FKBP52 deficiency–conferred uterine progesterone resistance is genetic background and pregnancy stage specific

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Immunophilin FKBP52 serves as a cochaperone to govern normal progesterone (P_4) receptor (PR) function. Using *Fkbp52*$^{-/-}$ mice, we show intriguing aspects of uterine P_4/PR signaling during pregnancy. Implantation failure is the major phenotype found in these null females, which is conserved on both C57BL6/129 and CD1 backgrounds. However, P_4 supplementation rescued implantation and subsequent decidualization in CD1, but not C57BL6/129, null females. Surprisingly, experimentally induced decidualization in the absence of blastocysts failed in *Fkbp52*$^{-/-}$ mice on either background even with P_4 supplementation, suggesting that embryonic signals complement uterine signaling for this event. Another interesting finding was that while P_4 at higher than normal pregnancy levels conferred PR signaling sufficient for implantation in CD1 null females, these levels were inefficient in maintaining pregnancy to full term. However, elevating P_4 levels further restored PR signaling to a level optimal for successful term pregnancy with normal litter size. Collectively, the results show that the indispensability of FKBP52 in uterine P_4/PR signaling is a function of genetic disparity and is pregnancy stage specific. Since there is evidence for a correlation between P_4 supplementation and reduced risks of P_4-resistant recurrent miscarriages and remission of endometriosis, these findings have clinical implications for genetically diverse populations of women.

Introduction

Progesterone (P_4) signaling is an absolute requirement for implantation and pregnancy maintenance in all eutherian mammals studied (1, 2). P_4 acts through its nuclear P_4 receptor (PR) to activate transcription of genes involved in ovulation, uterine receptivity, implantation, decidualization, and pregnancy maintenance. This is evident from the complete infertility of female mice lacking *Pgr*, the gene encoding PR (3). The failure of ovulation and implantation precludes using *Pgr*$^{-/-}$ mice to study potential new aspects of P_4 function during pregnancy. In contrast, the targeted deletion of the *Fkbp52* gene, which encodes FKBP52, an immunophilin cochaperone that optimizes PR signaling, has allowed us to address unique aspects of uterine P_4/PR signaling during pregnancy in mice.

Immunophilins are so named because of their ability to bind and mediate the actions of certain immunosuppressive drugs. They are grouped into 2 families, FK506-binding proteins (FKBPs) and cyclosporin A–binding proteins (cyclophilins [CyPs]). Some FKBP and CyP family members contain a tetratricopeptide repeat (TPR) domain that targets binding to the highly conserved C terminus of Hsp90. FKBP52, FKBP51, and CyP40 are 3 such TPR-containing cochaperones that have been identified in steroid receptor complexes (4). Like other steroid receptors, PR assembles in an ordered, multistep manner for hormone binding (5, 6); the final complex involves direct association of Hsp90 with PR, which stabilizes its ability to bind P_4 (7). At the mature stage, FKBPs and other TPR cochaperones dynamically exchange on Hsp90 such that there is a mixture of receptor complexes distinguished by which cochaperone is associated with Hsp90 (8, 9). Hormone binding stimulates interruption of the receptor/chaperone assembly cycle and promotes receptor activation. Recent findings show that cochaperones have unique influences on steroid receptor function. FKBP52 potentiates the responses of PR, androgen receptor, and glucocorticoid receptor to their respective ligands (10–12). In fact, only the mature PR complex bound to FKBP52 is capable of binding P_4 with high affinity and efficiency, although basal PR responsiveness to P_4 is still retained in the absence of FKBP52. Our recent study (12) revealed the previously unknown role of FKBP52/PR signaling in female reproduction.

The uterus consists of heterogeneous cell types that respond differentially to estrogen and P_4. For successful implantation, the uterus must be transiently receptive to implantation-competent blastocysts. For example, the prereceptive uterus on days 1–3 of pregnancy or pseudopregnancy becomes receptive to implantation on day 4 under the direction of P_4 and estrogen. By late day 5, the uterus becomes nonreceptive, since implantation-competent blastocysts transferred into uteri of pseudopregnant mice at this time fail to implant. Following implantation on day 4 evening (2200 to 2400 h), uterine stromal cells at the sites of blastocysts undergo extensive proliferation and differentiation, giving rise to decidual cells, a process termed decidualization (1, 13). Using proteomic analysis in *Hoxa10*$^{-/-}$ mouse uteri, we identified FKBP52 as an important signaling molecule in stromal cell function during the periimplantation period (14). Consistent with the role of

Nonstandard abbreviations used: *Areg*, amphiregulin; FKBP, FK506-binding protein; ICI, ICI 182,780; *Ihh*, Indian hedgehog; IS, implantation site; *Lif*, leukemia inhibitory factor; *Ltf*, lactoferrin; MEF, mouse embryonic fibroblast; P_4, progesterone; PR, P_4 receptor; TPR, tetratricopeptide repeat.

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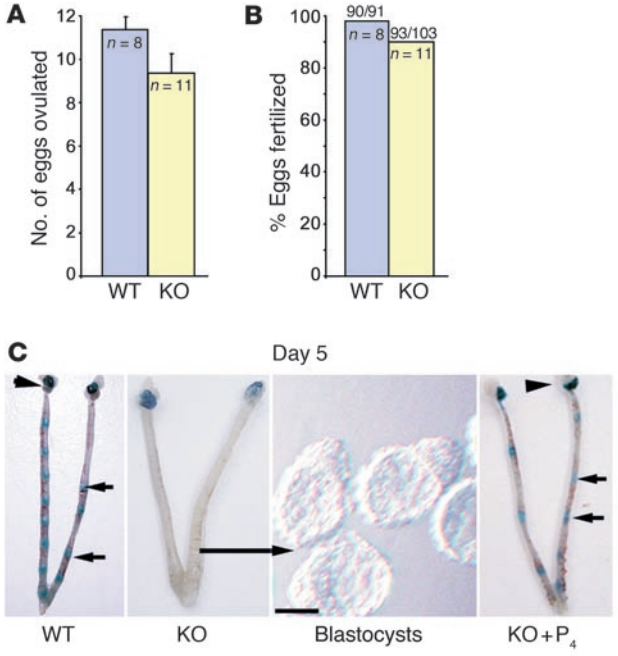

Figure 1
P_4 supplementation via Silastic implants rescues implantation failure in CD1 *Fkbp52*$^{-/-}$ females. Ovulation (**A**) and fertilization (**B**) were examined on day 2 of pregnancy. The number of ovulated eggs was not significantly different in WT and *Fkbp52*$^{-/-}$ (KO) females. Values are mean ± SEM; $P > 0.05$, unpaired Student's *t* test. Fertilization rate was determined by counting the number of 2-cell embryos after flushing oviducts. Numbers above the bars indicate the total number of 2-cell embryos per total number of eggs recovered. The fertilization rate was comparable between WT and KO females ($P > 0.05$; unpaired Student's *t* test). (**C**) Implantation fails in KO females but is rescued by P_4 as examined on day 5 of pregnancy. Implants containing P_4 were inserted s.c. in KO females (KO+P_4) on day 2 of pregnancy. Representative photographs of uteri with or without ISs as demarcated by blue bands in WT, KO, and KO+P_4 mice are shown. Representative photographs of blastocysts recovered from uteri of KO females without IS. Arrowheads and short arrows indicate the location of ovaries and ISs, respectively. The long arrow indicates the uterus from which unimplanted blastocysts were recovered. Scale bar: 50 μm.

FKBP52 as a PR cochaperone, uterine expression of *Fkbp52* and *Pgr* overlaps on days 4 and 5 of pregnancy (12). More importantly, we have shown that *Fkbp52*$^{-/-}$ females on a C57BL6/129 mixed background have complete implantation failure, with normal ovulation and slightly reduced fertilization rates (12). This phenotype has recently been confirmed by another group in independently generated *Fkbp52*$^{-/-}$ mice on the same background (15). The fact that the infertile phenotype of *Fkbp52*$^{-/-}$ females is primarily due to implantation defects suggests differential sensitivity of the ovary and uterus to FKBP52/PR-mediated P_4 action. This tissue-specific differential sensitivity is not noted in *Pgr*$^{-/-}$ females, in which severely compromised ovarian and uterine functions lead to complete female infertility (3, 16).

