

Galectin-1 Markedly Reduces the Incidence of Resorptions in Mice Missing Immunophilin FKBP52

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Progesterone (P_4) signaling is critical for pregnancy. We previously showed that immunophilin FK506 binding protein (FKBP)52 serves as a cochaperone to optimize progesterone receptor (PR) function in the uterus, and its deficiency leads to P_4 resistance in a pregnancy stage-specific and genetic background-dependent manner in mice. In particular, sc placement of SILASTIC implants carrying P_4 rescued implantation failure in CD1 *Fkbp52*^{-/-} mice, but the resorption rate was substantially high at midgestation due to reduced P_4 responsiveness. Because downstream targets of P_4 -FKBP52-PR signaling in the uterus to support pregnancy are not clearly understood, we performed proteomic analysis using *Fkbp52*^{-/-}, PR-deficient (*Pgr*^{-/-}), and wild-type (WT) uteri. We found that the expression of galectin-1 (Gal1), an evolutionarily conserved glycan-binding protein, was significantly down-regulated in both *Fkbp52*^{-/-} and *Pgr*^{-/-} uteri compared with WT uteri. During early gestation, *Lgals1*, which encodes Gal1, was distinctly expressed in stromal and decidual cells. *Lgals1* expression was much lower in d 4 *Fkbp52*^{-/-} uteri compared with WT uteri, and this reduction was reversed by P_4 supplementation. More interestingly, concomitant supplementation of recombinant Gal1 significantly suppressed the high resorption rate and leukocyte infiltration at implantation sites in CD1 *Fkbp52*^{-/-} females carrying P_4 SILASTIC implants. These findings suggest that uterine Gal1 is an important downstream target of P_4 -FKBP52-PR signaling in the uterus to support P_4 responsiveness during pregnancy. (*Endocrinology* 153: 2486–2493, 2012)

Pregnancy loss is a major complication in humans, occurring in more than 70% of all women trying to conceive (1). Most early losses occur before or with the next expected menses and remain unrecognized. Even after pregnancy is discernible, more than 15% of women experience spontaneous abortions or ectopic pregnancies. In addition, approximately 5% of couples trying to conceive have two consecutive miscarriages, and about 1% of them have three or more consecutive losses (1). One major cause of such pregnancy loss is considered to be progesterone (P_4) deficiency. P_4 supplements for the first trimester of

pregnancy are often prescribed for women who are diagnosed with low P_4 levels in the luteal phase during their menstrual cycles. P_4 signaling is an absolute requirement for implantation and pregnancy maintenance in humans and in most mammals studied (2). P_4 acts via its nuclear progesterone receptor (PR) to activate the transcription of genes involved in ovulation, uterine receptivity, implantation, decidualization, and pregnancy maintenance (3).

We recently found that immunophilin FKBP52 serves as a cochaperone to govern normal PR function in the mouse uterus, where its expression overlaps with PR ex-

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Abbreviations: 2D-DIGE, Two-dimensional fluorescence difference gel electrophoresis; E_2 , 17 β -estradiol; FKBP, FK506 binding protein; Gal1, galectin-1; P_4 , progesterone; PDZ, primary decidual zone; PR, progesterone receptor; SDZ, secondary decidual zone; WT, wild type.

pression. Immunophilins are so named because they bind to certain immunosuppressive drugs to mediate their actions. They are grouped into two families: FKBP binding (FKBP) and cyclosporin A binding (cyclophilin), proteins. Some FKBP and cyclophilin family members have a tetratricopeptide repeat domain that targets binding to the conserved C terminus of heat shock protein 90. FKBP52 is one such tetratricopeptide repeat-containing cochaperone that influences steroid hormone receptor function (4). The mature PR complex that is bound to FKBP52 binds to P_4 with high affinity and efficiency, although some basal PR responsiveness to P_4 is retained even in the absence of FKBP52 (5, 6).

