miR-200 family and targets, ZEB1 and ZEB2, modulate uterine quiescence and contractility during pregnancy and labor

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Throughout most of pregnancy, uterine quiescence is maintained by increased progesterone receptor (PR) transcriptional activity, whereas spontaneous labor is initiated/facilitated by a concerted series of biochemical events that activate inflammatory pathways and have a negative impact on PR function. In this study, we uncovered a previously undescribed regulatory pathway whereby micro-RNAs (miRNAs) serve as hormonally modulated and conserved mediators of contraction-associated genes in the pregnant uterus in the mouse and human. Using miRNA and gene expression microarray analyses of uterine tissues, we identified a conserved family of miRNAs, the miR-200 family, that is highly induced at term in both mice and humans as well as two coordinately down-regulated targets, zinc finger E-box binding homeobox proteins ZEB1 and ZEB2, which act as transcriptional repressors. We also observed up-regulation of the miR-200 family and down-regulation of ZEB1 and ZEB2 in two different mouse models of preterm labor. We further demonstrated that ZEB1 is directly up-regulated by the action of progesterone (P₄)/PR at the ZEB1 promoter. Excitingly, we observed that ZEB1 and ZEB2 inhibit expression of the contraction-associated genes, oxytocin receptor and connexin-43, and block oxytocin-induced contractility in human myometrial cells. Together, these findings implicate the miR-200 family and their targets, ZEB1 and ZEB2, as unique P₄/PR-mediated regulators of uterine quiescence and contractility during pregnancy and labor and shed light on the molecular mechanisms involved in preterm birth.

myometrium | parturition | lipopolysaccharide | RU486

Although premature labor is the leading cause of neonatal morbidity and mortality in developed countries, the signaling mechanisms that maintain uterine quiescence during pregnancy and promote increased uterine contractility leading to labor at term and preterm remain incompletely defined (1). In mammalian pregnancy, uterine quiescence is maintained by elevated circulating progesterone (P₄) acting via the progesterone receptor (PR). Conversely, parturition is associated with a decline in maternal circulating P₄ and/or a decrease in the function of the PR, termed “functional P₄ withdrawal,” (2, 3) and an increased inflammatory response within the uterus and cervix (4). Studies from a number of laboratories, including our own, suggest that P₄ and PR maintain uterine quiescence until term by inhibiting expression of contraction-associated genes [e.g., connexin-43 (CXN-43), oxytocin receptor (OXTR), cyclooxygenase 2 (COX-2)] in the myometrium, in part, via anti-inflammatory actions. For example, P₄ and PR inhibit activation of COX-2 expression in myometrial cells through direct interaction of PR with NF-κB p65 (5) and by P₄-induced expression of the NF-κB inhibitor, IκB-α (6).

Recently, it has been shown that micro-RNAs (miRNAs) play especially powerful roles in vascular smooth muscle cells and in female reproduction, wherein they have been implicated in proliferation, differentiation, and hormone responsiveness (7–9). The identification of miRNAs as hormonally regulated modulators of gene expression prompted us to investigate their roles in

P₄ and PR regulation of contraction-associated genes during pregnancy and labor.

In the present study, we show that members of the miR-200 family in both the mouse and human uterus are significantly induced during late gestation, repress the zinc finger E-box binding homeobox proteins ZEB1 and ZEB2, and mediate myometrial contractility. Through overexpression experiments, we show that miR-200s repress endogenous ZEB1 and ZEB2 expression in human myometrial cells. By overexpressing ZEB1 and ZEB2 in these cells, we establish that these transcription factors markedly suppress expression of the contraction-associated genes OXTR and CXN-43. Together, our findings implicate the miR-200 family and their targets, ZEB1 and ZEB2, as unique P₄- and PR-regulated modulators of uterine quiescence and contractility during pregnancy and in term and preterm labor.

Results

miR-200 Family Is Up-Regulated During Late Gestation and Labor. In an effort to identify miRNAs that mediate myometrial transition to a contractile phenotype in preparation for labor, we performed microarray analysis to compare the miRNA expression profile in RNA from isolated myometrium of three pools (six uteri each) from eighteen 15.5 d post-coitum (dpc) vs. three pools (six uteri each) from eighteen 18.5 dpc (with term labor being 19.0 dpc) pregnant mice. Among the regulated miRNAs (SI Dataset 1), we identified a family of miRNAs, the miR-200 family, that was highly expressed and significantly up-regulated between 15.5 and 18.5 dpc in all three arrays. Among the family members, miR-200b and miR-429 showed the most dramatic increases in expression between 15.5 dpc and term (Fig. S1A).

