FKBP52-PROGESTERONE RECEPTOR SIGNALING

DURING PREGNANCY

By

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To my parents, Lynn and Steve,
for their unwavering belief in me

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ABBREVIATIONS

AREG, amphiregulin

BMP, bone morphogenetic protein

HDC, histidine decarboxylase

EGF, epidermal growth factor

E_2, estrogen

HB-EGF, heparin-binding EGF-like growth factor

FKBP, FK506 binding protein

IHH, indian hedgehog

IS, implantation site

KO, knockout

LIF, leukemia inhibitory factor

P_4, progesterone

PR, progesterone receptor

PDZ, primary decidual zone

SDZ, secondary decidual zone

WT, wild-type

CHAPTER I

INTRODUCTION

Implantation is a process by which the blastocyst comes into intimate physical and physiological contact with the uterine endometrium. A two-way interaction between the implantation-competent blastocyst and receptive uterine luminal epithelium initiates this process (Dey et al., 2004; Wang and Dey, 2006). If an appropriate embryo-uterine dialogue at the molecular and cellular level is not established, the blastocyst will not implant leading to pregnancy failure. Coordinated effects of the ovarian steroid hormones estrogen and progesterone (P_4) guide these embryo-uterine interactions that are not only critical for determining correct implantation timing, but also for sustaining pregnancy. An increased understanding of mammalian implantation and pregnancy maintenance has been gained through the use of various genetically engineered mouse models, and this information will potentially lead to strategies to correct implantation failure and improve pregnancy rates in women.

The assembly of new life begins with the union between a sperm and an egg through the process of fertilization. The one-cell fertilized egg, termed embryo, undergoes several mitotic cell divisions, ultimately forming a ball of cells (16 cells or more) termed morula. The morula then differentiates into a blastocyst with two distinct cell populations, the inner cell mass (ICM) which will become the embryo proper and a layer of trophectoderm cells surrounding the

ICM which will form the placenta and extraembryonic membranes (Dey et al., 2004). Dysregulation of events prior, during, or after implantation can contribute to poor pregnancy rates in eutherian mammals. For example, for a successful pregnancy to occur, on the embryonic side, fertilized eggs must develop to blastocysts, transit through the oviduct to the uterus, establish an intimate contact with the uterine epithelium, be nurtured through embryogenesis, and ultimately delivered at birth. On the maternal side, the uterus must be receptive for implantation and undergo extensive remodeling for decidualization, pregnancy maintenance, and ultimately parturition (Dey, 1996; Dey et al., 2004). Therefore, studying implantation physiology and revealing the signaling pathways involved are necessary for alleviating problems with human infertility and ensuring the birth of quality offspring.

Stages of Embryo Implantation

Enders and Schlafke have classified the implantation process into three stages: apposition, adhesion, and penetration (Enders, 1976; Enders and Schlafke, 1969). Apposition is the stage when embryonic trophectoderm cells become closely apposed to the uterine luminal epithelium. This is followed by adhesion of the trophectoderm to the luminal epithelium. Ultimate attachment and penetration involves the invasion of the luminal epithelium by the trophectoderm.

The attachment reaction coincides with a localized increase in stromal vascular permeability at the site of the blastocyst, which can be demarcated by

intravenous injection of a macromolecular blue dye, also referred to as the uterine blue reaction (Psychoyos, 1973a). This is preceded by uterine luminal closure, which contributes to the intimate apposition of the blastocyst with the uterine epithelium (Enders, 1976; Psychoyos, 1973a). The first sign of the attachment reaction in the mouse occurs on the evening (2000-2400 h) of day 4 of pregnancy (day 1 = vaginal plug) (Psychoyos, 1973a). In mice and humans, while luminal epithelial cells undergo apoptosis after attachment (Parr et al., 1987), stromal cells surrounding the implanting blastocyst undergo decidualization eventually embedding the embryo within the antimesometrial stroma. In mice, blastocysts are oriented with their ICMs directed mesometrially, whereas in humans the ICM is directed antimesometrially (Dey et al., 2004). The mechanism by which the blastocyst is directed to the antimesometrial luminal epithelium or by which the orientation of the blastocyst is achieved at the time of implantation remains unknown. This introduction highlights the more recently defined signaling cascades involved in implantation, with specific regard to P_4 signaling, therefore focusing specifically on steroid hormones, homeotic transcription factors, and immunophilins.

Steroid hormonal regulation of early pregnancy

The uterus is comprised of heterogeneous cell types (luminal and glandular epithelium, stroma, and myometrium) that each respond differentially to estrogen and P_4. In mice, the coordinated effects of estrogen and P_4 regulate proliferation and/or differentiation of uterine cells in a spatiotemporal manner to

govern events of early pregnancy (Huet-Hudson et al., 1989). For example, on the first day of pregnancy (day 1) in mice, uterine epithelial cells undergo proliferation under the influence of preovulatory estrogen secretion. Rising levels of P_4 secreted from freshly formed corpora lutea initiate stromal cell proliferation from day 3 onward. This stromal cell proliferation is further stimulated by a small secretion of ovarian estrogen on the morning of day 4 of pregnancy. These coordinated effects of P_4 and estrogen result in the cessation of uterine epithelial cell proliferation, initiating differentiation (Huet-Hudson et al., 1989). During normal pregnancy, the presence of an active blastocyst in the uterus is the stimulus for the implantation reaction. After the attachment reaction is initiated on day 4 (2000–2400 h), stromal cells surrounding the implanting blastocyst begin to proliferate extensively and differentiate into decidual cells (Dey, 1996).

In pseudopregnant mice (female mice mated with vasectomized males), the steroid hormonal milieu within the uterus is similarly maintained due to the presence of corpora lutea. Therefore, the sensitivity of pseudopregnant uteri to implantation on days 1 through 4 is quite similar to that of normal pregnancy, and blastocyst transfer into the uterine lumen on day 4 of pseudopregnancy provokes normal implantation reactions and subsequent decidualization. Although blastocysts are the normal inducers of implantation events, various nonspecific stimuli such as intraluminal infusion of oil, air, and mechanical stimuli can also initiate certain aspects of the decidual cell reaction (deciduoma) in pseudopregnant or steroid hormonally prepared uteri (Dey, 1996). However, there is evidence that uterine reactions induced by nonspecific stimuli are

different than those induced by blastocysts (Lundkvist and Nilsson, 1982; Paria et al., 2001; Tranguch et al., 2007).

The window of uterine receptivity

Uterine sensitivity with respect to steroid hormonal requirements and implantation has been classified as prereceptive, receptive and nonreceptive (refractory) (Dey, 1996; Psychoyos, 1973b). These phases have been defined by blastocyst transfer experiments in pseudopregnant uteri. In the mouse, the uterus is considered prereceptive on days 1 through 3 of pregnancy or pseudopregnancy, fully receptive on day 4, and then progressively moves toward nonreceptivity thereafter. Evidence suggests that the uterus is most receptive on day 4 (Paria et al., 1993), and the efficiency of implantation decreases with time as the uterus enters the nonreceptive phase (Song et al., 2002). By day 6, the uterus is completely nonreceptive/refractory to blastocyst implantation.

The mouse uterus becomes receptive to blastocyst implantation only if exposed to a small amount of estrogen 24-48 h following P_4 priming (Huet-Hudson and Dey, 1990). The concentration of estrogen within a very narrow range determines the duration of the window of uterine receptivity in mice, i.e. uterine receptivity remains open for an extended period at lower estrogen levels but rapidly closes at higher levels. Uterine nonreceptivity induced at these higher estrogen levels is accompanied by aberrant uterine expression of genes, suggesting that careful regulation of estrogen levels could improve female fertility in fertility clinics (Ma et al., 2003).

Another critical factor determining the window of receptivity is the state of activity of the blastocyst. In mice, ovariectomy before the preimplantation estrogen secretion on the morning of day 4 of pregnancy induces delayed implantation (Paria et al., 1993; Yoshinaga and Adams, 1966). This status can be maintained for many days if P_4 treatment is continuously provided. Under this condition, blastocysts undergo zona hatching at a slower pace, but become dormant without initiating the attachment reaction and the P_4-primed uterus remains in the neutral stage. However, a single injection of estrogen promptly induces blastocyst activation with the initiation of implantation in the P_4-primed uterus. A recent global gene expression study shows that active and dormant blastocysts differ at the molecular and physiological level (Hamatani et al., 2004), suggesting that a complex network of signaling pathways distinctly regulate blastocyst dormancy and activation.

This model of delayed implantation also occurs naturally in many vertebrate species, but the underlying mechanisms that direct this process differ depending on species (Mead, 1993). For example, delayed implantation occurs during lactation after postpartum ovulation and fertilization of the egg in mice and rats (McLaren, 1968; Yoshinaga and Hosi, 1958). However, termination of the suckling stimulus readily initiates implantation. While many other species undergo obligatory seasonal delayed implantation, whether this process occurs in humans remains unknown. Regardless, the delayed implantation model in mice has been exploited in this and other studies to better understand the

molecular signaling events that emanate from the embryo to the uterus and vice versa.

The "ripple effect" theory

Recent evidence suggests that a short delay in the attachment reaction produces an adverse ripple effect throughout pregnancy with aberrant spacing of embryos, defective placentation, increased number of resorptions, and retarded fetal development, ultimately giving rise to a poor pregnancy outcome (Song et al., 2002; Wilcox et al., 1999; Ye et al., 2005). This phenotype was shown in mice lacking either cytoplasmic phospholipase $A_{2\alpha}$ ($cPLA_{2\alpha}$) or G-protein-coupled lysophosphatidic acid receptor (LPA3), illustrating the importance of these signaling pathways in determining the window of uterine receptivity and on-time implantation (Song et al., 2002; Ye et al., 2005). Interestingly, treatment of both $cPLA_{2\alpha}$ and *Lpa3* null females with prostaglandins (PGs) resumes on-time implantation, but embryo crowding persists (Song et al., 2002; Ye et al., 2005). The phenotypic similarity between $cPLA_{2\alpha}$ and *Lpa3* null females and the reduced levels of uterine COX-2 in *Lpa3* null females identifies COX-2 as a common signaling pathway in mediating these molecules. Overall, this concept of timing as a crucial component of normal feto-placental development and determinant of pregnancy outcome comprises a novel theme whereby early embryo-uterine interaction directs developmental programming for the remainder of the gestation.

Progesterone (P_4): discovering the 'pregnancy hormone'

While ovarian estrogen is essential for blastocyst implantation in the P_4-primed uterus in mice and rats, it is not essential for implantation in pigs, guinea pigs, rabbits or hamsters (Dey et al., 2004). One reason for this lack of requirement for estrogen could be that pig and rabbit blastocysts synthesize their own estrogen, while mouse embryos lack such machinery. Whereas ovarian estrogen is crucial in only some species, ovarian P_4, on the other hand, is required for pregnancy maintenance in most eutherians studied. The diverse actions of P_4 include balancing the effects of estrogen, regulating uterine smooth muscle contraction until labor, and modulating maternal immunological surveillance towards the embryo (Tranguch et al., 2006).

Since the discovery of the corpus luteum in 1672 by Regner DeGraaf, a Dutch scientist whose namesake was given to the antral "Graafian" follicle, much work followed to characterize the role of this structure during pregnancy. DeGraaf recognized that the presence of the corpus luteum was associated with pregnancy (Corner, 1943; Corner, 1974) and until 1900, many functions for the corpus luteum were proposed. One group postulated that its function was to prevent ovulation during pregnancy (August Prenant, France), while another group implicated its involvement in preparing the endometrium for implantation (Gustav Born, Germany) (Frobenius, 1999). Prior to his death, Gustav Born had a revelation that the corpus luteum must secrete some substance to protect the embryo and support implantation (Corner, 1974). In 1900, Ludwig Fraenkel, a disciple of Born, examined this hypothesis and provided proof for an endocrine

function of the corpus luteum, an amazing triumph considering the terms 'hormone' or 'endocrinology' had not yet been coined. In these experiments, Fraenkel used the rabbit as an animal model, a useful model since rabbits only ovulate post-mating with embryos residing in the oviducts and uterus for about seven days before implantation. Fraenkel found that pregnancy loss occurred if he removed the corpora lutea within those seven days post-mating (Fraenkel, 1903). It was also at this time that Paul Ancel and Paul Bouin found that the corpus luteum was responsible for changes occurring in the endometrium in both pregnant and pseudopregnant rabbits (Bouin and Ancel, 1910). From these experiments, it became clear that the corpus luteum does in fact secrete a substance to influence both the fate of the embryo and the integrity of the uterus. In 1934, the successful purification of this substance, progesterone, in its crystalline form was independently reported by several groups (Karl Slotta, Hans Ruschig, Eric Fels of Breslau; George W. Corner Sr. and Willard Allen of the United States; and Karl Butenandt and Oskar Wintersteiner of Germany) (Frobenius, 1999).

The discovery and isolation of P_4 coincided with experiments by several groups showing that pituitary or ovarian extracts stimulate ovulation in frogs (Heilbrunn et al., 1939; Rugh, 1935; Ryan, 1940; Wright, 1945). In the late 1950s and 1960s, while researchers began to elaborate on various roles for P_4 ligand, Gerald Mueller and Elwood Jensen discovered the estrogen receptor (ER) (Fannon et al., 2001). The field of steroid hormone action then quickly erupted when Jack Gorski and Angelo Notides linked estrogen action with uterine

protein synthesis (Notides and Gorski, 1966). Soon after, nuclear progesterone receptor (PR) was the first steroid hormone receptor to be purified, first from the chick oviduct (O'Malley et al., 1970) and later from humans (Smith et al., 1975). After purification of PR, ER and androgen receptor (AR), it was soon realized that steroid hormone receptors primarily affect gene transcription (Schwartz et al., 1977). It was not until 1985, however, with the advent of DNA sequencing technology, that a steroid hormone receptor, glucocorticoid receptor (GR), was cloned (Weinberger et al., 1985); the cloning of the remaining steroid hormone receptors followed shortly after (Arriza et al., 1987; Brinkmann et al., 1989; Greene et al., 1986; Jeltsch et al., 1986).

Mechanism of progesterone signaling

P_4 mediates its responses primarily through its nuclear PR to activate transcription of genes involved in uterine biology and pregnancy. Two isoforms of PR are expressed from one single gene: PRA and PRB. These two gene products are transcribed from separate promoters on the same gene, with PRB being the longer of the two proteins (120 kDa) compared with PRA (97 kDa) (**Figure 1**).

Figure 1. The PR gene, *Pgr*, encodes two isoforms, PRA and PRB, with separate translation initiation sites (AUG). AF, transactivation domain; DBD, DNA binding domain; LBD, ligand binding domain; N, amino terminal; C, carboxyl terminal.

The amino terminal portion of both isoforms is the most hypervariable region and contains the transactivation domains, AF3 (PRB only) and AF1 (PRA and PRB), responsible for the recruitment of coactivators and/or corepressors during transcriptional regulation. The DNA-binding domain (DBD) is the most conserved region, composed of two type II zinc finger structures responsible for binding to specific *cis*-acting DNA sequences (Freedman, 1992; Luisi et al., 1991). The carboxyl terminal region contains the ligand-binding domain (LBD) with an additional site, AF2, for interaction with transcriptional coregulators. PR interactions with heat shock proteins and other chaperones to localize to the LBD/AF2 (Schowalter et al., 1991). The larger segment of PRB, referred to as the B upstream sequence (Kelleher et al., 1999), contains the AF3 domain, which is thought to attenuate PRB activity. For this reason, PRB tends to be less transcriptionally active than the PRA isoform. In fact, it is thought that PRA acts as a dominant repressor of PRB, evidenced by coexpression studies in cultured cells (Kraus et al., 1995; Vegeto et al., 1993). PRA and PRB proteins also respond differentially to P_4 antagonists, e.g. RU-486, in that antagonist-bound PRA is inactive, while antagonist-bound PRB has increased transcriptional activity (Conneely et al., 2001). When expressed in equimolar concentrations in cells, PRA and PRB can homodimerize or heterodimerize, essentially existing as three entities: A:A, B:B, A:B. It is thought that each entity then will exhibit differential activity in regulating downstream target genes.

Mice lacking the *Pgr* gene encoding both PR isoforms have illustrated the essential roles of P_4 signaling through its nuclear steroid hormone receptor

during reproductive processes. Female mice lacking both PR isoforms have complete ovulation, fertilization, and implantation failure as well as impaired sexual behavior, defective responses to gonadotropins, and defective ductal branching morphogenesis and lobuloalveolar differentiation of the mammary gland (Chappell et al., 1997; Lydon et al., 1995). P_4 signaling through PR has also been shown to play a role in decidualization and maintaining the uterus in a quiescent state until delivery (Dey et al., 2004).

Mouse models lacking either PRA or PRB have been generated using the CRE-*loxP* system to introduce a point mutation at either of the two ATG translation initiation codons (Mulac-Jericevic et al., 2003; Mulac-Jericevic et al., 2000). While *PgrA* null female mice are infertile due to severe abnormalities in ovarian and uterine functions, *PgrB* null female mice show no apparent defects in ovarian and uterine functions but have significantly reduced mammary ductal side-branching and alveologenesis during pregnancy. These findings suggest that PRA and PRB act in a tissue-selective manner to regulate their specific actions, and also imply that PRA:PRB heterodimers are not required for uterine reproductive events.

It is also thought that P_4 can signal through a membrane receptor; however, the contribution of this signaling pathway to specific physiological processes has not yet been conclusively demonstrated. A role for this signaling was first shown in *Xenopus* oocytes. Quiescent *Xenopus* oocytes remain arrested in meiosis I until exposure to P_4, which triggers rapid non-transcriptional responses rather than the conventional transcriptional activation known to be a

signature of nuclear steroid hormone receptor signaling. Using the *Xenopus* model, one study identified XPR1 as a membrane PR necessary for *Xenopus* oocyte activation (Tian et al., 2000). Zhu and colleagues cloned a membrane progestin receptor from spotted sea trout ovaries, characterizing it as a G-protein coupled receptor expressed solely in the brain and reproductive organs that activates mitogen-activated protein kinase (MAPK) signaling pathways upon progestin binding (Zhu et al., 2003). Since then, three forms of putative membrane-bound progestin receptors have been identified in channel catfish (mPRα, β, and γ) (Kazeto et al., 2005), and another group has identified an adrenal cortex protein, inner zone antigen (IZAg), as a putative membrane PR (Raza et al., 2001). In the mouse, two potential membrane PRs are thought to play roles particularly in ovarian cells: membrane progesterone receptor (MPR) and PGR membrane component I (PGRMC1) (Peluso et al., 2006; Zhu et al., 2003). While MPRs and PGRMC1 are expressed in various cell types of the ovary, their roles in ovarian biology remain to be determined (Peluso et al., 2006). Whether a role for these membrane PRs exists in uterine biology also remains unexplored.