Although P_4 is commonly known as the "hormone of pregnancy," various aspects of its roles throughout pregnancy are not well understood. Therefore, *Fkbp52*$^{-/-}$ mice provide a unique opportunity for studying such roles of P_4 signaling throughout pregnancy. Using these null mice, we show in the present investigation that P_4/PR signaling is a function of genetic makeup and is pregnancy stage specific. For example, while the implantation failure phenotype was conserved in both C57BL6/129 and CD1 mice lacking *Fkbp52*, daily P_4 supplementation rescued implantation with subsequent decidualization in CD1 *Fkbp52*$^{-/-}$ females but not in C57BL6/129 *Fkbp52*$^{-/-}$ females. Surprisingly, experimentally induced decidualization failed to occur in *Fkbp52*$^{-/-}$ mice on either background even with exogenous P_4 supplementation, suggesting that embryonic signals complement the uterine signaling network. Another interesting finding is the differential requirement for P_4/PR signaling at specific stages of pregnancy in P_4-treated CD1 *Fkbp52*$^{-/-}$ females. For example, while P_4 at higher than normal levels conferred PR signaling sufficient for uterine receptivity and implantation in *Fkbp52*$^{-/-}$ females, these levels did not maintain adequate PR signaling to sustain pregnancy, resulting in reduced litter sizes due to in utero fetal resorption and restricted growth. However, increasing P_4 levels even further restored PR signaling, to a degree sufficient to maintain full-term pregnancy with normal litter size. Collectively, these findings show that FKBP52 deficiency confers uterine P_4 resistance during pregnancy, since null females have normal PR and P_4 levels with reduced PR activity (12, 17). This study also shows that the requirement for FKBP52 in optimizing PR activity is genetic background dependent and that levels of P_4/PR signaling required to ensure successful pregnancy differ depending on pregnancy stage.

Results

Implantation failure occurs in Fkbp52$^{-/-}$ *mice irrespective of genetic background.* There is increasing evidence that mutation of a gene often results in altered phenotypes depending on the genetic background of mice (18). These varying phenotypes are thought to be due to differential expression and/or regulation of modifier genes (19), although identification of such modifiers remains largely unknown. There is also evidence that compensatory function of genes among the same family is genetic background dependent (20). We have recently shown that C57BL6/129 *Fkbp52*$^{-/-}$ females have complete implantation failure, although ovulation is normal (12). To determine whether this phenotype is a function of genetic background, we established *Fkbp52* deletion in CD1 mice (see Methods). First, we examined ovulation and fertilization in CD1 *Fkbp52*$^{-/-}$ females after mating them with WT males, since *Fkbp52*$^{-/-}$ males, irrespective of genetic background, are infertile (10). We found ovulation and fertilization to be comparable to those of WT females (Figure 1, A and B). We then asked whether implantation occurs in these mice. Initiation of implantation accompanies an increased endometrial vascular permeability at sites of blastocysts, which are visualized as distinct blue bands after injection of a blue dye solution (13, 21). CD1 *Fkbp52*$^{-/-}$ females were examined for implantation sites (ISs) by this method on day 5 of pregnancy. We observed that only 2 of 14 CD1 *Fkbp52*$^{-/-}$ females showed very faint blue bands (Figure 1C and Table 1). Unimplanted blastocysts were recovered from uterine flushings of null mice with implantation failure (Figure 1C), suggesting that although embryos developed to blastocysts, they failed to implant. Our data indicate that optimal P_4/PR

Table 1
P_4 rescue of implantation failure in CD1 *Fkbp52*$^{-/-}$ female mice

Genotype	Day of pregnancy	No. of mice	No. of mice with IS (%)	No. of ISs	Weight of IS (mg)	No. of embryos recovered
WT	5	16	16 (100%)	12.2 ± 0.3	4.1 ± 0.3	NA
	6	9	9 (100%)	13.2 ± 0.9	10.7 ± 0.2	NA
	8	5	5 (100%)	10.8 ± 1.8	29.3 ± 1.0	NA
	12	11	11 (100%)	13.5 ± 0.8	214.7 ± 7.0	NA
KO	5	14	2 (14%)	7.0 ± 0.3	NE	66[A]
	6	9	4 (44%)	4.5 ± 1.2	6.3 ± 0.1[B]	21[C]
	8	8	1 (13%)	1	8.0	19[D]
	12	5	0	NA	NA	0
KO+P_4	5	11	9 (81%)	10.3 ± 0.5	3.75 ± 0.2	17[E]
	6	6	6 (100%)	10.8 ± 1.3	7.8 ± 0.3[B]	NA
	8	8	8 (100%)	11.4 ± 0.9	19.2 ± 0.9[B]	NA
	12	14	14 (100%)	11.6 ± 0.6	127.5 ± 8.8[B]	NA

WT and CD1 *Fkbp52*$^{-/-}$ females were mated with WT fertile males, and the number of ISs was determined on days 5, 6, 8, and 12 of pregnancy. *Fkbp52*$^{-/-}$ females were treated with Silastic implants containing P_4 (KO+P_4) from day 2 of pregnancy until the day of sacrifice. Uteri without ISs were flushed with saline to recover any unimplanted embryos. [A]Seven mice without ISs yielded 66 blastocysts, of which 6 showed signs of degeneration. [B]$P < 0.05$ compared with WT, unpaired Student's *t* test. [C]Twenty-one blastocysts were recovered from 5 mice, of which 5 showed signs of degeneration. [D]Nineteen blastocysts were recovered from 3 mice, of which 3 had signs of degeneration. [E]Seventeen blastocysts were recovered from 2 mice. The number and weights of ISs are presented as mean ± SEM. NE, not examined because ISs were very faint as demarcated by blue reaction, precluding their excision for weighing.

signaling imparted by FKBP52 is critical to uterine receptivity and implantation in mice, a phenotype conserved across these genetic backgrounds. Because ovulation is normal in *Fkbp52*$^{-/-}$ mice, our results suggest that uterine responsiveness to PR signaling differs from ovarian responsiveness. It is possible that relatively high local P_4 levels in the ovary (22), the site of its synthesis, enhance basal PR activity sufficient for ovulation and fertilization processes.

P_4 supplementation rescues implantation in CD1 Fkbp52$^{-/-}$ *females.* We have shown that PR activity, but not PR or P_4 levels, is compromised in C57BL6/129 *Fkbp52*$^{-/-}$ females (12, 17). It is possible that FKBP52 binding to the PR complex modulates the hormone responsiveness of PR not in an all-or-none fashion, but rather to fine-tune physiological responses to P_4. This would imply that while FKBP52 is necessary for optimal PR activity, PR signaling can still operate, albeit not as efficiently, in the absence of FKBP52. Since serum P_4 levels in CD1 *Fkbp52*$^{-/-}$ females are similar to those in WT mice on day 5 of pregnancy (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI31622DS1), we speculated that exposing *Fkbp52*$^{-/-}$ uteri to higher-than-normal P_4 levels would enhance PR activity to rescue pregnancy failure in the absence of FKBP52. This is consistent with our previous findings that PR activity in *Fkbp52*$^{-/-}$ mouse embryonic fibroblasts (MEFs) reached levels similar to those in WT MEFs exposed to higher P_4 concentrations in culture (12). This may also explain why ovulation, a P_4-regulated event, is normal in *Fkbp52*$^{-/-}$ females on both genetic backgrounds.

