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Involvement of epidermal growth factor receptor (EGFR) signaling in estrogen inhibition of oocyte maturation mediated through G protein-coupled estrogen receptor 1 (GPER) in zebrafish (*Danio rerio*)

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Involvement of epidermal growth factor receptor (EGFR) signaling in estrogen inhibition of oocyte maturation mediated through G protein-coupled estrogen receptor 1 (GPER) in zebrafish (*Danio rerio*)

by

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Dedication

This work is dedicated to my loving family. Without their support and encouragement my education would not be possible. Specifically, I'd like to thank Mom for always bringing peace when I was most anxious. Dad, thank you for always making me laugh out loud. Bridge and Nad, thanks for all of the sister pictures and visiting me in Port A when I needed family the most. JG, thanks for being the best little brother ever – I loved that you did a project about me when you were in second grade. I also love being able to tell "John Gabriel Stories" about how clever and funny you are! Aunt Marcia, thanks for helping me financially and emotionally get through the grad school years. I love you all!

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Abstract

Involvement of epidermal growth factor receptor (EGFR) signaling in estrogen inhibition of oocyte maturation mediated through G protein-coupled estrogen receptor 1 (GPER) in zebrafish (*Danio rerio*)

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Oocyte maturation (OM) in teleosts is under precise hormonal control by estrogens and progestins. We show here that estrogens activate an epidermal growth factor receptor (EGFR) signaling pathway through the G protein-coupled estrogen receptor (GPER) to maintain meiotic arrest of full-grown zebrafish (*Danio rerio*) oocytes in an *in vitro* germinal vesicle breakdown (GVBD) bioassay. A GPER-specific agonist decreased OM and a GPER-specific antagonist increased spontaneous OM, whereas specific nuclear estrogen receptor (ERα and ERβ) agonists did not affect OM, which suggests the inhibitory action of estrogens on OM are solely mediated through GPER. Furthermore, a peptide-bound estrogen, which cannot enter the oocyte, decreased GVBD, showing that these estrogen actions are mediated through a membrane receptor. Treatment of oocytes with actinomycin D, a transcription inhibitor, did not block the inhibitory effects of estrogens on OM,

indicating that estrogens act via a nongenomic mechanism to maintain oocyte meiotic arrest. EGFR mRNA was detected in denuded zebrafish oocytes by reverse transcription polymerase chain reaction (RT-PCR). Therefore, the potential role of transactivation of EGFR in estrogen inhibition of OM was investigated. The matrix metalloproteinase inhibitor, ilomastat, which prevents the release of heparin-bound epidermal growth factor (HB-EGF), increased spontaneous OM. Moreover, specific EGFR1 (ErbB1) inhibitors and inhibitors of extracellular-related kinase 1 and 2 (ERK1/2) increased spontaneous OM. Previously, estrogens have been shown to increase 3'-5'-cyclic adenosine mono phosphate (cAMP) levels through GPER in zebrafish oocytes during meiotic arrest. Taken together these present results suggest that estrogens also act through GPER to maintain meiotic arrest through a second signaling pathway involving transactivation of EGFR and activation of ERK 1 and 2.

Table of Contents

List of Figures

CHAPTER 1

INTRODUCTION: REGULATION OF OOGENESIS WITH SPECIAL REFERENCE TO THE MEMBRANE ESTROGEN RECEPTOR, GPER AND THE SIGNALING CASCADE THROUGH WHICH IT ACTS.

The timing and complex series of processes that occur during the reproductive cycle of fish are precisely controlled by hormones secreted by the hypothalamic-pituitary-gonadal (HPG) axis. Environmental stimuli are detected by sense organs that transmit this information through neural pathways to the hypothalamus, the region of the brain controlling reproduction (Thomas 2008). The hypothalamus integrates the diverse stimulatory and inhibitory stimuli mediated by neurotransmitters and neuropeptides and either promotes or inhibits transcription of the primary reproductive regulating neurohormone, gonadotropin-releasing hormone (GnRH). Only one of the three GnRH forms, seabream GnRH, causes release of gonadotropins in perciform fishes (Gothilf et al. 1996; Mohamed et al. 2005). Nerve terminals in the pituitary in the vicinity of the gonadotropes secrete GnRH. Gonadotropin-releasing hormone binds to specific G protein-coupled receptors (GPCRs) on the gonadotropes causing activation of second messengers resulting in the release of the gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) into the blood stream. The gonadotropins in turn bind to specific GPCRs on gonadal steroidogenic cells to regulate steroidogenesis and the secretion of growth factors and regulatory peptides. These gonadal hormones control the various stages of gametogenesis as well as exerting feedback effects on the hypothalamus and pituitary to regulate gonadotropin secretion. Both nuclear steroid receptors and

membrane steroid receptors have been shown to mediate steroid hormonal control of reproductive functions in vertebrates (Hammes and Levin 2007). The mechanisms of steroid hormonal action have been evolutionarily conserved throughout vertebrates from fish to humans (Thomas et al. 2005a, Pang and Thomas 2009). Therefore results in fish can reveal many actions of steroid hormones and hormone receptors that could provide a plausible explanation for the pleiotropic actions of steroids in vertebrates, including humans.

1.A. Genomic and Non-Genomic Mechanisms of Steroid Action

The classic, or genomic, model of steroid hormone action involves diffusion of the steroid hormone into the cell where it binds to a member of the nuclear steroid receptor family (ligand activated transcription factors) resulting in its activation, dimerization, and translocation to the nucleus where it binds to a hormone response element (HRE) on target genes resulting in alterations in their rates of transcription (Yamamoto 1985). This genomic mechanism of steroid action involving new mRNA and protein synthesis is relatively slow and occurs over several hours or even days. While this is the most recognized mechanism of steroid action, a study as early as 1942 showed that steroid hormones could also initiate a cellular response in seconds to minutes (Selye 1942).

Rapid actions of steroids can occur without gene transcription via the activation of intracellular signaling cascades. Lösel and Wehling (2003) summarize several criteria used to indicate nongenomic steroid action: 1, the absence of a 'normal, functional' nucleus in some cell types excludes genomic action (e.g.

spermatozoa and erythrocytes); 2, effects not blunted by inhibitors of transcription or translation cannot involve gene expression; 3, a time frame in the seconds to minutes range; and 4, effects elicited by steroid hormone analogues that do not access the cell interior exclude the classical mechanism steroid action. Using various assays based on the above-mentioned criteria, the identification and biochemical characterization of several membrane steroid hormone receptors has been possible (Zhu et al. 2003; Thomas et al. 2005b).

1.B Oogenesis

The ovarian cycle in fish functions to produce mature ova that are ready to be fertilized, via oogonial proliferation, primary oocyte growth, secondary oocyte growth, and final oocyte maturation, culminating in ovulation directly before the spawning event (Khan and Thomas 1999). Patiño and Sullivan (2002) have characterized the stages of oogenesis as follows: (1) formation of primordial germ cells (PGCs) (germ line segregation), (2) transformation of PGCs into oogonia (sex differentiation), (3) transformation of oogonia into oocytes (onset of meiosis), (4) growth of oocytes while under meiotic arrest, (5) resumption of meiosis (maturation), and (6) expulsion of the ovum from its follicle (ovulation). Stages 1-3 can also be termed pre-vitellogenic, stage 4 vitellogenic, stage 5 oocyte maturation, and stage 6 ovulation.

Oogonial proliferation correlates with the beginning of the seasonal ovarian cycle, which, in the case of the tropical fish zebrafish, occurs on a daily basis year round (Khan and Thomas 1999). Oogonial proliferation is characterized by the

oogonia, female germ cells, undergoing mitosis to give rise to primary oocytes. Primary oocyte formation occurs during the peak of oogonial proliferation and results in the arrest of meiosis at prophase I. This stage is immediately followed by the formation of a follicular wall around the oocyte, termed folliculogenesis (Khan and Thomas 1999).

The beginning of secondary oocyte growth is marked by the appearance of cortical alveoli, round muco-polysaccharide or glycoprotein-rich structures (Khan and Thomas 1999). Surrounding the oocyte plasma membrane is the acellular vitelline envelope (*zona radiata*), the future eggshell, which is surrounded by the follicle cells. Follicle cells contribute to steroidogenesis and consist of an inner layer of granulosa cells and at least one outer layer, sometimes more, of theca cells (Khan and Thomas 1999). During secondary oocyte growth the developing oocyte is said to be vitellogenic. Vitellogenesis, a female-specific function of the reproductive system, is the production of the yolk precursor protein, vitellogenin (VTG), which is synthesized in the liver in response to estrogen (Khan and Thomas 1999). The duration of vitellogenesis varies between species, depending on the rate of spawning particular to each species (Jalabert 2005), which is ultimately controlled by feedback-regulated pituitary gonadotropin release (Dickey and Swanson 1998). Estradiol-17β (E_2) is most widely accepted as the steroid hormone that induces the production of vitellogenin in fish hepatocytes (Bevelander et al. 2006; Montserrat et al. 2004). It is produced by the activity of cytochrome P-450 aromatase (P-450 arom), a steroid-metabolizing enzyme that converts testosterone to E_2 (Montserrat et al. 2004).

Follicle stimulating hormone (FSH, GTH I) is released by the pituitary in response to the negative feedback of E_2 (Bevelander et al. 2006) and other intragonadal autocrine/paracrine factors, such as inhibin and activin (Tyler et al. 1997), and mediates vitellogenic development of oocytes (Tyler et al. 1997). FSH functions to stimulate the expression and activity of P-450 arom, which increases ovarian production of E_2 that is secreted into the circulation (Montserrat et al. 2004).

Estradiol circulates through the blood from the ovaries to the liver and binds to hepatic nuclear estrogen receptors (nERs) to increase hepatic ER mRNA and protein, which in turn increases the hepatic sensitivity to estrogen stimulation of vitellogenesis (Khan and Thomas 1999). A recent study by Leaños-Castañeda and Van der Kraak (2007) looked at the effect of different estrogenic compounds known to preferentially interact with specific ER subtypes. They tested the ability of an ERα selective agonist (propyl-pyrazole-triol), ERα selective antagonist (methyl-piperidino-pyrazole), and an ERβ selective agonist (diarylpropionitrile) to induce or inhibit vitellogenin production in rainbow trout. Estradiol-17β and the ERβ selective agonist were the only compounds that induced a dose-dependent increase in vitellogenin synthesis and the ERα selective antagonist did not inhibit the estradiol-induced vitellogenin production. From these results they concluded that vitellogenin production is mediated via ERβ in this species.

In addition to the synthesis of VTG in response to E_2, the process of vitellogenesis requires the release of VTG into the blood, transport to the oocytes, uptake by the oocytes, and conversion to the yolk proteins lipovitellin and phosvitin.

