BIOLOGY OF REPRODUCTION (2014) **91**(5):104, 1–12
Published online before print 24 September 2014.
DOI 10.1095/biolreprod.114.122986

Progesterone Receptor Membrane Component-1 (PGRMC1) and PGRMC-2 Interact to Suppress Entry into the Cell Cycle in Spontaneously Immortalized Rat Granulosa Cells[1]

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ABSTRACT

Progesterone receptor membrane component 1 (PGRMC1) and PGRMC2 are expressed in rat granulosa cells and spontaneously immortalized granulosa cells (SIGCs) but their biological roles are not well defined. The present studies demonstrate that depleting either *Pgrmc1* or *Pgrmc2* in SIGCs increases entry into the cell cycle but does not increase cell proliferation. Rather, PGRMC1 and/or PGRMC2-deplete cells accumulate in metaphase and undergo apoptosis. Because both PGRMC1 and PGRMC2 localize to the mitotic spindle, their absence likely accounts for cells arresting in metaphase. Moreover, pull-down assays, colocalization studies and in situ proximity ligation assays (PLA) indicate that PGRMC1 binds PGRMC2. Disrupting the PGRMC1:PGRMC2 complex through the use of siRNA or the cytoplasmic delivery of a PGRMC2 antibody increases entry into the cell cycle. Conversely, overexpressing either PGRMC1-GFP or GFP-PGRMC2 fusion protein inhibits entry into the cell cycle. Subsequent studies reveal that depleting PGRMC1 and/or PGRMC2 reduces the percentage of cells in G_0 and increases the percentage of cells in G_1. These observations indicate that in addition to their role at metaphase, PGRMC1 and PGRMC2 are involved in regulating entry into the G_1 stage of the cell cycle. Interestingly, both PGRMC1 and PGRMC2 bind GTPase-activating protein-binding protein 2 (G3BP2) as demonstrated by pull-down assays, colocalization assays, and PLAs. *G3bp2* siRNA treatment also promotes entry into the G_1 stage. This implies that dynamic changes in the interaction among PGRMC1, PGRMC2, and G3BP2 play an important protein regulating the rate at which SIGCs enter into the cell cycle.

apoptosis, granulosa cells, mitosis, PGRMC1, PGRMC2

INTRODUCTION

Membrane-associated progesterone receptors (MAPRs) are a group of closely related proteins [1] that play important roles in regulating ovarian function [2, 3]. Progesterone receptor membrane component 1 (PGRMC1) is the first member of this group to be identified [4] and there are clinical data illustrating its importance in follicular development. Specifically, both deletion and point mutations in the *Pgrmc1* are linked to premature ovarian failure in women [5]. Similarly, PGRMC1 is expressed at very low levels in women with polycystic ovarian syndrome [5, 6]. Finally, poor follicular development is associated with elevated *Pgrmc1* mRNA levels in granulosa cells of women undergoing controlled ovarian stimulation as part of their infertility treatment [7]. All three of these clinical examples support a role for PGRMC1 in ovarian follicular development. PGRMC2 is the second member of the MAPR family [8] and its expression is elevated in women with diminished ovarian reserve [9], suggesting that PGRMC2 may also play a role in regulating ovarian follicle development.

Although there are clinical data implicating PGRMC1 and PGRMC2 as regulators of ovarian function, the mechanism through which these proteins influence ovarian function is just beginning to be investigated. It is known that both MAPR family members are highly expressed in granulosa cells [10–12] and may be involved regulating granulosa cell mitosis. For example, there is a 50% reduction in the number of antral follicles present within the immature ovary of conditional knockout mice in which PGRMC1 is depleted from granulosa cells [2, 3]. This suggests that PGRMC1 plays an essential role in granulosa cell mitosis during the transition of preantral follicles into antral follicles. PGRMC2 also seems to be involved in granulosa cells mitosis, as evidenced by initial studies using a granulosa cell line, spontaneously immortalized granulosa cells (SIGCs). In these cells, depleting PGRMC2 using siRNA promotes entry into the cell cycle but does not increase cell number [10]. Rather there is an increased incidence of apoptosis. It appears, then, that both PGRMC1 and PGRMC2 regulate granulosa cell mitosis, but their mode of action is basically unknown.

The function of PGRMC1 and PGRMC2 in the ovary is generally discussed in relationship to progesterone-mediated effects on mitosis and apoptosis, given that depleting either MAPR attenuates the antiapoptotic and/or antimitotic action of progesterone (P4) [2, 3, 10–14]. Although PGRMC2 is essential for P4's antimitotic action [10] *Pgrmc2* siRNA treatment does not reduce the capacity of SIGCs to bind P4 [10]. This is in contrast to *Pgrmc1* siRNA treatment, which virtually eliminates the ability of SIGCs to bind P4. Thus, PGRMC2's capability to regulate P4's actions in SIGCs is dependent on PGRMC1, although the nature of this dependency is unknown.

Finally, PGRMC1 and PGRMC2 may also have P4-independent actions. For example, in SIGCs, *Pgrmc1* siRNA alters gene expression, increasing several genes known to promote apoptosis in the absence of supplemental P4 [13, 15]. Similar *Pgrmc1* siRNA-based studies conducted on human

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Received: 30 June 2014.
First decision: 30 July 2014.
Accepted: 3 September 2014.

eISSN: 1529-7268 http://www.biolreprod.org
ISSN: 0006-3363

granulosa cells (i.e., hGL5 cells) suggest that PGRMC1 functions to suppress the expression of several genes involved in initiating or mediating apoptosis [15]. The ability of PGRMC1 to regulate gene expression may be mediated in part by its ability to regulate Tcf/Lef-based transcriptional activity [16]. Although PGRMC2's role in mitosis is just beginning to be assessed, recent data suggest that PGRMC2's action on mitosis involves an interaction with cyclin-dependent kinase 11b [10], which is involved in regulating the cell cycle cascade [17, 18].

Taken together, these data provide the rationale for the present series of studies, which is designed to define the functional relationship among PGRMC1, PGRMC2, and SIGC mitosis. Subsequent studies focused on identifying proteins that interact with PGRMC1 and/or PGRMC2 in order to gain insight into the mechanism through which PGRMC1 and PGRMC2 influence mitosis.

MATERIALS AND METHODS

SIGC Culture

All of the chemicals used in this study were purchased from Sigma Chemical Co. unless stated otherwise. Details regarding the antibodies used in this paper are provided in Supplemental Table S1 (Supplemental Data are available online at www.biolreprod.org). Spontaneous immortalized granulosa cells (SIGCs), which were derived from rat preovulatory follicles [19], were maintained in Dulbecco modified Eagle medium (DMEM)/F12 supplemented with 5% fetal bovine serum (FBS) as previously described [12, 14]. Unless otherwise stated, 4×10^5 cells were placed in 35-mm^2 dishes with or without cover glass in 2 ml of DMEM/F12 with 5% FBS. The protocol to obtain the granulosa cells that were used to generate the data shown in Supplemental Figure S1 was approved by the University of Connecticut Health Center Institutional Animal Care and Use Committee.

Depletion of PGRMC1 and/or PGRMC2 Levels

To determine the effect of depleting PGRMC1 and/or PGRMC2, SIGCs were cultured for 24 h and then transfected using Lipofectamine 2000 with *Pgrmc1* (siRNA ID #253165; Life Technologies) and/or *Pgrmc2* siRNA (siRNA ID #s168258; Life Technologies). As a control, a scramble siRNA (AM4611; Life Technologies) was used. Twenty-four hours after transfection, the cells from one 35-mm^2 dish were harvested, plated into two 35-mm^2 dishes, and cultured with DMEM/F12 supplemented with 5% steroid-free FBS for an additional 24 h. The cultures were then used either to measure *Pgrmc1*, *Pgrmc2*, and *Pairbp1* mRNA levels by real-time PCR as previously described [10, 12, 14, 20] or to monitor indicators of cell proliferation.

Assessment of Cell Proliferation

Several different methods were used to assess cell proliferation. These included 1) monitoring 5-bromo-2′-deoxyuridine (BrdU) incorporation and entry into metaphase, 2) determining the percentage increase in cell number and apoptotic cells, and 3) identifying specific stages of the cell cycle using a fluorescence ubiquitination cell cycle probe (FUCCI).

BrdU incorporation and entry into metaphase. SIGCs were plated and transfected with siRNA as described previously. For the last 24-h culture period, the culture medium was removed and replaced with DMEM/F12 with 5% steroid-free FBS. After 22 h of culture, the medium was supplemented with 0.1 μg/ml of colchicine and 1 μM BrdU. BrdU was not added to one culture dish, which served as a negative control. After 2 h of colchicine and BrdU treatment, the cells were washed with 0.1% bovine serum albumin in PBS and fixed in 70% ethanol for 30 min on ice, permeabilized with 2N HCl/0.5% Triton X-100 for 30 min at room temperature, and washed in PBS, pH 8.5, for 2 min and then in PBS, pH 7.0. The cover glasses were then incubated with a fluorescein isothiocyanate (FITC)-labeled BrdU antibody in 0.5% Tween-20 in PBS for 30 min at room temperature, washed with PBS, pH 7.0, stained with 4′,6-diamidino-2-phenylindole (DAPI; 1:5000 in PBS) and mounted in ProLong Anti-fade mounting medium. The cells that incorporated BrdU were revealed by green fluorescence and mitotic figures detected by DAPI staining (blue fluorescence). The number of DAPI-stained cells was counted and used to calculate the percentage of BrdU-incorporating cells and the percentage of metaphase cells.

