



Multifactorial Regulation of Myometrial Contractility During Pregnancy and Parturition

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The steroid hormones progesterone (P_4) and estradiol-17 β (E_2), produced by the placenta in humans and the ovaries in rodents, serve crucial roles in the maintenance of pregnancy, and the initiation of parturition. Because of their critical importance for species survival, the mechanisms whereby P_4 and its nuclear receptor (PR) maintain myometrial quiescence during pregnancy, and for the decline in P_4 /PR and increase in E_2 /estrogen receptor (ER) function leading to parturition, are multifaceted, cooperative, and redundant. These actions of P_4 /PR include: (1) PR interaction with proinflammatory transcription factors, nuclear factor κ B (NF- κ B), and activating protein 1 (AP-1) bound to promoters of proinflammatory and contractile/contraction-associated protein (CAP) genes and recruitment of corepressors to inhibit NF- κ B and AP-1 activation of gene expression; (2) upregulation of inhibitors of proinflammatory transcription factor activation ($I\kappa$ B α , MKP-1); (3) induction of transcriptional repressors of CAP genes (e.g., ZEB1). In rodents and most other mammals, circulating maternal P_4 levels remain elevated throughout most of pregnancy and decline precipitously near term. By contrast, in humans, circulating P_4 levels and myometrial PR levels remain elevated throughout pregnancy and into labor. However, even in rodents, wherein P_4 levels decline near term, P_4 levels remain higher than the K_d for PR binding. Thus, parturition is initiated in all species by a series of molecular events that antagonize the P_4 /PR maintenance of uterine quiescence. These events include: direct interaction of inflammatory transcription factors (e.g., NF- κ B, AP-1) with PR; increased expression of P_4 metabolizing enzymes; increased expression of truncated/inhibitory PR isoforms; altered expression of PR coactivators and corepressors. This article will review various mechanisms whereby P_4 acting through PR isoforms maintains myometrial quiescence during pregnancy as well as those that underlie the decline in PR function leading to labor. The roles of P_4 - and E_2 -regulated miRNAs in the regulation and integration of these mechanisms will also be considered.

Keywords: progesterone, gene regulation, transcription corepressor, inflammation, pregnancy, myometrium, NF- κ B

INTRODUCTION

Preterm birth (<37 weeks gestation), which affects $\sim 15 \times 10^6$ births globally each year, is a major cause of death within the first month of postnatal life (1). The highest rates of preterm birth ($\geq 15\%$ of all live births) occur in sub-Saharan Africa, Pakistan, and Indonesia. In the U.S., the preterm birth rate remains at $\sim 10\%$ of all overall live births. However, significant

racial disparities in preterm birth rates exist, with the incidence of preterm birth among African-Americans being 50% higher than that of the overall population. Notably, the underlying causes for these racial differences remain unknown (2). Astonishingly, the modalities used to treat and/or prevent preterm labor have changed little over the past 50 years. This is due, in part, to our incomplete comprehension of mechanisms that mediate myometrial quiescence and contractility as well as the reluctance of pharmaceutical companies to engage in drug discovery in this critical area.

Throughout pregnancy, myometrial quiescence is controlled by increased progesterone (P_4), secreted by the placenta and/or the ovarian corpus luteum, depending upon the species. In humans, two progesterone receptor (PR) isoforms, PR-A (94 kDa), and PR-B (114 kDa), alternative transcripts of a single gene (3, 4), mediate P_4 action to block myometrial contractility. Both PR-A and PR-B bind to progesterone response elements (PREs) in DNA; however, PR-A contains two of three transcriptional activation domains that are present in PR-B and is, therefore, less transcriptionally active. Thus, PR-A can repress PR-B transcriptional activity in a cell- and gene-specific context (5, 6). PR-A was also found to inhibit PR-B transcriptional activity in cultured human myometrial cells (7), suggesting a potential antagonistic role of PR-A on PR-B action in the myometrium. PR-A and PR-B are differentially regulated in the human myometrium during pregnancy (8); the ratio of PR-A to PR-B mRNA (9) and protein (7) was observed to increase significantly in the myometrium of women in labor when compared to those not in labor at term. In telomerase-immortalized human myometrial (hTERT-HM) cells stably expressing either PR-A or PR-B, P_4 treatment had increased anti-inflammatory activity in PR-B-expressing cells when compared to those expressing PR-A (10).

As described below, a number of unique and redundant mechanisms mediate the action of P_4 /PR to maintain uterine quiescence. In rodents and most other mammals, circulating maternal P_4 levels remain elevated throughout most of pregnancy and decline sharply prior to parturition (11). This has led to the concept that labor is associated with P_4 withdrawal. By contrast, in humans and guinea pigs circulating P_4 and myometrial levels of PR fail to decline during late pregnancy and into labor (12). However, treatment with PR antagonists can cause increased myometrial contractility, cervical ripening, and/or increased sensitivity to labor induction by contractile factors (13–16). Importantly, even in rodents, circulating maternal P_4 levels at term remain well above the equilibrium dissociation constant for binding to PR (17). Moreover, in rodents, local metabolism of P_4 within the cervix and myometrium to inactive products near term is essential for the normal timing of parturition. Thus, in mice deficient in 5α -reductase type I (expressed in cervix) (18, 19) or 20α -hydroxysteroid dehydrogenase (20α -HSD, expressed in myometrium) (20) parturition is severely delayed. Collectively, these findings have led to the concept that parturition in all placental mammals is initiated by a conserved sequence of molecular events that impairs the capacity of the PR to maintain uterine quiescence. These include: (1) direct interaction of transcription factor nuclear factor κ B

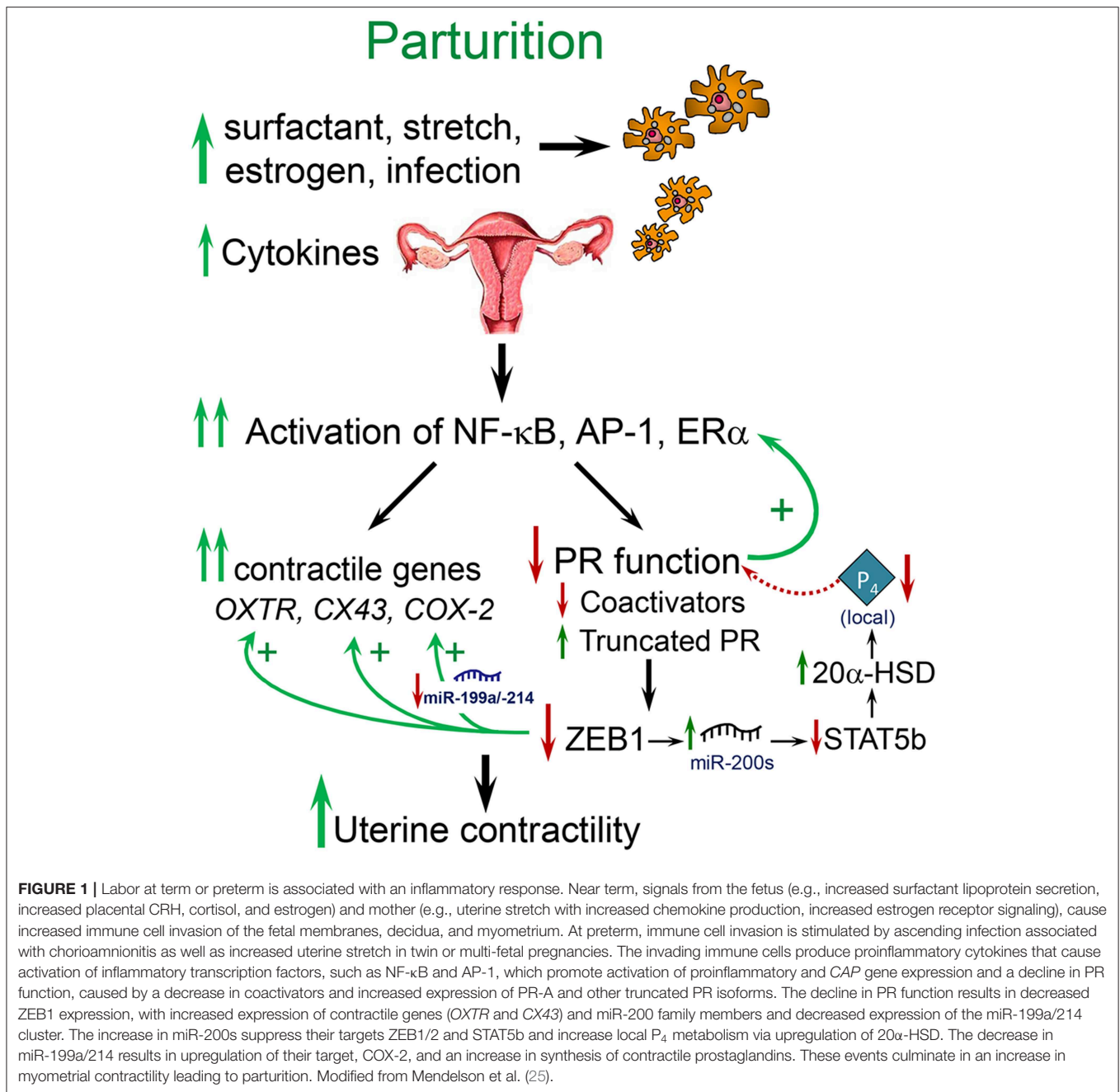
(NF- κ B) with PR; (2) upregulation of P_4 metabolizing enzymes within the uterus and cervix; (3) increased expression of truncated/inhibitory PR isoforms; (4) altered expression of key PR-interacting coactivators and corepressors. This article will review the various mechanisms whereby P_4 acting through PR isoforms maintains myometrial quiescence during pregnancy as well as those that underlie the decline in PR function leading to parturition.

