Non-canonical progesterone signaling in granulosa cell function

John J Peluso^{1,2} and James K Pru^{3,4}

Departments of ¹Cell Biology and ²Obstetrics and Gynecology, University of Connecticut Health Center, Farmington, Connecticut 06030, USA, ³The Center for Reproductive Biology and ⁴Department of Animal Science, Washington State University, Pullman, Washington 99164, USA

Correspondence should be addressed to J J Peluso; Email: peluso@nso2.uchc.edu

Abstract

It has been known for over 3 decades that progesterone (P₄) suppresses follicle growth. It has been assumed that P₄ acts directly on granulosa cells of developing follicles to slow their development, as P₄ inhibits both mitosis and apoptosis of cultured granulosa cells. However, granulosa cells of developing follicles of mice, rats, monkeys, and humans do not express the A or B isoform of the classic nuclear receptor for P₄ (PGR). By contrast, these granulosa cells express other P₄ binding proteins, one of which is referred to as PGR membrane component 1 (PGRMC1). PGRMC1 specifically binds P₄ with high affinity and mediates P₄'s anti-mitotic and anti-apoptotic action as evidenced by the lack of these P₄-dependent effects in PGRMC1-depleted cells. In addition, mice in which PGRMC1 is conditionally depleted in granulosa cells show diminished follicle development. While the mechanism through which P₄ activation of PGRMC1 affects granulosa cell function is not well defined, it appears that PGRMC1 controls granulosa cell function in part by regulating gene expression in T-cell-specific transcription factor/lymphoid enhancer factor-dependent manner. Clinically, altered PGRMC1 expression has been correlated with premature ovarian failure/insufficiency, polycystic ovarian syndrome, and infertility. These collective studies provide strong evidence that PGRMC1 functions as a receptor for P₄ in granulosa cells and that altered expression results in compromised reproductive capacity. Ongoing studies seek to define the components of the signal transduction cascade through which P₄ activation of PGRMC1 results in the regulation of granulosa cell function.

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Introduction and historical perspective

Progesterone (P₄) is synthesized and secreted from both the follicular and luteal components of the mammalian ovary (Monniaux *et al.* 1997). The corpora lutea secrete P₄ at the highest rate while P₄ synthesis gradually increases as the follicles develop (Roy & Greenwald 1987, 1996). Throughout the course of rat ovarian follicle development, the P₄ concentration within follicular fluid gradually increases from 5 µM on diestrus to 55 µM on the evening of proestrus (Fujii *et al.* 1983). These elevated P₄ levels play an essential role in regulating ovulation because ovulation is disrupted by treatment with P₄ receptor (PGR) antagonists, such as RU486, or in mutant mice in which the PGR is eliminated (Curry & Nothnick 1996, Lydon *et al.* 1996, Pall *et al.* 2000, Robker *et al.* 2000, Svensson *et al.* 2000, Conneely *et al.* 2002). Because these P₄-dependent effects are mediated to a large extent by PGR, which is expressed during the periovulatory period and not during folliculogenesis, this aspect of P₄'s actions will not be emphasized in this review.

While P₄ activation of PGR and its role in the peri-ovulatory period is well accepted, P₄ also influences

granulosa cell function in developing follicles prior to ovulation. These actions are controversial because granulosa cells of developing follicles do not express PGR (Park & Mayo 1991, Natraj & Richards 1993, Shao *et al.* 2003). This conclusion is based on the failure to detect PGR expression in rodent granulosa cells by northern blot, western blot, RT-PCR, *in situ* hybridization and immunocytochemistry. Also, rat luteal cells do not express PGR (Park & Mayo 1991). Thus, there is overwhelming evidence that rat and mouse granulosa and luteal cells do not express PGR. In addition, granulosa cells of monkeys do not express the PGR until the gonadotropin surge (Chandrasekher *et al.* 1991). Finally, immunohistochemical analysis fails to detect PGR in granulosa cells of human follicles, although PGR is readily detected in luteal cells (Suzuki *et al.* 1994).