Determination of the percentage increase in cell number and apoptotic cells. SIGCs were plated and transfected with siRNA as described above with the exception that after the initial 24-h culture period the cells were plated in 35-mm^2 dishes in which the bottom plate was scored with a diamond pen to identify four quadrants. Two hours later, the culture medium was removed and the dishes washed gently with DMEM/F12 to remove any cells that were not firmly attached. The culture medium was then replaced and pictures were taken of each quadrant. After 22 h of culture, pictures were taken of the same quadrants. The number of cells in each quadrant at 2 and 24 h of culture was counted and the percentage increase in cell number calculated. The cells were then stained with DAPI to calculate the percentage of metaphase cells and the percentage of apoptotic cells. A cell was considered to be apoptotic if its nucleus was condensed and/or fragmented [12, 14].

Identification of specific stages of the cell cycle using the FUCCI probe. For these studies, SIGCs were plated and transfected with siRNA as previously described. After 24 h, the cells were infected with FUCCI (20 viral particles per cell) and cultured for an additional 24 h. The cells were fixed and stained with DAPI, and images were taken of the same field of cells under phase and fluorescent optics, with the fluorescent images captured under the GFP, RFP, and DAPI filter sets.

FUCCI utilizes a baculovirus that infected virtually all the SIGCs [10] with DNA that encodes either the geminin-GFP fusion protein or cytolethal distending toxins (cdt)-RFP fusion protein. Geminin and cdt are expressed in G_2 and G_1, respectively, and both are expressed in the S phase of the cycle. Based on this, cells that fluoresced only blue (i.e., stained only with DAPI) and possessed interphase nuclei were considered to be in the G_0 stage, red fluorescent cells were in the G_1 stage, and green fluorescent cells were in the G_2 stage. S-stage cells were observed as fluorescing yellow after merging the green and red fluorescent images. Cells that fluoresced only blue (i.e., stained only with DAPI) and possessed chromosomes organized in a metaphase plate were considered to be in metaphase.

PGRMC1 and PGRMC2 Interaction

The interaction between PGRMC1 and PGRMC2 was assessed using three approaches. These include GFP-based pull-down, colocalization, and in situ proximity ligation assay (PLA).

GFP pull-down protocol. For the GFP pull-down, SIGCs were cotransfected with individual expression vectors that encode GFP-PGRMC2 [8, 21, 22] and PGRMC1-Flag [16]. The protocol to isolate GFP-PGRMC2 used GFP-antibody labeled magnetic beads as previously described [23]. The GFP-based isolate was used in a Western blot analysis that used a rabbit anti-GFP antibody or a mouse anti-Flag antibody to detect GFP-PGRMC2 and PGRMC1-Flag fusion proteins, respectively.

Colocalization protocol. PGRMC1 and PGRMC2 were colocalized using a rabbit polyclonal anti-PGRMC1 antibody and a mouse monoclonal anti-PGRMC2 antibody. PGRMC1 and PGRMC2 antibodies were detected using secondary antibody labeled with Alexa Fluor 488-labeled anti-rabbit antibody or an Alexa Fluor 546-labeled anti-mouse antibody, respectively. As negative controls cells were only incubated with secondary antibodies labeled with Alexa Fluor 488 and/or Alexa Fluor 546 (red fluorescence). The cells were then stained with DAPI (1:500 dilution) and mounted on a slide using ProLong Anti-fade mounting medium.

In situ PLA. The PLA enables the detection of protein-protein interactions in fixed cells using an immunocytochemical-based protocol [16, 20] per manufacturer's instruction (Olink Bioscience, http://www.olink.com). In this protocol, cells were fixed and then incubated with anti-PGRMC1 and anti-PGRMC2 antibodies as per the colocalization protocol. Then the cells were incubated with a pair of oligonucleotide-labeled secondary antibodies: anti-rabbit PLUS and anti-mouse MINUS. The secondary antibodies were labeled with complementary DNA oligonucleotides, which under the assay conditions are hybridized and amplified. The interaction between PGRMC1 and PGRMC2 was detected by hybridization of the fluorescent-labeled probe that detects the amplified DNA as a red fluorescent dot. At the completion of the PLA protocol, the cover glass was mounted onto a glass slide using PLA mounting medium, which contained DAPI. As a negative control one or both of the primary antibodies were omitted from the PLA reaction.

PGRMC1:PGRMC2 Interaction and Cell Cycle Traverse

In order to disrupt the interaction between PGRMC1 and PGRMC2, either normal goat IgG or a goat anti-PGRMC2 antibody was delivered into SIGCs using the Chariot transfection reagent (Active Motif) as previously described [24]. After 3 h of culture, colchicine (0.1 μg/m) and FITC-labeled BrdU (1 μM) were added and the cultures continued for an additional 2 h. The cells were then fixed and stained with DAPI. For each treatment at least 200 SIGCs were evaluated by sequentially imaging random fields of cells under the GFP filter

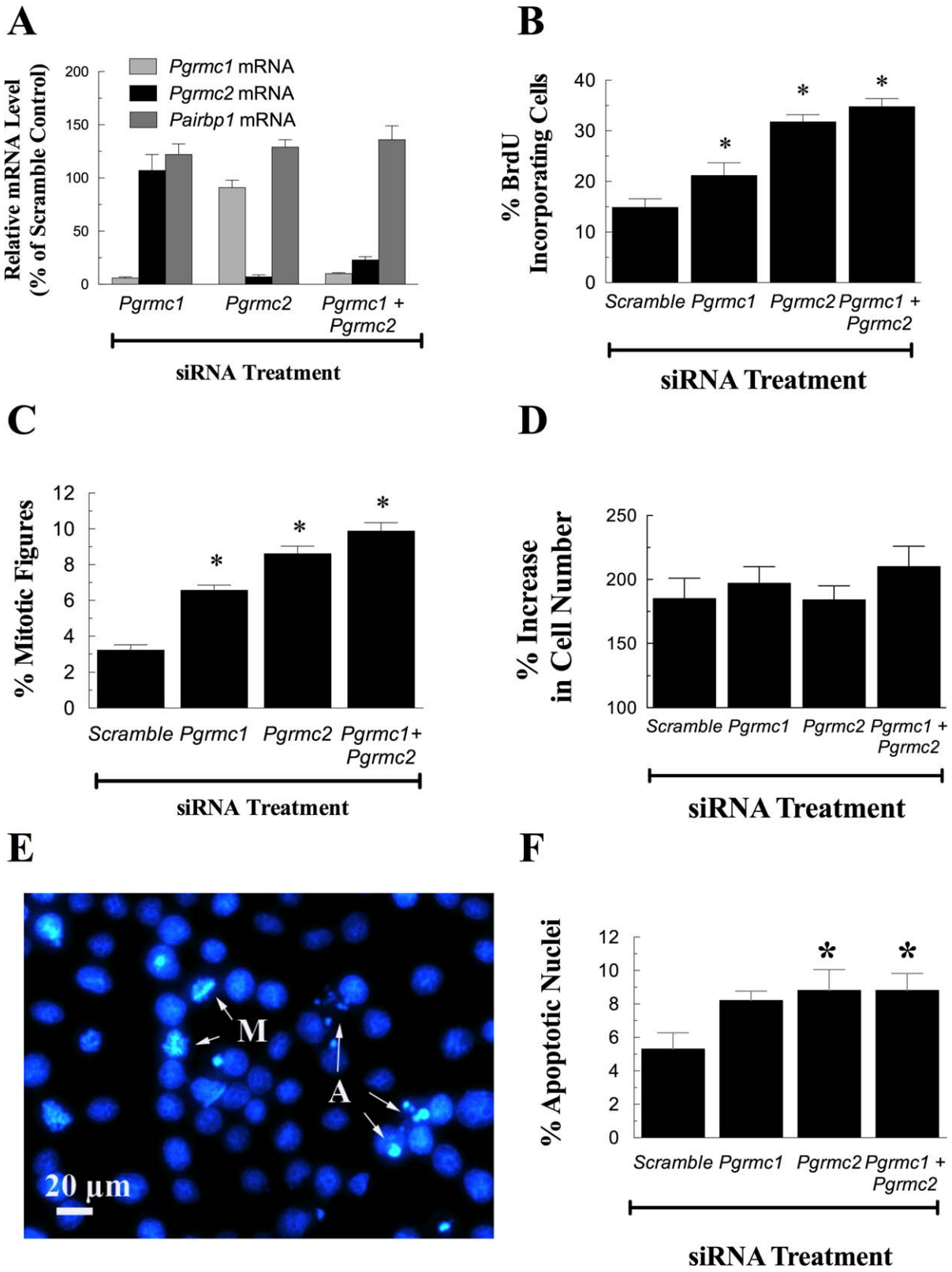


FIG. 1. The effect of *Pgrmc1* and/or *Pgrmc2* siRNA treatment on the mRNA levels of *Pgrmc1*, *Pgrmc2*, and *Pairbp1* (**A**); percentage of cells incorporating BrdU (**B**); percentage of cells arresting in metaphase in the presence of colchicine (**C**); and fold increase in the number of cells present after 24 h of culture (**D**). **E**) A DAPI-stained image of cells treated with *Pgrmc1* and *Pgrmc2* siRNA, which reveals the presence of cells in metaphase (marked by M arrows) and apoptotic cells (marked by A arrows). The effect of *Pgrmc1* and/or *Pgrmc2* siRNA treatment on the percentage of apoptotic nuclei is shown in **F**. In this and subsequent figures, the values are means ± standard error. *Value that is significantly different from scramble control ($P < 0.05$).

