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Progestin and AdipoQ Receptor 7, Progesterone Membrane Receptor Component 1 (PGRMC1), and PGRMC2 and Their Role in Regulating Progesterone's Ability to Suppress Human Granulosa/Luteal Cells from Entering into the Cell Cycle[1]

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ABSTRACT

The present studies were designed to determine the role of progesterone receptor membrane component 1 (PGRMC1), PGRMC2, progestin and adipoQ receptor 7 (PAQR7), and progesterone receptor (PGR) in mediating the antimitotic action of progesterone (P4) in human granulosa/luteal cells. For these studies granulosa/luteal cells of 10 women undergoing controlled ovarian hyperstimulation were isolated, maintained in culture, and depleted of *PGRMC1, PGRMC2, PAQR7*, or *PGR* by siRNA treatment. The rate of entry into the cell cycle was assessed using the FUCCI cell cycle sensor to determine the percentage of cells in the G_1/S stage of the cell cycle. *PGRMC1, PGRMC2, PAQR7*, and *PGR* mRNA levels were assessed by real-time PCR and their interactions monitored by in situ proximity ligation assays (PLAs). These studies revealed that *PGRMC1, PGRMC2, PAQR7*, and *PGR* were expressed by granulosa/luteal cells from all patients, with *PGRMC1* mRNA being most abundant, followed by *PAQR7, PGRMC2*, and *PGR*. However, their mRNA levels showed considerable patient variation. P4's ability to suppress entry into the cell cycle was dependent on PGRMC1, PGRMC2, and PAQR7 but not PGR. Moreover, PLAs indicated that PGRMC1, PGRMC2, and PAQR7 formed a complex within the cytoplasm. Based on these studies, it is proposed that these three P4 mediators form a complex within the cytoplasm that is required for P4's action. Moreover, P4's ability to regulate human follicle development may be dependent in part on the expression levels of each of these P4 mediators.

cell cycle, follicle, granulosa cells, mitosis, progesterone/progesterone receptor

INTRODUCTION

In order to optimize gonadotropin-based ovarian hyperstimulation protocols used to induce follicle growth, a more detailed understanding of the mechanism that regulates follicular development is required. To achieve this goal, genetic studies compared the gene expression profiles of granulosa cells isolated from women with poor ovarian response to gonadotropin with that observed in granulosa cells of women who had a good response to gonadotropin. This approach identified mutations in gonadotropin receptors, follicle-stimulating hormone receptor (FSHR) [1] and luteinizing hormone receptor (LHR) [2] and estrogen receptor (ESR1) [3], but these mutations are extremely rare. In addition, a comparison of the gene expression patterns of granulosa cells of women with normal and diminished ovarian reserve did not reveal major changes in the expression of these receptors [4–6]. Interestingly, two microarray-based studies detected significant decreases in the expression of components of the insulin-like growth factor (IGF) signaling pathway, which is known to promote granulosa cell mitosis [4, 5]. Moreover, the mitogenic action of gonadotropins is mediated in part through the IGF signaling pathway [7].

However, to completely understand the genetic changes that determine follicle growth, it is important to appreciate that gonadotropins stimulate not only granulosa cell mitosis but also the synthesis and secretion of progesterone (P4) [8]. In addition, P4 gradually increases in the follicular fluid as the follicle grows [9, 10]. Numerous in vivo [11–13] and in vitro [14–18] studies in laboratory rodents demonstrate that P4 inhibits granulosa cell mitosis and/or follicle growth. Importantly, P4 acts directly on rodent [14, 18] and human granulosa/luteal cells to limit their rate of proliferation [19, 20]. There is also compelling in vivo evidence for P4's antimitotic action in regulating the development of preovulatory follicles in primates. Briefly, studies on monkeys, conducted largely in the late 1970s and early 1980s, demonstrate that 1) follicle development is suppressed during the luteal phase of the menstrual cycle [21]; 2) the removal of the corpus luteum (the major source of P4) initiates follicle growth and the development of preovulatory follicles within 12–14 days [8, 22]; 3) supplemental P4 is sufficient to suppress follicle development after corpus luteum removal; 4) P4's action is not due to suppression of serum gonadotropins, because supplemental P4 treatment does not alter the increase in serum gonadotropins that occurs after corpus luteum removal [23]; and 5) gonadotropins can override P4's actions [23]. Interestingly, after corpus luteum removal, the dominant follicle always develops in the ovary that has the lower ovarian venous levels of P4, which is consistent with P4's antimitotic action [24, 25]. More recent studies in monkeys show that ≈50% of the granulosa cells of preovulatory follicles are undergoing mitosis as judged by the presence of the cell proliferation marker Ki-67 [26]. Within 12 h of human chorionic gonadotropin (hCG) treatment the percentage of Ki-67-positive cells decreases to ≈20% [26]. In addition, inhibiting P4 synthesis, using the 3β-HSD inhibitor trilostane, prevents hCG from suppressing mitosis, and this action can be reversed by supplemental progestin treatment [26].

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Although the signal pathways involved in the growth-promoting actions of gonadotropins have been and continue to be investigated, the molecular mechanisms through which P4 inhibits follicle growth have just begun to be studied. Interestingly, human granulosa/luteal cells express at least four potential mediators of P4's action: nuclear progesterone receptor (*PGR*), PGR membrane component 1 (*PGRMC1*), *PGRMC2*, and progestin and adipoQ receptor 7 (*PAQR7*; also known as membrane progestin receptor α) [27]. Therefore, in the first series of studies, the expression of each P4 mediator was assessed in human granulosa/luteal cells that were harvested at the time of oocyte retrieval and maintained in culture. The second set of experiments utilized siRNAs to deplete each of the potential P4 mediators in order to assess their involvement in mediating P4's ability to suppress mitosis. The final series of experiments used in situ proximity ligation assays (PLAs) to monitor the interaction amongst the four P4 mediators.

MATERIALS AND METHODS

Isolation and Culture of Human Granulosa/Luteal Cells

This study was approved by the University of Connecticut Health Center Institutional Review Board. The follicular aspirates were obtained from the clinical laboratory of the Center for Advanced Reproductive Services at the University of Connecticut Health Center as deidentified samples; therefore, a linkage between clinical information and the sample is not available. Briefly, after the oocytes were removed, the follicular aspirates were taken to the research laboratory, where the follicular fluid was centrifuged and the cell pellet was aspirated, layered onto a Histopaque-1077 gradient (Sigma-Aldrich) and centrifuged at 400 × *g* for 30 min [27, 28]. The red blood cells were pelleted and discarded, and the band of cells within the Histopaque was aspirated and pipetted onto an inverted 40-μm cell strainer (catalog number 08-771-1; Fisher Scientific), which was placed on top of a 50-ml tube [29]. After washing with PBS to remove the blood mononucleocytes (i.e., lymphocytes, monocytes, and macrophages), the strainer was placed into a new 50-ml tube, now in the correct position, and again washed with PBS to isolate the granulosa/luteal cells. The granulosa/luteal cells were centrifuged at 250 × *g* for 10 min and the cell pellet resuspended in trypsin (0.5%) to generate a highly enriched population of single granulosa/luteal cells. Fetal bovine serum (FBS)-supplemented medium was then added to stop trypsinization and the cells counted using a hemacytometer. The cells were plated at a concentration of 4×10^5 in 35-mm dishes in 2 ml of Dulbecco modified Eagle medium supplement with 10% FBS, 1% ITS (insulin-transferrin-selenium; catalog number I3146-5ML; Sigma-Aldrich Biochemical Co.), and 0.01% gentamicin (catalog number #G1397; Sigma-Aldrich Biochemical Co.), which had been previously coated with Matrigel (BD BioScience). Finally, these cells were cultured until confluent and then harvested and transferred into T-25 culture flasks. These cells proliferated at a relatively slow rate, requiring as much as 4 wk to reach confluence when cultured in a T-25 flask. After 5–10 passages, the cells were frozen and stored in liquid nitrogen. An equal number of cells from each patient was then pooled and maintained in culture. Cells from this pooled population of granulosa/luteal cells were used for all subsequent experiments, unless stated otherwise.

