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Downregulation of nuclear progesterin receptor (Pgr) and subfertility in double knockouts of progesterin receptor membrane component 1 (*pgrmc1*) and *pgrmc2* in zebrafish

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Abstract

The progesterin receptor membrane components (Pgrmcs) contain two paralogs, Pgrmc1 and Pgrmc2. Our previous research into single knockout of Pgrmc1 or Pgrmc2 suggests that Pgrmc1 and Pgrmc2 regulate membrane progesterin receptor or steroid synthesis and therefore female fertility in zebrafish. Additional roles of Pgrmcs may not be determined in using single Pgrmc knockouts due to compensatory roles between Pgrmc1 and Pgrmc2. To address this question, we crossed single knockout *pgrmc1* (*pgrmc1*^{-/-}) with *pgrmc2* (*pgrmc2*^{-/-}), and generated double knockouts for both *pgrmc1* and *pgrmc2* (*pgrmc1/2*^{-/-}) in a vertebrate model, zebrafish. In addition to the delayed oocyte maturation and reduced female fertility, significant reduced ovulation was found in double knockout (*pgrmc1/2*^{-/-}) in vivo, though not detected in either single knockout of Pgrmc (*pgrmc1*^{-/-} or *pgrmc2*^{-/-}). We also found significant down regulation of nuclear progesterin receptor (Pgr) protein expression only in *pgrmc1/2*^{-/-}, which was most likely the cause of reduced ovulation. Lower protein expression of Pgr also resulted in reduced expression of metalloproteinase in *pgrmc1/2*^{-/-}. With this study, we have provided new evidence for the physiological functions of Pgrmcs in the regulation of female fertility by regulation of ovulation, likely via regulation of Pgr, which affects regulation of metalloproteinase expression and oocyte ovulation.

Keywords

Progesterins; Pgrmc1; Pgrmc2; Pgr; Ovulation; Metalloproteinase

Introduction

Ovulation is a physiological process where a mature, fertilizable oocyte is released from the surrounding follicular cells. The degradation in extracellular matrix of the oocytes is paramount for follicular rupture to occur. In vertebrates, ovulation is triggered by a

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Author contribution

Y.Z. conceived the project and generated knockouts. X.W. and Z.Y. designed the experiments, performed the experiments and analyzed the data, and wrote the manuscript.

Declaration of interest

The authors have declared that no competing interests exist.

luteinizing hormone (Lh) surge from the pituitary, which signals via the receptor (Lhcgr) in the granulosa cell. This initial signal then amplified via progesterin synthesis in preovulatory follicles, which in turn activates nuclear progesterin receptor (Pgr). The role of Pgr in ovulation is highly conserved across vertebrate species, as is evidenced by the inability of Pgr knockout female zebrafish, rats, and mice to ovulate (Kubota et al 2016, Lydon et al 1995, Zhu et al 2015). Further studies indicated that Pgr is an upstream regulator important for the increased expression of proteolytic enzymes and therefore promotes the rupture of follicles for releasing mature oocytes during ovulation (Liu et al., 2017, 2018).

As the downstream targets of Pgr, several proteolytic enzymes that may be responsible for follicle rupture during ovulation have been identified (Ogiwara et al 2005, Takahashi et al 2013). Several members of matrix metalloproteinases (MMPs), a disintegrin and metalloproteinases (ADAMs), and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) family have been proposed to be important for ovulation. For example, MMPs and ADAMTS family were found to be induced in the ovarian follicles (Peluffo et al 2011, Piprek et al 2018, Rosewell et al 2015). In addition, inhibitors of MMPs using antibodies drastically suppressed in vitro ovulation in medaka (Ogiwara et al 2005). Pharmacologic inhibition of metalloproteases also indicates the importance of these proteinases in ovulation (Butler et al 1991, REICH et al 1985). However, since mice that lack metalloproteinases die either in utero or shortly after birth (Carmeliet et al 1993, Enomoto et al 2010, Holmbeck et al 1999, Kelly et al 2005, Peschon et al 1998, Vu & Werb 2000), the roles played by these proteases in ovulation remains unclear.

As in mammals, only two progesterin receptor membrane component (Pgrmc) paralogs (Pgrmc1 and Pgrmc2) have been identified in zebrafish. They have been suggested as progesterin receptors, or adaptor proteins, which facilitate other receptors to mediate progesterin signaling (Peluso et al 2008, Thomas 2008, Zhu et al 2008). Pgrmc1 take part in various physiological and biochemical processes that are important for normal reproduction. For instance, Pgrmc1 is regulated by progesterone and can also affect progesterone metabolism (Rohe et al 2009). In addition, Pgrmc1 may affect oocyte maturation via regulating expression and trafficking of mPR α to localize at cell surface (Thomas et al 2014, Wu et al 2018). Furthermore, Pgrmc1 has also been suggested to mediate antiapoptotic and antimitotic actions of progesterone in rat granulosa cells (Peluso et al 2006). Recent investigations in Pgrmc1 and Pgrmc2 knockout mice have suggested that Pgrmc1 are required for normal fertility in females. Conditional ablation of Pgrmc1 results in reduced fertility in female mice, while knocking out Pgrmc2 causes premature reproductive senescence in female mice, possibly due to post-implantation failure (Clark et al 2016, McCallum et al 2016). Similar reduced fertility was also observed in *pgrmc1*^{-/-} and *pgrmc2*^{-/-} zebrafish (Wu et al 2019, Wu et al 2018). However, whether Pgrmc1 and Pgrmc2 compensate each other is still unknown. In the present study, we generated double knockouts for Pgrmc genes in zebrafish (*Danio rerio*) using CRISPR/Cas9 gene editing technology, characterized phenotypes of these knockouts, and examined the molecular mechanisms underlying the actions of Pgrmc. We found reduced spawning frequencies and number of embryos in double knockouts *pgrmc1/2*^{-/-}. In addition, we observed reduced ovulation in vivo. Significant reduced Pgr protein expression in *pgrmc1/2*^{-/-} likely caused, at least partially, for attenuated ovulation in *pgrmc1/2*^{-/-}.