Progesterone-regulated target genes during early pregnancy

On day 4 of pregnancy in mice, the uterus is primarily under the influence of P_4, and P_4-target genes activated through PR include homeobox transcription factors, growth factors, and cytokines (Dey et al., 2004). These genes show robust expression on this day of pregnancy. Specific examples of P_4-regulated

or responsive genes include amphiregulin (*Areg*), histidine decarboxylase (*Hdc*), Indian hedgehog (*Ihh*), and *Hoxa-10*.

Amphiregulin is a glycosylated heparin-binding protein and a member of the epidermal growth factor (EGF) family. Under the influence of P_4, *Areg* is expressed in the luminal and glandular epithelia (Das et al., 1995) and is regulated by P_4 working via the PRA isoform (Mulac-Jericevic et al., 2003). While amphiregulin is associated with uterine cell-specific differentiation and proliferation, mice lacking the *Areg* gene appear to have normal fertility (Luetteke et al., 1999). This could result from compensation by other EGF family members, but precludes amphiregulin as an essential factor for implantation. Another P_4-regulated gene upregulated by P_4 working through the PRA isoform is histidine decarboxylase (*Hdc*) (Mulac-Jericevic et al., 2000). This gene is expressed primarily in the epithelium on day 4 of pregnancy (Paria et al., 1998), but its role in implantation biology has yet to be determined.

The components of the hedgehog signaling pathway are influenced by P_4, specifically those of Indian hedgehog (IHH) including the hedgehog-binding receptor, Patched, and its downstream transcription factor Gli1-3 (Matsumoto et al., 2002). Expression of *Ihh* increases in the luminal and glandular epithelium from day 3 of pregnancy onward. During the same time, the expression of its receptor and downstream transcription factors is upregulated in the underlying stroma. This suggests that IHH functions as a paracrine growth factor for stromal cell proliferation during early pregnancy. Roles for hedgehog signaling have

also been delineated in cancer, and recent evidence indicates roles for this conserved signaling pathway in uterine biology (Lee et al., 2006).

Hoxa10 encodes a transcription factor that belongs to a multigene family. *Hox* genes are developmentally regulated and share a common highly conserved sequence element called the *homeobox* that encodes a helix-turn-helix DNA binding domain (Krumlauf, 1994). *Hox* genes are organized into four clusters (A, B, C, and D) on four different chromosomes in mice and humans and follow a stringent pattern of spatial and temporal colinearity during embryogenesis (Krumlauf, 1994). Specifically, genes at the 3'-end of each cluster are activated during early embryogenesis in the anterior region of the developing embryo, whereas genes located toward the 5'-end are restricted to posterior regions of the embryo and are expressed during later stages of embryogenesis (Krumlauf, 1994). *Hoxa10* is located at the 5'end of the A cluster and is classified as an *AbdB-like Hox* gene because of its homology with the *Drosophila AbdB* gene. The *Abd* genes constitute a distinct subfamily of homeobox genes that exhibit posterior domains of expression including the genital imaginal disc in *Drosophila* and the developing genitourinary system in mammals (Dolle et al., 1991; Izpisua-Belmonte et al., 1991). Indeed, *Hoxa10* is highly expressed in the developing genitourinary tract and adult female reproductive tract, suggesting its roles in reproductive events (Benson et al., 1996; Gendron et al., 1997; Hsieh-Li et al., 1995).

Hoxa-10 null mice reveal FKBP52 as a critical mediator of implantation

Definitive evidence for roles of various genes in implantation and/or decidualization has been demonstrated with an array of mouse gene knockout models. *Hoxa10* is strongly expressed in the mouse uterine stroma during the receptive phase (day 4), implantation (day 5) and decidualization (day 6) (Satokata et al., 1995). Indeed, failure of decidualization in *Hoxa10* null uteri leads to pregnancy failure (Benson et al., 1996). Uterine stromal cells isolated from *Hoxa10* null uteri show reduced proliferation in response to P_4 contributing to decidualization defects, while epithelial cell proliferation remains normal in response to estrogen (Benson et al., 1996; Lim et al., 1999). Several P_4-responsive genes are dysregulated in uterine stroma of *Hoxa10* null uteri (Lim et al., 1999), suggesting that HOXA10 conveys P_4 responsiveness by regulating gene expression as a transcription factor. Furthermore, P_4 is a strong inducer of *Hoxa10* in mouse uterine stroma, inducing its expression within 4 h post-P_4 injection. This upregulation is attenuated by the PR antagonist RU-486, suggesting a requirement for PR in *Hoxa10* induction.

Since the mechanism by which HOXA10 regulates uterine stromal cell proliferation and differentiation during uterine receptivity, implantation, and decidualization remained unexplored, we used proteomics approach to identify downstream targets of HOXA10 in the periimplantation uterus. Specifically, we isolated stromal cells from wild-type and *Hoxa10* null uteri, cultured them for 24 h, and then analyzed them by 2D DIGE (two-dimensional difference gel electrophoresis). More than 1000 proteins were resolved with isoelectric points

between pH 4 and 7 and molecular masses between 10 and 150 kDa. Among these, less than 4% (n=36) of the detected features displayed changes in abundance that were consistent across all three measurements, supported by Student's *t*-test *P* values within the 99th percentile confidence interval (P<0.01, n=3) for the variance of mean changes. Twenty-two of the identified proteins showed upregulation in *Hoxa10* null stromal cells that primarily represented cytoskeletal, metabolic, and actin-related proteins (**Table 1**) (Daikoku et al., 2005).

Table 1. Proteomic identification of up-regulated proteins in uterine stromal cells of *Hoxa10* wild-type (+/+) and null (-/-) mice. Fold abundance changes are reported, whereby a fold increase is calculated directly from the -/-: +/+ volume ratio, and a fold decrease = 1/volume ratio.

Protein Name	Fold Change -/-: +/+ (n=3)	*t* Test *P* Value (n=3)	Functional Category
Nonmuscle caldesmon	2.65	0.00017	Actin-binding protein
Fetuin A	2.45	1.80E-0.5	Cytoskeletal protein, antiinflammation
Coronin-like protein and ATP-dependent RNA helicase DDX19	1.82	1.10E-05	Actin-related protein; RNA modification
Plasminogen/activator inhibitor-1	1.59	0.00018	Inflammation
Plasminogen/activator inhibitor-1	1.44	3.70E-0.5	Inflammation
Transaldolase	1.51	1.50E-05	Metabolism
Endoplasmic reticulum protein (ERp29)	1.48	4.30E-0.5	Chaperone
Tropomyosin	1.43	0.00026	Actin-binding protein
Gelsolin	1.40	1.70E-0.6	Actin-binding protein
Aldehyde dehydrogenase	1.37	0.00054	Metabolism
Lamin B1	1.35	0.0028	Glycoprotein, migration and proliferation
Transaldolase	1.33	8.30E-05	Metabolism
T complex protein 1 α-subunit B	1.32	0.0018	Actin production
HeteronuclearRNP K	1.30	0.0019	RNA modification
Annexin A4	1.30	0.00028	Cell aggregation
Annexin A1	1.29	0.004	Antiinflammation
Protein disulfide isomerase A6	1.27	0.018	Calcium-binding protein
Protein disulfide isomerase A6	1.26	0.00047	Calcium-binding protein
Swiprosin 1	1.25	0.00061	Unknown
Cytosolic nonspecific dipeptidase	1.24	0.00026	Protein modification
Eukaryotic initiation factor 4A-1	1.21	0.009	RNA translation

The seven down-regulated proteins in *Hoxa10* null stromal cells were mostly classified as metabolic with the exception of FK506-binding protein 4 (FKBP52) that displayed a statistically significant decrease (P = 0.00021) (**Table 2**) (Daikoku et al., 2005).

Table 2. Proteomic identification of down-regulated proteins in uterine stromal cells of *Hoxa10* wild-type (+/+) and null (-/-) mice. Fold abundance changes are reported, whereby a fold increase is calculated directly from the -/-: +/+ volume ratio, and a fold decrease = 1/volume ratio. CoA, Coenzyme A.

Protein Name	Fold Change -/-: +/+ (n=3)	*t* Test *P* Value (n=3)	Functional Category
Calreticulin (ERp60)	-1.39	0.00083	Chaperone
D-3-phosphoglycerate dehydrogenase	-1.34	1.90E-0.5	Metabolism
D-3-phosphoglycerate dehydrogenase	-1.30	0.002	Metabolism
Adenylate kinase isozyme 1	-1.31	0.0014	Metabolism
Succinly-CoA ligase	-1.24	2.30E-0.5	Metabolism
FKBP52	**-1.22**	**0.00021**	**Chaperone**
Asparagine synthetase	-1.20	0.0011	Metabolism

Peptide mass mapping and fragmentation spectra for FKBP52 are shown (**Figure 2**) (Daikoku et al., 2005).

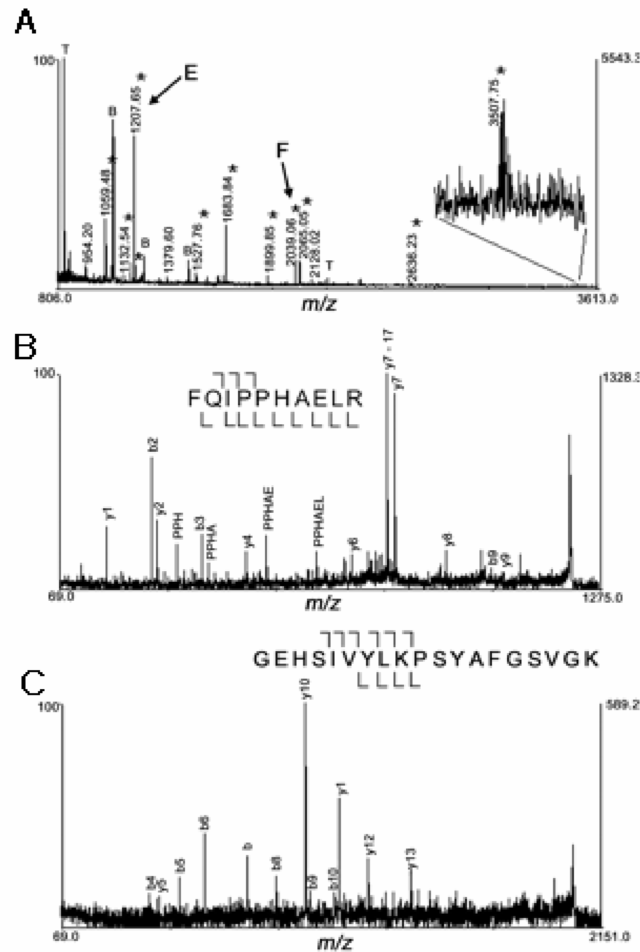

Figure 2. Mass spectrometry identifies FKBP52 protein as down-regulated in *Hoxa10* null stromal cell cultures. (A) MALDI-TOF mass spectrum representing a peptide mass map of FKBP52. Ions labeled with an asterisk indicate peptide masses (M+H) that match predicted peptide masses for FKBP52 identified using the MASCOT search algorithm. MALDI-TOF/TOF tandem MS of the indicated ions is shown in panels B and C, with b-ion and y-ion cleavages that are consistent with the predicted amino acid sequence of the FKBP52-derived peptides depicted above and below the amino acid sequences, respectively.

Uterine Fkbp52 expression is cell specific and downregulated in Hoxa10 null mice

We focused our attention to further characterize FKBP52 because of its known interaction with steroid receptors, in particular PR (Barent et al., 1998; Smith, 1993; Tai et al., 1986), its potential influence on receptor-hormone binding affinity (Riggs et al., 2003) and subcellular localization of receptors (Pratt et al., 2004). Immunostaining performed on sections of day 4 pseudopregnant mouse uteri show that FKBP52 protein is present in the stroma as well as the luminal and glandular epithelia in WT mouse uteri. However, as expected, its levels are lower in *Hoxa10* null uteri when compared to WT (**Figure 3A & B**). Localization of *Fkbp52* mRNA expression follows the pattern of immunolocalization (**Figure 3C**).

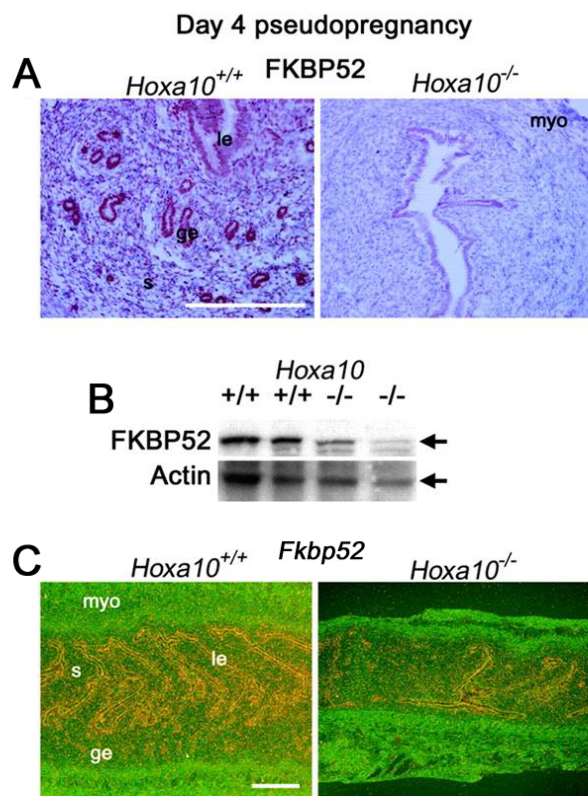

Figure 3. Uterine expression of FKBP52 in day 4 pseudopregnant wild-type (+/+) and *Hoxa10* null (-/-) mice. (A) Immunostaining of FKBP52 in uterine cross-sections. Bar = 200 µm. (B) Western blot analysis of FKBP52. Actin serves as a control. (C) In situ hybridization of *Fkbp52* expression in representative dark-field photomicrographs of longitudinal uterine sections from +/+ and -/- uteri. Bar = 400 µm. le, luminal epithelium; ge, glandular epithelium; s, stroma; myo, myometrium.

FKBP52 is expressed in a spatiotemporal manner in the periimplantation uterus

Fkbp52 is expressed in a unique temporal and cell-specific manner before implantation (days 1 and 4), at the time of implantation (day 5), and during the postimplantation (day 8) period (**Figure 4A & B**).

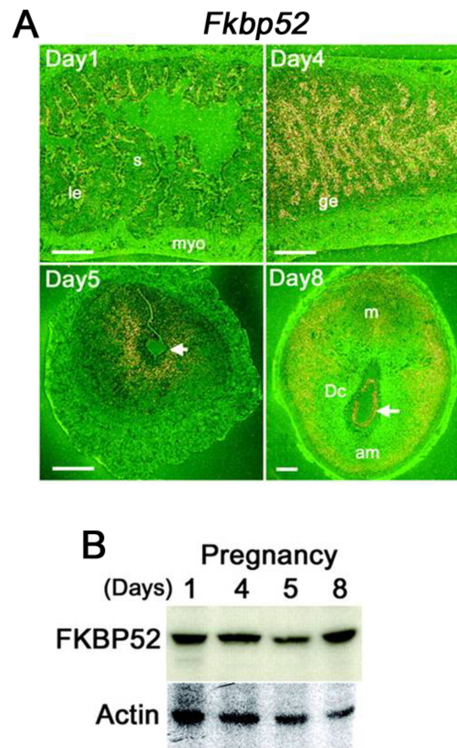

Figure 4. Uterine expression of FKBP52 in wild-type mouse uteri during early pregnancy. (A) In situ hybridization of *Fkbp52*. Hybridization signals in representative dark-field photomicrographs of longitudinal uterine sections on day 1 and day 4 and cross-sections on days 5 and 8 are shown. Bar = 400 µm. le, luminal epithelium; ge, glandular epithelium; s, stroma; myo, myometrium; m, mesometrial pole; am, anti-mesometrial pole; Dc, decidua. Arrows indicate location of embryo. (B) Western blotting of FKBP52 protein. Actin serves as a loading control.

As mentioned, the uterus is under the influence of preovulatory estrogen on day 1 of pregnancy or pseudopregnancy with heightened cell proliferation in the epithelium. In contrast, the day 4 uterus is exposed to rising levels of P_4 from the newly formed corpora lutea and fortified with a small amount of estrogen that results in epithelial cell differentiation with stromal cell proliferation. We observed that *Fkbp52* mRNA is detected mostly in the luminal epithelium on day 1 of

pregnancy when the uterus is primarily under the influence of estrogen. On day 4, the expression expands into the stroma with its persistent expression in the luminal and glandular epithelia, suggesting that ovarian steroids coordinate the differential expression of *Fkbp52*. Because P_4 effects are correlated with epithelial cell differentiation and stromal cell proliferation on day 4, it is speculated that FKBP52 in the epithelium and stroma directs these events in the context of cell types. This is consistent with a similar expression pattern of nuclear PR in the uterus on this day (Tan et al., 1999).

On the morning of day 5 of pregnancy, the attachment between the luminal epithelium and blastocyst trophectoderm is in the early stages marked by continued stromal cell proliferation and endometrial vascular permeability solely at the site of the blastocyst (Dey et al., 2004). On this day, expression of *Fkbp52* is uniquely displayed in the stroma surrounding the implanting blastocyst (**Figure 4A**). The localization of *Fkbp52* surrounding the embryo on day 5 suggests that the process of implantation has an impact on uterine expression of this immunophilin. On day 8 of pregnancy, the implantation process is well advanced with maximal stromal cell decidualization (Dey et al., 2004). In mice, the proliferating and differentiating stromal cells surrounding the implanting blastocyst begin to form the primary decidual zone (PDZ) on the afternoon of day 5. The PDZ is avascular and densely packed with decidual cells. By day 6, the PDZ is well formed and a secondary decidual zone (SDZ) is formed around the PDZ. At this time, cell proliferation ceases in the PDZ, but still continues in the SDZ (Dey et al., 2004), and a thin layer of undifferentiated stromal cells

establishes a boundary between the myometrium and the SDZ. The PDZ progressively degenerates through day 8; at this point, placental and embryonic growth gradually replaces the SDZ, which is reduced to a thin layer of cells called the decidua capsularis. The mesometrial decidual cells eventually form the decidua basalis (Dey et al., 2004). On day 8, expression of *Fkbp52* dramatically changes, lying in peripheral decidualizing cells more specifically at the mesometrial SDZ and the undifferentiated stroma situated between the deciduum and myometrium. Interestingly, *Fkbp52* is also evident in the growing embryo on this day (**Figure 4A**). Western blotting confirms translation of *Fkbp52* mRNA during these days of pregnancy (**Figure 4B**). Collectively, these results suggest that ovarian steroid hormones and implantation events differentially affect uterine *Fkbp52* expression.

