Molecular Regulation of Parturition: A Myometrial Perspective

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The molecular mechanisms that maintain quiescence of the myometrium throughout most of pregnancy and promote its transformation to a highly coordinated contractile unit culminating in labor are complex and intertwined. During pregnancy, progesterone (P_4) produced by the placenta and/or ovary serves a dominant role in maintaining myometrial quiescence by blocking proinflammatory response pathways and expression of so-called “contractile” genes. In the majority of placental mammals, increased uterine contractility near term is heralded by an increase in circulating estradiol-17 β (E_2) and/or increased estrogen receptor α ($ER\alpha$) activity and a sharp decline in circulating P_4 levels. However, in women, circulating levels of P_4 and progesterone receptors (PR) in myometrium remain elevated throughout pregnancy and into labor. This has led to the concept that increased uterine contractility leading to term and preterm labor is mediated, in part, by a decline in PR function. The biochemical mechanisms for this decrease in PR function are also multifaceted and interwoven. In this paper, we focus on the molecular mechanisms that mediate myometrial quiescence and contractility and their regulation by the two central hormones of pregnancy, P_4 and estradiol-17 β . The integrative roles of microRNAs also are considered.

Preterm birth, defined as birth at <37 wk gestation, is the leading cause of infant mortality during the first four weeks of life throughout the world (Blencowe et al. 2012). Globally, ~ 15 million babies are born prematurely each year and 1.1 million of these succumb to com-

plications of their untimely birth. The highest rates of preterm birth are found in parts of Africa and in North America. In the United States, the incidence of preterm birth has increased steadily over the past several decades and has recently leveled off at $\sim 11.5\%$ of all live births.

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N.E. Renthal et al.

Importantly, the incidence of preterm birth in the U.S. is even higher among certain racial and ethnic groups. Whereas the prematurity rate in white infants is ~11%, ~18% of black infants are born prematurely. The reasons for this racial disparity in the U.S. population are not understood (Peltier et al. 2012). The high incidence of preterm birth is due, in part, to our incomplete understanding of the signaling pathways that maintain quiescence of the uterine myometrium throughout pregnancy and mediate its conversion into a synchronous contractile unit culminating in parturition. Although spontaneous preterm birth has multiple pathologic associations, the only definitive causal link established thus far is to subclinical intraamniotic infections, which account for ~25% of preterm births (Romero et al. 2014).

The maintenance of myometrial quiescence throughout most of pregnancy is mediated by increased circulating levels of progesterone (P_4), produced by placenta and/or the ovarian corpus luteum, depending on the species. We believe that the predominant actions of P_4 to block myometrial contractility are mediated by its binding to two nuclear receptor isoforms, hPR-A (94 kDa) and hPR-B (114 kDa), which arise from a single gene by alternative transcription initiation from different promoters (Kastner et al. 1990) and by alternative translation initiation from different translation initiation (AUG) sites (Conneely et al. 1987). The mechanisms whereby P_4 /PR maintains uterine quiescence are diverse and involve the possible antagonistic interaction of PR with proinflammatory transcription factors (Hardy et al. 2006; Lee et al. 2012), as well as genomic actions, including increased expression of genes that block activation of proinflammatory transcription factors (Hardy et al. 2006; Chen et al. 2011) and/or by direct suppression of contractile genes (Renthal et al. 2010).

Both term and preterm labor are associated with an increased inflammatory response. This is characterized by elevated concentrations of proinflammatory cytokines (e.g., IL-1 β , IL-6) in amniotic fluid (Cox et al. 1997) and infiltration of the myometrium, cervix, and fetal membranes by neutrophils and macrophages

(Thomson et al. 1999; Osman et al. 2003; Condon et al. 2004). The invading immune cells secrete proinflammatory cytokines and chemokines (Romero et al. 2007), resulting in activation of NF- κ B and other proinflammatory transcription factors in the myometrium (Condon et al. 2004, 2006). Activated NF- κ B, in turn, increases expression of genes promoting myometrial contractility, including the prostaglandin $F_{2\alpha}$ receptor (Olson 2003), the gap junction protein, connexin 43 (CX43/Gja1) (Chow and Lye 1994), the oxytocin receptor (OXTR) (Fuchs et al. 1984), and cyclooxygenase-2 (COX-2) (Soloff et al. 2004; Hardy et al. 2006). In preterm labor, intra-amniotic infection associated with chorioamnionitis may provide the stimulus for increased amniotic fluid interleukins and inflammatory cell migration (Rauk and Chiao 2000). At term, mechanical stretch (Sooranna et al. 2004; Shynlova et al. 2008) caused by the growing fetus, as well as hormonal signals produced by the developing fetus near term (Mitchell et al. 1984; Challis et al. 2000; Shaw and Renfree 2001; Condon et al. 2004), promote production of chemokines leading to macrophage migration and up-regulation of inflammatory response pathways, as well as a decline in progesterone receptor (PR) function. Our findings suggest that SP-A, the major lung surfactant protein, which is up-regulated in fetal lung during the third trimester (Mendelson and Boggaram 1990) and secreted into amniotic fluid in large amounts near term (Snyder et al. 1988; Condon et al. 2004), may provide an important signal for macrophage migration, activation of uterine NF- κ B, and other inflammatory transcription factors, leading to the initiation of labor (Condon et al. 2004; Mendelson and Condon 2005; Montalbano et al. 2013).