In spite of the fact that granulosa cells of developing follicles do not express PGR, the rate at which follicles grow (i.e., rate of granulosa cell mitosis) is inversely associated with serum P₄ levels (Butcher *et al.* 1974, Hirshfield 1984, Pedersen 1984). Further, P₄ inhibits follicular development in hypophysectomized hamsters

(Moore & Greenwald 1974), gonadotropin-primed hamsters (Kim & Greenwald 1987), and rabbits (Setty & Mills 1987), as well as cycling rats (Buffler & Roser 1974). That intraovarian P_4 regulates follicle development is clearly shown in monkeys, in which intraovarian insertion of a P_4 implant into one ovary suppresses follicle development in that ovary without altering serum gonadotropin levels, which allowed follicles to continue to grow in the contralateral non- P_4 implanted control ovary (diZerega & Hodgen 1982). This is the best evidence that P_4 acts within the ovary to regulate follicle growth independent of its effects on serum gonadotropin levels.

Although these *in vivo* studies are consistent with a direct effect of P_4 on developing follicles, they do not reveal the cellular site of P_4 's action. However, P_4 affects the ability of granulosa cells of immature rats to secrete steroids *in vitro* (Schreiber *et al.* 1980, 1981, Fortune & Vincent 1983), as well as to undergo mitosis and apoptosis *in vitro* (Luciano & Peluso 1995, Peluso & Pappalardo 1998). Finally, P_4 inhibits mitosis (Peluso *et al.* 2002) and apoptosis (Peluso *et al.* 2001) of spontaneously immortalized granulosa cells (SIGCs), a cell line derived from rat granulosa cells (Stein *et al.* 1991) and, like freshly isolated primary rat granulosa cells, these cells do not express PGR (Peluso *et al.* 2002). These *in vitro* observations are difficult to reconcile with the findings that granulosa cells do not express PGR.

P_4 influences not only the function of granulosa cells but also luteal cells, which are derived from granulosa cells. Specifically, inhibiting P_4 synthesis results in structural changes similar to those associated with luteal regression (see review by Stouffer *et al.* (2013)). These changes are prevented by supplemental P_4 treatment (Stouffer *et al.* 2013). However, the effect of RU486 treatment is variable, at times increasing while at other times decreasing P_4 secretion. To explain these biological effects of P_4 and RU486, Rothchild (1996) put forth the concept that P_4 regulates the structure and steroidogenic capacity of luteal cells. His hypothesis, developed without the aid of molecular biological and genetic approaches, further states that P_4 can act through at least two different pathways but RU486 only acts through one of these pathways. While PGR is expressed by primate luteal cells (Chandrasekhar *et al.* 1991), PGR is not expressed in rodent luteal cells (Park & Mayo 1991, Natraj & Richards 1993, Cai & Stocco 2005). These observations raise the possibility of other receptors within luteal cells that mediate some of the P_4 's actions.

Identification of a novel P_4 binding protein in granulosa and luteal cells

How P_4 might then affect granulosa and luteal cell function in the absence of PGR? Ligand binding studies carried out in the late 1970s (Schreiber & Erickson 1979, Schreiber & Hsueh 1979) and early 1980s (Naess 1981)

reveal that immature rat ovaries, which are composed mainly of developing ovarian follicles that lack PGR, specifically bind P_4 . Rat luteal cells also bind P_4 (Cai & Stocco 2005). Therefore, these binding studies indicate that the granulosa cells of immature rat ovaries and rat luteal cells express a P_4 binding protein that is not the PGR and may function as a mediator of P_4 's actions.

Interestingly, there are two different families of progesterin binding proteins that could mediate P_4 's actions in these cells. The first family is progesterin AdipoQ receptors (PAQRs), initially identified in fish oocytes by Dr Peter Thomas's group (Zhu *et al.* 2003a,b). Thomas' studies reveal that the α type of the membrane progesterin receptor (PAQR7) is expressed in human testis and ovaries. Moreover, mRNA encoding PAQR7 is detected in human granulosa/luteal cells isolated from women undergoing IVF, a human granulosa cell line, hGL5 cells (Peluso *et al.* 2009), and rat luteal cells (Cai & Stocco 2005). Thus, a functional role for PAQRs in regulating P_4 's actions within the mammalian ovary is likely.

