3.2D-F).

In order to determine whether STAT5b might directly interact with the promoter region of 20α-HSD to cause these transcriptional changes within the pregnant myometrium, we assessed binding of endogenous STAT5b to the putative STAT5b response elements in the proximal

portion of the *20α-HSD* promoter using ChIP. In support of our hypothesis, we also found that endogenous STAT5b binding to the proximal portion of the *20α-HSD* promoter was relatively high at 15.5 dpc and declined markedly at 18.5 dpc, just prior to labor (Figure 2-3.3).

Discussion:

Collectively, these findings provide strong evidence for the inhibitory role of STAT5b in the transcriptional regulation of the *20α-HSD* gene within the myometrium during pregnancy.

In mice, STAT5b deficiency caused pregnancy loss during mid-gestation (Piekorz et al., 2005), whereas, *20α-HSD*$^{-/-}$ mice manifest delayed parturition and increased fetal demise at birth (Ishida et al., 2007). Although, 20α-HSD is expressed in a number of reproductive organs, the abortion phenotype in STAT5b-deficient mice was presumed to be due to enhanced 20α-HSD expression in the corpora lutea and to premature luteolysis, resulting in decreased circulating levels of P_4 (Piekorz et al., 2005). However, circulating P_4 levels in the STAT5b null mice still remained higher than the K_d for binding to the PR (Ishida et al., 2007). These data support our hypothesis that local P_4 metabolism in PR target tissues, such as the uterus, may be critical. Since the above mentioned studies entirely focused on the ovary and on circulating P_4 levels and did not assay for myometrial 20α-HSD and STAT5b expression or tissue P_4 levels, we found it imperative to investigate changes in STAT5b and 20α-HSD within the myometrium during late gestation and with labor in the pregnant mouse.

Interestingly, 20α-HSD mRNA has a unique expression pattern across mouse gestation with a sharp peak at 17.5 dpc, followed by a pronounced decline. Since there is significant homology among members of the AKR superfamily of NAD(P)(H)-dependent oxidoreductases, we performed sequence analysis of the qRT-PCR products to confirm our qRT-PCR results. In these studies we verified that the qRT-PCR primers for 20α-HSD were amplifying the transcript for the 20α-HSD gene, *AKR1C18*. Since 20α-HSD has such a distinctive pattern of expression during late gestation, we hypothesize that the transcriptional upregulation of 20α-HSD is tightly controlled by a number of regulatory factors, including STAT5b. Indeed, in previous studies of the regulation of 20α-HSD gene expression in the corpus luteum it was observed that the transcription factor nerve growth factor IB (Nur77) induced expression of 20α-HSD mRNA in mouse corpora lutea (Stocco et al., 2000). Intriguingly, in myometrial tissue samples from a mouse gestational series we observed a steady increase in the expression of Nur77 mRNA (Figure 2-3.4), suggesting that in the mouse uterus there are other transcription factors involved in its regulation during gestation. Additionally, although we did not observe a sustained increase in 20α-HSD mRNA levels during the transition to labor, the dramatic, but transient increase in 20α-HSD mRNA

(Figure 2-3.1A) was followed by a sustained increase in both 20α-HSD protein (Figure 2-3.1B) and enzymatic activity (Figure 2-3.1C).

Interestingly, in previous studies from our lab we observed that the macrophages that infiltrate the myometrium during the transition to labor express high levels of 20α-HSD. Furthermore, previous studies concerning 20a-HSD expression in the uterus have only focused on the endometrium. To confirm that myocytes of the myometrium contribute to the increase in 20α-HSD protein expression and activity during late gestation, we conducted immunofluorescence for 20α-HSD in sections of pregnant murine uterus (Figure 2-3.5). While we observed negligible staining for 20α-HSD at 15.5 dpc, we observed abundant 20α-HSD immunostaining at 18.5 dpc in the endometrium and modest expression within the myometrium. This finding confirmed our immunoblot results, which indicate an increase in 20α-HSD protein expression within the myometrium just before labor (Figure 2-3.1B). These data confirm the presence of 20α-HSD in the myometrium. Since the uterus forms a coordinated contractile unit with many intercellular junctions during the transition to labor, it is possible that the 20α-HSD produced in the endometrium also contribute to the metabolism of P_4 in the myometrium. Thus, our findings not only highlight 20α-HSD as an active player during the transition to labor, but also implicate STAT5b as negative regulator of this important P_4 metabolizing enzyme.

The induction of 20α-HSD expression within mouse myometrium at 17.5 dpc temporally coincides with the significant increase in miR-200a expression and decline in STAT5b protein levels. This time point also is associated with a decline in circulating levels of P_4, which is known to stimulate STAT5b expression (Lin et al., 2003). Moreover, the decrease in P_4 is associated with decreased ZEB1 expression and induction of miR-200 family expression in myometrium, which further suppresses STAT5b expression.

To further investigate the relationship between STAT5b and 20α-HSD, we observed that myometrial 20α-HSD was negatively regulated by STAT5b overexpression in human myometrial cells and noted a reciprocal relationship similar to that previously reported in the ovary (Piekorz et al., 2005). Our findings to reveal STAT5b as a bonafide target of miR-200a highlight the potential importance of miR-200a regulation of STAT5b in the local control of 20α-HSD and P_4 metabolism in myometrium. The role of miR-200a as a negative regulator of STAT5b was further supported by findings that miR-200a overexpression caused a decline in STAT5b and a coordinate increase in 20α-HSD mRNA expression in human myometrial cells (Figure 2-1.2C).

Together these data suggest that during most of pregnancy, STAT5b-mediated inhibition of 20α-HSD expression within the myometrium helps to maintain high local P_4 levels and sustains myometrial quiescence. Considering the essential role of P_4 in the maintenance of pregnancy and reported the influence of P_4 on STAT5b expression in mammary cells, it was of importance to investigate the effect of P_4 on miR-200a, STAT5b and 20α-HSD in the myometrium.

Materials and Methods

Reverse Transcriptase-Quantitative PCR (qRT-PCR) Analysis. RNA was DNase-treated (Invitrogen), and 2 μg were reverse-transcribed using the SuperScript III-RT kit (Invitrogen). Gene expression analysis was conducted using SYBR Green (Applied Biosystems) on an ABI Prism 7700 Detection System (Applied Biosystems). Relative gene expression was calculated using the comparative cycle threshold (ΔΔCt) method. qPCR cycling conditions were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C f or 15 sec and 60°C for 1 min. The cycle threshold was set at a level at which the exponential increase in PCR amplification was approximately parallel between all samples. All primer sets produced amplicons of the expected size and sequence. The primer sequences for analysis were as follows: mouse GAPDH (Forward: 5' AGG TCG GTG TGA ACG GAT TTG '3; Reverse: 5' TGT AGA CCA TGT AGT TGA GGT CA '3), mouse 20α-HSD (Forward: 5'GGA GGC CAT GGA GAA GTG TA '3; Reverse: 5'ATG GCA TTC TAC CTG GTT CG '3). Gene expression was normalized to GAPDH using the comparative cycle threshold (ΔΔCt) method.

Immunoblot Analysis. Tissues were lysed using the NE-PER extraction reagent kit (catalog no. 78833; Pierce) according to the manufacturer's

instructions. Protein concentration was determined by a Bradford assay (BCA Protein Assay Kit, catalog no. 23227; Pierce). Equivalent amounts of protein were added to 2× Laemmli buffer, and samples were heated to 95°C for 10 min, loaded on 10% (wt/vol) SDS-polyacrylamide gels, run at 70–110 V, and transferred to a PVDF membrane. Membranes were incubated in blocking buffer composed of Tris-buffered saline [0.15 M NaCl, 0.05 M Tris·HCl (pH 8.0), 0.05% (vol/vol) Tween 20 (TBST)] containing 3% (wt/vol) nonfat dry milk for 60 min at room temperature before addition of primary antibodies. The expression of proteins of interest was determined using primary polyclonal antibodies against 20α-HSD (graciously provided by Geula Gibori, Univ. Illinois) at a 1:5000 dilution and STAT5b (Abcam) at 1:1000 dilution. β-Actin (Abcam) was analyzed as a loading control. Antibodies were diluted in blocking buffer. Primary antibodies were incubated with membranes overnight at 4°C with rocking. Membranes were washed four times with TBST for 5 min each time at room temperature. Horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (catalog no. 62-1820; Zymed Invitrogen) was diluted in blocking buffer (1:6,000) and incubated with membranes for 60 min. Membranes were washed four times in TBST for 5 min each and visualized using SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific) according to the manufacturer’s protocol. Quantitative

measurement of immunoblots was performed using Image J (National Institutes of Health).

20α-HSD Enzyme Activity. Uteri were isolated from pregnant ICR mice from 15.5 dpc to 18.5 dpc. Tissues were homogenized in 10 mM potassium phosphate, 150 mM KCl and 1 mM EDTA. Protein concentrations were determined using a Bio-Rad Protein Assay Kit. P_4 metabolism was assessed by incubating tissue homogenates (5 µg protein) in 0.1 M Tris-citrate buffer, pH 7.0, containing 5 µM [^{14}C]P_4 (New England Nuclear Corp) and 5 mM NADPH (Sigma) in a total volume of 0.5 ml for 1 h at 37°C. Steroids were extracted into 5 ml methylene chloride and dried under a stream of nitrogen. Steroids were dissolved in 20 µl chloroform-methanol (2:1, vol/vol), spotted onto Silica Gel 150 TLC plates (4855-821, Whatman), and resolved by development in chloroform-ethylacetate (3:1, vol/vol). Radiolabeled steroids were visualized on a Bioscan imaging detector.