Granulosa/Luteal Cell Characterization

To confirm that these cells were in fact granulosa/luteal cells, the presence of steroid acute regulatory protein (STARD1), cholesterol side-chain cleavage enzyme (CYP11A1), and lipid droplets was detected by immunocytochemical and Nile red staining protocols, respectively. For the immunocytochemical studies, the granulosa/luteal cells were cultured on Matrigel-coated cover glass overnight, washed with PBS three times for 5 min each, and fixed with 4% paraformaldehyde for 7 min. The cells were permeabilized with 0.1% Triton X-100 in PBS for 7 min and incubated in 3% bovine serum albumin (BSA) for 1 h at room temperature. The cells were then incubated overnight at 4°C with a rabbit monoclonal anti-STAR antibody (Supplemental Table S1; Supplemental Data are available online at www.biolreprod.org). Negative controls were incubated with immunoglobulin G (IgG) in place of the primary antibody. Cells were washed with PBS three times for 5 min each. The cells, including those for the negative control, were then incubated for 1 h with the appropriate secondary antibody labeled with Alexa Fluor 488 (Supplemental Table S1). The cells were counterstained with DAPI solution (4′,6-diamidino-2-phenylindole, dihydrochloride, 0.2 μg/ml PBS for 5 min) and each cover glass mounted on a slide using ProLong Anti-fade mounting media (Invitrogen-Molecular Probes). The cells were then imaged using a Zeiss Axio Observer inverted microscope (Carl Zeiss MicroImaging Inc.) equipped with a Lumen 200 Fluorescence Illumination System (Prior Scientific Inc.) and a QImaging Retiga EXi CCD digital camera (QImaging).

Nile red staining was performed to detect lipid droplets, whose presence is characteristic of steroidogenic human granulosa/luteal cells [30]. For this study, human granulosa/luteal cells were plated at 2×10^5/2 ml in 35-mm dishes and cultured overnight. After the overnight culture period, Nile Red solution (10 μg/10 μl of dimethyl sulfoxide) was added for 5 min; the dish was washed with PBS and the cells were imaged using the microscope described above [30].

Real-Time PCR Measurements of mRNA Levels That Encode P4 Mediators

Total RNA was isolated from the human granulosa/luteal cells using RNeasy Plus Mini-Kit (Qiagen) per manufacturer's instructions and the amount of RNA isolated determined using a Nanodrop spectrophotometer. For each sample, 1 ng of RNA was converted to cDNA [31]. Real-time PCR was conducted using the cDNA as a template, primer pairs and probe for each P4 mediator, and actin (Supplemental Table S2) and SsoFast Probes Supermix (Bio-Rad Laboratories). All real-time PCR reactions were run in the CFX96 real-time PCR system. The cycling steps for enzyme activation were 30 sec at 95°C, denaturation for 5 sec at 95°C, and annealing/extension at 60°C for 1–10 sec; approximately 40 cycles were conducted. Gene expression was evaluated using the $2^{-\Delta\Delta CT}$ method provided by Bio-Rad CFX96 software. The mRNA level for each P4 mediator was expressed relative to actin.

In addition, an equal number of cells from each of the 10 individual patients were pooled and the mRNA level of the four P4 mediators (*PGRMC1*, *PGRMC2*, *PGR*, and *PAQR7*) determined by real-time PCR. The mRNA level for each P4 mediator was expressed relative to actin. The mRNA level of each P4 mediator for each patient was normalized to mean of the pooled control, which was set to 1.

Effect of Depleting P4 Mediators on Human Granulosa/Luteal Cell Function

Specific depletion of mRNA. To determine the specificity of each siRNA to deplete its targeted mRNA, 1×10^5 cells were cultured for 24 h in 35-mm dishes and transfected with either scramble (Silencer Negative Control) or an siRNA that targeted *PGRMC1*, *PGRMC2*, *PAQR7*, and *PGR* mRNA (Supplemental Table S3) using Lipofectamine 3000 according to manufacturers' instructions. The siRNAs were provided by Life Technologies (see Supplemental Table S3). After 48 h of incubation with the different siRNA treatments, total RNA was collected using RNeasy Plus Mini-Kit (Qiagen) per manufacturer's instructions and converted to cDNA. For each siRNA treatment, real-time PCR was performed as described previously to estimate the level of *PGRMC1*, *PGRMC2*, *PAQR7*, and *PGR* mRNA.

P4 secretion. One function of human granulosa/luteal cells is the ability to secrete P4. To assess the effect of P4 mediators, cells were cultured for 48 h with siRNA that targeted each P4 mediator. The medium was collected from the cultures, frozen, and stored at −80°C. Media samples were then thawed, diluted 1:50 with serum-free media, and assayed for P4 concentration, using the Progesterone EIA Kit (item #582601; Cayman Chemical Company). The detectable level of P4 of this assay was ≈8 pg/ml and all samples were assayed within the same assay.

Entry into the cell cycle. Human granulosa/luteal cells (1×10^5 cells in 250 μl of serum-supplemented media) were plated in each well of a four-well culture dish. After 24 h in culture, the cells were transfected with either siRNA targeted to one of the P4 mediators or Silencer Negative Control siRNA as described previously. After approximately 24 h, the cells were infected with baculovirus that encodes the ubiquitination domain of cytolethal distending toxin-1 (cdt-1)-RFP fusion protein (the red component of the FUCCI cell cycle sensor) and then cultured with nocodazole at a final concentration of 20 μM to arrest the cells in the G_2 stage of the cell cycle [32]. To assess the efficiency of the baculovirus to infect human granulosa/luteal cells, cells were infected with a baculovirus construct that encodes actin-GFP fusion protein. After overnight incubation, the nocodazole-containing medium was removed and replaced with fresh steroid-free serum (ATF 32351; HyClone)-supplemented medium in the presence of either 1 μM of P4 or equivalent volume of 100% ethanol. After 3 h, the medium was removed and the cells were washed three times with PBS, fixed in 4% paraformaldehyde for 7 min, washed with PBS, and stained with DAPI solution (0.2 μg/ml). The percentage of cells in the G_1/S stage of the cell cycle was determined by counting the number of cells expressing cdt-1-RFP fusion protein (red fluorescence), which is expressed by cells only in the G_1 or