Here, we review the cellular and molecular mechanisms whereby P_4 acting through nuclear PR regulates myometrial quiescence throughout most of pregnancy, and the mechanisms whereby proinflammatory cytokines and E_2 /ER α antagonize PR function near term. The hormonal regulation and roles of microRNAs (miRNA, miR) and their targets as important regulators of myometrial quiescence and contractility are also considered.

MECHANISMS FOR P₄/PR REGULATION OF MYOMETRIAL QUIESCENCE

The mechanisms whereby P₄/PR maintains the myometrium in a quiescent state throughout most of pregnancy are multifactorial and involve nongenomic and genomic actions. On the one hand, P₄/PR can exert a pronounced anti-inflammatory effect, by inhibiting activation/DNA binding of the proinflammatory transcription factors, NF- κ B (Hardy et al. 2006) and AP-1 (Dong et al. 2009). Alternatively, P₄/PR can block induction of the proinflammatory response by up-regulating expression of the NF- κ B inhibitor, I κ B α (Hardy et al. 2006), and the mitogen-activated kinase (MAPK) inhibitor, MAPK phosphatase-1 (MKP-1), also known as dual specificity phosphatase 1 (DUSP1) (Chen et al. 2011). Moreover, in recent studies, we observed that P₄/PR increases expression of the transcriptional inhibitor, ZEB1, which binds to the promoters of the contractile genes *CX43* and *OXTR* to suppress their expression throughout most of pregnancy (Fig. 1) (Renthal et al. 2010, 2013).

P₄/PR Maintains Myometrial Quiescence by Its Anti-Inflammatory Actions

P₄/PR maintains uterine quiescence, in part, by inhibiting activation of inflammatory response pathways and expression of “contractile” genes within the uterus and cervix and by blocking the production of chemokines that promote chemotaxis of immune cells. During the estrous cycle in rodents, migration of macrophages and neutrophils to the uterus is stimulated by E₂ and inhibited by P₄ (Hunt et al. 1998; Kaushic et al. 1998; Tibbetts et al. 1999). This antagonistic effect of P₄ on estrogen-induced macrophage/neutrophil migration fails to occur in mice with a deletion of the *PR* gene (*PRKO* mice) and is, therefore, PR-dependent (Tibbetts et al. 1999). Furthermore, *PRKO* mice manifest a massive uterine inflammatory response to estrogen (Tibbetts et al. 1999) or E₂ plus P₄ treatment (Lydon et al. 1995).

Expression of the β -chemokine, monocyte chemoattractant protein-1 (MCP-1/CCL-2), which attracts and activates macrophages, was

Figure 1. Molecular mechanisms for P₄/PR regulation of myometrial quiescence. PR maintains myometrial quiescence, in part, by blocking activation of NF- κ B and preventing its transcriptional activation of pro-inflammatory genes, such as *COX-2*. P₄/PR exerts this action by increasing expression of the NF- κ B inhibitor, I κ B α , which prevents activation and nuclear translocation of NF- κ B p50 and p65 and by induction of *MKP-1*/*DUSP1*, which inhibits p38 MAPK activation and NF- κ B p65 nuclear translocation. PR also inhibits activation of CAP genes (e.g., *COX-2*) by direct interaction/tethering to NF- κ B bound to response elements in the promoters of these genes. In addition, PR prevents myometrial contractility by increasing expression of ZEB1, which inhibits expression of *CX43* and *OXTR*.

N.E. Renthal et al.

found to be stimulated by NF- κ B and inhibited by P_4 /PR in choriodecidual and breast cancer cells (Kelly et al. 1997). Notably, MCP-1 was reported to be up-regulated in myometrium of women in labor, as compared with myometrium from term pregnant women not in labor (Esplin et al. 2005) and in pregnant rat myometrium before and during parturition (Shynlova et al. 2008). Furthermore, MCP-1 expression and macrophage infiltration were greatly increased in the pregnant rat uterus after PR blockade by RU486 treatment, which caused preterm parturition. MCP-1 and macrophage infiltration were inhibited by progestin treatment, which delayed parturition (Shynlova et al. 2008).

