Decidual cell FKBP51–progesterone receptor binding mediates maternal stress–induced preterm birth

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Edited by Jan-Ake Gustafsson, University of Houston, Houston, TX, and approved February 3, 2021 (received for review May 21, 2020)

Depression and posttraumatic stress disorder increase the risk of idiopathic preterm birth (iPTB); however, the exact molecular mechanism is unknown. Depression and stress-related disorders are linked to increased FK506-binding protein 51 (FKBP51) expression levels in the brain and/or *FKBP5* gene polymorphisms. *Fkbp5*-deficient (*Fkbp5*$^{-/-}$) mice resist stress-induced depressive and anxiety-like behaviors. FKBP51 binding to progesterone (P4) receptors (PRs) inhibits PR function. Moreover, reduced PR activity and/or expression stimulates human labor. We report enhanced in situ FKBP51 expression and increased nuclear FKBP51-PR binding in decidual cells of women with iPTB versus gestational age-matched controls. In *Fkbp5*$^{+/+}$ mice, maternal restraint stress did not accelerate systemic P4 withdrawal but increased *Fkbp5*, decreased PR, and elevated AKR1C18 expression in uteri at E17.25 followed by reduced P4 levels and increased oxytocin receptor (*Oxtr*) expression at 18.25 in uteri resulting in PTB. These changes correlate with inhibition of uterine PR function by maternal stress–induced FKBP51. In contrast, *Fkbp5*$^{-/-}$ mice exhibit prolonged gestation and are completely resistant to maternal stress–induced PTB and labor-inducing uterine changes detected in stressed *Fkbp5*$^{+/+}$ mice. Collectively, these results uncover a functional P4 withdrawal mechanism mediated by maternal stress–induced enhanced uterine FKBP51 expression and FKPB51-PR binding, resulting in iPTB.

maternal stress | preterm birth | FKBP51 | progesterone receptor | decidual cells

Preterm birth (PTB), characterized by parturition prior to 37 completed weeks of gestation, has a 5 to 18% prevalence and accounts globally for over 15 million births per year (1). In 2018, the PTB rate in the United States was 10.0% of livebirths (2), making it the leading cause of perinatal morbidity and mortality (3). It is also a major cause of childhood lung disease and neurodevelopmental disabilities, contributing to $26 billion/year in health care costs (4). Up to 80% of PTBs occur spontaneously (5), whereas ∼20% are indicated by deteriorating maternal or fetal conditions (5). While ascending genital tract infections, abruptions, and multiple gestations account for more than half of PTBs (6), maternal stress associated with depression and posttraumatic stress disorder, and fetal stress related to abnormal placentation are strongly implicated in many idiopathic PTBs (iPTBs) (7–9). However, the underlying molecular mechanism(s) responsible for stress-associated PTB remain unclear.

In all viviparous species, inhibition of progesterone (P4) production and/or function elicits labor (i.e., induces decidual inflammation, remodels the cervix, promotes fetal membrane rupture, and increases myometrial contractility). P4 signaling in target cells is achieved by binding to progesterone receptor (PR) isoforms, which belong to a ligand-activated nuclear transcription factor superfamily (10). Ligand binding to PR induces receptor dimerization, phosphorylation, and binding to PR response elements on DNA to modulate transcription of target genes (10, 11). Unlike most mammals in which parturition is initiated by declining maternal plasma P4 levels, during higher primate and guinea pig parturition, elevated P4 levels are sustained until after delivery of the placenta (12–14), indicating that a physiologic block of P4 signaling in target cells elicits "functional P4 withdrawal" (12, 14). Consistent with this hypothesis, treatment of women with PR antagonists (e.g., RU486) and prostanoids induces labor at any stage of pregnancy (15). Potential mechanisms contributing to functional P4 withdrawal include decreased PR levels (16–18), changes in PR coregulators, and/or indirect antagonism by other transcription factors (19).

In the uterus, PR expression appears to be greatest in the decidua, which is located between the fetal membrane and myometrium (20). Placental, chorionic, and amnionic cells fail to express demonstrable levels of PR (20–22), while expression of total PR in the myometrium is lower than in the decidua (21). Moreover, the decidua represents a major source of prostaglandins and proteases that are crucial to parturition (23). Thus, decidual cells appear to be a crucial arbiter of both term and PTB. Previously, we showed that decidual stromal cells in both the decidua basalis and parietalis display significantly reduced nuclear PR protein expression among patients in term labor (20) as well as those with chorioamnionitis- or abruption-associated

Significance

Preterm birth (PTB) is the leading cause of neonatal morbidity and mortality. The genetics and molecular characteristics of PTB remain unclear. FKBP51 is an important mediator of the stress response and is implicated in the etiology of stress-related disorders. By combining studies in humans and *Fkbp5*$^{-/-}$ mice, we show that maternal stress up-regulates FKBP51 levels and enhances binding of FKBP51 to progesterone receptors. FKBP51 binding to progesterone receptors reduces progesterone receptor activity by causing functional progesterone withdrawal that induces PTB. In contrast, *Fkbp5*$^{-/-}$ mice are resistant to maternal stress–induced PTB and exhibit prolonged gestation. These findings provide molecular insights into stress-related PTB, indicating the importance of the potential use of inhibitor(s) against FKBP51 to treat PTB in future studies.

Author contributions: O.G.-K., F.S., U.A.K., and C.J.L. designed research; O.G.-K., N.S., X.G., K.L., A.O., S.A., D.M., C.N., B.S., I.B., C.B., and U.A.K. performed research; O.G.-K. contributed new reagents/analytic tools; O.G.-K., N.S., X.G., K.L., A.O., S.A., D.M., C.N., B.S., U.A.K., and C.J.L. analyzed data; and O.G.-K., N.S., I.B., C.B., F.S., U.A.K., and C.J.L. wrote the paper.

The authors declare no competing interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2010282118/-/DCSupplemental.

Published March 8, 2021.

PTB compared to nonlaboring gestational age (GA)-matched controls (22, 24). In support of these in situ findings, our in vitro analysis revealed that primary third trimester decidual cell cultures display reduced *PGR* messenger RNA (mRNA) and protein levels in response to interleukin 1β or thrombin, the major molecular mediators of chorioamnionitis and abruption, respectively (22, 24). Collectively, these results indicate that decidual cells are the central target of P4 signaling and that decidual nuclear PR levels are critical in maintaining human pregnancy with a substantial decrease in PR expression inducing human parturition by causing functional P4 withdrawal.

The FK506-binding proteins, FKBP51 and 52, are immunophilins that assemble as cochaperones with heat shock protein 90 and p23 as well as steroid receptors including glucocorticoid receptor (GR) (25, 26) and PR (27). FKBP52 enhances, whereas FKBP51 attenuates PR- and GR-mediated transcription (25, 26). Robust upregulation of FKBP51 levels by ligand-activated GR, and to a lesser extent by ligand-activated PR, is well established (28–31). Our laboratory recently found (31) that 1) in cultured primary human decidual cells, FKBP51, but not FKBP52 levels, are up-regulated by either dexamethasone, a pure glucocorticoid, or medroxyprogesterone acetate, a mixed progestin-glucocorticoid (32, 33), whereas Organon 2058, a pure progestin agonist exerted minimal stimulatory effects; 2) overexpression of *FKBP5* resulted in inhibition of PR binding to progesterone response elements (PRE); and 3) at the maternal–fetal interface, FKBP51 is predominantly expressed in decidual cell nuclei with increased expression in term decidual cells among patients in labor compared with nonlaboring controls (31). Taken together, these findings provide an additional mechanism of functional P4 withdrawal via FKBP51-mediated inhibition of PR transcriptional activity.

As a stress-responsive protein, *FKBP5* gene polymorphisms are associated with increased FKBP51 expression levels and are linked to depression and stress-related disorders (34), known risk factors for PTBs (8). Depletion of *Fkbp5* in mice protects against stress-induced hormonal changes as well as depressive and anxiety-like behaviors (35), indicating FKBP51 as a promising new target for stress-related disorders. Therefore, we hypothesize that stress-induced increased FKBP51 expression promotes functional P4 withdrawal by inhibiting PR transcriptional activity to cause PTB. Conversely, depletion of *Fkbp5* prevents PTB in response to maternal stress. To test this hypothesis, 1) FKBP51 and PR levels were measured in decidual specimens collected from women with iPTB and in GA-matched controls, 2) the physical interaction between FKBP51 and PR was assessed in these samples, and 3) the effect of *Fkbp5* deletion was evaluated in a murine model of stress-induced PTB.

Results

Enhanced Decidual Expression of FKBP51 mRNA and Protein in iPTB. We previously showed decreased PR and increased FKBP51 expression in the nuclei of decidual cells in placental sections obtained from laboring versus nonlaboring women at term (20, 31), suggesting a contributing role for FKBP51 in mediating decidua-specific functional P4 withdrawal during parturition. To investigate if these molecular changes also occur in decidua obtained from iPTB specimens, we assessed *FKBP5* and *PGR* expression by qPCR in decidua basalis specimens obtained from women with iPTB compared with two control groups: 1) negative control—GA-matched women with elective PTB for placenta accreta (PA) or indicated PTB for preeclampsia (PE) or 2) positive control—women in labor at term. Decidual *FKBP5* mRNA levels were similarly elevated in both term labor (mean ± SEM: 4.01 ± 0.68) and iPTB (7.22 ± 2.01) specimens, and both were significantly higher than *FKBP5* mRNA levels found in PA (1.18 ± 0.22) or PE samples (1.98 ± 0.21; Fig. 1*A*). *PGR* mRNA levels were similarly decreased in term labor (0.50 ± 0.07) and iPTB (0.83 ± 0.11) specimens, and *PGR* mRNA expression term labor specimens were significantly lower compared with PA (1.12 ± 0.24) or PE (1.37 ± 0.44) specimens (Fig. 1*B*), whereas *PGR* mRNA levels in iPTB specimens (0.83 ± 0.11) trended lower without attaining statistical significance (Fig. 1*B*). The primers used to detect *PGR* mRNA levels recognized both *PGR* isoforms *A* and *B*; therefore, we performed another qPCR using the primer set that recognized only *PGR-B* levels, and the same pattern of mRNA expression was observed that compared with GA control (PA/PE) specimens. *PGR-B* mRNA levels were significantly lower in term but not in iPTB specimens (*SI Appendix*, Fig. S1), suggesting that PR isotype switching does not occur in decidual cells with labor.