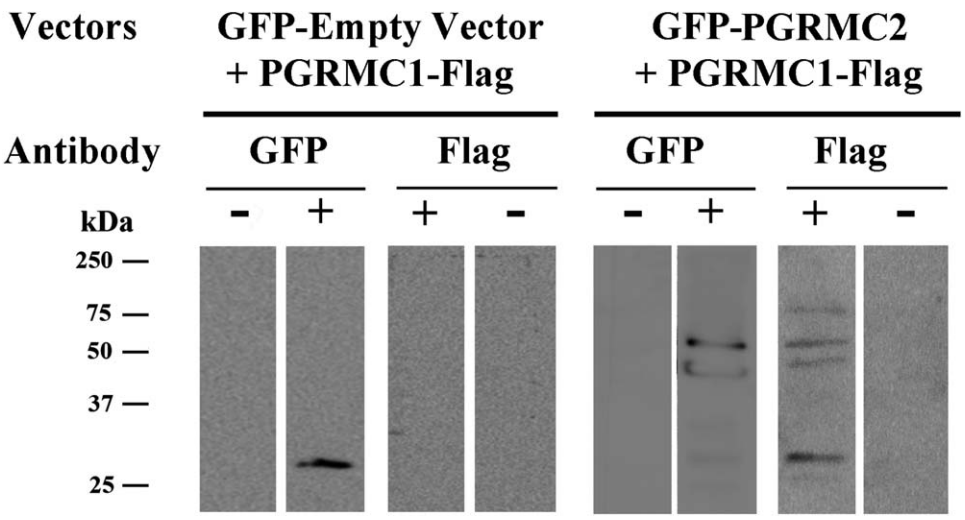


FIG. 2. Interaction between GFP-PGRMC2 and PGRMC1-Flag fusion proteins. In this study SIGCs were transfected with an expression vector that encodes PGRMC1-Flag fusion protein and either the GFP-empty vector or a vector that encodes GFP-PGRMC2 fusion protein. The GFP proteins were isolated and the isolates used in a Western blot probed with GFP and Flag antibodies. Note that the PGRMC1-Flag fusion proteins were detected among the proteins in the GFP-PGRMC2 fusion isolate but not with GFP isolate. The minus sign (–) and plus sign (+) over each lane mark a Western blot conducted in the absence or presence of the primary antibody.

set to detect BrdU-incorporating cells (i.e., green fluorescence) and the DAPI filter set to detect both the total number of cells and the number of mitotic figures. The total number of cells was used to calculate the percentage of BrdU incorporating cells and the percentage of mitotic figures. In addition other cultures were used to determine the effect of the goat PGRMC2 antibody on the interaction between PGRMC1 and PGRMC2 by using a PLA as described previously.

Overexpression of PGRMC1 and PGRMC2 on Cell Cycle Traverse

To assess the effect of overexpressing PGRMC1 or PGRMC2, SIGCs were transfected with expression vectors encoding GFP, PGRMC1-GFP [13], or GFP-PGRMC2 [10]. After 24 h of culture, cell lysates were made from some cultures and used to assess the expression of PGRMC1-GFP and GFP-PGRMC2 fusion proteins by Western blot analysis using the GFP antibody. Cells expressing GFP fusion proteins were revealed by their intrinsic green fluorescence when observed using the GFP filter set.

Once PGRMC1-GFP and GFP-PGRMC2 fusion protein expression was confirmed by Western blot, a similar study was conducted in which the cells were transfected with the expression vectors encoding GFP, GFP-PGRMC2, or PGRMC1-GFP and the cells cultured in DMEM with 5% steroid-free FBS overnight. Then the cells were infected with the dt-RFP vector of the FUCCI (20 viral particles per cell) and the cultures continued for an additional 24 h. The GFP-expressing cells in the G_1/S phases of the cell cycle were identified by comparing images taken under the GFP and RFP filter sets [10]. The percentage of GFP-expressing cells that were in the G_1/S phase was calculated for each treatment group.

GTPase-Activating Protein-Binding Protein 2 as a PGRMC1/2 Binding Partner

To gain insight into the mechanism through which PGRMC1 and 2 regulate entry into the cell cycle, GFP expression vectors that encode PGRMC1 or PGRMC2 were transfected into SIGCs and after 24 h, the GFP fusion proteins and the associated proteins were partially purified and sent to the Keck Foundation Biotechnology Resource Laboratory, Yale University, where the associated proteins were identified by mass spectrometry as previously described [23]. This analysis indicated that both PGRMC1 and PGRMC2 bound GTPase-activating protein-binding protein 1 (G3BP1) and G3BP2.

To demonstrate that the SIGCs express G3BP1 and G3BP2, lysates were prepared from SIGCs for Western blot analysis using either a G3BP1 or G3BP2 antibody. To confirm the proteomic analysis, partially purified preparations of PGRMC1-GFP or GFP-PGRMC2 fusion protein were analyzed for the presence of G3BP1 or G3BP2 by Western blot. In addition, the interaction between the PGRMC-GFP fusion proteins and G3BP2 was confirmed by PLA (OLINK Bioscience) per manufacturer instructions using GFP and G3BP2 antibodies. The interaction between two proteins was revealed as a single red fluorescent dot.

Finally, to demonstrate that endogenous G3BP2 interacts with endogenous PGRMC1 and/or PGRMC2, PLAs were conducted. For this assay PGRMC1-G3BP2 interaction was detected using a rabbit PGRMC1 antibody and a goat G3BP2 antibody, and PGRMC2:G3BP2 interaction was detected using a mouse PGMC2 antibody and a rabbit G3BP2 antibody. Using two different G3BP2 antibodies was necessary in order to use the available oligonucleotide-labeled probes provided by Olink, Inc.

Depletion of G3BP2 and Entry into the Cell Cycle

To determine the effect of depleting G3BP2 on entry into the cell cycle, SIGCs were cultured for 24 h and then transfected with either *G3bp2* siRNA (siRNA ID# S156617; Ambion) or scramble siRNA as described for the previous siRNA protocols. Twenty-four hours after transfection, the cells were infected with the dt-RFP vector of the FUCCI (20 viral particles per cell). The cultures were continued for an additional 24 h. Cells were then used to confirm the effectiveness of *G3bp2* siRNA by monitoring *G3bp2* mRNA by real-time PCR (forward primer sequence: AGCCTGCTTCTCTGCCTCAAG; reverse primer sequence: AGGTCGCTGTTCCCGTACAC; probe: 5′ d FAM-AACCG CCAAAGCCAAGGGTTGA-BHQ-1 3′). Lysates were also prepared for Western blot analysis of G3BP2. For this analysis glyceraldehyde phosphate dehydrogenase levels were also assessed to insure equal loading of the cells. Once the effectiveness of the *G3bp2* siRNA protocol was confirmed, studies were conducted in which G3BP2 was depleted and the cells infected with the dt-RFP vector of the FUCCI. Twenty-four hours after infection, the cells were fixed and stained with DAPI. The percentages of cells in the G_1/S phases of the cell cycle were identified by comparing images taken under the DAPI and RFP filter sets [10].

Statistical Analysis

All experiments were replicated at least three times. 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 Dunnett post hoc test if the means of three or more groups were compared. Regardless of the analysis, $P \leq 0.05$ was considered to be significantly different.

