PGRMC1/2 promotes luteal vascularization and maintains the primordial follicles of mice

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Abstract

To determine whether conditional depletion of progesterone receptor membrane component (PGRMC) 1 and PGRMC2 affected ovarian follicle development, follicle distribution was assessed in ovaries of young (≈ 3 -month-old) and middle-aged (≈ 6 -month-old) control (*Pgrmc1/2^{fl/fl}*) and double conditional PGRMC1/2-knockout (*Pgrmc1/2^{d/d}*) mice. This study revealed that the distribution of primary, preantral and antral follicles was not altered in *Pgrmc1/2^{d/d}* mice, regardless of the age. Although the number of primordial follicles was similar at ≈ 3 months of age, their numbers were reduced by $\approx 80\%$ in 6-month-old *Pgrmc1/2^{d/d}* mice compared to age-matched *Pgrmc1/2^{fl/fl}* mice. The *Pgrmc1/2^{d/d}* mice were generated using *Pgr-cre* mice, so ablation of *Pgrmc1* and *Pgrmc2* in the ovary was restricted to peri-ovulatory follicles and subsequent corpora lutea (CL). In addition, the vascularization of CL was attenuated in *Pgrmc1/2^{d/d}* mice, although mRNA levels of vascular endothelial growth factor A (*Vegfa*) were elevated. Moreover, depletion of *Pgrmc1* and *Pgrmc2* altered the gene expression profile in the non-luteal component of the ovary such that *Vegfa* expression, a stimulator of primordial follicle growth, was elevated; Kit Ligand expression, another stimulator of primordial follicle growth, was suppressed and anti-Mullerian hormone, an inhibitor of primordial follicle growth, was enhanced compared to *Pgrmc1/2^{fl/fl}* mice. These data reveal that luteal cell depletion of *Pgrmc1* and 2 alters the expression of growth factors within the non-luteal component of the ovary, which could account for the premature demise of the adult population of primordial follicles. In summary, the survival of adult primordial follicles is dependent in part on progesterone receptor membrane component 1 and 2.

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Introduction

Female fertility depends on primordial follicles developing into preovulatory follicles that are capable of ovulating fertilizable oocytes. While some primordial follicles transition into growing primary follicles, most remain in a non-growing state for long periods of time (Kerr *et al.* 2013, Monniaux *et al.* 2014). Whether through growth or atresia, the number of primordial follicles gradually decreases with age. When their numbers become limiting, follicular development does not proceed to the ovulatory stage, menstrual cycles become irregular and eventually cease and women enter menopause (Kerr *et al.* 2013, Monniaux *et al.* 2014). Typically, women enter menopause around 52 years of age. However, in about 1% of women, primordial follicles are reduced prematurely, and these women enter menopause before 40 years of age due to primary ovarian insufficiency (POI) (Maclaran & Panay 2015, Daan *et al.* 2016).

Understanding the mechanism that results in POI therefore centers around the factors that control the

growth and survival of the primordial follicles. One aspect of this mechanism relates to when primordial follicles are formed. The work from the Capel lab (Mork *et al.* 2012) reveals that pregranulosa cells derived from the surface epithelium of the developing gonads of a female fetus on embryonic day 11.5 interact with germ cells to form primordial follicles that ultimately move toward the center of the developing ovary. Primordial follicles that form during early embryonic development are often referred to as first wave follicles, because they grow and account for the follicles that develop throughout the neonatal and prepubertal periods. These follicles undergo atresia and form the interstitial component of the ovary. Importantly in mice, these first wave primordial follicles are virtually depleted by 3 months of age (Zheng *et al.* 2014a,b). However, there exists another population of primordial follicles that are formed around the time of birth that are also derived from surface epithelial cells (Mork *et al.* 2012). This second wave of primordial follicles remains within the ovarian cortex, and this population is often referred to as the adult population of primordial follicles. The concept

of two types of primordial follicles has been confirmed and expanded by a series of elegant studies published by Zheng *et al.* (2014a,b). Thus, the adult population of primordial follicles gives rise to all the follicles that develop throughout the reproductive lifespan.

