Role of Pgrmc1 in estrogen maintenance of meiotic arrest in zebrafish oocytes through Gper/Egfr

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Abstract

The regulation of receptor trafficking to the cell surface and its effect on responses of target cells to growth factors and hormones remain poorly understood. Initial evidence has been recently obtained using cancer cells that surface expression of the epidermal growth factor receptor (EGFR) is dependent on its association with progesterone receptor membrane component 1 (PGRMC1). Estrogen inhibition of oocyte maturation (OM) in zebrafish is mediated through G-protein-coupled estrogen membrane receptor 1 (Gper1) and involves activation of Egfr. Therefore, in this study, the potential roles of Pgrmc1 in the cell surface expression and functions of Egfr in normal cells were investigated in this *in vitro* OM model of Egfr action using an inhibitor of PGMRC1 signaling, AG205. A single ~60 kDa protein band, which corresponds to the size of the Pgrmc1 dimer, was detected on plasma membranes of fully grown oocytes by western blotting. Co-treatment with the PGRMC1 inhibitor AG205 (20 μM) blocked the inhibitory effects of 100 nM estradiol-17β and the GPER agonist, G-1, on spontaneous maturation of denuded zebrafish oocytes. Moreover, reversal of these estrogen effects on OM by the EGFR inhibitors AG1478 and AG825 (50 μM) was prevented by co-incubation with the PGRMC1 inhibitor. Inhibition of Pgrmc1 signaling with AG205 also caused a decrease in Egfr-dependent signaling and Egfr expression on oocyte cell membranes. These results indicate that maintenance of Pgrmc1 signaling is required for Egfr expression on zebrafish oocyte cell membranes and for conserving the functions of Egfr in maintaining meiotic arrest through estrogen activation of Gper.

Key Words
- PGRMC1
- E_2
- DHP
- EGFR
- GPER
- oocyte maturation
- zebrafish
- AG205

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Introduction

The single transmembrane protein progesterone receptor membrane component 1 (PGRMC1) is a 25–28 kDa protein that possesses a cytochrome *b*-like heme-binding domain and belongs to the membrane-associated progesterone receptor (MAPR) family (Cahill 2007). On the basis of results of structural analyses it is predicted that PGRMC1 has a N-terminal extracellular domain, a transmembrane domain, and a cytoplasmic region with a heme-binding domain (Peluso *et al*. 2006, Cahill 2007) and, based on the results of biochemical function studies using specific mutations, it has been confirmed that PGRMC1 is a heme-binding protein (Crudden *et al*. 2006). PGRMC1 is widely expressed in tissues as a monomer or as an approximately 60 kDa dimer and has been proposed to mediate a broad range of functions including cholesterol synthesis, drug and hormone metabolism, axonal guidance, regulation of the neuroendocrine system and uterine physiology, apoptosis in ovarian follicle cells, and

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initiation of the acrosome reaction in mammalian sperm (Cahill 2007, Peluso *et al*. 2008*a*, Thomas 2008, Intlekofer & Petersen 2011, Neubauer *et al*. 2013, Pru & Clark 2013). PGRMC1 has also been implicated in tumorigenesis and is over-expressed in many types of cancer, including those of the ovary, lung, colon, and breast as well as in a broad variety of cancer cell lines (Crudden *et al*. 2006, Neubauer *et al*. 2008, Peluso *et al*. 2008*b*, Dressing *et al*. 2012). PGRMC1 displays moderately high binding affinity for progesterone in porcine liver membranes, which is twofold to tenfold greater than that for testosterone and glucocorticoids, and can bind to other molecules such as heme, cholesterol metabolites, and proteins (Meyer *et al*. 1996, Thomas 2008). In addition, PGRMC1 has been recently proposed to be a putative sigma-2-binding site (Xu *et al*. 2011, Ahmed *et al*. 2012).

The fact that PGRMC1 binds to such a bewildering variety of ligands indicates that it functions as an adaptor protein (Cahill 2007, Thomas 2008). Results from a recent study by Thomas *et al*. (2014) indicating that PGRMC1 co-immunoprecipitates with membrane progesterone receptor alpha (mPRα) in several breast cancer cell lines and enhances its expression and function on the plasma membrane, and also enhances the membrane expression of estrogen receptor beta (ERβ), further support a role of PGRMC1 as an adaptor protein and suggests that it has an important function in regulating the cell-surface expression and membrane receptor functions of steroid receptors. Results described in a recent report by Ahmed *et al*. (2010*a*) which revealed that PGRMC1 associates with epidermal growth factor receptor (EGFR) and maintains EGFR at the plasma membrane indicate that PGRMC1 also acts as an adaptor protein for EGFR. This finding is likely to have broad implications for tumorigenesis because over-expression of EGFR in cancer cells is highly correlated with malignancy (Ono & Kuwano 2006).

A small aromatic compound, AG205, has been identified as a ligand for the heme-1 domain in the *Arabidopsis thaliana* PGRMC1 homolog, AtMAPR2, which is highly conserved with the human PGRMC1 heme domain (Yoshitani *et al*. 2005). Results of subsequent studies have indicated that AG205 binds to PGRMC1 and has a similar effect to that observed after treatment with *Pgmrc1* siRNA in lung cancer cells, decreasing phosphorylation of ERK (Ahmed *et al*. 2010*b*). Treatment with AG205 also decreases EGFR-dependent signaling in lung cancer cells, consistent with the proposed role of PGRMC1 as a binding partner with EGFR (Ahmed *et al*. 2010*a*). Thus, AG205 is a useful PGRMC1 antagonist for exploring its adaptor protein functions.

