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Conditional Ablation of Progesterone Receptor Membrane Component 2 Causes Female Premature Reproductive Senescence

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Ablation of *Pgrmc2* causes female infertility

The non-classical progesterone receptors, progesterone receptor membrane component (PGRMC) 1 and PGRMC2 have been implicated in regulating cell survival of endometrial and ovarian cells *in vitro* and are abundantly expressed in these cell types. The objective of this study was to determine if *Pgrmc1* and *Pgrmc2* are essential for normal female reproduction. To accomplish this objective, *Pgrmc1* and/or *Pgrmc2* floxed mice ($Pgrmc2^{fl/fl}$ and $Pgrmc1/2^{fl/fl}$) were crossed with *Pgr-cre* mice which resulted in the conditional ablation of *Pgrmc1* and/or *Pgrmc2* from female reproductive tissues (*i.e.*, $Pgrmc2^{d/d}$ and $Pgrmc1/2^{d/d}$ mice). A breeding trial revealed that conditional ablation of *Pgrmc2* initially led to subfertility with $Pgrmc2^{d/d}$ female mice producing 47% fewer pups/litter than $Pgrmc2^{fl/fl}$ mice (p=0.001). $Pgrmc2^{d/d}$ mice subsequently underwent premature reproductive senescence by parities 2-5, producing 37.8% fewer litters overall during the trial compared to $Pgrmc2^{fl/fl}$ mice (p=0.020). Similar results were observed with $Pgrmc1/2^{d/d}$ mice. Based on ovarian morphology and serum P4 the subfertility/infertility was not due to faulty ovulation or luteal insufficiency. Rather an analysis of mid-gestation implantation sites revealed that post-implantation embryonic death was the major cause of the subfertility/infertility. As with our previous report of $Pgrmc1^{d/d}$ mice, $Pgrmc2^{d/d}$ and $Pgrmc1/2^{d/d}$ mice developed endometrial cysts consistent with accelerated aging of this tissue. Given the timing of post-implantation embryonic demise, uterine decidualization may be disrupted in mice deficient in PGRMC2 or PGRMC1/2. Overall, this study revealed that *Pgrmc1* and/or *Pgrmc2* are required for the maintenance of uterine histoarchitecture and normal female reproductive lifespan.

INTRODUCTION

Progesterone (P4) is a female sex steroid that regulates tissue homeostasis and is essential for reproduction in the female. Disrupted P4 signaling is causally coupled to many reproductive diseases that result in subfertility or infertility including leiomyomas, endometriosis, irregular menstrual bleeding, miscarriage and preterm labor (1-9). In the United States, over 10% of women have impaired fecundity (10), and another 10% of women of reproductive age are clinically diagnosed with endometriosis (11). Infertility and reproductive diseases like endometriosis are multifaceted and difficult to successfully treat. Understanding the etiology of these diseases and how impaired P4 signaling contributes to their pathogenesis, is necessary for developing more effective therapeutic approaches.

Much of what is known about P4 actions is centered on the classical progesterone receptor (PGR). However, several putative non-classical progesterone receptors have been identified, and these include three members of the progestin and adipoQ receptor (PAQR) family (12,13) and two members of the progesterone receptor membrane component (PGRMC) family in PGRMC1

and PGRMC2 (14-17). Importantly, while *in vitro* studies using both primary and transformed reproductive cell lines that lack expression of PGR have established that PGRMC1 mediates at least some of the actions of P4, parallel findings have yet to be confirmed *in vivo*. As evaluated in several species, PGRMC1 and PGRMC2 are expressed throughout the female reproductive tract in both the non-gravid state and during pregnancy (18-29). Aberrant expression of PGRMC1 and PGRMC2 has been implicated in female reproductive diseases. These cumulative findings conceptually indicate that homeostatic expression of PGRMC1 and PGRMC2 is essential for normal female reproductive physiology and maintenance of reproductive tissue homeostasis. Recent findings from our lab demonstrate that conditional ablation of *Pgrmc1* from the uterus results in subfertility in female mice (30). These animals are subfertile at the time of sexual maturation, but then also develop endometrial cysts at 4-5 months of age. This phenotype is consistent with premature aging of the reproductive tract. Given that, as with PGRMC1, PGRMC2 is highly expressed in the uterus, the objective of this study was to evaluate the functional contribution of PGRMC2 to female reproduction. To accomplish this, we developed mutant mice in which *Pgrmc2* alone or in combination with *Pgrmc1* were conditionally ablated from the female reproductive tract in order to assess the function of these two genes on female fertility.

MATERIALS AND METHODS

Floxing the Pgrmc2 allele and genotyping

All procedures involving animals were approved by the Institutional Animal Care and Use Committees at Washington State University or the University of Connecticut Health Center. A *Pgrmc2* targeting vector was prepared by recombineering according to Lee *et al* (31). Briefly, a 13.6 kb section of the *Pgrmc2* genomic sequence containing both exon 2 and exon 3, as well as 4 kb of the 3'-downstream sequences was retrieved from the BAC, RP23-2C23 into pPL253 by gap repair. A 5' *LoxP* site was inserted into intron 1 approximately 1.3 bk upstream of exon 2 followed by insertion of *Frt-PGFneo-Frt-LoxP* into 3'-downstream sequence approximately 0.9 kb downstream of exon 3. The vector, which contained approximately 4.2 kb and 3.2 kb of the 5' and 3' arms, respectively, was linearized by NotI digestion, purified and electroporated into mouse ES cells derived from an F1(129Sv/C57BL6j) blastocyst. The cells were then cultured in the presence of 150 μg/ml of G418 and 2 μM ganciclovir. Drug resistant colonies were selected and screened by nested long range PCR using primers corresponding to sequences outside the arms and specific to the 5' and 3' *LoxP* sites to identify targeted ES clones. Targeted ES cells were used to generate chimeric mice by aggregation with CD1 morula. Chimeric male animals were then bred with *ROSA26-Flpe* mice to remove the PGKneo cassette to generate the *Pgrmc2* floxed ($Pgrmc2^{fl/fl}$) founder mice. Conditional *Pgrmc2* ablated ($Pgrmc2^{d/d}$) mice were produced by crossing $Pgrmc2^{fl/fl}$ (control) mice with $Pgr^{cre/+}$ mice (32). The floxed *Pgrmc1* allele as then included in the breeding scheme to generate $Pgrmc1/2^{fl/fl}$ and $Pgrmc1/2^{d/d}$ mice. Following DNA isolation from tail snips, PCR was completed to detect the presence of the floxed *Pgrmc1* and/or *Pgrmc2* allele(s) and cre recombinase using primer sets shown in Table 1.

Animals and Treatments

Six month fertility trials were completed for *Pgrmc2* and *Pgrmc1/2* mouse colonies on a *Pgr-cre* background in which six week old control ($Pgrmc2^{fl/fl}$ and $Pgrmc1/2^{fl/fl}$) and conditionally mutant ($Pgrmc2^{d/d}$ and $Pgrmc1/2^{d/d}$) female mice were placed with males of proven fertility. Six female mice were included in each colony for each genotype. Through the duration of the fertility trials, the following information was recorded for each litter: date of birth, number of pups born,

number of pups surviving to weaning, pup weights on postnatal day (pnd) 5 and pnd 21, number of days between parity, number of litters throughout the breeding trial, and number of surviving male and female pups. Female reproductive tracts were collected from mice at the end of the breeding trials, as well as from young and aged nulliparous female mice for histological analyses.

In order to evaluate estrogen receptor (ESR1) and PGR expression uterine tissues were obtained from *Pgrmc1/2*$^{fl/fl}$ and *Pgrmc1/2*$^{d/d}$ (*Pgr-cre*) synchronized mice under the following regimen. Sexually mature female mice were ovariectomized, rested for one week, and then given daily subcutaneous injections of estradiol (E2, 100 ng) diluted in sesame oil for three consecutive days. After a two day rest, mice were treated with P4 (1 mg) for two days and then E2 plus P4 for one day. Uterine tissues were collected 24h after the last injection and partitioned for RNA isolation and fixation in 4% paraformaldehyde in preparation for qPCR and paraffin embedding, respectively. Other mice were treated with 100 ng E2 for two days, and then after 4 days were treated with 50ng E2 and collected 18h later. Uterine tissues were collected for RNA isolation in preparation for qPCR. Expression of the vascular marker PECAM1 was evaluated in estrogen treated mice. Here, female *Pgrmc1*$^{fl/fl}$ and *Pgrmc1/2*$^{d/d}$ female mice were ovariectomized and allowed to rest for one week. The mice were then given three single daily injections of estradiol (100 ng) diluted in sesame oil. Uteri were collected and processed for PECAM1 immunohistochemistry and RT-PCR.