We found that *Fkbp52*^{-/-} females on C57BL/6/129 mixed and CD1 backgrounds have implantation failure, although they have normal ovulation (5, 6). However, P_4 supplementation rescues implantation and decidualization in CD1, but not in C57BL/6/129, *Fkbp52*^{-/-} females. In CD1 *Fkbp52*^{-/-} females, P_4 at higher than normal levels confers PR signaling sufficient for implantation, but even higher levels of P_4 are required to prevent pregnancy loss (6). Because FKBP52 positions PR in an optimal conformation for binding to P_4 , it is possible that excess P_4 in the absence of FKBP52 increases the probability of random binding of P_4 to the PR complex even under its less optimal configuration. Using proteomic analysis, we previously found that FKBP52 induces an antioxidant peroxiredoxin-6 independently of PR cochaperone activity and counters oxidative stress during implantation (7). The questions still remain as to how high doses of P_4 rescue pregnancy failure in CD1 *Fkbp52*^{-/-} mice with reduced PR responsiveness. We speculated that there are other factors in the PR-signaling pathway that could be important for pregnancy success. Our present study, using proteomic analysis, reveals an exciting finding that P_4 -FKBP52-PR signaling regulates the expression of galectin-1 (Gal1), an evolutionarily conserved glycan-binding protein, which has recently emerged as a critical regulator of feto-maternal interactions (8–11). Here we show that Gal1 significantly improves P_4 -PR signaling in the absence of FKBP52 to prevent embryo resorptions.

Materials and Methods

Mice

C57BL/6/129 and CD1 *Fkbp52*^{-/-} mice (6, 12) and C57BL/6/129 *Pgr*^{-/-} mice (13) were used in this study. All protocols for the present study were reviewed and approved by the Cincinnati Children's Research Foundation Institutional Animal Care and Use Committee in accordance with National Institutes of Health guidelines.

Two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) analysis and protein identification

Wild-type (WT), *Fkbp52*^{-/-}, and *Pgr*^{-/-} mice on a C57BL/6/129 background were ovariectomized, rested for 2 wk, and then treated with P_4 (2 mg/mouse/d) for 2 d. Mice were killed 24 h after the second P_4 injection, and their uteri were collected and processed for 2D-DIGE. Proteins were extracted from uterine tissues from three independent WT and *Pgr*^{-/-} mice and four *Fkbp52*^{-/-} mice, and 2D-DIGE and protein identification were performed as previously described by us (7, 14).

Analysis of resorption

WT and *Fkbp52*^{-/-} females (2–5 months old) were mated with WT fertile males to induce pregnancy (d 1=vaginal plug). To supplement *Fkbp52*^{-/-} females with exogenous P_4 , SILASTIC (Dow Corning Corp., Midland, MI) implants loaded with P_4 were sc implanted into CD1 *Fkbp52*^{-/-} mice on d 2 of pregnancy (6). Recombinant Gal1 (10 μ g/mouse/d, ip) was administered on d 10 and d 12 of pregnancy. Mice were killed on d 14, and number and weights of implantation sites and number of resorptions were examined.

Treatment with P_4 and 17 β -estradiol (E_2)

To assess the effects of ovarian hormones on uterine *Lgals1* expression, WT CD1 females were ovariectomized and rested for 2 wk. They were then given a single sc injection of P_4 (2 mg/mouse) and/or E_2 (100 ng/mouse) (15). The control group of mice received only vehicle (oil). Mice were killed 24 h later, and uteri were collected for *in situ* hybridization.

In situ hybridization

Paraformaldehyde-fixed frozen sections were hybridized with ³⁵S-labeled cRNA probes as described elsewhere (16).

Immunohistochemistry

Immunostaining of Gal1 was performed in formalin-fixed paraffin-embedded sections using a Gal1-specific rabbit polyclonal antibody as described previously (17).

Immunoblotting

Protein extraction and Western blotting were performed as described elsewhere (14). Antibodies to Gal1 (17) and actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were used. Actin served as a loading control.

Statistical analysis

Statistical analyses were performed using two-tailed Student's *t* test and ANOVA as appropriate. Values of *P* < 0.05 were considered statistically significant.