The miR-200 family of miRNAs includes miR-200b/c/429 and miR-200a/141 and maps to two clusters in the mouse and human genomes, each less than 2,000 bp in length. There is near-complete homology of the miR-200 family between the mouse and human; the genes cluster identically and differ in their mature sequences by only two nucleotides in the 3'-region of miR-429 (Fig. S1B).

Members of the miR-200 family bind and repress a highly similar set of mRNA targets in the mouse and human (10). To identify genes regulated by miRNAs during pregnancy and labor, we performed a gene expression microarray (SI Dataset 2) and compared sets of down-regulated uterine genes at 18.5 dpc with the strongest miR-200b/c/429 TargetScan (Whitehead Institute

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for Biomedical Research) context scores (11) (Fig. S1C). With five and six confirmed binding sites in their 3'-UTRs, respectively, ZEB1 and ZEB2 mRNAs (significantly down-regulated in the microarray) were predicted to be the strongest targets of the miR-200 family (10, 12–14) (Fig. S1C and D).

ZEB Transcriptional Repressors and Their Contraction-Associated Targets Are Reciprocally Regulated with miR-200s in Late Gestation and Labor. To compare their temporal expression in the mouse myometrium during late gestation, we next examined miR-200b/429 and ZEB1 and ZEB2 by quantitative RT-PCR (qRT-PCR) from 15.5 dpc to labor. miR-200b/429 were found to be up-regulated in the pregnant mouse uterus beginning at 17.5 dpc (Fig. 1A); this correlated with a significant decline in ZEB1 and ZEB2 mRNA, beginning at this time point (Fig. 1B). ZEB1 protein also was significantly decreased in the murine uterus during late gestation, declining to barely detectable in tissues from laboring mice (Fig. 1C). Several attempts to quantify ZEB2 protein with commercially available antibodies were unsuccessful.

To investigate whether expression of the miR-200 family and ZEB1 and ZEB2 during pregnancy and labor is conserved from the mouse to the human, we conducted qRT-PCR analysis of RNA from myometrial biopsies of women at term, either not in labor or in labor. The miR-200 family was found to be up-regulated (Fig. 1E) and ZEB1 and ZEB2 down-regulated in laboring human myometrium as compared with the myometrium of nonlaboring women at term (Fig. 1F). Protein expression of ZEB1 also was suppressed at term in human myometrial samples (Fig. 1G).

Given the temporal relationship of the decline in ZEB1 and ZEB2 to the induction of labor, we examined whether these transcriptional repressors regulate contraction-associated genes in the uterine myometrium. To this end, we conducted temporal expression analyses of CXN-43 and OXTR in the same myometrial samples used for analyses of miR-200b/429, ZEB1, and ZEB2. CXN-43 and OXTR mRNA showed temporal expression profiles that were coordinately up-regulated with down-regulation of ZEB1 and ZEB2 in the pregnant mouse uterus during late gestation (Fig. 1D) and were significantly up-regulated in myometrial biopsies from human subjects in labor as compared with those not in labor (Fig. 1H). Notably, the contraction-associated genes CXN-43 and OXTR were found to be colocalized in the murine myometrium with ZEB1 and ZEB2 during late gestation (Fig. S2).

miR-200b/429, ZEB1, and ZEB2 Are Differentially Regulated in Models of Preterm Labor. We also wanted to determine whether similar changes in miR-200b/429 and ZEB1 and ZEB2 expression were associated with preterm labor. To explore this, we evaluated two mouse models of preterm labor induced at 15.5 dpc, a time point before the observed changes in expression of miR-200b/429, ZEB1, ZEB2, and contraction-associated genes. Preterm labor was induced either by a single s.c. injection of the antiprogesterone/antiglucocorticosteroid RU486 (15) (Fig. 2A) or by intraamniotic injections of the bacterial endotoxin LPS (Fig. 2B). RU486 induced labor within 12 h in 7 of 7 injected mice; LPS induced preterm labor within 12–18 h in 7 of 10 injected mice. LPS- and RU486-treated mice were killed on the birth of one pup, and time-matched vehicle-injected mice were killed directly afterward. None of the vehicle-injected mice delivered preterm. Both RU486 and LPS significantly up-regulated miR-200b/429 expression in the maternal myometrium as compared with vehicle-injected controls (Fig. 2C and D). Consistent with the inverse regulation of miR-200b/429, ZEB1, and ZEB2 observed during late gestation in the mouse and human (Fig. 1), both models of preterm labor resulted in markedly decreased expression of ZEB1 and ZEB2 mRNA and ZEB1 protein (Fig. 2E–H). These findings suggest that alterations in myometrial expression of miR-200b/429, ZEB1, and ZEB2 may play a role in the induction of preterm labor.