RESULTS

Forty-eight-hour treatment with specific siRNAs selectively depleted *Pgrmc1* and/or *Pgrmc2* mRNA without altering the mRNA levels of *Pairbp1*, a known PGRMC1 binding partner [12, 20] (Fig. 1A). Depleting *Pgrmc1* and/or *Pgrmc2* increased

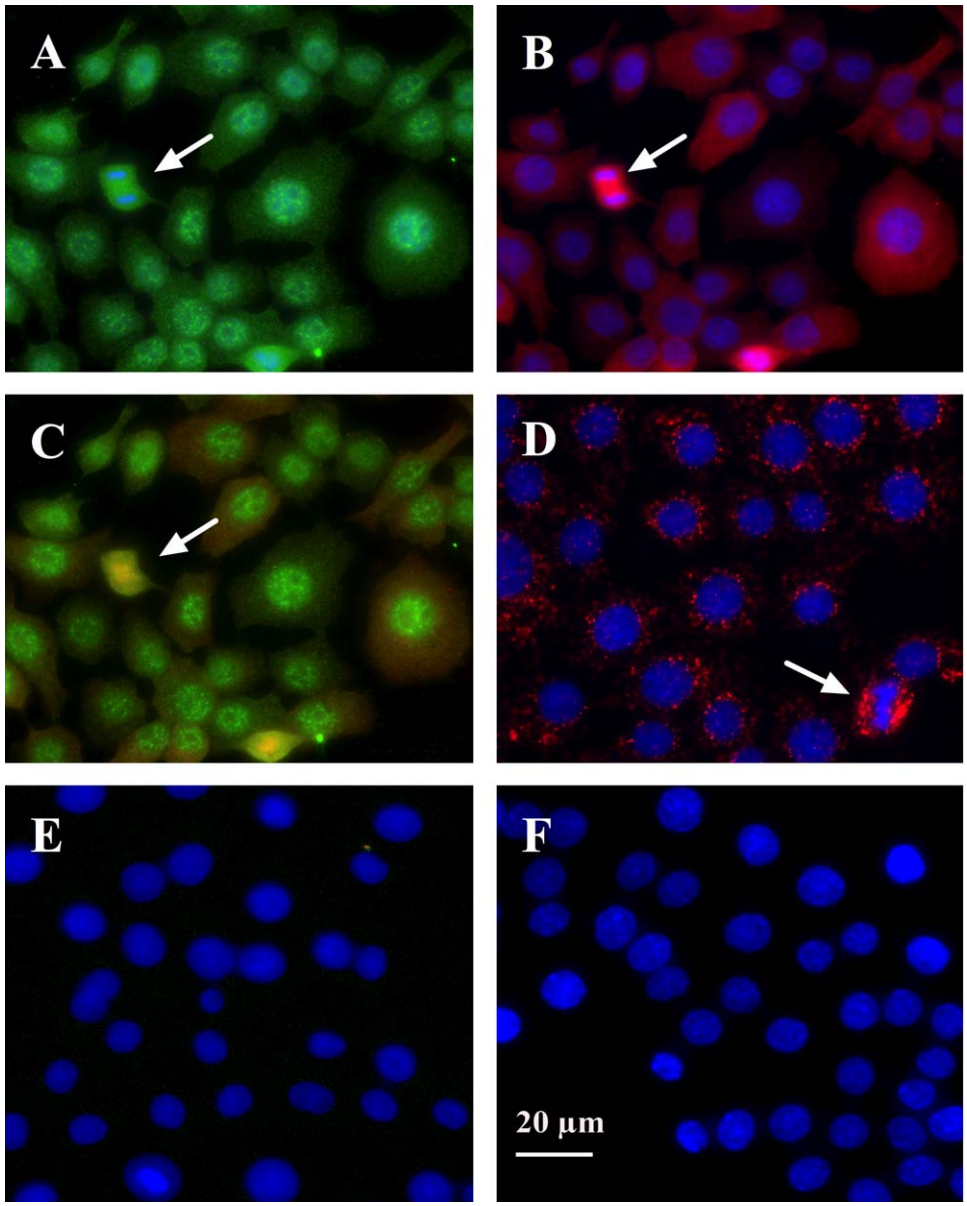
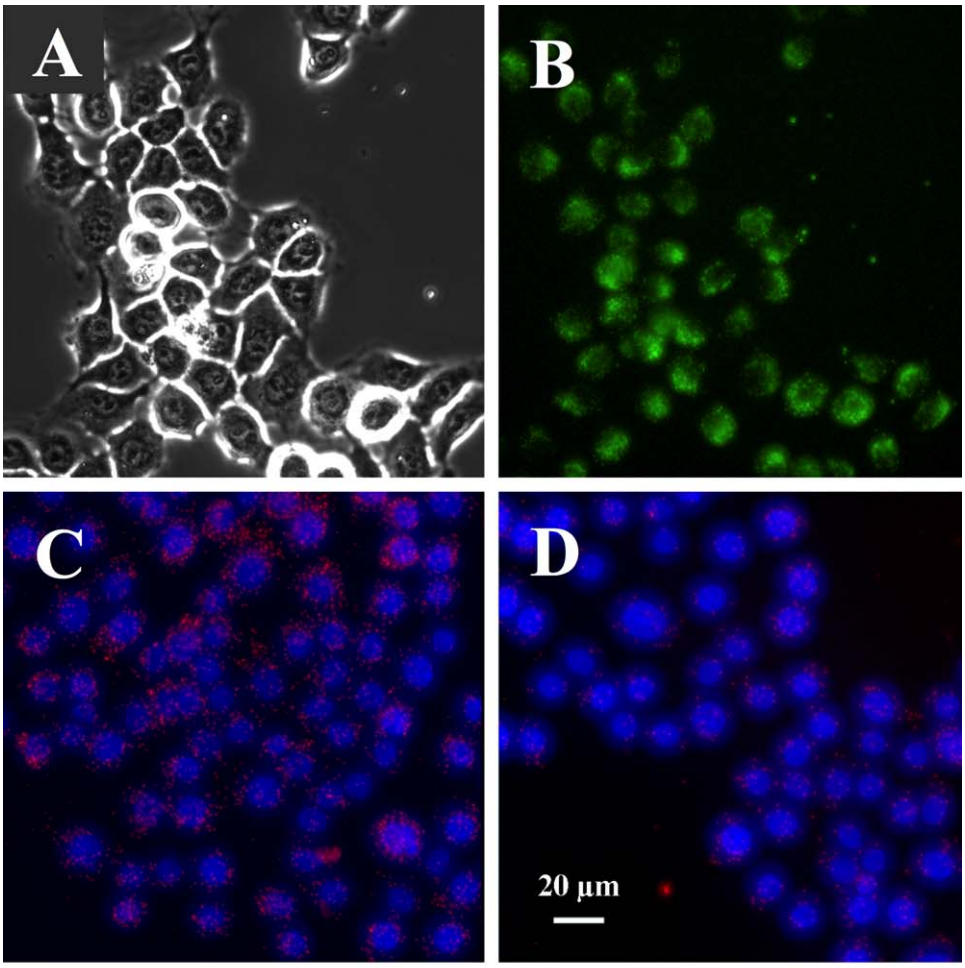


FIG. 3. The colocalization and interaction of PGRMC1 and PGRMC2. **A** and **B** show the localization of PGRMC1 (green fluorescence) and PGRMC2 (red fluorescence), respectively. **C**) Merged image that reveals the cellular sites to which PGRMC1 and PGRMC2 colocalize (orange-yellow fluorescence). In **D** a PLA reaction is shown in which PGRMC1:PGRMC2 interaction is detected by the red fluorescent dots. The arrows in **A**–**D** mark cells in metaphase. **E**) Negative control for the immunofluorescence in which the primary antibodies have been omitted. **F**) Negative control for the PLA in which the PGRMC1 antibody was omitted from the protocol. Note that all the cells were stained with DAPI (blue fluorescence) to reveal the nuclei. The DAPI image was not merged with the image shown in **C** in order to reveal the nuclear staining of PGRMC1.

the percentage of cells entering the cell cycle as judged by the increased percentage of cells either incorporating BrdU (Fig. 1B) or arresting in metaphase in the presence of colchicine (Fig. 1C) compared to scramble control values. However, depleting *Pgrmc1* and/or *Pgrmc2* in the absence of colchicine did not increase the number of cells present after 24 h of culture (Fig. 1D). Rather, there was an increase in the percentage of apoptotic cells (Fig. 1, E and F), although the increase in apoptosis after depleting *Pgrmc1* only approached statistical significance ($P = 0.06$) (Fig. 1F).

Because depleting both MAPR family members did not have an additive effect on the rate of entry into the cell cycle, this raised the possibility that PGRMC1 and PGRMC2 share a common mechanism, possibly by interacting with each other. To test this, cells were simultaneously transfected with expression vectors that encode either GFP-PGRMC2 or PGRMC1-Flag fusion proteins. Pull-down assays were then conducted in which GFP-PGRMC2 was isolated and the isolate run on a Western blot that was probed with a Flag antibody. This pull-down assay revealed that the PGRMC1-Flag fusion protein interacted with the GFP-PGRMC2 fusion protein (Fig. 2). In this pull-down assay the most intense band in the Flag Western blot was a ≈27-kDa protein, the expected sized of the PGRMC1-Flag protein. There are other bands ≥50 kDa, and these also represented PGRMC1-Flag fusion proteins that have undergone posttranslational modifications [16]. As a control, a study was conducted in which cells were simultaneously transfected with the GFP-empty vector and PGRMC1-Flag. In this pull-down assay the PGRMC1-Flag fusion protein was not detected in the GFP isolate (Fig. 2).