Results

Gal1 is down-regulated in *Pgr*^{-/-} and *Fkbp52*^{-/-} uteri

To examine which uterine proteins are differentially expressed in *Fkbp52*^{-/-} and *Pgr*^{-/-} mice compared with

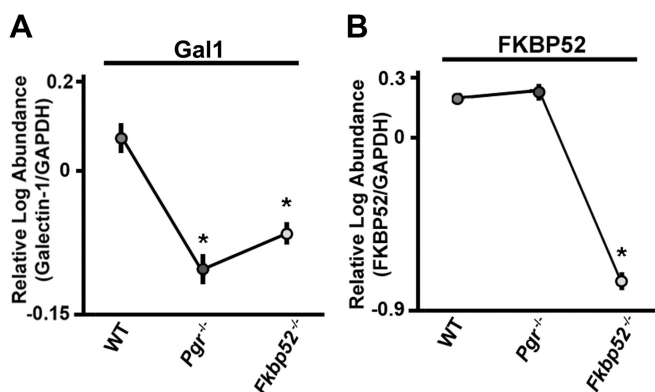


FIG. 1. Proteomic analysis shows down-regulation of Gal1 in *Pgr*^{-/-} and *Fkbp52*^{-/-} uteri compared with WT uteri. A, Protein levels of Gal1 in *P₄*-treated uteri of *Pgr*^{-/-} (n = 3) and *Fkbp52*^{-/-} (n = 4) ovariectomized mice are decreased compared with WT (n = 3) ovariectomized mice on a C57BL6/129 background. B, FKBP52 levels are reduced in uteri of *Fkbp52*^{-/-} mice compared with WT and *Pgr*^{-/-} mice as shown in our previous publication (7). Values are mean ± SEM. *, *P* < 0.05 compared with WT mice. GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

WT mice on a C57BL6/129 background, we performed 2D-DIGE analysis of uterine lysates obtained from ovariectomized mice treated with *P₄* after 2 wk of ovariectomy. We identified four proteins, two up-regulated and two down-regulated, in both *Pgr*^{-/-} and *Fkbp52*^{-/-} uteri

compared with WT uteri (Supplemental Tables 1 and 2 published on The Endocrine Society's Journals Online web site at <http://endo.endojournals.org>). Interestingly, Gal1 was identified as a protein significantly down-regulated in *Fkbp52*^{-/-} or *Pgr*^{-/-} uteri compared with WT uteri (Fig. 1A). As expected (7), our previous finding has shown that FKBP52 is reduced only in *Fkbp52*^{-/-} uteri (Fig. 1B). Because of Gal1's roles in pregnancy (8–11), we focused on Gal1 as a target molecule of *P₄*-FKBP52-PR signaling and to evaluate uterine expression of *Lgals1*, which encodes Gal1, during pregnancy.

Reduced *Lgals1* expression in d 4 *Fkbp52*^{-/-} uteri is reversed by *P₄*

We examined *Lgals1* expression in uteri of WT and *Fkbp52*^{-/-} mice on a C57BL6/129 background on d 4 when the uterus is under the *P₄* dominance, but superimposed with preimplantation ovarian estrogen. *Lgals1* localization is primarily restricted to the uterine stroma on d 4 in WT mice (Fig. 2A), and its expression in *Fkbp52*^{-/-} uteri markedly decreased compared with WT uteri (Fig. 2A); these results corroborate with our proteomics results (Fig. 1A). However, decreases in stromal *Lgals1* expression in *Fkbp52*^{-/-} females were rescued by *P₄* delivered by SILASTIC implants (Fig. 2A). These findings are consistent with the expression pattern of Gal1 protein in d 4 uteri of CD1 females. Likewise, down-regulated Gal1 expression in CD1 *Fkbp52*^{-/-} uteri was rescued by *P₄* released from SILASTIC implants (Fig. 2B).