Progesterone and estrogen differentially regulate Fkbp52 expression in the uterus

Our results showing differential expression of *Fkbp52* on days 1 and 4 of pregnancy suggested that this gene is regulated in the uterus by ovarian steroids in a cell-specific manner. Indeed, in situ hybridization was performed to determine the uterine cell-specific expression of *Fkbp52* in response to the ovarian steroid hormones P_4 and/or estrogen (**Figure 5**).

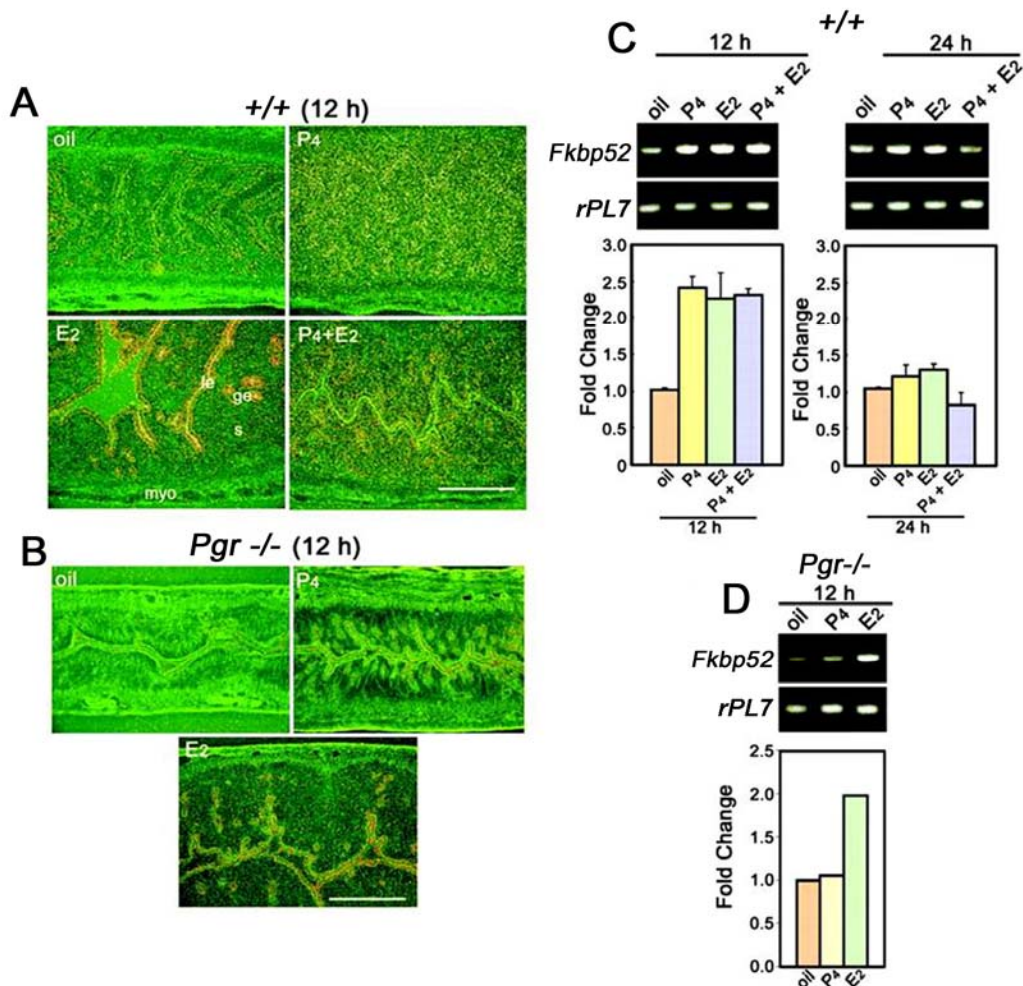

Figure 5. Uterine *Fkbp52* expression in ovariectomized mice after various steroid hormone treatments. (A) In situ localization of *Fkbp52* in wild-type uteri is shown in representative dark-field photomicrographs of longitudinal uterine sections. (B) In situ hybridization of *Fkbp52* in *Pgr*$^{-/-}$ uteri. Ovariectomized mice were treated with oil (control), E_2 (100 ng), P_4 (2 mg), or E_2+P_4 and killed 12 h following treatment. Bar = 200 µm. le, luminal epithelium; ge, glandular epithelium; s, stroma; myo, myometrium. (C) RT-PCR detection of *Fkbp52* in wild-type uteri. Ovariectomized mice were treated with P_4 and/or E_2 for the indicated times. (D) RT-PCR detection of *Fkbp52* in *Pgr*$^{-/-}$ uteri. *rPL7* is a housekeeping gene. The data presented as fold induction are relative to oil treatment and are mean ± SE.

We observed that *Fkbp52* expression is low in uteri of ovariectomized mice treated with oil (vehicle control) and that this expression is primarily restricted to the luminal and glandular epithelia with low to undetectable

expression in the stroma. An injection of estradiol-17β (E_2, 100 ng/0.1 ml/mouse) upregulated *Fkbp52* expression levels by 12 h with the pattern remaining unaltered. In contrast, an injection of P_4 (2 mg/0.1 ml/mouse) showed a prominent shift in *Fkbp52* expression from the luminal epithelium to the stromal compartment at 12 h and expression was downregulated by 24 h (**Figure 5A**). A combined injection of E_2 and P_4 showed an expression pattern similar to that of P_4 alone but at somewhat lower levels (**Figure 5A**). Interestingly, P_4 treatment markedly downregulated E_2-induced epithelial *Fkbp52* expression. Comparative RT-PCR analysis of total RNA isolated from uteri harvested from similarly treated mice confirmed our *in situ* hybridization results, showing an increase in *Fkbp52* expression in E_2, P_4, and E_2 + P_4 treatment at 12 h with a return to basal levels at 24 h (**Figure 5C**).

We next used mice lacking nuclear PR to further define the mechanism of steroidal regulation of *Fkbp52*. The induction of stromal *Fkbp52* expression that we observed in P_4-treated wild-type mice was virtually abolished in *Pgr* null mice (**Figure 5B**). However, the E_2 response remained intact, showing a similar increase in *Fkbp52* expression in the luminal epithelium as was observed in WT uteri (**Figure 5B**). In situ hybridization results were further confirmed by comparative RT-PCR analysis of total RNA isolated from uteri harvested from similarly treated *Pgr* null mice, showing an increase in *Fkbp52* expression with E_2 treatment, but no such increase in ovariectomized *Pgr* null mice treated with P_4 (**Figure 5D**). Collectively, these results suggest that steroid hormones regulate *Fkbp52* expression differentially and in a cell-specific manner. This observation of

differential regulation of FKBP52 by estrogen and P_4 points to a role for FKBP52 in epithelial-stromal cross-talk to mediate full complement of uterine functions in response to ovarian steroid hormones.

CHAPTER II

COCHAPERONE IMMUNOPHILIN FKBP52 IS CRITICAL TO UTERINE RECEPTIVITY AND IMPLANTATION

Abstract

Embryo implantation in the uterus is a critical step in mammalian reproduction, requiring preparation of the uterus receptive to blastocyst implantation. Uterine receptivity lasts for a limited period, and it is during this period that blastocysts normally implant. Ovarian steroid hormones estrogen and P_4 are the primary regulators of this process. FKBP52, an immunophilin, serves as a cochaperone for steroid hormone nuclear receptors to govern appropriate hormone action in target tissues. Here we show a critical role for FKBP52 in mouse implantation. This immunophilin has unique spatiotemporal expression in the uterus during implantation, and females missing the *Fkbp52* gene have complete implantation failure due to the lack of attainment of uterine receptivity. The overlapping uterine expression of FKBP52 with nuclear PR in wild-type mice together with reduced P_4 binding to PR, attenuated PR transcriptional activity and downregulation of several P_4-regulated genes in uteri of *Fkbp52* null mice, establishes this cochaperone as a critical regulator of uterine P_4 function. Interestingly, ovulation, another P_4-mediated event, remains normal in *Fkbp52* null animals. Collectively, the present investigation provides evidence for an *in vivo* role for this cochaperone in regulating tissue-specific hormone action and its critical role in uterine receptivity for implantation.

Introduction

Immunophilins are aptly named due to their ability to bind and mediate actions of certain immunosuppressive drugs. Immunophilins are grouped into two protein families: the FK506-binding proteins (FKBPs) and the cyclosporin A binding proteins termed cyclophilins (CyPs). FKBP and CyP typically exhibit peptidylprolyl *cis-trans* isomerase (PPIase) activity, and this PPIase domain also forms the drug-binding site (Fanghanel and Fischer, 2004). Some members of the FKBP and CyP families contain a tetratricopeptide repeat (TPR) domain that targets binding to the highly conserved C-terminal end of heat shock protein 90 (Hsp90), and thus participate as cochaperones with Hsp90 in its substrate interactions.

FKBP52 and its related immunophilins FKBP51 and CyP40 were initially characterized as Hsp90-binding proteins because of these conserved TPR domains (Pratt and Toft, 1997). Hsp90 is bound with high affinity to the PR complex in an ATP-bound state. ATP binding changes the conformation of the protein and therefore, the ATP turnover rate determines the amount of time Hsp90 remains bound to its target protein. This turnover rate is regulated by Hsp90's association with cochaperones, one being p23. This small, acidic co-chaperone is known to inhibit the ATPase activity of human Hsp90, and this inhibition is shown to be more robust in the presence of FKBP52 (McLaughlin et al., 2002); however, while various roles for p23 in these complexes have been proposed, its precise function and the molecular mechanism by which p23 mediates its functions are unknown.

The pathway for assembly of steroid receptor/chaperone complexes is complex, involving a variety of Hsps and cochaperones. For PR specifically, it is thought that this steroid hormone nuclear receptor first binds to Hsp40 and Hsp70 in the cytoplasm of the cell. This is followed by the binding of Hop-Hsp90 to the bound Hsp70 (Hernandez et al., 2002). Hsp70 and Hop then dissociate from this intermediate PR complex, while p23 is recruited to receptor-bound Hsp90. The PR complex with Hsp90 and p23 bound then recruits one of the cochaperone immunophilins (FKBP51, FKBP52, CyP40, or protein phosphatase 5 (PP5)) to an Hsp90 binding site. For PR, if this mature complex is bound to FKBP52, then it is capable of optimally binding P_4 ligand. The mature complex containing Hsp90 must be maintained so that the receptor can bind P_4 with high affinity and efficiency. Recent reports indicate that each steroid hormone nuclear receptor (GR, ER, and PR)-Hsp90 complex displays preferential binding with specific immunophilins (Barent et al., 1998).

FKBP52 and FKBP51 share a common binding site on Hsp90 and therefore compete for assembly with steroid receptor complexes (Riggs et al., 2003). The PR heterocomplex, however, preferentially assembles with FKBP51 despite its lower concentration in the cell (20 nM) when compared to FKBP52 (100 nM) (Schiene-Fischer and Yu, 2001). FKBP52 elevates the hormone binding affinity of PR in an Hsp90- and PPIase-dependent manner, while FKBP51 antagonizes the actions of FKBP52 and reduces PR binding affinity for ligand, thereby reducing secondary responses to hormones (Riggs et al., 2003).

It is surprising that functional differences exist between these two immunophilins, given the 70% homology in their amino acid sequences (Nair et al., 1997).

It has been suggested that while FKBP52 is necessary for potentiating PR activity through optimal ligand binding, basal PR activity can exist without FKBP52 presence, albeit not as efficiently. The mechanism by which FKBP52 potentiates receptor hormone-binding ability is still unclear. Point mutation of the FKBP52 TPR domain demonstrates that an association of FKBP52 with Hsp90 is critical (Riggs et al., 2003), suggesting that FKBP52 is obligatorily recruited to the receptor complex by Hsp90. In addition, point mutation of the FKBP52 PPIase domain or addition of the PPIase inhibitor, FK506, blocks receptor potentiation, which perhaps reflects a direct interaction between the PPIase domain and receptor sites. FKBP52 and FKBP51 have similar PPIase activity towards small peptide substrates (Pirkl and Buchner, 2001), and their respective PPIase domains share 80% sequence similarity; yet, FKBP51 fails to potentiate receptor activity. Chimeric constructs in which the PPIase domain has been exchanged confirms that the FKBP52 PPIase domain is unique in its ability to stimulate receptor activity (Riggs et al., 2003). However, the receptor site(s) putatively targeted by the FKBP52 PPIase domain have not been identified although it is known that they localize to the LBD (Riggs et al., 2003). Mapping of specific amino acid differences within the PPIase domains of FKBP52 and FKBP51 should provide further insight into mechanism(s) of receptor potentiation or inhibition.

There is additional evidence that FKBP52, through a dynein-binding ability lacking in FKBP51, can enhance nuclear translocation of hormone-bound GR (Davies et al., 2002; Pratt et al., 2004; Wochnik et al., 2005). This role for FKBP52 is based on evidence that FKBP52 binds both a nuclear localization signal (NLS) and dynein (Czar et al., 1995; Galigniana et al., 2004; Silverstein et al., 1999), and on *in vitro* studies demonstrating that FKBP52 facilitates the nuclear transport of both GR and p53 (Galigniana et al., 2004; Galigniana et al., 2001; Wochnik et al., 2005). Evidence suggests that FKBP52 binds dynein directly through its PPIase domain (Silverstein et al., 1999). It is debated whether FKBP52 facilitates a similar translocation of PR (Silverstein et al., 1999), and it is not yet clear what contribution dynein associations play relative to direct FKBP-mediated effects on hormone binding, especially with PR and other receptors that prior to hormone binding have a more predominant nuclear localization than GR. Steroid hormone binding prevents reinitiation of the heterocomplex assembly cycle in the cytoplasm, and while it is known that hormone binding is followed by heterocomplex dissociation, it is not known whether the complex dissociates during nuclear transport or within the nucleus. Once in the nucleus, however, as described earlier, nuclear PR forms homodimers to act as a transcription factor to either activate gene transcription by binding to its cognate DNA elements or to inhibit transcription through interaction with other transcription factors.

The purpose of this next study, therefore, was to examine a physiological role for FKBP52 and FKBP51 in an *in vivo* context, since roles for these cochaperones had not been examined with respect to P_4-dependent processes.

Methods

Generation of *Fkbp52* null mice

ES cells isolated from 129SvJ mouse were cultured in Knockout DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% FBS, penicillin/streptomycin, essential amino acids, ESGRO (10^3 U/ml; Chemicon, Temecula, CA) with irradiated embryonic fibroblast feeder cells. ES cells were electroporated at 0.2 kV, 950 µF (Gene Pulser II; Bio-Rad, Hercules, CA) with linearized targeting vectors and selected with G418 (300 µg/ml). DNA from G418-resistant clones was isolated for Southern blot analysis. A DNA probe was used to distinguish *Eco*RI restriction fragments from wild-type (17 kb) and mutant (9 kb) alleles. Appropriate homologous recombination in ES cell clones was confirmed by PCR using primers complementary to sequences within the neomycin cassette and to 3' *Fkbp52* sequences downstream from the recombination site. ES cell clones containing a mutant *Fkbp52* allele were injected into C57BL6/129 blastocysts and implanted into pseudopregnant 129SvJ females. Chimeric offspring were identified by coat patterns and mated to C57BL6/129 mice to obtain germline transmission of the mutant allele. These mice were created by David F. Smith (Mayo Clinic, Scottsdale, AZ) and are currently maintained in our animal facility

at Vanderbilt University (Nashville, TN). All mice were housed and used in the current investigation in accordance with the National Institutes of Health and institutional guidelines on the care and use of laboratory animals.

Mouse genotyping

To obtain genomic DNA, tail pieces (5–8 mm) collected from weaned mice were placed in 200 µl DirectPCR Lysis Reagent (Viagen Biotech Inc., Los Angeles, CA) containing freshly prepared 0.2-0.4 mg/ml Proteinase K (Sigma, St. Louis, MO). Tails were placed in 55°C water bath for > 6 h. Lysates were then incubated at 85°C for 45 min, spun down quickly for 10 sec, and stored at 4°C until use. Genotypes were determined by PCR using specific primers: neoF2, 5'-TCT ATC GCC TTC TTG ACG-3'; ex2F, 5'-AGA GAG GGT ACA GC-3'; ex3R, 5'-TAC AAG TGT GGC GTT GGG-3'; ex10R, 5'-ATG CAA CAG CGG TGT ACC C-3'. These are specific for the wild-type (1 kb product) or mutant (700 bp product) alleles. Cycle conditions are the following: 1 cycle of 95°C for 5 min, followed by 40 cycles of 95°C for 30 sec, 55°C for 30 sec, 68°C for 2.5 min, followed by 68°C for 10 min. Master mix contained 10x LA Taq PCR buffer, 2.5 mM dNTP mixture, 10 µM primer mix, and LA Taq polymerase (Takara Bio Inc, Shiga, Japan). Amplified fragments were separated by electrophoresis on 2% agarose gels and visualized by ethidium bromide staining.

Ovulation, fertilization, implantation, and blastocyst transfer

To examine ovulation and fertilization, wild-type or *Fkbp52*$^{-/-}$ mice were bred with fertile males. The morning of finding a vaginal plug was designated day 1 of pregnancy. Mice were killed on day 2 of pregnancy and oviducts were flushed with Whitten's medium to recover eggs and embryos. Egg and embryo morphology was examined under a dissecting microscope. Implantation sites on days 5 and 6 of pregnancy were visualized by an intravenous injection (0.1 ml per mouse) of a Chicago Blue B dye solution (1% solution in saline) and the number of implantation sites, as demarcated by distinct blue bands, was recorded. For blastocyst transfer, pseudopregnant recipients were generated by mating females with vasectomized males. Day 4 wild-type blastocysts were transferred into day 4 uteri of wild-type, heterozygous or *Fkbp52*$^{-/-}$ pseudopregnant recipients, and implantation sites were recorded 24 h later (day 5) by the blue dye method. All mice used were between 2 and 4 months of age.