How the fate of the primordial follicles within the adult ovary is controlled remains somewhat a mystery but likely involves changes in the hormonal/growth factor environment within the ovary. It is likely that the ovarian environment is influenced in part by genetic alterations in that mutations and/or changes in the expression of numerous genes have been proposed to promote POI (Pelosi *et al.* 2015a). Some of these genes are encoded by sequences within the X-chromosome (Pelosi *et al.* 2015a,b). One of these genes is progesterone receptor membrane component 1 (*Pgrmc1*) (Mansouri *et al.* 2008, Qin *et al.* 2015). Interestingly, progesterone (P4) signaling through PGRMC1 inhibits the formation of primordial follicles in cultured neonatal mouse ovaries without reducing the total number of oocytes (Guo *et al.* 2016). In addition, deletion mutations of *Pgrmc1* have been detected in women with POI. A point mutation in *Pgrmc1* has also been observed in women with POI, which converts the histidine residue at amino acid 165 to arginine, and this renders PGRMC1 less effective in mediating P4's anti-apoptotic action (Mansouri *et al.* 2008). A second point mutation in *Pgrmc1* was observed in Chinese women, but this point mutation did not alter the structure of PGRMC1 (Wang *et al.* 2014). In addition, decreased expression of PGRMC2 has been observed in the granulosa cells of young women (<35 years of age) with diminished ovarian reserve (DOR) compared to age-matched controls (Skiadas *et al.* 2012).

The decreased expression of PGRMC1 and PGRMC2 in some women with POI and DOR, respectively, suggests that these two proteins play a role in regulating the reproductive lifespan of women. To address this issue, we developed several unique transgenic mouse models in which progesterone receptor (*Pgr*)-cre driver mice were used to conditionally deplete *Pgrmc1* and/or *Pgrmc2* (Clark *et al.* 2017). *Pgrmc1*^{d/d}, *Pgrmc2*^{d/d} and *Pgrmc1/2*^{d/d} mice experience a reduction in fertility and premature reproductive senescence. Each group of mutant mice had a 50% decrease in litter size by the third or fourth parity (i.e., 4–5 months of age) with litter sizes declining more rapidly than in control mice with increasing age (Clark *et al.* 2017). The progressive decline in fertility was hypothesized to be due to changes in the uterus due to the presence of endometrial glandular cysts, but changes in ovarian function were not explored.

To determine whether the ovary played a role in the premature loss in fertility of the *Pgrmc1/2*^{d/d} mice, the present study was designed to assess age-related changes of follicle distribution. This study demonstrated that an ≈80% decrease in the number of primordial follicles occurred between 3 and 6 months of age, indicating

that PGRMC1/2 plays an essential role in maintaining the 'second wave' or adult population of primordial follicles. Importantly, the loss of the primordial follicle population starts shortly after puberty, which coincides with the initiation of estrous cycles and the formation of CL. Because *Pgr*-cre expression in the ovary is restricted to the CL (Soyal *et al.* 2005), these findings suggested that the adult population of primordial follicles is maintained by PGRMC1/2-directed signals originating from the CL. To assess this possibility, a second series of studies was conducted using pseudopregnant mouse model as the CL of pseudopregnant mice are functional and fully developed by day 4 (DOPP4) and then enter into the initial stages of regression around day 9 of pseudopregnancy (DOPP9) (Critser *et al.* 1982, Clark *et al.* 2017). Using the pseudopregnant mouse model, luteal morphology and the expression of various ovarian growth factors that could account for reduction in the primordial follicle populations were monitored in *Pgrmc1/2*^{d/d} mice and compared to that of *Pgrmc1/2*^{fl/fl} control mice.

Materials and methods

Animals and treatments

The generation of the *Pgrmc1/2*^{fl/fl} and *Pgrmc1/2*^{d/d} mice has been previously described (McCallum *et al.* 2016, Clark *et al.* 2017). All procedures involving animals were approved by the Institutional Animal Care and Use Committee at Washington State University. Briefly, *Pgrmc1/2* floxed (*Pgrmc1/2*^{fl/fl}) founder mice were mated with *Pgr*^{cre/+} mice (Soyal *et al.* 2005) to generate *Pgrmc1/2*^{d/d} mice. In the initial study, three sexually mature control (*Pgrmc1/2*^{fl/fl}) and four *Pgrmc1/2*^{d/d} mice autopsied at approximately 3 months of age and three control and four *Pgrmc1/2*^{d/d} mice were autopsied at approximately 6 months of age. The ovaries were removed, trimmed of fat, fixed in 4% paraformaldehyde and stored in 70% ethanol until processed for histological examination.