Uterine *Lgals1* is spatiotemporally expressed during early pregnancy

We assessed the expression patterns of *Lgals1* on d 1, d 4, d 6, and d 8 of pregnancy by *in situ* hybridization (Fig. 3A). The uterus is under the influence of preovulatory estrogen with increased epithelial cell proliferation on d 1 of pregnancy. In contrast, elevated *P₄* levels from the newly formed corpora lutea along with a small amount of estrogen secreted from the ovary on d 4 results in epithelial cell differentiation with stromal cell proliferation. Interestingly, we found that *Lgals1* is detected mostly in the myometrium on d 1 of pregnancy (Fig. 3A). On d 4, the expression domain moved to the stroma (Fig. 3A), suggesting that ovarian steroids modulate this differential expres-

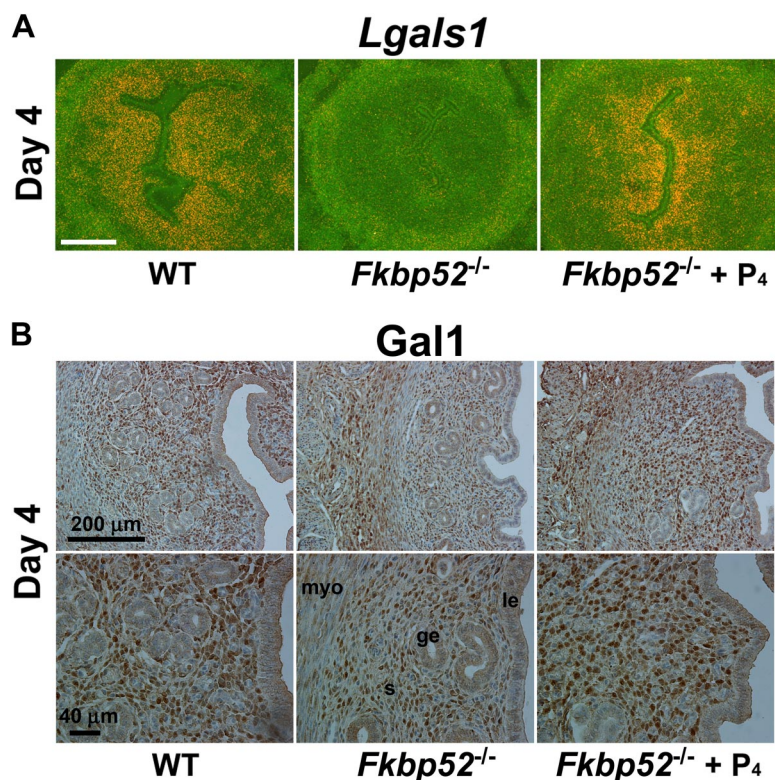


FIG. 2. *P₄* rescues down-regulated Gal1 expression in *Fkbp52*^{-/-} uteri on d 4 of pregnancy. A, Differential expression patterns of Gal1 mRNA (*Lgals1*) in d 4 uteri of WT, and *Fkbp52*^{-/-} mice treated with or without *P₄* on a C57BL6/129 background. Scale bar, 200 μm. B, Differential expression patterns of Gal1 protein in CD1 d 4 uteri of WT, and *Fkbp52*^{-/-} mice treated with or without *P₄*.