PARTURITION IS ASSOCIATED WITH AN INCREASED INFLAMMATORY RESPONSE

Term and preterm parturition is initiated by an enhanced inflammatory response, increased levels of proinflammatory cytokines in amniotic fluid (21) and the invasion of the fetal membranes, cervix and myometrium by neutrophils and macrophages ($M\phi$) (22–24) (**Figure 1**). The secretion of cytokines and chemokines by the invading immune cells (26) cause activation of NF- κ B and other inflammation-associated transcription factors (e.g., AP-1) (23, 27–30). These activated transcription factors promote increased expression of myometrial proinflammatory [e.g., interleukin (*IL*)-1 β , *IL*-8] and contractile/CAP [connexin-43 (*CX43/GJA1*), oxytocin receptor (*OXR*), and cyclooxygenase 2 (*COX-2/PTGS2*)] genes, leading to parturition (31–34). Whereas, intra-amniotic infection associated with chorioamnionitis can provide the stimulus for the inflammatory response leading to preterm labor (35), signals from both mother and fetus provide critical inflammatory stimuli leading to labor at term.

Increased Mechanical Stretch of the Uterus Results in Production of Chemokines, Enhanced Immune Cell Invasion, and Proinflammatory Signaling Leading to Labor

Near term, enhanced uterine stretch caused by the growing fetus provides an important stimulus for the initiation of labor (36, 37) (**Figure 1**). The increased incidence of preterm birth in twin and multiple, as compared to singleton, pregnancies implicates uterine over-distension as a causative factor (38). The expression of the β -chemokine, monocyte chemoattractant protein-1/C-C motif ligand 2 (MCP-1/CCL2), which attracts and activates $M\phi$, was found to be upregulated in the term pregnant myometrium of women in labor, as compared to myometrium from women not in labor (39) and of pregnant rats prior to and during parturition (36). In pregnant rats carrying pups only in one uterine horn, increased MCP-1 expression was observed only in the gravid horn, implying the potential role of the fetus and/or of uterine stretch (36) in the induction of MCP-1 expression. Furthermore, the findings that MCP1 expression and $M\phi$ infiltration were greatly increased in the pregnant rat uterus with preterm labor induction by the PR antagonist, mifepristone/RU486, and inhibited by progestin treatment to delay parturition (36) suggests a role of P_4 /PR in MCP-1 regulation. Preterm labor



was induced in non-human primates by intrauterine balloon inflation, in association with increased expression of IL-6, IL-8, and CCL-2 in the myometrium (40). Thus, enhanced inflammation associated with mechanical stress contributes to the initiation of term and preterm labor. In studies using human myometrial smooth muscle cells in culture, IL-1β and TNFα induced expression of MCP-1 and other chemokines; this was blocked by an inhibitor of the NF-κB signaling pathway (41). Likewise, in human choriondecidual and breast cancer cells, MCP-1 was stimulated by NF-κB activation and inhibited by P₄/PR (42).

Increased Estrogen Receptor Signaling Contributes to the Inflammatory Response Leading to Parturition

Across mammalian species, an increase in circulating estradiol-17β (E₂) (43, 44) and/or myometrial estrogen receptor α (ERα) activity (9, 45) precedes the increase in uterine contractility near term (Figure 1). Estrogens induce migration of immune cells to the uterus and antagonize anti-inflammatory actions of P₄/PR (9, 46). Moreover, ERα activation enhances transcription of the CAP genes, *OXTR* (47), *CX43* (48), and *COX-2* (9), and the resulting synthesis of prostaglandins that increase myometrial contractility

(49–51). These actions of estrogen may be mediated, in part, through interaction of ER α and p160 coactivators with the AP-1 transcription factors Fos and Jun at AP-1-regulated promoters, resulting in an increase in AP-1 transcriptional activity (52).

Interestingly, we observed that ER α is a direct target of the microRNA, miR-181a, which significantly declines in mouse myometrium near term and in term myometrial tissues from women in labor, compared to those not-in-labor (53). Furthermore, E₂ treatment inhibited miR-181a expression in uteri of ovariectomized mice and in human myometrial cells in primary culture. This revealed the presence of a feedback loop, wherein increased circulating E₂ near term causes suppression of miR-181a, resulting in upregulation of ER α with further downregulation of miR-181a (53). In human myometrial cells, overexpression of miR-181a mimics repressed TNF α , CCL-2 and CCL-8 expression, while expression of the anti-inflammatory cytokine, IL-10, increased (53). TNF α was confirmed as a direct target of miR-181a, while CCL-2 and CCL-8 are predicted targets of this miRNA (53). c-Fos, which increases in pregnant rat (54) and mouse (53) myometrium during late gestation and into labor, was validated as a target of miR-181a in dendritic cells (55). These collective findings suggest that, from early through mid-gestation, relatively low E₂/ER α levels allow increased expression miR-181a in myometrium, which represses ER α , c-FOS, TNF α , and several other proinflammatory cytokines, and increases the expression of anti-inflammatory cytokines. Moreover, near term increased circulating levels of E₂ inhibit miR-181a, which allows the upregulation of its targets, ER α , TNF α , other proinflammatory cytokines, and transcription factor, c-FOS. In turn, c-FOS mediates the proinflammatory effects of E₂/ER α and cytokines, which activate *CAP* genes and lead to labor.

We also previously observed that in concert with the increased expression of the miR-200 family in pregnant mouse myometrium between 15.5 days post-coitum (dpc) and term (18.5 dpc and in labor) (56), there was a decline in the expression of the miR-199a/miR-214 cluster of miRNAs (57) (**Figure 2**). This was mediated by increased E₂/ER α and the decrease in PR function, which inhibited expression of transcription factor ZEB1, a positive regulator of *miR-199a/miR-214* transcription (57, 58). Of note, miR-199a-3p and miR-214 directly target COX-2, which increases in the myometrium near term and during labor. Therefore, stimulatory effects of E₂ on COX-2 expression (50) are likely mediated, in part, by its inhibition of miR-199a-3p/miR-214. Since miR-181a targets both ER α and cFOS (53), we suggest that the coordinate decline in miR-181a and miR-199a-3p/214 in the myometrium toward term mediates the induction of COX-2 expression via indirect and direct mechanisms.

The Fetus Produces Signaling Molecules for the Initiation of Parturition

The fetus has been proposed to contribute to the initiation of parturition through the production of signaling molecules from its adrenals, placenta and lungs.