Materials and Methods

Zebrafish

The wildtype zebrafish (*Danio rerio*) strain used in this investigation, the Tübingen strain, was initially obtained from the Zebrafish International Resource Center, then propagated in our lab at East Carolina University. We used previously described methods to generate *pgrmc1*^{-/-} and *pgrmc2*^{-/-} single mutant lines (Wu et al 2019, Wu et al 2018). Double knockouts *pgrmc1/2*^{-/-} were generated by crossing of *pgrmc1*^{-/-} with *pgrmc2*^{-/-}. Fish were kept under a photoperiod of 14h light and 10h dark (lights on at 09:00, lights off at 23:00), with water temperature around 28.5°C, pH ~7.2, and salinity conductivity ranging from 700 to 1,200 µS in automatically controlled zebrafish rearing systems (Aquatic Habitats Z-Hab Duo systems, Florida, USA). Fish were fed twice a day and supplied with newly hatched brine shrimp. All the animal care and experimental protocols were approved by Institutional Animal Care and Use Committee (IACUC) at East Carolina University.

Spawning and fertility

After zebrafish reached their maturity at ~ 4-months of age, 10 homozygous *pgrmc1/2*^{-/-} female fish were crossed with fertility confirmed wildtype males. Number of offspring was recorded daily for a period of two weeks following a two-week acclimation period. Spawning frequency was defined as the number of times a female produce fertilized embryos in a two-week examination period.

Follicle isolation and quantification of different stage follicles

Oocyte maturation in zebrafish typically occurs prior to the onset of (day) light, while ovulation and spawning occurs within 1 hr following the onset of light. Therefore, adult females (n = 7) from different genotypes (*wt* and *pgrmc1/2*^{-/-}) were euthanized at 09:30am, thirty minutes after laboratory lights were turned on, by placing each fish in a lethal dose of MS-222 (300 mg/L buffered solution) for 10 minutes, then severing the spinal cord and blood supply using IACUC approved procedures. The ovaries of each fish were then immediately dissected out and rinsed in 60% L-15 media (Sigma-Aldrich, St. Louis, MO, USA) containing 15 mM HEPES (pH=7.2). Follicles of various sizes were isolated from the ovaries using fine forceps. Thereafter, ovaries were transferred to a 15-ml centrifuge tube and pipetted up and down to separate the follicles. Then the separated ovaries were transferred to a 90-mm petri dish containing 60% L-15 media. The diameter of each follicle was measured using a stereo microscope (SZX7, Olympus, Japan). The development of follicles was divided into five stages based on morphological criteria and on physiological and biochemical events (Selman et al 1993, Tyler & Sumpter 1996): Stage I (<140 µm) and II (140–340 µm) previtellogenic follicles; Stage III early vitellogenic follicles (340–690 µm); Stage IV late vitellogenic follicles (690–730 µm), which is comprised of two stages, immature Stage IVa oocytes (before germinal vesicle breakdown, i.e., GVBD) and mature Stage IVb (underwent GVBD but haven't yet gone through ovulation); and Stage V ovulated follicles (730–750 µm), characterized as ovulated eggs with no follicular cells attached.

RNA isolation and Real-time quantitative PCR

Based on the size standard mentioned above, total RNA was isolated from Stage III (21:00), Stage IVa (6:00), and Stage IVb (08:00) follicles using the RNazol reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's protocol. The quantity and quality of the RNA was determined using a Nanodrop 2000 (Thermo Fisher). RNA samples were then reverse transcribed to cDNA using High Capacity cDNA Reverse Transcription Kit (Thermo Fisher). Real-time quantitative PCR (qPCR) was performed using SYBR green with C1000 Touch Thermal Cycler (Bio-Rad). PCR efficiency was calculated from the equation of efficiency (EFF) = $10(-1/\text{slope}) - 1$ and authentic PCR products were confirmed by analyses of melting curve, gel electrophoresis, and DNA sequencing. PCR data was analyzed using the absolute quantitation method, expressed as copies/ μg RNA, and was determined using Ct values of samples and a standard curve from serial known concentrations of plasmids containing different cDNA fragment of target genes. Comparative Ct method was not used in this study because house-keeping-genes vary between different developmental stages of follicles (Liu et al 2018). The primers used in this study can be found in our previously published work (Liu et al 2018).