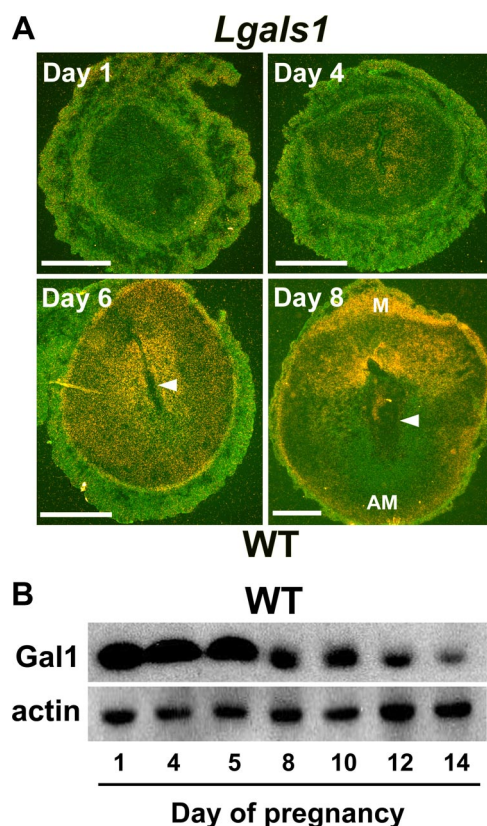


FIG. 3. Gal1 is spatiotemporally expressed in WT pregnant uteri. **A**, *In situ* hybridization showing spatiotemporal expression of *Lgals1* in WT CD1 pregnant uteri on d 1, d 4, d 6, and d 8 of pregnancy. Arrowheads indicate the locations of implanting embryos. M, Mesometrial pole; AM, antimesometrial pole. Scale bar, 500 μ m. **B**, Western blotting analyses show levels of uterine Gal1 protein during pregnancy. Whole uteri on d 1 and d 4, and implantation sites on d 5, d 8, d 10, d 12, and d 14 from WT CD1 mice were used for this experiment. Actin served as a loading control.

sion of *Lgals1*. The attachment reaction between the embryo and luminal epithelium occurs on d 4 night and continues through d 5 morning. The proliferating stromal cells surrounding the implanting blastocyst start to differentiate into decidual cells, which initiate the formation of the primary decidual zone (PDZ) on d 5 afternoon. By d 6, the PDZ is well formed and a secondary decidual zone (SDZ) is formed around the PDZ, when cell proliferation

ceases in the PDZ but still continues in the SDZ. On d 6, the expression of *Lgals1* was strongly displayed in the decidualizing stromal cells in both PDZ and SDZ. The PDZ progressively degenerates up to d 8, at which point the implantation process is well advanced with maximal stromal cell decidualization. On d 8, *Lgals1* was primarily expressed in decidual cells at the mesometrial pole. The results of Western blotting show that Gal1 protein levels are higher on d 1, d 4, and d 5 of pregnancy (Fig. 3B). Collectively, these findings suggest the importance of ovarian steroid hormones and events of decidualization in regulating Gal1 expression. A previous study showed that LGALS1 expression is up-regulated in the endometrium and decidua during the secretory phase (18), suggesting its role in human decidualization. Our unpublished results also showed that LGALS1 levels increase during *in vitro* decidualization in the human endometrial stromal cells.

The spatiotemporal *Lgals1* expression in uteri is regulated by P_4 and estrogen

The uterine biology on d 1 and d 4 of pregnancy is coordinated primarily by estrogen and P_4 , respectively, in a cell-specific manner. Because of differential expression patterns of *Lgals1* in uteri on d 1 and d 4, and induction of stromal *Lgals1* in *Fkbp52*^{-/-} mice by P_4 supplementation, we thought that ovarian hormones regulate uterine *Lgals1* expression. To assess whether estrogen and P_4 have major influences in regulating *Lgals1* expression, we examined the effects of P_4 and E_2 on *Lgals1* expression in uteri of ovariectomized mice. We found that whereas P_4 mainly induces *Lgals1* in the stroma, E_2 stimulates *Lgals1* expression in the stroma and myometrium (Fig. 4). These results corroborate with differential expression patterns of uterine *Lgals1* on d 1 when the uterus is under estrogen dominance and on d 4 when P_4 levels are higher due to newly formed corpora lutea.

Gal1 supplementation reverses increased rates of resorptions in *Fkbp52*^{-/-} mice

Gal1 has been shown to play immunotolerant roles in the pregnant mouse uterus, and its deficiency is associated with pregnancy loss at midgestation (8). Although CD1 *Fkbp52*^{-/-} mice can overcome implantation failure by P_4 released from SILASTIC implants starting on d 2 of pregnancy, they still experience a high rate of resorptions with significant leukocyte infiltration into implantation sites at midgestation due to reduced P_4 responsiveness (6). Because Gal1 is expressed in decidua and because of its