Cortisol Production by the Fetal Adrenal

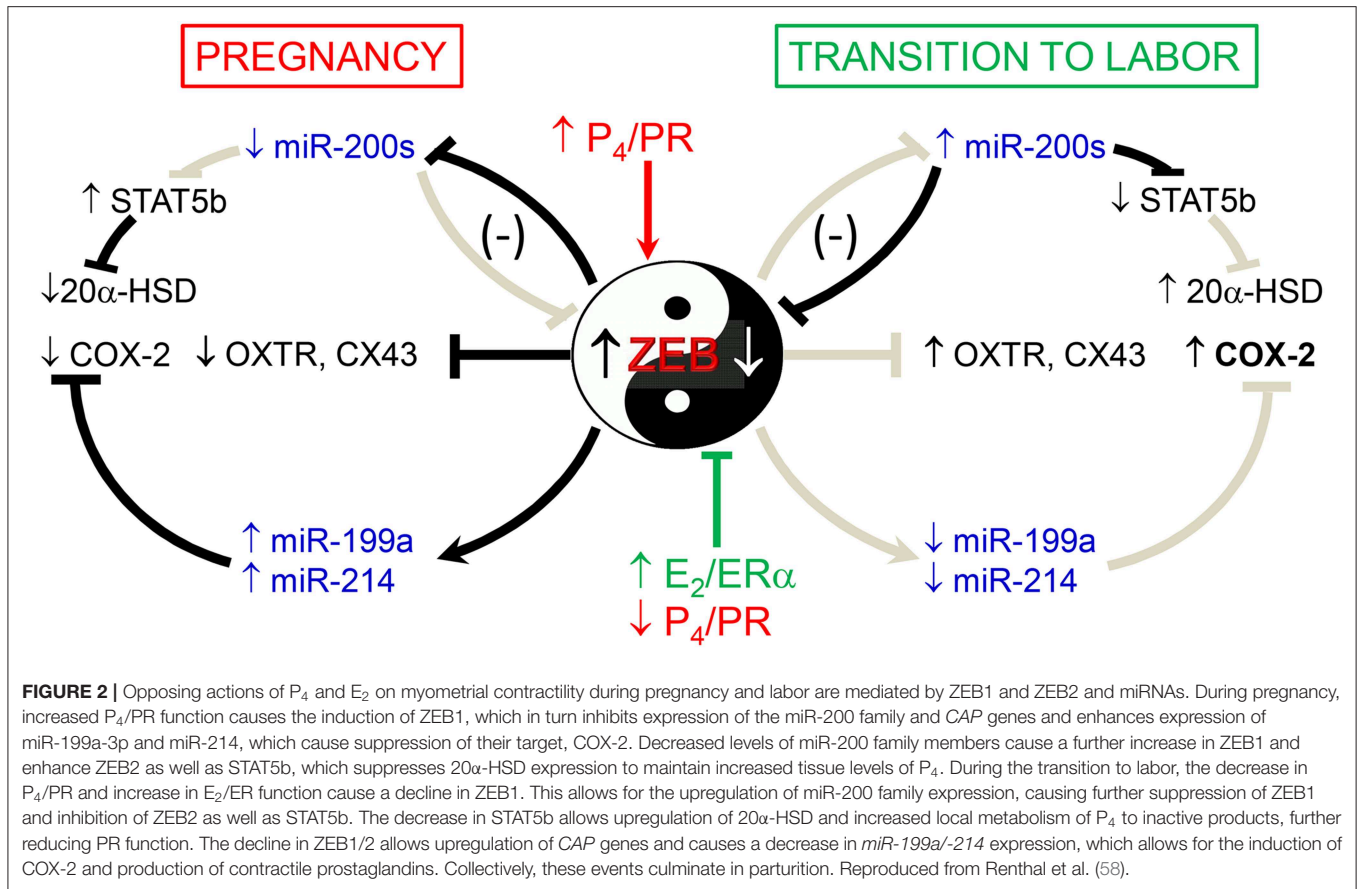
In sheep, increased cortisol production by the fetal adrenal has been implicated in the initiation of parturition via the activation of placental COX-2 and production of prostaglandins (59). The prostaglandins, in turn, stimulate 17 α -hydroxylase/17,20 lyase (CYP17) expression, resulting in increased placental production of C₁₉-steroids, which are metabolized to estrogens by placental aromatase P450 (CYP19). The increased estrogens may antagonize PR function (12) and upregulate *CAP* gene expression via the mechanisms described above. The surge of fetal cortisol also promotes fetal lung maturation and synthesis of surfactant components (60), which, as described below, also serve as fetal signals for the initiation of labor.

Corticotropin-Releasing Hormone (CRH)

In humans, the placenta lacks the capacity to express *CYP17* and produce C₁₉-steroids, which are instead synthesized in large quantities by the fetal adrenals (61). However, the human placenta is unique in its ability to secrete CRH (62), which is produced in increasing amounts near term and has been suggested to provide a fetal signal for the initiation of parturition (63, 64). Fetal placental CRH is proposed to upregulate secretion of adrenocorticotrophic hormone (ACTH) by the fetal pituitary, which stimulates production of cortisol and the C₁₉-steroid, dehydroepiandrosterone sulfate (DHEAS), by the fetal adrenals. DHEAS is subsequently metabolized within the placenta to estrogens, which, as mentioned, mediate inflammatory signaling leading to labor. CRH mRNA is also expressed at high levels in the bronchiolar epithelium of 13.5–17.5 dpc fetal mouse lung (65). It has been suggested that CRH may act directly within the fetal lung to promote synthesis of the major surfactant protein, surfactant protein (SP)-A. Accordingly, in *CRH* null mouse fetuses of *CRH*-deficient mothers, lung maturation and induction of *SP-A* expression were found to be delayed (66). Thus, CRH may act directly within the fetal lung to stimulate the production of surfactant components, and/or to increase fetal ACTH and adrenal cortisol production to enhance fetal lung development and surfactant synthesis. Accordingly, as described below, augmented surfactant production by the maturing fetal lung likely serves as an important fetal signal for the initiation of labor.

Surfactant Components Secreted by the Fetal Lung

Increased production of pulmonary surfactant components by the maturing fetal lung is proposed to signal the initiation of parturition (23, 67–70). Lung surfactant is a glycerophospholipid-rich surface-active lipoprotein produced specifically by type II cells of the pulmonary alveoli, which acts to reduce surface tension at the alveolar air-liquid interface after birth. Surfactant synthesis by the developing lung is initiated after ~85% of gestation is complete. Consequently, premature infants born prior to this time are at risk of developing respiratory distress syndrome due to surfactant deficiency. Dipalmitoylphosphatidylcholine (DPPC) is the major surfactant glycerophospholipid and most surface-active component. Approximately 10% of surfactant composition is comprised of the essentially lung-specific proteins SP-A, SP-B, SP-C, and



SP-D (71, 72). SP-B and SP-C are lipophilic peptides produced from larger precursors. SP-B serves an essential role, together with DPPC, in the reduction of alveolar surface tension (71, 73). SP-A and SP-D serve as C-type lectin components of the innate immune system (74) that enhance the uptake of a variety of microbes by M ϕ (74–77).

The role of surfactant components in the initiation of parturition was first suggested by the finding that surfactant isolated from human amniotic fluid stimulated prostaglandin synthesis in amnion discs (67). It was proposed that amniotic fluid surfactant phospholipids provide a source of arachidonic acid as a substrate for synthesis of contractile prostaglandins. Others suggested that a substance in human amniotic fluid secreted in urine from the fetal kidney enhanced PGE₂ production by human amnion cells (78); however, this amniotic fluid “substance” is likely derived from the fetal lung. Accordingly, Johnston and colleagues (79) suggested that platelet-activating factor (PAF), a potent proinflammatory phospholipid secreted into amniotic fluid with fetal lung surfactant near term, may enhance myometrial contractility leading to labor. Our laboratory has obtained extensive evidence for the roles of fetal lung SP-A and PAF as key fetus-derived inflammatory signals for the initiation of parturition (23, 69, 70).

