

# Uterine glucocorticoid receptors are critical for fertility in mice through control of embryo implantation and decidualization

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In addition to the well-characterized role of the sex steroid receptors in fertility and reproduction, organs of the female reproductive tract are also regulated by the hypothalamic–pituitary–adrenal axis. These endocrine organs are sensitive to stress-mediated actions of glucocorticoids, and the mouse uterus contains high levels of the glucocorticoid receptor (GR). Although the presence of GR in the uterus is well established, uterine glucocorticoid signaling has been largely ignored in terms of its reproductive and/or immunomodulatory functions on fertility. To define the direct *in vivo* function of glucocorticoid signaling in adult uterine physiology, we generated a uterine-specific GR knockout (uterine GR KO) mouse using the *PR<sup>cre</sup>* mouse model. The uterine GR KO mice display a profound subfertile phenotype, including a significant delay to first litter and decreased pups per litter. Early defects in pregnancy are evident as reduced blastocyst implantation and subsequent defects in stromal cell decidualization, including decreased proliferation, aberrant apoptosis, and altered gene expression. The deficiency in uterine GR signaling resulted in an exaggerated inflammatory response to induced decidualization, including altered immune cell recruitment. These results demonstrate that GR is required to establish the necessary cellular context for maintaining normal uterine biology and fertility through the regulation of uterine-specific actions.

glucocorticoid receptor | uterus | decidualization | implantation

**M**ammalian reproduction is a complex process where defects occurring at any of the sequential steps can lead to poor outcomes or pregnancy demise (reviewed in ref. 1). During early pregnancy, uterine receptivity, implantation, and endometrial remodeling (termed decidualization) are critical to supporting embryonic growth. Signaling pathways tightly governing these stages are coordinated through well-known and extensively studied actions of the ovarian steroid hormones estrogen and progesterone. However, one of the most complex and least understood factors contributing to the early stages of pregnancy is the maternal hypothalamic–pituitary–adrenal axis (HPA). Glucocorticoids are primary stress hormones synthesized in response to activation of the HPA axis that function to maintain homeostasis of a wide variety of physiological processes, including inflammatory and immune responses, development, and reproduction (2, 3). Previous studies implicating glucocorticoid signaling in the female reproductive tract have focused largely on their function in the hypothalamus and pituitary, where chronic stress or glucocorticoid exposure can suppress gonadotropin synthesis and release (reviewed in refs. 2, 4, 5). However, the potential direct actions of glucocorticoids on the uterus and its nurturing environment have been largely ignored.

At the cellular level, the actions of glucocorticoids are mediated by the glucocorticoid receptor (GR), which functions as a ligand-dependent transcription factor. Upon ligand activation, GR regulates the expression of many genes in a tissue- and context-specific manner. Due to the ubiquitous nature of the GR, glucocorticoids

can target cells throughout the body. Whole-animal knockouts of GR result in perinatal lethality, demonstrating the critical role GR plays in development but providing no insight into the role of GR in adult uterine physiology and fertility (6). Thus, to elucidate the role of glucocorticoid signaling in reproduction, we conditionally ablated GR in the uterus. These mice are subfertile, exhibiting defects in embryo implantation and subsequent decidualization. Moreover, the regulation of genes critical to decidualization was largely absent in uterine GR KO mice, revealing for the first time a crucial role for uterine GR signaling during the initiation of pregnancy.

## Results

Conditional ablation of GR was necessary to study its *in vivo* role in adult uterine function because global inactivation of GR leads to perinatal lethality (6). Therefore, *GR<sup>loxP,loxP</sup>* mice were crossed to the progesterone receptor (*PR<sup>cre</sup>*) mouse model, which recombines alleles in the PR-expressing epithelial and stromal compartments of the uterus (7) (Fig. 1A). Cre-negative littermates served as controls (*GR<sup>loxP,loxP</sup>*) in our studies. Compared with controls, GR mRNA was significantly reduced in the uteri but not ovaries or pituitaries from cycle-matched uterine GR KO mice (*GR<sup>loxP,loxP</sup>PR<sup>cre/+</sup>*) (Fig. 1B). Consistent with sites of cre expression in the *PR<sup>cre</sup>* mouse, immunohistochemistry revealed a loss of GR in the luminal epithelium and stromal cells but only a partial ablation in the uterine myometrium of uterine GR KO mice (Fig. 1C). Immunoblots of whole-uterine lysates showed a marked reduction in the level of GR protein from the

## Significance

Glucocorticoids are primary stress and immune mediators of several fundamental processes, including reproduction and development. However, the direct actions of glucocorticoid signaling in the uterus are largely unknown. We have discovered that the glucocorticoid receptor (GR) in the mouse uterus is essential for normal fertility. Female mice lacking GR specifically in the uterus are subfertile due to defects in implantation related to inadequate remodeling of the endometrial stroma. Moreover, the deficiency in uterine glucocorticoid signaling leads to aberrant gene regulation during tissue remodeling events. Our findings demonstrate previously unrecognized functions of GR in both uterine physiology and fertility.