To confirm that changes in *FKBP5* and *PGR* mRNA levels reflect parallel changes in protein expression, sections obtained from GA-matched patients with PA- or PE-associated PTBs and iPTBs were evaluated for FKBP51 and PR protein levels by immunohistochemistry. Double immunostaining for vimentin, to identify decidual cells, and either anti-FKBP51 or anti-PR antibodies was performed. Histological score (H-score) analysis detected significantly higher FKBP51 immunoreactivity in decidual cells from iPTB (242.92 ± 7.30) versus both PA (144.64 ± 18.29) or PE (168.16 ± 20.82) specimens (Fig. 1 *C* and *E*). Consistent with the mRNA findings, reductions in PR immunoreactivity in iPTB versus PA or PE specimens did not attain statistical significance (Fig. 1 *D* and *F*).

Increased Decidual Cell Nuclear FKBP51-PR Binding in iPTB Specimens. To detect direct interaction between FKBP51 and PR proteins, we performed in situ proximity ligation assays (PLA) in decidua basalis specimens from women with iPTB versus GA-matched women with PA- or PE-associated PTBs. Again, vimentin immunofluorescence staining was used to identify decidual cells in serial sections. We observed PLA-positive signaling, indicating an interaction between FKBP51 and PR, primarily located in the nucleus of decidual (vimentin positive) cells (Fig. 2*A* and *SI Appendix*, Fig. S2*A*) but not in trophoblasts (*SI Appendix*, Fig. S2*B*). No signal was detected in decidual cells used as a negative control by withholding primary antibodies (*SI Appendix*, Fig. S2*C*). Computer-based quantification of PLA-positive signals revealed that the number of PLA signals was significantly higher in iPTB (22.42 ± 3.46) versus GA-matched PE (10.07 ± 2.15) or PA specimens (9.85 ± 4.11; Fig. 2 *A* and *B*).

***Fkbp5* Deficiency Prolongs Normal Gestation and Counteracts Maternal Stress–Induced PTB.** To assess the role of *Fkbp5* in the onset of parturition at term as well as stress-induced PTB, we employed *Fkbp5* knockout ($Fkbp5^{-/-}$) and wild-type ($Fkbp5^{+/+}$) mice subjected to either a physiologic unrestrained normal state (NST) or maternal restraint-induced stress (ST). First, $Fkbp5^{+/+}$ and $Fkbp5^{-/-}$ female mice were mated with adult $Fkbp5^{+/+}$ male mice to examine the relative contribution of maternal genotype to gestational length under unrestrained or restrained conditions. Restraints were applied three times/day for 60 min starting at embryonic day 8 (E8) through E18. We measured plasma corticosterone and cortisol as biomarkers of chronic and acute stress, respectively (36), at E11. As anticipated, maternal stress increased serum corticosterone and cortisol levels by 3.8- and 8.4-fold, respectively, in $Fkbp5^{+/+}$ mice and 2.3- and 5.7-fold, respectively, in $Fkbp5^{-/-}$ mice (*SI Appendix*, Fig. S3 *A* and *B*). In unrestrained mice, gestational length was significantly prolonged in $Fkbp5^{-/-}$ (20.25 ± 0.29 d) versus $Fkbp5^{+/+}$ mice (19.22 ± 0.19 d; Fig. 3*A*). While maternal stress significantly reduced gestational length in $Fkbp5^{+/+}$ (18.74 ± 0.03) as well as in $Fkbp5^{-/-}$ (19.48 ± 0.19) mice, stress-associated gestational length was significantly longer in $Fkbp5^{-/-}$ versus $Fkbp5^{+/+}$mice (Fig. 3*A*).

To examine the net contribution of both maternal and fetal $Fkbp5^{-/-}$ genotype to gestational length, $Fkbp5^{-/-}$ female mice

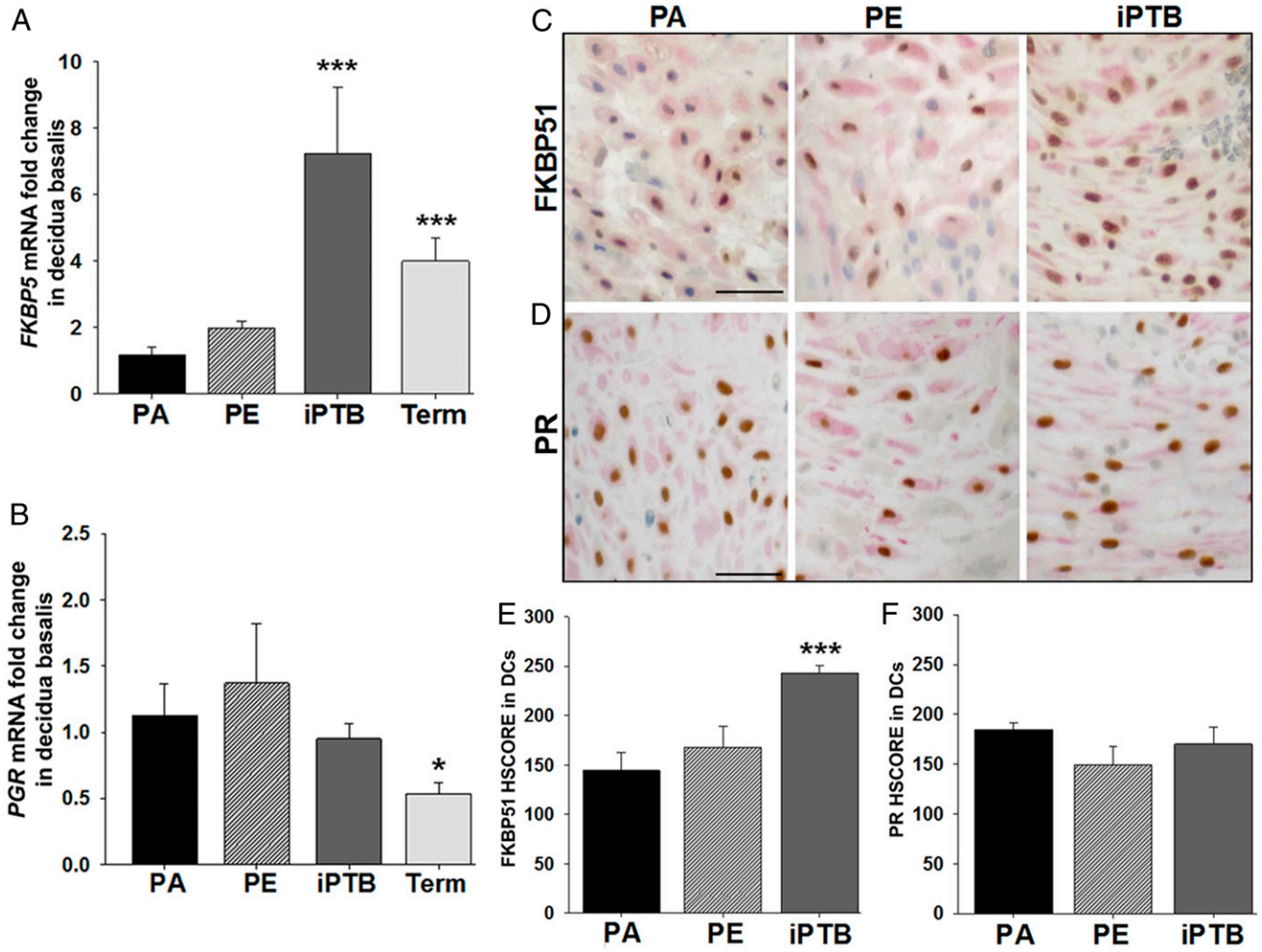

Fig. 1. Decidual cells at the maternal–fetal interface from idiopathic preterm birth display enhanced *FKBP5* mRNA and protein levels. (*A* and *B*) Decidual mRNA levels of *FKBP5* (*A*) and *PGR* (*B*) in PA ($n = 7$), PE ($n = 7$), iPTB ($n = 9$), and term specimens ($n = 10$). Data represent mean ± SEM; ***$P < 0.001$ versus PA or PE specimens and *$P < 0.05$ versus PA or PE specimens by one-way ANOVA with Dunn's method. (*C* and *D*) Double immunostaining for either FKBP51 (*C*, brown) or PR (*D*, brown) with vimentin (red) is displayed for decidual cells at the maternal–fetal interface in PA ($n = 8$), PE ($n = 8$), and iPTB ($n = 9$) specimens. (Scale bars: 30 μm.) (*E* and *F*) H-scores for either FKBP51 (*E*) or PR (*F*) nuclear immunoreactivity are presented in decidual cells (DCs). Bars represent mean ± SEM; ***$P < 0.001$ versus PA or PE specimens by one-way ANOVA with Student–Newman–Kuels method.

were also mated with adult *Fkbp5*$^{-/-}$ male mice, and pregnant mice were maintained under unrestrained and restrained conditions. In the absence of stress, *Fkbp5*$^{-/-}$ female mice mated with *Fkbp5*$^{-/-}$ males did not display longer gestations compared with in *Fkbp5*$^{-/-}$ females mated with *Fkbp5*$^{+/+}$ males (20.31 ± 0.24 versus 20.25 ± 0.29 d). However, restrained *Fkbp5*$^{-/-}$ mice mated with *Fkbp5*$^{-/-}$ males (19.93 ± 0.25) displayed longer gestations compared to restrained *Fkbp5*$^{-/-}$ females mated with *Fkbp5*$^{+/+}$ males. Moreover, there was no significant difference in gestational length among unrestrained versus restrained *Fkbp5*$^{-/-}$ mice mated with *Fkbp5*$^{-/-}$ males ($P = 0.18$; Fig. 3*A*). Collectively, these results indicate that 1) *Fkbp5*$^{-/-}$ mice have longer gestation than *Fkbp5*$^{+/+}$ mice, 2) maternal restraint stress induces PTB, and 3) *Fkbp5*$^{-/-}$ female mice mated with *Fkbp5*$^{-/-}$ males display maximal resistance to stress-induced PTB. While restraint stress elevated glucocorticoid levels (Fig. 3*B*) and shortened gestation in wild-type females and *Fkbp5*$^{-/-}$ females mated with *Fkbp5*$^{+/+}$ males (Fig. 3*A*), pharmacological doses of both corticosterone (CORT) and dexamethasone (DEX) did not shorten gestation in any of these mouse genotypes (Fig. 3 *C* and *D*).