Using telomerase-immortalized human myometrial cells, P_4 /PR was found to serve a major anti-inflammatory role via antagonism of both NF- κ B activation and COX-2 expression (Havelock et al. 2005; Hardy and Mendelson 2006). A similar phenomenon was observed in studies using amnion epithelial cells, lower uterine segment fibroblasts (Loudon et al. 2003), and human breast cancer cells (Hardy et al. 2008). By use of chromatin immunoprecipitation-quantitative PCR (ChIP-qPCR) analysis, we found that P_4 treatment of the human myometrial cells blocked IL-1 induction of in vivo binding of NF- κ B p65 to both proximal and distal NF- κ B response elements in the COX-2 promoter (Hardy et al. 2006). The P_4 -mediated decrease in p65 binding to the COX-2 promoter might be caused, in part, by a direct physical interaction of PR with p65 (Kalkhoven et al. 1996), which may, in turn, promote the recruitment of corepressors (Fig. 1). On the other hand, the anti-inflammatory effect of P_4 within the myometrium also may be mediated by increased expression of I κ B α , a crucial inhibitor of NF- κ B transactivation (Fig. 1). Glucocorticoids acting through the glucocorticoid receptor (GR), which is structurally related to PR, are known to induce I κ B α expression in a cell type- and promoter-specific manner (Auphan et al. 1995; Scheinman et al. 1995). Similarly, P_4 caused a rapid induction of I κ B α mRNA and protein expression in the immortalized human myometrial cells, which preceded its effect to

inhibit IL-1 β -induced COX-2 expression (Hardy et al. 2006). Moreover, co-incubation of IL-1 β with P_4 prevented the IL-1 β -induced decline in I κ B α protein levels, suggesting that P_4 /PR also blocks I κ B α degradation via the proteasome pathway (Baldwin 1996). As a consequence of the increased I κ B α expression, more NF- κ B is likely sequestered in an inactive state in the cytoplasm. P_4 inhibition of NF- κ B activation by induction of I κ B α has also been observed in macrophage cell lines (Miller and Hunt 1998) and in T47D breast cancer cells (De-roo and Archer 2002; Hardy et al. 2008).

In T47D human breast cancer cells, MKP-1/DUSP1 serves as a PR target gene that mediates P_4 /PR repression of ERK1/2 activation by serum growth factors and the subsequent increase in cell proliferation (Chen et al. 2011). PR acts to increase *MKP-1* expression and to inhibit MAPK activation in T47D cells both by classical and nonclassical mechanisms. Findings from ChIP-qPCR and luciferase reporter assays, suggest that PR up-regulates *MKP-1* promoter activity in a ligand-dependent manner through binding to two progesterone response elements (PREs) downstream from the *MKP-1* transcription start site (TSS). PR also acts in a ligand-independent manner to increase *MKP-1* expression by interaction with two putative Sp1 response elements downstream from the TSS (Chen et al. 2011). Because ERK and p38 MAPK can activate NF- κ B (Vanden Berghe et al. 1998) and MKP-1 overexpression decreases NF- κ B p65 nuclear translocation (Gil-Araujo et al. 2014), P_4 has the capacity to inhibit NF- κ B activation via induction of MKP-1.

Importantly, it appears that PR also inhibits NF- κ B activation and COX-2 induction via ligand-independent mechanisms. In studies using human breast cancer cells in which PR-A and PR-B protein levels were completely suppressed using siRNA-mediated knockdown, COX-2 expression was up-regulated >30-fold (Hardy et al. 2006). This phenomenon was observed in the absence of P_4 treatment. Conversely, up-regulation of PR protein expression in breast cancer cells, markedly inhibited IL-1 β induction of COX-2 expression in a P_4 -independent manner (Janowski et al. 2007).

P₄/PR Maintains Myometrial Quiescence throughout Pregnancy by Increasing Expression of the Gene Encoding ZEB1

Our recent findings have revealed that P₄/PR also maintains myometrial quiescence by direct genomic actions involving increased expression of the zinc finger E-box-binding transcriptional repressor, ZEB1/TCF8/ δ EF1 (Fig. 1) (Renthal et al. 2010). ZEB1 and the related transcription factor, ZEB2/SIP1, are E-box-binding transcription factors induced by TGF- β signaling that promote epithelial-to-mesenchymal transition (EMT) and increased metastasis of cancer cells (Peinado et al. 2007; Yang and Weinberg 2008; Gemmill et al. 2011). Zeb1 was previously reported to be highly expressed in mouse myometrium (Spoelstra et al. 2006) and to be up-regulated by P₄/PR (Cochrane et al. 2012). We observed that Zeb1 was expressed at relatively high levels in mouse myometrium at 15.5 d postcoitum (dpc) and declined precipitously toward term (Renthal et al. 2010). Zeb1 expression also declined with preterm labor induction by treatment of pregnant mice with the PR antagonist, RU486, or with the bacterial endotoxin, lipopolysaccharide (LPS) to mimic infection-induced preterm labor (Renthal et al. 2010). Analysis of human myometrial biopsies revealed that ZEB1 mRNA and protein were decreased in tissues from women in labor at term, as compared with those from women not in labor (Renthal et al. 2010).