Comparative RT-PCR and southern hybridization

Total RNA was extracted from mouse uteri using Trizol according to the manufacturer's instruction. Reverse transcription with oligo(dT) priming was performed to generate cDNAs from 4 µg total RNA using Superscript II following the instruction provided by the manufacturer. DNA amplification was carried out with Taq DNA polymerase (Invitrogen, San Diego, CA) using the following primers: *Fkbp52* (437 bp), 5'-AGT GTG GGG AAG GAG AGG TT-3' and 5'-GCT CTT GCC AGG TCA AAG TC-3'; *Pgr* (461 bp), 5'-GCC ATC ACT TCC TGG TGT

CT -3' and 5'-GCA ATG GGA GAG TCT TGC TC-3'; *Fkbp51* (402 bp), 5'-AAG GTG TTG GCA GTC AAT CC-3' and 5'-GGT GGT CAT TTG GGA AGC TA-3'; *β-actin* (246 bp), 5'-CTC TTT GAT GTC ACG CAC GAT TTC-3' and 5'-GTG GGC CGC TCT AGG CAC CAA-3'. PCR conditions were 95°C for 5 min and then 23 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 45 sec, followed by incubation at 72°C for 10 min. Amplified fragments were separated by electrophoresis on 2% agarose gels and visualized by ethidium bromide staining. The intensity of each band was measured by Scion Image (Scion Corp., Frederick, MD), and intensities of *Fkbp52*, *Pgr*, and *Fkbp51* were corrected by the intensity of *β-actin*. Nested primers used to make hybridization probes were as follows: *Fkbp52*, 5'-CAC GCT GAG CTG AGG TAT GA-3'; *Pgr*, 5'-GTC TCT TTG GGC CAG AGC TT-3'; *β-actin* 5'-CCA CGG GCA TTG TGA TGG AC-3'. Southern blots were prehybridized and hybridized at high stringency. Hybridization was carried out for ~16 h at 37°C in 3x SET (1x SET=150 mM NaCl, 5 mM EDTA and 10mM Tris-HCl, pH 8.0), 20 mM phosphate buffer (pH 7.2), 250 μg/ml tRNA, 10% dextran sulfate and ~2 x 10^{6} counts/min ^{32}P-labeled antisense RNA probe/ml of the hybridization buffer. After hybridization, the blots were washed once in 1x SSC, 0.1% SDS for 1 h at 37°C, followed by a second wash in 0.3x SSC, 0.1% SDS for 1 h at 37°C. Hybrids were detected by autoradiography.

P_4 binding assay

Ovariectomized wild-type or *Fkbp52*$^{-/-}$ mice were injected s.c with estradiol-17β (1 µg/mouse) or vehicle (sesame oil) for 14 days to increase PR levels. Aliquots of wild-type or *Fkbp52*$^{-/-}$ uterine cytosol (200 µg of total protein) were incubated overnight at 4°C in the presence of indicated concentrations of $[^3H]P_4$ (NEN, specific activity = 102.1 Ci/mmol; 1 Ci = 37 GBq), a 100-fold molar excess of cortisol plus or minus a 100-fold molar excess of unlabeled P_4. Unbound ligand was removed by incubation with dextran-coated charcoal, and bound radioactivity in the supernatant was measured by liquid scintillation counting.

Transfection and PR transcription activity assay

Cells (8×10^4 per well) were cotransfected with 20 ng of pCMV-galactosidase plasmid (Clontech), 0.3 µg of pCR3.1-hPRB expression plasmid (kindly provided by N. Weigel, Baylor College of Medicine, Houston, TX), 0.3 µg of pMMTV-luc reporter plasmid (provided by J. Scammell, University of South Alabama, Mobile, AL), and 0.3 µg of pCI-neo vector (Promega) lacking or containing human *Fkbp51* or *Fkbp52* cDNA. Cells were incubated overnight with P_4 as indicated. After transfection, 10 nM DHT was added, and cells were incubated an additional 24 h. Treated cells were washed three times with PBS and lysed with 100 µl of M-PER reagent. For luciferase (Luciferase Assay System; Promega) and β-galactosidase (Gal-Screen) assays, 30 µl and 20 µl,

respectively, of cell lysate were added to substrate mixtures and assayed according to suppliers' instructions.

In situ hybridization

In brief, frozen sections (12 μm) were mounted onto poly-L-lysine-coated slides and fixed in cold 4% paraformaldehyde in PBS. After prehybridization, sections were hybridized at 45°C for 4 h in 50% formamide hybridization buffer containing the ^{35}S-labeled antisense or sense cRNA probes. Ribonuclease A-resistant hybrids were detected by autoradiography. Sections were poststained with eosin and hematoxylin. Sections hybridized with sense probes did not exhibit any positive signals and served as negative controls.

Northern hybridization

Total RNA (6 μg) was denatured, separated by formaldehyde-agarose gel electrophoresis and transferred onto nylon membranes. Cross-linked blots were prehybridized, hybridized, and washed at conditions for high stringency. Briefly, hybridization was carried out at ~16 h at 68°C in 3x SET (1x SET=150 mM NaCl, 5 mM EDTA and 10mM Tris-HCl, pH 8.0), 20 mM phosphate buffer (pH 7.2), 250 μg/ml tRNA, 10% dextran sulfate and ~2 x 10^6 counts/min ^{32}P-labeled antisense RNA probe/ml of the hybridization buffer. After hybridization, the blots were washed once in 1x SSC, 0.1% SDS for 1 h at 68°C, followed by a second wash in 0.3x SSC, 0.1% SDS for 1 h at 68°C. Hybrids were detected by autoradiography.

Isolation and culture of embryonic fibroblasts

Day 13 embryos were isolated and genotyped by PCR as described. The head, limbs, and liver were excluded from each embryo, and remaining tissues were minced and digested with 0.25% trypsin at 37°C for 15 min, and plated in 60-mm plastic dishes in DMEM supplemented with 10% FBS plus essential amino acids and penicillin/streptomycin. Fibroblasts were immortalized by the 3T3 protocol of Todaro and Green (Todaro and Green, 1963). These cultures were performed in the laboratory of David F. Smith (Mayo Clinic, Scottsdale, AZ).

Histology and Immunostaining of Ki67

Frozen (10 μm) or formalin-fixed paraffin embedded uterine sections (5 μm) were subjected to hematoxylin and eosin staining or immunostaining by using a Ki67 antigen staining kit according to the manufacturer's instructions, respectively (Novacastra, Newcastle, U.K.) Nuclear brown color indicates positive Ki67 staining.

Results

Ovulation is normal in C57BL6/129 *Fkbp52* null females

To delineate whether FKBP51 or FKBP52 plays a critical role in ovarian and uterine functions, we generated mice with targeted deletion of either *Fkbp51* or *Fkbp52* genes. We found that, although *Fkbp51* deficient mice show no overt reproductive failures (unpublished observations), both males and females lacking

Fkbp52 are infertile (Cheung-Flynn et al., 2005; Hong et al., 2007), indicating the importance of this immunophilin in reproduction. The infertility status of *Fkbp52*$^{-/-}$ females is shown (**Table 3**).

Table 3. Fertility status of C57BL6/129 *Fkbp52* null (-/-) female mice. *Fkbp52*$^{-/-}$ females cohabitated with wild-type (+/+) fertile males failed to produce any offspring, while the average litter sizes from +/+ and heterozygous (+/-) females were comparable (mean ± SD).

Genotypes (males x females)	No. of breeders	No. of litters	Average litter size
+/+ x +/+	28	81	6.9 ± 3.0
+/- x +/-	63	195	6.7 ± 3.0
+/+ x -/-	53	0	0

Whereas male infertility is due to compromised androgen receptor function and decreased sperm fertilizing capacity (Cheung-Flynn et al., 2005; Hong et al., 2007), the site and cause of female infertility in *Fkbp52* null females remained unexplored.

Because *Pgr* null mice show ovulation failure and uterine refractoriness to implantation (Lydon et al., 1995; Mulac-Jericevic et al., 2003), and because FKBP52 associates with PR-Hsp90 complexes (Barent et al., 1998), we first examined whether ovulation is normal in *Fkbp52*$^{-/-}$ females. Because *Fkbp52*$^{-/-}$ males are infertile, normally cyclic or gonadotropin-stimulated *Fkbp52*$^{-/-}$ females were mated with fertile wild-type males. The number of normally ovulated or superovulated eggs in these mutant females is comparable to that of wild-type

females (**Figure 6A**); however, both *in vivo* and *in vitro* fertilization rates of eggs arising from mutant females are compromised (**Figure 6A**).

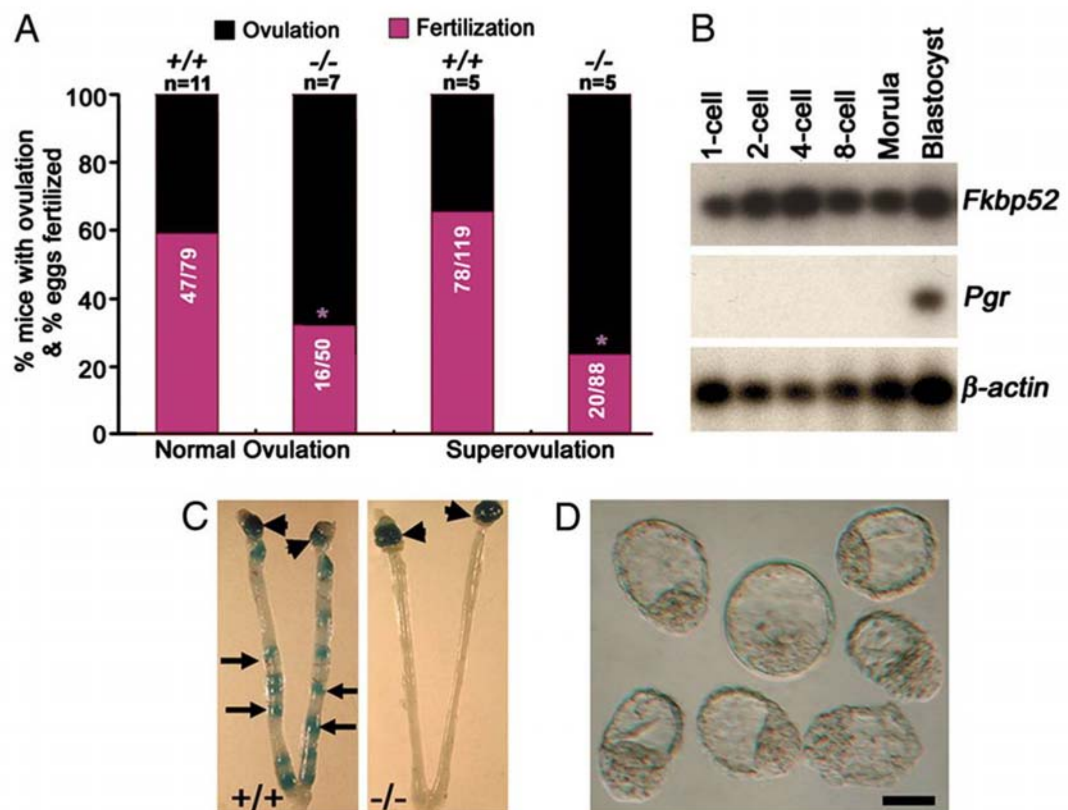

Figure 6. Female reproductive events in C57BL6/129 *Fkbp52*$^{-/-}$ mice. (A) Ovulation and fertilization were examined on day 2 of pregnancy. Numbers within the bars indicate number of fertilized eggs/total number of ovulated eggs. Normal and superovulation rates are not significantly different between +/+ and -/- mice (unpaired *t*-test); normal or in vitro fertilization rates are significantly different between +/+ and -/- mice (χ^2 analysis; *P = 0.01). (B) RT-PCR of *Fkbp52* and *Pgr* expression in preimplantation embryos. (C) Representative photographs of +/+ uteri with implantation sites and *Fkbp52*$^{-/-}$ uteri without on day 5. Arrowheads and arrows indicate sites of ovary and implantation, respectively. (D) Representative photomicrograph of blastocysts recovered from *Fkbp52*$^{-/-}$ mice on day 5. Bar = 50 μm.)

To determine whether oocyte FKBP52 contributes to the reduced fertilization rates, we examined the expression of *Fkbp52* and *Pgr* in preimplantation wild-type embryos from one-cell through blastocyst stages by RT-PCR. We found that, although embryos at all stages of development express *Fkbp52, Pgr* is expressed only at the blastocyst stage (**Figure 6B**); our observation of *Pgr* expression specifically in blastocysts and not in the other stages of preimplantation embryos is consistent with a previous report (Hou and Gorski, 1993). These results suggest that *Fkbp52* is maternally derived in the oocyte and may have a role in fertilization and preimplantation development independent of PR. Alternatively, oocyte maturation due to follicular deficiency arising from compromised PR function in the absence of FKBP52 may contribute to the reduced fertilization rate. Indeed, there is evidence that compromised PR function impairs the expansion of cumulus-oocyte complexes (Shimada et al., 2004). Further investigation is needed to address this question.

It is to be noted, however, that recovery of blastocysts from *Fkbp52*$^{-/-}$ uteri on day 4 of pregnancy suggests that FKBP52 is not an absolute requirement for fertilization or preimplantation embryo development. Collectively, these results imply that complete infertility observed in *Fkbp52*$^{-/-}$ females is not due to failure in ovulation as in *Pgr*$^{-/-}$ mice, or total absence of fertilization, but is the result of defective implantation and/or pregnancy failure following implantation.

Fkbp52$^{-/-}$ mice show implantation failure

To determine whether implantation occurs normally in *Fkbp52*$^{-/-}$ females, we mated wild-type, heterozygous and null females with fertile males of the same strain and examined implantation on day 5 of pregnancy by the described blue dye method. We observed that, although wild-type and heterozygous females had an expected number of implantation sites when examined on day 5 of pregnancy, none of the mutant females showed any sign of implantation (**Figure 6C & D; Table 4**). It is possible that blastocyst implantation is delayed in *Fkbp52*$^{-/-}$ mice like in $cPLA_{2\alpha}^{-/-}$ or *Lpa3*$^{-/-}$ mice (Song et al., 2002; Ye et al., 2005); however, no signs of implantation were evident even on day 6 of pregnancy (**Table 4**). In fact, a small number of unimplanted blastocysts were recovered from mutant females on this day of pregnancy (**Table 4**).

Table 4. C57BL6/129 *Fkbp52* null (-/-) female mice show implantation failure. +/+, +/-, and -/- females were mated with +/+ fertile males and the number of implantation sites (IS) was examined on days 5 and 6 of pregnancy by the blue dye method. Uteri without IS were flushed with saline to recover any unimplanted embryos. The results of +/+ and +/- mice were not significantly different and are presented as a pool (mean ± SD).

Day of pregnancy	Genotypes	No. of mice	No. of mice with IS (%)	No. of IS	No. of embryos recovered
5	+/+ / +/-	12	9 (75)	6.5 ± 2.0	0[a]
5	-/-	9	0	0	37[b]
6	+/+ / +/-	14	10 (71)	7.9 ± 2.0	2[c]
6	-/-	5	0	0	5[d]

[a] 3 mice without IS yielded no embryos.
[b] 15 embryos were at the blastocyst stage, and the remaining 22 were degenerating embryos and thus, their fertilization status could not be ascertained.
[c] 2 degenerating embryos were recovered from 1 mouse.
[d] 5 blastocysts were recovered from 1 mouse.

Compromised fertilization in *Fkbp52*$^{-/-}$ mice could explain the small number of recovered blastocysts in mutant mice. Therefore, we used blastocyst transfer experiments to confirm that FKBP52 is critical to uterine receptivity for implantation. Day 4 wild-type blastocysts were transferred to wild-type, heterozygous, or homozygous mutant day 4 pseudopregnant recipients. Similar to the results of natural mating, none of the transferred wild-type blastocysts showed implantation in any of the mutant recipients when examined 24 h post-transfer, whereas wild-type or heterozygous recipients showed an expected number of implantation sites. Again, a small number of blastocysts were recovered from mutant uteri (**Table 5**). Reciprocal embryo transfers with homozygous mutant embryos could not be performed because *Fkbp52*$^{-/-}$ males are infertile (Cheung-Flynn et al., 2005; Hong et al., 2007).

Table 5. Wild-type blastocysts fail to implant in C57BL6/129 *Fkbp52* null (-/-) recipients. Day 4 wild-type (+/+) blastocysts were transferred into uteri of +/+, +/-, or -/- recipients on day 4 of pseudopregnancy. Recipients were examined for implantation sites (IS) on day 5 by the blue dye method. Uteri without IS were flushed with saline to recover any unimplanted blastocysts. The results of +/+ and +/- mice were not significantly different and are presented as a pool (mean ± SD).

Genotype						
Blastocyst	Recipient	No. of blastocysts transferred	No. of recipients	No. of mice with IS (%)	No. of IS (%)	No. of blastocysts recovered
+/+	+/+ / +/-	178	10	8 (80)	74 (41)	2[a]
+/+	-/-	79	4	0 (0)	0	14[b]

[a] 2 mice without IS yielded 1 blastocyst each.
[b] 14 blastocysts were recovered from 3 recipients.

Collectively, these results show that, whereas P_4-mediated ovulation is normal in *Fkbp52*$^{-/-}$ mice, the mutant uterus is completely nonreceptive to blastocyst implantation, implying an essential role for this immunophilin specifically for governing uterine receptivity. Our next objective was to determine whether these phenotypes resulting from *Fkbp52* deficiency are caused by impaired P_4 function in the uterus.

FKBP52 and PR show overlapping uterine expression during implantation

We speculated that, if FKBP52 functions as a cochaperone to PR *in vivo*, cell-specific expression of these two genes must overlap. In situ hybridization results show that on day 4 of pregnancy, *Fkbp52* and *Pgr* expression overlaps in the stroma. However, *Fkbp52* is also expressed in the luminal and glandular epithelium. Both *Fkbp52* and *Pgr* become more localized in stromal cells surrounding the blastocyst on day 5 after initiation of attachment (**Figure 7A**). This mRNA expression translates to their protein expression (**Figure 7B**).