Since *Pgr*^{cre/+} mice were used to generate *Pgrmc1/2*^{d/d} mice, subsequent studies focused on the CL. This is because CLs are the only ovarian component that express cre recombinase in the *Pgr*^{cre/+} mice (Soyal *et al.* 2005). For the second series of studies, pseudopregnancy mice were used as they have functional CL, which are fully mature by the 4th day of pseudopregnancy and are in the initial stages of regression by the 9th day of pseudopregnancy (Critser *et al.* 1982, Clark *et al.* 2017). Pseudopregnancy was induced by mating 6-to-8-week-old female *Pgrmc1/2*^{fl/fl} (control) and *Pgrmc1/2*^{d/d} mice with a vasectomized male (van der Heijden *et al.* 2005). Four control and five *Pgrmc1/2*^{d/d} mice and three control and four *Pgrmc1/2*^{d/d} mice were autopsied on day of pseudopregnancy (DOPP) 4 and 9 respectively. Blood samples were obtained and assayed for serum P4 at the University of Virginia School of Medicine Ligand Assay and Analysis Core (Clark *et al.* 2017). Ovaries were collected and either fixed for histological and histochemical analysis or used for RNA isolation. In a separate experiment, CLs were dissected from the ovaries of three control and three *Pgrmc1/2*^{d/d} mice on DOPP4 and RNA

isolated from the CL. In the final experiment, CLs were isolated from the ovaries of four control and four *Pgrmc1/2^{dl/d}* mice on DOPP9. For this study, RNA was isolated from both the luteal and non-luteal components of the pseudopregnant ovaries.

While the CL of pseudopregnant mice is functional, histological analysis of the CL of pseudopregnant mice is complicated because CLs from the current cycle as well as previous cycles are present. The CLs derived in previous cycles are approximately 40–50% smaller than those formed during the current cycle, possess numerous necrotic areas and found in the medullary region (Sato *et al.* 2014). CL possessing these characteristics were not assessed and only fully developed CL as indicated by having diameters greater than 300 µm and without necrotic areas were analyzed. Note that some of these CL-possessed areas with vacuolated luteal cells, but these were not excluded as vacuoles often reflect an increase in steroid synthesis (Westwood 2008).

RNA isolation and real-time PCR measurements

Total RNA was isolated from the ovarian tissue using Tri Reagent (Sigma-Aldrich) according to the manufacturer's specifications and the amount of RNA in each sample was quantified using a NanoDrop Spectrophotometer. For each sample, 1 ng of RNA was converted to cDNA as described before (Sueldo *et al.* 2015). All real-time PCR reactions were run in the CFX96 real-time PCR system. Gene expression was evaluated using the $2^{-\Delta\Delta CT}$ method provided by Bio-Rad CFX96 software. The mRNA level for each gene of interest was expressed relative to actin. The primer sequences and probes used for the real-time PCR assays are shown in Table 1. A negative control (no reverse transcription) was included in each PCR reaction to confirm the absence of genomic DNA.

Histological and immunohistochemical analysis

The ovaries were collected from the nulliparous young (≈ 3 months of age) and middle-aged (≈ 6 -month-old) mice and embedded in paraffin, serially sectioned at 5 µm and stained with picric acid stain (Clark *et al.* 2017). Every fifth section of each ovary was examined and the number of primordial, primary, preantral and antral follicle was counted. Follicles were classified as described by Johnson *et al.* (2004).

Ovaries were collected from the ovaries of pseudopregnant mice that were 6–8 weeks of age and sectioned at 5 µm. Some sections were stained with hematoxylin and eosin to assess the histological characteristics of the pseudopregnant ovary. Other sections were used to identify vascular endothelial cells by detecting CD31 by immunohistochemistry. Briefly, ovarian sections were deparaffinized, incubated with sodium citrate buffer at 95°C for 20 min and incubated in 3% hydrogen peroxide in PBS for 10 min at room temperature to quench endogenous peroxidase activity. The sections were stained for the presence of CD31 using the anti-CD31 antibody at a 1:100 dilution (Cell Signaling Technology, Catalog Number 77699). The slides were then incubated with ImmPRESS peroxidase reagent (Vector Laboratories) for 30 min at room temperature and washed in PBS. The slides were developed using a DAB peroxidase substrate for 5 min. Finally, the slides were counter stained with hematoxylin for 10 s, rinsed in distilled water, dehydrated, cleared and mounted. The CD31 was revealed by the presence of a brown stain. Negative controls for each immunohistochemical reaction were conducted by excluding the primary antibody. The percentage of the area occupied by vascular endothelial cells was assessed in 20 CLs from 5 *Pgrmc1/2^{fl/fl}* and 15 CLs from 5 *Pgrmc1/2^{dl/d}* pseudopregnant mice as follows. The total area of each CL and the area stained for CD31 were determined using IVision imaging software. The percentage of the CL stained for CD31 was calculated and used to estimate the degree of luteal vascularization.