P₄ and PR Affect the miR-200–ZEB Contractile Axis via Direct Induction of ZEB1 Gene Expression in the Pregnant Myometrium. Although the mechanisms by which RU486 and LPS induce preterm labor are

Fig. 1. miR-200b/429, ZEB1, and ZEB2, and contraction-associated genes are coordinately regulated during late gestation. Mature miR-200b and miR-429 are significantly up-regulated (A) and ZEB1 and ZEB2 mRNA are significantly down-regulated (B) across late gestation in the mouse myometrium, beginning at 17.5 dpc. *mmu*, *Mus musculus*. (C) ZEB1 protein in nuclear extracts of murine myometrium mirrors the decline in ZEB1 mRNA. β -actin was used as a loading control. The immunoblot shown is representative of three independent gestational series of mice. Densitometry analysis of all three series revealed a significant decrease in ZEB1 protein between 15.5 dpc and labor (Student's *t* test, $P < 0.05$; $n = 3$ mice per group). (D) Contraction-associated genes CXN-43 and OXTR were significantly up-regulated in the same samples as in A and B, beginning at 18.5 dpc. Expression of each miRNA/mRNA was determined by qRT-PCR, normalized to GAPDH/U6, and expressed as the fold increase over 15.5 dpc. Mean $\pm$ SEM values are shown. For one-way ANOVA, miR-200b: $F(4,20) = 14.82$, $P < 0.0001$; miR-429: $F(4,20) = 17.49$, $P < 0.0001$; ZEB1: $F(4,20) = 12.71$, $P < 0.0001$; ZEB2: $F(4,20) = 5.37$, $P = 0.004$; CXN-43: $F(4,20) = 12.93$, $P < 0.0001$; OXTR: $F(4,20) = 11.03$, $P < 0.0001$. (Multiple comparison test compared with 15.5 dpc: $*P < 0.05$, $**P < 0.01$; $n = 10$ mice each for 15.5 dpc and laboring groups, 5 mice each for 16.5–18.5 dpc.) miR-200b/429 were significantly up-regulated (E) and ZEB1 and ZEB2 mRNA were significantly down-regulated (F) in laboring myometrium as compared with nonlaboring controls. *hsa*, *Homo sapiens*. (G) ZEB1 protein expression was decreased in nuclear extracts of laboring myometrium. β -actin was used as a loading control. The immunoblot shown is representative of three replicate experiments. Densitometry analysis of all blots comparing laboring myometrium with nonlaboring controls revealed a significant decrease in ZEB1 protein in labor (Student's *t* test, $P < 0.05$; $n = 9$ per group). (H) CXN-43 and OXTR in the same samples as in E and F were significantly up-regulated in laboring myometrium. Expression of each miRNA/mRNA was determined by qRT-PCR, normalized to U6/h36B4, and expressed as the fold increase over nonlaboring controls. Mean $\pm$ SEM values are shown (Student's *t* test, $*P < 0.05$; $n = 14$ for laboring myometrium, $n = 23$ for nonlaboring controls).

notably complex, both agents are known to attenuate PR signaling (16). To investigate whether P₄ and PR regulate expression of the miR-200 family and/or ZEBs during gestation, mice were injected daily with P₄ on 15.5–18.5 dpc at a dose known to delay parturition (Fig. 3A). P₄ injection had a modest effect to decrease myometrial expression of miR-200b/429 (Fig. 3B). By contrast, ZEB1 mRNA (Fig. 3C) and protein (Fig. 3D) were significantly increased in the myometrium of the P₄-injected mice, whereas ZEB2 mRNA was unaffected (Fig. 3C). Although the absence of a significant inhibitory effect of P₄ on miR-200/429 expression was somewhat

Fig. 2. miR-200b/429 are up-regulated and ZEB1 and ZEB2 are down-regulated in two models of preterm labor. Diagram of RU486 treatment (A) or LPS treatment (B) to induce preterm labor. Animals were injected with a single s.c. injection of RU486 (200 μ g) or intraamniotic injections of 1.5 μ g of LPS into each sac at 15.5 dpc. IL, laboring myometrium; NIL, nonlaboring control. Mice were considered in labor on birth of one pup. miR-200b/429 are significantly up-regulated in RU486-induced (C) and LPS-induced (D) preterm labor. *mmu*, *Mus musculus*. ZEB1 and ZEB2 mRNA levels in the same tissue samples as in C and D are decreased with RU486-induced (E) or LPS-induced (F) preterm labor. Expression of each miRNA/mRNA was determined by qRT-PCR, normalized to U6/GAPDH, and expressed as the fold change over vehicle-treated controls. Mean $\pm$ SEM values are shown (Student's *t* test, **P* < 0.05; ***P* < 0.01; *n* = 7 per group). Experiments were repeated twice with similar results. ZEB1 protein expression in nuclear extracts of murine myometrium is decreased in association with RU486-induced (G) or LPS-induced (H) preterm labor. β -actin was used as a loading control. Densitometry analysis of blots comparing uterine nuclear extracts from RU486- and LPS-treated mice with vehicle-injected controls revealed a significant reduction in ZEB1 protein with preterm labor (Student's *t* test, **P* < 0.05; *n* = 7 mice per group).