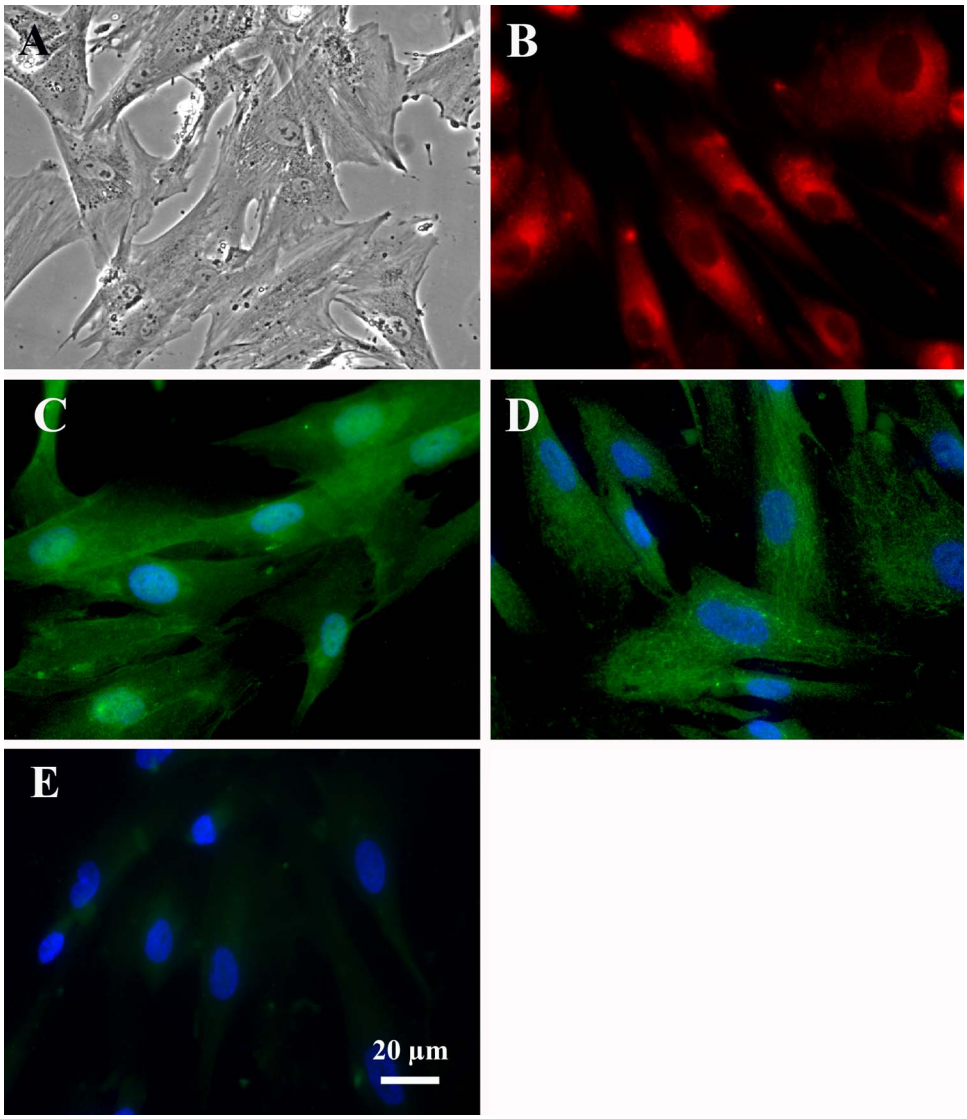

FIG. 1. The morphology (**A**) and the presence of Nile red-staining lipid droplets (**B**), CYP11A1 (**C**), and steroid acute regulatory protein, STARD1 (**D**), within cultured human granulosa/luteal cells. **A**) A phase image of cultured human granulosa/luteal cells. An image of human granulosa/luteal cells illustrating the presence of numerous lipid droplets (red) revealed by Nile red staining is shown in **B**. **C** and **D** show the presence of CYP11A and STARD1 as revealed by immunofluorescence (green). A negative control is shown in **E**. The nuclei in **C**, **D**, and **E** are stained with DAPI and shown in blue.

S stages of the cell cycle, and dividing the number of G_1/S stage cells by the total number of cells as revealed by DAPI staining (blue nuclei). This analysis was automated using the iVision Image Acquisition and Analysis Software (BioVision Technologies).

A similar study was conducted in which human granulosa/luteal cells were also infected with the geminin-GFP fusion protein construct, the green component of the FUCCI sensor that detects cells in the S/G_2 stage of the cell cycle. This study was conducted to determine if P4 accelerated the passage through the G_1/S stages of the cell cycle.

Localization and Interaction of the P4 Mediators

To better define the mechanism through which P4 acts it is important to first determine the cellular sites in which each P4 mediator resides. This was assessed by immunocytochemistry. In addition to cellular localization, in situ PLAs were conducted in order to determine if any of the P4 mediators interacted with each other.

Localization of P4 mediators by immunofluorescence. For the localization studies, human granulosa/luteal cells were plated on cover glass, cultured overnight, washed with PBS, fixed with 4% paraformaldehyde for 7 min, and treated with 0.1% Triton-X-100 and BSA, as previously described. The localization of each P4 mediator was detected by immunofluorescence using the appropriate primary and secondary antibodies (Supplemental Table S1). Negative controls were incubated using a nonspecific primary antibody (e.g., species-specific IgG).

In situ PLA. PLAs were used to assess the potential interactions between the different P4 mediators. The PLAs were performed according to the manufacturer's instructions (Olink Bioscience), using primary antibodies for each P4 mediator (Supplemental Table S1) and the appropriate secondary antibody pairs labeled with complementary oligonucleotide DNA sequences. These secondary antibodies were provided by Olink Bioscience. In this assay an interaction was detected if the two complementary oligonucleotide probes were in close enough proximity to hybridize and form a double-stranded DNA that could be amplified and detected by a fluorescent probe, which was visualized as an individual fluorescent (red) spot (http://www.olink.com/). For each assay, negative controls were performed by omitting one of the two primary antibodies.

Statistical Analysis

In each experiment all treatments were conducted in duplicate or triplicate and entire experiment replicated at least twice. Values from each experiment were pooled to generate a mean ± SEM. Differences between treatment groups were assessed by Student *t*-test if only means of two groups were compared or by an ANOVA followed by Fisher least significant difference post hoc test if more than two treatment groups were compared. Regardless of the analysis, *P*