G protein-coupled estrogen receptor 1 (GPER1), formerly known as GPR30, is a novel, specific seven-transmembrane-domain estrogen receptor coupled to a stimulatory G protein (G_s) that mediates rapid, nongenomic estrogen actions through activation of adenylyl cyclase and EGFR transactivation in various cell types, including breast cancer cells and fish oocytes (Filardo *et al*. 2000, Filardo 2002, Filardo & Thomas 2005, Thomas *et al*. 2005, Peyton & Thomas 2011). Activation of Gper on Atlantic croaker and zebrafish oocytes by estradiol-17β (E_2) or the specific GPER agonist, G-1, blocks spontaneous meiotic maturation of denuded oocytes and attenuates induction of oocyte maturation (OM) by the maturation-inducing steroid (Pang *et al*. 2008, Pang & Thomas 2009, 2010). These inhibitory actions of estrogens on OM are partially mediated through activation of membrane adenylyl cyclase to sustain high cAMP concentrations in the oocyte, which are essential for maintaining meiotic arrest (DeManno & Goetz 1987, Pang *et al*. 2008, Pang & Thomas 2010). They may also involve Egfr and Mapk3/1 because these inhibitory effects were blocked by co-treatment with several specific inhibitors of the EGFR pathway and MAPK (Peyton & Thomas 2011). Moreover, initial evidence was obtained from western blot analyses for the presence of Egfr on the plasma membranes of denuded oocytes (Peyton & Thomas 2011). However, additional research is required to clarify the roles of Egfr in OM, because EGF has been shown to exert an opposite action in teleost ovarian follicle cells to stimulate OM and the resumption of meiosis by increasing expression of activin (Wang & Ge 2004, Van Der Kraak & Lister 2011). Pgrmc1 has also been detected in fish oocytes (Mourot *et al*. 2006), but it is not known whether it has a similar function in oocytes to that observed in cancer cells of regulating the membrane expression and functions of EGFR and steroid receptors.

On the basis of the findings regarding PGRMC1 in cancer cells and from the studies with Egfr in fish oocytes, we propose that Pgrmc1 regulates expression of Egfr on zebrafish oocyte membranes and influences the inhibitory effects of estrogens on the resumption of meiosis through Gper. The expression pattern of the Pgrmc1 protein in oocytes and follicle cells during oogenesis and OM, and its localization on the oocyte membrane were investigated using a specific antibody for zebrafish Pgrmc1. A specific inhibitor of PGRMC1 adaptor protein functions, AG205, was used to determine the role of Pgrmc1 in inhibition of OM through E_2 and Egfr expression on the oocyte membrane. In addition, modulation by AG205 of the inhibitory effects of EGFR inhibitors on maintenance of meiotic arrest was examined to confirm the proposed

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role of Pgrmc1 in regulating the functions of Egfr on the oocyte membrane.

Materials and methods

Chemicals

Chemicals, including the AG205-specific PGRMC1 inhibitor and the EGFR (ErbB1) inhibitors, AG1478 and AG825, were purchased from Sigma–Aldrich unless otherwise stated. 17,20β-dihydroxy-4-pregnen-3-one (DHP) and E_2 were purchased from Steraloids (Newport, RI, USA). The GPER agonist, G-1, a nonsteroidal, dihydroquinoline compound (1-(4-(6-bromobenzo [1,3]dioxyl-5-yl)-3α,4,5,9β-tetrahydro-3H-cyclopenta[c]quinolin-8-y)-ethanone), was purchased from EMD Chemicals (Waltham, MA, USA). Specific antibodies for zebrafish Egfr (ErbB1) and zebrafish β-actin were purchased from AnaSpec (Fremont, CA, USA). p42/44 phosphorylated-ERK and p42/44 total-ERK antibodies were purchased from Cell Signaling (Danvers, MA, USA).

Animal care

Adult zebrafish were purchased from Segrest Farms (Gibsonton, FL, USA) and kept in ten-gallon recirculating tanks containing filtered freshwater at 28 °C with a 14 h light:10 h darkness photoperiod at the University of Texas Marine Science Institute for at least 1 week before experimentation. Fish were fed brine shrimp twice daily. All procedures with zebrafish in this study were approved by The Institutional Animal Care and Use Committee of the University of Texas at Austin.

Germinal vesicle breakdown assay

Ovarian follicles were collected and incubated according to procedures described previously (Pang & Thomas 2010, Peyton & Thomas 2011) for the zebrafish germinal vesicle breakdown (GVBD) assay. Briefly, ovarian follicles were separated and only those >560 μm in diameter were selected for subsequent enzymatic treatments with collagenase (50 μg/ml) to remove the follicle layers and obtain denuded oocytes for the GVBD bioassay. Ovarian follicles were treated with collagenase for 1 h followed by several washes in L-15 medium to ensure complete removal of the enzyme. For the GVBD assays, 20–30 oocytes were placed in each well of a 24-well culture plate containing 1 ml of L-15 culture medium and the various treatments, added in 1 μl aliquots dissolved in ethanol or DMSO. An equal volume (1 μl) of these solvents was added to the vehicle treatment control wells. The number of oocytes that had completed GVBD (i.e., for which the germinal vesicle no longer visible) was assessed after 3 h of incubation at 28 °C and the result expressed a percentage of the total number of oocytes in the well. To confirm that the oocytes were maturationally competent, the zebrafish maturation-inducing steroid, DHP, was used as a positive control in all the GVBD assays. The effects of E_2 (100 nM), G-1 (100 nM), and EGFR (ErbB1) inhibitors AG1478 (50 μM) and AG825 (50 μM) on GVBD were tested as described previously (Peyton & Thomas 2011) with or without co-treatment with the PGRMC1 inhibitor, AG205 (20 μM). All treatments were replicated three times in each experiment and all the GVBD experiments were repeated three times.