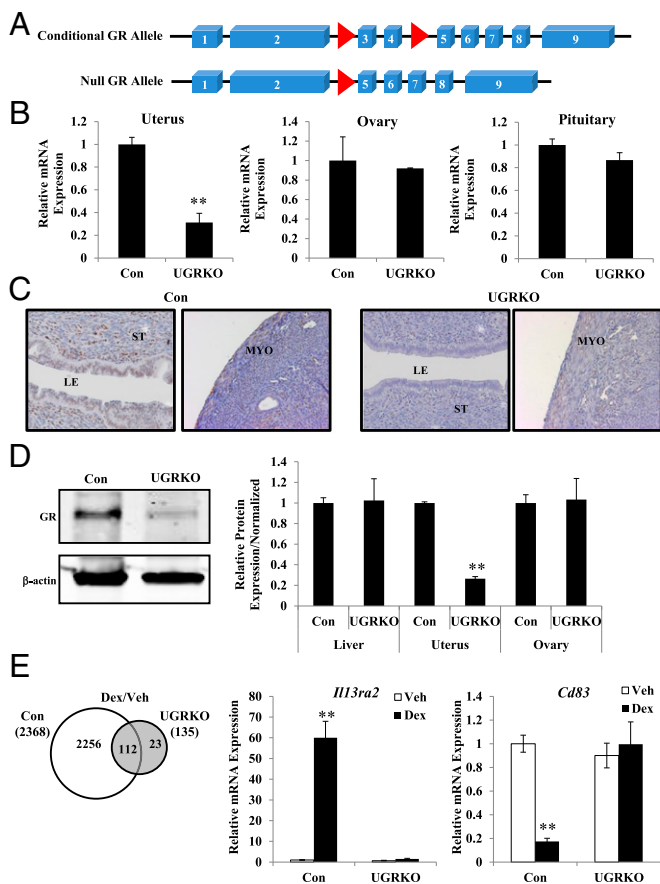
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**Fig. 1.** Generation of mice with a conditional knockout of *GR* in the uterus. (A) Mice deficient in *GR* in the uterus were created by crossing mice with a floxed *GR* allele (*GR<sup>loxP,loxP</sup>*; red arrows) with mice expressing *PR<sup>Cre/+</sup>* recombinase. Cre-mediated deletion of exons 3 and 4 results in a null *GR* allele. A frameshift mutation and premature stop codon result if splicing occurs between exons 2 and 5. (B) Quantitative RT-PCR of *GR* mRNA from the uterus, ovary, and pituitary of control and uterine *GR* KO mice ( $n = 3-5$  mice per genotype). The results represent the mean  $\pm$  SEM.  $**P < 0.01$ . (C) Representative immunohistochemistry staining of uterine sections from control and uterine *GR* KO mice with anti-*GR* antibody (200 $\times$  magnification). Counterstaining is hematoxylin. LE, luminal epithelium; MYO, myometrium; ST, stroma. (D) Representative immunoblot of *GR* from the uterus of control and uterine *GR* KO mice. *GR* protein expression in the liver, uterus, and ovary was quantified and normalized to levels of the housekeeping protein  $\beta$ -actin ( $n = 5-9$  mice per genotype). The results represent the mean  $\pm$  SEM.  $**P < 0.01$ . (E) Adrenalectomized/ovariectomized control and uterine *GR* KO mice were injected i.p. with vehicle (saline; white bar) or 1 mg/kg Dex (black bar) for 4 h, and microarray analysis was performed on isolated mRNA ( $n = 3$  mice per group). The number of genes statistically different ( $P < 0.01$ ) between control and uterine *GR* KO groups were sorted by treatment using Venn diagram. Quantitative RT-PCR was performed on independent mRNA samples ( $n = 4$  mice per group) to validate *Il13ra2* and *Cd83* mRNA levels in total RNA isolated from the uterus. Data are mean  $\pm$  SEM.  $**P < 0.01$ .

uterine *GR* KO mice (Fig. 1D). No differences were seen between genotypes in *GR* protein levels in the liver or ovary, and no changes were apparent in uterine expression of *ER $\alpha$*  and *PR* mRNA or protein, indicating the tissue and receptor specificity of the knockdown (Fig. S1 A and B). To determine whether glucocorticoid responsiveness was diminished in the uterus, adrenalectomized/ovariectomized control and uterine *GR* KO mice were treated with the synthetic glucocorticoid dexamethasone (Dex) for 4 h, and genome-wide gene expression analysis was performed (Fig. 1E). In the absence of uterine *GR*, dexamethasone treatment resulted in only 135 significantly regulated genes compared