Expression of OXTR and CX43, which are known to be up-regulated near term in a variety of species (Fuchs et al. 1984; Chow and Lye 1994; Ou et al. 1997, 1998; Sparey et al. 1999; Wu et al. 2001), are temporally up-regulated in mouse and human myometrium as ZEB1/2 expression declines (Renthal et al. 2010). CX43 is responsible for the formation of gap junctions in the myometrium, which mediate the intercellular communication required for the synchronous myometrial contractions necessary for successful labor. Mice with a smooth muscle-specific deletion of the *Cx43* gene manifest a significant delay in timing of parturition (Doring et al. 2006). Although the action of oxytocin as an uterotonic agent is widely accepted, the role of oxytocin and the OXTR in normal parturition is

uncertain, because *Oxtr* gene knockout mice undergo parturition normally and give birth to live young (Nishimori et al. 1996). Mice deficient in the *Oxtr* manifest normal timing and duration of parturition (Takayanagi et al. 2005). These unexpected phenotypes may be caused by a functional redundancy of the oxytocin/OXTR signaling system and/or to compensatory up-regulation of other uterotonic systems, such as COX-2 mediated prostaglandin synthesis.

ZEB1/2 may suppress myometrial contractility by negatively regulating OXTR and CX43 expression. Overexpression of ZEB1 or ZEB2 in human myometrial hTERT-HM cells markedly inhibited OXTR and CX43 mRNA levels (Renthal et al. 2010). Using ChIP-qPCR analysis of pregnant mouse myometrium, we observed that endogenous Zeb1 was bound at relatively high levels to E-box-containing regions of the mouse *Cxtr* and *Oxtr* promoters at 15.5 dpc, whereas binding was markedly reduced at term (Renthal et al. 2010). To assess the functional roles of ZEB1 and ZEB2 in myometrial contractility, hTERT-HM cells transduced with ZEB1 or ZEB2 expression vectors or with control vectors were embedded in 3D collagen gels. Oxytocin significantly increased contraction of collagen gel matrices embedded with untransduced and β -galactosidase-transduced hTERT-HM cells. However, this action of oxytocin was blocked in the hTERT-HM cells transduced with ZEB1 and ZEB2 expression vectors, demonstrating their inhibitory effect on myometrial contractility in vitro (Renthal et al. 2010).

Importantly, ZEB1 and ZEB2 are direct targets of members of the miR-200 family. In the following sections of this review, we will consider recent studies on the regulation and interactive roles of miRNAs and their targets in the control of myometrial quiescence and contractility in pregnancy and labor.

MicroRNAs SERVE IMPORTANT ROLES IN THE REGULATION OF MYOMETRIAL QUIESCENCE AND CONTRACTILITY

MicroRNAs are evolutionarily conserved regulators of gene expression that play important

N.E. Renthal et al.

roles in a variety of biological and pathological processes, including cell differentiation (van Rooij et al. 2008; Braun and Gautel 2011; Turner et al. 2011), immune regulation (Boldin and Baltimore 2012), female reproduction (Chakrabarty et al. 2007; Montenegro et al. 2007, 2009; Renthal et al. 2010; Hawkins et al. 2011; Williams et al. 2012a), and cancer (Mendell 2008; Garzon et al. 2009).

MicroRNAs are ~22 nucleotide, single-stranded RNAs that typically inhibit gene expression by binding through imperfect base-pairing via their “seed sequences” (nt 2–8 at their 5′-ends) to complementary sites, typically in the 3′-untranslated regions of target mRNAs. This results in inhibition of mRNA translation and/or mRNA degradation (Ruvkun 2008; Bartel 2009). A single miRNA can target multiple components of functionally related gene networks, whereas multiple miRNAs can target a single mRNA. They can act as rheostats or as on–off switches of gene expression. Although the effect of a specific miRNA on the regulation of a target can be subtle, combined actions of several related miRNAs on the same target can have pronounced phenotypic consequences. This is especially true when miRNAs with similar or identical seed sequences are expressed from the same polycistronic transcript and/or when miRNAs encoded within the same transcript have different seed sequences that bind to different sequences within the same target mRNA. It is estimated that ~1000 miRNAs are encoded by the human genome and these regulate approximately one-third of expressed human genes (van Rooij et al. 2008).

To identify miRNAs and targets that are differentially regulated in quiescent versus contractile pregnant mouse myometrium, we used an unbiased microarray approach. Using coordinated miRNA and gene expression microarray analyses of myometrial tissues from pregnant mice at 15.5 dpc (when the myometrium is quiescent) versus 18.5 dpc (just before labor at 19 dpc), 15 miRNAs were found to be significantly up- and down-regulated ($p < 0.05$), in association with a body of gestationally regulated genes. Intriguingly, the highly conserved miR-200 family was among the most signifi-

cantly up-regulated miRNAs at term, whereas the miR-199a/214 cluster was among the most significantly down-regulated (Renthal et al. 2010).

The miR-200 Family and Its Targets

The miR-200 family is comprised of five miRNAs arranged into two conserved, coordinately transcribed (Bracken et al. 2008) clusters (*miR-200b/200a/429* and *miR-141/200c*) that exist on two different chromosomes within the mouse and human genomes (Renthal et al. 2010). miR-200b, 200c, and 429 contain an identical seed sequence; miR-200a and 141 also share an identical seed sequence that differs by only a single nucleotide from those of miR-200b, 200c, and 429. Thus, all members of the miR-200 family likely share mRNA targets. From the results of our gene expression array, TargetScan prediction algorithms (Whitehead Institute for Biomedical Research) (Grimson et al. 2007), and published findings, we identified a pool of regulated miR-200 targets among those mRNAs down-regulated at term. The two most significantly down-regulated miR-200 targets were the transcriptional repressors, Zeb1 and Zeb2 (Renthal et al. 2010).