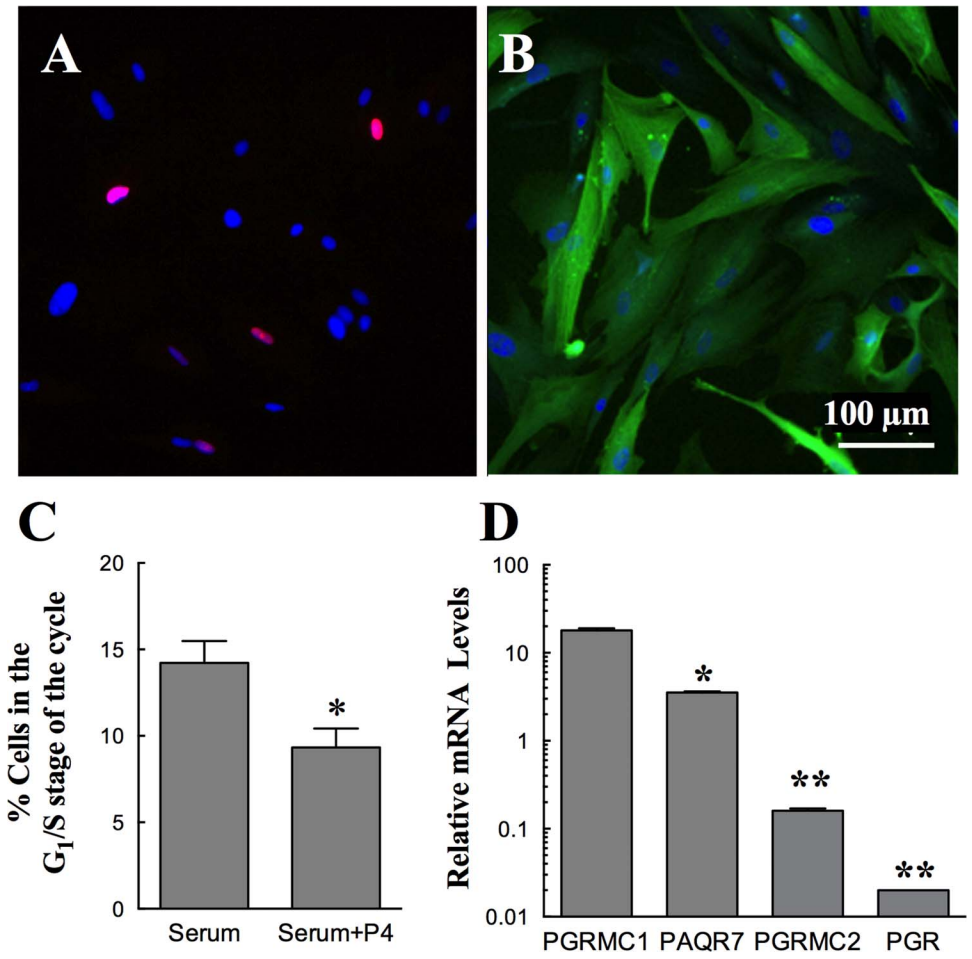

FIG. 2. **A**) Cells in the G_1/S stage of the cell cycle as identified by the presence of cdt-1-RFP (red nuclei); the nuclei of cells in other stages of the cell cycle are blue. Note that the limited number of cdt-1-RFP (red)-expressing cells is not due to inadequate infection, as nearly 100% of cells infected with a BacMan2.0 expression construct that encodes actin-GFP fusion protein are green (**B**). The ability of P4 to inhibit entry into the cell cycle is shown in **C**, and the relative mRNA levels of the P4 mediators in human granulosa/luteal cells as assessed by real-time PCR are shown in **D**. Values are expressed as a mean ± standard error. Data in **C** are based on nine paired observations obtained from three separate experiments. *Significantly less than serum control ($P < 0.05$). Values in **D** are means ± standard error (n = 3). *Significantly less than PGRMC1 mRNA ($P < 0.05$). **Less than both PGRMC1 and PAQR7 ($P < 0.05$).

≤ 0.05 was considered to be significantly different. The statistical analysis of the data was conducted using PRISM software (version 6.0; GraphPad).

RESULTS

Immunofluorescence analysis revealed that virtually 100% of cells isolated from follicular aspirates and maintained in culture contained numerous lipid droplets as detected by Nile red staining (Fig. 1 and Supplemental Fig. S1B). These cells also expressed the steroidogenic enzymes STARD1 and CYP11A1 (Fig. 1). These observations indicate that these cells retain many of the characteristics (i.e., steroidogenic capability) of luteinizing human granulosa cells.

Previous studies had demonstrated that P4 suppresses human granulosa/luteal cell proliferation [19, 20]. To determine if P4 suppresses proliferation by restricting entry into the cell cycle, human granulosa/luteal cells were infected with a baculovirus that encodes cdt-1-RFP and treated with nocodazole to arrest cells in the late G_2 stage of the cell cycle. After 24 h, the nocodazole was removed and the rate at which the cells entered the cycle assessed by determining the percentage of cells expressing cdt-1-RFP (a marker that identifies cells in the G_1/S stages of the cell cycle). As seen in Figure 2A, between 10% and 15% of the cells were observed to be in the G_1/S stage of the cell cycle as revealed by the red fluorescence. Importantly, virtually 100% of the cells expressed actin-GFP fusion protein after infection with a baculovirus construct that encodes actin-GFP fusion protein (Fig. 2B). Under these conditions, P4 suppressed the rate at which human granulosa/luteal cells transition from the G_2 stage and progressed to the G_1/S stage of the cell cycle (Fig. 2C). Studies were also conducted on human granulosa/luteal cells that were infected with geminin-GFP to detect cells in the G_2/S stage of the cell cycle. This study revealed that 2.16% ± 0.67% of the cells were in G_2/S stage of the cell cycle and this percentage was not affected by P4 (2.06% ± 0.35%, n = 5/group; $P > 0.05$).

Because there are at least four potential molecules that could mediate P4's action, real-time PCR was used to monitor the mRNA levels of the potential P4 mediators, *PGRMC1*, *PGRMC2*, *PGR*, and *PAQR7*. This study used mRNA that was isolated from a population of granulosa/luteal cells obtained by combining equal numbers of granulosa/luteal cells from each of the 10 patients. When expressed relative to actin mRNA, *PGRMC1* mRNA was the most abundant, followed by *PAQR7*, *PGRMC2*, and finally *PGR* (Fig. 2D). In addition, real-time PCR studies conducted on granulosa/luteal mRNA isolated from each of the 10 individual patients demonstrated

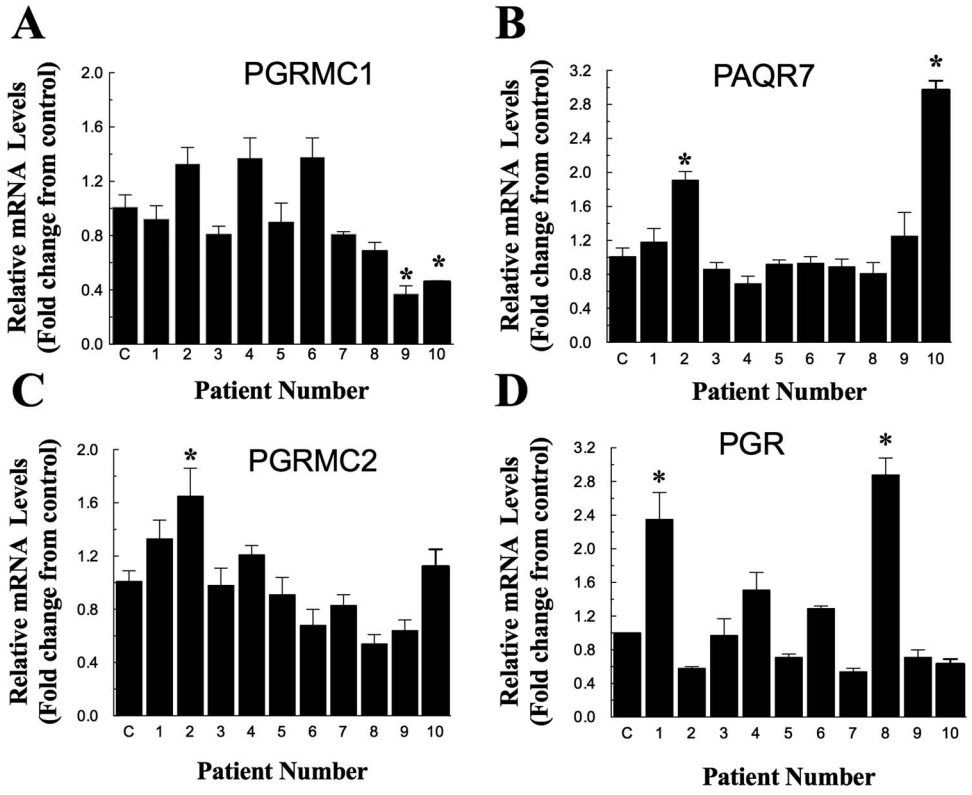

FIG. 3. **A–D**) The relative expression of each P4 mediator obtained from 10 individual patients. Values are normalized to the mean of the pooled control sample shown in Figure 2D. Values are means ± standard error (n = 3). *Significantly different from the control value.

that there was considerable patient variation in the expression profile of these P4 mediators (Fig. 3).