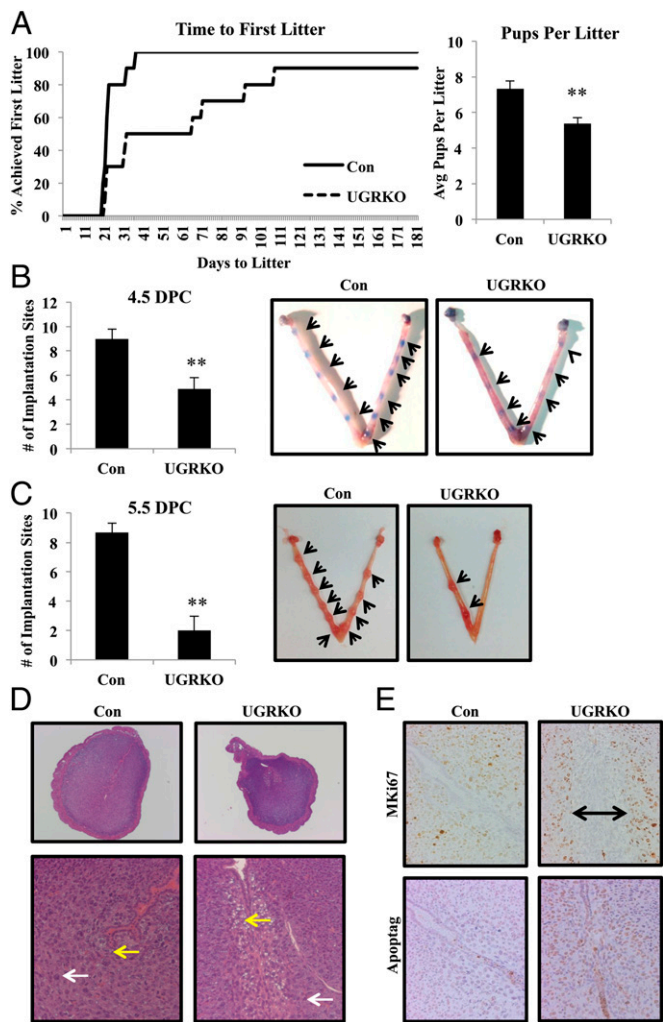
with 2,368 in *GR*-containing control mice. The expression of two identified glucocorticoid-regulated genes was validated in independent biological samples (Fig. 1E). Interleukin 13 receptor, alpha 2 (*Il13ra2*) was induced, and cluster of differentiation-83 (*Cd83*) was repressed in control mice in response to dexamethasone. In contrast, regulation of both genes was absent following Dex treatment in uterine *GR* KO mice. The absence of Dex-dependent gene regulation indicates uterine responsiveness to glucocorticoids is impaired in these mice.

To elucidate the impact of uterine *GR* ablation on fertility, 8-wk-old sexually mature control and uterine *GR* KO female mice ( $n = 10$  for each genotype) were mated with known fertile WT male mice for 6 mo (Fig. 2A). Whereas the control mice exhibited normal fertility and fecundity ( $25.2 \pm 1.78$  d to first litter and  $7.34 \pm 0.42$  pups per litter), the uterine *GR* KO mice were considerably subfertile ( $66 \pm 16.57$  d to first litter and  $5.37 \pm 0.33$  pups per litter). The estrous cycle was not significantly different in the uterine *GR* KO mice compared with controls (Fig. S2A), although one of the 10 uterine *GR* KO mice never completed an estrous cycle. Furthermore, vaginal plugs were found in uterine *GR* KO mice, indicating that the subfertility was not due to the absence of mating (Fig. S2A). To examine the ability to produce oocytes, 8-wk-old females were stimulated with gonadotropins and superovulated oocytes were retrieved from the oviduct. No differences in the number of oocytes present were observed between genotypes, suggesting that the subfertility observed is not due to impaired ovarian function (Fig. S2B). Histological examination of the uteri of 2-mo-old uterine *GR* KO mice showed no gross morphological changes, and no differences were observed in uterine weight (Fig. S2C).

Actions of the ovarian hormones estrogen and progesterone in the uterus are critical to the establishment of pregnancy (reviewed in ref. 8). To investigate whether the subfertility observed in the uterine *GR* KO mice was due to deficient estrogen and progesterone signaling, adrenalectomized/ovariectomized control and uterine *GR* KO mice were treated with *E<sub>2</sub>* or *P<sub>4</sub>*, and the estrogen-dependent induction of uterine weight (24 h postinjection) and target gene expression (4 h postinjection) were analyzed. The absence of uterine *GR* does not alter the mRNA expression of *ER* or *Pgr* (Fig. S3A). There was no significant difference in uterine weight gain (Fig. S3B) or *E<sub>2</sub>* target gene expression (Fig. S3C) in control mice compared with uterine *GR* KO mice, indicating that *GR* ablation does not impair estrogen responsiveness. The expression of acute progesterone target genes *Ihh*, *Sox17*, *Il13ra2*, *Fkbp5*, and *Gata2* was induced by progesterone injection in both control and uterine *GR* KO mice (Fig. S3D). However, there was modest attenuation of the extent of gene induction in uterine *GR* KO mice for some of these target genes, suggesting that progesterone action is preserved in a gene-specific fashion.