Chromatin Immunoprecipitation Assays. Uterine tissues were harvested from 8-week-old timed-pregnant ICR/CD1 female mice at 15.5 and 18.5 dpc. The uteri were cleared of all embryonic material and maternal decidua and were further enriched for myometrium by sterile

scraping and blotting with a paper towel. The remaining myometrial tissues were homogenized in cold 1× PBS, protease inhibitor mixture (Roche), and 10 mM PMSF. Homogenized samples were incubated with 1% formaldehyde, protease inhibitor mixture, and 10 mM PMSF for 15 min at room temperature to cross-link proteins and DNA. Samples were washed twice in cold 1× PBS, protease inhibitor mixture, and 10 mM PMSF and were then flash-frozen. ChIP was conducted using a ChIP Assay Kit (catalog no. 17-295; Millipore) according to the manufacturer's instructions. Briefly, samples were thawed by the addition of 1,000 μL of lysis buffer (catalog no. 20-163; Millipore). The lysates were sonicated on ice three times at 40% amplitude for 10 s to produce sheared soluble chromatin. The soluble chromatin was precleared with Protein A Agarose/Salmon Sperm DNA (75 μL, 50% slurry, catalog no. 16-157C; Millipore) at 4 °C for 30 min with rotation. The samples were microfuged at 14,000 × g to pellet the beads, and the supernatant containing the sheared chromatin was placed in different tubes. DNA concentration was determined using a spectrophotometer, and equivalent amounts of precleared chromatin were incubated with antibody for STAT5b (catalog no. ab7969, Abcam) at 4 °C overnight. An aliquot incubated with nonimmune IgG was used as a control. Protein A Agarose/salmon sperm DNA (60 μL, 50% slurry, catalog no. 16-157C; Millipore) was added to

each tube, the mixtures were incubated for 1 h at 4°C, and the immune complexes were collected by centrifugation. The beads containing the immunoprecipitated complexes were washed sequentially for 5 min in Low Salt Immune Complex Wash Buffer (catalog no. 20-154; Millipore), High Salt Immune Complex Wash Buffer (catalog no. 20-155;

Millipore), LiCl Immune Complex Wash Buffer (catalog no. 20-156; Millipore), and TE Buffer (catalog no. 20-157; Millipore). The beads were eluted with 250 µL of freshly prepared elution buffer at room temperature. Elution was repeated once, and eluates were combined. Cross-linking of the immunoprecipitated chromatin complexes and input controls (10% vol/vol of the total soluble chromatin) was reversed by heating the samples at 65°C for 4 h. Proteinase K (15 µg; Invitrogen) was added to each sample in buffer and incubated for 1 h at 45 °C. The DNA was purified by phenol-chloroform extraction and precipitated in ethanol overnight at −20°C. Samples and input controls were diluted in 10–400 µL of RNase/DNase-free water before qPCR. qPCR was employed, using at least three primer sets to confirm binding to the AKR1C18 promoter: mouse AKR1C18 promoter (Forward: 5’ GCTTCCTCACTGGGTGGAGTTGG ‘3; Reverse: 5’ GGGGCACAAAACCTCAGGAGCC ‘3),

Immunofluorescence. Cryosections of pregnant mouse whole uterus were postfixed in 3.7% formalin in PBS for 10 min. The sections were then blocked with normal goat serum for 30 min. Incubation with a primary antibody for 20α-HSD (1:1000 dilution, provided by Dr. G. Gibori) was performed overnight at 4°C in the dark. The sections were then incubated with anti-goat fluorophor-labeled secondary antibody (1:750 dilution, Jackson ImmunoResearch) for one hour at room temperature, followed by a counterstain with 4,6-diamino-2-phenylindole (DAPI, 1:100,000 dilution, Sigma) for 5 minutes at room temperature. A confocal microscope (Leica TCS SP5) was used to capture images of the slides and ImageJ software was used to generate z-projections of z-stacks.

CHAPTER 2 – Section 4

The miR-200a/STAT5b/20α-HSD Relationship is Conserved from Mice to Humans

Experimental Design

Because of the notable differences in circulating P_4 and estrogen levels during pregnancy and labor in mice and humans, we studied the expression pattern of miR-200a/STAT5b/20α-HSD in myometrial samples from women in labor *vs.* not in labor at term. Since miRNAs are evolutionary conserved across various species, including mouse and human, we hypothesized that the roles and relative expression pattern of miR-200a and its targets would be conserved, as well. Importantly, miR-200a is expressed both in mouse and human and the seed sequences of miR-200a are identical between the two species. Moreover, miR-200a is clustered together in a primary transcript with miR-200b and miR-429 in both mice and humans and in previous studies we observed that the miR-200b/miR-429/ZEB expression pattern and relative role in pregnancy is conserved from mouse to human. To determine whether this temporal pattern of regulation is conserved from mouse to human, we analyzed

expression of miR-200a, STAT5b and, 20α-HSD and 20α-HSD activity in myometrial biopsies from women at term who were either in labor or not in labor using qRT-PCR, immunoblotting and enzyme activity assays.

Results: The miR-200a/STAT5b/20α-HSD Relationship is Conserved from Mouse to Human

Similar to our findings in the mouse, myometrial miR-200a was increased (Figure 2-4.1A), while STAT5b mRNA (Figure 2-4.1B) and protein (Figure 2-4.1C) expression were decreased in myometrium from women in labor as compared to those not in labor. In contrast to the changes observed in STAT5b, 20α-HSD mRNA and protein expression (Figure 2-4.2A-B) and activity (Figure 2-4.2A) were increased in myometrium from women in labor, as compared to those not in labor. These findings indicate that miR-200a, STAT5b and 20α-HSD are regulated in similar fashion in both mice and humans during pregnancy and labor.

Discussion:

Excitingly, these findings stand in support of our hypothesis that the miR-200a/STAT5b/20α-HSD relationship in myometrium may serve a similar role during pregnancy and labor in mouse and human. These findings corroborate previous observations of increased levels of the 20α-HSD metabolite, 20α-OHP, relative to P_4 in the myometrium from pregnant women at term, implicating increase P_4 metabolism as a contributor to labor in humans (Runnebaum et al., 1971). Indeed, increased P_4 metabolism by the pregnant uterus approaching term has been observed in a number of species. In rats, mice, sheep and guinea pigs, there is a significant decline in local P_4 levels in uterine tissues near term (Puri et al., 1982)(Power et al., 1987)(Csapo et al., 1981). These findings are of great importance to our understanding of the mechanisms that underlie the decline in PR function in humans, since circulating P_4 levels remain extremely high throughout pregnancy and into labor in women (Mendelson et al., 2009). As mentioned previously, even in rodents where circulating P_4 levels plummet near term, they still remain higher than the K_d for binding of P_4 to its receptor. We propose that the decline in PR function that occurs in the pregnant myometrium near term is multifactorial in all species, and is caused by decreased expression of PR coactivators (Condon et al., 2003), increased levels and activity of inflammatory

transcription factors (Hardy et al., 2006), increased expression of truncated PR isoforms (Condon et al., 2006)(Merlino et al.) and increased local metabolism of P_4 to inactive hormones (Williams et al., 2012a).

Materials and Methods

Human Myometrial Preparations. Myometrial biopsies were obtained from the lower uterine segment of pregnant women undergoing cesarean section before and during the onset of active labor. After the tissues were collected, they were flash frozen and stored at -80° for subsequent protein and mRNA analysis.

Reverse Transcriptase-Quantitative PCR (qRT-PCR) Analysis. RNA was DNase-treated (Invitrogen), and 2 μg were reverse-transcribed using the SuperScript III-RT kit (Invitrogen). Gene expression analysis was conducted using SYBR Green (Applied Biosystems) on an ABI Prism 7700 Detection System (Applied Biosystems). Relative gene expression was calculated using the comparative cycle threshold (ΔΔCt) method. qPCR cycling conditions were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C f or 15 sec and 60°C for 1 min. The cycle threshold was set at a level at which the exponential increase in PCR amplification was approximately parallel between all samples. All primer sets produced amplicons of the expected size and sequence. Specific primers for human 36B4 were used as an internal standard. The primer sequences for analysis were as follows: human 36B4 (Forward: 5’ TGC ATC AGT ACC CCA TTC TAT CA ‘3; Reverse: 5’ AAG GTG TAA TCC

GTC TCC ACA GA ‘3), human STAT5b (Forward: 5’ GAA CAC CCG CAA TGA TTA CAG T ‘3; Reverse: 5’ ACG GTC TGA CCT AAT TCG T ‘3) and human 20α-HSD (Forward: 5’CAG CCA GGC TAG TGA CAG AA ‘3; Reverse: 5’ATT GCC AAT TTG GTG GC ‘3). For miRNA analysis, cDNA was reverse-transcribed from total RNA using specific miRNA primers from the TaqMan miRNA assay and reverse transcription kit (Applied Biosystems). PCR products were amplified from cDNA samples using the TaqMan miRNA assay. PCR for each sample was performed in triplicate using a miRNA-specific TaqMan probe and TaqMan Universal PCR Master Mix (Applied Biosystems) on an ABI Prism 7700 Detection System (Applied Biosystems). miRNA expression was normalized to U6 snRNA using the ΔΔCt method.

Immunoblot Analysis. Tissues were lysed using the NE-PER extraction reagent kit (catalog no. 78833; Pierce) according to the manufacturer’s instructions. Protein concentration was determined by a Bradford assay (BCA Protein Assay Kit, catalog no. 23227; Pierce). Equivalent amounts of protein were added to 2× Laemmli buffer, and samples were heated to 95°C for 10 min, loaded on 10% (wt/vol) SDS-polyacrylamide gels, run at 70–110 V, and transferred to a PVDF membrane. Membranes were incubated in blocking buffer composed of Tris-buffered saline [0.15 M

NaCl, 0.05 M Tris·HCl (pH 8.0), 0.05% (vol/vol) Tween 20 (TBST)] containing 3% (wt/vol) nonfat dry milk for 60 min at room temperature before addition of primary antibodies. The expression of proteins of interest was determined using primary polyclonal antibodies against STAT5b (Abcam) and 20α-HSD (graciously provided by Geula Gibori, Univ. Illinois) at 1:1000 and 1:5000 dilutions, respectively. β-Actin (Abcam) was analyzed as a loading control. Primary antibodies were incubated with membranes overnight at 4°C with rocking. Membranes were washed four times with TBST for 5 min each time at room temperature. Horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (catalog no. 62-1820; Zymed Invitrogen) was diluted in blocking buffer (1:6,000) and incubated with membranes for 60 min. Membranes were washed four times in TBST for 5 min each and visualized using SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific) according to the manufacturer's protocol. Quantitative measurement of immunoblots was performed using Image J (National Institutes of Health).

20α-HSD Enzyme Activity. Myometrial tissues were homogenized in 10 mM potassium phosphate, 150 mM KCl and 1 mM EDTA. Protein concentrations were determined using a Bio-Rad Protein Assay Kit. P_4 metabolism was assessed by incubating tissue homogenates (5 μg

protein) in 0.1 M Tris-citrate buffer, pH 7.0, containing 5 µM [^{14}C]P_4 (New England Nuclear Corp) and 5 mM NADPH (Sigma) in a total volume of 0.5 ml for 1 h at 37 C. Steroids were extracted into 5 ml methylene chloride and dried under a stream of nitrogen. Steroids were dissolved in 20 µl chloroform-methanol (2:1, vol/vol), spotted onto Silica Gel 150 TLC plates (4855-821, Whatman), and resolved by development in chloroform-ethylacetate (3:1, vol/vol). Radiolabeled steroids were visualized on a Bioscan imaging detector.