surprising, we reasoned that this may be attributable to the relatively high endogenous P_4 during late gestation in the mouse and to the postulated decline in PR function (1). To assess the effects of P_4 injection on miR-200b/miR-429 expression in the myometrium in the absence of endogenous P_4 , we conducted experiments using ovariectomized mice injected with P_4 (1 mg). As shown in Fig. S3, 24 h following P_4 injection, miR-200b/429 were significantly down-regulated in the myometrium as compared with oil-injected controls. These collective findings suggest that P_4 can inhibit miR-200b/429 expression and that this may be mediated, in part, via P_4 induction of ZEB1.

To investigate further the selective induction of ZEB1 by P_4 , we conducted experiments in T47D breast cancer cells because of their relatively high expression of endogenous PR. Treatment of the cells with P_4 (10^{-7} M) for 12 or 24 h caused a pronounced induction of ZEB1 mRNA, whereas no effect of P_4 on ZEB2 expression was evident (Fig. 3E). To determine whether the induction of ZEB1 expression by P_4 is attributable to the direct binding of PR to the ZEB1 promoter, we cotransfected HEK293 cells with a reporter construct containing 978 bp of the ZEB1 5'-flanking sequence subcloned upstream of the luciferase gene and with an expression vector containing either WT PR-B or PR-B with an inactivating mutation in the DNA-binding domain (mutPR-B_{DBD}). The cells were cultured with or without P_4 (10^{-7} M) for 24 h and assayed for luciferase activity. As can be seen, P_4 had a pro-

Fig. 3. P_4 affects the miR-200-ZEB contractile axis via direct induction of ZEB1 expression. (A) Diagram depicting P_4 injection of mice in late gestation. IL, laboring myometrium; NIL, nonlaboring control. (B) miR-200b/429 are only modestly down-regulated by exogenous P_4 treatment during late gestation. (C) ZEB1 but not ZEB2 mRNA is significantly increased by P_4 treatment (same samples as in B). Expression of each miRNA/mRNA was determined by qRT-PCR, normalized to U6/GAPDH, and depicted as the fold increase over vehicle-treated controls. Mean $\pm$ SEM values are shown (Student's *t* test, **P* < 0.05; *n* = 7 mice per group). Data are representative of three similar experiments. (D) ZEB1 protein in myometrial nuclear extracts is increased by P_4 treatment. β -actin was used as a loading control. Densitometry analysis of blots comparing P_4 -treated mice with vehicle controls revealed that P_4 causes a significant increase in ZEB1 protein (Student's *t* test, *P* < 0.05; *n* = 7 mice per group). (E) ZEB1 but not ZEB2 mRNA levels are induced in T47D cells treated with 10^{-7} M P_4 for 12 or 24 h. Expression of each gene was determined by qRT-PCR, normalized to h36B4, and expressed as the fold increase over vehicle-treated control cells. Data are the mean $\pm$ SD values from three replicate experiments (Student's *t* test, **P* < 0.05, ***P* < 0.01). (F) P_4 acting via PR induces ZEB1 promoter activity. HEK293 cells were transiently transfected with a ZEB1-Luciferase reporter construct containing 978 bp of the 5'-flanking sequence from the *hZEB1* gene and with empty expression vector (control) or with a CMV expression vector containing the wild-type mouse PR-B isoform or PR-B containing a mutation in the DNA-binding domain (mutPR-B_{DBD}). The cells were cultured with or without P_4 (10^{-7} M) for 24 h, and luciferase activity was assayed, normalized to β -gal, and expressed as the fold increase over vector-transfected control cells. Data are the mean $\pm$ SD values from three replicate experiments (Student's *t* test, **P* < 0.05). (G) Overexpression of ZEB1 causes induction of ZEB2 mRNA expression. Primary murine myometrial cells infected with recombinant adenoviruses expressing CMV/ZEB1 manifested induction of endogenous ZEB2 mRNA after 72 and 96 h. Expression of ZEB2 was determined by qRT-PCR, normalized to GAPDH, and expressed as the fold increase over ZEB2 expression in cells transfected with β -gal-expressing adenoviruses. Data are the mean $\pm$ SD values from two replicate experiments (Student's *t* test, **P* < 0.05).

nounced effect on the up-regulation of ZEB1 promoter activity in cells cotransfected with WT PR-B; however, this was largely prevented in cells cotransfected with mutPR-B_{DBD} (Fig. 3F). This suggests that PR predominantly enhances ZEB1 expression by direct binding to the ZEB1 promoter, although other mechanisms may be involved as well. Together, these data suggest that ZEB1 is a direct PR target gene.