Quantitative real-time PCR

Ovarian follicles were separated into the following diameter ranges: <400 μm (pre-vitellogenic (PV)), 400–450 μm (early-vitellogenic (EV)), 450–550 μm (late-vitellogenic (LV)), and 550–650 μm (full-grown (F)) as described previously (Pang & Thomas 2010) and pooled for mRNA and protein analyses.

TRI reagent was used to isolate total RNA following the manufacturer's instructions. RNA was then further treated with DNase according to the manufacturer's instructions (Applied Biosystems). Each sample contained 40 ovarian follicles of different sizes and at different developmental stages.

Real-time PCR was performed on a Mastercycler ep realplex (Eppendorf, Hamburg, Germany), with one-step Brilliant II SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA, USA) using a protocol described previously (Pang & Thomas 2010). The primers were designed on the basis of zebrafish *pgrmc1* (GenBank accession no. BC085558.1) sequences. *pgrmc1* primers were sense, 5′-GCG GAG ACA AGC CTG CAG AC-3′ and antisense, 5′-CCG CGA AGA CTC CTG AAG GG-3′. The zebrafish *ef1α* (GenBank accession no. NM_131263) gene was chosen as the control reference gene (Tang *et al.* 2007), the primers were: sense, 5′-AAG ACA ACC CCA AGG CTC TCA-3′ and antisense, 5′-CCT TTG GAA CGG TGT GAT TGA-3′. The relative abundance value of each RNA sample was normalized to the amount of *ef1α* by the comparative threshold cycle method ($2-\Delta Ct$).

Preparation of zebrafish ovarian cryosections and immunohistochemistry of Pgrmc1

Zebrafish ovaries were cryosectioned (thickness: 10–15 μm) at −20 °C in the cryostat chamber and the

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sections were distributed onto pre-cooled, gelatin-coated glass slides and fixed as described previously (Pang & Thomas 2010). Pgrmc1 was detected with a polyclonal antibody produced by Genescript (Piscataway, NJ, USA, 1:500) raised against zebrafish Pgrmc1 peptide (sequence: RGDKPADYGPVEEPC, which corresponds to the internal inner loop after the only transmembrane motif of the protein), and an IgG antibody (1:500) was used as a negative control. AlexaFluor 488 goat anti-rabbit IgG was used as a secondary antibody (1:2000, Invitrogen). Nuclei were visualized using 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen, ~0.1 μg/1 ml) staining. Sections were examined using a Nikon Eclipse E600 fluorescent microscope and the images were processed using the Nikon imaging system.

Western blot analysis

Plasma membranes were prepared from follicle-enclosed and denuded zebrafish oocytes as described previously (Pang & Thomas 2010). Membrane samples (10 μg membrane protein/lane) were transferred onto nitrocellulose membranes (Bio-Rad). Membranes were incubated with primary antibodies: zebrafish Egfr antiserum (catalog no. 55473, 1:2000; AnaSpec), zebrafish Pgrmc1 (1:2500) raised against a synthetic 14-mer peptide (RGDKPADYGPVEEPC), or with zebrafish β-actin antiserum (catalog no. 55339, 1:1000; AnaSpec). Procedures for all AnaSpec antibodies were performed following the manufacturer's instructions. Membranes were then incubated with a HRP-linked second antibody (1:5000) and the protein bands were visualized using Supersignal WestPico (Thermo, Waltham, MA, USA) and exposure to Hyperfilm ECL (Amersham). In order to confirm the specificity of the zebrafish Pgrmc1 immunoreaction, the antibody was pre-incubated with the peptide antigen (5 μg/μl) overnight before use in the western blot analyses. The Image J Software (Bethesda, MD, USA) was used to quantify the density of the protein bands.

Mapk3/1 phosphorylation

Denuded oocytes were incubated for 15–20 min in either vehicle, E_2 (100 nM), G-1 (100 nM), or a combination of AG205 (20 μM) with either E_2 or G-1 in L-15 medium. The L-15 medium was removed and 150 μl of RIPA buffer (Cell Signaling) containing protease inhibitor were added, followed by homogenization and centrifugation to obtain a lysate fraction as described previously (Pang & Thomas 2010, Peyton & Thomas 2011). Lysates (20 μg/lane) were loaded and run on SDS–PAGE gel samples, the proteins transferred onto nitrocellulose membranes, and western blot analyses performed as described previously. Membranes were probed with antibodies for phosphorylated Mapk3/1 and total Mapk3/1 (Peyton & Thomas 2011) and Image J was used to quantify the protein bands.

Statistical analyses

Data are expressed as the mean ± S.E.M. Significant differences between control and treatment were determined by one-way ANOVA followed by the Bonferroni's multiple-comparison test using the GraphPad Prism 5.03 Software (La Jolla, CA, USA).

Results

Pgrmc1 expression in ovarian follicles at different developmental stages and localization on the oocyte plasma membrane

The zebrafish Pgrmc1 antibody (Pgrmc1 ab) detected a single approximately 60 kDa band which probably represents the Pgrmc1 dimer on both western blots of ovarian lysates and ovarian membrane fractions (Fig. 1A). The immunoreaction was blocked by pre-incubation with the peptide antigen, confirming the specificity of the zebrafish Pgrmc1 ab (Fig. 1B). Single immunoreactive bands of the correct size for the Pgrmc1 dimer were observed in plasma membranes prepared from zebrafish follicle-enclosed oocytes and from denuded oocytes, indicating that Pgrmc1 is also expressed in oocyte membranes (Fig. 1C).