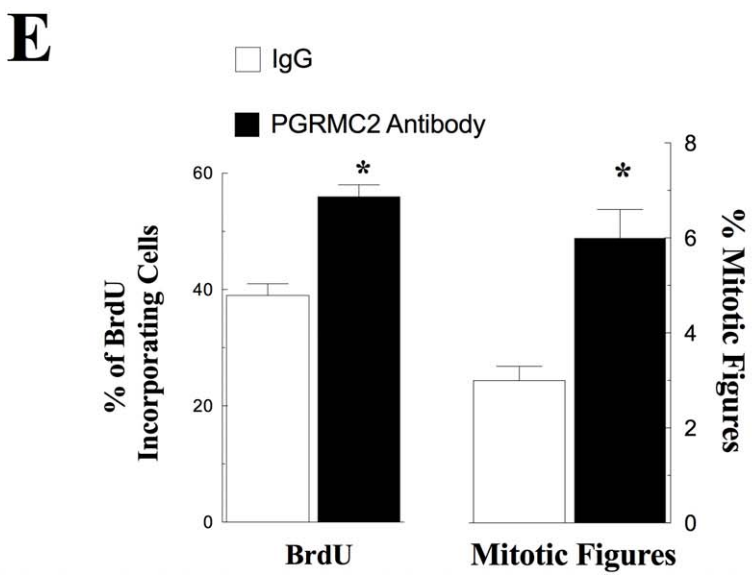


FIG. 4. The efficiency and effect of delivering a goat PGRMC2 antibody on the interaction between PGRMC1 and PGRMC2 and the rate of entry into the cell cycle. **A** and **B** are phase and fluorescent images, respectively, of the same field of cells after Chariot delivery of Alexa488-IgG. Note that virtually all of the cells shown in **A** fluoresce green (**B**). The PLA reactions shown in **C** and **D** illustrate the degree of PGRMC1:PGRMC2 interaction in the presence of either IgG (**C**) or PGRMC2 antibody (**D**). The reduction in the number of red dots/cells in **D** compared to that of **C** indicates a decrease in PGRMC1:PGRMC2 interaction. **E**) The effect of the PGRMC2 antibody on the percentage of cells incorporating BrdU and arresting in metaphase in the presence of colchicine. *Significant difference between treatment groups ($P < 0.05$).

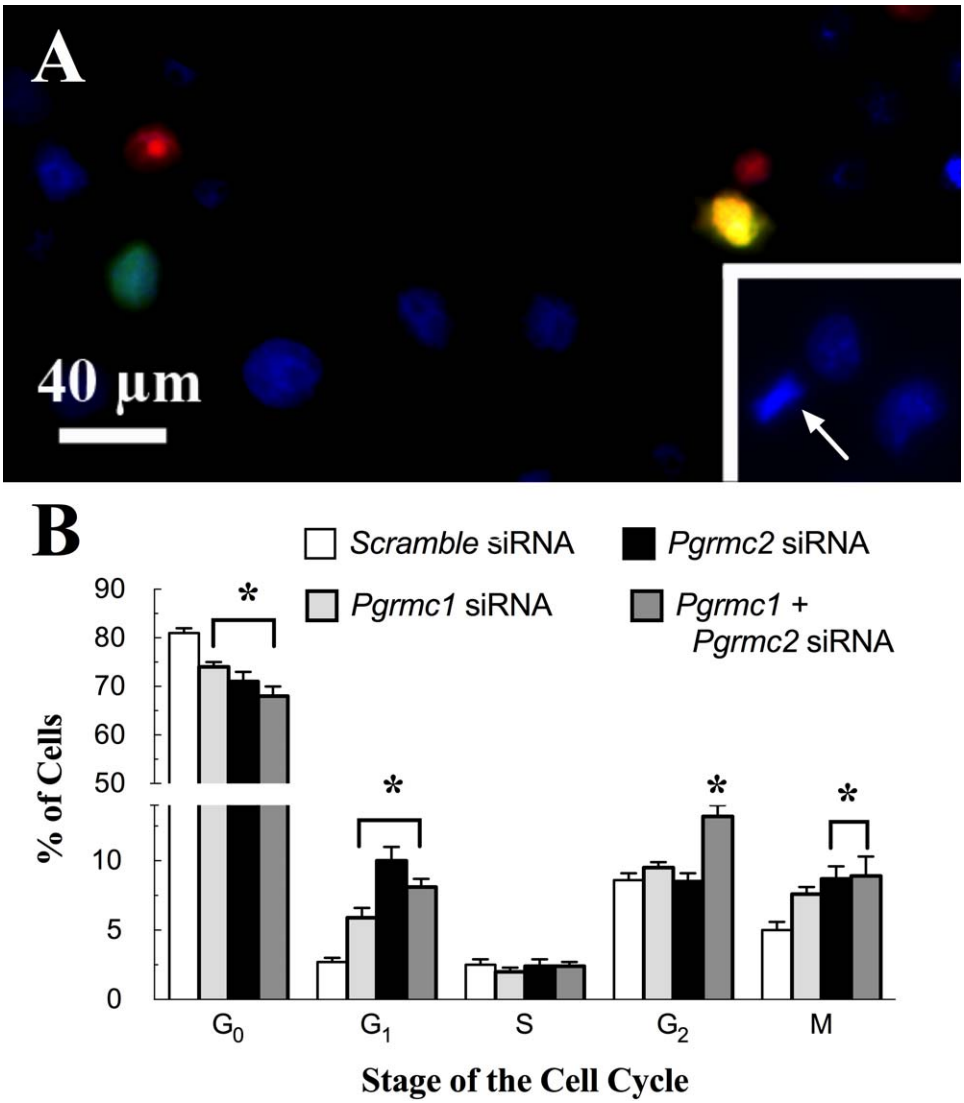


FIG. 5. The identification of cells in specific stages as revealed by FUCCI-infected cells. **A**) SIGCs that were infected with the FUCCI, fixed, and then stained with DAPI. Cells that fluoresced blue were considered to be either in G_0 or undergoing mitosis (marked by an arrow in the inset) depending on their nuclear structure. Red fluorescent cells were in G_1 stage and green fluorescent cells were in G_2 stage. S-stage cells were observed as fluorescing yellow after merging the green and red fluorescent images. The effect of PGRMC1 and/or PGRMC2 on the distribution of cells in each stage of the cell cycle is shown in **B**. Treatments enclosed by the bracket and marked by * are significantly different from the scramble control ($P < 0.05$).

Immunocytochemical studies showed that in interphase cells PGRMC1 was distributed throughout the cell, including the nucleus (Fig. 3A, green fluorescence), whereas PGRMC2 was predominately localized to the cytoplasm (Fig. 3B, red fluorescence). In addition, these two proteins colocalized to cytoplasm, as demonstrated by the orange-yellow fluorescence observed when the red and green images were merged (Fig. 3C). Moreover, PLA detected an interaction of endogenous PGRMC1 and PGRMC2 within the cytoplasm, as indicated by the presence of red fluorescent dots (Fig. 3D). For cells in metaphase, PGRMC1 and PGRMC2 appeared to colocalize and interact to the mitotic spindle, as judged by immunocytochemical and PLA assays (arrows mark metaphase cells in Fig. 3, A–D). Note that omitting the primary antibodies in the colocalization protocol (Fig. 3E) or in the PLA (Fig. 3F) eliminated any fluorescent signal, except for the blue fluorescence due to the DAPI staining of the nuclei. Similar PLA-based studies conducted on granulosa cells isolated from immature rats also revealed that endogenous PGRMC1 interacted with PGRMC2 (Supplemental Fig. S1).

