Involvement of Epidermal Growth Factor Receptor Signaling in Estrogen Inhibition of Oocyte Maturation Mediated Through the G Protein-Coupled Estrogen Receptor (Gper) in Zebrafish (Danio rerio)1

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ABSTRACT

Oocyte maturation (OM) in teleosts is under precise hormonal control by progestins and estrogens. We show here that estrogens activate an epidermal growth factor receptor (Egfr) signaling pathway in fully grown, denuded zebrafish (Danio rerio) oocytes through the G protein-coupled estrogen receptor (Gper; also known as GPR30) to maintain oocyte meiotic arrest in a germinal vesicle breakdown (GVBD) bioassay. A GPER-specific antagonist, G-15, increased spontaneous OM, indicating that the inhibitory estrogen actions on OM are mediated through Gper. Estradiol-17beta-bovine serum albumin, which cannot enter oocytes, decreased GVBD, whereas treatment with actinomycin D did not block estrogen’s inhibitory effects, suggesting that estrogens act at the cell surface via a nongenomic mechanism to prevent OM. The intracellular tyrosine kinase (Src) inhibitor, PP2, blocked estrogen inhibition of OM. Expression of egfr mRNA and Egfr protein were detected in denuded zebrafish oocytes. The matrix metalloproteinase (MMP) inhibitor, ilomastat, which prevents the release of heparin-bound epidermal growth factor, increased spontaneous OM, whereas the MMP activator, interleukin-Talpha, decreased spontaneous OM. Moreover, inhibitors of EGFR (ErB1) and extracellular-related kinase 1 and 2 (Erk1/2; official symbol Mapk3/1) increased spontaneous OM. In addition, estradiol-17beta and the GPER agonist, G-1, increased phosphorylation of Erk, and this was abrogated by simultaneous treatment with the EGFR inhibitor. Taken together, these results suggest that estrogens act through Gper to maintain meiotic arrest via an Src kinase-dependent G-protein betagamma subunit signaling pathway involving transactivation of egfr and phosphorylation of Mapk3/1. To our knowledge, this is the first evidence that EGFR signaling in vertebrate oocytes can prevent meiotic progression.

EGFR, estradiol/estradiol receptor, G protein-coupled estrogen receptor (GPER), GPR30, meiosis, oocyte maturation, zebrafish

INTRODUCTION

Oogenesis in teleosts and amphibians comprises two steroid hormone-dependent phases, a long period of oocyte growth followed by a much shorter period of oocyte maturation (OM). Estrogens produced by the ovarian follicle layer are of primary importance during the initial growth phase, because they regulate the hepatic production of vitellogenin, the yolk precursor protein that is sequestered by the oocytes, resulting in their dramatic enlargement. However, after they are fully grown (i.e., postvitellogenic), the oocytes cannot be fertilized, because the first meiotic division is incomplete and arrested at the G2/M border [1]. The resumption of meiosis and other processes during OM, resulting in a fully mature egg that can be fertilized, comprises the second steroid-dependent phase and is regulated by maturation-inducing steroids (MIS) [2]. OM is initiated in teleosts when the conditions are appropriate for spawning by a surge in luteinizing hormone (LH) secretion that “primes” the oocytes as well as induces the production of the MIS itself. The MIS has been identified as 17,20β-dihydroxy-4-pregnen-3-one (DHP) in salmonid and cyprinid fishes and 17,20β,21-trihydroxy-4-pregnen-3-one in sciaenid and other perciform fishes [3, 4].