To determine whether P_4 supplementation rescues implantation, we used P_4-containing Silastic implants to maintain steady-state hormone levels (23). WT and *Fkbp52*$^{-/-}$ females were mated with WT males, and Silastic implants containing P_4 were placed under the dorsal skin of *Fkbp52*$^{-/-}$ females from day 2 of pregnancy until the day of sacrifice. We were surprised to see that P_4 supplementation not only rescued implantation in CD1 *Fkbp52*$^{-/-}$ females examined on day 5, but the number of ISs was also comparable to that in WT mice (Figure 1C and Table 1). In contrast, exogenous P_4 supplementation was largely ineffective in rescuing implantation in C57BL6/129 *Fkbp52*$^{-/-}$ females; only 3 of 11 (27%) and 2 of 8 (25%) null females showed implantation when examined on days 5 and 8, respectively. In addition, the number of ISs was also remarkably low, especially on day 8, compared with that in WT females (Table 2). To circumvent any contribution arising from lower fertilization rates in C57BL6/129 *Fkbp52*$^{-/-}$ mice, we performed blastocyst transfer experiments. Day-4 WT blastocysts were transferred into uterine lumens of day-4 C57BL6/129 *Fkbp52*$^{-/-}$ pseudopregnant females carrying Silastic P_4 implants from day 2. Again, we noted extremely poor implantation rates in these mice (Table 3).

P_4 supplementation restores P_4- and implantation-regulated gene expression in CD1 Fkbp52$^{-/-}$ *uteri.* P_4-regulated events combined with preimplantation estrogen secretion guide the uterus from a prereceptive to receptive state, allowing blastocyst attachment in the uterine wall on day 4 evening. To determine whether rescue of implantation by P_4 is reflected in the restoration of P_4-dependent uterine gene expression, we placed a Silastic P_4 implant on day 2 in CD1 *Fkbp52*$^{-/-}$ females that had been mated with WT males and sacrificed them on day 4 of pregnancy. We selected P_4-regulated genes that encode *Hoxa10*, Indian hedgehog (*Ihh*), and amphiregulin (*Areg*) because of their participation in uterine receptivity (24–27). In situ hybridization results showed that exogenous P_4 treatment considerably restores expression of these genes (Figure 2A).

Lactoferrin (*Ltf*) and complement factor 3 (*C3*) are induced to a high degree in the uterus by estrogen and antagonized by P_4 (28, 29). They are abundantly expressed in the luminal epithelium on

Table 2
P_4 fails to rescue implantation failure in C57BL6/129 *Fkbp52*$^{-/-}$ female mice

Genotype	Day of pregnancy	No. of mice	No. of mice with IS (%)	No. of ISs	No. of embryos recovered
WT	5	11	11 (100%)	7.5 ± 0.5	NA
KO+P_4	5	11	3 (27%)	5.6 ± 1.0	23[A]
KO+P_4	8	8	2 (25%)	2 ± 0.5[B]	0[C]

WT and *Fkbp52*$^{-/-}$ females on a C57BL6/129 background were mated with WT fertile males, and the number of ISs was examined on days 5 and 8 of pregnancy by the blue dye method. *Fkbp52*$^{-/-}$ females were placed with P_4 containing Silastic implants from day 2 of pregnancy until the day of sacrifice. Uteri without ISs were flushed with saline to recover any unimplanted embryos. [A]Eight mice without ISs yielded 23 blastocysts, of which 21 showed signs of degeneration. [B]$P < 0.05$ compared with WT, unpaired Student's *t* test. [C]Six mice without ISs yielded no embryos. The number of ISs is presented as mean ± SEM.

Table 3
P_4 fails to rescue implantation failure of transferred WT blastocysts in C57BL6/129 *Fkbp52*$^{-/-}$ females

Genotype		No. of blastocysts transferred	No. of recipients	No. of mice with IS (%)	No. of ISs (%)	No. of embryos recovered
Blastocysts	Recipients					
WT	WT	82	6	6 (100%)	49/82 (59%)	NA
WT	KO+P_4	83	5	2 (40%)	6/83 (7%)	45[A]

Day-4 WT blastocysts were transferred into uteri of WT or KO recipients on day 4 of pseudopregnancy. *Fkbp52*$^{-/-}$ females were treated with Silastic implants containing P_4 from days 2 through 5 of pseudopregnancy. Recipients were examined for ISs on day 5 by the blue dye method. Uteri without ISs were flushed with saline to recover any unimplanted blastocysts. [A]Three mice without IS yielded 45 blastocysts, of which 2 showed signs of degeneration.

day 1 of pregnancy under the influence of a preovulatory estrogen surge but are dramatically downregulated on day 4 by rising P_4 levels from newly formed corpora lutea (1, 2). However, in CD1 *Fkbp52*$^{-/-}$ mice, uterine expression of these genes was aberrantly elevated on day 4 but downregulated with exogenous P_4 supplementation (Figure 2B). Together, these findings provide evidence that P_4 supplementation restores the expression of P_4-regulated genes and counters the expression of estrogen target genes in CD1 *Fkbp52*$^{-/-}$ uteri, shifting the uterus to a P_4-dominated milieu conducive to uterine receptivity as opposed to one of estrogenic dominance that is detrimental to uterine receptivity.

The fact that exogenous P_4 treatment fully rescues implantation in CD1 *Fkbp52*$^{-/-}$ females but is largely ineffective in C57BL6/129 *Fkbp52*$^{-/-}$ mice led us to examine whether P_4 treatment would restore the expression of P_4-regulated genes in C57BL6/129 *Fkbp52*$^{-/-}$ uteri, as was observed in CD1 *Fkbp52*$^{-/-}$ mice. We found that although exogenous P_4 treatment considerably restored *Hoxa10* and *Ihh* expression, *Areg* expression remained low to undetectable (Supplemental Figure 2A). More interestingly, exogenous P_4, which normally inhibits estrogen-responsive *Ltf* expression in CD1 *Fkbp52*$^{-/-}$ uteri on day 4, was not effective in attenuating *Ltf* expression in C57BL6/129 *Fkbp52*$^{-/-}$ uteri (Supplemental Figure 2B). Overall, these results imply that while C57BL6/129 *Fkbp52*$^{-/-}$ uteri are somewhat responsive to P_4 induction of target genes, they are less receptive to P_4's influence in antagonizing estrogen target genes. These findings are significant, since excess estrogenic influence leads to uterine nonreceptivity (30). Because implantation fails in most C57BL6/129 *Fkbp52*$^{-/-}$ mice even after P_4 treatment, we performed subsequent experiments on CD1 *Fkbp52*$^{-/-}$ mice.

Although expression of several P_4-regulated genes was considerably restored with P_4 supplementation in CD1 *Fkbp52*$^{-/-}$ uteri, the expression of leukemia inhibitory factor (*Lif*), normally expressed in day-4 WT pregnant uterine glands, was not restored by P_4 treatment in these null mice (Figure 3A). We next determined whether P_4 rescue of implantation on day 5 in CD1 *Fkbp52*$^{-/-}$ mice is accompanied by correct expression of implantation-related genes, such as *Lif*, *Ptgs2*, and *Bmp2* (31–33). We found that while these genes were not expressed at the site of blastocysts in CD1 *Fkbp52*$^{-/-}$ females with failed implantation in the absence of P_4, P_4 supplementation restored implantation with correct expression of these genes (Figure 3, A and B). The ability of P_4 to rescue both implantation and *Lif* expression on day 5 of pregnancy in *Fkbp52*$^{-/-}$ mice without salvaging *Lif* expression on day 4 agrees with previous observations that the first phase of *Lif* expression on day 4 is not as critical as its second phase of expression in stromal cells surrounding the implanting blastocyst (33, 34).