This mechanism takes place during ovarian recrudescence and the final products, yolk proteins, positively correlate with the plasma levels of FSH and E_2 (Khan and Thomas 1999). The receptor for VTG is a member of the low-density lipoprotein receptor (LDLR) superfamily and functions by transporting VTG into the oocytes (Davail et al. 1998). VTG receptor transcripts are abundant in the ovary, and are present in pre-vitellogenic oocytes, indicating that production of the receptor protein precedes the yolk deposition phase (Davail et al. 1998). At the end of yolk protein accumulation by the growing oocytes, the concentration of E_2 in rainbow trout decreases rapidly, triggering the release of LH, the gonadotropin responsible for inducing final oocyte maturation (Reis-Henriques et al. 2000). Reis-Henriques et al. (2000) suggested that the decrease in E_2 production in rainbow trout is due to a VTG-induced decrease in aromatase activity in thecal cells. However, estrogen levels do not show a similar decline in multiple spawning teleost species in which yolk accumulation is continuing in the smaller growing oocytes at the same time that final maturation is occurring in the largest full-grown oocytes (Khan and Thomas, 1999). These hormonal cues, regulated by the hypothalamic-pituitary-gonadal-liver (HPGL) axis, together with seasonal environmental cues, regulate the timing of oocyte production.

Final oocyte maturation (FOM), characterized by meiotic resumption, follows the period of oocyte growth. The timing of the induction of oocyte maturation, including germinal vesicle migration (GVM) and germinal vesicle breakdown (GVBD), correlates positively with a surge in circulating levels of a second

gonadotropin controlling the female reproductive system, luteinizing hormone (LH, GtH-II) (Thomas et al. 2001). Luteinizing hormone stimulates the production of a progestin hormone membrane receptor as well as an ovarian C21 steroid hormone, termed maturation-inducing steroid or substance (MIS) (Thomas et al. 2001). Depending on the species, the MIS in a teleost fish could be 17α,20β,21-trihydroxy-4-pregnen-3-one (20β-S) or 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P or DHP) (Khan and Thomas 1999). DHP is the MIS in zebrafish and other cyprinid fish.

Oocyte maturation occurs in two phases (Thomas et al. 2001). The first phase, the priming phase, involves the induction of oocyte maturational competence (OMC), which requires synthesis of MIS receptors, key components involved in the development of the ability of the oocyte to respond to progestins. The second phase, germinal vesicle breakdown (GVBD), completes oocyte maturation, which requires the presence of MIS. Following increases in LH concentrations, levels of the membrane progestin receptor protein are elevated during meiotic maturation of oocytes and decrease in ovulated oocytes, suggesting it is involved in the development of the ability of oocytes to respond to progestins (OMC) (Thomas et al. 2001).

While it has been observed for over a decade that the MIS acts via a nongenomic mechanism through a membrane progestin receptor to induce maturation of teleost oocytes (Patiño and Thomas 1990), it is only recently that the identity of the membrane progestin receptor for MIS has been discovered (Zhu et al. 2003). Inhibitors of RNA synthesis and protein synthesis do not prevent MIS from initiating

GVBD (Thomas et al. 2002). Injection of the oocytes with pertussis toxin (PTX) prevents GVBD, implicating the involvement of a PTX-sensitive inhibitory G protein (Thomas et al. 2002), which is consistent with previous findings that induction of oocyte maturation involves a decrease in intracellular cAMP (Yoshikuni and Nagahama 1994). The MIS receptor cDNA has been cloned from spotted seatrout ovaries (Zhu et al. 2003) and identified as a seven transmembrane receptor, named membrane progestin receptor alpha (mPRα), a member of the progesterone and adipoQ receptor (PAQR) subfamily (Thomas et al. 2007).

Luteinizing hormone control of FOM, which includes both the process of meiotic resumption and the structural changes that the oocytes undergo (eventually GVBD and the formation of a micropyle, a pore through which sperm enter (Patiño and Sullivan 2002)), can occur in a time span of <24 hours (Khan and Thomas 1999). Once GVBD is complete the micropyle has already formed and the oocytes swell due to the rapid influx of water, maintained by active ion transport via oubain-sensitive Na^+, K^+-ATPase, and proteolysis of yolk proteins (Khan and Thomas 1999). Ovulation occurs in response to swelling of the oocyte in addition to the actions of the nuclear progesterone receptor (PR) and the production of the prostaglandin PGF2α. In most fish, the completion of ovulation and formation of fully functional ova is followed within 2 to 3 hours by the spawning event (Khan and Thomas 1999).

1.C G protein-coupled estrogen receptor 1 – GPER

GPER has been identified as a seven transmembrane (7TM) estrogen receptor that initiates rapid second messenger signals via a stimulatory G protein (Thomas et

al. 2005). The receptor protein is expressed in testicular, ovarian, and oocyte membranes of fish as well as human breast cancer cells (Thomas et al. 2005; Pang et al. 2008). In addition to fish and humans, this membrane estrogen receptor is evolutionarily conserved in many other vertebrates including chicken, platypus, opossum, mouse, rat, dog, horse, cow, monkey, and chimpanzee (Pang et al. 2008).

GPER functions to inhibit oocyte maturation in fish when activated by estrogens. This action of GPER has been identified in different suborders of fish including Acanthopterigii (Pang et al. 2008) and Ostariophysi (Pang and Thomas 2009). In both Atlantic croaker and zebrafish GPER is localized to the oocyte membrane and not the follicular cell membranes. It has high-affinity, limited-capacity binding sites for E_2 (Pang et al. 2008), which are decreased by preincubation with GTPγS, suggesting that GPER is directly coupled to a G protein (Filardo et al. 2007). In addition, co-immunoprecipitation with a G_s α-subunit antibody, and increased cAMP production when incubated with GPER-specific agonists, suggests that the GPER is associated with a stimulatory G protein.

Pang et al. (2008) have also shown that 1,4,6-Androstatrien-3,17-dione (ATD), an aromatase inhibitor, causes an increase in spontaneous OM, and addition of the GPER agonists (including G-1, ICI 182 780, and tamoxifen) significantly reduced the amount of spontaneous OM. Furthermore, microinjection with GPER antisense oligonucleotides resulted in an increase in spontaneous OM. Taken together these results provide strong evidence that GPER exerts an inhibitory influence of OM in fish. Although the physiological significance of this function of GPER is not fully

understood, one possibility is that it prevents precocious maturation of oocytes in fish, delaying it until the MIS stimulatory signal is strong enough to induce maturation of all the competent oocytes, thereby synchronizing OM (Pang and Thomas 2009).

1.D Epidermal growth factor receptor – EGFR

Epidermal growth factor receptor is a transmembrane protein that is activated when EGF binds to the receptor and causes dimerization. Dimerization results in phosphorylation of the receptor resulting in its activation. There are four different types of EGFRs that all belong to the erythroblastic leukemia viral (v-erb-b) oncogene homolog (ErbB) family of receptors, which include EGFR (ErbB-1), HER2/c-neu (ErbB-2), Her 3 (ErbB-3) and Her 4 (ErbB-4). Oda et al (2005) report that 15 different ligands from the EGF family have been identified for the four different types of receptors, which includes amphiregulin, betacellulin, biregulin, EGF, epiregulin, HB-EGF, heregulin α/β, neuregulin (NRG) 1α/1β/2α/2β/3/4, and transforming growth factor alpha (TGFα). ErbB receptors function by activating different intracellular pathways including the MAP kinase cascade, Ca^{2+} cascade, phosphatidlinositol 4,5-bisphosphate (PIP) signaling, and transcription (Oda et al 2005). Wang and Ge (2004) identified EGFR in zebrafish, although their findings indicated that the receptor was mainly localized to the surrounding follicle cells, and they suggested that this resulted in a paracrine function of EGF. However, our data reported here indicates that EGFR is present in denuded oocytes.

Filardo et al. (2002) provided evidence that in human breast cancer cells GPER also acts through the βγ G protein subunit to activate the MAPK signaling

pathway through epidermal growth factor receptor (EGFR). Filardo et al. (2002) have proposed a mechanism for E_2 action via GPER to regulate growth factor receptors in mammalian cells via the Gβγ-subunit activation of an EGFR. Activation of ErbB1 increases the activity of Ras, Raf, Mek, and the final stage of the signal transduction pathway, ERK1/2 (Migliaccio et al. 1996).

1.E HYPOTHESIS AND GOALS OF RESEARCH

The purpose of this study is to investigate whether or not GPER acts through the same βγ G protein subunit pathway as in human breast cancer cells to regulate oocyte maturation in fish. My preliminary studies indicate that GPER activates the EGF pathway to inhibit oocyte maturation in zebrafish (*Danio rerio*). The role of the EGF pathway in OM will be investigated using a variety of molecular, biochemical, and immunological assays. This research tests the hypothesis that estrogens act through the EGFR pathway via GPER to inhibit the resumption of meiotic maturation. Three subhypotheses are investigated to address the above hypothesis: 1) Estrogens act through GPER, not through nuclear ERs, to inhibit oocyte maturation; 2) GPER inhibits oocyte maturation by activating the epidermal growth factor receptor 1 (ErbB1); 3) Activation of ErbB1 also upregulates the proteins and the activity of the proteins involved in the EGF signal transduction pathway.

1.F APPROPRIATENESS OF MODEL SPECIES

Zebrafish raised in the laboratory can provide maturationally competent oocytes daily. In addition, the oocytes are large (approximately 500μm), which makes them easy to denude and to perform biological assays. Since zebrafish go through the

cycle of mitosis, meiotic arrest, and resumption of meiosis, all of which are controlled by sex steroid hormones, on a daily basis, it makes zebrafish an excellent model to investigate the role of estrogen-mediated inhibition of oocyte maturation.

CHAPTER 2

ROLE OF DIFFERENT CLASSES OF ESTROGEN RECEPTORS IN INHIBITION OF OOCYTE MATURATION IN ZEBRAFISH, *DANIO RERIO*, WITH A FOCUS ON THE INVOLVEMENT OF THE EGFR SIGNALING PATHWAY

INTRODUCTION

Estrogens, like other hormones, exert their actions on target tissues by binding to and activating specific receptors to induce a biological response. The actions of estrogens were previously thought to occur only through classical nuclear estrogen receptors (ERs), of which two have been identified in mammals, ERα and ERβ (Kuiper et al. 1996), and three in fish ERα, ERβ1 and ERβ2 (Hawkins et al, 2000). Because estrogen is a lipophilic hormone, it can easily pass through the cell membrane in order to bind to and activate the classical ERs, located inside the cell. Once bound, these classical ERs dimerize and subsequently translocate into the nucleus where they bind to an estrogen response element (ERE) on the DNA to induce gene transcription (i.e. a genomic mechanism) (Marino et al. 2006). However, in the past 30 years nongenomic estrogen actions have been identified that are rapid, and mediated at the cell surface. Estrogens can function through a novel seven transmembrane (7TM) receptor, G protein-coupled estrogen receptor 1 (GPER), formerly known as GPR30, to regulate reproductive functions (Filardo and Thomas 2005; Prossnitz et al. 2008).