Extensive studies over the past 30 years have clarified the roles of LH and the MIS in the regulation of OM in teleosts [3, 4]. During the initial MIS-independent phase, LH induces the oocytes to become maturationally competent (i.e., able to respond to the MIS and complete OM) by upregulating MIS receptor concentrations and the steroidogenic enzymes required for MIS synthesis [5, 6]. In the second MIS-dependent phase, the MIS triggers the resumption of meiosis, migration of the nucleus to the animal pole, and its dissolution (germinal vesicle breakdown [GVBD]) in preparation for fertilization by sperm [5]. The MIS binds to specific receptors on the oocyte cell surface to activate intracellular transduction pathways that induce OM by a nongenomic mechanism [4]. Recently, a novel gene encoding a 40-kDa protein with a predicted seven-transmembrane domain, named membrane progestin receptor alpha (mPRα; official symbol Paqr7), with the characteristics of the MIS membrane receptor was discovered in a fish ovarian cDNA library [7]. The receptor has since been identified in other vertebrate groups and is a member of the progesterone adiponectin Q receptor (PAQR) family [8]. MIS binding to Paqr7 on the surface of fish oocytes causes activation of a pertussis toxin-sensitive inhibitory G protein (G) and subsequent downregulation of membrane-bound adenyl cyclase activity by the G-protein γ subunit. The decrease in adenyl cyclase activity causes cAMP levels in the ooplasm to decline, thereby releasing the oocyte from meiotic arrest [9]. The βγ subunit of the heterotrimeric G protein also appears to mediate a decrease in cAMP levels and promote OM through activation of a phoshatidylinositol 3-kinase/Akt pathway and a subsequent increase in phosphodiesterase activity [10].

 Whereas the important function of progestin hormones (MIS) in inducing OM has been recognized since the 1980s, a critical role of estrogens in regulating the onset of OM in teleosts has only been clearly demonstrated within the past few years [11–13]. Treatment with an aromatase inhibitor increased the spontaneous maturation of follicle-enclosed Atlantic croaker (Micropogonias undulatus) oocytes in vitro, which
could be reversed by estrogen treatment. Interestingly, estrogen treatment also attenuated the stimulatory effects of the MIS on OM in this species [11]. Similar effects of aromatase inhibitors and estrogens on OM were also observed in zebrafish oocytes [12]. Moreover, it was demonstrated that manual or enzymatic removal of the follicle layers caused a marked increase in the spontaneous maturation of zebrafish oocytes, which could be partially reversed by estrogen treatment [13]. It was concluded from these studies that estrogens produced by the ovarian follicle cells exert a powerful inhibitory influence on the resumption of OM in teleosts and that OM does not proceed until MIS levels and MIS receptor concentrations are high enough to override this inhibitory estrogen action [13].

Several lines of evidence suggest that this inhibitory estrogen action on fish oocytes is mediated through a seven-transmembrane receptor, G protein-coupled estrogen receptor (GPER; also known as GPR30) [14] that is a member of the G protein-coupled receptor (GPCR) superfamily. GPER was initially shown to be an intermediary in estrogen signaling and have the binding and signaling characteristics of a membrane estrogen receptor in human breast cancer SKBR3 cells [15]. Subsequently, Gpr was identified in croaker and zebrafish ovaries, and the receptor protein was localized on the plasma membranes of fully grown croaker oocytes capable of undergoing OM and displayed all the binding characteristics of a specific estrogen receptor [11]. Direct evidence for Gpr involvement in the regulation of OM was obtained from experiments showing that the inhibitory effects of estrogens were lost after downregulation of gper expression by microinjection of gper antisense oligonucleotides into zebrafish oocytes and after blocking estrogen activation of the receptor by incubation of the oocytes with a specific Gpr antibody [11, 13]. Croaker Gpr, like the human GPER, is coupled to a stimulatory G protein (Gs), causing increases in adenylyl cyclase activity and elevated cAMP levels, presumably through the G protein α subunit [11]. Therefore, maintenance of elevated cAMP levels in fish oocytes is one plausible mechanism by which estrogens maintain meiotic arrest through Gpr.

To our knowledge, the possibility that signaling through the βγ G protein subunit also contributes to the estrogen inhibition of OM in teleosts has not been examined. Filardo et al. [16] showed that GPER also acts through the βγ G protein subunit to activate the epididymal growth factor receptor (EGFR) and the mitogen-activated protein kinase (MAPK) signaling pathway in human breast cancer cells. Activation of EGFR increases the activity of RAS, RAF, MAP2K (also known as Mek), and the final stage of the signal transduction pathway, MAPK3/1 [16–18]. Therefore, the purpose of the present study was to investigate βγ subunit G protein-signaling through Gpr and transactivation of Egfr and their role in estrogen inhibition of OM in zebrafish.