The findings of the microarrays were validated in myometrial tissues from gestational series of timed pregnant mice (15.5 dpc–19.5 dpc), in which expression of miR-200b, miR-429 (Renthal et al. 2010), and miR-200a (Williams et al. 2012a) was found to be significantly up-regulated after 17.5 dpc; this corresponded to the time at which Zeb1 and Zeb2 were significantly down-regulated in the myometrium (Renthal et al. 2010). Notably, miR-200b and miR-429 were also significantly up-regulated and ZEB1/2 significantly down-regulated in myometrium of women in labor, as compared with those not in labor (Renthal et al. 2010). Thus, the relationship between miR-200 family members and ZEB1 and ZEB2 expression is conserved between mice and humans.

miR-200 family members and ZEB1 and ZEB2 exist in a double-negative feedback loop in a number of cancer cell lines (Bracken et al. 2008; Burk et al. 2008; Gregory et al. 2008; Bra-

bletz et al. 2011). An inverse relationship between ZEB1 and ZEB2 and the miR-200 family in myometrium was indicated by findings that overexpression of miR-200b/429 mimics in immortalized human myometrial cells caused inhibition of ZEB1 and ZEB2, whereas transduction of mouse myometrial cells in primary culture with recombinant adenoviruses containing ZEB1 and ZEB2 expression vectors caused repression of miR-200s (Renthal et al. 2010). Moreover, using ChIP-qPCR, in vivo binding of Zeb1 to the *miR-200b/a/429* promoter in mouse myometrium was observed at relatively high levels at 15.5 dpc; Zeb1 binding declined markedly at 18.5 dpc with the increase in miR-200b and miR-429 expression (Renthal et al. 2010).

The miR-200 family was up-regulated and Zeb1/2 down-regulated in pregnant myometrium using two mouse models of premature parturition, induced either by a single subcutaneous injection of RU486 (Dudley et al. 1996), or by intra-amniotic injection of LPS (Renthal et al. 2010). Conversely, daily injection of P₄ into pregnant mice from 15.5 through 18.5 dpc, which inhibited myometrial *Cx43* and *Oxtr* gene expression and delayed labor, caused in-

duction of Zeb1, compared with vehicle injected mice. Surprisingly, P₄ injection had no effect on Zeb2. Because both Zeb1 and Zeb2 are expressed at relatively high levels in the myometrium throughout most of pregnancy, this raised the question regarding the factor(s) that cause the pregnancy-associated induction of Zeb2. In studies using cultured mouse myometrial cells, it was observed that Zeb1 overexpression caused a time-dependent up-regulation of Zeb2. This suggests that P₄ induction of Zeb1 and the associated inhibition of the miR-200 family, in turn, relieves suppression of Zeb2, allowing its subsequent induction. A direct effect of P₄/PR on *ZEB1* promoter activity was supported by cotransfection studies of HEK293 cells with a *ZEB1-luciferase* reporter construct containing -978 bp of *ZEB1* 5'-flanking sequence (containing the two putative PREs). *ZEB1* promoter activity was induced by cotransfection of wild-type PR-B, but not by a PR DNA-binding domain mutant of PR (Renthal et al. 2010).

Altogether, these findings support an important role for the miR-200 family and ZEB1/2 in the regulation of myometrial contractility during pregnancy and labor from mice to humans (Fig. 2). Throughout most of gestation,

Figure 2. P₄/PR modulation of the ZEB1-miR-200 negative feedback loop regulates contractile gene expression in the pregnant myometrium. During pregnancy, P₄/PR increase expression of ZEB1, which acts to suppress the miR-200 family, as well as contraction-associated genes. Decreased expression of the miR-200 family relieves suppression of ZEB2 (as well as ZEB1), resulting in further down-regulation of contractile genes. Near term, a decrease in circulating P₄ and/or a decrease in PR function results in down-regulation of *ZEB1* expression, and, in turn, an up-regulation of the miR-200 family, further suppressing ZEB1 and ZEB2. This removes the brakes from contractile gene expression, resulting in increased uterine contractility and labor.

N.E. Renthal et al.

elevated circulating P_4 induces myometrial *ZEB1* expression via binding of P_4 /PR to the *ZEB1* promoter. Elevated *ZEB1* suppresses expression of *CX43* and *OXTR* (Renthal et al. 2010), as well as the *miR-200* gene clusters (Bracken et al. 2008; Burk et al. 2008) by binding to response elements within their promoters. The suppression of *miR-200* promotes further up-regulation of *ZEB1* and increases expression of *ZEB2*. Together, *ZEB1* and *ZEB2* inhibit *CX43* and *OXTR* to maintain myometrial quiescence. Near term, signals from mother and fetus, together with a decline in circulating P_4 and/or PR function result in an increased inflammatory response within the myometrium and a further decline in PR function, resulting in decreased *ZEB1* expression. The decline in *ZEB1* allows up-regulation of *miR-200* expression, resulting in a further suppression of *ZEB1* and inhibition of *ZEB2*. The combined decline in *ZEB1* and *ZEB2* then permits marked up-regulation of *OXTR* and *CX43* gene expression and induction of myometrial contractility.