Our findings in pregnant mice and cultured cells (Fig. 3B–F) indicate that P_4 selectively induces ZEB1 but does not have a direct effect to increase ZEB2 expression. Because both ZEB1 and

ZEB2 are up-regulated in the uterus throughout most of pregnancy, this raises the question as to what factor(s) cause the pregnancy-associated induction of ZEB2. We postulated that P₄ induction of ZEB1 causes suppression of the miR-200 family, which, in turn, relieves suppression of ZEB2, resulting in its subsequent induction. To test this hypothesis, primary cultures of mouse myometrial cells were infected with recombinant adenoviruses expressing ZEB1 or β -gal (control) and ZEB2 mRNA was analyzed as a function of time. As shown, overexpression of ZEB1 resulted in a time-dependent increase in ZEB2 mRNA (Fig. 3G). This was further supported by the finding that P₄ treatment of ovariectomized mice significantly suppressed miR-200/429 (Fig. S3). These collective findings may explain why both ZEB1 and ZEB2 are increased in the myometrium during pregnancy, whereas only ZEB1 is directly up-regulated by PR. Notably, ZEB1 has two putative progesterone response elements (PREs) in its promoter, although none are apparent in the ZEB2 promoter.

Manipulation of miR-200b/429, ZEB1, and ZEB2 Regulates Contraction-Associated Genes. Although previous studies indicate that miR-200 family members down-regulate expression of ZEB1 and ZEB2 in a variety of cells and cell lines (10, 12–14), this relation has not been investigated in the myometrium. Furthermore, the ability of ZEB1 and ZEB2 to repress expression of members of the miR-200 family, as reported elsewhere (17, 18), has not been explored in the myometrium. To investigate the regulation of ZEB1 and ZEB2 by members of the miR-200 family in the myometrium, we transfected mimics of miR-200b and miR-429 into an immortalized human myometrial cell line (hTERT-HM) (19). Transfection of hTERT-HM cells with miR-200b/429 mimics resulted in a significant reduction in endogenous ZEB1 and ZEB2 expression within 24 h (Fig. 4A). To explore the reciprocal repression of the miR-200 family by ZEB1 and ZEB2, primary cultures of mouse myometrial cells were infected with recombinant adenoviruses overexpressing ZEB1, ZEB2, or β -gal (control). Overexpression of ZEB1 or ZEB2 in primary myometrial cells resulted in a significant reduction in endogenous miR-200b/429 expression at 96 h posttransduction (Fig. 4B).

As mentioned, ZEBs act as transcriptional repressors by recruiting corepressors to the promoters of target genes (20). Therefore, it was of interest to examine the effects of ZEB1 and ZEB2 overexpression on contraction-associated genes in the uterine myometrium. Following transduction of myometrial cells with recombinant adenoviruses expressing either ZEB1 or ZEB2, CXN-43 and OXTR mRNA levels were found to be significantly suppressed at 48 h (Fig. 4C). This demonstrates regulation of these two contraction-associated genes by the ZEB family of transcription factors. To determine further whether the down-regulation of miR-200b/429 (Fig. 4B) or CXN-43 and OXTR mRNA (Fig. 4C) by ZEB1 and ZEB2 is attributable to direct binding of ZEB1 to the promoters of its targets, we conducted ChIP analysis using myometrial tissues from pregnant mice at 15.5 dpc vs. mice in labor. As can be seen, ZEB1 bound specifically to the promoters of *CXN-43* (Fig. 4D), *OXTR* (Fig. 4E), and the *miR-200b-a-429* cluster (Fig. 4F) at 15.5 dpc. Moreover, binding was significantly down-regulated in laboring mice (Fig. 4 D–F).