**MATERIALS AND METHODS**

**Chemicals**

Chemicals, including recombinant human epididymal growth factor (EGF), were obtained from Sigma Aldrich unless otherwise stated. The GPER agonist, G-1, a nonsteroidal, dihydroyquinoline compound (1-(4-(6-bromobenzo[1,3]-dioxyl-5-yl)-3α,5,9β-tetrahydro-3H-cyclopenta[c]quinolin-8-yl)-ethanone), was purchased from EMD Chemicals, and G-15, the GPER-specific antagonist, was a gift from Dr. Eric Prossnitz (University of New Mexico Health Sciences Center, Albuquerque, NM). Estradiol-17β conjugated to bovine serum albumin (E2-BSA) was purchased from Steraloids. Ilomastat was purchased from BIOMOL Research Laboratories, Inc. The MAP2K1/2 (MEK1/2) inhibitor, AZD6244, was purchased from Selleck Chemicals, and the MAP2K1/2 (MEK1/2) inhibitor, U0126, was purchased from Cell Signaling Technology, Inc.

**Animal Care**

Adult zebrafish were obtained from Segrest Farms and maintained in 10-gallon recirculating tanks containing filtered freshwater at 28°C with a 14:10D photoperiod at the University of Texas Marine Science Institute. Fish were fed a diet of brine shrimp twice daily and acclimated to laboratory conditions for approximately 1 wk before use in the OM bioassay, by which time the females were gravid.

**GVBD Assay**

Gravid females (n = 20–30) were removed from the aquaria at 1000 h and deeply anesthetized with 0.01% tricaine methanesulfonate solution for 2 min, then humanely killed by severing the spinal cord and blood supply using procedures in accordance with the Guiding Principles for the Care and Use of Laboratory Animals and approved by the University of Texas Institutional Animal Care and Use Committee. Ovaries were rapidly excised, and ovarian fragments were washed with 60% Leibovitz L-15 medium (L-15). Ovarian follicles were separated with fine forceps and scalpel blades under a binocular microscope followed by gentle pipetting through a Pasteur pipette approximately 50 times. Ovarian follicles greater than 500 μm in diameter were selected for subsequent enzymatic treatment and GVBD bioassay. Oocytes were denuded by enzymatic treatment with collagenase (50 μg/ml) for 1 h and washed several times with fresh L-15 medium. Completion of the follicle cells was confirmed by the absence of nuclei surrounding the denuded oocytes. Nuclei were visualized by staining with 4',6-diamidino-2-phenylindole (DAPI; 1 μg/ml in PBS) and examined under a fluorescent microscope. Approximately 20 oocytes were transferred to each well of a 24-well plate containing 1 ml of L-15 medium and various hormone and inhibitor treatments. Steroids were dissolved in ethanol (EtOH) or dimethyl sulfoxide and added to each well in 1 μl aliquots. An equal volume of the solvents (1 μl) was added to the vehicle control treatment wells. The oocytes were incubated for 3 h at 28°C, and at the end of the incubation period, the number of oocytes that underwent GVBD (i.e., germinal vesicle no longer visible) was counted and expressed as a percentage of the total. A short incubation period of 3 h was used, because spontaneous maturation of untreated denuded oocytes increased during longer incubation periods, thereby obscuring the treatment effects.