To evaluate implantation sites and serum hormone levels during early pregnancy, *Pgrmc2*$^{fl/fl}$ and *Pgrmc2*$^{d/d}$ females were bred with intact males of proven breeding capacity. Observation of a vaginal plug indicated day of pregnancy (DOP) 0.5. Blood was collected on DOP7.5 and 10.5 for E2 and P4 serum analysis, respectively, using the University of Virginia School of Medicine Ligand Assay and Analysis Core. Reproductive tracts were collected from these same animals and the number of normal and resorbed implantation sites was counted. Implantation sites were also isolated from *Pgrmc2*$^{fl/fl}$ and *Pgrmc2*$^{d/d}$ female mice on DOP6 for evaluation of the vascular marker PECAM1. Implantation sites and ovaries were fixed in 4% paraformaldehyde and processed for histological analysis.

RNA isolation and qPCR

PGRMC1 was previously demonstrated to be conditionally deleted from uteri, but not livers, of *Pgrmc1*$^{d/d}$ mice when using *Pgr-cre* driver mice (30). To next confirm conditional deletion of *Pgrmc2* from uteri, total cellular RNA was isolated from uteri and livers of E2-synchronized *Pgrmc1/2*$^{fl/fl}$ and *Pgrmc1/2*$^{d/d}$ mice using TRI-reagent. RNA samples were subjected to DNAse I digestion (Promega) and cDNA was synthesized with SuperScript II (Life Technologies) primer reverse transcriptase and oligo-dT or iScript Reverse Transcriptase Supermix (BioRad). Quantitative PCR was first performed to compare expression of *Pgrmc2* in cDNA samples generated from uteri and livers of *Pgrmc1/2*$^{fl/fl}$ and *Pgrmc1/2*$^{d/d}$ mice. *Rpl13a* was included for normalization. Primers were designed to amplify a region in exon 2 of *Pgrmc2* as listed in Table 1. *Esr1* and *Pgr* were similarly evaluated in the E2+P4-treated tissues. The vascular marker *Pecam1* was evaluated in E2 treated non-gravid uteri from *Pgrmc1/2*$^{fl/fl}$ and *Pgrmc1/2*$^{d/d}$ mice, as well as in implantation sites isolated from *Pgrmc2*$^{fl/fl}$ and *Pgrmc2*$^{d/d}$ female mice on DOP6. A negative control (no reverse transcriptase) was included to confirm the absence of genomic DNA.

Histology and Immunohistochemistry

All tissues were fixed in 4% paraformaldehyde and stored in 70% ethanol until paraffin embedding. Tissues were processed through an ethanol gradient and xylenes, embedded in paraffin, and sectioned at 5µm. Tissue sections were deparaffinized in xylenes and rehydrated in a series of decreasing ethanol washes. Picric acid stain or hematoxylin and eosin (H&E) staining (Scytek HAE-1-IFU kit, Logan, UT) were used for analysis of tissue architecture, including visualization of cytoplasmic versus nuclear morphology and histopathology analysis.

For immunohistochemistry (IHC), rehydrated tissue sections underwent quenching (10 minutes in 8% hydrogen peroxide) and antigen retrieval (boiling for 3 minutes in 0.1M sodium citrate followed by incubation in the heated solution for 20 minutes and then cooling to room temperature). Sections were then blocked (0.1% BSA, 0.1% NGS, and 1% Triton-X100 in PBS) for one hour at room temperature and incubated overnight at 4^{o}C in blocking solution containing primary antibody as outlined in Table 2. Slides were then washed in PBS (3 X 10 min) and incubated with biotinylated secondary antibody for 45 minutes at room temperature. Slides were washed as before and then incubated with horseradish peroxidase-conjugated streptavidin (Vector Laboratories, Burlingame, CA). After a third series of washes, sections were incubated with 3,3'-diaminobenzidine substrate (BD Biosciences, San Diego, CA). Sections were counterstained with hematoxylin, dehydrated in an ethanol gradient and xylenes, and mounted. The specificity of the secondary antibody was confirmed by incubating some sections with non-immune IgG or omitting the primary antibody. The E2+P4-synchronized uteri were stained for ESR1 and PGR expression using the antibodies listed in Table 2. Uterine natural killer cell recruitment to the uterine mesometrial compartment *Pgrmc2*$^{fl/fl}$ and *Pgrmc2*$^{d/d}$ female mice on DOP6 was evaluated by histochemical staining using biotinylated *Dolichos biflorus* (DBA) lectin (Sigma). Thin sections were prepared as described above for IHC with the exception that primary and secondary antibodies were replaced by incubation with DBA lectin (10 µg/ml) for one hour at room temperature.

Transmission electron microscopy

For transmission electron microscopy (TEM), tissues were fixed in 2.5% glutaraldehyde in 0.1M phosphate overnight at 4^{o}C, rinsed in 0.1M phosphate, and post-fixed in 1% osmium tetroxide overnight. Tissues were then dehydrated with a decreasing gradient of ethanol, infiltrated with acetone:SPURRs overnight followed by SPURRs overnight, and embedded in resin. Finally, the resin was polymerized and cured by baking at 65˚C overnight. Tissues were then sectioned and TEM images were taken at the Franceschi Microscopy and Imaging Center at Washington State University on a FEI Tecnai G2 20 Twin equipped with a 200KV LaB6 electron source and a 4K Eagle camera.

Data Analyses

Animals were randomly assigned to the various treatment groups. Unless otherwise noted, all data are presented as the mean ± SEM for n=3-6. Individual animals represent a single experimental replicate within each experiment. Differences between treatment groups were assessed by Student's *t*-test where the mean values of two groups were compared. A two-way analysis of variance was used to identify treatment effects in the breeding trials followed by a Bonferroni post-test. All data were analyzed using GraphPad 5.0 software (San Diego, CA) where a *p*-value of ≤0.05 was considered statistically significant.

RESULTS

Conditional ablation of Pgrmc2 or Pgrmc1/2 results in post-implantation embryonic death

Because PGRMC1 and PGRMC2 are expressed and regulated in the uterus, we hypothesized that they play an essential role in normal pregnancy. To test this hypothesis, the *Pgrmc2* allele was floxed by insertion of *loxP* sites before exon 2 and after exon 3 (Fig. 1A). *Pgrmc2* alone or in combination with *Pgrmc1* was conditionally ablated from the female reproductive tract using the tissue-specific *Pgr-cre* transgenic mouse. A PCR-based genotyping protocol was developed to identify control ($Pgrmc2^{fl/fl}$), heterozygous ($Pgrmc2^{fl/d}$), and conditional knockout ($Pgrmc2^{d/d}$) mice (Figure 1B, see primer sequences in Table 1). A similar genotyping strategy was previously reported for identifying the floxed *Pgrmc1* allele (30). Conditional ablation of PGRMC1 from the uterus was previously confirmed by Western blot analysis (30). Here, conditional ablation of *Pgrmc2* was confirmed by quantitative PCR in the uterus versus the liver of $Pgrmc1/2^{fl/fl}$ and $Pgrmc1/2^{d/d}$ mice (Fig. 1C).

To confirm that any phenotype resulting from the dual ablation of *Pgrmc1* and *Pgrmc2* was due to depletion of these receptors and not the classical sex steroid hormone receptors, we compared the expression of progesterone receptor (PGR) and estrogen receptor (ESR1) in uterine tissues isolated from synchronized $Pgrmc1/2^{fl/fl}$ and $Pgrmc1/2^{d/d}$ female mice by quantitative PCR and immunohistochemistry. As shown in Figure 2, there was no difference in the expression of *Pgr* and *Esr1* mRNAs or proteins in uterine tissues isolated from synchronized $Pgrmc1/2^{fl/fl}$ and $Pgrmc1/2^{d/d}$ female mice.