Expression and localization of P_4 membrane receptor component 1

In addition to PAQRs, a second purported mediator of P_4 's action is PGR membrane component 1 (PGRMC1). PGRMC1 is highly expressed in granulosa cells of developing follicles and luteal cells. Specifically, PGRMC1 is detected in virtually all granulosa cells of rat antral follicles (Peluso *et al.* 2006). While present at the plasma membrane of every granulosa cell, it is also detected in the nucleus of a limited number of granulosa cells of small antral follicles (Fig. 1A). Within 48 h of gonadotropin treatment, PGRMC1 localization changes to almost exclusively at or near select regions of the plasma membrane with few cells showing nuclear staining (Fig. 1B). After hCG induction of ovulation and luteinization, PGRMC1 expression increases with 100% of the luteal cells expressing high levels of PGRMC1 localized diffusely throughout the cytoplasm and nucleus (Peluso *et al.* 2006).

Figure 1 The expression of PGRMC1 in granulosa cells of antral (A) and preovulatory follicles (B) as assessed by immunohistochemistry. The antral follicle was obtained from an immature rat, while the preovulatory follicle was from an immature rat 48 h after PMSG injection. PGRMC1 was detected as a brown stain. The thecal layers at the base of each image show robust PGRMC1 expression. Data from Peluso *et al.* (2006).

It is important to appreciate that in the cytoplasm, PGRMC1 is mainly thought to localize to the endoplasmic reticulum (Meyer *et al.* 1996, Falkenstein *et al.* 1999, Losel *et al.* 2005). As steroids can enter the cell, it is not necessary for PGRMC1 to be at the extracellular surface of the plasma membrane to transduce P_4 's action. However, BSA-conjugated P_4 mimics P_4 's actions, suggesting that at least a fraction of P_4 's receptor is localized to the extracellular surface of the plasma membrane (Peluso & Pappalardo 1999). That some PGRMC1 localizes to the extracellular surface of the plasma membrane is conclusively demonstrated because PGRMC1 is detected among the plasma membrane proteins after biotinylation of these proteins (Peluso *et al.* 2006).

PGRMC1 as a P_4 binding protein

The expression studies imply that PGRMC1 could mediate P_4 's actions in granulosa cells of developing follicles. If so, then the first event in PGRMC1's mechanism is to bind P_4 . Interestingly, there are reports that PGRMC1 does not bind P_4 . These reports used bacterially expressed PGRMC1 fusion proteins (reviewed in Cahill (2007)) and not PGRMC1 fusion proteins expressed in mammalian cells. As a result, the bacterially expressed PGRMC1 may be unable to bind P_4 , as it may not be properly folded. By contrast, partially purified PGRMC1-fusion protein isolated from either SIGCs (Peluso *et al.* 2008) or human granulosa/luteal cells (hGL5 cells; Peluso *et al.* 2009) specifically binds P_4 with high affinity ($EC_{50} \approx 10$ nM) (Fig. 2A). In addition, the synthetic progestin, R5020, also binds PGRMC1 with the same affinity as P_4 (Fig. 2A). Further, siRNA treatment specifically depletes *Pgrmc1* mRNA and proteins levels and reduces the capacity of SIGCs to bind P_4 (Peluso *et al.* 2008; Fig. 2B). Collectively, these studies demonstrate that PGRMC1 binds P_4 and that it is required for P_4 binding protein in SIGCs. However, as its name implies, PGRMC1 likely binds P_4 as part of a complex with one member of the complex being membrane progesterin receptor α (PAQR7) (Thomas *et al.* 2014), which is also expressed by SIGCs, rat ovarian cells (Cai & Stocco 2005), and human granulosa/luteal cells (Peluso *et al.* 2009). These ligand-binding studies together with the expression data are supportive of PGRMC1 being a mediator of P_4 's action in both granulosa and luteal cells. However, this must be demonstrated by genetic manipulation of PGRMC1 levels.

Biological actions mediated by P_4 –PGRMC1 signaling in granulosa cells

As previously indicated, P_4 affects granulosa cell mitosis, apoptosis, and steroid synthesis in cells that do not express PGR. Given these actions, it is possible that

PGRMC1 is involved in each of these diverse aspects of granulosa cell biology. This concept was tested using both SIGCs and/or hGL5 cells as outlined in the following paragraphs.