Western blotting

Expression of Pgr in the fully-grown Stage IVa immature follicles was confirmed by Western blot analysis using a previously developed polyclonal antibody for Pgr (Hanna & Zhu 2011b). Total protein from 10 Stage IVa follicles (06:00) that was collected directly from freshly sacrificed fish was sonicated in 100 μl of 1 $\times$ SDS sample buffer (62.5 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 100 mM Dithiothreitol) on ice for about 10 short bursts (Sonic Dismembrator, Fisher Scientific). Samples were then immediately boiled for 10 minutes and stored in -20°C freezer until Western blot analysis. 10 μL of each sample was loaded onto 8% SDS PAGE gel and transferred to a nitrocellulose membrane. The membrane was first pre-incubated for 3 hrs with a blocking solution containing 5% BSA (albumin from bovine serum, Sigma A7906) in TBST (50 mM Tris, 100 mM NaCl, 0.1% Tween 20, pH 7.4), then with a primary antibody (Pgr, 1:250 dilution; α -Tubulin (Sigma, T6074), 1:3000 dilution) in the 1% BSA blocking solution overnight. The following day, the membrane was washed five times for a period of 5 minutes each with 1 $\times$ TBST, incubated for 2 hr with horseradish peroxidase conjugated secondary antibody (1:5000 dilutions, goat anti-rabbit antibody for Pgr detection or goat anti-mouse antibody for α -Tubulin), and finally washed five times for a period of 5 minutes each with 1 $\times$ TBST. The membranes were developed using Super Signal West Extended Dura Substrate (Pierce, Rockford, IL, USA) in a plastic wrap, then visualized using a Fluor Chem 8900 imaging station (Alpha Innotech, San Leandro, CA, USA). Protein size was determined by comparison to a biotinylated protein ladder (Cell Signaling Technology, Danvers, MA, USA) and a prestained protein ladder (Fermentas, Waltham, MA, USA). Finally, image analyzing software (ImageJ) was used to estimate relative densitometries (Schneider et al 2012).

Statistical analysis

All the results were presented as mean $\pm$ SEM. Significant differences among paired treatment groups was determined using unpaired Student's *t* test and among multiple

treatment group using One-way analysis of variance (ANOVA) followed by Turkey's test (GraphPad Prism 7.0a, San Diego, CA, USA). Statistical significance was set at $p < 0.05$.

Results

Reduced fertility in *pgrmc1/2^{-/-}* female zebrafish

To evaluate the reproductive capacities in *pgrmc1/2^{-/-}* female zebrafish, mature mutant females (n=10) at 4 months of age were mated with known fertile wildtype males during a minimum 4-week mating study period (two weeks of accommodating period followed by two weeks of quantification period). The fecundity of *pgrmc1/2^{-/-}* female zebrafish, determined as total number of embryos produced over two weeks following a two-week acclimation period, was recorded and compared to those in wildtype crossing (wildtype males crossed with wildtype females) that were treated exactly the same during the same time period. We found a significantly lower number of offspring produced by *pgrmc1/2^{-/-}* female zebrafish (*pgrmc1/2^{-/-}*, n=10, 828.5 ± 131.3 , $p < 0.0001$) in comparison to those produced by wildtype females (n=10, 1827 ± 63.2) (Fig. 1A). *pgrmc1/2^{-/-}* females also spawned with significantly less frequency (n=10, $60.71 \pm 8.06\%$) as compared with wildtype females (n=10, $92.14 \pm 1.67\%$) (Fig. 1B). In addition, embryos produced daily and embryos produced each time female spawned also were significantly lower in *pgrmc1/2^{-/-}* female zebrafish (Fig. 1C–1E). These results obtained in the double knockout of *Pgrmc* were similar as those we reported previously in the single knockout of *pgrmc1^{-/-}* or *pgrmc2^{-/-}* (Wu et al., 2018; Wu et al., 2019).

Reduced oocyte maturation and ovulation in *pgrmc1/2^{-/-}* in vivo

To determine the possible cause for reduced fertility in *pgrmc1/2^{-/-}*, ovaries from 4-month-old zebrafish were sampled 30 minutes after lights were turned on (09:30) and the number of different staged follicles were counted. No significant differences were found in the number of early stage, immature follicles (Stage II and Stage III) in the ovaries from *pgrmc1/2^{-/-}* females in comparison to those from wildtype female fish (Fig. 2). Typically, fully grown immature follicles (Stage IVa) would have already successfully completed processes of oocyte maturation and ovulation, and no Stage IVa and Stage IVb would be found in the ovaries from wildtype females after lights were on for half an hour (Fig. 2). However, a significantly large number of these Stage IVa and Stage IVb follicles still could be observed in *pgrmc1/2^{-/-}* females (Fig. 2). These results indicate that oocytes maturation and oocyte ovulation process were affected in *pgrmc1/2^{-/-}*.

Pgr was downregulated in *pgrmc1/2^{-/-}*

To identify the possible genes underlying the reduced ovulation found in *pgrmc1/2^{-/-}*, we examined the expression of *Pgr* in the *Pgrmc* mutants as *Pgr* is a key upstream regulator for ovulation. The RNA expression of *pgr* was significantly lower in *pgrmc1^{-/-}* single mutants in Stage III oocytes collected at 21:00, in Stage IVa oocytes sampled at 06:00 in both *pgrmc1^{-/-}* and *pgrmc1/2^{-/-}*, and in Stage IVb oocytes sampled at 08:00 in all *Pgrmc* mutants (Fig. 3A). The lowest RNA expression of *Pgr* was observed in *pgrmc1/2^{-/-}* of Stage IVa oocytes sampled at 06:00 (Fig. 3A). The protein levels of *Pgr* were significantly reduced

in Stage IVa follicles (06:00) of *pgrmc1/2^{-/-}*, but not in *pgrmc1^{-/-}* or *pgrmc2^{-/-}* single mutant (Fig. 3B).

Reduced expression of metalloproteinase

Based on the lower expression levels of Pgr protein in *pgrmc1/2^{-/-}*, we hypothesized that metalloproteinases required for ovulation would be low in the *pgrmc1/2^{-/-}*, since Pgr regulates metalloproteinases expression during oocyte ovulation. Therefore, we collected different stages of oocytes and determined expression of six representative metalloproteinase at critical time points. Several representative metalloproteinases including *adamts1*, *adamts8a*, *adamts9*, and *mmp2*, *mmp9* were significantly reduced in *pgrmc1/2^{-/-}* Stage IVb follicles (Fig. 4). Interestingly, *adamts9* and *mmp9* were also significantly lower in *pgrmc1^{-/-}* and *pgrmc2^{-/-}* than those in wildtype (Fig. 4).