The MIS in zebrafish, DHP, was used as a positive control in all the GVBD assays to confirm that the oocytes were maturationally competent. The inhibitory effects of either the endogenous estrogen, estradiol-17β (E2), or the synthetic specific GPER agonist, G-1, on GVBD in the presence of various inhibitors of the Egfr signaling pathway were investigated. Similar effects with both of these GPER agonists were only confirmed for several key downstream components of the pathway to limit animal use. The effects of an antagonist, G-15, on GVBD, as well as the effects of inhibitors of intracellular tyrosine kinase (SRC), matrix metalloproteinase (MMP), EGFRs (EGFR and ERBB2; formerly known as ErbB1 and ErbB2, respectively), and MAP2K1/2 on GVBD, were examined. The SRC inhibitor, PP2, was used at a concentration of 10 μM as outlined by Zheng et al. [20]. The MMP inhibitor, ilomastat (GM6001), was used at concentrations of 1 and 10 μM following the protocol from Quinn et al. [21], whereas the MMP activator, interleukin-1α (IL1α), was used at concentrations of 6 and 30 pM as described by Keller et al. [22]. The EGFR (ErbB1) inhibitors, AG1478 and AG825, and the ERBB2 (ErbB2) inhibitors, AG879 and BG13022, were tested at a concentration of 50 μM following the protocol of Filardo et al. [16], and the MAP2K1/2 inhibitor, U0126, was used at a concentration of 50 μM [23]. The effects of EGF on OM were not examined, because the results with the EGFR inhibitors indicated the presence of two Egfr pathways mediating opposite effects on OM, one (Egfr) causing inhibition of OM and the other (Erbb2) stimulating it. E2-BSA was used to remove any free E2 before use in the GVBD assay. Three microliters of 100 μM E2-BSA were added to 1.5 ml of L-15 medium containing 15 μg of Norit A activated charcoal, and the mixture was stirred for 30 min at 4°C, followed by centrifugation at 2000 × g for 2 min to remove the charcoal. All treatments were replicated four times (n = 4), and all the experiments were repeated at least three times.

**RT-PCR of egfr**

Total RNA was extracted from denuded and follicle-enclosed zebrafish oocytes with TRIzol reagent (Life Technologies), and 40 μl of RNA (4.68 μg/sample) were DNase treated with 10 μl of 10X Buffer (Applied Biosystems) and 2 μl of DNase at 37°C for 1 h. Four microliters of stop solution were added, and the samples were incubated at 65°C for 10 min. Reverse transcription was performed on 0.35 μg of total RNA using 2 μM dNTP, 0.5 μg/μl of oligo(dT)s, 0.1 M dithiothreitol, and 5X first-strand buffer. After 10 min of incubation at 70°C, 0.5 μl of Platinum Superscript III Reverse Transcriptase (Invitrogen) was added to the positive groups, with the negative-
control groups receiving none to confirm lack of DNA contamination, and the samples were incubated for 1 h at 50°C. PCR was performed using Platinum PCR SuperMix High Fidelity (Invitrogen) with 280 nM final primer concentrations. Primers were designed against zebrafish egfr (GenBank accession no. AY332223, sense: 5′-ATCTGAACACGTCCCACACA; antisense: 5′-TGGCTCTCTTACGCGCATCAC), and zebrafish β-actin (actb) for control (sense: 5′-GAGCAGGAGATGGGAACC; antisense: 5′-GATGGAGTTGAAAGTGGTAC) following the manufacturer’s instructions using an annealing temperature of 55°C for 35 cycles.

Western Blot Analysis of egfr

Plasma membranes were prepared from follicle-enclosed and denuded zebrafish oocytes by homogenization with a hand-held glass homogenizer in the presence of protease inhibitors followed by centrifugation at 20000 × g to pellet the plasma membranes as described previously [13]. Membrane samples were incubated with 5X loading buffer (Pierce) at room temperature for 20 min, separated by 10% SDS-PAGE, transferred to nitrocellulose membranes, blocked with Odyssey Infrared Imaging System blocking buffer (Li-Cor Biosciences), and then incubated overnight at 4°C with zebrafish EGFR antiserum (catalog no. 55473, 1:2000; AnaSpec). Membranes were incubated with a fluorescent dye-conjugated goat anti-rabbit secondary antibody and then stripped using Odyssey stripping buffer following the manufacturer’s protocol, and the process was repeated for the total Erk primary antibody (1:1000). Image J Software from Public Research Centre Henri Tudor (Luxembourg-Kirchberg) was used to gather densitometry data of the protein expression.

Statistical Analyses

All results are shown as the mean ± SEM. One-way ANOVA with Bonferroni multiple-comparison test were used to determine statistical differences between control and experimental treatments using GraphPad Prism 3.0 software (GraphPad Software).