Carmeci et al. (1997) discovered the orphan GPCR, GPR30 in MCF7 cells, and it has since been identified by Thomas et al. (2005) and Prossnitz et al. (2005) as

a membrane estrogen receptor, GPER. Estradiol-17β (E_2) has been shown to bind to GPER with high affinity in several different vertebrate cells including human breast and ovarian cancer cells and teleost oocytes (Filardo et al. 2000; Thomas et al. 2005b; Albanito et al. 2007; Pang et al. 2008; Pang and Thomas 2009). In addition, it has been shown that alterations in GPER expression by transfection with GPER cDNA or siRNA are accompanied by parallel changes in estrogen functions purported to be mediated by the receptor, such as inhibition of OM in teleosts (Pang et al., 2008). A GPER specific agonist, G-1, has also been useful for identifying functions mediated through GPER (Prossnitz et al. 2008).

G protein-coupled receptors couple to heterotrimeric G-proteins, consisting of an α subunit and a βγ subunit which dissociate from each other and the receptor upon ligand activation of the receptor to initiate alterations in second messengers such as cAMP (Clapham and Neer 1997). It has been demonstrated that GPCRs couple to a variety of different G proteins in vertebrates, including stimulatory G proteins ($G\alpha_s$ subunit) that increase the production of intracellular cAMP, and inhibitory G proteins ($G\alpha_i$ subunit) that inhibit cAMP production, resulting in decreased cAMP levels (Neves et al. 2002). In addition to regulation of cAMP production by the Gα subunit, there are also changes in second messengers due to initiation of different pathways by the Gβγ-subunit. These include pathways involving calcium mobilization, phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein kinase (Mapk1/3), extracellular related kinase 1 and 2 (ERK1/2), and Akt (Filardo et al. 2000; Filardo et al. 2002; Revankar et al. 2005; Dennis et al. 2009). Signaling from these second

messengers can result in cell adhesion, migration, survival, proliferation, and cancer (Revankar et al., 2005).

Production of cAMP in response to estrogen treatment in cells expressing GPER, suggested that GPER is coupled to a stimulatory G protein, which has since been confirmed by Thomas et al. (2005b). Additionally, the (ERK1/2) signal transduction pathway has been shown to be activated in response to GPER Gβγ-subunit activation of matrix metalloproteinase (MMP) in human breast cancer cells (Filardo et al. 2000; Filardo et al. 2008; Quinn et al. 2009). The MMP then releases heparin-bound epidermal growth factor (HB-EGF), which then binds to EGFR and elicits activation that results in ERK1/2 phosphorylation. In human breast cancer tissue this signal can cause formation of fibronectin assemblages as well as metastasis of the cancer itself (Filardo et al. 2000; Quinn et al. 2009). GPER also functions in cancer cell lines other than breast cancer, including ovarian cancer cells (Albanito et al. 2008), spermatogonial cells (Sirianni et al. 2008), endometrial cancer cells (Revankar et al. 2005), and thyroid carcinoma cell lines (Vivacqua et al. 2006). It is therefore very important to understand the different mechanisms of estrogen signaling, because modulation of these pathways may be of therapeutic value for treating cancer

However, reproductive functions of GPER have been identified in noncancerous cell lines, for example a role in maintaining meiotic arrest of oocytes in teleosts, including the marine sciaenid, Atlantic croaker (*Micropogonias undulatus*) (Pang et al. 2008) and the freshwater cyprinid, zebrafish (*Danio rerio*) (Pang and

Thomas 2009). These researchers have localized GPER in oocytes of both species and found that it displays high-affinity, limited-capacity, and displaceable binding specific for estrogens. Furthermore, treatment of oocytes with both E_2 and G-1, the GPER-specific agonist, inhibited OM, whereas treatment with an aromatase inhibitor, ATD, increased OM, which suggests that the induction of OM is under inhibitory control by endogenous estrogens. The inhibitory effects of estrogens were abrogated by injection of antisense oligonucleotides against GPER, implicating the receptor as an intermediary of this estrogen action. Furthermore, estrogen activation of GPER was associated with an increase in cAMP production, suggesting that, as in the case with human GPER, teleost GPER is also coupled to a stimulatory G protein (Gs) (Thomas et al. 2005).

The purpose of this study is to investigate the Gβγ subunit signaling of GPER to determine if it also functions similarly to human GPER, which has been characterized by Filardo and colleagues as transactivating EGFR. Data presented support the hypothesis that second messenger signaling through GPER is evolutionarily conserved in vertebrates. The results show that maintenance of meiotic arrest in zebrafish oocytes is at least partly regulated through activation of matrix metalloproteinase, EGFR (ErbB1), and ERK1/2 via estrogen-induced GPER signaling in zebrafish oocytes.

Materials and Methods

Chemicals

Chemicals were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise mentioned. G-1, GPER agonist was purchased from EMD Chemicals (San Diego, CA) and G-15, the GPER-specific antagonist, was a gift from the lab of Eric Prossnitz. Ilomastat was purchased from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA). The MEK1/2 inhibitor, AZD6244, was purchased from Selleck Chemicals (Houston, TX) and the MEK1/2 inhibitor, U0126, was purchased from Cell Signaling Technology, Inc. (Beverly, MA).

Animal care

Adult zebrafish were purchased from Segrest Farms (Gibsonton, FL) and maintained in 10-gallon re-circulating filtered freshwater tanks at 28 °C with a 14L:10D photoperiod at the University of Texas Marine Science Institute. Fish were fed a diet of brine shrimp twice daily.

Zebrafish were placed in an anesthetic and humanely sacrificed following procedures approved by the University of Texas IACUC. For all assays ovaries were removed and ovarian fragments containing oocytes were placed immediately in Leibovitz L-15 medium (60%).

Germinal Vesicle Breakdown (GVBD) Assay

20-30 fish were sacrificed for each assay between 10:00 and 10:30 AM. Ovarian follicles were removed and placed immediately in Leibovitz L-15 medium (60%) in a sterile 15 mL plastic centrifuge tube. The follicle-enclosed oocytes were separated under a binocular microscope with fine forceps and scalpel blades and

cleaned by pipetting approximately 50 times with a Pasteur pipette. Once cleaned and separated, ovarian follicles greater than 500 microns were selected and incubated in 10 mL of L-15 media in a petri dish with collagenase (50 μg/mL) for 1 H. After the enzyme treatment, which digests the connection between the oocytes and follicle cells, the oocytes were harvested and washed by pipetting up and down in fresh L-15 media. Approximately 20 oocytes were transferred to each well of a 24-well plate containing 1 mL of L-15 media. Each treatment was replicated four times (n=4) and each experiment was repeated 3 times. Steroids were dissolved in ethanol or DMSO and a total amount of 1μL was added to each well. The control groups had either 1 μL of ethanol or DMSO added without steroids. After 3 H of incubation in a shaker at 28 °C the number of oocytes that matured (i.e. germinal vesicle no longer visible) was counted under a binocular microscope and the percentage completing germinal vesicle breakdown was calculated. An incubation of 3 H was chosen because after that time point spontaneous maturation of untreated (control) oocytes began to increase, thereby obscuring the treatment effects.

The maturation-inducing steroid in zebrafish, 17,20β-dihydroxy-4-pregen-3-one (DHP), was always used as the positive control with the different treatments to ensure that the oocytes were, in fact, maturationally competent. GPER agonists and antagonists, nuclear receptor agonists and antagonists, as well as inhibitors of matrix metalloproteinase (MMP), inhibitors of epidermal growth factor receptor (EGFR), and inhibitors of MEK1/2 were tested to investigate their effects on germinal vesicle breakdown. Oocytes were treated with the following compounds, G-1, the GPER-

selective agonist; propyl-pyrazole-triol (PPT), an ERα-selective agonist; diarylpropronitrile (DPN), an ERβ-selective agonist; and diethylstilbestrol (DES), a non-selective estrogen, at a concentration of 100 nM following the protocol from Thomas et al. (2009). In addition, oocytes were treated with 100 nM of an estrogen (ethinylestradiol) conjugated to a 15 amino acid peptide, (EE2-PEP), which prevents the steroid from entering the oocyte, following the protocol from Thomas et al. (2009). ErbB1 inhibitors (AG1478 and AG825) and ErbB2 inhibitors (AG879 and RG13022) were used at a concentration of 50 μM following the protocol from Filardo et al. (2000) in human breast cancer cells. The MEK1/2 inhibitor, U0126, was used at a concentration of 50 μM following the *Xenopus* oocyte study from Mood et al. (2004). The MMP inhibitor, ilomastat or GM6001, was used at a concentration of 10 μM following the protocol from Quinn et al. (2009). Each experiment was repeated 3 times and similar results were obtained each time.

Detection of follicle cells on zebrafish oocytes by DAPI staining.

To confirm that the collagenase treatment removed the thecal and granulosa cells, ovarian follicles were harvested and half of them underwent the collagenase treatment while the other half was incubated in L-15 media alone for 1 H. At the end of the incubation, denuded and follicle-enclosed oocytes were rinsed and those greater than 500 μm were selected, placed on a microscope slide, and stained with the nuclear stain 4',6-diamidino-2-phenylindole (DAPI) (1μg/mL in PBS). Oocytes were examined for the presence of DAPI staining surrounding the oocytes, indicating the

presence of follicle cells surrounding the oocyte and micrographs were taken with a fluorescent microscope using RealPlex Software.

RT-PCR

Denuded zebrafish oocytes that were treated for 3 H with control – no treatment (NT), DHP (10 nM), G1, a GPR30-specific agonist, (50 nM), and EGF, epidermal growth factor (20 nM) were collected, placed in Tri-Reagent and RNA was isolated following the manufacturer's protocol (Sigma). Forty μL of the RNA product (4.68 μg per sample) was DNase treated with 10 μL of 10X Buffer and 2 μL of DNase at 37 °C for 1 H. Then 4 μL of stop solution was added and the samples were incubated at 65 °C for 10 min. Reverse transcription was performed on 0.35 μg total RNA using 2 mM dNTP, 0.5 μg/μL oligo(dT)s, 0.1 M DTT, and 5X first strand buffer. After a 10 min incubation at 70 °C 0.5 μL of Platinum Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) was added to the positive groups, while the negative control groups received none to confirm no 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, 5'ATCTGAACACGTCCCACACA, 5'TGCGTCTCTACTGGCATCAC), and zebrafish β-actin for control (5'GAGCAGGAGATGGGAACC, 5'GATGGAGTTGAAGGTGGTCT) following the manufacturer's instructions using an annealing temperature of 55 °C for 35 cycles. Image J Software from Public

Research Centre Henri Tudor (Luxembourg-Kirchberg) was used gather densitometry data of the mRNA expression.