Figure 2 The capacity of partially purified PGRMC1-GFP to bind progesterone (P_4) and R5020 (A) and the effect of PGRMC1 siRNA treatment on *Pgrmc1* mRNA levels and specific 3H - P_4 binding to SIGCs (B). Data shown in (A) are adapted from Peluso *et al.* (2009) and the data shown in (B) are unpublished observations (J Peluso, unpublished observations) that confirm our published data (Peluso *et al.* 2008). The effect of P_4 on serum-induced SIGC mitosis is shown in (C). Data shown in (C) are adapted from Peluso (2013). *indicates a value significantly different from control ($P < 0.05$).

P₄ and PGRMC1 as regulators of mitosis

P₄ attenuates mitogen-induced proliferation of rat granulosa cells isolated from both immature and preovulatory rat follicles (Peluso *et al.* 2006), human granulosa/luteal cells obtained from women undergoing ovulation induction for infertility treatment (Chaffkin *et al.* 1992), and SIGCs (Peluso *et al.* 2002). Further, P₄ does so in a dose-dependent manner (10–1000 nM). Furthermore, treatment with PGRMC1 siRNA attenuates P₄'s ability to suppress the percentage of SIGCs incorporating BrdU and the percentage of cells in metaphase (J Peluso, unpublished observations). Finally, PGRMC1 siRNA treatment ablates P₄'s ability to suppress the number of cells present after 22 h of culture (Peluso 2013; Fig. 2C), while forced expression of PGRMC1 blocks entry into the cell cycle (J Peluso, unpublished observation). Taken together, these studies support the concept that P₄–PGRMC1 signaling is involved in regulating the rate of granulosa cell proliferation.

PGRMC1's ability to influence cell cycle progression is complex in that it appears to play specific roles at different stages of the cell cycle. For example, PGRMC1 regulates the transition from G₀ to G₁ stage of the cell cycle and also prolongs the duration of metaphase through its ability to interact with the mitotic spindle (Lodde & Peluso 2011). These observations imply that PGRMC1 has different and specific modes of action that allow for its involvement in regulating the diverse signaling pathway that control different stages of the cell cycle.

P₄–PGRMC1 regulates apoptosis

Over the same dose range that inhibits mitosis, P₄ also suppresses the rate at which rat granulosa cells (Peluso *et al.* 2005), rat luteal cells (Peluso *et al.* 2005), human granulosa/luteal cells (Engmann *et al.* 2006), and SIGCs

(Peluso *et al.* 2004) undergo apoptosis (Fig. 3A). It is important to appreciate that P₄'s anti-apoptotic effects can only be detected in mature rat luteal cells if endogenous P₄ synthesis is inhibited with aminoglutethamide cells (Peluso *et al.* 2005), because the effect of supplemental P₄ treatment is not observed in the presence of the large amount of P₄ that is secreted by luteal cells (Peluso *et al.* 2006). As with mitosis, siRNA knockdown of PGRMC1 prevents P₄ from inhibiting SIGCs from undergoing apoptosis (Peluso *et al.* 2008; Fig. 3B). In addition, forced expression of PGRMC1 increases the ability of P₄ to suppress apoptosis (Peluso *et al.* 2008). These studies implicate P₄–PGRMC1 signaling as a regulator of apoptosis.

PGRMC1 and steroidogenesis

P₄ can also enhance its own synthesis (Rothchild 1996), and there is evidence to suggest that this action is mediated by PGRMC1. Specifically, this concept is based on the observation that PGRMC1 interacts with proteins sterol regulatory element binding protein cleavage-activating protein (SCAP) and insulin-induced gene 1 (INSIG1) that regulate cholesterol metabolism (Suchanek *et al.* 2005). Briefly, SCAP and INSIG1 control cholesterol metabolism by regulating the transcriptional action of sterol regulatory element binding protein, which induces the expression of StAR (Yang *et al.* 2002). P₄ induces the expression of StAR in MA-10 testicular interstitial cells (Schwarzenbach *et al.* 2003) and promotes cholesterol and P₄ synthesis in rat and human granulosa/luteal cells (Rung *et al.* 2005). Thus, the possibility exists that P₄–PGRMC1 interaction regulates a complex series of protein–protein interactions that promote cholesterol and P₄ biosynthesis. This concept is consistent with the observations that an antibody to PGRMC1 attenuates and forced PGRMC1 expression enhances adrenal steroidogenesis (Min *et al.* 2004).