Discussion

Pgrmc1 and *Pgrmc2* have been suggested as important adaptors in mediating progesterone signaling and steroid synthesis, but their functions in vivo have not been well established. To our knowledge, this is the first evidence that *Pgrmc1* and *Pgrmc2* play a role in the regulation of Pgr and oocyte ovulation. We found reduced oocyte ovulation in *pgrmc1/2^{-/-}* females in vivo but not in *pgrmc1^{-/-}* or *pgrmc2^{-/-}* (Wu et al 2019, Wu et al 2018). One main cause for reduced oocyte ovulation in *pgrmc1/2^{-/-}* fish is likely due to dramatically reduced protein expression of Pgr, which is the essential regulator of oocyte ovulation. Lower protein expression of Pgr subsequently caused abnormally low metalloproteinase expression and resulted in reduced ovulation. Our results indicate an interaction between two different progesterone receptor families, which operate with each other to regulate ovulation.

Low Pgr causes reduced ovulation

It is well established that Pgr is essential for ovulation across species. In agreement with other studies, *pgr* also increases prior to ovulation in zebrafish (Bayaa et al 2000, Hanna & Zhu 2011a, HILD-PETITO et al 1988, Liu et al 2018, Park & Mayo 1991, PRESS & GREENE 1988, Richards & Ascoli 2018). Selective PGR antagonist RU486 or CDB-2914 can reduce the number of ovulated oocytes in mice (Loutradis et al 1991, Palanisamy et al 2006). In addition, the oocytes remain trapped within follicles in *Pgr^{-/-}* mice and zebrafish because pre-ovulatory follicles are unable to undergo follicle-wall degradation (Lydon et al 1995, Zhu et al 2015). Our study demonstrated that low Pgr protein expression in *pgrmc1/2^{-/-}* zebrafish also resulted in ovulation reduction in vivo, supporting the notion that Pgr is a pivotal factor for oocyte ovulation.

Pgrmc1 and *Pgrmc2* affect gene expression of Pgr

A previous study in mice showed ablation of *Pgrmc1/2* did not alter the RNA expression of the Pgr (Clark et al 2016), but immunohistochemistry results showed less staining in the *Pgrmc1/2^{d/d}* uterine. Similarly, we also detected low Pgr transcription and protein level in *pgrmc1/2^{-/-}* zebrafish. The relationship between these two progesterone receptor families is still unclear. Previous research suggests a compensatory mechanism between *Pgrmc1* and Pgr since higher levels of *Pgrmc1* have been found in the brains of Pgr-KO female mice than

in their wild-type littermates (Krebs et al 2000). Also, activation of Pgr can represses expression of *Pgrmc1* during lordosis facilitation (Krebs et al 2000). In our results, we found a new relationship between *Pgrmcs* and Pgr; and *Pgrmcs* are important for normal expression of Pgr. But how *Pgrmc1* and *Pgrmc2* regulates the expression of Pgr remains unknown and needs further investigation. In our previous work, we showed *Pgrmc1* and *Pgrmc2* to be highly expressed in the follicular cells and denuded oocytes (Wu et al 2019, Wu et al 2018). In interphase cells, *Pgrmc1* was distributed throughout the cell including the nucleus (Peluso et al 2014). But whether *Pgrmcs* can bind to transcription factors and directly affect Pgr gene expression remains unclear. *PGRMC1* interacts with epidermal growth factor receptor (EGFR) (Ahmed et al 2010, Aizen & Thomas 2015, Kabe et al 2016) and membrane progesterone receptor α (mPR α) (Aizen et al 2018, Thomas et al 2014). The downstream signaling of EGFR and mPR α may result in gene expression differences of Pgr. *Pgrmc1* and *Pgrmc2* also co-localized to cytoplasm (Peluso et al 2014), indicating a close relationship between *Pgrmc1* and *Pgrmc2*. *Pgrmc1* and *Pgrm2* may have similar, but not identical, functions because double knockout (*pgrmc1/2^{-/-}*) female zebrafish had a more severe reproductive phenotype than those found in *pgrmc1^{-/-}* or *pgrmc2^{-/-}* single knockout female zebrafish, respectively. This unknown function from *Pgrmcs* may explain why oocyte ovulation delay and Pgr protein decrease only happens in *pgrmc1/2^{-/-}* but not single mutants.

Lower proteinase expression causes ovulation delay

In late stage follicles, such as Stage IVa and Stage IVb, the follicular cells surrounding the oocyte synthesize a variety of factors to induce ovulation, including several kinds of metalloproteinases. This process is under the control of Pgr (Liu et al 2017). The involvement of several metalloproteinases, including members of MMP (matrix metalloproteinase), ADAM (A Disintegrin And Metalloproteinases), and ADAMTS families, has been examined (Brown et al 2010, Peluffo et al 2011, Robker et al 2000, Sriraman et al 2008). In our *pgrmc1/2^{-/-}* follicles, we observed downregulation of several metalloproteinases. *Adamts1* has been identified as an important metalloproteinase exert functions in oocyte ovulation. *ADAMTS1* cleaves the surrounding versican-rich matrix of oocytes and allows the release of the oocyte (Brown et al 2010, Robker et al 2000). In *Adamts1^{-/-}* mice, both ovulation and subsequent fertilization were severely impaired as a result of the versican persistence (Brown et al 2010, Mittaz et al 2004, Shozu et al 2005). *Adamts1* transcripts increased in mice granulosa cells of periovulatory follicles, with this high expression being induced by hCG and progesterone. But this induction is impaired in *Pgr^{-/-}* ovaries, indicating that *Adamts1* acts downstream of Pgr (Robker et al 2000). In our *Pgrmc* mutant lines, low *adamts1* expression can only be seen in the Stage IVb oocytes of the *pgrmc1/2^{-/-}*. Besides *Adamts1*, *Adamts9* also plays an important role in oocyte ovulation. Due to embryonic lethality in *Adamts9^{-/-}* (Dubail et al 2014, Enomoto et al 2010), the function of this protein during ovulation is unknown. Some *adamts9^{-/-}* zebrafish can survive and grow to adulthood, but the ovaries are not well developed in mutants (Carter et al 2019). Interestingly, *Adamts9* expression is induced by LH and hCG in mature follicles in zebrafish, monkeys, and humans during early ovulation (Liu et al 2018, Peluffo et al 2011, Rosewell et al 2015). *Adamts9* is specifically expressed in follicular cells but not in the oocytes and its expression increase significantly prior to ovulation (Liu et al 2018). In