Upon closer examination of WT and null uterine histology, we came across an interesting observation. A significant decrease in the number of glands was noted in null uteri on both genetic backgrounds (Figure 3C), a phenotype not rescued by P_4 supplementation (data not shown). This decrease in gland numbers, however, does not fully account for the altered expression of *Areg*, *Ihh*, or *Lif* in null uteri, since the glands that were still present failed to show

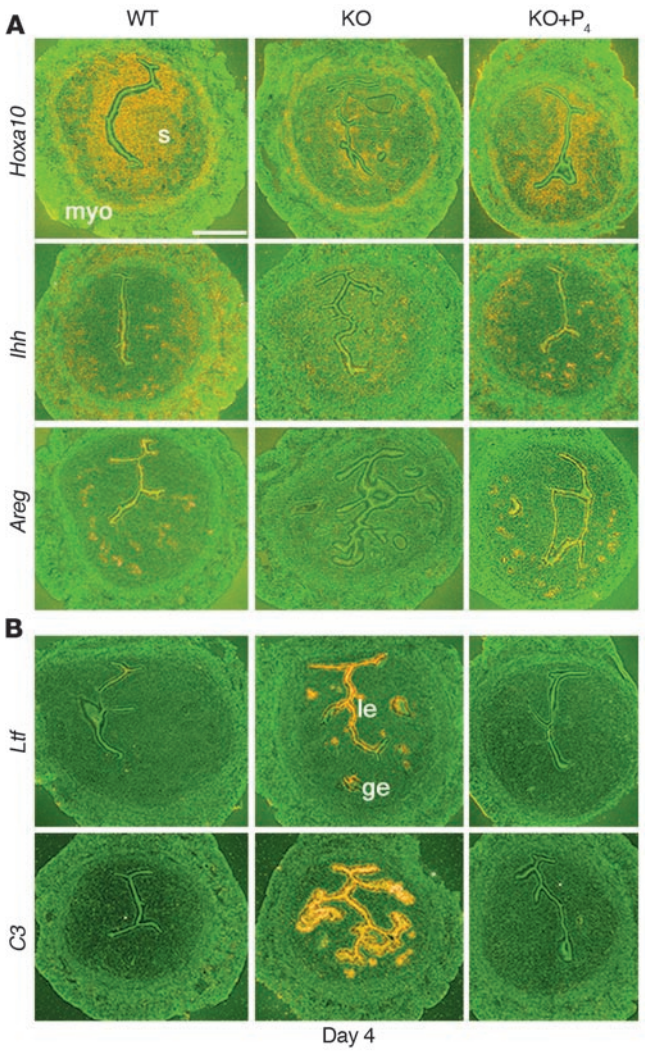

Figure 2
P_4 supplementation via Silastic implants corrects misexpression of genes in CD1 *Fkbp52*$^{-/-}$ uteri. (**A**) In situ hybridization of P_4-regulated genes *Hoxa10*, *Ihh*, and *Areg* in WT, KO, and KO+P_4 uteri on day 4 of pregnancy. (**B**) In situ hybridization of estrogen-target genes *Ltf* and complement factor 3 (*C3*) in WT, KO, and KO+P_4 uteri on day 4 of pregnancy. Implants containing P_4 were inserted s.c. in KO females on day 2 of pregnancy. ge, glandular epithelium; le, luminal epithelium; myo, myometrium; s, stroma. Scale bar: 200 μm.

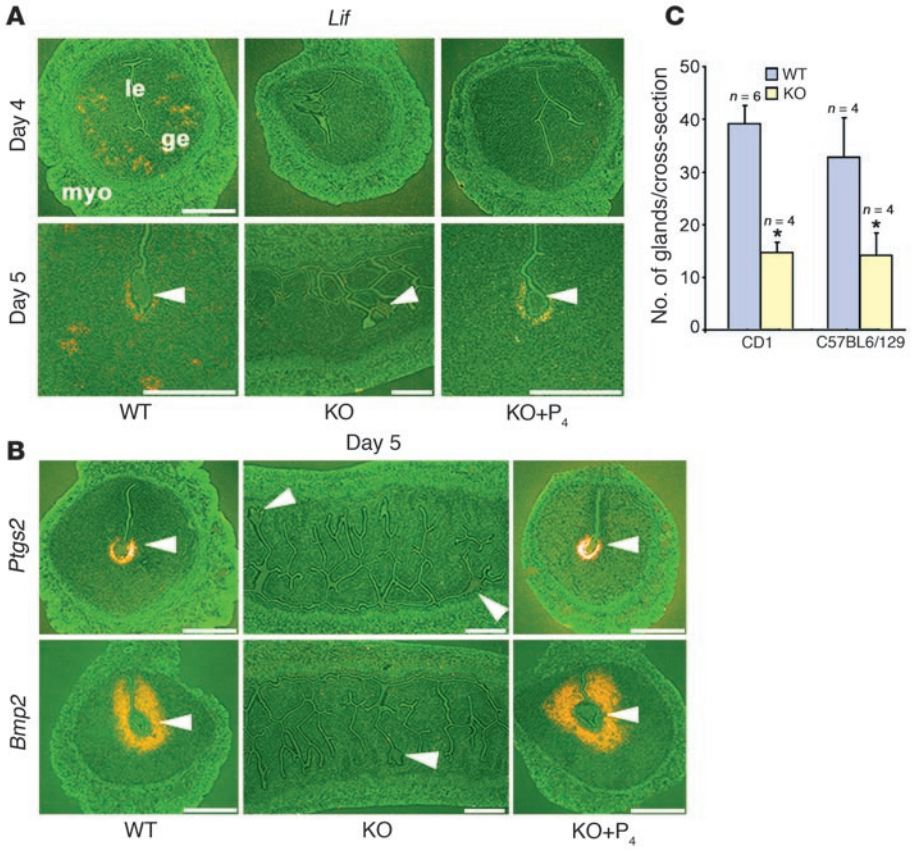

Figure 3
Delivery of P_4 via Silastic implants restores expression of implantation-related genes in CD1 *Fkbp52*$^{-/-}$ females. (**A**) In situ hybridization of *Lif* in WT, KO, and KO+P_4 uteri on days 4 and 5 of pregnancy. (**B**) In situ hybridization of *Ptgs2* and *Bmp2* in WT, KO, and KO+P_4 uteri on day 5 of pregnancy. P_4 implants were inserted s.c. in KO females on day 2 of pregnancy. Arrowheads in **A** and **B** indicate the location of embryos. Scale bars: 200 μm. (**C**) Number of glands per uterine cross-section of WT and KO uteri on CD1 and C57BL6/129 backgrounds. For each animal, glands were counted in 9–12 uterine sections. Numbers above bars indicate number of mice evaluated. Values are mean ± SEM. *$P < 0.05$, unpaired Student's *t* test.

normal expression patterns of these genes. There is clear evidence that both estrogen and P_4 participate in uterine gland formation in sheep (35, 36). Our data also implicate a potential role for FKBP52/PR signaling in mouse uterine gland formation and function. This is an exciting finding but warrants further investigation.

Postimplantation defects in P_4-treated CD1 Fkbp52$^{-/-}$ *females.* Our observation of unimplanted blastocysts recovered from CD1 *Fkbp52*$^{-/-}$ females examined on day 5 of pregnancy suggested deferral of implantation timing in these mice as is observed in *cPLA2a*$^{-/-}$ or *LPA3*$^{-/-}$ mice (37, 38). To test for this possibility, we sacrificed CD1 *Fkbp52*$^{-/-}$ mice on days 6 and 8 of pregnancy. We found that unlike in *cPLA2a*$^{-/-}$ or *LPA3*$^{-/-}$ mice, implantation timing was not altered; rather implantation drastically failed in *Fkbp52*$^{-/-}$ females (Figure 4, A and B, and Table 1). Again, blastocysts were recovered from CD1 *Fkbp52*$^{-/-}$ uteri on these days, confirming implantation as the major defect in these mice (Figure 4, A and B, and Table 1).

We next determined whether P_4 supplementation could sustain pregnancy beyond day 5. To our surprise, we found that placing Silastic P_4 implants in null females allowed progression of pregnancy in 100% of CD1 *Fkbp52*$^{-/-}$ females examined on days 6, 8, and 12 of pregnancy (Figure 4, A–C, and Table 1). However, ISs in P_4-treated CD1 *Fkbp52*$^{-/-}$ uteri were smaller and weighed less than those in WT uteri (Table 1), suggesting a somewhat compromised decidual response.