To assess the impact of each P4 mediator on human granulosa/luteal cell function, siRNAs that targeted each of the P4 mediators were used. As seen in Figure 4A, each siRNA depleted its targeted mRNA, reducing it by ≈80% of scramble control levels, while not significantly altering the mRNA levels on the other P4 mediators. Furthermore, P4 secretion was not affected by treatment with any of these siRNAs, with P4 levels typically ranging between 20 and 40 ng/ml after 48 h of culture (Figure 4B).

Treating cells for 48 h with scramble siRNA did not interfere with either the rate at which human granulosa/luteal cells entered the cell cycle or the ability of P4 to suppress cycle

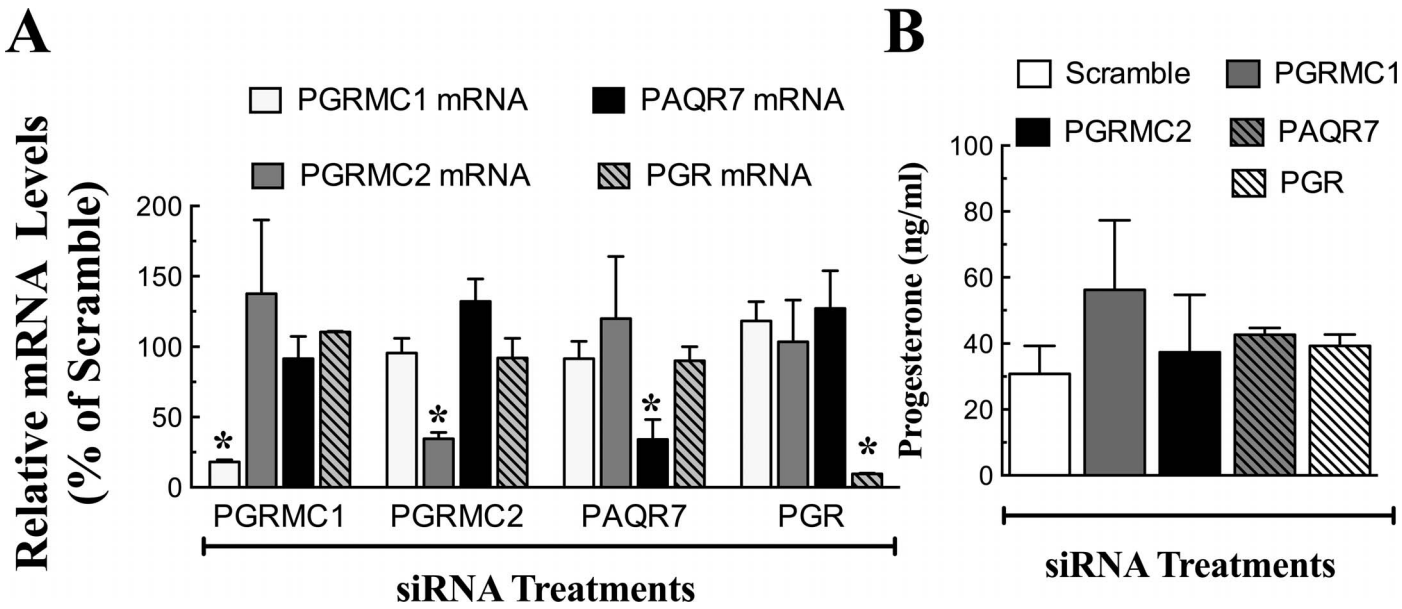

FIG. 4. The effect of a 48-h treatment with siRNA targeted to each P4 mediator on their mRNA levels is shown in **A**. In this panel the targeted siRNA treatment is plotted on the X-axis and the associated change in the mRNA level of each P4 mediator plotted on the Y-axis. Values are means ± standard error (n = 3). *Significantly different from scramble control value. **B**) The effect of each siRNA on P4 secretion. Values are means ± standard error (n = 3).

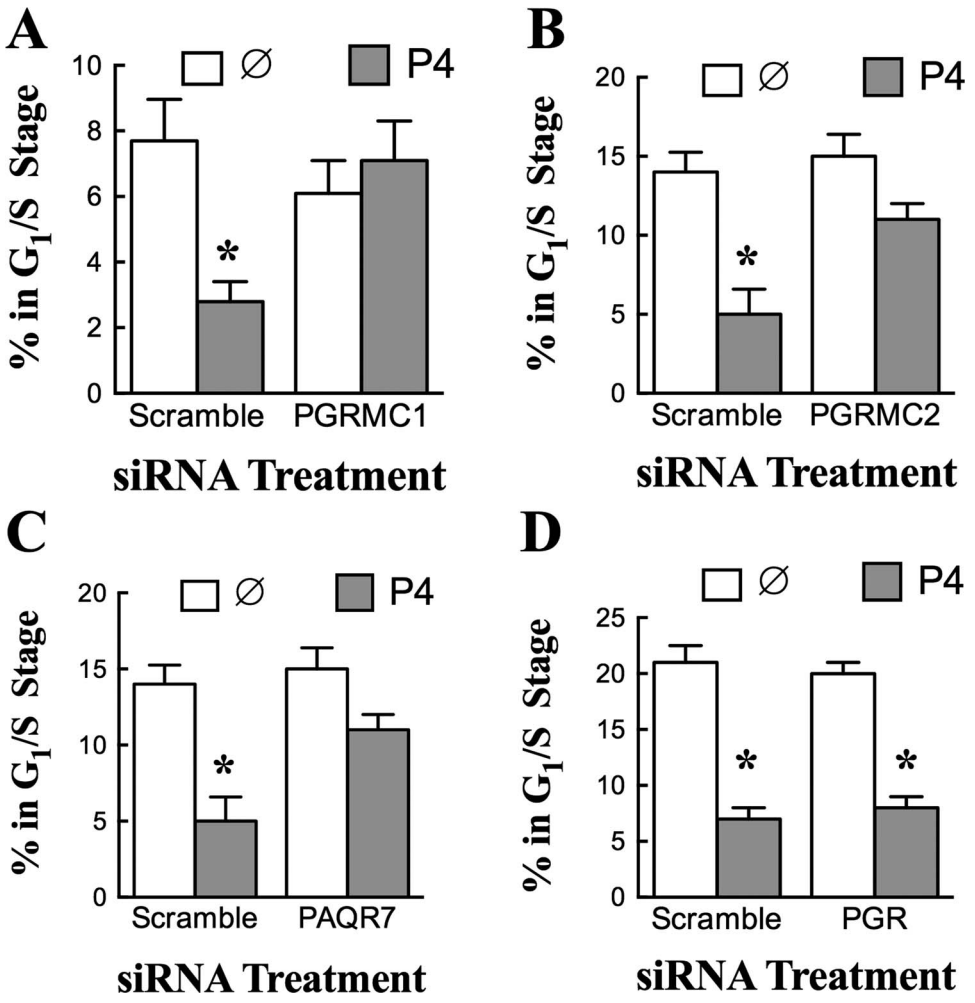

FIG. 5. The effect of PGRMC1 (**A**), PGRMC2 (**B**), PAQR7 (**C**), or PGR (**D**) siRNA on the ability of P4 to suppress entry into the cell cycle. As a control, cells were treated with scramble siRNA. Values represent means ± standard errors of four to six observations/treatment. *Significantly less than scramble control without P4 ($P < 0.05$).

cell entry (Fig. 5, A–D). However, depleting PGRMC1 (Fig. 5A), PGRMC2 (Fig. 5B), or PAQR7 (Fig. 5C) ablated P4's ability to suppress cell cycle entry. In contrast, PGR siRNA did not attenuate P4's ability to suppress the rate at which these cells entered the cell cycle (Fig. 5D).