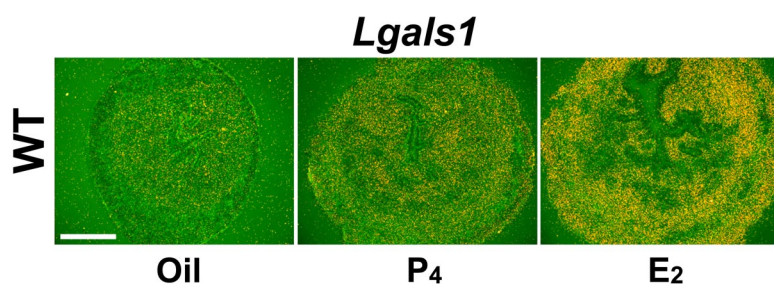


FIG. 4. P_4 and E_2 increase *Lgals1* expression in WT uteri of ovariectomized mice. Ovariectomized WT mice were injected sc with oil (vehicle), P_4 , or E_2 and killed 24 h later. Scale bar, 200 μ m.

role during midgestation, we hypothesized that pregnancy failure in *Fkbp52*^{-/-} mice with reduced P₄ responsiveness could be rescued by Gal1 supplementation after implantation. Therefore, we administered recombinant Gal1 (10 µg/mouse/d, ip) on d 10 and d 12 of pregnancy to *Fkbp52*^{-/-} females carrying SILASTIC implants loaded with P₄. As previously reported, *Fkbp52*^{-/-} mice without P₄ implants did not have any implantation sites, but mice carrying P₄ implants did rescue implantation (Fig. 5A). However, *Fkbp52*^{-/-} mice carrying only P₄ SILASTIC implants showed 75% higher resorption rate during the subsequent course of pregnancy (Fig. 5B) (6). Surprisingly, treatment with Gal1 significantly reduced the high inci-

dence of resorption rates in P₄-treated *Fkbp52*^{-/-} mice to 14% (Fig. 5B), although the number of implantation sites was comparable between the groups (Fig. 5C). In line with the reduction in resorption rate, average weight of implantation sites increased in P₄-treated *Fkbp52*^{-/-} mice superimposed with Gal1 supplementation (Fig. 5D). Furthermore, the decline in the number of resorption sites was reflected in disappearance of leukocyte infiltration at the implantation sites examined on d 14 of pregnancy (Fig. 6). These *in vivo* findings suggest that Gal1 complements the effects of P₄-PR signaling in the absence of FKBP52 to improve pregnancy outcome.

Discussion

In the present study, we used proteomic analysis to identify downstream targets of P₄-FKBP52-PR signaling and found lower expression levels of Gal1 in both *Fkbp52*^{-/-} and *Pgr*^{-/-} uteri compared with WT uteri. We also found that uterine *Lgals1* expression is induced by P₄ and E₂ in a spatiotemporal manner. Interestingly, administration of recombinant Gal1 in conjunction with P₄ supplementation via SILASTIC implants to pregnant *Fkbp52*^{-/-} females significantly reduced the incidence of resorptions that occurs due to reduced P₄-FKBP52-PR signaling.

Although P₄ has been argued to have antiinflammatory and immunomodulatory effects, the underlying mechanism is not fully understood. The present study shows that P₄-PR signaling promotes the expression of uterine Gal1, which should be beneficial to improve pregnancy. This is consistent with our findings that Gal1 supplementation significantly reduces the resorption rate and leukocyte infiltration seen in *Fkbp52*^{-/-} mice with reduced P₄ responsiveness (6). These results suggest that P₄-PR-Gal1 signaling influences immunoactivation and inflammation in the uterus. Our study showing Gal1-induced improvement of pregnancy events in *Fkbp52*^{-/-} females carrying P₄ SILASTIC implants also points toward a role for Gal1 in optimizing P₄-PR signaling in the absence of