Role of the *miR-200* Family in the Decline of PR Function Leading to Labor

As discussed above, in humans circulating P_4 levels remain elevated throughout pregnancy and into labor, owing to enhanced placental P_4 synthesis (Mendelson 2009). Although parturition in rodents is associated with 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 binding to PR (Pointis et al. 1981). Moreover, levels of PR remain relatively constant in the majority of species during pregnancy and into labor. Thus, a number of molecular and biochemical changes likely contribute in all species to the decline in PR function leading to parturition.

It has been suggested that increased local metabolism of P_4 and a local decline in PR function in the uterus and cervix near term are crucial for the initiation of parturition in all mammals (Runnebaum and Zander 1971; Csapo et al. 1981; Puri and Garfield 1982; Power and Challis 1987; Mahendroo et al. 1999). In

myometrium of pregnant women at term, a pronounced decrease was observed in the ratio of P_4 to 20α -dihydroprogesterone (20α -OHP) (Runnebaum and Zander 1971), an inactive metabolite of P_4 generated by the enzyme 20α -hydroxysteroid dehydrogenase (20α -HSD), a member of the aldo-ketoreductase (AKR) superfamily (Penning and Drury 2007). Furthermore, targeted deletion of *20\alpha-HSD* in mice caused a significant delay in the timing of labor (Piekorz et al. 2005). Notably, the transcription factor, signal transducer and activator of transcription (STAT)5b, is a P_4 -responsive transcriptional repressor of *20\alpha-HSD* in reproductive tissues (Richer et al. 1998; Piekorz et al. 2005). Consequently, *STAT5b*-deficiency in mice resulted in increased expression of ovarian 20α -HSD, decreased circulating P_4 , and pregnancy loss during mid-gestation (Piekorz et al. 2005). This mid-gestation pregnancy loss was partially corrected by combined *20\alpha-HSD* deficiency (Piekorz et al. 2005). Because 20α -HSD expression in the ovarian corpus luteum remains low throughout pregnancy and increases in association with $PGF_2\alpha$ -induced luteolysis at term (Stocco et al. 2001), it was suggested that delayed labor in *20\alpha-Hsd*-deficient mice was caused by an inhibition of luteolysis and sustained circulating P_4 levels. However, in one of the *20\alpha-Hsd* knockout studies, labor was delayed in pregnant gene-targeted mice, despite a fall in circulating P_4 levels similar to wild-type mice (Ishida et al. 2007). These findings suggest that mechanisms other than induction of ovarian 20α -HSD may contribute to the functional luteolysis near term and that actions of 20α -HSD to catalyze local metabolism of P_4 in the reproductive tract may be more critical for the decline in PR function leading to labor.

In consideration of the presumed importance of 20α -HSD and STAT5b in the timing of parturition, we were intrigued to discover that STAT5b is a bona fide target of *miR-200a* (Williams et al. 2012a), which together with other *miR-200* family members, increases dramatically at term in myometrium of mice and humans (Williams et al. 2012a). Remarkably, STAT5b mRNA and protein expression were decreased in myometrial tissues of pregnant mice

and women in association with the increase in miR-200a at term. Moreover, these gestational changes were associated with decreased binding of endogenous STAT5b to response elements in the 5'-flanking region of the *20 α -HSD* gene and an induction of *20 α -HSD* mRNA, protein, and enzyme activity (Williams et al. 2012a). An intermediary role of miR-200a in P_4 induction of STAT5b was suggested by the finding that P_4 treatment of ovariectomized mice inhibited miR-200a, induced STAT5b and inhibited *20 α -HSD* expression. Conversely, RU486 injection of timed pregnant mice caused induction of miR-200a, inhibition of STAT5b, and enhanced *20 α -HSD* mRNA levels. Because the seed sequence of miR-200a is nearly identical to that of miR-200b and miR-429, which are encoded within the same transcript (Renthal et al. 2010), it is likely

that other members of the miR-200 family also target myometrial STAT5b and regulate *20 α -HSD*. Moreover, miR-200a also directly targets ZEB1 and ZEB2 (Gregory et al. 2008), suggesting that near term, increasing levels of miR-200a act cooperatively with miR-200b/429 to inhibit ZEB expression and derepress contraction-associated genes.

Together, our findings suggest that the elevated P_4 and PR activity in the myometrium during most of pregnancy causes induction of ZEB1 and inhibition of miR-200 expression, which is permissive for increased expression of STAT5b. The increased STAT5b maintains low levels of *20 α -HSD* expression and allows tissue levels of P_4 and PR function to remain elevated (Fig. 3). The increased inflammatory response leading to term or preterm labor causes a de-

Figure 3. Increased miR-200 expression near term inhibits PR function by targeting STAT5b and enhancing P_4 metabolism in myometrium. During pregnancy, elevated P_4 /PR function increases expression of transcription factor ZEB1, which inhibits expression of miR-200a and other members of the miR-200 family. The decreased levels of miR-200 enhance expression of STAT5b, which represses *20 α -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, resulting in decreased levels of ZEB1, which releases repression of the miR-200 family. The increased levels of miR-200s inhibit STAT5b expression, releasing repression of *20 α -HSD*. The increase in *20 α -HSD* expression, 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 up-regulate *20 α -HSD*.