QRT-PCR

Zebrafish oocytes were prepared following the above protocol for RT-PCR experiments. Primers were designed against zebrafish EGFR (GenBank Accession No. AY332223, 5'GCCTCAGCATGTCAAGATCA, 5' ATCAACTCCCAGACGGTCAC). Final primer concentration was 400 nM and total RNA was 0.117 μg. QRT-PCR was performed using Brilliant II SYBR Green QRT-PCR Master Mix Kit, 1-Step (Stratagene, La Jolla CA) and Eppendorf RealPlex 2.0 software. The reactions were incubated in a 96-well optical plate at 50 °C for 30 min, followed by 95 °C for 10 min, followed by 35 cycles of 95 °C for 30 sec, 55 °C for 60 sec, and 68 °C for 30 sec. This was then followed by 95 °C for 15 sec, 60 °C for 15 sec, and 20 min for changes in temperature to reach 95 °C for 15 sec. The Ct (threshold cycle) data was then analyzed to determine any difference in EGFR concentration based on the different treatment groups.

W*estern Blot Analyses*

Denuded oocytes were incubated for 5- 15 min in either vehicle, E_2 (100 nM) or G-1 (100 nM) in L-15 medium. The L-15 medium was removed, 100 μL of RIPA buffer with protease inhibitor was added, and the oocytes were homogenized using a hand-held homogenizer for 1 min. The samples were vortexed followed by shaking for 30 min at 4 °C to obtain the lysate fraction. The samples were then centrifuged at 15,000-x g for 5 min at 4 °C to remove insoluble material. The supernatant was

transferred to a new tube and protein concentration was measured using the NanoDrop 2000 software (Thermo Scientific, Wilmington, DE). Protein (16 μg) was loaded and run on a 10% sodium dodecyl sulfate polyacrilamide gel electrophoresis (SDS-PAGE) gel and the protein bands were transferred to nitrocellulose membranes (Biorad, Hercules, CA). Membranes were blocked for 1 H at room temperature in Odyssey Infared Imaging System blocking buffer diluted 4 times with PBS, following the Li-Cor Biosciences (Lincoln, NE) protocol. Membranes were incubated with primary antibody for phosphorylated-Erk (1:1,000) in Odyssey blocking buffer diluted one time in PBS-T overnight at 4 °C. Following washing of the membranes with PBS-T they were incubated with goat anti-rabbit secondary antibody 1:5,000) in Odyssey buffer diluted one time in PBS-T at room temperature for 1 H. Following the detection of Phosphorylated-Erk with the Odyssey Infared Imaging System at 680nm, the membrane was stripped using Odyssey stripping buffer, following the manufacturer's protocol, and the process was repeated for the primary antibody Erk (1:1,000). Image J Software from Public Research Centre Henri Tudor (Luxembourg-Kirchberg) was used to gather densitometry data of the protein expression.

Statistical Analyses

One-way ANOVA with Bonferroni's multiple comparison test were used to determine statistical differences between control and experimental treatments using GraphPad Prism 3.0 software (GraphPad Software, San Diego, CA).

RESULTS

Collagenase treatment of zebrafish oocytes.

Treatment of zebrafish oocytes with 50 μg/mL collagenase for 1 H was an effective method of denuding oocytes as shown by a lack of DAPI staining near the surface of the oocytes (Fig. 1), whereas abundant DAPI staining of nuclei could be seen surrounding the oocytes that had not been treated with the enzyme, indicating the presence of nuclei of the surrounding follicular cells. By removing the thecal and granulosa cells, the steroid-producing cells that surround the oocyte, experiments may be conducted with out any interference from an additional steroid source.

Effects of estrogens on spontaneous maturation of denuded zebrafish oocytes.

Females whose ovaries contained large numbers of large full-grown oocytes that would have high rates of spontaneous OM if collected and incubated between 9:00am and noon were selected for this study**.** After defolliculating oocytes and treating them with different compounds for 3 hours, oocytes that completed OM were counted and the percentage that underwent GVBD was calculated. DHP, the maturation-inducing steroid (MIS) in zebrafish, at a concentration of 5 nM significantly increased maturation ($p < 0.001$) of denuded oocytes compared to the control group. E_2 (10 nM), an estrogen that binds with high affinity to zebrafish GPER, and G-1 (100 nM), the GPER-specific ligand, significantly decreased OM of denuded oocytes compared to vehicle controls ($p < 0.05$). In contrast, PPT and DPN, selective ER α and β agonists, respectively, as well as DES, a non-selective ER agonist, did not significantly affect spontaneous OM at concentrations of 100 nM (Fig. 2). G-15, a GPER-specific antagonist, at a concentration of 100 nM did not

affect OM alone but reversed the inhibitory effects of 10 nM E_2 on oocyte maturation of denuded zebrafish oocytes ($p < 0.05$) (Fig. 3). EE2-PEP that prevents the steroid from entering the oocyte, at a concentration of 100 nM mimicked the actions of E_2 and significantly decreased spontaneous OM compared to controls ($p < 0.05$), showing that this action of estrogens to inhibit OM is initiated on the surface of the oocytes) (Fig. 4). All of the GVBD bioassays were repeated three times and similar results were obtained.

Effects of the transcription inhibitor Actinomycin D on estrogen inhibition of spontaneous maturation of denuded oocytes.

Actinomycin D, a transcription inhibitor, at a concentration of 1μM did not significantly affect the inhibitory actions of 100 nM E_2 on OM, which suggests that these estrogen actions are nongenomic.

Effects of inhibitors of the EGFR pathway on maturation of denuded zebrafish oocytes.

Estrogens have been shown to activate EGFR through GPER, resulting in the release of heparin-bound EGF (Filardo et al. 2000; Filardo and Thomas 2005) via matrix metalloproteinase. Inhibition of matrix metalloproteinase (MMP) with ilomastat treatment (1 μM and 10 μM) alone did not alter spontaneous OM compared to vehicle controls, but partially reversed the inhibitory effects of E_2 (Fig. 6). The percent OM after co-treatment with ilomastat was higher than E_2 alone and not significantly different from the control group, whereas E_2 significantly decreased OM compared to the controls ($p < 0.05$) (Fig. 6). ErbB1-specific inhibitors, AG 1478 and

AG825, at a concentration of 50 μM alone significantly increased ($p < 0.001$) spontaneous OM to a percentage similar to that induced with the MIS, an effect which was not reversed by co-treatment with 100 nM E_2 (Fig. 7 and 8, respectively). In contrast, the ErbB2-specific inhibitors, AG879 and RG13022, did not increase spontaneous maturation of denuded zebrafish oocytes, and appeared to decrease it below vehicle control levels (Fig. 7 and 8, respectively).

Effects of the MEK1/2 inhibitors, U0126 and AZD6244, on OM were also examined. At a concentration of 50 μM, U0126 treatment reversed the inhibitory effects of E_2 (Fig. 9) and G-1 (Fig. 10), increasing the percentage of oocytes that underwent GVBD to levels not significantly different from vehicle controls. Similarly, another MEK1/2 inhibitor, AZD6244 reversed the effects of E_2 treatment on oocytes, significantly increasing the amount of OM compared to oocytes treated with E_2 only (Fig. 11).

Presence of EGFR message in denuded zebrafish oocytes and its hormonal regulation.

For RT- PCR, denuded zebrafish oocytes were treated with either E_2 (50 nM), G1 (50 nM), EGF (50 nM), or no treatment for a control and amplified using primers designed against zebrafish EGFR (Accession No. AY332223). EGFR message was present in the oocytes that lacked follicle cells (Fig. 12a) (βActin control, Fig. 12b) and the EGFR message appears to be up-regulated by EGF (Fig. 12c). Further examination of the Ct values from quantitative real time PCR did not reveal EGFR

regulation (data not shown), but the experiment confirmed that EGFR message is located in defolliculated oocytes.

Regulation of ERK1/2 activation in denuded zebrafish oocytes.

The results of our previous experiments suggest that E_2 acts through GPER to cause activation of the EGFR pathway in zebrafish oocytes. In order to further investigate the involvement of the EGFR pathway, we investigated whether E_2 treatment of defolliculated oocytes altered one of the final endpoints of the EGFR pathway, ERK1/2 phosphorylation. Additionally, treatment with the GPER-specific agonist, G-1 was tested to confirm that E_2 regulation of ERK1/2 phosphorylation is mediated through GPER. Oocytes treated for 15 min with E_2 and G-1 had increased levels of phosphorylated ERK1/2 compared to vehicle controls (Fig. 13a and 13b, respectively), which was confirmed by densitometry (Fig. 13c and 13d).

DISCUSSION

The present results support previous findings that estrogens inhibit meiotic maturation of zebrafish oocytes by binding to and activating the membrane estrogen receptor, GPER (Pang and Thomas 2009). By using a peptide-bound estrogen that is unable to cross the plasma membrane, EE2-PEP, this study confirms that estrogen's actions are occurring on the oocyte membrane to decrease spontaneous oocyte maturation (OM). Additionally, specific agonists of the nuclear receptors ERα and ERβ have no effect on spontaneous OM, indicating that these estrogen actions are not mediated by the nuclear ERs. Furthermore, Actinomycin D, a transcription inhibitor, did not alter the estrogen response, suggesting that the mechanism of estrogen

inhibition of oocyte maturation occurs nongenomically. Finally, the GPER-specific agonist G-1 mimics E_2 and decreases spontaneous OM, whereas the GPER-specific antagonist G-15 interferes with this estrogen action. Taken together, these data provide the first clear evidence that estrogen inhibition of OM in teleosts is mediated through GPER and not through full-length ERα, ERβ. The results also suggest that estrogens are not acting through a C-terminal truncated form of these ERs, which would also be expected to be activated by the ER-specific agonists, PPT and DPN.