Quantitative RT-PCR showed that *pgrmc1* mRNA expression did not vary significantly from the early follicle developmental stages (PV) through LV and F and after spontaneous GVBD. The Pgrmc1 protein was detected in ovarian follicles at all development stages (Fig. 1E). The immunoreactive Pgrmc1 band detected at the GVBD stage had a lower molecular weight (approximately 45 kDa), which may represent a partially degraded form of the zebrafish Pgrmc1 protein dimer (Fig. 1E), or a post-translational modification of the Pgrmc1 monomer. Expression of the Pgrmc1 protein was low during the PV, EV, and LV stages, higher in F follicles, and lowest after the completion of GVBD (Fig. 1F).

The localization of the follicle cell layers in cryosections of F follicles was visualized by staining their nuclei with DAPI (Fig. 2A and C). Immunohistochemical staining of the sections using the specific zebrafish Pgrmc1 ab resulted in green fluorescent staining in the periphery of oocytes near the oocyte plasma membrane, but not in the surrounding follicle cells (Fig. 2B and C). No signal was

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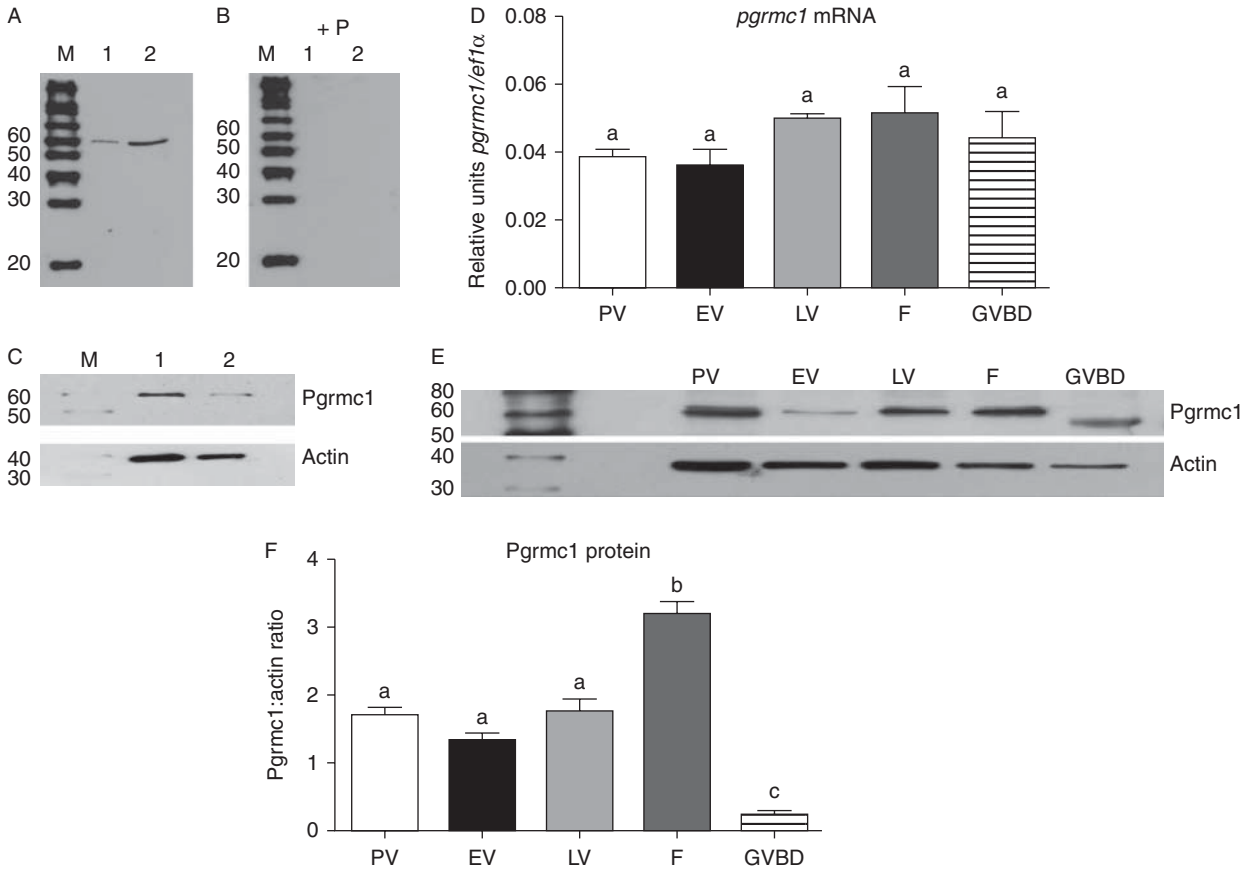

Figure 1

Expression of *pgrmc1* mRNA in zebrafish ovarian follicles and Pgrmc1 protein in ovarian and oocyte membranes at different developmental stages. (A and B) Western blot analysis using the zebrafish Pgrmc1 antibody (Pgrmc1 ab) of Pgrmc1 expression in zebrafish ovary (A, lane 1) and ovarian membrane (A, lane 2). (B) Antibody reaction blocked by preincubation with peptide antigen. (C) Western blot analysis of membranes of follicle-enclosed oocytes (C, lane 1) and denuded oocytes (C, lane 2). M, molecular weight marker; +P, peptide antigen; Pgrmc1, zebrafish Pgrmc1; Actin, zebrafish actin. (D) Quantitative RT-PCR measurement of *pgrmc1* mRNA levels in ovarian follicles at different developmental stages. PV, pre-vitellogenic; EV, early-vitellogenic; LV, late-vitellogenic; F, full-grown; GVBD, oocytes undergoing germinal vesicle breakdown, $n=9$. (E) Representative western blot of Pgrmc1 protein expression on plasma membranes of ovarian follicles at different developmental stages using a specific zebrafish Pgrmc1 ab. (F) Pgrmc1 protein levels relative to β-actin levels, $n=3$. Data collected from multiple westerns blots. Different letters denote significant differences from each other ($P<0.05$), one-way ANOVA, and nonparametric Bonferroni's test.

detected when the cryosections were incubated with control rabbit IgG (Fig. 2D). These immunohistochemistry results provide further evidence that the zebrafish Pgrmc1 ab is specific and that Pgrmc1 is localized on or near the plasma membrane of zebrafish oocytes.