We also evaluated whether either maternal stress or *Fkbp5*$^{-/-}$ deficiency affected litter size or gender ratio and found that pup numbers at parturition (*SI Appendix*, Fig. S4*A*) as well as the sex ratio of the pups did not differ among genotypes. The number of stillbirths also did not differ among groups (*SI Appendix*, Fig. S4*A*). Previous studies demonstrated that *Fkbp5*$^{-/-}$ mice have reduced body weights compared to wild-type littermates and that they are resistant to diet-induced obesity (37, 38). Therefore, we measured maternal weight gain as well as food and water intake during pregnancy under normal or stress conditions. Maternal weight gain did not differ among groups between E0 through E18 (*SI Appendix*, Fig. S4*B*). Moreover, no significant difference was observed in food consumption and water intake throughout gestation among the groups (*SI Appendix*, Fig. S4 *C* and *D*).

Maternal Stress–Induced PTB Is Not Associated with Systemic P4 Withdrawal. Luteal regression precedes labor in mice accompanied by a reduction in serum P4 levels (39). Thus, serum P4 levels were measured in the final 72 h of gestation in all groups by enzyme-linked immunosorbent assay. Both unrestrained and restrained *Fkbp5*$^{+/+}$ mice displayed similar serum P4 levels between E16 to E18, with a dramatic decrease on E18.25 (Fig. 4*A*).

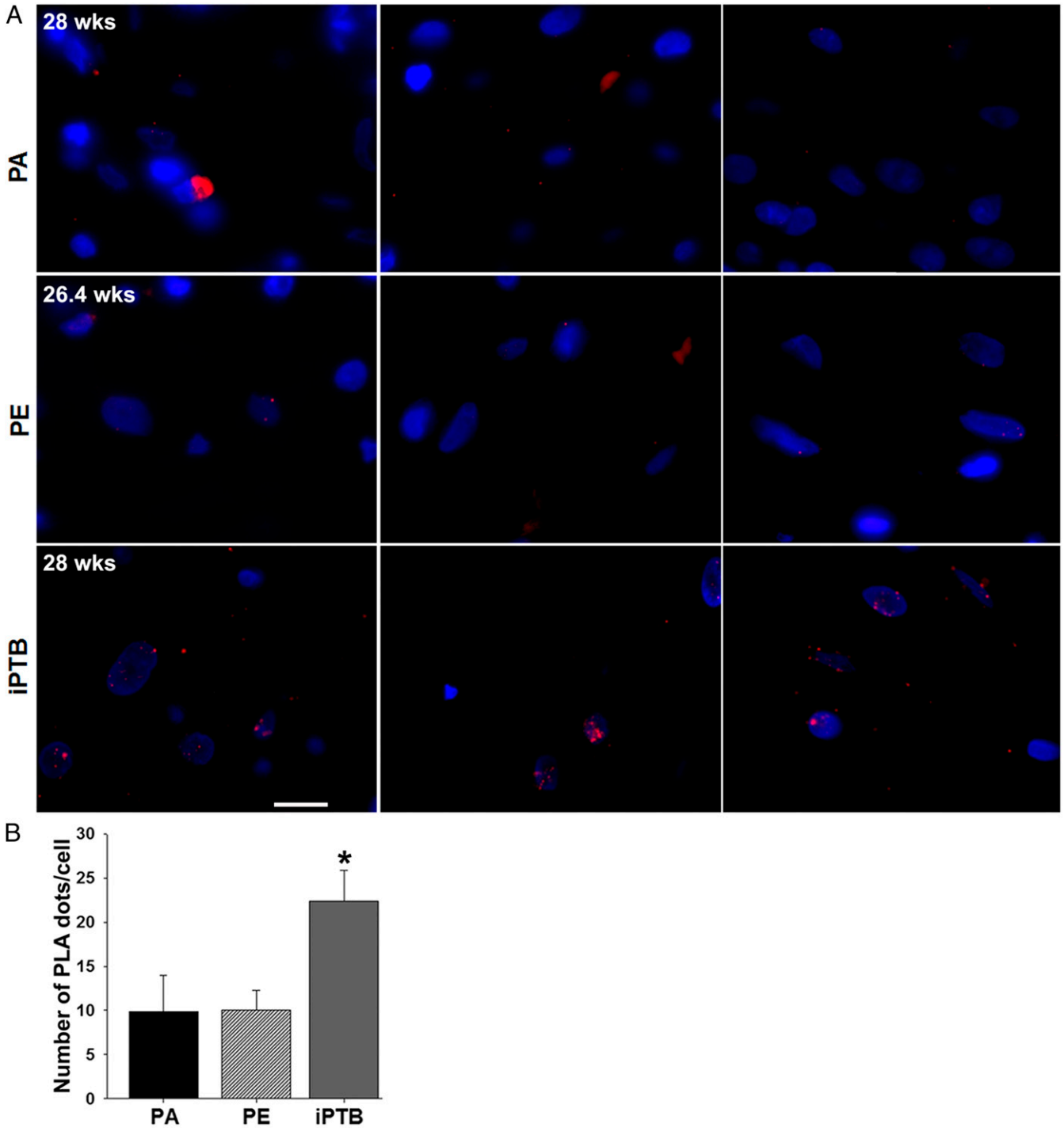

Fig. 2. Increased FKBP51 and PR interactions in nuclei of decidual cells in iPTB specimens. (*A*) Representative images of in situ PLA in decidua basalis specimens from women with iPTB versus GA-matched women with PA or PE between 28 and 34 gestational weeks. PLA signals (red) represent the interaction of FKBP51 with PR. DAPI (blue) is used for nuclear staining. (Scale bar: 20 μm.) (*B*) Higher numbers of PLA signals per cell are detected in iPTB versus PA or PE. Data represent mean ± SEM, $n = 6$/each group; $^*P < 0.05$ versus PA or PE specimens by one-way ANOVA followed by Student–Newman–Kuels method.

The decline in serum P4 levels in *Fkbp5*$^{-/-}$ mice was similar to *Fkbp5*$^{+/+}$ mice on E17 and E18 but not as great as in unrestrained *Fkbp5*$^{+/+}$ mice on E19. The increased rate of decline of serum P4 levels were not different between either restrained *Fkbp5*$^{+/+}$ versus unrestrained *Fkbp5*$^{+/+}$ or restrained *Fkbp5*$^{-/-}$ versus unrestrained *Fkbp5*$^{-/-}$ (Fig. 4*A*). Thus, systemic P4 withdrawal does not mediate maternal restraint-induced PTB. Further analysis of serum P4 levels according to time to delivery also supported the lack of an association between systemic P4 decline and maternal stress–induced PTB (Fig. 4*B*).

Ovarian luteal histology was evaluated followed by hematoxylin and eosin staining and the size and appearance of the corpus lutea were comparable among groups at E18.25 (*SI Appendix*, Fig. S5*A*). We also evaluated ovarian expression levels of enzymes involved in either P4 synthesis or metabolism, including steroidogenic acute regulatory protein (*Star*), cytochrome P450