To determine if these P4 mediators interacted with each other, immunofluorescence studies and PLAs were performed. The immunofluorescence studies showed that PAQR7 (Fig. 6A) and PGRMC2 (Fig. 6B) have a cytoplasmic localization, and PGRMC1 (Fig. 6C) and PGR (Fig. 6D) were localized to the cytoplasm and nucleus. In the nucleus PGRMC1 and PGR were not distributed evenly throughout the nucleoplasm but rather in a punctate pattern (insets in Fig. 6, C and D, respectively).

Interestingly, the PLAs revealed that in the cytoplasm PGRMC1 interacted with PAQR7 (Fig. 7A) and PGRMC2 (Fig. 7B). PGRMC2 also interacted with PAQR7 (Fig. 7C). All of these interactions appeared to occur frequently based on the PLA signal intensity (i.e., number of red dots) compared to that observed in the negative control (Fig. 7D). PGR also interacted with PGRMC1 in the cytoplasm (Fig. 8A). In the nucleus PGR interaction with PGRMC1 was limited based on the PLA signal intensity (Fig. 8A inset) and was not observed in all cells. PGR did not appear to interact with PGRMC2 (Fig. 8B) and PAQR7 (Fig. 8C), as the PLA signal for each of these interactions was similar to that observed in the negative control (Fig. 8D).

DISCUSSION

The present study confirms the findings of Ferrero et al. [29] that human granulosa/luteal cells can be isolated at the time of oocyte retrieval and cultured for several passages. Moreover, our studies demonstrate that these cells can be frozen, thawed, and then used in subsequent experiments. These cultured granulosa/luteal cells retain many characteristics of freshly isolated granulosa/luteal cells. Specifically, these cells maintain their capacity to synthesize and secrete steroids, as evidenced by the presence of 1) Nile red lipid droplets; 2) enzymes that are required for steroidogenesis, STARD1 and CYP11A1; and 3) P4 in the spent culture medium. Importantly, these cells also undergo mitosis, albeit at a slow rate, and are responsive to the antimitotic action of P4. The ability to proliferate in vitro appears to be related to the effective removal of the blood mononuclear cells (i.e., leukocytes, monocytes, and macrophages) prior to placing the granulosa/luteal cells in culture [29]. This concept is supported by the observation that the addition of leukocytes to the cultures of human granulosa/luteal cells greatly enhances their ability to differentiate and secrete P4 [33, 34], which in turn would reduce mitosis. Taken together, these findings indicate that if blood mononuclear cells are removed, human granulosa/

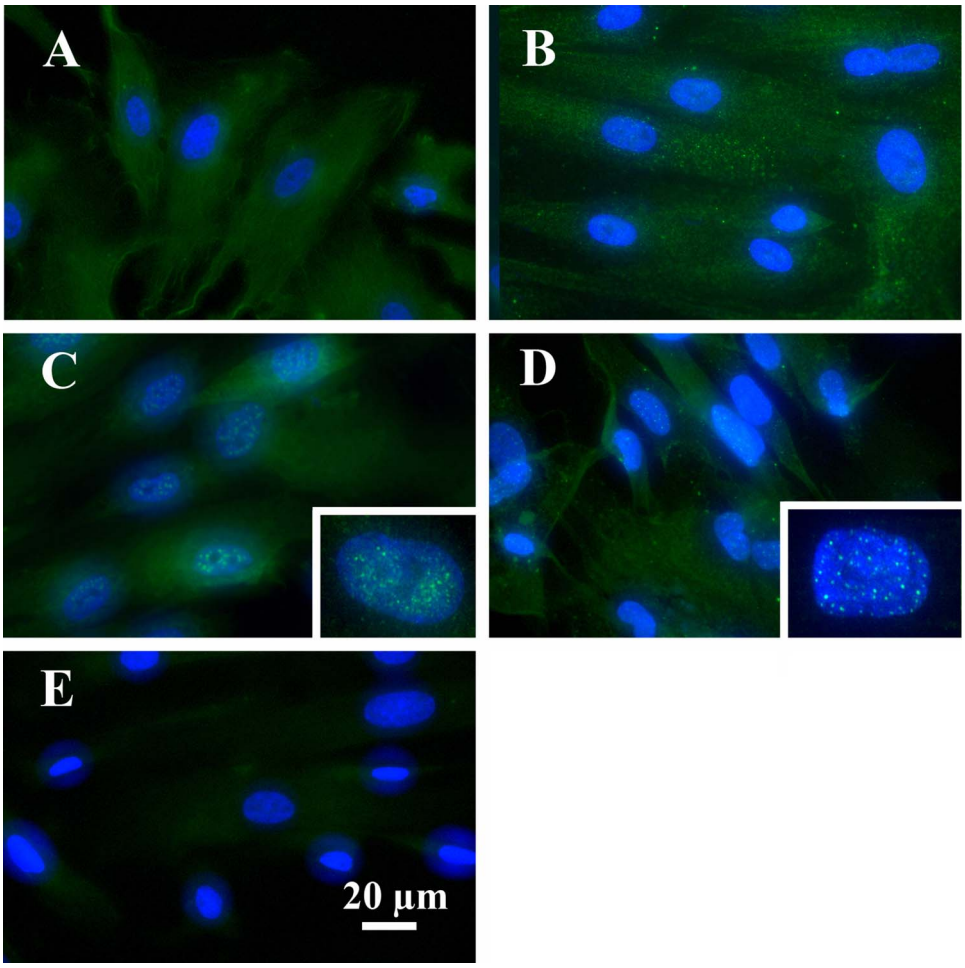

FIG. 6. Localization of PAQR7 (**A**) PGRMC2 (**B**), PGRMC1 (**C**), and PGR (**D**). A negative control is shown in **E**. The presence of each protein is shown in green and the nuclei are stained with DAPI and shown in blue. The insets in **C** and **D** are shown at three times the magnification of the other images.

luteal cells can be maintained in culture for long periods of time and retain their granulosa/luteal cell characteristics. Although not identical to freshly harvested granulosa/luteal cells, these cells provide a suitable model system to elucidate the mechanism through which P4 regulates human granulosa/luteal cell function.

To determine whether P4's antimitotic action is due to suppressing entry into the cell cycle, cells are infected with the red component of the FUCCI cell cycle sensor to detect cells in the G_1/S stages of the cell cycle, and shortly thereafter cells are treated with nocodazole to arrest cells in the G_2 stage of the cell cycle. Twenty-four hours later, nocodazole is removed and the percentage of cells that enter the cell cycle (i.e., in G_1/S stages of the cell cycle) monitored. The FUCCI cell cycle sensor is useful because it requires relatively few cells and yet provides data comparable to that obtained by FACS analysis [35]. Through the use of this protocol, the present studies reveal that P4's ability to suppress human granulosa cell/luteal mitosis is mediated in part by restricting entry into the cell cycle.