A six-month breeding trial was conducted in which six week old $Pgrmc2^{fl/fl}$, $Pgrmc2^{d/d}$, $Pgrmc1/2^{fl/fl}$ and $Pgrmc1/2^{d/d}$ female mice (n=6 per genotype) were individually placed with males of proven breeding capacity. The total number of pups produced during the breeding trial was significantly decreased in $Pgrmc2^{d/d}$ and $Pgrmc1/2^{d/d}$ female mice compared with their control counterparts (Tables 3 and 4). Overall, $Pgrmc2^{fl/fl}$ female mice generated an average of 47.67±8.06 pups during the trial while $Pgrmc2^{d/d}$ female mice had 13.5±2.705 pups (n=6, p=0.0024). Similarly, $Pgrmc1/2^{fl/fl}$ females had 51.33 ± 3.70 pups and $Pgrmc1/2^{d/d}$ females had 22.83±4.51 pups (n=6, p=0.0006). $Pgrmc2^{d/d}$ female mice had fewer litters than $Pgrmc2^{fl/fl}$ mice and this also tended (p=0.06) to be the case for $Pgrmc1/2^{d/d}$ versus $Pgrmc1/2^{fl/fl}$ mice (Figs. 3A and 3B). $Pgrmc2^{d/d}$ mice had fewer pups/litter than $Pgrmc2^{fl/fl}$ mice, and this was also the case for $Pgrmc1/2^{d/d}$ versus $Pgrmc1/2^{fl/fl}$ (Figs. 3C and 3D). As shown in Figures 3E and 3F where the number of pups/litter is plotted against parity, conditional ablation of *Pgrmc2* or *Pgrmc1/2* resulted in a steady decline in fecundity to the point of premature reproductive senescence. While all $Pgrmc2^{d/d}$ female mice experienced a significant reduction in lifetime fecundity, 50% of the $Pgrmc2^{d/d}$ mice became infertile after just the third parity. The decreased number of litters in the conditional mutant mice likely stems from faulty uterine function rather than ovarian function given that the parturition interval between control and conditional mutant mice was not different (Figs. 3G and 3H). Furthermore, conditional ablation of *Pgrmc2* or *Pgrmc1/2* likely did not affect prolactin production by the pituitary as indirectly assessed by two measures. First, an evaluation of pup weight at the time of weaning, an index of lactation efficiency, demonstrated that there was no difference (p=0.449) in weanling weights from $Pgrmc1/2^{fl/fl}$ (8.92 ± 0.621 grams) and $Pgrmc1/2^{d/d}$ (8.04 ± 0.892 grams) mothers. Second, corpora lutea, which require an intact prolactin:prolactin receptor signaling pathway for P4 production, functioned normally in that serum P4 levels were not decreased during pregnancy (see Fig. 5).

To further assess the relationship between the observed subfertility in $Pgrmc2^{d/d}$ mice and implantation, uterine tracts were examined on DOP10.5. There was no significant difference

between *Pgrmc2*$^{fl/fl}$ and *Pgrmc2*$^{d/d}$ females in the total number of implantation sites at DOP10.5 (Fig. 4A), suggesting that ovulation remained intact in *Pgrmc2*$^{d/d}$ females, that fertilization occurred, and that the embryos implanted normally. However, there was a significant increase in the percentage of post-implantation resorption sites in *Pgrmc2*$^{d/d}$ female mice (Figs. 4B and 4C). Histological examination of DOP10.5 embryos in *Pgrmc2*$^{d/d}$ females shows heavy necrosis of decidual and embryonic tissues (Figure 4D). Because the embryos had died and the sites began to resorb by DOP10.5, we evaluated implantation sites on DOP7.5. Upon histological examination, some of the embryos within sites from *Pgrmc2*$^{d/d}$ mice were not viable (Fig. 4E). The embryonic demise likely does not derive from altered uterine vasculature. As shown in Supplemental Figure 1, protein and mRNA expression of the vascular marker PECAM1 was not different between *Pgrmc1/2*$^{fl/fl}$ and *Pgrmc1/2*$^{d/d}$ female mice in response to estradiol treatment. Likewise, there was no difference in the expression of PECAM1 between female mice on DOP7. However, a reduction in the number of uterine natural killer cells was observed in *Pgrmc2*$^{d/d}$ female mice compared with *Pgrmc2*$^{fl/fl}$ mice on DOP6 consistent with disruption in the decidualization program (Supplemental Figure 1B).

An evaluation of ovaries isolated from DOP10.5 *Pgrmc2*$^{d/d}$ female mice revealed normal histology with large and abundant corpora lutea (Fig. 5A). *Pgrmc2*$^{d/d}$ females had marginally elevated serum P4 compared with *Pgrmc2*$^{fl/fl}$ females on DOP10.5 (Fig. 5B). Wildtype mice have between 25 and 40 ng/mL circulating P4 on DOP10.5 (33), so even though the *Pgrmc2*$^{d/d}$ mice have slightly elevated serum P4 compared with *Pgrmc2*$^{fl/fl}$ mice, serum P4 levels from *Pgrmc2*$^{fl/fl}$ and *Pgrmc2*$^{d/d}$ mice are within the normal range on DOP10.5. This indicates that luteal function remains intact in the *Pgrmc2*$^{d/d}$ mice. In addition, serum E2 was examined in *Pgrmc2*$^{fl/fl}$ versus *Pgrmc2*$^{d/d}$ females during early pregnancy. There was no difference in E2 between *Pgrmc2*$^{fl/fl}$ and *Pgrmc2*$^{d/d}$ females (Figure 5C). Overall, these results demonstrate that ovarian steroidogenesis in *Pgrmc2*$^{d/d}$ mice during early pregnancy is normal thus providing further evidence that the subfertility defect stems from faulty uterine function.

Conditional ablation of Pgrmc2 or Pgrmc1/2 results in premature aging of the uterus

Because the subfertility phenotype is due to a uterine defect, we next examined uterine histology. Unlike uteri from nulliparous *Pgrmc2*$^{fl/fl}$ & *Pgrmc1/2*$^{fl/fl}$, which appeared normal, uteri from nulliparous *Pgrmc2*$^{d/fl}$, *Pgrmc1/2*$^{d/fl}$, *Pgrmc2*$^{d/d}$ and *Pgrmc1/2*$^{d/d}$ mice developed abnormal uterine histoarchitecture with increased glandular content and enlarged amorphic and cystic glands (Fig. 6A). These features are commonly found in endometrium from aged (>8 months) female mice. These mice displayed varying degrees of abnormal histoarchitecture, with *Pgrmc2*$^{d/d}$ and *Pgrmc1/2*$^{d/d}$ mice having a higher overall epithelial-stromal ratio and more glandular epithelium than *Pgrmc2*$^{fl/d}$ and *Pgrmc1/2*^{fldl} mice. Examination of endometrial cellular ultrastructure using transmission electron microscopy revealed the breakdown of basement membrane and a disrupted epithelial-stromal interface in *Pgrmc1/2*$^{d/d}$ female mice (Fig. 6B). Glandular epithelial cells contained heavily vacuolated nuclei, accumulation of intracellular inclusion bodies, and excessive, highly convoluted plasma membrane.

To quantify the prevalence of the cystic phenotype, uteri from nulliparous and multiparous *Pgrmc2*$^{fl/fl}$, *Pgrmc1/2*$^{fl/fl}$, *Pgrmc2*$^{d/d}$ and *Pgrmc1/2*$^{d/d}$ mice of various ages were histologically examined. Some degree of abnormal uterine histoarchitecture was evident in 100% of nulliparous *Pgrmc2*$^{d/d}$ & *Pgrmc1/2*$^{d/d}$ mice by 4-5 months (Figure 7A). This is in contrast to control mice that showed greatly reduced development of endometrial cysts. Here, 20-30% of the mice developed cysts at 5-8 months of age. The cumulative incidence of cystic gland formation from all ages is shown in Figure 7B. Interestingly, parity prevented cyst formation in control

mice and greatly reduced the number and size of endometrial cysts in *Pgrmc2*$^{d/d}$ and *Pgrmc1/2*$^{d/d}$ mice (Fig. 7C).

DISCUSSION

We previously demonstrated the functional importance of the non-classical progesterone receptor PGRMC1 in female reproduction, where conditional ablation from the uterus resulted in subfertility and formation of endometrial cysts. The objective of the present study was to evaluate the functional contributions of the second member of the PGRMC family, PGRMC2, to female fertility. This was achieved by floxing exons 2 and 3 of the *Pgrmc2* allele. The resulting transgenic mice were crossed with *Pgr-cre* mice to conditionally ablate *Pgrmc1* and/or *Pgrmc2* from the female reproductive tract. We demonstrate here that conditional ablation of *Pgrmc2* alone or in combination with *Pgrmc1* from the female reproductive tract initially resulted in subfertility, and this then progressed to premature reproductive senescence. It was interesting to note that *Pgrmc2*$^{d/d}$ female mice had a more severe reproductive phenotype than *Pgrmc1/2*$^{d/d}$ female mice, suggesting that PGRMC1 deficiency may provide some protection against reproductive failure when *Pgrmc2* alone is ablated. This phenotype was accompanied by development of endometrial cysts starting around 4 months of age. This is the first functional evaluation of *Pgrmc2* in an *in vivo* setting.

