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 coordinate 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- and PR-regulated modulators of uterine quiescence and contractility during pregnancy and labor and shed light on the molecular mechanisms involved in preterm birth.

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 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-kB p65 (5) and by P4-induced expression of the NF-kB 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/414 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. 5C). 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. 5C 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 analyses 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. 1F) 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 mRNAs 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 antiprogestin/antiglucocorticosteroid RU486 (15) (Fig. 2A) or by intramammary 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. 2 C 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. 2C–E). 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 not only 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-200b/429 expression was somewhat
surprising, we reasoned that this may be attributable to the relatively high endogenous P4 during late gestation in the mouse and to the postulated decline in PR function (1). To assess the effects of P4 injection on miR-200b/miR-429 expression in the myometrium in the absence of endogenous P4, we conducted experiments using ovariectomized mice injected with P4 (1 mg). As shown in Fig. S3, 24 h following P4 injection, miR-200b/429 were significantly down-regulated in the myometrium as compared with oil-treated controls. These collective findings suggest that P4 can inhibit miR-200b/429 expression and that this may be mediated, in part, via P4 induction of ZEB1.

To investigate further the selective induction of ZEB1 by P4, we conducted experiments in T47D breast cancer cells because of their relatively high expression of endogenous PR. Treatment of the cells with P4 (10^{-7} M) for 12 or 24 h caused a pronounced induction of ZEB1 mRNA, whereas no effect of P4 on ZEB2 expression was evident (Fig. 3E). To determine whether the induction of ZEB1 expression by P4 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 a nonactivating mutation in the DNA-binding domain (mutPR-BDBD). The cells were cultured with or without P4 (10^{-7} M) for 24 h and assayed for luciferase activity. As can be seen, P4 had a pronounced 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-BDBD (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. 3 B–F) indicate that P4 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 subsequent induction. To test this hypothesis, primary cultures of mouse myometrial cells were infected with recombinant adeno-viruses expressing ZEB1 or β-gal (control) and ZEB2 mRNA was analyzed as a function of time. As shown, overexpression of ZEB2 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 established 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 adeno-viruses 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 posttransfection (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 adeno-viruses 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. 4D–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 adeno-viruses 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 OXTR, 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 up-regulation of ZEB1 caused up-regulation of ZEB2 mRNA (Fig. 3G), which 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
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 PR 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 anesthesia-anesthetized and uterus was exposed by laparotomy. LPS (1.5 µg in 50 µL of PBS; Sigma) or sterile PBS (50 µL) 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 i.c. in the right flank with RU486 [200 µg 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 (72 h post-RU486 and 18–24 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 i.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 mRNA 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 (ΔΔCt) method. Primer sets are listed in SI Materials and Methods.

**Immunoblot Analysis.** Antibodies directed against ZEB1 (generously provided by Douglas Darlington, 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 Fugene 6 transfection reagent (Roche) according to the manufacturer’s protocol. hTER-T-HM cells were cultured and transfected 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–lpPA–Ioxp–SSL adenoviral vectors as described in SI Materials and Methods.

**Collagen Matrix Contractility Assay.** hTER-T-HM cells were transfected with adenoviral vectors overexpressing ZEB1, ZEB2, or J-rgal (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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Supporting Information

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SI Materials and Methods

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

Tissue Preparation for Immunohistochemical Staining. For immunohistochemical staining of the uterus, pregnant mice at 15.5 and 18.5 dpc were deeply anesthetized with an injection of a ketamine-xylazine-acepromazine mixture and transcardially perfused with 4% (wt/vol) paraformaldehyde in PBS. The uteri were dissected, and pups and placental material were removed. Tissues were stored in 4% paraformaldehyde at 4 °C overnight and then transferred into a 30% (wt/vol) sucrose solution (in PBS) at 4 °C. After 2 d, the tissue was immersed in 2-methylbutan and flash-frozen in liquid N2. Twelve-micrometer-thick sections were cut on a cryostat and stored at −20 °C.