A recent study by Kang et al. (2010), has questioned the role of GPER as an intermediary in nongenomic estrogen signaling, and suggests, instead, that an estrogen receptor truncated variant ER-α36 is primarily responsible for mediating these estrogen actions in breast cancer SKBR-3 cells. However, recent data, including results presented in this study, suggest that estrogen inhibition of oocyte maturation in zebrafish is mediated by GPER, and not by a truncated ER homologous to ER-α36. First, no evidence has been obtained from searches of the zebrafish genome or zebrafish ESTs for the existence of an ER homologous to ER-α36, or any truncated form of ERα, in zebrafish. Second, Pang and Thomas (2009), have shown that silencing GPER with antisense oligonucleotides, or blocking activation of the receptor with specific GPER antibodies, causes the inhibitory actions of estrogens on OM to be lost, suggesting an involvement of GPER in this estrogen function. Furthermore, data in this study demonstrate that the mammalian GPER selective antagonist, G15, has similar estrogen antagonistic actions in fish, interfering with estrogen actions to inhibit spontaneous OM, resulting in a percent GVBD not

significantly different from vehicle controls. Finally, it is demonstrated here that human nuclear estrogen receptor agonists PPT and DPN, that have been shown to activate fish ERs (Leanos-Castaneda and Van der Kraak 2007), have no effect on oocyte maturation in zebrafish. If there is a truncated form of ERα in zebrafish, the specific ER agonists would be expected to recognize it and decrease spontaneous OM. Taken together, these results provide several lines of evidence that inhibition of OM is mediated through GPER and not ER-α36 or any other truncated form of ERα. While the study by Kang et al. (2010) does reveal that there may be other receptors mediating estrogen actions in human breast cancer cells, it cannot be concluded from these limited studies that this mechanism is universal in all other cells containing GPER. Certainly for zebrafish the results presented here support the hypothesis that the inhibitory action of E_2 is solely mediated through GPER.

The intracellular signaling pathways induced by estrogens through GPER have been extensively investigated in breast cancer cell lines (Filardo et al. 2000; Filardo et al. 2002). It has been demonstrated that GPER is coupled to a stimulatory G protein, $G_{s,}$ and that estrogen activation of the Gα subunit causes activation of membrane-bound adenylyl cyclase resulting in an increase in cAMP concentrations (Filardo and Thomas, 2006). It has also been proposed that estrogens also activate the βγ subunit of the stimulatory G protein through GPER. (Filardo et al. 2002; Filardo et al. 2008). Studies in mice and humans (Filardo and Thomas 2005; Migliaccio et al. 1996) have demonstrated that E_2 mimics EGF activity and causes downstream ERK1/2 activation. Filardo and co-workers used specific inhibitors of critical

components of the EGFR pathway from which they developed a model of the mechanism of EGFR transactivation through the βγ G protein subunit involving GPER (Filardo et al. 2002). These researchers additionally showed estrogen treatment also caused ERK1/2 phosphorylation these cells, suggesting it is a potential downstream target of EGFR transactivation (Filardo et al. 2000). Moreover they showed that MMP is involved in the induction of ERK1/2 phosphorylation through EGFR (Prenzel et al. 1999; Filardo et al. 2000).

In the present study we examined the hypothesis that estrogen actions mediated by GPER exert their effects through a similar signaling pathway in zebrafish oocytes to that described in breast cancer cells (Fig. 14). We used the same pharmacological tools as those used by Filardo and coworkers to determine if GPER transactivates EGFR by the MMP-induced release of heparin-bound EGF to induce activation of ERK1/2 in zebrafish oocytes to inhibit OM. The results provide the first evidence for a function of EGFR in the regulation of meiotic maturation in teleost oocytes. In a previous study only minor amounts of EGFR mRNA were found in zebrafish oocytes (Wang and Ge, 2004). However, high expression levels of EGFR mRNA were detected in denuded zebrafish oocytes using several different primer sets in the present study (other primers, data not shown). EGFR signaling has also been found to be required for induction of OM in mice, (Jamnongjit et al. 2005). These authors proposed that EGFR is in the thecal and granulosa cells and the signaling functions to prime the oocyte to respond to LH surges (Jamnongjit et al. 2005). Similarly, Wang and Ge (2004), identified EGFR in the follicle cells, and proposed it

has a paracrine function to influence zebrafish oocyte physiology. Thus, it is likely that EGFR signaling is important in both follicle cells and in the oocytes in the regulation of OM.

It has been shown that the MMP inhibitor ilomastat blocks activation of EGFR in human breast cancer cells by preventing the release of HB-EGF (Prenzel et al. 1999; Filardo et al. 2000; Filardo et al. 2008). The results provided here show that estrogens appear to be acting through the same mechanism in fish oocytes. Treatment with ErbB1 inhibitors AG1478 or AG825 increased spontaneous OM by blocking the inhibitory effects of estrogens. Interestingly these treatments increased spontaneous OM to levels induced by the MIS, DHP. This finding suggests that ErbB1 is involved in other OM inhibitory mechanism(s) in addition to that mediated by estrogens. The results suggest that multiple inhibitory pathways are involved in preventing the resumption of meiosis in zebrafish oocytes through activation of ErbB1. Other potential hormonal regulators of ErbB1 in zebrafish oocytes remain be investigated but possible candidates include members of the transforming growth factor-β (TGF-β) superfamily. Tan et al. (2009) found that oocyte maturation was partially inhibited by members of the TGF-β superfamily including bone morphogenic protein (BMP)-15 and TGF-β1 in zebrafish, and partially stimulated by a different member, activin-A, which is likely due to regulation of mPRα and mPRβ by these compounds. This raises the possibility that there are multiple upregulators of EGFR that could impact OM. Conversely, the MIS, DHP, could induce OM by downregulating EGFR transactivation. Treatment with ErbB2 inhibitors AG879 or RG13022 decreased

spontaneous OM. The present results suggest that treatment of oocytes with E_2 results in GPER specifically transactivating ErbB1 (EGFR), which together with other possible factors inhibits OM.

One of the likely downstream consequences of E_2 transactivation of EGFR via GPER is activation of the MAPK signaling cascade, and subsequent ERK1/2 phosphorylation. Treatment of the denuded oocytes with U0126, a MEK1/2 inhibitor, resulted in a significant increase in spontaneous OM by abrogating the effects of both E_2 and G-1. An additional MEK1/2 inhibitor, AZD6244, also increased spontaneous maturation even with E_2 treatment. Estrogen treatment of denuded oocytes for 15 min increased phosphorylated ERK compared to the control group. Migliaccio et al. (1996) studied responses to estrogen treatment in MCF-7 cells and found that E_2 activates the MAPK transduction pathway. At the time they could not determine whether it was acting through an ErbB receptor or its own receptor, but they knew that E_2 mimicked the actions of EGF. Since then, ErbB1 activation via GPER has been shown in mouse spermatogonial cells (Sirianni et al. 2008) and ovarian cancer cells (Albanito et al. 2007) in which cases the activation was abrogated by using ErbB1 inhibitors, MEK inhibitors, and GPER knockdowns. These results support the finding that E_2 mimics the actions of EGF by acting through GPER to transactivate EGFR resulting in an increase of ERK1/2 phosphorylation. In the case of zebrafish, transactivation of EGFR is a mechanism that helps maintain the meiotic arrest of oocytes to inhibit spontaneous OM.

In conclusion, the seven transmembrane (7TM) G protein-coupled receptor GPER is coupled to a stimulatory G protein, and as such activates adenylyl cyclase to increase cAMP concentration, a mechanism of GPER signaling that is conserved throughout evolutionary history between mammals and fish (Filardo et al. 2000; Filardo et al. 2002; Thomas et al. 2005; Pang et al. 2008, Pang and Thomas 2009; Thomas et al. 2009). In addition to the conserved Gα subunit signaling, this study presents data to support the hypothesis that the Gβγ subunit signaling through GPER is also conserved throughout vertebrates. Filardo and colleagues have previously demonstrated that estrogen activation of GPER results in transactivation of EGFR in mammals (Filardo et al. 2000; Filardo et al. 2002; Filardo and Thomas 2005). Here we provide evidence for transactivation of EGFR by GPER in a non-mammalian vertebrate species, the freshwater cyprinid zebrafish. This study confirms that estrogens maintain meiotic arrest of fish oocytes through activation of GPER, which in turn transactivates EGFR.

FIGURES & TABLE

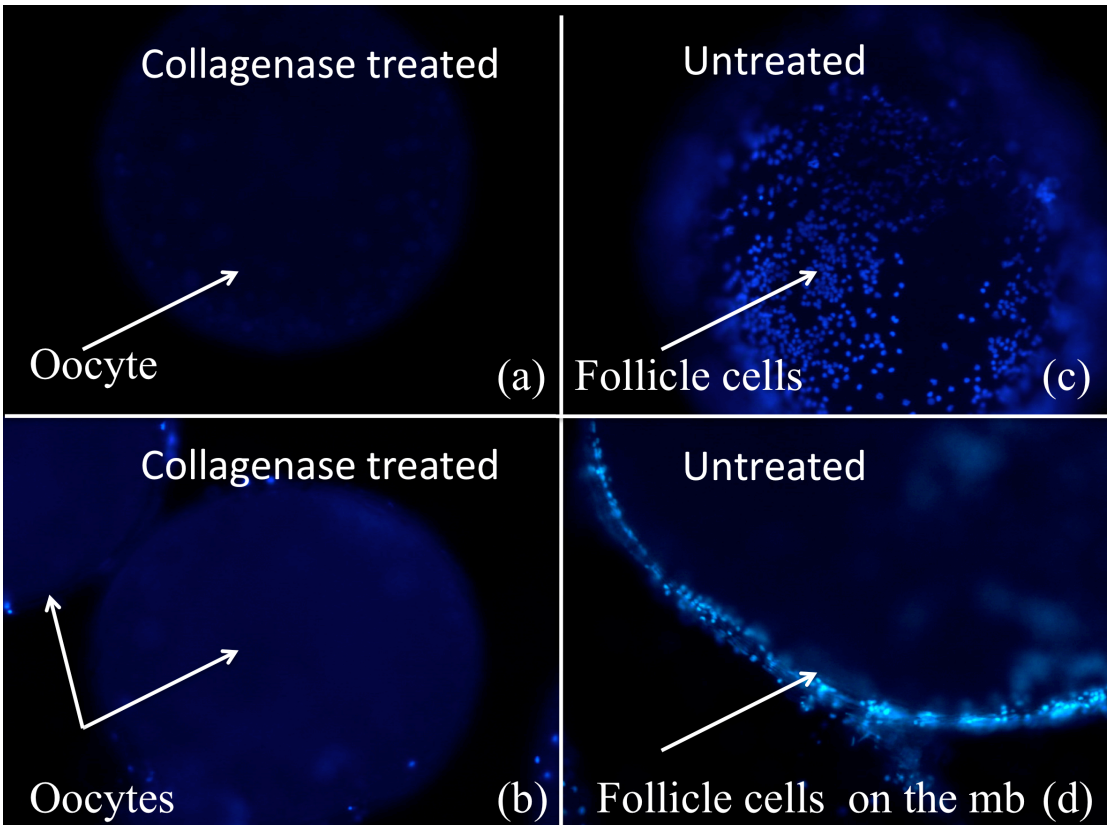

Fig. 1. Effect of collagenase treatment on the removal of follicle cell layers from zebrafish oocytes. DAPI staining of the follicle cell nucleus reveals that the treatment results in the removal of the thecal and granulosa cell layers, thus removing the endogenous source of estrogens. (a) One collagenase-treated oocyte showing no staining of follicle cell nuclei. (b) Two collagenase-treated oocytes showing no staining of follicle cell nuclei. (c) Untreated oocyte showing many follicle cells stained with DAPI. (d) Magnified view of the oocyte membrane showing the location of follicle cells on the oocyte.