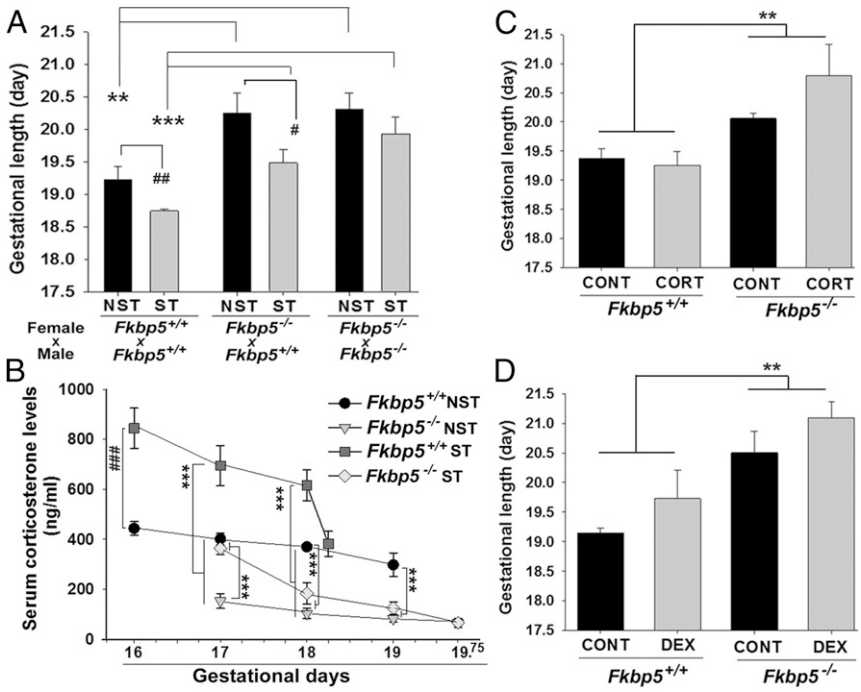

Fig. 3. Prolonged gestation and resistance to maternal stress–induced PTB in *Fkbp5*$^{-/-}$ mice. (*A*) Gestational length in *Fkbp5*$^{+/+}$ and *Fkbp5*$^{-/-}$ mice mated with either *Fkbp5*$^{+/+}$ *or Fkbp5*$^{-/-}$ male mice under NST or ST. Maternal stress reduces gestational length in *Fkbp5*$^{+/+}$ and *Fkbp5*$^{-/-}$ female mated with *Fkbp5*$^{+/+}$ male but not in *Fkbp5*$^{-/-}$ female mated with *Fkbp5*$^{-/-}$ male mice (n = 11/each, n = 13 for *Fkbp5*$^{-/-}$ × *Fkbp5*$^{-/-}$ mice). Data represent mean ± SEM; $^{\#\#}P < 0.01$ with Mann–Whitney *U* test; $^{\#}P < 0.05$ with *t* test; $^{**}P < 0.01$ and $^{***}P < 0.001$ with one-way ANOVA. (*B*) Serum corticosterone levels in *Fkbp5*$^{+/+}$ versus *Fkbp5*$^{-/-}$ mice at indicated day of gestation under NST or ST conditions. Mean ± SEM; n = 5/each group; $^{\#\#\#}P < 0.001$ with *t* test; $^{***}P < 0.001$ with one-way ANOVA followed by Student–Newman–Keuls test. (*C* and *D*) Gestational length in vehicle (control, CONT; n = 7) or CORT (n = 6; *C*) and vehicle (CONT; n = 7) or DEX (n = 7; *D*) administrated *Fkbp5*$^{+/+}$ or *Fkbp5*$^{-/-}$ mice. Data represent mean ± SEM; $^{**}P < 0.01$ with one-way ANOVA followed by Student–Newman–Keuls test.

family 11 subfamily a member 1 (*Cyp11a1*), and hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 2 (*Hsd3b2*), to represent key P4 synthetic enzymes as well as aldo-keto reductase family 1, member C18 (*Akr1c18;* 20 α-hydroxysteroid dehydrogenase, 20α-HSD), an enzyme that inactivates P4 by converting it to 20-α-dihydroprogesterone. Comparisons by qPCR analysis revealed similar ovarian expression levels of *Cyp11a1* and *Hsd3b2* mRNA among all groups at either E17.25 or E18.25 (*SI Appendix*, Fig. S5*B*), whereas a significant decrease in *Star* expression occurred in all groups at E18.25 versus E17.25 (Fig. 4*C*). Interestingly, the ovaries of unrestrained or restrained *Fkbp5*$^{-/-}$ mice displayed lower *Akr1c18* expression levels than either unrestrained or restrained *Fkbp5*$^{+/+}$ mice at both E17.25 and E18.25. However, *Akr1c18* levels were not significantly different between unrestrained and restrained *Fkbp5*$^{+/+}$ mice at E18.25 (Fig. 4*C*). Remarkably, comparison of ovarian and uterine expression of *Akr1c18* mRNA levels in unrestrained *Fkbp5*$^{+/+}$ mice revealed 79.6-fold higher *Akr1c18* levels in the uterus at E17.25 (*SI Appendix*, Fig. S6*A*). A similar pattern was also observed for restrained *Fkbp5*$^{+/+}$ or unrestrained *Fkbp5*$^{-/-}$ or restrained *Fkbp5*$^{-/-}$ mice at E17.25 (*SI Appendix*, Fig. S6*A*), indicating that local P4 metabolism is predominately initiated in the uterus at E17.25 and occurs subsequently in the ovary at E18.25.

Maternal Stress–Induced FKBP51 and Reduced Uterine PR Expression. A similar pattern of decline in serum P4 levels between restrained and unrestrained mice coupled with the predominant *Akr1c18* mRNA expression in uterine tissues led us to posit that functional P4 withdrawal and/or increased local P4 metabolism in uterine tissues contribute(s) to maternal stress–induced PTB. To test this hypothesis, we measured uterine expression of *Fkbp5* and *Fkbp4*, *Pgr* and P4 metabolizing enzymes *Akr1c18* and *Srd5a1* (5α-reductase, which metabolizes P4 to 5α-pregnane-3,20-dione and is primarily expressed in mouse cervix but also in the uterus) (40, 41). Consistent with our findings in human decidua (Fig. 1), uteri of unrestrained *Fkbp5*$^{+/+}$ mice displayed a significant increase in *Fkbp5* levels at E19 versus E17.25 or E18.25 (Fig. 5*A*), and maternal stress significantly elevated *Fkbp5* levels in *Fkbp5*$^{+/+}$ mice at both E17.25 and E18.25 compared with unrestrained *Fkbp5*$^{+/+}$ mice (Fig. 5*A*). In contrast to these findings for *Fkbp5* expression, levels of *Fkbp4*, which encodes FKBP52 protein, revealed no significant changes among the groups (*SI Appendix*, Fig. S6*B*), indicating no contribution of FKBP52 levels to maternal stress–induced PTB. Immunohistochemical analysis of decidual tissues from uterine sections confirmed increased FKBP51 protein levels in restrained versus unrestrained *Fkbp5*$^{+/+}$ mice (Fig. 5*B*). Maternal stress also significantly down-regulated *Pgr* mRNA levels in uterine tissues of *Fkbp5*$^{+/+}$ mice but not in *Fkbp5*$^{-/-}$ mice on both E17.25 and E18.25 (Fig. 5*C*). H-score analysis of immunostained uterine sections confirmed significantly lower PR protein levels in decidual cell nuclei in restrained *Fkbp5*$^{+/+}$ mice versus all other groups at both E17.25 and E18.25 (Fig. 5*D*). Significantly lower PR protein levels in decidual cell nuclei were detected in unrestrained *Fkbp5*$^{+/+}$ mice versus unrestrained or restrained *Fkbp5*$^{-/-}$ mice at E17.25. Moreover, at E18.25, restrained *Fkbp5*$^{-/-}$ mice displayed significantly higher PR protein levels than unrestrained or restrained *Fkbp5*$^{+/+}$ mice.

To evaluate the potential contribution of local uterine P4 metabolism to maternal stress–induced PTB, we assessed expression levels of *Akr1c18* and *Srd5a1*. Deficiency of either gene has been shown to prolong gestation independent of systemic P4 levels in mice (40, 42). Uterine *Srd5a1* expression was similar among all groups on either E17.25 or E18.25 (*SI Appendix*, Fig. S6*C*). In contrast, uterine *Akr1c18* levels were significantly up-regulated

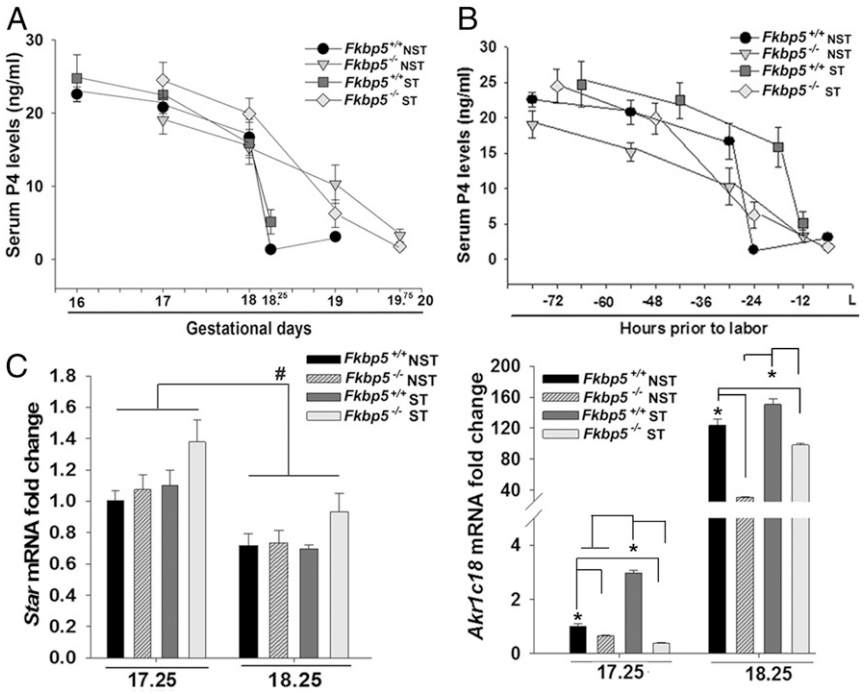

Fig. 4. Progesterone withdrawal is not impaired in *Fkbp5*$^{-/-}$ mice. (*A* and *B*) Serum progesterone (P4) levels in *Fkbp5*$^{+/+}$ and *Fkbp5*$^{-/-}$ mice for either days of gestation (*A*) or hours prior to delivery (*B*). Data represent mean ± SEM; n = 5/each, E16 analyzed by t test, others by one-way ANOVA with Student–Newman–Keuls test. (*C*) Ovarian expression levels of *Star* and *Akr1c18* mRNA in *Fkbp5*$^{+/+}$ and *Fkbp5*$^{-/-}$ mice under NST or ST conditions at E17.25 and E18.25. Data represent mean ± SEM; n = 4/each; $^{\#}P < 0.05$ versus *Star* mRNA levels of corresponding genotype at E17.25 by t test. $^{*}P < 0.05$ for *Akr1c18* mRNA levels with one-way ANOVA followed by Student–Newman–Keuls test.