Based on these findings we sought to determine if the effects of uterine *GR* signaling or deletion were critical for reproductive events of early pregnancy. Embryo attachment and subsequent invasion into a receptive endometrium are indispensable for the establishment of pregnancy. To determine whether circulating glucocorticoids are sufficient to activate the glucocorticoid receptor during implantation, we evaluated translocation of the glucocorticoid receptor at 4.5 d postcoitum (dpc) in intact and adrenalectomized mice (Fig. S4). Circulating levels of glucocorticoids in intact mice lead to nuclear translocation of the glucocorticoid receptor, and this effect was largely abolished in adrenalectomized mice. These data suggest that actions of the uterine glucocorticoid receptor during early pregnancy are mediated by glucocorticoids. Maternal stress (9) or exposure of pregnant mice to high levels of glucocorticoids can significantly reduce implantation sites at 4.5 dpc (corticosterone:  $5.36 \pm 1.45$ ; controls:  $8.89 \pm 1.45$ ), suggesting that altered glucocorticoid signaling can interfere with implantation (Fig. S5). However, this approach is subject to both indirect and direct actions of glucocorticoids at peripheral sites outside the uterus. Thus, we examined the number of implantation sites in uterine *GR* KO mice and found the number to be significantly lower





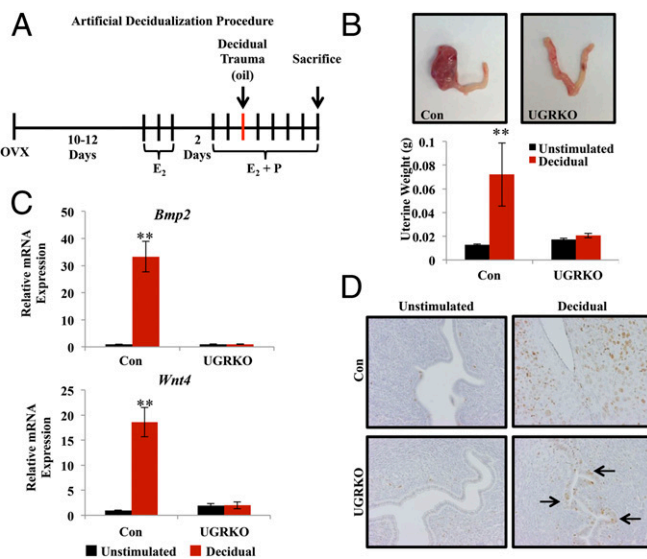
**Fig. 2.** Uterine GR deficiency results in subfertility. (A) A 6-mo breeding study reveals uterine GR KO mice are subfertile ( $n = 10$  mice per genotype). The results represent the mean  $\pm$  SEM. \* $P < 0.05$ ; \*\* $P < 0.01$ . (B and C) Gross morphology and quantification of implantation sites in pregnant uteri. Control and uterine GR KO mice were mated with wild-type males. The morning observance of a vaginal plug was considered 0.5 dpc. The average number of observed implantation sites at 4.5 dpc ( $n = 9$ –10 mice per genotype) (B) and 5.5 dpc ( $n = 6$ –11 mice per genotype) (C) in control and uterine GR KO mice was quantified. The results represent the mean  $\pm$  SEM. \*\* $P < 0.01$ . Images are representative uteri showing implantation sites. Arrows mark implantation sites. (D) Representative images of H&E-stained cross-sections of 5.5 dpc implantation sites (magnification: Upper, 40 $\times$ ; Lower, 200 $\times$ ). White arrows indicate decidualizing stromal cells. Yellow arrows denote cell vacuolization present in uterine GR KO mice but absent in control mice. (E) Measurement of uterine cell proliferation by MKI67 and cell death by TUNEL immunohistochemistry in control and uterine GR KO mice of 5.5 dpc implantation sites (200 $\times$  magnification). Counterstaining is hematoxylin. Bar with arrows designates region with no proliferation.

than in controls at 4.5 dpc (uterine GR KO:  $4.88 \pm 2.75$ ; controls:  $9.0 \pm 2.44$ ), indicating that uterine GR is necessary for successful implantation (Fig. 2B).

Moreover, the number of implanting embryos visualized was further decreased at 5.5 dpc (uterine GR KO:  $2.0 \pm 3.22$ ; controls:  $8.66 \pm 1.50$ ; Fig. 2C). Eight of 11 uterine GR KO mice that were plug-positive had no visible implantation sites at 5.5 dpc. Of those uterine GR KO mice able to implant, histological examination of the observed implantation sites at 5.5 dpc revealed altered endometrial architecture (Fig. 2D). At 5.5 dpc, the blastocyst has

implanted and decidualization of the stromal cell compartment is characterized by proliferation and differentiation. Compared with controls, stromal cells of the uterine GR KO uterus failed to equivalently decidualize. Furthermore, at day 5.5 uterine GR KO mice exhibit vacuoles in the stromal cell compartment lining the luminal epithelium. This area of vacuolization is devoid of proliferating stromal cells, unlike the stromal compartment of control mice (Fig. 2E). Interestingly, the stromal cells lining the luminal epithelium in the uterine GR KO mice were TUNEL positive, indicating that this region of the uterus is undergoing cell death (Fig. 2E). These findings suggest that uterine GR plays a major role in both embryo implantation and normal uterine decidualization.