In an attempt to disrupt the interaction between PGRMC1 and PGRMC2, a goat PGRMC2 antibody was transfected into cells using the Chariot protein transfection reagent. That Chariot delivered antibodies to nearly 100% of the cells was demonstrated by using Alexa488-labeled IgG (compare phase image of cells in Fig. 4A with the green fluorescing cells shown in Fig. 4B). The green fluorescence of the Alexa488-labeled IgG was not detected within the cells if the Chariot reagent was omitted from the protocol (data not shown). When goat PGRMC2 antibody was delivered using Chariot reagent, the interaction between PGRMC1 and PGRMC2 was disrupted, as judged by the relative absence of red dots after PLA (compare Fig. 4D with Fig. 4C). However, the presence of the goat PGRMC2 antibody could have prevented the mouse PGRMC2 antibody from detecting endogenous PGRMC2, which would have prevented the detection of a PGRMC1:PGRMC2 interaction by the PLA. This was not the case, because the mouse antibody detected PGRMC2 in cells transfected with goat PGRMC2 antibody (Supplemental Fig. S2). Importantly in the presence of the goat PGRMC2

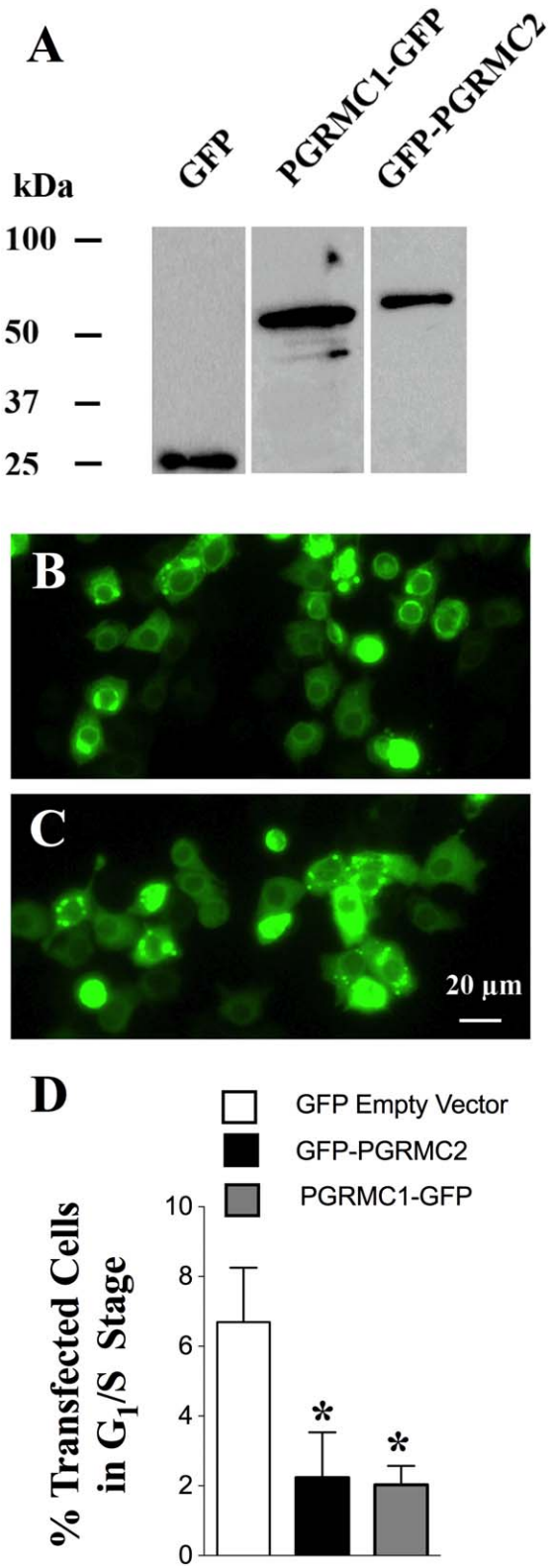


FIG. 6. The effect of transfecting expression vectors that encode GFP, PGRMC1-GFP, or GFP-PGRMC2 on their expression as judged by Western blot (**A**). The localization of PGRMC1-GFP and GFP-PGRMC2 is shown in **B** and **C**, respectively, and **D** illustrates the effect of overexpressing each of these GFP fusion proteins on the percentage of GFP-transfected cells that enter the cell cycle (i.e., in G_1/S stage of the cell cycle). *Value that is different from the GFP vector control ($P < 0.05$).

antibody, the percentage of cells incorporating BrdU and forming metaphase figures in the presence of colchicine increased compared to IgG controls (Fig. 4E).

In order to more precisely assess the effect of depleting MAPR family members on the rate at which cells enters mitosis, PGRMC1- and/or PGRMC2-depleted cells were infected with the FUCCI probe, which allows for the identification of cells in specific stages of the cell cycle based on their fluorescence (Fig. 5A). As shown in Figure 5B, depleting either or both MAPR family members decreased the percentage of cells in G_0 and increased the percentage in G_1 stage of the cell cycle. Depleting both PGRMC1 and PGRMC2 also increased the percentage of cells in G_2, whereas depleting PGRMC2 or PGRMC1 and PGRMC2 increased the percentage of cells in metaphase. The percentage of metaphase cells after *Pgrmc1* siRNA treatment was also greater than control but did not reach statistical significance ($P = 0.06$).

The transfection of SIGCs with expression vectors that encode each MAPR family member resulted in the expression of each PGRMC-GFP fusion protein as judged by Western blot (Fig. 6A). In addition, both GFP-fusion proteins localized mainly to the cytoplasm, although some PGRMC fusion proteins also appeared to be in the nucleus (Fig. 6, B and C). Overexpressing either PGRMC1-GFP or GFP-PGRMC2 fusion protein suppressed entry into the cell cycle compared to cells transfected with the GFP expression vector (Fig. 6D).

Given these findings, it is possible that PGRMC1 and PGRMC2 suppress entry into the cell cycle by interacting with a common protein. To test this concept, proteins that interact with both PGRMC1-GFP and GFP-PGRMC2 were identified by mass spectrometric analysis. This approach detected an interaction between G3BP1 and G3BP2 with each MAPR family member. Although Western blot analysis detected the presence of G3BP1 and G3BP2 in SIGCs (Fig. 7A), pull-down experiments only confirmed that G3BP2 interacted with both PGRMC-GFP fusion proteins (Fig. 7, B and C). To further confirm this interaction, PLA-based assays were conducted using a rabbit GFP antibody and a goat G3BP2 antibody to detect the interaction between the PGRMC1-GFP fusion protein and G3BP2. This PLA-based study detected an interaction only in cells that were transfected with the PGRMC1-GFP fusion proteins (compare the presence of red dots in Fig. 7d with the GFP-based fluorescence in Fig. 7D). Similarly, GFP-PGRMC2:G3BP2 interaction was only detected by PLA in cells that expressed GFP-PGRMC2 (compare the presence of red dots in Fig. 7e with the GFP-based fluorescence in Fig. 7E). Moreover, PLA-based assays detected an interaction between endogenous G3BP2 and either PGRMC1 or PGRMC2 (Fig. 8).

These studies implied that the interactions between G3BP2, PGRMC1, and PGRMC2 play important roles in the mechanism through which PGRMC1 and PGRMC2 regulate entry into the cell cycle. To directly implicate G3BP2 as part of this mechanism, siRNA was used to deplete G3BP2. This approach was effective in depleting G3BP2 as shown by real-time PCR (Fig. 9A) and Western blot (Fig. 9B). As seen in Figure 9C, depleting G3BP2 promoted entry into the cell cycle.

DISCUSSION

Previous studies have shown that depleting either PGRMC1 [2, 3] or PGRMC2 promotes entry into the cell cycle [10]. Although depleting either of these MAPRs stimulates entry into the cell cycle, the number of cells present after 24 h of culture is not increased. This is likely due to cells arresting in metaphase and eventually undergoing apoptosis. This possi-