Manipulation of miR-200b/429, ZEB1, and ZEB2 Affects Contraction of Myometrial Cells. Because of the pronounced inhibitory effect of ZEB1 and ZEB2 on expression of CXN-43 and OXTR, we examined the effects of ZEB1 and ZEB2 overexpression on contractility of uterine myocytes. To carry out these studies, we used collagen gel contraction assays in which hTERT-HM cells transduced with adenoviruses overexpressing ZEB1, ZEB2, or β -gal (control) were embedded into 3D collagen gel matrices (21–23) so that myocytes could contract within the matrix in response to oxytocin treatment. Oxytocin, a key hormone that enhances uterine contractility at term (24), significantly induced contraction of collagen gel matrices embedded with untransduced and β -gal-transduced hTERT-HM cells (Fig. 4G). This effect of oxytocin was blocked in the hTERT-HM cells transduced with ZEB1 and

ZEB2 expression vectors, demonstrating a direct effect of ZEB1 and ZEB2 to inhibit myometrial contractility in vitro (Fig. 4G).

Discussion

Our understanding of the mechanisms leading to the onset of parturition has increased considerably in recent years (1). Based on investigations from our laboratory and others, we postulate that the timing of parturition is mediated by integrated signaling pathways from both the mother and maturing fetus (25). These culminate in an inflammatory response within the uterus and cervix, which leads to a reduction in PR function (2, 3), exacerbated inflammatory signaling, cervical ripening, and activation of contraction-associated genes within the myometrium.

Despite our growing insight into the signals and pathways leading to the initiation of labor, much remains to be discovered regarding the mechanisms whereby the myometrium is transformed from a refractory near-quiescent state to a highly contractile unit capable of responding to a variety of signals from the fetus and mother. The present research sheds light on this extremely important transitional period within the maternal uterus. Our findings, which elucidate a unique regulatory pathway involving the miR-200 family and their targets, ZEB1 and ZEB2, provide insight into the mechanisms by which P₄ and PR maintain uterine quiescence throughout most of pregnancy and that mediate the myometrial transformation leading to parturition.

By using a multiple array-based approach, we were able to uncover gene networks outside the arsenal of genes known to be important in mammalian parturition. Specifically, we found that the miR-200 family of miRNAs is up-regulated in the murine myometrium beginning at 17.5 dpc and that two of its targets, ZEB1 and ZEB2, identified through bioinformatic analysis and gene expression microarrays, were coordinately suppressed. The temporal regulation of miR-200b/429, ZEB1, and ZEB2 beginning at 17.5 dpc is of particular interest because this marks an important time of transition in the maternal uterus, during which there is a switch from quiescence and resistance to inflammatory signals to increased receptivity to stimuli that trigger uterine contractility (26).

Alterations in P₄ and PR function have been suggested to play an important role in myometrial conversion to a contractile phenotype in the pregnant uterus (26). For example, at 20.5 dpc in the rat (which corresponds to 17.5 dpc in the mouse), a “synthetic to contractile switch” occurs during which myometrial proliferation declines, whereas myocyte attachment to the basement membrane and reorganization of myometrial cells increase (26). This is associated with a marked increase in basement membrane matrix synthesis and increased expression of genes encoding contraction-associated proteins. Notably, P₄ withdrawal appears to be a prime player in the initiation of this “switch,” because these same phenotypic changes in the myometrium are induced on RU486 injection and delayed by exogenous P₄ treatment (26). The temporal up-regulation of the miR-200 family and down-regulation of ZEB1 and ZEB2 expression in the pregnant mouse uterus at 17.5 dpc, which just precedes the time of induction of the contraction-associated genes *CXN-43* and *OXR*, marks a critical time for transition to a contractile myometrium.

Our findings suggest that elevated circulating P₄ throughout most of pregnancy directly up-regulates expression of *ZEB1* in the myometrium via binding of PR to the *ZEB1* promoter. Consequently, after 17.5 dpc, when P₄ levels and PR function decline (27), *ZEB1* mRNA and protein in the pregnant mouse uterus are significantly reduced. Because *ZEB1* directly binds and represses the miR-200b/429 promoter (17, 18) (Fig. 4F), a decline in *ZEB1* results in a reciprocal up-regulation of the miR-200 family. Interestingly, P₄ and PR do not directly regulate *ZEB2* in the mouse myometrium or in cultured human cells. However, we observed in primary cultures of mouse myometrial cells that overexpression of *ZEB1* caused up-regulation of *ZEB2* mRNA (Fig. 3G); this presumably occurs via *ZEB1* suppression of the miR-200 family. Interestingly, similar findings have been observed in stable MDCK cell lines overexpressing *ZEB1* (17). Because of these