in the uteri of restrained *Fkbp5*$^{+/+}$ mice compared to the other three groups at E17.25. At E18.25, uterine *Akr1c18* expression levels are similar in restrained *Fkbp5*$^{+/+}$ and restrained *Fkbp5*$^{-/-}$ mice and significantly higher than in unrestrained *Fkbp5*$^{+/+}$ and *Fkbp5*$^{-/-}$ mice (Fig. 6*A*). Immunohistochemical staining displays both nuclear and cytoplasmic localization of AKR1C18 protein in decidual cells in mice. H-score analysis of immunostained uterine sections confirmed significantly elevated AKR1C18 protein levels in the nuclei of decidual (Fig. 6*B*) and myometrial (*SI Appendix*, Fig. S7) cells of restrained *Fkbp5*$^{+/+}$ mice versus all other groups at both E17.25 and E18.25. To confirm that elevated AKR1C18 expression corelates with local reduction in P4 levels, H-score analysis was performed in uterine sections immunostained for P4. At E17.25, nuclear P4 levels are similar among all groups in decidua but significantly lower at E18.25 in unrestrained *Fkbp5*$^{+/+}$ and restrained *Fkbp5*$^{+/+}$ versus unrestrained *Fkbp5*$^{-/-}$ or restrained *Fkbp5*$^{-/-}$ mice (Fig. 7*A*), and among wild-type mice, there was a significant reduction observed in decidua of restrained *Fkbp5*$^{+/+}$ versus unrestrained *Fkbp5*$^{+/+}$ mice. However, restraint had no effect on nuclear P4 staining in either restrained or unrestrained *Fkbp5*$^{-/-}$ mice (Fig. 7*A*).

Signal transducer and activator of transcription 5b (*Stat5b)* deficiency shortens gestational length in mice by prematurely inducing AKR1C18 levels (43). Prolactin binding to its receptor in decidual cells activates STAT5B signaling to inhibit AKR1C18 expression preventing P4 metabolism, thereby contributing to the physiologic maintenance of pregnancy (44–46). Thus, we evaluated uterine *Stat5b* (Fig. 7*B*) and prolactin receptor (*Prlr*) expression (Fig. 7*C*) and found that *Stat5b* levels are significantly lower in unrestrained *Fkbp5*$^{+/+}$ and restrained *Fkbp5*$^{+/+}$ versus unrestrained *Fkbp5*$^{-/-}$ or restrained *Fkbp5*$^{-/-}$ mice at E18.25 (Fig. 7*B*), whereas *Prlr* mRNA levels did not change among the groups at either E17.25 or 18.25 (Fig. 7*C*). Finally, evaluation of expression of oxytocin receptor (*Oxtr*), which induces myometrial contraction, revealed significantly higher levels in unrestrained *Fkbp5*$^{+/+}$ and restrained *Fkbp5*$^{+/+}$ versus unrestrained *Fkbp5*$^{-/-}$ or restrained *Fkbp5*$^{-/-}$ mice at E18.25, with a significant increase in all groups at E18.25 versus E17.25 (Fig. 7*D*).

Discussion

Despite the initial promise that P4 therapy decreased recurrence risk of PTB as well as the risk of PTB among patients with a short cervix, recent large-randomized trials have not confirmed its efficacy (47–50). The failure of such therapy, coupled with observations that PR antagonist therapy ± prostaglandins induce labor at all gestational ages (51), indicates that functional P4 withdrawal mediated at the level of the PR triggers human labor. This hypothesis is further supported by our previous findings that term labor is associated with reduced decidual PR and increased decidual FKBP51 expression (20, 31) and that chorioamnionitis and abruption, major etiologies of early PTB, are associated with reduced PR expression (22, 24). We now demonstrate that idiopathic and stress-associated PTB, major causes of late PTB, are also triggered by functional P4 withdrawal, albeit mediated through increased FKBP51 expression.

Prior studies demonstrated that glucocorticoid exposure significantly upregulates decidual cell *FKBP51* mRNA and protein expression (28, 31). Moreover, overexpression of *FKBP5* inhibits PR binding to its canonical DNA response element in decidual cells (31), while PR binding to PRE is increased in decidual cells in which *FKBP5* has been silenced (31). Taken together, these findings suggest that rising fetal, placental, and/or maternal glucocorticoid levels prior to both term and iPTB trigger functional P4 withdrawal resulting from increased decidual cell FKBP51 expression. Significantly enhanced FKBP51-PR interaction in human decidual cell nuclei of iPTB specimens as revealed by PLA analysis in the current study provides further evidence for this pathway of functional P4 withdrawal causing iPTB.

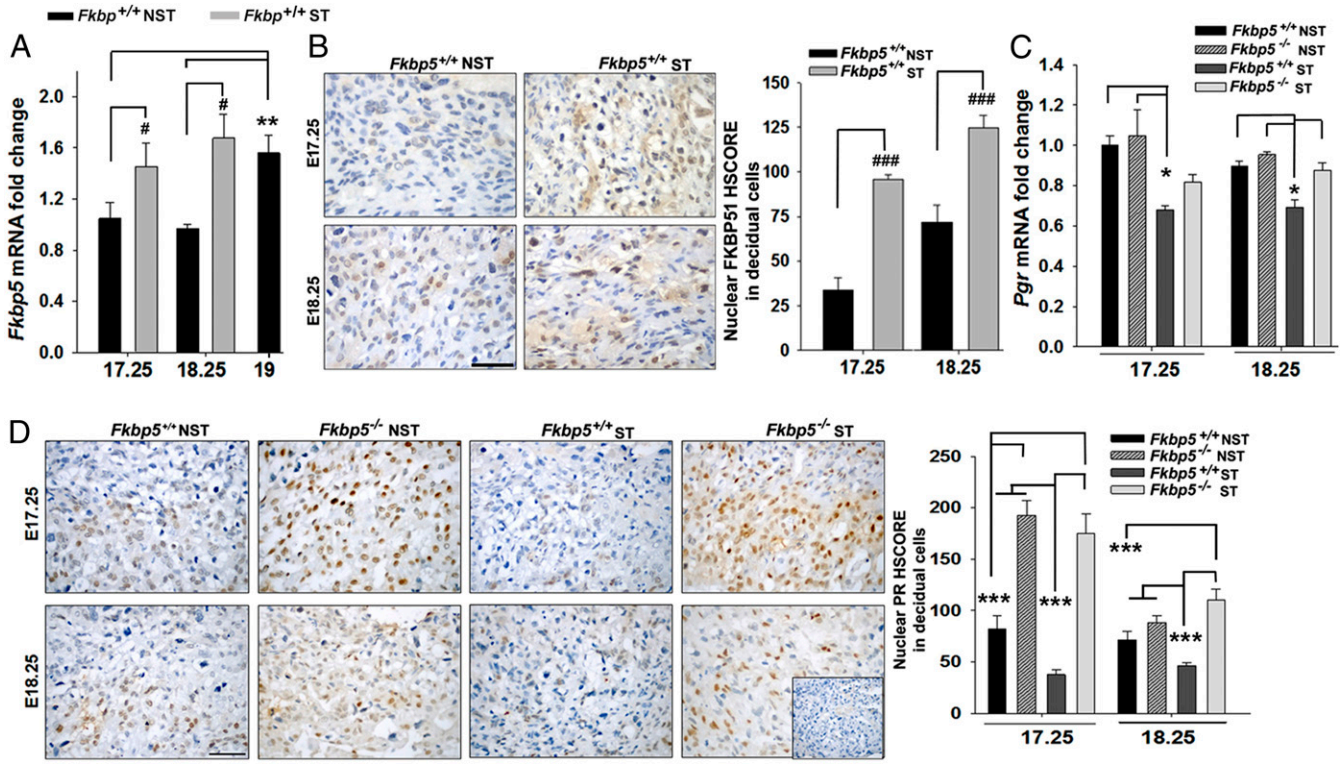

Fig. 5. Increased FKBP51 and reduced progesterone receptor expression in maternal stress–induced *Fkbp5*$^{+/+}$ mice. (*A*) *Fkbp5* mRNA expression in uterine tissues from *Fkbp5*$^{+/+}$ mice under NST or ST conditions collected at E17.25 and E18.25 as well as E19 for only NST-*Fkbp5*$^{+/+}$ mice. Bars represent mean ± SEM; n = 5/each; $^{\#}P < 0.05$ by t test and $^{**}P < 0.01$ by one-way ANOVA with Student–Newman–Keuls test. (*B*) Nuclear FKBP51 immunoreactivity (brown) and H-score for FKBP51 expression in decidual cells in NST-*Fkbp5*$^{+/+}$ versus ST-*Fkbp5*$^{+/+}$ mice at E17.25 and 18.25. Bars represent mean ± SEM; n = 6/each; $^{\#\#\#}P < 0.001$ by t test. (*C*) *Pgr* mRNA expression in uterine tissues from *Fkbp5*$^{+/+}$ and *Fkbp5*$^{-/-}$ mice under NST or ST conditions at E17.25 and E18.25. Bars represent mean ± SEM; n = 4/each; $^{*}P < 0.05$ with one-way ANOVA followed by Student–Newman–Keuls test. (*D*) Representative images of PR immunostaining (brown) in decidual cells and nuclear decidual PR H-scores of *Fkbp5*$^{+/+}$ and *Fkbp5*$^{-/-}$ mice under NST or ST conditions at E17.25 and 18.25. (Scale bar: 20 μm.) Inset picture represents negative control staining. Bars represent mean ± SEM; n = 6/each. $^{***}P < 0.001$ by one-way ANOVA with Student–Newman–Keuls test.