Much of what is known about members of the PGRMC family derives from *in vitro* studies of PGRMC1. PGRMC1 is expressed in many tissues within and outside the female reproductive system, suggesting that it has both P4-dependent and P4-independent functions as previously described (34,35). PGRMC1 and/or PGRMC2 are implicated in diverse cellular processes, including mitosis/cell cycle regulation (36,37), meiosis (23,38,39), sterol metabolism (40,41), cell survival/anti-apoptotic action (18,42), transcription (42,43), angiogenesis (44,45), immune regulation (46), autophagy (47), carbon monoxide sensing (48), and heme biosynthesis and transport (49). It is not clear from the present study if the fertility defect observed here is due to faulty P4 signaling or if it derives from disruption of any of the cellular processes with which PGRMC family members associate. The ability to conditionally ablate *Pgrmc1* and *Pgrmc2* in mice should be helpful in future studies in determining how these proteins function as P4 receptors.

The PGRMC1/2 deficient mice were generated using *Pgr-cre* to delete *Pgrmc1* and/or *Pgrmc2* from female reproductive tissues, including the hypothalamus, pituitary gonadotropes, ovarian periovulatory follicles and the majority of the female reproductive tract. Several lines of evidence suggest that the observed subfertility/infertility phenotype in *Pgrmc2*$^{d/d}$ and *Pgrmc1/2*$^{d/d}$ mice stems from faulty uterine function and that other elements of the reproductive system remain intact. First, the parturition interval did not differ between control and mutant mice suggesting that estrous cyclicity and the hypothalamic-pituitary unit is normal in mutant female mice when in the presence of a male with proven breeding capacity. Second, the number of implantation sites was not different between control and mutant mice indicating that ovulation, oviductal transport, fertilization, and implantation were normal in mutant mice. Third, a direct assessment of ovarian histology showed no difference in the presence of corpora lutea (CL) in control and mutant mice. CL from mutant mice were histologically indistinguishable from those of control mice. Fourth, CL steroidogenic functions were consistent between control and mutant mice in that mid-gestational serum P4 and E2 levels were not different. Finally, despite a normal number of implantation sites, we noted a significant increase in post-implantation resorptions in *Pgrmc2*$^{d/d}$ and *Pgrmc1/2*$^{d/d}$ female mice indicating that embryos are

able to implant, begin development, and initiate a decidual response by the mother, but then die prior to development of the placenta around DOP 6-7. With the data presented here, we cannot rule out the possibility that the progression from subfertility to infertility around parities 3 or 4 in mutant mice is caused by defects in other reproductive tissues.

As demonstrated by both qPCR and immunohistochemistry, ablation of *Pgrmc1/2* did not alter the expression of the classical estrogen and progesterone receptors indicating that the observed phenotypes in these mice does not result from disrupted ESR1 or PGR activity. The uterine defect causing embryonic death occurred after the decidualization program was fully engaged, but before placentation. Decidualized stromal cells produce nutrients and growth factors to promote embryonic growth and survival. Concurrently, pregnancy modifies maternal vasculature and the maternal immune system. Modifications of maternal vasculature facilitates the delivery of nutrients to the embryo prior to placentation, while modification of the maternal immune system provides an immune-privileged site that allows the histocompatibally distinct embryo to avoid rejection. PGRMC1/2 could be involved in regulating either of these processes. During decidualization, the uterus produces and/or acquires lipid, protein, and carbohydrate nutrients from the surrounding vasculature for the developing embryo (50), thus performing placenta-like functions. PGRMC1 may be involved in this process given that it interacts with lipid/sterol metabolic enzymes that may be involved in the production of lipid nutrients. For example, PGRMC1 participates in the biosynthesis of cholesterol and other lipids through its interactions with INSIG/Scap (41,51,52). Ablation of *Pgrmc1/2* may prevent normal nutrient production within the decidua and transport to the embryo, thus attenuating embryonic growth and survival. The decidua produces a variety of growth factors including vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), transforming growth factor beta (TGFβ) and others to support the embryo before placentation occurs (53). Aberrant production of such growth factors or uncoupling of their cognate receptors may contribute to the observed post-implantation embryonic loss. Interestingly, PGRMC1 has been implicated in growth factor signaling in several settings. For example, PGRMC1 has now been shown by several labs to be necessary for the exteriorization of the epidermal growth factor receptor (EGFR) from intracellular vesicular stores to the plasma membrane (54-56). EGFR plays an essential role in decidualization (57). It is perhaps not surprising that the *Pgrmc1/2* phenotype parallels that of the *Egfr* conditional mutant phenotype. The impact of *Pgrmc1/2* ablation on uterine decidualization is currently under investigation.

Progesterone regulates *Vegf* (vascular endothelial growth factor), which controls uterine decidual angiogenesis and vascular remodeling (58). Disruption of VEGF signaling leads to post-implantation embryonic death (59). PGRMC1 has been shown to mediate the influence of P4 on VEGF in breast cancer cells (45) and retinal glial cells (44), and it is feasible that PGRMC1 and/or PGRMC2 are necessary for mediating P4-dependent vascular remodeling during early pregnancy. However, preliminary evaluation of the vasculature in both the non-gravid and gravid uterus suggests that vascular development is not compromised by ablation of *Pgrmc1/2*. Progesterone is also involved in modifying the action of many components of the maternal immune system during early pregnancy (60), and abnormalities in this process have been implicated in recurrent pregnancy loss (61). Progesterone up-regulates the expression of PGRMC1 in immune cells in pregnant cows (46). While *Pgrmc1* and *Pgrmc2* are not ablated in immune cells when using *Pgr-cre* mice, aberrant P4 signaling in the uterus due to uterine ablation of *Pgrmc1/2* may prevent the uterus from properly signaling immune cells to generate an appropriate immune privileged environment. Of note, there was a reduction in the number of

uNK cells recruited to or proliferating in the mesometrial region of the implantation site. However, it is not clear if this reduction in uNK cell numbers is a direct response to PGRMC1/2 deficiency or if it occurs indirectly because of a faulty decidualization program. Further research is needed to understand why *Pgrmc2*$^{d/d}$ and *Pgrmc1/2*$^{d/d}$ mice have reduced fecundity and if this is due to a lack of nutrients, impaired vascularization, immune rejection of the embryo, or a combination of these factors.

PGRMC1/2 are implicated in regulating apoptosis and other processes in response to P4. There are multiple mechanisms whereby PGRMC1 could mediate the actions of P4. PGRMC1 is associated with both rapid P4 actions, as well as changes in gene expression (18,37). Despite lacking a P4-binding domain homologous to that of the classical PGR, PGRMC1 still directly binds P4. This was most recently demonstrated using NMR spectroscopy showing that the cytochrome b5 domain indeed binds P4 (62). This study supports initial receptor binding studies conducted over 15 years ago (63,64). PGRMC1 has also been shown to facilitate the binding of P4 to other purported P4 receptors, such as membrane progestin receptor α (mPRα) (65). PGRMC1 may mediate P4 signaling by any number of cell signaling pathways given that it harbors several SH2 and SH3 domains, tyrosine kinase binding sites, and interaction domains for ERK1, casein kinase 2, and PDK1 (17). Finally, PGRMC1 localizes to the nucleus to regulate gene expression in response to P4 (43). PGRMC1 does not have a DNA-binding domain; as such, PGRMC1 may function as a scaffold protein in a transcriptional complex. Further investigation is needed to understand the mechanisms by which PGRMC1 and PGRMC2 relay P4 signals to exert effects on the cell under normal uterine physiology, and if disruption in these pathways leads to uterine pathology.

While *Pgrmc2*$^{d/d}$ and *Pgrmc1/2*$^{d/d}$ mice do develop endometrial cysts, this does not account for the pregnancy defect, since cystic glands do not become evident until around four months of age despite delivery of fewer pups/litter from the first parity onward. However, the underlying molecular changes that may occur upon deletion of *Pgrmc1* and/or *Pgrmc2* may contribute to the initial subfertility phenotype that then later manifest in the development of cysts. The cystic glands are likely a result of faulty mesenchymal-epithelial communication. We previously demonstrated that ablation of *Pgrmc1* from the stromal, but not epithelial compartment, results in a similar phenotype to that described for *Pgrmc1* ablation from both compartments (30). Another interesting observation is that conditional ablation of *Pgrmc1*, *Pgrmc2* or *Pgrmc1/2* results in a similar, if not identical, histological phenotype. This finding suggests that PGRMC1 and PGRMC2 function distinctly within a similar pathway to maintain normal tissue homeostasis. These glands are indicative of bland endometrial cysts in women commonly associated with atrophic endometria or senile polyps (66). Such cysts, although smaller and less frequent than in *Pgrmc1*$^{d/d}$, *Pgrmc2*$^{d/d}$, and *Pgrmc1/2*$^{d/d}$ mice, are generally found in most mouse strains as part of the aging process. As such, the occurrence of endometrial cysts at an early age in *Pgrmc1*$^{d/d}$, *Pgrmc2*$^{d/d}$, and *Pgrmc1/2*$^{d/d}$ mice is consistent with a premature aging phenotype. The observed premature reproductive senescence may therefore more aptly be defined as premature uterine senescence.