CHAPTER 2 – Section 5

Regulation of miR-200a, STAT5b and 20α-HSD in the Myometrium by Hormones and Inflammatory Factors

Experimental Design

As mentioned, ZEBs and miR-200 family members exist in a double negative feedback loop, whereby miR-200s suppress expression of ZEB1/2 posttranscriptionally and ZEBs inhibit miR-200 transcription (Bracken et al., 2008)(Wellner et al., 2009). Previously, we observed that P_4 treatment of mice and cultured myometrial cells specifically induced ZEB1, resulting in inhibition of miR-200b/429 expression (Renthal et al., 2010). P_4 was previously reported to repress 20α-HSD expression in rat corpus luteum (Sugino et al., 1997)(Stocco et al., 2001) and to induce STAT5 expression in human breast cancer cells (Richer et al., 2008). To test the hypothesis that P_4/PR-mediated induction of ZEB1 and subsequent repression of miR-200a results in enhanced expression of STAT5b and inhibition of 20α-HSD in myometrium, we analyzed effects of P_4 injection in uterine tissues of ovariectomized mice after 24 h (Figure 2-5.1A).

The role of P_4, acting through PR, in the maintenance of uterine quiescence throughout most of pregnancy has long been appreciated in

the field of reproductive biology (Mendelson et al., 2009)(Smith et al., 2007). Although circulating maternal P_4 levels decline precipitously near term in the rodent (Virgo et al., 1974), in humans and guinea pigs, circulating P_4 levels remain elevated throughout pregnancy and into labor, as do myometrial levels of PR (Challis et al., 2000)(Smith et al., 2009). Nonetheless, treatment with PR antagonists, mifepristone (RU486) or onapristone, can cause increased cervical ripening and spontaneous labor or enhanced sensitivity to labor induction by oxytocin or prostaglandins (Frydman et al., 1992)(Elliott et al., 1998)(Stenlund et al., 1999)(Chwalisz et al., 1994)(Dudley et al., 1996). For these reasons, we sought to further investigate the role of P_4 in the regulation of miR-200a, STAT5b and 20α-HSD in a mouse model of preterm labor induced by 'progesterone-withdrawal.' In this mouse model, pregnant mice are subcutaneously injected with RU486; labor ensues approximately 12 h later (Figure 2-5.2B). Myometrial tissue samples were collected from RU486 treated pregnant mice in preterm labor and from time-matched vehicle-treated pregnant mice not in labor.

Additionally, in consideration of the fact that bacterial infection is associated with ~30% of all preterm births, we also analyzed effects of preterm labor induction in mice by intraamniotic lipopolysaccharide (LPS) injection, which is relevant to infection-induced preterm labor in humans.

In this model, timed-pregnant mice were injected with LPS into each amniotic sac; preterm birth occurred in the majority of injected mice within 12-16 h (Renthal et al., 2010) (Figure 2-5.2A). qRT-PCR and immunoblotting analysis were then used to analyze miR-200a, STAT5b and 20α-HSD expression.

Results: Progesterone Regulates miR-200a, STAT5b and 20α-HSD in the Uterus

We observed that P_4 treatment of ovariectomized mice inhibited miR-200a expression in uterus within 24 h (Figure 2-5.3A). This was associated with a P_4-mediated induction of STAT5b mRNA (Figure 2-5.3B) and protein (Figure 2-5.3C) and a coordinate repression of 20α-HSD mRNA (Figure 2-5.3D). By contrast, treatment of 15.5 dpc mice with the PR antagonist RU486 to induce preterm labor significantly increased miR-200a expression (Figure 2-5.3E), inhibited STAT5b mRNA (Figure 2-5.3F) and protein (Figure 2-5.3G) and increased 20α-HSD expression (Figure 2-5.3H) in the uterus. These findings suggest that P_4/PR-mediated repression of miR-200 expression caused by induction of ZEB1 (Renthal et al., 2010) maintains uterine quiescence, in part, via upregulation of STAT5b and subsequent repression of 20α-HSD. Conversely, the increase in myometrial miR-200 expression caused by RU486 treatment inhibits STAT5b and induces 20α-HSD, resulting in enhanced local metabolism of P_4 and a further decline in PR function leading to preterm labor. Identical findings to those observed using RU486 (Figure 2-5.3E-H) were obtained in an inflammatory preterm labor model using intraamniotic injection of lipopolysaccharide (LPS) (Figure 2-5.4A-C).

Discussion:

The molecular mechanisms that maintain quiescence of the myometrium throughout most of pregnancy and mediate its conversion into a synchronously contracting unit culminating in parturition remain incompletely understood. It is clear that P_4 acting through PR is essential for maintaining myometrial quiescence, while an enhanced inflammatory response caused by signals from mother and fetus promotes the progression to labor (Mendelson et al., 2009). P_4/PR mediates uterine quiescence, in part, by suppressing NF-κB activation of contraction-associated genes (Hardy et al., 2006). Our recent studies revealed that P_4/PR also contributes to the maintenance of uterine quiescence during pregnancy via upregulation of the transcription factor, ZEB1, which inhibits expression of the contraction-associated genes, *OXTR* and *CX43* and suppresses the miR-200 family to promote further upregulation of ZEB1 and ZEB2 (Renthal et al., 2010).

Our present findings suggest that miR-200a serves a critical role in the regulation of P_4 metabolism in the myometrium during pregnancy and labor. During pregnancy, high levels of P_4/PR function increase expression of transcription factor ZEB1, which inhibits expression of miR-200a and other members of the miR-200 family (Renthal et al. 2010), as described above. The decreased levels of miR-200 permit enhanced expression of

STAT5b, which represses 20α-HSD to sustain elevated P_4 levels within the myometrium. During the transition to term and preterm labor, an increased inflammatory response promotes a decline in PR function in myometrium (Condon et al., 2003)(Kalkhoven et al., 1996)(Condon et al., 2006)(Merlino et al. 2007), resulting in decreased levels of ZEB1, which releases repression of the miR-200 family. The increased levels of miR-200a inhibit STAT5b expression, releasing repression of 20α-HSD. The increase in 20α-HSD activity, in turn, catalyzes metabolism of P_4 to reduce local P_4 levels in the myometrium and further promote the progression of labor. The decline in PR function may act in a positive feed-forward manner to further increase miR-200 expression, suppress STAT5b and induce 20α-HSD. Taken together, these findings highlight another important function for the miR-200 family in the timing of parturition and emphasize its potential importance as a therapeutic target for prevention of preterm labor.

Materials and Methods:

P_4 Treatment Studies. Six to eight-week-old ovariectomized ICR mice were obtained from Harlan Laboratories. Two weeks after ovariectomy, the mice were subcutaneously injected with P_4 (1 mg in 0.25 mL sesame oil) or with sesame oil (vehicle). Uterine tissues were collected 24 h post-injection and flash frozen for subsequent protein and mRNA analysis.

Mouse Model of Preterm Labor Induced by Pharmacological P_4/PR Withdrawal. Induction of preterm labor using RU486 was implemented as described previously (Dudley et al., 1996)(Renthal et al., 2010). Briefly, 200 µg of RU486 (Sigma) or 5% ethanol (vehicle) were subcutaneously injected into the right flank of 15.5 dpc pregnant ICR mice. Preterm labor occurred in the RU486-injected mice within 12 h, while none of the vehicle-injected mice went into labor. The RU486 injected mice were sacrificed upon the birth of one pup; gestation-matched controls were sacrificed immediately afterwards.

Mouse Model of Preterm Labor by Lipopolysaccharide (LPS)-Induced Inflammation. Induction of preterm labor using LPS was implemented as described in detail previously (Renthal et al., 2010). Briefly, 15.5 dpc pregnant ICR/CD1 mice underwent laparotomy to expose the uterus after

anesthesia. LPS (1.5 µg in 50 µL PBS) or sterile PBS (vehicle) were injected into each amniotic sac and then the uterus was carefully reinserted into the abdominal cavity, the abdominal muscle wall and skin were sutured and the mouse was allowed to recuperate. There was a high rate of preterm labor in the LPS-injected mice (7 out of 10 mice), but no preterm labor occurred in the vehicle-injected mice. The LPS-injected mice were sacrificed upon the birth of one pup; gestation-matched controls were sacrificed immediately afterwards.

CHAPTER 3 – Section 1
Identifying MicroRNAs that are Downregulated in the Transition to Labor

Introduction and Experimental Design

Progesterone (P_4) and estradiol-17β (E_2) play critical and opposing roles in regulating myometrial quiescence and contractility during pregnancy and labor (Nissenson et al., 1978). While these contrasting hormonal effects are likely mediated via differential regulation of inflammatory and contractile genes, the underlying mechanisms remain incompletely understood. As mentioned in Chapter 2, we utilized a miRNA microarray assay comparing quiescent pregnant mouse myometrium at 15.5 dpc to contractile myometrium just before labor at 18.5 dpc in order to discover miRNAs that are differentially expressed during transition of the pregnant myometrium from a quiescent state to a highly contractile unit.

While these studies have established a compelling role for the members of the miR-200 family in pregnancy and labor, we next sought to identify what other miRNAs are temporally regulated during late gestation and control expression of genes in the pregnant uterus that control myometrial quiescence and contractility. Further analysis of the miRNA microarray results revealed that levels of the miR-199a cluster, miR-199a-

3p, miR-199a-5p and miR-214, were significantly decreased in laboring myometrium of pregnant mice across late gestation (Figure 1-1.6). From the miRNA microarray analysis it was also found that all three of the miRNAs within the miR-199a cluster were highly expressed in the pregnant mouse myometrium (Figure 1-1.6) at even greater levels than the miR-200 family members which are known to play a role in regulating the expression of contraction-associated genes (Renthal et al., 2010). This not only confirms the presence of these miRNAs within the myometrium, but also suggest these miRNAs may also play a role during pregnancy and labor.

Interestingly, miR-199a-3p and miR-214 are known (Chakrabarty et al., 2007) and predicted, respectively, to target the mRNA encoding COX-2, a key regulatory enzyme in synthesis of prostaglandins, which are potent stimulators of uterine contractility (Olson et al., 2007)(Rauk et al., 2000). Although, miR-199a-5p is not predicted to target COX-2, it has been shown to target one of the catalytic subunits of the IκB kinase (IKK) complex, IκB kinase beta (IKKβ), thereby, suppressing NF-κB pathway activation. Furthermore, studies revealed that inhibition of the NF-κB pathway resulted in a decrease in proinflammatory mediators.

In an effort to validate our miRNA microarray findings, we used qRT-PCR to assess miR-199a-3p, miR-199a-5p and miR-214 expression

within pregnant mouse myometrium collected from late gestation to active labor (which consists of gestation days 15.5, 16.5, 17.5, 18.5 and 19.0). In order to determine if the putative messenger RNA targets might be regulated by our miRNAs of interest, we conducted qRT-PCR and immunoblotting analysis of myometrial tissues from a gestational series in mice to assess mRNA and protein expression of putative miR-199a cluster targets relevant to pregnancy and labor. As previously mentioned in Chapter 2, the focus of our studies is to advance the understanding of mechanisms underlying the maintenance of pregnancy and the initiation of labor in humans and aid in the development of improved therapies for preterm labor. Therefore, we also conducted parallel studies in myometrial biopsies from women at term who were either in labor or not in labor in order to confirm that the expression pattern of the miR-199a cluster and their respective targets is conserved from mouse to human.