SP-A and Toll-like receptor 2 (TLR2)

In all species studied, synthesis of SP-A by the fetal lung is developmentally upregulated with surfactant

glycerophospholipids after ~85% of gestation is complete (80). Consequently, SP-A serves as a relevant marker of fetal lung maturity and surfactant production. SP-A expression in mouse fetal lung and its secretion into amniotic fluid are upregulated at 17.5 dpc and continue to increase toward term (19.5 dpc) (23, 81, 82). This is temporally associated with increased proinflammatory cytokine production by amniotic fluid M ϕ , their migration to the maternal uterus and the activation of uterine NF- κ B (23). In studies using *Rosa 26 Lac-Z* mice, we observed that fetal M ϕ migrated to the maternal uterus with the induction in SP-A expression by the fetal lung during late gestation. Moreover, an intra-amniotic injection of SP-A caused preterm delivery of fetuses, and was associated with the activation of uterine NF- κ B within 4.5 h. Conversely, injection of an SP-A antibody or NF- κ B inhibitor into amniotic fluid delayed labor by >24 h (23). These findings suggested that enhanced SP-A secretion by the fetal lung near term causes activation and migration of fetal AF M ϕ to the maternal uterus, where increased cytokine production activates NF- κ B and a signaling cascade, leading to labor. It should be noted that studies using laser capture of limited numbers of CD68⁺ or CD14⁺ (M ϕ markers) cells from the superficial portion of the myometrium from women carrying a male fetus at term failed to identify fetal mononuclear cells (83). Moreover, incubation of human amnion disks with SP-A resulted in upregulation of anti-inflammatory cytokines and cytokine receptors (84). However, SP-A can have both pro- and anti-inflammatory actions depending upon

the cellular environment, as well as the receptor to which it binds (85).

To further study the roles of SP-A, the related C-type lectin, SP-D, and their putative receptor, TLR2 (86–89), in the initiation of parturition, we utilized gene-targeted mice. In first pregnancies, *SP-A*^{-/-} and *SP-A*^{-/-}/*SP-D*^{-/-} female mice bred to genetically like males delivered at term (19.5 dpc). However, in subsequent pregnancies, these gene-targeted mice manifested a ~12 h delay in parturition, associated with significantly reduced levels of myometrial Cx43, Oxt, IL-1 β , and IL-6 mRNA at 18.5 dpc compared to wild-type (WT) mice (69). We postulated that the parturition timing difference in the deficient mice in first vs. second pregnancies was due to the dominant role of uterine mechanical stretch as a signal for parturition (36, 37, 90) in first pregnancies. However, in subsequent pregnancies, prior adaptation of the uterus to stretch (91) may allow other signals (e.g., surfactant proteins) to play a more significant role. *TLR2*^{-/-} females manifested a significant delay (~12 h) in parturition timing during first pregnancies, as well as reduced expression of *CAP* genes and the M ϕ marker, F4/80, in myometrium at term compared to WT (69). F4/80⁺ AF M ϕ s from *TLR2*^{-/-} and *SP-A/D*^{-/-} mice expressed significantly lower levels of both pro-inflammatory and anti-inflammatory activation markers, compared to those of gestation-matched WT mice (69). These findings suggested that SP-A and SP-D act via TLR2 on fetal-derived M ϕ to modulate parturition timing; their impact may depend upon parity.

Roles of SRC-1 and SRC-2 in production of fetal signals leading to labor

Previously, we observed that the p160 family members (92), steroid receptor coactivators, SRC-1, and SRC-2/TIF-2, are critical for transcriptional upregulation of *SP-A* gene expression in fetal lung type II cells (93–95). SRCs do not bind to DNA directly; however they regulate gene transcription by interacting with steroid receptors and other transcription factors and by recruiting other coregulators with histone-modifying activities (92, 96) to alter chromatin structure (97). Notably, gene-targeted mice that are singly-deficient in *Src-1*, *Src-2*, or *Src-3* manifest various reproductive phenotypes (96). Importantly, mice that were double-knockout (dKO) for *Src-1* and *Src-2* died at birth from respiratory distress (98), which is indicative of lung surfactant deficiency. This observation was of great interest to us, considering the critical roles of SRC-1 (94) and SRC-2 (93) in *SP-A* expression.

To characterize these mice further, we crossed *Src-1*^{+/-}/*Src-2*^{+/-} (*Src-1/2 dhet*) males and females. Remarkably, they manifested severely delayed parturition (~38 h). This parturition delay occurred with significant reductions in NF- κ B activation, as well as decreased expression of *Oxt*, *Cx43*, *PGF₂ α synthase/Akr1b3* and levels of the contractile prostaglandin, *PGF₂ α* , in the maternal myometrium. The decrease in myometrial *PGF₂ α* was associated with impaired luteolysis and elevated circulating P₄ (70). Notably, parturition timing was normal in *Src-1-KO* females and in *Src-2*^{+/-} females bred to genetically like males, revealing that *Src-1/2* double-deficiency is requisite for the delay in parturition. Importantly,

WT females bred to *Src-1/2* double-deficient males exhibited a parturition delay equivalent to that observed in our crosses of *Src-1/2* double-deficient males and females, with decreased myometrial NF- κ B activation and *CAP* gene expression and elevated circulating P₄. These findings indicated that the defect responsible for delayed parturition with *Src-1/2* double deficiency was fetal in origin. Because of the importance of fetal lung SP-A production in the timing of parturition, it was of great interest that SP-A levels were significantly reduced in the lungs and amniotic fluid of fetuses doubly deficient in *Src-1* and *Src-2* when compared to WT. On the other hand, levels of SP-A in lungs and amniotic fluid of *Src-1* or *Src-2* singly deficient fetuses were similar to WT.

Clearly, the increase in gestation length in mice carrying *Src-1/2* doubly deficient fetuses (~38 h) was significantly greater than what we observed in *SP-A*-deficient and in *SP-A/SP-D* double-deficient mice (~12 h). This suggested that signaling molecules, other than SP-A and SP-D, were affected by double-deficiency of *Src-1* and *Src-2*. As noted, *Src-1/2* dKO mice succumbed at birth to alveolar collapse/atelectasis (98), and this suggested that surfactant glycerophospholipids may also be altered. In this regard, we found that amniotic fluid levels of the major and most surface-active surfactant component, DPPC, were significantly reduced in *Src-1/2* dKO fetuses compared to WT (70).

We also considered the role of the glycerophospholipid, PAF, which is proinflammatory, produced by the developing fetal lung together with surfactant lipids and SP-A and secreted into amniotic fluid near term. PAF, which activates leukocytes and stimulates their migration, was suggested to contribute to the initiation of term and preterm labor (68, 79, 99–101). PAF also directly stimulated the contraction of myometrial strips (102–106). Intriguingly, we observed that PAF levels in fetal lungs and amniotic fluid of *SRC-1/2* double-deficient mice failed to increase toward term and were significantly reduced, compared to WT fetuses or those singly deficient in *Src-1* or *Src-2*.

Since both DPPC and PAF levels were significantly decreased in the amniotic fluid of *Src-1/2* double-deficient fetuses, we searched for glycerophospholipid metabolizing enzymes that might coordinately regulate the synthesis of both of these molecules. In so doing, we discovered that lysophosphatidylcholine acyltransferase 1 (*Lpcat1*), which serves a key role in the deacylation/reacylation of the *sn-2* position of both DPPC and PAF to make the surface-active and pro-inflammatory molecules, respectively (107–109), was significantly decreased in lungs of *Src-1/2* double-deficient fetuses compared to WT (70). *Lpcat1* was previously found to be expressed specifically in mouse lung type II cells, developmentally-induced in fetal lung toward term, and stimulated by glucocorticosteroids (108). The role of *Lpcat1* in surfactant synthesis by the developing lung was supported by the finding that mice carrying a hypomorphic allele of *Lpcat1* manifested atelectasis at birth and a deficiency in surfactant DPPC (107). Notably, the gestational increase of *Lpcat1* was blocked in lungs of *Src-1/2* double-deficient fetuses. Further, PAF or SP-A injection into the AF at 17.5 dpc rescued the parturition delay, enhanced uterine NF- κ B activation and *CAP* gene

expression and promoted luteolysis in *Src-1/2*-deficient mice (70). These collective findings further demonstrate the role of the fetal lung in producing signals for the initiation of labor when surfactant production is increased, and that SRC-1/2 coactivators serve crucial roles through enhanced production of SP-A and PAF (Figure 3).