Our current and previous in situ and in vitro results obtained from human decidual cells (28, 31) led us to employ an *Fkbp5* knockout mouse to define the in vivo function of FKBP51 on gestational length as well as its role in maternal stress–induced PTB. This study demonstrates prolonged gestation in *Fkbp5*$^{-/-}$ mice, indicating that FKBP51 plays a crucial role on gestational length and/or initiation of parturition. Both maternal and embryonic *Fkbp5* gene expression contributes to stress-associated reductions in gestational length since the combination of maternal and embryonic *Fkbp5* deficiency prolongs gestation compared to maternal deficiency alone in restrained mice. Compared to *Fkbp5*$^{+/+}$ mice, lower levels of ovarian and uterine *Akr1c18* (20α-HSD) at E17.25 through E19 in *Fkbp5*$^{-/-}$ mice correlate with the delayed decline in serum P4 observed in these knockout mice and help account for their prolonged gestation. Furthermore, our results revealing higher *Pgr* mRNA and protein levels at E17.25 and lower *Oxtr* levels at E18.25 in uteri of *Fkbp5*$^{-/-}$ mice represent additional factors contributing to their prolonged gestation. Thus, a combination of prolonged ovarian P4 production, reduced local uterine P4 metabolism, and sustained PR activity likely explain the prolongation of gestation in *Fkbp5*$^{-/-}$ mice.

Progesterone-induced decidualization is a potent inducer of prolactin levels. Previous studies demonstrated that prolactin inhibits 20α-HSD expression by activating STAT5B signaling in both mice and rats (43, 44) and that *Stat5b* deficient mice cannot maintain pregnancy beyond E12 as a result of a dramatic decline in serum P4 levels (43, 52). Our results showing higher uterine *Stat5b* levels in *Fkbp5*$^{-/-}$ versus *Fkbp5*$^{+/+}$ mice at E18.25 are consistent with *Fkbp5* deletion preventing premature inhibition of P4 signaling and maintenance of prolactin stimulated STAT5B expression, helping to account for the pregnancy prolonging effects of *Fkbp5* deletion. Alternatively, higher decidual P4/PR levels in *Fkbp5*$^{-/-}$ mice may directly contribute to the higher *Stat5b* levels and thereby lower *Akr1c18* expression, since P4/PR were reported to directly enhance STAT5 expression, activation, and nuclear localization in breast cancer cells (53).

Previously, two groups reported that mice with *Akr1c18 (*20α-HSD*)* deficiency also display prolonged gestation (42, 43). Moreover, administration of RU486, a PR inhibitor, to *Akr1c18*-deficient mice at E19 resulted in normal parturition on the following day, although sufficient serum P4 levels were detected to sustain pregnancy. These findings indicate that, as it occurs in humans, functional P4 withdrawal mediated by PR inhibition can also trigger parturition in mice. Thus, our finding of ∼80-fold greater expression of *Akr1c18* in the uterus versus ovary at E17.25, coupled with increased uterine FKBP51 levels and decreased PR levels at E18.25 in wild-type mice, suggest that local uterine P4 metabolism coupled with reduced PR activity rather than luteolysis initiates normal parturition in mice. Indeed, luteolysis is more likely a consequence than a cause of labor in mice, reflecting increasing uterine prostaglandin production, consistent with the findings of Roizen et al. (54). Thus, murine parturition is analogous to human parturition since both species display decreased PR and increased FKBP51 expression in decidual cells (20, 31). Moreover, consistent with our present findings in the mouse, two groups have shown increased myometrial 20α-HSD levels in term (55, 56) and preterm (56) human parturition.

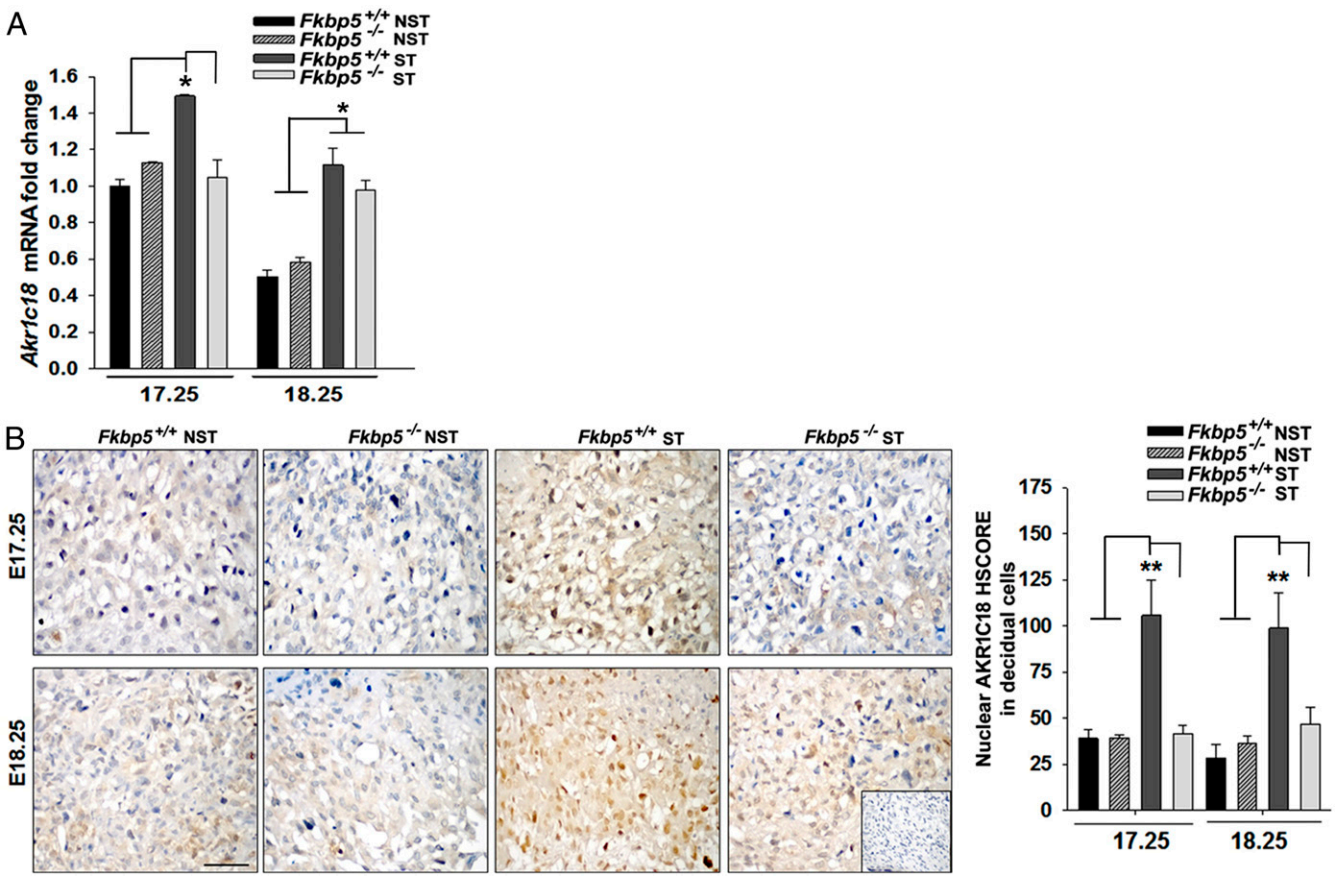

Fig. 6. Increased AKR1C18 expression in maternal stress–induced *Fkbp5*$^{+/+}$ mice. (*A*) *Akr1c18* mRNA expression in uterine tissues from *Fkbp5*$^{+/+}$ and *Fkbp5*$^{-/-}$ mice under NST or ST conditions at E17.25 and E18.25. Bars represent mean ± SEM; $n = 4$/each; $^{*}P < 0.05$ with one-way ANOVA followed by Student–Newman–Keuls test. (*B*) AKR1C18 immunoreactivity (brown) and H-scores in decidual cells at E17.25 and 18.25. Inset picture represents negative control staining. (Scale bar: 20 μm.) Bars represent mean ± SEM; $n = 7$/each at E17.25 and $n = 6$/each at E18.25; $^{**}P < 0.01$ by one-way ANOVA followed by Student–Newman–Keuls test.

Stressful circumstances directly stimulate the hypothalamus–pituitary–adrenal axis to promote glucocorticoid release (57). Glucocorticoids then bind to their receptor to initiate GR-mediated transcriptional induction of several genes, including up-regulation of *FKBP5* expression. Increased FKBP51 levels, in turn, lead to FKBP51 binding to GR, which generates an intracellular negative feedback loop to inhibit GR-mediated transcription, preventing excessive and persistent stress-induced GR responses (26, 57). Several studies reported strong evidence correlating *FKBP5* gene polymorphisms and/or increased FKBP51 expression with enhanced susceptibility to various psychiatric diseases including posttraumatic stress disorder, major depressive disorder, and anxiety (26, 57–61), all of which have been associated with PTB in humans. The current study demonstrates that maternal physiologic stress caused by immobilization of timed-pregnant wild-type mice increases uterine *Fkbp5* mRNA and protein expression at E17.25 and E18.25 and causes PTB. Moreover, combined maternal and fetal deficiency of the *Fkbp5* gene prevents such stress-mediated PTB. Consistent with our findings, Govindaraj et al. also showed that late restraint stress (three times/day and 45min/each) from E11 to parturition, but not early onset stress from E1 to E11, shortened gestation in pregnant rats, indicating that the restraint stress model is a reliable inducer of PTB among different rodent species (62). Moreover, while P4 levels decline earlier in both stressed and unstressed wild-type mice compared to stressed and unstressed *Fkbp5*$^{-/-}$ mice, stress-induced PTB does not appear to result from a premature decline in serum P4 (Fig. 4).