Results: miR199a-3p/miR-214 and Their Target COX-2 are Reciprocally Regulated During Late Gestation and Active Labor in Mouse and Human Myometrium

qRT-PCR of RNA isolated from myometrial tissues of pregnant mice at 15.5 dpc, 18.5 dpc and in active labor confirmed that miR-199a-3p and miR-214 were significantly downregulated at 18.5 dpc and during labor as compared to 15.5 dpc (Figure 3-1.1A). We did not observe a significant change in the expression of miR-199a-5p in the mouse myometrial samples during late gestation (Figure 3-1.1B). In situ hybridization and northern blot experiments confirmed that miR-199a-3p was expressed in the myometrium at 15.5 dpc (Figure 3-1.2)(Figure 3-1.4). Notably, COX-2 mRNA remained low in the pregnant mouse myometrium until active labor, when its expression was markedly induced (Figure 3-1.3A). On the other hand, a pronounced increase in COX-2 protein expression was evident by 18.5 dpc and remained elevated in laboring myometrium (Figure 3-1.3B).

In studies of myometrium from women in labor at term, miR-199a-3p and miR-214 were significantly decreased, as compared to myometrial samples from gestationally matched non-laboring women (Figure 3-1.1C). Interestingly, although we did not observe a significant change in COX-2 mRNA (Figure 3-1.3C), there was marked induction of COX-2 protein in

laboring, compared to non-laboring myometrium (Figure 3-1.3D), further suggesting potential translational regulation.

Discussion

In the present study, we have obtained compelling evidence for a role of miR-199a-3p and miR-214 in the regulation of myometrial COX-2 expression during pregnancy and labor in mice and humans. Since we did not observe a significant change in the expression of miR-199a-5p in our myometrial samples, we decided to solely focus our studies on the relationship between miR-199a-3p/miR-214 and COX-2. Our findings regarding the decline in miR-199a-3p and miR-214 during late gestation and with labor in myometrium of pregnant mice and women provide important insight into the regulation of COX-2 expression and synthesis of contractile prostaglandins in the myometrium. In studies of COX-2 mRNA expression in myometrium of women in labor *vs.* not in labor, it was previously observed that COX-2 mRNA levels were not induced in tissues from women in labor unless there was underlying chorioamnionitis (Havelock et al., 2004). Based on these findings, the authors suggested that COX-2 is only upregulated in the myometrium affected by underlying infection leading to increased inflammatory signaling and suggested that it does not play a role in normal parturition. In the present study, we observed that while COX-2 mRNA levels remained unchanged, COX-2 protein levels were markedly induced in human myometrium during labor in the absence of infection. We suggest that the induction of COX-2

protein expression may be due, in part, to the marked associated decline in expression of the miR-199a/214 cluster. In myometrium from pregnant mice, COX-2 mRNA remained relatively low during late gestation, but was markedly induced during labor. By contrast, COX-2 protein was upregulated by 18.5 dpc, in association with the temporal decline in miR-199a-3p and miR-214 expression.

The reciprocal regulation COX-2 protein and miR-199a-3p/miR-214 expression in the myometrium of mice and humans suggests that the role of these miRNAs in COX-2 regulation is conserved between these species and suggest that these miRNAs may inhibit COX-2 expression via translational repression rather than mRNA degradation. It was previously reported that miR-199a-3p is spatiotemporally expressed in mouse uterus during embryo implantation and posttranscriptionally regulates COX-2 (Chakrabarty et al., 2007). In those studies, they proved that COX-2 was a bonafide target of miR-199a.

It is important to note that the seed sequences of miR-199a-3p and miR-214 are nearly identical. This suggests that these miRNAs target a similar pool of messenger RNA targets. Therefore, a modest, yet significant, decrease in miR-199a-3p and miR-214 expression could collectively have a greater impact on the expression of their respective messenger RNA targets. Indeed, it appears that the decline in miR-199a-

3p and miR-214 during the transition to labor in mice and human myometrium may contribute to the surge in COX-2 protein expression prior to and during labor. Undoubtedly, there are other inflammatory factors present within the myometrium that could contribute to dramatic increase in COX-2 protein expression during labor, such as NF-κB activation in response to cytokine production by infiltrating macrophages and neutrophils (Thomson et al., 1999; Condon et al., 2003) and increased myometrial stretch (Shynlova et al., 2010). To confirm that myocytes of the myometrium contribute to the increase in COX-2 protein expression during late gestation, we conducted immunohistochemistry for COX-2 in sections of pregnant murine uterus (Figure 3-1.5). In these studies we observed a pronounced increase COX-2 immunostaining in uterine tissues at 18.5 dpc, as compared to 15.5 dpc. These findings confirmed our immunoblot results showing an increase in COX-2 protein expression in the myometrium just before labor. Therefore, we sought to investigate the ability of these miRNAs to modulate COX-2 expression within myometrial cells.

Material and Methods

Mouse and human tissue preparations. All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center (UT Southwestern). Eight-week-old timed pregnant ICR/CD1 mice were obtained from Harlan Laboratories. Myometrial tissues were isolated at 15.5 and 18.5 dpc and following the delivery of the first pup (in-labor) as previously described (Renthal et al, 2010).

All human studies and consent forms were approved by the Institutional Review Board of UT Southwestern in accordance with the Donors Anatomical Gift Act of the State of Texas. Informed consent was obtained in writing from each woman prior to surgery. Myometrial tissues were acquired from the lower uterine segment of pregnant women undergoing cesarean section before and after the onset of active labor. After the tissues were collected, they were flash frozen and stored at -80° for subsequent protein and mRNA analysis.

Quantitative RT-PCR (qRT-PCR). For gene expression analyses, total RNA was reverse-transcribed using SuperScript III-RT kit (Invitrogen) and subsequently amplified using SYBR Green (Applied Biosystems). The primer sequences for analysis are as follows: mouse GAPDH (Forward: 5’

AGG TCG GTG TGA ACG GAT TTG ‘3; Reverse: 5’ TGT AGA CCA TGT AGT TGA GGT CA ‘3), mouse COX-2 (Forward: 5’ CAG CCA GGC AGC AAA TCC ‘3; Reverse: 5’ ACA TTC CCC ACG GTT TTG AC ‘3), human 36B4 (Forward: 5’ TGC ATC AGT ACC CCA TTC TAT CA ‘3; Reverse: 5’ AAG GTG TAA TCC GTC TCC ACA GA ‘3), human COX-2 (Forward: 5’ TTC CAG ATC CAG AGC TCA TTA AA ‘3; Reverse: 5’ CCG GAG CGG GAA GAA CT‘3). For miRNA qRT-PCR, a TaqMan reverse transcription kit (Applied Biosystems) was used to reverse transcribe total RNA. We then used TaqMan Universal PCR Master Mix for miRNA analysis. All RNA samples were DNase treated prior to analysis (Invitrogen). The comparative cycle threshold ($\Delta\Delta$Ct) was used to determine relative gene and miRNA expression.

Immunoblotting. Cytoplasmic protein extracts were isolated from cellular and tissue samples using NE-PER extraction reagent kit (Pierce). The expression of proteins of interest was determined using primary polyclonal antibodies against COX-2 (Abcam) 1:500 dilution. β-Actin (Abcam) was used as a loading control.

In situ hybridization. At gestation day 18.5, pregnant mice were anesthesized and then perfused with paraformaldehyde, decapitated, and

their uterine tissues were frozen on dry ice in tissue TEK-OCT. 16-μM frozen tissue sections were cut and stored at -80°C until use. Prior to hybridization, sections were dried at room temperature for 5 min and then at 50°C for 10 min. The sections were then fixed in 4% paraformaldehyde and acetylated in acetic anhydride/triethanolamine. Then, the slides were washed in PBS and prehybridized at 48°C for 30 min in a hybridization solution consisting of 50% formamide, 5x sodium chloride and sodium citrate solution SSC, 500 μg/mL yeast RNA, 0.1%Tween, 50 μg/mL heparin. Hybridization of 25 μM 5' and 3' DIG-labeled probes (LNA-modified oligonucleotides, Exiqon) complementary to miR-199a-3p was then performed for 2 h at 53°C. For negative and positive controls, 5' and 3' DIG-labeled probes for scrambled oligonucleotide and U6, respectively, were hybridized to slides for 2 h at 47°C. Next, the slides were incubated in SSC washes of increasing stringency at 54°C. Anti-DIG-HRP antibody (Roche cat#11207733910) and a tyramide signal amplification system (Perkin Elmer) were then used to detect in situ hybridization signaling, according to the manufacturer's instructions. Lastly, the slides were counterstained with hematoxylin in order to visualize the different tissue layers of the uterus.

Immunofluorescence. Cryosections of whole pregnant mouse uterus were post-fixed in 3.7% formalin in PBS for 10 min. The sections were then blocked with normal goat serum for 30 min. Incubation with a primary antibody for COX-2 (1:1000 dilution, Abcam) was performed overnight at 4°C in the dark. Next, the sections were incubated with anti-goat fluorophore-labeled secondary antibody (1:750 dilution, Jackson ImmunoResearch) for 1 h at room temperature. Then, a counterstain with 4,6-diamino-2-phenylindole (DAPI, 1:100,000 dilution, Sigma) was performed for 5 min at room temperature. A confocal microscope (Leica TCS SP5) was used to capture images of the slides and ImageJ software was used to generate z-projections of z-stacks.

CHAPTER 3 – Section 2

miR-199a-3p and miR-214 Regulate COX-2 Expression

Experimental Design

In light of our interesting findings of differential regulation of COX-2 mRNA and protein in the gestational series myometrial tissues from mice and the myometrial biopsies from women at term, we sought to further examine the miR-199a-3p/miR-214/COX-2 relationship within the uterine myometrium. To determine whether miR-199a-3p and miR-214 directly regulate COX-2 in myometrium, we conducted studies using immortalized human myometrial cells (hTERT-HM) transfected with mimics for miR-199a-3p and miR-214. Since COX-2 mRNA and protein are undetectable in hTERT-HM under resting conditions, the cells were cultured ± the pro-inflammatory cytokine IL-1β to induce COX-2 expression (Rauk et al., 2000) (Fitzgibbon et al., 2009)(Soloff et al., 2004). Quantitative RT-PCR analysis was then conducted to assay for COX-2 mRNA expression and compared to immunoblot analysis of COX-2 protein within the same samples.

Although previous studies confirmed that miR-199a-3p directly targets COX-2 in the uterus (Chakrabarty et al., 2007), this had not been demonstrated for miR-214. To determine whether COX-2 is a direct target

of miR-214, we conducted luciferase reporter assays in COS-7 cells using *COX-2-3'-UTR-luciferase* reporters in which a portion of the COX-2 3'UTR containing the putative miR-214 binding site was subcloned downstream of *luciferase* (Figure 3-2.1C).