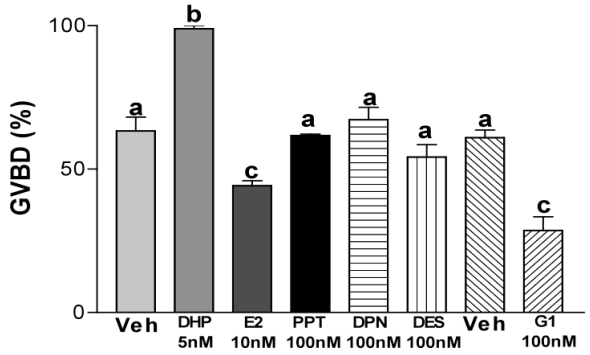

Fig. 2 Effects of selective estrogen receptor modulators on inhibition of spontaneous maturation of denuded zebrafish oocytes in the *in vitro* GVBD bioassay. Veh – Vehicle control; DHP-17,20β-dihydroxy-4-pregen-3-one; PPT – a selective ER alpha or beta agonist; DPN – a selective ER alpha or beta agonist; DES – nonselective ER agonist; G-1 – a selective GPER agonist. Different letters denote significant differences from each other ($p < 0.05$, one way ANOVA and nonparametric Bonferroni test).

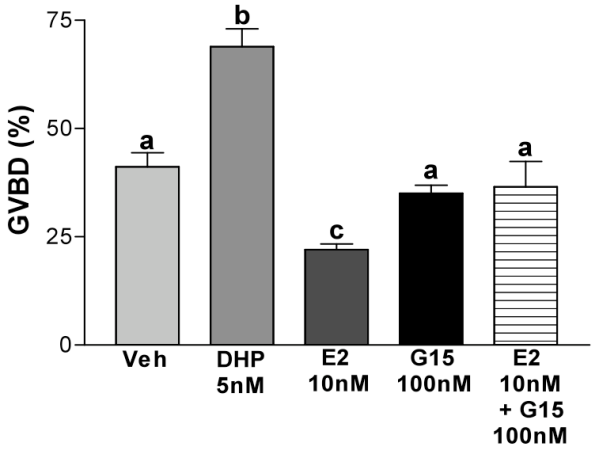

Fig. 3 Effects of 3hrs treatment with the GPER antagonist, G-15, on the estrogen inhibition of oocyte maturation in denuded zebrafish oocytes. Veh – Vehicle control; DHP-17,20β-dihydroxy-4-pregen-3-one; E2 –Estradiol-17β, a GPER agonist. Approximately 20 oocytes per well and 4 wells per treatment were counted and scored for germinal vesicle breakdown (oocyte maturation). Different letters denote significant differences from each other ($p < 0.05$, one way ANOVA and nonparametric Bonferroni test).

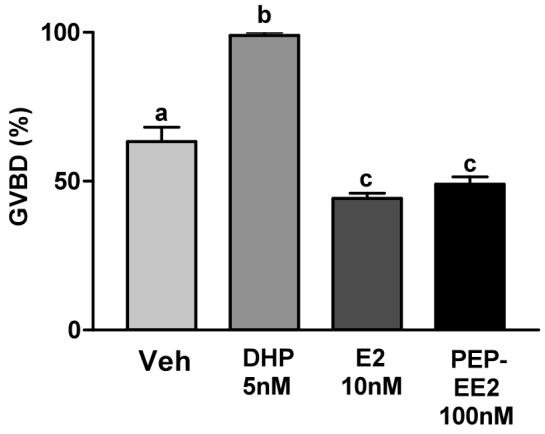

Fig. 4 Effects of treatment for 3hrs with an estrogen-peptide conjugate, ethinyl estradiol (PEP-EE2), on spontaneous oocyte maturation in denuded zebrafish oocytes in the in vitro GVBD bioassay. Veh – Vehicle control; DHP-17,20β-dihydroxy-4-pregen-3-one; E2 – Estradiol-17β, a GPER agonist. Different letters denote significant differences from each other ($p < 0.05$, one way ANOVA and nonparametric Bonferroni test).

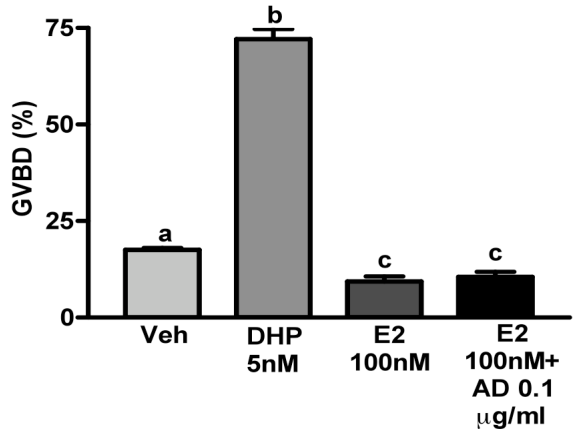

Fig. 5 Effects of 3hrs treatment with the transcription inhibitor, Actinomycin D (AD), on E2 inhibition action on oocyte maturation of denuded zebrafish oocytes in the in vitro GVBD bioassay. Veh – Vehicle control; DHP-17,20β-dihydroxy-4-pregen-3-one; E2 – Estradiol-17β, a GPER agonist. Approximately 20 oocytes per well and 4 wells per treatment were counted and scored for germinal vesicle breakdown (oocyte maturation). Different letters denote significant differences from each other ($p < 0.05$, one way ANOVA and nonparametric Bonferroni test).

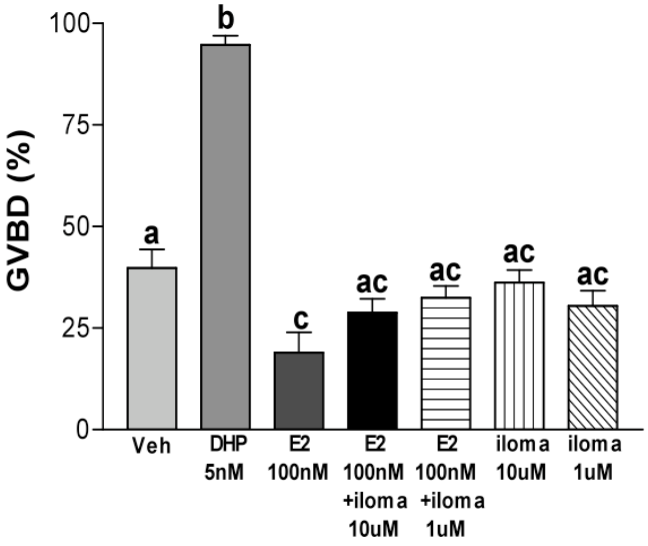

Fig. 6 Effects of 3 hrs treatment with the MMP inhibitor, ilomastat, on estrogen inhibition of spontaneous oocyte maturation of denuded zebrafish oocytes in the *in vitro* GVBD bioassay. Veh – Vehicle control; DHP-17,20β-dihydroxy-4-pregen-3-one; E2 – Estradiol-17β, a GPER agonist. Approximately 20 oocytes per well and 4 wells per treatment were counted and scored for germinal vesicle breakdown (oocyte maturation). Different letters denote significant differences from each other ($p < 0.05$, one way ANOVA and nonparametric Bonferroni test).

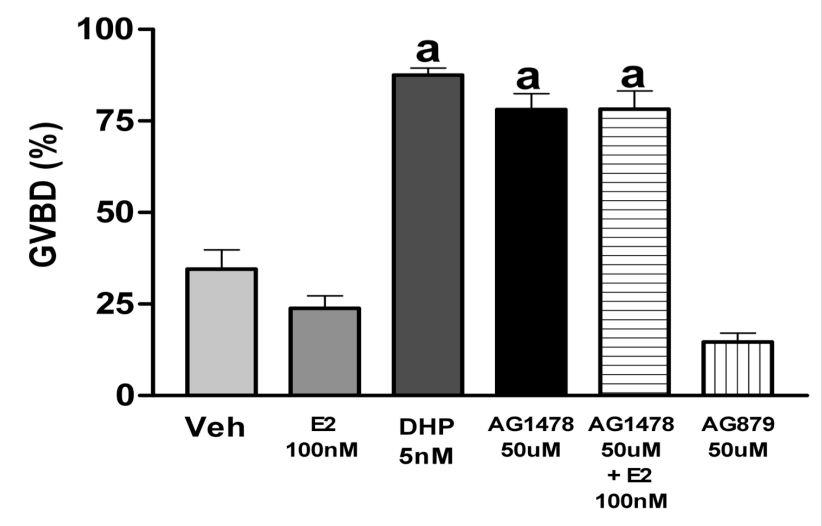

Fig. 7 Effects of an ErbB1 inhibitor, AG1478, and the ErbB2 inhibitor, AG879, on the induction of oocyte maturation in denuded zebrafish oocytes. Veh – Vehicle control; DHP-17,20β-dihydroxy-4-pregen-3-one; E2 – Estradiol-17β, a GPER agonist. Approximately 20 oocytes per well and 4 wells per treatment were counted and scored for germinal vesicle breakdown (oocyte maturation). Letters denote significant differences from the control (Veh) group ($p < 0.05$, one way ANOVA and nonparametric Bonferroni test).

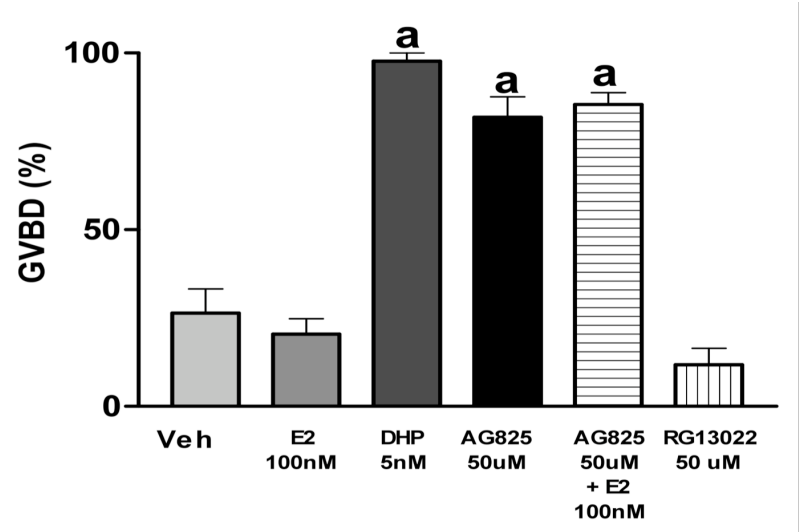

Fig. 8 Effects of an ErbB1 inhibitor, AG825, and the ErbB2 inhibitor, RG13022, on the induction of oocyte maturation in denuded zebrafish oocytes. Veh – Vehicle control; DHP-17,20β-dihydroxy-4-pregen-3-one; E2 – Estradiol-17β, a GPER agonist. Approximately 20 oocytes per well and 4 wells per treatment were counted and scored for germinal vesicle breakdown (oocyte maturation). Letters denote significant differences from the control (Veh) group ($p < 0.05$, one way ANOVA and nonparametric Bonferroni test).