P₄/PR MAINTAINS MYOMETRIAL QUIESCENCE VIA CONCERTED MECHANISMS

As described below, P₄/PR maintains myometrial quiescence through a number of cooperative mechanisms. These include: (1) inhibiting transcriptional activity of the pro-inflammatory transcription factors, nuclear factor κ B (NF- κ B) (110) and activating protein 1 (AP-1) (111), via direct interaction and recruitment of corepressors (10, 111, 112); (2) inducing inhibitors of proinflammatory transcription factor activation ($\text{I}\kappa\text{B}\alpha$, MKP-1) (110, 113–115); (3) upregulating expression of transcriptional repressors of *CAP* genes (e.g., ZEB1) (56, 116) (Figure 4).

P₄/PR Has Anti-inflammatory Actions in the Myometrium

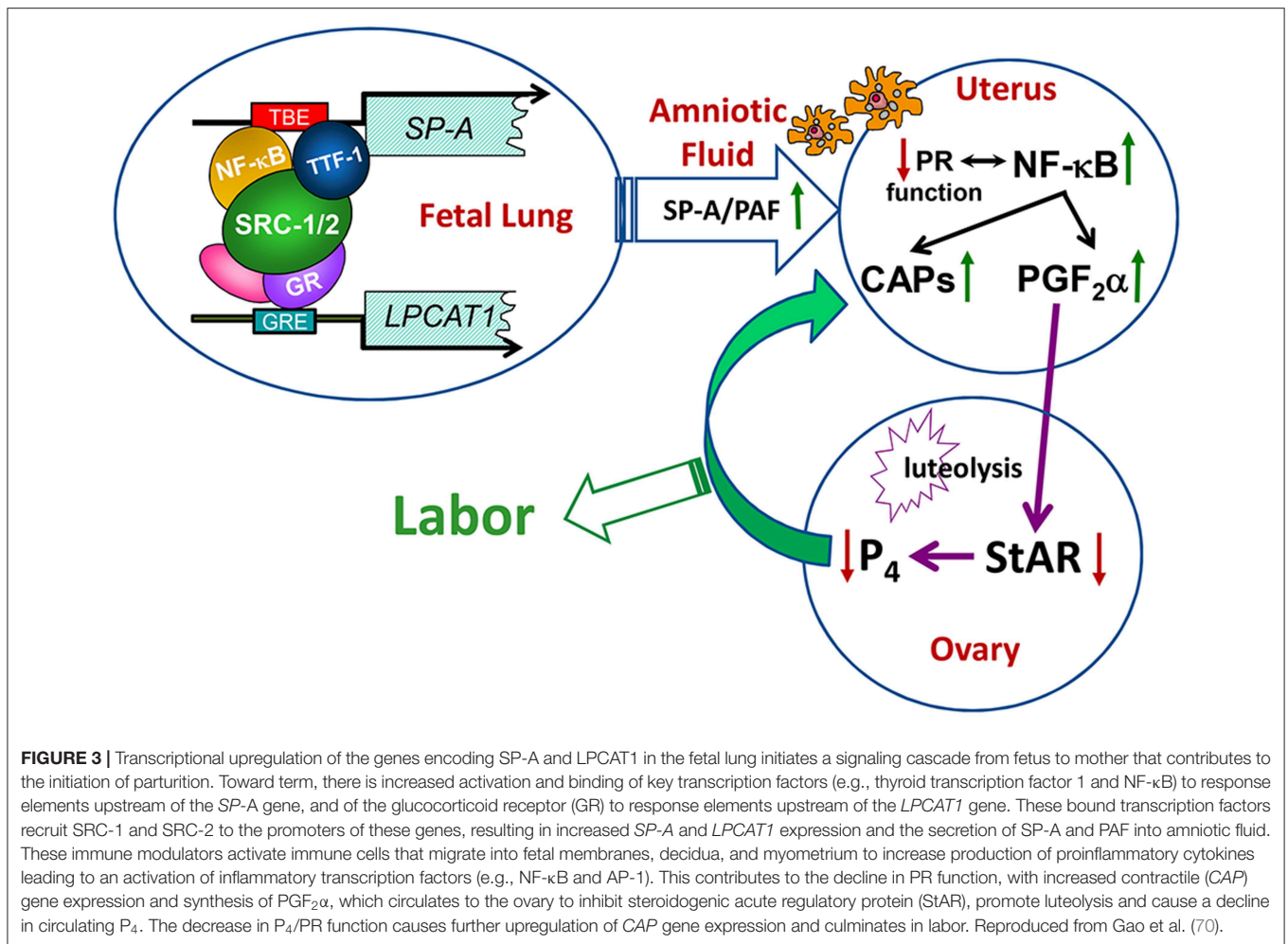
We (110, 114, 117) and others (46, 118–120) have obtained compelling evidence that P₄/PR maintains myometrial quiescence through its action to block inflammation (Figure 4). Using hTERT-HM cells, we observed that P₄/PR serves an anti-inflammatory role by antagonizing the activation of NF- κ B and preventing the induction of COX-2 (117, 121), proinflammatory cytokines (10) and *CAP* genes (56). Using chromatin immunoprecipitation (ChIP), we found that P₄ treatment of the hTERT-HM cells prevented interleukin-1 (IL-1)-induced binding of endogenous NF- κ B p65 to NF- κ B response elements in the COX-2 and *IL-8* promoters (10, 110). The P₄-mediated inhibition of proinflammatory and *CAP* gene transcription may be caused, in part, by the direct binding of PR to p65 (122) to inhibit NF- κ B DNA-binding and transcriptional activity.

P₄/PR can also block NF- κ B activation and inflammation by increasing the expression of $\text{I}\kappa\text{B}\alpha$, which sequesters NF- κ B in the cytoplasm and prevents its activation (110) (Figure 4). P₄ treatment of hTERT-HM cells rapidly induced expression of $\text{I}\kappa\text{B}\alpha$, which preceded the effect of P₄ to inhibit IL-1 β -induced COX-2 expression (110). Moreover, P₄ blocked the IL-1 β -mediated decrease in $\text{I}\kappa\text{B}\alpha$ protein, which suggested P₄/PR inhibition of the proteasome pathway (123). Consequently, more NF- κ B remains sequestered in the cytoplasm in an inactive state. Progesterone inhibition of NF- κ B activation by upregulation of $\text{I}\kappa\text{B}\alpha$ has also been reported in M ϕ (124) and breast cancer (113, 125) cell lines. The anti-inflammatory actions of P₄/PR were further mediated by the upregulation of the mitogen-activated protein kinase (MAPK) inhibitor, MAPK phosphatase-1/dual specificity phosphatase 1 (MKP-1/DUSP1) (114, 115). Inhibition of MAPK, in turn, inhibits activation of NF- κ B and AP-1 (126–128).