RESULTS

Collagenase Treatment of Zebrafish Oocytes

Treatment of zebrafish oocytes with 50 µg/ml of collagenase for 1 h effectively removed the theca and granulosa cells surrounding the oocyte, as shown by a lack of DAPI staining surrounding the surface of the oocytes (Fig. 1A), whereas abundant DAPI staining of nuclei could be seen surrounding the oocytes that had not been treated with the enzyme (Fig. 1B). The number of denuded oocytes after 3 h of incubation that had undergone spontaneous OM, as indicated by clearing of the ooplasm and disappearance of the nucleus (germinal vesicle) resulting in the oocytes becoming opaque, was reduced in the presence of 100 nM G-1 compared to vehicle-treated controls (Fig. 1, C and D).

Effects of Estrogens and a Transcription Inhibitor on Spontaneous Maturation of Denuded Zebrafish Oocytes

At a concentration of 5 nM, DHP significantly increased maturation (P < 0.001) of denuded oocytes compared to the vehicle control group, whereas E2 (10 nM) significantly
reduced spontaneous OM compared to controls. E2-BSA (100 nM) mimicked the actions of E2 and significantly decreased spontaneous OM compared to controls ($P < 0.05$), showing that E2 inhibition of OM is initiated on the surface of the oocytes (Fig. 2A). The transcription inhibitor actinomycin D (1 lM) did not significantly affect the inhibitory actions of 100 nM E2 on OM, which suggests that these estrogen actions are nongenomic (Fig. 2B). G-15, a GPER-specific antagonist, at a concentration of 100 nM did not affect spontaneous OM alone but reversed the inhibitory effects of 10 nM E2 on maturation of denuded zebrafish oocytes ($P < 0.05$) (Fig. 2C).

**Effects of Inhibitors of the Egfr Pathway on Maturation of Denuded Zebrafish Oocytes**

The specific GPER agonist, G-1, has previously been shown to inhibit spontaneous maturation of denuded zebrafish oocytes [13]. The inhibitory effect of G-1 on spontaneous OM was blocked by cotreatment with the Src inhibitor, PP2 (10 lM) (Fig. 3). The MMP inhibitor, ilomastat (1 and 10 lM), partially reversed the inhibitory effects of E2, and the percentages of OM for the combined treatment and ilomastat alone were not significantly different from the controls (Fig. 4A). In contrast, the MMP activator, Il1b, significantly decreased spontaneous OM compared to the controls (Fig. 4B).

**FIG. 2.** Characterization of the mechanism of estrogen inhibition of OM. Effects of a 3-h treatment with (A) an estrogen-protein conjugate, E2-BSA (100 nM); (B) the transcription inhibitor, actinomycin D (AD; 0.1 lM); and (C) the GPER antagonist, G-15 (100 nM), on the E2 (10 nM) inhibition of spontaneous maturation of denuded zebrafish oocytes in the GVBD bioassay. Approximately 80 oocytes per treatment were counted and scored for GVBD. Bars represent the mean ± SEM. Different letters denote significant differences from each other ($P < 0.05$, one-way ANOVA and nonparametric Bonferroni test). Veh, EtOH vehicle control; DHP, positive control (5 nM).
The MAP2K1/2 (MEK1/2) inhibitors, U0126 and AZD6244, at concentrations of 50 μM reversed the inhibitory effects of the estrogens G-1 (Fig. 6A) and E2 (Fig. 6, B and C), increasing the percentage of oocytes that underwent GVBD to levels not significantly different from that of vehicle controls.

Estrogen Regulation of Mapk3/1 Activation in Denuded Zebrafish Oocytes

Estrogen treatment of defolliculated oocytes altered MAPK signaling downstream of Egfr1 transactivation, resulting in Erk1/2 phosphorylation (Fig. 7, A and B). Oocytes treated for 15 min with E2 and G-1 had increased levels of phosphorylated Mapk3/1 compared to vehicle controls, which was confirmed by densitometry (Fig. 7, A and B, respectively). Treatment with EGF (positive control) also activated Mapk3/1 (Fig. 7C). Phosphorylation of Erk in response to E2 was blocked by cotreatment with the EGFR inhibitor, AG1478, confirming that activation of Mapk3/1 occurs via Egfr1 (Fig. 7D).