The synthetic progestin, R5020, stimulates P₄ secretion from cultured rat granulosa cells (Fanjul *et al.* 1983, Ruiz de Galarreta *et al.* 1985), luteal cells (Rothchild 1996), MA-10 cells (Schwarzenbach *et al.* 2003) and hGL5 cells (Peluso *et al.* 2009). R5020's ability to stimulate P₄ secretion is observed at concentrations equal to or >16 μM. While 16 μM may appear to be a pharmacological dose, it is within the physiological range, as women undergoing either natural or gonadotropin-stimulated cycles have P₄ follicular fluid levels between 15 and 60 μM (Tarlitzis *et al.* 1993, Kamel *et al.* 1994, Enien *et al.* 1995). However, human PGRMC1 binds R5020 with high affinity (EC₅₀ of ≈10 nM) (Peluso *et al.* 2009; Fig. 2A). Given this binding characteristic, all the R5020 binding sites within human PGRMC1 would be occupied by R5020 in the nanomolar range. Therefore, it is unlikely that the ability of R5020 to acutely promote P₄ secretion is mediated

Figure 3 The inhibitory effect of progesterone (P₄) on apoptosis of human granulosa/luteal cells, rat granulosa cells, and rat luteal cells from PMSG-hCG primed immature rats 4 days after hCG treatment (A). Note that to detect the effects of P₄ in luteal cells, endogenous P₄ secretion was blocked with aminoglutethamide. The effect of P₄ on the rate of spontaneously immortalized granulosa cells (SIGCs) apoptosis is shown in (B). The anti-apoptotic action of P₄ is ablated after PGRMC1 siRNA treatment. *A value that is significantly different from control ($P < 0.05$). In (A), all values under the bracket marked with an * are different from their respective control values. Data shown in (A) are redrawn from the following publications: (Engmann *et al.* 2006, Peluso *et al.* 2005, 2009). Data for (B) is from Peluso *et al.* (2008).

through its ability to interact with PGRMC1. Further, PGRMC1-deplete hGL5 cells respond to R5020 by increasing their rate of P_4 secretion (Peluso *et al.* 2009). Thus, these pharmacological and genetic-based observations make it clear that in hGL5 cells, PGRMC1 does not mediate P_4 's ability to acutely stimulate its own secretion. The mechanism through which R5020 promotes steroidogenesis in hGL5 cells is not known but could involve one of the other progesterin binding proteins that are expressed by both human granulosa cells and hGL5 cells (i.e., PGR and PAQR family members) (Peluso *et al.* 2009).

PGRMC1 and follicle growth in vivo

PGRMC1's role in regulating granulosa cell mitosis and apoptosis is supported by examining follicle development in a conditional knockout mouse in which PGRMC1 is conditionally deleted from granulosa cells using anti-Müllerian hormone type II receptor cre recombinase transgenic mouse (*Amhr2-cre*). This analysis shows that ovaries from immature (22–25 days old) *Pgrmc1* conditional knockout (cKO; *Amhr2^{cre/+}; Pgrmc1^{fl/fl}*) mice have fewer antral follicles compared to either the control (i.e., *Amhr2^{cre/+}; Pgrmc1^{fl/fl}*) or heterozygous (*Amhr2^{cre/+}; Pgrmc1^{fl/+}*) female mice (Fig. 4). Although the ovaries of heterozygous mice have the same number of antral follicles as control mice, they have a higher percentage of atretic antral follicles (Fig. 4). The reason for this is unclear but a reduced level of PGRMC1 likely results in an increase in granulosa cell apoptosis, which would account for the increase in atretic follicles in the heterozygous mice.