Experimentally induced decidualization fails in Fkbp52$^{-/-}$ *mice irrespective of P_4 treatment.* In pseudopregnant mice in the absence of embryos, the steroid hormonal milieu and responsiveness of the uterus on days 1 through 4 are similar to those of normal pregnancy. Various artificial stimuli, including intraluminal infusion of oil, can initiate many aspects of the decidual cell reaction in pseudopregnant mice if applied on day 4. Decidualization, characterized by stromal cell proliferation and differentiation into specialized types of cells with polyploidy, is critical to pregnancy establishment in many species (1). In fact, decidualization does not occur in *Pgr*$^{-/-}$ mice (3, 16), demonstrating an absolute requirement for P_4/PR signaling in this process.

We asked whether experimentally induced decidualization occurs in *Fkbp52*$^{-/-}$ females on both genetic backgrounds using the model of intraluminal oil infusion and if not, whether P_4 supplementation could rescue this phenotype. We observed severely compromised decidualization in both C57BL6/129 and CD1 *Fkbp52*$^{-/-}$ females when compared with WT littermates (Figure 4, D and E). Interestingly, P_4 treatment could not restore decidualization in *Fkbp52*$^{-/-}$ mice on either strain, with only a few swellings noted along the oil-infused uterine horn (Figure 4, D and E). That P_4 treatment rescues blastocyst implantation with decidualization in CD1 *Fkbp52*$^{-/-}$ females but not experimentally induced decidualization is remarkable. This agrees with previous observations that gene expression differs in the decidual bed induced by blastocysts from that induced experimentally (39). Differences in decidualization in these 2 models (oil-induced versus blastocyst-induced) were noted even at the ultrastructural level (40).

P_4 delivery via Silastic implants partially restores full-term pregnancy in CD1 Fkbp52$^{-/-}$ *females.* To investigate whether P_4 supplementation maintains full-term pregnancy in CD1 *Fkbp52*$^{-/-}$ females, P_4 implants placed on day 2 were removed on day 17, since P_4 withdrawal is necessary to initiate labor (41). Although 9 of 13 *Fkbp52*$^{-/-}$ mothers delivered pups of normal weight, litter sizes were sig-

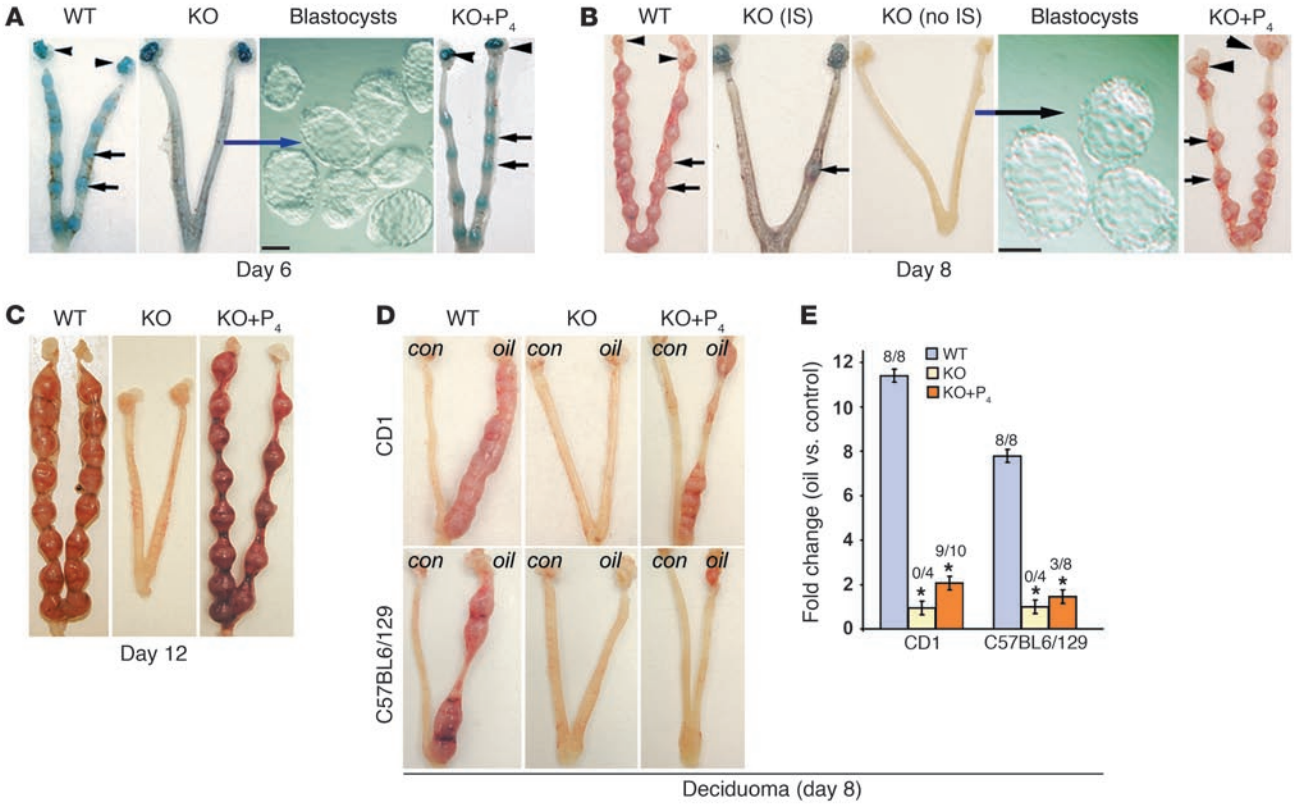

Figure 4

P_4 delivery via Silastic implants rescues blastocyst-induced, but not oil-induced, decidualization in *Fkbp52*$^{-/-}$ females. Representative photographs of WT, KO, and KO+P_4 uteri on day 6 (**A**), day 8 (**B**), and day 12 (**C**) of pregnancy are shown. Arrowheads and short arrows indicate the location of ovary and IS, respectively. Representative images of recovered unimplanted blastocysts from uteri (long arrow) of KO females without ISs are shown. Scale bars: 50 μm. (**D**) Oil-induced decidualization fails in KO females on both CD1 and C57BL6/129 backgrounds. Representative photomicrographs of WT, KO, and KO+P_4 uteri on day 8 of pseudopregnancy. On day 4 of pseudopregnancy, 25 μl of oil was infused intraluminally in one uterine horn (oil); the contralateral horn without oil infusion served as a control (con). (**E**) Fold changes in weight between oil-infused and noninfused (control) uterine horns. Numbers above the bars indicate the number of mice with decidual response per total number of mice examined. Fold changes are presented as mean ± SEM; *$P < 0.05$, unpaired Student's *t* test.

nificantly smaller (Figure 5A). This led us to determine when embryonic loss occurs in null females carrying P_4 implants. We observed that 70 of 106 ISs were resorbing in P_4 implant-treated *Fkbp52*$^{-/-}$ uteri when examined on day 14, compared with only 9 of 194 in WT uteri (Figure 5, B and C). Resorbing ISs in P_4-treated *Fkbp52*$^{-/-}$ females appeared dark blue and were infiltrated with a massive number of blood cells (Figure 5B). In addition, cytokeratin staining of sections of ISs with normal appearance from null females carrying P_4 implants showed placentas with less-developed and ill-defined spongiotrophoblast and labyrinth layers compared with those of WT females (Figure 5D).