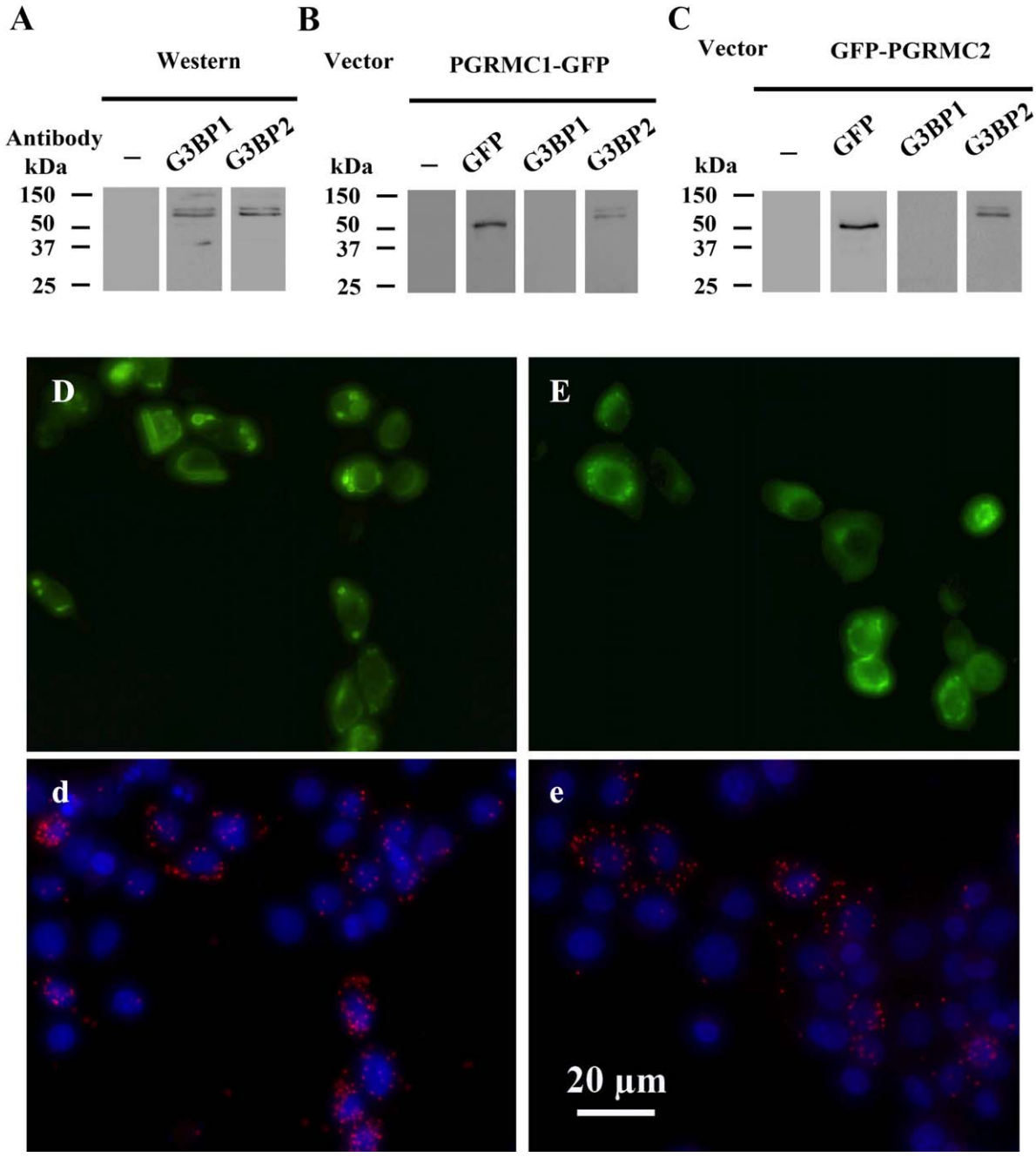


FIG. 7. A Western blot showing the expression of G3BP1 and G3BP2 in SIGCs (**A**). Pull-down assays are shown in **B** and **C**, which document the interaction between G3BP2 and PGRMC1-GFP or GFP-PGRMC2, respectively. Although G3BP1 is expressed by SIGCs, pull-down assays failed to detect its interaction with either PGRMC1-GFP or GFP-PGRMC2. In contrast, it was indicated that G3BP2 interacted with either PGRMC1-GFP or GFP-PGRMC2. The interaction between G3BP2 and PGRMC1-GFP or GFP-PGRMC2 was confirmed by PLA. Note the presence of the red dots associated with cells in **d** and **e** corresponds to those cells the express PGRMC1-GFP (green fluorescence) or GFP-PGRMC2 (green fluorescence) in **D** and **E**, respectively.

bility is supported by the present findings that depleting PGRMC1 and/or PGRMC2 increases the percentage of metaphase cells observed in the absence of colchicine. This is consistent with PGRMC1 and PGRMC2 localizing to and interacting within the mitotic spindle [10, 24], which implies that these MAPR members play essential roles during metaphase. In fact, PGRMC1 interacts with β-tubulin, one of the major proteins of the mitotic spindle, and enhances the stability of the mitotic spindle [24]. Based on these findings, future research on the mechanistic action of both MAPRs during metaphase seems to be warranted. However, the present studies also reveal that both PGRMC1 and PGRMC2 play significant roles in regulating entry into the cell cycle.

It is important to appreciate that each MAPR may influence specific pathways that regulate cell cycle traverse. For example, PGRMC1 localizes to the nucleus, where it is involved in regulating the gene expression [13, 15] likely mediated in part through interaction with Tcf/Lef sites [16]. Conversely, PGRMC2 is mainly cytoplasmic and selectively interacts with the active form of cyclin-dependent kinase 11b (p58) [10], a kinase involved in the G_0 to G_1 transition [17]. However, the previous studies depleted only one MAPR family member at a time; thus, these studies cannot determine if each

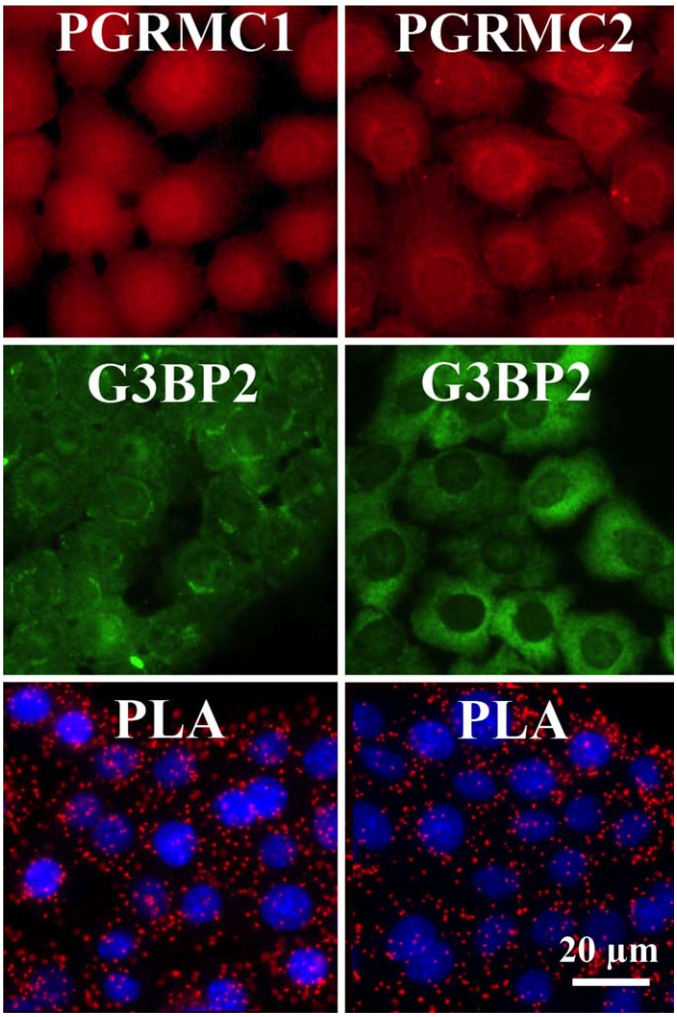


FIG. 8. The interaction between G3BP2 and either PGRMC1 or PGRMC2. The presence of PGRMC1 and PGRMC2 is shown in the upper panels and G3BP2 in the middle panels. The PLA reaction that illustrates the interaction between each MAPR and G3BP2 is shown in the lower panels.

MAPR family member mediates their action by regulating separate unique signal transduction pathways or a common signaling pathway.

The present study not only confirms previous work [3, 10, 12, 14] but also demonstrates that a simultaneous reduction in the levels of both MAPRs has a slight synergistic but not an additive effect on entry into the cell cycle. This raises the possibility that in addition to the ability to activate their respective specific pathways as cited above, the two MAPRs also may be components of a common signaling pathway. How these two MAPRs are organized into a common pathway that suppresses entry into the cell cycle is unclear. One option is that the two MAPRs bind to each other and this interaction is required to suppress entry into the cell cycle.

This concept is supported by five observations. First, pull-down studies demonstrate that GFP-PGRMC2 fusion protein interacts with PGRMC1-Flag fusion protein. Second, immunocytochemical studies reveal that endogenous PGRMC1 and PGRMC2 colocalize in the cytoplasm, although PGRMC1 also localizes to the nucleus. Third, endogenous PGRMC1 and PGRMC2 interact as judged by PLA. Fourth, the delivery of a goat PGRMC2 antibody into the cytoplasm disrupts PGRMC1:PGRMC2 interaction as assessed by PLA, results in an increase