addition, the expression of *adamts9* is significantly downregulated in the follicular cells of anovulatory *pgr^{-/-}* zebrafish (Liu et al 2018). The proteoglycans aggrecan and versican are known substrates of Adamts9. Therefore, Adamts9 is important for ovulation. Our results demonstrated that *adamts9* expression reduced in *pgrcm1^{-/-}*, *pgrmc2^{-/-}*, and *pgrmc1/2^{-/-}* when compared to *wt*. It is plausible that reduced ovulation in *pgrmc1/2^{-/-}* might be due to lower level of *adamts9* resulting from the lower levels of Pgr. MMPs may also play critical roles in ovulation through remodeling extracellular matrix (Cooke et al 1999). In rhesus monkeys, granulosa cells exposed to LH in vitro showed elevated RNA levels of MMP9 (Duffy & Stouffer 2003). MMP9 also plays a critical role in LH-induced steroidogenesis in mouse granulosa cells during ovulation (Light & Hammes 2015). In our *Pgrmc* mutant lines, transcription of *mmp9* showed a peak in Stage IVa oocytes and drop in Stage IVb oocytes. This *mmp9* peak is prior to that of Adams and Adamts expression, indicating a different role *Mmp9* play in oocyte ovulation. Compared to *wt*, both *mmp2* and *mmp9* are lower in the *pgrmc1/2^{-/-}*. In fact, the RNA expressions of *adamts9* and *mmp9* were also low in the *pgrmc1^{-/-}* and *pgrmc2^{-/-}*, but we did not observe ovulation delay in single-knockout *Pgrmc* mutants in vivo. Other proteinases such as *adamts1*, *adamts8a*, and *mmp2* may also contribute to abnormal ovulation in *pgrmc1/2^{-/-}*. However, we need to interpret these gene expression results with great caution since the protein expression and enzyme activities of these proteinases are missing due to lack of antibodies and bioassays. Further studies including follicular cell specific knockout of proteinases is required to determine role of proteinases in the ovulation

Oocyte maturation delay in *pgrmc1/2^{-/-}*

In *pgrmc1/2^{-/-}*, we also found some Stage IVa oocytes after lights were on for 30 mins; and GVBD experiments also show reduced oocyte maturation in vitro. Our previous studies show the different roles that *Pgrmc1* and *Pgrmc2* play in oocyte maturation. *Pgrmc1* promotes oocyte maturation through increased plasma localization and expression of mPR α (Thomas et al 2014, Thomas et al 2007, Wu et al 2018). Further, oocyte maturation reduced in *pgrmc1^{-/-}* in vivo and in vitro (Wu et al 2018). In contrast, the maturation rate of fully-grown immature oocytes from *pgrmc2^{-/-}* was similar to those found in wildtype zebrafish in vitro. Lower progesterin synthesis enzymes were observed in *pgrmc2^{-/-}*, which may cause reduced oocyte maturation delay (Wu et al 2019). Therefore, it is likely that the oocyte maturation delay found in *pgrmc1/2^{-/-}* in vivo and in vitro is caused by both *Pgrmc1* and *Pgrmc2*.

This is the first time it has been shown that both *Pgrmc1* and *Pgrmc2* are required for the regulation of Pgr signaling and function in ovulation. Losing both *Pgrmc*s ultimately leads to transcriptional and translational changes of Pgr. Information obtained from the present study will contribute to our understanding of the interaction between different progesterone receptors. Further research is needed to understand why *Pgrmc1* and *Pgrmc2* can affect the gene expression of Pgr.