Fig. 4. Overexpression of miR-200b/429 mimics, ZEB1, and ZEB2 regulates contraction-associated genes and human myometrial cell contractility. (A) Transfection of hTERT-HM cells with miRmimics of miR-200b/429 causes a significant reduction in endogenous ZEB1 and ZEB2 within 24 h. (B) Primary murine myometrial cells were infected with recombinant adenoviruses expressing ZEB1, ZEB2, or β-gal (control); levels of miR-200b and miR-429 were analyzed by qRT-PCR 96 h posttransduction. (C) hTERT-HM cells were infected with recombinant adenoviruses expressing ZEB1, ZEB2, or β-gal (control); CXN-43 and OXTR mRNA levels were analyzed by qRT-PCR 48 h posttransduction. The levels of each mRNA/miRNA were normalized to h36B4/U6 and expressed as the fold change over control. Data are the mean ± SD values of three replicate experiments (Student's *t* test, **P* < 0.05, ***P* < 0.01). ChIP studies of endogenous ZEB1 binding to the promoters of its targets reveals robust binding to *CXN-43* (D), *OXTR* (E), and the *miR-200b-a-429* cluster (F). Binding of endogenous ZEB1 was determined by qPCR, normalized to input, and expressed as the fold increase over binding by IgG. Data shown are the mean ± SEM values of two replicate experiments (Student's *t* test, **P* < 0.05; *n* = 10 mice per group). Diagrammed above each graph are the locations of E-boxes within the gene promoter. (G) Overexpression of ZEB1 and ZEB2 in hTERT-HM cells inhibits oxytocin-mediated contraction. Cells were infected with recombinant adenoviruses expressing ZEB1, ZEB2, or β-gal (control) and cultured for 48 h. These, as well as uninfected cells, were embedded into collagen gel matrices and treated with or without oxytocin (10 nM). Contraction of the gels was assessed after 24 h by measurement and calculation of mean gel area (mm²). A representative gel is shown below the data for each group. Data shown are the mean ± SD values from three replicate experiments (Student's *t* test, **P* < 0.05). (H) During pregnancy, P₄ and 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, 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.

collective findings, we suggest that the observed decline in ZEB2 during late gestation and in response to RU486 treatment is attributable to the rise in miR-200 family expression, which, in turn, represses both ZEB1 and ZEB2 (Fig. 4H). This negative feedback loop may explain the kinetics of miR-200b/429, ZEB1, and ZEB2 expression throughout gestation and in preterm labor.

In addition to defining the regulation of miR-200b/429 and their targets, ZEB1 and ZEB2, we wished to discern the functional role (s) of these players in the myometrium toward term. Through overexpression studies of ZEB1 and ZEB2 in cultured human myometrial cells, we have shown that ZEB1 and ZEB2 down-regulate the expression of CXN-43 and OXTR and that ZEB overexpression inhibits oxytocin-mediated contraction of myometrial cells embedded in a collagen matrix. In ChIP studies, we observed that endogenous ZEB1 binds to the promoters of the *OXTR*, *CXN-43*, and *miR200b-a-429* cluster and that ZEB1 binding significantly declines between 15.5 and 18.5 dpc, in concert with the gestational up-regulation of expression of these genes.

A role for CXN-43 and OXTR in myometrial contractility has long been appreciated, because expression of both of these genes enhances receptivity of the uterus to the contractile signals of labor. CXN-43 is a protein responsible for the formation of gap junctions in the myometrium, which mediate intercellular coupling to coordinate the synchronous myometrial contractions necessary for successful labor. In addition, mice with a smooth muscle-specific deletion of the *CXN-43* gene manifest a significant delay in the timing of parturition (28). The action of oxytocin as an uterotonic agent is widely accepted. Exogenous oxytocin can increase myometrial contractility and induce labor in mice and humans, whereas antagonists of OXTR are effective in maintaining uterine quiescence. However, the role of oxytocin and OXTR in normal parturition is uncertain, because oxytocin gene KO mice undergo parturition and give birth to live young (29) and mice deficient in OXTR manifest normal timing and duration of parturition (30). These unexpected phenotypes may be attributable to a functional redundancy of the oxytocin/OXTR signaling system and/or to compensatory up-regulation of other uterotonic systems, such as COX-2/prostaglandins.

Collectively, our findings suggest that ZEB1 is a key PR target gene in the myometrium that inhibits expression of contraction-associated genes and the miR-200 family throughout most of pregnancy (Fig. 4H). Near term, signals from the fetus and mother cause an increased inflammatory response, leading to a decline in local P₄ and/or PR function and the activation of contraction-associated genes. The decline in PR function near term causes a down-regulation of *ZEB1* gene expression, which, in turn, results in derepression and up-regulation of miR-200b/429 expression. The resulting elevated miRNAs can then feed back and repress both ZEB1 and ZEB2 (Fig. 4H). This negative feedback loop, which is supported by the present findings in the pregnant mouse and human myometrium and in cultured human myometrial cells as well as by published reports by others (17, 18), results in further induction of contraction-associated gene expression and labor. Taken together, our findings implicate a previously undiscovered pathway in the regulation of uterine contractility during pregnancy and parturition that is conserved from mice to humans and may ultimately open avenues for development of effective therapeutics for prevention of preterm labor.