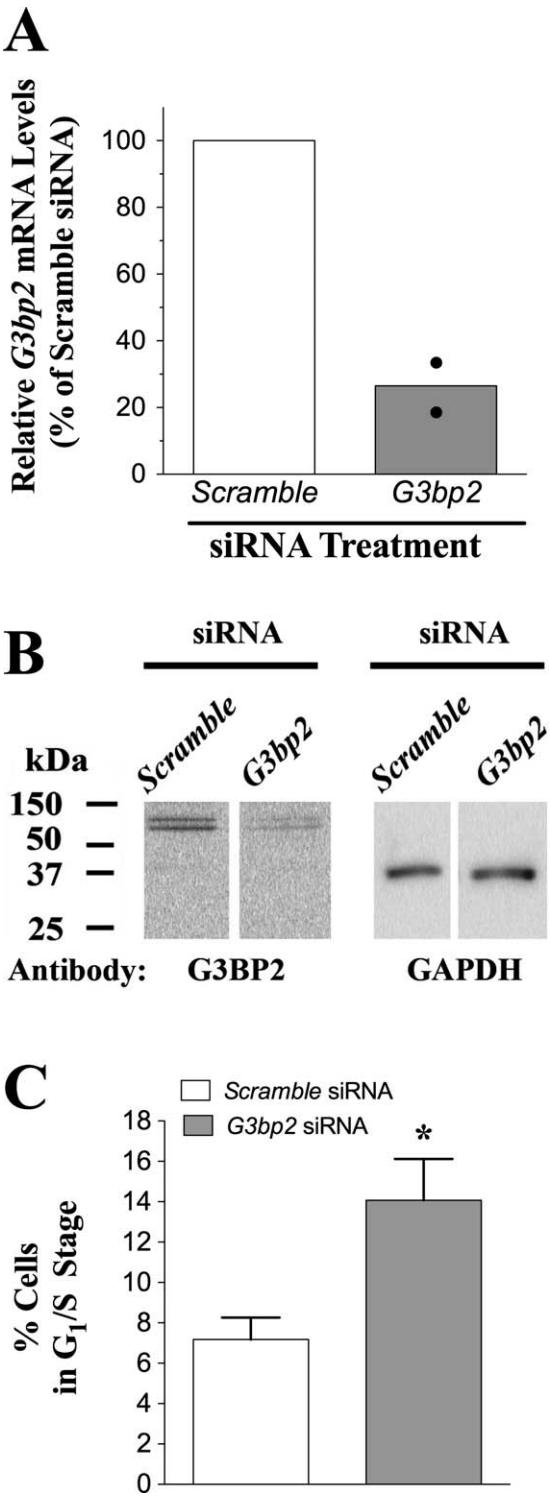


FIG. 9. The effect of *G3pb2* siRNA on the levels of *G3pb2* mRNA and protein as revealed by real-time PCR (**A**) and Western blot (**B**). The dots in **A** represent individual values obtained from two separate experiments. The effect of *G3pb2* siRNA on the percentage of cells entering the cell cycle (i.e., G_1/S) is shown in **C**. *Value that is significantly different from Scramble control ($P < 0.05$).

in the percentage of cells that incorporate BrdU, and increases the percentage of cells arrested in metaphase in the presence of colchicine. Disrupting PGRMC1:PGRMC2 interaction with the PGRMC2 antibody is a useful experimental approach because it does not alter the levels of either MAPR. Moreover,

failure of the PLA to detect an interaction between PGRMC1 and PGRMC2 is not an artifact due to the presence of the goat PGRMC2 antibody, because the goat PGRMC2 antibody does not interfere with the ability of the mouse antibody to detect endogenous PGRMC2, as required for the PLA to detect an interaction between PGRMC1 and PGRMC2. Finally, PLA reveals that PGRMC1:PGRMC2 interact in granulosa cells isolated from immature rats, which provide further evidence for a physiological role for this interaction. Taken together, these findings support the hypothesis that it is the interaction between PGRMC1 and PGRMC2 that controls in part the entry into the cell cycle. However, to conclusively demonstrate that the interaction between PGRMC1 and PGRMC2 is an essential factor in regulating entry into the cell cycle, future studies will have to identify the amino acid sequences that are required for PGRMC1:PGRMC2 interaction and that mutating these sites results in entry into the cell cycle.

In order to determine the molecular mechanism through which PGRMC1:PGRMC2 interaction influences entry into the cell cycle, it is essential to more precisely determine how depleting MAPRs affects the cell cycle. To this end, cells were depleted of one or both MAPRs and then infected with FUCCI probe. The FUCCI-base approach reveals that depleting either or both MAPRs decreases the percentage of cells in G_0 and increases the percentage of cells in G_1. This suggests that PGRMC1 and/or PGRMC2 suppresses the transition from G_0 to G_1. This raises the question as what allows for the mitogen-induced entry into the G_1 stage of the cell cycle in the presence of both MAPR family members. Although the complete answer to this question awaits further study, it appears that PGRMC2 expression is cell cycle dependent with its levels transiently reduced between G_0 and G_1 stages of the cell cycle [10]. This would reduce PGRMC2 levels as well as the amount of PGRMC1:PGRMC2 interaction. Either of these events could facilitate entry into the cell cycle. Unfortunately, very little is known about the factors that regulate the expression of PGRMC2. What is known is that in the immature rat ovary, gonadotropins decrease PGRMC2 expression [10], and this decrease coincides with an increase in granulosa cell proliferation and the development of preovulatory follicles [25, 26].

Although the knockdown studies provide strong evidence to support the hypothesis that PGRMC1 and PGRMC2 regulate entry into the G_1 stage of the cell cycle, additional evidence is provided by overexpressing of each of these MAPRs. As would be predicted, overexpression of either PGRMC1-GFP or GFP-PGRMC2 fusion protein suppresses entry into the cell cycle. This approach, however, does not reveal how increasing the expression of one MAPR family member inhibits entry into the cell cycle. One possibility is that overexpression increases an antimitotic signal cascade that is uniquely activated by each MAPR family member. Another possibility is overexpression of one MAPR member increases the amount of PGRMC1: PGRMC2 complex due to mass action. Although not mutually exclusive, the latter merits consideration because it appears that the unique action of PGRMC1 requires nuclear PGRMC1 and most of the PGRMC1-GFP is cytoplasmic and does not appear to mimic PGRMC1's genomic action [13].

The overexpression studies also provided an experimental approach to identify downstream components of the PGRMC1 and PGRMC2 signal transduction pathways by partially purifying each fusion protein and using mass spectrometry to identify proteins that interact with MAPR family members. This approach indicated that G3BP1 and G3BP2 interact with each MAPR family member. Although subsequent Western blot analysis demonstrates that both G3BP1 and G3BP2 are expressed by SIGC, pull-down experiments detected only an interaction between G3BP2 and each of the MAPR family members. Furthermore, PLA confirmed the ability of endogenous G3BP2 to interact with either of the MAPR-GFP fusion proteins. PLA studies also demonstrate that endogenous G3BP2 interacts with endogenous PGRMC1 and PGRMC2.

These findings imply that MAPR: G3BP2 interaction is essential in order to inhibit entry into the G_1 stage of the cell cycle. In fact, if this interaction is diminished by depleting G3BP2, then the percentage of cells in the G_1/S stage of the cell cycle doubles. How then might MAPRs: G3BP2 interaction function to inhibit entry into G_1? The answer to this question may be found be examining the proteins that interact with G3BP2. To this end, various approaches have detected physical interactions between G3BP2 and 59 different proteins (see [27]). Most of these interactions have been detected by high-throughput screening methods and have not been confirmed by more traditional biochemical approaches such as pull-down assays. Although any of the 53 putative G3BP2 binding partners could play a role in mediating the actions of G3BP2, one G3BP2 binding partner that has been confirmed by biochemical methods is nuclear factor kappa light polypeptide gene enhancer in B-cells inhibitor (NFKBIA; commonly referred to as IκBα) [28]. IκBα forms a complex with nuclear factor κB (NF-κB), which retains the IκBα:NF-κB complex within the cytoplasm. During the G_0 to G_1 transition, IκBα:NF-κB complex is disrupted and NF-κB translocates to the nucleus [28, 29], where it functions as a transcription factor that up regulates genes involved in the G_0-G_1 transition, such as c-Myc and Cyclin D [30].

Given the relationship among MAPRs, G3BP2, IκBα, and NF-κB, the following molecular framework is proposed to explain how these proteins might regulate entry into the G_1 stage of the cell cycle. That is, PGRMC1 and PGRMC2 acting either as separate molecules or through a PGRMC1:PGRMC2 complex localize within the cytoplasm, most likely to the endoplasmic reticulum [1], and this complex binds G3BP2. The presence of G3BP2 within the PGRMC1 and/or PGRMC2 complex recruits the IκBα:NF-κB complex, maintaining NF-κB within the cytoplasm and thereby preventing it from entering the nucleus and inducing genes that promote entry into the cell cycle. Mitogens act to transiently suppress PGRMC2, which reduces either the amount of PGRMC1:PGRMC2 complex or the number of PGRMC2 molecules available to bind G3BP2. Either of these events could result in the disruption of the IκBα:NF-κB complex and NF-κB being transported into the nucleus, where it could induce the expression of genes involved in the transition from the G_0 to the G_1 stage of the cell cycle. Interestingly, under oxidative stress, overexpression of PGRMC1 increases the phosphorylation of IκB [31], which would likely disrupt the IκB-NF-κB complex and lead to the translocation of NF-κB to the nucleus. This further supports the involvement of PGRMC1 in the NF-κB signaling pathway.

Finally, because progesterone's ability to suppress entry into the cell cycle is mediated through PGRMC1- and PGRMC2-dependent mechanisms, it is possible that it does so by enhancing the interactions among PGRMC1, PGRMC2, and G3BP2. However, the proposed role of P4 in influencing the interactions within the PGRMC1:PGRMC2:G3BP2 complex and ultimately the NF-κB signaling pathway is very speculative and merits further investigation.

ACKNOWLEDGMENT

The authors would like to acknowledge Dr. Martin Wehling of the University of Heidelberg-Mannheim for providing the GFP-PGRMC2 expression vector and Dr. Robert Burghardt of Texas A&M University for providing the spontaneously immortalized granulosa cells. We would also like to thank Dr. James Pru for his careful review of this manuscript.

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