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References

- Ahmed IS, Rohe HJ, Twist KE, Craven RJ. 2010 Pgrmc1 (progesterone receptor membrane component 1) associates with epidermal growth factor receptor and regulates erlotinib sensitivity. *Journal of Biological Chemistry* 285: 24775–82 [PubMed: 20538600]
- Aizen J, Pang Y, Harris C, Converse A, Zhu Y, et al. 2018 Roles of progesterone receptor membrane component 1 and membrane progestin receptor alpha in regulation of zebrafish oocyte maturation. *General and comparative endocrinology*
- Aizen J, Thomas P. 2015 Role of Pgrmc1 in estrogen maintenance of meiotic arrest in zebrafish oocytes through Gper/Egfr. *Journal of Endocrinology* 225: 59–68 [PubMed: 25720537]
- Bayaa M, Booth RA, Sheng Y, Liu XJ. 2000 The classical progesterone receptor mediates *Xenopus* oocyte maturation through a nongenomic mechanism. *Proceedings of the National Academy of Sciences* 97: 12607–12
- Brown HM, Dunning KR, Robker RL, Boerboom D, Pritchard M, et al. 2010 ADAMTS1 cleavage of versican mediates essential structural remodeling of the ovarian follicle and cumulus-oocyte matrix during ovulation in mice. *Biology of reproduction* 83: 549–57 [PubMed: 20592310]
- Butler TA, Zhu C, Mueller RA, Fuller GC, Lemaire WJ, Frederick Woessner J Jr. 1991 Inhibition of ovulation in the perfused rat ovary by the synthetic collagenase inhibitor SC 44463. *Biology of reproduction* 44: 1183–88 [PubMed: 1714777]
- Carmeliet P, Kieckens L, Schoonjans L, Ream B, Van Nuffelen A, et al. 1993 Plasminogen activator inhibitor-1 gene-deficient mice. I. Generation by homologous recombination and characterization. *The Journal of clinical investigation* 92: 2746–55 [PubMed: 8254028]
- Carter NJ, Roach ZA, Byrnes MM, Zhu Y. 2019 Adamts9 is Necessary for Ovarian Development in Zebrafish. *General and Comparative Endocrinology*
- Clark NC, Pru CA, Yee S-P, Lydon JP, Peluso JJ, Pru JK. 2016 Conditional ablation of progesterone receptor membrane component 2 causes female premature reproductive senescence. *Endocrinology* 158: 640–51
- Cooke RGI, Nothnick WB, Komar C, Burns P, Curry TE Jr. 1999 Collagenase and gelatinase messenger ribonucleic acid expression and activity during follicular development in the rat ovary. *Biology of reproduction* 61: 1309–16 [PubMed: 10529279]
- Dubail J, Aramaki-Hattori N, Bader HL, Nelson CM, Katebi N, et al. 2014 A new Adamts9 conditional mouse allele identifies its non-redundant role in interdigital web regression. *Genesis* 52: 702–12 [PubMed: 24753090]
- Duffy DM, Stouffer RL. 2003 Luteinizing hormone acts directly at granulosa cells to stimulate periovulatory processes. *Endocrine* 22: 249–56 [PubMed: 14709798]
- Enomoto H, Nelson CM, Somerville RP, Mielke K, Dixon LJ, et al. 2010 Cooperation of two ADAMTS metalloproteases in closure of the mouse palate identifies a requirement for versican proteolysis in regulating palatal mesenchyme proliferation. *Development* 137: 4029–38 [PubMed: 21041365]
- Hanna RN, Zhu Y. 2011a Controls of meiotic signaling by membrane or nuclear progestin receptor in zebrafish follicle-enclosed oocytes. *Molecular and cellular endocrinology* 337: 80–88 [PubMed: 21335056]
- Hanna RN, Zhu Y. 2011b Controls of meiotic signaling by membrane or nuclear progestin receptor in zebrafish follicle-enclosed oocytes. *Molecular and Cellular Endocrinology* 337: 80–88 [PubMed: 21335056]
- HILD-PETITO S, STOUFFER RL, BRENNER RM. 1988 Immunocytochemical localization of estradiol and progesterone receptors in the monkey ovary throughout the menstrual cycle. *Endocrinology* 123: 2896–905 [PubMed: 3197647]

- Holmbeck K, Bianco P, Caterina J, Yamada S, Kromer M, et al. 1999 MT1-MMP-deficient mice develop dwarfism, osteopenia, arthritis, and connective tissue disease due to inadequate collagen turnover. *Cell* 99: 81–92 [PubMed: 10520996]
- Kabe Y, Nakane T, Koike I, Yamamoto T, Sugiura Y, et al. 2016 Haem-dependent dimerization of PGRMC1/Sigma-2 receptor facilitates cancer proliferation and chemoresistance. *Nature communications* 7: 11030
- Kelly K, Hutchinson G, Nebenius - Oosthuizen D, Smith AJ, Bartsch JW, et al. 2005 Metalloprotease-disintegrin ADAM8: Expression analysis and targeted deletion in mice. *Developmental dynamics: an official publication of the American Association of Anatomists* 232: 221–31 [PubMed: 15580619]
- Krebs CJ, Jarvis ED, Chan J, Lydon JP, Ogawa S, Pfaff DW. 2000 A membrane-associated progesterone-binding protein, 25-Dx, is regulated by progesterone in brain regions involved in female reproductive behaviors. *Proceedings of the National Academy of Sciences* 97: 12816–21
- Kubota K, Cui W, Dhakal P, Wolfe MW, Rumi MK, et al. 2016 Rethinking progesterone regulation of female reproductive cyclicity. *Proceedings of the National Academy of Sciences* 113: 4212–17
- Light A, Hammes SR. 2015 LH-Induced steroidogenesis in the mouse ovary, but not testis, requires matrix metalloproteinase 2-and 9-mediated cleavage of upregulated EGF receptor ligands. *Biology of reproduction* 93: 65, 1–13 [PubMed: 26203177]
- Liu DT, Brewer MS, Chen S, Hong W, Zhu Y. 2017 Transcriptomic signatures for ovulation in vertebrates. *General and comparative endocrinology* 247: 74–86 [PubMed: 28111234]
- Liu DT, Carter NJ, Wu XJ, Hong W, Chen SX, ZHU Y. 2018 Gonadotropin and progestin jointly upregulate adamts9 proteinase in zebrafish preovulatory follicles. *Frontiers in Endocrinology* 9: 517 [PubMed: 30279677]
- Loutradis D, Bletsas R, Aravantinos L, Kallianidis K, Michalas S, Psychoyos A. 1991 Preovulatory effects of the progesterone antagonist mifepristone (RU486) in mice. *Human Reproduction* 6: 1238–40 [PubMed: 1752924]
- Lydon JP, DeMayo FJ, Funk CR, Mani SK, Hughes AR, et al. 1995 Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. *Genes & development* 9: 2266–78 [PubMed: 7557380]
- McCallum ML, Pru CA, Niikura Y, Yee S-P, Lydon JP, et al. 2016 Conditional ablation of progesterone receptor membrane component 1 results in subfertility in the female and development of endometrial cysts. *Endocrinology* 157: 3309–19 [PubMed: 27309940]
- Mittaz L, Russell D, Wilson T, Brasted M, Tkalcovic J, et al. 2004 Adamts-1 is essential for the development and function of the urogenital system. *Biology of reproduction* 70: 1096–105 [PubMed: 14668204]
- Ogiwara K, Takano N, Shinohara M, Murakami M, Takahashi T. 2005 Gelatinase A and membrane-type matrix metalloproteinases 1 and 2 are responsible for follicle rupture during ovulation in the medaka. *Proceedings of the National Academy of Sciences* 102: 8442–47
- Palanisamy GS, Cheon Y-P, Kim J, Kannan A, Li Q, et al. 2006 A novel pathway involving progesterone receptor, endothelin-2, and endothelin receptor B controls ovulation in mice. *Molecular endocrinology* 20: 2784–95 [PubMed: 16887885]
- Park O-K, Mayo KE. 1991 Transient expression of progesterone receptor messenger RNA in ovarian granulosa cells after the preovulatory luteinizing hormone surge. *Molecular endocrinology* 5: 967–78 [PubMed: 1840636]
- Peluffo MC, Murphy MJ, Talcott Baughman S, Stouffer RL, Hennebold JD. 2011 Systematic analysis of protease gene expression in the rhesus macaque ovulatory follicle: metalloproteinase involvement in follicle rupture. *Endocrinology* 152: 3963–74 [PubMed: 21791558]
- Peluso JJ, Griffin D, Liu X, Horne M. 2014 Progesterone receptor membrane component-1 (PGRMC1) and PGRMC-2 interact to suppress entry into the cell cycle in spontaneously immortalized rat granulosa cells. *Biology of reproduction* 91: 104, 1–12 [PubMed: 25253729]
- Peluso JJ, Liu X, Saunders MM, Claffey KP, Phoenix K. 2008 Regulation of ovarian cancer cell viability and sensitivity to cisplatin by progesterone receptor membrane component-1. *The Journal of Clinical Endocrinology & Metabolism* 93: 1592–99 [PubMed: 18319313]