Uterine decidualization is a key event in embryo implantation and is essential to supporting the developing embryo. Decidualization requires hormone priming and an inciting event, either embryo attachment or an inflammatory stimulus. To elucidate the cause of defective implantation and subsequent decidualization failure, female control and uterine GR KO mice were assessed for their ability to mount a decidual response following exogenous hormone treatment and decidual stimulus (Fig. 3A). For each genotype, one uterine horn received a decidual stimulus, and the contralateral horn served as the unstimulated control. Five days after receiving a decidual trauma, the decidualized horn of the control mice exhibited a robust response (Fig. 3B, Left). In stark contrast, the stimulated horn of the uterine GR KO mice showed no significant increase in uterine size compared with the unstimulated horn. Previous studies have determined molecular markers of decidualization, including induction of the genes bone morphogenetic protein 2 (*Bmp2*) and wingless-related MMTV integration site 4 (*Wnt4*) (10, 11). The expression of *Bmp2* and *Wnt4* mRNA was highly induced in the decidual horn of the control mice (Fig. 3C). However, neither *Bmp2* nor *Wnt4* were induced in the stimulated horn of the uterine GR KO

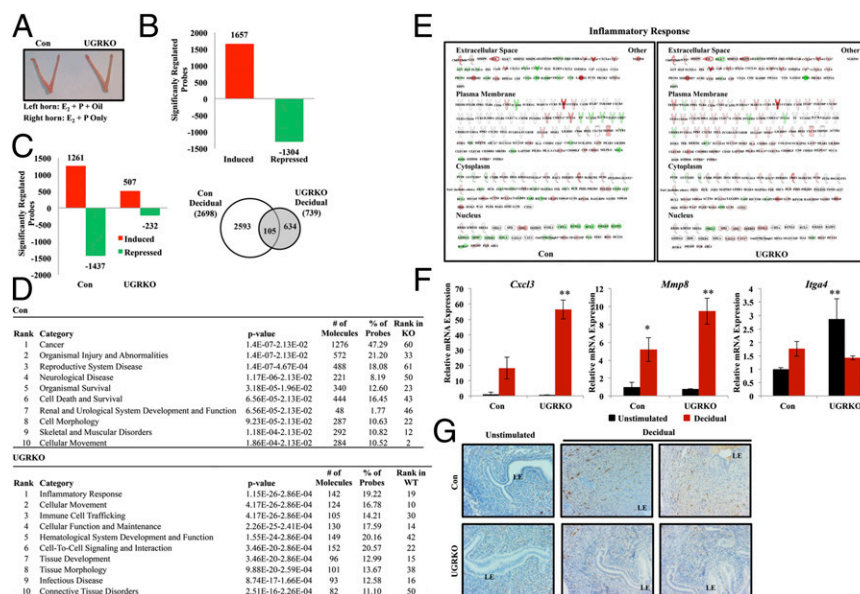


**Fig. 3.** Uterine GR is critical for decidualization of the mouse endometrial stroma. (A) Timeline of the artificial decidualization procedure. (B) Gross uterine morphology of uteri 5 d after decidual stimulus. (Left) Horn received decidual stimulus. (Right) Horn did not receive a stimulus (unstimulated) in both the control and uterine GR KO. The unstimulated (black bar) and decidualized horns (red bar) were weighed in the control and uterine GR KO mice ( $n = 4$ –5 mice per group). The results represent the mean  $\pm$  SEM. \*\* $P < 0.01$ . (C) mRNA measurement of known decidual regulators *Bmp2* and *Wnt4* on the fifth day following decidual stimulus by quantitative RT-PCR. The results represent the mean  $\pm$  SEM. \*\* $P < 0.01$ . (D) Proliferative marker MKI67 by immunohistochemistry of the unstimulated and decidual horn of control and uterine GR KO mice 5 d after decidual stimulus (magnification 200 $\times$ ). Counterstaining is hematoxylin.

mice, consistent with the absence of a decidual response in these mice. Stromal cell proliferation was assessed by MKi67 staining (Fig. 3D). Robust stromal cell proliferation was observed in the decidual horn of the control mice. Interestingly, in addition to the absence of stromal cell proliferation in the decidual horn of uterine GR KO mice, the luminal epithelium was undergoing unexpected proliferation. Persistent epithelial cell proliferation, an estradiol-driven function, indicates that the antagonism of the estrogenic response required during the window of implantation is deficient in the uterine GR KO mice, implicating GR in this process (12).

To investigate the role of GR in the coordinated orchestration of molecular events leading to decidualization, genome-wide microarray analysis was performed on the unstimulated and decidual horn of control and uterine GR KO mice 2 h following decidual trauma. Although no gross differences were apparent in uterine size at this early time point (Fig. 4A), gene expression differed vastly in the unstimulated and decidual horn of the control mice compared with the uterine GR KO mice (Fig. 4B and C). When the unstimulated horns were compared between genotypes, there were 2,691 genes that were identified as significantly different in the uterine GR KO mice compared with controls (1,657 induced; 1,304 repressed), indicating that the response to the priming hormones was vastly different in the absence of uterine GR. This alteration in gene expression likely contributes to these mice being unable to respond to the decidual stimulus (Fig. 4B). According to literature-based Ingenuity Pathway Analysis (IPA) analysis, the significantly different genes in the unstimulated horn were enriched for the biological functions organismal survival, reproductive system disease, and embryonic development (Table S1).