Glucocorticoid therapy in women with anti-phospholipid antibodies and recurrent fetal loss (63, 64) as well as rheumatoid arthritis (65) results in an increased rate of PTBs. However, we observed no decrease in gestational length in mice exposed to exogenous glucocorticoid therapy consistent with prior observations (66). We hypothesize that this reflects inhibition of uterine prostaglandin production preventing labor and impairing luteolysis. Thus, the elevated corticosterone levels observed in restrained *Fkbp5*$^{+/+}$ mice are likely insufficient to inhibit prostaglandin production but adequate to induce FKBP51. Our current results clearly show that uterine *Fkbp5* mRNA and protein levels are prematurely elevated in restrained *Fkbp5*$^{+/+}$ at E17, whereas this increase occurs physiologically at E19 in unrestrained *Fkbp5*$^{+/+}$ mice (Fig. 5 *A* and *B*). Thus, in wild-type mice, restraint stress induces both a 48-h earlier increase in *Fkbp5* together with a decrease in uterine *Pgr* mRNA and protein levels at E17.25, providing strong evidence that maternal stress initiates PTB by early inhibition of uterine PR signaling (Fig. 5 *C* and *D*).

Other potential mechanisms to account for the induction of PTB by restraint stress include increased uterine levels of *Srd5a1,* which metabolizes P4 to 5α-pregnane-3,20-dione and/or decreased expression of 15-hydroxyprostaglandin dehydrogenase (15-Hpgd), the main enzyme that catabolizes prostaglandin E_2 and $F_{2\alpha}$. While *Srd5a1*-deficient mice have only a 27% delivery

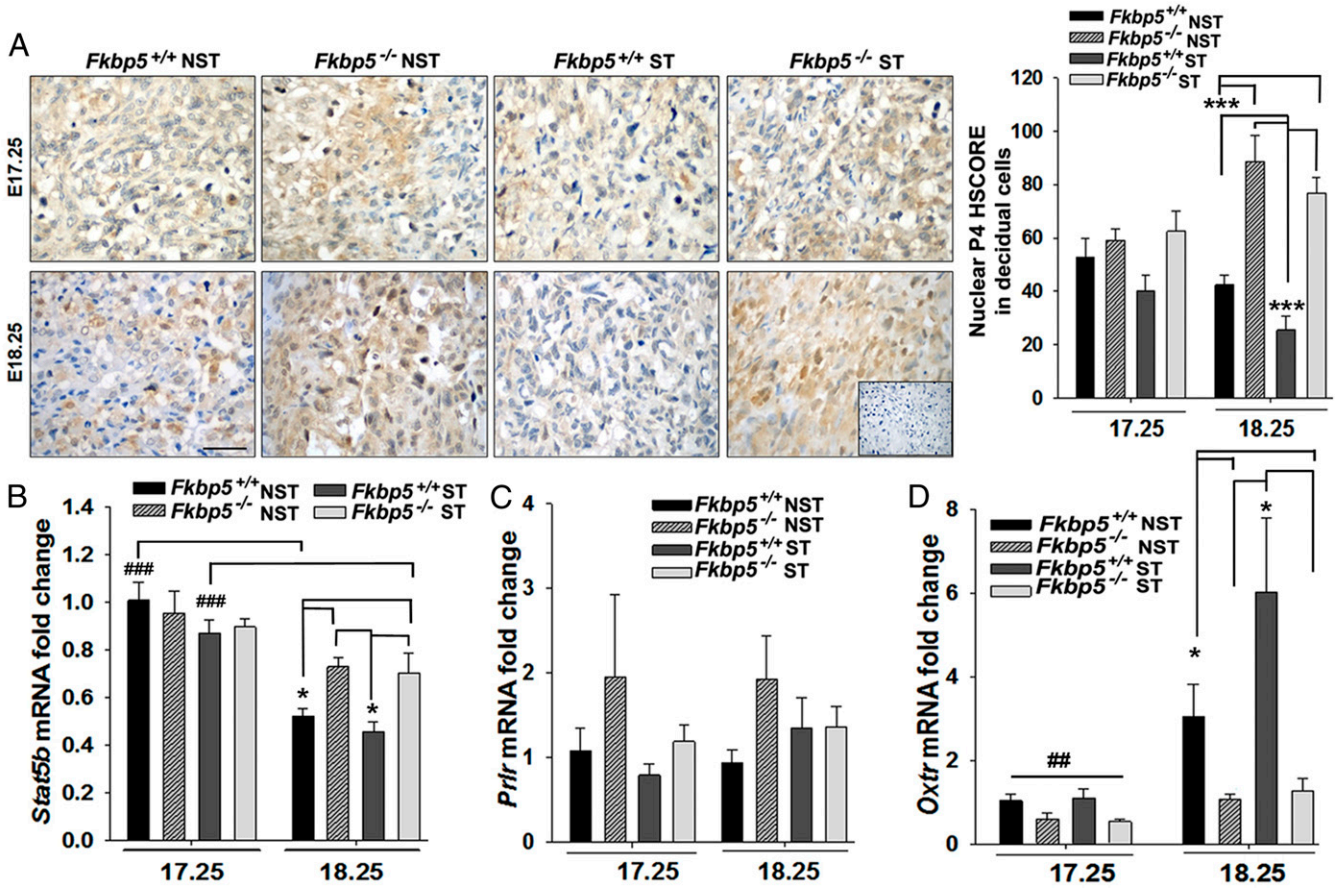

Fig. 7. Reduced progesterone (P4) and *Stat5b* levels and increased *Oxtr* levels in the uteri of *Fkbp5*$^{+/+}$ mice. (*A*) P4 immunostaining (brown) and nuclear P4 H-score levels in decidual cells in *Fkbp5*$^{+/+}$ and *Fkbp5*$^{-/-}$ mice under NST or ST conditions at E17.25 and E18.25. Inset displays negative control staining. (Scale bar: 20 μm.) Bars represent mean± SEM; $n = 6$/each. ***$P < 0.001$ by one-way ANOVA followed by Student–Newman–Keuls test. (*B–D*) Uterine mRNA levels of *Stat5b* (*B*) and *Prlr* (prolactin receptor; (*C*) as well as *Oxtr* (oxytocin receptor; (*D*) in *Fkbp5*$^{+/+}$ and *Fkbp5*$^{-/-}$ mice under NST or ST conditions at E17.25 and E18.25. Bars represent mean ± SEM, $n = 4$/each. ###$P < 0.001$ by *t* test; *$P < 0.05$ by one-way ANOVA followed by Student–Newman–Keuls test (*B*). ##$P < 0.01$ versus *Oxtr* levels in corresponding genotype at E18.25 by *t* test, and *$P < 0.05$ by one-way ANOVA with Student–Newman–Keuls test (*D*).

rate because of impaired cervical ripening, administration of the PR antagonists RU486 or ZK98299 elicits a 100% parturition rate (40). Moreover, *15-Hpgd* hypomorphic mice experience PTB without a decline in serum P4 levels [further evidence that luteolysis is not a cause of labor (54)]. However, since we did not detect alteration in the expression of uterine *Srd5a1* (*SI Appendix*, Fig. S6*C*) or *15-Hpgd* among these four groups, the current results do not support the involvement of *Srd5a1* or *15-Hpgd* in meditating maternal stress–induced PTB.

Further evidence of early inhibition of uterine progestational signaling resulting from restraint stress is indicated by increased mRNA and protein expression of uterine *Akr1c18* at E17.25 in restrained *Fkbp5*$^{+/+}$ mice. In contrast to wild-type mice, *Akr1c18* expression was significantly increased without changes in protein expression in restrained versus unrestrained *Fkbp5*$^{-/-}$ mice at E18.25 (Fig. 6 *A* and *B*). We hypothesize that FKBP51 may be an important inducer of AKR1C18 protein expression by enhancing translation and/or reducing degradation of stress-induced AKR1C18 protein expression, which require further studies to clarify such mechanisms. Consistent with this enhanced AKR1C18 expression, restraint stress significantly reduces P4 levels in decidual and myometrial cell nuclei in *Fkbp5*$^{+/+}$ mice at E18.25. This increased P4 metabolism likely elevates levels of unliganded PR and promotes PR-FKBP51 binding, leading to a further inhibition of PR-mediated transcriptional activity, which is supported by our PLA results in humans showing increased PR-FKBP51 interaction in iPTB specimens. These observations are also consistent with a recent study (56) showing that enhanced 20α-HSD expression reduces nuclear P4 levels, resulting in unliganded PR in the myometrium during mouse and human parturition. Furthermore, these findings are supported by reports indicating that in the absence of P4, FKBP51 exhibits higher PR affinity and that P4 switches FKBP51 binding in favor of FKBP52 to PR (67–70).

To further confirm this FKBP51-mediated mechanism, we compared expression of uterine *Oxtr*, which is physiologically suppressed by P4 and increased near parturition in all mammalian species, including primates and ruminants (71), and detected the highest *Oxtr* levels in the uteri of restrained *Fkbp5*$^{+/+}$ mice at E18.25, consistent with their earlier delivery. In contrast, *Fkbp5*$^{-/-}$ mice resist restraint-induced labor-related uterine changes by displaying higher *Pgr* expression and lower *Akr1c18* expression together with higher nuclear P4 levels and lower *Oxtr* levels. Thus, *Fkbp5*$^{-/-}$ mice are resistant to stress-induced functional P4 withdrawal in the uterus.