Statistical analysis

The data from all the experiments were expressed as a mean $\pm$ one standard error. Differences between the means of two groups was assessed by a Student's *t* test, while comparisons of means of three or more groups was assessed by a one or two-way ANOVA followed by Fisher LSD *post hoc* test. The statistical analysis was performed using GraphPad Prism 6.0. Regardless of the statistical test used, *P* values of <0.05 were considered to be significantly different.

Results

The ovaries of young sexually mature *Pgrmc1/2^{dl/d}* mice of approximately 3 months of age possessed follicles

Table 1 Primers and probes used to assess mRNA levels by real-time PCR. All primers and probes were designed and synthesized by LGC Biosearch Technologies, Petaluma, CA, USA.

Gene identifier	Sequence (5'–3')		Probe (5'–3')
	Forward	Reverse	
<i>Pgrmc1</i>	CTTTGCCGGAAGAGATGCA	GAGGTCAGAAAGGTCGTCATAC	FAM-CCTTGCCACATTTGCTGGACAA-BHQ-1
<i>Pgrmc2</i>	TGCCTGGATAAGGATGCACTTAG	CCCATTCTCGAACACTCTCCATT	FAM-ATGACGACCTCTCAGATTGAACGCA-BHQ-1
<i>Vegfa</i>	GCTGCACCCACGACAGAA	TACCTACAGATGGTCGCTTCG	FAM-AGAGCAGAAGTCCCATGAAAGTGATCA-BHQ-1
<i>Mmp9</i>	GGGCTACGTGACCTACGA	TGCACGGTTGAAGCAAAGAAG	FAM-CCTCCTGCACTGCCCTTGAACCTA-BHQ-1
<i>Timp1</i>	GTCCAGAACCGCAGTGAAG	GTACGCCAGGGAACCAAGAAG	FAM-AGTTTCTCATCAGGGCCGCTA-BHQ-1
<i>Lnc2</i>	CACCCGCTTTGCCAAGTCT	CACACTCACCACCCATTCAGT	FAM-TGACCTTCAGTCTCGGCAAGTGA-BHQ-1
<i>Kitl</i>	GGTGTGGGCTTAGGAGTGATC	GGAGATGGCAGTTGTGCATTAC	FAM-CAACAAGGGACAAGACC-BHQ-1
<i>Fgf2</i>	CCAACCGGTACCTTGCTATGAAG	TCCGTGACCGGTAAGTATTGTAG	FAM-AAGATGGACGGCTGCTGGCTTC-BHQ-1
<i>Lif</i>	CTGCGTCAGGCTCATCTCA	CCAGGCTGTGGCATCATCT	FAM-ACTGGCTGCCCTTCTCCAAGCT-BHQ-1
<i>Bmp4</i>	CTGGTGATCACCTCACTCAA	CACAACAGGCCTTAGGGATACTAG	FAM-CAACCATGCCATTGTGCAGACCC-BHQ-1
<i>Amh</i>	CGGGCAAGCTGCTCATCAG	GCACTCGGTGGTACCAT	FAM-CCTGTCCGAGGAGCGCATCCA-BHQ-1
<i>β-Actin</i>	TCACAGGATGCAGAAGGAGAT	GCGCTCAGGAGGAGCAAT	CAL FLUOR GOLD-ACTGCTCTGGCTCCTAGCACCAT-BHQ-1

ranging in size from primordial to antral follicles. The distribution of follicles was not statistically different than that observed in control *Pgrmc1/2^{fl/fl}* mice (Fig. 1A). By around 6 months of age, the ovaries of *Pgrmc1/2^{d/d}* mice possessed a similar number of primary, preantral and antral follicles compared to the aged-matched controls (*Pgrmc1/2^{fl/fl}*) (Fig. 1B). Roughly, equal numbers of CL were also observed in both control and *Pgrmc1/2^{d/d}* mice indicating that the *Pgrmc1/2^{d/d}* mice were ovulating and undergoing estrous cycles. However, at ≈ 6 months of age, the number of primordial follicles was reduced by approximately 80% in *Pgrmc1/2^{d/d}* mice compared to aged-match controls (Fig. 1B). This observation indicated that the population of primordial follicles was essentially depleted within a 3-month period.