PR Inhibits Proinflammatory and CAP Gene Expression by Recruitment of Corepressors

To define the mechanisms whereby P₄ inhibits proinflammatory and *CAP* gene expression in the myometrium, we analyzed the capacity of PR_{WT} vs. various PR mutants to mediate P₄ inhibition of proinflammatory gene expression in hTERT-HM human myometrial cells. These included a sumoylation mutant, a hinge domain mutation to prevent PR dimerization, and mutations of three amino acids in the DNA-binding domain (DBD). We observed that mutation of the PR-DBD had the most profound effect to prevent P₄-inhibition of proinflammatory genes (10). Consequently, P₄-mediated transrepression was significantly reduced in cells stably expressing a PR-A or PR-B DNA-binding domain mutant (PR_{mDBD}), compared to cells expressing PR_{WT}. ChIP analysis of the hTERT-HM cells revealed that P₄/PR_{WT} transrepressive activity was associated with P₄-induction of PR recruitment and inhibition of NF- κ B p65 and RNA Pol II recruitment to an NF- κ B response element in the COX-2 and *IL-8* promoters. Importantly, in response to P₄ treatment, equivalent recruitment of PR_{WT} and PR_{mDBD} to COX-2 and *IL-8* promoters was observed. This suggested that the inhibitory effects of P₄/PR on COX-2 and *IL-8* expression were not mediated by direct DNA binding, but most likely by tethering to NF- κ B. This led us to postulate that nuclear proteins interacting with the PR-DBD may mediate transrepression by P₄/PR. Using immunoprecipitation, followed by mass spectrometry, we identified proteins that interacted strongly with PR_{WT} and weakly with the PR_{DBD} mutants. Among these was the transcriptional repressor, GATA Zinc Finger Domain Containing 2B (GATAD2B), which interacted with the PR-DBD and was required for P₄/PR suppression of proinflammatory and *CAP* gene expression (10). Accordingly, P₄ treatment of PR_{WT} hTERT-HM cells increased recruitment of endogenous GATAD2B to COX-2 and *IL-8* promoters, whereas, siRNA knockdown of endogenous GATAD2B significantly reduced P₄/PR_{WT} transrepression of COX-2 and *IL-8*. Notably, GATAD2B expression decreased significantly in pregnant mouse and human myometrium during labor (10). Together, our findings suggest that GATAD2B serves as a novel mediator of P₄/PR suppression of proinflammatory and *CAP* genes during pregnancy. Thus, the decline in GATAD2B expression near term may contribute to the loss of PR function leading to labor.

The induction of *CX43* expression by proinflammatory stimuli in the pregnant myometrium is mediated, in part, by increased transcriptional activity of members of the AP-1 family, which comprises Fos/Jun heterodimers or Jun/Jun homodimers. Fos/Jun heterodimers were found to be strong inducers of *CX43* expression compared to Jun/Jun homodimers, which were relatively weak (129). P₄ acting through PR-B was found to repress *CX43* expression by recruiting inactive Jun/Jun homodimers and the P54^{trb}/Sin3A/HDAC corepressor complex to the *CX43* promoter (130). Near term, it was suggested that the increased metabolism of P₄ by 20 α -hydroxysteroid dehydrogenase (20 α -HSD) in myometrium (131) and an inflammation-induced increase in the PR-A/PR-B ratio (8, 132,



133) caused PR-A to become free of ligands. The unliganded PR is then proposed to recruit relatively active Fra2/JunD heterodimers (129, 130), resulting in the activation of *CX43* expression. This switch may allow transformation of PR-A to an activator of *CX43* expression.

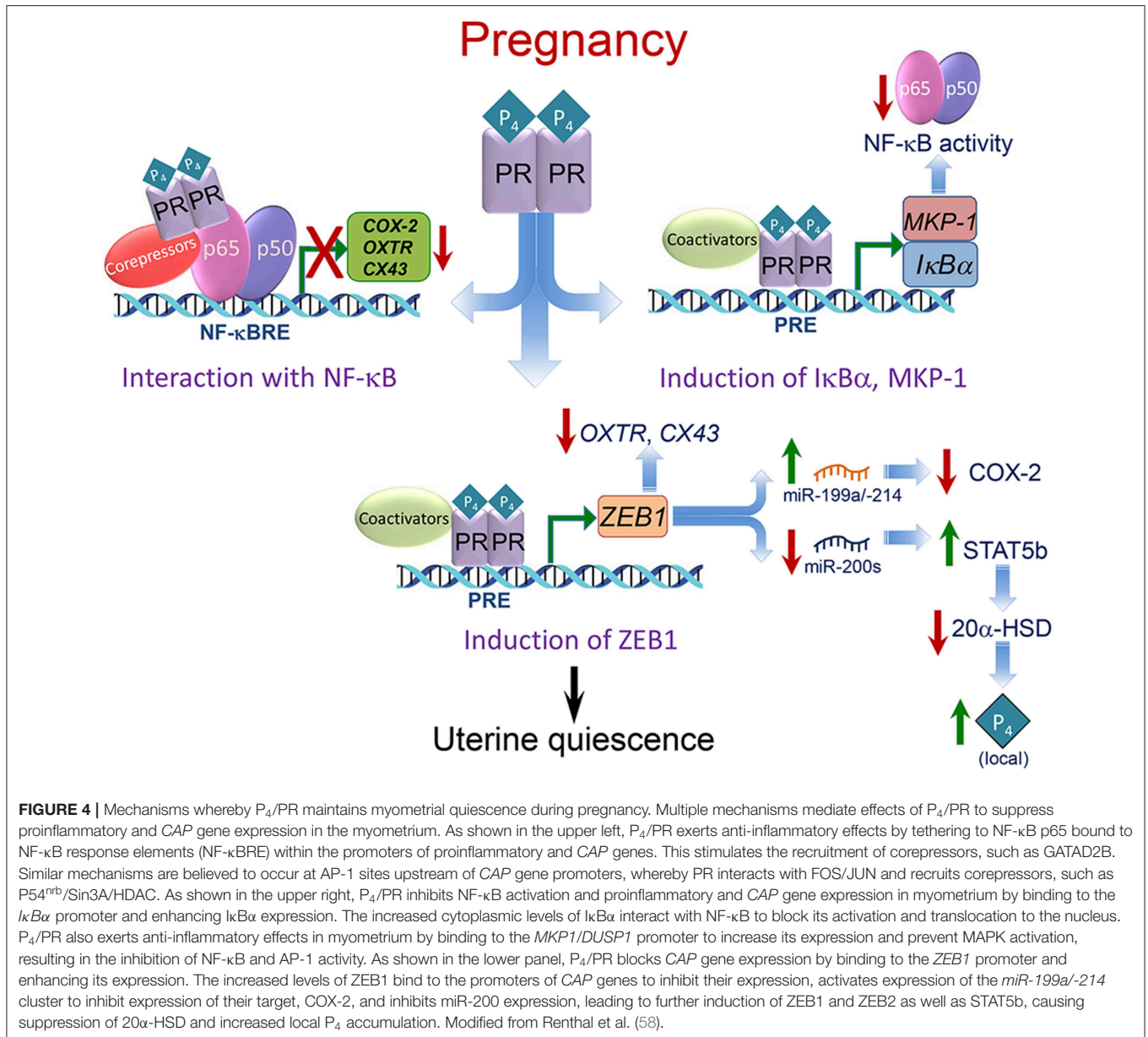
P_4/PR Maintains Myometrial Quiescence via Induction of ZEB1/2 and STAT5b

ZEB1 and ZEB2

Our findings reveal that P_4/PR maintains myometrial quiescence, in part, by the induction of the zinc finger E-box-binding transcriptional repressor, ZEB1/TCF8/ δEF1 (Figure 4), which binds to promoters of the *CAP* genes, *OXTR*, and *CX43* and the gene encoding members of the miR-200 family and represses their expression. Downregulation of miR-200s promotes further upregulation of ZEB1 and the related transcription factor, ZEB2/SIP2 (56) (Figure 2). Zeb1 was found to be expressed highly in mouse myometrium (116) and to be upregulated by P_4/PR (134). We observed that the expression of Zeb1 and Zeb2 was elevated in myometrial tissues of 15.5 dpc pregnant mice and decreased precipitously toward term with the decline in circulating P_4 and in PR function (56). Conversely, treatment

of pregnant mice with RU486 or with the bacterial endotoxin, lipopolysaccharide (LPS), to induce preterm labor (56) caused significant inhibition of Zeb1/2 expression. ZEB1 and ZEB2 levels also were decreased in term myometrial tissues from women in labor, vs. tissues from women not in labor at term (56). ChIP-qPCR analysis of pregnant mouse myometrium revealed that endogenous Zeb1 bound to E-box-containing regions of the mouse *Cx43* and *Oxtr* promoters at relatively high levels at 15.5 dpc and declined markedly at term (56). Importantly, ZEB1 overexpression in human myometrial hTERT-HM cells caused a pronounced inhibition of *OXTR* and *CX43* mRNA levels and blocked oxytocin-induced contraction of these cells using an *in vitro* contraction assay (56). Thus, the decrease in Zeb1 expression and binding to *CAP* gene promoters toward term likely promoted upregulation of *OXTR* and *CX43* leading to parturition.