The genes significantly regulated in the decidual horn were then compared with those in the unstimulated horn to determine the uterine transcriptional response to decidualization in the presence and absence of uterine GR. In control mice, 2,698 genes were induced or repressed in response to decidualization, whereas only 739 genes were significantly regulated in uterine GR KO mice (Fig. 4C). Though the ratio of induced to repressed genes was similar in control mice, the balance shifts in uterine GR KO mice to favor induction. Approximately 70% of the genes significantly regulated in the uterine GR KO mice were induced, suggesting that GR signaling is necessary to limit expression of these genes during decidualization. Due to the considerable reduction in the number of genes significantly regulated in response to decidualization in the uterine GR KO mice, the gene lists were compared to determine if the response to decidualization in uterine GR KO mice represented a diminished overall response or genes regulated uniquely in the absence of GR. A comparison of the genes significantly regulated by decidualization in the uterine GR KO mice revealed only 14% (105 genes) overlapped with those significantly regulated in control mice, defining uniquely regulated genes and indicating that GR signaling is necessary to maintain the transcriptional context required for decidualization. Analysis of the significantly expressed genes in each genotype by IPA software identified considerably different biological functions (Fig. 4D). In fact, only one of the top 10 biological functions (cellular movement) was common between control and uterine GR KO mice. Data from this microarray may shed light on the observed apoptosis in the cross-sections of 5.5 dpc uterine GR KO mice (Fig. S6A). In control mice, decidualization induced the expression of anti-apoptotic genes and repressed several proapoptotic genes (Fig. S6B). In response to decidualization in



**Fig. 4.** Transcriptional response to decidualization is blunted in uterine GR KO mice. (A) Gross uterine morphology of uteri 2 h after decidual stimulus. (Left) Horn received stimulus ( $E_2 + P + \text{oil}$ ). (Right) Control ( $E_2 + P$ ). (B) Microarray analysis was performed on mRNA isolated from both the unstimulated horn and horn receiving decidual stimulus (decidual) of control and uterine GR KO mice. Comparison of the unstimulated horn in control and uterine GR KO mice allowed the identification of gene probes differentially regulated in response to  $E_2 + P$ . (C) Probes differentially expressed during decidualization were identified by comparing the unstimulated horn from the control and uterine GR KO mice to the decidual horn of the same genotype. Significantly induced and repressed probes are graphed by genotype. The number of probes statistically different ( $P < 0.05$ ) between control and uterine GR KO were sorted by genotype using Venn diagram. (D) The differentially expressed decidual genes were analyzed by IPA software for each genotype. Shown are the top 10 diseases and biological functions most significantly associated with the genes differentially regulated in the uteri of control and uterine GR KO during decidualization. (E) Inflammatory response is the most significantly regulated pathway following the decidual stimulus in the uterine GR KO mice. The pathway is shown with genes regulated in control and uterine GR KO mice with expression values of each genotype overlaid (red indicates induced; green indicates repressed). (F) mRNA of significantly regulated genes *Cxcl3*, *Mmp8*, and *Itga4* that are associated with the inflammatory response was independently validated by quantitative RT-PCR ( $n = 3-4$  mice per group). Black bar, unstimulated horn; red bar, decidual horn. The results represent mean  $\pm$  SEM.  $*P < 0.05$ ;  $**P < 0.01$ . (G) Identification of macrophages by F4/80 immunoreactivity in the unstimulated and decidual horn of control and uterine GR KO mice 2 d after decidual stimulus. Counterstaining is hematoxylin. 200 $\times$  magnification. LE, luminal epithelium.

uterine GR KO mice, only two genes are regulated in the apoptosis signaling pathway, suggesting GR signaling is required to balance the expression of genes regulating cell death and survival in the uterus. Analysis of this microarray was inconclusive as to the contribution of autophagy regulated genes to the presence of vacuoles in the cross-sections of 5.5 dpc uterine GR KO mice.