The precise stage at which P4 acts remains to be determined. In our model nocodazole is used to block FUCCI-infected cells in the late G_2 stage of the cell cycle and then nocodazole is removed, the cells are cultured in the presence or absence of P4, and the percentage of cells in G_1/S determined. Given the limitations of this model, P4 could act to suppress entry into the cells cycle at several points in the cell cycle. One possibility is that P4 acts by restricting the transition from the G_2 stage of the cycle and/or at any point thereafter prior to entry into the G_1 stage of the cell cycle. Alternatively, P4 could accelerate passage through G_1/S, which would also result in few cells in the G_1/S of the cell cycle. However, our finding that the percentage of cells in the G_2 stage of the cell cycle is not affected by P4 does not support this possibility.

How P4 restricts entry into the cell cycle is complicated in that human granulosa/luteal cells express several potential P4 mediators, including PGRMC1, PGRMC2, PAQR7, and PGR. These potential P4 mediators are expressed at very different levels, with *PGRMC1* mRNA being the most abundant, followed by *PAQR7*, *PGRMC2*, and *PGR* mRNAs. Further, there is a rather large range of expression, with *PGRMC1* mRNA being 1000 times more abundant than PGR. This is consistent with the finding that PGR is only expressed in 20% of freshly isolated human granulosa/luteal cells, whereas PGRMC1 is detected in 100% of these cells [28]. That PGRMC1 is expressed at the highest level is potentially important in that low levels of PGRMC1 are observed in women with premature ovarian insufficiency [36] and polycystic ovarian disease [37], and elevated levels of *PGRMC1* [38] and *PGRMC2* [6] are associated with reduced follicle development in response to gonadotropins. It seems, then, that homeostatic levels of PGRMC1 and PGRMC2 are necessary for normal granulosa cell function.

The present studies also demonstrate that there is considerable patient variation in the relative expression of each of the

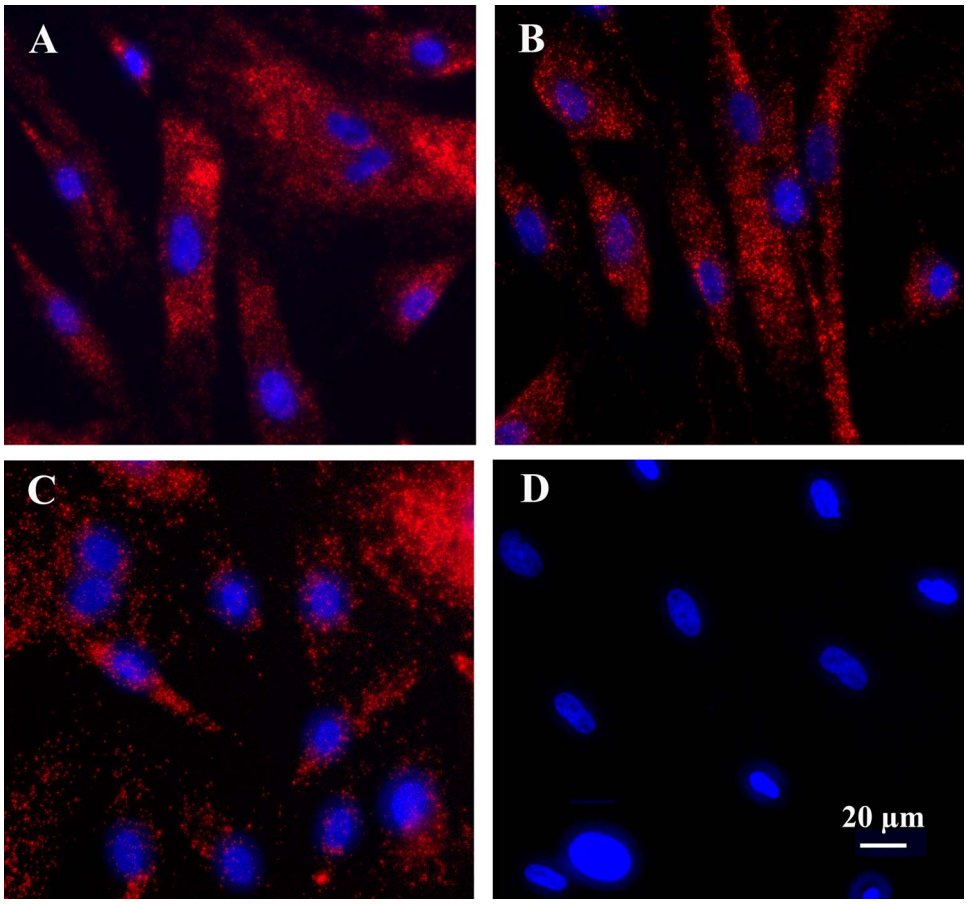

FIG. 7. Interactions between PGRMC1 and PAQR7 (**A**), PGRMC1 and PGRMC2 (**B**), and PGRMC2 and PAQR7 (**C**). A negative control is shown in **D**. The frequency of interaction is revealed by the number of red dots. The nuclei are stained with DAPI and shown in blue.

P4 mediators. For example, *PGR* mRNA levels of patients 1 and 8 are $\geq$2.5-fold greater than the mean of the pooled control (i.e., average value of all 10 patients). Similarly, PAQR7 mRNA levels are 2–3-fold greater than control levels in patients 2 and 10 and there is nearly a 2-fold increase in PGRMC2 levels in patient 2. Finally, PGRMC1 mRNA levels in patients 9 and 10 are significantly suppressed. The reasons for this variability are unknown and could include slight differences in ovulation induction protocols or epigenetic changes as observed previously [38]. Regardless of the source of variability, these studies are consistent with the concept that the expression profile of P4 mediators dictates the response to P4 and in part the number of follicles each patient develops in response to a gonadotropin-based ovarian stimulation protocol.

Although the concept that the expression profile of the P4 mediators plays an important role in regulating gonadotropin-induced follicular development is interesting, it is based solely on correlative expression data. To begin to gain insight into the functional role of each P4 mediator, siRNA treatments were used to selectively deplete the mRNA that encodes each P4 mediator. Although changes in protein levels were not assessed due to limited cell numbers, the siRNA-based approach allows for the depletion of the mRNA of one of the four P4 mediators without significantly altering the expression of the other three. Interestingly, the capacity of the granulosa/luteal cells to secrete P4 is not altered by depleting any of these receptors. This is somewhat surprising because PGRMC1 has been implicated in promoting cholesterol synthesis [39] and adrenal steroidogenesis [40, 41]. Specifically, PGRMC1 binds to and activates CYP51A1 family members that are involved in promoting cholesterol synthesis [39] and interacts with the proteins SCAP and INSIG1 [42]. The interactions with SCAP and INSIG1 control cholesterol metabolism by regulating the transcriptional activity of sterol regulatory element binding protein, which induces the expression of steroidogenic acute regulatory protein (STAR) [43]. In spite of its role in cholesterol synthesis, our present findings, as well as previous studies with hGL5 cells, a cell line derived from human granulosa/luteal cells [27], fail to show an effect of depleting PGRMC1 on P4 secretion. It is important to appreciate that PGRMC1 promotes cholesterol synthesis in response to low cellular levels of cholesterol [39]. Under the present culture conditions, cholesterol levels do not appear to be limiting, given the large number of lipid droplets detected by Nile red staining. This could explain the inability of PGRMC1 to influence P4 secretion. Therefore, a role for PGRMC1 in P4 synthesis in human granulosa/luteal cells remains to be conclusively demonstrated.