Preterm Labor Studies. Two mouse models were used for preterm labor studies: a model of intrauterine inflammation and a hormonal model of preterm birth. For inflammation-induced preterm labor, 15.5 dpc timed-pregnant ICR/CD1 female mice were placed briefly under isoflurane anesthesia and subsequently injected (i.p.) with 1.25 mL of Avertin (tribromoethanol; Sigma). Both uterine horns were gently pulled through a 1-cm midline abdominal incision, and LPS [1.5 µg of LPS in 50 µL of PBS; Sigma] or sterile PBS (50 µL) was carefully injected with a sterile, 24-gauge, half-inch needle through the exposed uterine wall into all amniotic sacs. The uterus was returned to the abdominal cavity, the abdominal muscle wall was closed with ETHICON 5-0 Chromic Gut sutures (Becton Dickinson), and skin was closed by using 9-mm wound clips (AUTOCLIP; Becton Dickinson). Mice were kept under a warming lamp for 1 h postoperatively. For the hormonally induced preterm labor, 15.5 dpc timed-pregnant ICR/CD1 female mice were placed briefly under isoflurane anesthesia. RU486 [200 µg in 1 mL of sterile 5% (vol/vol) EtOH; Sigma] or 5% EtOH (1 mL) was injected s.c. into the flank region of each mouse. Both models of preterm labor resulted in a high rate of preterm delivery (12 h post-RU486 treatment and 8–18 h post-LPS treatment) and did not result in maternal mortality (1). None of the vehicle-injected mice went into preterm labor. The mice were killed on the birth of one pup, and time-matched controls were killed directly afterward.

P2 Treatment Studies. Timed-pregnant ICR/CD1 female mice were injected s.c. daily with P2 (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 P2-treated animals were killed directly afterward. None of the P2-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. Informed consent was obtained in writing from each woman before surgery using protocols approved by the Institutional Review Board of the University of Texas Southwestern Medical Center in accordance with the Donors Anatomical Gift Act of the State of Texas. Myometrial biopsies were collected from two groups of subjects: (i) pregnant women who underwent cesarean section before the onset of labor at term with no evidence of infection and (ii) pregnant women in active labor at term who underwent cesarean section. Myometrial smooth muscle was dissected from each biopsy, flash-frozen in liquid nitrogen, and stored at −80 °C for subsequent protein and mRNA analysis.

miRNA Microarray Analysis. miRNA microarray assay was performed using a service provider (LC Sciences). Eighteen biological replicates of murine uterus at 15.5 dpc and an equal number of replicates at 18.5 dpc were pooled into three groups of six animals for each gestational time point, and two-color chips were run in triplicate. The assay was initiated with 5 µg of total RNA per sample, which was size-fractionated using a YM-10 Microcon centrifugal filter (Millipore); isolated small RNAs (<300 nt) were 3′-extended with poly(A) polymerase. An oligonucleotide tag was ligated to the poly(A) tail for later fluorescent dye staining with either Cy3 or Cy5 dye. Two different tags were used for 15.5 and 18.5 dpc samples, and dye swapping was conducted, alternating every other chip, to avoid dye bias. Hybridization was performed overnight on a μParaflo microfluidic chip using a microcirculation pump (Atactic Technologies). Each detection probe consisted of a chemically modified nucleotide coding segment complementary to target miRNA (mirBase Sequence Database v12.0, www.mirbase.org) or another RNA control sequence. The detection probes were made by in situ synthesis using photogenerated reagent chemistry. The hybridization melting temperatures were balanced, and hybridization images were collected using a laser scanner (GenePix 4000B; Molecular Device) and digitized using Array-Pro image analysis software (Media Cybernetics). Data were analyzed by first subtracting the background and then normalizing signals using a LOWESS filter (locally weighted regression). Because we conducted two-color experiments, the ratio of the two sets of detected signals (log2-transformed and balanced) and P values of the t test were calculated across all three chips; differentially detected signals were those with P < 0.05.

Gene Expression Microarray Analysis. Gene expression microarray analysis was performed on three pooled samples of murine uterus at 15.5 dpc and an equal number of pooled samples at 18.5 dpc. Each pool was composed of uterine tissues from 6 mice (total of 18 mice) for each gestational time point. RNA quality was assessed using a 2100 Bioanalyzer (Agilent), and RIN values were >9.3. In total, 260 of 280 values were >9.3. In total, 260 of 280 values were >1.85. After quality and purity were confirmed, 200 ng of total RNA was amplified and labeled by the University of Texas Southwestern Microarray Core Facility at the McDermott Center for Human Growth and Development using the TotalPrep RNA Amplification kit (Illumina/Ambion). cRNA (1.5 µg) from each sample was hybridized to Illumina Mouse 6-V1.1 microarrays using standard procedures. All microarray chips were Illumina Mouse V6-1.1 whole-genome arrays. The raw expression array data were extracted using BeadStudio v2.0 (Illumina). The data were background-sub-
tracted and median-normalized. The data were then analyzed by t test, and a false discovery rate <0.05 was used.