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Figure 1. Floxing the *Pgrmc2* allele. (**A**) Schematic of conditional mutagenesis approach used to ablate floxed regions of the *Pgrmc1* and *Pgrmc2* genes. (**B**) A PCR-based genotyping protocol was developed to identify control (*Pgrmc2*$^{fl/fl}$), heterozygous (*Pgrmc2*$^{fl/d}$), and conditional knockout (*Pgrmc2*$^{d/d}$) mice. (**C**) Quantitative PCR showing that *Pgrmc2* expression is reduced in uterine tissue isolated from *Pgrmc2*$^{d/d}$ female mice compared with tissue obtained from *Pgrmc2*$^{fl/fl}$ mice. *Pgrmc2* expressed does not change in the liver regardless of genotype. *p<0.05, n=3

Figure 2. Conditional ablation of *Pgrmc1/2* does not alter uterine expression of the PGR and ESR1. Quantitative PCR was used to compare the mRNA expression of *Pgr* (**A**) and *Esr1* (**B**) in uterine tissues isolated from ovariectomized E2+P4-synchronized *Pgrmc1/2*$^{fl/fl}$ and *Pgrmc1/2*$^{d/d}$ female mice. n=3 (**C**) Immunohistochemistry shows a comparable expression pattern for PGR and ESR1 protein between *Pgrmc1/2*$^{fl/fl}$ and *Pgrmc1/2*$^{d/d}$ ovariectomized E2+P4-synchronized female mice. n=4

Figure 3. Ablation of *Pgrmc2* or *Pgrmc1/2* causes subfertility that prematurely progresses to infertility. In a 6 month breeding trial, the number of pups/litter (**A**, **B**) and total number of litters during the 6 month trial (**C**, **D**) were determined in *Pgrmc2*$^{fl/fl}$, *Pgrmc2*$^{d/d}$, *Pgrmc1/2*$^{fl/fl}$ and *Pgrmc1/2*$^{d/d}$ female mice (n=6 per genotype). The number of pups/litter was plotted against parity to highlight the premature transition from subfertility to infertility (**E**, **F**). The parturition interval did not differ between floxed control and corresponding conditional mutant mice (**G**, **H**). *p<0.05

Figure 4. Subfertility of *Pgrmc2*$^{d/d}$ mice is uterine in origin. (**A**) There was no significant difference between *Pgrmc2*$^{fl/fl}$ and *Pgrmc2*$^{d/d}$ females in the number of implantation sites at DOP10.5. n=6-7 (**B**) There was a seven-fold increase in post-implantation embryonic death in *Pgrmc2*$^{d/d}$ females compared to *Pgrmc2*$^{fl/fl}$ females on DOP10.5. (**C**) Representative images of uteri from DOP10.5 *Pgrmc2*$^{fl/fl}$ and *Pgrmc2*$^{d/d}$ as indicated by black arrows. Picric acid stain showing histology of representative DOP10.5 (**D**) and DOP7.5 (**E**) implantation sites from *Pgrmc2*$^{fl/fl}$ and *Pgrmc2*$^{d/d}$ mice.

Figure 5. Ovarian function is not compromised in *Pgrmc2*$^{d/d}$ mice. (**A**) Ovaries from both *Pgrmc2*$^{fl/fl}$ and *Pgrmc2*$^{d/d}$ females display corpora lutea (*) on DOP10.5. (**B**) Serum P4 levels were not decreased in *Pgrmc2*$^{d/d}$ female mice on DOP10.5 indicating an absence of luteal

insufficiency. *p<0.05, n=5-6 (**C**) Serum E2 was not different between *Pgrmc2*$^{fl/fl}$ and *Pgrmc2*$^{d/d}$ female mice on DOP7.5. n=3

Figure 6. Conditional ablation of *Pgrmc2* or *Pgrmc1/2* results in formation of endometrial cysts after 3 months of age. (**A**) H&E stain showing histology of representative uterine cross sections from control (*fl/fl*), heterozygous (*d/fl*), and conditional knockout (*d/d*) mice at 5-8 months of age. n=3-10 per genotype. (**B**) High magnification light microscopy (*a*, *b*) and transmission electron microscopy (*c*, *d*) show that uteri from *Pgrmc1/2*$^{d/d}$ mice display abnormal uterine histoarchitecture. Note the breakdown of basement membrane and disrupted epithelial-stromal interface in *Pgrmc1/2*$^{d/d}$ female mice (*a*), heavily vacuolated nuclei in the glandular epithelium (*b*), accumulation of intracellular inclusion bodies (*c*) and excessive plasma membrane within the epithelial compartments (*d*). L, glandular lumen; E, epithelium; S, stroma

Figure 7. Conditional ablation of *Pgrmc2* or *Pgrmc1/2* accelerates uterine aging. (**A**) 100% of nulliparous *Pgrmc2*$^{d/d}$ and *Pgrmc1/2*$^{d/d}$ mice of young, medium, or older ages displayed abnormal uterine histoarchitecture including development of endometrial cysts, whereas *Pgrmc2*$^{fl/fl}$ and *Pgrmc1/2*$^{fl/fl}$ mice developed abnormal phenotype in an age-dependent manner, but at a much lower prevalence and severity starting at 5-8 months. (**B**) When combining all age groups, nulliparous *Pgrmc2*$^{d/d}$ and *Pgrmc1/2*$^{d/d}$ mice have a much higher prevalence of abnormal uterine histoarchitecture compared with *Pgrmc2*$^{fl/fl}$ and *Pgrmc1/2*$^{fl/fl}$ control mice, respectively (p<0.0001, n=3-4 per genotype per age group). (**C**) 67% of multiparous *Pgrmc2*$^{d/d}$ and 67% of *Pgrmc1/2*$^{d/d}$ mice displayed abnormal uterine histoarchitecture, while 0% for multiparous *Pgrmc1/2*$^{fl/fl}$ and *Pgrmc1/2*$^{d/d}$ mice displayed abnormal uterine histoarchitecture. n=3-6

Table 1. PCR primers

Gene identifier	Primer sequence
Pgr-cre P1	5'-ATGTTTAGCTGGCCCAAATG-3'
Pgr-cre P2	5'-TATACCGATCTCCCTGGACG-3'
Pgr-cre P3	5'-CCCAAAGAGACACCAGGAAG-3'
Pgrmc1_lox_gt F	5'-GGCTCAAGCACCCAGAATAG-3'
Pgrmc1_lox_gt R	5'-GCTTCCTTGCTTTCAACACC-3'
Pgrmc2_lox_gt F	5'-ATGGTGGATCATAACCATCTG-3'
Pgrmc2_lox_gt R	5'-CCTTGATTTCTAAGTGAAA-3'
Pgrmc2_exon2 F	5'-GGGTCCATATGGCATCTTTG-3'
Pgrmc2_exon2 R	5'-CTTTAAACTGCATTTCCCATTCT-3'
Pecam1 F	5'-TGATGTTTCTGGAAATGATGCAGT-3'
Pecam1 R	5'-GCTCAAGGGAGGACACTTCC-3'
Esr1 F	5'-CCAAAGCCTCGGGAATG-3'
Esr1 R	5'-CTTTCTCGTTACTGCTGG-3'
Pgr F	5'-ATGGTCCTTGGAGGTCGTAA-3'
Pgr R	5'-CACCATCAGGCTCATCC-3'
Rpl13a F	5'-TTGCTTACCTGGGGCGTCT-3'
Rpl13a R	5'-CCTTTTCCTTCCGTTTCTCCTCGC-3'

Table 2. Antibodies

Target	Clone	Manufacturer, catalog #, (lot #)	Species, clonal status	Dilution
ESR1	MC-20	Santa Cruz, SC-542, (#I1112)	rabbit, polyclonal	1:300
PGR	SP2	Thermo, RM-9102, (#9102s1007Z)	rabbit, monoclonal	1:200
PECAM1	D8V9E	Cell Signaling, CS-776995	rabbit, monoclonal	1:100
Anti-rabbit IgG	-	Santa Cruz, SC-2040	goat, polyclonal	1:1000
DBA lectin	-	Sigma, L6533	-	10 µg/ml

Table 3. Fertility trial from *Pgrmc2* colony

Genotype	Females (*n*)	Litters (*n*)	Pups (*n*)	Average pups/litter	Average litters/female
Pgrmc2$^{fl/fl}$	6	37	286	7.69 ± 0.80	6.16 ± 0.75
Pgrmc2$^{d/d}$	6	23	81	4.11 ± 0.43*	3.83 ± 0.65**