Since *Pgr*-directed cre recombinase expression in the ovary is restricted to the CL (Soyal *et al.* 2005), it is possible that signals from the CL are involved in maintaining the adult population of primordial follicles. To test this hypothesis, a pseudopregnant mouse model was used as the CL of pseudopregnant mice are functional and fully developed by day 4 (DOPP4) and then enter into the initial stages of regression around day 9 of pseudopregnancy (DOPP9) (Critser *et al.* 1982, Clark *et al.* 2017). The CL of pseudopregnant *Pgrmc1/2^{d/d}* mice had *Pgrmc1* and *Pgrmc2* mRNAs that were reduced by $\geq 80\%$. *Pgrmc1* and *Pgrmc2* mRNAs were only slightly reduced in the RNA isolated from whole ovaries of DOPP4 *Pgrmc1/2^{d/d}* mice (compare Fig. 2A with B). In spite of the reduced expression of *Pgrmc1* and *Pgrmc2*, the average size of the CL ($487 \pm 19 \mu\text{m}$ for control vs $460 \pm 31 \mu\text{m}$ for *Pgrmc1/2^{d/d}* mice; $P > 0.05$) and histological characteristics of the luteal cells of *Pgrmc1/2^{d/d}* pseudopregnant mice were similar to that of controls (compare Fig. 2C, E with D, F). However, there appeared to be fewer red blood cells among the luteal cells of the *Pgrmc1/2^{d/d}* mice compared to controls (compare Fig. 2E with F). There were also 'pools' of red blood cells in the vascular network at the basement membrane of the CL of *Pgrmc1/2^{d/d}* mice. These two observations implied that the vascularization of the CL was adversely affected by *Pgrmc1/2* depletion (compare Fig. 2E with F). To more precisely examine the vascularization of the ovaries, the vascular endothelial cells were detected using an immunohistochemical protocol using a CD31 antibody. The specificity of this approach was demonstrated by failing to detect CD31-stained cells throughout the CL when the CD31 antibody was not included in the staining protocol (compare Supplementary Fig. 1A, C with B, D, see section on supplementary data given at the end of this article). That the CL of *Pgrmc1/2^{d/d}* mice were not completely vascularized was further evidenced by a 30% reduction in the area occupied by CD31-stained vascular endothelial cells within the CL of *Pgrmc1/2^{d/d}* mice ($7.9 \pm 0.5\%$ for controls vs $5.5 \pm 0.6\%$ for *Pgrmc1/2^{d/d}* mice; $P < 0.05$) (compare Fig. 3A

Figure 1 The distribution of ovarian follicles in *Pgrmc1/2^{fl/fl}* control and *Pgrmc1/2^{d/d}* mice at ≈ 3 months of age (A) and 6 months of age (B). In this and all subsequent graphs, values are shown as a mean $\pm$ one standard error with an * indicating a value that is different from the *Pgrmc1/2^{fl/fl}* control group ($P < 0.05$).

with B). Note that in *Pgrmc1/2^{d/d}* mice CD31-stained vascular endothelial cells were mainly observed at the periphery of the CL. These changes in histology and the percentage of CD31-stained vascular endothelial cells were characteristic of the CL of *Pgrmc1/2^{d/d}* mice at DOPP4 and 9. Finally, the vasculature in the non-luteal component of the *Pgrmc1/2^{d/d}* ovary as judged by CD31 staining was not different than that observed in the *Pgrmc1/2^{fl/fl}* ovary (compare Fig. 3A with B). Even with these structural alterations in the CL of pseudopregnant *Pgrmc1/2^{d/d}* mice, serum P4 levels on DOPP4 ($23 \pm 3 \text{ ng/mL}$) were similar to that of controls ($29 \pm 1 \text{ ng/mL}$; $P > 0.05$) with serum P4 levels decreasing on DOPP9 in both *Pgrmc1/2^{d/d}* ($11 \pm 5 \text{ ng/mL}$) and *Pgrmc1/2^{fl/fl}* ($9 \pm 2 \text{ ng/mL}$; $P > 0.05$).

One factor that regulates vascularization of the CL is vascular endothelial growth factor A (VEGFA) (Lee *et al.* 2007). *Vegfa* mRNA levels were increased in the ovaries of DOPP4 and 9 by ≈ 2 -fold in *Pgrmc1/2^{d/d}* mice (Fig. 3C). Interestingly, *Vegfa* mRNA levels in the CL on DOPP4 increased by ≥ 4 -fold, accounting in part for the

Figure 2 The levels of *Pgrmc1* and *Pgrmc2* mRNA in the ovary (A) and CL (B) of *Pgrmc1/2^{fl/fl}* and *Pgrmc1/2^{d/d}* mice on DOPP4. Ovarian morphology of control *Pgrmc1/2^{fl/fl}* (C and E) and *Pgrmc1/2^{d/d}* (D and F) mice. Note the red blood cells (orange; marked by black arrows) among the luteal cells of controls (E) compared to *Pgrmc1/2^{d/d}* knockout mice (F). Some red blood cells in vessels outside the CL are marked by white arrows in (E) and (F).

increase in ovarian *Vegfa* mRNA on DOPP4 (Fig. 3D). However, on DOPP9, the CL levels of *Vegfa* mRNA in *Pgrmc1/2^{d/d}* mice were similar to control levels, while *Vegfa* expression in the non-luteal component of *Pgrmc1/2^{d/d}* mice increased by $\approx$ three-fold (Fig. 3D).