Finally, to determine whether miR-199a-3p and miR-214 overexpression cause an effective decline in COX-2 expression that would subsequently alter myometrial contractility, we employed collagen gel contraction assays (Renthal et al., 2010), in which cultured hTERT-HM cells transfected with miR-199a-3p or miR-214 mimics or with scramble miRNAs, were embedded into 3D collagen gel matrices. Contraction of the human myocytes within the matrix in response to treatment with tumor necrosis factor (TNF)-α, a pro-inflammatory cytokine that enhances uterine contractility (Fitzgibbon et al., 2009), was assessed by a reduction in the diameter of the collagen disc. As a positive control, untransfected hTERT-HM cells cultured ± TNF-α were treated with the cyclooxygenase inhibitor indomethacin, a tocolytic agent for early preterm labor.

Results: miR-199a-3p and miR-214 Inhibit COX-2 Expression at the Level of Protein Translation, Rather than mRNA Stability

Incubation of the hTERT-HM myometrial cells with IL-1β caused a marked induction of COX-2 mRNA and protein. Overexpression of miR-199a-3p or miR-214 in the hTERT-HM cells did not significantly affect COX-2 mRNA (Figure 3-2.1A), but caused a marked decline in COX-2 protein levels, as compared to cells transfected with scramble (control) mimic (Figure 3-2.1B). Remarkably, overexpression of miR-199a-3p and miR-214 repressed COX-2 protein expression to a similar degree suggesting that, like miR-199a-3p, miR-214 may directly target COX-2 protein expression. Indeed, the luciferase reporter studies revealed that miR-214 overexpression caused downregulation of *luciferase* via the COX-2 3'-UTR. Furthermore, this repression was lost when the putative miR-214 binding site in the COX-2 3'-UTR was mutated (Figure 3-2.1D). Taken together, these findings suggest that both miR-199a-3p and miR-214 directly target COX-2 in the myometrium.

In collagen gel contraction assays the inhibitory effects of miR-199a-3p/miR-214 on production of contractile prostaglandins was clearly evident. In these studies, we observed that TNF-α significantly induced contraction of collagen gel matrices embedded with hTERT-HM cells that were untransfected (Un) or transfected with the control (scramble) miRNA

(Con). By contrast, the contractile effect of TNF-α was significantly blocked in cells overexpressing miR-199a-3p or miR-214, in a manner comparable to the inhibitory action of indomethacin (Figure 3-2.2).

Discussion

The present findings support a role of miR-199a-3p/miR-214 in the translational repression of COX-2. In these studies, we clearly show that miR-214, as well as miR-199a-3p directly target COX-2 and that their overexpression selectively inhibits COX-2 protein expression without impacting COX-2 mRNA. This provides an explanation for previous findings that COX-2 mRNA fails to be upregulated in myometrium from women in labor (Havelock et al., 2005), while prostaglandin levels are increased (Olson et al., 2007). Our findings that miR-199a-3p/miR-214 overexpression in human myometrial cells embedded in collagen gels blocked TNF-α-induced myometrial contractions, also suggest the functional importance of this regulatory pathway in the control of myometrial contractility.

This study provides compelling evidence that miR-199a-3p and miR-214 play crucial roles in the maintenance of uterine quiescence by suppressing COX-2 and production of contractile prostaglandins throughout most of gestation. It is well established that uterine quiescence is maintained throughout most of pregnancy by progesterone acting via its nuclear receptors (PR) in the myometrium. This occurs, in part, via P_4/PR anti-inflammatory actions. On the other hand, parturition is associated with a decline in PR function, resulting in an increased inflammatory response

within the myometrium, as well as an increase in circulating estradiol (E_2) levels and/or estrogen receptor α (ERα) activation near term, which further contribute to the uterine inflammatory response and antagonize the anti-inflammatory actions of P_4/PR (Karmel et al., 2010). Given the delicate balance between estrogen, progesterone and inflammatory factors during pregnancy and labor and the role of inflammation in the induction of preterm labor, we next sought to investigate the role of inflammatory hormones and factors in the regulation of miR-199a-3p/miR-214 and COX-2 within the myometrium.

Materials and Methods

Luciferase reporter assay. TARGETSCAN was used to identify the putative miR-214 binding site(s) in the COX-2 3'UTR; one potential binding site was found. The 3'UTR of COX-2 containing the potential miR-214 binding site was amplified from human genomic DNA using the following primers: forward: 5'-GGGACTAGTCACAAAGAATATTGTCTCATTAGCCTGAAT-3', reverse: 5'-GGGAAGCTTTCAGAAAAGATCTGTCAATTTTTAAATAGT-3'. The 530 bp fragment was then cloned into the 3'UTR region of pMIR-REPORT (Invitrogen); the sequence of the recombinant plasmid was confirmed prior to transfection. For mutation analysis, the QuikChange II Site-Directed Mutagenesis Kit (Stratagene) was used to mutate three nucleotides in the putative miR-214 binding site (GATCTGCTGACA to GATCTGACAACA). COS-7 cells were co-transfected with these reporter plasmids and 20nM miR-214 or miR-1 (control) and then assayed for relative luciferase activity 48 h later.

Collagen matrix contraction assay. The collagen matrix contraction assay was carried out as described in detail, previously (Renthal et al., 2010). Briefly, hTERT-HM cells were transfected with miR-214, miR-199a-3p or scrambled mimics and cultured for 24 h, as described above. The

cells were then harvested, embedded into collagen matrices, and cultured in DMEM-F12 medium with or without TNF-α (10 ng/mL) for 24 h. As a positive control, untransfected hTERT-HM cells cultured ± TNF-α were treated with 5 μM indomethacin. Contractility of the collagen gels was assessed by analyzing the total area of the gel (mm^2) using Carestream Molecular Imaging Software.

Transfection of Cultured Human Myometrial Cells. hTERT-HM cells were cultured in DMEM-F12 medium (Gibco) with 10% FBS (vol/vol). For miR mimic studies, the cells were transfected with 20 nM scramble mimic, miR-199a-3p or miR-214 mimics (Qiagen) using HiPerFect Transfection Reagent, as described previously (Renthal et al., 2010). The cells were then cultured for 24 h and treated with IL-1β or vehicle 4 h prior to collection. The cells were harvested for mRNA and protein analysis 24 h post-transfection with miR mimics.

CHAPTER 3 – Section 3

Hormonal and Inflammatory Regulation of miR-199a-3p, miR-214 and COX-2

Experimental Design

The initiation of myometrial contractility leading to term, as well as preterm labor is mediated by an increased inflammatory response (Hardy et al., 2006), associated with enhanced migration and infiltration of immune cells (Thomson et al., 1999). While the downstream mechanisms leading to term and preterm labor appear to be similar, the inducing factors differ markedly. More than 30% of all preterm births are associated with bacterial infection with associated chorioamnionitis (Romero et al., 2007). To assess changes in miR-199a-3p/214 and COX-2 expression in mouse myometrium using an infection/inflammation-induced preterm labor model, we analyzed effects of intraamniotic injection of the gram-negative bacterial endotoxin, lipopolysaccaride (LPS), at 15.5 dpc using quantitative RT-PCR and immunoblotting.

As mentioned, progesterone maintains uterine quiescence throughout most of pregnancy, in part, by its anti-inflammatory actions (Hardy et al., 2006)(Loudon et al., 2003). Term labor is associated with a marked increase in circulating estradiol-17β (E_2) levels in a number of species

(Kamel et al., 2010)(Buster et al., 1979)(Challis et al., 1971). The increase in E_2 levels is associated with an increased inflammatory response and a decline in uterine PR function (Hardy et al., 2006)(Shynlova et al., 2009). Estrogens promote immune cell migration into the uterus and antagonize anti-inflammatory actions of P_4/PR (Tibbetts et al., 1999)(Mesiano et al., 2002). To investigate the effects of E_2 and P_4 on miR-199a-3p, miR-214 and COX-2 in the uterus, ovariectomized mice were subcutaneously injected within estrogen, progesterone, or vehicle (as a control); uterine tissues were removed 24 h later for subsequent qRT-PCR and immunoblot analysis. To investigate effects of P_4 on expression of miR-199a/214 within the myometrium during pregnancy, timed-pregnant mice were injected subcutaneously with P_4 (1.0 mg) or vehicle each day from 15.5 – 18.5 dpc (Figure 3-3.1). Myometrial tissues from a P_4-treated mouse were collected when a vehicle injected mouse went into labor, which we define as the birth of one pup.

Results: miR-199a-3p/miR-214 and COX-2 are Reciprocally Regulated in Mouse Uterine Tissues by Pro- and Anti-Inflammatory Hormones and Factors

Consistent with our findings from pregnant mice in labor at term, we found that in LPS treated mice that delivered preterm, myometrial miR-199a-3p and miR-214 were significantly reduced, as compared to gestation-matched vehicle injected controls (Figure 3-3.2A), while COX-2 protein levels were markedly upregulated (Figure 3-3.2B).

Similarly, E_2 treatment of ovariectomized mice significantly inhibited uterine miR-199a/214 expression (Figure 3-3.2C) and caused an associated induction of COX-2 protein (Figure 3-3.2D). In contrast, P_4 treatment, which delayed the timing of labor, caused a significant induction of miR-199a/214 expression (Figure 3-3.3A) and repression of COX-2 protein in the pregnant myometrium (Figure 3-3.3B). A similar upregulation of miR-199a/214 (Figure 3-3.3C) and inhibition of COX-2 expression (Figure 3-3.3D) was observed in uterine tissues from ovariectomized mice 24 h after P_4 treatment.

Discussion

The findings that miR-199a-3p and miR-214 as well as their respective target, COX-2, were reciprocally regulated in a model of preterm labor as well as a model of delayed labor suggest that these miRNAs play a crucial role in the appropriate timing of parturition. Furthermore, these data provide strong evidence that the miR-199a-3p/miR-214/COX-2 relationship is regulated by hormonal and inflammatory factors that are also important for the proper timing of parturition. Since we noted an increase in myometrial expression of miR-199a-3p/214 in the delayed labor mouse model in which we exogenously increased progesterone levels, we anticipated that RU486 treatment in pregnant mice would have the opposite effect. While the RU486 treatment did result in preterm labor in the majority of the pregnant mice, we did not note a decline in miR-199a-3p/214 or an increase in COX-2 expression in the myometrium of pregnant mice undergoing preterm labor due to RU486 injections (Figure 3-3.3). The lack of significant change in miR-199a-3p/214 and COX-2 expression in the RU486 mouse model of preterm labor highlights two important points: 1) the RU486 mouse model of preterm labor and the LPS mouse model of preterm model represent two different mechanistic components of preterm labor and 2) the increase in estrogen receptor activation near the end of term might be crucial

promoting the decline in miR-199a-3p/214 expression and the subsequent increase in COX-2 expression in the myometrium during the transition to labor. This is exciting because it suggests that therapeutic strategies to maintain elevated expression miR-199a-3p/miR-214 could be efficacious in preventing preterm labor.