N.E. Renthal et al.

cline in PR function and associated decrease in *ZEB1*. This allows up-regulation of miR-200a and other miR-200 family members, inhibition of STAT5b and induction of 20 α -HSD expression and activity. The increased local metabolism of P₄ to inactive products in the myometrium contributes to the further decline in PR function and progression to labor (Fig. 3). These studies have revealed a robust positive feed forward loop wherein an initially modest decline in PR function and induction of miR-200 expression can escalate to an intensity that effectively reduces tissue P₄ to levels significantly below the K_d for PR binding. This culminates in further decline of ZEBs, which permits the induction of *OXTR* and *CX43* gene expression in response to increased NF- κ B activation.

Other Mechanisms for the Decline in PR Function Leading to Parturition

In addition to increased metabolism of P₄ to inactive steroids, the decline in PR function near term also is associated with a decrease in PR coactivators (Condon et al. 2003; Leite et al. 2004), with increased expression of the inhibitory PR isoform, PR-C, an increase in the ratio of PR-A to PR-B (Mesiano et al. 2002; Condon et al. 2006; Tan et al. 2012) and a physical interaction of PR with NF- κ B p65 (Kalkhoven et al. 1996; Hardy et al. 2006, 2008), which is activated in the myometrium during late gestation (Condon et al. 2004, 2006). Furthermore, increased circulating E₂ levels (Challis 1971; Buster et al. 1979) and enhanced ER α activity (Wu et al. 1995; Mesiano et al. 2002; Mesiano and Welsh 2007; Welsh et al. 2012) near term also promote a cascade of proinflammatory events leading to the decline in PR function and parturition. Estrogens induce an influx of macrophages and neutrophils into the uterus and antagonize the anti-inflammatory actions of P₄/PR (Tibbetts et al. 1999; Mesiano et al. 2002). ER α activation also promotes labor by enhancing transcription of genes for *OXTR* (Murata et al. 2003), *CX43* (Piersanti and Lye 1995) and *COX-2* (Mesiano et al. 2002; Williams et al. 2012b), which is expressed at low to unde-

tectable levels in the uterus throughout most of pregnancy, but is highly up-regulated at term (Tsuboi et al. 2000; Engstrom 2001; Williams et al. 2012b).

miR-199a-3p and miR-214 Are Coordinately Down-Regulated in the Pregnant Myometrium Toward Term, Whereas Their Target COX-2 Is Increased

MicroRNAs that were significantly down-regulated near term in our microarray analysis included miR-199a-3p, known to target COX-2 mRNA (Chakrabarty et al. 2007) and miR-214, which targets PTEN and activates the Akt pathway (Yang et al. 2008; Yin et al. 2010). Mature miR-199a and miR-214 are processed from the same precursor/pri-miR-199a as part of a 6-kb antisense transcript (*Dnm3os*) from the intron of the *Dynamin3* (*Dnm3*) gene, which is highly expressed in pregnant uterus (Loebel et al. 2005). As noted, the expression of COX-2 increases in the pregnant myometrium during late gestation and is believed to be critical for the increase in contractile PGF₂ α (Olson et al. 2003) required for the progression to labor.

Our studies revealed that COX-2 also is a bona fide target of miR-214 (Williams et al. 2012b). In pregnant mouse myometrium, Cox-2 protein levels were significantly increased at 18.5 dpc and during labor, compared with 15.5 dpc, as miR-199a-3p and miR-214 reciprocally declined. However, Cox-2 mRNA levels remained low through 18.5 dpc and increased only during labor. Our findings suggested that miR-199a-3p/miR-214 may exert a direct effect on COX-2 mRNA translation, rather than on COX-2 mRNA stability. Accordingly, 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 (Williams et al. 2012b). Moreover, in myometrial samples from women in labor versus not in labor at term, in the absence of underlying infection, COX-2 mRNA was unchanged during labor, whereas COX-2 protein levels were markedly increased (Williams et al. 2012b). Thus, these findings suggest that COX-2 expression in the pregnant myometrium is regulated at

the level of mRNA translation and the miR-199a/214 cluster may serve an important role in this regulation. Our studies further revealed that myometrial levels of miR-199a-3p and miR-214 were significantly decreased in an LPS-induced mouse model of preterm labor, whereas Cox-2 expression increased. Moreover, the physiological relevance of the miR-199a/214-COX-2 relationship in the regulation of myometrial contractility was further supported by the finding that miR-199a/214 overexpression blocked TNF- α -induced myometrial cell contractility to the same extent as the cyclooxygenase inhibitor, indomethacin (Williams et al. 2012b).