The reduced vasculature observed in the CL of *Pgrmc1/2^{d/d}* mice occurred in the presence of elevated *Vegfa* mRNA levels. This suggests that there were other essential factors that act in conjunction with VEGFA to promote CL vascularization with their expression being dependent on the presence of PGRMC1 and/or PGRMC2. Potential factors include matrix metalloproteinase-9 (MMP9), tissue inhibitor of metalloproteinases 1 (TIMP1) and lipocalin 2 (LCN2). Real-time PCR analysis revealed that the ovarian mRNA levels of *Mmp9* (Fig. 4A) and *Timp1* (Fig. 4B) of pseudopregnant *Pgrmc1/2^{d/d}* mice

Figure 3 Changes in the ovarian vasculature of control (A) and *Pgrmc1/2^{d/d}* (B) mice as revealed by CD31 staining (brown stain), which is a marker for vascular endothelial cells. Insets are higher magnifications of CL. An image of negative control and positively stained section are shown in Supplementary Fig. 1. Alterations in the levels of *Vegfa* mRNA in the ovary of DOPP4 and DOPP9 mice (C) and the luteal and non-luteal components of *Pgrmc1/2^{fl/fl}* and *Pgrmc1/2^{d/d}* mice (D).

did not differ from those of pseudopregnant *Pgrmc1/2^{fl/fl}* mice. However, *Lcn2* mRNA levels were reduced in the ovaries of pseudopregnant *Pgrmc1/2^{d/d}* mice (Fig. 4C). Although ovarian levels were not dramatically reduced on DOPP4, *Lcn2* mRNA levels were reduced to $26 \pm 7\%$ in the CL of *Pgrmc1/2^{d/d}* mice compared to the CL of *Pgrmc1/2^{fl/fl}* mice (Fig. 4D).

Numerous ovarian growth factors that influence primordial follicle growth and/or survival were detected in the pseudopregnant ovaries of *Pgrmc1/2^{fl/fl}* mice, including Kit Ligand (*Kitl*) (Zhang et al. 2014, Saatcioglu et al. 2016), fibroblast growth factor 2 (*Fgf2*) (Chaves et al. 2012), leukemia inhibitory factor (*Lif*) (Nilsson et al. 2002, da Nobrega et al. 2012) and bone morphogenic protein 4 (*Bmp4*) (Skinner 2005) (Fig. 5A). *Kitl* mRNA was most abundant and its expression was the only one of these growth factors that was altered in the ovaries of pseudopregnant *Pgrmc1/2^{d/d}* mice. Specifically, in *Pgrmc1/2^{fl/fl}* mice, ovarian mRNA levels of *Kitl* increased on DOPP9, but this increase was not observed in DOPP9 *Pgrmc1/2^{d/d}* mice (Fig. 5B). The lower levels of ovarian *Kitl* mRNA were due to lower levels of *Kitl* mRNA in both the luteal and non-luteal components of DOPP9 ovary (Fig. 5C). An increase in anti-Müllerian hormone (*Amh*) mRNA levels was observed in the ovaries of DOPP9 *Pgrmc1/2^{fl/fl}* mice (Fig. 5D).

Discussion

The present studies reveal that conditional depletion of *Pgrmc1* and *Pgrmc2* using the *Pgr*-cre driver results

Figure 4 Effect of depleting *Pgrmc1* and *Pgrmc2* on the relative ovarian mRNA levels of *Mmp9* (A) *Timp1* (B) and *Lcn2* (C) on DOPP4 and DOPP9. Effect of depleting *Pgrmc1* and *Pgrmc2* on the relative *Lcn2* mRNA levels in CL on DOPP4 (D). In panel B, the ** indicates that values for DOPP9 are greater than DOPP4 in both *Pgrmc1/2^{fl/fl}* control and *Pgrmc1/2^{d/d}* mice ($P < 0.05$).

in the premature demise of primordial follicles, which coincides with the previously observed decrease in fertility (Clark *et al.* 2017). Because *Pgr-cre* driver mice were used to generate *Pgrmc1/2^{d/d}* mice, *Pgrmc1* and *Pgrmc2* were depleted in all cells that express PGR.