Although P_4/PR signaling is absolutely required for pregnancy maintenance in all eutherians thus far studied, uterine FKBP52 expression in later days of pregnancy has not yet been examined. We found that while FKBP52 is expressed in the mesometrial decidua with high expression in the placenta on days 10–14 of pregnancy, PR is mostly expressed in the decidua (Supplemental Figure 3, A and B, and data not shown). Embryonic signals direct normal decidual functions and development (39), which in turn govern placentation and embryonic growth (42). Therefore, the expression of FKBP52 and PR in the decidua suggests that maternally derived FKBP52-mediated PR signaling contributes to feto-placental well-being, while placental expression of FKBP52 suggests a PR-independent role for FKBP52.

Excessive estrogenic influence or complement activation does not contribute to pregnancy failure in P_4-implanted Fkbp52$^{-/-}$ *females.* We speculated that one possible explanation for the higher incidence of resorptions in *Fkbp52*$^{-/-}$ females carrying P_4 implants is the tipping of the balance between estrogen and P_4 signaling toward estrogenic dominance. Since high levels of estrogen are detrimental to pregnancy success (30), we examined whether combined treatment with P_4 implants and ICI 182,780 (ICI; 25 μg or 125 μg/0.1 ml oil/mouse, s.c.), an estrogen receptor antagonist (Tocris Bioscience), injected on days 8–13 improves pregnancy maintenance. Combined treatment with P_4 and the high dose of ICI increased the resorption rate to 100% in *Fkbp52*$^{-/-}$ mice, while treatment with a lower dose of ICI resulted in resorption rates similar to those observed in null mice receiving P_4 implants alone (Supplemental Figure 4). Our observation that treatment with a higher dose of ICI results in increased resorption rates suggests that appropriate estrogen signaling is also critical to pregnancy maintenance. This agrees with a previous study showing that pregnancy maintenance under the direction of P_4 is supported by low amounts of estrogen, especially on days 10 and 11 of pregnancy (43).

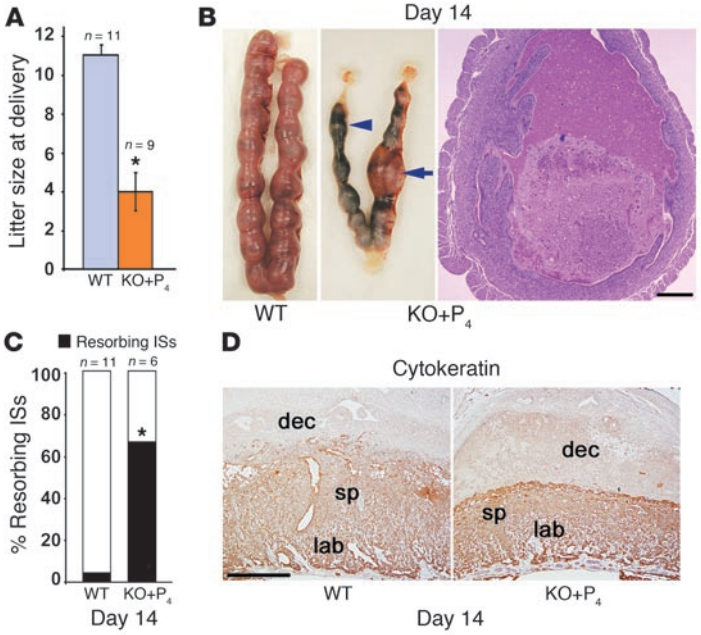

Figure 5

P_4 delivery by Silastic implants fails to sustain pregnancy to full term. (**A**) Average litter size of WT and KO+P_4 mothers. Litter size is presented as mean ± SEM; *$P < 0.05$, unpaired Student's *t* test. (**B**) Representative photomicrographs of WT and KO+P_4 uteri on day 14 of pregnancy. Arrowheads and arrows indicate resorbing and normal IS, respectively. A representative H&E-stained section of resorbing IS from KO+P_4 uterus shows massive infiltration of blood cells. Scale bar: 200 μm. (**C**) Percentage of resorption sites in WT and KO+P_4 mice on day 14 of pregnancy. *$P < 0.05$, unpaired Student's *t* test. (**D**) Cytokeratin staining of WT and KO+P_4 IS on day 14. Scale bar: 200 μm. dec, decidua; lab, labyrinth; sp, spongiotrophoblast.

While the underlying causes of recurrent pregnancy failure are not well understood, one possibility is that the maternal immune response mistakenly recognizes the fetus. A recent study shows that complement activation causes growth restriction and subsequent fetal rejection, leading to pregnancy failure (44). We speculated that this pathway is activated due to reduced PR signaling from FKBP52 deficiency, especially since P_4 has antiinflammatory roles within and outside the uterus (17, 45). Since low doses of heparin inhibit the complement pathway (44, 46), *Fkbp52*$^{-/-}$ mice carrying P_4 implants from day 2 were given a low dose of heparin (10 U/mouse) twice a day on days 8, 10, and 12 of pregnancy. However, this treatment also failed to rescue pregnancy maintenance in these mice (Supplemental Figure 4), suggesting that activation of the complement pathway is not a major contributing factor for pregnancy failure in P_4-treated *Fkbp52*$^{-/-}$ mice.

Differential P_4/PR signaling is required for successful full-term pregnancy. Normal serum P_4 levels during pregnancy in CD1 WT mice on days 5 and 14 of pregnancy range between 40 and 47 ng/ml (Supplemental Figure 1 and Figure 6A). In our experiments, Silastic P_4 implants provided an increase in serum P_4 levels sufficient to induce uterine receptivity and rescue implantation in CD1 *Fkbp52*$^{-/-}$ females but failed to maintain pregnancy to full term. We speculated that further increasing P_4 levels would rectify this failure. We injected P_4 s.c. at a dose of 2 mg/ml per mouse daily to further increase P_4 serum levels. We observed that in null mice, serum P_4 levels increased to approximately 156 ng/ml by daily injection as compared with approximately 100 ng/ml in mice treated with P_4 implants, as assessed on day 14 of pregnancy (Figure 6A). To our surprise, these elevated P_4 levels significantly improved pregnancy maintenance in null females; the number and weights of ISs on day 14 were comparable to those in WT mothers (Figure 6, B–D). Pregnancy maintenance in WT mice exposed to a similar P_4 injection regimen was normal (data not shown).

Our next objective was to examine whether daily P_4 injection results in pregnancy to full term in null females. CD1 *Fkbp52*$^{-/-}$ females mated with WT males were injected with P_4 daily from days 2 through 17 of pregnancy and monitored for term delivery on day 20. We observed that all P_4-injected null females carried pups to term, and the average litter size was comparable to that of WT mothers (Figure 6E). Pup weights from null and WT mothers at weaning and during early development were also similar (data not shown).

Discussion

Although P_4 signaling via PR is critical to ovulation, fertilization, implantation, postimplantation growth, and pregnancy maintenance, it is not known whether a similar P_4/PR signaling mechanism determines these target and stage-specific functions or whether genetic disparity influences this signaling. By using *Fkbp52*$^{-/-}$ females with compromised PR signaling as opposed to *Pgr*$^{-/-}$ females with total infertility (3, 12), we address these issues for the first time to our knowledge. Our previous and present investigations provide clear evidence that the major reproductive phenotype in mice lacking *Fkbp52* is unique to uterine deficiency in the context of implantation. The reason for the organ-specific dependence on FKBP52 for appropriate PR signaling is not clearly understood, but it is noteworthy, since ovulation that also requires P_4/PR signaling is normal in null females. It is possible that relatively higher P_4 levels locally in the ovary override the reduced PR signaling in the absence of FKBP52. We were also surprised to note that P_4/PR-regulated mating behavior appears normal, since null females mate and produce copulatory plugs. One possibility is that the uterus requires more robust P_4/PR signaling during pregnancy than other P_4 targets. Alternatively, FKBP52, in addition to its role in influencing PR signaling, may have a unique PR-independent role in the uterus not observed in other tissues.