As discussed previously, P_4 and E_2 exert opposing effects on myometrial quiescence/contractility. Accordingly, E_2 treatment of ovariectomized mice suppressed, whereas P_4 enhanced, uterine miR-199a-3p/214 expression. Interestingly, these opposing hormonal effects were

found to be mediated by Zeb1, which is induced by P_4 (Spoelstra et al. 2006; Renthal et al. 2010), inhibited by E_2 and activates miR199a/214 transcription (Williams et al. 2012b). Thus, these findings have uncovered an intriguing pivotal role of ZEB1 as a negative regulator of the miR-200b/200a/429 cluster and a positive regulator of the miR-199a/214 cluster that is under opposing control by P_4 and E_2 (Fig. 4).

CONCLUDING REMARKS

The opposing mechanisms that underlie the maintenance of myometrial quiescence throughout most of pregnancy and its transition to a contractile phenotype leading to labor are multifactorial and interrelated. It is clear that P_4 /PR serve a key role in the maintenance of pregnancy through anti-inflammatory actions and inhibition of contractile gene expression. These effects are mediated by PR antagon-

Figure 4. ZEB1 serves a pivotal role by mediating opposing actions of P_4 and E_2 on myometrial contractility during pregnancy and labor. During pregnancy, increased levels of P_4 and increased PR function promote up-regulation of ZEB1 in myometrium. ZEB inhibits expression of the miR-200 family and suppresses OXTR and CX43. The decline in miR-200 levels further up-regulates ZEB1 and increases ZEB2, which bind response elements upstream of the miR-199a/214 cluster to enhance its expression, causing suppression of COX-2, and preventing synthesis of contractile prostaglandins. The decreased levels of miR-200s allow up-regulation of another target, STAT5b, which inhibits expression of 20α -HSD, allowing local levels of P_4 in myometrium to remain elevated. During the transition to labor, the decline in myometrial P_4 /PR function and increase in circulating E_2 and $ER\alpha$ activity cause down-regulation of ZEB1. This leads to induction of the miR-200 family, which further suppresses ZEB1 and ZEB2, allowing up-regulation of OXTR and CX43 expression. The decline in ZEBs also causes decreased expression of the miR199a/214 cluster, which allows up-regulation of COX-2 and increased synthesis of contractile prostaglandins. The increase in miR-200 expression inhibits STAT5b, permitting increased transcription of 20α -HSD to promote increased metabolism of P_4 to inactive products in myometrium. Collectively, these molecular events contribute to the initiation of uterine contractility, leading to labor.

N.E. Renthal et al.

onism of inflammatory transcription factor activation (e.g., NF- κ B and AP-1) and by up-regulation of the transcriptional inhibitor, ZEB1, which acts together with ZEB2 to inhibit expression of contractile genes. Recent studies have provided intriguing evidence for the central role of ZEB1 in the regulation of two opposing pathways regulated by clusters of evolutionarily conserved miRNAs (Fig. 4). Throughout most of pregnancy, increased circulating and tissue levels of P₄ activate PR transcriptional activity resulting in up-regulation of ZEB1 expression. ZEB1 inhibits transcription of members of the *miR-200* family, as well as the contractile genes, *OXTR* and *CX43*. Elevated P₄/PR levels and suppression of the *miR-200* family also allow up-regulation of STAT5b, which inhibits expression of the P₄-metabolizing enzyme, 20 α -HSD. This permits myometrial tissue levels of P₄ to remain elevated, resulting in enhanced PR function to sustain myometrial quiescence, and to suppress NF- κ B activation of inflammatory genes, such as COX-2. The increased expression of ZEB1 and ZEB2 also cause up-regulation of miR-199a-3p and miR-214, which directly target COX-2, resulting in suppression of prostaglandin synthesis. Near term, the increased inflammatory response caused by uterine stretch, increased E₂ production and/or ER α activity and other signaling molecules produced by mother and fetus (Condon et al. 2004; Montalbano et al. 2013) initiate a decline in PR function. This further enhances NF- κ B activation and the inflammatory response. The decreased P₄/PR activity and increased in E₂/ER α signaling promote a decline in ZEB1, a reciprocal increase in miR-200 expression, with further inhibition of ZEB1 and of ZEB2. This allows for induction of *OXTR* and *CX43* gene expression. The increased levels of miR-200s cause suppression of STAT5b, allowing for induction of 20 α -HSD, which promotes increased metabolism of P₄ to inactive products within the myometrium, contributing to the decline in PR function. The decreased levels of ZEB1/2 also result in decreased expression of miR-199a-3p and miR-214, which allow for increased COX-2 protein expression with synthesis of contractile prostaglandins. Collec-

tively, these highly integrated molecular events coordinated by conserved miRNAs and their targets result in enhanced myometrial contractility leading to parturition. Thus, we are developing an understanding of the molecular events that underlie the maintenance of uterine quiescence during pregnancy and the activation of contractility leading to labor. In future investigations, we hope to apply these findings to development of therapeutic strategies to prevent preterm birth.

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N.E. Renthal et al.

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A Myometrial Perspective on Parturition

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N.E. Renthal et al.

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