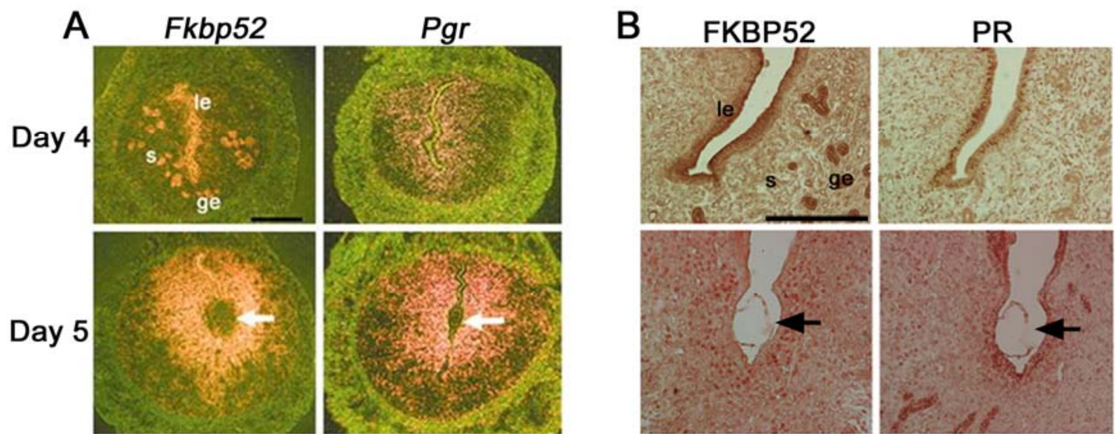

Figure 7. Uterine expression of FKBP52 and PR on days 4 and 5 of pregnancy. (A) In situ hybridization of *Fkbp52* and *Pgr* in wild-type uteri on days 4 and 5 of pregnancy. (B) Immunostaining showing FKBP52 and PR protein expression in serial sections on days 4 and 5 of pregnancy. Bar = 400 µm. Arrows indicate location of blastocysts. le, luminal epithelium; ge, glandular epithelium; s, stroma.

The overlapping expression pattern of FKBP52 and PR coincides with an increased stromal cell proliferation on day 4 and more intensely localized stromal cell proliferation at the implantation site on day 5. Uterine *Fkbp51*, although significantly lower compared to *Fkbp52*, is also expressed on days 4 and 5 (**Figure 8A**). The lower expression of *Fkbp51* on days 4 and 5 implies a limited role for this immunophilin during implantation and is consistent with normal fertility of *Fkbp51*$^{-/-}$ female mice.

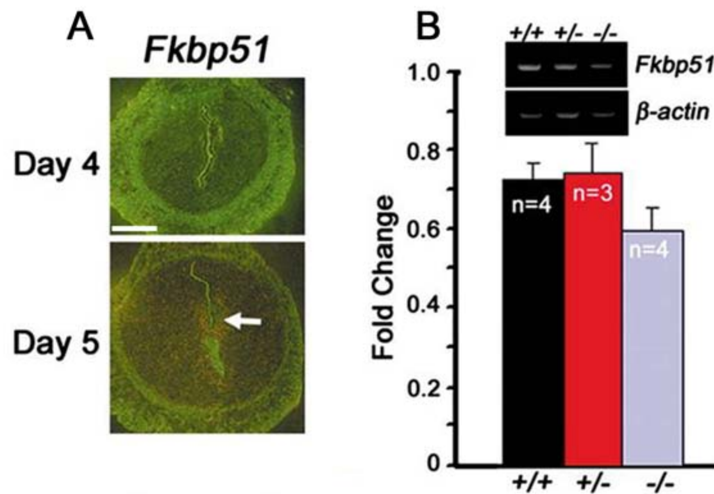

Figure 8. Uterine expression of *Fkbp51* in C57BL6/129 wild-type versus *Fkbp52*$^{-/-}$ uteri. (A) In situ hybridization of *Fkbp51* in wild-type uteri on days 4 and 5 of pregnancy. Arrow indicates blastocyst. Bar = 400 μm. (B) RT-PCR of *Fkbp51* expression in +/+, +/-, and -/- uteri on day 4 of pregnancy. Data are presented as fold changes (mean ± SEM). Fold changes are not significantly different between +/+, +/-, or -/- mice (unpaired *t*-test).

However, we wanted to determine whether an interaction between FKBP51 and FKBP52 modulates PR transcriptional activity. Using mouse embryonic fibroblasts (MEFs) in cellular assays, we found that FKBP51 is antagonistic to FKBP52 in modulating PR transcriptional activity (**Figure 9**).

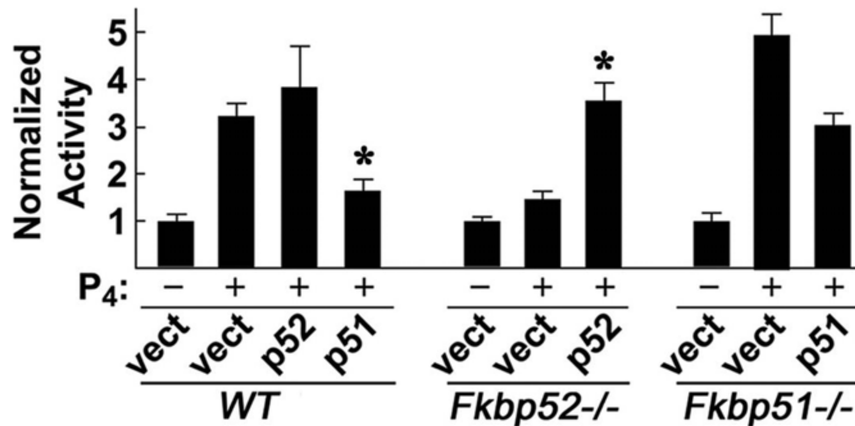

Figure 9. Differential modulation of PR transactivation by FKBP52 and FKBP51. Fibroblast cell lines prepared from wild-type (WT), *Fkbp52*$^{-/-}$, or *Fkbp51*$^{-/-}$ mouse embryos were cotransfected with four plasmids. Cells were treated with or without 0.5 nM P_4 for 16 h. The bars indicate the activity (mean ± SD; n = 3) normalized to uninduced levels of luciferase activity in each cell background. In WT cells expressing endogenous FKBP52 and FKBP51, overexpression of FKBP52 shows marginal increases in the reporter activity, whereas overexpression of FKBP51 decreases reporter activity (*P < 0.05). In *Fkbp52*$^{-/-}$ cells, P_4-induced reporter activity is minimal but restored by exogenous FKBP52 (*P < 0.05). In *Fkbp51*$^{-/-}$ cells retaining endogenous FKBP52, maximal reporter activity is induced; however, induced expression of FKBP51 decreases the activity. Statistical analysis, one-way ANOVA followed by paired *t* test.

Because FKBP51 attenuates PR responses due to FKBP52, we next wanted to determine whether *Fkbp51* is overexpressed in *Fkbp52*$^{-/-}$ uteri contributing to the observed reproductive defects. To address this, we used comparative RT-PCR experiments and found that *Fkbp51* expression is not exacerbated in mutant uteri on day 4 of pregnancy (**Figure 8B**). Collectively, these results indicate that *Fkbp52* null phenotypes are specific to *Fkbp52* deficiency and not due to overexpression of *Fkbp51*.

Given these results, we then compared *Pgr* expression in *Fkbp52* null uteri with wild-type uteri. In situ hybridization results show that *Pgr* is expressed in both the stroma and epithelium of null uteri similar to that in wild-type uteri

(**Figure 10A**), and Northern blot results show that *Pgr* expression is not compromised in *Fkbp52*$^{-/-}$ uteri, but levels are rather increased (**Figure 10B**). This is also reflected by apparently increased *Pgr* expression in the luminal epithelium in *Fkbp52* null uteri (**Figure 10A**). Along these same lines, serum P_4 levels between wild-type and *Fkbp52* null females on day 5 of pregnancy were similar (**Figure 10C**). Together, these results show that uterine refractoriness to implantation in *Fkbp52*$^{-/-}$ mice is not due to overexpression of *Fkbp51*, reduced *Pgr* expression, or reduced P_4 levels but instead could be due to reduced PR function.

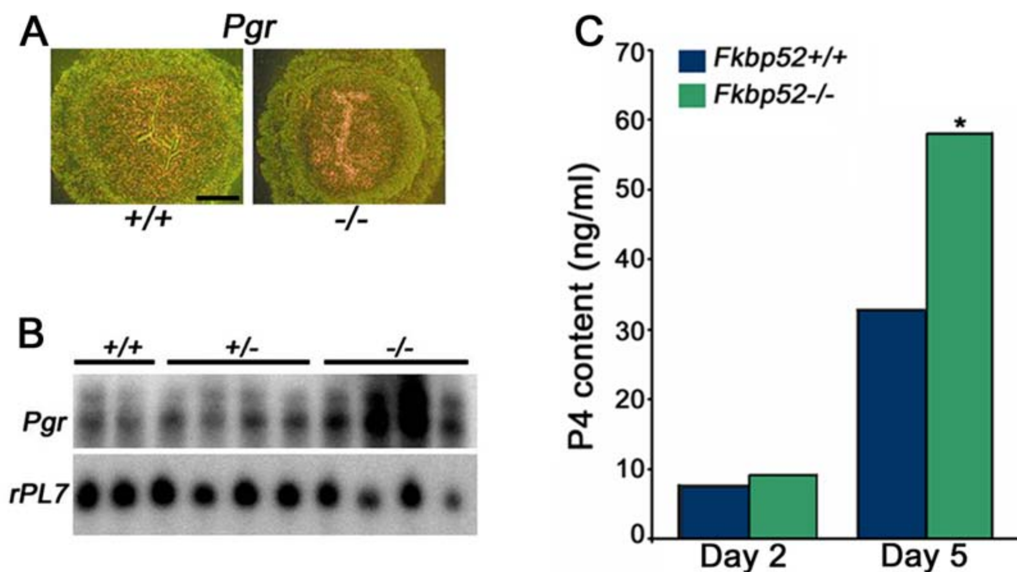

Figure 10. Uterine expression of *Pgr* and serum P_4 levels are not decreased in C57BL6/129 *Fkbp52* null (-/-) females. (A) In situ hybridization of uterine *Pgr* in wild-type (+/+) and -/- mice on day 4. Bar = 400 μm. (B) Northern blot analysis of uterine *Pgr* in +/+, +/-, and -/- mice on day 4. *rPL7* is a housekeeping gene. (C) Serum P_4 levels (ng/ml) in +/+ and -/- female mice on days 2 and 5 of pregnancy (*P < 0.05, unpaired *t*-test).

Uterine PR activity is compromised in *Fkbp52*$^{-/-}$ mice

If FKBP52 is required for optimal PR function, then uterine PR activity would be compromised in the absence of FKBP52. Indeed, binding assays using radiolabeled P_4 show that P_4 binding to PR is reduced ~2-fold in *Fkbp52*$^{-/-}$ uterine cytosol compared with that of wild-type mice. There is also a fractional reduction in the number of P_4 binding sites (**Figure 11A**). Reduced PR transcriptional activity in the absence of FKBP52 is also noted in a transfected cell system *in vitro*. Embryonic fibroblasts isolated from *Fkbp52*$^{-/-}$ mice were cotransfected with human PR-B plasmid, a luciferase reporter plasmid, and either an empty vector or a plasmid containing FKBP52 (p52). P_4-dependent reporter activity was measured over a range of P_4 concentrations. Cells expressing exogenous FKBP52 have enhanced P_4-dependent reporter gene activation as compared to cells lacking FKBP52 (**Figure 11B**).

PR-B results are shown because PR-A normally displays only modest reporter transactivation in cell culture systems (Daikoku et al., 2003); however, loss of FKBP52 did also reduce the modest activity of PR-A. These results provide evidence that FKBP52 is required for optimal uterine PR activity; thus, defects in uterine receptivity for implantation in *Fkbp52*$^{-/-}$ mice are likely caused by compromised uterine P_4 function.

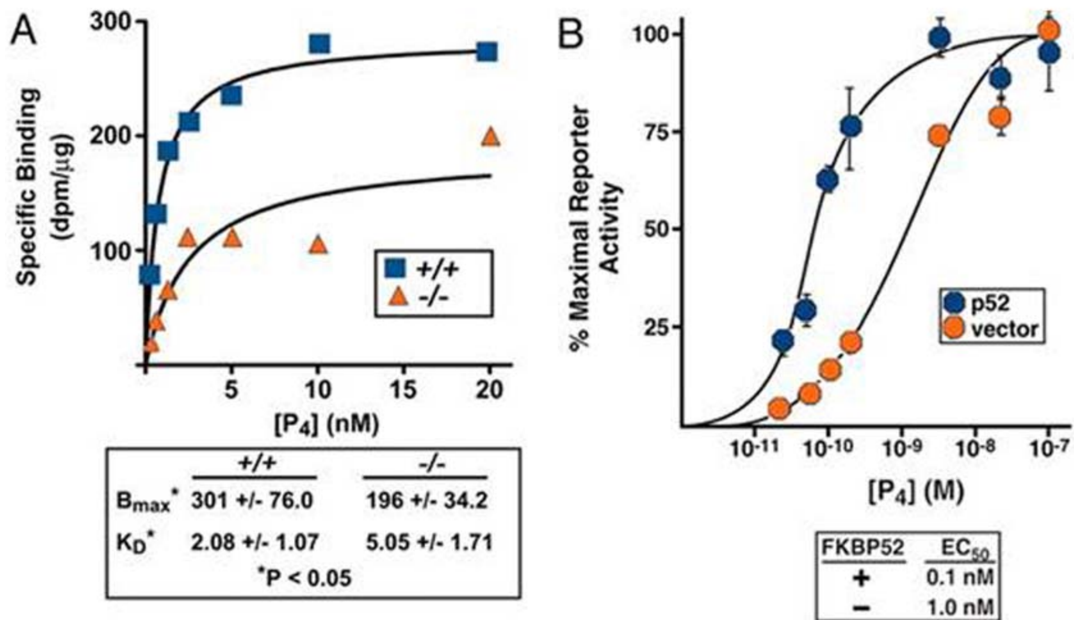

Figure 11. Uterine progesterone (P_4) binding and PR activity. (A) P_4 binding in uterine cytosol of +/+ and -/- mice. Both +/+ and -/- female mice were ovariectomized and treated with estrogen to increase uterine PR levels. Uterine cytosol samples from +/+ and -/- mice were used for binding assays. The data shown are representative of four independent experiments. (B) FKBP52 effects on reporter gene activation in embryonic fibroblasts isolated from -/- mice cotransfected with plasmid expressing human PR-B, a luciferase reporter plasmid, and an empty vector or plasmid expressing FKBP52 (p52). Each value represents the mean ± SD for three replicate samples.

PR responsive genes and functions are aberrant in uteri of *Fkbp52*$^{-/-}$ mice

We speculated that if PR functions are compromised in *Fkbp52*$^{-/-}$ uteri, uterine P_4-dependent genes would be aberrantly expressed. We selected P_4-regulated genes that encode *Hoxa-10*, Indian hedgehog (*Ihh*), amphiregulin (*Areg*), and histidine decarboxylase (*Hdc*) (Das et al., 1995; Lim et al., 1999; Matsumoto et al., 2002; Paria et al., 1998). Wild-type and *Fkbp52*$^{-/-}$ females were mated with wild-type males to collect uterine samples on day 4 of pregnancy,

when the uterus is under major P_4 influence and expresses these genes. Northern and *in situ* hybridization results show that expression of *Hoxa-10, Ihh*, and *Areg* are drastically down-regulated in *Fkbp52*$^{-/-}$ uteri, indicating compromised P_4 function (**Figure 12A, B & C**). As mentioned, there are two isoforms of PR, PRA and PRB. Genetic studies suggest that normal P_4-regulated ovarian and uterine functions are primarily mediated by PRA (Mulac-Jericevic et al., 2003). Because the mouse uterus expresses both PRA and PRB, we sought to examine whether FKBP52 differentially influences these isoforms. *Areg* and *Hdc* are known PRA and PRB regulated genes in the uterus, respectively (Mulac-Jericevic et al., 2000). Down-regulation of both *Areg* and *Hdc* in *Fkbp52*$^{-/-}$ uteri (**Figure 12A, B, C, & D**) provides evidence that FKBP52 influences both PRA and PRB functions. Because mice lacking PR-B have normal fertility (Mulac-Jericevic et al., 2003), we believe that compromised uterine PRA activity in *Fkbp52*$^{-/-}$ mice contributes to implantation failure.

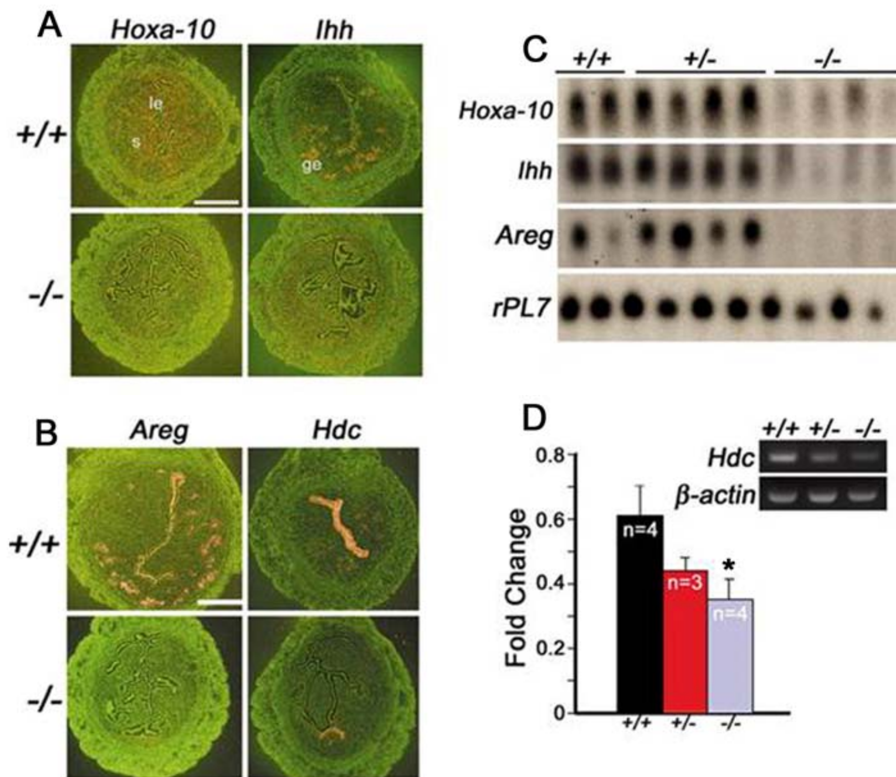

Figure 12. P_4-regulated genes are misexpressed in C57BL6/129 *Fkbp52* null uteri. In situ hybridization of *Hoxa-10* and *Ihh* (A) and *Areg* and *Hdc* (B) in +/+ and -/- mice on day 4 of pregnancy. (C) Northern blot analysis of uterine *Hoxa-10*, *Ihh*, and *Areg* in +/+, +/-, and -/- mice on day 4. *rPL7* is a housekeeping gene. le, luminal epithelium; ge, glandular epithelium; s, stroma. Bar = 400 μm. (D) Comparative RT-PCR of uterine *Hdc* in +/+, +/-, and -/- mice on day 4. The data are presented as fold changes (mean ± SEM) of three to four independent samples. Fold changes in *Fkbp52*$^{-/-}$ mice are significantly different compared to combined fold changes in +/+ and +/- mice (*P < 0.05, unpaired t test).