P_4 signaling via PR plays major roles at essentially all stages of pregnancy, from ovulation through parturition. It is surprising to see that genetic makeup of a species alters such a fundamental signaling pathway. While FKBP52 is essential to support implantation in both strains of mice in the absence of exogenous P_4, FKBP52's role becomes less significant in CD1 mice exposed to high levels of P_4, while still remaining crucial in C57BL6/129 mice under similar treatment conditions. The contrasting reproductive phenotypes of *Fkbp52*$^{-/-}$ mice on C57BL6/129 and CD1 backgrounds with respect to P_4 rescue provides the first evidence to our knowledge that P_4/PR/FKBP52 signaling is a function of genetic makeup. Coordinated interactions of P_4 and estrogen are essential to uterine receptivity and implantation. While one aspect of P_4 signaling is to correctly orchestrate P_4-responsive genes, another is to appropriately constrain and/or synergize estrogen-responsive genes in the uterus. One cause of implantation failure in C57BL6/129, but not in CD1, *Fkbp52*$^{-/-}$ females supplemented with P_4 could be the failure of the

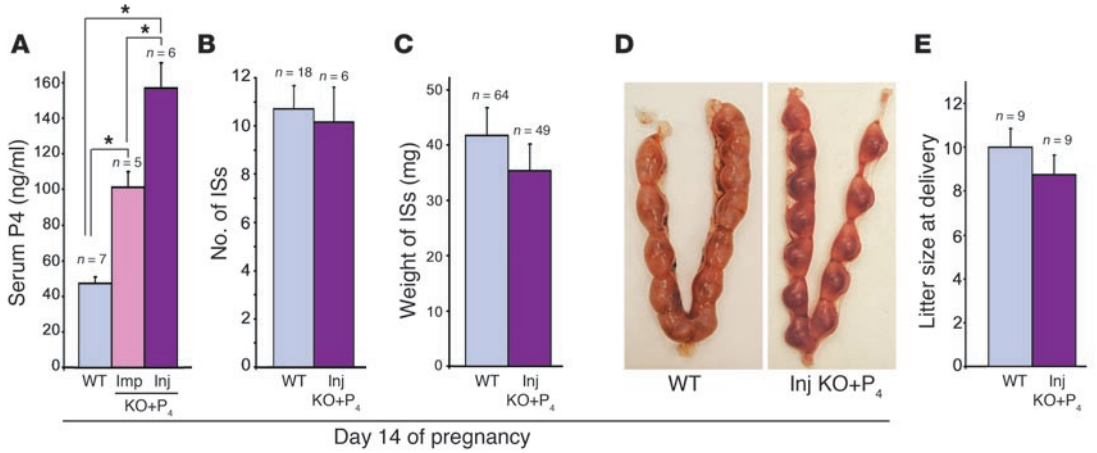

Figure 6

Daily P_4 injections restore pregnancy to full term in CD1 *Fkbp52*$^{-/-}$ females. (**A**) Serum P_4 levels in WT and KO+P_4 mice on day 14 of pregnancy. KO mice were exposed daily to P_4 either via Silastic implants (Imp KO+P_4) or s.c. injection (Inj KO+P_4; 2 mg/ml) from day 2 of pregnancy. *$P < 0.05$, univariate ANOVA. (**B**) The average number of ISs was not significantly different in WT and Inj KO+P_4 mice on day 14; $P > 0.1$, unpaired Student's *t* test. (**C**) Weights of ISs from WT and Inj KO+P_4 mice on day 14 of pregnancy were not significantly different; $P > 0.1$, unpaired Student's *t* test. (**D**) A representative photomicrograph of WT and Inj KO+P_4 uteri on day 14 of pregnancy is shown. (**E**) The average litter size of WT and Inj KO+P_4 mice was not significantly different; $P > 0.05$, unpaired Student's *t* test. All values are mean ± SEM.

uterus to attain optimal receptivity arising from altered expression of *Areg* and/or *Ltf*. This inappropriate gene expression in the presence of P_4 may reflect even lower basal PR activity in C57BL6/129 *Fkbp52*$^{-/-}$ uteri than in CD1 *Fkbp52*$^{-/-}$ uteri. Alternatively, differential expression of modifier genes in these 2 strains of mice could contribute to differential uterine responsiveness to P_4/PR signaling in the absence of FKBP52.

There is evidence that rodent blastocysts synthesize P_4 or structurally similar steroids (47–49) and that gene expression differs in embryo-induced decidua and experimentally induced deciduoma (39). Since our previous results show that *Fkbp52* and *Pgr* are expressed in blastocysts (12), it is possible that P_4 synthesized from blastocysts enhances PR/FKBP52 signaling locally at their sites of apposition in the uterus. This could explain the failure of oil-induced decidualization but not of that induced by implanting blastocysts in CD1 *Fkbp52*$^{-/-}$ mice supplemented with P_4. Alternatively, signaling arising from blastocysts may influence other uterine functions. For example, the gene encoding heparin-binding epidermal growth factor–like growth factor (HB-EGF) is expressed in implantation-competent blastocysts, which can induce uterine *Hbegf* to initiate the implantation cascade. In fact, Affi-Gel Blue beads (Bio-Rad) presoaked in HB-EGF, when transferred into uterine lumens of pseudopregnant mice on day 4, show implantation-like responses similar to those induced by living blastocysts, including upregulation of *Hbegf* and *Bmp2* (32, 50).

P_4/PR signaling is critical throughout pregnancy until its downregulation for the onset of parturition. Indeed, ovariectomizing mice at practically any stage of pregnancy causes resorptions and/or abortion (51). However, the magnitude of this signaling during various stages of pregnancy remains unknown. Although P_4 implants cannot rescue pregnancy in WT mice ovariectomized on day 8 unless they are given daily P_4 injections, similar P_4 injections alone maintain full-term pregnancy in mice ovariectomized on day 14 (51). The use of *Fkbp52*$^{-/-}$ females has enabled us to show that the requirement for P_4/PR signaling is different for uterine receptivity, implantation, and postimplantation growth. This signaling appears to be tightly regulated, since blood levels of approximately 100 ng/ml of P_4 are adequate to induce uterine receptivity and implantation, but levels above 150 ng/ml are required for full complement of pregnancy success in the absence of FKBP52. This suggests that more robust P_4/PR signaling is required for pregnancy maintenance than is required for uterine receptivity, implantation, and decidualization. It is also possible that a burst of P_4 levels as provided by daily injections is more amenable to pregnancy sustenance than the relatively constant levels maintained with Silastic implants.

The observation that differential P_4 levels are required for various stages of pregnancy implies that signaling targets are different. We believe that P_4/PR signaling for uterine preparation, implantation, and decidualization is primarily targeted to uterine epithelial and stromal cells. This is consistent with P_4's known roles in epithelial differentiation and stromal cell proliferation during the periimplantation period. On the other hand, P_4/PR signaling for pregnancy maintenance is directed more toward keeping the myometrium quiescent until parturition and providing sanctuary for the growing fetus from mother's immunological surveillance. P_4/PR signaling is also known to regulate angiogenesis (52), a process integral to placental development and pregnancy maintenance. Since the events during the course of pregnancy are very dynamic, P_4/PR signaling at various targets could be overlapping. We consider FKBP52's influence on P_4/PR signaling during pregnancy to be primarily of maternal origin, since both PR and FKBP52 are expressed in the decidua on days 10 and 12 of pregnancy. The high expression of FKBP52 in the placenta implies a PR-independent role for FKBP52 in placentation. This requires further investigation, but the putative PR-independent role is not critical, since pregnancies are completed to term with normal litter sizes in CD1 *Fkbp52*-null females receiving daily P_4 injections.

An interesting observation is the successful nursing of pups to weaning by P_4-injected CD1 *Fkbp52*$^{-/-}$ mothers. Mammary morphogenesis during pregnancy requires P_4/PR signaling (16, 53), but the latter stages of lactogenesis and lactation correspond to withdrawal

of this signaling (reviewed in ref. 54). It was recently reported that FKBP52 may not be critical for P_4/PR signaling in the mammary gland, since exogenously provided P_4 could stimulate mammary morphogenesis in *Fkbp52*$^{-/-}$ mice (15). This is similar to the findings of our studies in which *Fkbp52*$^{-/-}$ mothers were injected with exogenous P_4 prior to parturition. These results suggest that exogenously supplemented P_4 overcomes the P_4-resistant state in the mammary gland and uterus in the absence of FKBP52.