Involvement of Pgrmc1 in inhibitory actions of estrogen on OM

The effect of co-treatment with a PGRMC1 inhibitor and estrogens on spontaneous GVBD of denuded F oocytes was investigated to test the hypothesis that Pgrmc1 is involved in the inhibitory actions of estrogens on OM in zebrafish. The inhibitory effect of E_2 and G-1 on spontaneous OM was blocked by co-treatment with the PGRMC1 inhibitor, AG205 (20 μM; Fig. 3A), whereas treatment with AG205 alone had no effect. The hypothesis was further tested by examining the effects of incubating denuded oocytes with the zebrafish Pgrmc1 ab on the inhibitory actions of estrogens on OM. Incubation of denuded oocytes with the specific zebrafish Pgrmc1 polyclonal antibody (1:300) significantly attenuated the inhibitory effect of E_2 on OM, whereas incubation with control rabbit IgG was ineffective (Fig. 3B). Thus, the results of both of these studies indicate that Pgrmc1 is involved in estrogen inhibition of OM in zebrafish.

Estrogen inhibition of OM in zebrafish has previously been shown to involve Egfr and Mapk3/1 signaling (Peyton & Thomas 2011). Treatment with E_2 and G-1 for 15–20 min caused increases in Mapk1/3 phosphorylation

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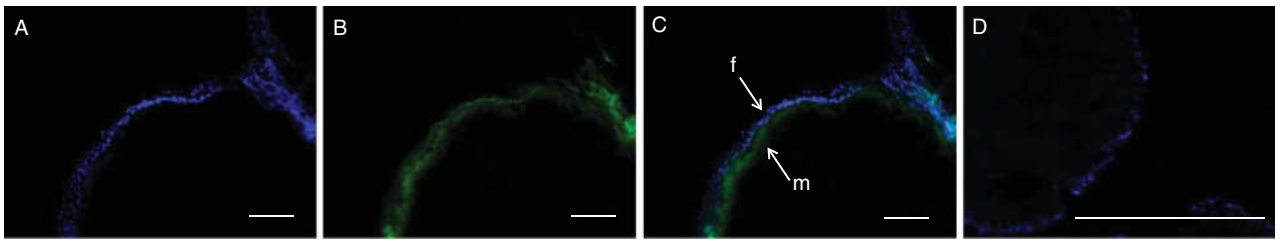

Figure 2
Immunohistochemical localization of Pgrmc1 in full-grown ovarian follicle cryosections using the specific Pgrmc1 antibody. (A) DAPI staining of nuclear DNA in follicle cells. (B) Localization of Pgrmc1 on or near oocyte membranes. (C) Merge of DAPI and Pgrmc1 images. f, follicle cells; m, oocyte plasma membranes. (D) Merge of DAPI and incubation with IgG as a negative control. Scale bars = 100 μm.

in denuded zebrafish oocytes, confirming these previous findings (Fig. 3C). Phosphorylation of Mapk1/3 in response to E_2 and G-1 was reduced by co-treatment with the PGRMC1 inhibitor, AG205, demonstrating that estrogen activation of Mapk3/1 involves Pgrmc1 (Fig. 3C and D).

Role of Pgrmc1 in localization of Egfr on the oocyte plasma membrane and Egfr inhibition of OM

Treatment with EGFR (ErbB1)-specific inhibitors, AG1478 and AG825 (50 μM), alone significantly increased the percentage of denuded oocytes undergoing spontaneous OM to a level similar to that induced with DHP (5 nM), as observed in a previous study (Peyton & Thomas 2011). However, this effect of the EGFR inhibitors was completely blocked by co-treatment with 20 μM AG205, which indicates that Pgrmc1 is required for the signaling functions of Egfr (Fig. 4A). Treatment of denuded oocytes with AG205 alone and in combination with the EGFR inhibitors for 6 h greatly reduced the expression of Egfr on the plasma membrane (Fig. 4B and C), suggesting that Pgrmc1 is required for the plasma membrane localization and signaling functions of Egfr. Treatment with AG205 and all the other treatments also decreased the surface expression of Pgrmc1 compared with that in vehicle-treated controls (Fig. 4B and D). In order to determine whether the inhibitory effects of AG205 on OM are specific and not due to a non-specific toxic effect, the oocytes were washed after 3 h of co-treatment with AG205 and treated with the EGFR inhibitors alone for an additional 3 h. The stimulatory effects of both EGFR inhibitors on OM that had been blocked with AG205 were restored after further incubation with the EGFR inhibitors alone, indicating that the oocytes were able to undergo OM after removal of the PGRMC1 inhibitor (Fig. 5).