Figure 4 The total number of antral follicles within the ovaries of controls (+/+), PGRMC1 heterozygous (+/-), and PGRMC1 conditional knockout (-/-) mice. Values are shown as means and s.e.m. with n of 6–8/treatment group. *A value is different from control ($P < 0.05$) as demonstrated by an ANOVA followed by Dunnett's multiple comparison test. The percentage of atretic antral follicles is shown in the base of each column. The percentage of atretic antral follicles is greater in the ovaries of *Pgrmc1^{+/-}* mice compared with the percentage in either the controls or *Pgrmc1^{-/-}* mice as assessed by Fisher's exact/ χ^2 tests ($P < 0.05$) (Peluso 2013).

Importantly, the heterozygous mouse mimics women with premature ovarian failure, who have $\approx 50\%$ reduction in the level of PGRMC1 (Mansouri *et al.* 2008). Taken together, these findings are consistent with PGRMC1's role in maintaining the viability of granulosa cells and thus the process of folliculogenesis.

P_4 –PGRMC1 signal transduction cascade

That PGRMC1 mediates P_4 's ability to regulate mitosis and apoptosis is clearly illustrated through the use of siRNA-based knockdown and forced expression experiments. Unfortunately, the mechanism through which P_4 activation of PGRMC1 regulates both mitosis and apoptosis of granulosa still remains to be determined. Specifically, how does P_4 binding to PGRMC1 either at the plasma membrane or within the cytoplasm activate a signal transduction cascade that affects the rate at which granulosa cells undergo mitosis and/or apoptosis? There are a few studies indicating that P_4 increases protein kinase G (PKG) activity (Peluso & Pappalardo 2004, Peluso *et al.* 2007) and suppresses MEK/ERK activity (Peluso *et al.* 2003). As activators of PKG inhibit and MEK/ERK activity induces apoptosis (Peluso *et al.* 2003), the ability of P_4 to regulate these two kinase pathways is probably a component of the anti-apoptotic P_4 –PGRMC1 signaling pathway. Unfortunately, siRNA-based studies have not been conducted, and therefore, it remains to be determined whether or not P_4 activation of PGRMC1 is directly linked to P_4 's effects on these kinase cascades.

Expression of genes in ovarian cells regulated by PGRMC1

What is clear is that P_4 activation of PGRMC1 alters gene expression, specifically genes that are involved in regulating mitosis and/or apoptosis. For example, P_4 promotes SIGC survival by suppressing Bad and increasing Bcl2a1d expression, thereby changing the Bcl21d:Bad ratio to favor cell survival (Peluso *et al.* 2010). In human granulosa cells (hGL5 cells), PGRMC1 suppresses mRNA levels that encode activators of apoptosis such as caspase-3 (Peluso *et al.* 2012a). These PGRMC1-dependent actions would make the cells less likely to undergo apoptosis and promote mitosis (Peluso *et al.* 2012a).

P_4 –PGRMC1 as a regulator of T-cell-specific transcription factor/lymphoid enhancer factor activity

While PGRMC1 knockdown studies are important in that they reveal a role for P_4 –PGRMC1 signaling in the regulation of gene expression, these types of studies provide little information about the mechanism through which P_4 -activated PGRMC1 alters gene expression.

Figure 5 The effect of P₄ and PGRMC1 on Tcf/Lef promoter in SIGCs as assessed by a luciferase reporter assay (A). In (B), the ability of P₄ to suppress Tcf/Lef activity in the presence or absence of PGRMC1-Flag protein was assessed by a luciferase reporter. In all graphs, * indicates a value that is significantly different than control ($P < 0.05$), ** indicates a value significantly less than cells transfected with an expression vector encoding PGRMC1-Flag (Peluso *et al.* 2012b).

As indicated, PGRMC1 localizes to the nuclei of many granulosa cells in developing ovarian follicles. Similarly, PGRMC1 is also present within the nuclei of SIGCs that are proliferating but not in the nuclei of mitotically arrested SIGCs (Peluso *et al.* 2012b). The mechanism that regulates the cellular localization is unknown but PGRMC1's nuclear localization suggests that nuclear PGRMC1 may be directly involved in regulating gene expression. Furthermore, the most likely mechanism through which PGRMC1 influences gene expression is by either increasing or decreasing the activity of transcription factors. Recent studies indicate that in SIGCs, P₄ significantly and faithfully suppresses T-cell-specific transcription factor/lymphoid enhancer factor (Tcf/Lef) activity and P₄'s ability to suppress Tcf/Lef activity is dependent on the presence of PGRMC1 (Peluso *et al.* 2012b; Fig. 5A). These observations provide some insight into how P₄-PGRMC1 signaling suppresses mitosis because the promoter region of genes involved in the initiation of mitosis, such as c-myc, possess Tcf/Lef consensus binding elements (Dang & Lewis 1997, Dang *et al.* 2006).