Aberrant expression of estrogen-responsive gene lactoferrin in *Fkbp52*$^{-/-}$ uteri on day 4 of pregnancy

To confirm that the observed changes in uterine gene expression are not due to aberrant responsiveness of the uterus to estrogen, we examined uterine expression of an estrogen-responsive gene lactoferrin (*Ltf*) on day 1 of pregnancy when the uterus is primarily under the influence of estrogen (**Figure 13A**). As

previously observed in the wild-type uterus (McMaster et al., 1992), normal expression of *Ltf* on day 1 in *Fkbp52*$^{-/-}$ uteri confirms that these null uteri appropriately respond to estrogen. On day 4 of pregnancy, however, when the uterus is under major P_4 influence, *Ltf* expression is undetectable in wild-type uteri as expected, but persists in *Fkbp52*$^{-/-}$ uteri, indicating reduced P_4 function with exaggerated estrogenic influence in the absence of FKBP52 (**Figure 13A**).

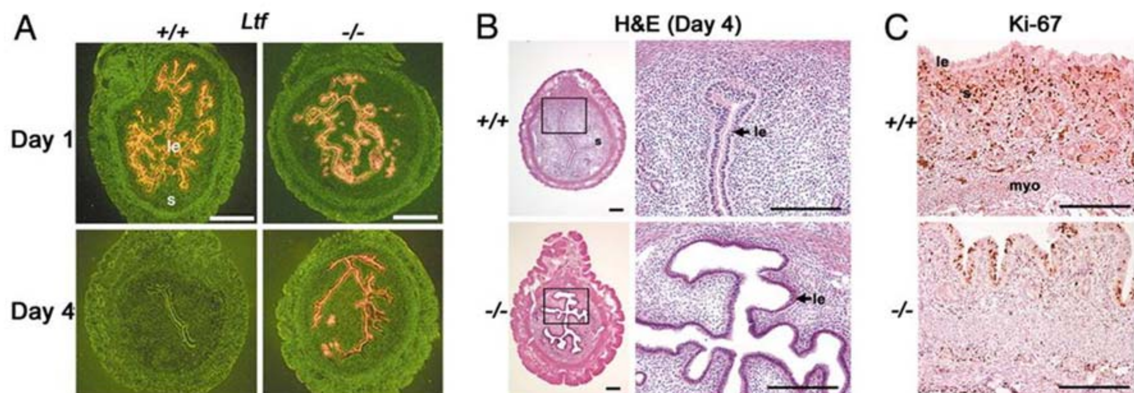

Figure 13. Differential expression of an estrogen-responsive gene lactoferrin (*Ltf*) and cell proliferation in C57BL6/129 *Fkbp52*$^{-/-}$ uteri. (A) In situ hybridization of *Ltf* in uteri of +/+ and -/- mice on days 1 and 4 of pregnancy. Bar = 400 µm. (B) Histological examination of +/+ and -/- uteri on day 4 of pregnancy. Bar = 200 µm. (C) Immunostaining of Ki67, a marker of cell proliferation, in uteri of +/+ and -/- mice on day 4. Note stromal cell proliferation in wild-type uterus versus luminal epithelial proliferation in -/- mice. Arrow points to luminal epithelial layer. Bar = 100 µm. le, luminal epithelium; s, stroma; myo, myometrium; H&E, hematoxylin

Abnormal pattern of cell proliferation in *Fkbp52*$^{-/-}$ uteri on day 4 of pregnancy

Another striking difference was observed between wild-type and *Fkbp52*$^{-/-}$ uteri at the histological and cellular level. Normally, uterine lumens in wild-type mice on day 4 of pregnancy under P_4 dominance show luminal closure with a slit-

like structure with nonproliferating differentiated cuboidal epithelia. In addition, stromal cell proliferation is intense on this day (Dey et al., 2004). Surprisingly, uteri of *Fkbp52*$^{-/-}$ mice have compromised luminal closure on day 4, displaying proliferating columnar luminal epithelia and nonproliferating stromal cells, consistent with defects in P_4-regulated events (**Figure 13B & C**). Collectively, these results suggest that responsiveness of *Fkbp52*$^{-/-}$ uteri to coordinated estrogen and P_4 action is skewed to favor estrogen.

Discussion

The present study identifies FKBP52 as a critical determinant of uterine P_4 action in preparing the uterus receptive for blastocyst implantation. Although genetic and pharmacological evidence has previously established that P_4 action via nuclear PR is essential for ovulation, implantation, and pregnancy maintenance, it is remarkable to see that FKBP52, an immunophilin cochaperone for PR, is so critical and specific to uterine preparation for implantation. Apparently normal ovulation and a less severe fertilization phenotype as compared to uterine preparation for implantation suggests differential sensitivity of the ovary and uterus to FKBP52-mediated P_4 action. This tissue-specific differential sensitivity is not noted in *Pgr* mutant mice in which both severely compromised ovarian and uterine functions are responsible for female infertility. The physiological significance of PR-associated immunophilins had been underappreciated until the present study, and the dramatic infertility of mice lacking *Fkbp52* lends credence to the significance of this immunophilin

cochaperone in female fertility. This study places FKBP52 as a target for improving female fertility or alternatively developing novel approaches to contraception.

CHAPTER III

FKBP52 DEFICIENCY-CONFERRED UTERINE PROGESTERONE RESISTANCE IS GENETIC BACKGROUND AND PREGNANCY STAGE SPECIFIC

Abstract

Immunophilin FKBP52 serves as a cochaperone to govern normal PR function. Using *Fkbp52*$^{-/-}$ mice, here 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 rescues implantation and subsequent decidualization in CD1, but not in C57BL6/129, null females. Surprisingly, experimentally-induced decidualization in the absence of blastocysts fails in *Fkbp52*$^{-/-}$ mice on either background even with P_4 supplementation, suggesting that embryonic signals complement uterine signaling for this event. Another interesting finding is that while P_4 at higher than normal pregnancy levels confers PR signaling sufficient for implantation in CD1 null females, these levels are inefficient in maintaining the full complement of term pregnancy. However, elevating P_4 levels further restores PR signaling optimal for successful term pregnancy with normal litter size. Collectively, our results show that the indispensability of FKBP52 in uterine P_4-PR signaling is a function of genetic disparity and 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

As mentioned, P_4 signaling is an absolute requirement for implantation and pregnancy maintenance in most eutherian mammals studied (Dey et al., 2004; Wang and Dey, 2006). The failure of ovulation and implantation, decidualization and pregnancy maintenance (Lydon et al., 1995) precludes using *Pgr*$^{-/-}$ mice to study potential new aspects of P_4 function during pregnancy. In contrast, the targeted deletion of the *Fkbp52* gene that 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 since these mice display uterine-specific infertility (Tranguch et al., 2005).

Our finding that *Fkbp52*$^{-/-}$ females on a C57BL6/129 mixed background have complete implantation failure with normal ovulation and slightly reduced fertilization rates has recently been confirmed by another group in independently-generated *Fkbp52*$^{-/-}$ mice on the same genetic background (Tranguch et al., 2005; Yang et al., 2006). 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, a tissue-specific differential sensitivity not noted in *Pgr*$^{-/-}$ females with complete female infertility (Lydon et al., 1995; Mulac-Jericevic et al., 2000).

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. By transferring C57BL6/129 *Fkbp52*$^{-/-}$ mice to a CD1 genetic background, we are able to exploit this mouse model to address these roles for the first time.

Methods

Mice

The *Fkbp52* gene was disrupted in mice by homologous recombination as previously described (Cheung-Flynn et al., 2005). Tail genomic DNA was used for PCR-based genotyping. Because genetic backgrounds of mice contribute to different phenotypes (Threadgill et al., 1995; Wang et al., 2004), we introduced *Fkbp52* deficiency in CD1 mice by crossing C57BL6/129 *Fkbp52* heterozygous males to CD1 wild-type (WT) females producing an F1 generation. F1 *Fkbp52*$^{+/-}$ males were then back-crossed to CD1 WT females, and the process was continued for ten generations. Crossing heterozygous females with heterozygous males of the same genetic background (CD1/F10) generated *Fkbp52* WT and null littermates for experiments. Mice on both genetic backgrounds were housed and used in the present investigation in accordance with the National Institutes of Health and institutional guidelines on the care and use of laboratory animals.

Ovulation, fertilization, implantation, blastocyst transfer and experimentally-induced decidualization

To examine ovulation and fertilization, CD1 WT or *Fkbp52*$^{-/-}$ mice were mated with fertile WT males. On day 2 of pregnancy (day 1 = vaginal plug), oviducts were flushed with Whitten's medium to recover ovulated eggs, and fertilization was assessed by counting the number of 2-cell embryos. Implantation sites on days 5 and 6 of pregnancy were visualized by an intravenous injection (0.1 ml/mouse) of Chicago Blue B dye solution (1% in saline) and the number of implantation sites as 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 *Fkbp52* WT or null pseudopregnant recipients, and implantation sites were observed 24 h (day 5) or 96 h (day 8) later by the blue dye method (Paria et al., 2001).

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

Exogenous P_4 supplementation and other treatments

To observe whether exogenous P_4 treatment rescues the infertility phenotype of *Fkbp52*$^{-/-}$ females, null females were mated with WT males, and a silastic implant (4 cm length x 0.31 cm diameter) containing P_4 was placed under the dorsal skin on day 2 of pregnancy. Implants were either 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. For pregnancy rescue with P_4 injection, null female mice were injected subcutaneously with P_4 (2 mg/0.1 ml saline/mouse) from days 2 through 14 to monitor pregnancy maintenance or through day 17 to allow labor to ensue on day 20.

To determine contribution of complement pathway to pregnancy maintenance, silastic P_4 implants were placed under the dorsal skin on day 2 of pregnancy, and heparin (10U/0.1ml/mouse in saline) was injected subcutaneously twice a day on days 8, 10, and 12 of pregnancy. To determine whether the P_4 to estrogen ratio influences pregnancy maintenance, silastic P_4 implants were placed under the dorsal skin on day 2 of pregnancy and ICI 182,780 (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 exogenous 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, one uterine horn was infused with sesame oil (25 µl), and the contralateral horn served as control. Mice were sacrificed on day 8 of pseudopregnancy. Uterine weights of infused (oil) and non-infused (control) horns were recorded, and fold increase in weight was used 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 (3,000 rpm, 15 min) and stored at -80°C until analysis. Serum P_4 levels were measured by radioimmunoassay (University of Virginia Center for Research in Reproduction, Ligand Assay & Analysis Core, Charlottesville, VA).

In situ hybridization

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

Immunohistochemistry

Immunolocalization of cytokeratin was performed by using a polyclonal rabbit anti-cow antibody (DakoCytomation, Carpinteria, CA). A Histostain-Plus

(DAB) kit (Zymed) was used to visualize antigen. Brown deposits indicate sites of positive immunostaining.

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 (Threadgill et al., 1995; Wang et al., 2004). These varying phenotypes are thought to be due to differential expression and/or regulation of modifier genes (Bonyadi et al., 1997), 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 (Wang et al., 2004). We have recently shown that C57BL6/129 *Fkbp52*$^{-/-}$ females have complete implantation failure, although ovulation is normal (Tranguch et al., 2005). 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 with WT males since *Fkbp52*$^{-/-}$ males, irrespective of genetic background, are infertile (Cheung-Flynn et al., 2005; Hong et al., 2007). We found ovulation and fertilization in null females to be comparable with WT (**Figure 14A & B**). CD1 *Fkbp52*$^{-/-}$ females were examined for implantation sites on day 5 of pregnancy by the blue dye method. We observed that only 2 of 14 CD1 *Fkbp52*$^{-/-}$ females showed very faint blue bands (**Figure 14C & Table 6**).

Unimplanted blastocysts were recovered from uterine flushings of null mice with implantation failure (**Figure 14C**), suggesting that although embryos developed to blastocysts, they fail to implant.

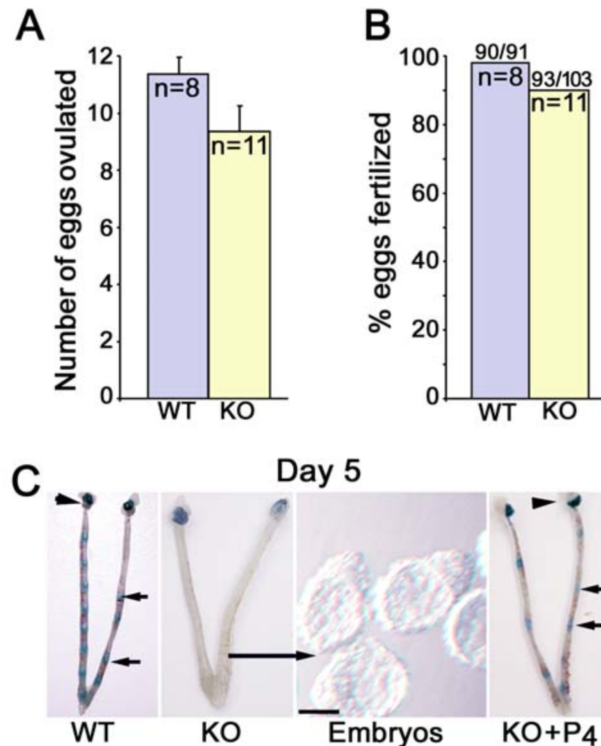

Figure 14. P_4 supplementation via silastic implants rescues implantation failure in CD1 *Fkbp52*$^{-/-}$ females. Ovulation (A) and fertilization (B) were examined on day 2 of pregnancy. The number of ovulated eggs is not significantly different between wild-type (WT) and null *(*KO) females. Values are mean ± SE (P>0.05; unpaired *t*-test). Numbers above the bars indicate the total number of 2-cell embryos/total number of eggs recovered. Fertilization rate is comparable between WT and KO females (P > 0.05; unpaired *t*-test). C. Implantation fails in KO females, but is rescued by P_4 as examined on day 5 of pregnancy. Representative photographs of uteri with or without implantation sites (IS) as assessed by blue dye reaction 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 ovary and IS, respectively. Long arrow indicates the uterus from which unimplanted blastocysts were collected. Bar = 50 µm.

Our data signify that optimal P_4-PR 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 that of ovarian responsiveness. It is possible that relatively high local P_4 levels in the ovary (Pointis et al., 1981), the site of its synthesis, enhance basal PR activity sufficient for ovulation and fertilization processes.

Progesterone 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 (Tranguch et al., 2005; Tranguch et al., 2006). 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 tenet implies 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 of WT on day 5 of pregnancy (**Figure 15**), we speculated that exposing *Fkbp52*$^{-/-}$ uteri to higher than normal P_4 levels will 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

(**Figure 10C)** (Tranguch et al., 2005). This may also explain why ovulation, a P_4-regulated event, is normal in *Fkbp52*$^{-/-}$ females on both genetic backgrounds.

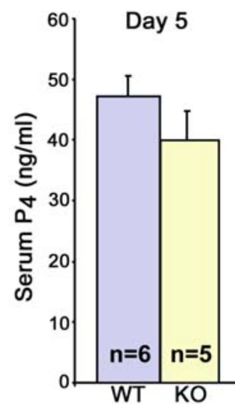

Figure 15. Peripheral serum P_4 levels in CD1 WT and KO mice on day 5 of pregnancy. Values are not significantly different (P = 1.0, univariate ANOVA).

To determine whether P_4 supplementation rescues implantation, we used P_4 containing silastic implants to maintain steady-state hormone levels (Milligan and Cohen, 1994). 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 implantation sites was also comparable to those of WT mice (**Figure 14C & Table 6**).

Table 6. P_4 rescues implantation in CD1 *Fkbp52*$^{-/-}$ mice. Wild-type (WT) and CD1 *Fkbp52*$^{-/-}$ (KO) females were mated with WT fertile males and the number of implantation sites (IS) was examined 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. The number and weights of IS are presented as mean ± SE.

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

[a]7 mice without IS yielded 66 blastocysts of which 6 showed signs of degeneration
[b]21 blastocysts were recovered from 5 mice of which 5 showed signs of degeneration
[c]19 blastocysts were recovered from 3 mice of which 3 had signs of degeneration
[d]17 blastocysts were recovered from 2 mice
*$P<0.05$ compared to WT; unpaired *t*-test.

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 implantation sites was also remarkably low, especially on day 8, compared to WT females (**Table 7**).

Table 7. P_4 fails to rescue implantation failure in C57BL6/129 *Fkbp52*$^{-/-}$ mice. WT and *Fkbp52*$^{-/-}$ (KO) females on C57BL6/129 background were mated with WT females and the number of implantation sites (IS) was examined on days 5 and 8 of pregnancy by the blue dye method. *Fkbp52*$^{-/-}$ females were placed with P_4 containing silastic implants (KO+P_4) from day 2 of pregnancy until the day of sacrifice. Uteri without IS were flushed with saline to recover any unimplanted embryos. The number of implantation sites is presented as mean ± SE.

Genotype	Day of pregnancy	No. of mice	No. of mice with IS (%)	No. of IS	No. of embryos recovered
WT	5	11	11 (100)	7.5 ± 0.5	n/a
KO + P_4	5	11	3 (27)	5.6 ± 1.0	23[a]
KO + P_4	8	8	2 (25)	0.5 ± 0.2*	0[b]

[a]8 mice without IS yielded 23 blastocysts of which 21 showed signs of degeneration
[b]6 mice without IS yielded no embryos
*P < 0.05 compared to WT; unpaired *t*-test.

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 8**).

Table 8. P_4 fails to rescue implantation failure of transferred WT blastocysts in C57BL6/129 *Fkbp52*$^{-/-}$ females. 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 (KO+P_4) from days 2 through 5 of pseudopregnancy. Recipients were examined for implantation sites (IS) on day 5 by the blue dye method. Uteri without IS were flushed with saline to recover any unimplanted blastocysts.