**qRT-PCR Analysis of ZEB1, ZEB2, and Contraction-Associated Genes.** RNA was DNase-treated (Invitrogen), and 2 μg was reverse-transcribed using the SuperScript III-RT kit (Invitrogen). Gene expression analysis was conducted using SYBR Green (Applied Biosystems) on an ABI Prism 7700 Detection System (Applied Biosystems). Relative gene expression was calculated using the comparative cycle threshold (ΔΔCt) method.

**Immunoblot Analysis.** Immunoblot analysis was conducted on nuclear extracts of hTERT-HM cells or flash-frozen myometrial tissues. Cells or tissues were lysed using the NE-PER extraction reagent kit (catalog no. 78833; Pierce) according to the manufacturer’s instructions. Protein concentration was determined by a Bradford assay (BCA Protein Assay Kit, catalog no. 23227; Pierce). Equivalent amounts of protein were added to 2x Laemmli buffer, and samples were heated to 95 °C for 10 min, loaded on 10% (wt/vol) SDS-polyacrylamide gels, run at 70–120 V, and transferred to a PVDF membrane. Membranes were incubated in blocking buffer composed of Tris-buffered saline (0.15 M NaCl, 0.05 M Tris-HCl (pH 8.0), 0.05% (vol/vol) Tween 20 (TBST)) containing 3% (wt/vol) nonfat dry milk for 60 min at room temperature before addition of primary antibodies. Antibodies directed against ZEB1 (obtained from Douglas Darling, University of Louisville, Louisville, KY) (2) were diluted in blocking buffer (1:5,000), β-Actin (Abcam) diluted in blocking buffer (1:5,000) was detected as a loading control. Primary antibodies were incubated with membranes overnight at 4 °C with rocking. Membranes were washed four times with TBST for 5 min each at room temperature. Horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (catalog no. 62-1820; Zymed Invitrogen) was diluted in blocking buffer (1:6,000) and incubated with membranes for 60 min. Membranes were washed four times in TBST for 5 min each and visualized using SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific) according to the manufacturer’s protocol. Quantitative measurement of immunoblots was performed using Image J (National Institutes of Health).

**Cell Culture.** hTERT-HM cells were maintained in DMEM-F12 (Gibco) with 10% (vol/vol) FBS. HEK-293 cells were maintained in DMEM (Gibco) supplemented with 10% (vol/vol) FBS. Human breast cancer T47D cells were maintained in RPMI medium with 10% (vol/vol) FBS and 1.7 μM insulin. Primary cultures of mouse myometrial cells were prepared using a modification of a previously described method (3). Nonpregnant female ICR mice were injected s.c. with 50 μg of 17β-estradiol (Sigma) diluted in 10% (vol/vol) ethanol and 90% (vol/vol) sesame oil. After injection (24 h), uteri were excised and placed in buffer A [HBSS (pH 7.4) containing 0.098 g/L MgSO4 and 0.185 g/L CaCl2, 25 mM Hepes, 100 U/mL penicillin/streptomycin (Gibco), and 2.5 μg/mL amphotericin B (Sigma)]. The uterine horns were cleaned of fat, minced, and washed three times with buffer B (buffer A without Mg2+ or Ca2+). Enzymatic digestion of the tissue was performed at 37 °C with agitation for 30 min by adding 10 mg/l tissue containing 1 mg/mL collagenase type II (Sigma), 0.15 mg/mL DNase I (Boehringer Mannheim), 0.1 mg/mL soybean trypsin inhibitor, 10% (vol/vol) FBS (Cansera), and 1 mg/mL BSA (Sigma) in buffer B. Following incubation, the uterine tissues were gently titrated for 1–2 min to aid enzymatic dispersion. Equal amounts of buffer B supplemented with 10% (vol/vol) FBS were added, and the mixture was passed through a cell strainer (40 μm) and stored on ice. Fresh enzyme mix was added to the remaining undigested tissue, and the incubation-aspiration process was repeated a total of five to six times. The first cell solution was discarded because it contained mostly debris and damaged cells, and the remaining solutions were combined. Dissociated cells were collected by centrifugation (200 x g for 15 min), and the cell pellet was resuspended in sterile phenol red-free DMEM (Gibco), supplemented with 10% (vol/vol) FBS, 25 mM Hepes, 100 U/mL penicillin/streptomycin, and 2.5 μg/mL Amphotericin B. To enrich selectively for uterine myocytes, the freshly isolated cell mixture was subjected to a differential attachment step, whereby dissociated cells were pelleted on polystyrene dishes for 30–45 min at 37 °C to allow quickly adhering nonmyocytes (mostly fibroblasts) to attach to the bottom of the dish. Following attachment of these cells, the supernatant containing the more slowly adhering myometrial smooth muscle cells was collected. Cell numbers and viability were assessed by trypan blue exclusion using a Countess Automated Cell Counter (Invitrogen) before plating in 60-mm
dishes at a density of $3 \times 10^5$ cells per dish. Media were changed 24 h after plating and every other day thereafter. Cells were used on days 2–5 following isolation.