Figure 5 Relative expression of growth factors that regulate primordial follicle growth and/or survival as expressed as growth factor mRNA level normalized to β -actin mRNA (A). Effect of the depletion of *Pgrmc1* and *Pgrmc2* on *Kitl* mRNA levels within the whole ovary (B) as well as the luteal and non-luteal components of the DOPP9 ovary (C). Effect of the depletion of *Pgrmc1* and *Pgrmc2* on *Amh* mRNA levels within the whole ovary on DOPP9 (D).

Importantly, neuroanatomical mapping studies reveal that high levels of *Pgrmc1*, *Pgrmc2* and *Pgr* are expressed in the same regions of the hypothalamus that control GnRH secretion (Intlekofer & Petersen 2011). Although GnRH neurons express PGR, P4s does not act through PGR to suppress *in vitro* secretion of GnRH (Sleiter *et al.* 2009). Rather P4 acts through membrane progesterin receptor α and/or β (Sleiter *et al.* 2009) and/or PGRMC1 (Bashour & Wray 2012, Mittelman-Smith *et al.* 2017). It is possible then that in the *Pgrmc1/2^{d/d}* mice, *Pgrmc1* and *Pgrmc2* would be reduced in GnRH-secreting neurons and this would attenuate P4's ability to suppress GnRH secretion *in vivo*. This would likely result in an increase serum gonadotropin levels. However, for this to occur, the individual neurons that regulate GnRH would have to express *Pgr* and *Pgrmc1*, but this has not been demonstrated *in vivo*.

Regardless of whether serum gonadotropin levels are elevated, the present study demonstrates that in *Pgrmc1/2^{d/d}* mice, preovulatory follicles develop, ovulate and differentiate into functional CL capable of secreting P4 at the same level as the control mice. In addition, the age at which the mice obtain sexual maturity and the interval between pregnancies in *Pgrmc1/2^{d/d}* mice is the same as controls (Clark *et al.* 2017). Therefore, the hypothalamic–pituitary–ovarian axis in *Pgrmc1/2^{d/d}* mice matures and results in normal ovarian function in the expected time frame. However, the mechanism that maintains the population of adult primordial follicle in *Pgrmc1/2^{d/d}* mice after puberty is altered.

The mechanism that governs the fate of primordial follicles in adult animals is not well defined, but it is unlikely to involve gonadotropins, since primordial follicles are maintained and transition into primary follicles in the absence of the pituitary (Hirshfield 1991). We propose that the CL plays an essential but not necessarily an exclusive role in preserving the adult population of primordial follicles throughout adulthood. We further propose that the capacity of the CL to maintain the primordial follicle population is regulated by PGRMC1 and/or PGRMC2 and that these PGRMC family members do so by influencing two aspects of luteal function.

The first function involves the vascular development within the CL. This is evidenced by the reduction in the number of vascular endothelial cells within the CL of *Pgrmc1/2^{d/d}* mice. VEGFA is a well-known factor that regulates vascularization in the CL (Lee *et al.* 2007) and its expression in the CL is inhibited by *Pgrmc1* and/or *Pgrmc2* as demonstrated by *Vegfa* mRNA levels in the CL on DOPP4 in *Pgrmc1/2^{d/d}* mice increasing by ≥ 4 -fold. The failure of the CL to vascularize fully in the presence of elevated levels of *Vegfa* mRNA suggests that there are other PGRMC1/2-dependent luteal-derived factors that act in conjunction with VEGFA to promote CL vascularization. One such factor is LCN2 as its

expression in the CL is dependent on the presence of PGRMC1 and/or PGRMC2. That LCN2 expression in luteal cells is dependent on PGRMC1 is consistent with previous findings in breast cancer cells (Mir *et al.* 2012). LCN2 promotes vascular development by binding and activating MMP9 (Leng *et al.* 2009, Mir *et al.* 2012). MMP9 activity plays several roles in vascular development, one of which is to cleave the extracellular matrix thereby releasing VEGFA allowing it to be more biologically active (Hawinkels *et al.* 2008). Thus, the decrease in *Lcn2* mRNA observed in the CL of *Pgrmc1/2^{d/d}* mice could result in reduced MMP9 activity and a subsequent reduction in VEGFA's biological activity. This would in part account for the limited vascularization of the CL of *Pgrmc1/2^{d/d}* mice even in the presence of elevated *Vegfa* mRNA. This concept merits further consideration.