Similarly, depleting PGR did not alter P4 secretion. This contradicts previous work on rat and human granulosa/luteal cells in which the ability of the PGR antagonists, Org 31710 and RU486, to suppress P4 synthesis was attributed to their ability to inhibit the expression of genes involved cholesterol synthesis [44]. In this study, an attempt to override the effect of the PGR antagonists with supplemental P4 was not made. Thus, a definitive role for PGR in regulating P4 synthesis remains to be established and requires further investigation.

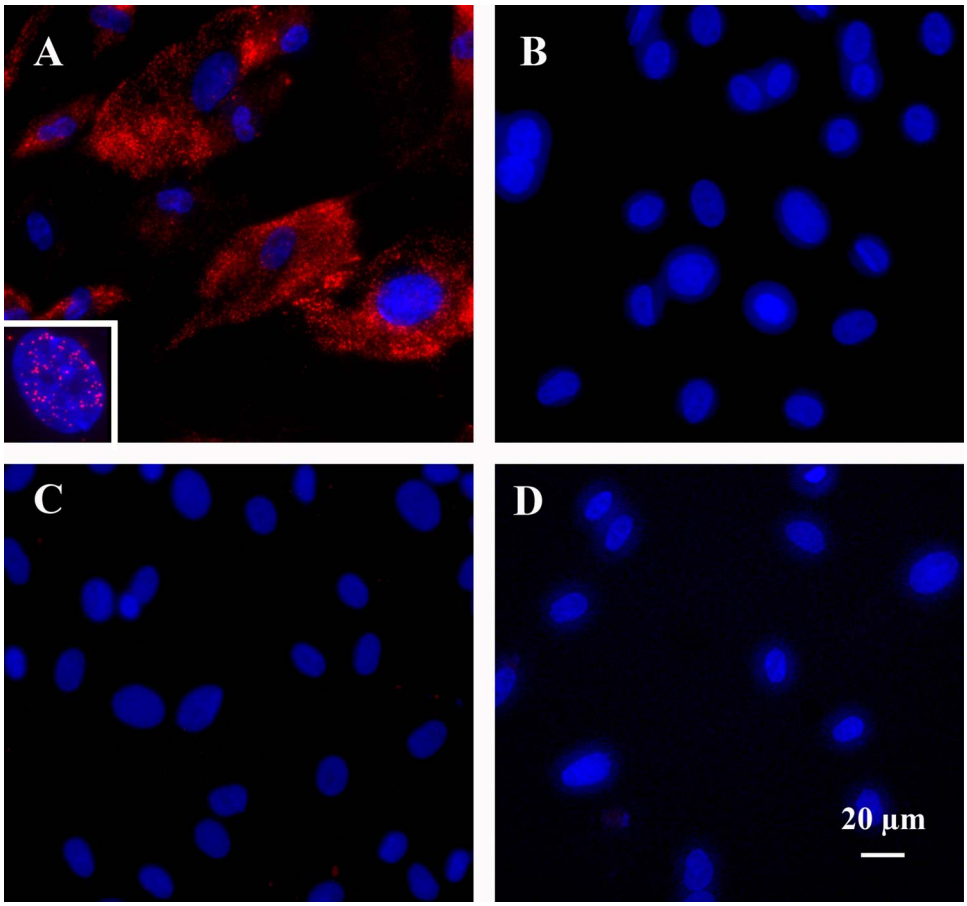

FIG. 8. Interactions between PGR and PGRMC1 (**A**), PGR and PGRMC2 (**B**), and PGR and PAQR7 (**C**). A negative control is shown in **D**. The frequency of interaction is revealed by the number of red dots. The inset in **A** is shown at three times the magnification of the other images. The nuclei are stained with DAPI and shown in blue.

Although altering the expression of each P4 mediator does not influence P4 secretion, depleting PAQR7, PGRMC1, or PGRMC2 completely attenuates P4's ability to suppress entry into the cell cycle. In contrast, PGR depletion does not alter the capacity of P4 to restrict cell cycle entry. The inability to detect an effect of PGR on entry into the cell cycle may be surprising, but it is consistent with the work of Lydon et al. [45]. These investigators show that PGR is absolutely necessary for ovulation but follicular development proceeds normally in PGR-null mice. This suggests that granulosa cell proliferation is independent of the presence or absence of PGR.

That PAQR7, PGRMC1, or PGRMC2 is essential for P4's action on cell cycle traverse raises the question as to the mechanism through which each of these P4 mediators act to regulate this aspect of P4's action. There are two obvious possibilities that could account for P4's action being dependent on PAQR7, PGRMC1, and PGRMC2. The first possibility is that each of these three mediators regulates separate and independent signal transduction pathways with each being required for P4's action. The second possibility is that the three proteins are components of the same P4-regulated signal transduction pathway. Although the mode through which each P4 mediator transduces P4's actions awaits extensive research, determining their cellular localization and assessing whether they interact with each other can provide insight into their mechanism of action.

Interestingly, the present studies indicate PAQR7, PGRMC1, and PGRMC2 localize to the cytoplasm. Although there is evidence that PAQR7 [46] and PGRMC1 [47] also localize to the plasma membrane, the immunofluorescence approach used in these studies is not sufficiently precise to distinguish between a purely cytoplasm localization versus a plasma membrane localization. Importantly, PLA experiments suggest that these three proteins interact in the cytoplasm and/or possibly at the plasma membrane. Moreover, previous coimmunoprecipitation assays confirm the interaction between PAQR7 and PGRMC1 [48] and PGRMC1 and PGRMC2 [16]. Although coimmunoprecipitation studies will have to be conducted to confirm these interactions in human granulosa/luteal cells, the present PLA data support the concept that these three P4 mediators interact with each other. If so, then it is likely that they are essential components of the same P4-signaling pathway. Additional support for this concept comes from Thomas et al., who show that PAQR7 and PGRMC1 interact to form a complex that is required to bind P4 [48]. Although these three P4 mediators interact, this does not exclude the possibility that each of these three proteins could also have independent actions. This is particularly true for PAQR7, because it activates G proteins and regulates intracellular cAMP levels depending on cell type [49].

The observations that depleting PGR neither alters P4 secretion nor affects P4's ability to restrict entry into the cell cycle should not be taken to indicate that PGR does not play a role in regulating human granulosa/luteal function. PGR is known to have both cytoplasmic [50, 51] and nuclear sites of action [52]. In the cytoplasm PGR interacts with c-src and

promotes MAP kinase signaling [50]. In the nucleus PGR functions as a transcription factor [52]. In mouse granulosa cells, PGR regulates the transcription of over 100 genes that are involved in various aspects of ovarian function [53]. Interestingly, PGR-PGRMC1 interaction is observed mainly in the cytoplasm and to a lesser extent in the nucleus of many granulosa/luteal cells. Because both the cytoplasmic and the nuclear PGRMC1 interact with PGR as judged by PLA, it is possible that their interaction plays a role in modulating PGR-dependent actions that are not involved in regulating entry into the cell cycle or P4 secretion in human granulosa/luteal cells. One such action may involve P4's ability to suppress apoptosis of human granulosa/luteal cells, because both PGRMC1 [27, 28] and PGR [54] have been implicated in this P4-dependent action. This possibility merits further investigation.

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