The PGRMC1 siRNA study illustrates that P₄'s ability to inhibit Tcf/Lef activity is dependent on PGRMC1, but it does not demonstrate that nuclear PGRMC1 interacts with the Tcf/Lef site. One way to demonstrate that nuclear PGRMC1 interacts with the Tcf/Lef site is to use electrophoretic mobility shift assay (EMSA). Nuclear extract from SIGCs not treated with P₄ contains proteins that bind to the Tcf/Lef probe. Moreover, nuclear extract from P₄-treated SIGCs reduces the amount of Tcf/Lef probe bound to nuclear protein by $\approx 50\%$ (J Peluso, unpublished observation). Unfortunately, the PGRMC1 antibody is not suitable for use in a super-shift EMSA, thus, PGRMC1 could not be shown to be one of the proteins that binds Tcf/Lef response elements.

This obstacle can be overcome by transfecting cells with an expression construct that encodes a PGRMC1-Flag fusion protein. Importantly, the presence of PGRMC1-Flag increases Tcf/Lef activity by nearly

twofold compared with controls and the PGRMC1-Flag-induced increase in Tcf/Lef activity is suppressed by P₄ (Fig. 5B). After transfection with PGRMC1-Flag construct, a super-shift EMSA using an anti-Flag antibody reveals that PGRMC1-Flag is among the proteins that bind to the Tcf/Lef response element and P₄ reduces the amount of PGRMC1 that is bound to this transcription factor site. Taken together, these studies demonstrate that P₄ inhibits Tcf/Lef activity in part by suppressing the ability of nuclear PGRMC1 to interact with this transcription factor response element (Peluso *et al.* 2012b).

Expression and function of other PGRMC family members

Although progress is being made to define PGRMC1's role and mechanism of action in ovarian function, there are other members of the PGRMC family, including PGRMC2 and neudesin (NENF). As can be seen in Fig. 6, granulosa cells also express these other two PGRMC family members. Among these PGRMC family members, the relative level of expression is considerably different with PGRMC1 having the lowest level of expression, although *Pgrmc1* mRNA levels in granulosa cells are higher compared with their levels in whole ovary. The higher *Pgrmc1* mRNA levels in granulosa cells are consistent with its known function in granulosa cells. Interestingly, PGRMC2 is expressed at a five- to tenfold greater levels than PGRMC1, while NENF is expressed at nearly 100 times that of PGRMC1. This pattern of PGRMC family expression is also observed in mRNA levels obtained from the entire immature mouse ovary, as well as SIGCs (J Pru & J Peluso unpublished observation).

Given the structural similarity between PGRMCs (Kimura *et al.* 2013), it is likely that PGRMC2 influences granulosa cell function. There are several ways that PGRMC2 could influence granulosa cell function including it: i) functioning as an antagonist to

Figure 6 Expression of PGRMC family members in the immature mouse ovary and granulosa cells as assessed by real-time PCR. Note that the level of expression for the PGRMC family member is dramatically different with the lowest expression for PGRMC1, followed by PGRMC2 and then NENF, which has the highest level of expression (J Pru, unpublished observations).

PGRMC1, ii) synergizing with PGRMC1, or iii) regulating a redundant pathway. Each of these possibilities merits experimental investigation. The likely key to understanding PGRMC2's function will be to determine the proteins to which it binds. It is probable that PGRMC2 will bind a unique set of intracellular proteins as well as some of the same proteins that bind PGRMC1. To date, the only published study on the function of PGRMC2 involves its role in promoting the migrations of ovarian cancer cells (Wendler & Wehling 2013).