The reduced vasculature of CL of *Pgrmc1/2^{d/d}* mice would not be expected to affect P4 secretion as P4 is lipophilic and diffuses from the luteal cell into the extracellular space and eventually entering the circulation (Niswender & Nett 1988). Nor would P4 synthesis be expected to be reduced in *Pgrmc1/2^{d/d}* mice, since ablation of *Pgrmc1* (McCallum *et al.* 2016) or *Pgrmc2* (Clark *et al.* 2017) does not suppress P4 secretion. However, KITL is a luteal cell growth factor that is not lipophilic and regulates primordial follicle growth (Skinner 2005, Zheng *et al.* 2014a,b, Saatcioglu *et al.* 2016). The secretion of KITL could be attenuated due to poor vascularization of the CL.

The second function that luteal PGRMC1/2 affects is gene expression, as evidenced by the reduced levels of *Kitl* mRNA in DOPP9 CL of *Pgrmc1/2^{d/d}* mice. It is likely then that ablating *Pgrmc1/2* in luteal cells suppresses both the expression and secretion of KITL. If so, this could alter the intraovarian environment in a manner that is not supportive of the adult population of primordial follicles and account at least in part to the premature demise of the adult population of primordial follicles.

Interestingly, ablation of luteal *Pgrmc1/2* also alters the expression profile in the non-luteal component of the ovary on DOPP9, which could also contribute to the premature demise of the primordial follicles. For example, *Vegfa* mRNA levels are elevated, while *Kitl* mRNA levels are suppressed in the non-luteal component of the ovary. Both these growth factors are known to stimulate primordial to primary follicle transition (Roberts *et al.* 2007, Yang & Fortune 2007, McFee *et al.* 2009, Saatcioglu *et al.* 2016). In addition, the ovarian *Amh* mRNA levels are also elevated in the DOPP9 ovary of *Pgrmc1/2^{d/d}* mice. Elevated levels of AMH are important in that AMH is expressed and secreted by developing follicle and acts to maintain primordial follicle viability but inhibits their transition into primary follicles (Durlinger *et al.* 1999, Kano *et al.* 2017). Thus, ablation of *Pgrmc1/2* alters the non-luteal

component of the ovary such that one intraovarian factor that promotes primordial follicle growth (*Vegfa*) is overexpressed; the expression of another factor that promotes primordial follicle growth (*Kitl*) is suppressed and a factor that suppresses primordial follicle growth (*Amh*) is elevated. It is possible then that this imbalance in growth-promoting and -suppressing factors within the non-luteal component of the ovary induces an incomplete or altered signal cascade that results in primordial follicle death as opposed to its transition into a primary follicle. Importantly, this imbalance in the non-luteal ovarian growth factor environment would be transient, only occurring during luteal regression. This would likely result in a gradual decrease in the number of primordial follicles starting with the initiation of the estrous cycles. While this hypothesis is consistent with our data, caution must be exercised in accepting it as it is based on mRNA measurements. Thus, this hypothesis must be confirmed by considerably more experimentation. Such future experiments include demonstrating that the proteins encoded by these mRNAs are changing in parallel with mRNA levels as well as generating knockout mice in which each growth factor is conditionally ablated.

Finally, the present data are consistent with the possibility that in normal cycling mice, PGRMC1 and/or PGRMC2 direct changes in the gene expression profile during luteal regression that allow for an increase in ovarian *Kitl* mRNA and a suppression of *Amh* mRNA. As such, this would be conducive to increasing the number of primordial follicles that transition into growth primary follicles. The expression of *Amh* is important because it inhibits the growth of primordial follicles (Durlinger *et al.* 1999, Kano *et al.* 2017) by suppressing the growth-promoting actions of KITL (Nilsson *et al.* 2007). Since serum AMH levels are maintained at a relatively constant level in mature mice ≤ 8 months of age (Kevenaar *et al.* 2006), there must be a mechanism to override AMH's growth-inhibiting action. We propose that the transient 2-fold increase in *Kitl* mRNA, which is observed throughout the ovary of control mice during the initial stages of luteal regression (i.e. DOPP9) could override AMH's actions and stimulate a limited number of the primordial follicles to transition into growing primary follicles. Since the increase in ovarian *Kitl* mRNA occurs while the CL is regressing, the time that *Kitl* mRNA levels are elevated would be limited to the life of the CL. This CL-dependent increase in *Kitl* expression would account for the relatively constant number of primary follicles that start to grow in mature adult mice (Pedersen 1972). This proposed mechanism provides for the efficient and controlled utilization of the adult population of primordial follicles and a prolonged female reproductive lifespan and therefore merits further investigation.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-18-0155>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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