DISCUSSION

The present results are consistent with a proposed nonclassical mechanism of estrogen action to inhibit meiotic maturation of zebrafish oocytes through binding to Gper and activation of second messengers [12]. Experiments with a cell
membrane-impermeable estrogen conjugate, E2-BSA, show this estrogen action is at the cell surface, whereas those using actinomycin D demonstrate it is nongenomic. The importance of various second messengers as intermediaries in estrogen inhibition of OM was demonstrated using a variety of pharmacological agents. The finding that one of these intermediaries, Mapk3/1, is rapidly phosphorylated in zebrafish oocytes after treatment with the GPER-specific agonist, G-1, suggests this estrogen action is mediated by Gper. Finally, the finding that a GPER-specific antagonist, G-15 [19], increases spontaneous OM provides further evidence that these cell surface-initiated, rapid, nongenomic (i.e., nonclassical) inhibitory actions of estrogens are mediated through Gper. Previous studies have demonstrated that estrogen inhibition of OM in zebrafish is blocked after interference with Gper signaling by incubation of denuded oocytes with a Gper antibody and after knockdown of gper levels by antisense oligonucleotide microinjections, whereas knockdown of esr1 (ERα) with antisense oligos does not affect the estrogen response [11, 13]. Moreover, the GPER-specific agonist, G-1, has been shown to mimic the actions of E2 and decrease spontaneous OM, whereas specific agonists of the nuclear estrogen receptors Esr1 (ERα) and Esr2 (ERβ) are ineffective [14]. Taken together, these findings demonstrate that estrogen inhibition of OM in teleosts is solely mediated through Gper and not through full-length nuclear estrogen receptors.

Recently, the role of GPER as an intermediary in nongenomic estrogen signaling in human breast cancer cells has been questioned by Kang et al. [24] on the basis of their results showing that an N-terminal truncated variant of ESR1 (ERα36), is present in GPER-positive SKBR3 cells that do not express full-length ESRs and appears to be involved in estrogen signaling. However, the results of all the studies reported so far in zebrafish suggest that estrogen inhibition of OM in this species is solely mediated by Gper and does not involve a truncated variant of Esr1 homologous to ER-α36. Our extensive searches of the zebrafish genome and zebrafish expressed sequence tags as well as screening of a zebrafish ovarian cDNA library have not provided any evidence for the existence of an estrogen receptor homologous to ER-α36, or any N-terminal truncated variant form of the nuclear estrogen receptor, in this species (unpublished observations). If zebrafish oocytes expressed an N-terminal truncated form of the nuclear estrogen receptor containing the ligand-binding domain of Esr1 or Esr2, then specific ESR agonists would be expected to recognize it and decrease spontaneous OM. However, neither the mammalian ESR1-selective agonist, propyl-pyrazole-triol, nor the ESR2-selective agonist, diarylpropionitrile, which have been shown to activate fish Esrs [25], shows any estrogenic activity in the zebrafish GVBD bioassay [14]. In contrast, the present finding that the mammalian GPER-selective antagonist, G-15, antagonizes estrogen inhibition of OM in fish is consistent with a role for Gper in mediating this estrogen action. Whereas the study by Kang et al. [24] reveals that N-terminal truncated ESR1 receptor variants may be involved in mediating some estrogen actions in human breast cancer cells, it cannot be concluded from these limited studies that this mechanism is universal in cells containing GPER. Certainly, for zebrafish, the results presented here support the hypothesis that the inhibitory actions of estrogens on OM are solely mediated through Gper.