To further assess the effects of P_4 on Zeb expression, timed-pregnant mice were injected with P_4 or vehicle daily between 15.5 and 18.5 dpc. P_4 treatment, which delayed labor, caused a significant upregulation of Zeb1 and inhibited myometrial *CX43* and *OXTR* gene expression, compared to vehicle injected mice. A similar inductive effect of P_4 on ZEB1 expression was observed in cultured T47D breast cancer cells. The stimulatory effect of P_4 on



ZEB1 expression was mediated at the level of gene transcription by the direct binding of PR to response element(s) within the ZEB1 promoter (56). Notably, ZEB2 is not directly regulated by P₄/PR (56).

ZEB1 and ZEB2 are directly targeted by all five members of the highly conserved miR-200 family (135–138), which significantly increase in mouse and human myometrium toward term and in labor, in association with the decline in ZEB1 and ZEB2 expression (56). ZEB1 and ZEB2 also negatively regulate miR-200 expression. Accordingly, ZEB1/2 and miR-200s exist in a hormonally-regulated negative feedback loop (135–138). Thus, during pregnancy, elevated P₄/PR increases the expression of ZEB1, which suppresses the miR-200 family,

as well as contraction-associated genes. The decrease in miR-200 expression further upregulates ZEB1 and increases the expression of ZEB2. Near term, the decline in circulating P₄ and/or PR function causes the downregulation of ZEB1 expression, and consequent upregulation of the miR-200 family, further suppressing ZEB1 and ZEB2. This de-represses CAP gene expression, resulting in increased uterine contractility and labor (Figure 2).

In parallel with our discovery of the gestational upregulation of the miR-200 family, we found that the conserved miR-199a/-214 cluster was significantly downregulated in mouse myometrium near term (56, 57), and in myometrial biopsies from women in-labor, compared to those not-in-labor at

term (57). The *miR-199a/-214* cluster, comprised of miR-199a-5p, miR-199a-3p, and miR-214, are encoded within a 6-kb anti-sense transcript of the *Dynammin3* gene (*Dnm3os*), which is highly expressed in pregnant uterus (139). We observed that E₂ treatment of ovariectomized mice suppressed, whereas P₄ enhanced, uterine miR-199a-3p/-214 expression (57). Interestingly, these opposing hormonal effects were mediated by ZEB1, which, as described above, is induced by P₄ (56, 116) and inhibited by E₂ (57). ZEB1 binds directly to the *miR199a/-214* promoter to activate its transcription (57) (**Figure 2**). Importantly, miR-199a-3p and miR-214 both target the mRNA for COX-2 (57, 140). Thus, miR-199a-3p and miR-214 maintain uterine quiescence by suppressing the synthesis of contractile prostaglandins. These collective findings revealed the intriguing central role of ZEB1 as an inhibitor of the *miR-200* family and an inducer of the *miR-199a/-214* cluster, which are oppositely regulated by P₄ and E₂ (**Figure 2**).

Signal Transducer and Activator of Transcription (STAT)5b

Increased local metabolism of P₄ to inactive products near term in the uterus and cervix has been suggested to contribute to the decline in PR function that is crucial for the initiation of parturition in all mammals (19, 141–144). The finding that mice with a deletion of the gene encoding the P₄-metabolizing enzyme, 20 α -hydroxysteroid dehydrogenase (20 α -HSD), manifested a significant delay in the timing of parturition, indicates its important role (20). In myometrium of pregnant women at term, a pronounced decrease in the ratio of P₄ to 20 α -dihydroprogesterone (20 α -OHP), an inactive metabolite of P₄ generated by 20 α -HSD, were observed (144). In the course of our studies, we discovered that miR-200s directly target the P₄-induced transcription factor STAT5b (131), which is a negative regulator of 20 α -HSD in reproductive tissues (20, 145). Thus, the upregulation of miR-200 expression in mouse myometrium near term and in human myometrium during labor (56) was associated with suppression of STAT5b and induction of 20 α -HSD (131). In contrast, throughout most of pregnancy, increased P₄ levels upregulate ZEB1 and inhibit miR-200 expression in the myometrium (56). This, in turn, allows upregulation of STAT5b, which inhibits 20 α -HSD expression (131) to maintain elevated endogenous levels of P₄ and myometrial quiescence (**Figure 2**).

P₄/PR Induction of Caspases in the Pregnant Myometrium During Mid-Gestation Maintains Quiescence

During early to mid-pregnancy in rats there is a high rate of myometrial cell proliferation and hyperplasia, exemplified by increased BrdU incorporation and PCNA staining in the longitudinal smooth muscle layer (146). This declines precipitously by 17 dpc (term = 23 dpc) and is accompanied by an increase in cellular hypertrophy. From 12 to 15 dpc, there was a remarkable induction of the stress-induced caspase cascade (cleaved caspases 9, 3, 6, and 7) (146). However, this was not accompanied by evidence for apoptosis. Rather, it was suggested that caspase activation may cause inhibition of

myometrial proliferative activity and promote the transition to hypertrophy and smooth muscle cell differentiation (146). Similar inductive changes in activation of the caspase cascade were observed in pregnant mouse myometrium from 12 to 15 dpc (term = 19 dpc) (147). Importantly, the activation of caspase 3 was found to be stimulated by P₄ treatment. Moreover, caspase 3 activation was accompanied by the cleavage of myocyte contractile proteins, smooth muscle α - and γ -actins (147) as well as downregulation of the gap junction protein Cx43 (148). Thus, an important mechanism whereby P₄/PR maintains myometrial quiescence during pregnancy is via its action to induce the active caspase cascade and cause degradation of proteins involved in myometrial contractility. The decline in PR function in the pregnant myometrium toward term results in decreased caspase activation and allows for the increase in contractile protein accumulation. Notably, caspase activation in uterine myocytes was also associated with induction of the endoplasmic reticulum stress response (ERSR), which is likely enhanced by physiological/mechanical stimuli (149). The ERSR was reduced near term by upregulation of the adaptive unfolded protein response (UPR), resulting in a decline in active caspase 3 and for the induction of contractile proteins (149).

MECHANISMS FOR THE DECLINE IN PR FUNCTION LEADING TO PARTURITION

The decline in PR function leading to parturition, which is fundamental and critical for species survival, is mediated by multiple complementary mechanisms, several of which are discussed below. It is likely that all of these processes are regulated by an increased inflammatory response within the myometrium at term, resulting in activation of NF- κ B and AP-1 transcription factors. In various cell types, activation of NF- κ B represses PR transcriptional activity, while PR activation also represses NF- κ B-mediated transcription (28, 122).

Altered PR Isoform Expression and Posttranslational Modification in the Myometrium Toward Term

As mentioned, PR-A, which is truncated at its N-terminus, contains only two of the three transcriptional activation domains that are present in PR-B. Thus, in certain cell- and gene-specific contexts, including human myometrial cells, PR-A has been found to repress PR-B transcriptional activity (5–7). PR-A and PR-B are differentially regulated in the human myometrium during pregnancy (8); the ratio of PR-A to PR-B mRNA (9) and protein (7) was observed to be significantly higher in term myometrium from women in labor compared to those not in labor. Moreover, in hTERT-HM myometrial cells stably expressing PR-A or PR-B, it was observed that P₄ had decreased anti-inflammatory activity in PR-A-expressing cells when compared to those expressing PR-B (10). Furthermore, proinflammatory stimuli specifically increased phosphorylation of the PR-A isoform on Ser-344/345 in a P₄-dependent manner and enhanced its ability to antagonize the anti-inflammatory activity of PR-B (133). Notably, in term myometrium from

women in-labor vs. not-in-labor, phosphorylation of Ser-345 occurred exclusively on PR-A, and the abundance of phospho-Ser-345-PR-A relative to total PR-A was increased significantly in laboring vs. non-laboring myometrium in association with increased NF- κ B activation (133).