Discussion

The present results clearly indicate that Pgmrc1 is involved in estrogen maintenance of meiotic arrest of zebrafish oocytes through Gper. Estrogen inhibition of spontaneous maturation of denuded oocytes was blocked by treatment with the PGRMC1 inhibitor, AG205, and also by incubation of oocytes with the zebrafish Pgrmc1 ab. The Pgrmc1 protein is expressed in the periphery of full-grown oocytes and on the oocyte membrane. Several lines of evidence indicate that Pgrmc1 modulates OM by regulating the membrane expression and functions of Egfr. Treatment with the PGRMC1 inhibitor blocked the stimulatory effects of two EGFR inhibitors, AG1478 and AG825, on GVBD, indicating that Pgrmc1 is required for maintaining the inhibitory influence of Egfr on spontaneous OM. Phosphorylation of the downstream signal of Egfr transactivation, Mapk3/1, was also blocked by treatment with AG205. Finally, treatment with the PGRMC1 inhibitor was shown to prevent expression of Egfr on the oocyte plasma membrane. Collectively, these results indicate that Pgrmc1 acts as an adaptor protein to maintain meiotic arrest of zebrafish oocytes through regulating the expression and functions of Egfr on the oocyte plasma membrane. To our knowledge, the involvement of PGRMC1 in the regulation of oocyte meiotic arrest has not been reported previously.

The finding that Pgrmc1 is expressed in zebrafish ovarian follicles throughout the period of follicular development, when meiosis is arrested at prophase I and circulating estrogen levels are elevated, is consistent with its proposed role in estrogen maintenance of meiotic arrest. *pgrmc1* mRNA is also present in rainbow trout ovaries throughout oogenesis, but the pattern of expression differs from that for zebrafish in that it declines during the later developmental stages (Mourot *et al.* 2006). The fact that Pgrmc1 protein levels are highest in F zebrafish follicles immediately before OM is in accordance

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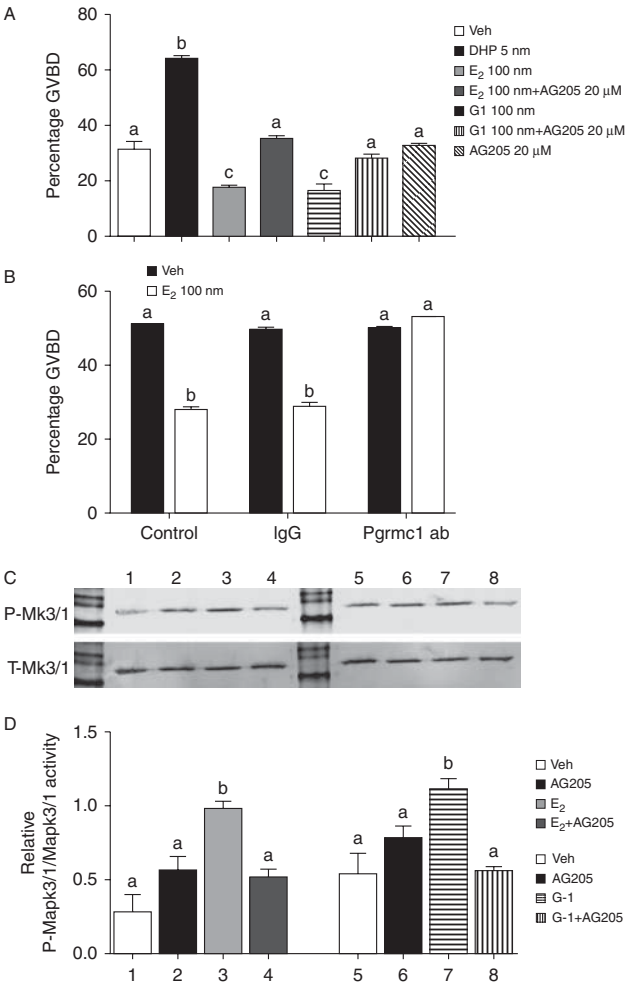

Figure 3
Involvement of Pgrmc1 in estrogen inhibition of OM through activation of Gper and Mapk3/1. (A) Effects of a 3-h co-treatment with 20 μM AG205, a PGRMC1 inhibitor, on E_2 and G-1 (100 nM) inhibition of spontaneous maturation of full-grown denuded zebrafish oocytes in the GVBD bioassay. Veh, spontaneous maturation control; DHP, positive control used to confirm that oocytes are competent to undergo DHP-induced GVBD. (B) Effects of co-treatment with the zebrafish Pgrmc1 antibody (Pgrmc1 ab, 1:300) and IgG on E_2 inhibition of spontaneous OM of denuded full-grown oocytes. (C) Effects of 15 min co-treatment with estrogens and AG205 (20 μM) on phosphorylation of Mapk3/1 in denuded zebrafish oocytes by western blot analysis. P-Mk3/1, phosphorylated Mapk3/1; T-Mk3/1, total Mapk3/1. Representative western blot shown. (D) Relative amounts of p-Mapk3/1 compared with their loading controls, T-Mk3/1, determined from densitometry of western blots. The same lane numbers indicate the same treatments in C and D. All data represent means±S.E.M. of the results compiled from three replicate samples per experiment and from three experiments, $n=9$. Similar results were obtained for each of the three experiments. Different letters denote significant differences from each other ($P<0.05$), one-way ANOVA, and nonparametric Bonferroni's test.

with the experimental results indicating that Pgrmc1 is required for maintaining meiotic arrest of F oocytes by estrogens through the Gper/Egfr pathway, delaying the onset of OM. Importantly, the immunohistochemical and western blot results indicating that Pgrmc1 is expressed in zebrafish oocytes and is localized to the plasma membrane provide the cellular context for a role of Pgrmc1 in oocyte physiology and as an adaptor protein, regulating the expression of receptors on the oocyte cell surface. In agreement with these results, PGRMC1 has also been detected in rainbow trout, rat, and bovine oocytes (Mourot *et al.* 2006, Peluso *et al.* 2006, Luciano *et al.* 2010). Nonetheless, PGRMC1 has been implicated in the