Interestingly, many of the top biological functions enriched following the decidual stimulus in uterine GR KO mice were related to inflammatory and immune responses. These findings were further substantiated using an independent functional annotation clustering analysis. Gene Ontology (GO) analysis found no common biological processes in control and uterine GR KO mice. The top biological functions identified by GO analysis of the genes regulated in uterine GR KO mice included chemotaxis, inflammatory response, defense response, immune response, and immune cell migration (Tables S2 and S3). As one of the most notably affected functions according to both IPA and GO analysis, the inflammatory response pathway was compared in control and uterine GR KO mice. There was a pronounced shift in the number of genes regulated and the ratio of induced to repressed genes following decidualization in uterine GR KO mice (Fig. 4E). In control mice, 102 genes (representing 4% of the total genes regulated in control mice) were significantly regulated with 45% induced and 55% repressed. In the uterine GR KO mice, 134 genes (representing 18% of the total genes regulated in uterine GR KO mice) were significantly regulated with 85% induced and 15% repressed. Furthermore, of the 212 genes annotated as part of the inflammatory response pathway, only 49 (23%) were in common in control and uterine GR KO mice, and 10% of these commonly regulated genes were anti-correlated in direction of regulation. Among the genes identified with differential expression between control and uterine GR KO mice were chemokine ligand 3 (*Cxcl3*), matrix metalloproteinase-8 (*Mmp8*), and integrin alpha 4 (*Itga4*). To independently confirm the changes in these three genes, we performed quantitative RT-PCR on the uteri from four additional control and uterine GR KO mice 2 h following the decidual stimulus (Fig. 4F). The magnitude of induction of *Cxcl3* and *Mmp8* following the stimulus was significantly greater in the uterine GR KO mice, suggesting that uterine GR may dampen the inflammatory response during decidualization. Interestingly, expression of *Itga4* mRNA, an integrin expressed in the uterus during early pregnancy, was significantly higher in the unstimulated horn of uterine GR KO mice compared with control mice (13). In response to decidualization, *Itga4* was repressed in uterine GR KO mice, whereas it was induced in controls.

Because the regulation of genes involved in the inflammatory response and immune cell trafficking is abnormal following the decidual stimulus in uterine GR KO mice, we wanted to determine whether immune cell populations differ in the decidual horn of control and uterine GR KO mice. Macrophage infiltration, apparent in the decidualizing control horn 2 d following the decidual stimulus, is largely absent in the stimulated horn of the uterine GR KO mice (Fig. 4G). Together, these data indicate that disruption of GR signaling in the uteri of uterine GR KO mice not only leads to the disruption of the anti-inflammatory actions of GR but also dysregulation of multiple genes and pathways with critical roles during decidualization.

## Discussion

Glucocorticoids are critical regulators of a wide variety of physiological processes largely due to their actions as transcriptional regulators, allowing signal amplification across many molecular networks. Initially recognized for their potent anti-inflammatory properties, glucocorticoids and the ubiquitous GR mediate transcriptional regulation and signal transduction throughout the body. Glucocorticoid signaling has been suggested to play a role centrally in regulating female fertility through modulation of hypothalamic and pituitary functions. However, only indirect evidence has indicated that glucocorticoids are able to modulate the actions of

estradiol in the uterus (14, 15). To elucidate the direct contribution of glucocorticoid signaling in the uterus, GR was conditionally ablated using the *PR<sup>Cre</sup>* mouse model (7). We have demonstrated that GR KO in the uterus results in early pregnancy failure, indicating that GR is required for appropriate uterine endometrial receptivity vital to implantation and decidualization. In the absence of uterine GR, the incidence of implantation was reduced at 4.5 dpc. A further decrease in the number of implantation sites was observed at 5.5 dpc, indicating that GR is necessary for implantation and the uterine response to implantation to support pregnancy. Early pregnancy loss is of considerable concern clinically, where it is estimated that ~30–60% of conceptuses are lost before 12 wk (16, 17).

Successful implantation requires both interaction between the luminal epithelium and synchronous development of the endometrial stroma. The etiology of subfertility in the uterine GR KO mice suggests GR may play a role in both of these processes and is likely due to the combined dysregulation of many different genes. Integrins are important adhesion molecules present at the surface of the luminal epithelium during implantation. Immediately following the activation of the uterus by a decidualization stimulus expression of *Itga4* was induced in control mice, though this same stimulus resulted in *Itga4* repression in uterine GR KO mice. At the initiation of implantation, expression of *Itga4* is induced in the uterus, and in vivo blocking of *Itga4* in the uterus of pregnant mice on the day of implantation leads to implantation failure (13). Dexamethasone has been shown to induce *Itga4* expression in eosinophils and bone-marrow cultures, although to our knowledge this is the first study implicating GR regulation of *Itga4* in the uterus during pregnancy (18). In addition to *Itga4*, many other plasma membrane receptors exhibited altered expression levels in uterine GR KO mice. Prostaglandins also have an important role as signaling molecules in the early events of implantation and decidualization, and both prostaglandin I<sub>2</sub> receptor (*Ptgir*) and prostaglandin E receptor 4 (*Ptger4*) were down-regulated in uterine GR KO mice following the decidualization stimulus (19). Although neither of these genes has been previously shown to be regulated by glucocorticoids, we found that *Ptgir* was significantly regulated by dexamethasone in our microarray in the uterus of control mice. Failure of glucocorticoids to maintain appropriate expression of these and other cell surface receptors may underlie defective implantation in the absence of uterine GR.