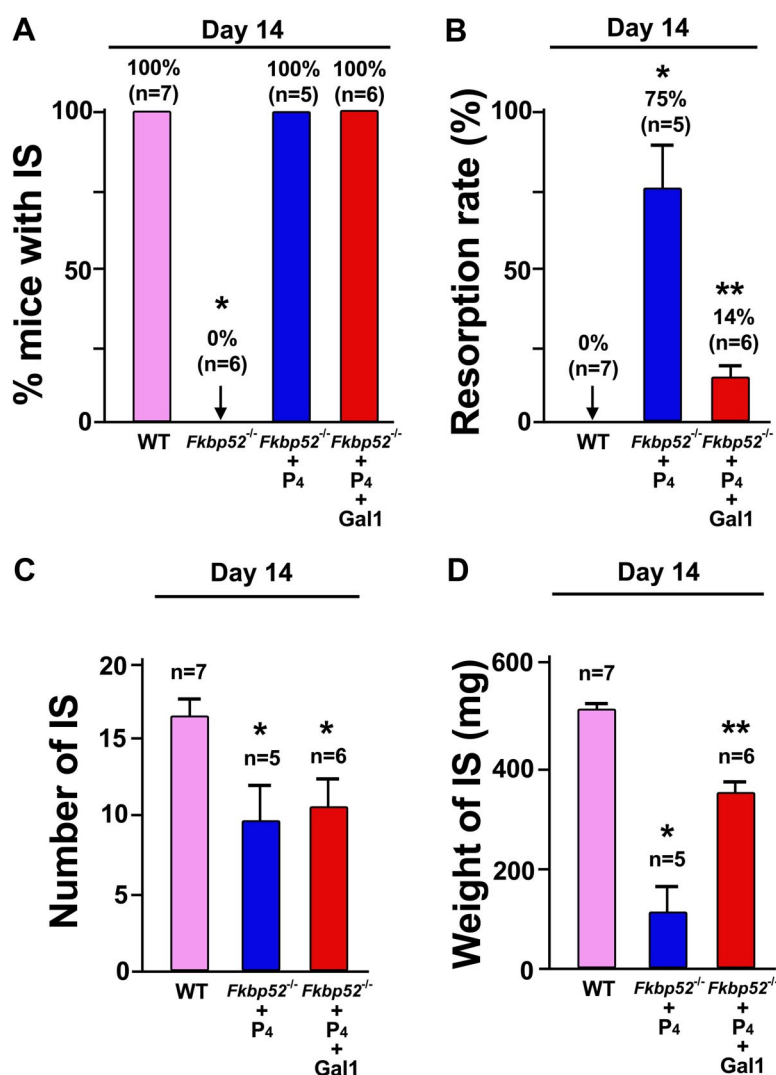


FIG. 5. Injection of recombinant Gal1 reduces the high rate of resorptions in *Fkbp52*^{-/-} females. SILASTIC implants carrying P₄ were implanted sc into CD1 *Fkbp52*^{-/-} mice on d 2 of pregnancy. Recombinant Gal1 (10 µg/mouse/d) was injected ip on d 10 and d 12 of pregnancy. Mice were killed on d 14, and the number and weights of implantation sites (IS) and the number of resorptions were examined. A, *, *P* < 0.05 vs. WT, *Fkbp52*^{-/-} females with P₄ treatment and *Fkbp52*^{-/-} females with P₄ and Gal1 treatment; B, *, *P* < 0.05 vs. WT; **, *P* < 0.05 vs. *Fkbp52*^{-/-} with P₄ treatment; C, *, *P* < 0.05 vs. WT; D, *, *P* < 0.05 vs. WT, **, *P* < 0.05 vs. *Fkbp52*^{-/-} with P₄ treatment.

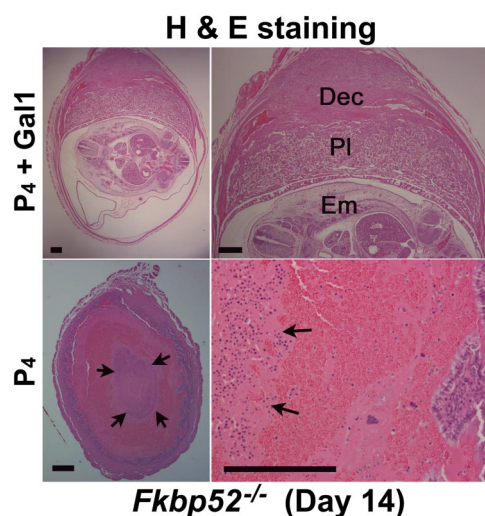


FIG. 6. Recombinant Gal1 supplementation rescues leukocyte infiltration in *Fkbp52*^{-/-} females carrying P₄ SILASTIC implants. *Fkbp52*^{-/-} females carrying P₄ SILASTIC implants were treated with or without recombinant Gal1 on d 10 and d 12 and were killed on d 14 of pregnancy. Representative photomicrographs of hematoxylin and eosin (H&E)-stained implantation sites are shown. Arrows indicate the location of leukocyte infiltration. Em, Embryo; Pl, placenta; Dec, decidua. Scale bars, 500 μ m.