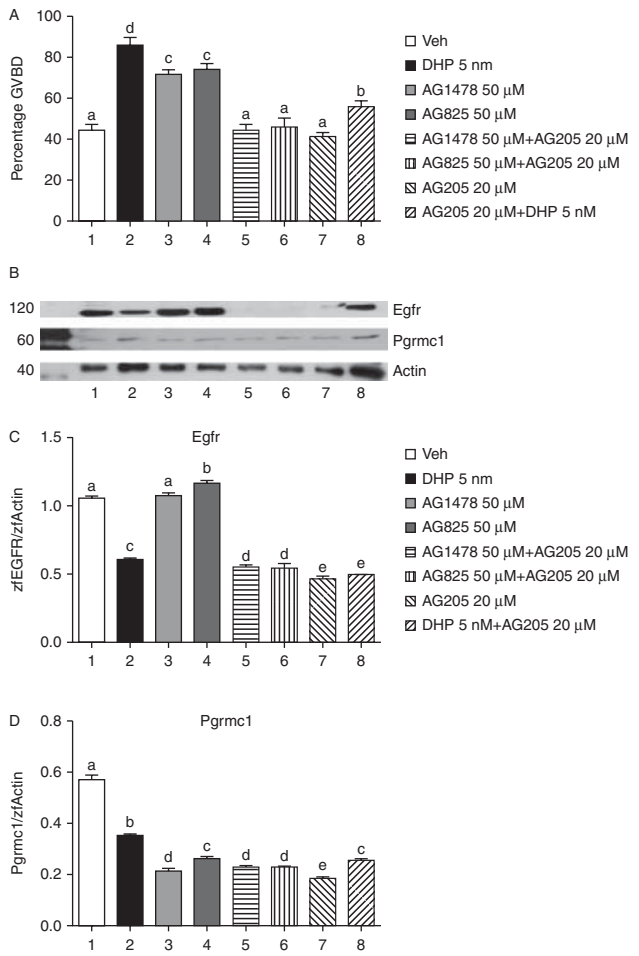

Figure 4
Effects of EGFR inhibitors in the presence or absence of AG205 on OM and role of Pgrmc1 in Egfr expression on oocyte membranes. Effects of a 3 h treatment with DHP and EGFR inhibitors alone or with AG205 on OM of full-grown denuded oocytes (A). Detection of plasma membrane expression of Egfr, Pgrmc1, and β-actin (B) proteins after 6 h of treatment with EGFR inhibitors and AG205, alone and in combination, by western blot analysis. Representative western blot shown. Protein levels of Egfr (C) and Pgrmc1 (D) relative to β-actin levels. The same lane numbers indicate the same treatments in all the graphs. All data represent means±S.E.M. of the results compiled from three replicate samples per experiment and from three experiments, $n=9$. Similar results were obtained for each of the three experiments. Different letters denote significant differences from each other ($P<0.05$), one-way ANOVA, and nonparametric Bonferroni's test.

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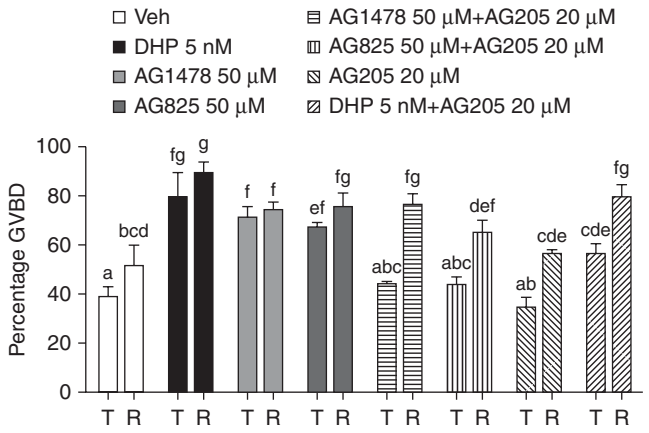

Figure 5
Effects of a 3 h treatment (T) with DHP and EGFR inhibitor alone or with AG205 for 3 h and rescue (R) for 3 h on GVBD of full-grown oocytes. All data represent means±S.E.M. of the results compiled from three replicate samples per experiment and from three experiments, $n=9$. Similar results were obtained in each of the three experiments. Different letters denote significant differences from each other ($P<0.05$), one-way ANOVA, and nonparametric Bonferroni's test.

regulation of an extraordinary variety of functions in vertebrate tissues (Cahill 2007), hence it probably participates in multiple processes in oocytes, in addition to its involvement in estrogen inhibition of OM. In support of this, recent results of experiments with bovine oocytes have indicated that PGRMC1 is also required for induction of OM and after GVBD is co-localized with active phosphorylated aurora kinase B on chromosomes and centromeres, on the basis of which the authors suggested that PGRMC1 is involved in the mechanism of chromosomal segregation (Luciano *et al*. 2010). Surprisingly, PGRMC1 was not detected in either zebrafish or rainbow trout follicle cells, although it is expressed in an immortalized rat granulosa cell line (Peluso *et al*. 2008*a*, Thomas *et al*. 2014), in both granulosa and luteal cells in rat ovary (Peluso *et al*. 2006), and in isolated primary co-cultures of Atlantic croaker granulosa and theca cells (Dressing *et al*. 2010). PGRMC1 has been recently shown to act as an adaptor protein in immortalized rat granulosa cells, increasing the cell membrane expression of mPRα, as well as its [^{3}H]-progesterone binding, signaling, and anti-apoptotic functions (Thomas *et al*. 2014). Taken together, the results from these studies indicate that PGRMC1 has important and diverse roles in ovarian and oocyte physiology. Additional research will be required, however, to determine whether they are solely mediated by the adaptor protein functions of PGRMC1.