**Analysis of P2 and PR Regulation of ZEB1 Promoter Activity.** For investigation of the effect of P2 and PR on ZEB1 promoter activity, 978 bp of 5′-flanking region of the hZEB1 gene (−978 to +7 bp) was amplified from human genomic DNA and cloned into pGL4 plasmid to produce a ZEB1-Luciferase reporter construct. To analyze the effects of P2 and PR on hZEB1 promoter activity, HEK293 cells were seeded in 24-well plates and transiently transfected with pGL4 reporter constructs (100 ng), Renilla luciferase plasmid (20 ng; Promega), and WT CMV-PR-B expression vector (100 ng, kindly provided by Donald McDonnell, Duke University, Durham, NC) or an expression vector containing mutPR-BDMP with three point mutations in the DNA-binding domain (G585E-S586G-V589A) that abrogate PR-B DNA-binding activity (4). One day after transfection, cells were treated with DMSO (vehicle) or P2 (100 nM) for 24 h in medium without phenol red or FBS. Cells from each experiment were then harvested in 100 μL of 1× Passive lysis buffer (Promega). Firefly luciferase and Renilla luciferase activities were assayed using the Dual-Luciferase assay system (Promega). The relative luciferase activity was calculated by normalizing Firefly luciferase activity to Renilla luciferase activity obtained from the same sample to correct for transfection efficiency.

**ZEB1 and ZEB2 Plasmid Construction.** Genomic fragments for ZEB1 and ZEB2 were each released from vectors provided by Yujiro Higashi (Osaka University, Osaka, Japan) using KpnI (Roche) and XbaI (Roche) restriction enzymes. Fragments were subcloned into a pAC-CMVpLA−llox-SPS shuttle vector. Recombinant viruses were constructed by Cre-lox recombination in vitro with cAd5di7011loxP and transfected into 911 cells using Lipofectamine 2000. Viral clones were propagated in 911 cells; viral supernatants were clarified by centrifugation and titered by plaque assay. Aliquots of virus were stored frozen at −80 °C until use.

**miR mimic Studies.** On reaching ~75% confluency, hTERT-HM cells were trypsinized, counted, appropriately diluted, plated, and transfected with HiPerFect Transfection Reagent (Qiagen) according to the manufacturer’s instructions for transfection of smooth muscle cells. Briefly, 5 × 10^5 hTERT-HM cells plated in 2 mL of culture medium per well of a six-well plate were transfected on plating with miR mimics for both miR-200b and miR-429 (to achieve a final mimic concentration of 10 nM for each miRNA). Cells were allowed to adhere to culture dishes in the presence of miR mimics and harvested at 12 and 24 h post-transfection for subsequent qRT-PCR analysis.

**Adenoviral Overexpression Assays.** hTERT-HM cells were plated in 30-mm dishes at 500,000 cells per well. Later (24 h), recombinant adenoviruses overexpressing ZEB1, ZEB2, or β-gal were added in minimal medium (0.5 mL of complete medium per well) at a multiplicity of infection of 85–500 pfu per cell. After overnight infection, the cells were washed once with PBS, fed with complete medium, and harvested 48–72 h later. Cells were centrifuged at 1,000 g for 5 min, and resuspended in serum-free DMEM/F12. The number of cells was ascertained, and the viability of cells was checked by trypan blue exclusion using a Countess Automated Cell Counter. A type I rat tail collagen solution (catalog no. 356236; BD Biosciences) was adjusted to pH 7.2 with 1 M NaOH. The final concentration of collagen was 1.0 mg/mL. The appropriate number of tERT-HM cells was then added to the neutralized collagen solution to achieve 2 × 10^5 cells per gel, mixed, and incubated on ice for 5 min. Collagen gel-cell suspensions were pipetted into untreated 12-well culture dishes that had been prescored with a circle (0.5-mm radius) using a plate scorer (Four Seasons Decorations) and were incubated for 1 h at 37 °C to allow gelling. One milliliter of fresh DMEM, either unsupplemented (control) or supplemented with 10 nM oxytocin, was added over the cell-collagen matrix. The gel matrices were then gently detached from the sides of the scored circle and lifted off the bottom of the well. The areas of the gels were measured periodically for up to 48 h. Maximum effects on gel contraction were obtained within 48 h of culture. Images of the floating gels were captured and digitized using a flatbed scanner (Hewlett Packard). The area of each gel was measured using Adobe Illustrator CS2, and the final areas (mm^2) of each gel were compared.