FKBP52. Alternatively, Gal1 provides protection toward immunological responses that are up-regulated in the absence of FKBP52 deficiency, which confers reduced uterine P₄-PR signaling. Interestingly, a previous study has shown that Gal1-deficient (*Lgals1*^{-/-}) female mice experiencing allogeneic, but not syngeneic, matings have higher rates of fetal loss. Collectively, these findings suggest that the immunoprotective role of P₄ is mediated, at least partially, through Gal1 to counter pathological inflammation, such as bacterial infection, during pregnancy.

There is evidence for a heightened expression of Gal1 in human decidua (18) and placentas (19–22). In mouse uteri, Gal1 expression increased in both the deciduum and myometrium in early pregnancy and after treatment with E₂ or P₄ in ovariectomized mice (23). Our results show that *Lgals1* is highly expressed in myometrium on d 1 when the uterus is under the influence of ovarian estrogen, and in stromal and decidual cells on d 4, d 6, and d 8, when ovarian P₄ primarily governs uterine functions. At present, the role of myometrial expression of *Lgals1* is not understood. Nonetheless, these findings suggest the involvement of estrogen and P₄ regulating Gal1 expression via their nuclear receptors. Because a phylogenetic analysis has demonstrated the presence of estrogen-responsive elements in the *Lgals1* promoter (19), the uterine induction of Gal1 by an E₂ injection, as seen in this study, could be due to the transcriptional regulation by estrogen receptors. Although it remains unclear how P₄ induces Gal1 in

the uterus, it is likely that Gal1 plays an important role in immune-endocrine cross talk during pregnancy.

In addition to the function of optimizing PR activity, FKBP52 induces the expression of an antioxidant peroxiredoxin-6 to regulate oxidative stress (7). In fact, FKBP have several physiological roles including binding and sequestration of calcineurin, protein folding and assembly, protein trafficking, and direct regulation of protein activity (24). The present study identifies Gal1 as a new downstream target of P₄-FKBP52-PR signaling. Because FKBP52 has various immunoregulatory functions the mechanisms of which are still unknown (25), it is possible that Gal1-glycan interactions play a role in such functions.

Gal1 is abundant in the human and mouse female reproductive tracts (18, 26) and in the human placenta (19–22). A previous study has shown that human decidual NK cells secrete a considerable amount of Gal1, which induces apoptosis of decidual but not peripheral T cells (9). Similar to Th1 and Th17 subsets (27), decidual T cells express a repertoire of cell surface glycans that are critical for Gal1 binding and cell death (9), suggesting that this lectin may preferentially control the fate of decidual T cells. Remarkably, Gal1 is abnormally expressed in placental tissues of failed pregnancy and preeclampsia (19, 28, 29). In addition to the immunomodulatory activity, Gal1 also recognizes glyco-epitopes on trophoblast cells and stimulates syncytium formation (30). The role of Gal1 in pregnancy preservation is supported by phylogenetic analysis, showing not only the acquisition of estrogen-responsive elements in the *Lgals1* promoter, but also selective gain of cysteine residues involved in redox regulation occurring in early mammalian evolution (19). Interestingly, the most intense selection process in the *Lgals1* gene was found on residues localized within the Gal1 carbohydrate recognition domain and the dimerization interface (19), suggesting the adaptation of these biochemical features to immune regulatory effects. In fact, we and others have shown that P₄ markedly up-regulates Gal1 expression during pregnancy in mice (8). Collectively, these findings underscore an evolutionarily conserved function of P₄-regulated Gal1 in establishing fetomaternal tolerance through the modulation of a hierarchy of regulatory pathways. In conclusion, the uterine P₄-PR-Gal1 axis is critical for successful pregnancy. These findings emphasize a potential approach for therapeutic intervention aimed at reestablishing immune cell homeostasis in troubled pregnancies.

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