The implanting blastocyst is the stimulus for normal decidualization in mice. However, in humans, stromal cells undergo decidualization during the receptive phase in each menstrual cycle in the absence of blastocysts. This predecidualization is thought to be critical for blastocyst implantation in the pregnant uterus (55). Our findings that P_4 supplementation fails to rescue oil-induced decidualization in *Fkbp52*-null uteri may therefore have implication for predecidualization events in P_4-resistant women.

P_4 resistance is also a hallmark of endometriosis, a condition that affects an estimated 5 million women of childbearing age in the United States (National Women's Health Information Center, National Institute of Child Health and Human Development [NICHD], NIH) (56–58). In fact, a recent study examining global gene expression profiles in endometria of women with or without endometriosis found dysregulation of many known P_4 target genes during the window of uterine receptivity (59). While the mechanism(s) of P_4 resistance remain unclear, one speculation is that downregulation of PR contributes to P_4 resistance; however, other studies refute this concept (57). Whether FKBP52 expression differs in normal and endometriotic tissues has not been examined.

The finding that human FKBP52 interacts with and potentiates human PR activity in MEFs (12) and our preliminary observation on a limited number of samples showing FKBP52 and PR expression in human endometria in proliferative and secretory phases (data not shown) suggest a potential role for uterine P_4/PR/FKBP52 signaling in women. In fact, 3 separate clinical trials found that P_4 treatment resulted in a statistically significant decrease in miscarriages in women with a history of 3 or more consecutive pregnancy losses (60). In this respect, pregnancy rescue in CD1 *Fkbp52*$^{-/-}$ mice by daily P_4 injections is clinically relevant for women who are infertile due to P_4 resistance. These are exciting results, especially since no significant differences in adverse effects were found between P_4 treatment and control groups. These findings in humans are similar to our findings in mice that exogenous P_4 injections in WT females do not adversely affect pregnancy (data not shown). *Fkbp52*$^{-/-}$ mice with normal PR and P_4 levels but reduced PR activity constitute a unique model for studying P_4 resistance specifically in uterine biology, and it is hoped that our findings will encourage the development of human studies to determine whether FKBP52 status influences P_4 resistance in the uterus.

Methods

Mice. The *Fkbp52* gene was disrupted in mice by homologous recombination, as previously described (10). Tail genomic DNA was used for PCR-based genotyping. Because genetic backgrounds of mice contribute to different phenotypes (18, 20), we introduced *Fkbp52* deficiency in CD1 mice by crossing C57BL6/129 *Fkbp52* heterozygous males to CD1 WT females producing an F_1 generation. F_1 *Fkbp52*$^{+/-}$ males were then back-crossed to CD1 WT females, and the process was continued for 10 generations. Crossing heterozygous females with heterozygous males of the same genetic background (CD1/F_{10}) generated *Fkbp52*-null and WT littermates for experiments. Mice on both backgrounds were housed and used in the present investigation in accordance with NIH, and animal protocol was approved by the Vanderbilt Institutional Animal Care and Use Committee.

Ovulation, fertilization, implantation, blastocyst transfer, and experimentally induced decidualization. Mice were examined for ovulation, fertilization, and implantation as described previously (37). To examine ovulation and fertilization, CD1 WT or *Fkbp52*$^{-/-}$ mice were mated with fertile WT males. On day 2 of pregnancy (the day the vaginal plug was first observed was considered day 1), oviducts were flushed with Whitten's medium to recover ovulated eggs, and fertilization was assessed by the number of 2-cell embryos. ISs on days 5 and 6 of pregnancy were visualized by an i.v. injection (0.1 ml/mouse) of Chicago blue B dye solution (1% in saline), and the number of ISs demarcated by distinct blue bands was recorded. For blastocyst transfer, pseudopregnant recipients were generated by mating females with vasectomized WT males. Day-4 WT blastocysts were transferred into day-4 uteri of C57BL6/129 WT or *Fkbp52*$^{-/-}$ pseudopregnant recipients, and ISs were examined 24 hours (day 5) or 96 hours (day 8) later by the blue dye method (32).

To determine whether experimentally induced decidualization occurs in null females, WT or *Fkbp52*$^{-/-}$ females were mated with vasectomized WT males. On day 4, one uterine horn was infused with sesame oil (25 μl), while the contralateral horn served as control. Mice were sacrificed on day 8 of pseudopregnancy. Weights of infused (oil) and noninfused (control) uterine horns were recorded, and fold increase in weight was used as an index of decidualization. All mice used were between 2 and 5 months of age.

Exogenous P_4 supplementation and other treatments. To see whether P_4 supplementation rescues the infertility phenotype of *Fkbp52*$^{-/-}$ females, null females were mated with WT males, and a Silastic implant (4 cm length × 0.31 cm diameter) containing P_4 was placed under the dorsal skin on day 2 of pregnancy. Implants were removed upon sacrifice on days 5, 6, and 8 to examine implantation; days 12 or 14 to examine pregnancy maintenance; or day 17 to allow labor to complete full-term pregnancy. Alternatively, null female mice were given a daily injection of P_4 (2 mg/0.1 ml/mouse, s.c.) from days 2 through 14 to monitor pregnancy maintenance or through day 17 to allow labor to ensue on day 20 for full-term pregnancy.

To determine the contribution of the complement pathway to pregnancy maintenance, Silastic P_4 implants were placed under the dorsal skin on day 2 of pregnancy, and heparin (10 U/0.1 ml/mouse in saline) was injected s.c. twice a day on days 8, 10, and 12 of pregnancy. To determine whether the P_4/estrogen ratio influences pregnancy maintenance, Silastic P_4 implants were placed under the dorsal skin on day 2 of pregnancy, and ICI (25 μg or 125 μg/0.1 ml/mouse in sesame oil), an estrogen receptor antagonist, was injected once a day on days 8 through 13 of pregnancy.

To determine whether P_4 supplementation rescues experimentally induced decidualization in null females, WT or *Fkbp52*$^{-/-}$ females were mated with vasectomized WT males, and P_4 was either injected daily from day 2 or Silastic P_4 implants placed under the dorsal skin on day 2 of pseudopregnancy. On day 4, while the uterine lumen of one horn was infused with sesame oil (25 μl), the noninfused contralateral horn served as a control. Mice were sacrificed on day 8 of pseudopregnancy. Uterine weights of oil-infused and noninfused horns were recorded, and fold increases in weight were recorded as an index of decidualization.

P_4 assay. Blood samples from mice were collected on the indicated days of pregnancy. Serum was separated by centrifugation (850 *g* for 15 minutes) and stored at –80°C until analysis. Serum P_4 levels were measured by radioimmunoassay.

In situ hybridization. Sense or antisense ^{35}S-labeled cRNA probes for *Areg*, *Ihh*, *Hoxa10*, *Ltf*, *C3*, *Lif*, *Bmp2*, *Ptgs2*, *Pgr*, and *Fkbp52* generated using appropriate polymerases from respective cDNAs were used for hybridization as described previously by us (61). Sections hybridized with sense probes showed no positive signals and served as negative controls.

Immunohistochemistry. Immunolocalization of cytokeratin was performed using a polyclonal rabbit anti-cow antibody (Dako). A Histostain-Plus (DAB) kit (Zymed) was used to visualize antigen. Brown deposits indicated sites of positive immunostaining.

Statistics. Statistical significance was determined as $P < 0.05$ by 1-tailed Student's t test. To compare serum P_4 levels in WT versus KO+P_4 mice on day 14 of pregnancy, statistical significance was determined as $P < 0.05$ by univariate ANOVA. All values are presented as mean ± SEM.

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