*p=0.002 versus *Pgrmc2*$^{fl/fl}$, **p<0.05 versus *Pgrmc2*$^{fl/fl}$

Table 4. Fertility trial from *Pgrmc1/2* colony

Genotype	Females (*n*)	Litters (*n*)	Pups (*n*)	Average pups/litter	Average litters/female
Pgrmc1/2$^{fl/fl}$	6	39	308	8.43 ± 0.28	6.5 ± 0.42
Pgrmc1/2$^{d/d}$	6	29	137	5.21 ± 0.67*	4.83 ± 0.65**

*p=0.0007 versus *Pgrmc1/2*$^{fl/fl}$, **p<0.05 versus *Pgrmc1/2*$^{fl/fl}$

Figure 1

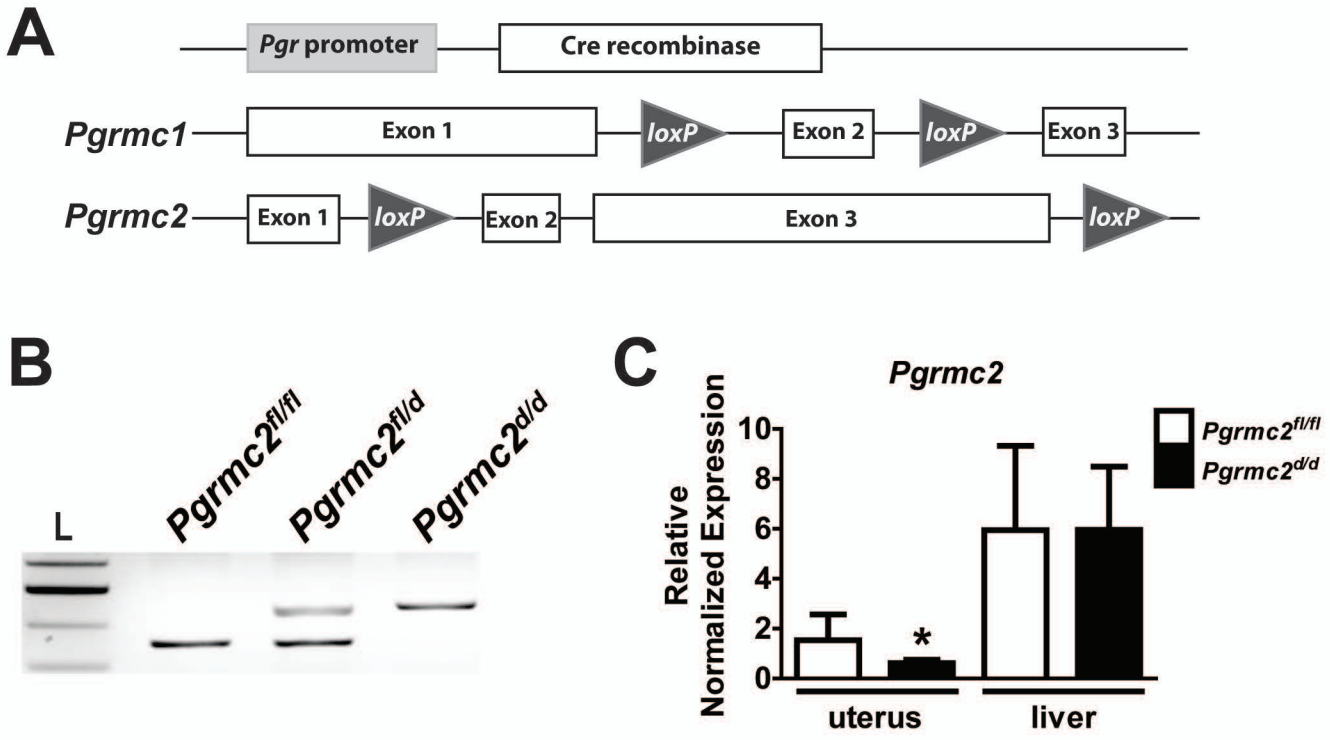


Figure 2

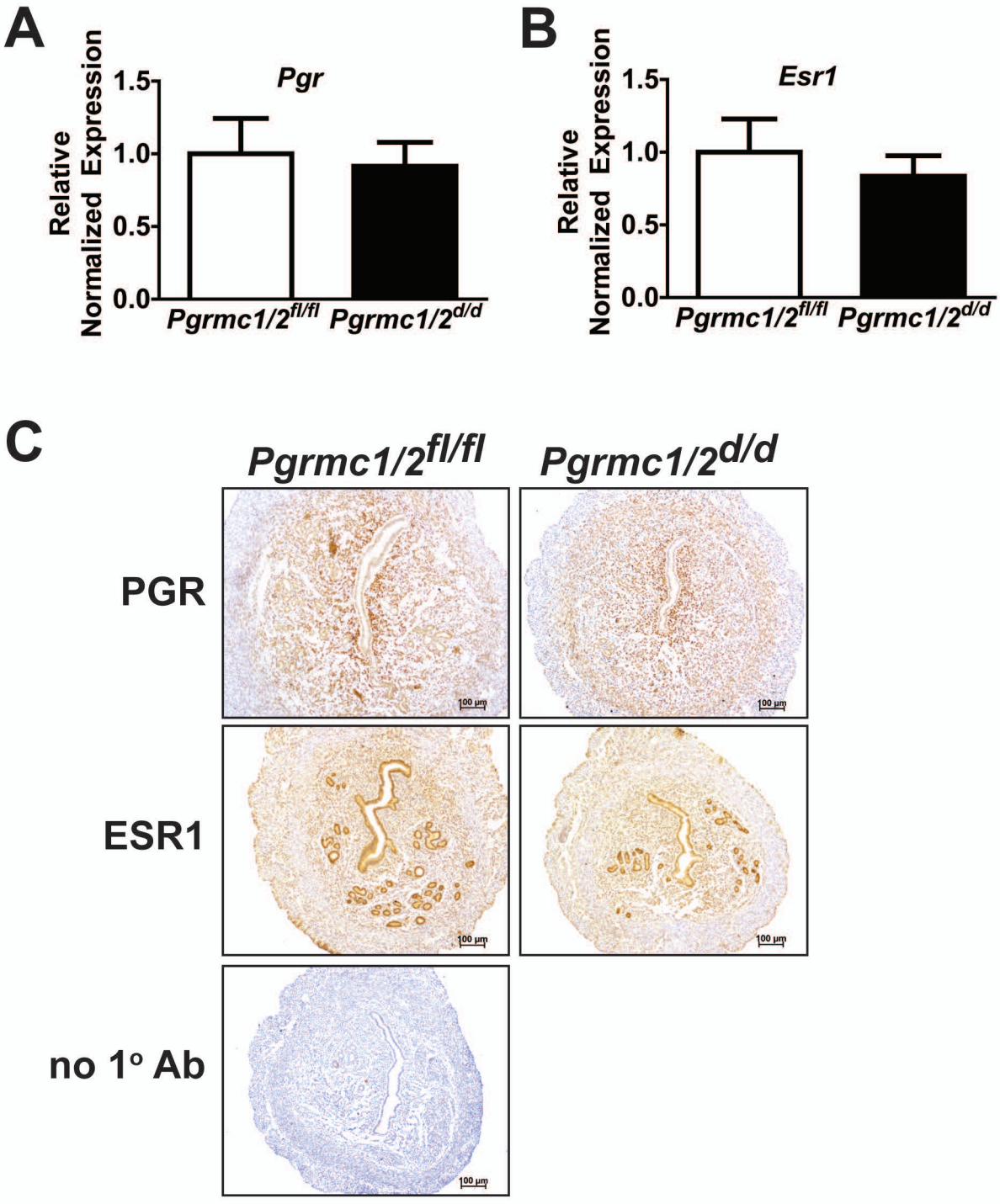


Figure 3

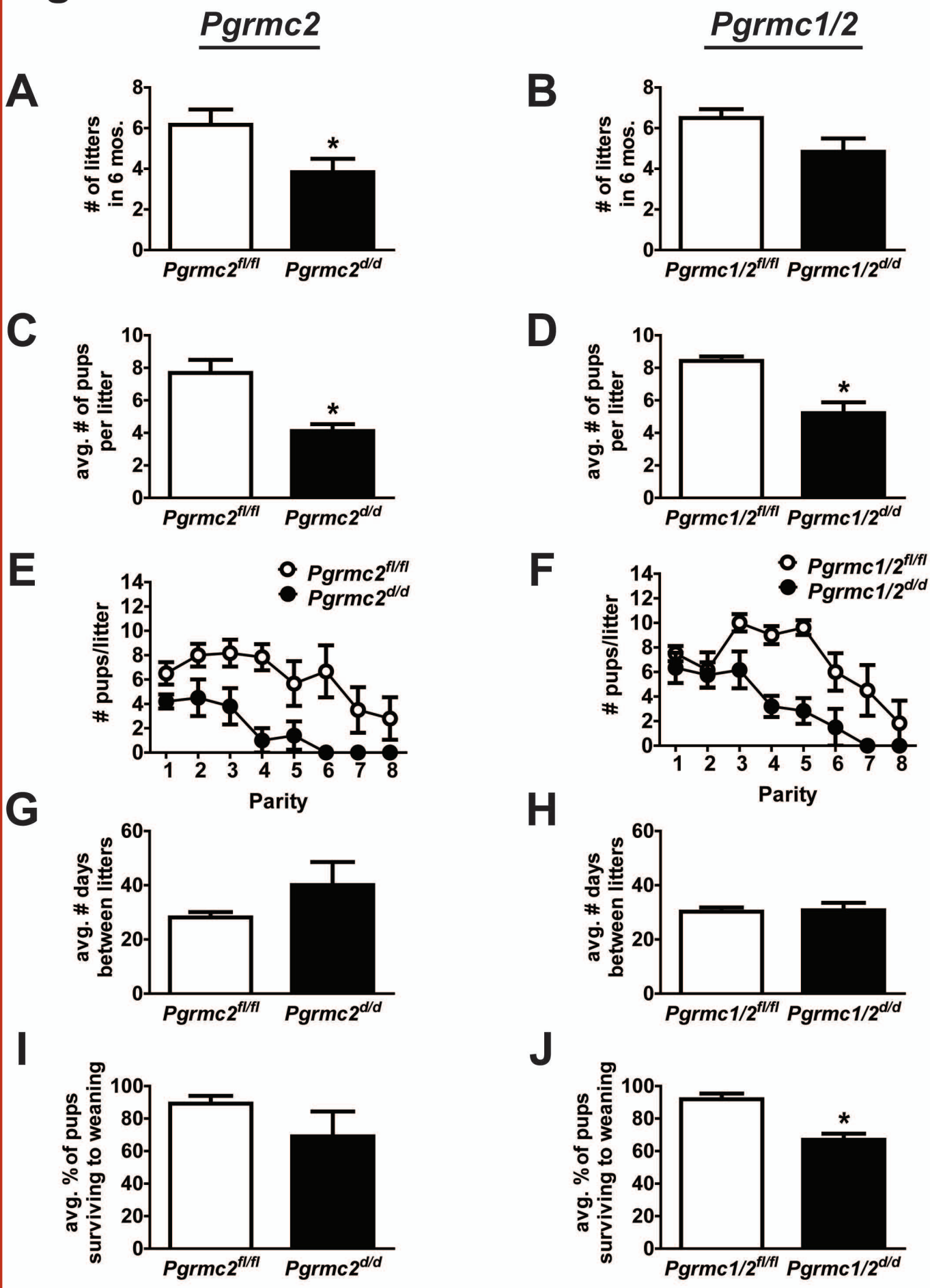


Figure 4