**ChIP.** Uterine tissues were harvested from 8-wk-old timed-pregnant ICR/CD1 female mice at 15.5 and 18.5 dpc. The uteri were cleared of all embryonic material and maternal decidua and were further enriched for myometrium by sterile scraping and blotting with a paper towel. The remaining myometrial tissues were homogenized in cold 1× PBS, protease inhibitor mixture (Roche), and 10 mM PMSF. Homogenized samples were incubated with 1% formaldehyde, protease inhibitor mixture, and 10 mM PMSF for 15 min at room temperature to cross-link proteins and DNA. Samples were washed twice in cold 1× PBS, protease inhibitor mixture, and 10 mM PMSF and then flash-frozen. ChIP was conducted using a ChIP Assay Kit (catalog no. 17-295; Millipore) according to the manufacturer’s instructions. Briefly, samples were thawed by the addition of 1,000 μL of lysis buffer (catalog no. 20-163; Millipore). The lysates were sonicated on ice three times at 40% amplitude for 10 s to produce sheared soluble chromatin. The soluble chromatin was pre-clear and proteinase K incubation at 4 °C overnight. An aliquot incubated with nonimmune IgG was used as a control. Protein A Agarose/Salmon Sperm DNA (75 μL, 50% slurry, catalog no. 16-157C; Millipore) at 4 °C for 30 min with rotation. The samples were microfuged at 14,000 g × 10 min to pellet the beads, and the supernatant containing the sheared chromatin was placed in different tubes. DNA concentration was determined using a spectrophotometer, and equivalent amounts of pre-clearved chromatin were incubated with antibody for ZEB1 (H-102, SC-25388; Santa Cruz) at 4 °C overnight. An aliquot incubated with nonimmune IgG was used as a control. Protein A Agarose/Salmon Sperm DNA (60 μL, 50% slurry, catalog no. 16-157C; Millipore) was added to each tube, the mixtures were incubated for 1 h at 4 °C, and the immune complexes were collected by centrifugation. The beads containing the immunoprecipitated complexes were washed sequentially for 5 min in Low Salt Immune Complex Wash Buffer (catalog no. 20-154; Millipore), High Salt Immune Complex Wash Buffer (catalog no. 20-155; Millipore), LiCl Immune Complex Wash Buffer (catalog no. 20-156; Millipore), and TE Buffer (catalog no. 20-157; Millipore). The beads were eluted with 250 μL of freshly prepared elution buffer at room temperature. Elution was repeated once, and eluates were combined. Cross-linking of the immunoprecipitated chromatin complexes and input controls (10% vol/vol of the total soluble chromatin) was reversed by heating the samples at 65 °C for 4 h. Proteinase K (15 μg; Invitrogen) was added to each sample in buffer and incubated for 1 h at 45 °C. The DNA was purified by phenol-chloroform extraction and precipitated in ethanol overnight at −20 °C. Samples and input controls were diluted in 10–400 μL of RNase/DNase-free water before qPCR, qPCR was employed, using...
at least two primer sets per gene to confirm binding to the CXN-43, OXTR, and miR-200b-a-429 cluster promoters:

<table>
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<th>Gene</th>
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<th>Primer Sequence 2</th>
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Immunohistochemistry. Cryosections were postfixed with formalin (3.7% vol/vol in PBS) for 10 min and blocked with normal goat serum or normal donkey serum for 30 min. Incubation with the primary antibodies was performed overnight at 4 °C. The following antibodies were used: mouse-α-smooth muscle actin (1:1,000; Sigma), rabbit-α-ZEB1 (1:100; Santa Cruz), rabbit-α-ZEB2 (1:100; Santa Cruz), rabbit-α-CXN-43 (1:200; Invitrogen), goat-α-CXN-43 (1:50; Santa Cruz), and goat-α-OXTR (1:1,000; Santa Cruz). Subsequently, the sections were incubated with the appropriate, fluorophore-labeled secondary antibodies (goat-α-mouse-DyLight488, goat-α-rabbit-Cy3, donkey-α-rabbit-DyLight549, and donkey-α-goat-DyLight488, all diluted 1:750 in PBS; Jackson ImmunoResearch) at room temperature for 1 h. Nuclei were counterstained with DAPI (1:100,000 in PBS; Sigma) for 5 min at room temperature, and the sections were mounted with fluorescence mounting medium (Dako). For the OXTR/ZEB1 double labeling, the sections were first incubated in a mixture of both primary antibodies, followed by incubation with a mixture of both secondary antibodies. For the CXN-43/ZEB1 double labeling, staining was first performed for ZEB1, followed by staining for CXN-43. Images were acquired with a confocal microscope (Leica TCS SP5), and z-projections of z-stacks were generated with ImageJ software (National Institutes of Health).

miR-200b  miR-429  miR-200c  miR-200a  miR-141

A

-2.0  +2.0

15.5 dpc  18.5 dpc

miR-200b

miR-429

miR-200c

miR-200a

miR-141

B

mouse:Chr 4  155428K  155430K
human:Chr 1  155429K  155430K

miR-200b

mmu-miR-200b
hsa-miR-200b

mmu-miR-200c
hsa-miR-200c

mmu-miR-429
hsa-miR-429

5' UAAUACUGCCUGGUAAUGAUGA 3'

C

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<th>Gene Symbol</th>
<th>Gene Name</th>
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D

3'UTR of Murine ZEB1: 1845 nucleotides long

3'UTR of Murine ZEB2: 4938 nucleotides long

Fig. S1. Microarray analyses reveal significant up-regulation of the miR-200 family and down-regulation of their targets, ZEB1 and ZEB2, in the murine uterus at term. (A) Heat map comparing miRNA expression between 15.5 and 18.5 dpc (n = 18 per group) for the miRNAs significantly up-regulated at term: miR-200b and miR-429 (P < 0.05) and their related family members, miR-200a, miR-200c, and miR-141 (P < 0.1). (B) Chromosomal location and mouse-human homology of members of the miR-200 family. Seed sequences for each miRNA are underlined. (C) Gene expression microarray was performed on the same samples as in A. Significantly down-regulated genes (false discovery rate <0.05) were filtered against the TargetScan 5.1 mouse database to identify the strongest predicted targets of the miR-200 family. The top seven down-regulated genes are shown, with transcriptional repressors ZEB2 and ZEB1 first and second on the list. (D) Diagram depicting the known experimentally validated binding sites for members of the miR-200 family within the 3'UTR of murine ZEB1 and ZEB2 mRNA transcripts (1, 2).

Fig. S2. ZEB1 and ZEB2 are colocalized with CXN-43 and OXTR in the myometrium of the murine uterus during pregnancy. (A) Immunostaining for smooth muscle actin (green) marked the circular and longitudinal muscle layers of the myometrium. Both layers were also immunopositive for the transcription factors ZEB1 (E) and ZEB2 (F) as well as for CXN-43 (G) and OXTR (H). (I and J) Higher magnification revealed that ZEB1 was localized in the nuclei of smooth muscle cells (red staining). Smooth muscle cells that were immunopositive for ZEB1 also showed signals for CXN-43 (I, green staining) and OXTR (J, green staining). Control sections that were incubated with mouse IgG (B), rabbit IgG (C), and goat IgG (D) instead of the primary antibodies did not manifest any staining. Uterine tissues were obtained from mice late in pregnancy. Nuclei were counterstained with DAPI (blue staining). (Scale bars: A and E–H, 100 μm; B–D, 50 μm; I and J, 10 μm.)

Fig. S3. Myometrial miR-200b/429 are significantly suppressed by P4 treatment of ovariectomized mice. Expression of mature miR-200b and miR-429 was significantly decreased by P4 treatment in ovariectomized mice. Eight-week-old ICR/CD1 virgin female mice were ovariectomized and rested for 2 wk. Animals were then injected with 1 mg of P4 in oil or an equal volume of oil alone (400 μL) and assayed for miR-200b/429 expression in myometrial tissues isolated 24 h later. Expression of each miRNA was determined by qRT-PCR, normalized to U6, and depicted as the fold increase over vehicle-treated controls. Mean ± SEM values are shown (Student’s t test, *P < 0.05; n = 5 mice per group). mmu, Mus musculus.
Other Supporting Information Files

SI Dataset 1 (XLS)
SI Dataset 2 (XLS)