Genotype		No. of blastocysts transferred	No. of recipients	No. of mice with IS (%)	No. of IS (%)	No. of embryos recovered
Blastocyst	Recipient					
WT	KO + P_4	82	6	6 (100)	49/82 (59)	n/a
WT	KO + P_4	83	5	2 (40)	6/83 (7)	45[a]

[a]3 mice without IS yielded 45 blastocysts of which 2 showed signs of degeneration.

P_4 supplementation restores P_4- and implantation-regulated gene expression in CD1 *Fkbp52*$^{-/-}$ uteri

P_4-regulated events superimposed with preimplantation estrogen secretion guide the uterus from a prereceptive to the receptive state, allowing blastocyst attachment to 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, CD1 *Fkbp52*$^{-/-}$ females mated with WT males were placed with a silastic P_4 implant on day 2 and sacrificed on day 4 of pregnancy. We selected P_4-regulated genes that encode *Hoxa-10, Ihh,* and *Areg* because of their participation in uterine receptivity (Das et al., 1995; Lee et al., 2006; Lim et al., 1999; Matsumoto et al., 2002). In situ hybridization results show that exogenous P_4 treatment considerably restores expression of these genes (**Figure 16A**).

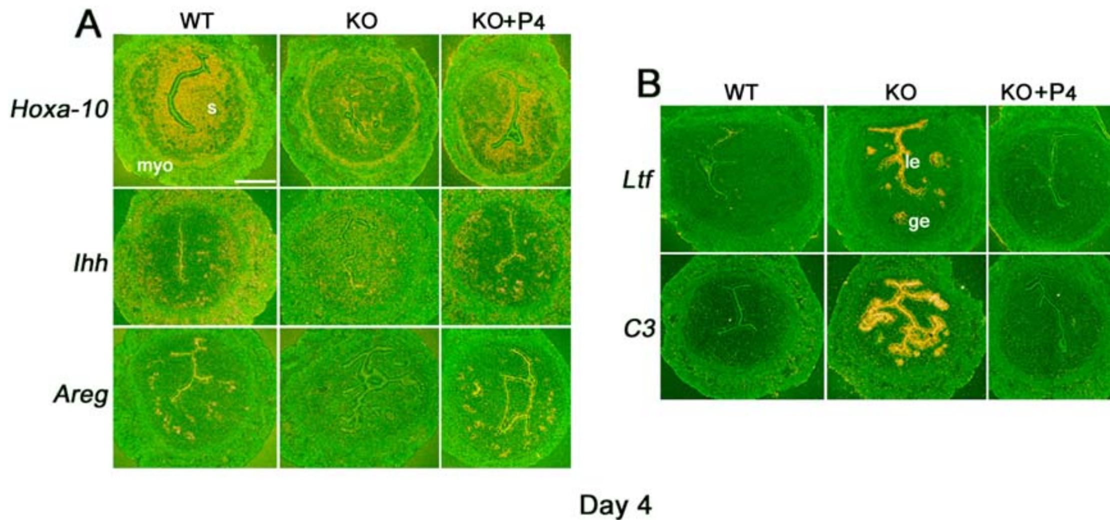

Figure 16. P_4 supplementation via silastic implants corrects misexpression of genes in CD1 *Fkbp52*$^{-/-}$ uteri. A. In situ hybridization of P_4-regulated genes *Hoxa-10, Ihh,* and *Areg* in WT, KO and KO + P_4 uteri on day 4 of pregnancy B. In situ hybridization of estrogen-target genes *Ltf* and *C3* in WT, KO and KO + P_4 uteri on day 4 of pregnancy. le, luminal epithelium; ge, glandular epithelium; s, stroma; myo, myometrium. Bar, 400 µm.

Lactoferrin (*Ltf)* and complement factor 3 (*C3*) are highly induced in the uterus by estrogen and antagonized by P_4 (Heikaus et al., 2002; McMaster et al., 1992). They are abundantly expressed in the luminal epithelium on 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 (Dey et al., 2004; Wang and Dey, 2006). However, in CD1 *Fkbp52*$^{-/-}$ mice, uterine expression of these genes is aberrantly elevated on day 4, but downregulated with exogenous P_4 supplementation (**Figure 16B**). 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 provoked us to examine whether P_4 treatment restores the expression of P_4-regulated genes in C57BL6/129 *Fkbp52*$^{-/-}$ uteri similar to that observed in CD1 *Fkbp52*$^{-/-}$ mice. We found that although exogenous P_4 treatment considerably restores *Hoxa-10* and *Ihh* expression in mice on this genetic background, *Areg* expression remains low to undetectable with P_4 treatment (**Figure 17A**). 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 (**Figure 17B**).

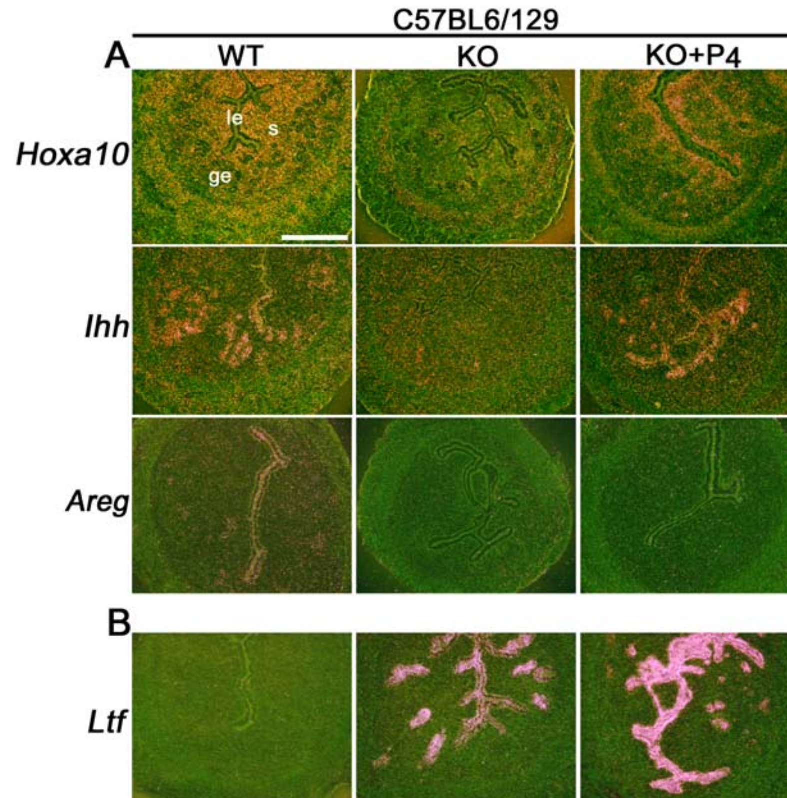

Figure 17. P_4 supplementation via silastic implants partially restores the expression of P_4-target genes, but fails to counter an estrogen-responsive gene in C57BL6/129 *Fkbp52*$^{-/-}$ uteri. (A) In situ hybridization of P_4-target genes *Hoxa-10, Ihh* and *Areg* in WT, KO and KO + P_4 uteri on day 4 of pregnancy. (B) In situ hybridization of an estrogen-target gene *Ltf* in WT, KO and KO + P_4 uteri on day 4 of pregnancy. le, luminal epithelium; ge, glandular epithelium; s, stroma. Bar = 200 μm.

Overall, these results implicate that while C57BL6/129 *Fkbp52*$^{-/-}$ uteri are somewhat responsive to P_4 induction of its 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 (Ma et al., 2003). Because implantation fails in most C57BL6/129 *Fkbp52*$^{-/-}$ mice even after P_4 treatment, we performed subsequent experiments in CD1 *Fkbp52*$^{-/-}$ mice.

Although expression of several P_4-regulated genes is 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, is not restored by P_4 treatment in these null mice (**Figure 18A**). We therefore determined whether P_4 rescue of implantation on day 5 in CD1 *Fkbp52*$^{-/-}$ mice is accompanied by correct expression of implantation-related genes, including *Lif* as well as *Ptgs2* and *Bmp2* (Lim et al., 1997; Paria et al., 2001; Song et al., 2000). We found that while these genes are not expressed at the site of blastocysts in CD1 *Fkbp52*$^{-/-}$ females with failed implantation in the absence of P_4, P_4 supplementation restores implantation with correct expression of these genes (**Figure 18A & 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 (Lim et al., 1997; Paria et al., 2001; Song and Lim, 2006; Song et al., 2000).

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 18C**). This decrease in gland numbers, however, does not fully account for the altered expression of *Areg, Ihh* or *Lif* in null uteri, since glands that are still present fail to show normal expression patterns of these genes. While there is clear evidence that both estrogen and P_4 participate in uterine gland formation in the sheep (Carpenter et

al., 2003; Gray et al., 2001), 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.

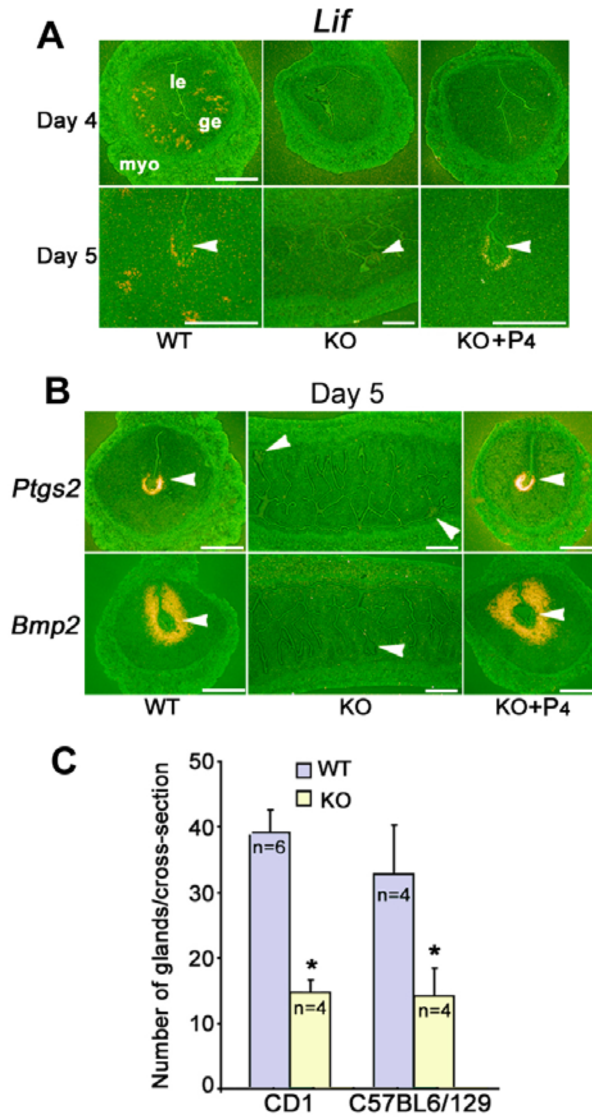

Figure 18. Delivery of P_4 via silastic implants restores expression of implantation-related genes in CD1 *Fkbp52*$^{-/-}$ females. (A) In situ hybridization of *Lif* in WT, KO and KO + P_4 uteri on days 4 and 5 of pregnancy. (B) In situ hybridization of *Ptgs2* and *Bmp2* in WT, KO and KO + P_4 uteri on day 5 of pregnancy. Arrowheads indicate the location of embryos. le, luminal epithelium; ge, glandular epithelium; myo, myometrium. Bar = 400 µm. (C) Number of glands/uterine cross-section of WT and KO uteri on both CD1 and C57BL6/129 backgrounds. For each animal, glands were counted from 9-12 uterine sections. Numbers within the bars indicate number of mice evaluated. Values are mean ± SE, (* $P<0.05$; unpaired *t*-test).

Post-implantation 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 *cPLA*$_{2\alpha}^{-/-}$ or *Lpa*$_{3}^{-/-}$ mice (Song et al., 2002; Ye et al., 2005). To test for this possibility, CD1 *Fkbp52*$^{-/-}$ mice were sacrificed on days 6 and 8 of pregnancy. We found that unlike *cPLA*$_{2\alpha}^{-/-}$ or *Lpa*$_{3}^{-/-}$ mice, implantation timing is not altered; rather the process still drastically fails in *Fkbp52*$^{-/-}$ females (**Figure 19A, B & Table 6**). Again, blastocysts were recovered from CD1 *Fkbp52*$^{-/-}$ uteri on these days, confirming implantation as the major defect in these mice (**Figure 19A, B & Table 6**).

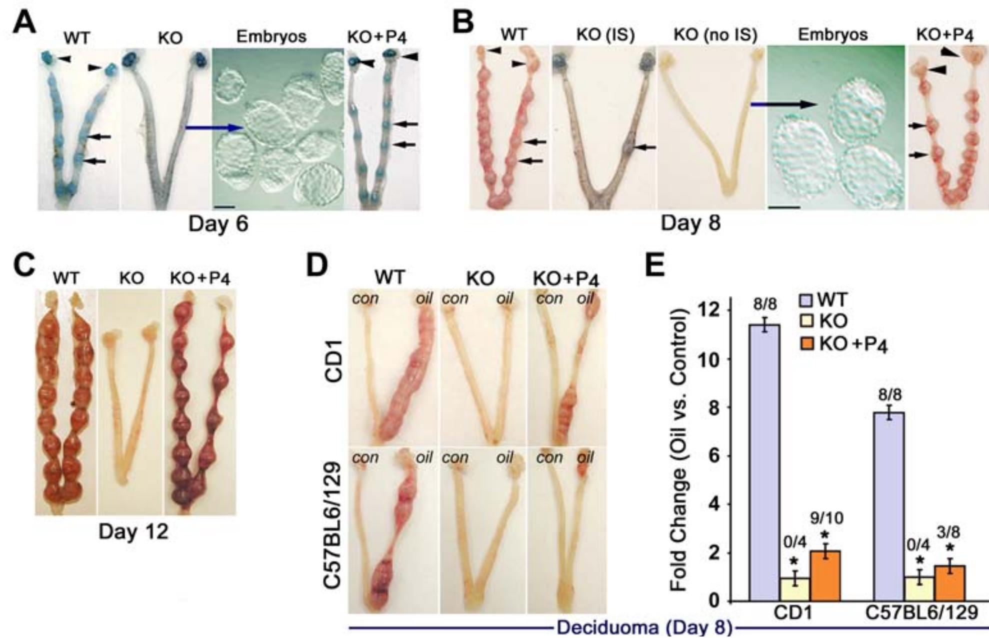

Figure 19. P_4 delivery via silastic implants rescues blastocyst-induced, but not oil-induced, decidualization in *Fkbp52*$^{-/-}$ females. Representative photographs of WT, KO and KO + P_4 uteri on day 6 (A), day 8 (B) and day 12 (C) of pregnancy are shown. Arrowheads and short arrows indicate the location of ovary and IS, respectively. Representative images of recovered unimplanted blastocysts from uteri (long arrow) of KO females without IS are shown. Bar = 50 µm. (D) Experimentally induced decidualization fails in KO females on both genetic 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 non-infused control uterine horns. Numbers above the bars indicate the number of mice with decidual response/total number of mice examined. Fold changes are presented as mean ± SE (* $P<0.05$; unpaired *t*-test).

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 19A, B, C & Table 6**). However, implantation sites in P_4-treated CD1 *Fkbp52*$^{-/-}$ uteri were smaller and weighed less than WT uteri (**Table 6**), 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 that 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 (Dey et al., 2004). Decidualization does not occur in *Pgr*$^{-/-}$ mice (Lydon et al., 1995; Mulac-Jericevic et al., 2000), 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 utilizing 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 to WT littermates (**Figure 19D & 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 19D & E**). That P_4 treatment rescues blastocyst implantation with decidualization in CD1 *Fkbp52*$^{-/-}$ females but not experimentally-induced decidualization is remarkable. This may explain why gene expression differs in decidual beds induced by blastocysts from that induced experimentally (Bany and Cross, 2006). Differences in decidualization between these two models also have been noted at the ultrastructural level (Lundkvist and Nilsson, 1982).

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 (McCormack and Greenwald, 1974). Although 9 of 13 *Fkbp52*$^{-/-}$ mothers delivered pups of normal weight, litter sizes were significantly smaller (**Figure 20A**). This provoked us to determine when embryonic loss occurs in null females carrying P_4 implants. We observed that 70 of 106 implantation sites were resorbing in P_4 implant-treated *Fkbp52*$^{-/-}$ uteri when examined on day 14, compared to only 9 of 194 in WT uteri (**Figure 20B & C**). Resorbing implantation sites in P_4-treated *Fkbp52*$^{-/-}$ females appeared dark blue and were infiltrated with a massive number of blood cells (**Figure 20B**). In addition, cytokeratin staining of sections of implantation sites with normal appearance from null females carrying P_4 implants showed placentas with less

developed and ill-defined spongiotrophoblast and labyrinth layers compared to those of WT females (**Figure 20D**).