Materials and Methods

Timed Pregnancies. Female mice were housed with male mice overnight beginning at 1800 hours. Mice found to have vaginal plugs at 0600 hours the following day were considered to be 0.5 dpc.

Microarray Analysis. RNA was purified from mouse myometrium (miRNeasy kit; Qiagen). The miRNA microarray was performed (LC Sciences) on 18 biological replicates of murine myometrium at 15.5 dpc and on an equal number of replicates at 18.5 dpc. Gene expression microarray assays were performed (University of Texas Southwestern Medical Center) on the same 36 samples, as detailed further in *SI Materials and Methods*.

Preterm Labor Studies. For LPS-induced preterm labor, 15.5-dpc timed-pregnant ICR/CD1 (Institute for Cancer Research) female mice were anesthetized and the uterus was exposed by laparotomy. LPS (1.5 μ g in 50 μ L of PBS; Sigma) or sterile PBS (50 μ L) was carefully infused into each amniotic sac, the uterus was reinserted into the abdominal cavity, and the mouse was allowed to recover. For RU486-induced preterm labor, 15.5-dpc timed-pregnant ICR/CD1 female mice were injected s.c. in the right flank with RU486 [200 μ g of RU486 in 1 mL of sterile 5% (vol/vol) ethyl alcohol (EtOH); Sigma] or 5% EtOH (1 mL). Both models of preterm labor resulted in a high rate of preterm delivery (12 h post-RU486 and 8–18 h post-LPS treatment) and did not cause maternal mortality (31). None of the vehicle-injected mice went into labor. The mice were killed on the birth of one pup, and time-matched controls were killed directly afterward.

P₄ Treatment Studies. Timed-pregnant ICR/CD1 female mice were injected s.c. daily with P₄ (1 mg in 0.5 mL of sterile sesame oil) or with sesame oil (0.5 mL) in the flank region from 15.5–18.5 dpc. For these experiments, vehicle-injected mice were killed in labor (on the birth of one pup) and time-matched P₄-treated animals were killed directly afterward. None of the P₄-treated animals progressed to labor at 19.0 dpc.

Human Subjects and Tissue Acquisition. Lower uterine segment myometrial tissues were biopsied at term from pregnant women undergoing cesarean section as detailed in *SI Materials and Methods*.

qRT-PCR. RNA was DNase-treated (Invitrogen) and reverse-transcribed using the SuperScript III-RT kit (Invitrogen) or TaqMan miRNA reverse transcription kit (Applied Biosystems). Gene expression analysis was conducted using SYBR Green (Applied Biosystems) or TaqMan Universal PCR Master Mix (Applied Biosystems). Relative gene expression was calculated using the comparative cycle threshold ($\Delta\Delta C_t$) method. Primer sets are listed in *SI Materials and Methods*.

Immunoblot Analysis. Antibodies directed against ZEB1 (generously provided by Douglas Darling, University of Louisville, Louisville, KY) (32) were used to determine protein levels by immunoblotting. β -Actin (Abcam) was detected as a loading control. Details are presented in *SI Materials and Methods*.

Cell Culture and Luciferase Assays. HEK293 cells were transfected with various PR expression plasmids and ZEB1-Luciferase reporter constructs using Eugene 6 transfection reagent (Roche) according to the manufacturer's protocol. hTERT-HM cells were cultured and transduced with recombinant adenoviruses or transfected with miRNA mimics (Qiagen). Primary cultures of mouse myometrial cells were prepared as previously described (33). Details are presented in *SI Materials and Methods*.

ZEB1 and ZEB2 Plasmid Construction. Genomic fragments containing ZEB1 and ZEB2 were each released from vectors (kindly provided by Yujiro Higashi, Osaka University, Osaka, Japan) and subcloned into pACCMV-pLP Δ (-)loxP-SSP adenoviral vectors as described in *SI Materials and Methods*.

Collagen Matrix Contractility Assay. hTERT-HM cells were transduced with adenoviral vectors overexpressing ZEB1, ZEB2, or β -gal (control) and embedded into collagen matrices. Cell-embedded matrices were treated with 10 nM oxytocin and assayed for contractility as described in *SI Materials and Methods*.

ChIP. Using a ChIP Assay Kit (catalog no. 17-295; Millipore) according to the manufacturer's instructions and ZEB1 antibody (H-102, SC-25388; Santa Cruz), myometrial tissues from timed-pregnant ICR mice (15.5 dpc and in labor) were subjected to ChIP. DNA was purified and analyzed by qPCR as detailed in *SI Materials and Methods*.

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