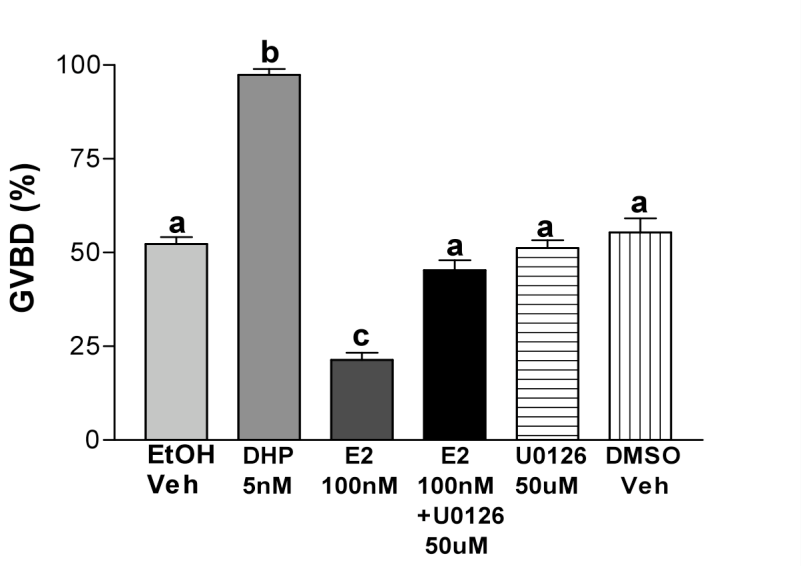

Fig. 9 Effects of the MEK1/2 inhibitor, U0126, on the induction of oocyte maturation in denuded zebrafish oocytes. Veh – Vehicle control; DHP-17,20β-dihydroxy-4-pregen-3-one; E2 – Estradiol-17β, a GPER agonist. Approximately 20 oocytes per well and 4 wells per treatment were counted and scored for germinal vesicle breakdown (oocyte maturation). Different letters denote significant differences from each other ($p < 0.05$, one way ANOVA and nonparametric Bonferroni test).

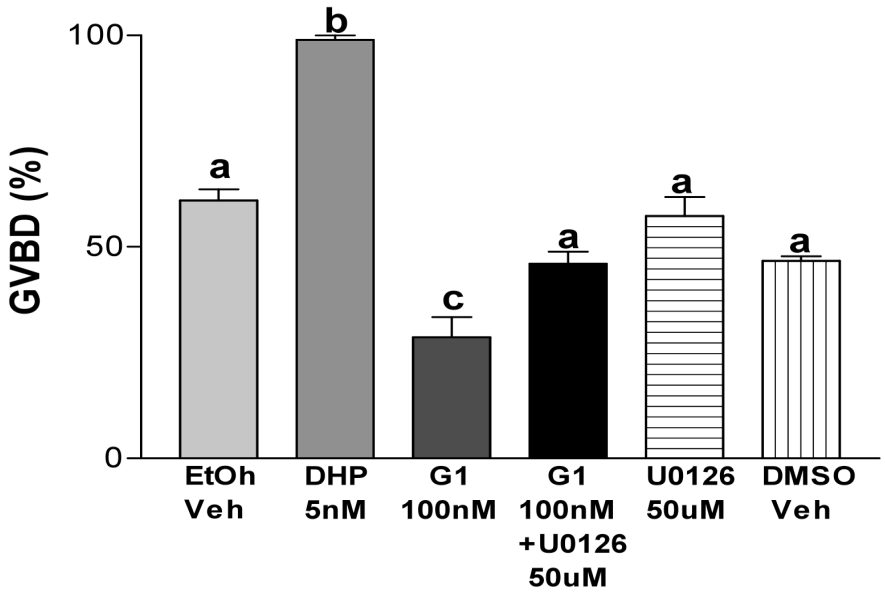

Fig. 10 Effects of the MEK1/2 inhibitor, U0126, on the G-1 inhibition of oocyte maturation in denuded zebrafish oocytes. Veh – Vehicle control; DHP-17,20β-dihydroxy-4-pregen-3-one; G1 – a GPER selective agonist. Approximately 20 denuded oocytes per well and 4 wells per treatment were counted and scored for germinal vesicle breakdown (oocyte maturation). Different letters denote significant differences from each other ($p < 0.05$, one way ANOVA and nonparametric Bonferroni test).

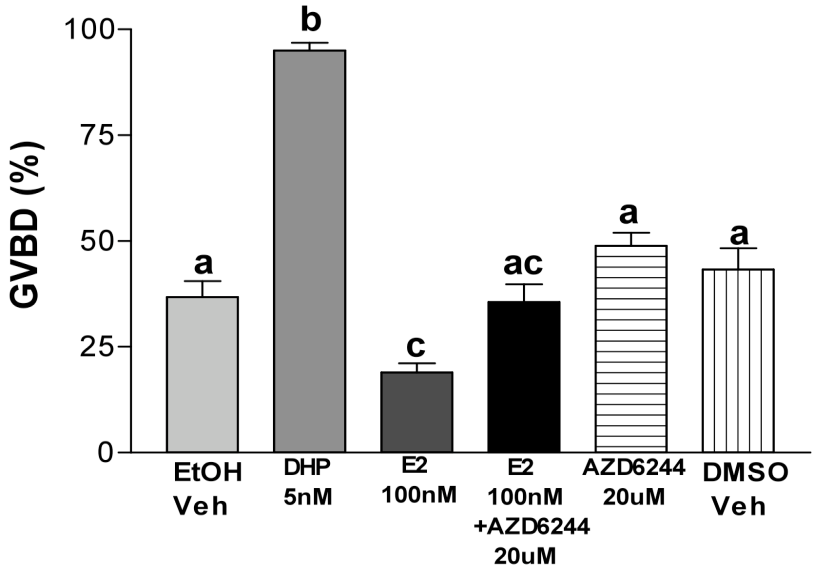

Fig. 11 Effects of the MEK1/2 inhibitor, AZD6244, on the estrogen inhibition of oocyte maturation. Veh – Vehicle control; DHP-17,20β-dihydroxy-4-pregen-3-one; E2 – Estradiol-17β, a GPER agonist. Approximately 20 denuded oocytes per well and 4 wells per treatment were counted and scored for germinal vesicle breakdown (oocyte maturation). Different letters denote significant differences from each other ($p < 0.05$, one way ANOVA and nonparametric Bonferroni test).

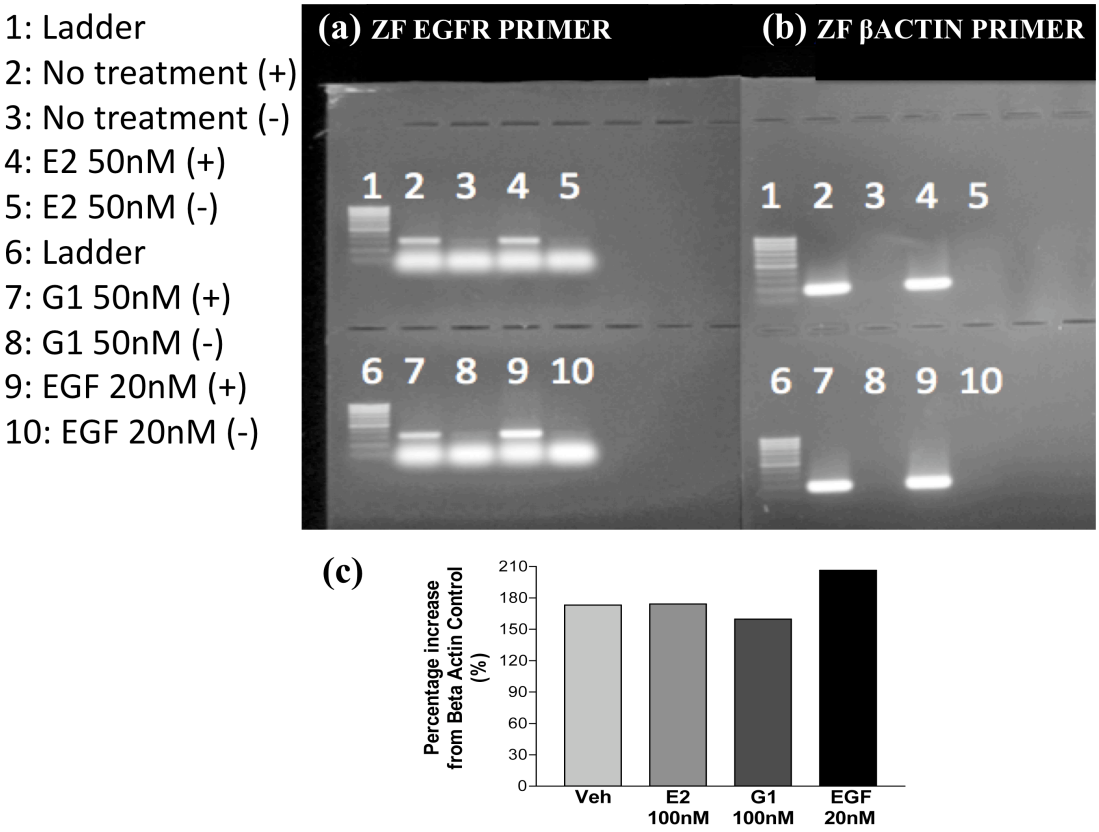

Fig. 12 Presence of EGFR mRNA transcripts in defolliculated oocytes and effects of hormonal treatments. (a) Detection of EGFR in oocytes using sense and antisense primers designed against zebrafish EGFR mRNA. (b) Loading control βActin. (c) Percent change in EGFR mRNA calculated from densitometry of EGFR mRNA bands. Values were determined from increase in message compared to the loading control: (EGFR/βActin)*100 = Percentage increase in message.