Like PGRMC2, NENF is highly expressed in granulosa cells and SIGCs and shares sequence homology to PGRMC1 (Kimura *et al.* 2013). As NENF is secreted by neural cells (Kimura *et al.* 2005, 2008), it is hypothesized that NENF binds P_4 , thereby reducing the amount of P_4 available to interact with PGRMC1. However, there are reports indicating that the NENF does not bind P_4 , but in these studies, P_4 binding was assessed using dot blots and bacterially expressed NENF (Kimura *et al.* 2005, 2008). Given that this approach is relatively insensitive and it is unclear if NENF bacterial protein is folded to allow for P_4 binding, the capacity of NENF to bind P_4 still remains to be determined.

There is another way for NENF to affect granulosa cell function. In SIGCs, P_4 suppresses MAP kinase activity (Peluso *et al.* 2003), which likely accounts in part for both P_4 's anti-mitotic and anti-apoptotic actions (Peluso *et al.* 2003). Interestingly, NENF has neurotrophic actions and these actions are mediated through the activation of MAP kinase (Kimura *et al.* 2005, 2008). Thus, the balance between P_4 and NENF could influence the overall activity of MAP kinase and thereby the fate of granulosa cells. However, a receptor for NENF has not been identified (Kimura *et al.* 2013) and this is required before any mechanism for NENF's action can be completely developed.

Clinical correlations

Although it has only been known that PGRMC1 is expressed in the mammalian granulosa cells since 2006 (Peluso *et al.* 2006), there are now three publications suggesting that it plays a clinically relevant role in regulating follicular function in women. The first report is from Dr Dahl's group in Sweden in which he identified a mother and daughter with an X; autosome translocation (Mansouri *et al.* 2008). This translocation results in a reduced expression of PGRMC1 by 50%. The same publication describes a missense mutation in PGRMC1 that is observed in one of 67 women with premature ovarian failure/insufficiency. This mutation at amino acid 165 reduces the ability of PGRMC1 to transduce P_4 's anti-apoptotic action by 50% and also attenuates its ability to interact with several cytochrome P450 enzymes (Mansouri *et al.* 2008). In their second publication, Dahl's group reports that PGRMC1 levels are relatively constant throughout the menstrual cycle

but are lower in postmenopausal women and women with premature ovarian failure and polycystic ovary syndrome (Schuster *et al.* 2010). Finally, an examination of the granulosa/luteal cells obtained from women undergoing ovulation induction as part of their infertility treatment reveal that women with elevated levels of *Pgrmc1* mRNA levels have 30% fewer follicles and few oocytes retrieved (Elassar *et al.* 2012). The reason for the association between higher levels of *Pgrmc1* mRNA is not completely known but may be related to the finding that there is an increase in the methylation of the PGRMC1 promoter (Elassar *et al.* 2012).

Like PGRMC1, PGRMC2 appears to be associated with altered follicle development in women. This is based on the finding that young women with diminished ovarian reserve (i.e., fewer ovarian follicles) have elevated *Pgrmc2* mRNA levels compared to normal young women (Skiadas *et al.* 2012). To date, there are no studies on ovarian function and NENF. Taken together, these correlative clinical studies provide new insight by demonstrating that genetic changes or altered expression of PGRMC family members affects follicle growth in women. These findings are consistent with actions of PGRMC family members observed in the studies on rodent granulosa cells both *in vitro* and *in vivo*.

Summary and future research perspectives

The biological actions of P_4 on developing ovarian follicles have been known for several decades, but the receptors that mediate these effects remain a point of contention, as the granulosa cells of these follicles do not express PGR. The application of biochemical and genetic approaches reveal that PGRMC1 and possibly other PGRMC family members play important roles in this aspect of ovarian follicle function. Recent studies implicate PGRMC1 as a regulator of granulosa cell apoptosis and mitosis. Moreover, PGRMC1 functions to regulate gene transcription, in part through regulating Tcf/Lef transcription factor activity. Thus, the basic elements of the P_4 -PGRMC1 signal transduction pathway have been identified in granulosa cells. Future research must now be directed toward determining the molecular steps that link P_4 -activated PGRMC1 to Tcf/Lef-dependent transcription and the subsequent regulation of mitosis, apoptosis, and steroidogenesis.

Declaration of interest

J K Pru has nothing to disclose. J J Peluso was awarded a patent on non-genomic regulators of progesterone's action.

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