Extensive investigations of the intracellular signaling pathways induced by estrogens through GPER in breast cancer cell lines show that the receptor is coupled to a Gs and
that activation of the G-protein α subunit enhances membrane-bound adenyl cyclase activity, resulting in increased cAMP concentrations [15, 17]. Experiments using specific inhibitors of critical components of EGFR signaling have demonstrated that estrogen activation of GPER also causes EGFR transactivation in mammalian cells via a G-protein βγ subunit-dependent signaling pathway [16, 26]. In addition, estrogen treatment causes Mapk3/1 phosphorylation in these

FIG. 7. Effects of estrogens on phosphorylation of Mapk3/1 in denuded zebrafish oocytes. A) Treatment (15 min) with E2 (100 nM). B) Treatment (15 min) with G-1 (100 nM), positive control. C) Treatment (15 min) with EGF (50 nM), positive control. D) Effect of cotreatment with the EGFR inhibitor, AG1478 (50 μM), on the response to G-1. Relative phosphorlyated (p) Mapk3/1/Mapk3/1 (%) represents the percentage increase p-Mapk3/1 compared to the loading control, total Mapk3/1. Bars represent the mean ± SEM. Asterisks denote significant differences from respective controls (P < 0.05, Student t-test). Veh, vehicle controls (EtOH for steroids, dimethyl sulfoxide for AG1478, and PBS for EGF).

FIG. 8. Proposed model of estrogen signaling through Gper to maintain meiotic arrest of zebrafish oocytes. The sites of action of the inhibitors used in the present study are also shown. Estrogen binding to Gper results in activation of a Gs. The G-protein βγ subunit transactivates Egfr through Src and MMP, which results in the phosphorylation of Mapk3/1 to inhibit OM.
cells that is dependent on release of heparin-bound EGF and EGFR transactivation, suggesting Mapk3/1 is a downstream target of EGFR [16]. On the basis of their findings in breast cancer cells, Filardo et al. [17] as well as Filardo and Thomas [26] proposed a model of estrogen regulation of Mapk3/1 activation through GPER via a G-protein βγ subunit-dependent pathway involving EGFR transactivation. The results of the present study using the same inhibitors show that estrogens activate a similar G-protein-mediated signaling pathway in zebrafish oocytes involving Src, MMP, Egfr1, and Mapk3/1, presumably through activation of the G-protein βγ subunit (Fig. 8). Previous studies have shown that estrogen activation of the G-protein α subunit through Gper in teleost oocytes initiates the same second-messenger pathway as in human breast cancer cells, increasing membrane-bound adenylyl cyclase activity and cAMP levels [11, 13, 15, 26]. The finding that estrogen binding to GPER in representatives of two distantly related vertebrate groups, fish and mammals, results in stimulation of the same G-protein α and βγ subunit-mediated second messengers involving activation of adenylyl cyclase and EGFR transactivation suggests that GPER signaling pathways are evolutionarily conserved in vertebrates.

The present results provide strong evidence for a role of Egfr in zebrafish oocytes in maintaining oocyte meiotic arrest. In addition, preliminary evidence was obtained that Erbb2 signaling in oocytes is involved in DHP stimulation of OM in this species. EGFR signaling in follicle cells has been shown to be necessary for induction of OM in a variety of mammalian species [27–30]. Similarly, egfr mRNA was identified in zebrafish follicle cells, and Egfr was proposed to have a paracrine mode of action to influence zebrafish oocyte physiology and promote OM [31, 32]. Only minor amounts of egfr mRNA were found in zebrafish oocytes in a previous study [32], whereas in the present study, high expression levels of egfr mRNA were detected in denuded zebrafish oocytes using two different sets of primers, and the Egfr protein was detected on oocyte plasma membranes using a zebrafish Egfr antibody. Previous studies have identified EGFR in both oocytes and follicles in other vertebrates [33, 34]. Therefore, EGFR signaling in both the oocytes and follicle cells through multiple EGFR subtypes appears to be involved in the regulation of OM.