- Peluso JJ, Pappalardo A, Losel R, Wehling M. 2006 Progesterone membrane receptor component 1 expression in the immature rat ovary and its role in mediating progesterone's antiapoptotic action. *Endocrinology* 147: 3133–40 [PubMed: 16513825]
- Peschon JJ, Slack JL, Reddy P, Stocking KL, Sunnarborg SW, et al. 1998 An essential role for ectodomain shedding in mammalian development. *Science* 282: 1281–84 [PubMed: 9812885]
- Piprek RP, Kolasa M, Podkowa D, Kloc M, Kubiak JZ. 2018 Transcriptional profiling validates involvement of extracellular matrix and proteinases genes in mouse gonad development. *Mechanisms of development* 149: 9–19 [PubMed: 29129619]
- PRESS MF, GREENE GL. 1988 Localization of progesterone receptor with monoclonal antibodies to the human progestin receptor. *Endocrinology* 122: 1165–75 [PubMed: 3342750]
- REICH R, TSAFRIRI A, MECHANIC GL. 1985 The involvement of collagenolysis in ovulation in the rat. *Endocrinology* 116: 522–27 [PubMed: 2981665]
- Richards JS, Ascoli M. 2018 Endocrine, paracrine, and autocrine signaling pathways that regulate ovulation. *Trends in Endocrinology & Metabolism* 29: 313–25 [PubMed: 29602523]
- Robker R, Russell D, Espey LL, Lydon J, O'malley B, Richards J. 2000 Progesterone-regulated genes in the ovulation process: ADAMTS-1 and cathepsin L proteases. *Proceedings of the National Academy of Sciences* 97: 4689–94
- Rohe HJ, Ahmed IS, Twist KE, Craven RJ. 2009 PGRMC1 (progesterone receptor membrane component 1): a targetable protein with multiple functions in steroid signaling, P450 activation and drug binding. *Pharmacology & therapeutics* 121: 14–19 [PubMed: 18992768]
- Rosewell KL, Al-Alem L, Zakerkish F, McCord L, Akin JW, et al. 2015 Induction of proteinases in the human preovulatory follicle of the menstrual cycle by human chorionic gonadotropin. *Fertility and sterility* 103: 826–33 [PubMed: 25516084]
- Schneider CA, Rasband WS, Eliceiri KW. 2012 NIH Image to ImageJ: 25 years of image analysis. *Nature methods* 9: 671 [PubMed: 22930834]
- Selman K, Wallace RA, Sarka A, Qi X. 1993 Stages of oocyte development in the zebrafish, *Brachydanio rerio*. *Journal of morphology* 218: 203–24 [PubMed: 29865471]
- Shozu M, Minami N, Yokoyama H, Inoue M, Kurihara H, et al. 2005 ADAMTS-1 is involved in normal follicular development, ovulatory process and organization of the medullary vascular network in the ovary. *Journal of molecular endocrinology* 35: 343–55 [PubMed: 16216914]
- Sriraman V, Eichenlaub-Ritter U, Bartsch JW, Rittger A, Mulders SM, Richards JS. 2008 Regulated expression of ADAM8 (a disintegrin and metalloprotease domain 8) in the mouse ovary: evidence for a regulatory role of luteinizing hormone, progesterone receptor, and epidermal growth factor-like growth factors. *Biology of reproduction* 78: 1038–48 [PubMed: 18287572]
- Takahashi T, Fujimori C, Hagiwara A, Ogiwara K. 2013 Recent advances in the understanding of teleost medaka ovulation: the roles of proteases and prostaglandins. *Zoological science* 30: 239–48 [PubMed: 23537233]
- Thomas P. 2008 Characteristics of membrane progestin receptor alpha (mPR α) and progesterone membrane receptor component 1 (PGRMC1) and their roles in mediating rapid progestin actions. *Frontiers in neuroendocrinology* 29: 292–312 [PubMed: 18343488]
- Thomas P, Pang Y, Dong J. 2014 Enhancement of cell surface expression and receptor functions of membrane progestin receptor α (mPR α) by progesterone receptor membrane component 1 (PGRMC1): evidence for a role of PGRMC1 as an adaptor protein for steroid receptors. *Endocrinology* 155: 1107–19 [PubMed: 24424068]
- Thomas P, Pang Y, Dong J, Groenen P, Kelder Jd, et al. 2007 Steroid and G protein binding characteristics of the seatrout and human progestin membrane receptor α subtypes and their evolutionary origins. *Endocrinology* 148: 705–18 [PubMed: 17082257]
- Tyler C, Sumpter J. 1996 Oocyte growth and development in teleosts. *Reviews in fish biology and fisheries* 6: 287–318
- Vu TH, Werb Z. 2000 Matrix metalloproteinases: effectors of development and normal physiology. *Genes & development* 14: 2123–33 [PubMed: 10970876]
- Wu X-J, Williams MJ, Patel PR, Kew KA, Zhu Y. 2019 Subfertility and reduced progestin synthesis in Pgrmc2 knockout zebrafish. *General and Comparative Endocrinology*: 113218 [PubMed: 31301284]