In control mice, activation of the uterus by the decidualization stimulus resulted in a transcriptional profile favoring functions related to cell survival and morphology. The biological response to decidualization in the control mice was characterized by abundant stromal cell proliferation and morphological changes. As indicated by the pathway analysis of genes regulated by decidualization in the uterine GR KO mice, cell survival and morphology were not highly regulated biological functions. Accordingly, stromal cell proliferation was significantly blunted in the GR-deficient uterus. Both bone morphogenic protein 2 (*Bmp2*) and wingless-related MMTV integration site 4 (*Wnt4*) are key regulators of stromal cell proliferation and differentiation because deletion of either gene confers fertility defects in early pregnancy (10, 11). In the absence of an intact GR signaling pathway in the uterus, the induction of these genes during decidualization was abolished. Uterine deletion of *Wnt4* results in aberrant apoptosis in the stroma adjacent to the luminal epithelial cells, and this phenotype was recapitulated in the uterine GR KO mice. Despite the similarities in phenotypes, it is not clear whether *Wnt4* and *Bmp2* are direct GR target genes or alterations in their expression profiles are secondary to the defect in decidualization.

Strikingly, the genes that were significantly regulated in response to decidualization in the uterine GR KO mice were enriched for biological functions related to the immune system and inflammatory response. Historically, maternal tolerance of the semiallogenic fetus was thought to occur through immune suppression (20). It is now well recognized that the maternal immune system not only adapts



during pregnancy but is also actively involved in all stages of the reproductive process. Animal models have shown that appropriate levels of immune cell activity in the uterus are necessary to establish and maintain a healthy pregnancy (21–23). Macrophages play an important role in the endometrium during early pregnancy, including uterine remodeling and inducing the expression of epithelial glycoproteins required for embryo attachment and implantation (24, 25). Macrophage depletion in mice results in early pregnancy loss due to implantation failure, which can be corrected by macrophage transfer (26). Macrophage recruitment into the endometrium is largely absent in the uterine GR KO mice, suggesting that altered expression of the signaling and transcriptional networks regulating the immune and inflammatory response may underlie subfertility in uterine GR KO mice.

Infertility in women may arise from a variety of causes, including inappropriate hormone levels, ovulation defects, damage or obstruction of the female reproductive tract, or an inadequate uterine environment. Understanding the mechanisms governing uterine receptivity, implantation, and uterine support of the developing embryo has been aided by the development of gene-targeting experiments in mice and uterine-specific deletion models. Such model systems have exposed the complex and redundant nature in which the uterus regulates early stages of pregnancy (27). Historically, stress or high levels of glucocorticoids have been suggested to regulate reproductive function through modulating hypothalamic and pituitary actions on the gonadotropins (5, 28). Our model indicates that uterine GR signaling plays a crucial role in establishing uterine receptivity, as evidenced by microarray analysis demonstrating deficiencies in the programming required to induce decidualization in the uterine GR KO mice. As a transcriptional regulator, GR is able to regulate thousands of genes and impact numerous signaling pathways. Identification and characterization of master regulators, such as GR, that are able to integrate many aspects of physiology are of significant clinical relevance and may lead to strategies to improve pregnancy rates.

## Materials and Methods

Please see detailed methods in *SI Material and Methods*.

**Generation of the Uterine GR KO Mice and Treatments.** Mice homozygous for the floxed GR allele ( $GR^{loxP,loxP}$ ) (29) were mated with mice expressing Cre recombinase under the direction of the progesterone receptor ( $PR^{Cre}$ ) (7), provided by Franco DeMayo and John Lydon, Baylor College of Medicine, Houston (UGRKO; Fig. 1A). The artificial decidualization response was performed as previously described (30). All experiments were performed according to a protocol approved by the Institutional Animal Care and Use Committee at the National Institute of Environmental Health Sciences.

**Real-Time RT-PCR.** Total RNA was extracted from mouse tissue using the Qiagen RNeasy Mini Kit (Qiagen), according to the manufacturer's instruction. The abundance of mRNAs was determined from at least three biological replicates on a 7900HT sequence detection system (Applied Biosystems).

**Immunohistochemistry.** Uterine sections from paraffin-embedded tissue (5  $\mu$ m) were deparaffinized and rehydrated in graded alcohol series. Sections were either stained with H&E for histological analysis or with antibodies to GR (1:500) (31), MKI67 (1: 400; Cell Signaling Technology), or F4/80 (1:500; BioLegend).

**Immunoblotting.** Tissues from control and uterine GR KO mice were lysed in RIPA buffer. Membranes with equivalent amounts of protein were incubated with polyclonal anti-GR antibodies (1:1,000), ER $\alpha$  (HC-20) antibodies (1:1,000; Santa Cruz Biotechnology), PR (C-19) antibodies (1:1,000; Santa Cruz Biotechnology), or monoclonal anti- $\beta$ -actin antibodies (1:10,000; EMD Millipore).

**Microarray Analysis.** Gene expression analysis was performed on RNA from uteri of control and uterine GR KO mice using the Agilent Whole Mouse Genome oligonucleotide arrays (014868; Agilent Technologies). Significant changes in gene expression were defined on the basis of  $P$  value ( $P < 0.01$ ).

**Statistical Analysis.** Data are presented as means  $\pm$  SEM. Statistical significance was determined by ANOVA with Tukey's post hoc analysis. Statistical significance was defined as  $*P < 0.05$  or  $**P < 0.01$ .

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