In conclusion, increased mRNA and protein expression of decidual *FKBP5* together with significantly enhanced nuclear FKBP51-PR interaction in decidual cells of iPTB specimens provide a novel mechanism for functional P4 withdrawal as a trigger of iPTB in humans. This study also reports that *Fkbp5*$^{-/-}$ mice display a prolonged gestational length, accompanying a slower decline in systemic P4 levels. That functional P4 withdrawal is delayed by

Fkbp5 deletion is indicated by lower *Oxtr* levels at E18.25 in $Fkbp5^{-/-}$ versus $Fkbp5^{+/+}$mice. While maternal restraint stress causes PTB in $Fkbp5^{+/+}$ mice by inducing uterine expression of *Akr1c18* and *Fkbp5* levels, resulting in increased local P4 metabolism and an enhanced FKBP51-PR interaction, which blocks PR-mediated transcriptional activity, maternal and fetal *Fkbp5* deletion renders mice completely resistant to maternal stress–induced PTB. Collectively, these results suggest that FKBP51 plays a pivotal role in both term and preterm parturition and that FKBP51 antagonists may prove to be a novel therapy to prevent iPTB.

Materials and Methods

Study Approval. Collection of placental specimens was approved by the University of South Florida Institutional Review Board (#19472). Written informed consent was received from patients prior to inclusion in the study and patients were de-identified. Breeding and all experimental procedures were conducted with prior approval of the Animal Care and Use Committee at the University of South Florida (USF #2235R and #3063M).

Collection of Human Tissues. Placental specimens containing decidua basalis were obtained from patients with iPTB ($n = 9$) or from patients undergoing cesarean delivery in the absence of labor due to PE ($n = 8$) or PA ($n = 8$) or from term placentas after the onset of labor ($n = 10$). The decidua basalis was obtained by dissecting basal plate from cotyledons of the placenta and by trimming excess villous tissues. Collected tissues were rinsed with phosphate-buffered saline (PBS) to remove blood. iPTB specimens displayed no clinical or histological evidence of either abruption or chorioamnionitis. Placental specimens obtained from patients with PE or PA served as GA-matched controls. The GA for iPTB specimens (mean ± SEM; 31.54 ± 0.74 wk) did not significantly differ from that of control PE or PA patients (31.10 ± 0.88 or 33.22 ± 0.31 wk, respectively, $P = 0.32$).

Animals. The $Fkbp5^{-/-}$ mice were generated and genotyped as described (72). Female and male $Fkbp5^{+/-}$ breeders, a mixed C57BL/6–129/SvJ background (kindly gifted by the late Chad Dickey, Byrd Alzheimer's Research Institute, University of South Florida), were house-mated to generate $Fkbp5^{+/+}$ and $Fkbp5^{-/-}$ littermates. Mice were housed in a temperature-controlled environment with 12 h light/dark cycles with ad libitum access to food and water.

Timing of Labor in $Fkbp5^{+/+}$ and $Fkbp5^{-/-}$ Mice. To examine the effect of deletion of the *Fkbp5* gene on birth timing, female $Fkbp5^{+/+}$ and $Fkbp5^{-/-}$ mice (6 to 8 wk old) were mated with an adult $Fkbp5^{+/+}$ male (12 to 16 wk old) by housing 1:1 for 4 h (9:00 AM to 1:00 PM). Pregnancy was confirmed by the presence of a vaginal plug or sperm in vaginal smears; gestational day (E) was designated at that time (10:00 AM) as 0 d post coitum of pregnancy. The timing of delivery was monitored from E18 through E21 by observing mice every 4 h upon completion of delivery, and gestational length was calculated. Litter size and numbers of live and dead pups were recorded. Additionally, to determine whether there was a relative contribution of fetal genotype on birth timing, female $Fkbp5^{-/-}$ mice were also mated with an adult male $Fkbp5^{-/-}$ mouse as described above, and the timing of labor was recorded.

Experimental Procedures. Time-mated pregnant $Fkbp5^{+/+}$ and $Fkbp5^{-/-}$ mice ($n = 11$) were randomly assigned into NST and ST groups. Restraint stress was administered in three sessions for 1 h using standard restraint chambers (1.5″ × 4″; Braintree Scientific, Inc.), starting on E8 through E18, while the control group was left undisturbed during the whole pregnancy. This time frame was chosen to eliminate the potential impact of maternal stress on implantation and on neuronal development. The stress schedule was varied day to day to reduce a possible habituation to restraint stress. Pregnant mice were weighed every 3 d, and water/food intake were measured weekly.

Maternal DEX or CORT Injection. DEX (Sigma-Aldrich) suspended in sterile PBS was administered intraperitoneally at 200 μg/kg (73). CORT (Sigma-Aldrich) was dissolved in ethyl alcohol and diluted in PBS before injection and administered subcutaneously at 10 mg/kg (74). The drugs were administered daily to time-mated $Fkbp5^{+/+}$ and $Fkbp5^{-/-}$ mice × $Fkbp5^{+/+}$ male mice DEX ($n = 7$) or corresponding vehicle control (PBS; $n = 7$) as well as CORT ($n = 6$) or corresponding vehicle control (0.5% ethanol; $n = 6$), starting E8 through E18.

Mice Tissue Collection. Animals were euthanized and uterine and ovarian tissues were collected from timed-pregnant unrestrained and restrained $Fkbp5^{+/+}$ and $Fkbp5^{-/-}$ mice at E17.25 and 18.25 as well as at E19 from unrestrained $Fkbp5^{+/+}$ mice. Under dissection microscopy, uterine tissues containing myometrial and endometrial tissues were carefully collected after removal of all fetal tissues and stored for RNA studies. Placental tissues including decidua and myometrium were collected and processed for paraffin embedding for histochemical and immunohistochemical studies to evaluate relevant protein expression in different maternal and fetal tissue compartments. Ovaries were dissociated from surrounding fat and oviduct. In each mouse, one ovary was used for RNA studies, whereas the other ovary was used for paraffin embedding. All collected tissues were rinsed in PBS and stored at −80 °C until analysis.

Immunohistochemistry and H-score Analysis. Immunostaining was performed on 4% paraformaldehyde (PFA)-fixed, paraffin-embedded sections from GA-matched iPTB ($n = 9$) and PE ($n = 8$) and PA-complicated ($n = 8$) pregnancies as well as mouse ovarian and uterine samples. As previously described (22), deparaffinization, antigen retrieval using citrate buffer (pH: 6.0), and endogenous peroxidase quenching in 3% hydrogen peroxide solution was performed. Following washing steps with PBS, slides were incubated with 5% normal goat or horse serum (Vector Labs) for blocking and then with the primary antibodies presented in *SI Appendix*, Table S2 either overnight or at room temperature. After washing, the slides were incubated with biotinylated secondary antibodies (Vector Labs) for 30 min, then incubated with streptavidin-conjugated peroxidase complex (Vector Labs) for 30 min. Following several rinses with tris-buffered saline with Tween-20, immunoreactivity was developed using diaminobenzidine (Vector Labs) as the chromogen, and sections were counterstained with hematoxylin.

To identify decidual cells, human tissues were double immunostained sequentially with vimentin (a decidual marker, Abcam) (20) as described above. Vector Red (Vector Labs) was used as chromogens. For negative control, appropriate nonspecific IgG isotype was used at the same concentrations as the primary antibody. Immunoreactivity for each antibody was assessed by H-score analysis, a semiquantitative method that evaluates the intensity and the number of immunostained cells by two blinded investigators as described (22).

In Situ PLA. A Duolink In Situ PLA Detection Kit (Sigma-Aldrich) was used to detect the interaction between FKBP51 and PR. 4% PFA-fixed paraffin-embedded decidua basalis specimens from women with iPTB ($n = 6$) versus GA-matched control PA or PE ($n = 6$/each) were deparaffinized and rehydrated, and then, antigen retrieval was carried out by incubating the slides in citrate buffer (pH = 6.0) in microwave for 20 min. After washing steps, the slides were incubated with blocking solution for 1 h at 37 °C and then incubated with primary antibodies (PR rabbit monoclonal antibody; Cell Signaling and FKBP51 goat polyclonal antibody; R&D Systems) overnight at 4 °C. Primary antibodies were carefully selected from different species sources based on negligible background. Hybridization with anti-rabbit MINUS and goat PLUS PLA probes, ligation, and amplification of conjugants were performed as per the manufacturer's instructions. Negative controls were included in all experiments in the absence of primary antibodies. Slides were mounted with in situ mounting medium with DAPI (Sigma-Aldrich). A total of 10 different areas were randomly selected per slide, and photographs were obtained with 40× or 100× magnification, and images were analyzed using a ZEISS fluorescence microscope with the ZEN 2012 software system. The interaction of FKBP51 with PR was calculated as the number of PLA-positive dots per cell.

Statistics. Normality of data were investigated using the Kolmogorov–Smirnov test. Results that were normally distributed were analyzed by *t* test or one-way ANOVA, whereas data not normally distributed were analyzed by the Mann–Whitney *U* or Kruskal–Wallis test followed by the Student Newman–Keuls or Dunn's test. Analyses were performed using SigmaStat version 3.0 (Systat Software); $P < 0.05$, $^{\#}P < 0.01$, or $P < 0.001$ were considered statistically significant in pairwise comparison, whereas $P < 0.05$, $P < 0.01$, or $P < 0.001$ were considered statistically significant in multiple comparison groups.

Data Availability. All study data are included in the article and/or *SI Appendix*.

ACKNOWLEDGMENTS. This study was supported by The March of Dimes Prematurity Research Center Ohio Collaborative Grant #134266. D.M. and S.A. were supported by a postdoctoral scholarship from Scientific and Technological Research Council of Turkey. We thank Dr. Ronald Magness (Morsani College of Medicine, University of South Florida, Tampa, FL) for his contribution to discussions and suggestions during evaluation of results of this manuscript.

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