Materials and Methods

Mouse models of LPS/inflammation-induced preterm labor. Induction of preterm labor via LPS has been previously described (Renthal et al., 2010). Briefly, 15.5 dpc pregnant ICR mice underwent laparotomy to expose the uterus after anesthetization. LPS (1.5 μg in 50 μl) or sterile PBS (vehicle) were injected into each amniotic sac; the uterus was then carefully reinserted into the abdominal cavity, the abdominal muscle wall and skin were sutured and the mouse was allowed to recuperate. There was a high rate of preterm delivery in this mouse model of preterm labor; none of the vehicle-injected mice manifested preterm labor. The LPS-injected mice were sacrificed upon the birth of one pup; gestation-matched vehicle-injected controls were sacrificed immediately afterwards.

Progesterone and estradiol treatment studies. Six- to eight-week-old ovariectomized mice were obtained from Harlan Laboratories. Two weeks after ovariectomy, the mice were subcutaneously injected with either estradiol-17β (1 μg in 0.3 mL sesame oil vehicle), with P_4 (1 mg in 0.25 mL sesame oil) or with vehicle alone. The uterus was harvested 24 h post-injection and flash frozen for subsequent protein and mRNA analysis.

Studies also were conducted to assess the effects of P_4 on expression of miR-199a/214 in myometrium of timed-pregnant mice. For these

studies, timed-pregnant mice were injected subcutaneously with P_4 (1.0 mg) or vehicle each day from 15.5 – 18.5 dpc. None of the P_4-injected mice manifested spontaneous labor at term. At the time that a vehicle-injected mouse delivered the first pup, a P_4 treated mouse also was sacrificed.

Mouse Model of Preterm Labor Induced by Pharmacological P_4/PR Withdrawal. Induction of preterm labor using RU486 was implemented as described previously (Dudley et al., 1996)(Renthal et al., 2010). Briefly, 200 µg of RU486 (Sigma) or 5% ethanol (vehicle) were subcutaneously injected into the right flank of 15.5 dpc pregnant ICR mice. Preterm labor occurred in the RU486-injected mice within 12 h, while none of the vehicle-injected mice went into labor. The RU486 injected mice were sacrificed upon the birth of one pup; gestation-matched controls were sacrificed immediately afterwards.

CHAPTER 3 – Section 4

Transcriptional Regulation of miR-199a Cluster by ZEB1

Experimental Design

To begin to define mechanisms for the opposing effects of P_4 and E_2 on miR-199a-3p/214 expression, we considered the possible roles of ZEB1 and ZEB2, which decline in the pregnant uterus near term (Renthal et al., 2010) in a temporal pattern highly similar to miR-199a/214. We previously observed that P_4 specifically induced expression of ZEB1, which represses miR-200 family expression, allowing for upregulation of ZEB2 (Renthal et al., 2010). Importantly, ZEBs are structurally related to Twist1, a transcription factor reported to increase *miR-199a/214* cluster expression (Lee et al., 2009). Twist1 and ZEBs both bind to E-boxes and have several overlapping functions (Lee et al., 2009)(Postigo et al., 1997)(Peinado et al., 2007)(Yang et al., 2008)(Hebrok et al., 1994)(Hijantoniou et al., 1994). We, therefore, postulated that ZEB1 might coordinately induce *miR-199a-3p/214* expression and inhibit miR-200 family expression in the pregnant uterus. To investigate this hypothesis, we transduced cultured human myometrial cells (hTERT-HM) with a recombinant adenovirus expressing ZEB1 and assayed for the expression of the primary transcript of miR-199a-3p and miR-214, miR-199a-2, as

well as the mature miRNA forms of miR-199a-3p/miR-214 using qRT-PCR. Additionally, we performed similar analyses in cultured hTERT-HM cells transfected with siRNA against ZEB1. To further examine whether this action of ZEB1 is mediated through its interaction with the E-box-containing region of the *Dnm3os* promoter, COS-7 cells were cotransfected with a luciferase reporter controlled by the *Dnm3os* promoter region previously observed to drive miR-199a/214 expression and be regulated by Twist1 (Lee et al., 2009).

As previously stated, ZEB1 functions by binding to E-box regions within the promoter of its target genes. Notably, the proximal promoter region of *Dnm3os*, the anti-sense transcript that encodes miR-199a-3p and miR-214, contains several E-boxes. To determine whether endogenous myometrial ZEB1 binds to E-boxes in the region upstream of the miR-199a/214 cluster previously reported to bind Twist1, and to assess gestational changes in binding activity, ChIP analysis of the E-box containing region of the *Dnm3os* promoter was conducted (Figure 3-4.2B).

In addition to its action to increase ZEB1 expression in mouse myometrium, in the present study, we observed that P_4 treatment of mice also increase myometrial expression of miR-199a-3p and miR-214 (Chapter 3 – Section 3). In light of the opposing effects of E_2 on uterine miR-199a-3p/214 levels and the relevance of the antagonistic relationship

between progesterone and estrogen in the pregnancy and labor, we investigated effects of E_2 treatment of ovariectomized mice on ZEB1 expression using qRT-PCR and immunoblotting.

Results: The Hormonally-Regulated Transcription Factor ZEB1 Binds to the *Dnm3os* promoter and Stimulates Expression of the miR-199a-3p/214 Cluster at the Transcriptional Level

We observed that ZEB1 overexpression in cultured human myometrial hTERT-HM cells (Figure 3-4.1B) markedly induced miR-199a-3p/214 (Figure 3-4.1A). Conversely, siRNA-mediated knockdown ZEB1 (Figure 3-4.1D) caused a significant downregulation of miR-199a-3p/214 levels (Figure 3-4.1C). Moreover, ZEB1 overexpression in hTERT-HM cells upregulated expression of the primary transcript of miR-199a/214, pri-miR-199a, (Figure 3-4.1E), further suggesting that ZEB1 enhances miR-199a/214 expression at the transcriptional level. In support of these findings, we observed in luciferase promoter assay studies that cotransfection of an CMV-driven ZEB1 expression plasmid caused induction of a luciferase reporter under control of the *Dnm3os* promoter. Moreover, induction was lost when the *Dnm3os* promoter region containing the putative ZEB1 binding sites was deleted (Figure 3-4.2A). In studies using ChIP to investigate binding of endogenous ZEB1 to the E-box-containing region of the *Dnm3os* promoter upstream of the miR-199a/214 cluster, we found ZEB1 bound specifically to this genomic region in pregnant mouse myometrium at 15.5 dpc and that ZEB1 binding was dramatically reduced during labor (Figure 3-4.2C).

Interestingly, in studies to assess the effects of E_2 treatment in ovariectomized mice, we observed that E_2 caused significant inhibition of uterine ZEB1 mRNA and protein expression within 24 h (Figure 3-4.1G-H). This suggests that E_2 inhibition of miR-199a-3p/214 expression in myometrium may be meditated by the inhibitory effect of E_2 on ZEB1 expression.

Discussion

Our collective findings point to the key pivotal roles of myometrial ZEB1 and its miRNA targets as a hormonally-controlled regulators of inflammatory and contractile gene expression in the pregnant uterus.

As noted, ZEB1 and Twist1 are members of a E-box binding transcription factor family. Previous reports indicate that Twist1 positively regulates the expression of miR-199a-3p and miR-214 in embryonic tissues; however, we observed that myometrial Twist1 mRNA and protein were not altered during the transition to labor (Figure 3-4.3). On the other hand, ZEB1 expression declines in pregnant uterus toward term in a pattern highly similar to that of miR-199a-3p, suggesting that ZEB1 might positively regulate miR-199a-3p/miR-214 expression (Renthal et al., 2010). In support of this hypothesis, we observed that enhanced expression of ZEB1 in human myometrial cells induced both the expression of the mature forms of miR-199a-3p and miR-214, as well as the primary transcript, miR-199a-2, suggesting that ZEB1 enhances miR-199a/214 expression at the transcriptional level. In agreement with these data, we also found that ZEB1 enhanced *Dnmos/miR-199a/214* cluster promoter activity. The physiological importance of this finding in the molecular events leading to the induction of COX-2 and the initiation of labor is emphasized by the finding that endogenous ZEB1 binding to the

miR-199a/214 promoter is markedly decreased in myometrial tissues from mice just prior to labor. Furthermore, our data suggest that ZEB1 serves a key pivotal role in the opposing actions of progesterone and estrogen in the regulation of myometrial quiescence and contractility through transcriptional regulation of miR-199a/214 expression and translational regulation of COX-2.

Collectively, our findings point to the key central role of myometrial ZEB1, which is upregulated by P_4/PR throughout most of pregnancy (Renthal et al., 2010) and declines with the decrease in P_4/PR function and the increase in E_2/ERα activity during the progression to labor (Figure 3-4.4). ZEB maintains myometrial quiescence by suppressing both the miR-200 family (Bracken et al., 2008) and expression of contraction-associated protein (CAP) genes, such as the oxytocin receptor and connexin-43 (Renthal et al., 2010), on the one hand, and by enhancing miR-199a-3p/214 expression and causing suppression of COX-2, on the other. Near term, enhanced ERα activity and the increased inflammatory response in myometrium promote a decline in PR function cause decreased expression of ZEB1. This results in de-repression of CAP genes and downregulation of *miR-199a/214* expression with an associated increase in COX-2, causing further upregulation of the inflammatory response and active labor (Figure 3-4.4). The combinatorial

role of miR-199a-3p and miR-214 in the selective suppression of myometrial COX-2 may have important therapeutic implications, since overexpression of these miRs could attenuate premature uterine contractions and prevent preterm labor.

Materials and Methods

qRT-PCR. For analysis of Twist1 mRNA expression in murine uterus during late gestation and in labor, total RNA was reverse-transcribed using SuperScript III-RT kit (Invitrogen) and subsequently amplified using SYBR Green (Applied Biosystems). The primer sequences for analysis were as follows: mouse Twist1 (Forward: 5' GGA CAA GCT GAG CAA GAT TCA '3; Reverse 5' CGG AGA AGG CGT AGC TGA G '3).

Transfection and transduction. For ZEB1 overexpression experiments, hTERT-HM cells were transduced with recombinant adenoviruses expressing β-galactosidase or ZEB1 at a multiplicity of infection of 500 pfu per cell and processed 48 h after infection. To assess transduction efficiency, β-gal staining (β-Gal Staining Kit, Invitrogen) was performed on those cells transduced with the β-gal virus. The hTERT cells were at ~80% confluence for these experiments. In the ZEB1 knockdown studies, hTERT cells were either transfected with 20nM control siRNA or ZEB1 siRNA and harvested 72 h later.