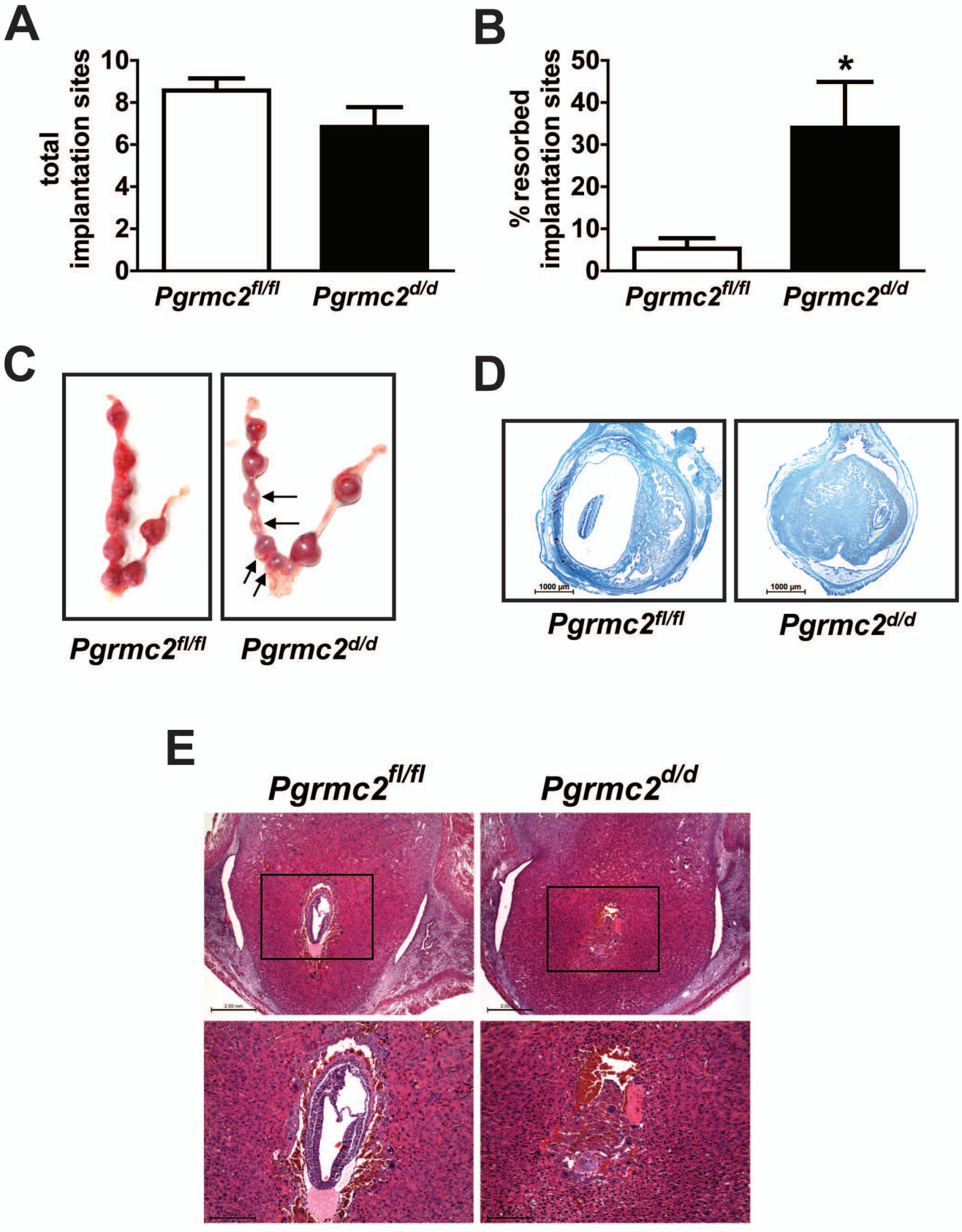


Figure 5

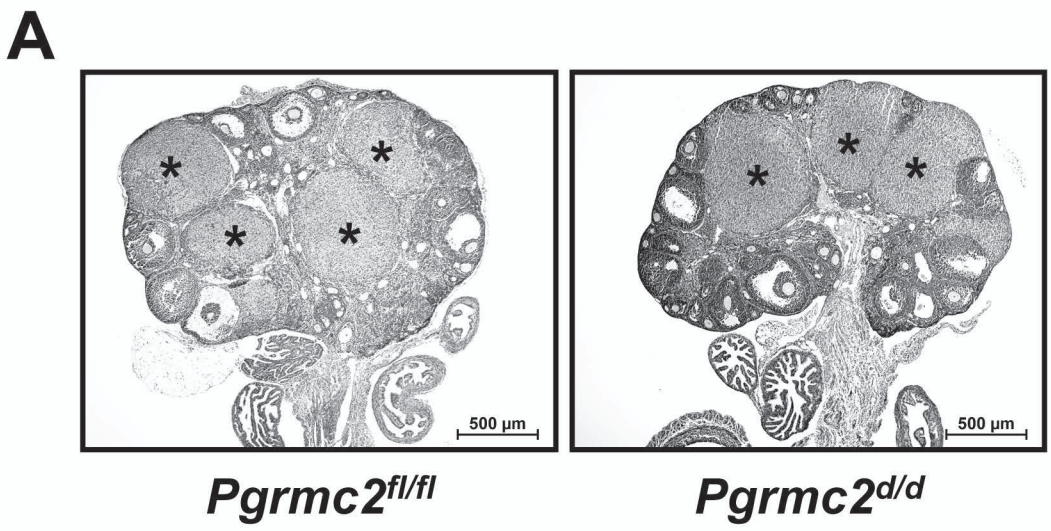
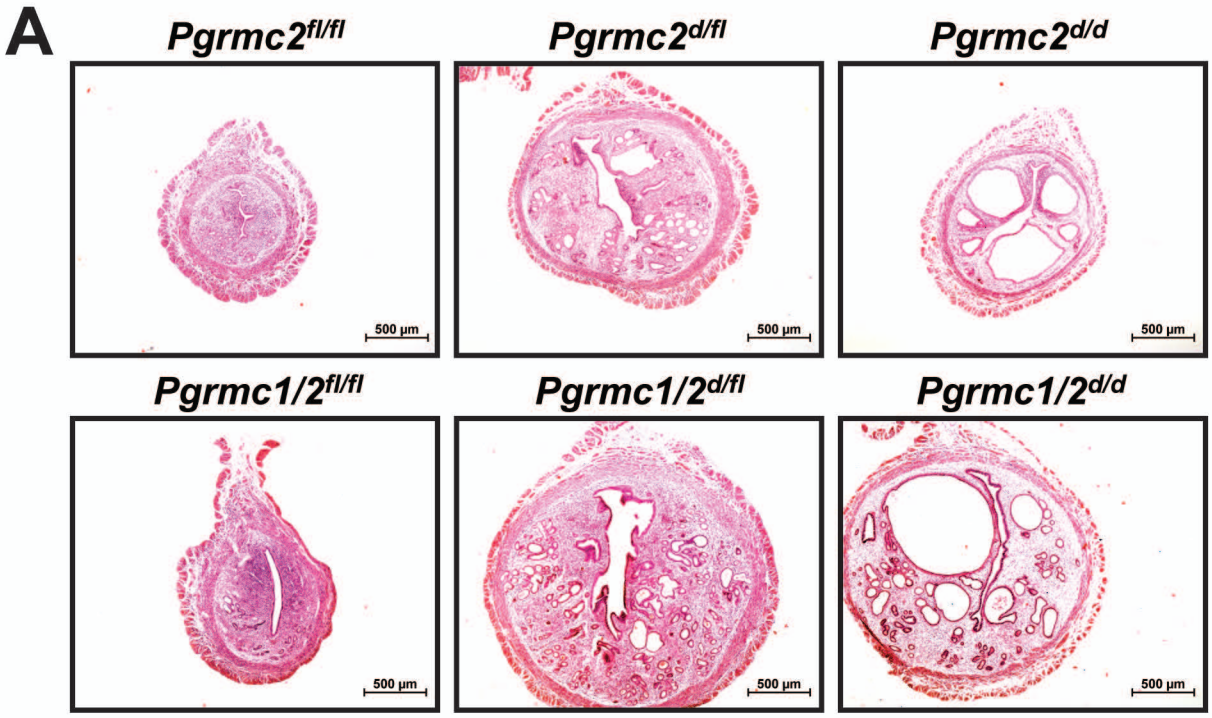


Figure 6



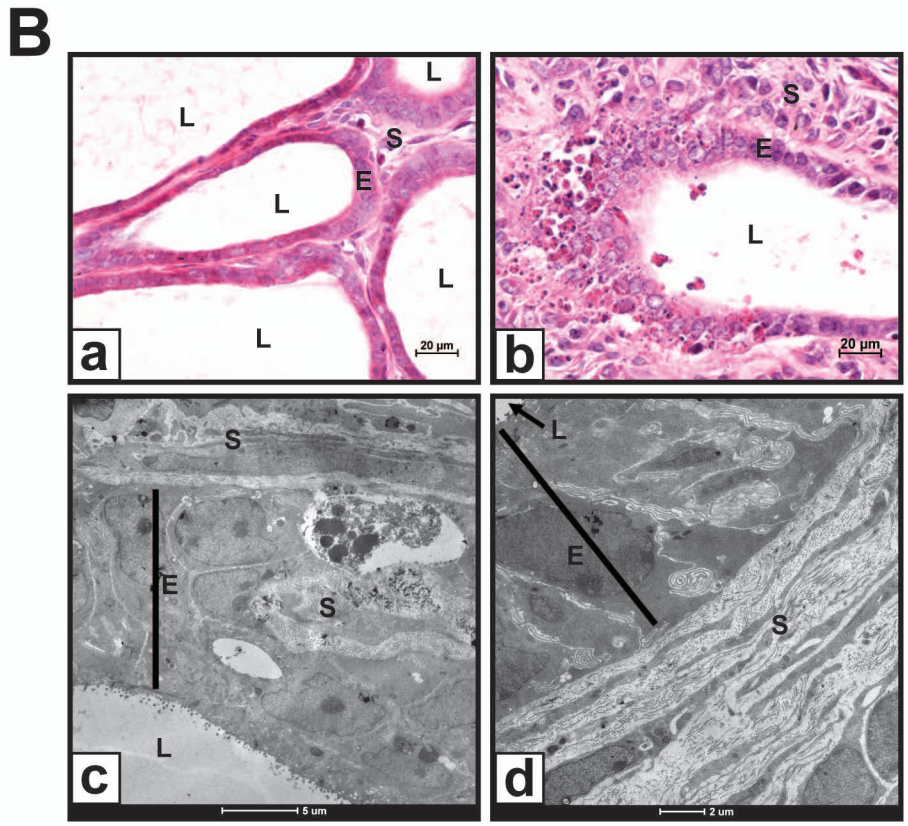
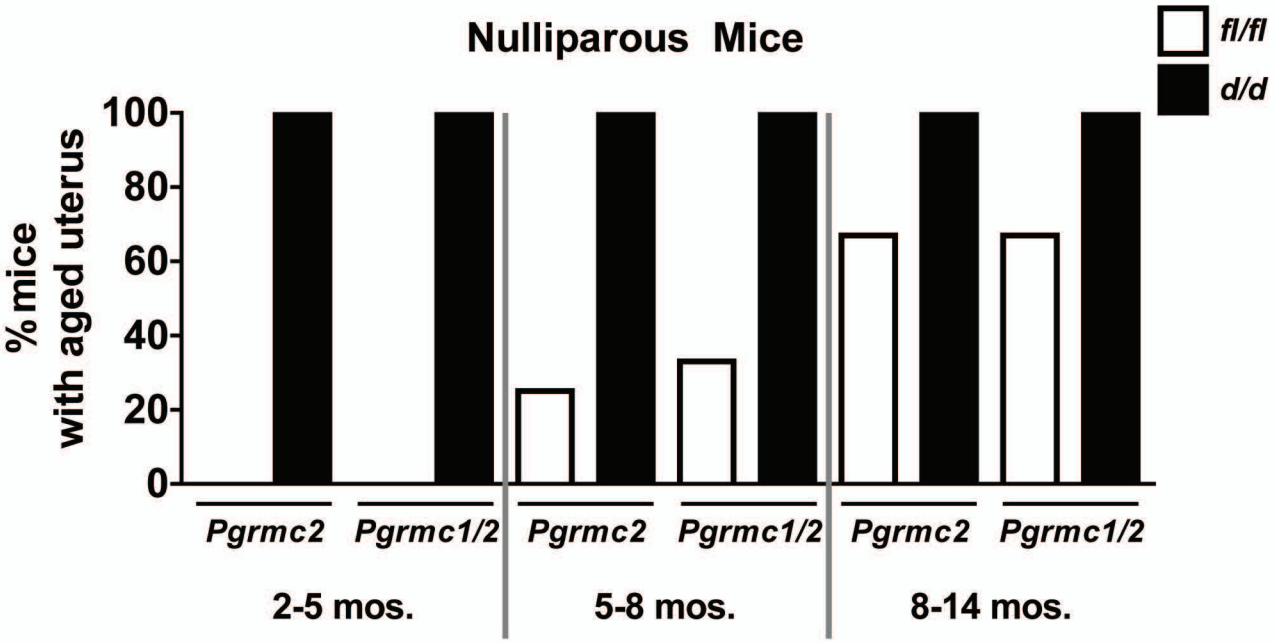
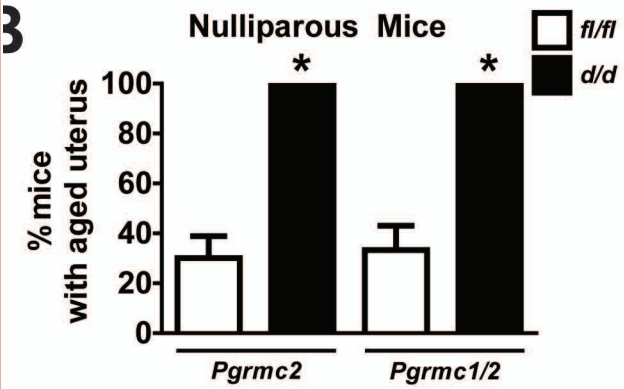


Figure 7

A



B



C