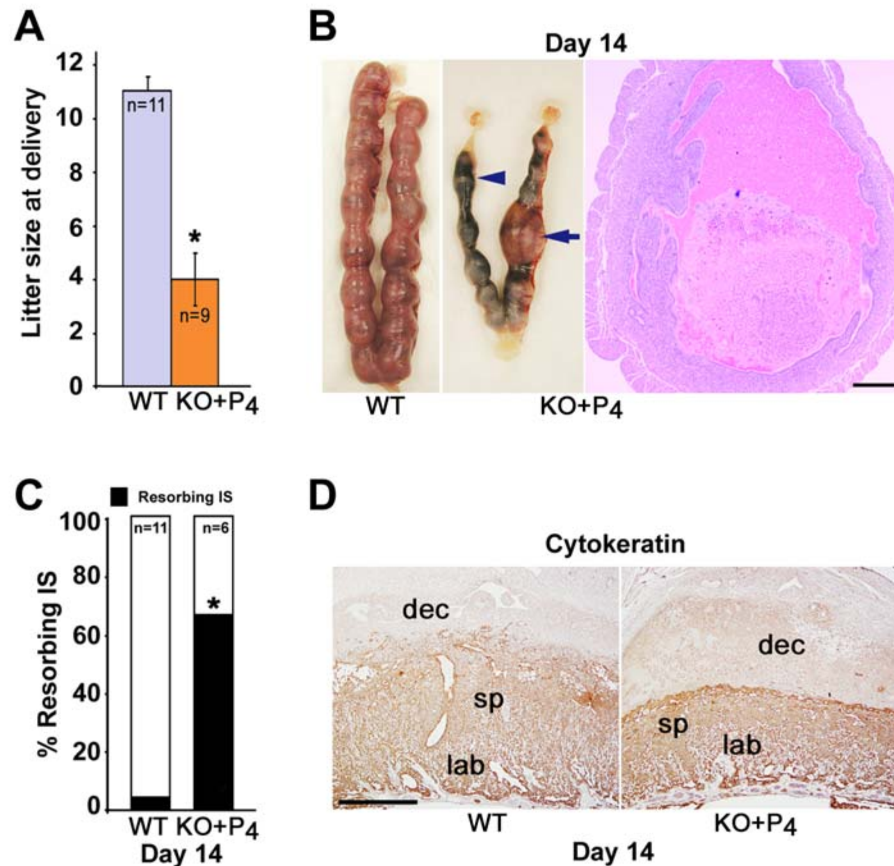

Figure 20. P_4 delivery by silastic implants fails to sustain full complement of term pregnancy. (A) Average litter size from WT and KO + P_4 mothers. Litter size is presented as mean ± SE (*P<0.05; unpaired *t*-test). (B) Representative photomicrographs of WT and KO + P_4 uteri on day 14 of pregnancy. Arrowheads and arrows indicate resorbing and normal IS, respectively. A representative H&E stained section of resorbing IS from KO + P_4 shows massive infiltration of blood cells. Bar = 200 µm. (C) Percentage of resorption sites in WT and KO+P_4 mice on day 14 of pregnancy (*P<0.05; unpaired *t*-test). (D) Cytokeratin staining of WT and KO + P_4 IS on day 14. Bar = 200 µm. dec, decidua; sp, spongiotrophoblast; lab, labyrinth.

Although P_4-PR signaling is absolutely required for pregnancy maintenance in all eutherians thus far studied, uterine FKBP52 expression on later days of pregnancy had not yet been examined. We found that while *Fkbp52* is expressed in the mesometrial decidua with high expression in the placenta on days 10 and 12 of pregnancy, *Pgr* is mostly expressed in the decidua (**Figure 21A & B**).

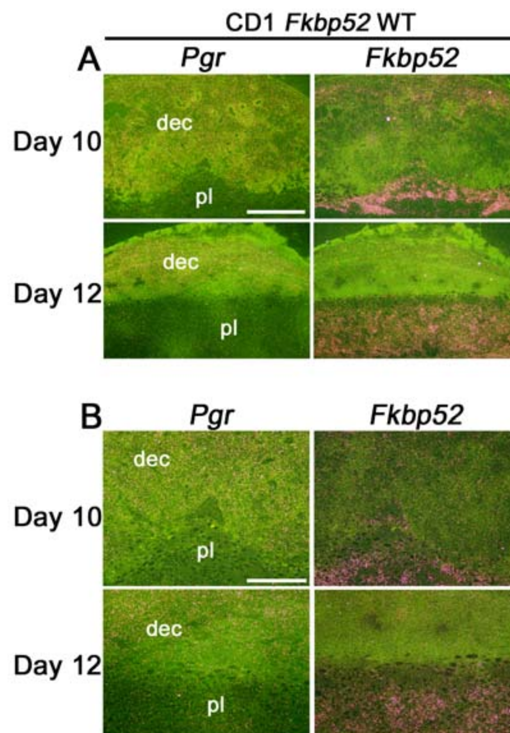

Figure 21. In situ hybridization of *Pgr* and *Fkbp52* in sections of implantation sites (IS) on days 10 and 12 of pregnancy in CD1 *Fkbp52* WT uteri shown in two magnifications. Bar = 200 µm (A) and 100 µm (B). dec, decidua; pl, placenta. Notice heavy expression (pink signal) of *Fkbp52* present in the placenta.

Embryonic signals direct normal decidual functions and development (Bany and Cross, 2006), which in turn govern placentation and embryonic growth (Bilinski et al., 1998). Therefore, expression of *Fkbp52* and *Pgr* in the decidua

suggests that maternally derived FKBP52-mediated PR signaling contributes to feto-placental wellbeing, 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 possibility for the higher incidence of resorptions in *Fkbp52$^{-/-}$* females carrying P_4 implants is due to tipping of the balance between estrogen and P_4 signaling towards estrogenic dominance. Since high levels of estrogen are detrimental to pregnancy success (Ma et al., 2003), we examined whether a combined treatment of P_4 implants with ICI 182,780 (ICI, 25 µg or 125 µg/0.1ml oil/mouse, sc), an estrogen receptor antagonist (Tocris, Avonmouth, UK), injected on days 8-13 improves pregnancy maintenance. However, this combinatorial treatment of P_4 with the high dose of ICI instead increased the resorption rate to 100% in *Fkbp52$^{-/-}$* mice, while treatment with a lower dose of ICI showed resorption rates similar to those observed in null mice receiving P_4 implants alone (**Figure 22**). Our observation that treatment with a higher dose of ICI results in increased resorptions 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 10-11 of pregnancy (Milligan and Finn, 1997).

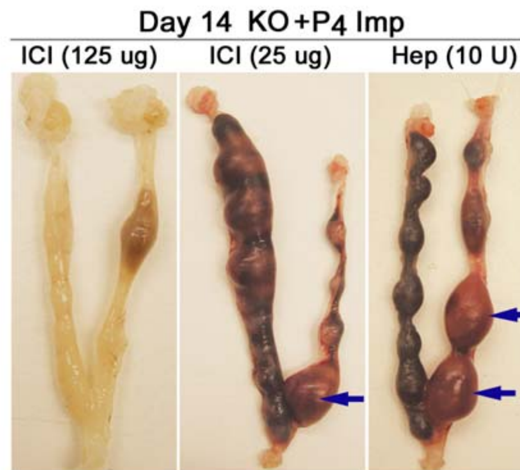

Figure 22. Anti-estrogen or heparin fails to rescue implantation in P_4-treated CD1 KO mice. Representative photomicrographs of uteri on day 14 of pregnancy in CD1 KO mice treated with P_4-containing silastic implants from day 2 and receiving injections of ICI 182,780 (ICI, 125µg or 25µg) or heparin (Hep, 10 U). Arrows denote morphologically normal implantation sites (IS) among numerous resorption sites.

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 (Girardi et al., 2006). We speculated that this pathway is activated due to reduced PR signaling from FKBP52 deficiency especially since P_4 has anti-inflammatory roles within and outside the uterus (Hardy et al., 2006; Tranguch et al., 2006). Since low doses of heparin inhibit the complement pathway (Girardi et al., 2004; Girardi et al., 2006), *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 (**Figure 22**), 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 40-47 ng/ml (**Figure 15 & 23A**). 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 full complement of term pregnancy. We speculated that increasing P_4 levels even further would rectify this failure. We injected P_4 subcutaneously 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 ~156 ng/ml by daily injection as compared to ~100 ng/ml in mice treated with P_4-implants, assessed on day 14 of pregnancy (**Figure 23A**). To our surprise, these elevated P_4 levels significantly improved pregnancy maintenance in null females; the number and weights of implantation sites on day 14 were comparable to those in WT mothers (**Figure 23B, C & D**). Pregnancy maintenance in WT mice exposed to a similar P_4 injection regimen was normal.

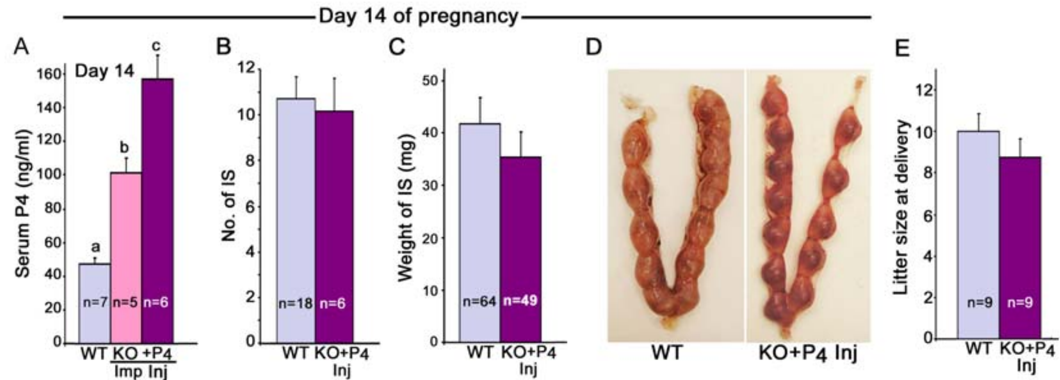

Figure 23. Daily P_4 injections restore full complement of term pregnancy in CD1 *Fkbp52*$^{-/-}$ females. (A) Serum P_4 levels in WT and KO + P_4 mice on day 14 of pregnancy. KO mice were exposed daily to P_4 either via silastic implants (Imp) or subcutaneous injection (Inj; 2 mg/ml) from day 2 of pregnancy. Bars with different letters are significantly different ($P<0.05$, univariate ANOVA). (B) Average number of IS between WT and KO + P_4 Inj on day 14 is not significantly different ($P>0.1$; unpaired *t*-test). (C) Weights of IS from WT and KO + P_4 Inj mice on day 14 of pregnancy are not significantly different ($P>0.1$;unpaired *t*-test). (D) A representative photomicrograph of WT and KO + P_4 Inj uteri on day 14 of pregnancy is shown. (E) The average litter size from WT and KO + P_4 Inj mice is not significantly different ($P>0.05$; unpaired *t*-test). All values are mean ± SE.

Our next objective was to examine whether daily P_4 injections results in full complement of term pregnancy in null females. CD1 *Fkbp52*$^{-/-}$ females mated with WT males were injected daily with P_4 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 those of WT mothers (**Figure 23E**). Pup weights from null and WT mothers at the age of weaning and during early development were also similar (data not shown).

Discussion

Although P_4 signaling via PR is critical for ovulation, fertilization, implantation, post-implantation 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 utilizing *Fkbp52*$^{-/-}$ females with compromised PR signaling as opposed to *Pgr*$^{-/-}$ females with total female infertility (Lydon et al., 1995; Tranguch et al., 2005), we for the first time address these issues. Our present investigations provide clear evidence that the major reproductive phenotype in mice missing *Fkbp52* is unique to uterine deficiency in the context of implantation (Tranguch et al., 2005; Tranguch et al., 2007). The reason for the organ specific dependence on FKBP52 for appropriate PR signaling is not clearly understood, but 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. This hypothesis also agrees with our finding that increasing P_4 levels higher via injection rescues full complement of pregnancy, while P_4 implants that increased P_4 levels moderately only rescue pregnancy through day 12. A proposed model for how this could occur at the molecular level is proposed in **Figure 24**.

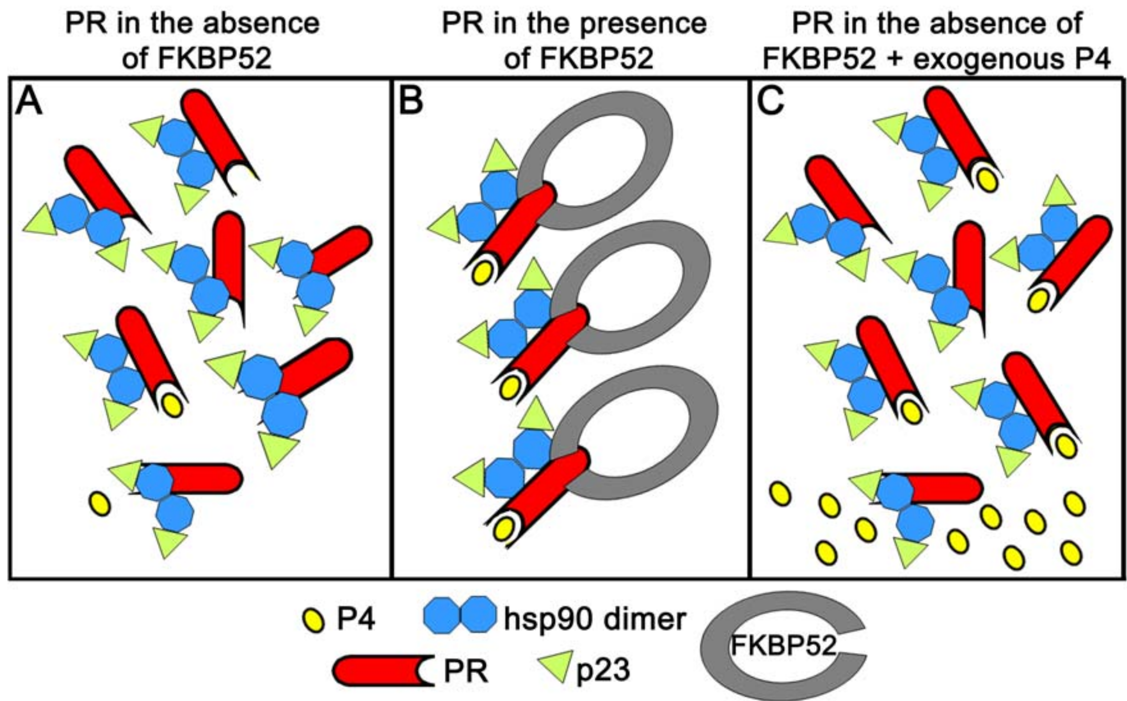

Figure 24. Putative model for how exogenous P_4 treatment rescues PR activity in the absence of FKBP52. (A) In the absence of FKBP52, PR is not held in proper conformation to optimally bind P_4, resulting in minimal PR-mediated response to ligand. (B) In the presence of FKBP52, PR is held in proper conformation and efficiently binds hormone for optimal P_4-PR signaling. (C) In the absence of FKBP52 but with exogenous ligand, even though PR is not held in the optimal conformation for ligand binding, the increased ligand availability increases its probability of binding to PR, resulting in P_4-PR signaling analogous to (B).

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 a 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. PR-independent roles for FKBP52 are now being examined in our laboratory.

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 indeed provides first evidence 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 aspect 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 due to the failure of the 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 compared to CD1 *Fkbp52*$^{-/-}$ uteri. Alternatively, differential expression of modifier genes in these two 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 (Carson et al., 1982; Wu, 1987; Wu and Liu, 1990) and that gene

expression differs in embryo-induced decidua versus experimentally induced deciduoma (Bany and Cross, 2006). Since our previous results show that *Fkbp52* and *Pgr* are expressed in blastocysts (Tranguch et al., 2005), 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 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 (*Hbegf*) is induced in implantation-competent blastocysts which can induce uterine *Hbegf* to initiate the implantation cascade. In fact, Affi-Gel Blue beads presoaked in rHB-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* (Hamatani et al., 2004; Paria et al., 2001).

P_4-PR signaling is critical throughout pregnancy until its downregulation for the onset of parturition. Indeed, ovariectomizing mice practically at any stage of pregnancy causes resorptions and/or abortion (Sharma and Bulmer, 1983). However, the magnitude of this signaling during various stages of pregnancy remains unknown. 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 post-implantation growth. This signaling appears tightly regulated since blood levels of ~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 a 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 insinuates 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 towards keeping the myometrium quiescent until parturition and providing sanctuary to the growing fetus from mother's immunological surveillance. P_4-PR signaling is also known to regulate angiogenesis (Ma et al., 2001), 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 primarily to be 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 this 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 (Mulac-Jericevic et al., 2003; Mulac-Jericevic et al., 2000), but the latter stages of lactogenesis and lactation correspond to withdrawal of this signaling (Neville et al., 2002). 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 (Yang et al., 2006). This is similar to 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 pre-decidualization is thought to be critical for blastocyst implantation in the pregnant uterus (Chobotova et al., 2005). Our findings that P_4 supplementation fails to rescue oil-induced decidualization in *Fkbp52* null uteri may therefore have implications for pre-decidualization events in P_4-resistant women.

P_4 resistance is also a hallmark of endometriosis, a condition that affects an estimated 5 million women in the United States alone (Berkley et al., 2005; Bulun et al., 2006; Fang et al., 2004). 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 (Kao et al., 2003). While the mechanism(s) of P_4 resistance remain unclear, one speculation is that downregulation of PR contributes to P_4 resistance, but other studies refute this concept (Bulun et al., 2006). Whether FKBP52 expression differs in normal and endometriotic tissues has not been examined. However, the finding that human FKBP52 interacts with and potentiates human PR activity in mouse embryonic fibroblasts (Tranguch et al., 2005), and our preliminary observation on a limited number of samples showing FKBP52 and PR expression in human endometria in proliferative and secretory phases (**Figure 25A & B**) suggest a potential role for uterine P_4-PR-FKBP52 signaling in women.

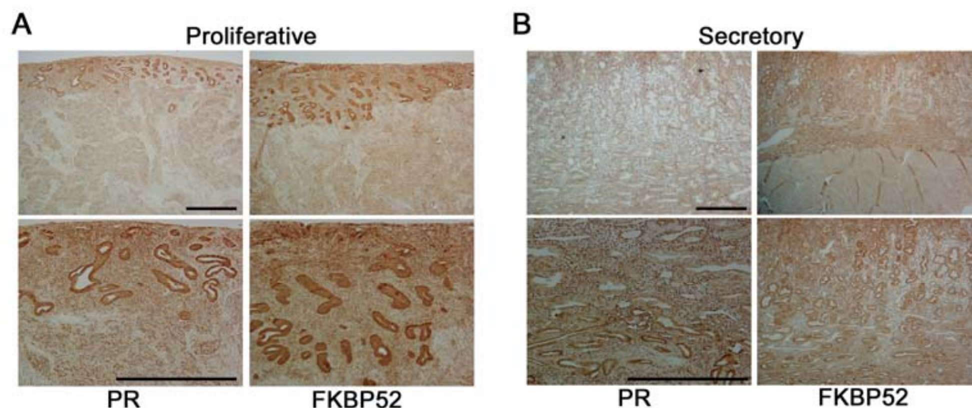

Figure 25. PR and FKBP52 expression in human proliferative (A) and secretory (B) endometrium shown at two magnifications. Bar = 100 μm (A) and 200 μm (B).

In fact, three separate clinical trials found that P_4 treatment resulted in a statistically significant decrease in miscarriages in women with history of three or

more consecutive pregnancy losses (Oates-Whitehead et al., 2003). 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. *Fkbp52* null mice with normal PR and P_4 levels but reduced PR activity constitute a unique model to study P_4 resistance specifically in uterine biology, and our findings hopefully will encourage human studies to determine whether FKBP52 status influences P_4 resistance in the uterus.

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