A third PR isoform, PR-C (~60 kDa), which is truncated from the N-terminus and lacks part of the DNA binding domain, is primarily cytoplasmic in its localization (150, 151). Since PR-C can bind P_4 (152), it may inhibit PR function by sequestering P_4 and/or by physically interacting with PR-B to reduce its DNA-binding capacity (150). In fundal myometrium from women before and after the initiation of labor at term, we observed a labor-associated increase in PR-C mRNA and a ~60 kDa immunoreactive PR protein. This was temporally and spatially associated with activation of NF- κ B (27). A temporal increase in a truncated 60 kDa PR isoform was also observed in mouse uterus near term. Notably, the identity of truncated PR isoforms in the pregnant uterus (8, 153) requires further study.

Decreased Expression of Selected Coregulators in the Myometrium Toward Term

P_4 /PR activation of target gene expression is dependent upon recruitment of coactivator complexes, which contain histone acetyltransferases and cause an opening of chromatin structure (154, 155). Previously, we observed that expression of selected steroid receptor coactivators (SRC) and the histone acetyltransferase, CREB-binding protein (CBP), declined in myometrium of women in labor, compared to non-laboring myometrium, and were profoundly decreased in myometrial tissues of pregnant mice at term (156). This was associated with decreased levels of acetylated histone H3. Remarkably, treatment of pregnant mice with the histone deacetylase (HDAC) inhibitor, trichostatin A (TSA), during late gestation increased myometrial histone acetylation and delayed parturition by 24–48 h (156). Subsequently, it was reported that HDAC inhibitors suppressed proinflammatory gene expression in cultured human myometrial cells (157) and strongly inhibited contractility of human myometrial strips (158). Collectively, these findings suggest that decreased expression of PR coactivators and in histone acetylation in the myometrium during late gestation may impair the capacity of P_4 /PR to upregulate genes that maintain myometrial quiescence and increase sensitivity of the uterus to prostaglandins and other contractile factors. In cultured human myometrial cells, the effect of TNF α to inhibit SRC-1 and SRC-2 expression mediated TNF α inhibition of PR-B transcriptional activity (159). Thus, the decline in coactivators in the myometrium near term may be caused by induction of proinflammatory mediators. Moreover, corepressors, GATAD2B (10) and p54nrb (non-POU-domain-containing, octamer binding protein) (111), which interact with PR and mediate its capacity to repress proinflammatory and *CAP* gene expression, were found to decrease in rodent myometrium at term, further contributing to the decline in PR function.

Increased Metabolism of P_4 Toward Term Contributes to the Decline in PR Function Leading to Parturition

As mentioned, in women, circulating P_4 levels remain elevated throughout pregnancy and labor due to the maintenance of placental P_4 production (160). Furthermore, levels of PR remain elevated in reproductive tissues during pregnancy and into labor (12). Even in rodents, where P_4 production by the corpus luteum declines precipitously near term, the levels of P_4 in circulation remain higher than the K_d for binding to PR (17). As mentioned, in the myometrium of pregnant women at term, there is a pronounced decrease in the ratio of P_4 to 20α -dihydroprogesterone (144), an inactive P_4 metabolite generated by the enzyme 20α -HSD. In mice, the initiation of parturition is accompanied by increased expression of the P_4 -metabolizing enzymes, 20α -HSD in the uterus (131) and 5α -reductase type I in the cervix (18, 19). Accordingly, gene-targeted mice lacking 5α -reductase type I fail to deliver because of impaired cervical ripening, even though maternal circulating P_4 levels decline normally (18, 19). Similarly, 20α -HSD knockout mice manifested severely delayed parturition (20, 161). Thus, increased local metabolism of P_4 in the uterus and cervix near term contributes to the decline in PR function and is crucial for the initiation of parturition (19, 141–144).

CONCLUSIONS

Throughout pregnancy, the critical role of P_4 /PR in maintaining myometrial quiescence is principally mediated by its capacity to inhibit inflammatory pathways and to suppress *CAP* gene expression. As depicted in **Figure 4**, this occurs via several cooperative mechanisms that include: tethering of PR to the inflammatory transcription factors, NF- κ B or AP-1, with recruitment of corepressors (10, 110–112, 130); PR promoter binding and transcriptional activation of genes encoding the NF- κ B suppressor, I κ B α (110), and the MAPK inhibitor, MKP1 (114). Increased P_4 /PR also upregulates expression of transcription factor ZEB1, which interacts directly with the promoters of the *OXT*R and *CX43* genes to inhibit their expression and suppresses expression of the miR-200 family (56). Decreased miR-200 expression allows upregulation of its target, STAT5b, a transcriptional inhibitor of 20α -HSD, so that P_4 metabolism in the myometrium is prevented (131). The increased ZEB1 also upregulates the expression of members of the miR-199a/-214 cluster, which directly target COX-2, resulting in the suppression of contractile prostaglandin synthesis (57) (**Figures 2, 4**). P_4 also contributes to myometrial quiescence by activation of the caspase cascade, which maintains low levels of myocyte contractile proteins through increased caspase-mediated degradation (147).

The transition of the pregnant myometrium to an inflammatory, contractile state at term is affected by cooperative signals from the mother and fetus (**Figure 1**). Our findings suggest that appropriate timing for parturition is mediated, in part, by the induction of surfactant signals, SP-A and PAF, produced by the fetal lung and secreted into amniotic fluid where they interact with fetal M ϕ to alter their phenotypic

state (23, 69, 70). These activated immune cells then migrate to the maternal uterus (23) where they promote an inflammatory response with activation of NF- κ B and AP-1, leading to a decline in PR function and increased expression of proinflammatory and CAP genes in the myometrium (Figures 1, 3). The decline in PR function in human myometrium is thought to be mediated by: an inflammation-induced increase in PR-A, relative to PR-B isoform expression (133), with possible upregulation of other truncated PR isoforms (27); a decreased expression of ZEB1 and coordinate induction of miR-200 family expression (56), resulting in suppression of STAT5b, upregulation of 20 α -HSD and with increased local metabolism of P₄ (131); the direct interaction of NF- κ B p65 with PR (122); a decline in PR coactivators (156). The decline in ZEB1 also contributes to upregulation of OXTR and CX43 (56) and a decrease in miR-199a/-214 expression with the induction of their target, COX-2 (57), and of PGF₂ α synthesis (Figure 1). Toward term, the increase in E₂ production and ER α activation in the myometrium results in increased proinflammatory and CAP gene expression, which is mediated, in part, by inhibition of ZEB1/2 (57) and by the decreased expression of miR-181b, allowing for the upregulation of its targets, ER α , TNF α and c-FOS (53). These highly coordinated molecular events, together with increased myometrial stretch and a decline in PR corepressor expression culminate in the increased myometrial contractility leading to parturition.

Our extensive knowledge of the mechanisms that underlie myometrial quiescence during pregnancy and its transition to a contractile state prior to parturition has led to the identification of a number of conserved potential therapeutic targets for the prevention of preterm labor and its consequences. Several of these targets include miRNA clusters and families that

are coordinately upregulated or downregulated toward term and target a number of signaling molecules and pathways. Of considerable importance is the miR-200 family, which is markedly upregulated in pregnant human and mouse myometrium toward term. miR-200 family members target and downregulate expression of ZEB1 and ZEB2, leading to increased contractile gene expression and the suppression of STAT5b, which results in increased local metabolism of P₄ by 20 α -HSD (131). The decline in ZEB1/2 also causes decreased miR-199a and miR-214 expression, which both independently target the contractile gene, PTGS2/COX2, resulting in an increase in prostaglandin synthesis (57). The induction of miR-200s also directly targets PR (162), which may contribute to the loss of its function. Thus, anti-miR-200 therapy could form the basis for a comprehensive, multifactorial and highly effective therapeutic strategy for prevention of preterm birth.

AUTHOR CONTRIBUTIONS

CM wrote the manuscript. LG and AM critiqued and edited the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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