Luciferase reporter assays. A portion of the *Dnm3os* promoter (–640 to 0 bp) and an E-Box deleted promoter region of *Dnm3os* (–640 to-357) were amplified from mouse genomic DNA using previously published

primers (Renthal et al., 2010). These PCR fragments were then cloned upstream of *luciferase* in PGL4.23; sequence of the recombinant plasmid DNA was confirmed prior to transfection. Using Lipofectamine 2000 (Invitrogen), COS-7 cells were transfected with a recombinant plasmid expressing *CMV-ZEB1* or with an empty vector for 24 h prior to transfection with the luciferase reporters. Relative luciferase activity was assayed 24 h later. To normalize for efficiency of transfection, the cells were cotransfected with a *CMV* expression vector containing β-galactosidase.

Chromatin immunoprecipitation studies. ChIP was performed using a ChIP Assay Kit (catalog no. 17-295; Millipore) to assess binding of endogenous ZEB1 to the *Dnm3os* promoter in myometrial tissues from timed-pregnant mice at 15.5 and 18.5 dpc. Briefly, mouse myometrial tissues were isolated, homogenized, crosslinked with formaldehyde (1%) and sonicated to produce sheared soluble chromatin, as described in detail previously (Renthal et al., 2010). Pre-cleared chromatin was incubated with ZEB1 antibody (generously provided by Douglas Darling, PhD, University of Louisville School of Dentistry) or non-immune IgG, as control, at 4°C overnight. Immune complexes were collected on Protein A agarose beads (ChIP Assay Kit, Millipore). Chromatin complexes were eluted from the beads and crosslinking was reversed. DNA purified from

the samples and input controls was analyzed for *Dnm3os* promoter sequences containing putative ZEB1 response elements using qPCR and the following primers: forward: 5'- GAC AGG CTC TCC CCA GCC CC -3'; reverse: 5'- CAG CCG TCC ATG GCG TTG CT -3'.

CHAPTER FIVE
Conclusions and Future Directions

The molecular mechanisms that maintain quiescence of the myometrium throughout most of pregnancy and mediate its conversion into a synchronously contractile unit culminating in parturition remain incompletely understood. Due to extensive research from our laboratory and those of others, we understand that the timing of labor involves a delicate balance between hormonal, inflammatory and physical factors that regulate integrated signaling pathways between the mother and the fetus (Mendelson et al., 2009). Specifically, it is apparent that progesterone acting through PR serves an essential and complex role in the maintenance of uterine quiescence throughout most of pregnancy. This effect of progesterone is mediated, in part, by its anti-inflammatory actions (Hardy et al., 2006)(Loudon et al., 2003) and its capacity to repress expression of genes encoding contraction-associated proteins (CAPs), including *OXTR*, *CX43* and *COX-2*. On the other hand, increased myometrial contractility leading to labor is associated with enhanced inflammatory signaling, which reduces PR function (Hardy et al., 2006), resulting in further intensification of the inflammatory response. This results in activation of expression of contraction-associated genes within

the myometrium. Notably, near term, increased circulating E_2 levels and/or increased activation of ERα within the uterus act to antagonize anti-inflammatory actions of P_4/PR (Kamel et al., 2010). In this manner, estradiol-17β opposes the anti-inflammatory actions of P_4 within the uterus and promotes an increased inflammatory response. In an effort to obtain a deeper understanding of the molecular events that underlie the transition of the pregnant myometrium from a refractory to a contractile state leading to labor, we further investigated the roles of miRNAs and their transcriptional and hormonal regulation.

MicroRNAs are regulatory molecules that have been shown to be involved in a number of complex physiological processes (Bartel et al., 2009). Since miRNAs are evolutionarily conserved across vertebrate species, their roles in lower mammals, such as the mouse, are often preserved in higher organisms, such as the human (Lee et al., 2007). The coordinated regulation of miRNAs allows cells to either fine-tune the regulation of functionally related set of genes or cause a pronounced change in gene expression. While the involvement of different clusters and families of miRNAs has been evaluated in a number of female reproductive processes, including implantation, menstruation, endometriosis, and leiomyomas (Creighton et al., 2010), the role of

miRNAs has not been explored in pregnancy and labor until recently (Renthal et al., 2010).

In previous studies, using a microarray based approach and a number of mouse models, we uncovered a role for the miR-200 family and its targets, transcription factors ZEB1 and ZEB2, as mediators of progesterone suppression of CAP gene expression in the pregnant myometrium. We observed that P_4/PR maintains uterine quiescence during pregnancy, in part, via upregulation of the ZEB1, which inhibits expression of the contraction-associated genes, *OXTR* and *CX43* and suppresses the miR-200 family, which promotes further upregulation of ZEB1 and increases ZEB2 (Renthal et al., 2010). Toward term, the decline in PR function results in a decline in ZEB1 and derepression of the miR-200 family and of CAP genes. Similar findings were obtained upon preterm labor induction with RU486 or LPS and in myometrium from women in labor at term, as compared to gestation matched myometrium from women not in labor (Renthal et al., 2010).

miR-200a Regulates a Novel Mechanism in the Decline of PR Function

To date, the mechanisms for the decline in PR function near term have not been fully understood, since in humans levels of circulating P_4 remain elevated throughout pregnancy and into labor, due to increased

placental P_4 synthesis (Mendelson et al., 2009) (Creighton et al., 2010). Moreover, while labor in rodents is heralded by a pronounced decline in P_4 production by the corpus luteum, the levels of circulating P_4 at term still remain higher than the K_d for its binding to PR (Pointis et al., 1981). These paradoxical observations suggest that increased local metabolism of P_4 and a local decrease in PR function near term are of paramount importance for the initiation of parturition in all mammals. In fact, pregnancy is significantly prolonged when progesterone levels remain elevated throughout gestation due to the targeted deletion of progesterone-metabolizing enzyme 20*α*-HSD or 5*α*-Reductase type 1 (Piekorz et al., 2005)(Mahendroo et al., 1999). Similarly, exogenous administration of progesterone prolongs labor in pregnant mice (Hirsch et al., 2002). Alternatively, the use of RU486 in a variety of species causes phenotypic changes in the myometrium similar to those that occur during term labor and induces labor prematurely (Shynlova et al., 2009).

Importantly, in the present study, we demonstrated that miR-200a, a member of the miR-200 family, directly targets STAT5b, which is crucial to the regulation of PR function because of its established role to inhibit expression of the P_4-metabolizing enzyme, 20α-HSD (Piekorz et al., 2005). Specifically, we found that miR-200a expression increased markedly in the pregnant mouse myometrium after 17.5 dpc in association

with a significant downregulation of STAT5b and upregulation of expression and activity of 20α-HSD. Notably, in studies using cultured human myometrial cells, we obtained evidence that miR-200a overexpression suppressed STAT5b and enhanced expression of 20α-HSD. This suggests that the enhanced local metabolism of P_4 in myometrium to the inactive metabolite, 20α-OHP, contributes to the reduction in PR function during the progression to labor and that miR-200a – and likely other members of the miR-200 family that share the same seed sequence - plays an important role in this process.

Interestingly, miR-200a exists in a cluster with miR-200b and miR-429, which we previously observed to be negatively regulated by ZEB1 in myometrium during pregnancy and labor (Renthal et al., 2010). Our findings suggest that during late pregnancy, an increased inflammatory response causes a decline in PR function by a number of mechanisms (Condon et al., 2003)(Hardy et al., 2006)(Condon et al., 2006). This decrease in PR function leads to a reduction in ZEB1, resulting in an increase in expression of the miR-200b/a/429 cluster. The increased levels of miR-200a decrease expression of STAT5b and the decline in STAT5b expression allows for an increase in the expression of 20α-HSD, which causes a local decline in P_4 levels within the myometrium. This further decline in PR function may act in a positive feed-forward manner to

further increase miR-200 expression, suppress STAT5b and induce 20α-HSD. This further enhances the inflammatory response and upregulation of COX-2, which promotes synthesis of, contractile prostaglandins, such as prostaglandin $F_2\alpha$ ($PGF_2\alpha$). Notably, $PGF_2\alpha$ upregulates expression of 20α-HSD to further increase P_4 metabolism (Stocco et al, 2000)(Callegari et al., 2005). Together, these data highlight a novel mechanism in the local decline of PR function in the face of the paradoxically high circulating levels of progesterone.

miR-199a-3p/miR-214 Regulates Myometrial Contractility via Inhibition of COX-2 Protein Expression

The importance of prostaglandins as uterotonic agents has been appreciated since the early 1970s (O'Brien et al., 1995). Studies investigating the temporal expression of prostaglandins indicate an increase in the bioavailability of PGE_2 and PGF_2 just prior to labor and during active labor (Romero et al., 1996). Furthermore, labor-inducing signals, such as mechanical stretch, platelet activating factor (PAF), and oxytocin, have been known to stimulate prostaglandin production in reproductive tissues (Hertelendy et al., 2004). In fact, the binding of oxytocin to OXTR induces and activates precursors for prostaglandins in the myometrium (Hertelendy et al., 2004). In addition, in labor there are

increased levels of the prostaglandin precursor, arachidonic acid (Keirse et al., 1977). The role of prostaglandins in labor is emphasized by the fact that the prostaglandin E_2 (PGE_2) and vaginal misoprostol, a synthetic prostaglandin E_1 (PGE_1) analogue, are more effective than oxytocin in the induction of vaginal delivery (Mozurkewich et al., 2011). In addition, inhibitors of prostaglandin synthesis, such as indomethacin and celecoxib, have been reported to be as effective as magnesium sulfate, the gold standard tocolytic agent, in delaying early preterm labor in humans (Borna et al., 2007). Since Celecoxib is a COX-2 specific inhibitor, this finding also highlights the importance of COX-2 in the upregulation of myometrial contractility leading to labor.

The importance of myometrial COX-2 induction in the initiation of labor has been questioned by Word and colleagues (Havelock et al., 2004) who previously reported that COX-2 mRNA levels were not increased in myometrial tissues from women in labor, as compared to not in labor, unless there was underlying chorioamnionitis. However, in those studies the investigators failed to assay for the expression of COX-2 protein. In the present study, we also found that COX-2 mRNA levels remained unchanged when comparing myometrium from women at term who were in labor *vs.* not in labor, but noticed a dramatic induction of COX-2 protein levels in human myometrium during labor, in the absence

of underlying infection. Similarly, in myometrium from pregnant mice, COX-2 mRNA remained relatively low during late gestation, but was markedly induced during labor. By contrast, COX-2 protein was upregulated by 18.5 dpc in association with a marked induction in myometrial $PGF_2\alpha$ levels (unpublished observations). In the present study, we obtained compelling evidence to suggest that COX-2 expression in the myometrium is regulated at the level of protein translation and that the miR-199a/214 cluster may serve an important role in this regulation.