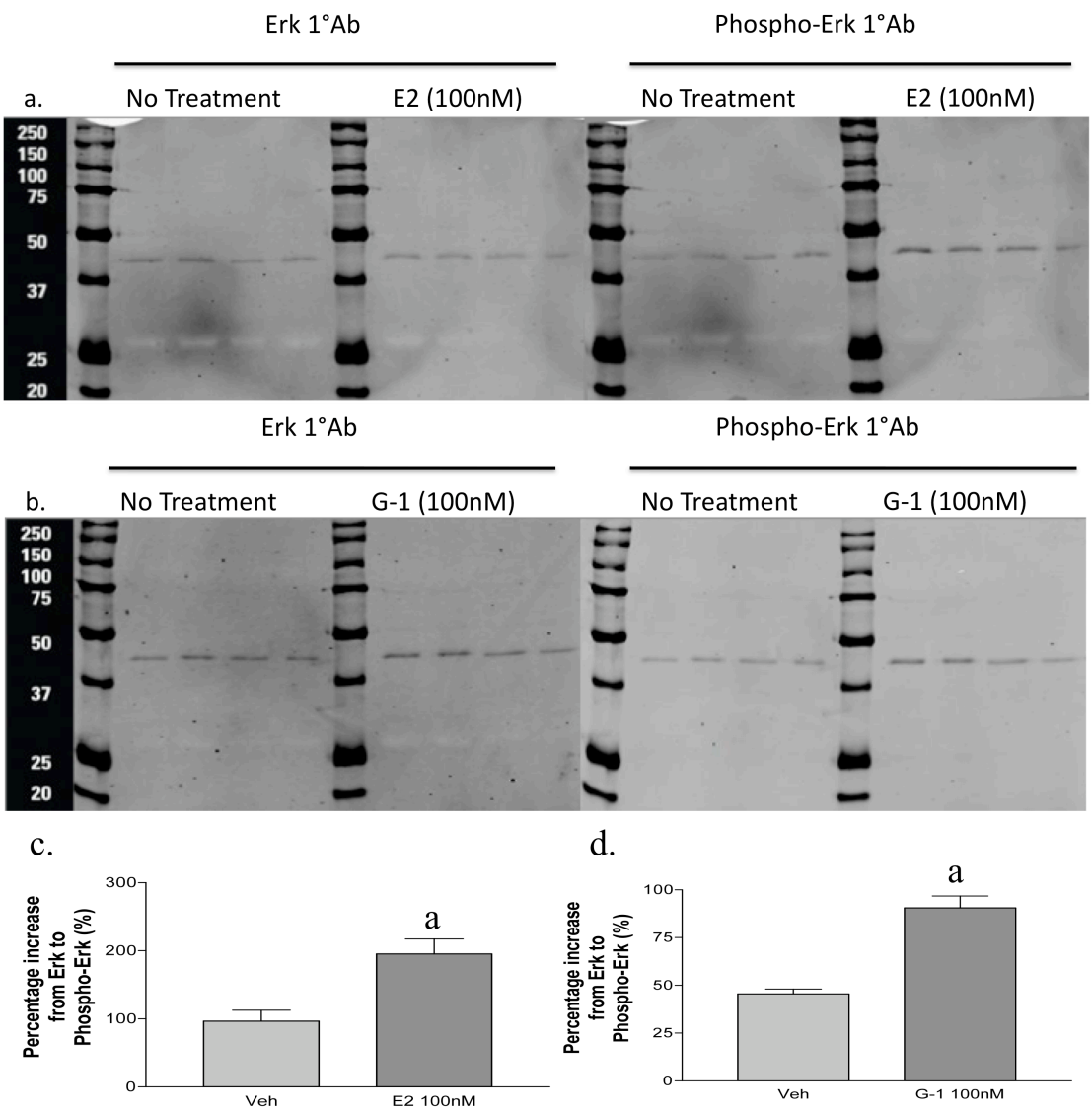

Fig. 13 Effects of hormonal treatments on Erk and phosphorylated Erk levels in defolliculated oocytes. (a) Effects 15 minute treatment with E2 (100nM). (b) Effects 15 minute treatment with G-1 (100nM) (c,d) Percent increase in phosphorylated Erk levels compared to total Erk levels determined by densitometric analysis of immunoreactive bands detected on the Western blots. (Phospho-Erk/Erk)*100 = Percentage increase compared to the loading control, Erk. Different letters denote significant differences from control ($p < 0.05$), Student's t test.

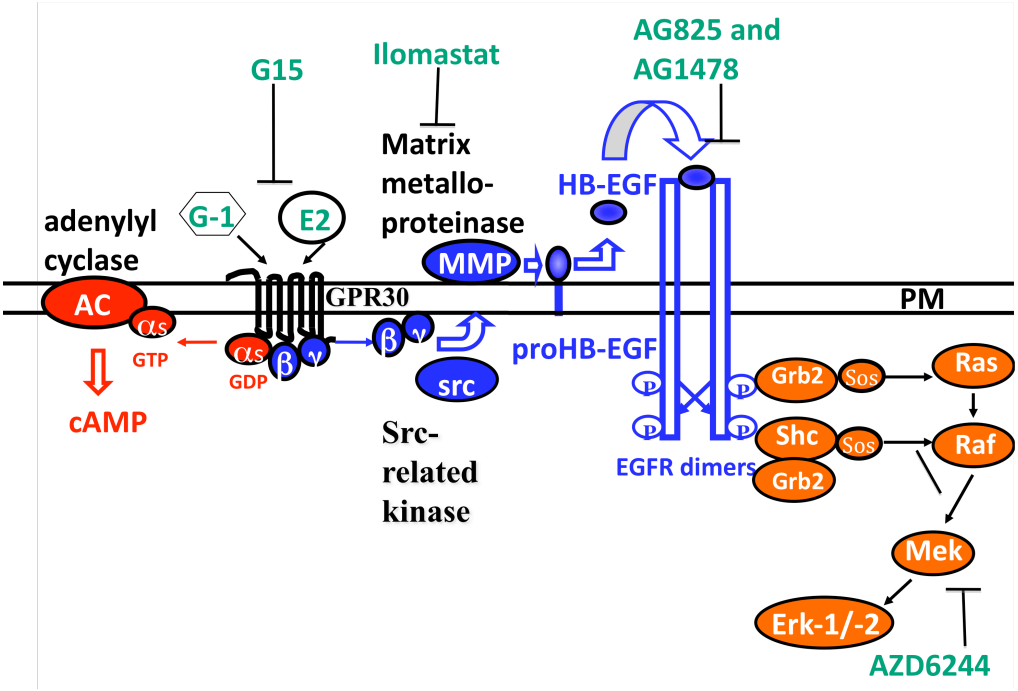

Fig. 14 Proposed model of estrogen signaling through GPER to maintain meiotic arrest of zebrafish oocytes. Red indicates the pathway stimulated by the Gα subunit. Blue indicates the pathway stimulated by the Gβγ subunit. Compounds highlighted in green were used as inhibitors or stimulators of the estrogen signaling mechanism that inhibits OM.

CHAPTER 3

SUMMARY AND CONCLUSIONS

A recent review by Thomas et al. (2009) investigated the similarities between the estrogen binding and signaling characteristics of GPER in mammals and fishes. According to the research, GPER found in human breast cancer cells as well as in teleost fishes displays high binding affinity to E_2, which elicits the downstream effect of increased cAMP production by coupling to a stimulatory G protein. The binding and signaling of E_2 therefore appears to be conserved for over 200 million years. Recent studies from Filardo et al. (2000 and 2002) show that in human breast cancer cells there is activation of an additional pathway in response to estrogen binding to GPER. His lab has demonstrated that upon GPER activation, matrix metalloproteinase (MMP) causes heparin-bound epidermal growth factor (HB-EGF) release, which binds to EGFR and activates the mitogen-activated protein kinase (MAPK) signaling pathway that ultimately results in the phosphorylation of extracellular-signal-regulated kinases 1 and 2 (ERK1/2). By using various inhibitors of MMP, EGFR, and ERK1/2, we demonstrate here that the activation of the EGFR signal transduction pathway resulting from estrogen binding to GPER is an additional function that remains conserved throughout evolutionary history from fishes to mammals, and in the case of fishes, functions to maintain the meiotic arrest of oocytes to prevent precocious maturation. Additionally, this study is the first to

confirm that the inhibitory actions of estrogens are mediated through GPER and not through ER-α36 or any other truncated form of ERα.

Since this study has established the role of EGFR in maintaining meiotic arrest of oocytes by using certain inhibitors of the pathway to see if oocyte maturation would increase, it would be useful to use certain stimulators of the pathway to see if oocyte maturation would decrease. Therefore, future experiments should include any activators of MMP such as interleukin-1α (Keller et al. 2000), overexpression of EGFR, or addition of any factor that would phosphorylate Erk including pervanadate (Ratz 2001). To get even greater confirmation of the transactivation, an experiment that inhibits the Gβγ subunit from activating MMP is necessary. The Gβγ subunit is essentially made up of two different subunits, a beta subunit and a gamma subunit. However, it acts as a monomer because it can only function when the two are bound together. Therefore, specific denaturants could disable the βγ signaling and should result in a loss of function, loss of transactivation of EGFR. Moreover, knockout of GPER should also prevent phosphorylation of Erk resulting from E_2 treatment. Additionally, it would be beneficial to further explore the regulation of expression of GPER and EGFR in the oocytes, perhaps even by looking at the TGF-β superfamily of gonadotropins proposed by Tan et al. (2009) to have a role in OM regulation.

Finally, preliminary data shows that certain compounds act as endocrine disruptors, specifically Bis-phenol A (BPA). It would be useful to confirm that it is acting through GPER and transactivating EGFR. This would provide insight into whether or not other endocrine disruptors can act in the same manner and this data

could be useful in making regulatory decisions of the discharge of xenoestrogens into the environment since vertebrates from fishes to humans can be affected in like manners.

APPENDIX

ABBREVIATIONS:

17α,20β-dihydroxy-4-pregnen-3-one .....DHP

Adenylate Cyclase .....AC

Cyclic adenosine 3', 5' monophospate .....cAMP

Follicle Stimulating Hormone.....FSH

G protein-coupled receptor .....GPCR

G protein-coupled estrogen receptor 1.....GPER

GPCR membrane progestin receptor .....mPR

Germinal vesicle breakdown.....GVBD

Heterotrimeric GTP binding protein.....G protein

Leibovitz L-15 medium… L-15

Luteinizing hormone.....LH

Maturating inducing steroid.....MIS

Maturation promoting factor.....MPF

Mitogen-activated protein kinase.....MAPK

Oocyte Maturation .....OM

Oocyte Maturational Competence .....OMC

Pertussis toxin .....PTX

Phosphatidylinositol 3-kinase PI3K

Protein kinase A (cAMP-dependent protein kinase) .....PKA

Sodium dodecyl sulfate.....SDS

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