Results from previous studies by Peyton & Thomas (2011) have provided strong evidence that Egfr has an important role in maintaining zebrafish oocyte meiotic arrest. Results obtained using inhibitors of the EGFR signaling pathway indicated that E_2 acts through Gper to transactivate Egfr, causing an increase in Mapk3/1 phosphorylation. Taken together, the results from these studies supported the hypothesis that the Egfr/Mapk signaling pathway participates in the estrogen-mediated inhibition of OM in the zebrafish (Peyton & Thomas 2011). The present results, obtained using inhibitors of EGFR and its plasma membrane expression, further support a role for Egfr in the maintenance of meiotic arrest in zebrafish oocytes. Although only low levels of expression of Egfr mRNA were initially detected in zebrafish oocytes (Wang & Ge 2004), recent evidence has indicated that significant amounts of the *Egfr* transcript are present in denuded oocytes (Peyton & Thomas 2011). Moreover, the Egfr protein was present on the oocyte plasma membrane (Peyton & Thomas 2011), which was confirmed in this study.

One of the most important observations from this study is that treatment of denuded oocytes with a PGRMC1 inhibitor, AG205, causes a dramatic decline in Egfr protein expression on the plasma membrane and blocks its signaling, as demonstrated by a complete loss of the stimulatory effects of EGFR inhibitors on OM as well as attenuation of estrogen stimulation of Mapk3/1 phosphorylation. These results are in broad agreement with those obtained by Ahmed *et al*. (2010*a*,*b*) using cancer cells, where PGRMC1 was shown to bind to EGFR and stabilize it on the plasma membrane, whereas AG205 decreased EGFR expression. Treatment with AG205 also inhibited cancer cell growth in these studies which is consistent with the known roles of both PGRMC1 and EGFR in promoting cancer growth and progression (Ono & Kuwano 2006, Ahmed *et al*. 2010*b*). Thus, PGRMC1 acts as an adaptor protein regulating EGFR expression on the cell membranes of normal and malignant cells and promotes EGFR-dependent signaling in both health and disease. The effects of pgrmc1 on estrogen signaling in this study are at least partially indirect through regulation of membrane expression of Egfr. However, PGRMC1 can also directly regulate the membrane expression and receptor functions of the steroid receptors, mPRα and ERβ (Thomas *et al*. 2014). Therefore, the adaptor protein functions of PGRMC1 can be mediated through multiple pathways to modulate steroid hormone signaling, by interacting with the steroid receptors themselves or with their downstream target, EGFR. Additional research will be required to determine whether Pgrmc1 regulates both pathways in the estrogen maintenance of meiotic arrest in fish oocytes.

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The decline in Pgrmc1 protein expression on oocyte membranes observed after treatment with DHP and the EGFR inhibitors is probably associated with the completion of meiotic maturation in the majority of the oocytes, because a dramatic decline in Pgrmc1 levels occurs at GVBD. Treatment with AG205 alone, and in combination with these treatments, also decreased membrane Pgrmc1 expression, but this was not accompanied by increases in GVBD, which indicates that the inhibitor directly influences cell surface expression of Pgrmc1. In contrast, a concentration-dependent increase in whole-cell PGRMC1 protein concentrations was observed in experiments on lung cancer cells after treatment with AG205 (Ahmed *et al*. 2010*b*). The mechanisms by which AG205 alters PGRMC1 expression are unclear but may be associated with alterations in signaling, trafficking, and degradation of the protein once it interacts with AG205. However, these AG205-induced changes in PGRMC1 expression are unlikely to have any functional significance because, as shown previously (Ahmed *et al*. 2010*b*), AG205 inhibits PGRMC1 functions directly through binding to its heme-binding domain, so that the immunoreactive PGRMC1 detected in cells largely represents an inactive form of the protein.

A novel finding from this study is that PGRMC1 is involved in GPER signaling through EGFR. These results indicate that PGRMC1 is probably involved in regulating GPER-dependent estrogen functions mediated through transactivation of EGFR in other tissues. GPER is expressed in a broad range of malignancies, including breast, ovarian, endometrial, prostate, and testicular cancers, as well as in tumors of non-reproductive tissues (Filardo *et al*. 2006, Lappano *et al*. 2013), and mediates estrogen actions such as cell growth through EGFR-dependent signaling in cancer cells (Filardo *et al*. 2000, 2002). Collectively, results from these recent studies have implicated GPER in estrogen-dependent tumor progression through cross-talk with growth-factor signaling pathways, particularly EGFR (Lappano *et al*. 2013). Both GPER and EGFR are currently under investigation as potential targets for treating estrogen-dependent malignancy and also hormone-resistant malignancy. Our results indicate that the role of PGRMC1 in GPER/EGFR signaling in cancer cells is worthy of investigation and may have led to the identification of an additional therapeutic target for treating estrogen-dependent malignancies.

Taken together, the results indicate that Pgrmc1 has a role in maintaining oocyte meiotic arrest and that this involves regulating Egfr membrane expression mediating Gper-dependent estrogen signaling. Information on other potential adaptor protein functions of Pgrmc1 during OM, including up-regulation of Gper and mPRα expression on the oocyte membrane, will be required, however, to obtain a comprehensive understanding of its physiological functions in regulating the onset of OM.

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

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