The demonstration that the MMP inhibitor, ilomastat, blocks estrogen inhibition of OM suggests the estrogen signaling pathway in zebrafish oocytes involves MMP activation and transactivation of Egfr, because ilomastat has been shown to block activation of EGFR in human breast cancer cells by preventing the release of HBEGF [16, 35, 36]. The experiment showing that an MMP activator, III1x, significantly decreased spontaneous OM is consistent with a requirement for MMP activation for induction of OM in zebrafish. The results with the EGFR inhibitors suggest that transactivation of the Egfr1 member of this family of receptor tyrosine kinases, but not the Egfr2 member, is necessary to maintain meiotic arrest of zebrafish oocytes through GPER. This is consistent with previous findings in mammals showing that estrogen causes ErbB1 transactivation in mouse spermatogonial cells [37] and ovarian cancer cells [38] through GPER. An interesting finding was that treatment with the ErbB1 (EGFR) inhibitors, AG1478 or AG825, not only blocked the inhibitory effect of E2 but also increased spontaneous OM to levels induced by the MIS, DHP. This suggests that Egfr is an intermediary of other OM inhibitory mechanism(s) in addition to that mediated by estrogens. Members of the transforming growth factor-β (TGF-β) superfamily, including bone morphogenetic protein 15 and TGFβ1, have been shown to partially inhibit OM in zebrafish [39]. In addition, two orphan GPCRs, Gpr3 and GPR12, are involved in maintaining oocyte meiotic arrest in zebrafish and rodent oocytes, respectively, through activation of Gi [40, 41]. However, none of these TGF-β family members or orphan GPCRs has been shown to act through EGFR1, so the identities of the other potential regulators of Egfr in zebrafish oocytes remain unclear. Preliminary data were also obtained in the present study suggesting a stimulatory influence of Erbb2 on OM, because slight decreases in spontaneous OM were observed after treatment with ERBB2 inhibitors AG879 or RG13022. Possible transactivation of erbb2 in zebrafish oocytes by the MIS, DHP, through binding to mPrxγ/β is a plausible mechanism to induce OM, because the stimulatory effects of DHP on OM were attenuated by treatment with these inhibitors in the present study and progesterone has been recently shown to transactivate EGFR via mPrxγ/β in breast cancer cells [42]. Alternatively, the resumption of meiosis could be partially mediated by a DHP-induced inhibition of egfr transactivation. Although extensive evidence indicating an involvement of other hormones or receptors in the regulation of OM through EGFRs is lacking, the present results clearly demonstrate that egfr transactivation is regulated by estrogens through GPER to maintain meiotic arrest in zebrafish oocytes.

Activation of the MAPK signaling cascade and subsequent Mapk3/1 phosphorylation are major downstream effects of E2 transactivation of EGFR via GPER in breast cancer cells [17]. The results show the same signaling pathway is activated by estrogens through Gper/Egfr in zebrafish oocytes. Both of the MAP2K1/2 (MEK1/2) inhibitors tested, U0126 and AZD6244, blocked estrogen inhibition of the maturation of denuded oocytes, suggesting Map2k1/2 and Mapk3/1 are components of this estrogen signaling pathway. The involvement of Mapk3/1 was confirmed in an experiment showing that Erk1/2 phosphorylation was increased after treatment with E2 and the GPER-specific agonist, G-1. The fact that the stimulatory effect of G-1 on Mapk3/1 phosphorylation was blocked by cotreatment with AG1468 suggests that Egfr is upstream of the MAPK signaling cascade. Thus, these results indicate that estrogens act through GPER to transactivate Egfr, resulting in an increase of Mapk3/1 phosphorylation. It is concluded from these experiments that maintenance of meiotic arrest in zebrafish oocytes is at least partly regulated through activation of Src, MMP, Egfr (ErbB1), and Mapk3/1 via estrogen-induced GPER signaling (Fig. 8).

Although the importance of EGFR signaling in the regulation of OM has been demonstrated in a wide variety of vertebrate models [27–30], the results of the present study provide the first evidence, to our knowledge, for a role of an EGFR in maintaining oocyte meiotic arrest. Moreover, no previous reports have described an involvement of EGFR and EGFR-dependent signaling in denuded oocytes in the regulation of OM in any vertebrate species. Previous studies have shown a requirement for EGFR-dependent signaling in follicle cells for the induction of OM [27–30]. EGFR expression has also been reported in mammalian and avian oocytes, but its physiological functions are unknown [33, 34]. The possibility that EGFR has a similar function in the maintenance of meiotic arrest in mammalian oocytes to that identified in zebrafish is currently being investigated.

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