In our studies, we found that levels of the clustered miRNAs, miR-199a-3p and miR-214, were significantly decreased in laboring myometrium of pregnant mice and humans and in an inflammatory mouse model of preterm labor, while the miR-199a-3p/miR-214 target, COX-2, was coordinately increased. Overexpression of miR-199a-3p and miR-214 in cultured human myometrial cells inhibited COX-2 protein, but had no effect on COX-2 mRNA levels. miR-199a/214 overexpression also blocked TNF-α-induced myometrial cell contractility, suggesting their physiological relevance. Notably, E_2 treatment of ovariectomized mice suppressed, whereas P_4 enhanced uterine miR-199a-3p/214 expression. Interestingly, these opposing hormonal effects were mediated by ZEB1, which is induced by P_4, inhibited by E_2 and activates miR199a/214 transcription. Thus, in the course of these studies, we uncovered an intriguing pivotal

role of ZEB1 as a regulator of both the miR-200b/200a/429 cluster and the miR-199a cluster.

The Pivotal Role of the Hormonally-Regulated Transcription Factor, ZEB1

Intriguingly, we found that the miR-200b/200a/429 and miR-199a/214 clusters are opposingly regulated by ZEB1. Previous studies from our lab revealed that progesterone induced ZEB1 expression though activation and binding of PR to response elements in its 5'-flanking region (Renthal et al., 2010). Progesterone induction of ZEB1, in turn, inhibits expression of the miR-200b/miR-200a/miR-429 cluster via ZEB1 interaction with the miR-200 cluster promoter (Bracken et al., 2008). On the other hand, in this study, we observed that progesterone increases miR-199a/214 expression via induction ZEB1. As mentioned, estradiol-17β opposes the actions of P_4 within the uterus to promote an inflammatory response. Interestingly, estrogen treatment inhibited expression of ZEB1 and miR-199a-3p/miR-214 in uterine tissues from ovariectomized mice. Thus, increased E_2/ERα activation, coupled with the decline in PR function near term leads to the repression of ZEB1, the decline in miR-199a-3p/miR-214 and the induction of COX-2. Collectively, these studies highlight an important pivotal role of ZEB1 as a mediator of the opposing actions of progesterone and estradiol-17β on uterine quiescence and contractility via temporal regulation of the miR-200 and miR-199a clusters.

The temporal changes in miR-200b/200a/429 and miR-199a-3p/miR-214 expression highlight three important stages during gestation. Challis et al. describes these phases as myometrial quiescence (Phase 0), myometrial activation (Phase 1) and myometrial stimulation (Phase 2) (Challis et al., 2000). Phase 0 is a stage of uterine quiescence in which the uterus is remains refractory to contractile stimuli (Challis et al., 2000). During this phase, P_4/PR function remains relatively high. This allows PR to induce high expression of ZEB1, which in turn, inhibit miR-200 family expression, and upregulate miR-199a-3p/miR-214 expression within the myometrium. The low levels of miR-200 family help to maintain high ZEB1 and ZEB2 levels, thereby, further increasing miR199a-3p/214, which actively represses COX-2 expression and prostaglandin synthesis in the myometrium. The elevated ZEB1 and ZEB2 also repress expression of OXTR and CX43. In addition, the reduced levels of miR-200a expression allow upregulation of its target, STAT5b, which maintains repression of the P_4-metabolizing enzyme, 20α-HSD and increased local levels of P_4 within the myometrium.

Phase 1, which occurs after 17.5 dpc, involves the transition of the uterus from a quiescent state to one more receptive to contractility-inducing factors due to the decline in P_4/PR function. During this phase, the decline in P_4/PR function leads to decreased expression of myometrial

ZEB1. This releases ZEB1 repression from the miR-200 family. The induction of the miR-200 cluster leads to further repression of its targets, ZEB1, as well as ZEB2 and STAT5b. The decline in ZEBs releases repression of the CAP genes, OXTR and CX43 (Renthal et al., 2010), while the decline in STAT5b causes upregulation of 20α-HSD resulting in increased P_4 metabolism within the myometrium, leading to a further decline in PR function. The induction of these CAP genes enhances the receptivity of the myometrium to the contractile signals of labor, such as prostaglandins, thereby further promoting myometrial activation.

Phase 2 occurs just before labor when the uterus switches to an increasingly contractile state. During this phase, the progressive increase in local P_4 metabolism and decline in PR function, coupled with the increase in circulating E_2 and myometrial ERα activation further contribute to the decrease in ZEB1/2, the resulting downregulation of *miR-199a/214* expression and associated increase in COX-2 expression. The marked increases in myometrial contractile prostaglandin production, coupled with the increase in CAP gene expression collectively promote coordinated myometrial contractility, culminating in active labor. Together with our previous studies, the current findings further implicate miRNAs as evolutionarily conserved crucial mediators in the hormonal regulation of uterine quiescence and contractility during pregnancy and labor.

Future Directions

In future experiments, we will further explore additional roles of the miR-200 family and the miR-199a cluster by investigating other potential mRNA targets of these miRNAs, as well as other gene targets of the transcription factors that these miRNAs repress, STAT5b and ZEB1. In doing so, we may uncover novel factors and pathways in the maintenance of pregnancy and the timing of labor.

Notably, miR-214 is predicted to target 17β-hydroxysteroid dehydrogenase type 8 (17β-HSD8), an enzyme that catalyzes the conversion of inactive estrone to active estradiol in the reproductive tissues (Fomitcheva et al., 1998)(Kasai et al., 2004)(Pelletier et al., 2005). Therefore, in upcoming experiments, we will investigate whether the miR-214 directly inhibits this enzyme. In this manner, the decline in miR-214 during late gestation could lead to increased expression of 17β-HSD to which would enhance the metabolism of estrone to estradiol-17β and promote estrogen receptor activation within the myometrium near term.

We also noted changes in the expression of other miRNAs in our original microarray of mouse myometrial tissues that did not reach statistical significance, but certainly showed a trend towards up- or downregulation during the transition to labor. For example, miR-30 appeared to be upregulated, whereas, there was a decrease miR-29a

expression. These miRNAs are either known or predicted to target genes that influence the contractility of the uterus during parturition. miR-30 is predicted to bind within the 3'UTR of the prostaglandin $F_2\alpha$ receptor negative regulator (PTGFRN). As implied by the gene name, PTGFRN inhibits the actions of prostaglandins by inhibiting the prostaglandin $F_2\alpha$ receptor. On the other hand, miR-29a is predicted to repress expression of the OXTR. Thus, a decline in miR-29a may contribute to the upregulation of OXTR expression that is induced by the increase in miR-200 and decline in ZEB1/2 that occur in the myometrium near term. Confirmation of direct regulation of these miRNAs on their prospective uterine contractility gene targets would provide further evidence for the role of miRNAs in the preparation of the myometrium for labor.

Additionally, ZEB1 is known to target several genes involved in basement membrane structure and epithelial phenotype. By targeting these structural genes, ZEB1 may contribute to the physical changes that occur within the myometrium during the crucial switch from quiescence to contractility near term (Shynlova et al., 2009). One particular ZEB1 target gene of interest in this area is desmoplakin (DSP). Interestingly, DSP functions in a manner similar to connexin-43, a structural protein previously shown to be targeted by ZEB1 in the myometrium during labor (Renthal et al., 2010). Specifically, desmoplakin is an essential component

of desmosomes, which are intercellular junctions that tightly link adjacent cells (Hobbs et al., 2011). In preliminary qRT-PCR studies in myometrial tissues from mouse gestational series, we found a significant increase in the expression of DSP that was inversely correlated with the decline in myometrial ZEB1 expression, and positively correlated with the decrease in myometrial CX43 expression (Figure 4.1-1). Therefore, in future studies we could also investigate the effect of miR-200 family on DSP expression via ZEB-meditated inhibition in the myometrium during parturition. In this manner, we will further define pathways within the myometrium that promote mesenchymal to epithelial transition, allowing the myometrial cells to electrically couple and effectively contract during labor.

Furthermore, STAT5b has been linked to several physiological processes that are altered during gestation, such as cell proliferation, differentiation, and immune function. Interestingly, STAT5b has been reported to upregulate transcription of peroxisome proliferator-activated receptor gamma (PPARγ) (Wakao et al., 2011). Findings suggest that high levels of PPARγ may play an important role during implantation and decidualization in the rat uterus (Ding et al., 2003). In the placenta, PPARγ plays a key role in hormone secretion and vascularization. Specifically, PPARγ may help to maintain uterine quiescence during pregnancy by decreasing COX-2 expression, stimulating the secretion of progesterone

and reducing endometrial inflammation (Froment et al., 2006). Indeed, PPARγ is known to have anti-inflammatory effects and activation of PPARγ was found to inhibit COX-2 transcription in human epithelial cells (Subbaramiah et al., 2001). Also, in our gene expression microarray comparing refractory myometrium from 15.5 dpc to contractile myometrium at 18.5 (Renthal et al., 2010), we detected a 2-fold decrease in the peroxisome proliferator-activated receptor gamma, coactivator 1β, suggesting that both expression and activation of PPARγ may be decreased within the myometrium during the transition to labor. Since STAT5b is known to induce PPARγ expression, it would be of interest to investigate whether miR-200a down regulation of STAT5b similarly affects PPARγ expression and function within the myometrium during gestation.

It is also crucial that we investigate the functional roles of miR-200b/miR-200a/miR-429 cluster and miR-199a-3p/miR-214 in myometrium during pregnancy and parturition using *in vivo* mouse models. Thus, it is of interest to generate mice overexpressing miR-199a-3p/miR-214 and determine whether this results in a delay in the timing of labor. Since miR-199a-3p/214 target COX-2 and COX-2 is involved in prostaglandin synthesis, it would be of interest to measure prostaglandin levels in these transgenic mice as well as assay for the expression of 15-hydroxy prostaglandin dehydrogenase (PGDH), the critical enzyme in

prostaglandin breakdown. Similarly, we could determine whether intrauterine injections of antagomiRs for the miR-200b/miR-200a/miR-429 cluster delay labor. Given the central role of ZEB1 in the regulation of these two miRNA clusters, it is of particular interest to generate transgenic mice overexpressing ZEB1 within the myometrium. Based on our findings, we anticipate that ZEB1 overexpression would result in enhanced myometrial expression of the miR-199a cluster with suppression of COX2, as well as a repression of the miR-200 family, CAP genes and 20α-HSD. Collectively, this would result in a significant delay in the timing of parturition. In order to assess the effects of overexpression of ZEB1 or miR-199a-3p/miR-214 in preterm labor, we could apply either the LPS- or RU486-induced preterm labor model to each of these transgenic mouse models in order to determine whether labor is prolonged.

In conclusion, considering the abundance of potential and known targets of the miR-200 family, miR-199a-3p/miR-214 cluster, ZEB1 and STAT5b that are relevant to the maintenance of pregnancy and the initiation of labor, we propose that these factors may collectively play an extensive and immensely important role in regulating myometrial quiescence and contractility.

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