- Wu XJ, Thomas P, ZHU Y. 2018 *Pgrmc1* Knockout Impairs Oocyte Maturation in Zebrafish. *Frontiers in Endocrinology* 9: 560 [PubMed: 30319543]
- Zhu Y, Hanna RN, Schaaf MJ, Spaink HP, Thomas P. 2008 Candidates for membrane progesterin receptors—past approaches and future challenges. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* 148: 381–89 [PubMed: 18602498]
- Zhu Y, Liu D, Shaner ZC, Chen S, Hong W, Stellwag EJ. 2015 Nuclear progesterin receptor (*pgr*) knockouts in zebrafish demonstrate role for *pgr* in ovulation but not in rapid non-genomic steroid mediated meiosis resumption. *Frontiers in endocrinology* 6: 37 [PubMed: 25852646]

Highlights:

1. Subfertility in double knockout of *pgrmc1* and *pgrmc2* (*pgrmc1/2^{-/-}*) female zebrafish
2. Reduced ovulation in *pgrmc1/2^{-/-}*.
3. Reduced expression of nuclear progesterin receptor (Pgr) in *pgrmc1/2^{-/-}*.
4. Reduced expression of metalloproteinases in *pgrmc1/2^{-/-}*.

Fig 1. Reduced fertility in *pgrmc1/2*^{-/-} homozygous female zebrafish.

(A) Mutant female zebrafish produced fewer embryos over a two-week mating period than *wt* females. (B) Mutant females spawned with less frequency than wildtype. (C) *pgrmc1/2*^{-/-} females spawned less embryos per day. (D) *pgrmc1/2*^{-/-} females spawned less embryos each time they spawned. (E) Wildtype females produced more embryos daily than *pgrmc1/2*^{-/-} females. **, $p<0.01$; ****, $p<0.0001$.

Fig 2. Quantification of different stages of oocytes in *wt* and *pgrmc1/2*^{-/-} female ovaries. Significant numbers of fully-grown immature Stage IVa follicles and matured but not ovulated Stage IVb oocytes were observed in *pgrmc1/2*^{-/-} females 30 minutes after room lights were switched. At the same time point, no Stage IVa and Stage IVb follicles could be observed in wildtype females. Further, *pgrmc1/2*^{-/-} females had less Stage V oocytes.

Fig 3. Reduced Pgr expression in the most advanced follicles of *pgrmc1/2*^{-/-}.

The night prior to spawning at 21:00, the most advanced stage of oocytes sampled at 21:00 are Stage III oocytes. These oocytes will grow further and develop into Stage IVa oocytes (fully grown but immature) at 06:00. Then, these advanced oocytes will undergo oocyte maturation to become Stage IVb oocytes around 08:00 about 1 hour prior to lights on and ovulation. (A) Expression of *pgr* transcripts in most advanced stage oocytes from *wt* or *Pgrmc* mutants over different developmental time points (21:00, Stage III; 06:00, Stage IVa; 08:00, Stage IVb). (B) Reduced expression of Pgr protein in Stage IVa follicles (06:00) only in *pgrmc1/2*^{-/-}. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

Fig 4. Expression of metalloproteinase in *Pgrmc* knockouts in comparison to wt in most advanced follicles

(21:00, Stage III; 06:00, Stage IVa; 08:00, Stage IVb.). Low *adamts9* expression can be observed in Stage IVb oocytes of all genotypes (*pgrmc1*^{-/-}, *pgrmc2*^{-/-}, and *pgrmc1/2*^{-/-}). In addition, expression of *adamts1*, *adamts8a*, and *mmp2* was significantly reduced in Stage IVb follicles in *pgrmc1/2*^{-/-}. Asterisks indicate a significant difference of transcripts compared to wt at the same time point. *adam8b*, a disintegrin and metalloproteinase domain 8b; *adamts1*, a disintegrin and metalloproteinase with thrombospondin type 1 motif 1; *adamts8a*; *adamts9*; *mmp2*, matrix metalloproteinase 2; and *mmp9*, matrix metalloproteinase 9. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.